JOURNAL OF NATURAL PRODUCTS

NOTE

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Viridaphin A_1 Glucoside, a Green Pigment Possessing Cytotoxic and Antibacterial Activity from the Aphid *Megoura crassicauda*

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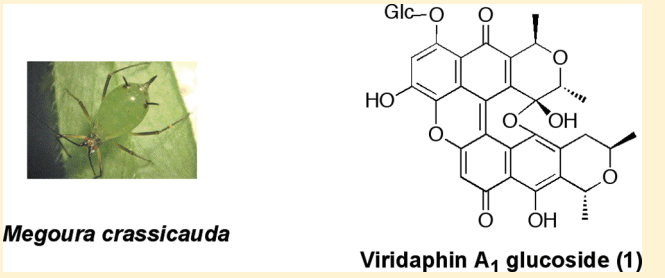
Supporting Information

ABSTRACT: A green pigment, viridaphin A_1 glucoside (**1**), was isolated from the green aphid *Megoura crassicauda*. One- and two-dimensional NMR spectrometric analyses of **1** and its aglycone established the structure as an octacyclic compound. Viridaphin A_1 glucoside exhibited cytotoxicity against HL-60 human tumor cells with an IC_{50} of 23 μM and antibacterial activity against *Bacillus subtilis* NBRC 3134 with a minimum inhibitory concentration of 10.0 μg/mL. These results suggested that aphid pigments may protect aphids from invasive species, including viruses and bacteria.



Todd, Cameron, and co-workers revealed that a number of aphids produced novel red and yellow pigments.[1] They also studied a group of green pigments, collectively called aphinins, isolated from the green aphid *Aphis sambuci*.[1c,d] However, they were unsuccessful in determining the structures of those green pigments, and their studies ended in the early 1960s.[2]

Our previous investigations showed that some aphid pigments possessed cytotoxicity.[3−5] This led to the hypothesis that aphid pigments may be chemopreventive agents that aid in resisting infection or attack. Furthermore, pigments are important for aphid body color, and it is presumed that body colorations affect predator−prey interactions.[6] These properties have generated interest in the unique structures and potentially important biological activities of aphid pigments.

In earlier studies, we examined the chemical structures of pigments in aphids.[3,4,7] Herein, we describe the isolation and structure of a green pigment, viridaphin A_1 glucoside (**1**), isolated from the green aphid *Megoura crassicauda* (Figure 1). We demonstrated that this pigment is cytotoxic against HL-60 (leukemia) human tumor cells and that it exhibits antibacterial activity.

Green aphids were collected in Utsunomiya, Tochigi Prefecture, Japan. Aphids were removed from *Vicia sativa* leaves with a soft paintbrush and collected in a plastic Erlenmeyer flask equipped with a plastic funnel. In the laboratory, the aphids were crushed with a pestle and washed repeatedly with a mixture of *n*-hexane and MeOH. The MeOH-soluble portion was separated and evaporated under reduced pressure. The green pigment, compound **1**, was isolated by repeated chromatographic purification on columns of reversed-phase silica gel and Sephadex LH-20.

Compound **1** was a green, unstable, amorphous solid. It exhibited a large specific rotation of −1231 (*c* 0.008, MeOH).[8]

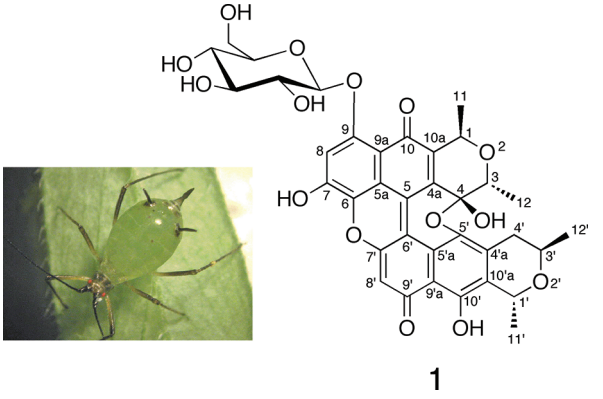


Figure 1. (Left) Photograph of *Megoura crassicauda*; (right) structure of viridaphin A_1 glucoside (**1**).

Its molecular formula was established as $C_{36}H_{34}O_{15}$ by FAB-HRMS (*m*/*z* 706.1867, $[M]^-$). The IR spectrum of **1** indicated the presence of hydroxy groups (3392 cm^{-1}, br) and a conjugated carbonyl moiety (1660 cm^{-1}). The chemical shift values of the ^1H and ^{13}C NMR spectra of **1** in methanol-d_4 were subject to variation;[9] however, we achieved reproducible results with the addition of a small amount of acetic acid-d_4. The ^1H and ^{13}C NMR data for **1** (7.2 mg) in methanol-d_4 (700 μL) were recorded with 5 μL of acetic acid-d_4 (125 mM; Table 1). The ^1H and ^{13}C NMR data in DMSO-d_6 are also shown in Table 1.

Received: November 7, 2010
Published: August 10, 2011

dx.doi.org/10.1021/np2001286 | *J. Nat. Prod.* 2011, 74, 1812–1816

Table 1. ^{13}C and ^{1}H NMR Data of 1 and 3

	1 in methanol-d_4[a]		**1** in DMSO-d_6[b]		**3** in DMSO-d_6[b]		**3** in acetone-d_6[b]	
position	δ_C, mult.	δ_H (J in Hz)	δ_C, mult.	δ_H (J in Hz)	δ_C, mult.	δ_H (J in Hz)	δ_C, mult.	δ_H (J in Hz)
1	68.2, CH	4.73, q (6.6)	66.7, CH	5.05, q (6.6)	65.8, CH	4.76, q (6.8)	67.2, CH	4.78, br q (6.8)
3	74.4, CH	4.15, q (6.4)	72.9, CH	4.20, q (6.6)	72.3, CH	4.20, q (6.6)	74.0, CH	4.35, q (6.6)
4	93.7, C		93.1, C		92.0, C		93.4, C	
4-OH				not observed		7.31, s		not observed
4a	139.5,[c,d] C		141.9,[c] C		138.3, C		139.3, C	
5	130.8,[c] C		134.0,[c,e] C		128.1,[c] C		129.0, C	
5a	120.4,[c] C		118.1,[c] C		117.1,[c] C		118.4	
6	135.6, C		139.3, C		132.6, C		133.1, C	
7	156.3, C		169.4,[c] C		153.8, C		153.9, C	
7-OH				not observed		not observed		not observed
8	109.4, CH	6.99, s	109.8, CH	6.44, s	105.4, CH	6.64, s	106.7, CH	6.68, s
9	159.0, C		163.1, C		162.3, C		163.6 C	
9-OH						13.55, s		13.50, s
9a	108.7, C		99.8, C		102.7, C		104.6, C	
10	181.5, C		174.4, C		183.2, C		184.9, C	
10a	137.8,[c,d] C		134.4,[c,e] C		136.1, C		137.7, C	
11	19.6, CH_3	1.33, d (6.6)	20.3, CH_3	1.29, d (6.6)	19.0, CH_3	1.38, d (6.8)	19.5, CH_3	1.45, d (6.8)
12	15.2, CH_3	1.51, d (6.4)	14.5, CH_3	1.38, d (6.6)	14.4, CH_3	1.43, d (6.6)	15.0, CH_3	1.55, d (6.6)
1′	69.7, CH	5.16, q (6.6)	67.2, CH	5.10, q (6.6)	67.3, CH	5.11, q (6.8)	68.7, CH	5.16, q (6.8)
3′	64.1, CH	4.23, m	62.1, CH	4.04, m	62.0, CH	4.10, m	63.1, CH	4.18, m
4′	33.8, CH_2	3.10, br d (15.1)	31.6, CH_2	2.89, dd (17.2, 3.3)	31.9, CH_2	2.95, dd (17.2, 2.9)	33.2, CH_2	3.07, dd (16.9, 2.7)
		2.50, dd (17.0, 10.9)		2.47−2,53[f]		2.48−2.52[f]		2.55, dd (16.9, 10.9)
4′a	135.1, C		132.0, C		133.9, C		135.4, C	
5′	145.0,[c] C		142.0,[c] C		143.1, C		144.3, C	
5′a	121.6,[c] C		121.1,[c] C		120.1,[c] C		121.3, C	
6′	125.2, C		119.8, C		124.0, C		125.5, C	
7′	161.7, C		161.2, C		160.6, C		161.6, C	
8′	106.4, CH	6.23, s	100.7, CH	6.33, s	104.2, CH	6.29, s	105.8, CH	6.32, s
9′	188.1, C		184.0, C		185.6, C		187.3, C	
9′a	112.4, C		111.7, C		110.5, C		111.9, C	
10′	154.9,[c] C		154.1, C		154.8, C		156.6, C	
10′−OH				15.97, s		15.44, s		15.40, s
10′a	133.7, C		129.9, C		132.4, C		134.4, C	
11′	19.3, CH_3	1.64, d (6.6)	18.6, CH_3	1.50, d (6.6)	18.6, CH_3	1.57, d (6.8)	19.0, CH_3	1.62, d (6.8)
12′	22.1, CH_3	1.41, d (5.9)	21.9, CH_3	1.31, d (5.9)	21.7, CH_3	1.34, d (6.0)	22.2, CH_3	1.37, d (6.0)
1″	104.9, CH	4.73, d (7.3)	105.4, CH	4.63, (7.3)				
2″−5″	71.0, 74.2,	3.65, t (8.4); 3.54, t (9.2);	69.8, 73.3,	3.26−3.50[g] (3H);				
	77.2, 78.6	3.44−3.46 (2H)	75.4, 77.7	3.19, dd (9.9, 9.2)				
6″	62.5, CH_2	3.99, d (11.8)	60.9, CH_2	3.77, br d (11.7)				
		3.84, dd (11.8, 4.8)		3.53, m				

[a] ^{13}C (125 MHz) and ^{1}H NMR (500 MHz) data for **1** (7.2 mg) in methanol-d_4 (700 μL) were recorded in the presence of 5 μL of acetic acid-d_4. [b] ^{13}C (150 MHz) and ^{1}H NMR (600 MHz) data. [c] Assignments were based on data from compound **3** in acetone-d_6. [d,e] Assignments are interchangeable. [f] This signal overlapped with a solvent. [g] These signals overlapped with H_2O in DMSO-d_6.

The presence of a β-glucopyranosyl linkage was suggested by the ^{1}H NMR (methanol-d_4) signals at δ 4.73 (J = 7.3 Hz); by the ^{13}C NMR (methanol-d_4) signals at δ 62.5, 71.0, 74.2, 77.2, 78.6, and 104.9; and by the detection of D-glucose in an aqueous solution after acidic hydrolysis of **1**. The HMBC correlation between the anomeric proton (δ_H 4.73) of the glucose and C-9 (δ_C 159.0) of the aglycone moiety revealed the position of the sugar linkage (segment A in Figure 2).

The HMQC spectra (Table 1) revealed the presence of four methyl groups on carbons bearing oxygen [δ_H/δ_C 1.51/15.2, 1.64/19.3, 1.33/19.6, and 1.41/22.1], four oxygen-bearing methine carbons [δ_H/δ_C 4.23/64.1, 4.73/68.2, 5.16/69.7, and 4.15/74.4], two aromatic protons [δ_H/δ_C 6.23/106.4 and 6.99/109.4], and a methylene [δ_H/δ_C 2.50 and 3.10/33.8] in the aglycone moiety. Moreover, the ^{13}C NMR (methanol-d_4) spectra indicated the presence of one oxygen-bearing quaternary carbon (δ_C 93.7), 16 quaternary carbons (δ_C 108.7, 112.4, 120.4,

dx.doi.org/10.1021/np2001286 | J. Nat. Prod. 2011, 74, 1812–1816

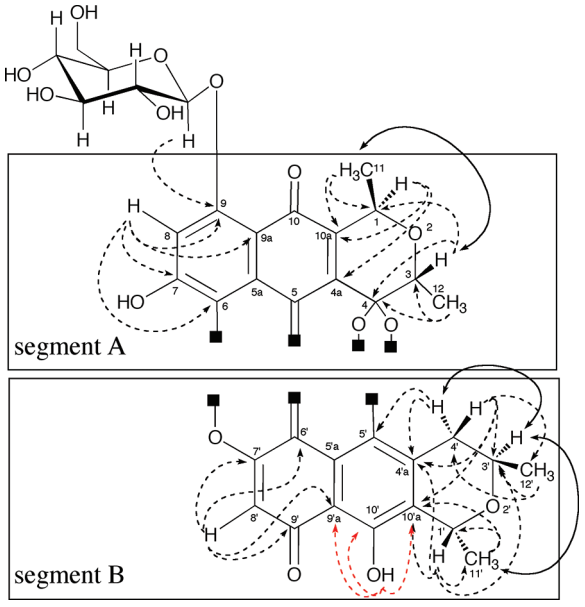
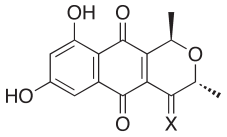


Figure 2. HMBC (black dots: in methanol-d_4; red dots: in DMSO-d_6) and NOE correlations (black solid lines: in methanol-d_4) in segments A and B of viridaphin A_1 glucoside (**1**).



2a: X=H, H
b: X=H, OH
c: X=O

Figure 3. Structures of quinone A (**2**) derivatives.

121.6, 125.2, 130.8, 133.7, 135.1, 135.6, 137.8, 139.5, 145.0, 154.9, 156.3, 159.0, and 161.7), and two carbonyl carbons (δ_C 181.5 and 188.1). A signal at δ_C 93.7 could be assigned to a hemiacetal or acetal carbon.[10] Analyses of 1D and 2D NMR spectra that included $^1H-^1H$ COSY, NOESY, HMQC, and HMBC measurements suggested that the structure of **1** comprises a dimeric compound involving quinone A (**2**) derivatives, particularly **2a**[11] and **2c**[12] (Figure 3).

In segment A (Figure 2), HMBC correlations of H-3 to C-1 and C-4, H-1 to C-4a and C-10a, H-11 to C-1 and C-10a, and H-12 to C-3 and C-4 suggested the presence of a dihydropyran ring with two methyl groups. HMBC correlations of H-8 to C-6, C-7,[13] C-9, and C-9a also suggested the presence of an aromatic ring with oxygen functionalities. The hemiacetal (acetal) carbon at δ_C 93.7 was C-4, which must connect to segment B (Figure 2) via an ether linkage. In segment B, HMBC correlations suggested another dihydropyran ring system with two methyl groups and another aromatic ring with oxygen functionalities. HMBC cross-peaks of H-4′ to C-5′ observed in methanol-d_4,[14] and OH-10′ to C-9′a, C-10′, and C-10′a observed in DMSO-d_6, elucidated a fused ring system between the naphthoquinone moiety and the dihydropyran ring. Although the NMR spectra were prone to variation in methanol-d_4 without the addition of acetic acid-d_4, each individual NOESY spectrum made up for missing NOE correlations and suggested the relative configuration of each dihydropyran ring.

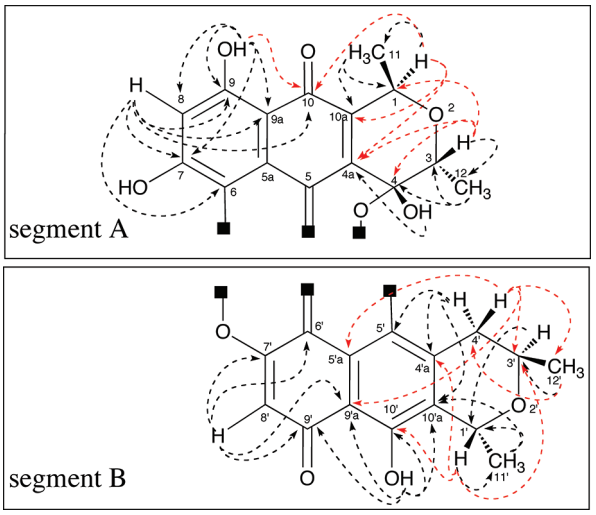


Figure 4. HMBC correlations (black dots: in DMSO-d_6; red dots: in acetone-d_6) in segments A and B of viridaphin A_1 (**3**).

Because there were only a few protons on the aromatic rings, it was difficult to elucidate which part of the carbon skeleton of **1** linked the two segments, A and B (Figure 2). To obtain further information, compound **1** was hydrolyzed in an aqueous solution of 10-camphorsulfonic acid to afford viridaphin A_1 (**3**), an aglycone of **1**, which was a green, amorphous solid. Its molecular formula was established as $C_{30}H_{24}O_{10}$ by FAB-HRMS (m/z 544.1361, $[M]^-$). The IR spectrum of **3** indicated the presence of hydroxy groups (3451 cm^{-1}, br). The ^1H and ^{13}C NMR data of **3** are shown in Table 1. For these samples, reproducible results were obtained without the addition of acetic acid-d_4.

We gained more information on **3**, particularly on the OH-9 and OH-4 signals in DMSO-d_6, with detailed 1D and 2D NMR spectra analyses involving $^1H-^1H$ COSY, ROESY, HMQC, and HMBC measurements. In segment A, we observed HMBC correlations of H-1 and OH-9 to C-10 in acetone-d_6 and a correlation of H-8 to C-10 in DMSO-d_6; this sufficiently elucidated the fused ring system of segment A (Figure 4). A correlation of OH-4 to C-4a supported this partial structure. In segment B, HMBC measurements of **3** provided structural information that reinforced the proposed partial structure for glucoside **1** (*vide supra*).

ROESY experiments with **3** in DMSO-d_6 (Figure 5) gave more correlations than in acetone-d_6. The correlations between H-1/H-12, H-3/H-11, and H-3′/H-11′ revealed that the two methyl groups were in the *anti* configuration, and the OH-4 and H-3 were in the *syn* configuration. Furthermore, the correlations between H-4′α/H-12, H-4′β /H-3, and H-4′β /H-12 suggested that the two dihydropyran rings in segments A and B were in close proximity.

HMBC correlations of H-1 and H-8′ to C-4a, C-5, and C-6′ observed in acetone-d_6 were sufficient to suggest the linkage between segments A and B (Figure 5). Finally, only the C−C bond between C-5 and C-6′ led to a feasible structure; on this basis, the ether linkages between C-6 and C-7′ and between C-4 and C-5′ were fixed in a logical manner.

Thus, the structure of viridaphin A_1 glucoside was proposed, as shown for **1** (Figure 1), to contain two units of quinone A derivatives that were fused through six- and seven-membered cyclic ethers, which led to the octacyclic structure. However, we

dx.doi.org/10.1021/np2001286 |*J. Nat. Prod.* 2011, 74, 1812–1816

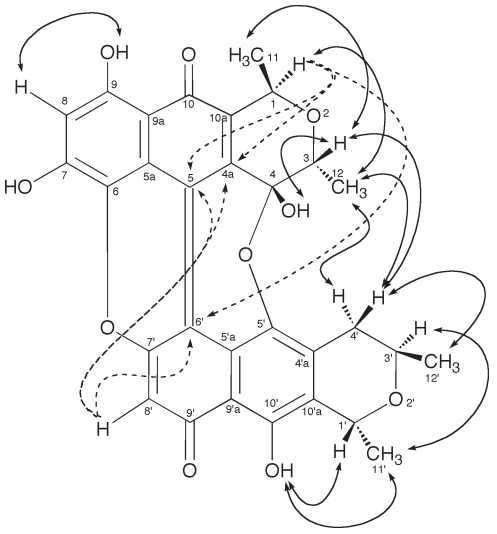


Figure 5. Linkage between segments A and B of viridaphin A_1 (**3**), revealed by HMBC correlations (dotted lines: in acetone-d_6) and ROESY correlations (solid lines: in DMSO-d_6).

were unsuccessful in the X-ray analysis of **3** and its derivatives including acetate, bromobenzoate, methyl ether, MTPA ester, and camphorsulfonyl ester.

Pigment **1** exhibited cytotoxic activity against human promyelocytic leukemia HL-60 cells[15] with an IC_{50} value of 23 μM. This value was comparable to the IC_{50} of antimycin A (45 μM)[16] observed in the same analysis.[17] Compound **1** exhibited moderate antimicrobial activity against *Bacillus subtilis* NBRC3134 with an MIC value of 10.0 μg/mL. This biological activity suggested that **1** may act as a chemopreventive agent against bacteria.

We could not confirm whether **1** was the same compound as the aphinin described earlier by Todd and Cameron.[1c,d,18] The deep green color of aphids[19] has now attracted a lot of attention from natural scientists; aphid coloration has generated interest from the perspectives of host−endosymbiont and evolutionary−coevolutionary relationships.[20,21] On the basis of their cytotoxic and antibacterial activities, we hypothesize that aphid pigments may protect aphids from invasive species, such as viruses, bacteria, and fungi.

EXPERIMENTAL SECTION

General Experimental Procedures. Melting points were determined on a Yanaco MP-3 apparatus and are uncorrected. Optical rotations were obtained on JASCO DIP-1000 and P-1030 polarimeters. UV−visible spectra were measured on Shimadzu UV-1650pc and JASCO V-650 spectrophotometers. IR spectra were measured on a JASCO FT/IR-410 spectrophotometer. ^{1}H NMR spectra were recorded on Varian Unity-600 (600 MHz) and Unity-500 (500 MHz) NMR spectrometers with TMS as an internal standard in solvent. ^{13}C NMR spectra were recorded with Varian Unity-600 (150 MHz) and Unity-500 (125 MHz) NMR spectrometers; chemical shifts were referenced to the residual solvent signal (DMSO-d_6: δ_C 39.5; methanol-d_4: δ_C 49.0; acetone-d_6: 29.8; $CDCl_3$: δ_C 77.0). Signal multiplicities were established with distortion enhancement by polarization transfer (DEPT) experiments. Mass spectra including HRMS were recorded on a JEOL JMS-700 spectrophotometer. For column chromatography, stationary phases of silica gel (Kanto Chemical Co., Inc., 60N 63-210 μm) and C_{18} reversed-phase silica gel (Nacalai Tesque Inc., Cosmosil 75C_{18} OPN) were used. For the TLC analysis, Merck precoated silica gel plates (60F and RP-18 WF_{254S}) were used. Acetic anhydride and pyridine were purchased from Nacalai Tesque Inc. Pyridine was used after distillation from CaH_2.

Materials. The aphids, *Megoura crassicauda*, were collected as they fed on *Vicia sativa* in Utsunomiya, Tochigi Prefecture, Japan, in April 2010; the species was authenticated by one of the authors (S.T.). A voucher specimen of this aphid was prepared by S.T. and has been deposited in the Faculty of Agriculture of Utsunomiya University under the code no. 86005.

Extraction and Isolation. See Supporting Information.

Viridaphin A_1 glucoside (1) properties: green, amorphous powder; $[\alpha]^{21}_D$ −1231 (*c* 0.008, MeOH); UV [MeOH containing AcOH (125 mM)] λ_{max} (log ε) 222 (4.36), 246 (4.42), 293 (4.04), 331 (3.92), 418 (3.94) 637 (4.03) nm; UV (MeOH) λ_{max} (log ε) 254 (4.09), 303 (3.83), 360 (3.80), 671 (3.64) 730 (3.61) nm; IR (neat) ν_{max} 3392 (−OH), 1660, 1592, 1494, 1426, 1363, 1239 cm^{-1}; ^{1}H NMR (500 MHz, methanol-d_4) and ^{13}C NMR (125 MHz, methanol-d_4) data are provided in Table 1; FAB-MS *m*/*z* 706 ([M]$^-$); FAB-HRMS *m*/*z* 706.1867 (calcd for $C_{36}H_{34}O_{15}$, 706.1898).

Viridaphin A_1 (3). 10-Camphorsulfonic acid (12 mg) was added to a suspension of **1** (14 mg) in MeOH (5 mL). The resulting mixture was stirred at ambient temperature for 6.5 h, and then, saturated $NaHCO_3$-(aq) was added. After stirring for 15 min, the resulting mixture was extracted with EtOAc. The organic layer was washed with NH_4Cl(aq) and dried over Na_2SO_4. The solvent was evaporated to give 10.4 mg of aglycone **3**, with the following properties: green, amorphous powder; $[\alpha]^{22}_D$ −1785 (*c* 0.014, MeOH); UV (MeOH) λ_{max} (log ε) 256 (4.26), 302 (3.90), 369 (3.96), 681 (3.75) 743 (3.73) nm; IR (ATR) ν_{max} 3451 (−OH), 1612, 1423, 1385, 1300, 1266, 1233, 1193, 1049 cm^{-1}; ^{1}H NMR (600 MHz, DMSO-d_6) and ^{13}C NMR (150 MHz, DMSO-d_6) data are provided in Table 1; FAB-MS *m*/*z* 544 ([M]$^-$); FAB-HRMS *m*/*z* 544.1361 (calcd for $C_{30}H_{24}O_{10}$, 544.1369).

Hydrolysis of Viridaphin A_1 Glucoside (1) and Determination of the Resulting Sugar Structure. A mixture of compound **1** (7.3 mg) in 0.5 M H_2SO_4 (1 mL) and dioxane (1 mL) was heated at 100 °C for 1.5 h. After cooling, the reaction mixture was neutralized with the addition of Amberlite IRA96SB. The resin was filtered off and washed with a small amount of water. *n*-Butanol (1 mL) was added to the filtrate, and the mixture was evaporated. The residue was dissolved in H_2O (3 mL), and the aqueous layer was washed with EtOAc and separated. After the addition of a small amount of *n*-butanol, the aqueous layer was evaporated *in vacuo* to give the crude sugar. A pyridine (400 μL) solution of the resulting sugar was treated with 100 μL (large excess) of Ac_2O at ambient temperature for 22 h. After the addition of 2 M HCl (2 mL), the mixture was extracted with CH_2Cl_2 (3 mL × 3) and the combined organic layers were dried over Na_2SO_4. After evaporation of the solvent, the residue was purified by silica gel column chromatography (2 g, *n*-hexane/EtOAc, 3:1) to give 2.1 mg of the pentaacetate of the sugar as a colorless powder; this was identified as D-glucose by comparison with the ^{1}H NMR data of standard pentaacetate and the $[\alpha]_D$ value {$[\alpha]^{22}_D$ +34.8 (*c* 0.18, $CHCl_3$) vs pentaacetate of standard L-glucose, $[\alpha]^{22}_D$ −43.8 (*c* 2.1, $CHCl_3$)}.

MTT Assay for Cytotoxic Activity and Antimicrobial Assay. See Supporting Information.

ASSOCIATED CONTENT

Supporting Information. ^{1}H NMR, ^{13}C NMR, and UV−visible spectra of viridaphin A_1 glucoside (**1**) and viridaphin A_1 (**3**) and the IR spectrum of **1** are available in supporting tables. The procedure for extraction and isolation of **1**, the MTT assay for cytotoxic activity of **1**, and the antimicrobial assay of **1** are described. This material is available free of charge via the Internet at http://pubs.acs.org.

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ACKNOWLEDGMENT

This work was supported partially by a Grant-in-Aid for Scientific Research (C) from MEXT (the Ministry of Education, Culture, Sports, Science, and Technology of Japan). We also thank MEXT-Senryaku, 2008−2012.

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(8) The optical rotation was not measured with a more concentrated solution of **1**, because deeply colored solutions block the transmission of light.

(9) Although we had no evidence to explain the variation in chemical shift values, we suspected that some chelation had arisen from contamination by external metal cations, for example, from the SiO_2 column chromatography. These contaminants caused variable chemical shift values for glucoside **1**, but had no effect on aglycone **3**. The addition of acid eliminated the variability; we speculated that the effect of contamination was removed by fully protonating a susceptible moiety, and this stabilized the spectra.

(10) Although the value of the ^{13}C NMR chemical shift for C-4 was lower than expected for ordinary acetals and hemiacetals, the shift appeared to be more acceptable in comparison with that of preussomerin F. See: Weber, H. A.; Gloer, J. B. *J. Org. Chem.* **1991**, *56*, 4355–4360.

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