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Makomotines A to D from Makomotake, *Zizania latifolia* infected with *Ustilago esculenta*

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ABSTRACT

Four novel compounds, makomotines A to D (1-4), along with two known ones (5, 6) were isolated from Makomotake, *Zizania latifolia* infected with *Ustilago esculenta*. The structures were determined or identified by the interpretation of spectroscopic data. Compound **1** suppressed the formation of osteoclast without toxicity.

Introduction

The fungus, *Ustilago esculenta*, penetrates into the aquatic perennial grass, *Zizania latifolia*. After penetration, the fungus incites the formation of an edible gall and inhibits inflorescence and seed production in the plant. The gall is called Makomotake in Japanese, and people eat the gall in Japan, China and other Asian countries. We have already reported osteoclast-forming suppressive nortriterpenes and makomotindoline from it.[1,2] In the course of further search for new biologically active compounds from Makomotake, we succeeded in purifying four novel compounds, makomotines A to D, along with two known ones. Here we describe the isolation and structure determination of the new compounds, and biological activity of makomotine A.

Results and discussion

Fresh Makomotake was extracted with EtOH and then with acetone. After the solutions were combined and concentrated under reduced pressure, the concentrate was partitioned between CH_2Cl_2 and H_2O, EtOAc and H_2O, and then n-BuOH and H_2O. Compound **1** was purified from EtOAc soluble part and compounds **2**-**6** were obtained from the n-BuOH soluble part. By comparison of the ^1H and ^{13}C NMR and mass spectra of **5** and **6** with those reported in previous papers, the two compounds were identified as shown in Figure 1.[3,4] Although **5** has been synthesized, this is the first report of isolation from nature.[3]

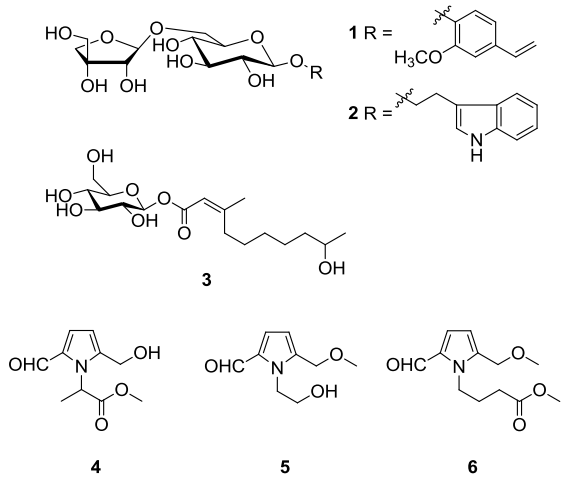

Figure 1. The structures of **1** to **6** from Makomotake.

Makomotine A (**1**) was purified as a white amorphous powder. Its molecular formula was determined as $C_{20}H_{28}O_{11}$ by HRESIMS at *m/z* 467.1499 $[M+Na]^+$ (calcd for $C_{20}H_{28}O_{11}Na$, 467.1529), indicating the presence of seven degrees of unsaturation in the molecule. The structure of **1** was elucidated

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by interpretation of NMR spectra including DEPT, COSY, HMQC, and HMBC (Figure 2).

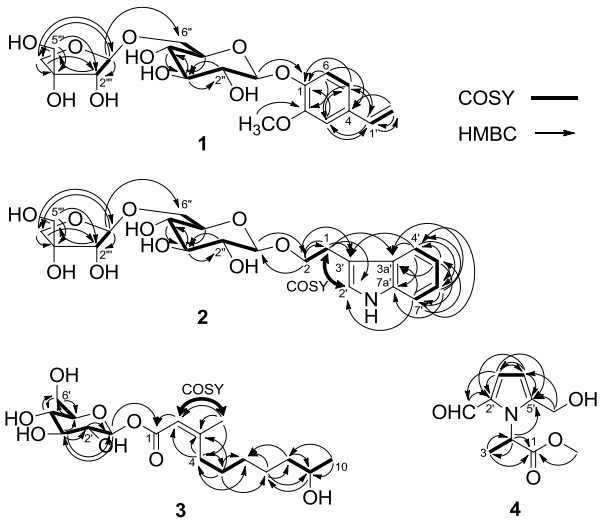

Figure 2. COSY and HMBC correlations of **1** to **4**.

The complete assignment of the protons and carbons was accomplished as shown in Table 1. The DEPT experiment indicated the presence of a methyl, four methylenes, eleven methines, and four quaternary carbons. The presence of a disaccharides including a β-glucopyranosyl group was suggested by the characteristic chemical shifts and coupling constants of its ^{1}H NMR spectrum: β-glucopyranosyl group, δ_C 102.8 (C1″), 74.9 (C2″), 77.9 (C3″), 71.6 (C4″), 77.1 (C5″), 68.7 (C6″); δ_H 4.80 (d, *J*=8.5 Hz, H1″), 3.44 (dd, *J*=8.5, 8.5 Hz, H2″), 3.40 (dd, *J*=8.5, 9.7 Hz, H3″), 3.30 (m, H4″), 3.51 (m, H5″), 3.57 (dd, *J*=11.0, 7.3 Hz, H6″a), 3.95 (d, *J*=11.0 Hz, H6″b): the other sugar, δ_C 111.0 (C1‴), 78.0 (C2‴), 80.5 (C3‴), 75.0 (C4‴), 65.6 (C5‴); δ_H 4.92 (d, *J*=3.4, Hz, H1‴), 3.85 (d, *J*=3.4 Hz, H2‴), 3.69 (d, *J*=9.8 Hz, H4′a), 3.90 (d, *J*=9.8 Hz, H4′b), 3.53 (s, H5′). These chemical shifts of each carbon and protons in the sugar part are very similar to those of manglieside B isolated from the leaves of *Manglietia phuthoensis*,[5] which possesses 6-*O*-β-D-apiofuranosyl-β-D-glucosyl moiety as the sugar part. The HMBC correlations confirmed the structure (in apiose; H1‴/C2‴, H1‴/C4‴, H2‴/C5‴, H4‴/C1‴, H4‴/C2‴, H4‴/C3‴, H5‴/C2‴, H5‴/C3‴, H5‴/C4‴: in Glc; H2″/C3″, H3″/C2″, H3″/C4″, H4″/C3″, H5″/C3″; between apiose and Glc; H1‴/C6″). The presence of 2-methoxy-4-vinylphenol as the aglycon was also suggested by the NMR data: δ_C 147.9 (C1), 150.9 (C2), 111.2 (C3), 134.3 (C4), 120.7 (C5), 118.0 (C6), 56.8 (C2O–Me), 137.7 (C1′), 112.9 (C2′) ; δ_H 7.04 (s, H3), 6.94 (d, *J*=6.9 Hz, H5), 7.07 (d, *J*=6.9 Hz, H6), 3.30 (s, Me–OC2), 6.22 (dd, *J*=18.3, 11.0 Hz, H1′), 5.10 (d, *J*=11.0 Hz, H2′a), 5.63 (d, *J*=18.3 Hz, H2′b). The HMBC correlations supported the structure of the aglycone and the linkage position between the aglycone and the sugar; Me–OC2/C2, H3/C1, H3/C5, H3/C1′, H5/C1, H5/C3, H5/C1′, H6/C1, H6/C2, H6/C4, H1′/C3, H1′/C5, H1′/C2′, H2′/C4, H2′/C1′, H1″/C1). By comparison of the specific rotation of **1** ($[\alpha]_D^{28}$ – 20, *c* 0.10, MeOH) with its analogues, methyl β-D-apiofuranosyl-(1→6)-β-D-glucopyranoside ($[\alpha]_D^{21}$ – 81, *c* 0.40, MeOH) and ethane-1,2-diol 1-*O*-β-D-apiofuranosyl-(1→6)-β-D-glucopyranoside ($[\alpha]_D^{21}$ – 47, *c* 0.70, MeOH),[6] the structure of **1** including its absolute configuration was established to be 2-methoxy-4-vinylphenyl β-D-apiofuranosyl-(1→6)-β-D-glucopyranoside.

Table 1. ^{1}H and ^{13}C NMR data for **1** to **4** (in CD_3OD)

Position	**1**		**2**		**3**		**4**	
	^{1}H δ (multiplicity, J in Hz)	^{13}C δ	^{1}H δ (multiplicity, J in Hz)	^{13}C δ	^{1}H δ (multiplicity, J in Hz)	^{13}C δ	^{1}H δ (multiplicity, J in Hz)	^{13}C δ
1		147.9	3.07 (dd, 9.5, 9.5)	26.8		161.1		172.5
2		150.9	3.84 (dd, 15.0, 9.5)	71.4	5.73 (br s)	116.1	5.43 (br q, 7.0)	55.8
			4.12 (dd, 15.0, 9.5)					
3	7.04 (s)	111.2				165.4	1.67 (d, 7.0)	18.0
4		134.3			2.63 (m)	34.5		
					2.67 (m)			
5	6.94 (d, 6.9)	120.7			1.50 (m)	29.2		
6	7.07 (d, 6.9)	118.0			1.36 (m)	30.8		
7					1.36 (m)	26.7		
					1.44 (m)			
8					1.38 (m)	40.1		
					1.43 (m)			
9					3.70 (m)	68.5		
10					1.13 (d, 6.1)	23.5		
2-OMe	3.30 (s)	56.8						
3-Me					1.93 (d, 1.2)	25.4		
1-OMe							3.66 (s)	52.8
1′	6.22 (dd, 18.3, 11.0)	137.7			5.47 (d, 8.2)	95.2		
2′	5.09 (d, 11.0)	112.9	7.12 (s)	123.7	3.34 (m)	74.0		133.4
	5.63 (d, 18.3)							
3′				112.5	3.42 (dd, 8.9, 8.5)	78.1	7.05 (d, 4.0)	127.4
3a′				128.9				
4′			7.53 (br d, 7.9)	119.3	3.35 (m)	78.8	6.27 (d, 4.0)	111.4
5′			6.99 (ddd, 7.9, 7.2, 0.9)	119.5	3.36 (m)	71.1		144.6
6′			7.06 (ddd, 7.2, 8.2, 0.9)	122.2	3.67 (dd, 12.0, 4.7)	62.4		
					3.83 (dd, 12.0, 2.0)			
7′			7.31 (br d, 8.2)	112.2				
7a′				138.0				
2′-CHO							9.31 (s)	180.4
5′-CH_2							4.60 (d, 13.7)	56.7
							4.63 (d, 13.7)	
1″	4.80 (d, 8.5)	102.8	4.32 (d, 7.6)	104.5				
2″	3.44 (dd, 8.5, 8.5)	74.9	3.21 (dd, 7.6, 8.2)	75.1				
3″	3.40 (dd, 8.5, 9.7)	77.9	3.35 (dd, 8.2, 8.8)	78.0*				
4″	3.30 (m)	71.6	3.30 (m)	71.7				
5″	3.51 (m)	77.1	3.40 (ddd, 8.2, 6.1, 2.0)	76.9				
6″	3.57 (dd, 11.0, 7.3)	68.7	3.61 (dd, 11.3, 6.1)	68.6				
	3.95 (d, 11.0)		3.98 (dd, 11.3, 2.0)					
1‴	4.92 (d, 3.4)	111.0	5.00 (d, 2.4)	111.0				
2‴	3.85 (d, 3.4	78.0	3.89 (d, 2.4)	78.1*				
3‴		80.5		80.5				
4‴	3.69 (d, 9.8)	75.0	3.74 (d, 9.5)	75.0				
	3.90 (d, 9.8)		3.95 (d, 9.5)					
5‴	3.53 (s)	65.6	3.55 (s)	65.6				

*interchangeable

Makomotine B (**2**) was isolated as a yellow amorphous powder. Its molecular formula, $C_{21}H_{29}NO_{10}$, which was determined by HRESIMS at *m/z* 478.1709 $[M+Na]^+$ (calcd for $C_{21}H_{29}NO_{10}Na$, 478.1689), indicated the presence of eight degrees of unsaturation in the molecule. The ^1H and ^{13}C NMR data of the sugar part of **2** are very similar to those of **1** and manglieside B,[5] suggesting that this compound also possessed the same disaccharide as **1** (Table 1). The aglycon, tryptophol moiety, was determined by HMBC and COSY correlations (Figure 2); H1/C2, H1/C2′, H1/C3′, H1/C3a′, H2/C1, H2/C3′, H2′/C1, H2′/C3′, H2′/C3a′, H2′/C7a′, H4′/C3′, H4′/C3a′, H4′/C6′, H4′/C7a′, H5′/C3a′, H5′/C4′, H5′/C6′, H5′/C7′, H5′/C7a′, H6′/C3a′, H6′/C4′, H6′/C7′, H6′/C7a′, H7′/C2′, H7′/C3a′, H7′/C4′, H7′/C5′ and H1/H2, H1/H2′, H4′/H5′, H5′/H6′, H6′/H7′. The connection between the sugar and the tryptophol moiety was confirmed by the HMBC correlations (H2/C1′′ and H1′′/C2). By comparison of the specific rotation of **2** ($[\alpha]_D^{22}$ – 34, *c* 0.10, MeOH) with **1** and its analogues mentioned above,[6] the structure of **2** was determined to be 2-(3-indole)ethyl β-D-apiofuranosyl-(1→6)-β-D-glucopyranoside.

Makomotine C (**3**) was isolated as a white amorphous powder. Its molecular formula was determined as $C_{17}H_{30}O_8$ by HRESIMS at *m/z* 385.1858 $[M+Na]^+$ (calcd for $C_{17}H_{30}O_8Na$, 385.1838). The presence of the β-glucopyranosyl group was suggested by the characteristic chemical shifts and coupling constants of its ^1H NMR spectrum: δ_C 95.2 (C1′), 74.0 (C2′), 78.1 (C3′), 78.8 (C4′), 71.1 (C5′), 62.4 (C6′); δ_H 5.47 (d, *J*=8.2 Hz, H1′), 3.34 (m, H2′), 3.42 (dd, *J*=8.9, 8.5 Hz, H3′), 3.35 (m, H4′), 3.36 (m, H5′), 3.67 (dd, *J*=12.0, 4.7 Hz, H6′a), 3.83 (dd, *J*=12.0, 2.0 Hz, H6′b). The presence of the 9-hydroxy-3-methyldec-2-enoic acid in the aglycon was elucidated by the HMBC correlations (H2/C1, Me-C3/C2, Me-C3/C3, Me-C3/C4, H4/C2, H4/C3, H4/C5, H4/C6, H5/C3, H5/C4, H5/C6, H5/C7, H7/C6, H7/C8, H7/C9, H8/C6, H8/C9, H9/C7, H10/C8, H10/C9), COSY correlations (H2/Me-C3, H4/H5, H5/H6, H8/H9, H9/H10) (Figure 2), the molecular formula and the chemical shift of the hydroxyl group at C9 (δ_C 68.5). A significant NOE was observed between the methyl proton at Me-C3 (δ_H 1.93) and H2 (δ_H 5.73, br, s) in the NOE difference experiment, indicating that the stereochemistry of the olefin is *Z* (Figure 2). The connection of the glucosyl group and aglycon was determined by the HMBC correlation (H1′/C1). As a result, the plane structure of **3** was determined as shown in Figure 1. Its absolute configuration remains undetermined.

Makomotine D (**4**) was isolated as colorless oil. Its molecular formula was determined as $C_{10}H_{13}NO_4$ by HRESIMS at *m/z* 234.0766 $[M+Na]^+$ (calcd for $C_{10}H_{13}NO_4Na$, 234.0742). The structure of **4** was elucidated by interpretation of NMR spectra including DEPT, COSY, HMQC, and HMBC (Figure 2). The complete assignment of the protons and carbons was accomplished as shown in Table 1. The DEPT experiment indicated the presence of two methyls, a methylene, four methines, and three quaternary carbons. The 5-hydroxymethyl-pyrrole-2-carbaldehyde moiety was suggested by the HMBC correlations (OHC-C2′/C2′, H3′/C2′, H3′/C4′, H3′/C5′, H3′/CHO-C2′, H4′/C2′, H4′/C3′, H4′/C5′, CH_2-C5′/C4′, CH_2-C5′/C5′) and COSY correlation (H3′/H4′). The methyl propionate group was constructed by the HMBC and COSY correlations (Figure 2); MeO-C1/C1, H2/C1, H2/C3, H3/C1, H3/C2, and H2/H3. The connection between the pyrrole moiety and the propionate group was confirmed by the HMBC correlation (H2/C5′). However, the absolute configuration of **4** remains undetermined.

Biological activity of the isolated compounds was tested by using some bioassays and compound **1** showed the osteoclast-forming inhibitory activity. The assay is based on the principle that osteoclast-like multinucleated cells can be formed *in vitro* from co-cultures of mouse bone marrow cells and osteoblastic cells by treatment with osteotropic factors. By adding suppressive agents, the formation of osteoclast is inhibited during the differentiation. As shown in Figure 3, **1** inhibited osteoclast formation at 25 μg/mL with no cytotoxicity.

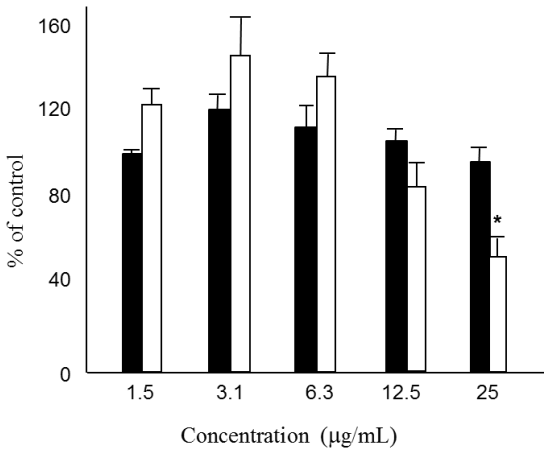

Figure 3. Inhibition of osteoclast formation by **1**.
Closed and open columns indicate cell viability and osteoclast formation, respectively. TRAP-positive multinucleated cells that had more than three nuclei were counted. Cell viability was determined by MTT assay. Data are the mean±SE of two cultures (**P*<0.01 vs control using Student's *t*-test).

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http:

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