ISOLATION AND IDENTIFICATION OF AN ODOR COMPOUND
PRODUCED BY A SELECTED AQUATIC ACTINOMYCETE

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ISOLATION AND IDENTIFICATION OF AN ODOR COMPOUND
PRODUCED BY A SELECTED AQUATIC ACTINOMYCETE

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By

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CHAPTER I

INTRODUCTION

The need for a potable water supply has been recognized for over fifty years. In earlier times, the primary criteria for water quality were turbidity, color, taste, and odor. Biological quality as a standard was non-existent until it was shown that cholera was a water-borne infection.

Chemical treatment by flocculation has solved many of the problems of turbidity and color. Likewise, chlorine treatment for biological quality has, in general, made finished water safe for consumption. The problems of tastes and odors in tap water, however, are still to be solved.

Taste and odor compounds in a water supply result from industrial and domestic wastes, organic decomposition of both plant and animal matter, and metabolites produced by certain microorganisms. Efforts to control those tastes and odors arising from industrial and domestic wastes have met with some success. In such instances, those materials entering a water supply which would cause taste and odor problems may be removed by treatment prior to the discharge of the waste into a water supply. Taste and odor problems arising
from organic decomposition of plant and animal matter and from metabolites which are normally produced by these plants and animals during their active growth stages are still paramount. Little noteworthy success in the elimination of such problems has been accomplished by any prescribed treatment.

Many microorganisms have come under scrutiny as being possible producers of taste and odor metabolites. The first such microorganisms to be so implicated were algae (American Public Health Association, 1960).

**History of the Problem**

One of the early references to actinomycetes as being the causal agents of tastes and odors in water supplies was by Adams (1929). Burger and Thomas (1934) studied the tastes and odors of the Delaware River and while not denying the involvement of actinomycetes, preferred to believe that the tastes and odors resulted from the activity of a mixture of microorganisms. Thaysen (1936) reported that salmon of the Thames River acquired an earthy or muddy taint as the result of actinomycete-produced compounds, which in concentrated form assumed a brown amorphous appearance and produced a penetrating manurial odor. This same substance when dissolved in water in a concentration of two-tenths of a part per billion imparted a taint like the one observed in the river and in the salmon. Issatchenko and Egorova (1944) recounted the results of works done earlier on several rivers and
reservoirs in Russia where earthy odors were attributed to actinomycetes growing predominantly in soil of the river bank. Silvey et al. (1950) showed that certain actinomycetes were responsible for a variety of tastes and odors by comparing the odor produced from pure cultures of the organisms with those odors found in water supplies. The odors produced were classified as earthy, woody, and musty and were occasionally accompanied by taints of potato-bin, grassy, hay-like, and possibly, putrescible odors.

Once it had been established that aquatic actinomycetes did impart tastes and odors to water supplies, numerous investigators assumed the task of trying to isolate those metabolic compounds responsible for the tastes and odors. In laboratory investigations, Pipes (1951) applied the carbon-chloroform extraction techniques to raw water possessing tastes and odors of actinomycete origin in an attempt to isolate those compounds responsible for the tastes and odors. Infrared and ultraviolet spectral analyses of the concentrates revealed aromatic, amine, and aldehyde functional groups to be most concentrated, while alcohol, ester, carboxylic acid, and ketone functional groups were much less concentrated. Dill (1954) and McCormick (1954) found that amines, aldehydes, and carboxylic acids were produced as by-products during actinomycete growth. Silvey et al. (1950) demonstrated that isoamylamine, isobutyl amine, valeric acid, betahydroxybutyric acid, and isovaleraldehyde could be identified from actinomycete cultures.
Romano and Safferman (1963) isolated a brown amorphous solid from actinomycete cultures that possessed a threshold odor of $2 \times 10^9$. There was, however, no attempt made to identify the substance. Morris et al. (1963) isolated a pale yellow oil-like material from actinomycete cultures. The oil was characterized by gas chromatography, infrared spectro-photometry, per cent carbon, hydrogen, and oxygen as well as boiling point and molecular weight determinations.

Gains and Collins (1963) employed steam distillation and extraction techniques in order to isolate certain volatile substances from *S. odorifer* cultures. The major odor producing compounds were identified as acetic acid, acetaldehyde, ethanol, isobutyl alcohol, isobutyl acetate, and ammonia.

Gerber and Lechevalier (1965) isolated an earthy-smelling substance from actinomycete cultures by using whole broth steam distillation and extraction techniques. Gerber and Lechevalier named the compound they recovered geosmin. It was found to be a neutral oil containing carbon, hydrogen, and oxygen, but no nitrogen. The investigators posed an empirical formula, $C_{12}H_{22}O$, but no structural formula.

Dougherty et al. (1966) also employed steam distillation and extraction techniques in order to isolate a musty-smelling material from an actinomycete culture. The investigators gave a molecular weight, a discrete structural formula of $\Box_{\text{C}_6\text{H}_9(\text{CH}_3)_2}$, and named the compound mucidone.
Silvey et al. (1968) investigated the suitability of various liquid phases in a gas chromatographic study of actinomycete-produced tastes and odors in aqueous samples. It was found that FFAP (free fatty acid phase) gave the best results as regards the number of odor peaks resolved.

Purpose

The purpose of this study was to develop an efficient method for the concentration of specific volatile taste and odor compounds produced by a selected aquatic actinomycete. A second purpose was to isolate and identify the specific taste and odor compound present in the highest concentration.
CHAPTER II

METHODS

Culture and Control Chambers

Actinomycetes were grown in batteries of culture chambers. Each culture chamber, as shown in Figure 1, consisted of a four foot by four inch pyrex glass tube inside of which was placed a sufficient number of porcelain saddles to occupy approximately one-half the volume of the tube. The ends of the tube were closed by inserting rubber stoppers. Each stopper contained three holes into which had been placed a four inch by one-quarter inch piece of glass tubing. These tubes served as injection ports, gas ports (inlet and exit), and drainage ports.

Once assembled, the culture chamber was sterilized by evacuating the air and then adding ethylene oxide in sufficient volume to produce a pressure of twenty-five pounds per square inch. The ethylene oxide remained inside the chamber for a period of twenty-four hours. It was then removed by aspiratory evacuation. Fifteen hundred milliliters of culture medium were then added to the culture chamber through the injection port by gravity feed. The culture medium was allowed to stand in any individual culture chamber for an additional twenty-four
Fig. 1--Culture chamber and cryostatic-trap system
hours prior to inoculation with the actinomycete organism in order to insure sterility of the medium. The chamber was then inoculated with ten milliliters of the desired actinomycete spore suspension which had been prepared twenty-four hours prior to inoculation. The gas inlet and exit ports were subsequently opened so that sterile air could be swept through the chamber. The air was sterilized by passing through a muffle furnace at 1200°C. and then traveling a sufficient distance in one-fourth inch copper tubing to allow for its cooling to room temperature before entry into the chamber. This air carried the volatile odor compounds being produced by the growing actinomycetes. The odor-laden air was conducted by means of glass tubing with tygon joints to the cryostatic trap (Figure 1). The collection process continued for approximately one to two weeks for any single chamber. This period of time allowed the actinomycete to develop in its life cycle from the spore stage through the secondary mycelial stage. Maximum odor production began with the secondary mycelium development. The intensity of the odor being swept from any individual chamber was a function of the air flow rate after the actinomycete organism had reached the secondary mycelial stage of development. Thus, a flow rate was established such that the maximum intensity of odor could be swept from the chamber into the cryostatic trap in the shortest possible period of time. This flow rate was two hundred milliliters per minute per chamber. Since each
chamber varied in the actual number of actinomycete spores injected, the period of maximum odor production also varied within the range of one to two weeks. Thus, the collection period was initiated and terminated to reflect this range. The control chambers were treated exactly as the other culture chambers with the exception that they were not inoculated with the actinomycete culture.

The Actinomycete Organism and the Culture Media

The actinomycete used in this study was from the North Texas State University Actinomycete Culture Collection. The organism had been designated as strain number SH-9 by the laboratory. This particular actinomycete strain was utilized due to its desirable characteristics. These characteristics were its rapid growth on synthetic culture medium and its production of a strong earthy odor during its active growth phase.

The culture media utilized in this study were of two types: a liquid nutrient broth medium and a solid nutrient broth medium. The liquid nutrient broth medium contained four grams of NH₄NO₃, forty grams of dark brown sugar, and eight grams of nutrient broth. The ingredients were dissolved and made up to a volume of 1000 milliliters with distilled water. The pH of the medium was adjusted to 8.3 with KOH pellets before autoclaving. After autoclaving, the pH dropped to 8.2 and upon termination of the collection period,
the pH had risen to 8.6. The solid nutrient broth medium was prepared exactly in the same manner as the liquid nutrient broth medium except that sixteen grams of agar-agar was also added. Use of the latter medium necessitated its immediate injection into the culture chamber after autoclaving since, upon cooling, it would solidify. In instances where the latter medium was utilized, the porcelain saddles were omitted from the culture chambers during their assembly.

Taste and Odor Concentration

Silvey and Roach (1959) have shown that tastes and odors of actinomycete origin can be concentrated in aqueous solution utilizing the acetone-dry ice trap. The freeze-stir technique, another method for concentration of actinomycete-produced tastes and odors, was also reported by Silvey et al. (1968). The technique resembled that described by Shapiro (1963) and later modified by Wilson (1964) and Baker (1965). The freeze-stir technique allowed the production of a threshold odor of \(4 \times 10^4\) to \(5 \times 10^4\) in an aqueous medium.

This study utilized a refrigeration unit as a cryostatic trap in order to concentrate the taste and odor compounds from the actinomycete culture chambers. The unit contained four matching stainless steel wells, each having a two foot depth and a diameter of ten inches. The volume capacity of each well was thirty-two liters and each was equipped with a separate refrigeration condenser and thermostatic control.
The temperature of each well used for collection purposes was dropped to minus ten degrees centigrade and 2000 milliliters of dichloromethane (methylene chloride) were then added to each well. This particular solvent system was used since it possessed a wide spectrum of solubility characteristics, that is, it would dissolve both polar and nonpolar compounds.

The exhaust of effluent gas from the actinomycete columns was conducted into the cryostatic trap by one-quarter inch glass tubing with tygon joints. The odor-laden gas was discharged near the bottom of each well by means of a Sedgewick-Rafter funnel attached to the gas line as illustrated in Figure 1. The concentration process was continued for a period of one to two weeks from any given culture column.

After the collection period, the dichloromethane solvent was removed from the well of the cryostatic trap. The solvent at this point contained a considerable volume of water which had been exhausted from the culture chambers and condensed during the collection period. A major portion of this water was removed by means of a separatory funnel. The remaining portion of the water was removed by adding five per cent (weight to volume) sodium chloride crystals. The raw concentrate resulting from this treatment was then referred to as the final concentrate. The final concentrate was stored at minus twenty degrees centigrade for later use in gas chromatographic analysis and collection, infrared and ultraviolet
spectrophotometric analyses, nuclear magnetic resonance, and mass spectral analyses.

The Gas Chromatograph and Other Analytical Instruments

An Aerograph Autoprep, Model 705 gas chromatograph, equipped with a hydrogen flame detector and linear splitter was utilized in this study. The effluent of this unit, as mentioned above, had the added advantage of a linear splitter so that a small fraction of the effluent gas from the column could be shuttled to the detector while the remainder could be split off for collection purposes.

The column packing employed in this study was SE-30, 30 per cent on 60/80 mesh Chromosorb G acid washed. Two types of columns were used: an analytical column, ten feet by one-eighth inch, for detailed analysis and resolution, and a preparative column, twenty feet by three-eighths inch for collection purposes.

A sample of the final odor concentrate was injected into the gas chromatograph utilizing the analytical column as previously described. A chromatogram was produced, as is shown in Figure 2. Peak number one resulted from the dichloromethane solvent. Peak number two resulted from an impurity in the dichloromethane, as was determined by comparison with a chromatogram of the concentrate from a control chamber (Figure 3). Peak number three of Figure 2 was an odor peak which produced a very acrid odor upon sensing by olfaction at the exit port.
CS 1 min./in.
Det. flame
N 77 cc/min.
H 24 cc/min.
Air 270 cc/min.
DA see below
Sens. 0.1
Col. SE-30, 30% 10' X 1/8"
Col. Temp. 110 °C
In. Temp. 140 °C
Det. Temp. 190 °C

Fig. 2--Gas chromatogram of odor concentrate
Fig. 3—Gas chromatogram of control concentrate
of the gas chromatograph during peak elution. No perceptible odor could be sensed from peak number four. This was due, no doubt, to the small concentration of this particular component as was evidenced by the small peak area in the chromatogram. Peak number five also produced an odor and resulted from the actinomycete metabolism, as was determined when compared with the control chromatogram (Figure 3). The odor peak produced a very sweet smell when sensed by olfaction. This particular odor component was present in very high concentration and was selected for collection.

It was necessary to inject a rather large sample of the final odor concentrate at one time in order to collect enough of peak number five for further instrumental analyses. The preparative column would handle a three hundred microliter injection sample at any one time without excessive overloading, tailing, or column bleeding.

Effective collection of a given peak, provided it was present in sufficient concentration, was achieved by a micro-adaptation method, as is shown in Figure 4. Before collection was initiated, the middle portion of a polyethylene tube (.045 inches in diameter by 12 inches in length) was submerged in an acetone-dry ice bath. At the precise instant of peak elution from the column, as was evidenced by a response on the recorder of the instrument, one end of the polyethylene tube was slipped over the exit port of the gas chromatograph.
Fig. 4—Micro-adaptation method for collection
The remaining free end of the tube was open to the atmosphere. The eluant, present in an aerosol form, was eluted from the column, entered the tube, and condensed on the cold inner surface. The procedure was continued until a twenty-five-microliter volume of the odor compound had been collected. The sample was then removed from the tube by means of a microliter syringe. A small known volume of the compound was then used to prepare a standard solution by diluting with n-hexane. The final concentration of the solution was $7.6 \times 10^{-4}$ microliters of the odor compound per milliliter of solution. A ten-microliter sample of this solution was then injected into the gas chromatograph, utilizing the analytical column, in order to determine if there were other compounds contaminating the sample. A chromatogram was produced, as is shown in Figure 5. The odor compound had a retention time of three minutes. No peaks, other than number five, were resolved by temperature and flow rate variations. It was determined that the compound was rather pure in terms of the resolution capacity of the column. The remaining portion of the collected sample was stored at a minus twenty degrees centigrade to await further instrumental analyses.

Mass spectra were recorded with a Hitachi-Perkin-Elmer RMU-6E double focusing spectrometer. Liquid samples of the odor compound were injected into the heated inlet system with a microliter syringe.
Fig. 5--Gas chromatogram of standard odor solution
Infrared spectra were recorded as thin films between sodium chloride plates with a Perkin-Elmer 237 spectrophotometer.

Nuclear magnetic resonance spectra were recorded with a Varian A-60 spectrometer. Due to the small quantities of the odor compound sample, a microcell supplied by Nuclear Magnetic Resonance Specialities, Incorporated, was utilized. Deuterated chloroform was the solvent, with one per cent tetramethylsilane as the internal standard.

Ultraviolet absorption spectra were obtained with a Cary 14 spectrophotometer. Solutions of the odor compound in reagent grade n-hexane were prepared on a weight to volume basis for quantitative measurement. The absorbance was then measured by placing the solutions into one-centimeter quartz cells and recording.
CHAPTER III

RESULTS

Infrared spectral results of the odor compound, represented by peak number five of Figure 2, are shown in Table I. Only the major peaks of the spectrum are listed in Table I. The table illustrates the wavelength (µ) at which absorption occurred, along with the functional group assignment for that particular wavelength. The spectrum suggests a branched-chain aliphatic ketone and precludes other carbonyl-containing species such as carboxylic acids, esters, and lactones.

TABLE I

INFRARED SPECTRUM OF ODOR COMPONENT

<table>
<thead>
<tr>
<th>Wavelength (µ)</th>
<th>Assignment</th>
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<tr>
<td>3.38</td>
<td>(s) C-H stretch (aliphatic)</td>
</tr>
<tr>
<td>3.41</td>
<td>(s) C=O stretch (saturated ketone)</td>
</tr>
<tr>
<td>3.48</td>
<td></td>
</tr>
<tr>
<td>5.83</td>
<td>(s) C=O stretch (saturated ketone)</td>
</tr>
<tr>
<td>6.85</td>
<td>(m) C-H bend (aliphatic with chain branching)</td>
</tr>
<tr>
<td>7.09</td>
<td>(m) C-H bend (aliphatic with chain branching)</td>
</tr>
<tr>
<td>7.23-7.42</td>
<td>(m) C-H bend (aliphatic with chain branching)</td>
</tr>
<tr>
<td>8.55</td>
<td>(w) H₃C-CH-CH₂-CH₃ bend</td>
</tr>
<tr>
<td>8.74</td>
<td>(m) C-G stretch and skeletal modes</td>
</tr>
<tr>
<td>9.05</td>
<td>(m) C-G stretch and skeletal modes</td>
</tr>
<tr>
<td>9.71</td>
<td>(w) C-G stretch and skeletal modes</td>
</tr>
<tr>
<td>10.35</td>
<td>(w) C-G stretch and skeletal modes</td>
</tr>
<tr>
<td>10.95</td>
<td>(w) C-G stretch and skeletal modes</td>
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The results of mass spectral analysis are shown in Table II. The major peaks (fragments), along with their respective relative abundance, are listed. Also listed in this table are five metastable ions. The fragmentation scheme illustrated in Figure 6 accounts for the major peaks in Table II as well as the five metastable ions ($m^*$). Although the base peak at 43 strongly suggests a methyl ketone, closer examination of the major peaks suggests an alternative structure, 5-methyl-3-heptanone.

**TABLE II**

**MASS SPECTRUM OF ODOR COMPONENT**

<table>
<thead>
<tr>
<th>M/E**</th>
<th>Relative Abundance</th>
<th>M/E**</th>
<th>Relative Abundance</th>
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<tr>
<td>128</td>
<td>15.0</td>
<td>50</td>
<td>8.0</td>
</tr>
<tr>
<td>100</td>
<td>3.4</td>
<td>44</td>
<td>6.5</td>
</tr>
<tr>
<td>99</td>
<td>21.2</td>
<td>43</td>
<td>100</td>
</tr>
<tr>
<td>73</td>
<td>13.5</td>
<td>42</td>
<td>5.5</td>
</tr>
<tr>
<td>72</td>
<td>20.5</td>
<td>41</td>
<td>13.2</td>
</tr>
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<td>71</td>
<td>21.5</td>
<td>39</td>
<td>4.2</td>
</tr>
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<td>69</td>
<td>3.4</td>
<td>30</td>
<td>5.4</td>
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<td>58</td>
<td>5.4</td>
<td>29</td>
<td>17.3</td>
</tr>
<tr>
<td>57</td>
<td>22.7</td>
<td>28</td>
<td>5.8</td>
</tr>
<tr>
<td>56</td>
<td>8.8</td>
<td>27</td>
<td>5.4</td>
</tr>
<tr>
<td>55</td>
<td>4.3</td>
<td>26</td>
<td>10.2</td>
</tr>
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*Metastable ion peaks at 14.7, 18.7, 25.5, 26.1, 37.2, 51.0, and 76.7.

**M/E** = Mass to charge ratio.

Analysis of the odor compound sample by ultraviolet spectrophotometry resulted in a maximum absorption at a
Fig. 6—Fragmentation scheme of odor compound
wavelength (\(\mu\)) of 282 millimicrons and with an extinction coefficient (\(E_{\text{max.}}\)) of 21 (Figure 7).

The nuclear magnetic resonance spectrum of the odor compound is shown in Figure 8. The spectrum is complicated but is entirely consistent with the spectrum to be expected for 5-methyl-3-heptanone. The multiplet around 7.7 ppm contains the \(-\text{CH}_2\text{CH}_2\)- group as well as the methinyl proton of the isopentyl group. The absence of a \(\text{CH}_3\)- group is also confirmed. Aldehydes and other unsaturated compounds are eliminated by the absence of low field resonances.
Fig. 7--Ultraviolet spectrum of odor compound

\[ \lambda_{\text{max.}} : 282 \text{ mp} \]

\[ E_{\text{max.}} : 21 \]
<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
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<td>Temp.</td>
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<td>Solvent</td>
<td>CD013</td>
</tr>
<tr>
<td>Sweep Width</td>
<td>500</td>
</tr>
<tr>
<td>Sweep Time</td>
<td>500</td>
</tr>
<tr>
<td>Filt. Band</td>
<td>1</td>
</tr>
<tr>
<td>R. F.</td>
<td>0.2</td>
</tr>
<tr>
<td>Spec. Amp.</td>
<td>25</td>
</tr>
<tr>
<td>Int. Amp.</td>
<td>10</td>
</tr>
</tbody>
</table>

Fig. 8—Nuclear magnetic resonance spectrum of odor compound
CHAPTER IV

DISCUSSION

Earlier investigations (Dougherty et al., 1966; Gains and Collins, 1963; Gerber and Lechevalier, 1965) involving actinomycete taste and odor compounds, have utilized organic solvents in order to extract the taste and odor components from steam-distilled samples of either the spent medium or the whole cultures of the actinomycetes. The solvent extracts were then subjected to gas chromatographic analysis. Gains and Collins (1963) published inconclusive results based upon the interpretation of the gas chromatograms. Dougherty et al., (1966) and Gerber and Lechevalier (1965) followed up gas chromatographic analysis with collection of at least one of the odor components for further chemical studies.

One objective of this study was to couple the organic-cryostatic trap method of concentrating the volatile taste and odors of actinomycete origin (Silvey et al., 1968) and by gas chromatographic analysis, collect the primary odor constituent into a concentrate. Another objective was to characterize the odor constituent chemically by defining a discrete chemical structure.
Infrared spectrophotometric analysis of the compound (Table I) suggested that the odor compound was a branched-chain aliphatic ketone which eliminated the possibility of other carbonyl-containing species such as carboxylic acids, esters, and lactones. Ultraviolet spectrophotometric analysis results (Figure 7) also lent credence to the proposed branched-chain aliphatic ketone structure. The final decision concerning the proposed structure of the compound was confirmed by examination of the mass spectrum (Table II). The fragmentation scheme (Figure 6) accounts for all major peaks as well as the five metastable ions. The nuclear magnetic resonance spectrum (Figure 8) allowed the elimination of aldehydes and other unsaturated compounds due to the absence of low field resonance. The multiplet at 7.7 ppm contained the $\text{-CH}_2\text{CH}_2\text{-}$ group as well as the methinyl proton of the isopentyl group. The absence of a $\text{CH}_3\text{O}$ group was also confirmed. On the basis of these data, a complete structural formula of 5-methyl-3-heptanone was proposed. A reagent grade sample of the compound was obtained from Aldrich Chemical Company. Infrared and ultraviolet spectrophotometric analysis, as well as mass and nuclear magnetic resonance spectra of the known compound, confirmed the proposed structure. It was noted also, that the spectra of 5-methyl-3-heptanone, as reported in Sadtler Standard Spectra (IR 32062, NMR 3896), were identical with those of the isolated odor compound.
1. It can be seen from these data that the aquatic actinomycete, strain SH-9, does produce volatile organic odor compounds as a result of its metabolic activities under laboratory conditions.

2. Certain of these compounds are amenable to concentration by the organic cryostatic trap method. Collection of one odor compound from the concentrate was achieved by gas chromatographic methods.

3. The non-polar liquid phase, SE-30, is very useful in the gas chromatographic analysis of such compounds since the compounds are eluted from the column in the order of their boiling points. Parameters of the gas chromatograph can easily be adjusted in order to analyze compounds having boiling points in the low range, intermediate range, or high range.

4. The micro-adaption method of collection from the gas chromatograph is quite efficient when applied to the collection of volatile, aerosol-forming constituents.

5. The compound isolated and identified in this study was 5-methyl-3-heptanone, a branched-chain aliphatic ketone.
6. The metabolic origin of 5-methyl-3-heptanone is unknown. Metabolic pathway studies, utilizing $^{14}$C labelled precursors in the growth media, may help elucidate this question.

7. Gas chromatographic analysis has shown that 5-methyl-3-heptanone is not the only constituent present in the concentrates. Constituents have been shown to be present in the concentrates whose retention times are either shorter or longer than the retention time of 5-methyl-3-heptanone. Efforts are to be continued in order to identify those constituents.

8. The methods utilized in this study have yielded results which differ from the results obtained by steam distillation and extraction techniques of other investigators. It should be realized, therefore, that steam distillation and extraction techniques are not the sole methods by which tastes and odors of actinomycete origin can be isolated. Evidence has indicated that these compounds possess a wide spectrum of chemical and physical properties.
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