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HCV-NS3/4A Protease Inhibitory Iridoid Glucosides and Dimeric Foliamenthoic Acid Derivatives from *Anarrhinum orientale*

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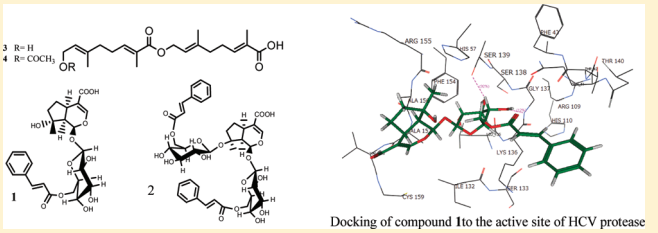
Supporting Information

ABSTRACT: Four new compounds were isolated from the methanol extract of the aerial parts of *Anarrhinum orientale*: 6′-*O*-cinnamoylmussaenosidic acid (**1**), 6′-*O*-cinnamoyl-8-*O*-(6‴-*O*-cinnamoylglucopyranosyl)mussaenosidic acid (**2**), (2*E*,6*E*)-8-{[(2*E*,6*E*)-8-hydroxy-2,6-dimethylocta-2,6-dienoyl]oxy}-2,6-dimethylocta-2,6-dienoic acid (**3**), and (2*E*,6*E*)-8-{[(2*E*,6*E*)-8-acetoxy-2,6-dimethylocta-2,6-dienoyl]oxy}-2,6-dimethylocta-2,6-dienoic acid (**4**). The known 8-*O*-cinnamoylmussaenosidic acid (**5**) was also identified. All five compounds were tested for inhibition of the hepatitis C virus (HCV) protease. Compounds **1** and **5** exhibited moderate activity, while **2** and **3** showed weak effects. No inhibitory activity on the human serine protease was observed for any of these compounds, which may infer the selectivity toward the viral protease. A computational docking study of the isolated compounds against HCV protease was used to formulate a hypothetical mechanism for the inhibitory activity of the active compounds on the enzymes tested.



Docking of compound **1** to the active site of HCV protease

Hepatitis C virus (HCV) is a leading cause of life-threatening liver illnesses such as cirrhosis and cancer.[1−3] However, no vaccine is available for the treatment of this virus. The current treatment for HCV relies on administration of interferon alone or in combination with ribavirin.[3] Interferon-based therapy shows a sustained response in 40−50% of HCV-infected patients and is often accompanied by side effects such as depression, psychoses, and extreme fatigue.[4] Few treatment options exist for patients who either do not respond to interferon therapy or respond and later relapse. Although two recent clinical trials have shown improved rates of sustained virologic response to as high as 65% when telaprevirin (a specific inhibitor of the HCV protease) was used in combination with peginterferon and ribavirin, it was shown to increase the rate of anemia, nausea, diarrhea, pruritis, and rash.[5]

HCV protease (HCV PR) inhibition is considered to be one of the important targets for designing drugs to treat HCV.[6] HCV NS3/4A is a serine protease, which is a heterodimeric enzyme responsible for the proteolytic processing of four out of five junctions between nonstructural protein regions along the HCV polyprotein.[7] It also plays a role in silencing the host's antiviral immune response by interfering with interferon production as a natural defense against infections.[8]

Iridoids make up an important class of natural products in plants. Some naturally occurring iridoids were reported to be effective against HCV infections by acting as entry inhibitors.[9] The aerial parts of *Anarrhinum orientale* (Scrophulariaceae) have been investigated by Dawidar et al. for their iridoid content, and two iridoid glucoside esters, 6-*O*-nerol-8-oylantirrinoside and 6′-*O*-cinnamoylantirrinoside,[10] were identified. No other reports on iridoid glycosides from the genus *Anarrhinum* were traced, in spite of such compounds being reported in several members of the family Scrophulariaceae.[11−13] Therefore, we investigated the iridoid content of *A. orientale* in search of HCV protease inhibitors.

RESULTS AND DISCUSSION

The methanol extract of the aerial parts of *A. orientale* was resuspended in H_2O and partitioned with *n*-hexane, $CHCl_3$, EtOAc, and *n*-BuOH. Repeated chromatography of the EtOAc fraction on silica gel and HPLC (RP, C-18) yielded four new compounds (**1**−**4**) as well as a known iridoid glucoside (**5**). Compound **5** was identified as 8-*O*-cinnamoylmussaenosidic acid by comparison of observed and published spectroscopic data.[14]

Compound **1** was obtained as a yellowish oil, $[\alpha]_D^{23}$ −19.7 (*c* 0.15, MeOH). The HRFABMS indicated the molecular formula $C_{25}H_{29}O_{11}$, which is consistent with the ^1H and ^{13}C NMR data. The spectroscopic data of **1** revealed the presence of a typical conjugated carboxylic enol−ether system of iridoids.[15−17] 1D NMR spectra showed the presence of a group of characteristic signals for an iridoid skeleton at δ_H 7.42 (s, H-3), 5.13 (d, *J* = 6.0, H-1),

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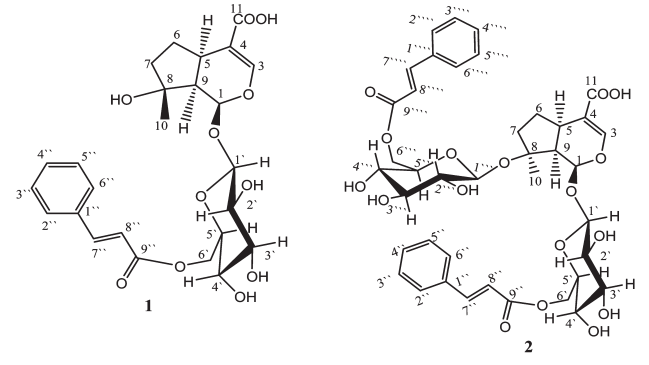

1

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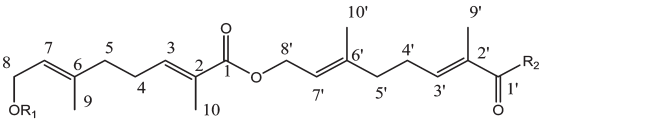
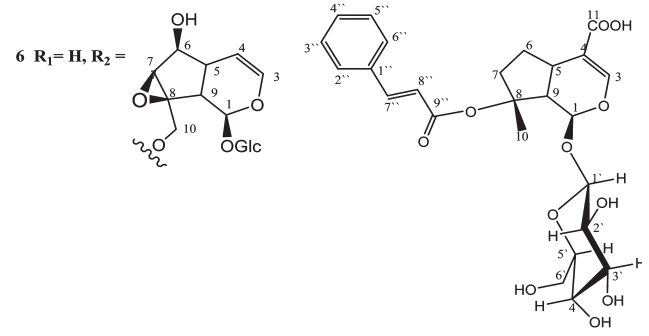


3 R_1=H R_2 = OH
4 R_1= $COCH_3$, R_2 = OH



6 R_1= H, R_2 =

5

3.06 (m, H-5), 2.14 (m, H-6b), 2.02 (dd, J = 6.0, 8.4, H-9), 1.57 (t, J = 8, H-7), 1.27 (m, H-6a), and 1.20 (s, H-10). In addition, an anomeric proton signal at δ_H 4.63(d, J = 7.6, 1H) together with proton signals at δ_H 3.14 (t, J = 7.6, 1H), 3.29 (m, 1H), 3.30 (m, 1H), 3.46 (m, 1H), 4.45 (dd, J = 12.0, 2.4, 1H), and 4.30 (dd, J = 12.0, 6.0, 1H) indicated the presence of one sugar moiety. The ^{1}H NMR spectrum also showed resonances at δ_H 6.44 (d, J = 16.0, 1H) and 7.59 (d, J = 16.0, 1H), indicating the presence of an (*E*)-C═C bond conjugated with an aromatic ring. The ^{13}C NMR spectrum (Table 1) in combination with the HMQC spectrum displayed resonances for an (*E*)-C═C bond at δ_C 146.6 and 118.6, for an aromatic ring at δ_C 135.6, 129.3, and 130.0, for an acyl group at δ_C 168.2, for a sugar moiety at δ_C 99.8, 77.9, 75.6, 74.8, 71.7, and 64.4, and for a pair of olefinic C-atoms at δ_C 152.0 and 113.3. In addition, signals of three methine C-atoms at δ_C 95.6, 33.1, and 52.1, two methylene C-atoms at δ_C 30.9 and 40.0, and one oxygenated quaternary C-atom at δ_C 81.0 were observed. The ^{1}H and ^{13}C NMR data suggested that **1** should be an iridoid glycoside with a structure similar to that of the known iridoid glucoside mussaenosidic acid[18] esterified with a cinnamoyl moiety. In the ^{1}H NMR spectrum of **1**, downfield shifts of the sugar protons H-6′a and H-6′b (at δ_H 4.45 and 4.30, respectively) were observed, which indicated the esterification at C-6′ of the β-glucopyranosyl moiety. The HMBC confirmed the position of the cinnamoyl residue by showing a clear long-range correlation peak between its carbonyl carbon (δ_C 168.2) and H-6′ at δ_H 4.45 and 4.30 of the sugar unit.[19]

The ^{13}C NMR chemical shifts, TLC, and GC comparison of the sugar resulting from the acid hydrolysis of **1** with those of authentic samples revealed that the sugar moiety was D-glucose.[19,20]

Table 1. ^{1}H and ^{13}C NMR Data of Compounds 1 and 2 (methanol-d_4)

	1		2	
position	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C
1	5.13, d (6.0)	95.6	5.22, d (5.6)	95.7
3	7.42, s	113.3	7.42, s	113.1
4		152.0		152.3
5	3.06, m	33.1	3.16, m	33.0
6	1.27, m (a), 2.14, m (b)	30.9	1.33, m (a), 2.22, m (b)	30.9
7	1.57, t (8.0)	40.0	1.63, t (8.0)	40.1
8		81.0		80.9
9	2.02, dd (6.0, 8.4)	52.1	2.11, dd (5.6, 8.4)	52.1
10	1.20, s	25.0	1.28, s	24.9
11		170.0		170.0
Glycosyl				
1′	4.63, d (7.6)	99.8	4.73, d (8.0)	99.7
2′	3.14, t (7.6)	74.8	3.24, t (8.0)	74.7
3′	3.29, m	77.9	3.45, m	77.8
4′	3.30, m	71.7	3.37, m	71.6
5′	3.46, m	75.6	3.47, m	75.6
6′	4.45, dd (12.0, 2.4) (a)	64.4	4.38, dd (6.4, 12.0) (a)	64.0
	4.30, dd (12.0, 6.0) (b)		4.53, dd (2.8, 12.4) (b)	
1′′′			5.55,d (8.0)	95.8
2′′′			3.24, t (7.8)	73.9
3′′′			3.41, m	77.8
4′′′			3.39, m	71.5
5′′′			3.47, m	76.3
6′′′			4.19, dd (6.4, 12.4) (a)	64.4
			4.45, dd,(1.6, 11.6) (b)	
Cinnamoyl[a]				
1′′		135.6		135.5
2′′, 6′′	7.49, m	129.3	7.60, m	129.2
3′′, 5′′	7.30, m	130.0	7.38, m	130.1
4′′	7.30, m	131.6	7.38, m	131.5
7′′	7.59, d (16.0)	146.6	7.77, d (16.0)	146.6
8′′	6.44, d (16.0)	118.6	6.51, d (16.0)	118.5
9′′		168.2		168.2
1′′′′				135.5
2′′′′, 6′′′′			7.54, m	129.4
3′′′′, 5′′′′			7.38, m	130.1
4′′′′			7.38, m	131.5
7′′′′			7.66, d (16.0)	147.7
8′′′′			6.54, d (16.0)	118.1
9′′′′				166.9

[a] The values of the cinnamoyl moieties are overlapped.

The relative configuration at C-1, C-9, and C-5 was assigned on the basis of the NOE experiments and biogenetic considerations.[21] The NOE experiment indicates the presence of H-1, H-9, and H-5 in an α-orientation, since H-1 is known to be α-oriented in naturally occurring iridoid glucosides.[21] No correlation was observed to the C-10 methyl protons, suggesting its β-orientation. Moreover, the J value of 6 Hz between H-1 (δ_H 5.13) and H-9 (δ_H 2.02) confirmed the α-orientation of H-9, as the calculation of the dihedral angle between H-1 and H-9 was found to be near 30°. Additionally, the large coupling constant of H-9 (J = 8.4 Hz) with H-5 suggested the α-orientation of H-5 and a ca. 0° dihedral angle between H-9 and H-5, thus demonstrating that the ring fusion was *cis*. From the aforementioned results, compound **1** was identified as 6′-*O*-cinnamoylmussaenosidic acid.

Compound **2** was obtained as a yellowish oil with an $[\alpha]_D^{23}$ −23.8 (*c* 0.2, MeOH). The spectroscopic data of compound **2** were similar to those of compound **1**, but possessed an additional cinnamoyl-substituted β-glucopyranosyl moiety. This additional group was assigned to C-8 due to the HMBC correlations

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between the anomeric proton H-1‴ (δ_H 5.55) and C-8 (δ_C 80.9) (see Supporting Information). The downfield shift of C-6‴ (δ_C 64.4) and H-6‴a and H-6‴b (δ_H 4.19 and 4.45, respectively) and the upfield shift of C-5‴ (δ_C 73.9) are all consistent with the esterification of C-6 of the sugar moiety.[22]

Similarly to compound **1**, the sugar moiety of **2** was identified as D-glucose and its relative configuration was determined using the NOE experiment, which indicated the presence of H-1, H-9, H-5, and the methyl group in the same α-orientation. Compound **2** was determined to be 6′-*O*-cinnamoyl-8-*O*-(6‴-*O*-cinnamoylglucopyranosyl)mussaenosidic acid.

Compound **3** was obtained as a yellowish oil and had the molecular formula $C_{20}H_{29}O_5$, as deduced from the HRFABMS and ^{13}C NMR data. The ^{13}C NMR spectrum exhibited signals for two conjugated carbonyls, which showed an upfield shifted resonance at δ_C 171.6, four quaternary olefinic carbons at δ_C 138.4, 138.6, 142.8, and 143.1, four vinylic methyl groups at δ_C 12.5 (×2), 16.2, and 23.5, and two CH_2OR groups at δ_C 59.1 and 59.4. These data suggested the presence of two monoterpene units. The ^1H NMR and ^{13}C NMR data of **3** were similar to those of the 10-bisfoliamenthoyl moiety of 10-bisfoliamenthoylcatalpol (**6**), previously isolated from *Penstemon newberryi*.[23] A comparison of the ^{13}C NMR data of **3** with those of the 10-bisfoliamenthoyl moiety of **6** showed a difference in chemical shift at C-1′ in **3** (δ_C 171.6), which is upfield shifted compared to **6** (δ_C 169.4), indicating the esterification with an iridoid moiety in **6**. Analysis of the HMBC data revealed a correlation between H-8′ (δ_H 4.04) and C-1 (δ_C 171.6), indicating that the latter is attached to C-8′ via an oxygen atom. A signal at δ_H 1.80 (H-10, H-9′) showed a correlation in the HMBC spectrum with four olefinic carbons at δ_C 129.1 (C-2), 129.3 (C-2′), 142.8 (C-3), and 143.1 (C-3′). The signals at δ_H 1.68 and 1.75 (H-9, H-10′) were correlated with signals at δ_C 138.4 (C-6), 125.5 (C-7), 138.6 (C-6′), and 126.5 (C-7′). Additionally, the use of prediction software such as ACD/Laboratories (version: 6.0, 2006) and MestReNova (version: 6.0.2-5475, 2009) assisted in the prediction of the structure and in assigning the closely similar carbon resonances. From the aforementioned data, compound **3** was identified as (2*E*,6*E*)-8-{[(2*E*,6*E*)-8-hydroxy-2,6-dimethylocta-2,6-dienoyl]oxy}-2,6-dimethylocta-2,6-dienoic acid.

Compound **4** was obtained as a yellowish oil with the molecular formula $C_{22}H_{31}O_6$, as deduced from the HRFABMS and ^{13}C NMR data. The spectroscopic data of compound **4** showed similarity with those of **3**, except the presence of an additional signal at δ_H 2.00 (3H, s), which exhibited a correlation in the HMBC spectrum with δ_C 172.9, which is in turn correlated with a signal at δ_H 4.53 (H-8), indicating that compound **4** is an *O*-acetyl derivative of **3**. Thus compound **4** was determined to be (2*E*,6*E*)-8-{[(2*E*,6*E*)-8-acetoxy-2,6-dimethylocta-2,6- dienoyl]-oxy}-2,6-dimethylocta-2,6-dienoic acid.

Compounds **3** and **4** are dimers of foliamenthoic acid, which is formed by oxidation of geraniol at C-8 to yield rare monoterpenoids oxidized at both ends. Foliamenthoic acid, a monomeric unit of compound **3**, was previously reported as a side chain of a limited number of iridoid glycosides but not as free acid.[10,24−26] The presence of these dimeric terpenoid acids could be utilized to establish taxonomic relations between species, genera, or families such as Scrophulariaceae and the closely related family Plantaginaceae.

Compounds **1**−**5** (with purity about 90−92%) were tested for their inhibitory activity against HCV protease using hepatitis virus C NS3 protease inhibitor 2 as a positive control (Table 3). Compounds **1** and **5** showed moderate activity against HCV NS3/4A with IC_{50} values of 100 and 109 μM, respectively (Table 3), while compounds **2** and **3** were less potent, with IC_{50} values of 125 and 126 μM, respectively, and **4** was inactive. This indicates that acetylation of **4** results in complete loss of activity, while glycosylation of the hydroxy group at C-8 of **2** reduces the activity. The selectivity of the active compounds against HCV PR was tested via their abilities to inhibit the human protease trypsin. Trypsin is a serine protease similar to HCV PR.[27,28] None of the tested compounds exhibited an inhibitory activity against human trypsin at concentrations up to 200 μM. These findings suggested that the compounds selectively inhibited HCV PR with moderate inhibitory activity and may not interfere with human physiological processes requiring trypsin activity.

Docking Study. It is well established that interactions between HCV protease active site and conventional electrophiles such as aldehydes, ketones, α-ketoacids, and α-ketoamides followed by trapping of the resulting covalently bonded intermediate by the active site triad (Ser139, His57, and Asp81) would provide effective inhibition.[28] Additionally, it has been reported that the fitting of ligand functional groups to the shallow, solvent-exposed active site of the protease through other forces such as van der Waals, aromatic, hydrogen-bonding, and hydrophobic interactions plays an important role in the inhibition of the HCV PR.[29] The cocrystal structure ligand used in this study (2A4Q) inhibited HCV PR by forming a reversible covalent bond between the enzyme active site Ser139 hydroxy group and the ketone carbonyl of the inhibitor (code 2A4Q). This compound also forms multiple hydrogen bonds with the protease through its amide chain.[30] Due to the lack of previous reports about the binding of other compounds to HCV protease, we performed a computational docking study of compounds **1**−**4** to the active site of the enzyme.

Compound **1** forms a hydrogen bond between the hydroxy groups of the sugar moiety and Ser139 as well as Gly137 (Figure 1a) and an aromatic interaction between the cinnamoyl aromatic moiety and His110. Compound **2** binds in a similar way to **1**, but the bulky additional substituent of **2** failed to fit well into the pocket and protruded out of it (Figure 1b), which could be one reason for its lower activity. Compound **5** showed a binding orientation in the active site similar to that of compound **1**.

Docking of compound **3** to the HCV protease active site showed hydrogen bonding between the Ser139 and the ester carbonyl moiety of compound **3**. In addition, a hydrogen bond is formed between Lys136 and Thr42 and its acid and terminal OH moieties. On the other hand, the closely related acetyl derivative **4** (Figure 1d) failed to form such a bond with Ser139, which may be the reason for the loss of its activity.

Although most of the active compounds in this study showed hydrogen bonding with Ser139, the distance between the functional group and the hydroxy group of Ser139 was not sufficient to permit the formation of a covalent bond (mostly 2.8−3.2 Å). This may be why these compounds are not as potent as the positive control or the inhibitor used in the docking study,[30] as those compounds are covalently linked to the active site.

EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotations were measured using a DIP-360 automatic polarimeter (Jasco Co., Tokyo, Japan). HRFABMS was measured using a JEOL JMX-AX 505HAD mass

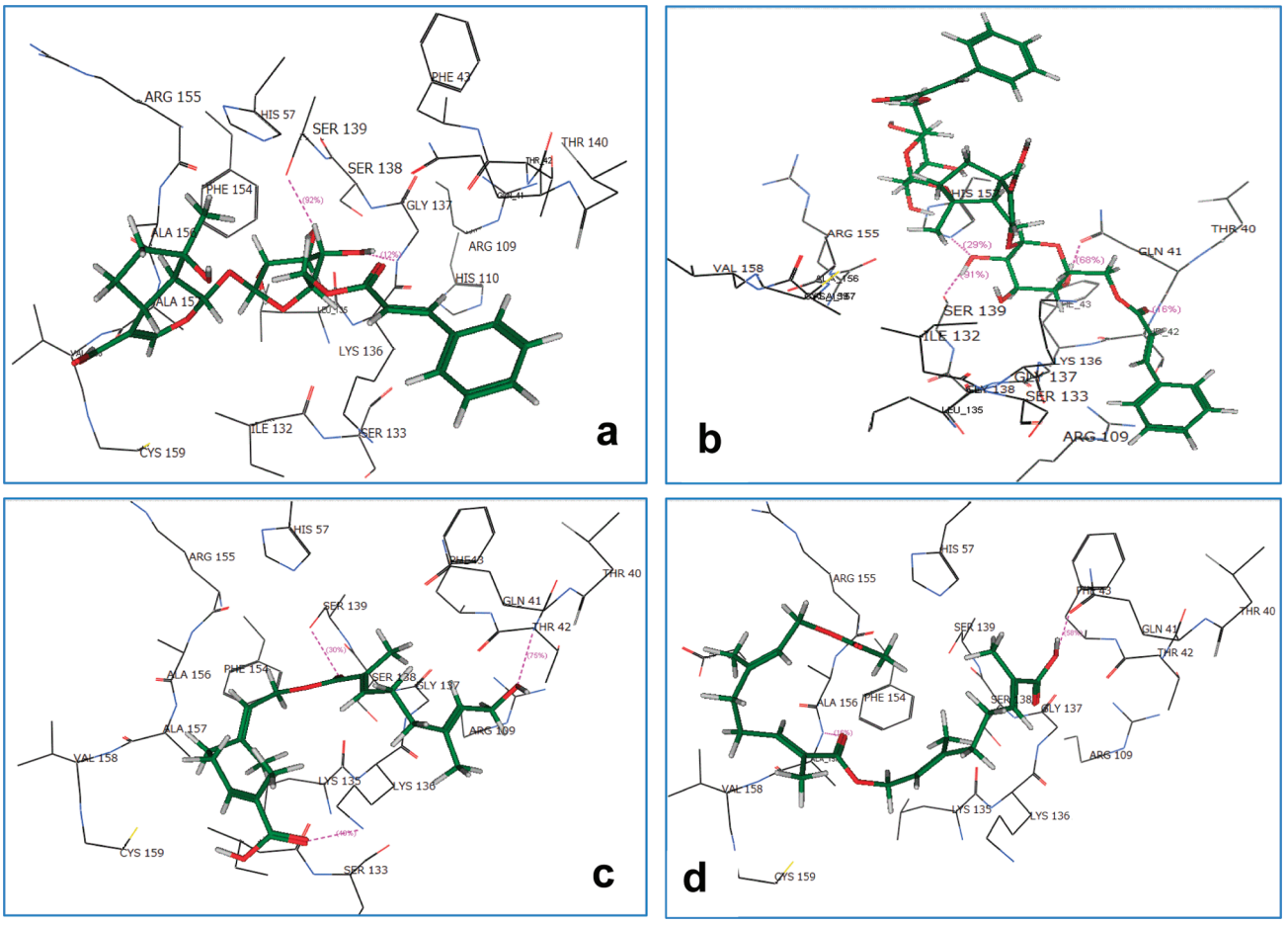

Figure 1. Docking of the isolated compounds to the HCV NS3/4A protease active site. H-bonds are represented by dashed lines, H-bonding strength is represented as a percentage score, and the compounds are colored green: (a) compound **1**, (b) compound **2**, (c) compound **3**, (d) compound **4**.

spectrometer at an ionization voltage of 70 eV. ^{1}H and ^{13}C NMR spectra were recorded in methanol-d_4 with a Jeol JNA-LAA 400WB-FT (^{1}H, 400 MHz; ^{13}C, 100 MHz) NMR spectrometer. Column chromatography (CC) was carried out using silica gel (Kieselgel 60, 70−230 mesh, Merck, Germany).

Fractions were monitored by TLC on precoated silica gel 60 F_{254} and RP-18 F_{254S} plates (both 0.25 mm; Merck), and spots were detected under UV illumination and by heating after spraying with *p*-anisaldehyde/H_2SO_4. Preparative HPLC was performed on a Tosoh CCPM-CCPM-II system (Tosoh Co., Tokyo, Japan) equipped with a UV 8020 detector, TSK gel ODS-80Ts column (21.5 × 300 mm, Tosoh Co., Tokyo, Japan), and Cosmosil 5C18-MS-II (20.0 × 250 mm, Nacalai tesque Inc., Kyoto, Japan). GC conditions were as follows: column, DB-1, J & W Scientific, 0.25 mm × 30 m; column temperature, 50−250 at 10 °C/min, then 10 min at maximum temperature; carrier gas, helium.

Plant Material. *Anarrhinum orientale* was collected from Al-Hadda Road, Saudi Arabia, in April 2007 and identified by the staff members of the Department of Biology (Botany), College of Science, King Abdulaziz University, Saudi Arabia. A voucher specimen (#AF 1185) has been deposited in the Herbarium of the Faculty of Pharmacy, King Abdulaziz University, Jeddah, Saudi Arabia.

Extraction and Isolation. Dried aerial parts of *A. orientale* (450 g) were extracted with MeOH (3 × 3 L) at room temperature for 3 days. The combined extracts were evaporated under reduced pressure (≤60 °C) to yield 40 g of a green residue. The MeOH extract was suspended in H_2O and fractionated with *n*-hexane (5.3 g), $CHCl_3$ (1.1 g), EtOAc (3.2 g), and *n*-BuOH (23 g) to yield the respective fractions. The EtOAc fraction (3.2 g) was subjected to a Sephadex LH-20 column, which was eluted first with H_2O, with a stepwise gradient of aqueous MeOH (10% MeOH until 100% MeOH). The homogeneity of the collected fractions (100 mL each) was monitored by TLC (solvent systems: $CHCl_3$−MeOH, 9:1, 4:1, 7:3, and 1:1 v/v). The spots were visualized by heating the plates after spraying with *p*-anisaldehyde−H_2SO_4. Fractions showing similar TLC profiles were combined to give four pools (frs. I−IV).

Fraction II (300 mg), was further separated on a Si gel column (20 cm × 2.2 cm) starting with $CHCl_3$−MeOH (98:2) as the eluent, then increasing the polarity by addition of 2% MeOH until 40% MeOH to obtain subfractions A−G.

Subfraction B (39 mg) was further purified on a Si gel column (20 cm × 1.5 cm) by elution with $CHCl_3$−MeOH (98:2), leading to the isolation of compound **4** (12 mg, purity 91%). Subfraction D (133 mg) was subjected to silica gel column chromatography (20 cm × 1.5 cm) eluted with $CHCl_3$−MeOH (94:6 to 92:8) to afford compound **3** (45 mg, purity 90%). Subfraction F (37.2 mg) was rechromatographed by HPLC on a TSK gel ODS-80Ts (C_{18}) column using 0.1% TFA in H_2O as solvent A and MeOH as solvent B with gradient elution starting from 30% until 90% MeOH during 1 h. Compound **1** (5 mg, purity 92%) was eluted with 85% MeOH at t_R = 55 min. Subfraction G (64.7 mg) was rechromatographed by HPLC on a Cosmosil (C_{18}) column using the above-mentioned eluting system. Compound **5** (3 mg, purity 92%) was eluted with 78% MeOH at t_R = 48 min.

Fraction IV (32 mg), eluted from a Sephadex LH-20 column with 80% MeOH, was chromatographed on a silica gel column using $CHCl_3$−MeOH

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Table 2. ^{1}H and ^{13}C NMR Data of Compounds 3 and 4 (methanol-d_4)

	3		4	
position	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C
1		171.6[a]		171.6[a]
2		129.1		129.4
3	6.73, m[a]	142.8	6.71, m[a]	142.4
4	2.28, m[a]	27.9	2.32, m[a]	27.8
5	2.15, m	39.1	2.18, m	39.1
6		138.4		142.6[a]
7	5.38, m[a]	125.5	5.36, m[a]	121.2
8	4.04, m[a]	59.1	4.53, m[a]	61.9
9	1.68, s	12.5[a]	1.73, s	16.2
10	1.80, s[a]	16.2	1.80, s[a]	12.6
1′		171.6[a]		171.6[a]
2′		129.3		129.6
3′	6.73, m[a]	143.1	6.71, m[a]	142.6
4′	2.21, m	28.2	2.27, m	28.0
5′	2.28, m[a]	31.6	2.32, m[a]	31.6
6′		138.6		142.6[a]
7′	5.38, m[a]	126.5	5.36, m[a]	121.4
8′	4.04, m[a]	59.4	4.53, m[a]	62.1
9′	1.80, s[a]	12.5[a]	1.80, s[a]	12.4
10′	1.75, s	23.5	1.76, s	23.3
OCO$\underline{CH_3}$			2.00, s	20.8
O$\underline{CO}CH_3$				172.9

[a] Overlapping signals.

(9:1) to yield compound **2** (6.5 mg, purity 92%), which was further purified by HPLC on a Cosmosil column using the aforementioned system.

*6′-O-Cinnamoylmussaenosidic acid (**1**):* yellowish oil; $[\alpha]_D^{23}$ −19.7 (*c* 0.15, MeOH); ^{1}H and ^{13}C NMR, see Table 1; HRFABMS (negative ion mode) *m/z* 505.17215 [M-1] (calcd for $C_{25}H_{29}O_{11}$, 505. 17103).

*6′-O-Cinnamoyl-8-O-(6′′′-O-cinnamoyl-β-D-glucopyranosyl)mussaenosidic acid (**2**):* yellowish oil; $[\alpha]_D^{23}$ −23.8 (*c* 0.2, MeOH); ^{1}H and ^{13}C NMR, see Table 1; HRFABMS (negative ion mode) *m/z* 797.26373 [M-1] (calcd for $C_{40}H_{45}O_{17}$, 797.26563).

*(2E,6E)-8-{[(2E,6E)-8-Hydroxy-2,6-dimethylocta-2,6-dienoyl]oxy}-2,6-dimethylocta-2,6-dienoic acid (**3**):* yellowish oil; for ^{1}H and ^{13}C NMR, see Table 2; HRFABMS (negative ion mode) *m/z* 349.19874 [M − 1] (calcd for $C_{20}H_{29}O_5$, 349.20150).

*(2E,6E)-8-{[(2E,6E)-8-Acetoxy-2,6-dimethylocta-2,6-dienoyl]oxy}-2,6-dimethylocta-2,6-dienoic acid (**4**):* yellowish oil; for ^{1}H and ^{13}C NMR, see Table 2; HRFABMS (negative ion mode) *m/z* 391.21439 [M − 1] (calcd for $C_{22}H_{31}O_6$:, 391.21207).

Acid Hydrolysis of Compounds 1 and 2. A solution of **1** and **2** (2 mg of each) in 1% H_2SO_4 (0.2 mL) in a sealed tube was heated on a boiling water bath for 1 h. The solution was extracted with EtOAc and concentrated under vacuum.[31] The aqueous layer was neutralized with Na_2CO_3 and freeze-dried. The sugar component of the residue was deduced by TLC and ^{13}C NMR through comparison with authentic samples to be glucose. The absolute configuration of the sugar was determined as D-glucose by GC according to the method described by Hara et al.[20]

Enzymes and Chemicals. Sensolyte 520 HCV protease assay kit fluorimetric (lot #AK71145-1020), HCV NS3/4A protease (lot #091-019), hepatitis virus C NS3 protease inhibitor **2** (cat #25346), and Sensolyte Green protease assay kit fluorimetric (lot #AK 71124-1011) were purchased from AnaSpec Inc., San Jose, CA, USA. Soybean trypsin−chymotrypsin inhibitor was purchased from Sigma Aldrich Co. Falcon Microtest 384-well 120 μL black assay plates, nonsterile, no lid, were purchased from Becton Dickinson Inc., Tokyo, Japan.

Table 3. Anti HCV NS3/4A Protease and Anti-human Trypsin Activities[a]

	HCV protease inhibitory activity	trypsin inhibitory activity
compound	IC_{50} (μM)	IC_{50} (μM)
1	100 ± 2.3	>200
2	125 ± 1.3	>200
3	126 ± 1.3	>200
4	>200	nt
5	109 ± 3.6	>200
HCV-I_2	1.65 ± 1.5	nt
T-I	nt	0.04

[a] HCV-I_2: Hepatitis virus C NS3/4A protease inhibitor 2 (positive control for HCV PR). T-I: Soybean trypsin−chymotrypsin inhibitor (positive control for trypsin). nt: not tested.

Assay for HCV Protease Inhibitory Activity. Two microliters of a compound solution (DMSO as solvent) was placed in each well of a 384-well microplate; then 8 μL of recHCV-protease (0.5 μg/mL) was added to the well containing the sample, and the plate was briefly agitated. Finally, 10 μL of the freshly prepared substrate (Ac-Asp-Glu-Dap (QXLTM520)-Glu-Glu-Abu-COO-Ala-Ser-Cys(5-FAMsp)-NH2) (100× dilution of a DMSO stock solution) was added with sequential rotational shaking. The reaction mixture was incubated for 30 min at 37 °C. The fluorimetric analyses were performed on an automated TECAN GENios plate reader with excitation wavelength at 485 nm and emission at 530 nm. Each test compound was carried out in triplicate. The HCV-PR inhibition (%) was calculated by using the following equation:

$$\%\text{inhibition} = (F_{\text{substrate}} - F_{\text{test compound}}) \times 100/F_{\text{substrate}}$$

($F_{\text{substrate}}$ is the fluorescence value of the substrate and enzyme without test compounds; $F_{\text{test-compounds}}$ is the fluorescence value of the test compound dissolved in DMSO).

Green Protease Assay. Compounds **1**−**5** were dissolved in DMSO (2.5 μL; final concentration, 10%) and placed in wells of the 384-well microplate. Then 17.5 μL of assay buffer and 2.5 μL of trypsin (0.1 U/μL) were added, and the plate was briefly agitated. Finally, 2.5 μL of the freshly diluted protease substrate HiLyte Fuor 488-labeled casein was added under sequential rotary shaking and incubated at 37 °C for 30 min. The positive control was soybean trypsin−chemotrypsin inhibitor. The calculation method was the same as under HCV protease assay.

Docking Study. The docking study was carried out using MOE software 10/2008 (see Supporting Information). The crystal structure of HCV NS3/4A protease (2A4Q) was downloaded from the protein data bank (www.pdb.org). The 3D structures of the tested compounds were generated using the ligx function of the MOE program followed by energy minimization of the generated structures. The crystal structure of HCV protease was prepared for the docking study using the protonate 3D function adjusting the temperature to 300 K and pH to 7. The electrostatics functional form was used for calculation; electrostatic interactions are computed using GB/VI (generalized Born/volume integral formalism) between two atoms if their separation distance is smaller than the cutoff value of 10 Å. The electrostatic constants of the solute and solvent were set to 1 and 80, respectively. The van der Waals functional was set to 800R3. The energy was minimized using the MMFF94x forcefield. The active site was detected using the site finder function of the program. Docking was carried out by setting the

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placement to Alpha PMI, rescoring 1 to Affinity dG and its retain to 10, refinement to force field and rescoring 2 to Affinity dG and its retain to 10. The previous parameters were selected, as they gave the best redocking result for the cocrystal ligand with root-mean-square deviation (rmsd) = 2 Å.

■ ASSOCIATED CONTENT

Supporting Information. NMR data (^{1}H, ^{13}C NMR, HMQC, and HMBC) for new compounds as well as high-resolution mass data are provided. This material is available free of charge via the Internet at http://pubs.acs.org.

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