NOTE

Cytotoxic anthrasteroid glycosides, malsterosides A–C, from *Malbranchea filamentosa*

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Malbranchea species belong to the family *Onygenaceae* and are taxonomically close to human and animal pathogenic fungi.¹ The fact prompted us to investigate the chemical constituents of *Malbranchea* fungi. We already have reported the isolation and structural characterization of 4-benzyl-3-phenyl-5H-furan-2-one as a vasodilator, malfilanols A and B as antifungal and cytotoxic sesquiterpenes, malbrancheosides A–D as triterpene glycosides and malfilamentosides A and B as furanone glycosides, from the fungus *Malbranchea filamentosa* IFM41300.^{2–5} Further purification of extracts of rice cultivated by the above fungus allowed us to isolate three new cytotoxic anthrasteroid glycosides, designated malsterosides A (1), B (2) and C (3) (Figure 1). This paper deals mainly with the structure determination and cytotoxic activity of 1–3.

The fungus *M. filamentosa* Sigler and Carmichael IFM41300 was provided from Medical Mycology Research Center, Chiba University in Japan.

The fungus was cultivated on moisturized rice (1120 g) using 8 Roux flasks (Kimura laboratory glass, Tokyo, Japan) at 25 °C for 21 days. The cultivated rice was extracted with methanol after extracted with acetone and the solvent was evaporated *in vacuo*. The residue was chromatographed on DIAION HP20 (Mitsubishi chemical, Tokyo, Japan) with H₂O, 20% MeOH, 40% MeOH, 60% MeOH, 80% MeOH, MeOH and acetone, in turn. The evaporated acetone elute was purified by centrifugal partition chromatography (Senshu Scientific, Tokyo, Japan) using the solvent system of CH₂Cl₂–MeOH–H₂O (5:6:4) to obtain seven fractions. The second fraction (169 mg) was purified using HPLC on ODS-4 (5 μm, 10 × 250 mm, GL Sciences, Tokyo, Japan) (70% MeOH, CH₃CN–MeOH–H₂O (7:7:6), in turn) to obtain malsteroside B (2: 10 mg) and malsteroside C (3: 30 mg). The sixth fraction was purified using HPLC on ODS-4 (85% MeOH) and Inertsil SIL 100 A (5 μm, 10 × 250 mm, GL Sciences) (CH₂Cl₂–MeOH (7:1)) to obtain malsteroside A (1: 37 mg).

Malsteroside A (1) was obtained as a colorless crystalline powder (m.p. 129.5 °C from MeOH); molecular formula C₃₄H₅₂O₇ by direct ion analysis in real time-time of flight-MS (m/z: 573.3762 [M + H]⁺, calculated for C₃₄H₅₃O₇, m/z: 573.3791); [α]_D²⁰ –12° (c 1.0, MeOH);

UV λ_{max} nm (log ε): 263.5 (3.47), 254.5 (3.46), 227.0 (sh, 4.07), 213.0 (4.29); IR ν_{max}^{ATR} cm^{–1}: 3360, 1650, 1450. ¹H-NMR spectrum showed three singlet methyl protons (δ 0.43, δ 1.80 and δ 2.06), three doublet methyl protons (δ 0.78 (d, 7.0), δ 0.85 (d, 6.7) and δ 0.92 (d, 6.9)), two methine protons (δ 5.34 (d, 9.3), 6.56 (s)), nine oxygenated protons including an anomeric proton (δ 4.54 (bs)) (Table 1). ¹³C-NMR spectrum showed 6 methyl carbons, 8 sp³ methylene carbons including an oxygenated methylene (δ 62.5), 11 sp³ methine carbons, 6 of which were bearing oxygen functions (δ 67.9, δ 68.9, δ 72.7, δ 75.3, δ 77.8 and δ 79.7) and 1 of which was anomeric carbon (δ 100.9), an sp³ quaternary carbon (δ 44.1), 2 sp² methine carbons (δ 124.5 and δ 126.2) and 6 sp² quaternary carbons (Table 1). The contiguous 2D-INADEQUATE correlations from C-1 to C-21, C-22 to C-28 and C-1' to C'-6 of 1 suggested that structure of 1 consisted of a hexose moiety, a 1(10→6)*abeo*-5,7,9-androstatriene skeleton and a 2,3-dimethylpentane unit (Figure 2a). The HMBC correlations from 21-H₃ to C-22 suggested that C-20 position of the tetracyclic structure and C-22 position of side chain were linked by a carbon double bond, and 1'-H to C-23 also suggested that the C-1 position of sugar was attached to C-23 position of side chain in ether linkage. These results suggested that 1 was an anthrasteroid mono-glycoside, which should be rearranged such that the C-1–C-10 linkage of ergostane skeleton cleaved, followed by the C-1–C-6 linkage was newly formed by ring-closing reaction. The relative stereochemistry of 1 was confirmed by the analysis of ROESY spectrum (Figure 2b). The ROESY correlations between the methyl group of 18-H₃ and methine proton of 16α-H, between the methine proton of 14-H and the methine proton of 16β-H and 17-H suggested that the stereochemistry between C and D ring was *trans*-form, and two protons of 14-H and 17-H were on same side. The geometric position at olefinic bond of C-20 and C-22 was confirmed to *E*-configuration by the ROESY correlations from 21-H₃ to 18-H₃ and 23-H, and from 22-H to 28-H₃. Acid hydrolysis of 1 gave the sugar moiety of 1, but the genin could not be obtained for the production of complicated compounds unfortunately. The sugar moiety was determined D-mannose as by the comparison to commercial chemical with

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^1H -NMR spectrum and the value of $[\alpha]_{\text{D}}$ (D-mannose, $+15.3^\circ$ (H_2O)). The glycoside linkage of D-mannose in **1** was confirmed to β -anomer from the ROESY correlation between $1'\text{-H}$ and $5'\text{-H}$. Given that biosynthetic intermediate of **1** is an ergosterol, it is

Figure 1 Structure of malsterosides A (1)–C (3).

supposed that the stereochemistry of C-13 and C-17 is 'R' and 'R' configuration, respectively.

Malsteroside B (**2**) was obtained as a colorless crystalline powder: molecular formula $\text{C}_{42}\text{H}_{65}\text{NO}_{12}$ by direct ion analysis in real time-time of flight-MS (m/z : 776.4615 $[\text{M} + \text{H}]^+$, calculated for 776.4585); $[\alpha]_{\text{D}}^{20} + 56^\circ$ (c 1.0, MeOH); UV λ_{max} nm (log ϵ): 271.0 (3.14), 249.0 (3.03), 226.5 (sh, 3.93); m.p. 160.8°C (from MeOH); IR $\nu_{\text{max}}^{\text{ATR}}$ cm^{-1} : 3330, 1645, 1550, 1430. Malsteroside B (**2**) showed two anomeric protons (δ 4.54 (bs) and δ 5.07 (d, 3.7)) in the ^1H -NMR spectrum (Table 1), and the contiguous ^1H – ^1H COSY correlations from $1'\text{-H}$ to $6'\text{-H}_2$ and $1''\text{-H}$ to $6''\text{-H}_2$ of **2**. Therefore, malsteroside B (**2**) should be a diglycoside. Observation of HMBG correlations from $1\text{-H}''$ to C-5'' and from $2''\text{-H}$ to carbonyl carbon of acetylated group, NOESY correlation between $3''\text{-H}$ and $5''\text{-H}$, and the J value of 11.0 Hz between $2''\text{-H}$ and $3''\text{-H}$ and between $3''\text{-H}$ and $4''\text{-H}$ in **2** suggested that one sugar contained within **2** was *N*-acetyl-D-glucosamine (Figure 3). Acid hydrolysis of **2** gave one sugar and complicated decomposed products. This sugar was determined D-mannose from the comparison with its standard chemical. *N*-acetyl-D-glucosamine, which was another sugar

Table 1 ^1H - and ^{13}C -NMR data of malsterosides A (1)–C (3) in methanol- d_4

Atom	Malsteroside A (1)		Malsteroside B (2)		Malsteroside C (3)	
	δ_{C}	δ_{H} (J in Hz)	δ_{C}	δ_{H} (J in Hz)	δ_{C}	δ_{H} (J in Hz)
1	29.3	2.72m, 2.83m	27.6	2.67m, 2.84m	28.8	2.76ddd (5.4, 9.7, 16.6), 2.87td (5.5, 16.6)
2	32.4	1.64m, 1.97m	27.8	1.80m, 1.90m	31.9	1.66m, 1.97m
3	68.9	3.99m	74.0	4.11m	68.5	4.04m
4	37.3	2.45dd (8.2, 16.2), 2.98dd (5.4, 16.2)	35.3	2.71m, 2.99dd (4.7, 16.4)	37.0	2.47dd (7.8, 16.4), 2.99m
5	131.1	—	131.0	—	132.4	—
6	133.5	—	133.9	—	135.8	—
7	124.5	6.56s	124.6	6.58s	124.2	6.61s
8	138.3	—	138.5	—	138.9	—
9	132.2	—	132.6	—	134.1	—
10	134.6	—	135.0	—	137.4	—
11	26.7	2.64m, 2.77dd (7.8, 17.7)	26.7	2.65m, 2.78dd (7.5, 17.5)	67.7	5.07t (7.6)
12	37.6	1.67m, 2.11m	37.9	1.71m, 2.13m	49.7	1.77dd (6.8, 12.8), 2.61dd (8.5, 12.8)
13	44.1	—	44.6	—	47.3	—
14	52.0	2.68m	52.6	2.72m	51.4	2.97m
15	25.2	1.56m, 2.11m	25.3	1.59m, 2.13m	25.5	1.61m, 2.14m
16	26.4	1.86m, 2.08m	26.5	1.88m, 2.07m	26.0	1.86m, 2.03m
17	59.3	2.24t (9.6)	59.6	2.26t (9.3)	59.7	2.38t (9.6)
18	12.8	0.43s	12.8	0.43s	14.1	0.33s
19	15.0	2.06s	14.7	2.06s	15.6	2.27s
20	139.1	—	139.5	—	139.0	—
21	20.0	1.80s	19.7	1.81s	19.8	1.82s
22	126.2	5.34d (9.3)	126.5	5.34d (9.7)	126.5	5.34d (9.4)
23	79.7	4.42dd (7.5, 9.3)	79.7	4.43dd (7.3, 9.1)	79.6	4.43dd (7.8, 9.2)
24	44.4	1.56m	44.5	1.55m	44.4	1.57m
25	28.5	1.93m	28.8	1.93m	28.6	1.93m
26	17.8	0.85d (6.7)	17.8	0.85d (6.5)	17.9	0.85d (6.8)
27	22.1	0.92d (6.9)	22.0	0.92d (6.6)	22.1	0.92d (6.8)
28	10.7	0.78d (7.0)	10.6	0.77d (7.3)	10.7	0.77d (7.1)
1'	100.9	4.54bs	101.0	4.54bs	100.9	4.54bs
2'	72.7	3.83d (3.2)	72.8	3.83bd (3.6)	72.7	3.83d (3.2)
3'	75.3	3.42dd (3.2, 9.5)	75.6	3.41dd (3.6, 9.3)	75.3	3.42dd (3.2, 9.5)
4'	67.9	3.63t (9.5)	68.3	3.63t (9.3)	68.0	3.62t (9.5)
5'	77.8	3.13ddd (2.6, 4.5, 9.5)	78.1	3.14ddd (2.8, 3.6, 9.3)	77.8	3.15ddd (2.6, 4.6, 9.5)
6'	62.5	3.74dd (4.6, 11.6), 3.81dd (2.6, 11.6)	62.7	3.74m, 3.80m	62.6	3.74dd (4.6, 11.7), 3.82dd (2.6, 11.7)
1''	—	—	96.5	5.07d (3.7)	—	—
2''	—	—	55.7	3.86dd (3.7, 11.0)	—	—
2''-NHCOCH ₃	—	—	173.6	—	—	—
2''-NHCOCH ₃	—	—	22.6	1.97s	—	—
3''	—	—	74.0	3.64t (11.0)	—	—
4''	—	—	72.6	3.75m	—	—
5''	—	—	72.4	3.33m	—	—
6''	—	—	62.7	3.66m, 3.77m	—	—

part of **2**, was not detected in hydrolysate of **2**. The reasons for this are not clear, but we suppose that *N*-acetyl-D-glucosamine was degraded by hydrolysis procedure. The ^1H - and ^{13}C -NMR spectra of genin part in **2** were similar to that of **1**, except for the downfield shift of C-3 (**1**: δ 68.9, **2**: δ 74.0). From the analysis of ^1H - ^1H COSY and HMBC correlations in **2**, the genin of **2** was determined to be the same as the anthrasteroid skeleton of **1** (Figure 3a). Observation of the HMBC correlations from the anomeric proton ($1'\text{-H}$) of D-mannose to C-23 and anomeric proton ($1''\text{-H}$) of *N*-acetyl-D-glucosamine to C-3 in **2** suggested that the D-mannose and the *N*-acetyl-D-glucosamine linked at C-23 and C-3 in the genin of **2**, respectively. The glycoside linkage of *N*-acetyl-D-glucosamine was determined as α -configuration from the *J* of the anomeric proton (3.7 Hz for $1''\text{-H}$), and that of D-mannose was determined as β -configuration from the NOESY correlation between $1''\text{-H}$ and $5''\text{-H}$ (Figure 3b).

Malsteroside C (**3**) was obtained as a colorless crystalline powder (m.p. 137.9 °C from MeOH); molecular formula $\text{C}_{34}\text{H}_{52}\text{O}_8$ by direct ion analysis in real time-time of flight-MS (m/z : 589.3778 $[\text{M} + \text{H}]^+$,

calculated for 589.3740); $[\alpha]_{\text{D}}^{20} -33^\circ$ (c 1.0, MeOH); UV λ_{max} nm (log ϵ): 272.0 (3.00), 246.0 (2.49), 226.0 (sh, 4.04), 214.0 (4.25); IR $\nu_{\text{max}}^{\text{ATR}}$ cm^{-1} : 3280, 1645, 1500. The ^1H - and ^{13}C -NMR spectra of genin part in **3** were similar to that of **1**, except for the downfield shift of C-11 (**1**: δ 26.7, **3**: δ 67.7). From the results of MS spectra, it was clear that malsteroside C (**3**) has more than one oxygen atom than malsteroside A (**1**). These results revealed that the genin of **3** was 11-oxygenated of that of **1**. This was confirmed by the detail analysis of 2D-INADEQUATE and HMBC spectra (Figure 4a). Acid hydrolysis of **3** gave D-mannose, which was determined by the comparison to standard compound, and analysis of 2D-INADEQUATE and NOESY spectra of **3** (Figure 4b). Observation of the NOESY correlation between methyl group of 18-H_3 and oxygenated methine proton of 11-H in **3** suggested that the stereochemistry of C-11 is 'R' configuration.

Malsterosides A (**1**)–C (**3**) were tested for cytostatic activity against human malignant epithelial cells (Hela) and human lung cancer cells (A549) using a modified method.³ Malsteroside A (**1**) inhibited the cell proliferation of Hela and A549 with IC_{50} values of 28.1 and

Figure 2 2D-NMR correlations of malsteroside A (**1**). (a) Correlations of 2D-INADEQUATE and selected HMBC spectra. (b) Correlations of NOESY spectrum.

Figure 4 2D-INADEQUATE and HMBC correlations of malsteroside C (**3**). (a) Correlations of 2D-INADEQUATE and selected HMBC spectra. (b) Selected correlations of NOESY spectrum.

Figure 3 2D-NMR correlations of malsteroside B (**2**). (a) Selected correlations of ^1H - ^1H COSY and HMBC spectra. (b) Correlations of selected NOESY spectrum.

38.6 μM , respectively. Malsteroside C (**3**) showed moderate cytostatic activity for HeLa cell line (IC_{50} value: 76.9 μM) and malsteroside B (**2**) showed no inhibition of these cell growths.

Compounds with anthrasteroidal skeletons are rare in nature. Isolation of anthrasteroids has been first reported by Hussler and Albrecht⁶ from Cretaceous black shale. Then, citreanthrasteroids A–B from fungi⁷ and other anthrasteroidal structure from sheep tick⁸ have been isolated. From our knowledge, malsterosides A (**1**)–C (**3**) are the first examples of anthrasteroidal glycosides.

Acid hydrolysis of malsterosides A (**1**)–C (**3**) were carried out as follows. Malsterosides A (**1**)–C (**3**) (each 10 mg) were dissolved in 4 M HCl (10 ml) and the solution was refluxed for 2 h. After cooling, the reaction mixture was extracted with CHCl_3 and then concentrated *in vacuo*. The aqueous layer obtained after extraction of reaction mixture was chromatographed using HPLC on amino column with 60% CH_3CN to give D-mannose (**1**: 2 mg, **2**: 1 mg and **3**: 2 mg).

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