

Genotoxicity of Microbial Volatile Organic Compounds

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Luminescent and light absorption *umu* tests were used to investigate the genotoxicity of microbial volatile organic compounds (MVOCs), which have been reported to occur in conjunction with the growth of filamentous fungi. Investigation of 20 types of MVOC samples confirmed the SOS-inducing activity of 1-octen-3-ol, 2-methyl-1-propanol, 2-heptanone, 3-octanol, 1-pentanol, 1-butanol, 2-methyl-1-butanol, 3-methyl-1-butanol, 3-methyl-2-butanol, 3-octanone, 2-hexanone, 2-butanone, 3-methyl-2-butanone, 2-pentanol, ethyl isobutyrate, and terpinen-4-ol. Of these materials, 3-methyl-2-butanone and 3-methyl-2-butanol, which were positive in both the luminescent and light absorption *umu* tests, were clearly shown also to be mutagenic based on the results of the Ames test. Each of these 20 MVOCs is known to be produced by microorganisms commonly detected in indoor environments, and long-term exposure could be a health hazard.

Key words — microbial volatile organic compound, fungi, *umu* test, Ames test, mutagenicity

INTRODUCTION

There are many types of indoor air pollutants. Examples include volatile organic compounds (VOCs), such as formaldehyde and toluene, which have drawn attention in recent years as causes of sick building syndrome; phthalate esters (plasticizers); agents used to treat wood, such as termite-proofing agents and fire retardants; combustion exhaust gases in conjunction with heating and cooking; and even agents such as mold spores, mites, pet fur, and pollen, which are causes of allergies. Most recently, microbial VOCs (MVOCs) produced by microorganisms have been added to the list. There have been reports of more than 100 types of chemical compounds produced by several species of microorganisms,^{1–3} as well as reports of MVOCs being detected in rooms with severe mold damage. De-

spite reports of various chemical analyses of MVOCs in the air from indoor environments, few reports have discussed the actual damage to health caused by MVOCs or their toxicity. To date, reports on the health effects of MVOCs have been limited to demonstrating the possibility that they cause nasal inflammation⁴ and a study using the microculture tetrazolium assay and methylene blue assay to examine general toxicity in human lung epithelial cells (A549).⁵ To the best of our knowledge, the only report to address genotoxicity specifically is that of Kreja and Seidel,⁶ who subjected human lung carcinoma epithelial A549 cells, V79 Chinese hamster fibroblasts, and human peripheral blood pB cells to the comet assay. Because various microorganisms are present in ordinary living environments at various concentrations, there is the possibility of human exposure to MVOCs. Exposure from indoor air can be generally considered long-term exposure, and even at low concentrations there is a possibility of health risks that should not be ignored. Therefore in this study we performed *umu* tests with microorganisms to investigate the SOS-inducing activity of representative MVOC samples. We report the results

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not only for the light absorption *umu* test, but also for the more sensitive luminescent *umu* test, which we additionally used to investigate genotoxicity.

MATERIALS AND METHODS

Reagents and Test Materials — The following 20 types of MVOC samples were tested: 2-butanone, 2-methyl-1-propanol, ethyl isobutyrate, 2-heptanone, and 3-octanone (Wako Pure Chemical Industries, Osaka, Japan, special grade); 2-hexanone and 2-nonanone (Wako Pure Chemical Industries, grade 1); terpinen-4-ol (Wako Pure Chemical Industries, highly pure); 3-methyl-2-butanone, 2-pentanol, 3-methyl-1-butanol, 1-pentanol, and 1-hexanol (Nacalai Tesque, Kyoto, Japan, special grade); 2-methyl-1-butanol, 1-decanol, and 1-butanol (Nacalai Tesque, grade 1); 2-*n*-pentylfuran and 1-octen-3-ol (Lancaster Synthesis, Lancashire, U.K., highly pure); and 3-octanol and 3-methyl-2-butanol (Tokyo Kasei Kogyo, Tokyo, Japan, highly pure). All of these compounds have been reported to be produced by filamentous fungi.^{2,3,7-9} Sample dilution was performed with both dimethyl sulfoxide (DMSO), (Dojindo, Kumamoto, Japan) for luminescent measurement and methanol (Wako Pure Chemical Industries) for testing agricultural chemical residue.

Luminescent *umu* Test — In the luminescent *umu* microplate method, luminescent genes *luxA-E* extracted from the luminescent bacterium *Vibrio fischeri* are joined downstream from the *umu D, C* gene of plasmid pSK1002 to construct plasmid pTL210.¹⁰ *Salmonella typhimurium* (*S. typhimurium*) TL210, which is a phenotypic transformation of *S. typhimurium* TA1535, is used in the plasmid. After overnight culturing of the bacterial strain, DMSO was mixed into and dispersed through the bacterial suspension to create a final concentration of 7%; the frozen material was used on each test day. Tryptone-glucose-ampicillin (TGA) medium was used to dilute the frozen and cultured bacteria and for the test bacterial suspension. The TGA medium was prepared with 1% bacto-tryptone, 0.5% NaCl, 0.2% glucose, and ampicillin. The luminescent *umu* microplate method was performed as follows. An *S. typhimurium* TL210 strain of frozen bacteria was thawed and mixed with TGA medium in an Erlenmeyer flask in a 1-to-25 dilution. The suspension underwent shaking culture in an incubator at 37°C at 120 rpm for 2 hr. Optical density at 600 nm (OD₆₀₀) was diluted to 0.1 with TGA me-

dium. DMSO or methanol was used to dilute test materials and environmental samples.¹¹ A 96-well, clear-bottomed, black plate (Matrix Technologies, Hudson, NH, U.S.A.) was used; 4 μ l of sample solution was added to each well. Next, 200 μ l of prepared bacterial suspension was mixed and left to stand in an incubator at 30°C. Immediately after the bacterial suspension was mixed, the luminescence was measured with a Luminescencer-JNR AB2100 (Atto, Tokyo, Japan). Turbidity at 595 nm was measured with a Wallac 1420 ARVOsx plate reader (Perkin Elmer, Wellesley, MA, U.S.A.), and the number of live bacteria estimated. The shortest time between measurement periods was once per 20 min, and the longest approximately 5 hr. In addition, when measuring luminescence, an estimated value for 1 sec per well was sought. When genotoxicity and mutagenicity testing is done *in vitro*, the mammalian metabolic system is included by adding rat liver S9 mix. However, when S9 mix is added to luminescent *umu* tests, the value of the control solvent is reduced, and attention is required to make a determination of positive or negative, particularly for samples with a weak SOS response.^{10,12} Therefore S9 mix was not added in the current study.

Light Absorption *umu* Test^{11,13} — The light absorption *umu* test was performed using the *S. typhimurium* strain TA1535/pSK1002. The frozen preserved bacterial strain was rapidly dissolved, inoculated in luria-bertani (LB) medium (1% bacto-tryptone, 0.5% NaCl, 0.5% yeast extract), and cultured by undergoing shaking culture at 37°C at 120 rpm for 12 hr. The culture suspension was diluted 100 times with TGA medium, and shaking culture continued for an additional 2 hr. The bacterial suspension obtained and the test materials were put into a microplate, sealed, and after thorough stirring with a plate mixer, underwent shaking culture at 37°C at 900 rpm for 2 or 4 hr. The number of live bacteria in the bacterial suspension was then estimated based on the OD₅₉₅. Ten microliters of bacterial suspension was transferred to each of the holes in another 96-well microplate and diluted with 140 μ l of Z buffer solution. A Z buffer solution of 2-mercaptoethanol was added immediately before. Ten microliters (4 mg/ml) of chlorophenol red- β -D-galactopyranoside (CPRG) was added to this. After culturing for 30 min at 37°C, 100 μ l of 1 mol/l sodium bicarbonate aqueous solution was added to stop coloration. Then β -galactosidase activity was measured based on OD₅₇₀ (*umu* unit = OD₅₇₀/OD₅₉₅).

Ames Test¹⁴ — *S. typhimurium* TA98 and

TA100¹⁵⁾ were used as the test strains. To prevent solvent vaporation, we used the screw-capped test tube for preincubation. To sterilized screw-capped test tubes containing the bacterial suspension and the DMSO solution of the test substances, we added either S9 mix (+S9) or sodium phosphate buffer 0.1 mol/l (-S9). After preincubation at 37°C for 20 min, soft agar was added and mixed, and the mixture spread on the plates. After culturing for 48 hr at 37°C, the number of revertant colonies was counted. The S9 mix was prepared with freeze-dried material containing a set of cofactors and S9 solution (S9/Cofactor A Set for Ames Tests; Oriental Yeast Co., Tokyo, Japan). The sample solutions, including the solvent control, were diluted with DMSO to seven different strengths. The test used two plates for each strength. The positive control for +S9 tests was benzo[*a*]pyrene (BaP), and for -S9 tests, 2-(2-furyl)-3-(5-nitro-2-furyl)acrylamide (AF2). Microscopic examination of plate surfaces was done as needed to determine inhibited bacterial growth.

Judgment of Results—— Determination of each bioassay was done for samples with a good dose response: samples for which the number of revertant colonies was 1.5 to 2 times that of spontaneous revertant colonies were deemed pseudopositives, and those that were 2 times or more positive. It is often observed that dose-response curves immediately decrease at high doses in bioassays such as the *umu* test or Ames test. This is because potent cytotoxicity occurs. As a result, although the response is not positive, a comparatively strong dose-response curve is often seen, unlike the case of a negative response. Therefore not only positive results but also pseudopositive results are indicated in this report.

RESULTS AND DISCUSSION

The dose-response curve from the luminescent *umu* test exhibits good dose dependency in the case of 3-methyl-2-butanol (Fig. 1). In this example, because the luminescence is more than double that of the control solvent, the sample was determined to be positive for SOS-inducing activity. Among the MVOCs examined in the current study, their influence on bacterial growth differed greatly, resulting in some cases in which turbidity was extremely low at MVOC concentrations above a certain amount. Accordingly, the concentration in the samples was appropriately reduced, and testing was done in a range in which the growth inhibition of the bacteria

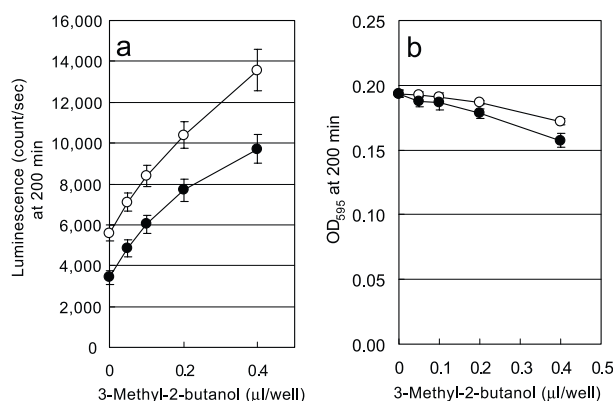


Fig. 1. Example of a Dose-Response Curve in the Luminescent *umu* Test (3-Methyl-2-butanol)

a, Luminescence; b, light absorption at 595 nm; ○, dilution with DMSO; ●, dilution with methanol.

Table 1. Response of MVOCs in the Luminescent *umu* Test

Compound	DMSO	MeOH
	(count/μl·sec)	
1 1-Decanol	N.D.	N.D.
2 1-Octen-3-ol	N.D.	(18500)
3 3-Octanol	(30000)	14000
4 1-Hexanol	N.D.	N.D.
5 1-Pentanol	(25000)	(42800)
6 1-Butanol	9600	9800
7 2-Methyl-1-butanol	18700	22900
8 3-Methyl-1-butanol	14600	18600
9 3-Methyl-2-butanol	19400	15100
10 2-Methyl-1-propanol	12300	11900
11 2-Nonanone	N.D.	N.D.
12 3-Octanone	(17900)	12800
13 2-Heptanone	28300	22500
14 2-Hexanone	(18600)	23300
15 2-Butanone	5300	5300
16 3-Methyl-2-butanone	21000	32000
17 2-Pentanol	9800	14300
18 Ethyl isobutyrate	(3600)	3700
19 2- <i>n</i> -Pentylfuran	N.D.	N.D.
20 Terpinen-4-ol	(44000)	(30000)

N.D., not detected. Numbers in parentheses are pseudopositives.

did not exceed 50%. Test results for the chemical compounds are summarized in Table 1. Generally, in bioassays like the Ames test in which test materials are not dissolved in water, DMSO is used as the solvent, but it is known that in the light absorption *umu* test, DMSO itself shows an SOS response to some extent.¹⁶⁾ Similar phenomena have also been observed in the luminescent *umu* test, but the SOS

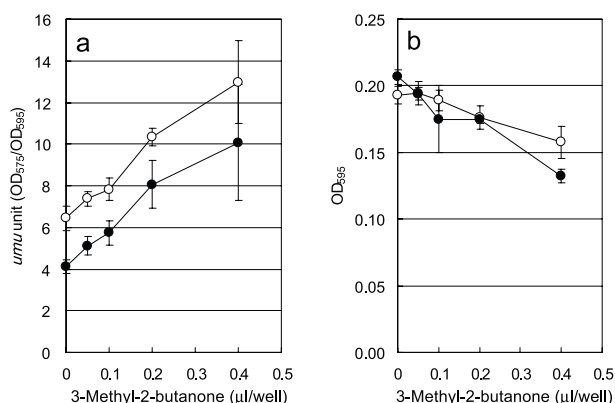


Fig. 2. Example of a Dose-Response Curve in the Light Absorption *umu* Test (3-Methyl-2-butanone)

a, *umu* unit; b, light absorption at 595 nm; ○, dilution with DMSO; ●, dilution with methanol.

response of methanol and similar agents is low, and it is possible that the background is suppressed to a greater extent than it is DMSO.¹¹⁾ Therefore in this study tests were done using both DMSO and methanol as the solvent. When DMSO was used as the solvent, 9 of the 20 MVOCs tested positive for SOS-inducing activity, and 6 displayed pseudopositive results then methanol was used as the solvent, 13 MVOCs tested positive, and 3 were pseudopositive. Of the positive MVOCs, 3-methyl-2-butanone showed the strongest activity (32000 count/sec·μl when using methanol). Other compounds that showed comparatively strong activity included 2-heptanone, 2-methyl-1-butanol, 3-methyl-2-butanol, and 3-methyl-1-butanol, followed by 3-octanone, 3-octanol, 2-methyl-1-propanol, 2-hexanone, 2-pentanol, 1-butanol, ethyl isobutyrate, and 2-butanone.

A dose-response example from the light absorption *umu* test is shown in Fig. 2, and the results obtained are given in Table 2. In this test, the number of live bacteria was also estimated by measuring the turbidity of the culture medium in the wells at 595 nm. When growth inhibition of 50% or greater was seen, the sample was appropriately diluted and retested. In the light absorption *umu* test as well, both DMSO and methanol were used as dilution solvents; results are shown for each. In addition, the incubation time for both the bacterial suspension and the test materials in *umu* tests is usually 2 hr, but because the SOS response of each MVOC was comparatively weak, the period was extended to 4 hr in this study. When DMSO was used as the solvent, only 3-methyl-2-butanone was positive for SOS-in-

Table 2. Response of MVOCs in the Conventional Light Absorption *umu* Test

Compound	DMSO	MeOH
	(umu unit/μl)	
1 1-Decanol	N.D.	N.D.
2 1-Octen-3-ol	N.D.	N.D.
3 3-Octanol	N.D.	N.D.
4 1-Hexanol	N.D.	N.D.
5 1-Pentanol	N.D.	N.D.
6 1-Butanol	N.D.	N.D.
7 2-Methyl-1-butanol	N.D.	N.D.
8 3-Methyl-1-butanol	N.D.	N.D.
9 3-Methyl-2-butanol	(6.34)	14.3
10 2-Methyl-1-propanol	N.D.	N.D.
11 2-Nonanone	N.D.	N.D.
12 3-Octanone	N.D.	N.D.
13 2-Heptanone	N.D.	N.D.
14 2-Hexanone	N.D.	N.D.
15 2-Butanone	N.D.	N.D.
16 3-Methyl-2-butanone	16.6	15.1
17 2-Pentanol	N.D.	N.D.
18 Ethyl isobutyrate	N.D.	N.D.
19 2- <i>n</i> -Pentylfuran	N.D.	N.D.
20 Terpinen-4-ol	N.D.	N.D.

N.D., not detected. Numbers in parentheses are pseudopositives.

ducing activity (3-methyl-2-butanol was a pseudopositive). When methanol was used, 3-methyl-2-butanol was also positive. As in the luminescent *umu* test, there was a tendency for more samples to show positives when using methanol as the dilution solvent. 3-Methyl-2-butanone, which displayed the greatest activity in the luminescent *umu* test, also had the greatest activity in the light absorption *umu* test, but other MVOCs that showed comparable activity at the same level or greater than 3-methyl-2-butanol in the luminescent *umu* test were not positive in the light absorption *umu* test.

It is thought that in the carcinogenesis initiation process, genetic damage occurs first, leading to mutation. The *umu* test is a primary screening method for detecting the SOS response induced by genetic damage. Therefore the 2 MVOCs that were positive in the light absorption *umu* test, 3-methyl-2-butanone and 3-methyl-2-butanol, were tested for mutagenicity using the Ames preincubation method (Fig. 3). 3-Methyl-2-butanone displayed good dose dependency with both TA98 and TA100 when S9 mix was not added (-S9) and with TA98 when S9 mix was added (+S9). Because under these condi-

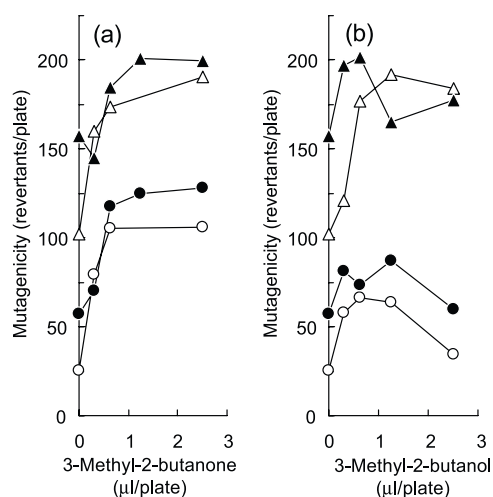


Fig. 3. Results of Ames Test of 3-Methyl-2-butanone and 3-Methyl-2-butanol

(a), 3-Methyl-2-butanone; (b), 3-methyl-2-butanol; ○, TA98 -S9; ●, TA98 +S9; △, TA100 -S9; ▲, TA100 +S9.

tions it showed 2-fold or more revertant colonies than did the control solvent, it was determined to be positive for mutagenicity. With TA100 when S9 mix was added (+S9), an increase in the number of revertant colonies corresponding to the dose was also seen, but the number was not 2-fold that of the control solvent. With 3-methyl-2-butanol on the other hand, good dose dependency was only displayed for TA98 and TA100 when S9 mix was not added (-S9). The number of revertant colonies was 2-fold or more that of the control solvent with TA98 and 1.5-fold or more with TA100, resulting in a determination of positive and pseudopositive, respectively.

Kreja and Seidel investigated the genotoxicity of 16 types of MVOCs with the comet assay using A549, V79, and pB cells.⁶ According to their results, DNA damage was caused by 1-decanol, 1-octen-3-ol, 2-methyl-1-propanol, and 2-heptanone, but because of the strong concentration range (near or greater than the IC_{50} value) for cytotoxicity, they reported that genotoxicity was not seen under the test conditions. In the current study performed with the luminescent *umu* test, among the MVOCs tested 1-octen-3-ol, 2-methyl-1-propanol, and 2-heptanone showed positive or pseudopositive results in concentration ranges at which 50% or more of bacterial propagation was not inhibited. However, 1-decanol had strong growth inhibitory effects on the test bacterium *S. typhimurium* TL210, with growth inhibition of 50% or greater seen at 4 nl/well, and detecting the SOS response was difficult. This suggests

that a similar problem exists in light absorption *umu* tests because detection is difficult when the sample has potent growth-inhibitory effects on bacteria.

In the current study, in addition to the 3 MVOCs mentioned above, SOS-inducing activity was also seen in the following 13 types of MVOCs: 3-octanol, 1-pentanol, 1-butanol, 2-methyl-1-butanol, 3-methyl-1-butanol, 3-methyl-2-butanol, 3-octanone, 2-hexanone, 2-butanone, 3-methyl-2-butanone, 2-pentanol, ethyl isobutyrate, and terpinen-4-ol. Of these, 3-methyl-2-butanone and 3-methyl-2-butanol, which were positive in both the luminescent *umu* and light absorption *umu* tests, clearly showed mutagenicity in Ames test results. These MVOCs are reported to originate in *Penicillium brevicompactum*, *Eurotium amstelodami*, and *Chaetomium globosum*.⁸ Also, with the addition of 2-methyl-1-propanol, which showed a pseudopositive result on the luminescent *umu* test, these MVOCs are known to be produced during the growth of a comparatively large number of types of filamentous fungi, including *Cladosporium*, *Aspergillus*, *Alternaria*, and *Stachybotrys*.⁷ All of these are fungi that are commonly detected in indoor environments.⁹ Therefore it is possible that these MVOCs are present in indoor environments and that long-term exposure may pose health risks. In the future, steps such as detection of known SOS-inducing MVOCs from actual environmental air and periodic monitoring are needed to understand the facts concerning contamination by such agents.

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