

Two New Naphthoquinones with Antiviral Activity from *Rhinacanthus nasutus*

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Two new naphthoquinones, rhinacanthin-C (**1**) and rhinacanthin-D (**2**), exhibit inhibitory activity against cytomegalovirus (CMV), with EC₅₀ values of 0.02 and 0.22 $\mu\text{g/mL}$, respectively, against human CMV. They were isolated from the medicinal plant *Rhinacanthus nasutus* (Acanthaceae). The structures of the compounds were determined by analysis of their spectroscopic data, in particular, 2D NMR.

The shrub *Rhinacanthus nasutus* (L.) Kurz (Acanthaceae) is a medicinal plant that is widely distributed in Southeast Asia. On the basis of ethnomedical field research by Shaman Pharmaceuticals, extracts of the aerial parts of *R. nasutus* were identified as being potentially useful against herpes virus infections. Indigenous medical practitioners in the country of Thailand use the roots and leaves, which are pounded with vinegar or alcohol and applied to herpes infections and other skin eruptions. The literature reports that extracts of various parts of *R. nasutus* and *Rhinacanthus communis* are used for treatments against ringworm and other fungus derived skin diseases, as well as eczema, pulmonary tuberculosis, and cancer.^{1–6} Although flavonoids, anthraquinones, triterpenes, sterols, and other compounds have been isolated from *R. nasutus*,^{6–8} the only biologically active constituents reported are naphthoquinones.¹⁹ Rhinacanthin-A (**3**) and -B (**4**) were isolated from the leaves and stems of *R. nasutus* by bioassay-guided fractionation using a cytotoxicity assay; compound **3** was shown to be the major cytotoxic agent.⁹ These compounds are distinguished by the presence of a tricyclic ring system containing a naphtho-1,4-quinone and a 2,2-dimethyldihydropyran ring. A related naphthoquinone, rhinacanthone, isolated from *R. nasutus*, has been shown to have potent in vitro antifungal activity;¹ the original structure (**5**) of this compound was recently revised to **6**.¹⁰ Using an in vitro antiviral screen, we found that an extract of *R. nasutus* exhibited significant activity against murine cytomegalovirus (mCMV, EC₅₀ = 21 $\mu\text{g/mL}$) with no measurable activity against herpes simplex virus types 1 or 2. We have used an anti-CMV-activity-guided fractionation to isolate two new biologically active naphthoquinones, named rhinacanthin-C (**1**) and rhinacanthin-D (**2**). Both **1** and **2** are potent inhibitors of human CMV, and their core structures resemble the tricyclic system seen for **3** and **4**, but are differentiated from the 1,2-diketone **6**. Their structures were eluci-

dated by detailed analysis of their spectroscopic data. We describe here the isolation, structure elucidation, and biological activity of **1** and **2**.

Rhinacanthin-C (**1**) was isolated as a yellow oil and gave a molecular ion peak at m/z 410.2097, corresponding to the formula C₂₅H₃₀O₅ (dev + 0.95 ppm). The ¹³C- and DEPT-NMR spectra were consistent with this formula, showing the presence of 25 carbon signals with 29 attached protons. The ¹H-NMR spectrum of **1** showed 7 downfield (δ 5.20–8.71, including one D₂O exchangeable signal at 7.20) and 8 upfield (δ 1.02–3.90) signals, accounting for all 30 protons. Signals at δ 8.12 (d, 1 H, J = 7 Hz), 8.09 (d, 1 H, J = 7 Hz), 7.77 (t, 1 H, J = 7 Hz), and 7.70 (t, 1 H, J = 7 Hz) in the ¹H-NMR spectrum of **1** were similar to signals due to the naphthoquinone moiety in lapachol (**7**).¹¹ The UV spectrum of **1** was also consistent with a naphtho-1,4-quinone moiety.^{12–14} The IR spectrum contained absorption bands due to carbonyl groups at 1710 (ester), 1670, and 1650 (p -quinone) cm⁻¹ and a hydroxyl group at 3400 (br) cm⁻¹.

The NMR spectra (COSY, HMQC, HMBC) of **1** reveal signals due to two methylenes [δ 2.71 (s, 2 H), 3.9 (s, 2 H)] and two methyl groups [1.02 (s, 6 H)] assigned to a 2,2-dimethylpropyl group (see Table 1). HMBC correlations also established the naphthoquinone moiety; key correlations between δ 8.12 (H-6) and δ 184.0 (C-5) and between δ 8.09 (H-9) and δ 181.0 (C-10) require a p -quinone as shown in Figure 1. Correlations between the signal at δ 2.71 (H-4) and three signals [δ 121.8 (C-4a), 154.3 (C-10a), 184.0 (C-5)] in the HMBC spectrum of **1** established the connection of the 2,2-dimethylpropyl group to the naphthoquinone at C-4a. The presence of a 2,6-dimethyl-1,2-octadienoic acid ester in **1** was shown by analysis of its NMR spectra. The ¹H-NMR spectrum contained signals due to an olefinic proton [δ 6.70 (t, 1 H, J = 7 Hz, H-3')] coupled to a methylene signal [δ 2.17 (dt, 2 H, J = 7 Hz, H-4')] which was in turn coupled to a second methylene signal [δ 2.02 (t, 2 H, J = 7 Hz, H-5')], an olefinic proton signal [δ 5.20 (d, 1 H, J = 7 Hz, H-7')] coupled to a signal due to a vinyl methyl group [δ 1.56 (d, 3 H, J = 7 Hz, H-8')], and two more vinyl methyl groups [δ 1.80 (br s, 3 H, H-9') and 1.59 (br s, 3

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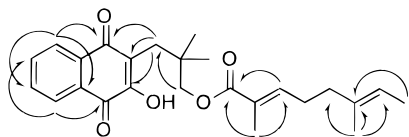
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Table 1. ^1H - and ^{13}C -NMR Shift Values and Long-Range C–H Connectivities in Compound **1** Established by HMBC (400 MHz, CDCl_3)

position	δ_{H} (mult., J) ^a	δ_{C} (mult.) ^{b,c}	$^2J_{\text{CH}}$	$^3J_{\text{CH}}$
2	3.90 (2 H, s)	72.9 (CH_2)		C-4, C-11,12
3		37.0 (C)		
4	2.71 (2 H, s)	32.2 (CH_2)	C-3, C-4a	C-2, C-5, C-10a, C-11,12
4a		121.8 (C)		
5		184.0 (C)		
5a		133.1 (C)		
6	8.12 (d, 8)	126.1 (CH)		C-5, C-8, C-9a
7	7.77 (t, 8)	134.9 (CH)	C-6	C-5a, C-9
8	7.70 (t, 8)	132.9 (CH)	C-9	C-9a
9	8.09 (d, 8)	127.1 (CH)		C-7, C-10
9a		129.2 (C)		
10		181.0 (C)		
10a		154.3 (C)		
11, 12	1.02 (6 H, s)	25.2 ($2 \times \text{CH}_3$)		C-2, C-4
1'		168.5 (C)		
2'		127.7 (C)		
3'	6.70 (t, 7)	142.3 (CH)		C-1'
4'	2.17 (2 H, dt, 7)	27.2 (CH_2)	C-3'	
5'	2.02 (2 H, t, 7)	38.2 (CH_2)		
6'		134.5 (C)		
7'	5.20 (d, 7)	119.3 (CH)		
8'	1.56 (3 H, d, 7)	13.3 (CH_3)	C-7'	C-6'
9'	1.80 (3 H, br s)	12.2 (CH_3)	C-2'	C-1', C-3'
10'	1.59 (3 H, br s)	15.5 (CH_3)	C-6'	C-5', C-7'

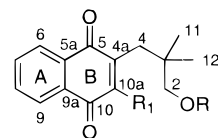
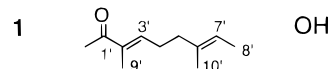
^a 1 H, unless noted. ^b Multiplicities were determined by DEPT. ^c Assignments are based on HMQC correlations.

**Figure 1.** Structure of **1** showing key HMBC correlations ($^1\text{H} \rightarrow ^{13}\text{C}$).

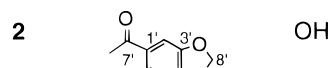
H, H-10'). Inspection of the ^{13}C , DEPT, COSY, HMQC, and HMBC NMR spectra allowed assignment of these signals to the structure as shown in Figure 1 and Table 1. The configuration of the double bonds in **1** was established on the basis of the chemical shifts of the vinyl methyl groups in the ^{13}C -NMR spectrum: δ 12.2 (C-9'), 15.5 (C-10').¹⁵

The NMR spectra of **1** and **3** are very similar, the only significant difference being the signals assigned to C-2, C-3, and C-4 [δ 3.90 (s, 2 H, H-2), 72.9 (t, C-2), 37.0 (s, C-3), 2.71 (s, 2 H, H-4), 32.2 (t, C-4) for **1**; δ 79.0 (s, C-2), 5.13 (t, 1 H, $J = 4.6$ Hz, H-3), 69.1 (d, C-3), 2.91 and 2.77 (each 1 H, dd, $J = 19.5, 4.6$ Hz, H-4), and 38.0 (t, C-4) for **3**]. Comparison of the ^1H -NMR spectra of **1** with the spectra of **3** and the synthetic compounds 2-(3-hydroxy-2,2-dimethylpropyl)-1,4-naphthoquinone (**8**) and 1-hydroxy- β,β -dimethyl-2-naphthalenepropanol (**9**)¹⁶ established the position of the ester chain in **1**. The chemical shift of the protons assigned to C-2 in **1** is 3.90 ppm. The chemical shift of these protons in **6** is also 3.90 ppm, while in the underivatized alcohols **8** and **9** they are at 3.12 and 3.19 ppm, respectively, such that esterification is at C-2 in **1**.

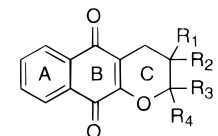
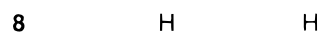
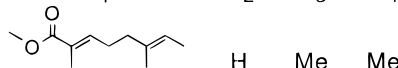
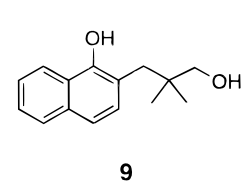
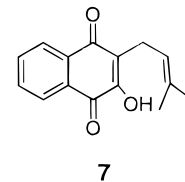
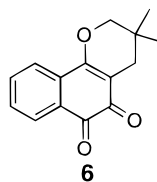
The EIMS of **1** fully supported the designated structure. Two key diagnostic peaks at m/z 259 [naphthoquinone - O]⁺ ($\text{C}_{15}\text{H}_{15}\text{O}_4$) and m/z 151 [2,6-dimethyl-1,2-octadienyl]⁺ ($\text{C}_{10}\text{H}_{15}\text{O}$) accounted for all of the five oxygen atoms and are α -cleavage products adding up to the M_r . A comparison of the mass spectral data of **1**

R R₁

OH



OH

R₁ R₂ R₃ R₄**4** OH H Me Me**5** Me Me H H

with that of **3** [m/z 408 (M^+), $\text{C}_{25}\text{H}_{28}\text{O}_5$]⁹ disclosed that **1**, like **3**, is a 1,4-naphthoquinone derivative having a 10-carbon unit ester side chain. The difference of 2 Da (equivalent to 2H by HRMS) and the presence of a hydroxyl band in the IR spectrum of **1** suggested that the pyran moiety (ring C) in **3** has been opened and modified in **1**. Two abundant hydrocarbon fragments at m/z 123 (C_9H_{15}), a daughter ion of m/z 151-CO, and m/z 69 (C_5H_9), an allylic 4',5'-bond cleavage product, and other ions [m/z 150 (base) and 82] common to **1** and **3** clearly supported that **1** possesses the same 10-carbon ester side chain as in **3**. Other diagnostic peaks associated with the two halves of the molecule (m/z 259 and 151), represented by their daughter ions, are summarized in Figure 2. The occurrence of the base peak at m/z 225 in **3**, via McLafferty elimination of the side chain unit (R_1) from M^+ followed by extrusion of the Me group, was not seen in **1**. Instead, a prominent peak at m/z 188 was observed; its formation could only be rationalized via a simple elimination of the side chain as $\text{RCOOCH}=(\text{CH}_3)_2$, if the *gem*-dimethyl group were at C3 and the side chain at C2. Similarly, the locus of the OH group in **1** follows from the presence of m/z 242 and 228 ions; these ions could only be explained if the OH group were at C-10a of the quinone ring B.

The molecular formula of rhinacanthin-D (**2**), $\text{C}_{23}\text{H}_{20}\text{O}_7$, was determined by HREIMS [m/z 408.1206 (M^+ , dev -0.7 ppm)]. The UV spectrum of **2** was similar to that of **1** and indicated a naphtho-1,4-quinone. The IR spectrum contained bands due to carbonyl groups at 1718 (ester), 1680, and 1660 (*p*-quinone) cm^{-1} and a

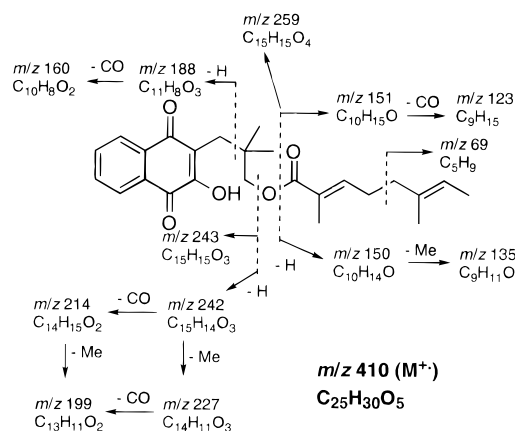


Figure 2. Major EIMS fragments of **1**, with their molecular formulae as determined by HRMS.

Table 2. ^1H - and ^{13}C -NMR Shift Values and Long-Range C–H Connectivities in Compound **2** Established by HMBC (400 MHz, CDCl_3)

position	δ_{H} (mult., J) ^a	δ_{C} (mult.) ^{b,c}	$^2J_{\text{CH}}$	$^3J_{\text{CH}}$
2	4.06 (2 H, s)	74.6 (CH ₂)	C-3	C-4, C-11, 12
3		38.0 (C)		
4	2.71 (2 H, s)	33.4 (CH ₂)	C-3, C-4a	C-2, C-4a, C-5, C-10a, C-11, 12
4a		123.2 (C)		
5		186.9 (C)		
5a		134.1 (C)		
6	8.03 (d, 8)	127.3 (CH)	C-7	C-8
7	7.70 (t, 8)	135.4 (CH)		
8	7.71 (t, 8)	133.9 (CH)		
9	7.90 (d, 8)	126.1 (CH)	C-8	C-7
9a		131.5 (C)		
10		182.3 (C)		
10a		153.1 (C)		
11, 12	1.12 (6 H, s)	25.9 (2 × CH ₃)		
1'		126.7 (C)		
2'	7.24 (d, 2)	109.9 (CH)	C-4'	C-1', C-5', C-7'
3'		149.1 (C)		
4'		153.1 (C)		
5'	6.71 (d, 8)	108.8 (CH)	C-7'	C-4'
6'	7.49 (dd, 8, 2)	126.8 (CH)	C-6'	C-5'
7'		167.4 (C)		
8'	5.98 (2 H, s)	103.3 (CH ₂)	C-4', C-5'	

^a ^1H , unless noted. ^b Multiplicities were determined by DEPT. ^c Assignments are based on HMQC correlations.

hydroxy group at 3400 cm^{-1} . As in **1**, a 2,2-dimethylpropyl group attached to a naphtho-1,4-quinone moiety at C-4a was established after analyzing 2D NMR experiments and comparing them with the NMR data for **1** (see Table 2). However, the signals in the NMR spectra of **1** that were due to the diterpene ester unit R were missing in the spectra of **2**. Instead, signals due to a 1,3,4-trisubstituted benzoate and a methylene dioxy carbon were observed: δ 7.24 (d, $J = 2\text{ Hz}$, H-2'), 6.71 (d, $J = 8\text{ Hz}$, H-5'), 7.49 (dd, $J = 8, 2\text{ Hz}$, H-6'); 5.98 (s, 2 H, H-8'). Correlations in the HMBC spectrum of **2** between the signal at δ 5.98 (H-8') and signals 149.1 (C-3') and 153.1 (C-4') and between the signals at 7.24 (H-2') and 167.4 (C-7') confirmed the 3,4-methylenedioxybenzoate moiety. These signals suggested structure **2**. The complete structure of **2** was determined using two dimensional NMR experiments. The NMR assignments of **2** are shown in Table 2.

The EIMS of **2** showed the presence of structural elements of **1** except for the 10-carbon unit ester side chain at C-2, which is replaced by a 3,4-(methylenedioxy)benzoate in **1**. This finding was clearly evident

from two complementary halves of the molecule: the α -cleavage products at m/z 259 [naphthoquinone – O]⁺ and at m/z 149 (base, $\text{C}_8\text{H}_5\text{O}_3$) [3,4-(methylenedioxy)benzoyl]⁺ that add up to the M_r . Another distinguishing feature between **1** and **2** came from the presence of two peaks at m/z 166 [$\text{C}_8\text{H}_6\text{O}_4$] and 165 [$\text{C}_8\text{H}_5\text{O}_4$] in **2**, not observed in **1**, shown by HRMS to be the β -cleavage products, $[\text{RCOOH}]^+$ and $[\text{RCOO}]^+$, with and without hydrogen abstraction, respectively. These key fragments together with the further loss from m/z 149 of CO [m/z 121, $\text{C}_7\text{H}_5\text{O}_2$]⁺ strongly support structure **2**.

Using a cell culture-based assay, **1** and **2** exhibit potent inhibitory activity against both human and murine strains of CMV with selective indices ranging from 3 to 28 (Table 3). No activity was detected against respiratory syncytial virus (RSV), influenza A virus (Flu-A), or herpes simplex virus type-2 (HSV-2); however, under these assay conditions, the two compounds were cytotoxic.

The unique drug discovery process pioneered by Shaman Pharmaceuticals emphasizes ethnobotany, the study of plants that have a history of medicinal use. Using ethnobotany as a platform, we have identified a plant that contains two new compounds, **1** and **2**, with potent *in vitro* activity against CMV. Rhinacanthin-D (**2**) is the first example of a naphthoquinone with a 3,4-methylenedioxybenzoate group. The only previous report of a naphthoquinone containing a *gem*-dimethyl group flanked by two methylene carbons, as in **1** and **2**, was compound **6**;¹⁰ this structure type may be limited to *Rhinacanthus* spp.

Experimental Section

General Experimental Procedures. All NMR spectra were recorded at 400 MHz for ^1H and 100 MHz for ^{13}C on a Varian Unity 400 using TMS as an internal reference. MS were obtained on a Kratos MS-50 mass spectrometer; UV spectra, on a Perkin-Elmer UV-vis spectrometer; and IR spectra, on a Perkin-Elmer Model 1600 FTIR spectrometer. All solvents used were HPLC grade obtained from Fisher Scientific. Column chromatography was carried out using HP-20 obtained from Mitsubishi Kasei Corporation. A Rainin Dynamax C18 column (20 × 250 mm, MeCN/H₂O gradient) was used for HPLC on a Rainin Dynamax system.

Antiviral and Cytotoxicity Assays. The antiviral activities of compounds **1** and **2** as well as control antiviral compounds were determined using the viral cytopathic effect (CPE) assay, the plaque-neutralization (plaque) assay, and the hemadsorption-inhibition (HAI) assay. The procedures used for the antiviral and cytotoxicity assays have been previously described.^{17–20}

Plant Material. The whole plant of *Rhinacanthus nasutus* (Acanthaceae) was collected on Jan 1, 1993, in Thailand by Michael Balick of the New York Botanical Garden, on Dec. 10, 1993, in Huay Kaew, Chiang Mai, by Rachan Pooma of the Royal Forest Department of Thailand, and on Dec 29, 1993, in the Central Botanical Garden, Thailand, by Thawatchai Wongpraseart of the Royal Forest Department of Thailand. The plant was identified by Weerachai Nanakorn of the Botanical Garden Organization, Chaing Mai, Thailand, and by Mary Merello of the Missouri Botanical Garden. Voucher specimens are deposited in the reference collection, Department of Ethnobotany and Conservation, Shaman Pharmaceuticals.

Table 3. *In Vitro* Antiviral Activity of **1** and **2**

compound	virus	assay	EC ₅₀ (μg/mL) ^a	IC ₅₀ (μg/mL) ^b	SI ^c	n
1	mCMV ^d	CPE ^e	1.1 ± 0.2	8.0 ± 3.0	7.3	4
	mCMV	plaque ^f	0.57	2.6	4.6	1
	hCMV ^g	plaque	0.02	0.56	28	1
	Flu-A ^h	HAI ⁱ	none	0.2 ± 0.2	NOSI	2
	HSV-2 ^j	CPE	none	0.03	NOSI	1
	RSV ^k	CPE	none	0.3	NOSI	1
2	mCMV	CPE	9.5 ± 1.6	49 ± 4.8	5.2	4
	mCMV	plaque	9.5	35	4	1
	hCMV	plaque	0.22	0.75	3	1
	FluA	HAI	none	0.78	NOSI	2
	HSV-2	CPE	none	<0.8	NOSI	1
	mCMV	CPE	5.0 ± 0.4	>100	>20	20
gancyclovir	mCMV	plaque	13.8 ± 5.2	>100	>7.2	2
	hCMV	plaque	3.4 ± 1.1	>1000	>290	4
	Flu-A	HAI	0.054 ± 0.004	56 ± 10	1040	12
amantadine	Flu-A	HAI	0.054 ± 0.004	56 ± 10	1040	12
acyclovir	HSV-2	CPE	2.3 ± 0.3	>10	>4.3	14
ribavirin	RSV	CPE	1.8 ± 0.2	35 ± 4.6	19	23

^a Antiviral activity. ^b Cytotoxicity. ^c Selective Index = IC₅₀/EC₅₀. ^d Murine CMV. ^e Cytopathic effect. ^f Plaque-neutralization. ^g Human CMV. ^h Influenza virus type A. ⁱ Hemadsorption inhibition. ^j Herpes simplex virus type 2. ^k Respiratory syncytial virus.

Extraction and Isolation. The above-ground parts from *R. nasutus* (226 g) were ground to a powder and extracted with 1:1 CH₂Cl₂-2-propanol with gentle stirring for ca. 24 h. After filtration, the extract was concentrated to dryness (4.64 g), suspended in 90% aqueous MeOH (100 mL), and extracted successively with hexane (3 × 100 mL). The MeOH-soluble fraction was concentrated (1.1 g), suspended in H₂O, and purified on an HP-20 column (200 g, 3.5 × 100 cm) eluting with a H₂O/MeOH/Me₂CO gradient. Fractions eluting with MeOH had activity against mCMV; the active fractions were combined, evaporated, and purified by HPLC on C18 (Dynamax, 20 × 250 mm) using a MeCN/0.1% TFA/H₂O gradient (60–70% MeCN, 0–20 min; 70–95% MeCN, 20–30 min; 15 mL/min) to give **1** (11.6 mg, 0.005%) and **2** (1.8 mg, 0.0007%) as the only components having activity against CMV.

Rhinacanthin-C (1): obtained as a yellow oil; UV (MeOH) λ max (log ε) 214 (3.17), 247 (sh, 3.69), 251 (3.71), 277 (3.67), 288 (sh, 3.66), 330 (2.95), and 380 (sh, 2.69) nm; IR (film) ν max 3400 (br, OH), 2930–2860 (CH), 1710 (C=O, ester), 1670, 1650 (C=O, *p*-quinone), 1595, 1461, 1371, 1346, 1275, 1218, 1183, 1127, 1080, 1050, 957, 920, 727 cm⁻¹; ¹H NMR see Table 1; ¹³C NMR see Table 2; eims *m/z* 410 (16), 342 (4), 262 (15), 259 (5), 244 (70), 243 (74), 242 (18), 230 (50), 227 (22), 214 (18), 199 (8), 188 (62), 160 (34), 151 (68), 150 (100), 149 (62), 135 (51), 123 (57), 82 (47), 69 (76), 55 (43); HRMS *m/z* 410.2906 (calcd 410.2903 for C₂₅H₃₀O₅).

Rhinacanthin-D (2): isolated as a yellow powder; UV (MeOH) λ max (log ε) 217 (3.33), 253 (3.02), 275 (2.96), 283 (sh, 2.95), 326 (sh, 2.13), 387 (1.84), and 486 (1.57) nm; IR (KBr) ν max 3422 (br, OH), 2970–2900 (CH), 1718 (C=O, ester), 1680, 1660 (C=O, *p*-quinone), 1438, 1375, 1277, 1260, 1215, 1185, 1155, 1072, 1038, 802, 761, 726 cm⁻¹; ¹H NMR see Table 1; ¹³C NMR see Table 2; eims *m/z* 408 (6), 259 (2), 242 (32), 227 (17), 214 (9), 166 (19), 165 (23), 150 (12), 149 (100), 121 (18), 77 (13), 65 (11), 60 (10), 57 (13), 55 (19); HRMS *m/z* 408.1206 (calcd 408.1209 for C₂₃H₂₀O₇).

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