J Appl Biol Chem (2014) 57(2), 175–178
http://dx.doi.org/10.3839/jabc.2014.028

Online ISSN 2234-7941
Print ISSN 1976-0442

Short Communication: Bioactive Materials

Phytosterols from the Rice (*Oryza sativa*) Bran

Ye-Jin Jung · Ji-Hae Park · Sabina Shrestha · Myoung-Chong Song · Suengmok Cho · Chang-Ho Lee · Daeseok Han · Nam-In Baek*

Received: 12 August 2013 / Accepted: 9 December 2013 / Published Online: 30 June 2014

Abstract Three phytosterols of rare occurrence, schleicheol 2 (**1**), 7β-hydroxysitosterol (**2**), and 7α-hydroxysitosterol (**3**), were isolated from the *n*-hexane fraction of rice (*Oryza sativa*) bran, for the first time. Some nuclear magnetic resonance (NMR) assignments in the literatures are inaccurate. This study employed two-dimensional NMR experiments to identify exact peak assignments.

Keywords *Oryza sativa* L. · phytosterol · rice bran · schleicheol 2 · 7α-hydroxysitosterol · 7β-hydroxysitosterol

Rice (*Oryza sativa*) is one of the most important agricultural products of Korea. Rice milling yields 70% of rice (endosperm) as the major product, and 8% of rice bran as the by-products. The main component of rice bran is proteins (38.1%), fats (30.0%) and γ-oryzanol (0.12%) (Parrado et al., 2006). In comparison with common vegetable oils, rice bran oil (RBO) has a qualitatively different composition of several bioactive components such as γ-oryzanol, tocotrienols, and phytosterols (McCaskill and Zhang, 1999; Kim and Godber, 2000). γ-Oryzanol and phytosterols of RBO have the capacity to lower blood cholesterol and decrease cholesterol absorption (Berger et al., 2005; Revilla et al., 2009; Wilson et al., 2000). However, the active compounds of rice bran are not investigated sufficiently. Feruloyl esters of triterpene alcohols and sterols such as cycloartenol ferulate, 24-methylene-cycloartanol ferulate, and sitosterol ferulate also have been reported from the rice bran (Tanaka et al., 1971; Akihisa et al., 2000; Fang et al., 2003). The present study was conducted to search for phytosterols and yielded three phytsterols, schleicheol 2, 7β-hydroxysitosterol, and 7α-hydroxysitosterol, which were relatively rarely found in plant system. Moreover, there are many variances and inaccuracies found in the published NMR data of the above described phytosterols, or there are a few reports for NMR data (Roh et al., 2010; Santana et al., 2012). The present study identified with certainty individual proton and carbon signals using 2D NMR techniques, which include correlation spectroscopy (COSY), heteronuclear single quantum correlation (HSQC), and gradient heteronuclear multiple bond connectivity (gHMBC).

Y.-J. Jung · J.-H. Park · S. Shrestha · N.-I. Baek
Graduate School of Biotechnology, Institute of Life Sciences & Resources, Kyung Hee University, Yongin 446-701, Republic of Korea

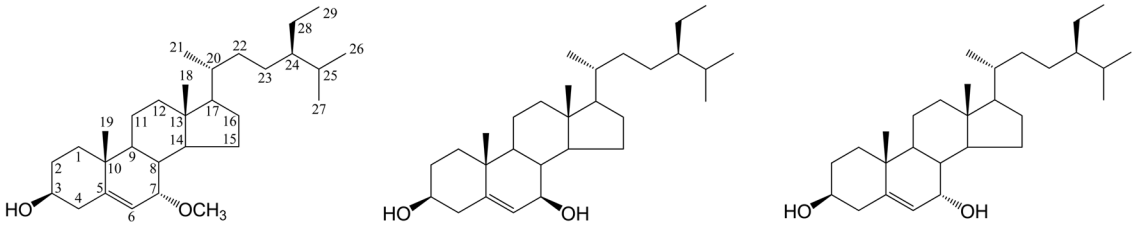
M.-C. Song
Intelligent Synthetic Biology Center, KAIST, Daejeon 305-701, Republic of Korea

S. Cho · C.-H. Lee · D. Han
Korea Food Research Institute, Seongnam 463-746, Republic of Korea

*Corresponding author (N.-I. Baek: nibaek@khu.ac.kr)

Materials and Methods

Dried rice bran (200 kg) of *O. sativa* was extracted at 50°C with 60% aqueous ethanol (EtOH, 2,000 L×2) for 43 h, which resulted in a concentrated extract (12 kg). The EtOH extract was successively partitioned with water (27 L), *n*-hexane (27 L×2), EtOAc (27 L×2) and *n*-BuOH (25 L×2), yielding concentrated extract in *n*-hexane (RBH, 2.2 kg), EtOAc (RBE, 1.5 kg), *n*-BuOH (RBB, 1.2 kg), and H_2O (RBW, 6.74 kg) fractions. The concentrated rice bran *n*-hexane fraction (RBH, 365 g) was subjected to a silica gel (SiO_2) column chromatography (c.c.) (Ø 13×15 cm) and eluted with *n*-hexane-EtOAc (10:1, 14 L→7:1, 12 L→2:1, 4 L) resulting in 12 fractions (RBH-1 to RBH-12). Fraction RBH-10 [9.5 g, V_e/V_t (elution volume/total volume) 0.81–0.87] was subjected to the silica gel c.c. (Ø 6×17 cm), and eluted with *n*-hexane-EtOAc (10:1, 10 L→6:1, 2.5 L→4:1, 3 L→2:1, 2 L→1:1, 3 L) and $CHCl_3$-MeOH (8:1, 1.8 L→1:1, 1 L) to provide 23 fractions (RBH-10-1 to RBH-10-23). Fraction RBH-10-16 (774 mg, V_e/V_t 0.71–0.74) was subjected to an ODS c.c. (Ø 4×5 cm), and eluted with



Schleicheol 2 (**1**) 7β-hydroxysitosterol (**2**) 7α-hydroxysitosterol (**3**)

Fig. 1 Chemical structures of the isolated compounds from the rice (*Oryza sativa*) bran.

acetone-H_2O (3:1, 910 mL) resulting in 12 fractions (RBH-10-16-1 to RBH-10-16-12). The fraction RBH-10-16-9 (45 mg, V_e/V_t 0.48–0.77) was subjected to the SiO_2 c.c. (Ø 3×12 cm), and eluted with *n*-hexane-EtOAc (3:1, 300 mL→2:1, 460 mL) resulting in 7 fractions (RBH-10-16-9-1 to RBH-10-16-9-7), with isolation of compound **1** [RBH-10-16-9-2, 12.0 mg, V_e/V_t 0.13–0.24, TLC (SiO_2 F_{254}) R_f 0.63 in *n*-hexane-EtOAc =1:3], compound **2** [RBH-10-16-9-3, 12.0 mg, V_e/V_t 0.24–0.49, TLC (SiO_2 F_{254}) R_f 0.45 in *n*-hexane-EtOAc =1:3], and compound **3** [RBH-10-16-9-5, 8.6 mg, V_e/V_t 0.53–0.32, TLC (SiO_2 F_{254}) R_f 0.35 in *n*-hexane-EtOAc =1:3].

Compound **1**: white powder, m.p. 92-95°C, $[\alpha]_D$ −58.4° (*c* 0.50, $CHCl_3$); EI/MS *m/z* 444 $[M]^+$; IR (CaF_2 window, ν) 3452, 1673 cm^1; ^{1}H-NMR (400 MHz, $CDCl_3$, δ_H) 5.71 (1H, d, *J* =4.8 Hz, H-6), 3.59 (1H, m, H-3), 3.33 (3H, s, 7-OCH_3), 3.26 (1H, m, H-7), 0.96 (3H, s, H-19), 0.90 (3H, d, *J* =6.4 Hz, H-21), 0.83 (3H, t, *J* =7.6 Hz, H-29), 0.80 (3H, d, *J* =7.6 Hz, H-26), 0.78 (3H, d, *J* =6.8 Hz, H-27), 0.64 (3H, s, H-18); ^{13}C-NMR (100 MHz, $CDCl_3$, δ_C) 146.13 (C-5), 120.72 (C-6), 73.92 (C-7), 71.38 (C-3), 56.80 (7-OCH_3), 55.65(C-17), 48.46 (C-14), 45.84 (C-24), 42.75 (C-9), 42.30 (C-4), 42.08 (C-13), 39.03 (C-12), 37.43 (C-10), 37.18 (C-8), 36.73 (C-1), 36.13 (C-20), 33.94 (C-22), 31.44 (C-2), 29.15 (C-25), 28.24 (C-16), 25.98 (C-23), 24.26 (C-15), 23.07 (C-28), 20.80 (C-11), 19.82 (C-27), 19.02 (C-21), 18.97 (C-26), 18.24 (C-19), 11.99 (C-29), 11.46 (C-18).

Compound **2**: white powder, m.p. 157-158°C, $[\alpha]_D$ −13.3° (*c* 0.50, $CHCl_3$); EI/MS *m/z* 430 $[M]^+$; IR (CaF_2 window, ν) 3343, 1653 cm^1; H-NMR (400 MHz, $CDCl_3$, δ_H) 5.27 (1H, br s, H-6), 3.83 (1H, d, *J* =8.0 Hz, H-7), 3.52 (1H, m, H-3), 1.03 (3H, s, H-19), 0.90 (3H, d, *J* =6.4 Hz, H-21), 0.83 (3H, t, *J* =7.6 Hz, H-29), 0.81 (3H, d, *J* =7.6 Hz, H-26), 0.79 (3H, d, *J* =6.8 Hz, H-27), 0.67 (3H, s, H-18); ^{13}C-NMR (100 MHz, $CDCl_3$, δ_C) 143.47 (C-5), 125.46 (C-6), 73.35 (C-7), 71.43 (C-3), 55.98 (C-14), 55.41 (C-17), 48.29 (C-9), 45.88 (C-24), 42.94 (C-12), 41.73 (C-4), 40.93 (C-13), 39.57 (C-8), 36.96 (C-1), 36.44 (C-10), 36.09 (C-20), 34.00 (C-22), 31.58 (C-2), 29.19 (C-23), 28.53 (C-16), 26.38 (C-15), 26.17 (C-25), 23.08 (C-28), 21.09 (C-11), 19.79 (C-27), 19.14 (C-19), 19.03 (C-21), 18.83 (C-26), 11.97 (C-29), 11.81 (C-18).

Compound **3**: white powder, m.p. 219-220°C, $[\alpha]_D$ −72.7° (*c* 0.50, $CHCl_3$); EI/MS *m/z* 430 $[M]^+$; IR (CaF_2 window, ν) 3261, 1652 cm^1; ^{1}H-NMR (400 MHz, $CDCl_3$, δ_H) 5.59 (1H, d, *J* =5.2 Hz, H-6), 3.83 (1H, br s, H-7), 3.55 (1H, m, H-3), 0.97 (3H, s, H-19), 0.90 (3H, d, *J* =6.4 Hz, H-21), 0.83 (3H, t, *J* =7.6 Hz, H-29), 0.81 (3H, d, *J* =7.6 Hz, H-26), 0.79 (3H, d, *J* =6.8 Hz, H-27), 0.66 (3H, s, H-18); ^{13}C-NMR (100 MHz, $CDCl_3$, δ_C) 146.24 (C-5), 123.86 (C-6), 71.33 (C-3), 65.36 (C-7), 55.70 (C-17), 49.42 (C-14), 45.82 (C-24), 42.26 (C-9), 42.13 (C-4), 42.01 (C-13), 39.17 (C-12), 37.51 (C-10), 37.39 (C-1), 37.00 (C-8), 36.10 (C-20), 33.91 (C-22), 31.37 (C-2), 29.12 (C-23), 28.27 (C-16), 25.91 (C-25), 24.30 (C-15), 23.05 (C-28), 20.69 (C-11), 19.79 (C-27), 19.02 (C-21), 18.79 (C-26), 18.23 (C-19), 11.98 (C-29), 11.62 (C-18).

Results and Discussion

Compound **1**, a white powder, showed the absorbance bands of the hydroxy (3452 cm^{-1}) and olefin (1673 cm^{-1}) groups, and the molecular weight was determined as 444 from the molecular ion peak *m/z* 444 $[M]^+$ in the EI/MS spectrum. The ^{1}H-NMR (400 MHz, $CDCl_3$) spectrum showed an olefin methine proton signal at δ_H 5.71 (1H, d, *J* =4.8 Hz, H-6). Two oxygenated methine proton signals and a methoxy signal were also observed at δ_H 3.59 (1H, m, H-3), 3.33 (3H, s, 7-OCH_3), and 3.26 (1H, m, H-7), respectively. In upfield region, several methylene and methine proton signals were observed. Additionally, two singlet methyl proton signals at δ_H 0.96 (3H, s, H-19) and 0.64 (3H, s, H-18); three doublet methyl proton signals at δ_H 0.90 (3H, d, *J* =6.4 Hz, H-21), 0.80 (3H, d, *J* =7.6 Hz, H-26), and 0.78 (3H, d, *J* =6.8 Hz, H-27); and a triplet methyl proton signal at δ_H 0.83 (3H, t, *J* =7.6 Hz, H-29) were observed. The above mentioned proton signals indicated that compound **1** was a sterol. The ^{13}C-NMR (100 MHz, $CDCl_3$) spectrum of compound **1** showed 29 carbon signals except for a methoxy carbon signal, which confirmed that compound **1** had a stigmane skeleton moiety. Among them, an olefin quaternary carbon signal at δ_C 146.13 (C-5); an olefin methine carbon signal at δ_C 120.72 (C-6); two oxygenated methine carbon signals at δ_C 73.92 (C-7) and 1.38 (C-3); and a methoxy carbon signal at δ_C 56.80 (7-OCH_3) were detected. In upfield region, several methylene and methine carbon signals along with six methyl carbon signals are observed at δ_C 19.82 (C-27), 19.02 (C-21), 18.97 (C-26), 18.24 (C-19), 11.99 (C-29), and 11.46 (C-18). The gHMBC spectrum enabled us to establish the locations to be connected

among the methoxy group, hydroxyl group, double bond, and methyl group of compound **1**. The olefin methine proton signal (δ_H 5.71, H-6) was correlated with the oxygenated methine carbon signal (δ_C 73.92, C-7), the methylene carbon signal (δ_C 42.30, C-4), and the methine carbon signal (δ_C 37.18, C-8). The methoxy proton signal (δ_H 3.33, 7-OCH_3) was correlated with the oxygenated methine carbon signal (δ_C 73.92, C-7). The methyl proton signals showed cross peaks with several carbon signals; H-19 (δ_H 0.96) with the olefin quaternary carbon signal (δ_C 146.13, C-5), the methine carbon signal (δ_C 42.75, C-9), the quaternary carbon signal (δ_C 37.43, C-10), and the methylene carbon signal (δ_C 36.73, C-1); H-21 (δ_H 0.90) with the methine carbon signals [(δ_C 55.65, C-17), (δ_C 36.13, C-20)] and the methylene carbon signal (δ_C 33.94, C-22); H-29 (δ_H 0.83) with the methine carbon signal (δ_C 45.84, C-24) and the methylene carbon signal (δ_C 23.07, C-28); H-26 (δ_H 0.80) and H-27 (δ_H 0.78) with the methine carbon signals [(δ_C 45.84, C-24), (δ_C 29.15, C-25)] and the methyl carbon signals of each other [(δ_C 19.82, C-27), (δ_C 18.97, C-26)]; and H-18 (δ_H 0.64) with the methine carbon signals [(δ_C 55.65, C-17), (δ_C 48.46, C-14)], the quaternary carbon signal (δ_C 42.08, C-13), and the methylene carbon signal (δ_C 39.03, C-12). Thus, from the above data, compound **1** was identified as 3-hydroxy-7-methoxy-stigmast-5-ene. The stereo structure of the hydroxyl (3-OH) and methoxy group (7-OCH_3) are confirmed as β and α-configuration, respectively, by comparing the NMR data and specific optical rotation ($[\alpha]_D$ -58.4° (*c* 0.50, $CHCl_3$) with those described in the literature (Pettit et al., 2000; Santana et al., 2012). Finally, on the basis of comparison of the physicochemical and spectroscopic data with those of the literatures (Pettit et al., 2000; Niu et al., 2001; Santana et al., 2012), compound **1** was identified as 3β-hydroxy-7α-methoxystigmast-5-ene, schleichenol 2. Some carbon chemical shifts such as C-23 and C-25 in the literature (Santana et al., 2012) are partly different from those obtained in this study, but they are certainly determined by 2D-NMR.

Compound **3**, a white powder, showed the absorbace bands of the hydroxy (3,261 cm^{-1}) and olefin (1,652 cm^{-1}) groups, and the molecular weight was determined as 430 from the molecular ion peak *m/z* 430 $[M]^+$ in the EI/MS spectrum. The NMR data of compound **3** was similar with those of compound **1** except for the absence of a methoxy group. Based on the ^{1}H- and ^{13}C-NMR data as well as by comparison of the data with those of the literatures (Zhang et al., 2005; Cui et al., 2011; Roh et al, 2012; Santana et al., 2012), compound **3** was determined as 7α-hydroxysitosterol (3β,7α-dihydroxystigmast-5-ene).

Compound **2**, a white powder, showed the absorbance bands of the hydroxy (3,343 cm^{-1}) and olefin (1,653 cm^{-1}) groups, and the molecular weight was determined as 430 from the molecular ion peak *m/z* 430 $[M]^+$ in the EI/MS spectrum. The NMR data of compound **2** was very similar to those of compound **3**, with the exception of the stereosturucture of the hydroxyl group at C-7. The ^{1}H-NMR (400 MHz, $CDCl_3$) spectrum of compound **2** showed the proton signals as δ_H 5.27 (1H, br s, H-6) and 3.83 (1H, d, *J*=8.0 Hz, H-7), instead of the proton signala as δ_H 5.59 (1H, d, *J*=5.2 Hz, H-6) and 3.83 (1H, br s, H-7) in the spectrum of compound **3**. In the ^{13}C-NMR (100 MHz, $CDCl_3$) spectrum of compound **2**, carbon signals were observed as δ_C 125.46 (C-6), 73.35 (C-7), and 39.57 (C-8), which were shifted towards the downfield region compared to the carbon signals of compound **3** as δ_C 123.86 (C-6), 65.36 (C-7), and 37.00 (C-8). Additionally, the specific rotation of compound **2** was observed as $[\delta]_D$ −13.3° (*c* 0.50, $CHCl_3$), instead of $[\alpha]_D$ −72.7° (*c* 0.50, $CHCl_3$) of compound **3**. Therefore, the configuration of the hydroxyl group at C-7 was identified as a β form (Chang et al., 2003; Cui et al., 2011). Consequently, by comparison of the data with those of the literatures (Zhang et al., 2005; Lee et al., 2009; Roh et al., 2010; Cui et al., 2011), compound **2** was identified as 7β-hydroxy-sitosterol (3β,7β-dihydroxystigmast-5-ene). Some carbon chemical shifts such as C-12, 13, 8, 23, and 25 in the literature (Roh et al., 2010) are partly different from those obtained in this study, but they are certainly determined by 2D-NMR. Compounds **1-3** were isolated for first time from rice bran.

Even though NMR is the most typically employed instrument to identify the sterols, there are many variances and inaccuracies in the published NMR data. Especially, lots of the methine and methylene proton signals for the sterol moiety overlapped at upfield. Thus, one-dimensional-NMR techniques do not give enough information for identification of each. To date, peak assignments in NMR data for these types of materials have been based on previously reported data. However, much of the earlier data may be erroneous due to instrument-resolution limitations. The definite assignments of the NMR data were established by the intensive examination of the extensive NMR experiments including COSY, HSQC, and gHMBC.

As a result, the phytosterols **1-3** were isolated and identified in this study together with the exact assignment of their NMR data. The isolated sterols have been reported in the literatures for antitumor (Pettit et al., 2000; Rho et al., 2010), anti-inflammatory (Cui et al., 2011), anti-cancer (Roussi et al., 2005), and antifeedant (Santana et al., 2012) activities. These findings may facilitate the use of by-product of rice processing in the fields of functional food and pharmaceutical preparation.

Acknowledgment This study was supported by the Mental Health Food Development Program from the Korea Food Research Institute (20130352U0054101S000100).

References

Akihisa T, Yasukawa K, Yamaura M, Ukiya M, Kimura Y, Shimizu N et al. (2000) Triterpene alcohol and sterol ferulates from rice bran and their anti-inflammatory effects. *J Agric Food Chem* **48**, 2313–9.

Berger A, Rein D, Schäfer A, Monnard I, Gremaud G, Lambelet P et al. (2005) Similar cholesterol-lowering properties of rice bran oil, with varied γ-oryzanol, in mildly hypercholesterolemic men. *Eur J Nutr* **44**, 163–73.

Chang KC, Duh CY, Chen IS, and Tsai IL (2003) A cytotoxic butenolide, two new dolabellane diterpenoids, a chroman and a benzoquinol derivative Formosan *Casearia membranacea*. *Planta Med* **69**, 667–72.

Cui EJ, Park JH, Park HJ, Chung IS, Kim JY, Yeon SW et al. (2011) Isolation of sterols from cowpea (*Vigna sinensis*) seeds and their promotion

activity on HO-1. *J Korean Soc Appl Biol Chem* **54**, 362–6.

Fang N, Yu S, and Badger TM (2003) Characterization of triterpene alcohol and sterol ferulates in rice bran using LC-MS/MS. *J Agric Food Chem* **51**, 3260–7.

Kim JS and Godber JS (2001) Oxidative stability and vitamin E levels increased in restructured beef roasts with added rice bran oil. *J Food Qual* **24**, 17–26.

Lee DY, Lee SJ, Kwak HY, Jung LK, Heo J, Hong SY et al. (2009) Sterols isolated from *Nuruk* (*Rhizopus oryzae* KSD-815) inhibit the migration of cancer cells. *J Microbiol Biotechnol* **19**, 1328–32.

McCaskill DR and Zhang F (1999) Use of rice bran oil in foods. *Food Technol* **53**, 50–4.

Niu XM, Li SH, Peng LY, Lin ZW, Rao GX, and Sun HD (2001) Constituents from *Limonia crenulata*. *J Asian Nat Prod Res* **3**, 299–311.

Parrado J, Miramontes E, Jover M, Gutierrez JF, Collantes L de Terán, and Bautista J (2006) Preparation of a rice bran enzymatic extract with potential use as functional food. *Food Chem* **98**, 742–8.

Pettit GR, Numata A, Cragg GM, Herald DL, Takada T, Iwamoto C et al. (2000) Isolation and structures of schleicherastatins 1-7 and schleicheols 1 and 2 from the teak forest medicinal tree *Schleichera oleosa*. *J Nat Prod* **63**, 72–8.

Revilla E, Maria CS, Miramontes E, Bautista J, García-Martínez A, Cremades O et al. (2009) Nutraceutical composition, antioxidant activity and hypocholesterolemic effect of a water-soluble enzymatic extract from rice bran. *Food Res Int* **42**, 387–93.

Rho EM, Jin Q, Jin HG, Shin JE, Choi EJ, Moon YH et al. (2010) Structural Implication in cytotoxic effects of sterols from *Sellaginella tamariscina*. *Arch Pharm Res* **33**, 1347–53.

Roussi S, Winter A, Gosse F, Werner D, Zhang X, Marchioni E et al. (2005) Different apoptotic mechanisms are involved in the antiproliferative effects of 7β-hydroxysitosterol and 7β-hydroxycholesterol in human colon cancer cells. *Cell Death Differ* **12**, 128–35.

Santana O, Reina M, Fraga BM, Sanz J, and González-Coloma A (2012) Antifeedant activity of fatty acid esters and phytosterols from *Echium wildpretii*. *Chem Biodiv* **9**, 567–76.

Tanaka A, Kato A, and Tsuchiya T (1971) Isolation of methyl ferulate from rice bran oil. *J Am Oil Chem Soc* **48**, 95–7.

Wilson TA, Ausman LM, Lawton CW, Hegsted DM, and Nicolosi RJ (2000) Comparative Cholesterol Lowering Properties of Vegetable Oils: Beyond Fatty Acids. *J Am Coll Nutr* **19**, 601–7.

Zhang X, Geoffroy P, Miesch M, Julien-David D, Raul F, Aoudé-Werner D et al. (2005) Gram-scale chromatographic purification of β-sitosterol synthesis and characterization of α-sitosterol oxides. *Steroids* **70**, 886–95.