Structural Determination of Glucosylceramides in the Distillation Remnants of Shochu, the Japanese Traditional Liquor, and Its Production by *Aspergillus kawachii*

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Ⓢ *Supporting Information*

ABSTRACT: Shochu is traditional Japanese liquor produced from various crops and fungi *Aspergillus kawachi* or *A. awamorii*. The amount of unutilized shochu distillation remnants is increasing because of the recent prohibition of ocean dumping of these remnants. In this Article, we first describe the structures of glucosylceramides contained in shochu distillation remnants by fragment ion analysis using ESI−tandem mass spectrometry. Shochu distillation remnant produced from barley contained glucosylceramides d18:2/C16:0h, d18:2/C20:0h, d19:2/C18:1h, and d18:2/C18:0h. Koji (barley fermented with *A. kawachii*) contained the same glucosylceramides. Shochu distillation remnants produced from rice contained glucosylceramides d18:2/C18:0h and d19:2/C18:1h. The culture broth of *A. kawachii* contained glucosylceramides d19:2/C18:1h and d19:2/C18:0h. These results indicate that the glucosylceramides contained in crops and those produced by *A. kawachii* transfer through the processes of fermentation with yeast and distillation to the shochu distillation remnant. This information will enable utilization of shochu distillation remnants and koji as novel sources of sphingolipids.

KEYWORDS: glucosylceramide, fermentation, alcohol beverage, Aspergillus kawachii, shochu, fungi, barley, rice, ESI−tandem mass spectrometry, fragment ion analysis

■ INTRODUCTION

Shochu is a distilled alcohol beverage produced from barley, rice, sweet potato, sugar cane, or buckwheat traditionally produced in the southern islands of Japan near the Asian continent. For the purposes of saccharification of starch and production of citric acid to prevent microbial spoilage, *Aspergillus luchuensis* including *A. kawachii* and *A. awamorii* are first propagated on the surface of steamed crops, which becomes "koji". After pitching yeast to koji and steamed crops, ethanol fermentation occurs in the mash, and the mash is distilled. During the process of distilling, shochu distillation remnant is separated from the fermented mash. Shochu produced in Okinawa, the southernmost island in Japan, is designated Awamori and is produced from rice. Approximately 1 million tons of shochu distillation remnants is produced per year, and has been traditionally dumped into the ocean. Because of a recent prohibition against ocean dumping after ratification of the London Treaty, shochu manufacturers have developed methods to utilize shochu distillation remnants. For example, shochu distillation remnants have been applied to methane fermentation, ensiled for animal feed, composted, or used as culture media for production of a saccharifying enzyme,[1] protease,[2] chitosan,[3] phenolic compounds,[4] polyunsaturated fatty acids, xanthophylls,[5] and nisin.[6] However, sufficient profitable utilization of shochu distillation remnants has not occurred, perhaps because detailed chemical studies on its inherent value have never been conducted.

Sphingolipids have gained attention for their utility in cosmetics because of their moisture-holding ability.[7−9] Moreover, sphingolipids have anticancer activities through their role in maintaining membrane structure and exerting effects on cell signaling and apoptosis.[10−12] Furthermore, sphingosine-1-phosphate is a ligand of the G-protein-coupled receptor and stimulates growth of quiescent fibroblasts.[13] Therefore, sphingolipids are becoming significant targets in the development of anticancer drugs. Sphingolipids are also downstream signaling molecules of adiponectin, a hormone that ameliorates insulin resistance in pancreatic β-cells.[14] Glucosylceramide-synthesizing enzymes regulate energy metabolism and insulin resistance.[15,16] Therefore, sphingolipids are emerging as a diabetes-improving molecule. Sphingolipids reportedly improve cognitive function in dementia model rats.[17] Thus, the potential utility of sphingolipids as cosmetics, functional foods, and anticancer medicines is likely to expand. Commercial sphingolipids are extracted from bovine brain. However, since the bovine spongiform encephalopathy crisis, extraction of sphingolipids from bovine brain has become difficult, and several alternative sources of sphingolipids have been explored.

Sphingolipids contain various chemical structures. Serine and palmitoyl-CoA are conjugated to give 3-keto-sphinganine and, eventually, sphinganine (dihydrosphingosine, d18:0). Sphinganine receives diverse chemical modifications such as hydroxylation or desaturation. These conversions give rise to various sphingoid bases such as sphingosine (trans-4-sphingenine, d18:1[4]), 4,8-sphingadienine (d18:2[4,8]), 4,8,10-sphingatrienine (d18:3[4,8,10]), phytosphingosine (4-hydroxysphinganine, t18:0), 4-hydroxy-8-sphingenine (t18:1[8]), 9-methyl-4,8-sphingadienine (d19:2[4,8]), and 9-methyl-4,8,10-sphingatrienine (d19:3[4,8,10]).[18] These sphingoid bases are acylated to fatty acids via amidic linkage to generate ceramides. Some fatty acids are hydroxylated or desaturated. Ceramide receives two kinds of chemical modifications on the 1-hydroxyl bond. Single or multiple sugar (glucose, galactose, mannose, neuraminic acid, or syalic acid) units are acetal-linked to the hydroxyl bond of ceramide to give neutral glycosphingolipids.[18,19] Alternatively, inositol, mannosylinositol, or choline is phosphodiester-linked to the hydroxyl bond of ceramides, to give inositolphosphorylceramide, mannosylinositolphosphorylceramide, mannose-(inositol-P)$_2$-ceramide, or sphingomyelin.[20] To analyze these complex chemical structures of sphingolipids, new analytical methods using tandem mass spectrometry have recently been developed.[21−32] Mass spectrometry is able to measure the mass of the target molecule. Several ionization techniques, such as electron ionization,[33,34] chemical ionization,[35] electrospray ionization (ESI),[32,36] atmospheric pressure chemical ionization,[37] and fast atom bombardment,[25,27] have been adopted to analyze sphingolipids by mass spectrometry. Although mass spectrometry alone may not achieve the resolution to determine the complete structures of the sphingolipids such as the positions of unsaturated bonds, methyl bases, and the hydroxyl bases, coupling the fragmentation patterns obtained through tandem mass spectrometry and accumulated literature information is now enabling researchers to obtain structural information on sphingolipids with this approach.

In this Article, the determination of the molecular structures of glucosylceramides contained in shochu distillation remnants and its source by ESI−tandem mass spectrometry is described. It turned out that glucosylceramides contained in crops and those produced by *A. kawachii* transfer to the shochu distillation remnants. This is the first report of the molecular structures of glucosylceramides contained in shochu distillation remnants and *A. kawachii*, and the first to describe the contribution of *A. kawachii* to the glucosylceramides contained in shochu distillation remnants. This information will contribute to future utilization of shochu distillation remnants and bioresources in the fermentation industry, which uses filamentous fungi.

■ MATERIALS AND METHODS

Samples. The shochu distillation remnants produced from barley and *A. kawachii* and from rice and *A. awamorii* were kind gifts from shochu manufacturers. Shochu produced from barley and *A. kawachii* was distilled under decompression, and 30% of the surface of the barley was polished. Awamori was distilled under atmospheric pressure, and 10% of the surface of the rice was polished. Mycelium powder of *A. kawachii* was obtained from Higuchi Matsunosukeshoten Co. Ltd. (Osaka, Japan).

Media and Culture. Mycelium powder of *A. kawachii* (5 mg) was inoculated with steamed barley (30% of its surface was polished, 16 g) and incubated at 37 °C for 48 h. Koji (barley fermented with *A. kawachii*) was lyophilized and applied for further analyses. *A. kawachii* was inoculated with 200 mL of 24 g/L Difco Potato dextrose broth (Beckton Dickinson, Sparks, U.S.). The incubated culture was centrifuged,

Table 1. Ions Provided by Tandem ESI−MS/MS of Glucosylceramides Purified from Barley Shochu Distillation Remnant[a]

| MS m/z (%)[b]	MS/MS m/z (%)[b]
792.7 (46.2)	482.4 (100), 483.4 (23.7), 484.4 (22.6), 789.5 (19.1), 630.6 (17.6), 685.5 (17.0), 774.7(13.7), 612.6 (12.8), 525.3 (12.6), 510.4(9.4), 630.6 (17.6), 376.4 (8.5)
776.7 (23.8)	496.4 (100), 758.6 (47.7), 437.3 (43.8), 497.4 (24.3), 759.6 (23.3), 614.6 (20.4), 612.6 (18.1), 524.4 (18.0), 346.3 (15.5), 399.3 (15.4), 346.3 (15.3)
764.7 (25.6)	482.4 (100), 569.3 (25.5), 686.5 (23.0), 483.4 (21.1), 553.4 (14.3), 602.6 (14.2), 591.3(12.4), 584.6 (12.1), 685.5 (11.9), 746.6 (11.5), 348.3 (8.3)
736.6 (28.8)	482.3 (100), 493.2 (32.4), 640.3 (30.3), 718.5 (21.2), 574.5 (20.6), 556.5 (20.4), 483.3 (19.8), 594.3 (18.3), 538.5 (14.2), 717.4 (12.2), 320.3 (8.7)
701.4 (55.0)	
657.4 (84.6)	
613.3 (95.2)	
569.3 (100)	
525.3 (53.3)	
413.4 (41.4)	
360.4 (67.3)	
288.4 (60.6)	

[a]The spectra of ESI−MS and ESI−MS/MS are shown in Figures 1−5. [b]% compared to base peak.

washed with sterile distilled water, and the pellet was lyophilized and used for further analyses.

Extraction and Fractionation of Sphingolipids. Samples were freeze-dried, and 0.3 g of the freeze-dried samples was extracted with 2.0 mL of chloroform/methanol. The extracted lipid was mixed with 2.0 mL of 0.8 M KOH−methanol and incubated at 42 °C for 30 min. The samples were mixed with 5.0 mL of chloroform and 2.5 mL of water and centrifuged at 3000 rpm for 5 min. The organic phase was collected and used for further analyses. The amounts of glucosylceramides were calculated on the basis of the standard curve of signal intensities of spots in TLC using image J software and a standard material (Cerebroside, Matreya Inc., Pleasant Gap, PA).

Purification of Sphingolipids. The extracted lipids were dried in a centrifugal evaporator and dissolved in 4 mL of hexane. The sample was applied to silicagel chromatography, eluated with ethyl acetate: methanol (9:1), and fractionated by checking the elution positions of glucosylecramide with TLC. The fractions containing glucosylceramide were dried in a centrifugal evaporator and dissolved in 4.5 mL of chloroform:methanol (2:1 v/v). Four milliliters of the samples was injected into the 500 μL injection loop of an HPLC system. Purification of sphingolipid by HPLC was performed as follows: column, Inertsil SIL 100 A, 5 μm, diameter 4.6 mm × length 250 mm (GL Science Inc., Tokyo, Japan). The mobile phase consisted of buffer A (chloroform) and buffer B (95% methanol/5% water), and separation was achieved using the following gradient program: 0 min A 100%/B 0%, 15 min A 75%/B 25%, 20−40 min A 10%/B 90%. Flow rate was 0.7 mL/min, and the volume of the sample injection loop was 500 μL. Fractions were collected at 0.5 or 1 min intervals. The collected fractions were applied to thin layer chromatography (TLC) analysis followed by orcinol-sulfate visualization.

Electrospray Ionization Tandem Mass Spectrometry (ESI−MS/MS). Mass spectrometry was performed on an ion trap mass spectrometer (HC Ultra, Bruker Daltonics, Bremen, Germany) with an electrospray ion source. The sample was dissolved in 50 μL of chloroform:methanol (1:1 v/v), and 950 μL of methanol was added. The sample was infused into ion trap mass spectrometry by an online syringe pump at a constant flow rate of 3 μL/min. Nitrogen at 4 L/min and 300 °C was employed for desolvation and as a nebulizer gas at 10 psi. The instrument was set to operate in the positive ion mode with capillary voltage 4 kV and the end plate offset 0.5 kV. All spectra were acquired in the mass range 50−1500 m/z, with a scan
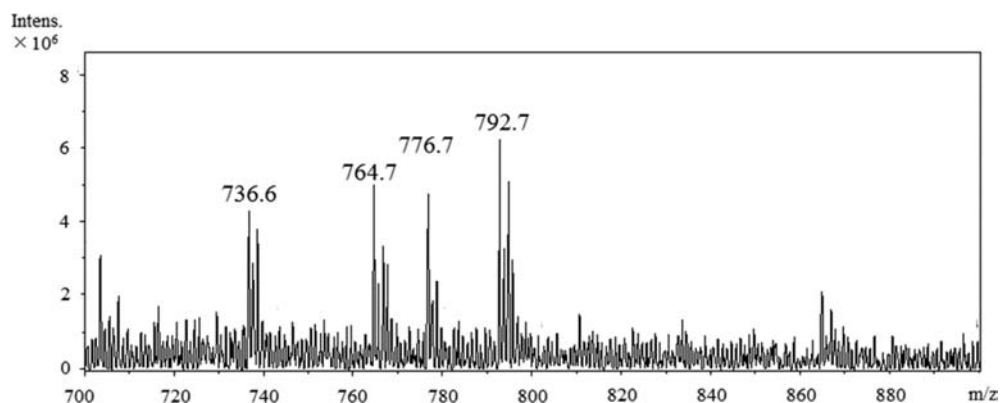
Figure 1. Positive ESI−MS spectrum of glucosylceramide purified from barley shochu distillation remnants. Glucosylceramide purified from mild alkali-resistant lipids of barley shochu distillation remnants was applied to ESI−MS in the positive-ion mode.
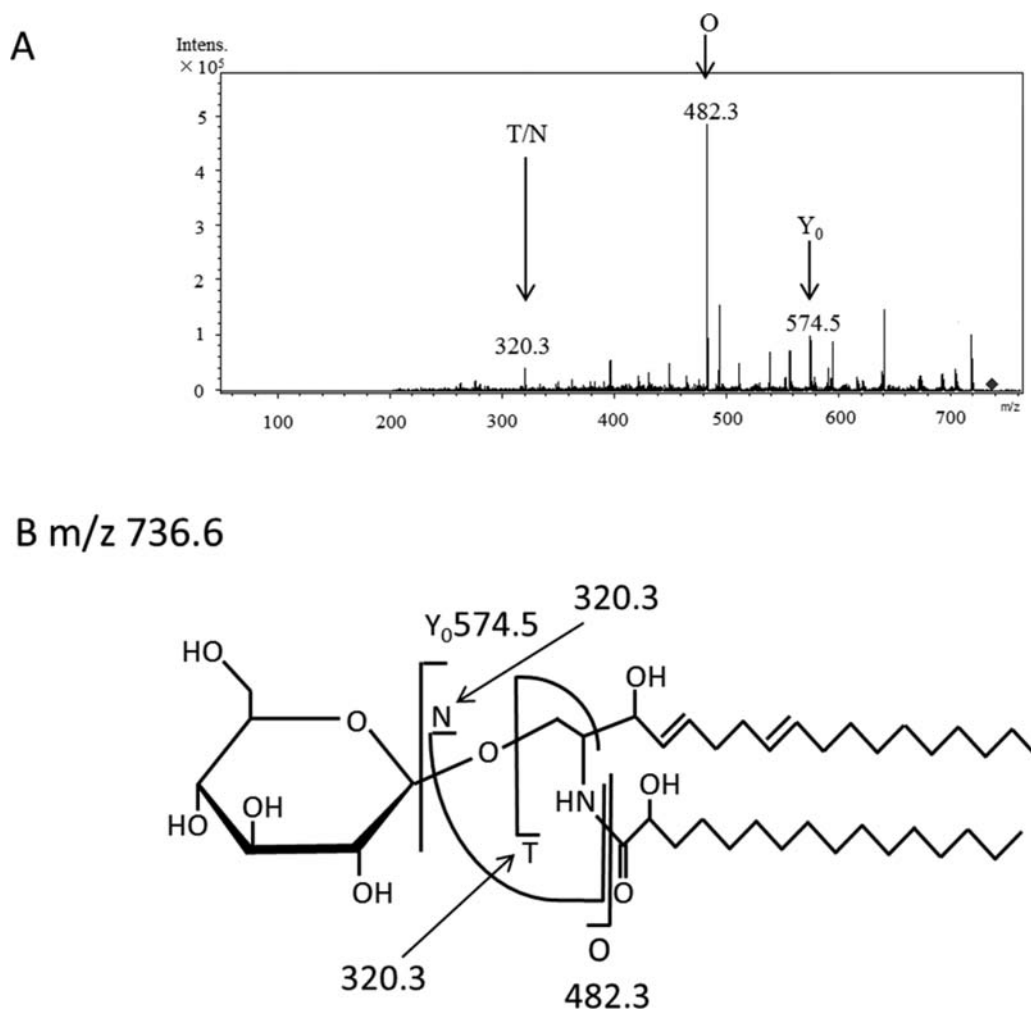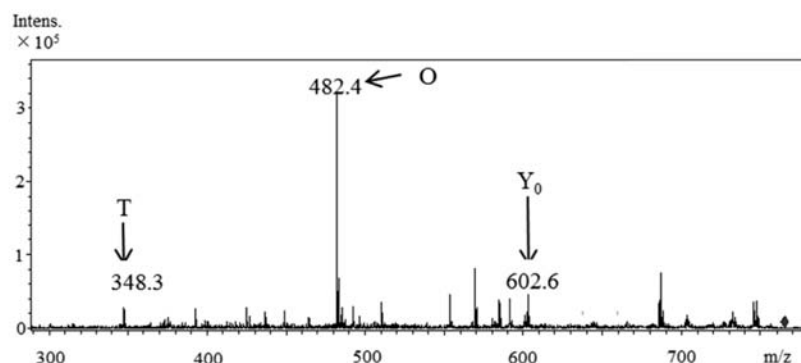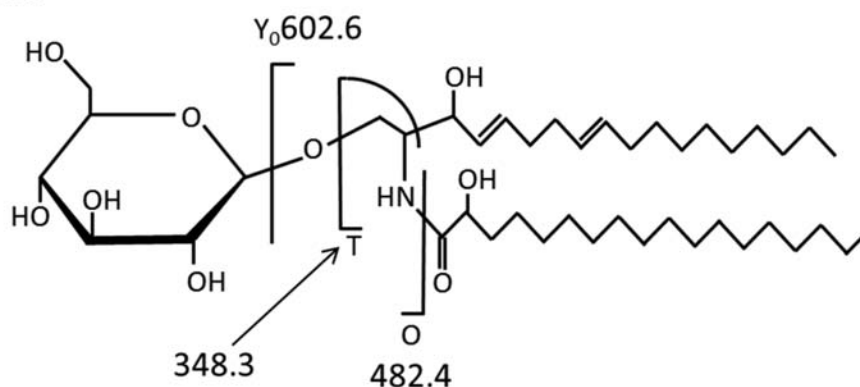

Figure 2. Structural determination of the molecule having m/z of 736.6 derived from barley shochu distillation remnants. (A) Positive ESI−MS/MS spectrum of the ion m/z of glucosylceramide purified from barley shochu distillation remnants. Ions trapped in ESI−MS of glucosylceramide were applied to ESI−MS/MS. (B) Structure of glucosylceramide predicted from detected precursor and fragment ions of the molecule having m/z of 736.6.

speed of 4000 m/z per second. Multiple stage MS was performed by collision-induced dissociation using helium as the collision gas. The precursor ion was selected within an isolation width of 4 u. The multiple stage sequencing up to MS/MS was carried out using a fragmentation amplitude of 1.0 V, ramped from 30% to 200% within 40 ms for each single spectrum, and a fragmentation cutoff default of 27% of the precursor ion m/z.

■ RESULTS AND DISCUSSION

Structural Determination of Glucosylceramide Contained in Shochu Distillation Remnants Produced from Barley and *A. kawachii*. In the former study, we have elucidated that glucosylceramide is contained at 2.7 mg/g dry weight of barley shochu distillation remnant.[38] To elucidate

Figure 3. Structural determination of the molecule having m/z of 764.7 derived from barley shochu distillation remnants. (A) Positive ESI−MS/MS spectra of the ion m/z of glucosylceramide purified from barley shochu distillation remnants. Ions trapped in ESI−MS of glucosylceramide were applied to ESI−MS/MS. (B) Structures of glucosylceramides predicted from detected precursor and fragment ions of the molecule having m/z of 764.7.

the molecular structure of glucosylceramide contained in barley shochu distillation remnant, glucosylceramide was purified and analyzed by ESI−MS. ESI−MS of the glucosylceramide generated four major singly charged, sodium adduct ions, m/z 736.6, 764.7, 776.7, and 792.7 (Table 1 and Figure 1).

One of the molecules having m/z of 736.6 was isolated and submitted to the first stage of fragmentation analysis proposed by a previously described method.[25] As shown in Table 1 and Figure 2A, the precursor molecule having m/z of 736.6 provided several abundant fragment ions. For structure assignment, the data were converted to those of lithium adduct ions and compared to fragmentation patterns deduced from the previously reported fragmentation ions of glucosylceramides.[25] The most abundant ion having m/z of 482.3 corresponded to O (deacyl) ion, a fragment ion of glucosylceramide N-2′-hydroxyhexadecanoyl-l-O-β-D-glucopyranosyl-4,8-sphingadienine (d18:2/C16:0h), which yields by the loss of the fatty acid acyl chain (neutral loss 267 u ($C_{18}O_1H_{35}$)) as a result of the cleavage of the amide N−CO bond. The other abundant ion having m/z of 574.5 corresponded to Y_0 (ceramide) ion, a fragment ion of the glucosylceramide, which yields by the loss of the glucosyl headgroup (neutral loss 163 u ($C_6H_{11}O_5$)) as a result of the cleavage of the acetal C−O bond (Figure 2B). Another abundant ion having m/z of 320.3 corresponded to T (fatty acid) and N (sphingoid base) ions of the same glucosylceramide molecule. T ion yields by the loss of the glucosyl headgroup and part of sphingoid base (neutral loss 416 u ($C_{22}O_7H_{40}$)) (Figure 2B).

N ion yields by the loss of glucosyl headgroup and fatty acid acyl chain (neutral loss 416 u ($C_{22}O_7H_{40}$)) of the glucosylceramide molecule (Figure 2B). These results firmly assign this molecule as N-2′-hydroxyhexadecanoyl-l-O-β-D-glucopyranosyl-4,8-sphingadienine (d18:2/C16:0h) (Figure 2B).

Similarly, the precursor molecule having m/z of 764.7 provided major fragment ions having m/z values of 602.6, 482.4, and 348.3 (Table 1 and Figure 3A), which coincided with Y_0, O, and T ions of glucosylceramide with a molecular structure of N-2′-hydroxyoctadecanoyl-l-O-β-D-glucopyranosyl-4,8-sphingadienine (d18:2/C18:0h) (Figure 3B). In addition, the precursor molecule having m/z of 776.7 provided major fragment ions having m/z values of 614.6, 496.4, and 346.3 (Table 1 and Figure 4A). These fragment ions coincided with m/z of Y_0, O, and T ions of glucosylceramide with a molecular structure of N-2′-hydroxy-3′-octadecenoyl-l-O-β-D-glucopyranosyl-9-methyl-4,8-sphingadienine (d19:2/C18:1h) (Figure 4B). The precursor molecule having m/z of 792.7 provided major fragment ions having m/z values of 630.6, 482.4, and 376.4 (Table 1 and Figure 5A), which coincided with Y_0, O, and T ions of glucosylceramide with a molecular structure of N-2′-hydroxyicosanoyl-l-O-β-D-glucopyranosyl-4,8-sphingadienine (d18:2/C20:0h), respectively (Figure 5B).

These results indicate that shochu distillation remnant (barley) contains glucosylceramides with the molecular structures d18:2/C16:0h, d18:2/C18:0h, d19:2/C18:1h, and d18:2/C20:0h. In addition to these ions, minor ions of $[M+Na+1]^+$, $[M+Na+2]^+$,
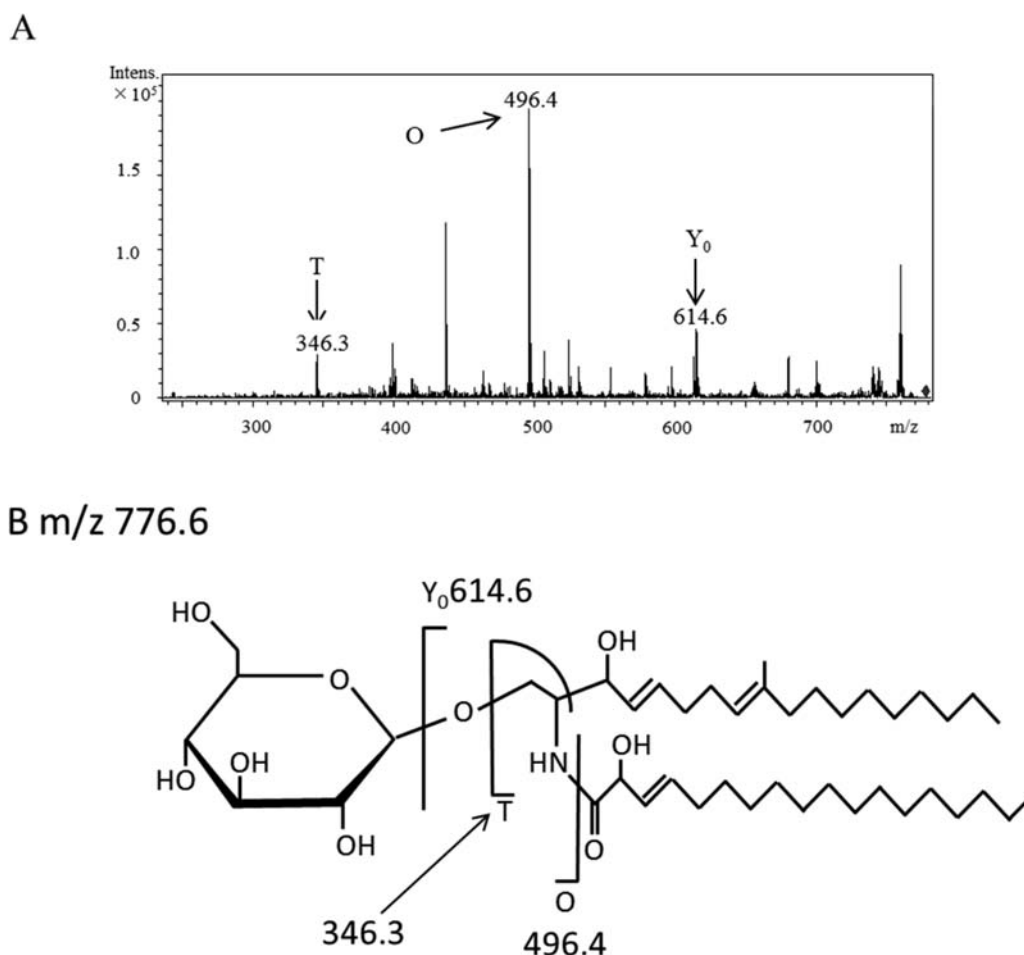
Figure 4. Structural determination of the molecule having m/z of 776.7 derived from barley shochu distillation remnants. (A) Positive ESI–MS/MS spectra of the ion m/z of glucosylceramide purified from barley shochu distillation remnants. Ions trapped in ESI–MS of glucosylceramide were applied to ESI–MS/MS. (B) Structures of glucosylceramides predicted from detected precursor and fragment ions of the molecule having m/z of 776.7.

[M+Na+3]$^+$, and [M+Na+4]$^+$ of the major [M+Na]$^+$ ions were observed in the ESI–MS spectrum (Figure 1). [M+Na+2]$^+$ and [M+Na+4]$^+$ ions are considered to be the molecules that have one or two saturated bonds instead of unsaturated bonds in the sphingoid base or fatty acid moiety of glucosylceramide [M+Na]$^+$ ion or ^{18}O mass isotopomers of [M+Na]$^+$ and [M+Na+2]$^+$ ions.[39] [M+Na+1]$^+$ and [M+Na+3]$^+$ ions are considered to be ^{13}C or ^{2}H mass isotopomers of [M+Na]$^+$ and [M+Na+2]$^+$ ions. Therefore, these ions were not analyzed further.

Structural Determination of Glucosylceramides Contained in the Rice Shochu Distillation Remnant. We sought to determine the source of glucosylceramide contained in barley shochu distillation remnant by investigating glucosylceramide contained in distillation remnants of shochu produced from other crops. Awamori is a kind of shochu produced from rice and *Aspergillus awamorii*. *A. kawachii* and *A. awamorii* are genetically close; *A. kawachii* is considered an albino mutant of *A. awamorii*.[39] Glucosylceramide, which was contained at 1.7 mg/g dry weight of the distillation remnant of the shochu produced from rice (Awamori), was purified and analyzed by ESI–MS/MS technique. ESI–MS analysis provided ions corresponding to glucosylceramides having m/z values of 776.6 and 791.7 (Table 2 and Figure S1A).

The precursor molecule having m/z of 776.6 provided major fragment ions having m/z values of 614.6, 496.4, 346.3, and 334.3, which correspond to Y_0, O, T, and N ions of d19:2/C18:1h, respectively (Table 2, Figure 6A, and Figure S1B). The precursor molecule having m/z of 791.7 provided major fragment ions having m/z values of 630.5, 482.4, and 376.4, which correspond to Y_0, O, and T ions of d18:2/C20:0h, respectively (Table 2, Figure 6B, and Figure S1C).

These results indicate that rice shochu distillation remnant contains glucosylceramides d19:2/C18:1h and d18:2/C20:0h, which were also detected in barley shochu distillation remnant.

Structural Determination of Glucosylceramides Contained in Koji (Barley Fermented with *A. kawachii*). To determine the origin of the glucosylceramides contained in shochu distillation remnants, glucosylceramide contained in koji (barley fermented with *A. kawachii*), which is used for production of shochu, was analyzed. Glucosylceramide was contained at 1.3 mg/g dry weight of koji. The glucosylceramide contained in koji was purified and analyzed by ESI–MS/MS technique. ESI–MS analysis provided ions having m/z values of 736.6, 764.6, 776.6, and 792.7 (Table 3 and Figure S2A).

The precursor molecule having m/z of 736.6 provided major fragment ions having m/z values of 574.5, 482.4, and 320.3, which correspond to Y_0, O, and T ions of glucosylceramide having a structure of d18:2/C16:0h (Table 3, Figure 7A, and Figure S2B), respectively. The precursor molecule having m/z of 764.6 provided major fragment ions having m/z values of
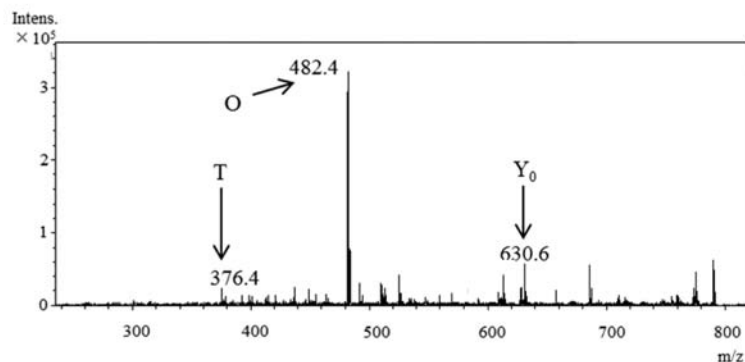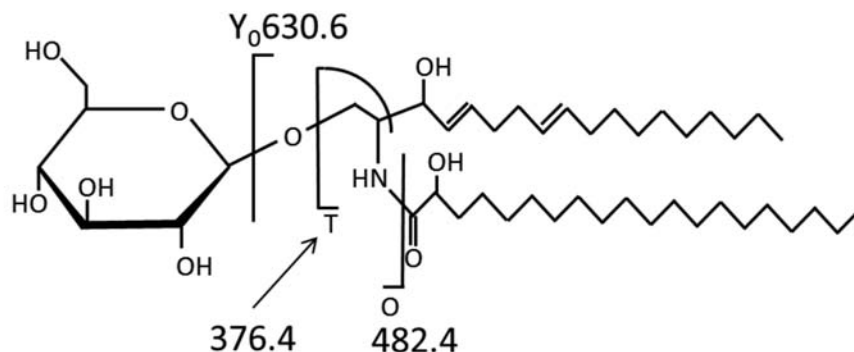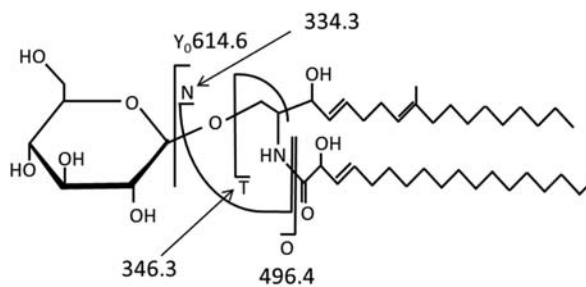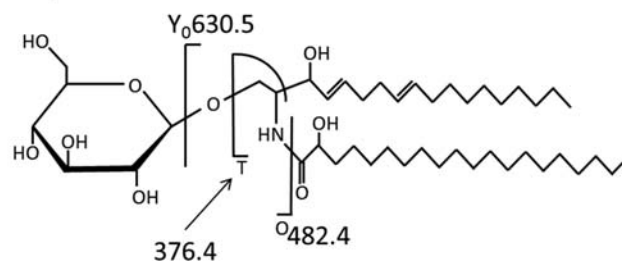
Figure 5. Structural determination of the molecule having m/z of 792.7 derived from barley shochu distillation remnants. (A) Positive ESI−MS/MS spectra of the ion m/z of glucosylceramide purified from barley shochu distillation remnants. Ions trapped in ESI−MS of glucosylceramide were applied to ESI−MS/MS. (B) Structures of glucosylceramides predicted from detected precursor and fragment ions of the molecule having m/z of 792.7.

Table 2. Ions Provided by Tandem ESI−MS/MS of Glucosylceramides Purified from Rice Shochu Distillation Remnant[a]

MS m/z (%)[b]	MS/MS m/z (%)[b]
791.7 (1.4)	679.5 (100), 591.4 (79.2), 592.4 (21.2), 677.5 (20.0), 547.4 (12.8), 503.4 (12.4), 773.6 (11.2), 459.3 (10.0), 678.5 (9.7), 415.3 (8.9), 482.4 (7.8), 630.6 (2.2), 376.4 (1.1)
776.6 (2.2)	496.4 (100), 758.6 (52.1), 346.3 (32.2), 497.4 (20.4), 596.5 (19.0), 759.6 (16.0), 575.5 (15.2), 506.3 (15.0), 614.6 (14.2), 524.3 (14.2)
663.5 (55.4)	
619.5 (76.4)	
589.5 (39.1)	
575.5 (94.5)	
561.4 (37.9)	
545.4 (39.6)	
531.4 (100)	
517.4 (38.1)	
487.4 (94.9)	
443.4 (65.9)	

[a]The spectra of ESI−MS and ESI−MS/MS are shown in Figure S1.
[b]% compared to base peak.

Figure 6. Structures of glucosylceramides predicted from detected precursor and fragment ions of rice shochu distillation remnant. (A) m/z 776.6 (N-2′-hydroxy-3′-octadecenoyl-l-O-β-D-glucopyranosyl-9-methyl-4,8-sphingadienine), (B) m/z 791.7 (N-2′-hydroxyicosanoyl-l-O-β-D-glucopyranosyl-4,8-sphingadienine).

602.6, 482.4, and 348.4, which correspond to Y_0, O, and T ions of glucosylceramide with a structure of d18:2/C18:0h, respectively (Table 3, Figure 7B, and Figure S2C). The precursor molecule having m/z of 776.6 generated fragment ions of m/z 614.6, 496.4, and 346.3, which correspond to Y_0, O, and T ions

of glucosylceramide having a structure of d19:2/C18:1h, respectively (Table 3, Figure 7C, and Figure S2D). The precursor molecule with m/z 792.7 generated fragment ions of m/z 630.6, 482.4, and 376.4, which correspond to Y_0, O, and T ions of

glucosylceramide with a structure of d18:2/C20:0h, respectively (Table 3, Figure 7D, and Figure S2E).

These results indicate that koji contains glucosylceramides with structures of d18:2/C16:0h, d18:2/C18:0h, d19:2/C18:1h, and d18:2/C20:0h, which correspond to the glucosylceramides contained in the barley shochu distillation remnants. The common glucosylceramides shared between barley shochu distillation remnants, rice shochu distillation remnants, and koji (barley fermented

Table 3. Ions Provided by Tandem ESI−MS/MS of Glucosylceramides Purified from Koji (Barley Fermented with _A. kawachii_)[a]

MS m/z (%)[b]	MS/MS m/z (%)[b]
792.7 (39.4)	510.4 (100), 774.6 (83.2), 772.6 (73.0), 628.6 (62.1), 773.6 (60.2), 512.4 (53.4), 482.4 (52.9), 315.3 (47.8), 630.6 (46.2), 477.3 (43.3), 376.4 (15.1)
776.6 (27.4)	496.4 (100), 758.6 (53.0), 437.3 (48.8), 693.6 (27.5), 614.6 (24.8), 759.6 (23.2), 497.4 (20.7), 694.1 (18.8), 524.4 (17.8), 346.3 (17.5)
764.6 (24.9)	569.3 (100), 482.4 (73.5), 591.3 (53.0), 744.6 (39.8), 570.4 (33.9), 425.3 (31.1), 746.5 (31.0), 393.2 (30.4), 454.4 (27.8), 629.3 (25.5), 602.6 (11.9), 348.4 (9.3)
736.6 (24.1)	345.2 (100), 482.4 (96.0), 538.6 (64.4), 601.3 (60.0), 565.5 (52.9), 718.5 (51.7), 610.9 (43.1), 540.4 (40.6), 581.9 (39.9), 600.3 (39.6), 574.5 (25.8), 320.3 (12.0)
657.4 (37.8)	
613.3 (48.2)	
569.3 (53.7)	
413.3 (51.5)	
360.4 (100)	
301.2 (68.2)	
280.2 (51.8)	
266.1 (42.5)	

[a]The spectra of ESI−MS and ESI−MS/MS are shown in Figure S2.
[b]% compared to base peak.

Table 4. Ions Provided by Tandem ESI−MS/MS of Glucosylceramides Purified from Culture Broth of _A. kawachii_[a]

MS m/z (%)[b]	MS/MS m/z (%)[b]
779.7 (44.0)	
778.7 (100)	496.4 (100), 758.6 (21.5), 497.4 (18.0), 524.4 (13.4), 614.6 (12.6), 506.4 (11.2), 616.6 (10.3), 463.3 (7.9), 759.6 (7.7), 348.3 (7.4), 334.3 (1.0)
776.6 (42.2)	496.4 (100), 758.6 (47.3), 694.6 (42.8), 693.6 (31.3), 695.1 (30.2), 614.6 (26.4), 694.1 (24.1), 759.6 (20.2), 497.4 (19.9), 524.4 (16.7), 346.3 (15.3), 334.3 (2.2)
531.4 (30.6)	
487.4 (29.4)	
443.4 (30.5)	
360.4 (43.0)	
301.2 (26.0)	
258.3 (44.8)	
230.3 (47.9)	

[a]The spectra of ESI−MS and ESI−MS/MS are shown in Figure S3.
[b]% compared to base peak.
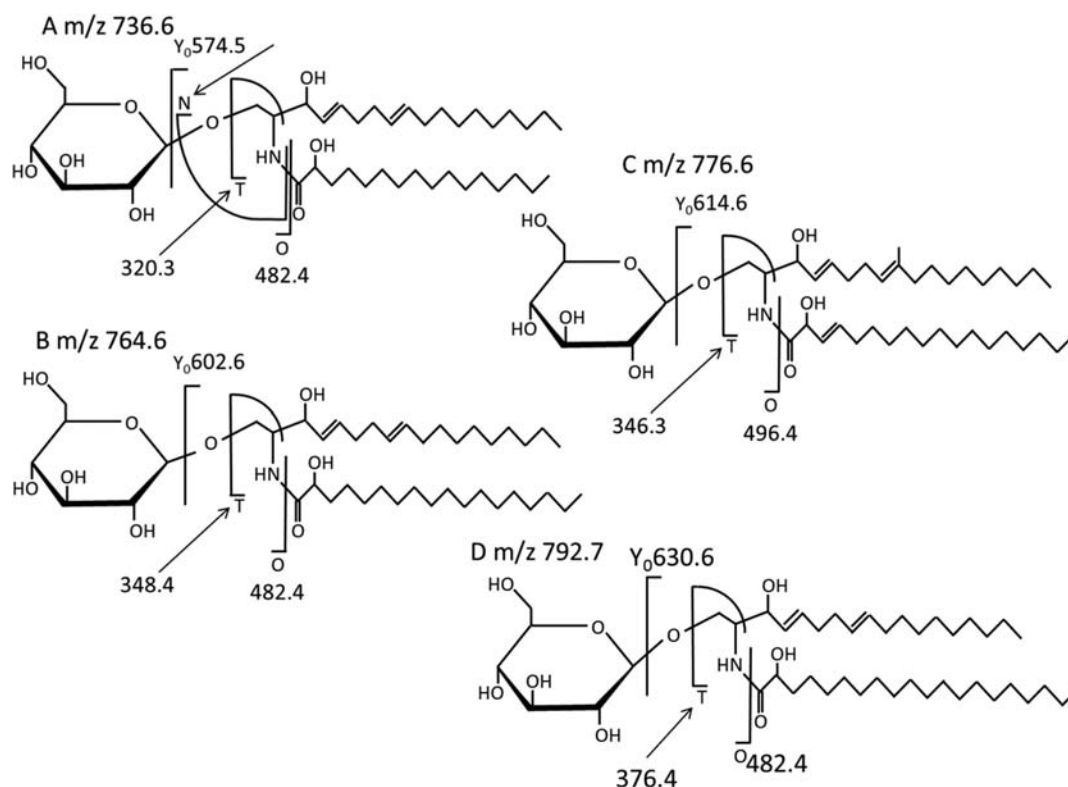
Figure 7. Structures of glucosylceramides predicted from detected precursor and fragment ions of koji (barley fermented with _A. kawachii_). (A) m/z 736.6 (_N_-2′-hydroxyhexadecanoyl-l-_O_-β-_D_-glucopyranosyl-4,8-sphingadienine), (B) m/z 764.6 (_N_-2′-hydroxyoctadecanoyl-l-_O_-β-_D_-glucopyranosyl-4,8-sphingadienine), (C) m/z 776.6 (_N_-2′-hydroxy-3′-octadecenoyl-l-_O_-β-_D_-glucopyranosyl-9-methyl-4,8-sphingadienine), (D) m/z 792.7 (_N_-2′-hydroxyicosanoyl-l-_O_-β-_D_-glucopyranosyl-4,8-sphingadienine).

with *A. kawachii*) led us to hypothesize that d19:2/C18:1h is produced by *A. kawachii*.

Structural Determination of Glucosylceramides Contained in the Culture Broth of *A. kawachii*. Finally, to determine whether glucosylceramide with a structure of d19:2/C18:1h contained in the shochu distillation remnants and koji is derived from *A. kawachii*, glucosylceramide, which was contained at 3.1 mg/g dry weight of the culture broth of *A. kawachii*, was purified and investigated by ESI−MS/MS technique. Two major molecules, m/z 776.6 and 778.7, were detected in the ESI−MS spectrum (Table 4 and Figure S3A). The precursor molecule with m/z 776.6 provided major fragment ions having m/z values of 614.6, 496.4, and 346.3, which correspond to Y_0, O, and T ions of glucosylceramide with the structure of d19:2/C18:0h, respectively (Table 4, Figure 8A, and Figure S3B). The
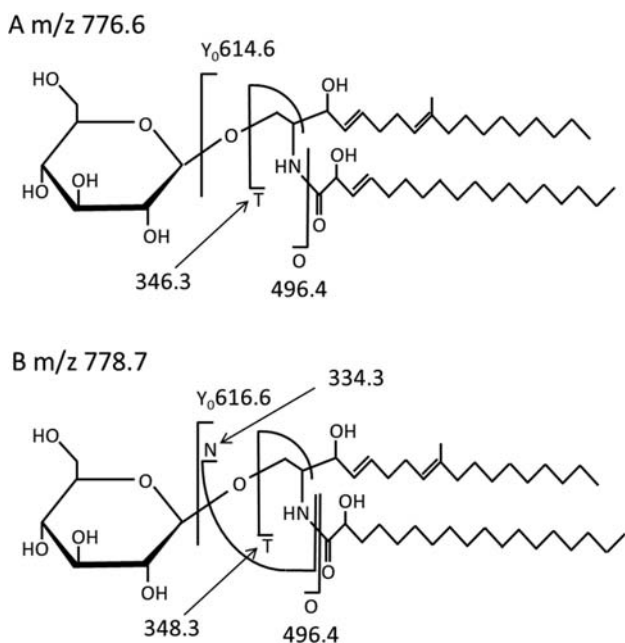
Figure 8. Structures of glucosylceramides predicted from detected precursor and fragment ions of culture broth of *A. kawachii*. (A) m/z 776.6 (N-2′-hydroxy-3′-octadecenoyl-l-O-β-D-glucopyranosyl-9-methyl-4,8-sphingadienine), (B) m/z 778.7 (N-2′-hydroxyoctadecanoyl-l-O-β-D-glucopyranosyl-9-methyl-4,8-sphingadienine).

precursor molecule with m/z 778.7 provided major fragment ions having m/z values of 616.6, 496.4, 348.3, and 334.3, which correspond to Y_0, O, T, and N ions of glucosylceramide with a structure of d19:2/C18:1h, respectively (Table 4, Figure 8B, and Figure S3C). These results indicated that culture broth of *A. kawachii* contains glucosylceramides with structures of d19:2/C18:0h and d19:2/C18:1h and that glucosylceramide with a structure of d19:2/C18:1h contained in shochu distillation remnants (barley and rice) and koji is derived from *A. kawachii*.

To our knowledge, this is the first report to describe that (1) barley shochu distillation remnants contain glucosylceramides d18:2/h16:0, d18:2/C20:0h, d19:2/C18:1h, and d18:2/C18:0h, (2) rice shochu distillation remnants contained glucosylceramides d19:2/C18:1h and d18:2/C20:0h, (3) koji (barley fermented with *A. kawachii*) contained glucosylceramides d18:2/C16:0h, d18:2/C18:0h, d19:2/C18:1h, and d18:2/C20:0h, and (4) the culture broth of *A. kawachii* contained glucosylceramides d19:2/C18:1h and d19:2/C18:0h (Table 5). These results first indicate that glucosylceramides having structures of d18:2/h16:0, d18:2/C20:0h, and d18:2/C18:0h in shochu distillation remnant are derived from crops, that having a structure of d19:2/C18:1h is produced by *A. kawachii*, and these glucosylceramide are contained in shochu distillation remnants. Furthermore, this is the first report of structural determination of glucosylceramides produced by *A. luchuensis* including *A. kawachii* and *A. awamorii*.

Specificity of Glucosylceramides of *Aspergillus luchuensis*. The structures of glucosylceramides of *A. luchuensis*, which we first elucidated in the present study, share some similarities but specificities as compared to those of other *Aspergillus* genus. *A. luchuensis*, which contains *A. kawachii* and *A. awamorii*, is grouped in the *Aspergillus* genus, and is often categorized into black *Aspergilli* (e.g., *A. niger* and *A. luchuensis*), but is genetically discriminated from *A. niger* based on RFLP analysis.[40,41] The glucosylceramides of *A. luchuensis* are N-2′-hydroxy-3′-octadecenoyl-l-O-β-D-glucopyranosyl-9-methyl-4,8-sphingadienine and N-2′-hydroxyoctadecanoyl-l-O-β-D-glucopyranosyl-9-methyl-4,8-sphingadienine, as elucidated in this study. To the contrary, the predominant glucosylceramide species of *A. oryzae* are reported to be N-2′-hydroxyoctadecanoyl-1-O-β-D-glucosyl-17-methylsphingadienine and N-2′-hydroxy-3′-octadecenoyl-1-O-β-D-glucosyl-17-methylsphingadienine.[42] The predominant glucosylceramide species of *A. fumigatus* was N-2′-hydroxy-3′-octadecenoyl-l-O-β-D-glucopyranosyl-9-methyl-4,8-sphingadienine.[43] The structures of glucosylceramides in *A. niger* are reported to be N-2′-hydroxy-3′-octadecenoyl-1-O-β-D-glucosyl-4,8-sphingadienine[44] and N-2′-hydroxy-3′-hexadecenoyl-1-O-β-D-glucopyranosyl-9-methyl-4,8-icosadien-1,3-diol.[45] Therefore, the structures of glucosylceramides of *A. luchuensis* including *A. kawachii* and *A. awamorii* share some similarities with those of *A. fumigatus* and *A. niger*, but are distinct from those of *A. oryzae*.

Stability of Glucosylceramides in Shochu Distillation Remnant. Although four glucosylceramide species of barley shochu distillation remnant and koji were detected, only two glucosylceramides were detected from rice shochu distillation remnant. Furthermore, a peak having m/z of glucosylceramides containing sphingatriene (d18:3[4,8,10]), which has been reported to be contained in rice (m/z of the sodium adduct ions of d18:3/C18:0h and d18:3/C20:0h correspond to 762 and 790, respectively),[21] was not clearly observed in the ESI−MS spectrum. We consider that the difference in distillation can explain

Table 5. Fragment Ions of Glucosylceramides Contained in Shochu Distillation Remnant (Barley or Rice), Koji, and Culture Broth of *A. kawachii*

m/z	structure	barley shochu distillation remnant	rice shochu distillation remnant	koji (barley fermented with *A. kawachii*)	culture broth of *A. kawachii*
736	d18:2/C16:0h	Y_0, 574.5; O, 482.3; T/N, 320.3		Y_0, 574.5; O, 482.4; T/N, 320.3	
764	d18:2/C18:0h	Y_0, 602.6; O, 482.3; T, 348.3		Y_0, 602.6; O, 482.4; T, 348.4	
776	d19:2/C18:1h	Y_0, 614.6; O, 496.4; N, 346.3	Y_0, 614.6; O, 496.4; N, 346.3	Y_0, 614.6; O, 496.4; N, 346.3	Y_0, 614.6; O, 496.4; N, 346.3
778	d19:2/C18:0h				Y_0, 616.6; O, 496.4; N, 348.3; T, 334.3
792	d18:2/C20:0h	Y_0, 630.6; O, 482.4; T, 376.4	Y_0, 630.5; O, 482.4; T, 376.4	Y_0, 630.6; O, 482.4; T, 376.4	

the variable detection of glucosylceramides in rice distillation remnants. While barley shochu is produced by distilling the mash under decompression and the mash is heated to approximately 50 °C, rice shochu (Awamori) is produced by distilling the mash at atmospheric pressure and the mash is heated to approximately 100 °C. Therefore, glucosylceramides that are contained in rice and that are produced by *A. awamorii* are likely to be oxidized and degraded during the distillation process.

Transfer of Glucosylceramides to the Shochu Distillation Remnants from Crops and *A. kawachii*. By comparing the structures of glucosylceramides from various sources, it was indicated that glucosylceramides d19:2/C18:1h and d19:2/C18:0h are produced by *A. kawachii*, and glucosylceramides d18:2/C16:0h, d18:2/C18:0h, and d18:2/C20:0h are contained in barley, and glucosylceramide d18:2/C20:0h is contained in rice. Because sphingolipids having 9-methyl-C18-sphingosine moiety are widely found in fungi,[19,41,43,44] and glucosylceramides d18:2/C16:0h, d18:2/C18:0h, and d18:2/C20:0h are found in the family *Poaceae* including wheat and rice[21,25] and related crop species,[36] it is estimated from the current study that the glucosylceramides that are already contained in these crops and those produced by *A. kawachii* during fermentation on steamed crops are transferred through the processes of fermentation with yeast and distillation to the shochu distillation remnant.

In conclusion, we purified and determined molecular structures of glucosylceramides contained in barley and rice shochu distillation remnants, koji (barley fermented with *A. kawachii*), and the culture broth of *A. kawachii*. It turned out that glucosylceramides d18:2/C16:0h, d18:2/C18:0h, and d18:2/C20:0h, which are contained in crops, and glucosylceramide d19:2/C18:1h, which is produced by *A. kawachii*, comprise the major glucosylceramides in shochu distillation remnants. This information will be valuable to facilitate utilization of shochu distillation remnants and *A. kawachii* for cosmetics, functional foods, and medicines.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information
Complete spectra of ESI−MS and ESI−MS/MS in this study provided as Figures S1−S3. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes
The authors declare no competing financial interest.

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