Three Novel Crustulinol Esters, Saponaceols A—C, from *Tricholoma saponaceum*

Kazuko YOSHIKAWA,* Mina KUROBOSHI, Seiko AHAGON, and Shigenobu ARIHARA

Faculty of Pharmaceutical Sciences, Tokushima Bunri University; Yamashiro-Cho, Tokushima 770–8514, Japan.
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Three novel triterpene esters, saponaceols A (1), B (2), and C (3) were isolated from the fruiting body of the fungus *Tricholoma saponaceum*, and their structures were elucidated on the basis of extensive NMR experiments. Saponaceol A (1) exhibited moderate inhibitory activity against HL-60 cells.

Key words *Tricholoma saponaceum*; Tricholomataceae; saponaceol; crustulinol; lanostane

In a previous paper, we reported novel triterpenes, saponaceolides E—G, and lanostane triterpenes, saponaceoic acids I—III, from the fruit bodies of *Tricholoma saponaceum*.[1] Further investigation of the more polar fraction of the 70% EtOH extract of the fungus afforded three novel crustulinol esters,[2,3] designated saponaceols A (**1**), B (**2**), and C (**3**), in which phenylalanine or its derivatives form an amide bond with the 3-hydroxy-3-methylglutaryl (HMG) moiety attached at C-2-OH or C-3-OH. They were identified as the amides of deacyl hebelomic acid A.[4] Hebelomic acids H and I reported to be an inseparable mixture by Dossena *et al.*,[3] and fasciculol esters, fasciculols D and E, as reported by Takahashi *et al.*,[5] are analogues of saponaceols A (**1**)—C (**3**) with amide bonds. In this paper, we report the isolation, structural elucidation, and biological activities of **1**—**3**.

Saponaceol A (**1**) was obtained as an amorphous powder and its IR spectrum showed the presence of hydroxyl (3400 cm^{-1}), carbonyl (1740 cm^{-1}), and amide (1670 cm^{-1}) groups. The molecular formula of **1** was established to be $C_{46}H_{69}NO_{11}$ by analysis of NMR and high-resolution (HR)-FAB-MS data [*m*/*z* 834.4775, M+Na], indicating 13 degrees of unsaturation. Analyses of ^1H–^1H correlation spectroscopy (COSY), ^1H-detected multiple quantum coherrence connectivity (HMQC), and ^1H-detected heteronuclear multiple-bond connectivity (HMBC) correlation spectra completed the definition of all of functional groups in the lanostane-type triterpene, that is, with four hydroxy groups at C-2, C-3, C-12, and C-25, and with the characteristic hemiacetal pyran ring between C-21 and C-24 (Fig. 2). The relative stereochemistry was determined on the basis of rotating frame nuclear Overhauser and exchange spectroscopy (ROESY) correlations and the coupling constants from ^1H-NMR data. The angular methyl groups [Me-19 (δ 1.05), Me-18 (δ 0.70), and Me-30 (δ 1.34)] showed strong ROSEY correlations with H-2 (δ 4.16), H-12 (δ 4.10) and H-20 [δ 1.75 (m)], and H-17 [δ 2.55 (m)], respectively. Nuclear Overhauser effects (NOEs) were also observed between H-21 (δ 5.91) and H-12 (δ 4.10), and between 21-OH (δ 8.99) and H-24 (δ 4.22). All these data required the structure of 21,24-epoxy-2α,3β,12α,21,25-pentahydroxylanost-8-ene, with an E ring in the hemiacetal pyran form to adopt the chair conformation, which was identified as crustulinol from the coincidence of the NMR data with those reported in the literature.[2] The ester part must account for 7 of the 13 degrees of unsaturation, as indicated by the molecular formula. The tertiary methyl (δ_C 28.5, δ_H 1.69) attached to the carbon atom [δ 70.8 (s)] bearing a hydroxy group and the signals of two methylene groups [δ_C 47.1, δ_H 2.96 (2H, s), and δ_C 46.9, δ_H 3.05 (1H, d, J=13.7 Hz), 3.14 (1H, d, J=13.7 Hz)], and two carbonyls (δ 172.1, 172.0) indicated the presence of a 3-hydroxy-3-methylglutaryl (HMG) moiety.[3] Furthermore, in the remaining 10 car-

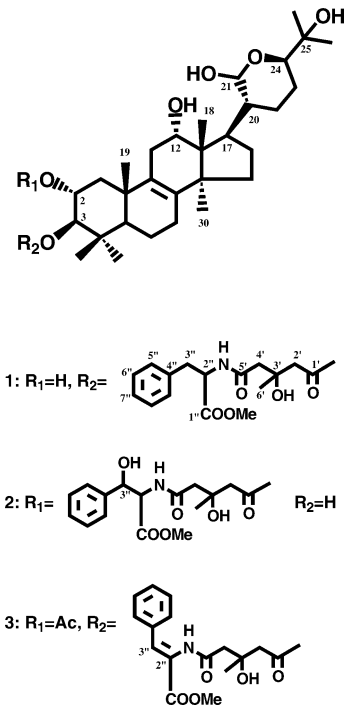

Fig. 1. Structures of **1**—**3**

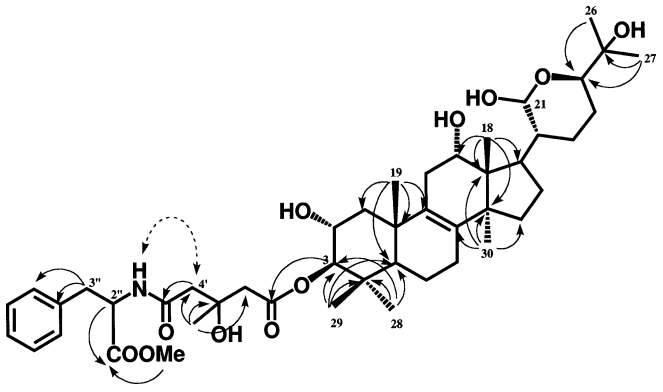

Fig. 2. Key HMBC (↷) an NOE (⇢) Correlations of **1**

* To whom correspondence should be addressed. e-mail: yosikawa@ph.bunri-u.ac.jp

Table 1. NMR Data for Saponaceols A (**1**)—C (**3**) (in Pyridine-d_5)

Position	**1**		**2**		**3**	
C-1	44.3	1.58 (m), 2.34 (dd, 12.6, 4.4)	41.1	1.35 (m), 2.24 (dd, 12.0, 4.2)	40.9	1.37 (m), 2.20 (dd, 12.1, 4.3)
2	66.8	4.16 (m)	74.4	5.45 (ddd, 11.5, 10.5, 4.2)	70.4	5.43 (ddd, 11.5, 10.5, 4.3)
3	85.3	5.07 (d, 9.9)	79.5	3.49 (d, 11.5)	80.2	5.20 (d, 11.5)
4	39.2	—	40.2	—	39.5	—
5	50.6	1.40 (m)	50.8	1.27 (m)	50.1	1.35 (m)
6	18.4	1.51 (m), 1.66 (m)	18.9	1.52 (m), 1.70 (m)	18.3	1.47 (m), 1.63 (m)
7	26.1	1.43 (m), 2.06 (m)	26.9	1.44 (m), 2.00 (m)	26.4	1.38 (m), 2.10 (m)
8	134.8	—	135.0	—	134.5	—
9	133.4	—	133.3	—	132.8	—
10	38.1	—	38.7	—	38.2	—
11	32.2	2.40 (br d, 18.7), 2.72 (dd, 18.7, 8.6)	32.4	2.30 (br d, 18.4), 2.59 (m)	32.1	2.27 (br d, 18.4), 2.60 (m)
12	72.7	4.10 (br d, 8.6)	73.0	4.09 (br d, 8.0)	72.7	4.10 (br d, 8.2)
13	50.0	—	50.3	—	50.0	—
14	50.5	—	50.8	—	50.5	—
15	32.2	1.24 (m), 1.72 (m)	32.4	1.23 (m), 1.72 (m)	32.2	1.22 (m), 1.74 (m)
16	27.6	1.40 (m), 2.16 (m)	27.9	1.37 (m), 2.04 (m)	27.6	1.38 (m), 2.10 (m)
17	39.9	2.55 (m)	40.2	2.56 (m)	39.9	2.58 (m)
18	17.2	0.70 (s)	17.5	0.69 (s)	17.2	0.68 (s)
19	20.1	1.05 (s)	20.3	1.10 (s)	19.8	1.05 (s)
20	43.9	1.75 (m)	44.2	1.76 (m)	43.9	1.77 (m)
21	93.2	5.91 (br s)	93.4	5.93 (br s)	93.2	5.93 (br s)
22	24.4	1.76 (m), 1.88 (m)	24.8	1.77 (m), 1.90 (m)	24.5	1.77 (m), 1.89 (m)
23	26.5	1.72 (m), 2.00 (m)	26.5	1.74 (m), 2.00 (m)	26.1	1.73 (m), 2.04 (m)
24	75.0	4.22 (dd, 11.6, 2.2)	75.2	4.22 (dd, 11.7, 2.0)	75.0	4.23 (dd, 11.7, 2.0)
25	71.2	—	71.4	—	71.2	—
26	26.1	1.44 (s)	26.5	1.44 (s)	26.1	1.45 (s)
27	26.8	1.44 (s)	27.1	1.44 (s)	26.8	1.45 (s)
28	28.6	1.04 (s)	29.1	1.21 (s)	28.3	1.02 (s)
29	17.9	1.01 (s)	17.5	1.08 (s)	17.7	0.97 (s)
30	24.2	1.34 (s)	24.6	1.35 (s)	24.3	1.36 (s)
1′	172.1	—	171.8	—	171.8	—
2′	46.9	3.05, 3.14 (each d, 13.7)	47.1	3.07, 3.16 (each d, 13.4)	46.8	3.17, 3.23 (each d, 14.3)
3′	70.8	—	71.0	—	70.7	—
4′	47.1	2.96 (2H, s)	47.5	2.91, 2.94 (each d, 14.3)	47.5	3.23, 3.28 (each d, 14.2)
5′	172.0	—	171.7	—	171.5	—
6′	28.5	1.69 (s)	29.0	1.60 (s)	28.2	1.83 (s)
1″	172.7	—	172.3	—	166.4	—
2″	54.3	5.21 (ddd, 8.4, 8.0, 5.8)	59.7	5.47 (dd, 8.8, 2.9)	135.8	—
3″	37.9	3.17 (dd, 14.0, 8.4), 3.32 (dd, 14.0, 5.8)	73.0	5.84 (br d, 2.9)	132.2	7.56 (s)
4″	137.5	—	142.9	—	134.5	—
5″	129.6	7.34 (2H, d, 7.5)	126.8	7.77 (2H, d, 7.5)	130.4	7.87 (2H, d, 7.5)
6″	128.9	7.30 (2H, t, 7.5)	128.6	7.37 (2H, t, 7.5)	129.0	7.37 (2H, t, 7.5)
7″	127.2	7.22 (d, 7.5)	127.8	7.27 (t, 7.5)	129.5	7.28 (t, 7.5)
OMe	52.0	3.63 (s)	52.3	3.60 (s)	52.3	3.80 (s)
MeCO					21.3	2.11 (s)
MeCO					170.7	—
NH		9.15 (d, 8.0)		9.24 (d, 8.8)		10.9 (s)

bons, each phenyl, methylene (δ 37.9), methine [δ_C 54.3, δ_H 5.21 (ddd, J=8.4, 8.0, 5.8 Hz)], and methoxylcarbonyl signal [δ_H 3.63 (s), δ_C 172.7, 52.0] was made by a phenylalanine methyl ester based on HMBC and COSY data. The NOE observed between the proton signal at δ 9.15 assignable to CONH and H_2-4′ (δ 2.96) of the HMG moiety suggested the presence of the amide bond. Finally, it was clear that C-3 (δ 85.3) was esterified with the HMG moiety based on the key HMBC correlation from H-3 [δ_H 5.07 (d, J=9.9 Hz)] to the carboxyl carbon [δ 172.1 (C-1′)]. Thus, the structure of saponaceol A was formulated as **1**.

Saponaceol B (**2**) showed an $[M(C_{46}H_{69}NO_{12})+Na]^+$ ion peak at *m*/*z* 850.4720 in its HR-FAB-MS, which differs from **1** by one oxygen atom (O). The ^{1}H- and ^{13}C-NMR spectra of **2** exhibited features resembling those of saponaceol A (**1**), that is, a crustulinol HMG ester. The major differences in the ^{1}H-NMR spectra were the appearance of one methine signal at δ 5.84 (br d, J=2.9 Hz), which was correlated with the carbon signal at δ 73.0 in the HMQC spectra, and the downfield chemical shift of H-2 [δ 5.45 (ddd, J=11.5, 10.5, 4.2 Hz)] judging from splitting patterns. The COSY connectivity from the oxymethine proton at δ 5.84 to H-2″ [δ 5.47 (dd, J=8.8, 2.9 Hz)] assigned to the α-position in the amino acid indicated the presence of a β-hydroxy-phenylalanine methylester. The formation of the amide bond was deduced by NOE correlation between CONH (δ 9.24) and H_2-4′ (δ 2.91, 2.94) in the HMG moiety. The combined position of the above-mentioned ester was confirmed at C-2 (δ 74.4) based on extensive HMBC correlations from H-2 (δ 5.45) of genin to C-1′ (δ 172.1) in the HMG moiety. Detailed analysis of the COSY, HMQC, HMBC, and ROESY data of **2** led to the assignment of the structure and relative stereochemistry of

saponaceol B as shown in **2**.

Saponaceol C (**3**) showed a quasimolecular $[M(C_{48}H_{69}NO_{12})+Na]^+$ ion peak at *m/z* 874.4719 in its HR-FAB-MS. Saponaceol C is also an analogue of saponaceols A and B based on its ^{1}H- and ^{13}C-NMR spectra. However, the appearance of an olefinic proton at δ 7.56 (s) of the acetyl group [δ 2.11 (s)], and the downfield shifts of H-2 [δ 5.43 (ddd, J=11.5, 10.5, 4.3 Hz)] and H-3 [δ 5.20 (d, J=11.5 Hz)] were observed in the ^{1}H-NMR data. The newly occurring olefinic proton (δ 7.56), which connected to the carbon (C-3″) at δ 132.2 in the HMQC spectrum, showed the HMBC long-range correlations to carbomethoxy (δ 166.4) and to C-5″ (δ 130.4). Therefore the new double bond was defined at the α,β-position (C-2″–C-3″) in the phenylalanine methyl ester. The stereochemistry was confirmed to be *Z* by the NOE observed between CONH (δ 10.9) and H-5″ [δ 7.87 (2H, d, J=7.5 Hz)] of the phenyl group. Based on the NOE correlation between CONH (δ 10.9) and H_2-4″ (δ 3.23, 3.28) in the HMG moiety, the formation of an amide bond was also confirmed. The combined position of the above-mentioned ester was limited to C-3 (δ 80.2) and that of acetyl was limited to C-2 (δ 70.4) in the HMBC correlations, respectively. The relative stereochemistry was independently assigned on the basis of a ROESY experiment and found to be analogous to that of saponaceols A and B. Hence, the structure of saponaceol C was formulated as **3**.

To the best of our knowledge, saponaceols A—C are the first crustulinol esters in which the phenylalanine derivative was condensed with an HMG moiety.[3,5]

The inhibitory effect of compound **1** on cell growth was evaluated in HL-60 human leukemia cells,[6,7] and moderate active was found in this assay (IC_{50} 8.9 μM).[8]

Experimental

General Experimental Procedures Optical rotations were recorded on a JASCO DIP-360 polarimeter. IR spectra were recorded on a Hitachi IR-27G, and NMR spectra on a Varian UNITY 600 spectrometer in C_5D_5N solution, using TMS as an internal standard. NMR experiments included the COSY, HMQC, HMBC, DEPT, and ROESY pulse sequences. Coupling constants (*J* values) are given in Hz. FABMS (Xe gun, 10 kV, *m*-nitrobenzyl alcohol as the matrix) was measured on a JEOL JMS-HX-100 mass spectrometer. Silica gel 60 (230—400 mesh, Merck) and silica gel 60F-254 (Merck) were used for column chromatography and TLC, respectively.

Material *T. saponaceum* was collected in Nagano, Japan, in autumn 2000. A specimen (TB 2085) is deposited in the Herbarium of the Department of Pharmacognosy, Tokushima Bunri University, Tokushima, Japan.

Extraction and Isolation The fresh fruit bodies (1.15 kg) of *T. saponaceum* were extracted with 70% EtOH at room temperature for 6 weeks. The ethanolic extract was partitioned between EtOAc and H_2O. The EtOAc-soluble portion (7.1 g) was repeatedly subjected to silica gel column chromatography with $[(CH_3)_2CH]_2O$–MeOH (50 : 1—25 : 6) to afford six fractions (frs. 1—6). Fraction 5 was further subjected to silica gel column chromatography with $[(CH_3)_2CH]_2O$–MeOH–H_2O (25 : 2 : 0.1—25 : 6 : 0.1) to give four fractions (frs. 5.1—4). Fraction 5-2 was purified by preparative HPLC (ODS, 82—85% MeOH) to afford saponaceols B (**2**, 9.41 mg) and C (**3**, 6.25 mg). Fraction 5-3 was purified by preparative HPLC (ODS, 88% MeOH) to afford saponaceol A (**1**, 6.3 mg).

Saponaceol A (**1**): Amorphous powder; $[\alpha]_D^{25}$ −10.4° (c=0.6, MeOH); IR (KBr) ν_{max} 3400, 1740, 1670, 1080 cm^{-1}; FAB-MS *m/z* 810 $[M-H]^-$; HR-FAB-MS *m/z* 834.4775 (Calcd for $C_{46}H_{69}NO_{11}$+Na, 834.4769); ^{1}H-NMR (600 MHz) and ^{13}C-NMR (150 MHz) spectra: Table 1.

Saponaceol B (**2**): Amorphous powder; $[\delta]_D^{25}$ −4.45° (c=0.4, MeOH); IR (KBr) ν_{max} 3450, 1740, 1670, 1050 cm^{-1}; FAB-MS *m/z* 826 $[M-H]^-$; HR-FAB-MS *m/z* 850.4720 (Calcd for $C_{46}H_{69}NO_{12}$+Na, 850. 4717); ^{1}H-NMR (600 MHz) and ^{13}C-NMR (150 MHz) spectra: Table 1.

Saponaceol C (**3**): Amorphous powder; $[\alpha]_D^{25}$ −20.6° (c=0.5, MeOH); IR (KBr) ν_{max} 3450, 1750, 1660, 1050 cm^{-1}; FAB-MS *m/z* 850 $[M-H]^-$; HR-FAB-MS *m/z* 874.4719 (Calcd for $C_{48}H_{69}NO_{12}$+Na, 874.4718); ^{1}H-NMR (600 MHz) and ^{13}C-NMR (150 MHz) Table 1.

Cytotoxic Assay against HL-60 Cells The MTT Cell Growth Assay Kit (Chemicon International Inc, CA, U.S.A.) was used in this assay. HL-60 cells were maintained in RPMI-1640 medium (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) supplemented with 10% fetal bovine serum (ICN Biomedicals Inc, Ohio, U.S.A.) in a humidified atmosphere of 5% CO_2 at 37 °C throughout study. HL-60 cells (90 μl) at a density of 5×10^5 cells/ml in the exponential growth phase were plated in 96-well flat-bottomed microplates with various drug concentrations (10 μl). After 24 h, 10 μl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) solution well was added to each culture medium. After a further 4 h of incubation, 100 μl of isopropanol with 0.04 N HCl solution was added to each well, and the formazen crystals in each well were dissolved by stirring with a pipette. The optical density measurements were made using a microplate reader (BIO-RAD Co., Ltd., Tokyo, Japan) at 570 nm. In these experiments, three replicate wells were used to determine each data point.

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