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Screening and Identification of Precursor Compounds of Dimethyl Trisulfide (DMTS) in Japanese Sake

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Dimethyl trisulfide (DMTS) is involved in the unpalatable aroma of stale sake, called "hineka"; however, the mechanism underlying the formation of DMTS during the storage of sake has not been elucidated. This paper investigates the precursors of DMTS in sake. An experiment using [methyl-d_3]-methionine showed that Strecker degradation of methionine plays a minor role in the formation of DMTS. Separation of components in sake by cation exchange resin revealed that DMTS precursors are present in the acidic/neutral fraction rather than in the basic one. Purification of the DMTS precursor compounds was carried out through several chromatographic steps, measuring DMTS-producing potential as an index. High-resolution ESI-MS and 1D/2D NMR experiments enabled the identification of one of the precursor compounds as 1,2-dihydroxy-5-(methylsulfinyl)pentan-3-one.

KEYWORDS: Dimethyl trisulfide; DMTS; 1,2-dihydroxy-5-(methylsulfinyl)pentan-3-one; sake; hineka; flavor precursor

INTRODUCTION

Storing sake for a certain period (from several months to a few years) causes deterioration of its aroma. This stale sake aroma is called "hineka" in Japanese. It is considered that hineka consists of many kinds of volatile compound (*1*). Recently, we demonstrated that dimethyl trisulfide (DMTS) is one of the main components that contribute to hineka (*2*, *3*). DMTS presents a sulfury, onion-like odor and is widely distributed in foods and beverages such as cooked onion (*4*), broccoli (*5*), milk (*6*), cheese (*7*), whiskey (*8*), beer (*9*), and wine (*10*).

Several pathways of DMTS formation have been reported, and different compounds have been presented as precursors of DMTS. One of the precursor compounds of DMTS is *S*-methylcysteine sulfoxide, a compound that is widely distributed in plants such as *Allium* and *Brassica* vegetables (*11*, *12*). Degradation of this compound by the action of cysteine sulfoxide lyases or heat processes results in the formation of DMTS (*11*, *13*). Another precursor of DMTS in plants is sulforaphane (4-methylsulfinylbutyl isothiocyanate), a compound that is contained in *Brassica* vegetables such as broccoli (*14*). Thermal or enzymatic degradation of this compound results in various sulfur-containing compounds, including dimethyl disulfide (DMDS) and DMTS (*15*, *16*).

DMTS also contributes to the "ripened" aroma of cheeses such as Camembert and Cheddar. The origin of DMTS in cheese is the oxidation of methanethiol, a product of the degradation of methionine by bacteria. It has been reported that many kinds of microorganisms are able to produce methanethiol from methionine (*7*).

During the distillation of whiskey, methional in the wash is converted to DMDS and DMTS via methanethiol (*17*). Methional has also been reported as a precursor of DMTS formed during the storage of beer (*18*). Strecker degradation of methionine is considered to be a source of methional (*8*, *9*). Heat processes such as wort boiling promote Strecker degradation of methionine (*18*). *S*-Methylcysteine sulfoxide from hop is another precursor of DMTS in beer (*19*).

The formation of DMDS during the storage of sake was investigated by Sato et al. (*20*). They separated the components of sake into acidic, basic, and neutral fractions and assessed their contribution to the formation of DMDS. They found that DMDS was produced from each fraction; however, precursor compounds other than methionine and cysteine were not

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identified. Furthermore, the mechanism of the formation of DMTS in sake has not been studied at all. The elucidation of the mechanism of DMTS formation is necessary for controlling the generation of hineka in sake.

In this study, we separated the components in sake and examined the responsibility for the formation of DMTS. We also identified one of the precursor compounds of DMTS in sake.

MATERIALS AND METHODS

Chemicals. L-Methionine, ammonia solution (25%), succinic acid, formic acid, ethanol, methanol (HPLC grade), CH_3CN (HPLC grade), and D_2O (NMR grade) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). [*methyl*-d_3]-Methionine and acetonitrile-d_3 were from Cambridge Isotope Laboratories, Inc. (Andover, MA). Dowex 50WX4 (200−400 mesh) was purchased from Muromachi Technos Co. Ltd. (Tokyo, Japan). Ultrapure water was obtained through a Milli-Q water purification system (Millipore, Bedford, MA).

Sake Samples. Commercial sake samples were purchased from sake companies. The alcohol content was 15.5−18.0%. Five samples were used. Three of them (J1, J2, and J3) were Junmai shu (sake made from rice only), and the other two samples (H1 and H2) were Honjozo shu (sake made from rice and alcohol). The production year was 2004−2007.

Accelerated Aging of Sake Spiked with [*methyl*-d_3]-Methionine. The concentration of methionine in sake sample H1 was measured. An equal concentration of either methionine or [*methyl*-d_3]-methionine was then added to sample H1. Nine milliliters of the 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 (*2*) except that ions with *m*/*z* 129 and 132 were used to monitor the deuterated DMTS in SIM mode.

Fractionation on Cation Exchange Resin and Measurement of DMTS-Producing Potential. The components of sake were fractionated according to the method of Sato et al. (*20*) with slight modification. One hundred milliliters of sake was diluted 1:1 with ultrapure water and passed through a cation exchange resin (Dowex 50WX4, H^+ form, 200−400 mesh, 60 mL) filled in a glass column. Fraction N (components not adsorbed to the cation exchange resin) was obtained as the effluent after the column had been washed with 3 column volumes of ultrapure water. Fraction A (components adsorbed to the cation exchange resin) was obtained as the effluent from the column eluted with 10 column volumes of 0.5 M aqueous ammonia. The fractions were concentrated in vacuo, lyophilized, and dissolved in 20 mL of ultrapure water. A portion (1.8 mL) of the dissolved fraction and ethanol (15.5−18.0%, the same concentrations as in sake samples) were put in a 10-mL glass vial, and the volume was adjusted to 9 mL with ultrapure water. Then pH (4.2−4.4) was adjusted to match that of sake, and accelerated aging was conducted as mentioned above. Nine milliliters of untreated sake was also put in the vial, and accelerated aging was conducted as well. The concentration of DMTS was determined as reported (*2*). The coefficient of variation of the measurement was 2.9%. The quantification limit and the detection limit were 0.04 and 0.01 μg/L, respectively. DMTS-producing potential was defined as the nanograms of DMTS formed from 1 mL of sake or an equivalent amount of the fraction after accelerated aging.

Purification of DMTS Precursor Compounds from Sake. DMTS precursor compounds were purified from sake sample J3 as shown in the scheme of **Figure 1**. Two HPLC systems were used. One was a Gilson model 305 equipped with a 5 mL loop (Gilson, Middleton, WI) and used for reversed-phase chromatography. The other was a Shimadzu LC-10ADvp equipped with a 50 μL loop and a UV−vis detector SPD-$10A_{VP}$ (Shimadzu, Kyoto, Japan), which was used for ion exclusion chromatography and normal phase chromatography. Fractions were collected using fraction collector Frac-920 (Amersham Biosciences, Buckinghamshire, U.K.).

Fraction N was obtained by passing 1 L of the sample through 500 mL of Dowex 50WX4 resin filled in a glass column (50 × 500 mm). It was concentrated, lyophilized, dissolved in 120 mL of ultrapure water,

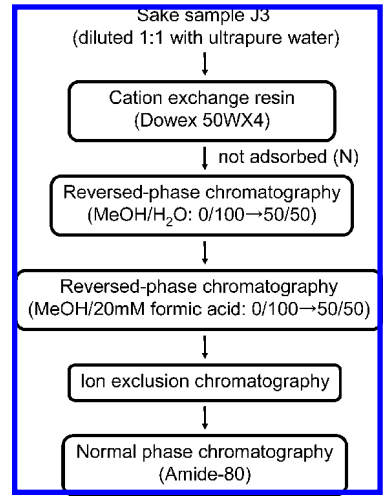

Figure 1. Scheme of the purification of DMTS precursor compound (DMTS-P1) from sake.

and then fractionated on a reversed-phase column (TSKgel ODS-80Ts, 21.5 × 300 mm, TOSOH, Tokyo, Japan). The chromatography was performed under the following conditions: ultrapure water was run for 75 min, and then the concentration of methanol was increased linearly to 50% in 62.5 min and held for 75 min. The flow rate was 4 mL/min. The injection volume was 4 mL. The fraction size was 10 mL/tube. This procedure was repeated 29 times. A portion (2.7 mL) of the fraction was added to succinate buffer solution, and the DMTS-producing potential was measured for each fraction as mentioned below. The fractions from which DMTS was produced (DMTS-producing fractions) were combined, concentrated, lyophilized, and dissolved in 12 mL of 20 mM formic acid solution (pH 2.8). Reversed-phase chromatography was then performed again using 20 mM formic acid instead of water as mobile phase. The DMTS-producing fractions were concentrated, lyophilized, and dissolved in 3 mL of ultrapure water. Aliquots (50 μL) were then applied to an ion exclusion column (IC-Pak Ion-Exclusion, 7.8 × 300 mm, Waters, Milford, MA). The eluent was 20 mM formic acid, and the flow rate was 0.6 mL/min. The fraction size was 0.3 mL/tube. The chromatography was repeated 55 times. The DMTS-producing fractions were combined, concentrated, lyophilized, dissolved in 1.5 mL of 80% aqueous CH_3CN, and applied to an Amide-80 column (4.6 × 250 mm, TOSOH). The chromatography was performed isocratically with 80% aqueous CH_3CN at a flow rate of 0.5 mL/min, which was repeated 29 times, and pure DMTS precursor (DMTS-P1) was obtained.

Measurement of DMTS-Producing Potential of Chromatography Fraction. A portion of the chromatography fraction, 1.6 mL of ethanol, and 0.9 mL of buffer solution (100 mM succinate buffer, pH 4.0) were put in a 10-mL glass vial, and the volume was adjusted to 9 mL with ultrapure water. When the fraction contained organic solvent, it was removed by evaporation and lyophilization, and the residue was dissolved in ultrapure water. The amount of addition of the fraction was determined by dividing the total volume of incubation (9 mL) by the condensation rate of the fraction, which was calculated on the assumption that a compound was eluted in one chromatography fraction with the recovery rate of 100%. Accelerated aging was carried out, and DMTS-producing potential was determined as described above.

High-Resolution LC-MS Analysis. A JMS-T100LP mass spectrometer (JEOL, Tokyo, Japan) coupled to an Agilent-1200 HPLC system (Agilent Technologies, Palo Alto, CA) was used. An aqueous solution of purified DMTS-P1 was applied to a Unison UK-C18 column (2.0 × 150 mm, Imtakt, Kyoto, Japan). Ultrapure water was used as a mobile phase at a flow rate of 0.2 mL/min. The eluent was introduced for ESI (positive)-TOF mass spectrometry. For the accurate mass measurement, sodium formate was injected as an internal mass reference.

Nuclear Magnetic Resonance Spectroscopy (NMR). The NMR spectra were acquired on a JEOL ECP-500 spectrometer with Delta NMR operating and processing software. The spectrometer was

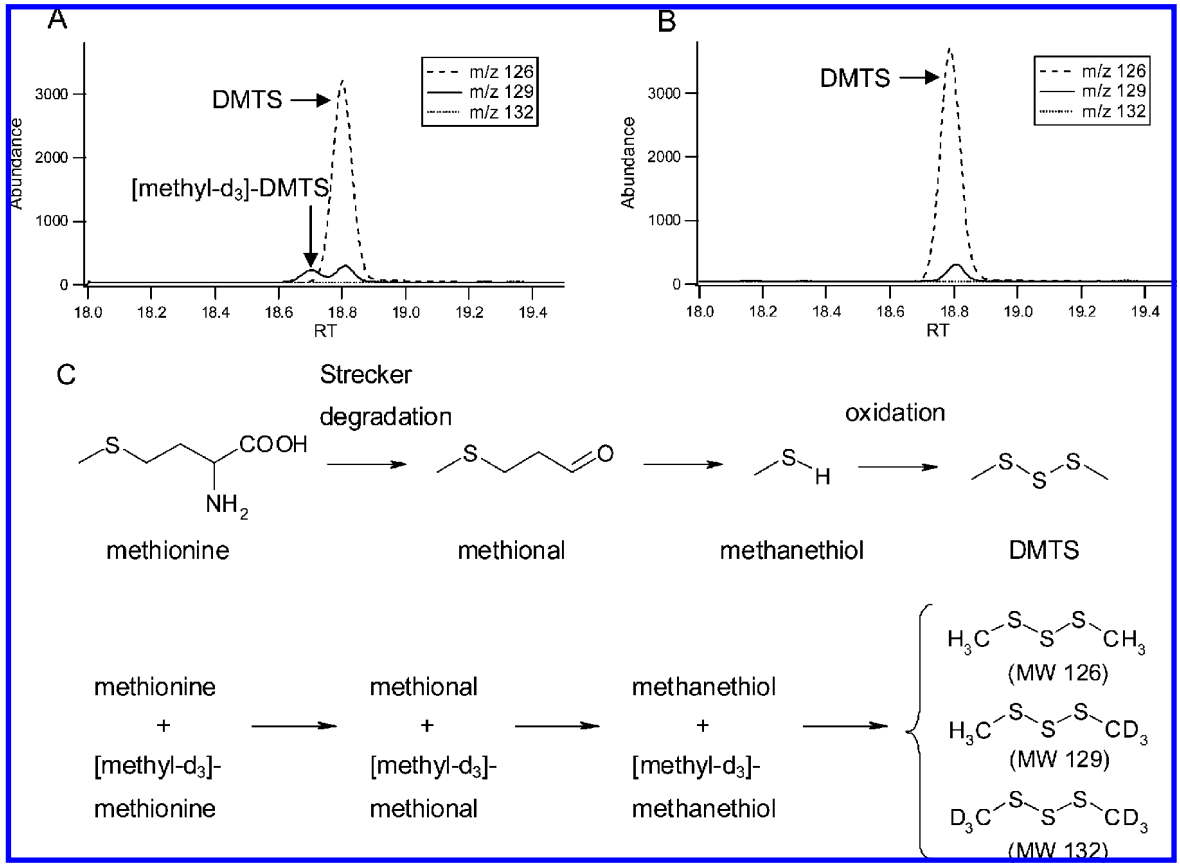

Figure 2. GC-MS chromatograms of sake samples spiked with [*methyl-d*$_3$]-methionine (**A**) and control (**B**) after accelerated aging. (**C**) Pathway of DMTS formation from methionine via Strecker degradation.

equipped with a 5 mm direct detection pulsed field *z*-axis gradient probe, operating at 500.16 MHz for ^{1}H and 125.77 MHz for ^{13}C. The measurement was conducted in CD_3CN/D_2O (80:20) at 5 °C. No sample rotation was applied.

^{1}H NMR. The 1D ^{1}H spectra using a standard pulse sequence were acquired with 32K data points, 8 scans, a spectral width of 7507 Hz, an acquisition time of 4 s, and a relaxation delay of 4 s. Spectra were Fourier transformed with a single exponential function and a line broadening of 0.2 Hz.

^{13}C NMR. ^{13}C spectra using a standard pulse sequence were acquired with 32K data points, 30000 scans, a spectral width of 31446 Hz, an acquisition time of 1 s, a relaxation delay of 1 s, and proton decoupling. ^{13}C distortionless enhancement by polarization transfer spectra (DEPT) (90° and 135°) were acquired with 32K data points, 15000 scans, a spectral width of 31446 Hz. ^{13}C NMR spectra and DEPT spectra were processed with a single exponential function and a line broadening of 2 Hz.

2D NMR. Gradient double quantum filtered shift ^{1}H−^{1}H correlation spectroscopy (DQF-COSY) spectra using a standard pulse sequence were acquired with field gradient pulse, 16 scans, 1024 data points in F2, and 254 data points in F1 over a 7507 Hz bandwidth. Spectra were processed with sine bell shift function in F2 and F1 dimensions. Gradient heteronuclear multiple quantum coherence (HMQC) spectra using a standard pulse sequence were acquired with field gradient pulse, 32 scans, 1024 data points over a 4502 Hz bandwidth in F2, 256 data points over a 31446 Hz bandwidth in F1, and a relaxation delay of 2 s. Spectra were processed with a sine bell shift function in F2 and F1 dimensions. Gradient heteronuclear multiple bond correlation (HMBC) using a standard pulse sequence were acquired with field gradient pulse, 32 scans, 1024 data points over a 4502 Hz bandwidth in F2, 256 data points over a 31446 Hz bandwidth in F1, long-range coupling of 8 Hz, and a relaxation delay of 4 s. Spectra were processed with a sine bell shift function in F2 and F1 dimensions. The resonances of CD_3CN at δ_H 1.93 and δ_C 1.28 were used as internal standards for NMR spectra.

1,2-Dihydroxy-5-(methylsulfinyl)pentan-3-one: ^{1}H δ 2.57 (s, 3H, H-6), 2.89 (m, 1H, H-5$_\alpha$), 3.00 (m, 2H, H-4), 3.05 (m, 1H, H-5$_\beta$), 3.70 (dd, 1H, J = 3.0, 12.0 Hz, H-1$_\alpha$), 3.79 (dd, 1H, J = 3.0, 12.0 Hz, H-1$_\beta$), 4.21 (t, 1H, J = 3.5 Hz, H-2); ^{13}C NMR δ 31.5 (C-4, CH_2), 37.2 (C-6, CH_3), 46.2 (C-5, CH_2), 63.2 (C-1, CH_2), 77.8 (C-2, CH), 210.4 (C-3, C=O); COSY data, H-1 → H-2; H-2 → H1; H-4 → H5; H-5 → H-4; HMQC data, H-1 → C-1; H-2 → C-2; H-4 → C-4; H-5 → C-5; H-6 → C-6; 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.

Other Chemical Analyses. The concentration of methionine was measured using a JLC-500 amino acid analyzer (JEOL). The analysis of methional was carried out as described (*2*). The concentrations of organic acids were measured by an HPLC ($LC\text{-}10AD_{VP}$) equipped with a SPR-H column and a conductivity detector $CDD\text{-}10A_{VP}$ (Shimadzu). The concentration of glucose was determined by using a Glucose Auto and Stat GA-1150 (Arkray, Kyoto, Japan).

RESULTS AND DISCUSSION

Accelerated Aging of Sake Spiked with [*methyl-d*$_3$]-Methionine. We first investigated the contribution of methionine, the precursor of methional, to the formation of DMTS by spiking a sample of sake with the labeled compound [*methyl-d*$_3$]-methionine. We reasoned that if DMTS is formed through Strecker degradation of methionine, then [*methyl-d*$_3$]-DMTS and [*dimethyl-d*$_6$]-DMTS should be generated (**Figure 2C**). The concentration of natural methionine in sake sample H1 was 66 μM, and the same amount of [*methyl-d*$_3$]-methionine was added to it. As a control, an equal amount of unlabeled methionine was added. DMTS was not detected before aging (data not shown). After accelerated aging, a small peak at *m*/*z* 129, corresponding to [*methyl-d*$_3$]-DMTS, was detected just before the peak of unlabeled DMTS (*m*/*z* 126) in the sample spiked with [*methyl-d*$_3$]-methionine (**Figure 2A**). The peak of [*methyl-d*$_3$]-DMTS was not detected in the control (**Figure 2B**). The results show that the methyl group of methionine was incor-

Table 1. DMTS-Producing Potential before and after Fractionation by Cation Exchange Resin

	DMTS-producing potential (ng of DMTS/mL of sake)		
fraction	J1	J2	H2
before fractionation	14	10	0.2
adsorbed (A)	0.1	1.1	nd[a]
not adsorbed (N)	112	22	22
A + N	26	8.8	3.2

[a] nd, not detected.

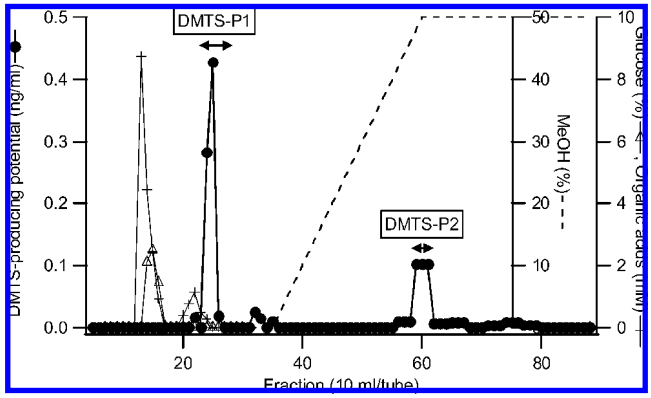

Figure 3. Reversed-phase chromatogram with MeOH/H_2O as eluent. DMTS-producing potential of fractions 5−40 was measured by directly adding the fractions to the buffer solution. For fractions 41−89, every three fractions were combined, lyophilized, and dissolved in 10 mL of ultrapure water. The potential was measured in the same way as for fractions 5−40 and represented by dividing the measured value by 3.

porated into DMTS. However, the peak area of [*methyl-d*$_3$]-DMTS was only 6% of that of unlabeled DMTS. In addition, a peak at *m*/*z* 132, corresponding to [*dimethyl-d*$_6$]-DMTS, was not detected. Assuming that DMTS is produced absolutely from Strecker degradation of methionine, the ratio of unlabeled DMTS to [*methyl-d*$_3$]-DMTS to [*dimethyl-d*$_6$]-DMTS should be 1:2:1. These results indicate that Strecker degradation of methionine plays a minor role in the formation of DMTS during the storage of sake and that other precursor compounds or mechanisms exist. Although a small amount of methional (10 μg/L) was detected in sample H1, it could account for only about 10% of the DMTS produced (0.2 μg/L) because the reported conversion rate of methional to DMTS is 0.1% (*18*).

Fractionation on Cation Exchange Resin. To screen for DMTS precursor compounds, the components of three sake samples (J1, J2, and H2) were separated on cation exchange resin, and the DMTS-producing potential was analyzed. **Table 1** shows the DMTS-producing potential of the untreated sake samples and their fractions. For all sake samples tested, the DMTS-producing potential of the fraction that did not adsorb to the cation exchange resin (fraction N) was greater than that adsorbed (fraction A). Fraction A contained basic components such as amines and amino acids including methionine, whereas fraction N contained acidic and neutral components. These results suggest that DMTS precursor compounds are mainly present in the acidic/neutral fraction rather than in the basic one. Unexpectedly, the DMTS-producing potential of fraction N was greater than that of untreated sake (i.e., before fractionation). The addition of fraction A to fraction N (A + N) decreased the DMTS-producing potential of fraction N, suggesting that fraction A contained components that suppress the formation of DMTS from fraction N.

Purification of DMTS Precursor Compounds from Sake. To separate DMTS precursor compounds from other components, fraction N was fractionated by column chromatography using reversed-phase silica gel (ODS). As shown in **Figure 3**, two peaks possessing the DMTS-producing potential were detected. They were efficiently separated from organic acids (citric, malic, lactic, and succinic acids) and glucose, the main components of fraction N. The DMTS precursor compound in the first peak (DMTS-P1) eluted with only water, whereas that in the second peak (DMTS-P2) eluted during the linear gradient of methanol, suggesting that the polarity of DMTS-P1 is higher than that of DMTS-P2.

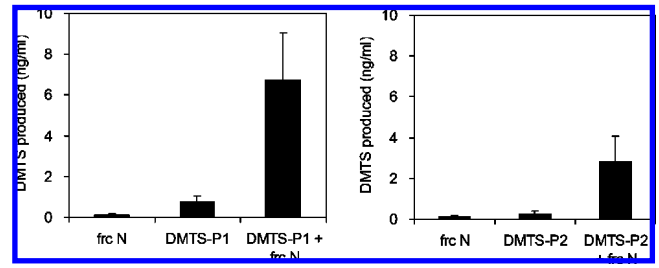

Figure 4. Combination effect of DMTS-P1 or DMTS-P2 with diluted fraction N. DMTS-P1 or DMTS-P2 was incubated with and without fraction N (1/16 dilution) in 10 mM succinate buffer (pH 4, containing 18% EtOH), and the amount of generated DMTS was measured. Bars indicate SE (n = 3).

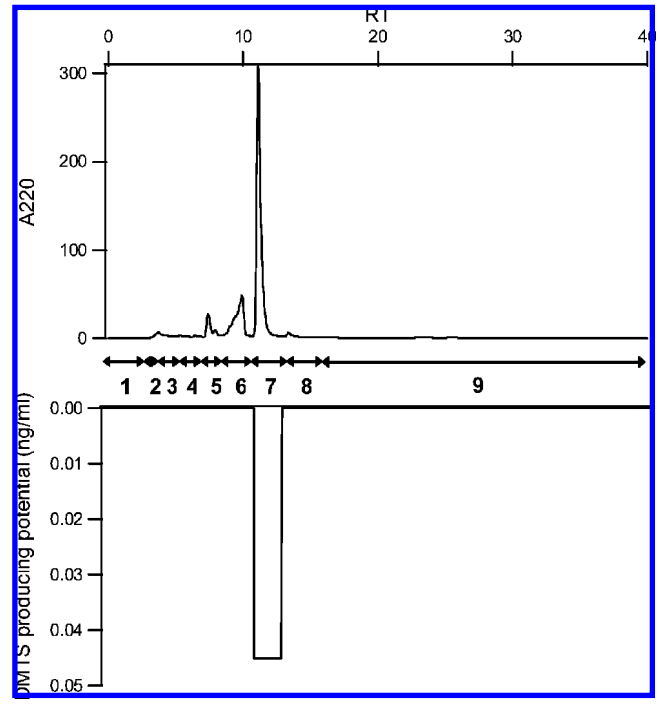

Figure 5. Separation by the Amide-80 column. The effluent was collected in nine fractions, as indicated by the arrows.

We also observed a synergetic effect of DMTS-P1 and DMTS-P2 with fraction N (1/16 dilution). As can be seen in **Figure 4**, DMTS produced from DMTS-P1 or DMTS-P2 in the presence of fraction N, respectively, was about 10 times higher than that of DMTS-P1 or DMTS-P2 alone. These results indicate that DMTS-P1 and DMTS-P2 are involved in the formation of DMTS.

As DMTS-P1 in the first fraction had higher potential, it was further purified by column chromatography. On the reversed-phase column using 20 mM formic acid solution (pH 2.8) as the mobile phase, DMTS-P1 eluted at a similar retention time as when water was used as the mobile phase (data not shown). According to its chromatographic behavior using eluents with

Table 2. Summary of the Purification of DMTS-P1 from Sake

chromatography step	DMTS-producing potential[a] (ng/mL)
sake	4.3
cation exchange	182
reversed-phase	2.4
reversed-phase (20 mM formic acid)	0.82
ion exclusion	0.04
Amide-80	0.04

[a] DMTS-producing potential of each chromatography step was measured after combining the DMTS-producing fractions. It was expressed as nanograms of DMTS produced from the combined fraction equivalent to 1 mL of sake.

different pH values, DMTS-P1 was considered to be a neutral compound rather than an acidic one. DMTS-P1 was further purified by HPLC on an ion exclusion column and a normal phase column (Amide-80). As shown in **Figure 5**, DMTS-P1 was isolated as one peak in fraction 7 in the HPLC separation on the Amide-80 column.

The purification of DMTS-P1 is summarized in **Table 2**. The DMTS-producing potential gradually decreased during the purification steps, suggesting that DMTS-P1 is unstable in the column separation.

Structure Determination of DMTS-P1. The structure of DMTS-P1 was elucidated on the basis of spectroscopic data obtained by MS and NMR. DMTS-P1 was determined to have the molecular formula $C_6H_{12}O_4S$ by positive-mode high-resolution ESI-MS (*m*/*z* 181.0534, $[M + H]^+$, Δ −0.1 mmu) as shown in **Figure 6**. A peak at *m*/*z* 361.0988 was considered to be a dimer $[2\ M + H]^+$, and a peak at *m*/*z* 117.0566, which was assigned as $C_5H_9O_3$ (calcd. 117.0552), most probably corresponded to the loss of a methyl sulfoxide moiety $[M - S(O)Me]^+$.

Analysis of the ^1H and ^{13}C NMR, $^1H-^1H$ COSY, and HMQC spectra of DMTS-P1 allowed assignment of all of the ^{13}C and ^1H signals. The ^{13}C NMR and DEPT spectra revealed that the six carbons in DMTS-P1 comprise a carbonyl carbon, two methylenes, two oxygenated sp3 carbons, and a methyl group. The results of high-resolution ESI-MS analysis indicated that DMTS-P1 contains a methyl sulfoxide moiety. A singlet signal

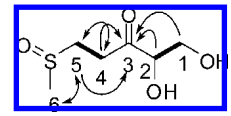

Figure 7. Key HMBC (indicated by arrows from ^1H to ^{13}C) and $^1H-^1H$ COSY (indicated by bold lines) for 1,2-dihydroxy-5-(methylsulfinyl)pentan-3-one.

at δ_H 2.57 was assigned as methyl protons at C-6 of the methyl sulfoxide moiety. In the $^1H-^1H$ COSY spectra, cross peaks between δ_H 3.70 and 3.79 (H-1$_\alpha$ and H-1$_\beta$) and δ_H 4.21 (H-2) were observed; thus, two hydroxyl groups are connected at C-1 (δ_C 63.2) and C-2 (δ_C 77.8), respectively.

The observation of HMBC correlations between the carbonyl carbon (C-3) and the H-1, H-2, H-4, and H-5 protons and between the methylene carbon (C-5) and the methyl protons at H-6 indicated that the C-2 and C-4 carbons are linked to the carbonyl carbon (C-3) and that the methyl sulfoxide moiety is attached to C-5 (**Figure 7**).

Thus, the structure of DMTS-P1 was determined to be 1,2-dihydroxy-5-(methylsulfinyl)pentan-3-one, as shown in **Figure 7**. To the best of our knowledge, this compound has not previously been reported in the literature.

Pathway of the Formation of DMTS from DMTS-P1. In the high-resolution ESI-MS analysis, a daughter ion with *m*/*z* 117.0566, consistent with the loss of a methyl sulfoxide moiety, was detected. It seems likely that the methyl sulfoxide moiety is easily removed from DMTS-P1 and is responsible for the formation of DMTS. Ostermayer et al. (*21*) reported that hydrolysis of *S*-methylcysteine sulfoxide generates unstable methanesulfenic acid through β-elimination, and Chin et al. (*13*) reported that the formation of DMDS occurs by the oxidation of methanethiol formed from methanesulfenic acid. The oxidation of methanethiol produces DMTS as well as DMDS (*7*). It is possible that the methyl sulfoxide moiety of DMTS-P1 is removed in the form of methanesulfenic acid through β-elimination, as it is in *S*-methylcysteine sulfoxide. Thus, the estimated pathway of the formation of DMTS can be summarized as depicted in **Figure 8**.

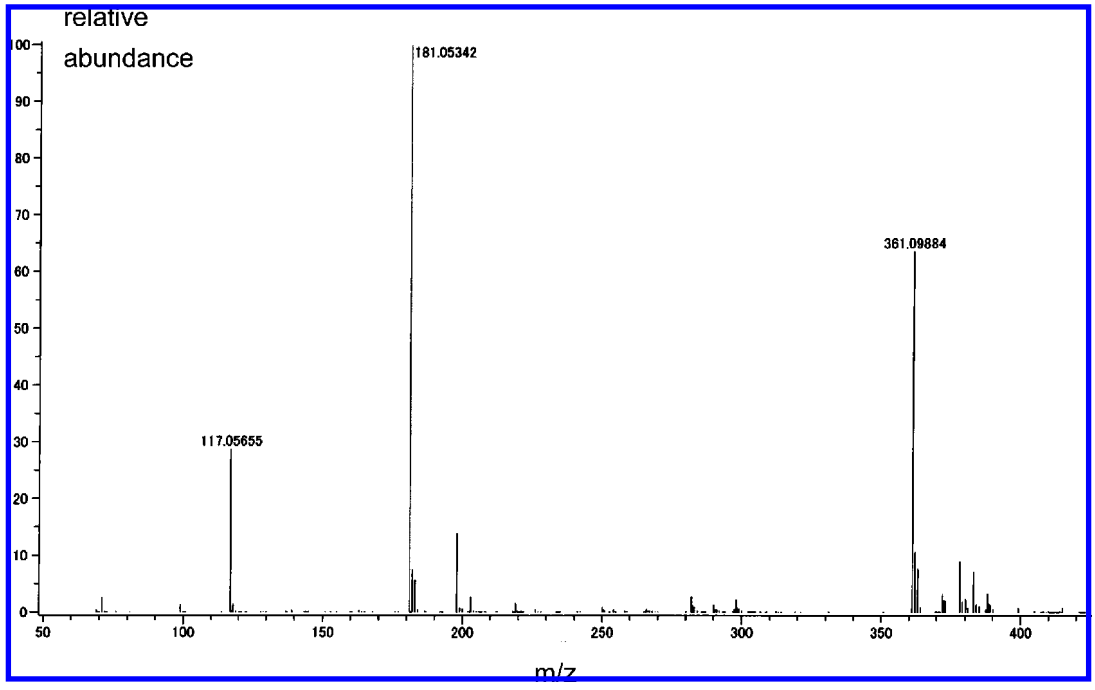

Figure 6. High-resolution mass spectra of DMTS-P1.

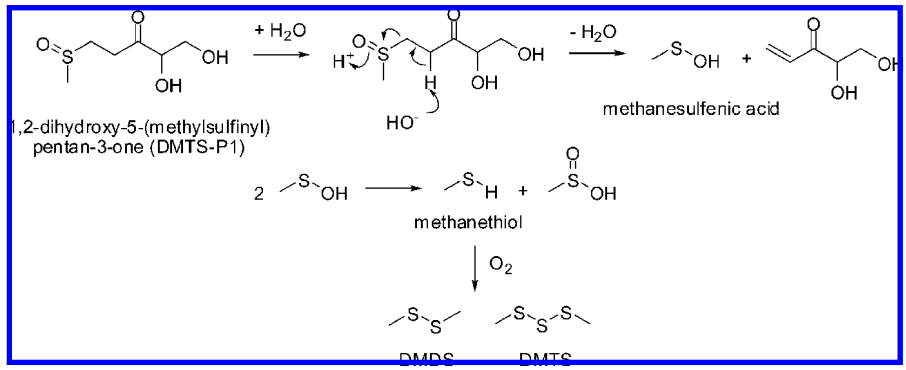

Figure 8. Estimated mechanism of the formation of DMDS and DMTS from DMTS-P1.

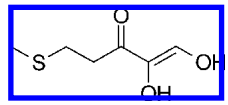

Figure 9. Structure of 1,2-dihydroxy-5-(methylthio)-1-penten-3-one, an intermediate compound of the methionine salvage pathway.

The synergetic effect was observed between DMTS-P1 and fraction N (**Figure 4**). It is possible that highly reactive methansulfenic acid, formed through the degradation of DMTS-P1, reacted with other sulfur-containing compounds in fraction N and produced DMTS.

Possible Origin of DMTS Precursors. Because DMTS-P1 is a new compound, it is not known how it is generated in the process of sake making. One possible origin might be the methionine salvage pathway, in which methylthioadenosine (MTA) regenerates methionine. This pathway is widely distributed among bacteria (*22*, *23*), yeast (*24*), plants (*25*), and animals (*26*). In this pathway (*23*), methylthioribose (MTR), a hydrolyzed product of MTA, is converted into MTR-1-phosphate (MTR-1-P), and then MTR-1-P undergoes successive enzymatic reactions by an isomerase, dehydratase, enolase, and phosphatase to yield 1,2-dihydroxy-5-(methylthio)-1-penten-3-one (**Figure 9**). This unstable compound either spontaneously decomposed to 2-keto-4-methylthiobutyrate or 3-methylthiopropionate under aerobic conditions (*27*) or underwent enzymatic conversion by a dioxygenase into 2-keto-4-methylthiobutyrate (*26*). Finally, this keto acid is transaminated to methionine. Some of the genes encoding the pathway have been identified in the *Saccharomyces cerevisiae* genome (*28*, *29*).

The chemical structure of 1,2-dihydroxy-5-(methylthio)-1-penten-3-one seems to resemble DMTS-P1 except that the sulfide group of the former is oxidized to sulfoxide in the latter and the enediol structure of the former is replaced by diol in the latter (**Figures 7** and **9**). Thus, the relationship of DMTS-P1 to the methionine salvage pathway was suspected. However, further studies are needed to confirm the hypothesis.

We also found another precursor compound, DMTS-P2. The purification of DMTS-P2 is in progress, and a lower affinity with the Amide-80 column compared with DMTS-P1 was observed (data not shown), which was consistent with a more hydrophobic property than DMTS-P1 (**Figure 3**). As the metabolites in the methionine salvage pathway are hydrophilic compounds containing hydroxyl groups or phosphate group, it is not likely that DMTS-P2 is a member of the pathway. Structure determination of DMTS-P2 is necessary to elucidate its origin.

Although further studies are needed, the discovery of DMTS-P1 and DMTS-P2, through the elucidation of their formation mechanism in the sake-making process, will offer the possibility to regulate the formation of "hineka" in sake.

ABBREVIATIONS USED

DMTS, dimethyl trisulfide; DMDS, dimethyl disulfide; CH_3CN, acetonitrile; CD_3CN, deuterated acetonitrile; D_2O, deuterium oxide; MeOH, methanol; ESI, electron spray ionization; NMR, nuclear magnetic resonance; DEPT, distortionless enhancement by polarization transfer; COSY, correlation spectroscopy; HMQC, heteronuclear multiple quantum coherence; HMBC, heteronuclear multiple bond correlation; MTA, methylthioadenosine; MTR, methylthioribose; MTR-1P, methylthioribose-1-phosphate.

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LITERATURE CITED

(1) Takahashi, K. The aroma of aged sake. *J. Soc. Brew. Japan* **1980**, *75*, 463–468.
(2) 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.
(3) Isogai, A.; Utsunomiya, H.; Kanda, R.; Iwata, H.; Nalano, S. Aroma compounds responsible for "hineka" in commercial sake. *J. Soc. Brew. Japan* **2006**, *101*, 125–131.
(4) Tokimoto, Y. Volatile components of cooked onions. *Nippon Shokuhin Kagaku Kogaku Kaishi* **1995**, *42*, 279–287.
(5) Maruyama, F. T. Identification of dimethyl trisulfide as a major aroma component of cooked brassicaceous vegetables. *J. Food Sci.* **1970**, *35*, 540–543.
(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) Lee, K.-Y. M.; Paterson, A.; Piggott, J. R. Origins of flavour in whiskies and a revised flavour wheel: a review. *J. Inst. Brew.* **2001**, *107*, 287–313.
(9) Gijs, L.; Chevance, F.; Jerkovic, V.; Collin, S. How low pH can intensify β-damascenone and dimethyl trisulfide production through beer aging. *J. Agric. Food Chem.* **2002**, *50*, 5612–5616.
(10) Guth, H. Identification of character impact odorants of different white wine varieties. *J. Agric. Food Chem.* **1997**, *45*, 3022–3026.
(11) Marks, H. S.; Hilson, J. A.; Leichtweis, H. C.; Stoewsand, G. S. *S*-Methylcysteine sulfoxide in *Brassica* vegetables and formation of methyl methanethiosulfinate from *Brassica* sprouts. *J. Agric. Food Chem.* **1992**, *40*, 2098–2101.
(12) 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.
(13) Chin, H.-W.; Lindsay, R. C. Mechanism of formation of volatile sulfur compounds following the action of cysteine sulfoxide lyases. *J. Agric. Food Chem*. **1994**, *42*, 1529–1536.
(14) Nakagawa, K.; Umeda, T.; Higuchi, O.; Tsuzuki, T.; Suzuki, T.; Miyazawa, T. Evaporative light-scattering analysis of sulforaphane in broccoli sample: quality of broccoli products regarding sulforaphane contents. *J. Agric. Food Chem*. **2006**, *54*, 2479–2483.
(15) Nishiwaki, T. Deodorizing activity of yeast fermentation against volatile compounds of pickled radish. *J. Soc. Brew. Jpn*. **2007**, *102*, 432–440.
(16) Jin, Y.; Wang, M.; Rosen, R. T.; Ho, C. T. Thermal degradation of sulforaphane in aqueous solution. *J. Agric. Food Chem*. **1999**, *47*, 3121–3123.
(17) 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.
(18) 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.
(19) Peppard, T. L. Dimethyl trisulfide, its mechanism of formation in hop oil and effect on beer flavour. *J. Inst. Brew*. **1978**, *84*, 337–340.
(20) Sato, S.; Tadenuma, M.; Takahashi, K.; Koike, K. Flavor changes during sake aging (part 5). *J. Soc. Brew. Jpn*. **1975**, *70*, 588–591.
(21) Ostemayer, F.; Tarbell, D. S. Products of acid hydrolysis of *S*-methylcysteine sulfoxide; the isolation of methyl methanethiosulfonate, and mechanism of the hydrolysis. *J. Am. Chem. Soc*. **1960**, *82*, 3752–3755.
(22) Sekowska, A.; Denervaud, V.; Ashida, H.; Michoud, K.; Haas, D.; Yokota, A.; Danchin, A. Bacterial variations on the methionine salvage pathway. *BMC Microbiol*. **2004**, *4*, 9.
(23) Ashida, H.; Saito, Y.; Kojima, C.; Kobayashi, K.; Ogasawara, N.; Yokota, A. A functional link between RuBisCO-like protein of *Bacillus* and photosynthetic RuBisCO. *Science* **2003**, *302*, 286–290.
(24) Marchitto, K. S.; Ferro, A. J. The metabolism of 5′-methylthioadenosine and 5-methylthioribose-1-phosphate in *Saccharomyces cerevisiae*. *J. Gen. Microbiol*. **1985**, *131*, 2153–2164.
(25) Kushad, M. M.; Richardson, D. G.; Ferro, A. J. Intermediates in the recycling of 5-methylthioribose to methionine in fruits. *Plant Physiol*. **1983**, *73*, 257–261.
(26) Wray, J. W.; Abeles, R. H. The methionine salvage pathway in *Klebsiella pneumoniae* and rat liver: identification and characterization of two novel dioxygenases. *J. Biol. Chem*. **1995**, *270*, 3147–3153.
(27) Myers, R. W.; Wray, J. W.; Fish, S.; Abeles, R. H. Purification and characterization of an enzyme involved in oxidative carbon-carbon bond cleavage reactions in the methionine salvage pathway of *Klebsiella pneumoniae*. *J. Biol. Chem*. **1993**, *268*, 24785–24791.
(28) Subhi, A. L.; Diegelman, P.; Porter, C. W.; Tang, B.; Lu, Z. J.; Markham, G. D.; Kruger, W. D. Methylthioadenosine phosphorylase regulates ornithine decarboxylase by production of downstream metabolites. *J. Biol. Chem*. **2003**, *278*, 49868–49873.
(29) Bumann, M.; Djafarzadeh, S.; Oberholzew, A. E.; Bigler, P.; Altmann, M.; Trachsel, H.; Baumann, U. Crystal structure of yeast Ypr118w, a methylthioribose-1-phosphate isomerase related to regulatory eIF2B subunits. *J. Biol. Chem*. **2004**, *279*, 37087–37094.

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