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Structure Elucidation and Chemical Profile of Sphingolipids in Wheat Bran and Their Cytotoxic Effects against Human Colon Cancer Cells

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ABSTRACT: Sphingolipids are known to have diverse properties and physiological functions. These distinctive lipids have been identified in wheat bran, a food well-known for its chemopreventive activity. However, the complete profile of sphingolipids in wheat bran and their contributions to the cancer preventive effect of wheat bran have not been fully explored until this study. Twelve sphingolipids (**1**−**12**) were purified from wheat bran extract and characterized by analyzing their 1D and 2D NMR spectra, and seven sphingolipids (**13**−**19**) were characterized based on their tandem mass spectra (MS^n: $n = 2-4$). To the best of our knowledge, this is the first report of sphingolipids **1**, **6**−**9**, **11**−**14**, and **16**−**19** in wheat bran. In particular, 2-*N*-(2′-hydroxy-15′-tricosenoyl)-4-hydroxysphinganine (peak **17**) is a novel compound. Additionally, compounds **2**−**4** were reported with complete NMR data for the first time. Sphingolipids (**1**−**12**) showed little growth inhibition against human colon cancer cell lines (HCT-116 and HT-29) in vitro.

KEYWORDS: *sphingolipids, wheat bran, chemical profile, cytotoxic effects, colon cancer*

INTRODUCTION

Sphingolipids constitute a class of distinct compounds that are both endogenous to mammalian cells and available exogenously through dietary consumption. These unique lipids are composed of a sphingoid long-chain base, a fatty acid tethered to the amino group of the sphingosine, and a variable polar headgroup. The sphingosine base and the fatty acid alone constitute ceramide, and the linked head groups can vary from phosphocholine (sphingomyelin), to sugars (glycosphingolipids), to complex carbohydrates.[1] Mammalian cells usually contain sphingomyelin, a component not found in plants, and some neutral and acidic glycolipids as complementary constituents to phosphoglycerolipids and cholesterol in the plasma membrane structure.[2] Sphingolipids found in plant cells show more variety in their head groups, positions of alkene double bonds on the structure, and location of the hydroxyl groups on the sphingoid base.[3] The predominant sphingolipids in plant extracts are glucosylceramide and inositolphosphorylceramide (IPC) derivatives.[4]

Given the complexity and great variety of sphingolipid structures, it is not surprising that these compounds exert a multitude of bioactivities. In addition to their contribution to membrane structure, a growing body of literature suggests that dietary sphingolipids have protective effects against colon cancer. Dietary sphingomyelin supplementation at 0.1% of the diet (w/w) reduced the number of aberrant crypt foci (ACF), an early marker of colon carcinogenesis, by ∼70% in 1,2-dimethylhydrazine (DMH)-treated mice.[5] With longer feeding in the same model, sphingomyelin treatment suppresses the conversion of adenomas to adenocarcinomas.[6] Mazzei et al. found that dietary sphingomyelin suppressed intestinal inflammation and inflammation-driven colon cancer in dextran sodium sulfate and azoxymethane-treated mice.[7] It has been reported that sphingadienes derived from soy and other natural sphingolipids are cytotoxic to colon cancer cells and reduce adenoma formation in $Apc^{min/+}$ mice.[8,9] Aida et al. observed that DMH-treated mice fed diets containing 0.1 or 0.5% maize cerebroside had greatly suppressed ACF formation, with about 60% less ACF than the control group.[10] The activity of sphingolipids from plant-derived sources is extended to other grains, including wheat, a major dietary source of these compounds.[11] For example, biological effects of sphingoid bases prepared from wheat flour were evaluated in DLD-1 human colorectal cancer cells, and it was found that apoptosis was induced in a dose-dependent manner.[12]

The consumption of wheat bran products has been correlated with a lower incidence of colon cancer in epidemiological studies as well as in vivo cancer models.[5−7,13,14] For instance, Reddy et al. established that populations with a low risk for colon cancer consume foods high in dietary grains, such as wheat bran.[5] Our group showed that $Apc^{min/+}$ mice fed a wheat bran oil-fortified diet had significantly fewer tumors than animals in the control group. Whether sphingolipids in wheat bran contribute to its observed colon cancer preventive effects is still unclear. Although there have been studies implicating ceramide mono-, di-, tri-, or tetrahexosides in wheat flour or wheat grain by FD-MS or GC-MS,[9,15,16] there is a dearth of information on the complete chemical profile of sphingolipids in wheat bran as well as their anticancer activities. Therefore, this study is designed to provide the structures of the specific sphingolipids found in wheat bran and to investigate the cytotoxic activities of these compounds against human colon cancer cells. Our efforts are to evaluate whether sphingolipids in wheat bran contribute to the colon cancer preventive properties displayed by this grain.

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Table 1. ^{1}H (700 MHz) and ^{13}C (175 MHz) NMR Spectroscopic Data of 2−4 (in Py-d5)

	2		3		4	
position	δ_H, J	δ_C	δ_H, J	δ_C	δ_H, J	δ_C
1	4.74, dd, 10.5, 6.1 4.25, m	70.7 CH_2	4.74, m 4.25, m	70.9 CH_2	4.74, m 4.25, m	70.9 CH_2
2	4.83, m	55.1 CH	4.73, m	55.0 CH	4.73, m	55.0 CH
3	4.79, m	72.8 CH	4.17, m	71.7 CH	4.16, m	71.7 CH
4	6.02, dd, 15.3, 6.8	132.7 CH	1.88, m 1.86, m	35.4 CH_2	1.88, m 1.86, m	35.4 CH_2
5	5.95, dt, 15.3, 6.1	132.6 CH	1.90, m 1.57, m	26.8 CH_2	1.90, m 1.57, m	26.8 CH_2
6	2.18, m	33.4 CH_2	1.29, m	30.6 CH_2	1.29, m	30.6 CH_2
7	2.21, m	27.8 CH_2	2.16, m	28.2 CH_2	2.16, m	28.2 CH_2
8	5.50, m	129.9 CH	5.49, m	130.7 CH	5.51, m	130.7 CH
9	5.50, m	131.2 CH	5.49, m	130.7 CH	5.51, m	130.7 CH
10	2.09, m	28.1 CH_2	2.11, m	28.1 CH_2	2.11, m	28.1 CH_2
11	1.37, m	30.5 CH_2	1.40, m	30.6 CH_2	1.40, m	30.6 CH_2
12−15	1.35−1.24, m	30.5−30.1 CH_2	1.36−1.23, m	30.7−30.0 CH_2	1.35−1.23, m	30.6−30.0 CH_2
16	1.25, m	32.6 CH_2	1.25, m	32.6 CH_2	1.25, m	32.6 CH_2
17	1.26, m	23.4 CH_2	1.28, m	23.4 CH_2	1.28, m	23.4 CH_2
18	0.88, t, 7.0	14.8 CH_3	0.88, t, 7.1	14.8 CH_3	0.88, t, 7.0	14.8 CH_3
2′	4.60, m	73.0 CH	4.63, m	72.9 CH	4.63, m	72.9 CH
3′	2.23, m 2.03, m	36.2 CH_2	2.24, m 2.08, m	36.1 CH_2	2.24, m 2.08, m	36.1 CH_2
4′	1.82, m 1.74, m	26.4 CH_2	1.81, m 1.77, m	26.3 CH_2	1.81, m 1.77, m	26.3 CH_2
5′	1.37, m	30.5 CH_2	1.40, m	30.6 CH_2	1.40, m	30.6 CH_2
6′−15′	1.35−1.24, m	30.5−30.1 CH_2	1.36−1.23, m	30.7−30.0 CH_2	1.35−1.23, m	30.6−30.0 CH_2
16′	1.25, m	32.6 CH_2	1.36−1.23, m	30.0 CH_2	1.25, m	32.6 CH_2
17′	1.26, m	23.4 CH_2	1.36−1.23, m	30.0 CH_2	1.28, m	23.4 CH_2
18′	0.88, t, 7.0	14.8 CH_3	1.25, m	32.6 CH_2	0.88, t, 7.0	14.8 CH_3
19′			1.28, m	23.4 CH_2		
20′			0.88, t, 7.1	14.8 CH_3		
1″	4.94, d, 7.8	106.2 CH	4.93, d, 7.8	106.2 CH	4.94, d, 7.8	106.2 CH
2″	4.06, m	75.7 CH	4.06, m	75.7 CH	4.05, m	75.7 CH
3″	4.25, m	79.0 CH	4.23, m	79.0 CH	4.25, m	79.0 CH
4″	4.25, m	72.0 CH	4.23, m	72.1 CH	4.25, m	72.1 CH
5″	3.93, m	79.1 CH	3.94, m	79.1 CH	3.94, m	79.1 CH
6″	4.52, dd, 11.8, 6.0 4.38, dd, 11.8, 5.7	63.2 CH_2	4.55, m 4.38, m	63.2 CH_2	4.54, m 4.38, m	63.2 CH_2
−C═O		176.2 C		176.0 C		176.1 C
−NH	8.39, d, 8.7		8.43, d, 8.8		8.44, d, 8.7	

MATERIALS AND METHODS

Materials. Analytical (250 μm thickness, 2−25 μm particle size) TLC plates were purchased from Sigma (St. Louis, MO). Silica gel (230−400 mesh, Sorbent Technologies Inc., Atlanta, GA) was used in open column chromatography (CC) fractionations. Medium pressure CC was carried out over silica gel (350−600 mesh, 60 Å, Fisher Scientific, Fair Lawn, NJ) or ODS (60 Å, Sigma), equipped with LabAlliance Series I pump and Spectra/chrom fraction collector CF-2. LC/MS-grade solvents and other reagents were obtained from Thermo Fisher Scientific (Waltham, MA). HCT-116 and HT-29 human colon cancer cells were obtained from American Type Tissue Culture (Manassas, VA). McCoy's 5A medium was purchased from Thermo Fisher Scientific (Waltham, MA). Supplements of fetal bovine serum (FBS) and penicillin/streptomycin were purchased from Gemini Bio-Products (West Sacramento, CA). MTT [3-(4,5-dimethylthiaxol-2-yl)-2,5-diphenyltetrazolium bromide] was procured from Calbiochem-Novabiochem (San Diego, CA) via Thermo Fisher Scientific.

LC/MS Analysis. LC/MS analysis was carried out with a Thermo-Finnigan Spectra System, which consisted of an Accela high speed MS pump, an Accela refrigerated autosampler, and an LCQ Fleet ion trap mass detector (Thermo Electron, San Jose, CA) incorporated with an electrospray ionization (ESI) interface. A Gemini-NX C_{18} column (150 mm × 3.0 mm i.d., 5 μm, Phenomenex) was used to analyze authentic sphingolipids and the sphingolipids-enriched fraction (fraction 8) with a flow rate of 0.3 mL/min. The binary mobile phase system consisted of water with 0.2% acetic acid as phase A and methanol with 0.2% acetic acid as phase B. The column was eluted by a gradient progress (80−90% B from 0 to 5 min; 90−95% B from 5 to 10 min; 95−99% B from 10 to 20 min; 99−100% B from 20 to 35 min; 100% B from 35 to 55 min, and then 80% B from 55 to 60 min). The injection volume was 10 μL for each sample. The column temperature was maintained at 20 °C. To optimize the mass spectrometric parameters, each standard dissolved in MeOH (10 μg/mL) was infused in the ESI source by a syringe pump (flow rate, 10 μL/min) and analyzed in negative ion mode. The optimized parameters were as follows: capillary voltage, −13 V; spray voltage, 5 kV; tube lens offset, −60 V; capillary temperature, 274 °C; and sheath gas (nitrogen) flow rate, 40 (arbitrary units). The structural information of compounds **1**−**19** were obtained by tandem mass spectrometry (MS/MS) through collision-induced dissociation (CID)

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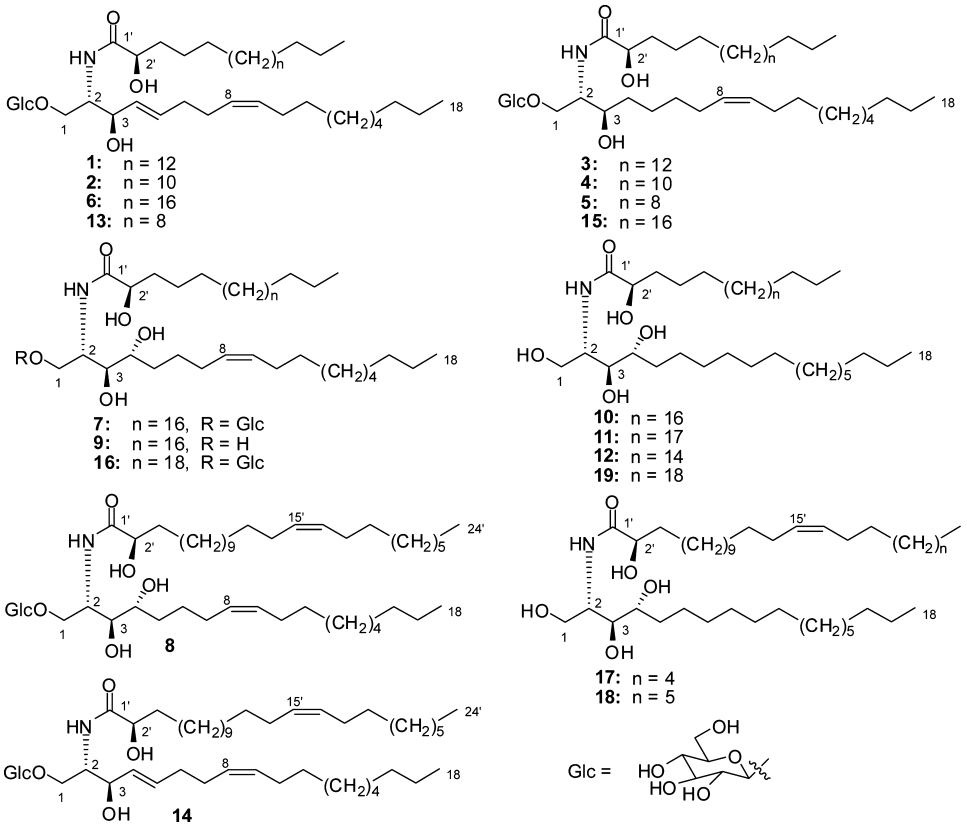

Figure 1. Structures of sphingolipids (**1**–**19**) identified from wheat bran.

with a relative collision energy setting of 35%. Data acquisition was performed with Xcalibur version 2.0 (Thermo Electron).

NMR Analysis. ^{1}H (700 MHz), ^{13}C (175 MHz), ^{1}H–^{1}H COSY (correlation spectroscopy), ^{1}H–^{13}C HMQC (heteronuclear multiple quantum correlation), and HMBC (heteronuclear multiple bond correlation) NMR spectra were acquired on a Bruker 700 MHz instrument. All compounds were analyzed in pyridine-d_5, with TMS as the internal standard.

Purification of Compounds 1–12. Wheat bran (423 kg) was extracted by 95% ethanol to produce 42.4 kg of ethanol extract. The ethanol extract (1 kg) was then suspended in water and partitioned with ethyl acetate. The organic solvent was concentrated in vacuo to give 800 g of wheat bran oil. The wheat bran oil (250 g) was separated into eight subfractions (fraction 1–fraction 8) by normal phase CC eluted with a stepwise gradient of hexane and ethyl acetate, as described in our previous work.[8] Fraction 8 (29.7 g) was identified as the sphingolipids-enriched fraction based on the analysis of its LC-MS data. This fraction (29.0 g) was subjected to CC over silica gel (230–400 mesh, Sorbent Technologies Inc., Atlanta, GA) and was eluted with a gradient of $CHCl_3$–MeOH (100:0, 100:3, 20:1, 9:1, 4:1, 3:2, and 0:100, 6000 mL each), yielding 13 subfractions ($F8_1$–$F8_{13}$). Repeated purification of $F8_6$ by reverse phase C_{18} silica gel medium pressure CC (30 mm × 360 mm, 60 Å, Sigma) eluted with MeOH (containing 0.2% formic acid) gave **9** (5 mg), **10** (10 mg), **11** (3 mg), and **12** (3 mg). $F8_8$ (0.3 g) was loaded to reverse phase C_{18} medium pressure column, eluted with a gradient of MeOH in H_2O (85, 95, and 98%), and further purified by normal phase medium pressure CC (15 mm × 300 mm) over silica gel (350–600 mesh, 60 Å, Fisher Scientific) eluted with a mixture of $CHCl_3$ and MeOH (6:1), producing **6** (5 mg). Repeated purification of $F8_9$ (1.3 g) by reverse phase C_{18} medium pressure CC (15 mm × 300 mm, 60 Å, Sigma) eluted with a gradient of MeOH–H_2O (80:20, 90:10, 95:5, and 100:0) afforded **1** (20 mg), **2** (4 mg), **3** (10 mg), **4** (3 mg), **5** (8 mg), and **8** (5 mg). $F8_{10}$ (1.4 g) was subjected to reverse phase C_{18} medium pressure CC, eluted with a gradient of MeOH in H_2O (70:30, 80:20, 90:10, 95:5, and 100:0), and further purified by normal phase medium pressure CC (15 mm × 300 mm) over silica gel (350–600 mesh, 60 Å, Fisher Scientific) eluted with a mixture of $CHCl_3$ and MeOH (6:1), giving **7** (5 mg). The spectroscopic data of compounds **2**–**4** are listed below.

1-*O*-*β*-Glucopyranosyl-(2*S*,3*R*,4*E*,8*Z*)-2-*N*-[(2′*R*)-hydroxyoctadecanoyl]-4,8-sphingadienine (2). White powder. ^{1}H (700 MHz, Py-d_5) and ^{13}C (175 MHz, Py-d_5) NMR, see Table 1; negative ESIMS, m/z 740 $[M - H]^-$ and 800 $[M + HOAc - H]^-$.

1-*O*-*β*-Glucopyranosyl-(2*S*,3*R*,8*Z*)-2-*N*-[(2′*R*)-hydroxyicosanoyl]-8-sphingenine (3). White powder. ^{1}H (700 MHz, Py-d_5) and ^{13}C (175 MHz, Py-d_5) NMR, see Table 1; negative ESIMS, m/z 770 $[M - H]^-$ and 830 $[M + HOAc - H]^-$.

1-*O*-*β*-Glucopyranosyl-(2*S*,3*R*,8*Z*)-2-*N*-[(2′*R*)-hydroxyoctadecanoyl]-8-sphingenine (4). White powder. ^{1}H (700 MHz, Py-d_5) and ^{13}C (175 MHz, Py-d_5) NMR, see Table 1; negative ESIMS, m/z 742 $[M - H]^-$ and 802 $[M + HOAc - H]^-$.

Growth Inhibition of Human Colon Cancer Cells. Cell growth inhibition was determined by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay.[17] Human colon cancer cells HCT-116 and HT-29 were plated in 96-well microtiter plates with 3000 cells/well and allowed to attach for 24 h at 37 °C. The test compounds (in DMSO) were added to cell culture medium to desired final concentrations (final DMSO concentrations for control and treatments were 0.1%). After the cells were cultured for 48 h, the medium was aspirated, and cells were treated with 200 μL of fresh medium containing 2.41 mmol/L MTT. After incubation for 3 h at 37 °C, the medium containing MTT was aspirated, 100 μL of DMSO was added to solubilize the formazan precipitate, and the plates were shaken gently for an hour at room temperature. Absorbance values were derived from the plate reading at 550 nm on a Biotek microtiter plate reader. The reading reflected the number of viable cells and was expressed as a percentage of viable cells in the control. Both HCT-116 and HT-29 cells were cultured in McCoy's 5A medium. All of the above media were supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, and 1% glutamine, and the cells were kept in a 37 °C incubator with 95% humidity and 5% CO_2.

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Statistical Analyses. IC_{50} values were obtained using Microsoft (Redmond, WA) Excel functions. Significance of variances was determined using GraphPad Prism (GraphPad Software, San Diego, CA) and a Student's t test, and $p < 0.05$ was regarded as significant.

RESULTS AND DISCUSSION

Structure Elucidation of Sphingolipids (1−12) Purified from Wheat Bran. As an ongoing search for bioactive components against colon cancer in wheat bran, the elaborate separation of a sphigolipids-enriched fraction (fraction 8) yielded 12 individual compounds, including eight cerebrosides (**1**−**8**) and four ceramides (**9**−**12**) (Figure 1). Among them, sphingolipids **1**, **6**−**9**, **11**, and **12** were reported for the first time as individual components from wheat bran. The structures of sphingolipids **1** and **5**−**12** were identified using 1D and 2D NMR techniques (data not shown) and confirmed by comparison of their NMR data with those already published. The compounds' identities are as follows: 1-*O*-*β*-glucopyranosyl-(2*S*,3*R*,4*E*,8*Z*)-2-*N*-[(2′*R*)-hydroxyicosanoyl]-4,8-sphingadienine (**1**),[18] 1-*O*-*β*-glucopyranosyl-(2*S*,3*R*,8*Z*)-2-*N*-[(2′*R*)-hydroxypalmitoyl]-8-sphingenine (**5**),[19] 1-*O*-*β*-glucopyranosyl-(2*S*,3*R*,4*E*,8*Z*)-2-*N*-[(2′*R*)-hydroxytetracosanoyl]-4,8-sphingadienine (**6**),[20] 1-*O*-*β*-glucopyranosyl-(2*S*,3*S*,4*R*,8*Z*)-2-*N*-[(2′*R*)-hydroxytetracosanoyl]-4-hydroxy-8-sphingenine (**7**),[21] 1-*O*-*β*-glucopyranosyl-(2*S*,3*S*,4*R*,8*Z*)-2-*N*-[(2′*R*,15′*Z*)-2′-hydroxy-15′-tetracosenoyl]-4-hydroxy-8-sphingenine (**8**),[21,22] (2*S*,3*S*,4*R*,8*Z*)-2-*N*-[(2′*R*)-hydroxytetracosanoyl]-4-hydroxy-8-sphingenine (**9**),[23] (2*S*,3*S*,4*R*)-2-*N*-[(2′*R*)-hydroxytetracosanoyl]-4-hydroxysphinganine (**10**),[9,24] (2*S*,3*S*,4*R*)-2-*N*-[(2′*R*)-hydroxypentacosanoyl]-4-hydroxysphinganine (**11**),[25] and (2*S*,3*S*,4*R*)-2-*N*-[(2′*R*)-hydroxydocosanoyl]-4-hydroxysphinganine (**12**).[25]

Compound **2**, previously predicted by analysis of mass spectrometry or rough comparison of its spectroscopic data with other known homologues,[18,26,27] was reported with complete NMR data for the first time in our current study. The structures of compounds **3** and **4** were previously described in wheat grain by using mass spectrometry or chemical decomposition methods.[9,16,26] This is the first report for compounds **3** and **4** on the purification and structure elucidation using 1D and 2D NMR techniques.

Compound **2** had a molecular formula of $C_{42}H_{79}NO_9$ based on negative ESI-MS at m/z 800 $[M + HOAc - H]^-$ due to the formation of an adduct with acetic acid. Its ^1H and ^{13}C NMR data (Table 1) were very close to those of 1-*O*-*β*-glucopyranosyl-(2*S*,3*R*,4*E*,8*Z*)-2-*N*-[(2′*R*)-hydroxyicosanoyl]-4,8-sphingadienine (**1**), previously reported from *Arisaema amurense*.[18] The main difference between **2** and **1** was that **2** had 28 mass units less than **1**, corresponding to two methylenes losses. The $^1H-^1H$ COSY and HMBC correlations of **2** indicated that it consisted of a hexanose residue, a long-chain base, and a long-chain fatty acid (Figure 2), which was also supported by fragmentation ions at m/z 299 and 352 on its tandem mass due to the typical neutral losses shown in Figure 3. The coupling constant of an anomeric proton at δ_H 4.94 (d, J = 7.8 Hz, H-1″) indicated the *β*-configuration of the sugar moiety.[18] The alkene bond at Δ^4 was found to be trans, as evidenced by the large vicinal coupling constant ($J_{4,5}$ = 15.3 Hz) and the chemical shift of an allylic methylene at C-6 (δ_C 33.4) (Table 1). It has been reported that the chemical shifts of carbons next to a trans (*E*) double bond appear at δ_C 32−33, whereas those of a cis (*Z*) double bond appear at δ_C 27−28.[28] The geometry of the double bond at Δ^8 was determined to be cis, as evidenced by allylic methylenes at δ_C 27.8 (C-7) and δ_C 28.1 (C-10) (Table 1). Because the chemical shifts of C-2 (δ_C 55.1) and C-3 (δ_C 72.8) in **2** were in agreement with those of synthetic *N*-octadecanoyl-D-*erythro*-sphingosine (δ_C 54.7 and δ_C 73.1)[29] and glucosyl-*erythro*-ceramide (δ_C 53.8 and δ_C 72.6),[30] the relative stereochemistry of **2** at C-2 and C-3 was predicted to be 2*S* and 3*R*. Thereafter, compound **2** was identified as 1-*O*-*β*-glucopyranosyl-(2*S*,3*R*,4*E*,8*Z*)-2-*N*-[(2′*R*)-hydroxyoctadecanoyl]-4,8-sphingadienine, which was previously found from wheat grain by analysis of mass spectrometry[26] and also reported from *Arisaema amurense* or *Psychotria correae* based on rough comparison of its spectroscopic data with other known analogues,[18,27] whereas the complete NMR data of compound **2** as well as its relative configurations were not specified.

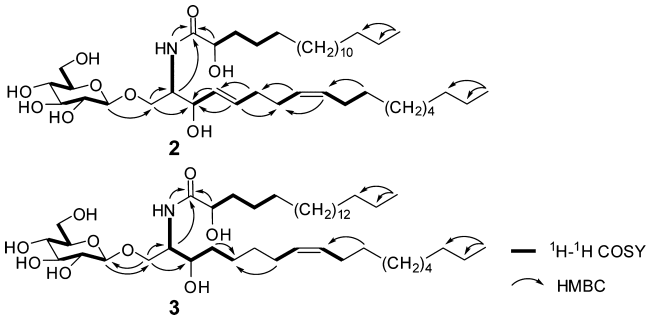

Figure 2. Key $^1H-^1H$ COSY (—) and HMBC correlations (↷) of compounds **2** and **3**.

Compound **3** showed a molecular formula of $C_{44}H_{85}NO_9$ based on negative ESI-MS at m/z 830 $[M + HOAc - H]^-$, because of the formation of an adduct with acetic acid. Its ^1H and ^{13}C NMR data (Table 1) were similar to those of 1-*O*-*β*-glucopyranosyl-(2*S*,3*R*,4*E*,8*Z*)-2-*N*-[(2′*R*)-hydroxyicosanoyl]-4,8-sphingadienine (**1**).[18] The main difference between **3** and **1** was that only one double bond at δ_H 5.49 (m, 2H) was found in **3**, unlike **1** with two double bonds in its structure. The $^1H-^1H$ COSY and HMBC correlations of **3** confirmed the location of the alkene bond at Δ^8 (Figure 2). The geometry of the double bond was determined to be cis, as evidenced by allylic methylenes at δ_C 28.2 (C-7) and δ_C 28.1 (C-10) (Table 1).[28] The relative stereochemistry of **3** at C-2 and C-3 was predicted to be 2*S* and 3*R* and correlated with the literature.[29,30] Therefore, the structure of **3** was proposed to be 1-*O*-*β*-glucopyranosyl-(2*S*,3*R*,8*Z*)-2-*N*-[(2′*R*)-hydroxyicosanoyl]-8-sphingenine, which was previously predicted from wheat grain as a mixture of analogues based on GC mass spectrometry and chemical decomposition methods,[9,16] whereas the complete NMR data of **3** as well as its relative stereochemistry, including the geometry of alkene double bonds, have never been described before.

Compound **4** displayed a molecular formula of $C_{42}H_{81}NO_9$ based on negative ESI-MS at m/z 802 $[M + HOAc - H]^-$, attributing to the formation of an adduct with acetic acid. Its ^1H and ^{13}C NMR data (Table 1) were almost identical to those of **3**. The main difference between **4** and **3** was that **4** had 28 mass units less than **3**, indicating two methylenes losses in **4**, which was also supported by a fragmentation ion at m/z 299 on its tandem mass spectra due to the neutral loss of a carboxylic acid (Figure 3). Therefore, the structure of **4** was determined to be 1-*O*-*β*-glucopyranosyl-(2*S*,3*R*,8*Z*)-2-*N*-[(2′*R*)-hydroxyoctadecanoyl]-8-sphingenine, which was also previously reported from wheat grain as a mixture of analogues based on the analysis of

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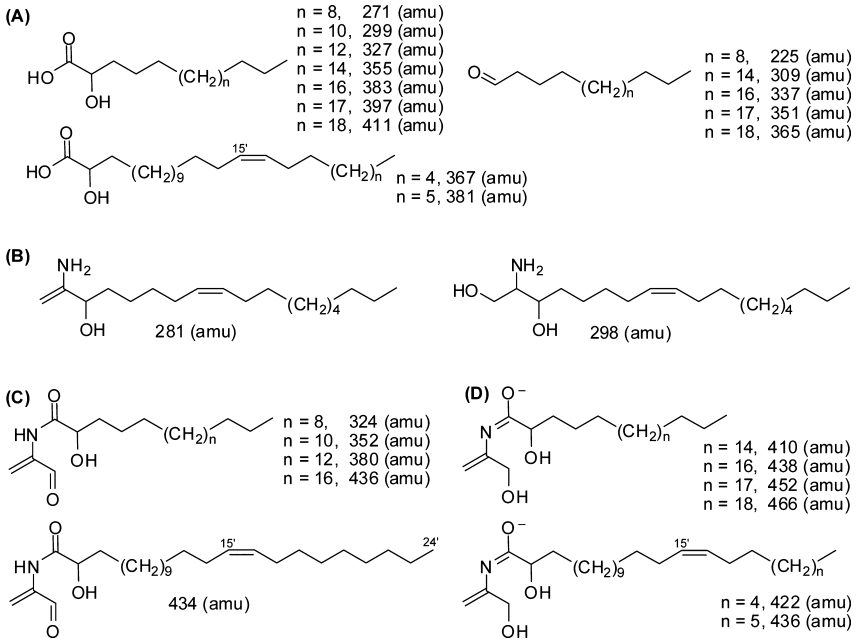

Figure 3. Typical fragment ions observed from sphingolipids purified from wheat bran. (A) A neutral loss of carboxylic acid for acyl-chain residue in sphingolipids; (B) a neutral loss of a sphingoid base at *m/z* 281 or 298 in 8-sphingenine-type sphingolipids; (C) a characteristic neutral loss, containing fatty acyl chain bounded to part of a sphingoid base in 4,8-sphingadienine-type sphingolipids, formed by cleavage between C-3 and C-4; and (D) a characteristic anion, containing fatty acyl chain bounded to part of the sphingoid base, in 4-hydroxysphinganine- or 4-hydroxy-8-sphingenine-type sphingolipids, formed by cleavage between C-3 and C-4.

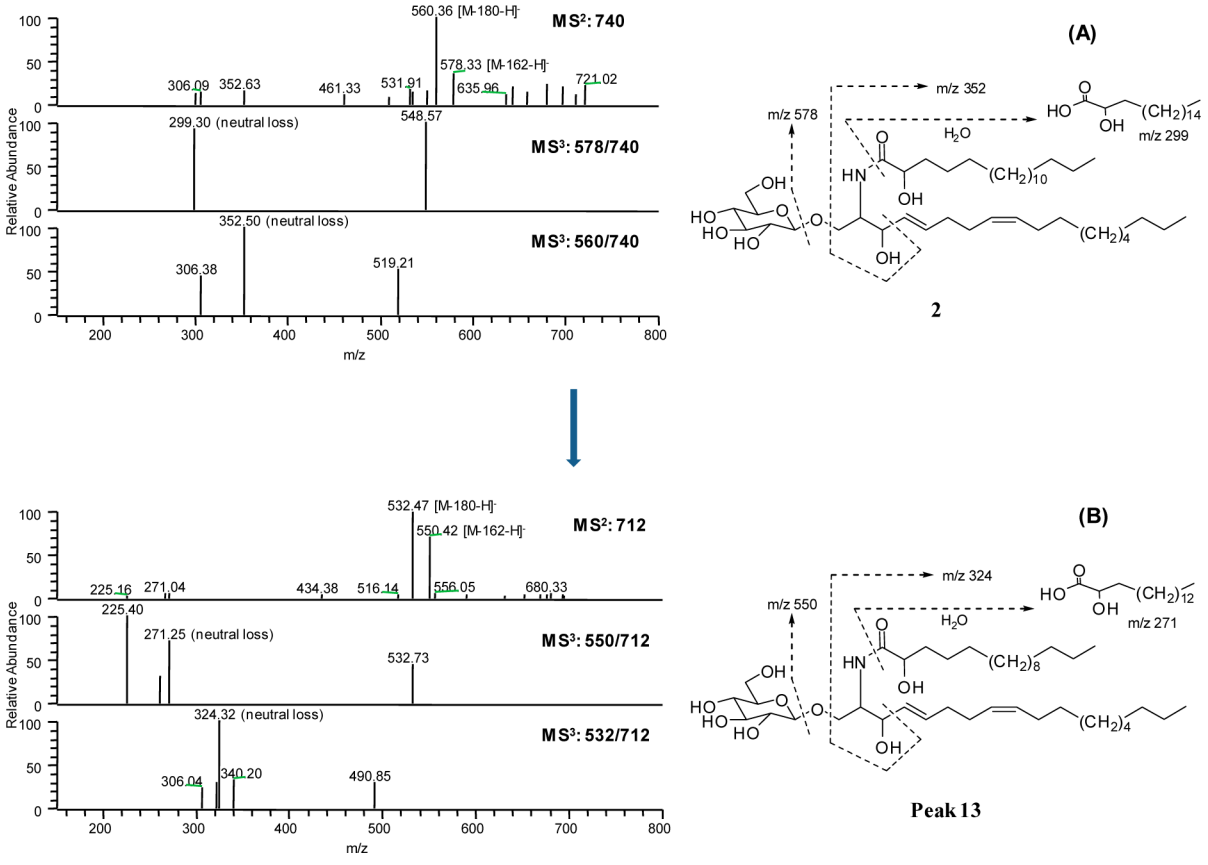

Figure 4. ESI/MS^n (n = 2−3) spectra (left) and fragmentation pattern (right) of compound **2** (A) and ESI/MS^n (n = 2−3) spectra (left) and fragmentation pattern (right) of peak **13** (B).

GC-MS,[26] whereas the NMR data of compound **4** as well as its relative configurations were not included.

Fragmentation Patterns of Authentic Sphingolipids Using ESI Negative Ionization Multistage Mass Spec-

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Table 2. ESI-MS and ESI-MS^n (n = 2−4) Fragment Ions of Sphingolipids (1−19) in Wheat Bran

	$[M - H]^-$	$[M + HOAc - H]^-$	MS^2	MS^3
1	768	828	**768**/606 $[M - 162 - H]^-$, 588 (B)	**606**/576, 558 (B), 327; **588**/560, 380 (B), 288
2	740	800	**740**/578 $[M - 162 - H]^-$, 560 (B)	**578**/548, 299 (B); **560**/519, 352 (B), 306
3	770	830	**770**/608 $[M - 162 - H]^-$, 590 (B)	**608**/590, 334, 327, 309 (B), 298, 281; **590**/352, 281 (B)
4	742	802	**742**/580 $[M - 162 - H]^-$, 562 (B)	**580**/299
5	714	774	**714**/552 $[M - 162 - H]^-$ (B), 534	**552**/534, 297, 271; **534**/281 (B), 237, 225
6	824	884	**824**/662 $[M - 162 - H]^-$, 644 (B), 382	**662**/630, 424 (B), 383, 351, 337; **644**/603, 464, 452, 436 (B), 383, 337, 278
7	842	902	**842**/680 $[M - 162 - H]^-$, 662, 438 (B)	**680**/383 (B), 365; **662**/420; **438**/420, 410, 393, 365, 337 (B)
8	840	900	**840**/678 $[M - 162 - H]^-$, 660, 436 (B), 418, 381	**678**/381; **436**/408, 406, 363, 335 (B); **381**/335
9	680	740	**689**/662, 438, 426, 383 (B)	**662**/408 (B), 382; **426**/408, 378, 337 (B); **383**/337
10	682	742	**682**/438, 426, 383 (B)	**438**/410, 365, 337; **426**/365, 337; **383**/337
11	696	756	**696**/452, 440, 397 (B)	**452**/434, 396, 351 (B); **440**/351; **397**/351
12	654	714	**654**/410, 355 (B), 309	**410**/309; **355**/309
13	712	772	**712**/550 $[M - 162 - H]^-$, 532 (B)	**550**/532, 271, 261, 225 (B); **532**/340, 324 (B), 322, 306
14	822	882	**822**/660 $[M - 162 - H]^-$, 642 (B)	**642**/434, 381, 363, 306
15	826	886	**826**/664, 646 (B), 448, 429, 383, 309	**664**/298; **383**/337
16	870	930	**870**/690, 466 (B), 445, 411	**466**/365
17	666	726	**666**/584, 422, 410, 367 (B)	**422**/404
18	680	740	**680**/662, 644, 436 (B), 381	**436**/418, 394
19	710	770	**710**/626, 466, 454, 411 (B), 365	**411**/365

trometry. The purified 12 sphingolipids **1**−**12** (Figure 1) could be classified into four types of sphingolipids based on the nature of the sphingoid long-chain base: 4,8-sphingadienine-type (**1**, **2**, and **6**), 8-sphingonine-type (**3**−**5**), 4-hydroxy-8-sphingenine-type (**7**−**9**), and 4-hydroxysphinganine-type (**10**−**12**). Further study of their multistage mass spectra led to the observation of the following four different fragmentation patterns: (a) a neutral loss of a carboxylic acid, which can be used to determine the length of the fatty acyl-chain moiety (from all sphingolipids) (Figure 3A); (b) a neutral loss of a sphingoid base at m/z 281 or 298 (from 8-sphingonine-type cerebrosides) (Figure 3B); (c) a neutral loss containing a fatty acyl-chain bounded to part of the sphingoid base formed from product ion $[M - 180 - H]^-$ by cleavage between C-3 and C-4 (from 4,8-sphingadienine-type cerebrosides) (Figure 3C);[31] and (d) formation of a fragment anion containing a fatty acyl-chain bounded to part of the sphingoid base, formed from parent ion $[M - H]^-$ by cleavage between C-3 and C-4 (from 4-hydroxy-8-sphingenine- and 4-hydroxysphinganine-type sphingolipids) (Figure 4D).[31] In addition, all sphingolipids produced predominant $[M + HOAc - H]^-$ ions under ESI negative ionization mode (Table 2), due to the formation of adducts with acetic acid, being useful diagnostic ions as sphingolipid selective markers,[31] and all cerebrosides gave daughter ions $[M - 162 - H]^-$ and $[M - 180 - H]^-$ in their MS^2 spectra (Table 2).

Identification of Additional Sphingolipids (13−19) in WB by Analyses of Tandem Mass Spectra. As per the knowledge of the fragmentation patterns of 12 authentic sphingolipids (**1**−**12**), an additional seven sphingolipids (**13**−**19**) were identified from fraction 8 of wheat bran extract by analyses of their tandem mass spectra (Figure 1). As far as we know, this is the first report of sphingolipids **13**, **14**, and **16**−**19** in wheat bran. Particularly, 2-*N*-(2′-hydroxy-15′-tricosenoyl)-4-hydroxysphinganine (peak **17**) is a novel compound.

A representative example is shown in Figure 4 to deduce the structure of peak **13** following the fragmentation patterns observed in the tandem mass spectra of compound **2** (Figure 3). In brief, compound **2**, as an 4,8-sphingadienine-type cerebroside, showed a carboxylic acid loss at m/z 299 (Figure 4A) in the MS^3 spectrum of m/z 578/740 $[M - H]^-$ following fragmentation pattern a and a characteristic neutral loss at m/z 352 in the MS^3 spectrum of m/z 560/740 $[M - H]^-$ following fragmentation pattern c (Figure 4C and Table 2). In a similar manner, peak **13** gave a neutral loss at m/z 324 as a base peak originating from the MS^3 spectrum of m/z 532/712 $[M - H]^-$ (Figure 4C) following fragmentation pattern c, demonstrating that peak **13** had 4,8-sphingadienine as its sphingoid base residue. In addition, the MS^3 spectrum of m/z 550/712 $[M - H]^-$ had a neutral loss at m/z 271 following fragmentation pattern a (Figure 4A), suggesting that peak **13** had an acyl-chain moiety possessing 16 carbons. Its relative stereochemistry was assumed to be the same as other homologues found in this study. Therefore, peak **13** was tentatively proposed to be 1-*O*-glucopyranosyl-2-*N*-(2′-hydroxyhexadecanoyl)-4,8-sphingadienine, which was previously reported from *Psychotria correae*.[27] Peak **14** was also identified as a known 4,8-sphingadienine cerebroside (Figure 1) based on the observation of neutral loss at m/z 434 in the MS^3 spectrum of m/z 642/822 $[M - H]^-$ (Table 2). In addition, neutral losses at m/z 381 and 363 in the MS^3 spectrum of m/z 642/822 $[M - H]^-$ (Table 2) demonstrated an unsaturated fatty acid residue in a length of 24 carbons in peak **14**, and the location of the alkene double bond was proposed tentatively at $\Delta^{15'}$ by comparing its key fragments with those of its homologue, compound **8**.[21,22] Therefore, peak **14** was identified as 1-*O*-*β*-glucopyranosyl-(2*S*,3*R*,4*E*,8*Z*)-2-*N*-[(2′*R*,15′*Z*)-2′-hydroxy-15′-tetracosenoyl]-4,8-sphingadienine, which was previously found from beer waste.[32]

Similarly, MS^3 spectrum of m/z 664/826 $[M - H]^-$ of peak **15** showed a product ion at m/z 298, allowing the recognition of a cerebroside having 8-sphingonine as its sphingoid base based on fragmentation pattern b (Figure 3B). The presence of a neutral loss at m/z 383 agreed with an acyl-chain residue with 24 carbons in length according to fragmentation pattern a (Figure 3A). Thus, peak **15** was concluded as 1-*O*-glucopyranosyl-2-*N*-(2′-hydroxytetracosanoyl)-8-sphingenine,

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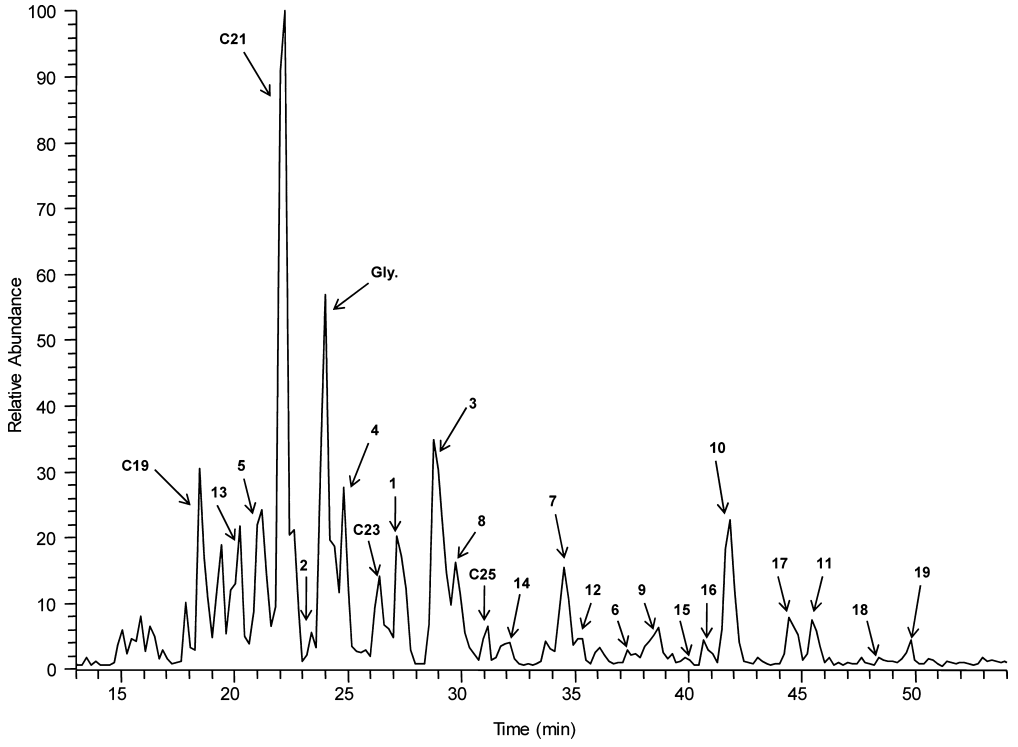

Figure 5. Chemical profile of sphingolipids in fraction 8 of wheat bran extract generated from negative HPLC-ESI/MS. C19, 5-*n*-nonadecylresorcinol; C21, 5-*n*-heneicosylresorcinol; C23, 5-*n*-tricosylresorcinol; C25, 5-*n*-pentacosylresorcinol; and Gly, glycerides.

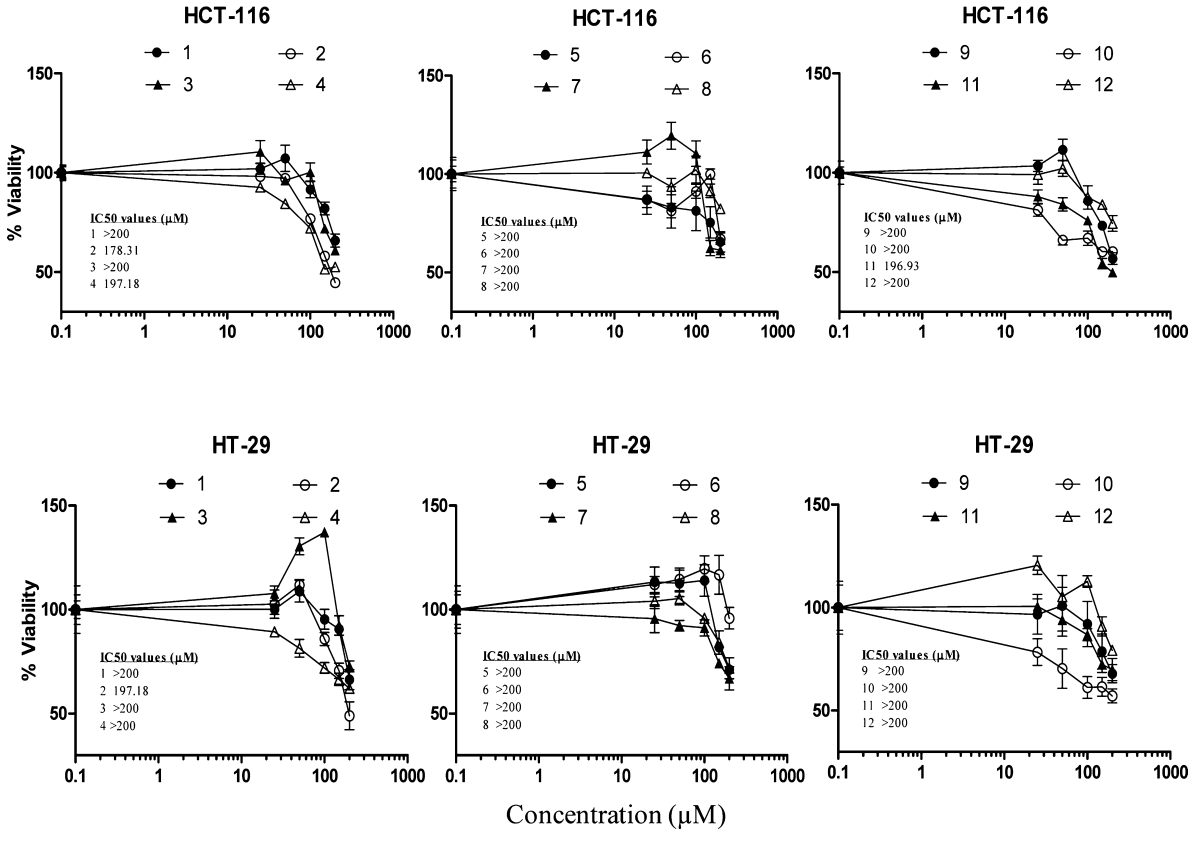

Figure 6. Cell growth inhibition in HCT-116 and HT-29 human colon cancer cell lines by purified sphingolipids **1**−**12**. Cells were treated with 25, 50, 100, 150, and 200 μM concentrations of the test compounds for 48 h in the presence of 10% FBS and 1% streptomycin/penicillin at 37 °C. Cell growth inhibition was determined by MTT assay (n = 6 ± SD).

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which was previously reported from *Euphorbia nicaeensis*[33] and wheat bran.[34]

Peak **16** had similar tandem mass spectra as those of compounds **7** and **9** (Figure 3 and Table 2). The MS^2 spectrum of peak **16** had the product ion at m/z 466, suggesting it has 4-hydroxysphinganine as its sphingoid base based on fragmentation pattern d (Figure 3D) and a neutral loss at m/z 411, indicating the presence of an acyl-chain reside with 26 carbons in length according to fragmentation pattern a (Figure 3A). Therefore, peak **16** was identified as 1-*O*-*β*-glucopyranosyl-(2*S*,3*S*,4*R*,8*Z*)-2-*N*-[(2′*R*)-2′-hydroxyhexaconasoyl]-4-hydroxy-8-sphingenine, which was previously separated from the leaves of *Clinacanthus nutans*.[35]

Peaks **17**−**19**, showing typical fragment anions at m/z 422, 436, or 466 generated from respective parent ions m/z 666, 680, or 710 $[M - H]^-$ (Figure 3D and Table 2), were defined as 4-hydroxysphinganine-type ceramides (Figure 1). Peaks **17** and **18** had neutral losses at m/z 367 and 381 in their MS^2 spectra, respectively, suggesting the existence of unsaturated fatty acyl residues in lengths of 23 carbons in peak **17** and 24 carbons in peak **18** based on fragmentation pattern a (Figure 3A). The location of the double bond was tentatively proposed at $\Delta^{15'}$ upon comparison to the fragmentation profile of its homologue **8**.[21,22] Peaks **17** and **18** were identified as 2-*N*-(2′-hydroxy-15′-tricosenoyl)-4-hydroxysphinganine (Figure 1), a novel compound, and 2-*N*-(2′-hydroxy-15′-tetracosenoyl)-4-hydroxysphinganine (Figure 1), previously reported from *Hygrophorus eburnesus*,[36] respectively. Peak **19** had similar fragmentation patterns to its homologues **10**−**12** (Figure 3A and Table 2). The neutral loss at m/z 411 in the MS^2 spectrum of peak **19** (m/z 710 $[M - H]^-$) indicated a fatty acyl moiety in a length of 26 carbons in peak **19** according to fragmentation pattern a (Figure 3A), suggesting peak **19** was 2-*N*-(2-hydroxyhexacosanoyl)-4-hydroxysphinganine, previously reported from *Grifola frondosa*.[25]

At this point, we must emphasize that the relative stereochemistries of sphingolipids **13**−**19** were tentatively considered similar to those of their known homologues **1**−**12**, attributing to their common enzymatic biosynthesis pathway in plants.[37] Their relative configurations were also in agreement with reports in literature.[25,27,32,33,35,36] As a result, a total of 19 sphingolipids (**1**−**19**) were identified and characterized from fraction 8 of wheat bran extract, using NMR techniques or HPLC-ESI/MS^n experiments, eventually leading to the sphingolipids profile shown in Figure 5.

Cell Growth Inhibition by Purified Compounds. All 12 purified sphingolipids (**1**−**12**) were evaluated for growth inhibitory effects against human colon cancer cells HCT-116 and HT-29 and showed weak but statistically significant activity at 200 μM in both cell lines (Figure 6). However, at low concentrations (25−50 μM), compounds **3** and **7** in HCT-116 cells and compounds **3** and **12** in HT-29 cells could promote cancer cell growth (Figure 6).

Dietary sphingolipids are enzymatically hydrolyzed to ceramides and free sphingoid bases, which have greater activity in this form, in the gastrointestinal tract.[38,39] Hence, the sphingolipids observed in this study may show greater potency in vivo, thereby contributing to the chemopreventative properties associated with wheat bran consumption. For instance, free sphingoid bases have been proven as inhibitors of colon carcinogenesis,[40] showing apoptosis-inducing effects in human colon cancer cells.[12,41] Studies also outline an effective chemopreventative strategy by which nonsteroidal anti-inflammatory drug are taken in tandem with foods rich in sphingolipids and exert therapeutic activity through activation of lipid-hydrolyzing enzymes, converting dietary sphingomyelin to its base ceramide.[42] Further study of the enzymatic metabolism of sphingolipids would be useful for simulating in vivo conditions in preclinical diagnostics.

In conclusion, 19 sphingolipids (**1**−**19**) were characterized by NMR experiments or HPLC-ESI/MS^n techniques. This is the first report of sphingolipids **1**, **6**−**9**, **11**−**14**, and **16**−**19** in wheat bran. In particular, 2-*N*-(2′-hydroxy-15′-tricosenoyl)-4-hydroxysphinganine (peak **17**) has not been reported before. As far as we know, this is also the first report of the complete NMR data for compounds **2**−**4**. As a result, we have outlined the comprehensive sphingolipid profile in wheat bran for the first time and have found that sphingolipids in wheat bran may be implicated in colon cancer prevention as a component of this bioactive food. Further study on the metabolism of wheat bran sphingolipids in vivo is necessary and may elucidate the importance of sphingolipids in contributing to chemoprevention.

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS USED

ACF, aberrant crypt foci; CC, column chromatography; COSY, correlation spectroscopy; DMH, 1,2-dimethylhydrazine; HMBC, heteronuclear multiple bond correlation; HMQC, heteronuclear multiple quantum correlation; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

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