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Dibenz[*b*,*f*]oxepin and Antimycobacterial Chalcone Constituents of *Empetrum nigrum*

Haoxin Li,[†] Stéphanie Jean,[‡,§] Duncan Webster,[⊥] Gilles A. Robichaud,[‡,§] Larry A. Calhoun,[∥] John A. Johnson,[†] and Christopher A. Gray*[,†,∥]

[†]Department of Biology, University of New Brunswick, Saint John, New Brunswick, Canada E2L 4L5

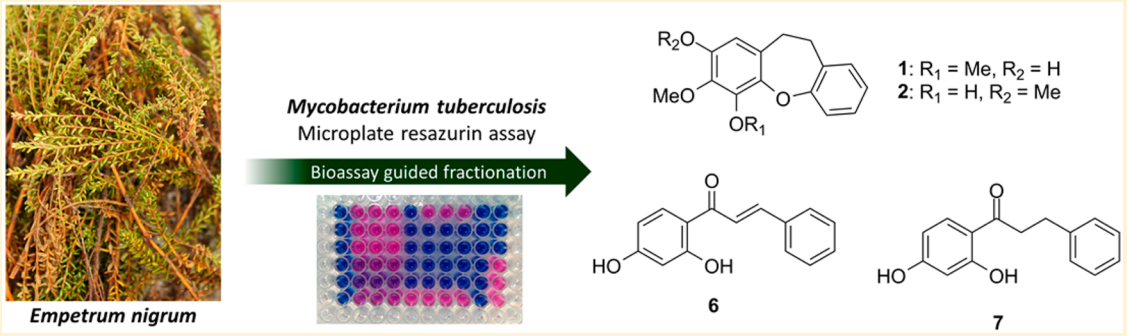
[‡]Department of Chemistry and Biochemistry, Université de Moncton, Moncton, New Brunswick, Canada E1A 3E9

[§]Atlantic Cancer Research Institute, Moncton, New Brunswick, Canada E1C 8 × 3

[⊥]Department of Medicine, Division of Infectious Diseases, Saint John Regional Hospital, Saint John, New Brunswick, Canada E2L 4L2

[∥]Department of Chemistry, University of New Brunswick, 30 Dineen Drive, Fredericton, New Brunswick, Canada E3B 5A3

Supporting Information



ABSTRACT: Two new dibenz[*b*,*f*]oxepins, empetroxepins A and B (**1** and **2**), and seven known compounds (**3**–**9**) were isolated from an extract of the Canadian medicinal plant *Empetrum nigrum* that significantly inhibited the growth of *Mycobacterium tuberculosis* H37Ra. The structures of **1** and **2** were established through analysis of NMR and MS data. The antimycobacterial activity of the plant extract was attributed primarily to the presence of two chalcone derivatives (**6** and **7**) that exhibited selective antimycobacterial activity (IC_{50} values of 23.8 and 32.8 μM, respectively) in comparison to mammalian (HEK 293) cells (IC_{50} values of 109 and 249 μM, respectively).

The black crowberry, *Empetrum nigrum* L. (Ericaceae), is widely distributed in the higher latitudes of the Northern hemisphere.[1] The plant is used medicinally by Canadian First Nations communities as an antidiarrheal drug and a cold remedy (Upper Tanana),[2] a pediatric aid (Cree),[2] and a tuberculosis remedy (Haida Gwaii).[3] The ethnopharmacological significance of *E. nigrum* has prompted numerous investigations of the plant's secondary metabolites.[3−9] Compounds previously isolated from *E. nigrum* include bibenzyls,[4−6] chalcones,[6,9] dihydrochalcones,[6,9] flavonoids,[6,7] dihydrophenanthrenes,[3,9] and terpenoids.[6,8] However, only the bibenzyls and dihydrophenanthrenes have exhibited antimicrobial activities,[3] and none of the previously isolated compounds have been reported to possess antimycobacterial activities.

Extracts of *E. nigrum* have exhibited promising antimycobacterial activities in preliminary screenings of First Nations medicinal plants,[10,11] which prompted us to further investigate this species. Bioassay-guided fractionation employing a combination of solvent partitioning, flash chromatography, and normal- and reversed-phase HPLC resulted in the isolation of two new dibenz[*b*,*f*]oxepins, empetroxepins A (**1**) and B (**2**), in addition to seven known compounds (**3**–**9**).

Compound **1** was isolated as a colorless, amorphous solid that gave a protonated molecular ion $[M + H]^+$ at m/z 273.1126 in the positive-ion HRESIMS, consistent with a molecular formula of $C_{16}H_{16}O_4$ (calcd 273.1121) that implied nine degrees of unsaturation. The ^{13}C NMR spectrum of **1** (Table 1) revealed 16 resonances that were assigned to a bibenzyl skeleton:[4,5] 12 aromatic carbons [δ_C 144.8 (C-1), 138.4 (C-2), 145.3 (C-3), 109.3 (C-4), 130.2 (C-4a), 130.9 (C-6a), 131.2 (C-7), 123.8 (C-8), 127.3 (C-9), 121.4 (C-10), 157.0 (C-10a), and 144.5 (C-11a)]; two benzylic methylenes [δ_C 30.3 (C-5) and 31.6 (C-6)]; and two oxymethyls [δ_C 62.0 (OMe-1) and 61.5 (OMe-2)]. The ^1H NMR spectrum and multiplicity-edited HSQC spectrum provided further evidence to support a bibenzyl skeleton through the presence of five aromatic methines [δ_H 7.10 (H-7, m), 7.01 (H-8, m), 7.15 (H-9, m), 7.22 (H-10, m), and 6.51 (H-4, s)], two benzylic

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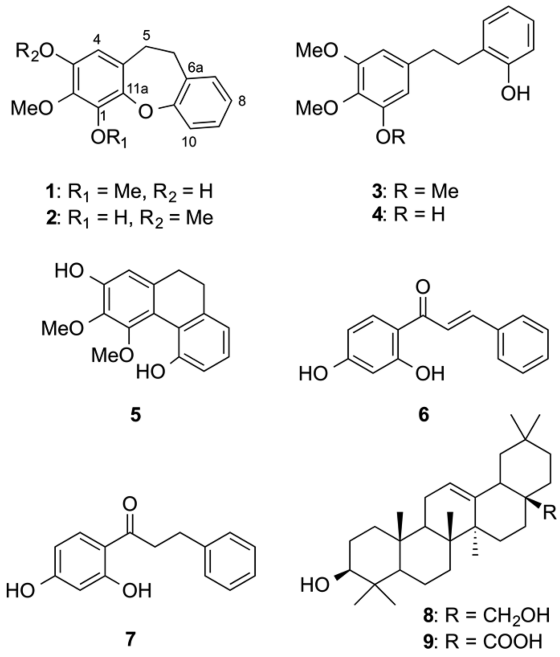

methylenes [δ_H 3.07 (H_2-5, m) and 3.10 H_2-6, m)], two methoxy groups [δ_H 4.00 (OMe-1, s) and 3.93 (OMe-2, s)], and one phenolic hydroxy group proton [δ_H 5.47, (OH-3, brs)]. The carbon skeleton of **1** was assembled from analysis of the HMBC data (Figure 1), which showed correlations from H-4 to C-1, C-2, and C-5; H-5 to C-4, C-4a, C-6, and C-6a; H-6 to C-4a, C-5, C-6a, and C-7; H-7 to C-6, C-9, and C-10a; H-10 to C-6a, C-8, and C-10a; OMe-1 to C-1; and OMe-2 to C-2, and the COSY data, which revealed a conjugated spin system between H-7, H-8, H-9, and H-10 (Figure 1). However, as the HMBC spectrum (optimized for heteronuclear coupling of 8 Hz) did not show any correlations for the OH-3 proton, we relied on a constant time inverse-detection gradient accordion rescaled HMBC experiment (CIGAR-HMBC) to reveal two- and three-bond correlations from OH-3 to C-2, C-3, and C-4.

The presence of a third ring in **1** was suggested by only eight degrees of unsaturation being accounted for within the carbon

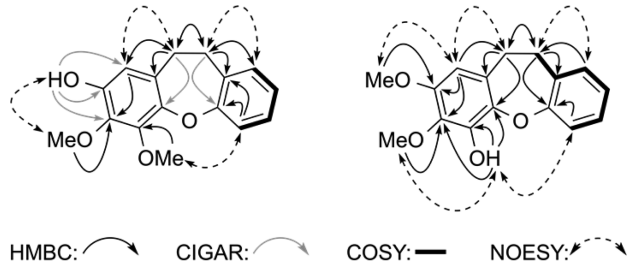

Figure 1. Key NMR correlations observed for empetroxepins A (**1**) and B (**2**).

skeleton, and the ^{13}C NMR shifts of C-10a and C-11a were consistent with oxygenated aromatic carbons implying that they are joined by an ether linkage. This inference was supported by the NOESY data, with NOEs being observed between OMe-1 and H-10. NOEs also confirmed the placement of C-1, C-2, C-3, and C-4 through correlations between H-4 and H-5; OH-3 and OMe-2; and H-10 and OMe-1. On the basis of the spectroscopic data obtained, **1** was therefore identified as 5,6-dihydro-1,2-dimethoxy-3-hydroxydibenz[*b*,*f*]oxepin and is named empetroxepin A.

Compound **2** was isolated as a colorless, amorphous solid that was found to be isomeric with **1** on the basis of a protonated molecular ion $[M + H]^+$ that was observed at *m*/*z* 273.1122 (calcd for $C_{16}H_{17}O_4$, 273.1121) in the positive-ion HRESIMS. The NMR data of **2** were similar to those of **1** (Table 2), although HMBC correlations indicated that the substituents at C-1 (methoxy group) and C-3 (hydroxy group) in **1** were transposed in **2**. This was supported by the observation of NOE correlations between H-4 [δ_H 6.18 (1H, brs)] and OMe-3 [δ_H 3.81 (3H, s)] and between OH-1 [δ_H 5.89 (1H, brs)] and OMe-2 [δ_H 3.89 (3H, s)] and a weak NOE between OH-1 and H-10 [δ_H 7.19 (1H, m)] (Figure 1). On the basis of this evidence, **2** was identified as 5,6-dihydro-2,3-dimethoxy-1-hydroxydibenz[*b*,*f*]oxepin and named empetroxepin B.

The known compounds batatasin V (**3**),[12] 2′-hydroxy-3-hydroxy-4,5-dimethoxybibenzyl (**4**),[4] 9,10-dihydro-2,5-dihydroxy-3,4-dimethoxyphenanthrene (**5**),[13] 2′,4′-dihydroxychalcone (**6**),[9] 2′,4′-dihydroxydihydrochalcone (**7**),[9] erythrodiol (**8**),[14] and

Table 1. NMR Spectroscopic Data Obtained for Empetroxepin A (1)[a]

position	δ_C, mult.	δ_H (int., mult., *J* in Hz)	HMBC	CIGAR HMBC	NOE	COSY
1	144.8, s					
2	138.4, s					
3	145.3, s					
4	109.3, d	6.51 (1H, s)	2, 3, 5, 11a		5	
4a	130.2, s					
5	30.3, t	3.07 (2H, m)	4, 4a, 6, 6a	11a	4	
6	31.6, t	3.10 (2H, m,)	4a, 5, 6a, 7	10a	7	
6a	130.9, s					
7	131.2, d	7.10 (1H, m)	6, 9, 10a		6	8
8	123.8, d	7.01 (1H, m)	6a, 10			7, 9
9	127.3, d	7.15 (1H, m)	7, 10a			8, 10
10	121.4, d	7.22 (1H, m)	6a, 8, 10a		OMe-1	9
10a	157.0, s					
11a	144.5, s					
OMe-1	62.0, q	4.00 (3H, s)	1, 2, 11a		10	
OMe-2	61.5, q	3.93 (3H, s)	2		OH-3	
OH-3		5.47 (1H, brs)		2, 3, 4	OMe-2	

[a]Recorded in $CDCl_3$ at 400 MHz for ^1H and 100 MHz for ^{13}C.

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Table 2. NMR Spectroscopic Data Obtained for Empetroxepin B (2)[a]

position	δ_C, mult.	δ_H (int. mult., J in Hz)	HMBC	NOE	COSY
1	141.5, s				
2	134.9, s				
3	149.1, s				
4	103.4, d	6.18 (1H, brs)	2, 3, 5, 11a	5, OMe-3	5, OMe-3
4a	132.3, s				
5	31.03, t	3.08 (2H, m)	4a, 6, 6a, 11a	4	4, 6
6	30.97, t	3.14 (2H, m)	4, 5, 6a, 10a	7	5, 7
6a	126.7, s				
7	130.6, d	7.16 (1H, m)	6, 8, 10a	6	6, 9
8	127.5, d	7.18 (1H, m)	10a		9
9	124.5, d	7.06 (1H, m)	10, 10a		7, 8, 10
10	120.9, d	7.19 (1H, m)	10a	OH-1	9
10a	157.2, s				
11a	139.0, s				
OH-1		5.89 (1H, brs)	1, 2, 11a	10, OMe-2	
OMe-2	61.1, q	3.89 (3H, s)	2	OH-1	
OMe-3	56.3, q	3.81 (3H, s)	3	4	4

[a]Recorded in $CDCl_3$ at 400 MHz for ^1H and 100 MHz for ^{13}C.

oleanolic acid (**9**)[14] were also isolated from *E. nigrum*. Compounds **3**–**9** were identified by comparison of their respective NMR and HRESIMS data with literature values, and, of these, only **9** has previously been reported from the *Empetrum* genus.[6]

Empetroxepins A (**1**) and B (**2**) are new members of a small family of dibenz[*b*,*f*]oxepins that are relatively rare in nature[15] and isolated predominantly from the genus *Bauhinia* (e.g., pacharin from *B. racemosa*,[16] the bauhinoxepins from *B. saccocalyx*[17] and *B. purpurea*,[18] and the bauhiniastatins from *B. purpurea*[15]). Indeed, prior to the present work only two dibenz[*b*,*f*]oxepins, both derivatives of pacharin isolated from *Cercis chinensis*,[19] have been reported outside the genus *Bauhinia*. Within the dibenz[*b*,*f*]oxepins, **1** and **2** present additional examples of a subclass lacking a benzylic methyl group at C-2 that was previously only represented by bauhinoxepin J.[18]

Although dibenz[*b*,*f*]oxepins have displayed notable antimalarial, antimycobacterial, and cytotoxic activities,[15,18] **1** and **2** displayed only weak antimycobacterial activity and low selectivity (see Table 1). The significant antimycobacterial activity initially observed for *E. nigrum* was primarily due to the presence of the chalcone **6** and the dihydrochalcone **7**, which were the principal antimycobacterial constituents of the extract (Table 1).

EXPERIMENTAL SECTION

General Experimental Procedures. All solvents for extraction and isolation were purchased from Fisher Scientific (Ottawa, ON, Canada). Optical rotations were determined using a Rudolph Autopol III polarimeter equipped with a halogen lamp (589 nm) and a 5 cm sample cell. IR spectra were recorded on a PerkinElmer Spectrum Two FT-IR spectrometer as thin films on sodium chloride disks. NMR solvents were purchased from Sigma-Aldrich (Oakville, ON, Canada). NMR spectra were recorded on an Agilent 400-MR DD2 instrument in $CDCl_3$ and CD_3OD and were calibrated to residual protonated solvent resonances (δ_H 7.260 and 3.310; δ_C 77.160 and 49.000, respectively). HRMS was recorded on a Thermo LTQ Exactive instrument with an ESI source. Flash chromatography was performed using a Biotage Flash + chromatography system and KP-Sil 25+S silica cartridges (40–63 μm, 60 Å). Size-exclusion chromatography was performed with Sephadex LH-20 (25–100 μm). Semipreparative normal-phase HPLC was performed on a Waters 510 pump, a Waters R401 refractive index detector, and a Phenomenex Luna silica column (250 × 10 mm, 10 μm, 100 Å). Semipreparative reversed-phase HPLC was performed on a Waters 600 system, a 2487 dual-λ absorbance detector, and a Phenomenex Luna C_{18} column (250 × 10 mm, 10 μm, 100 Å).

Plant Material. *Empetrum nigrum* (identified by Dr. Stephen Clayden, New Brunswick Museum) was collected by hand from Prince of Wales, New Brunswick, Canada (45°11.932′ N, 066°13.803′ W) in May 2010. The aerial parts of the plant were washed with water to remove debris, freeze-dried, and stored at −20 °C. A voucher specimen has been deposited in the New Brunswick Museum Herbarium (NBM VP-37479).

Extraction and Isolation. Freeze-dried plant material (40 g) was ground into a powder and exhaustively extracted (8 h) with MeOH in a Soxhlet apparatus. Removal of the MeOH in vacuo gave a crude extract (10.3 g) that exhibited significant antimycobacterial activity and was subjected to bioassay-guided fractionation. A portion of the crude extract (10.0 g) was fractionated using a modified Kupchan solvent–solvent partition to give five fractions.[20] The CH_2Cl_2 fraction (2.3 g) exhibited significant bioactivity and was separated by silica gel flash chromatography using a stepwise gradient from 100% hexanes to 100% EtOAc (10% increments of EtOAc) to afford 11 fractions (fractions A–K).

Fraction B (279 mg) was further fractionated by silica gel flash chromatography (column eluted with 100% hexanes, 19:1, 23:2, 9:1, 87:13, 83:17, and 4:1 hexanes–EtOAc, and 100% EtOAc) to afford 11 fractions (fractions B_1–B_{11}). Fraction B_7 (24 mg) was subjected to normal-phase HPLC (17:3 hexanes–EtOAc) to afford empetroxepin A (**1**, 6 mg). Fraction B_8 (25 mg) was subjected to normal-phase HPLC (9:1 hexanes–EtOAc) to afford empetroxepin B (**2**, 1 mg). Fraction B_9 (26 mg) was subjected to normal-phase HPLC (17:3 hexanes–EtOAc) and reversed-phase HPLC (3:1 MeOH–H_2O) to afford 2′,4′-dihydroxydihydrochalcone (**7**, 4 mg) and 9,10-dihydro-2,5-dihydroxy-3,4-dimethoxyphenanthrene (**5**, 3 mg). Fraction B_{10} (64 mg) was subjected to normal-phase HPLC (17:3 hexane–EtOAc) and reversed-phase HPLC (3:1 MeOH–H_2O) to afford 2′,4′-dihydroxychalcone (**6**, 2 mg).

Fraction C (610 mg) was fractionated by silica gel flash chromatography (column eluted with 100% hexanes, 19:1, 23:2, 9:1, 17:3, 41:9, 4:1, and 3:1 hexanes–EtOAc, and 100% EtOAc) to afford eight fractions (fractions C_{1-8}). Fraction C_4 was separated by normal-phase HPLC (83:17 hexanes–EtOAc) and reserved-phase HPLC (47:3 MeOH–H_2O) to afford oleanolic acid (**9**, 2 mg). Fraction C_5 (308 mg) was subjected to size-exclusion chromatography over Sephadex LH-20 (1:1 CH_2Cl_2–MeOH) to afford batatasin V (**4**, 15 mg) and 2′-hydroxy-3-hydroxy-4,5-dimethoxybibenzyl (**3**, 135 mg).

Fraction K (64 mg) was subjected to normal-phase HPLC (17:3 hexanes–EtOAc) and reserved-phase HPLC (19:1 MeOH–H_2O) to afford erythrodiol (**8**, 2 mg).

*Empetroxepin A (**1**):* colorless, amorphous solid; IR (thin film) ν_{max} 3398, 2936, 2840, 1590, 1481, 1268, 1206, 1111, 1082 cm^{-1}; ^1H and ^{13}C NMR, see Table 1; HRESIMS m/z 273.1126 $[M + H]^+$ (calcd for $C_{16}H_{17}O_4$, 273.1121).

*Empetroxepin B (**2**):* colorless, amorphous solid; IR (thin film) ν_{max} 3429, 2933, 2846, 1598, 1486, 1230, 1201, 1125, 1076 cm^{-1}; ^1H and ^{13}C NMR, see Table 1; HRESIMS m/z 273.1122 $[M + H]^+$ (calcd for $C_{16}H_{17}O_4$, 273.1121).

*Batatasin V (**3**):* colorless oil; IR (thin film) ν_{max} 3417, 2937, 1591, 1506, 1456, 1130 cm^{-1}; ^1H and ^{13}C NMR data were consistent with literature values;[12] HRESIMS m/z 289.1434 $[M + H]^+$ (calcd for $C_{17}H_{21}O_4$, 289.1434).

*2′-Hydroxy-3-hydroxy-4,5-dimethoxybibenzyl (**4**):* colorless oil; IR (thin film) ν_{max} 3322, 2940, 1593,1509, 1458, 1096 cm^{-1}; ^1H and ^{13}C NMR data were consistent with literature values;[4] HRESIMS m/z 275.1279 $[M + H]^+$ (calcd for $C_{16}H_{19}O_4$, 275.1278).

*9,10-Dihydro-2,5-dihydroxy-3,4-dimethoxyphenanthrene (**5**):* colorless, amorphous solid; IR (thin film) ν_{max} 3250, 2940, 1580, 1458, 1345, 1003 cm^{-1}; ^1H and ^{13}C NMR data were consistent with

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literature values;[13] HRESIMS m/z 273.1121 $[M + H]^+$ (calcd for $C_{16}H_{17}O_4$, 273.1121).

*2′,4′-Dihydroxychalcone (**6**):* yellow, amorphous solid; IR (thin film) ν_{max} 3264, 3028, 2930, 1752, 1630, 1138 cm^{-1}; ^1H and ^{13}C NMR data were consistent with literature values;[9] HRESIMS m/z 241.0859 $[M + H]^+$ (calcd for $C_{15}H_{13}O_3$, 241.0859).

*2′,4′-Dihydroxydihydrochalcone (**7**):* white, amorphous solid; IR (thin film) ν_{max} 2925, 1739, 1633, 1360, 1228, 1138 cm^{-1}; ^1H and ^{13}C NMR data were consistent with literature values;[9] HRESIMS m/z 241.1016 $[M + H]^+$ (calcd for $C_{15}H_{15}O_3$, 244.1016).

*Erythrodiol (**8**):* white, amorphous solid; $[\alpha]^{25}_D$ −12.5 (c 0.032, CH_2Cl_2); IR (thin film) ν_{max} 3393, 2925, 1641, 1096 cm^{-1}; ^{13}C NMR data were consistent with literature values;[14] HRESIMS m/z 443.3883 $[M + H]^+$ (calcd for $C_{30}H_{51}O_2$, 443.3884).

*Oleanolic acid (**9**):* white, amorphous solid; $[\alpha]^{25}_D$ +37.3 (c 0.16, CH_2Cl_2); IR (thin film) ν_{max} 3406, 2932, 2906, 2869, 1686, 1090 cm^{-1}; ^{13}C NMR data were consistent with literature values;[14] HRESIMS m/z 457.3679 $[M + H]^+$ (calcd for $C_{30}H_{49}O_3$, 457.3676).

Biological Assays. Antimycobacterial activity against *M. tuberculosis* H37Ra (ATCC 25177) was evaluated using the microplate resazurin assay, as previously described.[11] Cytotoxicity was evaluated against HEK 293 cells (ATCC CRL-1573) using the CellTiter-Blue cell viability assay, as previously described.[21] All assays were run in triplicate. The MIC of a compound was considered to be the lowest concentration at which it inhibited mycobacterial growth by more than a mean value of 90%.[22] Absolute median inhibitory concentrations (IC_{50}s) were estimated by four-parameter logistic (4PL) regression[23] using GraphPad Prism (version 6.0).

Table 3. Biological Activities (MIC and IC_{50} Values in μg/mL [μM]) of Compounds 1−9[a]

	Mycobacterium tuberculosis H37Ra		human embryonic kidney 293 cells
compound	MIC	IC_{50} (95% CI)	IC_{50} (95% CI)
1	100 [367]	25.7 (23.6, 28.0) [94.4 (86.7, 103)]	45.6 (35.0, 59.4) [167 (129, 218)]
2	100 [367]	28.5 (27.1, 30.0) [105 (100, 110)]	96.7 (81.6, 115) [355 (300, 421)]
3	200 [694]	51.7 (47.6, 56.2) [179 (165, 195)]	32.5 (27.6, 38.3) [113 (95.7, 133)]
4	>400 [>1458]	247 (234, 261) [900 (853, 951)]	81.7 (55.1, 121) [298 (215, 389)]
5	200 [734]	30.7 (29.3, 32.1) [113 (108, 118)]	78.8 (58.6, 106) [289 (215, 389)]
6	25 [104]	5.71 (5.44, 5.98) [23.8 (22.6, 24.9)]	26.2 (16.9, 40.6) [109 (70.3, 169)]
7	50 [206]	7.95 (7.53, 8.39) [32.8 (31.1 34.6)]	60.3 (43.9, 82.9) [249 (181, 342)]
8	>400 [>903]	69.6 (54.6, 75.0) [157 (123, 169)]	272 (218, 338) [613 (493, 763)]
9	>400 [>876]	97.9 (82.1, 117) [214 (180, 256)]	150 (107, 211) [329 (234, 463)]

[a]Bioassay data were obtained in triplicate. 95% confidence intervals.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jnatprod.5b00627.

NMR spectra of compounds **1** and **2** (PDF)

■ AUTHOR INFORMATION

Corresponding Author

*Tel: +1 (506) 648-5576. Fax: +1 (506) 648-5650. E-mail: cgray@unb.ca.

Notes

The authors declare no competing financial interest.

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