Synthesis of Glycols by Microbial Transformation of Some Monocyclic Terpenes

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The transformation of three monocyclic terpenes by three soil microorganisms have been studied. The organisms were isolated on, and grew rapidly in, mineral salts medium containing the appropriate terpene substrates as sole carbon sources. These organisms belong to the class Fungi Imperfecti, and two of them have been tentatively identified as Cladosporium species. A Cladosporium species designated T1 was isolated from terpene-soaked soil, using 1-methene as the sole source of carbon. The major catabolic product isolated from the growth medium of this organism was found to be a cyclic 1,2-diol identified as trans-p-methane-1,2-diol. A similar but biochemically distinct Cladosporium sp. designated T2 was isolated on p-limonene. After growth, the medium of this organism contained 1.5 g/liter of the analogous product, trans-limonene-1,2-diol. Minor quantities of the corresponding cis-1,2-diol were also isolated. The third organism, designated as laboratory culture T3, was isolated on 3-methene and yielded a diol identified as trans-p-methane-3,4-diol. From these results it is concluded that the formation of diols is a common intermediate in the fungal metabolism of monocyclic terpenes.

The last few years have produced notable advances in our understanding of the biological transformation of various classes of compounds, namely steroids, alkaloids, and various types of aliphatic and aromatic compounds (3, 5, 6, 7, 14, 17). Interest in microbial conversion has become increasingly significant in research and industry since the pioneering work of Peterson and Murray (15) on the hydroxylation of steroids. Since then, a large volume of literature has appeared on the microbial transformations of aliphatic and aromatic hydrocarbons and their derivatives. Very little work has been reported, however, on the microbial transformations of simple alicyclic hydrocarbons.

Among the most common alicyclic hydrocarbons from natural sources are the terpenes and their related oxidized forms, the terpenoids. Microbial attack of the terpenoids, particularly camphor, has been extensively studied by Gunsalus and his co-workers (2). Bhattacharyya and co-workers have recently reported the transformation of various types of monocyclic and bicyclic monoterpenes by Aspergillus niger and a pseudomonad (1, 4, 16). Except for this terpene hydrocarbon work, only passing reference is made in the literature to microbial attack of alicyclic hydrocarbons. Most of these refer either to the difficulty or impossibility of isolation of microbes on such hydrocarbons. In view of this, we found it quite interesting when we began to isolate large numbers of bacterial and fungal organisms capable of excellent growth on one or more of some seven alicyclics. We would like to describe here three soil microbes which attack three monocyclic terpenes in the hope that it will throw some light on the mode of attack of the hydrocarbons. (This work was presented in part at the 69th Annual Meeting for The American Society for Microbiology, Miami, Fla., in 1969.)

MATERIALS AND METHODS

Analytical methods. Melting points determined on a capillary melting point apparatus (Thomas Hoover) were uncorrected. Ultraviolet absorption spectra were determined with a Spectronic 505 recording spectrophotometer (Bausch and Lomb) using methanol as solvent. The infrared spectra were recorded on a Perkin-Elmer Grating Infracord spectrophotometer (model 337 or 521). Samples were run either in liquid films, solid KBr discs, Nujol mull, or...
in solutions of carbon tetrachloride. The NMR spectra were recorded in CDCl₃, D₂O, and C₆D₆ with a model A-60-A spectrophotometer (Varian Ass.) using tetramethylsilane as the internal reference standard. Mass spectra of all samples were taken on a CEC-103-C mass spectrometer operating at an ionization voltage of 70 V, employing a heated glass inlet system at 250 C.

Methyl esters of acids were prepared by using diazomethane. Diazomethane was prepared from diazald by the method of Moore and Reed (12).

Thin-layer chromatography (TLC) was carried out on glass plates (20 by 20 cm) using plain silica gel as the adsorbent layer. The developing solvent for the neutral compounds was a mixture of n-hexane and ethyl acetate in proportions of 8:2 (Method A). For acidic compounds, a mixture of methyl ethyl ketone, methanol, and ammonia was used in the ratio of 3:6:1 (Method B). For detecting the separated compounds, the plates were sprayed with ammonium molybdate reagent and heated for 10 min at 110 C. In other instances, a 0.4% solution of 2,4-dinitrophenyl hydrazine in 2.0 N HCl was used to detect any carbonyl compounds. Gas-liquid chromatography (GLC) was carried out either on a Victoreen gas chromatograph or a Perkin-Elmer instrument (model 820) at a temperature of 90 C. Helium gas was used as the carrier gas (3 liters/hour), and a column with Carbowax 4000 as stationary phase on Chromosorb W support was employed.

Terpene hydrocarbons. The terpenes chosen for our work were 1-menthene, 3-menthene, and D-limonene. These were obtained from the Newport division of Tenneco Chemicals, Inc., at Pensacola, Fla.. Before using them as substrates, the oxidized impurities were removed by passing the liquid hydrocarbon through a column of either silica gel or alumina. The eluted terpene had a purity of 99.95% determined by vapor phase chromatography.

Organisms. Three biochemically distinct microorganisms were isolated from soil samples which had previous contact with terpenes. These three microbes can use the three terpene hydrocarbons as sole source of carbon. The soil samples were plated on mineral salts medium (Table 1) containing 0.01% yeast extract. The pH of the media were adjusted in increments between 5.6 to 6.4. Terpenes were applied to these plates by a modification of the Gibson wick technique, where only a small wick surface is exposed, limiting the volatilization of the terpene (D. T. Gibson, 1967, personal communication). According to this technique, a piece of wool yarn is threaded through a 5.0-cm piece of a glass tube (0.5-cm thick) and clipped so that only 2 to 4 cm of wick is exposed. The wick is then soaked with terpene which volatilizes slowly due to the small surface exposure.

All isolates were subcultured twice to demonstrate purity and substantiate terpene utilization. These microorganisms belong to the class Fungi Imperfecti and will be trivially designated as T₁, T₂, and T₃. Two of them, T₁ and T₂, have been tentatively identified as Cladosporium species.

Fermentation. The organism was grown in shaker flasks at a suboptimal temperature of 30 C to minimize losses of the hydrocarbon as well as of some transformation products because of evaporation. A fresh inoculum was prepared by transferring loopfuls of the culture from the slants into 500-ml shaker flasks containing 100 ml of sterile mineral salts medium. The hydrocarbon was normally added at levels of 1% by volume every day over the 4 days of the fermentation period. Samples of 10 ml of this inoculum were used to inoculate 100 ml of identical medium containing 1% of the terpene hydrocarbon. In all cases, two sets of controls were included along with the experimental flasks, one without the microbe and the other without the terpene.

Table 1. Composition of mineral salts medium

| Reagent          | Quantity  |
|------------------|-----------|
| MgSO₄             | 0.2 g/liter |
| FeCl₃             | 0.01 g/liter |
| Trace elements    | 1.0 ml/liter |
| H₂BO₃, 2.9 g/liter |          |
| MnCl₂, 4H₂O, 1.8 g/liter |         |
| ZnSO₄, 7H₂O, 0.2 g/liter |         |
| CuSO₄, 5H₂O, 0.1 g/liter |         |
| (NH₄)₂MoO₄, 4H₂O, 0.02 g/liter |         |
| FeCl₃, 0.01 g/liter |                 |
| Phosphate buffer (pH 5.4) 1.0 M | 50.0 ml/liter |
| Yeast extract     | 0.01%     |
| Urea              | 0.75%     |

Extraction of transformation products. At the end of the fermentation period, the broth from the shaken flasks was pooled and centrifuged at 10,000 x g for 5 to 10 min at a temperature of 5 C. The clear supernatant fluid was acidified with 6 N HCl to pH 2.0 and extracted twice with half the volume of diethyl ether. The combined ether extracts were dried over anhydrous sodium sulfate, and solvent was evaporated under a slow stream of nitrogen gas. The residue after removal of solvent was examined for fermentation products by GLC and TLC and compared with the residues obtained from the proper controls.

RESULTS

Microbial transformation of 1-menthene by T₁. The first Cladosporium, called T₁, was originally isolated using 1-menthene as the sole source of carbon. However, when this organism was grown in presence of a cosubstrate such as glucose plus 1-menthene, some crystalline material appeared in the ether extract of the fermentation broth. The crystals could easily be separated by filtration, and the last traces of other impurities were removed by washing the solids with cold petroleum ether. The yield of the crystals was approximately 200.0 mg/liter.
of the fermentation broth. Thin-layer chromatography of these crystals showed the presence of a single spot in solvent A \((R_f = 0.14)\). Duplicate analysis for carbon and hydrogen gave: carbon, 69.47%, 69.54%; hydrogen, 11.14%, 11.34%. Assuming that the compound did not contain any nitrogen or halogens, the oxygen content was calculated to be: oxygen, 19.39%, 19.12%. The empirical formula consistent with this composition is \(C_{10}H_{16}O\). Other analytical data on this compound are presented in Table 2. The infrared spectra and GLC data indicated that the fermentation product contained more than one hydroxyl function and an isopropyl group in the molecule. The NMR data indicated that it contained two hydroxyl groups, one of which was secondary and the other tertiary in nature.

Further insight into the structure of the fermentation product was provided by the mass spectrum (Fig. 1). The mass spectral molecular weight was found to be 172. Two prominent peaks were observed at 154 (M-H\(_2\)O) and at 136 (M-2H\(_2\)O), respectively, demonstrating the presence of two hydroxyl groups in the molecule. From all these data it can be concluded that the fermentation product from 1-menthene was 1,2-dihydroxy \(p\)-menthane.

**Trans relationship of the two hydroxyl groups in the fermentation product of 1-menthene.** To establish the relative spatial disposition of the two hydroxyl groups in 1,2-dihydroxy-\(p\)-menthane obtained from the fermentation extract, infrared analysis of the compound in chloroform was performed at different dilutions. It can be seen from Fig. 2 that in dilute solutions the main hydroxyl peak of the diol was shifted toward shorter wave length, and the ratio of the polyhydroxy peak to the monohydroxy peak decreased. This is typical of intermolecular hydrogen bonding, and intermolecular hydrogen bonding is more prevalent in trans-1,2-diols than cis-1,2-diols (11). Hence, a trans-diol structure was assigned to the fermentation product.

**Microbial transformation of \(p\)-limonene by \(T_1\).** The second *Cladosporium*, called \(T_1\), was isolated using \(p\)-limonene as the sole source of carbon. When this organism was grown in regular mineral salts medium with \(p\)-limonene as the sole source of carbon, the fermentation residue showed the presence of two compounds, one major at 1.5 g/liter and the other in minor quantities at <0.2 g/liter. However, when the crude fermentation extract was left in the refrigerator for a few hours, the major component separated as needle-shaped crystals. The crystals were removed by filtration and thoroughly washed with cold \(n\)-hexane to remove the last traces of impurities.

The physicochemical properties of the major product are presented in Table 3. Infrared bands at 3,400 cm\(^{-1}\), 3,620 cm\(^{-1}\), and 3,630 cm\(^{-1}\) are indicative of monomeric and polymeric hydroxyl groups. Retention time of 18.0 min in the GLC suggested the presence of the hydroxyl groups. The band at 1,640 cm\(^{-1}\) suggested the presence of an isopropyl group in the molecule. The NMR spectra indicated the presence of two hydroxyl groups, one secondary and the other tertiary. It also indicated the presence of an isopropenyl group in the molecule. The mass spectral fragments at 152 corresponded to M-H\(_2\)O (170-18) and the fragment at 134 corresponded to M-2H\(_2\)O. All these data are consistent with the proposed structure for the major fermentation product from \(p\)-limonene. Further proof of the structure of this product came from the isolation of carvone after the following series of reactions:

\[
\text{HO} \quad \text{HO} \\
\begin{array}{c}
\text{OH} \quad \text{OH} \\
\end{array}
\rightarrow \quad \text{t-butyl chromate} \\
\rightarrow \quad \text{oxalic acid} \\
\rightarrow \quad \text{(carvone)}
\]

### Table 2. Properties of the transformation product of 1-menthene formed by *Cladosporium* sp.

| Property       | Determination          |
|----------------|------------------------|
| Melting point  | 72 to 80°C             |
| GLC/Carbowax 4000 (190°C) | Rt = 18 min |
| IR spectrum   | Bands at 3,400, 1,380 (split), 1,125 cm\(^{-1}\) |
| NMR spectrum  | Peaks at 8.7 \(\tau\) (3H), 8.3 \(\tau\) (1H), 6.3 \(\tau\) (1H), 9.05 \(\tau\) (doublet, 6H) |

(1-Menthene) (trans-1,2-Dihydroxy-\(p\)-menthane)
An authentic sample of carvone and the product from the above series of reactions had identical $R_f$ values in TLC and identical retention times in GLC.

By using the dilution technique in the infrared analysis, it was shown that the two hydroxyl functions in this diol were in trans relationship to one another.

The minor product was isolated from the same fermentation extract by column chromatography over alumina. The column was eluted sequentially with 100 ml each of $n$-hexane (fraction 1), $n$-hexane: benzene (1:1) (fraction 2), benzene (fraction 3), benzene: ether (8:2) (fraction 4), benzene: ether (1:1) (fraction 5), and ether (fraction 6), respectively. The minor product appeared almost exclusively in fraction 4, whereas the major product appeared in fractions 5 and 6. The final purification of the
minor product was achieved by preparative GLC.

The analytical data on this compound were very similar to those of *trans*-limonene glycol (the major product) except for two minor differences; a lower retention time of GLC (14.0 min) and some dissolving of the infrared pattern in the fingerprint region of the spectra. These discrepancies were explained by the fact that the two hydroxyl groups in the limonene glycol were in cis relationship to one another. This was proved by infrared spectroscopy at three different concentrations (Fig. 3). As can be seen from this figure, there was no shifting of the major hydroxyl band and there was no change in the ratio of the polyhydroxy peaks to the monohydroxy peaks.

**Transformation product of 3-menthene by T₄.** When a similar imperfect fungus, T₄, was grown on 3-menthene as the sole source of carbon, it resulted in the accumulation of a major metabolite in yields of about 0.5 g/liter. This was isolated by passing the fermentation residue through a column of silica gel and eluting it with (i) *n*-hexane, (ii) *n*-hexane:benzene (1:1), (iii) benzene, (iv) benzene:ether (1:1), and (v) ether. Most of the unconverted 3-menthene was eluted in the *n*-hexane fraction, and the fermentation product was eluted in the benzene:ether fraction. Like the other transformation products, this compound also proved to be a cyclic vicinal glycol. The structural data for this compound are presented in Table 4. The infrared and the NMR data are consistent with the proposed structure for the diol. Further evidence to the above structure of the diol was obtained from the conversion of

**Table 3. Properties of the major transformation product of d-limonene formed by Cladosporium sp.**

| Property             | Determination             |
|----------------------|---------------------------|
| Melting point        | 65 to 68 °C               |
| GLC/Carbowax 4000 (185 °C) | Rt = 18.0 min           |
| IR spectrum          | Bands at 3,400, 3,620, 3,630, 3,080, 1,640 cm⁻¹ |
| NMR spectrum         | Peaks at 8.8 τ (3H), 8.6 τ (2H), 8.3 τ (3H), 6.4 τ (1H) 5.4 τ (2H) |
| Mass spectrum        | Major fragments (m/e) 152,134 |

**Table 4. Properties of the transformation product of 3-menthene formed by a member of the Fungi Imperfecti**

| Property             | Determination             |
|----------------------|---------------------------|
| Melting point        | 62 °C                     |
| GLC/Carbowax 4000 (190 °C) | Rt = 10 min             |
| IR spectrum          | Bands at 3,600, 3,400, 1,380 cm⁻¹ |
| NMR spectrum         | Peaks at 9.1 τ (doublet, 6H), 9.0 τ (3H), 5.9 τ (doublet, 1H), 6.7 τ (1H), 6.5 τ (1H), splits at 6.2 τ (1H) |
the diol to menthone via a pinacol pinacolone
type of transformation:

\[
\begin{align*}
\text{OH} & \quad \text{conc. } \text{H}_2\text{SO}_4 \quad \text{OC} \\
\text{OH} & \quad \text{K} \\
\end{align*}
\]

An authentic sample of DL-menthone and the
product obtained from the diol have identical
\(R_t\) values. Infrared analysis at two dilutions
again showed that the two hydroxyl groups
were in trans relationship to one another.

**DISCUSSION**

In the present study we have isolated three
different cyclic vicinal glycols as major mi-
crobial transformation products of three differ-
ently substituted cycloalkanes. In each case,
the two hydroxyl functions are trans oriented
with respect to one another. Only in the case of
\(\alpha\)-limonene have we observed the formation
of both cis- and trans-diols. Whether the cis-diol
is truly a fermentation product or a chemically
isomerized product of the trans-diol during the
work-up procedure is difficult to say with the
available data. However, in view of the iso-
lation of three species in respectable quantities,
we are inclined to consider trans as the cata-
bolic form. The initial step in the formation of
1,2-diol probably proceeds through the forma-
tion of an epoxide and the hydrolysis of the
same.

These trans diols could either be active inter-
mediates or end products of a minor path-
way in the transformation of the hydrocarbon.
The results of some preliminary growth experi-
ments indicated that the organism \(T\), can grow
more readily on trans-limonene glycol than it
can on \(\alpha\)-limonene alone (B. B. Mukherjee
and J. D. Hill, 1969, unpublished data). This is
a strong indication that these glycols are not the
end products of the degradative pathway of the
terpene. Isolation of some other intermediates
would throw more light on the glycol pathway
of these microorganisms.

In contrast to the trans glycols reported here,
in the aromatic series, Gibson and co-workers
recently demonstrated the formation of a cis-
glycol from benzene by *Pseudomonas putida*
(8). However, in mammalian system, Jerina
and co-workers (9) have reported the forma-
tion of trans 1,2-dihydro-1,2-dihydroxy naph-
thalene from naphthalene. In 1961, Foster and
Ooyama (13) predicted the formation of trans-
glycols in the alicyclic series based on an anal-
ogy to the trans-diol formed from benzene in
rabbits (13). They were unable to demonstrate
this, finding instead a surprising series of satu-
rated ketones. Considering the fact that the
aromatic molecule is planar while the alicycles
are not, the isolation of the trans-glycols in our
case is consistent with the present state of
knowledge.

Diol formation as an early intermediate is
well described in the case of open chain al-
kanes. It has been demonstrated that epoxi-
dation and diol formation are initial steps in
the biodegradation of alkenes (10). Thus it
appears that the microbial attack upon terpene
cycloalkenes, like the open chain alkenes, can
proceed via an initial attack at the double bond
with the formation of a vicinal glycol. It should
be even more evident from the microbial
growth and the quantities of the intermediates
accumulated that the alicyclic hydrocarbons
are not necessarily resistant to microbial at-
attack.

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