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Note

Two novel oligosaccharides isolated from a beverage produced by fermentation of a plant extract

Naoki Kawazoe,[a,b] Hideki Okada,[a] Eri Fukushi,[c] Akira Yamamori,[a] Shuichi Onodera,[b] Jun Kawabata[c] and Norio Shiomi[b,*]

[a]*Ohtakakohso, Co. Ltd, Otaru 047-0193, Japan*
[b]*Department of Food and Nutrition Sciences, Graduate School of Dairy Science Research, Rakuno Gakuen University, Ebetsu 069-8501, Japan*
[c]*Graduate School of Agriculture, Hokkaido University, Sapporo 060-8589, Japan*

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Abstract—An extract from 50 kinds of fruits and vegetables was fermented to produce a new beverage. Natural fermentation of the extract was carried out mainly by lactic acid bacteria (*Leuconostoc* spp.) and yeast (*Zygosaccharomyces* spp. and *Pichia* spp.). Two new saccharides were found in this fermented beverage. The saccharides were isolated using carbon–Celite column chromatography and preparative high performance liquid chromatography. Gas liquid chromatography analysis of methylated derivatives as well as MALDI-TOF MS and NMR measurements were used for structural confirmation. The ^{1}H and ^{13}C NMR signals of each saccharide were assigned using 2D-NMR including COSY, HSQC, HSQC-TOCSY, CH_2-HSQC-TOCSY, and CT-HMBC experiments. The saccharides were identified as β-D-fructopyranosyl-(2→6)-β-D-glucopyranosyl-(1→3)-D-glucopyranose and β-D-fructopyranosyl-(2→6)-[β-D-glucopyranosyl-(1→3)]-D-glucopyranose.

Keywords: β-D-Fructopyranosyl-(2→6)-β-D-glucopyranosyl-(1→3)-D-glucopyranose; β-D-Fructopyranosyl-(2→6)-[β-D-glucopyranosyl-(1→3)]-D-glucopyranose; β-D-Fructopyranosyl-(2→6)-D-glucopyranose; Fructopyranoside; Oligosaccharides; Fermented beverage

A beverage was produced by fermentation of an extract from 50 kinds of fruits and vegetables. The extract was obtained using sucrose-osmotic pressure in a cider barrel for seven days and was fermented by lactic acid bacteria (*Leuconostoc* spp.) and yeast (*Zygosaccharomyces* spp. and *Pichia* spp.) for 180 days. The fermented beverage showed scavenging activity against 1,1′-diphenyl-2-picrylhydrazyl (DPPH) radical, and significantly reduced the ethanol-induced damage of gastric mucosa in rats.[1] In a previous paper, we showed that this beverage contained high level of saccharides, containing mainly D-glucose and D-fructose, and a small amount of disaccharides, laminaribiose, maltose and a novel saccharide: β-D-fructopyranosyl-(2→6)-D-glucopyranose.[2]

Recently, our preliminary studies showed that two trisaccharides were also produced during fermentation. Now, we have confirmed the structures of these two novel saccharides as β-D-fructopyranosyl-(2→6)-β-D-glucopyranosyl-(1→3)-D-glucopyranose and β-D-fructopyranosyl-(2→6)-[β-D-glucopyranosyl-(1→3)]-D-glucopyranose (Fig. 1) through the use of GC–MS, MALDI-TOF-MS, and NMR measurements.

The synthesis of saccharides by the fermentation of the plant extract was investigated using HPLC using the *p*-aminobenzoic acid ethyl ester (ABEE) derivatization method.[3,4] The plant extract was fermented at 37 °C for 180 days in the dark. HPLC analysis showed that saccharides **1** and **2** were produced during fermentation.

After fermentation, the beverage (1000 g) was loaded onto a carbon–Celite column and was successively eluted with water (14 L), 5% ethanol (30 L), and 30%

* Corresponding author. Tel.: +81 11 388 4754; fax: +81 11 387 5848; e-mail: n-shiomi@rakuno.ac.jp

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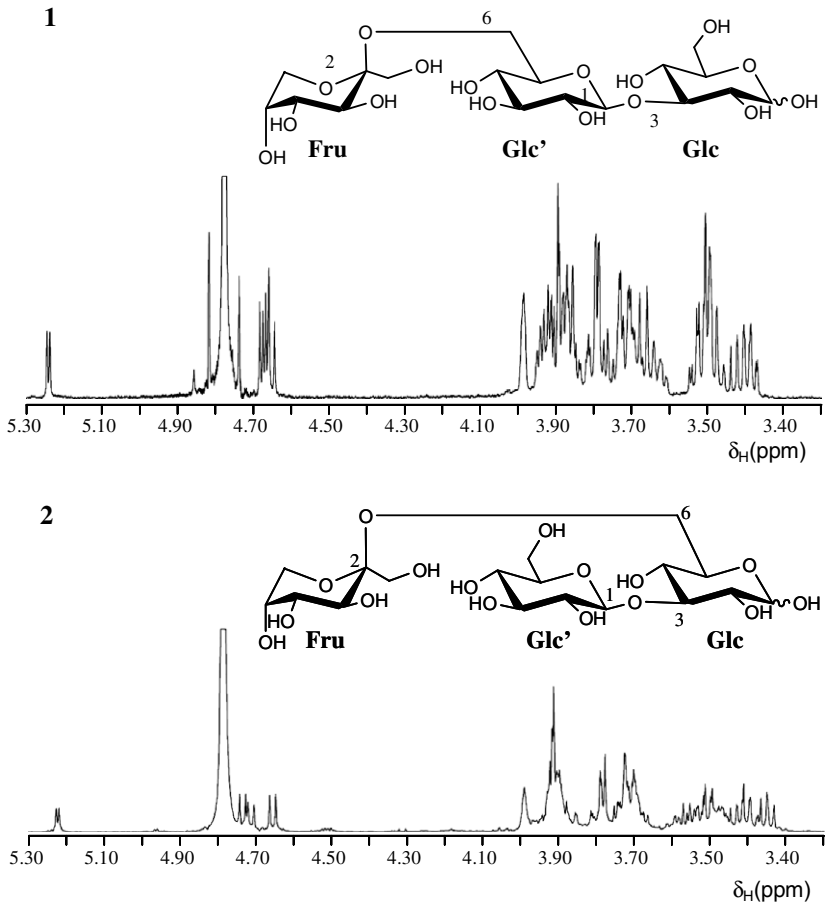

Figure 1. Structures and ^{1}H NMR spectra of the two novel oligosaccharides. Compound **1**: β-D-fructopyranosyl-(2→6)-β-D-glucopyranosyl-(1→3)-D-glucopyranose; compound **2**: β-D-fructopyranosyl-(2→6)-[β-D-glucopyranosyl-(1→3)]-D-glucopyranose.

ethanol (10 L). Almost all of D-glucose and D-fructose were eluted with water (4 L) and then saccharides **1** and **2** were eluted with 30% ethanol (1–2 L). The 30% ethanol fraction containing **1** and **2** was concentrated in vacuo and freeze-dried to give 22 mg. Subsequently, the 30% ethanol fraction was successfully purified repeatedly by HPLC (Tosoh, Tokyo, Japan) on an Amide-80 column (7.8 mm × 30 cm, Tosoh, Tokyo, Japan) at 80 °C, and eluted with 80% acetonitrile at 2.0 mL/min, using refractive index detection. Saccharides **1** and **2** were separated in order of elution in a short time using HPLC. Purified saccharides **1** (10 mg) and **2** (8 mg) were obtained as white powders.

Saccharides **1** and **2** were shown to be homogeneous using anion exchange HPLC ($t_{R,sucrose}$ (relative retention time; relative retention time of sucrose = 1.0): 1.74 and 1.72). The relative retention time of **1** and **2** did not correspond to that of any authentic samples. The degree of polymerization was established as three by mass spectrometric measurements $[M+Na]^{+}$ ions (*m*/*z*: 527) using TOF-MS, and analyses of the molar ratios of D-glucose to D-fructose in the acid hydrolysates. Yeast β-D-fructofuranosidase did not hydrolyze **1** and **2** nor were they hydrolyzed by yeast α-glucosidase. However, **2** was hydrolyzed by β-glucosidase to release glucose and β-D-fructopyranosyl-(2→6)-glucose. Partial acid hydrolysis of **1** and **2** liberated D-glucose, D-fructose, and laminaribiose.

By the GLC analysis, the retention times of the methanolysate of the permethylated saccharides were investigated (*t*, retention time of methyl 2,3,4,6-tetra-*O*-methyl-β-D-glucopyranoside; 8.70 min). The methanolysate of permethylated saccharide **1** exhibited six peaks, four of which corresponded to the α/β isomers of methyl 2,3,4-tri-*O*-methyl-D-glucopyranoside (*t*, 21.8 and 31.6 min) and methyl 2,4,6-tri-*O*-methyl-D-glucopyranoside (*t*, 28.4 and 41.5 min). Because the other two peaks (*t*, 8.44 and 12.4 min) were estimated to correspond to the α/β isomers of methyl 1,3,4,5-tetra-*O*-methyl-D-fructopyranoside, the methanolysate of permethylated saccharide **1** was analyzed by GC–MS. This methanolysate exhibited two peaks (14.2 and 15.4 min) corresponding to methyl 1,3,4,5-tetra-*O*-methyl-D-fructopyranoside.[5] No peaks corresponding to methyl 1,3,4,6-tetra-*O*-methyl-D-fructofuranoside were detected. On the other hand, the methanolysate of permethylated **2** exhibited three peaks, which corresponded to the α/β

isomers of methyl 2,3,4,6-tetra-*O*-methyl-D-glucopyranoside (*t*, 8.96 and 12.3 min) and methyl 1,3,4,5-tetra-*O*-methyl-D-fructopyranoside (*t*, 8.00 and 12.3 min). The methanolysate of permethylated **2** also gave other two peaks that were confirmed to be methyl 2,4-di-*O*-methyl-D-glucopyranoside (α/β anomers, 14.3 and 15.8 min) from the retention time and pattern of fragmentation with GC–MS.[6] No peaks corresponding to methyl 1,3,4,6-tetra-*O*-methyl-D-fructofuranoside and methyl 2,4,6-tri-*O*-methyl-D-glucopyranoside were detected. From these findings, saccharides **1**and **2** were shown to be D-fructopyranosyl (2→6)-β-D-glucopyranosyl-(1→3)-D-glucose and D-fructopyranosyl-(2→6)-[β-D-glucopyranosyl (1→3)]-D-glucose, respectively.

The structural confirmation of saccharides **1** and **2** by ^{1}H and ^{13}C NMR analyses and the subsequent complete assignment of ^{1}H and ^{13}C NMR signals of the two saccharides were carried out using 2D-NMR techniques. The NMR spectra of **1** showed that it was anomeric mixture at the glucose residue. The β anomer was predominant and some signals of the α anomer were distinct and could be assigned. The HSQC-TOCSY[7,8] and CH2-HSQC-TOCSY[9] spectrum revealed the ^{1}H-spin systems of each Glc′, β-Glc, α-Glc, and Fru residues. The COSY[10,11] spectrum assigned from H-1 to H-3 in each Glc′, β-Glc, and α-Glc, and from H-3 to H-5 in Fru. The corresponding ^{13}C signals were assigned from the HSQC[7] spectrum. The quaternary carbon was assigned as C-2 of Fru. The isolated methylene was assigned as H-1 and C-1 in Fru. The HMBC[12,13] correlations of C-3/H-1 and C-2/H-1 in Fru confirm the assignment of these signals. The other four methylene carbons were assigned as C-6 in these residues (Fig. 2A-a). The HMBC correlations of C-5/H-1 in each Glc′ and β-Glc revealed the assignment of C-5 in each residue. The resting ^{1}H and ^{13}C signals in each Glc′, β-Glc, and α-Glc residues could be assigned as H-4 and C-4. These results clarified the assignment of ^{1}H and ^{13}C NMR signals of each residue.

The position of glycosidic linkage, and pyranoside form of Fru were analyzed as follows: The C-3 in β-Glc and α-Glc showed the HMBC correlations between H-1 in Glc′, and C-1 in Glc′ showed the HMBC correlations to H-3 in β-Glc and α-Glc (Fig. 2A-b). The *J* (H-1, H-2) value in Glc′ was 7.7 Hz. These results indicate the Glc′-(1→3)-Glc linkage, namely, the laminaribiose moiety. The C-2 of Fru showed the HMBC correlations to H-6 of Glc′ and H-6 of Fru (Fig. 2A-b). These results revealed **1** had fructopyranoside residue and Fru*p*-(2→6)-Glc′ linkage. All ^{1}H and ^{13}C NMR signals were assigned as shown in Table 1.

The coupling patterns of overlapped ^{1}H-signals of the major anomer were analysed by the SPT method. Due to strong coupling between H-3 and H-4 and between H-5 and H-6 in Glc′, between H-4 and H-5 in β-Glc, and between H-3 and H-4 and between H-1 methylene protons in Fru, these couplings could not be analyzed as first order. The δ_C values of Fru indicated its β anomer form, by comparing those of α and β form of methyl-D-fructopyranoside.[13] The δ_H and δ_C of Glc′, β-Glc, and α-Glc are in good agreement with those of laminaribiose except for the position at which the Fru was attached.

The NMR spectra of **2** were analysed in the same manner as those of **1**. Saccharide **2** was also an anomer mixture at the glucose residue. Here too, the β anomer was predominant and some distinct signals of α anomer could be assigned. The methylene signals were assigned by HSQC spectrum (Fig. 2B-a). The HMBC spectrum (Fig. 2B-b) revealed a laminaribiose moiety having a fructopyranoside residue at Glc-6. NMR signals were assigned as shown in Table 1. The δ_C values of Fru indicated its β anomer form, by comparing those of α and β form of methyl-D-fructopyranoside.[14]

From these findings, the fructose residue of the nonreducing termini of this saccharide was in the pyranose form, and **1** and **2** were confirmed to be the new trisaccharides: β-D-fructopyranosyl-(2→6)-β-D-glucopyrano-

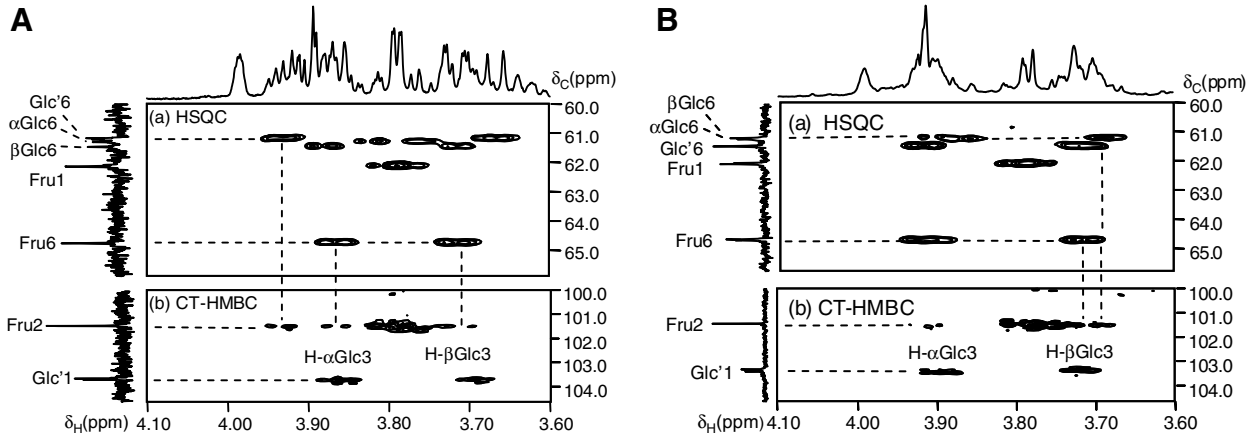

Figure 2. Partial HSQC (A-a and B-a) and CT-HMBC (A-b and B-b) spectra of **1** (A) and **2** (B).

Table 1. ^{1}H and ^{13}C NMR spectral data (δ[a] in ppm, j in Hz) of **1** and **2**

Saccharide **1**						Saccharide **2**					
		δ_C	δ_H		J_{HH}			δ_C	δ_H		J_{HH}
Fru	1	62.18	3.79	m[b]		Fru	1	62.16	3.79		
			3.79	m					3.79	m	
	2	101.58					2	101.55			
	3	69.50	3.89	m			3	69.28	3.92	m	
	4	70.36	3.90	m			4	70.33	3.92	m	
	5	69.95	3.99	dd	3.2, 1.5		5	69.95	3.99	br s[c]	
	6	64.86	3.87	m			6	64.86	3.93	m	
			3.72	m					3.72	m	
Glc′	1	103.95	4.67	d	7.7	Glc′	1	103.58	4.74	d	8.0
	2	74.08	3.38	dd	9.8, 7.7		2	74.26	3.35	dd	9.4, 8.0
	3	76.19	3.53	m			3	76.37	3.52	dd	9.4, 8.7
	4	70.45	3.47	m			4	70.39	3.41	dd	8.9, 8.7
	5	75.80	3.63	m			5	76.83	3.47	m	
	6	61.20	3.93	m			6	61.52	3.92	m	
			3.67	m					3.72	m	
Glcβ	1	96.44	4.68	d	8.0	Glcβ	1	96.50	4.66	d	8.1
	2	74.11	3.42	dd	9.4, 8.0		2	74.63	3.43	dd	8.3, 8.1
	3	87.02	3.69	m			3	85.03	3.73	dd	8.3, 8.3
	4	69.16	3.50	m			4	68.91	3.56	m	
	5	76.26	3.50	m			5	75.57	3.59	m	
	6	61.51	3.88	m			6	61.23	3.90	m	
			3.72	m					3.69	m	
Glcα[d]	1	92.71	5.25	d	3.7	Glcα	1	92.79	5.23	d	3.6
	2	71.27	3.72	dd	9.6, 3.7		2	71.81	3.73	dd	9.6, 3.6
	3	84.83	3.85				3	82.83	3.90	dd	9.6, 9.6
	4	69.13	3.50				4	69.03	3.55		
	5	71.92	3.87				5	71.46	3.96		
	6	61.34	3.82				6	61.97	3.87		
			3.67						3.69		

[a] Chemical shifts of ^{1}H (δ_H) and ^{13}C (δ_C) are in ppm and were determined relative to an external standard of sodium [2,2,3,3-$^{2}H_4$]-3-(trimethylsilyl) propanoate in D_2O (δ_H 0.00 ppm) and 1,4-dioxane (δ_C 67.40 ppm) in D_2O, respectively.
[b] m: multiplet.
[c] br s: broad singlet.
[d] Some of the signals of the minor anomer (α-anomer of glucose) were separated and could be assigned.

syl-(1→3)-D-glucopyranose, β-D-fructopyranosyl-(2→ 6)-[β-D-glucopyranosyl-(1→3)]-D-glucopyranose (Fig. 1). Furthermore, these novel saccharides were confirmed to be produced by fermentation. No saccharides containing fructopyranoside residues have been found in natural sources except the saccharides in this fermented beverage.

1. Experimental

1.1. Materials

The ABEE Labeling Kit was purchased from Seikagaku Co. (Tokyo, Japan). Glucose, fructose, maltose, isomaltose, melibiose, nigerose, sophorose, lactose, gentiobiose, cellobiose, kojibiose, laminaribiose, and raffinose were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals used in this study were of analytical grade.

1.2. Preparation of fermented beverage of plant extract

For preparation of the initial juice, 50 different fruits and vegetables were used to produce the final extract as described in a previous paper.[1,2] The 50 fruits and vegetables were cut, sliced or diced into small pieces, mixed and put in cedar barrels. Afterwards, an equivalent weight of sucrose was added, mixed well to allow high contact between the samples and the sucrose, then the barrels were left for one week at room temperature. The juice exudate was then separated without compression from the solids and used for fermentation. The beverage was obtained by incubation of the juice at 37 °C in the dark by natural fermentation using yeast (*Zygosaccharomyces* spp. and *Pichia* spp.) and lactic acid bacteria (*Leuconostoc* spp.). After 7 days, the fermented beverage was kept in a closed enameled tank at 37 °C for 180 days for additional maturation and ageing, obtaining finally a brown and slightly sticky liquid.

1.3. *p*-Aminobenzoic acid ethyl ester (ABEE) derivatization method

Derivatization of the saccharides at reducing end with ABEE was carried out according to the method of Yasuno et al.[3,4] The saccharide solution (10 μL) was added to the ABEE reagent solution (40 μL). The mixture was incubated at 80 °C for 1 h. Distilled water (0.2 mL) and chloroform (0.2 mL) were added and the mixture was centrifuged at 2000*g* for 1 min. The aqueous layer was diluted 100-fold with water and subjected to HPLC analysis. The ABEE-derivatized saccharide was detected by UV at 305 nm.

1.4. Carbon–Celite column chromatography

Carbon–Celite [1:1; charcoal (Wako Pure Chemical Industries, Ltd; Osaka, Japan) and Celite-535 (Nacalai Tesque Inc, Osaka, Japan)] column (4.5 × 35 cm) was used to isolate **1** and **2**. The column was eluted with water, 5% and 30% ethanol.

1.5. High performance anion-exchange chromatography (HPAEC)

The oligosaccharides were analyzed using a Dionex Bio LC Series apparatus (Sunnyvale, CA) equipped with an HPLC carbohydrate column (Carbo Pack PA1, inert styrene divinyl benzene polymer, Sunnyvale, CA) and a pulsed amperometric detector (PAD)[15,16] in the same way as described in a previous paper.[17,18]

1.6. Hydrolysis

The oligosaccharides (0.5 mg) were dissolved in 0.05 N oxalic acid (0.5 mL) and partially hydrolyzed by heating at 60 °C for 15 min.

The oligosaccharides (0.5 mg) were incubated with 7.0 U of α-glucosidase (from yeast, Oriental Yeast Co., Ltd, Tokyo, Japan), 7.4 U of β-glucosidase (from Almond, Oriental Yeast Co., Ltd, Tokyo, Japan) or 5.0 U of β-fructofuranosidase (from yeast, Sigma Chemical Co., St. Louis, MO, USA) in 0.2 mL of McIlvaine buffer (pH 5.0) at 37 °C for 60 min.

1.7. Methylation and methanolysis

Methylation of the oligosaccharides was carried out by the method of Hakomori.[19] The permethylated saccharides were methanolyzed by heating with 1.5% methanolic hydrochloric acid at 96 °C for 10 or 180 min. The reaction mixture was treated with Amberlite IRA-410 (OH^-) to remove hydrochloric acid, and evaporated in vacuo to dryness. The resulting methanolysate was dissolved in a small volume of MeOH and analyzed using gas liquid chromatography.

1.8. Gas liquid chromatography (GLC)

For the analysis of methanolysate, GLC was carried out using a Shimadzu GC-8A gas chromatograph equipped with a glass column (2.6 mm × 2 m) packed with 15% butane 1,4-diol succinate polyester on acid-washed Celite at 175 °C. Flow rate of the nitrogen gas carrier was 40 mL/min.

1.9. GC–MS analysis

GC–MS analysis was performed using JMS-AX500 mass spectrometer (JEOL, Japan) using a DB-17HT capillary column (30 m × 0.25 mm I.D., J&W Scientific, USA). The injection temperature was 200 °C. The column temperature was kept at 50 °C for 2 min after sample injection, increased to 150 °C at 50 °C/min, kept at 150 °C for 1 min, then increased to 250 °C at 4 °C/min. The mass spectra were recorded in the positive ion electron ionization (EI) mode.

1.10. MALDI-TOF MS

Matrix assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF MS) spectra were obtained on a Shimadzu-Kratos mass spectrometer (KOMPACT probe) using 2,5-dihydroxybenzoic acid matrix.

1.11. NMR measurement

The saccharide (ca. 5 mg of **1** or **2**) was dissolved in 0.4 mL D_2O. NMR spectra were recorded at 27 °C with a Bruker AMX 500 spectrometer (^1H 500 MHz, ^{13}C 125 MHz) equipped with a 5 mm diameter C/H dual (1D spectra) and TXI proof (2D spectra). Chemical shifts of ^1H (δ_H) and ^{13}C (δ_C) in ppm were determined relatively to the external standard of sodium (2,2,3,3-$^2H_4$)-3-(trimethylsilyl) propanoate in D_2O (δ_H 0.00 ppm) and 1,4-dioxane (δ_C 67.40 ppm) in D_2O, respectively. ^1H–^1H COSY[10,11], HSQC,[7] and CT-HMBC[12,13] spectra were obtained using gradient selected pulse sequences. The phase sensitive HSQC-TOCSY[7,8] and CH_2-selected E-HSQC-TOCSY[9] spectra were determined with the sequence including inversion of direct resonance (IDR). The TOCSY mixing time (108 ms) was composed of MLEV-17 composite pulses guarded by trim pulse (2.5 ms).

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