Tetrahedron 66 (2010) 9332–9335

Contents lists available at ScienceDirect

Tetrahedron

journal homepage: www.elsevier.com/locate/tet

Applanatines A–E from the culture broth of *Ganoderma applanatum*

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ARTICLE INFO

Article history:
Received 27 August 2010
Received in revised form 7 October 2010
Accepted 8 October 2010
Available online 14 October 2010

ABSTRACT

Five novel compounds, applanatines A–E (**1**–**5**), and a known one (**6**) were isolated from the culture broth of *Ganoderma applanatum*. Their structures including the relative configurations were determined by the interpretation of spectroscopic data. Compounds **3** and **4** suppressed the growth of *Fusobacterium nucleatum*, that is, a prominent member of the oral microflora implicated in periodontitis.

1. Introduction

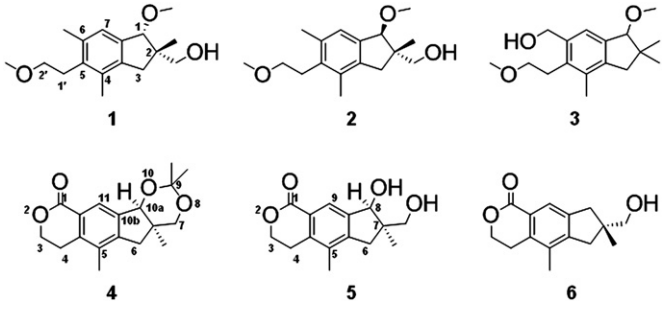
Chronic-degenerative dental diseases, including periodontal diseases, are widespread in human populations and represent a significant problem for public health.[1] *Fusobacterium nucleatum* is a Gram-negative obligate anaerobe and a prominent member of the oral microflora implicated in periodontitis, a disease affecting 5–15% of most populations worldwide.[2–4] The primary role of *F. nucleatum* in promoting the onset of periodontal disease is associated with its ability to co-aggregate with different bacterial species in oral biofilms, leading to plaque formation and permanent establishment of pathogenic strains within the oral cavity.[2,5–8] Therefore, inhibition of the growth of *F. nucleatum* is effective in prevention and progression of the disease.

During screening for the antibiotic activity of extracts of various mushrooms against *F. nucleatum* growth, we found strong inhibitory activity in the extract of the culture broth of a fungus *Ganoderma applanatum* (Japanese name, Kofukisarunokoshikake), and tried to isolate the active molecules from the culture broth. This mushroom is from bracket and wood-decay fungi class and grows in broadleaf forests and almost anywhere around the world.

Here we describe the isolation, structural determination, and biological activity of five novel compounds, applanatines A–E, and a known one from the culture broth of the fungus.

2. Results

The culture broth of *G. applanatum* was extracted with hexane, EtOAc, and then H_2O. Since EtOAc-soluble fraction showed antibacterial activity against *F. nucleatum*, the fraction was subjected to column chromatography, being guided by the result of the bioassay. As a consequence, five novel compounds (**1**–**5**), and a known one (**6**) were purified (Scheme 1).



Scheme 1. Structures of **1**–**6**.

Applanatine A (**1**) was purified as colorless oil. Its molecular formula was determined as $C_{17}H_{26}O_3$ by HRESIMS *m*/*z* 301.1761 $[M+Na]^+$ (calcd for $C_{17}H_{26}NaO_3$, 301.1780). The structure of **1** was elucidated by interpretation of NMR spectra including DEPT, COSY, HMBC, and HMQC (Fig. 1). The complete assignment of the protons and carbons was accomplished as shown in Table 1. The presence of the benzene skeleton (C3a–C7a) was suggested by the characteristic chemical shifts at δ_C 125.1, 133.7, 134.7, 135.3, 138.5, and 140.8. The structure of methoxyethyl moiety (C1′–C2′) was constructed by the COSY correlations (H1′/H2′), the HMBC correlations (H1′/C2′; H2′/C1′, C2′-OMe; C2′-OMe/C2′). The position of the methoxyethyl at the benzene ring was elucidated by the HMBC correlations (H1′/C4, C5, C6), and the positions of the two methyls were also assigned by the HMBC correlations (C4–Me/C3a, C4, C5; C6–Me/C5, C6, C7;

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doi:10.1016/j.tet.2010.10.014

H7/C5, C6–Me). The structure of the cyclopentane moiety (C1–C3) and the other parts were constructed by the HMBC correlations (H1/C1–OMe, C2, C2–Me, C2–CH_2OH, C3, C3a, C7; C1–OMe/C1; C2–Me/C1, C2, C2–CH_2OH, C3; C2–CH_2OH/C1, C2, C2–Me, C3; H3/C1, C2, C2–Me, C2–CH_2OH, C3a, C4, C7a; H7/C1, C3a) and the chemical shifts (C1–OMe, δ_H 3.42, δ_c 57.2; C2-CH_2OH, δ_H 3.63, 3.76, δ_c 68.2). The relative stereochemistry of C1 and C2 in **1** was determined by the NOE difference experiment; an NOE was observed between H1 and C2–Me and there was no NOE between C1–OMe and C2–Me. As a result, the structure of **1** was determined as ((1*S**,2*S**)-1-methoxy-5-(2-methoxyethyl)-2,4,6-trimethyl-2,3-dihydro-1*H*-inden-2-yl)methanol.

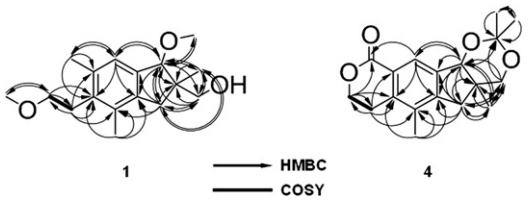

Figure 1. HMBC correlations in **1** and **4**.

Applanatine B (**2**) was purified as colorless oil. Its molecular formula was determined as $C_{17}H_{26}O_3$ by HRESIMS *m*/*z* 301.1763 $[M+Na]^+$ (calcd for $C_{17}H_{26}NaO_3$, 301.1780). The formula was the same as that of **1** and the NMR data of **2** were very similar to those of **1** (Table 1), suggesting that **2** must be a diastereomer of **1**. The relative stereochemistry of **2** was confirmed by the observed NOE between H1 and C2–CH_2OH in the NOE difference experiment. As a result, the structure of **2** was determined as ((1*R**,2*S**)-1-methoxy-5-(2-methoxyethyl)-2,4,6-trimethyl-2,3-dihydro-1*H*-inden-2-yl) methanol.

Applanatine C (**3**) was purified as colorless oil. Its molecular formula was determined as $C_{17}H_{26}O_3$ by HRESIMS *m*/*z* 301.1780 $[M+Na]^+$ (calcd for $C_{17}H_{26}NaO_3$, 301.1780) and the same as those of **1** and **2**. The NMR data of **3** were similar to those of **1** and **2** (Table 1). The HMBC cross peaks (two of C2–Me/C1, C2, C3, the other C2–Me; C6–CH_2OH/C5, C6, C7) indicated that the positions of the hydroxymethyl and the methyl in **3** are opposite to those in **1** and **2**. As a result, the planar structure of **3** was determined as (1-methoxy-5-(2-methoxyethyl)-2,2,4-trimethyl-2,3-dihydro-1*H*-inden-6-yl)methanol.

Applanatine D (**4**) was purified as colorless oil. Its molecular formula was determined as $C_{18}H_{22}O_4$ by HRESIMS *m*/*z* 303.1619 $[M+H]^+$ (calcd for $C_{18}H_{23}O_4$, 303.1596). The structure of **4** was elucidated by interpretation of NMR spectra including DEPT, COSY, HMBC, and HMQC (Fig. 1). The complete assignment of the protons and carbons was accomplished as shown in Table 1. The presence of the benzene ring (4a–5a and 10b–11a) was suggested by the characteristic chemical shifts at δ_C 124.3, 125.7, 131.9, 138.8, 140.8, and 150.0. The presence of the δ-lactone moiety (1–4a and 11a) and its linkage to the benzene ring was constructed by the COSY correlations (H3/H4), the HMBC correlations (H3/C1, C4, C4a; H4/C3, C4a, C5, C11a; H11/C1, C4a), the chemical shifts (C1, δ_c 165.7; C3, δ_H 4.47, δ_c 66.5; C4, δ_H 2.95, δ_c 25.3), and the IR absorption at 1718 cm^{-1}. The position of the methyl at the aromatic ring was also assigned by the HMBC correlations (C5–Me/C4a, C5, C5a). The structure of the 2,2-dimethyl-1,3-dioxane moiety (6a to 10a) and the other parts were constructed by the HMBC correlations (H6/C5, C5a, C6a, C6a-Me, C7, C10a, C10b; C6a-Me/C6, C6a, C7, C10a; H7/C6, C6a, C6a-Me, C9, C10a; two of C9-Me/C9, the other C9-Me; H10a/C5a, C6, C6a, C6a-Me, C9, C10b, C11; H11/C5a, C10a) and the chemical shifts (C7, δ_H 3.77, 3.81, δ_c 66.8; C9, δ_c 98.1). The relative stereochemistry of **4** was determined by the NOE difference experiment; an NOE was observed between H10a and C6a–Me. As

Table 1
^1H and ^{13}C NMR data for **1**–**5** (in $CDCl_3$)

Position	**1**		**2**		Position	**3**		Position	**4**		Position	**5**	
	^1H	^{13}C	^1H	^{13}C		^1H	^{13}C		^1H	^{13}C		^1H	^{13}C
	δ (multiplicity, *J* in Hz)	δ	δ (multiplicity, *J* in Hz)	δ		δ (multiplicity, *J* in Hz)	δ		δ (multiplicity, *J* in Hz)	δ		δ (multiplicity, *J* in Hz)	δ
1	4.25 (s)	93.8	4.39 (s)	88.0	1	4.11 (s)	92.3	1		165.7	1		166.0
2		47.6		49.1	2		43.8	3	4.47 (m)	66.5	3	4.48 (dd, 5.5, 5.5)	66.7
3	2.34 (d, 15.9)	39.2	2.62 (d, 15.9)	40.3	3	2.51 (d, 15.6)	45.1	4	2.95 (m)	25.3	4	2.96 (dd, 5.5, 5.5)	25.2
	3.22 (d, 15.9)		2.66 (d, 15.9)			2.75 (d, 15.6)		4a		138.8	4a		138.5
3a		140.8		138.6	3a		142.3	5		131.9	5		131.5
4		133.7		133.0	4		133.2	5a		150.0	5a		148.2
5		135.3		134.6	5		135.5	6	2.51 (d, 16.2)	40.7	6	2.53 (d, 16.8)	40.0
6		134.7		135.1	6		138.3		3.38 (d, 16.2)			3.13 (d, 16.8)	
7	7.00 (s)	125.1	7.01 (s)	124.6	7	7.19 (s)	124.7	6a		40.4	7		47.1
7a		138.5		139.9	7a		140.9	7	3.77 (d, 12.1)	66.8	8	4.89 (s)	83.6
1-OCH_3	3.42 (s)	57.2	3.49 (s)	58.4	1-OCH_3	3.46 (s)	58.0		3.81 (d, 12.1)				
2-CH_3	1.00 (s)	23.7	1.12 (s)	17.6	2-CH_3	1.11 (s)	22.3	9		98.1	8a		143.1
2-CH_2OH	3.63 (d, 11.6)	68.2	3.47 (d, 10.7)	69.8		1.11 (s)	28.2	10a	4.68 (s)	80.5	9	8.02 (s)	124.6
	3.76 (d, 11.6)		3.50 (d, 10.7)		4-CH_3	2.17 (s)	15.8	10b		140.8	9a		124.3
4-CH_3	2.22 (s)	15.8	2.18 (s)	15.6	6-CH_2OH	4.56 (d, 11.9)	64.1	11	8.01 (s)	125.7	5-CH_3	2.20 (s)	15.1
6-CH_3	2.34 (s)	20.4	2.32 (s)	20.3		4.62 (d, 11.9)		11a		124.3	7-CH_3	1.11 (s)	23.0
1′	2.95 (t, 7.9)	30.1	2.93 (t, 8.1)	29.9	1′	3.03 (t, 5.5)	29.6	5-CH3	2.21 (s)	15.3	7-CH_2OH	3.72 (d, 11.3)	68.1
2′	3.42 (t, 7.9)	71.4	3.40 (t, 8.1)	71.4	2′	3.57 (t, 5.5)	71.8	6a-CH_3	0.91 (s)	22.5		3.77 (d, 11.3)	
2′-OCH_3	3.36 (s)	58.6	3.35 (s)	58.6	2′-OCH_3	3.28 (s)	58.8	9-CH_3	1.31 (s)	28.1			
									1.53 (s)	20.4			

a result, the structure of **4** was determined as (6a*S**,10a*S**)-5,6a,9,9-tetramethyl-3,4,6a,7,9,10a-hexahydrocyclopenta[*d*][8,10]dioxono[*g*]-isochromen-1(6*H*)-one.

Applanatine E (**5**) was purified as colorless oil. Its molecular formula was determined as $C_{15}H_{18}O_4$ by HRESIMS *m*/*z* 285.1098 $[M+Na]^+$ (calcd for $C_{15}H_{18}NaO_4$, 285.1103). The NMR data of **5** were similar to those of **4** (Table 1). However, **5** lacks three carbons and has no isopropyl compared with **4**. In addition, all the HMBC correlations in **4** (Fig. 1) except for those of the isopropyl could be also observed in the HMBC experiment of **5** (data not shown). Based on the NOE between H8 and C7–Me, the structure of **5** was determined as (7*R**,8*R**)-8-hydroxy-7-(hydroxymethyl)-5,7-dimethyl-3,4,7,8-tetrahydrocyclopenta[*g*]isochromen-1(6*H*)-one.

The absolute configurations of all the novel compounds remain unknown.

Compound **6** has been reported as a plant growth promoter, echinolactone D from the culture broth of *Echinodontium japonicum* Imazeki (Japanese name, Kouyaku-mannen-haritake).[9]

The antibiotic effects of the compounds on the growth of *F. nucleatum* were tested in vitro. In this experiment, thymol was used as the positive control and its MIC was 100 ppm (667 μM). Compounds **1** (MIC, 3.13 ppm, 11.3 μM), **2** (MIC, 3.13 ppm, 11.3 μM), and **4** (MIC, 3.13 ppm, 10.4 μM) were stronger inhibitors than the control, though **3** and **6** inhibited at higher concentrations, 100 ppm (11.3 μM) and 200 ppm (11.3 μM), respectively.

3. Experimental

3.1. General

^{1}H NMR spectra (one- and two-dimensional) were recorded on a JEOL lambda-500 spectrometer at 500 MHz, while ^{13}C NMR spectra were recorded on the same instrument at 125 MHz. The HRESIMS spectra were measured on a JMS-T100LC mass spectrometer. A JASCO grating infrared spectrophotometer was used to record the IR spectra. The specific rotation values were measured by using a JASCO DIP-1000 polarimeter. HPLC separations were performed with a JASCO Gulliver system using reverse-phase HPLC columns (CAPCELL PAK C18 AQ, Shiseido, Japan; COSMOSIL Cholester Waters, Nacalai tesque, Japan; Develosil C30-UG-5, Nomura Chemical, Japan; Develosil C30-UG-15/30, Nomura Chemical, Japan). Silica gel plate (Merck F_{254}) and silica gel 60N (Merck 100–200 mesh) were used for analytical TLC and for flash column chromatography, respectively.

3.2. Fungus materials and incubation

The strains of *G. applanatum* and *F. nucleatum* have been deposited at the culture collection of Forestry and Forest Products Research Institute and Central Laboratory, Lotte Co. Ltd., respectively.

The culture medium (24 g/L) of *G. applanatum* was prepared containing potato dextrose broth (Difco). The medium was packed in each glass bottle (6 g/500 mL flask) and autoclaved. The pre-incubated mycelia were inoculated to the bottle and incubated under the condition (22 °C, shaking with 130 rpm) for 4 weeks in an incubator (NR-30, Tietech, Japan).

3.3. Extraction and isolation

The culture broth of *G. applanatum* (30 L) was filtrated and then concentrated under reduced pressure. The filtrate was successively extracted with hexane (three times), EtOAc (five times) and then H_2O. The EtOAc-soluble part (15.5 g) was fractionated by silica gel flash column chromatography (CH_2Cl_2/EtOAc 90:10, 70:30, 50:50; EtOAc; EtOAc/MeOH, 70:30, 50:50; and MeOH) to obtain 13 fractions.

Fraction 8 (2.2 g) was adsorbed to ODS gel and eluted with 50% MeOH and then MeOH. The eluent with 50% MeOH, fraction 8-1 (1.4 g), was fractionated by reverse-phase HPLC (Develosil C30-UG-15/30, 50% MeOH) to obtain 12 fractions. Fraction 8-1-8 (50.7 mg) was further separated by reverse-phase HPLC (Develosil C30-UG-5, 40% MeOH) to afford compound **6** (31.8 mg). Fraction 9 (3.7 g) was fractionated by silica gel flash column chromatography (CH_2Cl_2; CH_2Cl_2/acetone 95:5, 90:10, 80:20, 60:40, 30:70; acetone; acetone/MeOH 50:50; and MeOH) to obtain 15 fractions. Each fraction 9-2 (20.4 mg), 9-3 (30.4 mg), and 9-4 (52.7 mg) was further separated by reverse-phase HPLC (Develosil C30-UG-5, 60% MeOH) to afford compounds **1** (4.0 mg, from fraction 9-2), **2** (9.0 mg from fraction 9-3), **3** (2.3 mg from fraction 9-4), and **4** (2.2 mg from fractions 9-2 and 9-3), respectively. Fraction 9-5 (126.2 mg) was further separated by reverse-phase HPLC (CAPCELL PAK C18 AQ, 70% MeOH) to obtain 9 fractions, and compound **5** (2.0 mg) was obtained from fraction 9-5-4 (8.0 mg) by reverse-phase HPLC (CAPCELL PAK C18 AQ, 50% MeOH).

*3.3.1. Applanatine A (**1**).* Colorless oil; $[\alpha]_D^{25}$ −26 (*c* 0.2, MeOH); IR (neat): 3160 cm^{-1}; ^{1}H and ^{13}C NMR, see Table 1; ESIMS *m*/*z* 301 $[M+Na]^+$; HRESIMS *m*/*z* 301.1761 $[M+Na]^+$ (calcd for $C_{17}H_{26}NaO_3$, 301.1780).

*3.3.2. Applanatine B (**2**).* Colorless oil; $[\alpha]_D^{25}$ −20 (*c* 0.9, MeOH); IR (neat): 3457 cm^{-1}; ^{1}H and ^{13}C NMR, see Table 1; ESIMS *m*/*z* 301 $[M+Na]^+$; HRESIMS *m*/*z* 301.1763 $[M+Na]^+$ (calcd for $C_{17}H_{26}NaO_3$, 301.1780).

*3.3.3. Applanatine C (**3**).* Colorless oil; $[\alpha]_D^{25}$ −9.5 (*c* 0.2, MeOH); IR (neat): 3421 cm^{-1}; ^{1}H and ^{13}C NMR, see Table 1; ESIMS *m*/*z* 301 $[M+Na]^+$; HRESIMS *m*/*z* 301.1780 $[M+Na]^+$ (calcd for $C_{17}H_{26}NaO_3$, 301.1780).

*3.3.4. Applanatine D (**4**).* Colorless oil; $[\alpha]_D^{25}$ +45 (*c* 0.1, MeOH); IR (neat): 1718 cm^{-1}; ^{1}H and ^{13}C NMR, see Table 1; ESIMS *m*/*z* 303 $[M+H]^+$; HRESIMS *m*/*z* 303.1619 $[M+H]^+$ (calcd for $C_{18}H_{23}O_4$, 303.1596).

*3.3.5. Applanatine E (**5**).* Colorless oil; $[\alpha]_D^{23}$ +24 (*c* 0.2, MeOH); IR (neat): 1703, 3400 cm^{-1}; ^{1}H and ^{13}C NMR, see Table 1; ESIMS *m*/*z* 285 $[M+Na]^+$; HRESIMS *m*/*z* 285.1098 $[M+Na]^+$ (calcd for $C_{15}H_{18}NaO_4$, 285.1103)

*3.3.6. Echinolactone D (**6**).* Colorless oil; $[\alpha]_D^{29}$ +1.0 (*c* 0.5, MeOH); IR (neat): 1715, 3410 cm^{-1}; ^{1}H NMR ($CDCl_3$): δ 1.09 (C7–Me, s), 2.10 (C5–Me, s), 2.54 (H6, d, 14.5), 2.59 (C8, d, 13.5), 2.87 (H4, dd, 12.5, 14.5), 2.88 (H6, d, 14.5), 2.88 (H8, d, 14.5), 3.44 (C7–CH_2OH, s), 4.38 (H3, dd, 4.0, 4.5), 7.67 (H9, s); ^{13}C NMR ($CDCl_3$): δ 15.1 (C5–Me), 24.1 (C7–Me), 24.8 (C4), 42.1 (C6), 42.4 (C8), 44.4 (C7), 66.6 (C3), 69.8 (C7–CH_2OH), 123.3 (C9a), 124.0 (C9), 130.8 (C5), 136.2 (C4a), 141.5 (C8a), 148.6 (C5a), 166.3 (C1); ESIMS *m*/*z* 269 $[M+Na]^+$

3.4. Bioassay

The antibiotic activity against *F. nucleatum* was examined as follows. *F. nucleatum* ATCC25586 strain was maintained on brain heart infusion agar plates (BBL). The agar was inoculated to liquid culture containing trypticase soy broth (3.0 g, BBL), yeast extract (0.3 g, BD), hemin-1 N NaOH (0.1 mL, Acros organics) and menadione-50% EtOH (100 mL, Sigma) in 500 mL flasks and incubated at 37 °C for 2 days in an incubator. After the incubation, the cultures were diluted 10 times. The diluted culture of the *F. nucleatum* (100 μl) was poured into each well of 96-well plates and concentration of the samples (100 μl in 2% DMSO) was added to the wells. Thymol was used as a positive control. After the incubation under

the anaerobically condition at 37 °C for 3 days, the minimum inhibitory concentration of the samples were measured.

Acknowledgements

We thank V.K. Deo (Shizuoka University) for valuable discussion.

Supplementary data

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.tet.2010.10.014.

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