Anaerobic Formation of the Aromatic Hydrocarbon
$p$-Cymene from Monoterpenes by Methanogenic
Enrichment Cultures

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The fate of monoterpenes in methanogenic habitats was investigated in enrichment cultures using defined mineral media inoculated with activated sludge and R(-)-α-phellandrene, (+)-2-carene, (−)-α-pinene, or (+)-sabinene. The anaerobic biodegradation of α-pinene and 2-carene supported methanogenesis. In contrast, consumption of α-phellandrene and sabinene was accompanied by formation of the aromatic hydrocarbon $p$-cymene. This aromatization reaction did not occur in pasteurized or autoclaved cultures or in the absence of microorganisms. The monoterpene γ-terpinene accumulated transiently. We conclude that anaerobic microorganisms exist that are able to aromatize monoterpenes with a cyclohexadiene structure. Similar biological reactions may contribute to the formation of aromatic biomarker molecules, e.g., monoaromatic steroids and hopanoids.

Keywords aromatization, biodegradation, biomarker, biotransformation, methanogenesis

Monoterpenes are hydrocarbons almost only synthesized in plants. The annual global formation rate is expected to exceed $1 \times 10^{15}$ g, based on an annual emission from trees of $4.8 \times 10^{14}$ g (Zimmermann et al. 1978) and the ubiquitous presence of monoterpenes in leaves and fruits. Transport processes cause the presence of monoterpenes in anoxic habitats, i.e., fall foliage in lake sediments. Recently we discovered that anaerobic bacteria are able to utilize these natural hydrocarbons as electron donors and carbon source. Thus, monoterpenes previously considered to be recalcitrant to microbial degradation in the absence of molecular oxygen were completely mineralized to carbon dioxide by denitrifying β-Proteobacteria, Alcaligenes defragrans and Thauera terpenica (Harder and Probian 1995; Foß and Harder 1998; Foß et al. 1998).

The solubility of monoterpenes, 20 to 50 μM in water (Weidenhamer et al. 1993), is significant and can explain the removal of these hydrocarbons from subsurface sediment layers in geological timescales. Thus, organic geochemical analyses of rock samples rarely encounter monoterpenes. Peat-covered pine stumps still contained monoterpenes (Maxwell et al. 1972). The monoterpene pattern found in these stumps provided evidence for a disproportionatereaction of α-pinene into $p$-menthane, an alkane, and $p$-cymene ($p$-isopropylmethylbenzene). This reaction and the presence of aromatic compounds in crude oil have been considered nonbiological catagenic processes (Tissot and Welte 1984). However,
we noticed the formation of traces of \( \textit{p} \)-cymene in denitrifying enrichment and pure cultures (Harder and Probian 1995; Heyen and Harder 1998). This observation led us to initiate methanogenic enrichment cultures on monoterpenes to investigate whether anaerobic microorganisms are able to aromatize monoterpenes to \( \textit{p} \)-cymene during such conditions.

**Materials and Methods**

**Enrichment Culture Conditions**

Anaerobic cultivation techniques were applied and anoxic media were prepared as described by Widdel and Bak (1992). One liter of mineral freshwater medium contained in extreme pure water of type I, according to the American Society For Testing and Materials, 1.0 g of NaCl, 0.4 g of MgCl\(_2 \cdot 6\)H\(_2\)O, 0.1 g of CaCl\(_2\), 0.5 g of KCl, 0.2 g of KH\(_2\)PO\(_4\), 0.25 g of NH\(_4\)Cl, and 0.2 g of NaSO\(_4\). After autoclaving, 11 of medium received 1 ml of the following sterile solutions: chelated trace element mixture (per liter of distilled water: 2100 mg of FeSO\(_4 \cdot 7\)H\(_2\)O, 60 mg of H\(_3\)BO\(_3\), 1.0 g of MnCl\(_2 \cdot 4\)H\(_2\)O, 0.38 g of CoCl\(_2 \cdot 6\)H\(_2\)O, 0.24 g of NiCl\(_2 \cdot 6\)H\(_2\)O, 2 mg of CuCl\(_2 \cdot 2\)H\(_2\)O, 0.29 g of ZnSO\(_4 \cdot 7\)H\(_2\)O, 72 mg of NaMoO\(_4 \cdot 7\)H\(_2\)O, and 7.8 g of Na\(_2\)EDTA, pH 6.0), selenite-tungstate solution (per liter: 0.4 g of NaOH, 32 mg of Na\(_2\)WO\(_4 \cdot 2\)H\(_2\)O, 24 mg of Na\(_2\)MoO\(_4 \cdot 2\)H\(_2\)O, and 6 mg of Na\(_2\)SeO\(_3 \cdot 5\)H\(_2\)O), vitamin solution (4 mg of 4-aminobenzoic acid, 2 mg of \( \textit{d} \)-(+)-biotin, 10 mg of nicotinic acid, 5 mg of calcium \( \textit{d} \)-(+)-pantothenate, 15 mg of pyridoxin hydrochloride, 4 mg of folic acid, and 1.5 mg of lipoic acid in 100 ml of 10 mM NaH\(_2\)PO\(_4\), pH 7.1), cyanocobalamin solution (5 mg of 4-aminobenzoic acid, 2 mg of \( \textit{d} \)-(+)-biotin, 10 mg of nicotinic acid, 5 mg of calcium \( \textit{d} \)-(+)-pantothenate, 15 mg of pyridoxin hydrochloride, 4 mg of folic acid, and 1.5 mg of lipoic acid in 100 ml of 10 mM NaH\(_2\)PO\(_4\), pH 7.1), cyanocobalamin solution (5 mg of 4-aminobenzoic acid, 2 mg of \( \textit{d} \)-(+)-biotin, 10 mg of nicotinic acid, 5 mg of calcium \( \textit{d} \)-(+)-pantothenate, 15 mg of pyridoxin hydrochloride, 4 mg of folic acid, and 1.5 mg of lipoic acid in 100 ml of 10 mM NaH\(_2\)PO\(_4\), pH 3.4), and riboflavin solution (2.5 mg in 100 ml of 25 mM NaH\(_2\)PO\(_4\), pH 3.2). After addition of 2 mL of 0.5 M cysteine and 30 mL of 1 M NaHCO\(_3\), the pH was adjusted to 7.0 with sterile 2.0 M HCl.

Activated sewage sludge was collected in January 1997 in a local wastewater treatment plant (Lintel, Osterholz-Scharmbeck, Germany) and incubated for 6 days in a closed polyethylene container to allow a biological consumption of endogeneous electron acceptors such as oxygen and nitrate. Enrichment cultures were prepared by using 50-ml portions of sewage sludge in 156-ml serum bottles that contained 50 ml of the anoxic medium, 10 ml of 2,2,4,4,6,8,8-heptamethylnonane (HMN) as organic carrier phase, and 20 \( \mu \)l of monoterpenes \([R-(\textit{\textdollar})-(\textit{\textdollar})-\textit{a}-\)phellandrene, \((\textit{\textdollar})+(\textit{\textdollar})-2\)-carene, \((\textit{\textdollar}-)(\textit{\textdollar})-\alpha\)-pinene, or \((\textit{\textdollar})+(\textit{\textdollar})-sabinene\)] under an oxygen-free N\(_2\)-CO\(_2\) atmosphere (90:10, vol/vol, \(<7\) ppm O\(_2\)). HMN is a nearly water-insoluble alkane with a very low toxicity to microorganisms and extremely low biodegradability (Alexander 1994; Rontani and Giusti 1986). The monoterpenes are dissolved in the organic phase, causing a reduced partial pressure and toxicity of the monoterpenes. The bottles were closed with thick black butyl rubber stoppers (hardness of 40–45 Shore A; Maag Technic, Dübendorf, Switzerland) to exclude oxygen. The control experiment contained 50 ml of autoclaved sewage sludge, 50 ml of the anoxic medium, and a mixture of the aforementioned monoterpenes (5 \( \mu \)l of each) in 10 mL of HMN. Sulfide-free FeS (0.2 M suspension) was prepared according to Hanert (1981) and added as chemical reductant to a final concentration of 0.2 mM. Solid FeS served as a redox indicator: The presence of black precipitates indicated a reduced, oxygen-free medium. Thus FeS substituted the combination of resazurin as redox-indicator and sodium sulfide as reductant with the advantage of a low, nontoxic free sulfide concentration in the enrichment culture (Dolfini et al. 1990; Harder and Probian 1995). The limited amount of sulfate in the medium (1.4 mM) may also contribute via a small population of sulfate-reducing bacteria to the reduced state of the medium. The enrichment cultures were incubated at 28°C in the dark on a horizontal shaker circuiting with 60 rpm.
Two cultivation systems were used for transfers of the enrichments. Besides the aforementioned 156-ml serum bottle, a 250-ml round bottle with two additional sample ports for the HMN and the aqueous phase was used, containing 200 ml of mineral medium and 20 ml of HMN. A small amount of acetate (2 mM) was added as carbon source into all transfer cultures. These cultures contained also FeS (0.2 mM) and were stirred with an internal magnetic bar at 50 rpm during the incubation at 28°C in the dark. Control experiments in the first transfer involved heat inactivation of the inoculated culture bottle at 60°C for 16 h prior to monoterpene addition.

Gas formation was measured as overpressure with a gas-tight glass syringe. The gas formed was analyzed by gas chromatography. Aliquots of the HMN phase (100 µl) were taken in regular intervals and analyzed to witness the changes in monoterpene content.

**Chemical Analyses**

Gases were analyzed with a Shimadzu GC-8A gas chromatograph equipped with thermal conductivity detectors and connected to a digital data analyzing system (Turbochrom 4.0; Perkin Elmer, Überlingen, Germany). Separation was obtained on a Poraplot Q column (3.2 mm × 2 m) at 40°C with nitrogen as carrier gas (32 ml min⁻¹). Injection and detection were performed at 110°C.

Monoterpene contents were analyzed by single- and dual-column capillary-column gas chromatography (GC) (Harder and Probian 1995; Foß and Harder 1997). Separation occurred on long columns (0.32 mm × 50 m, 0.5 µm film thickness) with hydrogen at a flow rate of 2 ml min⁻¹. The following temperature program was applied with heat-stable columns: injection port temperature, 270°C; column temperature, 60°C for 2 min, increasing to 140°C at a rate of 4°C min⁻¹, 140°C for 0.1 min, increasing to 320°C at a rate of 40°C min⁻¹, 320°C for 5 min; detection temperature, 350°C. Compounds formed from the substrates in the methanogenic enrichment cultures were identified by GC analyses of commercial available monoterpene standards, retention time analysis (Kovâts indices), and GC–mass spectrometric (MS) analysis with an ion-trap system as described by Harder and Probian (1995). Coinjection analyses of standards and samples confirmed the identification of *p*-cymene. Response factors of the flame ionization detector for monoterpenes including *p*-cymene deviated < 10%, as expected for organic compounds with similar molecular mass and structure (Gottwald 1995). Thus quantification could be based on comparison of peak areas.

Free energy changes of the reactions considered were calculated according to the increment method of Mavrovouniotis (1991).

**Results**

The distribution of monoterpenes in the aqueous, organic, and gas phase of the enrichment cultures was tested by GC analysis. More than 95% of the substance applied was observed to occur in the HMN phase. Thus, toxic effects on microorganisms were low, and the monoterpene concentration could be measured reliably by analysis of small subsamples from the organic phase. Control experiments without microorganisms or with autoclaved inoculate showed a slow loss of monoterpene over time without *p*-cymene formation. This can be due to diffusion into the butyl rubber stopper.

**Sabinene**

The disappearance of sabinene in the enrichment culture started after 3 weeks of incubation. The consumption was accompanied by the formation of several organic substances in different amounts (Figure 1A). *p*-Cymene, γ-terpinene, and α-thujene were identified.
FIGURE 1 Formation of \( \alpha \)-thujene (triangle pointing up), \( p \)-cymene (diamond), and \( \gamma \)-terpinene (inverted triangle) from sabinene (solid circle) in a methanogenic enrichment culture (A) and after the first transfer in the enrichment procession (B).

as major compounds by retention time analysis, coinjection with the authentic compound (\( p \)-cymene), and GC-MS analysis. In an incubation period of 74 days, 84% of sabinene was depleted, and more than 90% of the loss was balanced by the formation of \( p \)-cymene, \( \gamma \)-terpinene, and \( \alpha \)-thujene (61%, 11%, and 4% of sabinene provided, respectively). The gas atmosphere of the culture contained finally more than 20% vol/vol methane.

The first transfer involved an inoculation size of 5% vol/vol and a 10-fold increase in the sabinene concentration (400 \( \mu \)l in 20 ml of HMN with 200 ml of mineral medium). Sabinene transformation started without a lag phase. After 134 days of incubation, a mixture of \( p \)-cymene (41%), \( \gamma \)-terpinene (18%), and \( \alpha \)-thujene (7.4%) had been formed. The content of \( \gamma \)-terpinene exhibited a maximum at day 106 (Figure 1B). The gas volume formed during the incubation at 28°C was 54.2 ml, and the methane content approached 30% vol/vol. After 134 days of incubation, the culture was placed at 8°C without stirring. An analysis of the culture after 429 days in the cold room revealed an increase in \( p \)-cymene (58%) accompanied by a decrease of \( \gamma \)-terpinene (2.6%), and \( \alpha \)-thujene (4.4%) (Figure 2, spectrum A). These changes suggested a transient accumulation of \( \gamma \)-terpinene and \( \alpha \)-thujene during the transformation of sabinene into \( p \)-cymene.

Heat inactivation caused a strong inhibition of sabinene transformation. After an incubation time of 105 days, the sabinene content was reduced by 85% in the active culture,
FIGURE 2 GC analyses of the monoterpenes present in the organic phase of sabinene cultures after incubation. (A) active culture of the first transfer; (B) pasteurized culture of the first transfer; (C, D) active cultures of the third transfer; (E) chemical control without inoculum of the third transfer.
but only by 25% in the heat-treated culture. The chemical control without inoculum had a loss of 10%. The transformation product pattern in the heat-treated culture shifted toward menthadienes: α-thujene, α-terpinene, p-cymene, γ-terpinene, and α-terpineol were observed (Figure 2, spectrum B). Hence the p-cymene-synthesizing catalyst was more heat-sensitive than other monoterpene-transforming enzymes.

The second transfer was used to increase the sabinene amount slightly (0.5 ml of sabinene in 20 ml of HMN, 200 ml of methanogenic medium) and to vary the inoculation size (1 and 5% vol/vol). After an incubation time of 206 days, more than 90% of the sabinene content was gone in active cultures, whereas only 20% had disappeared in control experiments without or with autoclaved inocula. Over 70% of the sabinene used by active cultures was recovered as transformation products. The active culture with a large inoculum (5% vol/vol) exhibited a fourfold higher gas formation and contained more methane in the gas phase than did the active culture with a small inoculum (Table 1). This observation questioned a catabolic use of the aromatization reaction by the microorganisms.

A third transfer (5% vol/vol inoculum) was performed in duplicate. After 156 days of incubation, analysis of the organic phases yielded the same product pattern as seen before in the enrichment culture, and the first and second transfers: p-cymene, γ-terpinene, and α-thujene were major products (Figure 2, spectra C and D). Methane formation occurred in both cultures, accumulating to more than 2 mmol methane (Table 1).

**α-Phellandrene**

α-Phellandrene disappeared in the enrichment culture. The depletion coincided with the formation of p-cymene (Figure 3). In contrast to sabinene, the p-cymene synthesizing activity could not be transferred efficiently. The first transfer yielded a complete consumption of α-phellandrene, and α-terpinene, p-cymene, and β-phellandrene were found as transformation products in a ratio of 3:1:3. No biotransformation was performed by a heat-inactivated inoculum. To overcome the failure of p-cymene formation, the first transfer was repeated. This time, it was performed with an amendment of glucose (20 mM) or with a mixture of fatty acids in the medium. These carbon sources enabled an intensive methanogenesis. Partial or complete α-phellandrene depletion took place without the formation of p-cymene or other hydrocarbons.

**FIGURE 3** Formation of α-terpinene (triangle pointing up), p-cymene (diamond), and γ-terpinene (inverted triangle) in a methanogenic enrichment culture on α-phellandrene (solid circle).
**TABLE 1** Methane formation in transfer cultures

<table>
<thead>
<tr>
<th>Culture</th>
<th>Transfer no.</th>
<th>Inoculum size (% vol/vol)</th>
<th>Monoterpene provided (μmol)</th>
<th>Measured CH$_4$ formation (μmol)</th>
<th>Theoretical CH$_4$ formation (μmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>From acetate and cysteine$^a$</td>
</tr>
<tr>
<td>Sabinene</td>
<td>2</td>
<td>1</td>
<td>3068</td>
<td>529</td>
<td>650</td>
</tr>
<tr>
<td>Sabinene</td>
<td>2</td>
<td>5</td>
<td>3068</td>
<td>3511</td>
<td>650</td>
</tr>
<tr>
<td>Sabinene</td>
<td>3</td>
<td>5</td>
<td>1534</td>
<td>2136</td>
<td>325</td>
</tr>
<tr>
<td>Sabinene</td>
<td>3</td>
<td>5</td>
<td>1534</td>
<td>2438</td>
<td>325</td>
</tr>
<tr>
<td>α-Pinene</td>
<td>1</td>
<td>5</td>
<td>125</td>
<td>635</td>
<td>325</td>
</tr>
<tr>
<td>α-Pinene</td>
<td>1</td>
<td>5</td>
<td>624</td>
<td>2960</td>
<td>325</td>
</tr>
<tr>
<td>2-Carene</td>
<td>1</td>
<td>5</td>
<td>123</td>
<td>1050</td>
<td>325</td>
</tr>
<tr>
<td>2-Carene</td>
<td>1</td>
<td>5</td>
<td>614</td>
<td>3614</td>
<td>325</td>
</tr>
</tbody>
</table>

$^a$Acetate (2 mM) and cysteine (1 mM) were provided with the medium. Methane formation from these carbon sources was calculated based on the reactions C$_2$H$_5$O$_2$ → CH$_4$ + CO$_2$ and 4 C$_3$H$_7$NO$_2$S + 6 H$_2$O → 5 CH$_4$ + 7 CO$_2$ + 4 NH$_3$ + 4 H$_2$S.

$^b$The difference between monoterpenes supplied and recovered was defined as total hydrocarbon consumption and was related to a theoretical maximum methane formation according to the reaction C$_{10}$H$_{16}$ + 6 H$_2$O → 7 CH$_4$ + 3 CO$_2$. 
2-Carene and α-Pinene

The bicyclic monoterpenes 2-carene and α-pinene were transformed in the enrichment cultures to 70% and 98%, respectively, after incubation for 135 days. No transformation products were observed. The gas phases contained more than 40% vol/vol methane. The first transfer of the enrichment cultures involved an inoculum of 5% vol/vol and two different monoterpene concentrations (20 and 100 µL in the 156-ml serum flask system). 2-Carene and α-pinene disappeared in active cultures but not in heat-inactivated and chemical control experiments. Measurements of gas formation (Figure 4) and methane content (Table 1) showed that methane was formed. The major carbon sources for methanogenesis were the inoculum, acetate and cysteine in the medium, and different amounts of monoterpene. The correlation of methanogenesis with the amount of monoterpene supplied identified α-pinene and 2-carene as substrates for methanogenic populations.

Discussion

Anoxic habitats have been identified as significant sources of biogenic toluene (Jüttner and Henatsch 1986) because of the activity of fermenting microorganisms that transform phenyl-substituted acids into toluene (Fischer-Romero et al. 1996). Here we report a second biogenic source of aromatic hydrocarbons, the formation of p-cymene from monoterpenes in methanogenic enrichment cultures. So far, aromatization has been reported only for oxygen-functionalized alicyclic compounds by aerobic and denitrifying bacteria (Blakley 1974; Kaneda 1974; Dangel et al. 1988). In plants, the biosynthesis of p-cymene is considered to be an aromatization reaction of γ-terpinene (Poulose and Croteau 1978). The transient formation of α-thujene and γ-terpinene during sabinene consumption suggests a similar pathway involving an allyllic rearrangement of sabinene to α-thujene and a ring-opening reaction of the reactive cyclopropane ring (Figure 5).

A thermodynamic analysis of the aromatization reaction of menthadienes to p-cymene and molecular hydrogen shows an accompanying release of free energy:

\[ \alpha\text{-Phellandrene} \rightarrow p\text{-cymene} + \text{H}_2 \quad \Delta G^\circ = -18 \text{ kJ mol}^{-1} \]

\[ \text{Sabinene} \rightarrow p\text{-cymene} + \text{H}_2 \quad \Delta G^\circ = -129.8 \text{ kJ mol}^{-1} \]
FIGURE 5 Monoterpenes utilized as carbon source and proposed pathway of $p$-cymene formation.

The low hydrogen concentration present in methanogenic habitats causes a free energy increase of $-22.8$ to $-28.6 \text{ kJ mol}^{-1}$ at hydrogen concentrations of 100 to 10 $\mu\text{l/l}$, respectively. Hence, the $p$-cymene formation from monoterpenes presents an opportunity for bacteria to perform the aromatization reaction catabolically. To support this reaction, we used a mineral medium with carbonate as major electron acceptor that enables fermenting and methanogenic growth. However, in second-transfer cultures containing sabinene, we observed a correlation of methanogenesis with the inoculum size, an indication for a cometabolic nature of the aromatization reaction. Still, the fact that the physiological capacity of $p$-cymene formation by monoterpen oxidation was successfully subcultured three times with sabinene as monoterpen supports a contribution of the reaction to the microbial energy metabolism.

Björkqvist et al. (1998) observed $p$-cymene as a major volatil nonmethanehydrocarbon in biogas that originated from household solid waste in a two-step anaerobic digestion system with a fermentation tank and a packed-column methanogenic reactor. Gas analyses of the different process stages indicated a decreasing limonene content from the first stage, air in a solid-waste drum sieve, to the biogas. $p$-Cymene was already observed in the gas of the fermentation tank. The authors theorized that the unexpected appearance of $70 \mu\text{l/l}$ $p$-cymene in the biogas (71% vol/vol methane, 27% vol/vol carbon dioxide) might be explained by nonbiological rearrangement and dehydrogenation of limonene and other monoterpenes at the low pH in the fermentation tank (pH 4–6). Now, our results explain these observations as a biological contribution: Anaerobic microorganisms can form $p$-cymene from monoterpenes in fermenting and methanogenic reactors.

A biological catalysis of the aromatization of cyclohexadiene systems may also contribute to the occurrence of aromatic biomarker molecules, e.g., monoaromatic steroids and hopanoids (Peters and Moldovan 1993). Clearly, further research is desired on the biological aromatization reaction to identify the organisms and the mechanisms involved and to assess diagenetic biological and catagenic chemical contributions to the formation of aromatic biomarker molecules.

Hydrocarbons that support methanogenic communities as carbon source and electron donor include, according to earlier laboratory studies, the alkenes hexadecene and squalene (Schink 1985) and the aromatic compounds benzene (Weiner and Lovley 1998), toluene, and o-xylene (Edwards and Grbic-Galic 1994). Now our observation of monoterpe
transformation in active methanogenic enrichment cultures points toward the existence of microbial populations with the capacity to utilize monoterpenes via bicarbonate respiration.

References


