Two New Lignans with Activity against Influenza Virus from the Medicinal Plant *Rhinacanthus nasutus*

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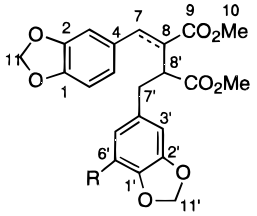
Two new lignans, rhinacanthin E (**1**) and rhinacanthin F (**2**), were isolated from the aerial parts of the plant *Rhinacanthus nasutus*. Their structures were established by detailed spectroscopic analysis. These compounds show significant antiviral activity against influenza virus type A.

We have recently reported[1] the isolation and identification of two new naphthoquinones from the medicinal plant *Rhinacanthus nasutus* (L.) Kurz (Acanthaceae) that have antiviral activity against human cytomegalovirus. In our continuing investigation of antiviral agents from *R. nasutus*, a plant used for a variety of illnesses including cancer, fungal infections, eczema, pulmonary tuberculosis, and herpes virus infections,[1−7] we have isolated and identified two new lignans, rhinacanthin-E (**1**) and rhinacanthin-F (**2**). Both compounds have significant *in vitro* activity against influenza virus, with EC_{50} values of 7.4 μg/mL for **1**, and 3.1 μg/mL for **2** in an anti-Flu-A cytopathic effect (CPE) assay. Herein, we describe the isolation, structure elucidation, and antiviral activity of the new compounds.



1 R = OCH_3, Δ 7E
2 R = OCH_3
3 R = H, Δ 7E
4 R = H
5 R = H, Δ 7Z

Compounds **1** and **2** were obtained as amorphous powders after liquid/liquid partition, reversed-phase chromatography, and HPLC of a dichloromethane−2-propanol extract of *R. nasutus*. The molecular formula, $C_{23}H_{22}O_9$, for **1** (*m*/*z* 442, deviation 1.4 ppm) and $C_{23}H_{24}O_9$ for **2** (*m*/*z* 444, deviation −1.9 ppm) were determined from high-resolution EI mass spectra. The EIMS and ^{1}H NMR spectra of **1** and **2** were suggestive of lignan structures and indicated that **2** is the dihydro derivative of **1**. The structure of **1** was deduced from the NMR spectra, assigned from 2D NMR data (COSY, HMQC, HMBC) and from mass spectral analyses. The ^{1}H NMR spectrum of **1** was very similar to that of (7*E*)-7,8-dehydroheliobuphthalmin **3**,[8] except that three of the aromatic signals in **3** (δ 6.35, br s, H-3′; 6.37 br d, H-5′; 6.59, d, H-6′) were replaced by two signals in **1** (δ 6.09, br s; 6.05, br s), and there was a signal due to a third methoxy group (δ 3.73, s) in the spectrum of **1**. This suggested that **1** was the 6′-*O*-methoxy derivative of **3**.

The HMBC spectrum of **1** (Table 1) was consistent with the assigned structure. For example, the C-10 carboxymethyl signal (δ 3.82) showed a $^3J_{H,C}$ correlation to the C-9 ester carbonyl signal (δ 167.1) that had additional correlations to signals at δ 7.67 (H-7) and 4.04 (H-8′). This indicated the presence of an α,*β*-disubstituted α,*β*-unsaturated methyl ester unit with a methine at the α position. The C-8′ methine proton signal (δ 4.04) also showed a $^2J_{H,C}$ correlation to a second ester carbonyl signal [δ 173.0, (C-9′)] that in turn had a $^3J_{H,C}$ correlation to a carboxymethyl signal [δ 3.74 (H-10′)]. These correlations indicated that the second methyl ester was attached to the C-8′ methine (δ 45.5). This, together with additional correlation data from the HMBC spectrum of **1**, allowed unambiguous assignment of the structure as shown. Particularly revealing were the correlations from H-7 to C-3, C-5, C-8, and C-9 and from H-5 to C-7, indicating that **1** has a double bond between C-7 and C-8. The complete assignments of the proton and carbon chemical shifts are shown in Table 1.

Due to the paucity of material obtained, only $[\alpha]_D$, UV, ^{1}H NMR, and EIMS data were obtained for **2**. The structure of **2** followed from the ^{1}H NMR spectrum, which contained signals due to five aromatic protons, three methoxy groups, and two methylenedioxy groups. The signals due to the four aliphatic and ethylene protons in **1** are replaced by an overlapping 6 H multiplet between δ 2.8 and 3.0, similar to that observed for heliobuphthalmin **4**.[9] These data, along with the molecular formula, suggest that **2** is a 7,8-dihydro derivative of **1**. The new lignans **1** and **2** are closely related to **3**−**5**[8,9] and have very similar ^{1}H NMR spectra. The chemical shifts of H-7 (δ 7.67) and H-8′ (δ 4.04) in **1** as compared to **3** [δ 7.66 (H-7) and 4.06 (H-8′)] and **5** [δ 6.51(H-7) and 3.56 (H-8′)] clearly indicate that the

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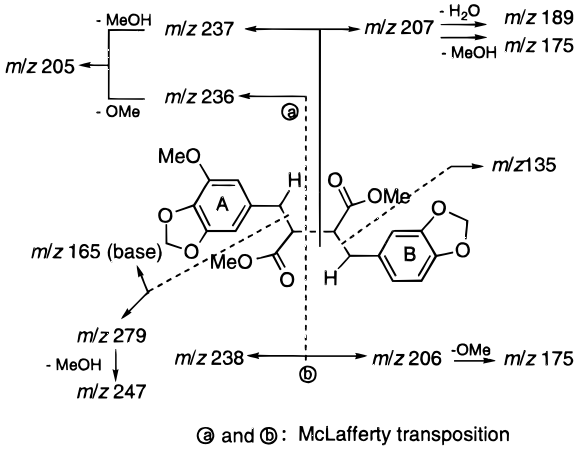
Table 1. NMR Assignments ($CDCl_3$) for Compounds **1** and **2** and HMBC Correlations for **1**

position	δ ^{13}C[a] **1**	δ ^{1}H (mult) **1**[b]	HMBC (H to C)	**2**[c]
1	147.7			
2	147.6			
3	108.2	6.44 (br s)	C-1, C-5, C-7	6.56 (d)
4	128.9			
5	122.5	6.53 (br d)	C-1, C-3, C-7	6.58 (dd)
6	108.4	6.73 (d)	C-2, C-4, C-5	6.70 (d)
7	142.5	7.67 (s)	C-3, C-5, C-8, C-9, C-8′	2.8−3.0 (m)[d]
8	129.5			2.8−3.0 (m)[d]
9	167.1			
10	52.0	3.82 (s)	C-9	3.66 (s)
11	101.2	5.97 (s)	C-1, C-2	5.93 (s)
1′	134.0			
2′	148.4			
3′	103.3	6.09 (s)	C-1′, C-4′, C-5′, C-7′	6.42 (s)
4′	133.5			
5′	107.9	6.05 (s)	C-1′, C-3′, C-4′, C-6′, C-7′	6.56 (s)
6′	144.8			
7′	36.2	3.34 (dd); 2.87 (dd)	C-8, C-3′, C-5′, C-8′	2.8−3.0 (m)[d]
8′	45.5	4.04 (dd)	C-8, C-9, C-9′	2.8−3.0 (m)[d]
9′	173.0			
10′	52.2	3.74 (s)	C-9′	3.66 (s)
11′	101.1	5.92 (d); 5.89 (d)	C-1′, C-2′	5.95 (d); 5.94 (d)
12′	56.2	3.73 (s)	C-6′	4.02 (s)

[a] Multiplicity determined by DEPT 90° and 135°. [b] J (Hz): 5,6 = 8; $7_a'$,$7_b'$ = 14; $7_a'$,8′ = 4.8; $7_b'$,8′ = 10; $11_a'$,$11_b'$ = 1.5. [c] J (Hz): 3,5 = 2; 5,6 = 8; $11_a'$,$11_b'$ = 1.5. [d] Overlapping signal, 6 H.

Scheme 1. Most Significant Ions in the EIMS of **2**



configuration of the 7,8-double bond in **1** is *E* as in **3**; however, the relative stereochemistry of **2** was not determined.

The EIMS of **1** and **2** fully support the designated structures. Lignans **1** and **2** exhibit molecular ion peaks at *m/z* 442 ($C_{23}H_{22}O_9$) and *m/z* 444 ($C_{23}H_{24}O_9$), respectively. The presence of two COOMe groups in both **1** and **2** was clearly recognized by the fragmentation peaks at *m/z* 411 (M−OMe), shifted to *m/z* 413 in **2**, and *m/z* 379 (411−MeOH), shifted to *m/z* 381 in **2**. Both **1** and **2** exhibit *m/z* 165 ion as the base peak with the same elemental composition ($C_9H_9O_3$); this is obtained by the allylic cleavage to ring A as shown in Scheme 1. However, the other half at *m/z* 277 ($C_{14}H_{13}O_6$) in **1** is shifted in **2** to *m/z* 279 ($C_{14}H_{15}O_6$). These findings together with the fragment at *m/z* 135 with insignificant intensity in **1**, compared to 85% in **2**, strongly support that **2** is the dihydro derivative of **1** and that the double bond in **1** is conjugated to ring B. The presence of this double bond in **1** affected the fragmentation in such a way that none of the McLafferty rearrangement ions (*m/z* 238, 236, and 206) and their daughter ions observed in **2** (Scheme 1) were observed in **1**.

Table 2. Antiviral Activity of Compounds **1** and **2** and Control Compounds

compd	virus	EC_{50}[a]	IC_{50}[b]	SI[c]	n[d]
1	Flu-A[e]	1.7	44	26	1
1	Flu-A[f]	7.4 ± 2.0	102 ± 64	15	2
1	HSV-2[g]	none	17		1
2	Flu-A[e]	<0.94	17	>18	1
2	Flu-A[f]	3.1	21	6.8	1
2	HSV-2[g]	none	4.4		1
amantadine[h]	Flu-A[e]	0.054 ± 0.004	56 ± 10	1000	12
ribavirin[h]	Flu-A[f]	3.7 ± 1.2	>200	>59	4
acyclovir[h]	HSV-2[g]	1.5 ± 0.2	>100	>60	2

[a] Antiviral activity, μg/mL, 50% effective concentration. [b] Cytotoxicity, μg/mL, 50% inhibitory concentration. [c] Selective index = IC_{50}/EC_{50}. [d] Number of assays. [e] Influenza virus type A, hemadsorption inhibition assay. [f] Influenza virus type A, cytopathic effect assay. [g] Herpes simplex virus type 2, CPE assay. [h] Antiviral reference controls.

Both compounds **1** and **2** exhibited significant antiviral activity against influenza type A virus (Flu-A) when tested in a hemadsorption-inhibition assay and in a viral cytopathic effect assay, as shown in Table 2. The compounds were also tested against herpes simplex virus type 2 (HSV-2) but had no activity. Under the assay conditions used, both compounds exhibited some cytotoxicity, but at concentrations well above the antiviral endpoints. Thus, although only one non-Flu virus was tested, these data suggest that lignans **1** and **2** have selective antiviral activity against Flu-A. The lack of any anti-HSV activity was surprising, because other lignans, including podophyllotoxin and derivatives, have been shown to have antiviral activity against HSV and measles virus.[10−13] It has been proposed that such compounds prevent viral replication by inhibition of microtubule formation or nucleic acid metabolism, which explains why these compounds are active against several different viruses.[10,14] Although it is possible that compounds **1** and **2** inhibit influenza virus via a similar mechanism, this seems unlikely since the compounds are inactive against HSV. Therefore, our findings suggest that lignans **1** and **2** are selective in their antiviral effect against Flu-A and indicate that **1** and **2** inhibit a specific influenza biosynthetic process that is

unrelated to the anti-herpes activity of podophyllotoxin analogs. This is further supported by the fact that some dibenzylbutyrolactones, structurally more similar to **1** and **2**, are considerably less active (1/1000) against HSV-2 than the corresponding cyclic analogs.[12] Further investigation of the antiviral activity of **1** and **2**, and the inhibition of influenza virus by these lignans, is warranted.

Experimental Section

General Experimental Procedures. The general procedures have been described previously.[1] Column chromatography was carried out using HP-20 polystyrene-divinylbenzene gel obtained from Mitsubishi Kasei Corp. A Rainin Dynamax C18 column (20 × 250 mm, MeCN/H_2O gradient) was used for HPLC on a Rainin Dynamax system.

Antiviral and Cytotoxicity Assays. The antiviral activities and cytotoxic effects of compounds **1** and **2** as well as control antiviral compounds were determined using the viral cytopathic effect (CPE) assay and the hemadsorption-inhibition (HAI) assay. The procedures used for the antiviral and cytotoxicity assays have been previously described.[15−18] Influenza virus type A, NJH1N1 strain, was used for the antiviral Flu-A HAI assay in MDCK cells; both the antiviral assay and a separate cytotoxicity assay in MDCK cells employed visual endpoints. A neutral red endpoint was used for both the antiviral Flu-A CPE assay (NWS-33 strain) and cytotoxicity assay in MDCK cells. Herpes-simplex virus type 2, MS strain, was used in a visual antiviral CPE assay in Vero cells; a cytotoxicity assay in Vero cells used an MTT endpoint. The antiviral activity of each sample was expressed in μg/mL as 50% effective concentration (EC_{50}), and cytotoxicity was expressed as 50% inhibitory concentration (IC_{50}).

Plant Material. The whole plant of *R. nasutus* (Acanthaceae) was collected on January 1, 1993, in Thailand by Michael Balick of the New York Botanical Garden and on December 10, 1993, in Huay Kaew, Chiang Mai, by Rachan Pooma of the Royal Forest Department of Thailand. The plant was identified by Weerachai Nanakorn of the Botanical Garden Organization, Chiang 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, 213 E. Grand Ave., South San Francisco, CA 94080.

Extraction and Isolation. The aerial parts from *R. nasutus* (226 g) were ground to a powder and extracted with 1:1 dichloromethane−2-propanol with gentle stirring for ~24 h. After filtration, the extract was concentrated to dryness (4.64 g), suspended in 90% aqueous methanol (100 mL), and extracted successively with hexane (3 × 100 mL). The methanol-soluble fraction was concentrated to dryness (1.1 g), suspended in water, and purified on an HP-20 column (3.5 × 10 cm) eluting with a water, methanol, acetone gradient. Fractions eluting with methanol had activity against Flu-A; the active fractions were combined, evaporated and purified by HPLC on C18 (Dynamax, 20 × 250 mm) using a MeCN− 0.1% TFA/H_2O gradient (60−70% MeCN, 0−20 min; 70−95% MeCN, 20−30 min; 15 mL/min) to give **1** (1.8 mg, 0.0008%) and **2** (1.1 mg, 0.0005%).

Compound **1** was obtained as an amorphous powder: $[\alpha]_D$ −30.2° (*c* 0.076, MeOH); UV (MeOH) λ_{max} (log ϵ) 217 (3.17), 252 (3.71), 278 (3.67), 328 (3.66) nm; IR (film) ν_{max} 1740, 1710, 1600, 1490, 1480 cm^{-1}; ^1H and ^{13}C NMR, see Table 1; EIMS *m/z* 442 (9.4), 411 (3.9), 382 (3), 379 (2), 351 (4), 339 (5), 308 (8), 307 (48), 277 (4), 275 (3), 246 (4), 242 (3), 227 (2), 225 (5), 218 (7), 217 (50), 189 (2), 188 (3), 175 (3), 167 (3), 166 (22), 165 (100), 77 (15), 70 (11), 69 (13), 57 (18), 55 (15), 45 (18), 43 (14), 41 (14); HRMS *m/z* 442.1270 (calcd 442.1264 for $C_{23}H_{22}O_9$).

Compound **2** was isolated as an amorphous powder: $[\alpha]_D$ −5.2° (*c* 0.06, MeOH); UV (MeOH) λ_{max} (log ϵ) 219 (3.02), 280 (3.42), 335 (3.15) nm; ^1H NMR, see Table 1; EIMS *m/z* 444 (59), 413 (5), 381 (9), 279 (12), 249 (17), 247 (23), 238 (12), 237 (21), 236 (18), 207 (14), 206 (32), 205 (44), 189 (14), 176 (11), 175 (24), 166 (23), 165 (100), 152 (62), 150 (14), 135 (83), 122 (41), 113 (12), 79 (13), 77 (29), 50 (17), 44 (17), 43 (18); HRMS *m/z* 444.1412 (calcd 444.1420 for $C_{23}H_{24}O_9$).

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References and Notes

(1) Sendl, A.; Chen, J. L.; Jolad, S. D.; Stoddart, C.; Rozhon, E.; Kernan, M.; Nanakorn, W.; Balick, M. *J. Nat. Prod.* **1996**, *59*, 808−811.
(2) Kodama, O.; Ichikawa, H.; Akatsuka, T.; Santisopasri, V.; Kato, A.; Hayashi, Y. *J. Nat. Prod.* **1993**, *56*, 292−294.
(3) Nagaraju, N.; Rao, K. N. *J. Ethnopharmacol.* **1990**, *29*, 137−158.
(4) Rojanapo, W.; Tepsuwan, A.; Siripong, P. *Basic Life Sci.* **1990**, *52*, 447−452.
(5) Lewis, W. H.; Elvin-Lewis, M. P. F. *Medical Botany*; Wiley-Interscience: New York 1977; pp 328−329, 351.
(6) Mokkhasmit, M.; Ngarmwathana, W.; Sawasdimongkol, K.; Permphiphat, U. *J. Med. Assoc., Thailand* **1971**, *54*, 490−504.
(7) Subramanian, N. S.; Nagarajan, S. *J. Indian Chem. Soc.* **1981**, *58*, 926−927.
(8) Jakupovic, J.; Schuster, A.; Bohlmann, F.; King, R. M.; Robinson, H. *Planta Med.* **1986**, 18−20.
(9) Bohlmann, F.; Lonitz, M.; Knoll, K. H. *Phytochemistry* **1978**, *17*, 330−331.
(10) MacRae, W. D.; Towers, G. H. N. *Phytochemistry* **1984**, *23*, 1207−1220.
(11) Bedows, E.; Hatfield, G. M. *J. Nat. Prod.* **1982**, *45*, 725−729.
(12) Markkanen, T.; Makinen, M. L.; Maunuksela, E.; Himanen, P. *Drugs Expl. Clin. Res.* **1981**, *7*, 711−716.
(13) May, G.; Willuhn, G. *Drug Res.* **1978**, *28*, 1−7.
(14) Seif, R. *J. Virol.* **1980**, *36*, 421−428.
(15) Dowdle, W.; Kendal, A.; Noble, G. In *Diagnostic Procedures for Viral, Rickettsial, and Chlamydial Infections*, 5th Ed.; Lenette, E. H., Schmidt, N. J., Eds.; American Public Health Association: Washington, D.C. 1979; pp 585−609.
(16) Rozhon, E.; Cox, S.; Buontempo, P.; O'Connell, J.; Slater, W.; De Martino, J.; Schwartz, J.; Miller, G.; Arnold, E.; Zhang, A.; Morrow, C.; Jablonski, S.; Pinto, P.; Versace, R.; Duelfer, T; Girijavallabhan, V. *Antiviral Res.* **1993**, *21*, 15−35.
(17) Ubillas, R.; Jolad, S. D.; Bruening, R. C.; Kernan, M. R.; King, S. R.; Sesin, D. F.; Barrett, M.; Stoddart, C. A.; Flaster, T.; Kuo, J.; Ayala, F.; Meza, E.; Castañel, M.; McMeekin, D.; Rozhon, E.; Tempesta, M. S.; Barnard, D.; Huffman, J.; Smee, D.; Sidwell, R.; Soike, K.; Brazier, A.; Safrin, S.; Orlando, R.; Kenny, P. T. M.; Berova, N.; Nakanishi, K. *Phytomedicine* **1994**, *1*, 77−106.
(18) Wyde, P.; Meyerson, L.; Gilbert, B. *Drug Dev. Res.* **1993**, *28*, 467−472.

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