STREPTOMYCES sp: A SOURCE OF ODOROUS SUBSTANCES IN POTABLE WATER

JOHN D. WEETE*
Department of Botany and Microbiology, Auburn University, Auburn, AL 36830 U.S.A.
WEN Y. HUANG and JOHN L. LASETER
Center for Bioorganic Studies, University of New Orleans, New Orleans, LA 70122 U.S.A.

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Abstract. Growth medium containing an aquatic actinomycete *Streptomyces* sp. was analyzed by gas-liquid chromatography and mass spectrometry for the purpose of identifying substances that may represent potential odorous contaminants of water. Twenty-one odorous and nonodorous compounds were identified. Phenyl, C_2, and C_4 structures were predominant. Free fatty acids from C_{13} to C_{16} were detected and in some cases accompanied by their ethyl (C_{16}) and butyl (C_{15} and C_{16}) esters. The long-chain acids and esters had no detectable odor. Although goesmin, an earthy smelling substance with a low odor threshold, was detected, butyric acid was the principal metabolite under the growth conditions employed and represented the greatest potential as an odorous water pollutant under natural conditions.

1. Introduction

The occurrence of objectionable odors of biological origin in water used for drinking and commercial purposes is well documented in certain regions of the United States (27,28,36) and other parts of the world (1,17,18,23,26). These odors have been described as earthy, musty, wood-like, potato-bin, camphoric, leathery, fishy, and others, suggesting that they are due to a variety of chemical substances. Actinomycetes are among the organisms held responsible for these odors. Although several volatile actinomycete metabolites have been identified, relatively few have been associated with a particular odor problem. The most common of these, particularly in the southern United States, is the earthy odor. The chemical agent responsible for this odor is geosmin (*trans*-1,10-dimethyl-*trans*-9-decalol) (7) which is a product of certain aquatic actinomycetes (3,7,17,18,24) and blue-green algae (24,30,32). The chemical agent responsible for a severe musty odor episode in the Cedar River near Cedar Rapids, Iowa (U.S.A.) (4,5), originally called mucidone, was identified as 6-ethyl-3-isobutyl-2-pyrone by UV, IR, NMR, and MS (33). More recently, 2-isopropyl-3-methoxy-pyrazine was identified as a potato-like odor from a geosmin producing *Streptomyces* species (12).

Other volatile metabolites of actinomycetes including isoamylamine, isobutylamine, valeric and isovaleric acids, ß-hydroxybutyric acid, and isovaleraldehyde were identified as products of *Streptomyces* species by GLC (32) and 5-methyl-3-heptanone was identified by IR, NMR, and MS (14). A series of low molecular weight C_2 to C_4

*hom correspondence should be addressed.

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alcohols, acids, and esters (6) and H_2S (2) were identified in extracts of *S. odorifer* culture media. 2-Methyl-isoborneol, a menthol–camphor smelling substance, was identified by IR and GLC–MS techniques as a product of several *Streptomyces* species (2,8,17,25,37). Gerber (11) detected a series of C_9 to C_{12} lactones in extracts of *S. odorifer* IMRV 3344. Five sequiterpenoid mono-alcohols were detected in *Streptomyces* sp. B-7 medium extracts, two of which were identified as the odorous cadin-4-ene-1-ol (9) and the faintly odorous selina-4(14),7(11)-diene-9-ol (10). 1-Phenyl-2-propanone and 2-phenylethanol were identified as odorous but not badly smelling products of a strain resembling *S. platensis* (19).

Based on data from the literature and our previous study (37) it is evident that aquatic actinomycetes produce a large variety of odorous substances and that the production of these compounds is influenced by the composition of the growth medium. This study was initiated to characterize additional natural products of *Streptomyces* species that may represent potential water pollutants.

2. Materials and Methods

2.1. MATERIALS

The following chemicals used as reference standards were purchased from Eastman Chemicals (Rochester, NY): diphenylamine, 2-phenylethanol, 2,5-hexanediol, 4-*n*-butylphenol, benzothiazole, 2-*tert*-butylphenol. The following were synthesized in our laboratory: *n*-pentyl butyrate, benzyl acetate, *ß*-phenylethyl acetate, and *n*-butyl palmitate. Gas chromatography (GC) supplies were purchased from Applied Science Laboratories.

2.2. CULTURE CONDITIONS

Inocula of *Streptomyces* sp. 33L isolated on Actinomycete Isolation Agar (Difco) from mud of Lake Ogletree, Lee County, Alabama, were grown in 500 ml flasks containing 150 ml medium consisting of succinic acid (10 g l^{-1}), yeast extract (10 g l^{-1}), and 1 pellet NaOH l^{-1} water. After two days on a reciprocal shaker at room temperature, the inocolum was added to a 2.5 l Fernbach flask containing 1 l of the following medium: succinic acid (20 g l^{-1}), Bacto-soytone (10 g l^{-1}), Bacto-peptone (5 g l^{-1}), NaCl (5 g l^{-1}), and $CaCO_3$ (2 g l^{-1}) in distilled water at about pH 7.5. The cultures were incubated for 5 to 7 days at 25°C on a rotary shaker.

2.3. EXTRACTION AND SAMPLE PREPARATION

Media containing mycelium from three Fernbach flasks were combined and washed three times with diethyl ether (1.5 l ether/3 l medium). The combined extracts were concentrated under a stream of N_2 gas, dried over anhydrous Na_2SO_4, and stored at 5°C prior to analysis. Initial fractionation of the crude extract was accomplished by placing a portion of the extract on a 15 cm × 3 cm silica gel (60 to 200 mesh) column

and washing it sequentially with the following solvents: 400 ml petroleum ether (PE) collected in 100 ml and 300 ml fractions, 300 ml 2% diethyl ether in PE (EPE), 10% EPE, 25% EPE, 50% EPE, 100% diethyl ether, ethyl acetate, and 600 ml methanol.

Autoclaved uninoculated medium was extracted as described above and the extract analyzed as described below to distinguish between actinomycete products and components of the medium. The 25% EPE fraction was screened initially on a Hewlett–Packard (HP) 5710A gas chromatograph equipped with a variable all glass splitter and 3 m × 2 mm stainless steel column packed with 12% diethyleneglycol succinate (DEGS). A portion of the column effluent was vented into the atmosphere so that the odor of each component could be determined. Mass spectra were obtained using a computerized (HP 5933A data system) HP 5882A mass spectrometer coupled with a HP 5708A gas chromotograph (GC) equipped with a 25 m × 0.3 mm i.d. glass capillary column coated with SE–52, or the above described packed column. The following operating conditions were used: Ionization voltage, 70 eV; source temperature, 150°C; injection port and interface line temperature, 250°C; scanning mass range, 40 to 300; scan speed 208 a.m.u. s^{-1}; carrier gas (He) flow rate, 3 ml min^{-1}; G.C. oven temperature, 60 to 240°C at 4°C min^{-1}. Due to the extremely short retention times of sample components in EPE 25% extract on glass capillary column, a separate stainless steel column packed with 12% DEGS phase was used for the GC–MS analysis of this fraction. The conditions for the analysis were identical except the oven temperature ranged between 25 to 190°C and the flowrate was 40 ml min^{-1}. Identifications were made on the basis of comparison of mass spectra and GLC retention data of sample compounds with authentic standards when available. Tentative structural assignments are made for substances for which no reference compounds were available.

3. Results and Discussion

Geosmin is generally considered to be the product of aquatic actinomycetes and blue–green algae and is responsible for the earthy odor periodically occurring in freshwater supplies (13,30). It is a major product of the actinomycete isolate used in this study, *Streptomyces* sp. 33L, which was isolated from a local reservoir, used as a municipal drinking water source, that experiences annual earthy odor episodes. Geosmin was produced when the fungus was grown in culture (37) and was detected in the present study (data not given).

It is apparent that the unpleasant odor of an aquatic actinomycete culture medium is not due solely to geosmin; in fact, the earthy odor may be partially or entirely masked by other odorous substances that may vary with the culture medium (12). The ether extract of medium in which *Streptomyces* sp. 33L had grown had a strong unpleasant odor. The extract was fractionated on a silica gel column and, with the exception of the 100% PE (100 ml fraction), all fractions contained odorous substances. The 100% PE (300 ml fraction) and 25% EPE fraction contained the

most intense odors and, after further fractionations, many of the components were characterized by gas chromatography and mass spectrometry. Compounds of these two fractions generally have one or two of four common structural features. Many of the esters, acids, and alcohols contained a branched (one compound) or non-branched C_4 structure; both butyric acid and 1-butanol were detected. Butyric acid was the most abundant substance detected and had the strongest and most unpleasant (rancid) odor. The mass spectrum and retention time of the sample compound matched closely with that of an authentic standard, with *m/e* 60 $[CH_2COOH_2]^+$ being the base peak. In addition, the 100% PE fraction contained a homologous series of free fatty acids ranging in chain-length from C_{13} to C_{16} (Table I). Mass spectra of these acids also contained *m/e* 60 as the base or very prominent fragment ion as well as M–OH and fragment series typical of saturated hydrocarbon chains (31). The fatty acids were typical of those for most organisms (15) and mycelium of other *Streptomyces* species (20).

In addition to the free fatty acids, ethyl hexadecanoate and the butyl esters of penta- and hexadecanoic acids were detected (Table I). The mass spectra of the sample ethyl ester was similar to that previously published (21), except *m/e* 88 was not the base peak. Naturally occurring methyl and ethyl fatty acid esters have been reported for a variety of organisms, but they are not widely occurring (21,22,35,38). Butyl esters of fatty acids, on the other hand, to our knowledge have not been reported as naturally occurring microbial products. The mass spectrum and GLC retention time of a synthetic C_{16} butyl ester matched closely with that of sample compound number 87 (Figure 1). Mass spectra of saturated fatty acid methyl esters have characteristic ion fragments at *m/e* 74 and 87, corresponding to $[CH_3–O–COH–CH_2]^+$ and $[CH_3–O–CO–CH_2–CH_2]^+$, respectively (34). The mass spectra of butyl esters are characterized by a few prominent ions; namely, M–55 $[CH_3(CH_2)_nCO]^+$, *m/e* 56 of $[C_4H_8]^+$, and the butyl analog of the γ hydrogen rearrangement ion of a methyl ester at *m/e* 116 of $[CH_2COHCOC_4H_9]^+$ (29). These ions were observed in the spectra of samples No. 76 and No. 87 which gave molecular ions at *m/e* 298 and 312 for butyl pentadecanoate and butyl hexadecanoate, respectively. The ethyl and butyl esters of long chain aliphatic acids had no detectable odor.

Other noncyclic compounds containing a C_4 structure were pentyl butyrate and 1,1-dibutoxyethane in the 100% PE fraction and 3-methyl-1-butanol in the 25% EPE fraction (Table I).

A fragment ion at *m/e* 91 $[C_6H_5CH_2]^+$ was present as the base or very prominent peak in the spectra of several components of the 100% PE fraction. The ion fragment is indicative of an alkyl substituted benzene ring (31). The spectrum of one of these substances matched that of 2-phenylethanol (benzyl carbinol) with *m/e* 91 as the base peak, 104 (M^+–18) and 122 (M^+) as predominant fragment ions. 2-Phenylethanol was also detected in the 25% EPE fraction. This compound was identified as a volatile product of an actinomycete strain resembling *S. platensis* together with 1-phenyl-2-propanone (16). They were not considered to have a disagreeable odor.

TABLE I

Some metabolites of *Streptomyces* sp. 33L

GLC Peak No.	Compound	Major ion fragments *m/e*
100% PE[a,b]		
4	Pentyl butyrate	43, 55, 70, 71 (base peak), 89, 101, 115 (M^+43)
5	1,1-Dibutoxy ethane[d]	41, 45, 57, 101 (base peak), 159 (M^+–15)
7	2-Phenyl ethanol (benzyl carbinol)	65, 91 (base peak), 92, 104, 122 (M^+)
9	*ß*-phenylethyl formate[d]	65, 91, 104 (base peak) (M–60)
11	Benzothiazole	45, 63, 69, 81, 82, 108, 135 (base peak, M^+)
12	Ethyl phenylacetate	43, 65, 89, 91 (base peak), 92, 105, 164
13	*ß*-Phenylethyl acetate	43, 57, 65, 73, 91, 104 (base peak, M–60)
26	*n*-Butyl phenol (*para*?)[d]	39, 41, 43, 67, 105, 107 (base peak), 150 (M^+)
48	Tridecanoic acid	41, 43 (base peak), 55, 73, 83, 97, 129, 171
53	Tetradecanoic acid	41, 43 (base peak), 55, 60, 73, 83, 97, 129, 185, 228 (M^+)
63	Pentadecanoic acid	41 (base peak), 43, 55, 57, 60, 69, 73, 83, 97, 129, 188, 242 (M^+)
69	Ethyl hexadecanoate	41, 43, 55, 60, 73 (base peak), 83, 88, 97, 101, 129, 157, 213, 257, (M^+–29), 284 (M^+)
75	Hexadecanoic acid	41, 43, 55 (base peak), 60, 73, 83, 97, 129, 157, 213, 256 (M^+)
76	Butyl pentadecanoate	41, 43, 55, 56, 57, 60, 73, 83, 97, 101, 116, 115, 116, 117, 129, 243 (M^+–55), 256, 298 (M^+)
87	Butyl hexdecanoate	41, 43, 55, 56, 57, 60, 73, 101, 116, 117, 129, 239, 257 (M^+–55), 312 (M^+)
25% EPE[c]	Benzyl alcohol	50, 51, 63, 65, 77, 79 (base peak), 91, 107, 108 (M^+)
	2-Phenyl ethanol[e]	(see above)
	1-Butanol	41, 43, 56 (M^+–18, base peak), 74 (M^+)
	3-Methyl-1-butanol	41, 55 (base peak), 70 (M^+–18)
	2,3-Hexanediol[d]	45 (base peak), 57, 73
	Butyric acid	60 (base peak), 73 (M^+–15)

[a]See Figure 1.

[b]Second cut 100% PE fraction of medium extract.

[c]25% EPE fraction of medium extract.

[d]Tentative identification.

[e]Present in the 100% PE (300 ml fraction) and 25% EPE fractions.

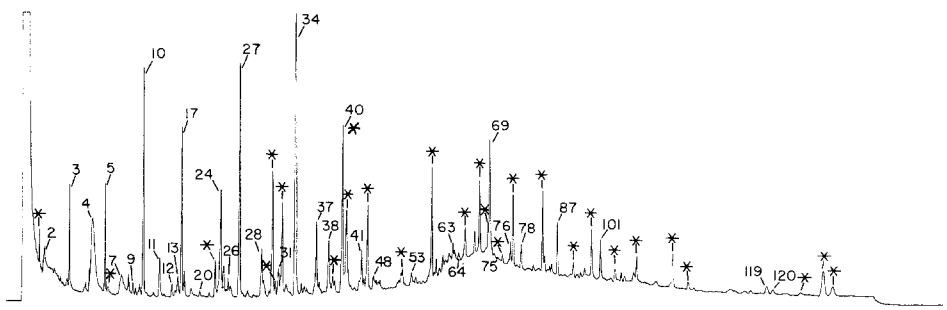

Fig. 1. Gas chromatogram illustrating components of the second (300 ml) cut 100% PE fraction of crude extract of medium in which *Streptomyces* sp. 33L was grown. Peaks with asterisks were contaminants for the medium.

Two other compounds with a prominent fragment *m/e* 91 and base peak at *m/e* 104 $[(C_6H_5C_2H_4)^+-H]$ were identified in the 100% PE fraction as *ß*-phenylethyl formate (tentative) and *ß*-phenylethyl acetate (Table I). The mass spectrum and GLC retention time of the latter compound matched closely with that of synthetic *ß*-phenylethyl acetate, which had a sweet chrysanthemum-like odor. A third alkyl substituted benzene ester was identified as ethylphenyl acetate by comparison of its mass spectrum and GLC retention time with an authentic standard which has a pleasant (39) or initially licorice and then fruit-like odor. Benzyl alcohol, with a faint aromatic odor (39), was also identified in the 25% EPE fraction from its mass spectrum which had *m/e* 79 as the base peak and *m/e* 108 as the molecular ion.

p-n-Butylphenol was also tentatively identified from its mass spectrum which contained a base peak at *m/e* 107 and molecular ion at *m/e* 150 (Table I). The mass spectrum of peak 11 (Figure 1) was very similar to that of the quinoline-like-smelling (39) substance, benzothiazole, with the base peak and molecular ion at *m/e* 135 (Table I). The mass spectrum and GLC retention time confirm the presence of this compound in the 100% PE fraction. 2,3-Hexanediol was also tentatively identified as a component of the 25% EPE fraction of the *Streptomyces* sp. medium extract.

Streptomyces isolate 33L produces a large variety of substances that are liberated into the culture medium, some of which are odorous and represent potential water pollutants. Although the odorous substances produced by this actinomycete were not quantitatively analyzed, butyric acid was produced in much greater quantities than the other substances and under the growth conditions employed represents the greatest potential as an odorous pollutant.

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