Chlorochimaphilin: A New Antibiotic from *Moneses uniflora*

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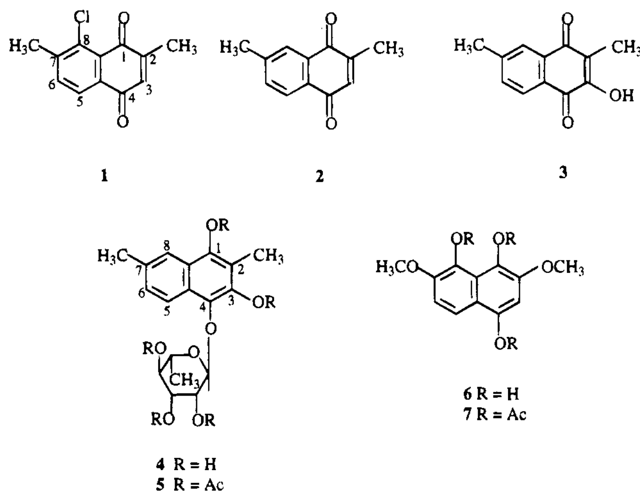
Received May 22, 1995®

A study of the antimicrobial compounds from *Moneses uniflora* resulted in the isolation of a novel compound, 8-chloro-2,7-dimethyl-1,4-naphthoquinone (8-chlorochimaphilin) (**1**), together with chimaphilin (**2**) and 3-hydroxychimaphilin (**3**) as the antimicrobial components. 2,7-Dimethyl-1,3-dihydroxynaphthyl 4-*O*-α-L-rhamnopyranoside (**4**) and 2,7-dimethoxy-1,4,8-trihydroxynaphthalene (**6**) were also isolated and identified.

In our continuing search for antibacterial and antifungal compounds from British Columbian medicinal plants, we found that extracts of *Moneses uniflora* A. Gray (Ericaceae) exhibited good activity against fungi and a number of Gram-positive and Gram-negative bacteria.[1,2] This species has been reported to be used by aboriginals in Canada to alleviate cough and cold, reduce pain, and cure cancer and paralysis.[3,4]

We report herein the isolation and the structure of an active chlorinated derivative of chimaphilin from *Moneses uniflora*, 8-chlorochimaphilin (**1**), together with chimaphilin (**2**) and 3-hydroxychimaphilin (**3**), using bioactivity-guided fractionation procedures. In addition, two naphthalene derivatives, **4** and **6**, were also identified.



Chimaphilin (**2**), a well-known naphthoquinone, has long been known to have antifungal, antibacterial, antihemorrhagic, and vitamin K-like activities[5] and to display anti-inflammatory and analgesic properties.[6]

Results and Discussion

The MeOH extract of the aerial parts of *M. uniflora*, when assayed for antimicrobial activity, gave a wide inhibition zone against *Candida albicans*, *Aspergillus fumigatus*, and *Microsporum gypseum*.

Chromatography of the MeOH extract on a Si gel column yielded 290 mg of chimaphilin (**2**). The physical and spectral data of **2** matched those of previously isolated chimaphilin in all respects.[6,7] Chimaphilin from *Pyrola rotundifolia* was reported to have anti-inflammatory and analgesic effects,[6] while from *Chimaphila umbellata*[5] it was reported to have antibacterial, antifungal, antihemorrhagic, and vitamin K-like activity. Kagawa *et al.* isolated this compound from *Pyrolae herba* and reported platelet aggregation inhibition and other activities.[7]

Compound **1** was slightly less polar than chimaphilin (**2**). EIMS of **1** revealed a molecular ion of m/z 220/222 with a ratio of about 3:1 due to the characteristic abundance of the two chlorine isotopes.[8,9] HRMS measurements provided the exact mass 220.0291, indicating a molecular formula of $C_{12}H_9O_2Cl$. This molecular weight was 34 mass units more than that for **2**. Its ^1H-NMR spectrum was almost superimposable with that of **2**, except for the disappearance of a one-proton doublet (δ7.88 for H-8) with a small coupling of 1 Hz, and there was a downfield shift of about 0.5 ppm for H-6 (δ 7.87), which appears as a doublet instead of a double doublet with respect to **2**, which indicated the presence of a chlorine atom at the C-8 position. ^{13}C-NMR assignments of **1**, when compared with those of **2**, also showed a downfield shifting of 7.4 ppm for C-8. These ^1H- and ^{13}C-NMR assignments together with COSY ($^1H-^1H$) and MS data proved compound **1** to be 8-chloro-2,7-dimethyl-1,4-naphthoquinone. To our knowledge, **1** is a novel natural product.

Compound **3** was more polar than chimaphilin (**2**). EIMS revealed a molecular ion of m/z 202, 16 mass units more than **2**, indicating the possibility of one more oxygen atom in **3**. The IR spectrum indicated the presence of a hydroxyl group (3410 cm^{-1}). The ^{13}C-NMR spectrum was similar to that of compound **2**, but in the ^1H-NMR spectrum a three-proton doublet at δ 2.17 was replaced by a singlet at δ 2.18, the H-3 quartet at δ 6.78 had disappeared, and a singlet due to a hydroxyl group appeared at δ 7.32 (D_2O exchangeable). The rest of the peaks were superimposable on the spectrum of **2**, indicating that the C-3 position was substituted by a hydroxyl group in compound **3**. This was further supported by its ^{13}C-NMR assignments in which a downfield shift of 8.9 ppm for C-3 was noticed. The EIMS spectrum of **3** gave major ions at m/z 174, 146, and 145,[10] further confirming the structure of **3** as 3-hydroxy-2,7-dimethyl-1,4-naphthoquinone (3-hydroxychimaphilin).

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® Abstract published in *Advance ACS Abstracts,* December 1, 1995.

0163-3864/96/3259-0062$12.00/0 © 1996 American Chemical Society and American Society of Pharmacognosy

Table 1. MIC Values (μg/mL) for Antibacterial Activity of the Crude Extract and Pure Compounds of *Moneses uniflora*

	microorganisms[14,15] used							
	E. coli		*P. aeruginosa*		*S. aureus*		*B. subtilis*	*C. albicans*
compound[a]	UB1005	DC2	K799	Z61	SAP00017	RN4220	Vernon	CAND105
MeOH extract	>800.0	>800.0	>800.0	>800.0	200.0	200.0	400.0	800.0
compound **1**	>800.0	800.0	>800.0	400.0	25.0	12.5	12.5	50.0
compound **2**	>800.0	>800.0	>800.0	>800.0	25.0	25.0	50.0	100.0
compound **3**	>800.0	>800.0	>800.0	>800.0	800.0	400.0	800	800.0
polymyxin B	2.0	1.0	2.0	2.0	32.0	32.	>32.0	>32.0
gentamicin	0.5	1.0	1.0	0.3	>32.0	0.3	16.0	>32.0
methicillin	>512.0	<4.0	256.0	<4.0	256.0	<4.0	128.0	>512.0
fungizone	>5.0	>5.0	>5.0	>5.0	>5.0	>5.0	>5.0	0.1

[a] Compounds **4**–**7** are inactive against the above-mentioned test organisms at the concentration of 800 μg/mL and below.

Compound **4**, obtained as a white powder, showed M^+ m/z 350.1370 in the HRMS spectrum, corresponding to a formula of $C_{18}H_{22}O_7$ (calcd 350.1365). This was confirmed by the CIMS spectrum, which showed a peak at m/z 368 MNH_4^+. The ^{1}H-NMR spectrum of **4** was similar to that of **3** except for additional peaks due to the sugar moiety. The presence of a downfield anomeric proton doublet at δ 3.98 (J = 2.0 Hz), a heavily overlapped five-proton multiplet in the δ 3.50–4.30 region, and a three-proton doublet at δ 0.85 indicated the presence of a 6-deoxy sugar attached with a (α-L)-glycosidic linkage. The glycosidic nature of **4** was supported by acid hydrolysis and by comparing the nature of the sugar moiety with an authentic sample that was proven to be L-rhamnose by co-TLC. The structure of **4** was further supported by its mass fragmentation, which gave m/z 350 for the glycoside; 188 for the aglycon moiety, and 163, 145, and 127 as sugar fragments. The presence of five hydroxyl groups in compound **4** was confirmed via pentaacetate derivative **5**. Thus, compound **4** was 1,3-dihydroxy-2,7-dimethylnaphthalene-4-*O*-α-L-rhamnopyranoside, a novel naturally occurring naphthalene glycoside.

A molecular formula of $C_{12}H_{12}O_5$ for **6** was disclosed by HRMS. Its IR spectrum showed a broadened hydroxyl absorption band centered at 3400 cm^{-1}. Its ^{1}H-NMR spectrum differed from that of **4** by the presence of two methoxy signals at δ 3.85 and 3.32, along with the disappearance of methyl peaks observed in **4**. A singlet at δ 6.8 and two coupled doublets at δ 7.9 and 6.4 (J = 8.0 Hz) of one proton each indicated that there is no substitution at C-3, C-5, and C-6 in the naphthalene nucleus. The presence of three hydroxyl groups in **6** was confirmed by the formation of a triacetate derivative **7**. Hence, compound **6** is 2,7-dimethoxy-1,4,8-trihydroxynaphthalene, also a new natural product (to our knowledge).

Oleanolic acid was also identified by ^{1}H NMR and direct comparison with an authentic sample.

Compounds **1**–**7** were evaluated for antimicrobial activity by an agar overlay bioautography method.[10] For those compounds that exhibited activity, MIC values were determined by a twofold serial microbroth dilution assay previously described.[11] The results of these assays are summarized in Table 1. 8-Chlorochimaphilin (**1**) and chimaphilin (**2**), the most active of the compounds isolated, showed good activity against *Staphylococcus aureus*, *Bacillus subtilis*, and *Candida albicans* when checked by the TLC overlay bioassay method. MICs in antifungal assays were obtained using a range of pathogenic fungi (Table 2). The order of activity was chlorochimaphilin (**1**) > chimaphilin (**2**) > hydroxy chimaphilin (**3**). None of the other isolated compounds was active. It is known that naphthoquinone derivatives, in contrast to naphthalene derivatives, can act as inhibitors of bacterial growth by participating competitively in electron transport, as described by Ambrogi and co-workers using synthetic naphthoquinone derivatives.[12]

Table 2. MIC (μg/mL) for Antifungal Activity of the Crude Extract and Pure Compounds of *Moneses uniflora*

microorganisms used	MeOH extract	**1**	**2**	**3**	nystatin
Candida albicans	>400	100	50	50	10
C. lipolytica	>400	50	50	25	>10
Saccharomyces cervisiae	>400	100	50	>100	10
Microsporum gypseum	50	3.1	6.2	12.5	2.5
Fusarium tricuictum	50	12.5	12.5	25	5
Aspergillus flavus	400	25	25	100	>10
A. fumigatus	400	25	50	50	5

Crude fractions as well as compounds **1**, **2**, and **3** were also tested against Herpes simplex virus-1 and Respiratory Sincytial virus at 200 μg/mL concentration and found to be inactive.

Sheth and co-workers[13] reported that Wasco Indians of the Warm Springs Reservation of Oregon as well as the Sahaptin-speaking Indians were using decoctions of whole plant of *Chimaphila umbellata* for "Consumption" or "tuberculosis". We tested the activity of crude extracts and of compounds **1**, **2**, and **3** against *Mycobacterium tuberculosis* 107 and *Mycobacterium avium* 724 and found that there was no inhibition at concentrations of 100 μg/disk.

Experimental Section

General Experimental Procedures. NMR spectra (in $CDCl_3$ if not otherwise stated) {δ values in ppm relative to internal Me_4Si(= 0 ppm) and J values in Hz}: Bruker WH-400 spectrometer {^{1}H-^{1}H COSY experiments}, Varian XL-300 spectrometer {^{1}H at 300 MHz and ^{13}C at 75 MHz}, and Bruker AC-200E spectrometer. UV spectra (MeOH) were taken on a PU8007 UV/VIS spectrophotometer. IR spectra were recorded on Perkin-Elmer 1710 spectrometer. Low resolution and high resolution EIMS were obtained on a Kratos MS 50 mass spectrometer at 70 eV. DCI on a Delsi Nermag R10-10 C mass spectrometer with NH_3 as the CI gas.

Microorganisms Used. Bacterial strains[14,15]: *B. subtilis* vernon, *Escherichia coli* UB1005, *E. coli* DC2, *Pseudomonas aeruginosa* Z61, *P. aeruginosa* K799, *S. aureus* meth^S RN4220, *S. aureus* meth^R P00017, *M. tuberculosis* 107, and *M. avium* 724.

Fungal strains: *A. fumigatus*, *Aspergillus flavus*, *C. albicans*, *Candida lipolytica*, *Fusarium tricuictum*, *M. gypseum*, and *Saccharomyces cerevisiae*.

Viral strains: Herpes simplex virus-1, Respiratory Sincytial virus.

Antibacterial and Antifungal Assay. The disk diffusion assay[16] was used for screening of antibacterial and antifungal activity of the crude plant extract. Sterile Mueller-Hinton agar plates (for bacterial cultures) and sterile yeast morphology agar (Difco) plates (for fungal strains) were used. These plates were inoculated with the desired microorganisms before placing the extract-impregnated paper disks on the plates. Gentamicin (for antibacterial testing) and Nystatin (for antifungal testing) were used as positive controls, and MeOH was used as a negative control. The plates were incubated for different times and at temperatures suitable to the specific strains tested.[1,2]

Antiviral Assay. Cell culture and cytotoxicity assays were performed using a cell culture system of the monolayer-forming vero cell line grown in 96-well trays. In the antiviral assays 2-fold serial dilutions of the plant extracts were used, starting with non-cytotoxic concentrations. The infectivity of two test viruses was assayed qualitatively through microscopic observations of characteristic viral cytopathic effect (CPE). The cell layers were exposed to the extract mixture for 1 h at 37 °C, and then the viruses were added at the concentration of 100 plaque-forming units (PFU)/well and incubated for 3 days.[17]

Antituberculosis Assay. *M. tuberculosis* 107 (American Type Culture Collection No. 35801) and *M. avium* 724 (ATCC No. 25291) were grown, stored, and assessed for viability as previously described.[15] Screening was performed using a modified disk diffusion assay. Disks were loaded with 10 μL of extract in MeOH (representing 100 μg of compound) and allowed to dry. Disks were placed in quadrant petri dishes and overlaid with Difco's Middlebrook 7H10 agar. Plates were incubated overnight at 4 °C to allow for the diffusion of the compound. Each quadrant was then inoculated with 100 μL of bacterial suspension. For *M. tuberculosis* the inoculum was 1000 CFU, for *M. avium* the inoculation was 200 CFU. Plates were incubated for 4 weeks (*M. tuberculosis*) or 2 weeks (*M. avium*) at 37 °C to allow for bacterial growth. Compounds were screened for complete inhibition of growth as compared to controls, which received only MeOH (negative control) or 10 μg/mL Isoniazid (positive control) [*M. tuberculosis* is susceptible to Isoniazid; *M. avium* is resistant].

Minimum Inhibitory Concentration (MIC). MIC values for antibacterial activity were determined by the broth microtitre dilution method using an inoculum of 10^4-10^5 cells/mL, and results were read after 24-h incubation at 37 °C. All bacterial strains were grown at 37 °C in Muller-Hinton broth. Fungal strains were grown in yeast and mold broth (Difco) at 30 °C for 48 h with an inoculum of 10^3-10^5 cells/mL.

Extraction and Isolation. The aerial parts of *M. uniflora* were collected in Graham Island, Queen Charlotte Island, British Columbia, Canada, in June/July 1993, and identified by A. R. McCutcheon of the Department of Botany, University of British Columbia. A voucher specimen was deposited in the herbarium of the Department of Botany, University of British Columbia, Vancouver. Air-dried, powdered plant material (215.5 g) was extracted with MeOH (2.5 L × 3) and evaporated to dryness, first on a rotaevaporator and then on a freeze dryer (26.1 g). The MeOH extract (10 g) was chromatographed on a Si gel column (60−230 mesh), which was eluted with petroleum ether/EtOAc of increasing polarity followed by increasing polarity of MeOH in EtOAc. Finally, the column was washed with a mixture of EtOAc/MeOH/Me_2CO. In all, 28 fractions were collected. Isolation of active components was carried out by activity-guided purification using a bioautography agar overlay method.[10] Fractions 7−9 gave pure compound **2** (290 mg), while other fractions were subjected to further purification by CC, VLC, or prep. TLC. Fractions 5 and 6, when subjected to VLC using petroleum ether and petroleum ether/EtOAc (98:2, v/v), gave pure compound **1** (17.8 mg). Compound **3** (42.5 mg) was isolated by VLC using petroleum ether/EtOAc (90:10, v/v) from fractions 11−13. Fractions 15−17 applied to a Si column and eluted with increasing polarity of EtOAc in petroleum ether afforded oleanolic acid (29 mg), which was further purified by prep. TLC ($CHCl_3$\ MeOH, 95:5). Fraction 24 when eluted with $CHCl_3$ and increasing polarity of MeOH on a Si column gave compound **4** (138.9 mg), which was further purified by VLC. Compound **6** (28 mg) and methyl α-D-glucopyranoside (36 mg) were isolated from fractions 25 and 26 by CC using increasing polarity of MeOH in $CHCl_3$ and crystallizing with MeOH. Methyl α-D-glucopyranoside is possibly an artifact found during isolation.

8-Chloro-2,7-dimethyl-1,4-naphthoquinone (1). Green needles; mp 58−61 °C; IR ν_{max} ($CHCl_3$) 1662, 1603, 1297, 1225 cm^{-1}; EIMS m/z 220/222 (M^+, 16.7/6.2), 188 (100), 186 (81.2), 171 (7.4), 158 (37), 129 (29.2), 118 (41.4); HRMS m/z M^+ 220.0291 (calcd for $C_{12}H_9O_2Cl$, 220.0291); ^1H NMR δ ($CDCl_3$) 8.08 (d, J = 8.3 Hz, H-5), 7.87 (d, 8.3, H-6), 6.53 (q, 1.5, H-3), 2.48 (d, 1.5, 2-CH_3), 2.32 (s, 7-CH_3); ^{13}C NMR δ($CDCl_3$) 187.4 (C-1), 145.5 (C-2), 139.0 (C-3), 186.2 (C-4), 126.2 (C-5), 127.5 (C-6), 134.0 (C-7), 134.2 (C-8), 132.8 (C-9), 130.2 (C-10), 17.8 (2-CH_3), 24.2 (7-CH_3).

2,7-Dimethyl-1,4-naphthoquinone (2). Yellow needles; mp 99 °C; IR ν_{max} ($CHCl_3$) 1662, 1603 cm^{-1}; EIMS m/z 186 M^+ (100), 171 (15.3), 158 (25.8), 144 (2.5), 129 (12.7), 118 (25.2); HRMS m/z M^+ 186.0673 (calcd for $C_{12}H_{10}O_2$, 186.0681); ^1H NMR δ ($CDCl_3$) 7.92 (d, J = 8.0 Hz, H-5), 7.88 (d, 1.0, H-8), 7.48 (dd, 8.0, 1.0, H-6), 6.78 (q, 1.5, H-3), 2.48 (s, 7-CH_3), 2.17 (d, 1.5, 2-CH_3); ^{13}C NMR δ ($CDCl_3$) 184.5 (C-1), 148.2 (C-2), 144.6 (C-3), 185.9 (C-4), 126.2 (C-5), 135.7 (C-6), 134.3 (C-7), 126.8 (C-8), 132.0 (C-9), 131.1 (C-10), 16.4 (2-CH_3), 21.8 (7-CH_3). ^1H-NMR, ^{13}C-NMR, and MS data were identical with those of reported values for chimaphilin.[6,7]

3-Hydroxy-2,7-dimethyl-1,4-naphthoquinone (3). Bright yellow needles; mp 171 °C; IR ν_{max} ($CHCl_3$) 3410, 1661, 1600 cm^{-1}; EIMS m/z 202 M^+(100), 174 (35.6), 146 (18.0), 145 (17.8), 119 (44.0), 118 (27.3), 91 (33.0), 90 (18.5); ^1H NMR δ ($CDCl_3$) 7.92 (d, J = 8.0 Hz, H-5), 7.89 (d, 1.0, H-8), 7.45 (dd, 8.0, 1.0, H-6), 7.32 (s, 3-OH) (D_2O exchange), 2.49 (s, 7-CH_3), 2.18 (s, 2-CH_3); ^{13}C NMR δ ($CDCl_3$) 185.5 (C-1), 145.2 (C-2), 153.5 (C-3), 181.5 (C-4), 126.3 (C-5), 132.8 (C-6), 133.4 (C-7), 127.2 (C-8), 127.0 (C-9), 120.0 (C-10), 8.64 (2-CH_3), 22.06 (7-CH_3).

1,3-Dihydroxy-2,7-dimethylnaphthyl 4-*O*-α-L-Rhamnopyranoside (4). White crystals, mp 205 °C; EIMS *m/z* M^+ 350 (0.6), 188 (100), 163 (30.0), 145 (7.4), 127 (1.4); HRMS *m/z* M^+ 350.1370 (calcd for $C_{18}H_{22}O_7$); ^1H NMR δ (CD_3OD) 6.62 (d, J = 1.0 Hz, H-8), 6.42 (d, 8.0, H-5), 5.62 (dd, 8.0, 1.0, H-6), 3.98 (d, 1.5, H-1′), 1.68 (s, 7-CH_3), 0.90 (s, 2-CH_3), 0.85 (d, 8.0, C-6′); ^{13}C NMR δ (CD_3OD) 108.78 (C-1), 149.0 (C-2), 104.5 (C-4), 127.5 (C-5), 134.7 (C-7), 128.0 (C-8), 125.0 (C-9), 120.5 (C-10), 20.1 (7-CH_3), 5.6 (2-CH_3), 100.6 (C-1′), 69.9 (C-2′), 74.0 (C-3′), 60.9 (C-4′), 76.2 (C-5′), 15.9 (C-6′).

Acetate of 4. Acetylation of **4** with acetic anhydride and pyridine gave penta-acetate derivative **5**; EIMS *m/z* M^+ 562 (0.2), 502 (0.1), 458 (0.2), 415 (0.2), 373 (0.7), 331 (28.7), 271 (4.2), 230 (4.8), 211 (4.1), 188 (29.8), 169 (98.5), 145 (7.7), 139 (9.2), 127 (21.07); ^1H NMR δ ($CDCl_3$) 7.9 (s, H-8), 7.63 (d, J = 8.0 Hz, H-5), 7.25 (dd, 8.0, 1.0, H-6), 4.98 (d, 8.0, H-1′), 2.5 (s, 7-CH3), 2.42 (d, C-6′), 2.35 (s, OAc), 2.32 (s, OAc), 2.12 (s, OAc), 2.0 (s, OAc), 1.85 (s, OAc), 1.55 (s, 2-CH_3).

Acid Hydrolysis of 4. Compound **4** (10 mg) was refluxed with 4% H_2SO_4 (5 mL) in MeOH for 1 h. The reaction mixture was then concentrated under reduced pressure to remove MeOH, diluted with H_2O, and extracted with Et_2O. The aqueous layer was adjusted to pH 7 with $BaCO_3$ and filtered. The filtrate was concentrated and examined by TLC using $CHCl_3$/MeOH/Me_2CO/H_2O (50:40:10:2, v/v). L-Rhamnose was identified by comparison with an authentic sample.

2,7-Dimethoxy-1,4,8-trihydroxynaphthalene (6). White crystals, mp 120 °C; EIMS *m/z* M^+ 236 (28.2), 222 (8.2), 208 (100), 188 (56.3); HRMS *m/z* {M^+} 236.0691 (calcd for $C_{12}H_{12}O_5$, 236.0685); ^1H NMR δ (CD_3OD) 7.9 (d, J = 8.0 Hz, H-5), 6.8 (s, H-3), 6.4 (d, 8.0, H-6), 3.85 (s, OCH_3), 3.32 (s, OCH_3).

Acetate of 6. Acetylation of **6** with acetic anhydride and pyridine gave triacetate derivative **7**; EIMS *m/z* M^+ 362 (0.2), 331 (0.4), 302 (0.3), 271 (0.6), 238 (3.2), 208 (3.7), 206 (22.0), 187 (3.4), 149 (72.6); ^1H NMR δ ($CDCl_3$) 7.63 (d, J = 9.5 Hz, H-5), 6.70 (s, H-3), 6.35 (d, 9.5, H-6), 3.86 (s, OCH_3), 3.40 (s, OCH_3), 2.35 (s, $OCOCH_3$), 2.10 (s, $OCOCH_3$), 2.05 (s, $OCOCH_3$).

Acknowledgements. This work was supported by grants from the Canadian Bacterial Diseases Network and the Natural Sciences and Engineering Research Council of Canada. We are grateful to Ms. Robin S. Taylor, Department of Botany, University of British Columbia, and Prof. J.B. Hudson, Department of Medical Microbiology, University of British Columbia, for the antiviral testing and to Prof. David Speert's group, especially Ms. Lisa Thorson and Dr. Richard Stokes, Department of Pediatrics, University of British Columbia, for testing anti-tuberculosis activity. We also thank the NMR and MS laboratories of the Department of Chemistry, University of British Columbia, for acquisition of spectra.

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NP960006V