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ARTICLE

Contribution of 1,2-Dihydroxy-5-(methylsulfinyl)pentan-3-one (DMTS-P1) to the Formation of Dimethyl Trisulfide (DMTS) during the Storage of Japanese Sake

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Dimethyl trisulfide (DMTS) is involved in the unpalatable aroma of stale Japanese sake, called "hineka". Recently, we isolated one of the precursor compounds of DMTS in sake and identified it as 1,2-dihydroxy-5-(methylsulfinyl)pentan-3-one (DMTS-P1), a previously unknown compound. In this work, the contribution of DMTS-P1 to the formation of DMTS was investigated. DMTS-P1 was chemically synthesized from methional in three steps, consisting of the Grignard reaction, followed by oxidation by MnO_2 and an immobilized osmium oxide catalyst. The formation of synthetic DMTS-P1 was confirmed by a comparison of the liquid chromatography−mass spectrometry (LC−MS) and nuclear magnetic resonance (NMR) data to that of natural DMTS-P1. Quantitative analysis of DMTS-P1 in sake was developed using LC−MS/MS, and a positive correlation was observed between the concentration of DMTS-P1 in sake and the production of DMTS during storage. These results indicate that DMTS-P1 contributes to the formation of DMTS in sake.

KEYWORDS: Dimethyl trisulfide; DMTS; 1,2-dihydroxy-5-(methylsulfinyl)pentan-3-one; DMTS-P1; sake; hineka; LC−MS/MS

INTRODUCTION

Dimethyl trisulfide (DMTS) presents a sulfury, onion-like odor with a very low odor threshold: 0.18 μg/L in sake (*1*), 0.1 μg/L in beer (*2*), and 4 μg/L in grain whiskey (*3*). DMTS is widely distributed in vegetables and fermented foods, such as cooked broccoli (*4*), cooked onion (*5*), milk (*6*), cheese (*7*), whiskey (*3*), beer (*8*), wine (*9*), and sake (*10*), among others. Its contribution to flavor is important in some kinds of cheese (*7*) and a new-make spirit (*11*). However, excess amounts of the compound impart a characteristic off odor to whiskey, pasteurized milk (*6*), aged beer (*8*), and aged sake. The off odor resulting from the storage of sake is called "hineka" in Japanese. We previously reported that 65% of commercial sake in which "hineka" was perceived contained a level of DMTS higher than its odor threshold (*12*).

Controlling the level of DMTS has been an important issue for the producers of the above-mentioned beverages. The mechanism of the formation of DMTS has been well-studied for beer and whiskey. During the distillation of whiskey, methional in the wash is converted to DMTS via methanethiol (*11*). In the same way, DMTS is formed from methional during the storage of beer (*13*). Heat processes, such as wort boiling, promote Strecker degradation of methionine, which results in the formation of methional. *S*-methylcysteine sulfoxide derived from hops is another precursor of DMTS in beer (*2*). The following reaction mechanism has been postulated: β-elimination of *S*-methylcysteine sulfoxide results in the formation of unstable methanesulfenic acid, which forms DMTS.

The formation of DMTS is usually concomitant with the formation of dimethyl disulfide (DMDS), although the sensory contribution of DMDS to sake is not as significant as that of DMTS because of its higher odor-threshold value (*10*). These polysulfides are considered to have a common pathway of formation (*11*).

Sato et al. studied the mechanism of the formation of DMDS in sake (*14*). They separated the components of sake into acidic, basic, and neutral fractions and found that DMDS was produced from each fraction. Furthermore, they found promoting and inhibitory effects among all fractions. However, a precursor compound other than methionine and cysteine was not identified.

Recently, we demonstrated that Strecker degradation of methionine played a minor role in the formation of DMTS through the experiments using [methyl-d_3]-methionine. Thus, we explored precursor compounds of DMTS by fractionating sake and measuring the DMTS-producing potential of the fractions. As a result, we identified one of the precursors as 1,2-dihydroxy-5-(methylsulfinyl)pentan-3-one (DMTS-P1)(*15*). It was estimated that the

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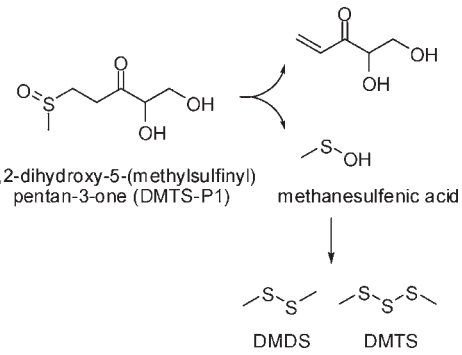

Figure 1. Proposed pathway of the formation of polysulfides from DMTS-P1.

Table 1. Profiles of Samples

sample	category[a,b]	alcohol[a] (%)	polishing rate of rice[a,c] (%)
A	junmai ginjo shu	15−16	50
B	junmai ginjo shu	15−16	50
C	junmai ginjo shu	15−16	50
D	junmai shu	15−16	65
E	junmai shu	15	65
F	ginjo shu	15	55
G	honjozo shu	15−16	70
H	honjozo shu	15−16	65
I	josen	15−16	
J	josen	13−14	
K	gousei shu	13−14	
L	gousei shu	15−16	

[a] Values stated on the labels. [b] The details of the category are as follows: "Junmai shu" and "junmai ginjo shu" are only made from rice and koji. "Ginjo" means that sake was brewed with highly polished rice and fermented at low temperature. In "ginjo shu" and "honjozo shu", a small amount of alcohol is added. More alcohol is added in "josen". "Gousei shu" is made by mixing alcohol, glucose, amino acids, and organic acids, among others. [c] The polishing rate is the rate of the weight of polished rice/brown rice. The polishing rate of the rice used for "ginjo shu" is lower than 60%.

methylsulfoxide moiety of DMTS-P1 was removed in the form of methanesulfenic acid through β-elimination and is involved in the formation of DMTS (**Figure 1**), as it is in *S*-methylcysteine sulfoxide (*2*).

However, DMTS-P1 was not commercially available because it was a previously unknown compound. We therefore devised a synthetic route of DMTS-P1 to study the contribution of DMTS-P1 to DMTS formation. Here, we report the preparation of synthetic DMTS-P1 and the development of a method for quantifying DMTS-P1. Using this developed method, we have investigated the contribution of DMTS-P1 to the formation of DMTS during the storage of sake.

MATERIALS AND METHODS

Sake Samples. Commercial sake samples were purchased from a local market. The profiles of the samples are listed in **Table 1**.

Chemicals. Osmium oxide immobilized catalyst I (Os IC-I), 4-methylmorpholine 4-oxide monohydrate (NMO), sodium sulfate, yttrium standard solution (1 mg/mL), 1,5-pentanediol, L-methionine, succinic acid, ethanol, acetone, acetonitrile (CH_3CN, HPLC grade), and D_2O (NMR grade) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Deuterated acetonitrile (CD_3CN) was from Cambridge Isotope Laboratories, Inc. (Andover, MA). Dowex 50W-X4 (200−400 mesh) was purchased from Muromachi Technos Co. Ltd. (Tokyo, Japan). Ultrapure water was obtained by a MilliQ water purification system (Millipore, Bedford, MA). Natural DMTS-P1 was prepared from sake as previously described (*15*).

Liquid Chromatography−Mass Spectrometry (LC−MS). A Surveyor high-performance liquid chromatography (HPLC) system, coupled with a LCQ Advantage ion-trap mass spectrometer equipped with electrospray ionization (ESI), was used (Thermo Fisher Scientific, Waltham, MA). A total of 10 μL of the sample was injected onto a BDS Hypersil C18 column (3.0 × 150 mm, Thermo Fisher Scientific). Ultrapure water was used as the mobile phase at a flow rate of 0.3 mL/min. The eluent was introduced to ESI (positive)-ion-trap mass spectrometry. Ion spray voltage was set to 5.0 kV. The sheath gas pressure was 30 arb ("arbitrary units" according to the equipment procedure), and the capillary temperature was 250 °C. The mass spectrometer was operated in full-scan mode, monitoring positive ions ranging from 100 to 500 amu.

Nuclear Magnetic Resonance (NMR) Spectroscopy. ^{1}H NMR spectra of 5-(methylsulfanyl)pent-1-en-3-ol and 5-(methylsulfanyl)pent-1-en-3-one were recorded with a JEOL JMM-PM × 60 spectrometer, using tetramethylsilane (TMS) as an internal standard. The solvent used was $CDCl_3$. One- and two-dimensional NMR experiments for DMTS-P1 were performed on a JEOL ECP-500 spectrometer according to a procedure described previously (*15*). Both natural and synthetic DMTS-P1 were dissolved in CD_3CN/D_2O (80:20). The resonances of CD_3CN at δ 1.93 and 1.28 ppm were used as internal standards for NMR spectra.

Gas Chromatography (GC). A shimadzu GC-17A gas chromatograph was used to check the purity of 5-(methylsulfanyl)pent-1-en-3-ol and 5-(methylsulfanyl)pent-1-en-3-one. A flame photometric detector (FID) or a mass spectrometer QP-5000 was used as a detector. The conditions of the analysis were as follows: column, DB-5MS (30 m × 0.25 mm inner diameter, 0.25 μm film thickness); oven, 50 °C for 3 min, raised at 10 °C/min to 280 °C, followed by a 5 min isotherm; injector temperature, 270 °C; FID temperature, 250 °C.

Synthesis of DMTS-P1. The synthesis pathway of DMTS-P1 from methional is summarized in **Figure 2**. 5-(Methylsulfanyl)pent-1-en-3-one was synthesized by Chemical Soft R&D, Inc. (Kyoto, Japan).

5-(Methylsulfanyl)pent-1-en-3-ol. To a stirred solution of methional (6.04 g, 58.0 mmol) in tetrahydrofuran (THF) (80 mL), a solution of vinylmagnesium bromide (1 M, 69.6 mmol) was slowly added under cooling from −5 to 0 °C. The solution was stirred for 2 h under argon at room temperature. Saturated NH_4Cl (500 mL) was added to quench the reaction, and the pH was adjusted to 3 with 0.1 N HCl (about 1 L). The reaction mixture was extracted with ethyl acetate (3 L). The organic portion was washed with brine (1.5 L), dried over anhydrous Na_2SO_4, and filtrated. The solvent was concentrated to a residue, which was purified by distillation (81 °C, 3−5 mmHg). 5-(Methylsulfanyl)pent-1-en-3-ol was obtained as a colorless liquid. The purity of the compound obtained, determined by GC−FID, was 98%. The amount of obtained counpound was 3.73 g (yield 49%).

^{1}H NMR δ: 1.53−1.90 (m, 2H, H-4), 2.06 (s, 3H, H-6), 2.40−2.60 (m, 2H, H-5), 3.30 (s, 1H, hydroxyl proton), 4.15 (dd, 1H, J = 6, 12 Hz, H-3), 4.96 (dd, 1H, J = 2, 6 Hz, H-1$_\alpha$), 5.20 (dd, 1H, J = 2, 12 Hz, H-1$_\beta$), 5.67 (dd, 1H, J = 6, 12 Hz, H-2).

5-(Methylsulfanyl)pent-1-en-3-one. To a solution of 5-(methylsulfanyl)-pent-1-en-3-ol (7.23 g, 54.7 mmol) in methylene chloride, MnO_2 (102 g, 1.2 mol) was added. The mixture was stirred under argon at room temperature for 24 h. The precipitate was filtered, and the solvent was removed to obtain a red liquid containing 5-(methylsulfanyl)pent-1-en-3-one. The purity and amount of the compound obtained was 96% (GC−FID) and 5.10 g (yield 73%), respectively.

^{1}H NMR δ: 2.13 (s, 3H, H-6), 2.60−3.10 (m, 4H, H-4, H-5), 5.66 (m, 1H, H-1$_\alpha$), 6.13−6.30 (m, 2H, H-1$_\beta$, H-2).

GC−MS (EI) m/z: 130 $[M]^+$, 115 $[M - CH_3]^+$, 82 $[M - CH_3SH]^+$, 75 $[M - CH_2{=}CHC{=}O]^+$.

DMTS-P1. To a solution of 5-(methylsulfanyl)pent-1-en-3-one (429 mg, 3.3 mmol) and NMO (672 mg, 5.0 mmol) in acetone/water/acetonitrile (40 mL, 1:1:1), Os IC-I (444 mg, 0.16 mmol) was added and the solution was stirred for 24 h at room temperature. The catalyst was filtered with a glass filter, and the filtrate was applied to 20 mL of Dowex 50W-X4 resin packed in a plastic column to absorb NMO. The resin was washed with ultrapure water. The eluate was combined, concentrated *in vacuo*, and lyophilized to obtain a crude product. The crude product was dissolved in 5 mL of ultrapure water and analyzed by LC−MS. As shown in **Figure 3**, the presence of DMTS-P1 and byproduct was observed. Thus, the crude product was purified by column chromatography using a reversed-phase column (TSKgel ODS-80Ts, 21.5 × 300 mm, TOSOH, Tokyo, Japan) and an ion-exclusion column (IC-Pak Ion-Exclusion, 7.8 × 300 mm, Waters,

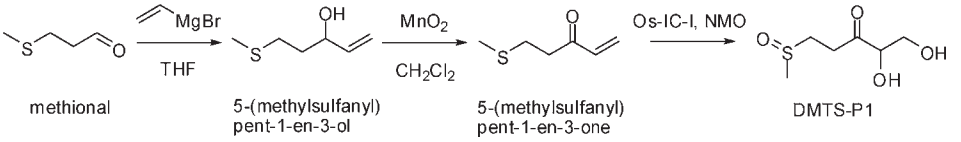

Figure 2. Synthetic pathway from methional to DMTS-P1.

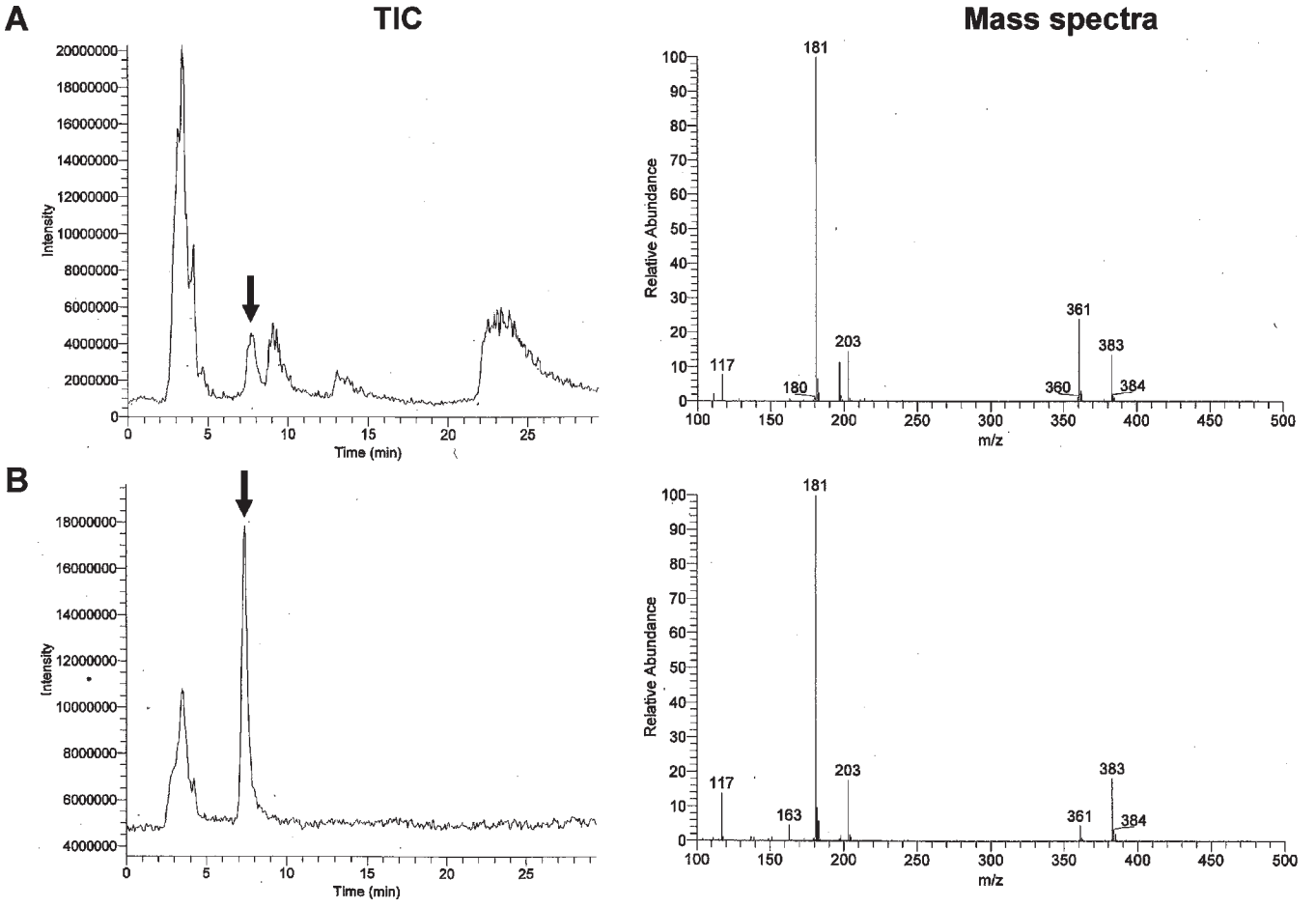

Figure 3. Comparison of LC−MS data of (A) synthesized crude DMTS-P1 and (B) natural DMTS-P1. Mass spectral data represent the peak indicated by arrows on the total ion chromatogram (TIC).

Milford, MA). The conditions of the chromatography have been reported previously (*15*). The presence of DMTS-P1 in the fractions was monitored by LC−MS following the procedure described above. After separation by the ion-exclusion column, the fractions containing pure DMTS-P1 were combined and lyophilized to give the title compound as a colorless syrupy solid. The amount of DMTS-P1 obtained was determined to be 0.028 mmol (0.8% yield) by an inductively coupled plasma−atomic emission spectrometer (ICP−AES) as described below. The spectral data of synthetic DMTS-P1 were as follows. ^{1}H NMR δ: 2.57 (s, 3H, H-6), 2.87 (m, 1H, H-5$_\alpha$), 3.01 (m, 2H, H-4), 3.06 (m, 1H, H-5$_\beta$), 3.70 (dd, 1H, J = 3.1, 12.0 Hz, H-1$_\alpha$), 3.78 (dd, 1H, J = 3.1, 12.0 Hz, H-1$_\beta$), 4.21 (t, 1H, J = 3.8 Hz, H-2). ^{13}C NMR δ: 31.0 (C-4, CH_2), 37.0 (C-6, CH_3), 46.1 (C-5, CH_2), 63.1 (C-1, CH_2), 77.7 (C-2, CH), 210.3 (C-3, C=O). Correlation spectroscopy (COSY) data: H-1 → H-2; H-2 → H1; H-4 → H5; H-5 → H-4. Heteronuclear multiple-quantum coherence (HMQC) data: H-1 → C-1; H-2 → C-2; H-4 → C-4; H-5 → C-5; H-6 → C-6. Heteronuclear multiple-bond correlation (HMBC) data: H-1 → C-3; H-2 → C-3; H-4 → C-3, 5; H-5 → C-3, 4, 6; H-6 → C-5. LC−MS (ESI$^+$) m/z: 181 [M + H]$^+$, 203 [M + Na]$^+$, 361 [2 M + H]$^+$, 383 [2 M + Na]$^+$, 117 [M − S(O)Me]$^+$.

Determination of the Concentration of Synthetic DMTS-P1 by ICP. On the basis of its molecular formula containing one sulfur atom, the amount of the synthetic DMTS-P1 was calculated by measuring sulfur content using an ICPS 1000IV instrument (Shimadzu, Kyoto, Japan). Lyophilized DMTS-P1 was dissolved in ultrapure water and diluted. Then, yttrium (1 mg/L) was added as an internal standard. The calibration curve for sulfur was established with a standard solution containing sodium sulfate (0.02−0.2 mM). The conditions of the analysis were as follows: frequency, 27.12 MHz; forward power, 1.2 kW; reflected power, 0 kW; argon gas flow rate: coolant, 14.0 L/min; plasma, 1.2 L/min; carrier gas, 0.8 L/min; purge gas flow, 0.8 L/min; axial observation: height, 15 mm; analytical wavelength: *S*, 180.731 nm; *Y*, 371.029 nm.

Quantitative Analysis of DMTS-P1 by LC−MS/MS. A total of 1 mL of sake sample was diluted 2-fold with ultrapure water, and 50 μL of a solution of 1000 mg/L 1,5-pentanediol was added as an internal standard. The solution was applied to 1 mL of Dowex 50W-X4 resin (H^+ form) packed in a 3 mL plastic column. The resin was washed with 6 mL of ultrapure water. The eluate was combined, lyophilized, and dissolved in 1 mL of ultrapure water. After filtration, 10 μL of the sample was applied to the LC−MS system mentioned above. For the MS/MS analysis, the collision energy was set to 30%. The ion transitions from m/z 181 to 117 and m/z 105 to 87 were selected for monitoring DMTS-P1 and 1,5-pentanediol, respectively. The calibration curve was determined using sake sample K (gousei shu) supplemented with DMTS-P1 ranging from 0 to 2.0 mg/L. Only a trace amount of DMTS-P1 was detected in sample K. The quantification and detection limits of DMTS-P1 were 0.07 and 0.02 mg/L, respectively, which were determined from eight analyses of the lowest calibration sample. The relative standard deviation (RSD) was calculated to be 12% from five analyses of sake sample H. The recovery

rate, determined by spiking the sake samples A−J with 1 mg/L of DMTS-P1, ranged from 45 to 67%, and the recovery rate of sample L (gousei shu) was 91%. The concentration calculated from the calibration curve was corrected by the recovery rate of each sample. The amount of DMTS-P1 in dried koji was determined as follows: 1 g of koji was extracted with 5 mL of ultrapure water overnight; the extract was then lyophilized and diluted in 1 mL of ultrapure water. The extract from dried koji was analyzed in the same way as the sake sample.

Accelerated Aging of Sake or Buffer Solution Supplemented with DMTS Precursors. The concentration of methionine, methional, and DMTS-P1 in sake sample A was measured. An equal concentration of each compound was added to sake sample A and a buffer solution (10 mM succinate buffer at pH 4.0 containing 16% ethanol) with a composition similar to that of sake. As for DMTS-P1, both natural and synthetic compounds were used. These samples contained no detectable amount of DMTS before aging. To investigate the relationship between the added DMTS-P1 and the production of DMTS during storage, different concentrations of DMTS-P1 were added to sake samples A, D, and G and to the buffer solution. A total of 9 mL of each sample was put in a 10 mL glass vial and sealed with a PTFE/silicon septum. Accelerated aging was carried out by incubating the vial at 70 °C for a week. DMTS was analyzed by GC−MS as reported previously (*10*), except that the injection was carried out in splitless mode.

Accelerated Aging of Commercial Sake Samples. The samples listed in **Table 1** were placed in a vial as described above. Accelerated aging was carried out by incubating the vial at 70 °C for a week or 45 °C for a month.

Small-Scale Sake Brewing. Small-scale sake brewing was carried out using 300 g of total rice, including dried rice and dried koji (Tokushima Seikiku, Awa, Japan), and *Saccharomyces cerevisiae* K-901 yeast (Nippon Jozokyokai, Tokyo, Japan) according to a method reported by Nanba et al. (*16*). Yeast (9×10^8 cells) was added to a mixture of 15 g of dried koji, 86 mL of water, and 1.68 mL of 7% lactic acid at 15 °C ("mizu-koji" in Japanese). The next day, 38 g of dried rice and 11 mL of water were added (the first addition, "soe"). After 2 days, 83 g of dried rice, 15 g of dried koji, and 140 mL of water were added and the mixture was fermented at 9 °C (the second addition, "naka"). The following day, 120 g of dried rice, 30 g of dried koji, and 237 mL of water were added and the mixture was fermented at 7 °C (the third addition, "tome"). The fermentation temperature was increased by 1.0 °C/day to a maximum temperature of 15 °C. The sake was separated from the solid by centrifugation (9040*g* for 20 min) on day 23 after the third addition. After 4 weeks, the sake was filtered using a membrane filter (0.45 μm) and pasteurized at 63 °C for 5 min. Two batches of mash were prepared: one for the measurement of weight loss as the amount of carbon dioxide emission and the other for the measurement of DMTS-P1. For the analysis of DMTS-P1, samples of mash were taken every 4 days and centrifuged (9850*g* for 15 min). The supernatant was used for DMTS-P1 measurement.

Other Chemical Analysis. The concentration of methionine was measured using a JLC-500 amino acid analyzer (JEOL). Analysis of methional was carried out as described (*10*).

RESULTS AND DISCUSSION

Synthesis of DMTS-P1. DMTS-P1 contains diol, carbonyl, and sulfinyl groups. Therefore, we designed a route for the synthesis of DMTS-P1 from methional using the different oxidizing agents shown in **Figure 2**. According to the synthesis of alkenyl alcohol (*17*), the Grignard reaction of methional and vinylmagnesium bromide produced 5-(methylsulfanyl)pent-1-en-3-ol in 49% yield. Selective oxidation of the secondary alcohol of 5-(methylsulfanyl)-pent-1-en-3-ol by manganese(IV) oxide yielded the corresponding ketone. This ketone, 5-(methylsulfanyl)pent-1-en-3-one, was further converted to the corresponding 1,2-diol by treatment with osmium-(VIII) oxide (OsO_4), which is one of the most reliable and efficient methods for the synthesis of 1,2-diols. Instead of highly volatile and toxic OsO_4, we used immobilized OsO_4 on the polymer (Os IC-I) (*18*). Fortunately, the use of Os IC-I as an oxidizing reagent in the reaction of the ketone led directly to the formation of DMTS-P1, as confirmed by LC−MS analysis of the crude products (**Figure 3**).

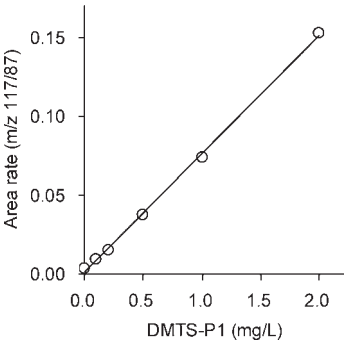

Figure 4. Calibration curve for the analysis of DMTS-P1 in sake.

The reaction mixture was then purified by a cation-exchange resin, a reversed-phase column, and an ion-exclusion column to give DMTS-P1 in 0.8% yield. NMR experiments were carried out for the purified synthetic DMTS-P1, and the results were compared to those of natural DMTS-P1(*15*). The ^{1}H NMR, ^{13}C NMR, ^{1}H−^{1}H COSY, HMQC, and HMBC signals were consistent between synthetic and natural DMTS-P1 (data not shown).

Development of a Quantification Method for DMTS-P1 by LC−MS/MS. In the LC−MS analysis of DMTS-P1, a peak at *m*/*z* 117 was observed (**Figure 3**); this peak was considered to be a breakdown product, most likely corresponding to the loss of a methylsulfoxide moiety $[M - S(O)Me]^+$ (*15*). Thus, the ion transition from *m*/*z* 181 $[M + H]^+$ to 117 was monitored for the detection of DMTS-P1. 1,5-Pentanediol was chosen as an internal standard, showing an ion transition from *m*/*z* 105 $[M + H]^+$ to 87 $[M - H_2O]^+$ in its mass spectra.

When a sake sample was pretreated with a cation-exchange resin before LC−MS/MS analysis, the peak of DMTS-P1 was about 2-fold higher as compared to a sample without the pretreatment with cation-exchange resin (data not shown). We considered that some cation components inhibited ionization of DMTS-P1 in the LC−MS/MS analysis. Thus, each sample was passed through a cation-exchange resin (Dowex 50W-X4) before being applied to LC−MS/MS analysis.

A calibration curve for DMTS-P1 was established by analyzing six sample solutions of sake sample K spiked with a known amount of DMTS-P1 and the internal standard. The base sake sample, sample K, contained only a trace amount of DMTS-P1. A linear correlation was observed between the concentration of DMTS-P1 and the ratio of the area of DMTS-P1/1,5-pentanediol over the concentration range examined (**Figure 4**).

Contribution of DMTS-P1 to the Formation of DMTS. The contribution of DMTS-P1 to the formation of DMTS was compared to that of methionine and methional, which have been reported as precursors of DMTS (*13*, *14*). The concentration of DMTS-P1, methionine, and methional in commercial sake A was 0.65 mg/L, 6 mg/L, and 4 μg/L, respectively. An equal concentration of these compounds was separately added to the buffer solution and sake A.

In the case of the buffer solution, the formation of DMTS after accelerated aging was confirmed only in the samples spiked with DMTS-P1 (**Table 2**). The amount of DMTS produced from synthesized DMTS-P1 added to the buffer solution was 0.09 ± 0.03 μg/L (mean ± SD), and this amount was almost the same as that produced from natural DMTS-P1 (0.08 ± 0.03 μg/L). No detectable amount of DMTS was produced in buffer samples spiked with methionine or methional.

When sake sample A was spiked with these compounds, the production of DMTS by accelerated aging was approximately 2-fold higher in the sample spiked with DMTS-P1 (**Table 2**),

Table 2. Production of DMTS by Accelerated Aging (70 °C for a Week) of the Buffer Solution and Sake Sample A Supplemented with DMTS Precursors

		DMTS production[a] (μg/L)	
added compounds	added amount (mg/L)	buffer	sake
none		nd[b]	0.8 ± 0.06
methionine	6.0	nd	1.0 ± 0.11
methional	0.0042	nd	0.9 ± 0.11
DMTS-P1 (natural)	0.65	0.08 ± 0.03	1.6 ± 0.19
DMTS-P1 (synthesized)	0.65	0.09 ± 0.03	1.5 ± 0.12

[a] Values are means ± SD (n = 3). [b] Not detected.

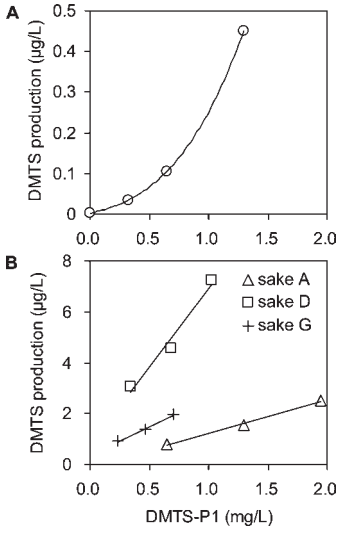

Figure 5. Production of DMTS by accelerated aging (70 °C for a week) of (A) the buffer solution and (B) sake supplemented with various concentrations of DMTS-P1.

which indicated that DMTS production was doubled (0.8 → 1.6 μg/L) when the concentration of DMTS-P1 in sake sample A was doubled (0.65 → 1.3 mg/L). The addition of methionine to sake sample A slightly increased the production of DMTS. Methional did not affect the formation of DMTS at the concentration added to sake sample A.

Although the contribution of DMTS-P1 to DMTS production was greater than that of methionine or methional contained in sake sample A, only 0.2% (molar ratio) of the DMTS-P1 added to sake sample A was converted to DMTS. When we analyzed the sake sample after accelerated aging, only a trace amount of DMTS-P1 was detected (data not shown). These results indicated that DMTS-P1 was converted not only to DMTS but also to other compounds. Such an observation has also been reported for *S*-methylcysteine sulfoxide, a primary precursor of DMTS in *Brassica* and *Allium* vegetables. The conversion ratio of this compound into DMTS was below 2% when it was heated under various conditions, and many kinds of volatile compounds were generated (*19*).

Difference in DMTS Production between Buffer Solution and Sake. Next, we investigated DMTS production during storage by spiking both sake and buffer solution with various concentrations of DMTS-P1. As shown in **Figure 5A**, a quadratic relationship was observed between the production of DMTS and the concentration of DMTS-P1 in the buffer solution. Considering the structure of DMTS, which contains three sulfur atoms, it seems reasonable that more than one molecule of DMTS-P1 is responsible for the formation of one molecule of DMTS. On the other hand, DMTS production increased linearly with the concentration of DMTS-P1 when DMTS-P1 was added to sake samples A, D, and G (**Figure 5B**). In addition, the increment in DMTS production that followed the increase in the concentration of DMTS-P1 from 0.65 to 1.3 mg/L was compared between the buffer solution and sake sample A. As shown in **Figure 5**, a greater increment in DMTS production was observed in sake sample A (0.77 μg/L) than in the buffer solution (0.34 μg/L). Furthermore, the increment in DMTS production following the addition of DMTS-P1 to sake samples D and G was even greater than that in sake sample A (**Figure 5B**). These results suggest the presence of some components that promote the formation of DMTS from DMTS-P1 in sake. The elucidation of such components may provide another tool to regulate DMTS formation.

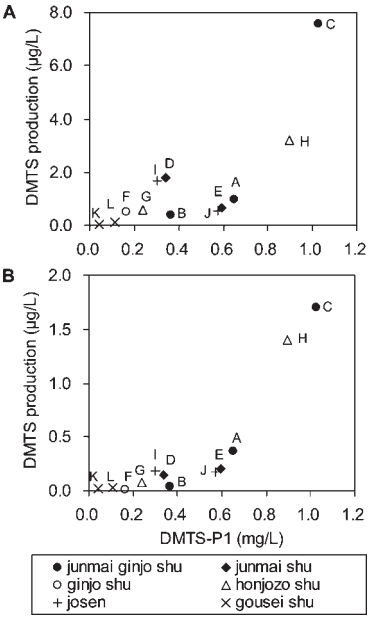

Figure 6. Relationship between the DMTS-P1 concentration and DMTS production by accelerated aging at (A) 70 °C for a week or (B) 45 °C for a month.

Relationship between the Concentration of DMTS-P1 in Commercial Sake and DMTS Production by Accelerated Aging. The concentration of DMTS-P1 in 12 commercial sake samples, as listed in **Table 1**, ranged from trace (below 0.07 mg/L) to 1.0 mg/L. These 12 samples were incubated under two conditions, 70 °C for a week and 45 °C for a month, and the production of DMTS was measured. As shown in **Figure 6**, the higher the concentration of DMTS-P1 in sake, the higher the production of DMTS by accelerated aging. The correlation coefficient between the DMTS-P1 concentration and DMTS production at 45 °C (r = 0.87) was higher than that at 70 °C (r = 0.75). We considered that DMTS-P1 had more influence on the formation of DMTS under conditions closer to those of the normal storage.

Recently, Okuda et al. reported that the total sulfur content in rice grain correlated with the production of polysulfides during the storage of sake (*20*). Rice with a lower polishing rate, that is, highly polished rice, usually contains a lower amount of sulfur because the protein content decreases with polishing. In this study, however, sake sample C (junmai ginjo shu) showed the highest level of both DMTS-P1 and DMTS production among the samples investigated (**Figure 6**), although the polishing rate was relatively low (**Table 1**). These results suggested the presence of factors affecting the level of DMTS-P1 in addition to rice components.

Formation of DMTS-P1 during the Sake-Brewing Process. Although DMTS-P1 was barely detected in koji (steamed rice

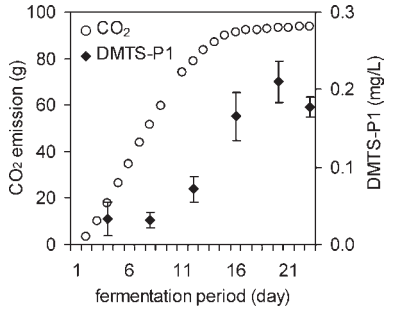

Figure 7. Changes in the concentration of DMTS-P1 (means ± SD; $n = 3$) and CO_2 emission during sake fermentation.

inoculated with *Aspergillus oryzae*), its concentration gradually increased during the fermentation period (**Figure 7**). Therefore, it seems that DMTS-P1 is mainly produced during fermentation. This may be why there was a low level of DMTS-P1 in gousei shu (**Figure 6**), which is made by mixing alcohol, glucose, amino acids, and organic acids, among others, and adding a little amount of sake for flavoring. These data made us speculate about the involvement of yeast in the formation of DMTS-P1. We previously pointed out the structural similarity between DMTS-P1 and 1,2-dihydroxy-5-(methylsulfanyl)pent-1-en-3-one, an intermediate compound of the methionine salvage pathway (*15*). All of the genes encoding the enzymes of this pathway have been identified in the *S. cerevisiae* genome (*21*). Recently, Kanai et al. reported that the addition of magnesium sulfate to a moromi mash reduced both the concentration of amino acids, especially methionine, and the production of DMTS during the storage of sake (*22*). The correlation between the production of DMTS and the content of sulfur-containing amino acids in sake has also been reported by Okuda et al. (*23*). When these findings are taken together, they suggest the involvement of the metabolism of sulfur-containing amino acids in yeast in the formation of DMTS precursors. Genetic strategies, such as gene disruption and gene chip analysis, will be needed to confirm this hypothesis. Elucidation of the mechanism of DMTS-P1 formation will enable the control of "hineka" in sake.

ABBREVIATIONS USED

DMTS, dimethyl trisulfide; DMDS, dimethyl disulfide; Os IC-I, osmium oxide immobilized catalyst I; NMO, 4-methylmorpholine 4-oxide; CH_3CN, acetonitrile; CD_3CN, deuterated acetonitrile; D_2O, deuterium oxide; THF, tetrahydrofuran; ICP−AES, inductively coupled plasma−atomic emission spectrometer; LC−MS, liquid chromatography−mass spectrometry; ESI, electron spray ionization; TIC, total ion chromatogram; NMR, nuclear magnetic resonance; COSY, correlation spectroscopy; HMQC, heteronuclear multiple-quantum coherence; HMBC, heteronuclear multiple-bond correlation.

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LITERATURE CITED

(1) Utsunomiya, H.; Isogai, A.; Iwata, H. Measurement of thresholds for reference compounds for the sensory profiling of sake (part 1). *J. Soc. Brew. Jpn.* **2004**, *99*, 652–-658.

(2) Peppard, T. L. Dimethyl trisulfide, its mechanism of formation in hop oil and effect on beer flavour. *J. Inst. Brew.* **1978**, *84*, 337–340.

(3) Lee, K.-Y. M.; Paterson, A.; Piggott, J. R. Measurement of thresholds for reference compounds for sensory profiling of Scotch whisky. *J. Inst. Brew.* **2000**, *106*, 287–294.

(4) Maruyama, F. T. Identification of dimethyl trisulfide as a major aroma component of cooked brassicaceous vegetables. *J. Food Sci.* **1970**, *35*, 540–543.

(5) Tokimoto, Y. Volatile components of cooked onions. *Nippon Shokuhin Kagaku Kogaku Kaishi* **1995**, *42*, 279–287.

(6) Vazquez-Landaverde, P. A.; Torres, J. A.; Qian, M. C. Quantification of trace volatile sulfur compounds in milk by solid-phase microextraction and gas chromatography-pulsed flame photometric detection. *J. Dairy Sci.* **2006**, *89*, 2919–2927.

(7) Curioni, P. M. G.; Bosset, J. O. Key odorants in various cheese types as determined by gas chromatography−olfactometry. *Int. Dairy J.* **2002**, *12*, 959–984.

(8) Williams, R. S.; Gracey, D. E. F. Beyond dimethyl sulfide: The significance to flavor of thioesters and polysulfides in Canadian beers. *J. Am. Soc. Brew. Chem.* **1982**, *40*, 68–71.

(9) Guth, H. Identification of character impact odorants of different white wine varieties. *J. Agric. Food Chem.* **1997**, *45*, 3022–3026.

(10) Isogai, A.; Utsunomiya, H.; Kanda, R.; Iwata, H. Changes in the aroma compounds of sake during aging. *J. Agric. Food Chem.* **2005**, *53*, 4118–4123.

(11) Prentice, R. D.; McKernan, G.; Bryce, J. H. A source of dimethyl disulfide and dimethyl trisulfide in grain spirit produced with a coffey still. *J. Am. Soc. Brew. Chem.* **1998**, *56*, 99–103.

(12) Isogai, A.; Utsunomiya, H.; Kanda, R.; Iwata, H.; Nakano, S. Aroma compounds responsible for "hineka" in commercial sake. *J. Soc. Brew. Jpn.* **2006**, *101*, 125–131.

(13) Gijis, L.; Perpete, P.; Timmermans, A.; Collin, S. 3-Methylthiopropionaldehyde as a precursor of dimethyl trisulfide in aged beers. *J. Agric. Food Chem.* **2000**, *48*, 6196–6199.

(14) Sato, S.; Tadenuma, M.; Takahashi, K.; Koike, K. Flavor changes during sake aging (part 5). *J. Soc. Brew. Jpn.* **1975**, *70*, 588–591.

(15) Isogai, A.; Kanda, R.; Hiraga, Y.; Nishimura, T.; Iwata, H.; Goto-Yamamoto, N. Screening and identification of precursor compounds of dimethyl trisulfide (DMTS) in Japanese sake. *J. Agric. Food Chem.* **2009**, *57*, 189–195.

(16) Nanba, K.; Obata, T.; Kayashima, S.; Yamazaki, Y.; Murakami, M.; Shimoda, T. The setting of the small sake brewing. *J. Soc. Brew. Jpn.* **1978**, *73*, 295–300.

(17) Tonari, K.; Gotoh, S.; Furukawa, H. Synthesis and flavor evaluation of matsutake alcohol homologs. *Kinki Shokugyo Noryoku Kaihatsu Daigakko Kiyo* **2001**, *9*, 13–15.

(18) Kobayashi, S.; Endo, M.; Nagayama, S. Catalytic asymmetric dihydroxylation of olefins using a recoverable and reusable polymer-supported osmium catalyst. *J. Am. Chem. Soc.* **1999**, *121*, 11229–11230.

(19) Kubec, R.; Drhova, V.; Velisek, J. Thermal degradation of *S*-methylcysteine and its sulfoxide-important flavor precursors of *Brassica* and *Allium* vegetables. *J. Agric. Food Chem.* **1998**, *46*, 4334–4340.

(20) Okuda, M.; Isogai, A.; Joyo, M.; Goto-Yamamoto, N.; Mikami, S. Influence of sulfur and nitrogen content of rice grains on flavor in stored sake. *Cereal Chem.* **2009**, *86*, 534–541.

(21) Pirkov, I.; Norbeck, J.; Gustafsson, L.; Albers, E. A complete inventory of all enzymes in the eukaryotic methionine salvage pathway. *FEBS J.* **2008**, *275*, 4111–4120.

(22) Kanai, M.; Seo, Y.; Murakami, Y.; Isogai, A.; Iwata, H.; Mukai, N.; Fujii, T.; Iefuji, H. Proceedings of the Convention of the Brewing Society of Japan, **2008**; p 10.

(23) Okuda, M.; Isogai, A.; Joyo, M.; Goto-Yamamoto, N.; Mikami, S. Relationship between the concentrations of nitrogen/sulfur compounds in sakes before storing and those of polysulfides in sakes after storing. *J. Soc. Brew. Jpn.* **2009**, *104*, 131–141.

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