[Chem. Pharm. Bull. 31(2) 659—663 (1983)]

Odorous Metabolites of Fungi, *Chaetomium globosum* KINZE ex FR. and *Botrytis cinerea* PERS. ex FR., and a Blue-green Alga, *Phormidium tenue* (MENEGHINI) GOMONT[1)]

TOHRU KIKUCHI,*[,a] SHIGETOSHI KADOTA,[a] HISASHI SUEHARA,[a] ARASUKE NISHI,[b] KEISUKE TSUBAKI,[c] HIROSHI YANO,[d] and KENJI HARIMAYA[d]

Research Institute for Wakan-Yaku [Oriental Medicines], Toyama Medical and Pharmaceutical University,[a)] Sugitani 2630, Toyama 930-01, Japan, Faculty of Pharmaceutical Sciences, Toyama Medical and Pharmaceutical University,[b)] Sugitani 2630, Toyama 930-01, Japan, Institute of Biological Sciences, The University of Tsukuba,[c)] Sakura-mura, Niihari-gun, Ibaraki 305, Japan and Kobe City Water Supply Bureau,[d] Kusudani-cho 65, Hyogo-ku, Kobe 652, Japan

(Received July 23, 1982)

Two strains of earthy-musty smelling fungi, identified as *Chaetomium globosum* KINZE ex FR. and *Botrytis cinerea* PERS. ex FR., were isolated from soil samples collected at Suga-daira, Nagano Prefecture. Geosmin, having a strong earthy-musty smell, and 2-phenylethanol were detected as volatile metabolites of *Chaetomium globosum*, while furfural, benzaldehyde, phenylacetaldehyde, and benzyl cyanide were identified as metabolites of *Botrytis cinerea*. On the other hand, 2-methylisoborneol (with an earthy-muddy smell) was detected in the culture of *Phormidium tenue* (MENEGHINI) GOMONT, a blue-green alga isolated from water in Sengari Reservoir (Kobe City Water Supply Bureau). Another compound was also detected and identified as 2,6-di-*tert*-butyl-*p*-benzoquinone, which would be a minor component in tap water.

The present results suggest that, besides actinomycetes and blue-green algae, fungi may also be responsible for the objectionable earthy-musty odor and taste sometimes apparent in public water supplies.

Keywords——geosmin; 2-methylisoborneol; benzyl cyanide; fungi; *Chaetomium globosum*; *Botrytis cinerea*; blue-green algae; *Phormidium tenue*; earthy-musty odor; 2,6-di-*tert*-butyl-p-benzoquinone

Studies on the causual agents of unpleasant odors and tastes, especially earthy-musty odor, in public water supplies have been greatly advanced in the last decade by several groups of workers[2)] and a number of odorous compounds such as geosmin[3)] (I) and 2-methylisoborneol[4)] (II) have been reported so far. These substances are known to be produced by a variety of aquatic actinomycetes and algae, which multiply remarkably in rivers and lakes from time to time when pollution of the water is severe. Some kinds of fungi may also be responsible for the putrid earthy-musty odor and taste in water,[5)] but no report on fungal metabolites with a smell of this kind has been found in the literature. Previously we reported the identification of 6-pentyl-α-pyrone from two strains of fungi, *Trichoderma* and *Aspergillus* species,[6)] and of 2-phenylethanol and phenylacetaldehyde from several fungi such as *Chaetomium, Penicillium,* and *Robillarda* species, isolated from the bottom deposits of Sengari Reservoir (Kobe City Water Supply Bureau).[2f)] We could not detect any earthy-musty metabolite from these fungi, although they had a weak smell when cultured. Recently we have isolated two strains of earthy-smelling fungi, *Chaetomium globosum* KINZE ex FR.[1)] and *Botrytis cinerea* PERS. ex FR., from the soil at Suga-daira, Nagano Prefecture, and also succeeded in obtaining a blue-green alga, identified as *Phormidium tenue* (MENEGHINI) GOMONT, from water in Sengari Reservoir (Kobe City Water Supply Bureau). In this paper we describe the volatile metabolites of these organisms.

Experimental

Chaetomium globosum KINZE ex FR. and *Botrytis cinerea* PERS. ex FR. were isolated from soil samples collected at Suga-daira, Nagano Prefecture.

Phormidium tenue (MENEGHINI) GOMONT was isolated from water in Sengari Reservior (Kobe City Water Supply Bureau).

Laboratory Culture and Separation of the Volatile Metabolites of the Fungi———The fungi were grown on a stationary liquid medium (yeast extract 4 g, malt extract 4 g, peptone 1 g, and glucose 4 g in water 1000 ml) at 26°C for 25—30 days (during this period the cultures smelled strongly, and after that the smell diminished gradually). The whole culture (10 l for each fungus) was then subjected repeatedly to steam distillation in order to concentrate the volatile substances. The final distillate (*ca.* 500 ml) was saturated with sodium chloride and extracted with methylene chloride. The extract was dried over $MgSO_4$ and the solvent was removed by careful distillation. The residue (15—30 g) was further purified by silica gel chromatography (0.8×4 cm column) with ether–pentane (3:97) and the eluted solution was concentrated carefully to give a small amount of oily substance (10—20 mg). This sample was analyzed by GC and GC–MS methods.

Laboratory Culture and Separation of the Metabolites of *Phormidium tenue*———The alga was cultured on a synthetic medium without shaking at 25°C for 70 days. The medium was prepared according to Takeda *et al.*[7] by dissolving $NaNO_3$ 100 mg, $K_2HPO_4 \cdot 3H_2O$ 40 mg, $MgSO_4 \cdot 2H_2O$ 380 mg, $CaCl_2 \cdot 2H_2O$ 20 mg, and a trace element (P IV) solution 6 ml in water 1000 ml. The whole culture (25 l) was then extracted with methylene chloride (three times). After being dried over $MgSO_4$, the extract was concentrated carefully to give a small amount of oily substance with an earthy-muddy odor.

Gas Chromatography (GC)———GC analyses were done on a Shimadzu gas chromatograph Model GC-6A with a flame ionization detector using a 5% Thermon-1000 column (on Chromosorb W-AW DMCS, 1 m×3 mm i.d. glass tube) at a column temp. of 100°C, or a 10% PEG-20M column (on Chromosorb WNAW, 2 m×3 mm i.d. glass tube) at a column temp. of 150°C. Nitrogen was used as the carrier gas at a flow rate of 35 ml/min.

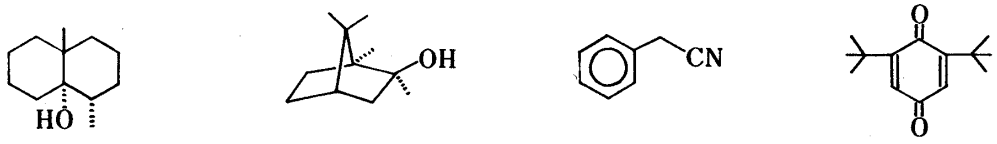
Mass Spectrometry combined with Gas Chromatography (GC–MS)———GC–MS measurements were done on a JEOL D-300 mass spectrometer using a GC injection system. GC was carried out with a 5% Thermon-1000 column (1 m×2 mm i.d. glass tube) at 100—160°C (3°C/min) or a 10% PEG-20M column (2 m×2 mm i.d. glass tube) at 145°C. Helium was used as the carrier gas (1 kg/cm^2). Mass spectra were measured under the following conditions: ionization energy, 70 ev; accelerating voltage, 3 kV.

Results and Discussion

The oily substance obtained from *Chaetomium globosum* had an earthy-musty smell. The GC showed a very complicated pattern, but mass chromatographic analyses revealed the presence of a compound having the same retention time as geosmin (I) (Fig. 1, peak A). As shown in Fig. 2 a), the GC–mass spectrum corresponding to peak A gave the molecular ion peak at m/z 182 ($C_{12}H_{22}O$) together with characteristic peaks at m/z 167, 164, 149, and 112. This pattern is superimposable on that of authentic *dl*-geosmin and thus peak A was identified as I.

Peak B, on the other hand, was considered to be 2-phenylethanol from the mass chromatography based on the ions of m/z 122, 104, and 91 (Fig. 1), and this was confirmed by direct comparison of the GC–mass spectrum (Fig. 2, b)) with that of an authentic sample. This compound is a known fungal metabolite, as mentioned before.[2f] Peak C might be phenylacetaldehyde, but this could not be confirmed because of the incomplete separation (see Fig. 2, c)).

On the other hand, the oily substance obtained from *Botrytis cinerea* showed a somewhat



I II III IV

Chart 1

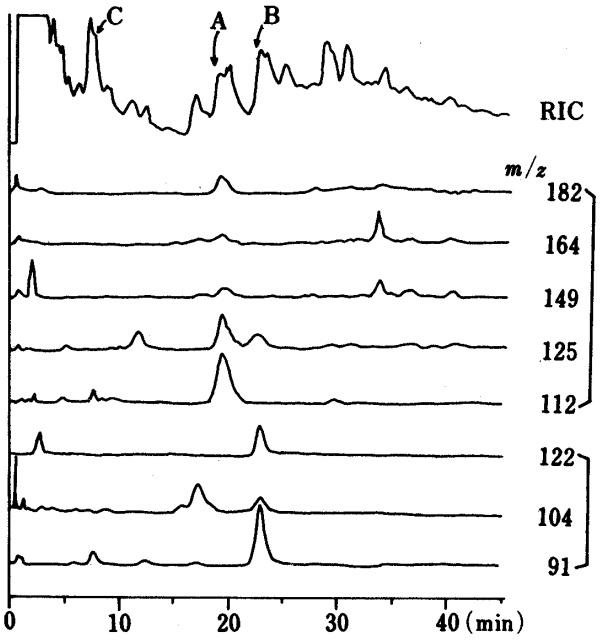


Fig. 1. Gas Chromatogram and Mass Chromatogram of the Volatile Metabolites of *Chaetomium globosum* (5% Thermon 1000 column) (peak A, geosmin; peak B, 2-phenylethanol; peak C, phenylacetaldehyde).

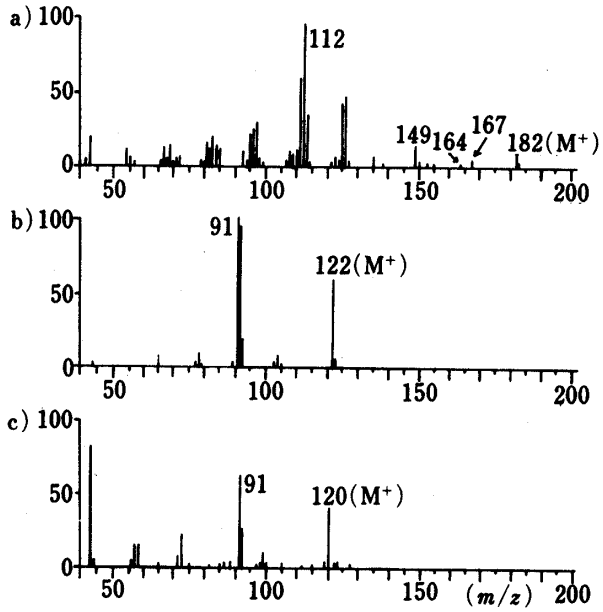


Fig. 2. Mass Spectra of the Volatile Metabolites of *Chaetomium globosum:* a) peak A (geosmin), b) peak B (2-phenylethanol, and c) peak C (impure phenylacetaldehyde).

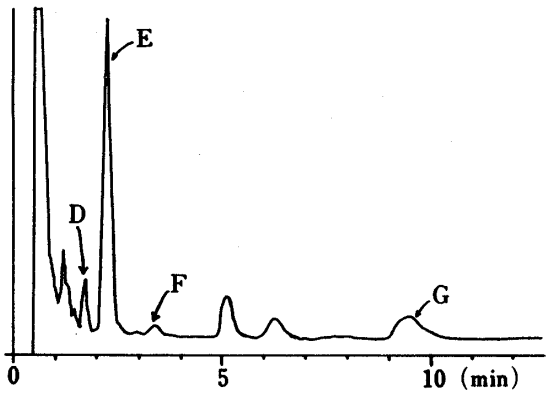


Fig. 3. Gas Chromatogram of the Volatile Metabolites of *Botrytis cinerea* (10% PEG-20M column) (peak D, furfural; peak E, benzaldehyde; peak F, phenylacetaldehyde; peak G, benzyl cyanide).

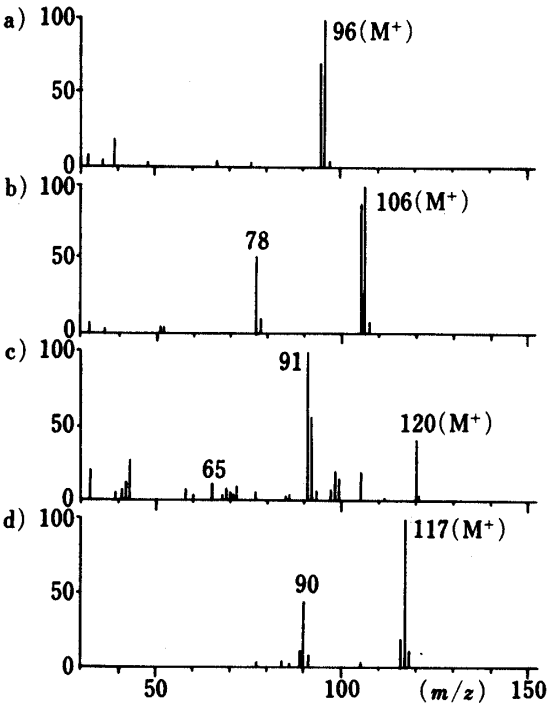


Fig. 4. Mass Spectra of the Volatile Metabolites of *Botrytis cinerea:* a) peak D (furfural), b) peak E (benzaldehyde), c) peak F (phenylacetaldehyde), and d) peak G (benzyl cyanide).

putrid grassy, not earthy, smell, although the intact fungi had a weak earthy-musty odor. This substane exhibited many peaks on GC (Fig. 3), among which peaks D, E, and F were assigned to furfural, benzaldehyde, and phenylacetaldehyde,[2f] respectively, by GC-MS analyses.

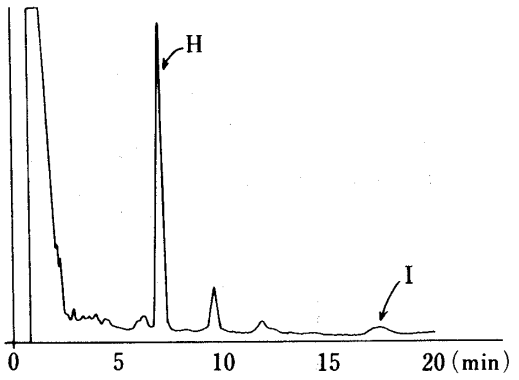


Fig. 5. Gas Chromatogram of the Metabolites of *Phormidium tenue* (10% PEG-20M column) (peak H, 2-methylisoborneol; peak I, 2,6-di-*tert*-butyl-*p*-benzoquinone).

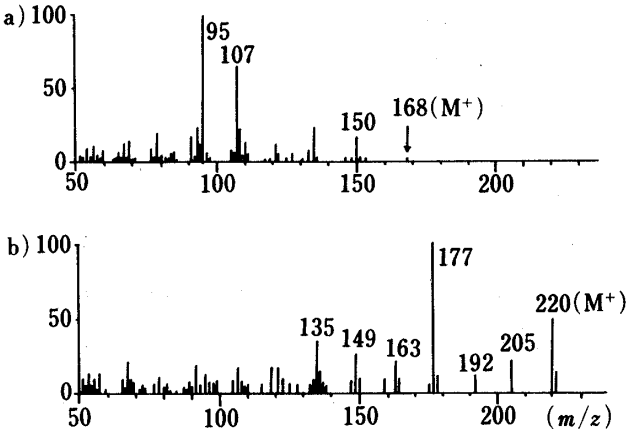


Fig. 6. Mass Spectra of the Metabolites of *Phormidium tenue*: a) peak H (2-methylisoborneol) and b) peak I (2,6-di-*tert*-butyl-*p*-benzoquinone).

Another significant peak appeared in Fig. 3 (peak G); the GC–mass spectrum (Fig. 4, d)) gave the molecular ion peak at m/z 117 (C_8H_7N) and a strong peak at m/z 90. This spectral pattern suggests that the compound is an unsaturated cyanide. Eventually, peak G was ascribed to benzyl cyanide (III) on the basis of direct GC and GC–MS comparisons with an authentic sample (III).

Next, we examined the metabolites of *Phormidium tenue*.[8] The oily substance obtained showed an earthy-muddy smell, and its GC gave a rather simple pattern as illustrated in Fig. 5, in which the most prominent peak (peak H) could be assigned to 2-methylisoborneol (II). The GC–mass spectrum (Fig. 6, a)) corresponding to peak H was identical with that of authentic II, measured under the same conditions.

We would like to mention here another small, but significant peak (peak I) observed in this experiment. As shown in Fig. 6 b), the GC–mass spectrum obtained from peak I exhibited the molecular ion peak at m/z 220 (Calcd for $C_{14}H_{20}O_2$: 220.1463, Found: 220.1469) and characteristic fragment peaks at m/z 205 (M^+-15), 192 (M^+-28), 177 (base peak, Calcd for $C_{12}H_{17}O$: 177.1279, Found: 177.1270), 163 (M^+-57), 149 ($M^+-15-28-28$), and 135 ($M^+-57-28$), suggesting that the compound might be an unsaturated diketone. It was finally identified as 2,6-di-*tert*-butyl-*p*-benzoquinone (IV) by GC and GC–MS comparisons with an authentic sample of IV.

The production of this compound, however, was not reproducible when the culture experiment was repeated. Therefore it is not clear whether or not IV is a real metabolite of this blue-green alga. However, IV was isolated and identified from tap water in New Orleans, U.S.A.[9] It is well known that the quinone IV is easily derived by air oxidation from 2,6-di-*tert*-butyl-*p*-cresol, which is widely used as an antioxidant for various food products. Thus, compound IV in our case could have come from the water, which had been prepared by distillation of tap water and used for the culture experiment. Thus, tap water from rivers or lakes in Japan, as well as in the U.S.A., may contain the quinone IV.

Summarizing the foregoing findings, geosmin (I) with a characteristic earthy-musty odor was first identified as a volatile metabolite of *Chaetomium globosum,* which is one of very common fungi, widely distributed in nature. Possibly other species of fungi may also produce geosmin and it follows that fungi,as well as actinomycetes and algae, may be responsible for the earthy-musty odor and taste sometimes occurring in public water supplies. Benzyl cyanide (III), identified from *Botrytis cinerea,* shows a somewhat aromatic, grassy odor, but could be partly responsible for complex unpleasant odors and tastes in water in combination with other volatile substances.

Acknowledgment This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture, which is gratefully acknowledged.

References and Notes

1) This work was presented at the 101st Annual Meeting of the Pharmaceutical Society of Japan (Kumamoto, April 1981). A preliminary communication of a part of this work has appeared: T. Kikuchi, S. Kadota, H. Suehara, A. Nishi, and K. Tsubaki, *Chem. Pharm. Bull.*, **29**, 1782 (1981).
2) *a*) A.A. Rosen, C.I. Mashni, and R.S. Safferman, *Water Treatment and Examination,* **19**, 106 (1970); *b*) T. Kikuchi, T. Mimura, Y. Moriwaki, K. Negoro, S. Nakazawa, and H. Ono, *Yakugaku Zasshi,* **91**, 1255 (1971); *c*) T. Kikuchi, *Japan Analyst (Bunseki Kagaku),* **22**, 1530 (1973); *d*) T. Kikuchi, T. Mimura, Y. Masada, and T. Inoue, *Chem. Pharm. Bull.*, **21**, 1847 (1973); *e*) T. Kikuchi, T. Mimura, K. Asoh, Y. Masada, and T. Inoue, *J. Japan Water Works Assoc.*, **491**, 45 (1975); *f*) T. Kikuchi, T. Mimura, Y. Itoh, K. Harimaya, Y. Matsuda, and Y. Masada, *ibid.*, **492**, 23 (1975); *g*) T. Kikuchi, T. Mimura, Y. Kawashima, Y. Moriwaki, Y. Masada, and T. Inoue, *Yakugaku Zasshi,* **95**, 1517 (1975); *h*) J.L. Tabachek and M. Yurkowski, *J. Fish. Res. Board Can.*, **33**, 25 (1976); *i*) M. Otsuhata and M. Suwa, *J. Water & Waste,* **19**, 1483 (1977); *j*) Y. Tsuchiya and T. Okamoto, *Yakugaku Zasshi,* **98**, 124 (1978); *k*) Y. Tsuchiya, K. Shudo, and T. Okamoto, *Eisei Kagaku,* **26**, 77 (1980); *l*) A. Yasuhara, *J. Odor Control,* **9**, No. 43, 28 (1980).
3) N.N. Gerber, *Tetrahedron Lett.*, **1968**, 2971.
4) N.N. Gerber, *J. Antibiotics* (Tokyo), **22**, 508 (1969).
5) K. Ikeda, *Research Reports Osaka City Water Works Bureau* (Suido-Jigyo Kenkyu), **76**, 70 (1973).
6) T. Kikuchi, T. Mimura, K. Harimaya, H. Yano, T. Arimoto, Y. Masada, and T. Inoue, *Chem. Pharm. Bull.*, **22**, 1946 (1974).
7) H. Takeda, N. Takada, S. Sakai, Y. Katsuyumi, and Y. Oshima, The lst Annual Meeting of the Society of Japan Fermentation Technology, Abstract Papers, p. 249 (Osaka, November 1979).
8) No bacteria were detected by culture experiments with a nutrient broth or a peptone-glucose-yeast extract medium.
9) K. Urano, *Kagaku No Ryoiki,* **35**, 675 (1981).