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

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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 $\text{C}_{46}\text{H}_{69}\text{NO}_{11}$ by analysis of NMR and high-resolution (HR)-

FAB-MS data [m/z 834.4775, $\text{M}+\text{Na}$], indicating 13 degrees of unsaturation. Analyses of ^1H – ^1H correlation spectroscopy (COSY), ^1H -detected multiple quantum coherence 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-2 α ,3 β ,12 α ,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 (—) and NOE (---) Correlations of 1

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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 methoxycarbonyl 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 H₂-4' (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(C₄₆H₆₉NO₁₂)+Na]⁺ ion peak at m/z 850.4720 in its HR-FAB-MS, which differs from **1** by one oxygen atom (O). The ¹H- and ¹³C-NMR spectra of **2** exhibited features resembling those of saponaceol A (**1**), that is, a crustulinol HMG ester. The major differences in the

¹H-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 H₂-4' (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 1H - 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 1H -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 H₂-4'' (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; $[\alpha]_D^{25} -10.4^\circ$ ($c=0.6$, 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); 1H -NMR (600 MHz) and ^{13}C -NMR (150 MHz) spectra: Table 1.

Saponaceol B (2): Amorphous powder; $[\delta]_D^{25} -4.45^\circ$ ($c=0.4$, 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); 1H -NMR (600 MHz) and ^{13}C -NMR (150 MHz) spectra: Table 1.

Saponaceol C (3): Amorphous powder; $[\alpha]_D^{25} -20.6^\circ$ ($c=0.5$, 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); 1H -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 formazan 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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