Antibacterial Neurymenolides from the Fijian Red Alga *Neurymenia fraxinifolia*

E. Paige Stout, Adam P. Hasemeyer, Amy L. Lane, Theresa M. Davenport, Sebastian Engel, Mark E. Hay, Craig R. Fairchild, Jacques Prudhomme, Karine Le Roch, William Aalbersberg, and Julia Kubanek

Supporting Information

Contents: **Pages:**

Additional Acknowledgments:

We thank W. Kelly for thorough discussions and insights; M. Sharma, K. Feussner, and A. Wang for technical assistance; S. Franzblau and B. Wan for antitubercular assays; M.C. Sullards and D. Bostwick for mass spectral analyses; L. Gelbaum for NMR assistance; A. Bommarius and T. Rogers for use of their polarimeter; and U. Bunz and S. Brombosz for use of their IR spectrophotometer.

Experimental Section

Chemicals

Chemicals used for extractions and synthesis were reagent grade, and those used for HPLC and LC-MS were HPLC or Optima grade (Fisher Scientific). NMR solvents were purchased from Cambridge Isotope Laboratories.

Collection

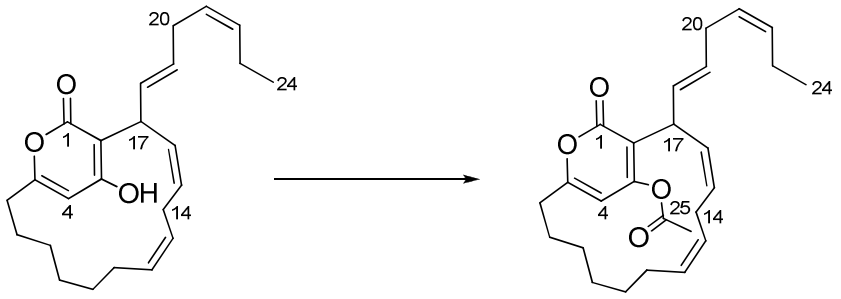
Neurymenia fraxinifolia (family Rhodomelaceae, order Ceramiales, class Florideophyceae, phylum Rhodophyta) was collected at depths of 20-25 m from Taveuni, Fiji, offshore from Waitabu Marine Protected Area near Lavena Village (16° 48' 97" S, 179° 50' 84" W) in July 2006. *N. fraxinifolia* was stored at -60 °C until extraction 15 months later. One sample was placed directly in methanol and stored until further analysis. One voucher specimen (G-0086) was deposited at the University of the South Pacific, Fiji, and the second was stored at Georgia Institute of Technology.

Isolation

N. fraxinifolia was exhaustively extracted with methanol and methanol/dichloromethane (1:1, 1:2). The extracts were combined, filtered, and reduced *in vacuo.* The crude extract was separated with HP20ss resin into four fractions, eluting with (1) 1:1 methanol: water, (2) 4:1 methanol: water, (3) 100% methanol, and (4) 100% acetone. The antibacterial HP20ss fractions 3 and 4 were separated by C_{18} reversed-phase HPLC (Alltech Alltima C_{18}, 5 μm, 10 × 250 mm) using a gradient of methanol and water, followed by normal phase silica gel HPLC (Agilent Zorbax RX-SIL, 5 μm, 9.4 × 250 mm) with 85:15 hexanes: ethyl acetate to yield neurymenolides A and B (**1-2**). Because **1** appeared to isomerize on normal phase silica gel, separation by chiral HPLC (Astec Chirobiotic T, 5 μm, 4.6 × 250 mm) was attempted with 78:12 hexanes: ethyl acetate; again, **1** eluted as two peaks. When the material from each peak was reinjected onto the chiral column, the same two peaks consistently reappeared. The two HPLC peaks from **1** were

stored separately under N_2 at -80 ^oC, along with **2**, until characterization and pharmacological testing was conducted.

Acetylation of neurymenolide A (1)



1

To a solution of **1** (10 mg, 0.027 mmol) in pyridine (0.5 mL) was added acetic anhydride (20 μL, 0.21 mmol) under a nitrogen atmosphere. The reaction was stirred at room temperature for 12 h and then diluted with cold water (10 mL) and extracted with ethyl acetate (10 mL). The organic layer was dried over $MgSO_4$ and evaporated. The residue was separated via reversed-phase HPLC (Alltech Alltima C_{18}, 5 μm, 10 × 250 mm), eluting with 100% acetonitrile, yielding 9.8 mg (88% yield) of 3-*O*-acetylneurymenolide A, acetylated atropisomers of **1**. The two atropisomers were present in a 3:2 ratio, based on HPLC-UV peak integration and ^1H NMR spectral integration. Only ^1H chemical shifts differing from that of **1** (Table 1, manuscript; Figure 3, SI) are listed. ^1H-NMR ($CDCl_3$, 500 MHz) for major atropisomer δ: 5.98 (H-4, s); 4.49 (H-17, br s); 2.80 (H-14b, m); 2.72 (H_2-20, m); 2.43 (H-14a, br d, J = 17); 2.21 (H_3-26, s) ppm. ^{13}C-NMR ($CDCl_3$, 250 MHz) for major atropisomer δ: 166.6; 164.2; 163.7; 158.5; 132.5; 130.2; 130.0; 129.8; 129.4; 129.3; 127.2; 126.3; 117.3; 103.7; 37.1; 33.7; 30.0; 27.2; 27.1; 26.9; 26.4; 26.2; 25.5; 21.2; 20.5; 14.2 ppm. ^1H-NMR ($CDCl_3$, 500 MHz) for minor atropisomer δ: 6.0 (H-4, s); 4.14 (H-17, m); 2.80 (H-14b, m); 2.72 (H_2-20, m); 2.43 (H-14a, br d, J = 17); 2.34 (H_3-26, s) ppm. ^{13}C-NMR ($CDCl_3$, 250 MHz) for minor atropisomer δ: 167.1; 165.0; 162.1; 157.2; 132.6; 130.1; 129.8; 129.7; 129.4; 129.4; 127.5; 126.3; 116.9; 102.5; 37.5; 33.8; 30.0; 27.6; 27.4;

27.1; 26.9; 25.8; 25.5; 20.9; 20.5; 14.2 ppm. ESI-MS for both atropisomers $[M + H]^+$ *m/z* 411.3, 100%, $[M + H - COCH_3]^+$ *m/z* 369.3, 15%.

Analytical and spectroscopic methods

UV spectra were recorded in acetonitrile with a Spectronic 21D spectrophotometer using quartz cuvettes. Optical rotations were measured on a Jasco P-1010 spectropolarimeter with a 10 cm (1.5 mL) cell, with compounds dissolved in methanol. IR spectra were generated from thin films on a Shimadzu FTIR 84005 spectrophotometer. High-resolution mass spectra were generated using electrospray ionization with an Applied Biosystems QSTAR-XL hybrid Quadrupole-Time-of-Flight tandem mass spectrometer and Analyst QS software. LC-MS analyses were conducted using a Waters 2695 HPLC with Waters 2996 photodiode array UV detection and Micromass ZQ 2000 mass spectrometer with electrospray ionization. LC-MS chromatography was achieved with an Alltech Alltima C_{18} (3 μm, 2.1 × 150 mm) column, using gradient mobile phases of aqueous acetonitrile with 0.1% acetic acid. NMR spectra were recorded on a Bruker DRX-500 instrument, using a 5 mm inverse detection probe for ^1H, COSY, HSQC, and HMBC experiments, and a 5 mm broadband probe for ^1H, ^{13}C, DEPT-135, and ROESY experiments, and referenced to residual $CHCl_3$ (7.24 and 77.0 ppm, for ^1H and ^{13}C, respectively).

Pharmacological assays

All pharmacological assays were performed as previously described.[1,2] Briefly, antibacterial assays were performed using methicillin-resistant *Staphylococcus aureus* (MRSA, ATCC#10537) and vancoymcin-resistant *Enterococcus faecium* (VREF, ATCC#12952) as test pathogens. These bacterial strains were grown overnight at 37 ^oC in nutrient broth (BD Difco™) and brain heart infusion (BD Bacto™) media, respectively. Assay cultures were diluted to an optical density of 0.05, diluted 10-fold, and added to 96-well microliter plates. Treatments and controls were suspended in DMSO, added to the top row of assay plates (final concentration 250

μg/mL), and serially diluted 1:1 down each column. Vancomycin and chloramphenicol were used as positive controls for MRSA and VREF, respectively, and DMSO was used as negative control. All assay plates were sealed with parafilm and incubated overnight at 37 ^{o}C. The optical density was measured at 600 nm using a microplate reader, and the IC_{50} of each compound was calculated using the dose concentration at 50% inhibition on a sigmoidal dose response curve generated using GraphPad Prism version 4.00 for Windows, GraphPad software, San Diego, CA, USA.

For the antifungal assay, amphotericin B-resistant *Candida albicans* (ATCC#90873) was grown overnight at 37 ^{o}C in YPM media (2 g yeast extract, 2 g peptone, 4 g D-Mannitol, 1 L DI water). A hemocytometer was used to determine cell density, and the assay culture was diluted to 1×10^4 cells/mL, before it was added to 96-well microliter plates. Treatments and controls were suspended in DMSO, added to the top row of the assay plate (final concentration 250 μg/mL), and serially diluted 1:1 down each column. A mixed nystatin/amphotericin B solution was used as a positive control, and DMSO was used as a negative control. All assay plates were incubated overnight at 37 ^{o}C. The optical density was then measured at 600 nm using a microplate reader and the IC_{50} was calculated for each in the same method as the antibacterial assays.

Neurymenolides A and B (**1-2**) were evaluated against a panel of 12 tumor cell lines including breast, colon, lung, prostate, and ovarian cancer cells. Specific cell lines were: BT-549, DU4475, MDA-MD-468, PC-3, SHP-77, LNCaP-FGC, HCT116, MDA-MB-231, A2780/DDP-S, Du145, CCRF-CEM, and A549. *In vitro* cytotoxicity was tested with the (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxylmethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt) MTS dye conversion assay as described previously.[3]

Antitubercular activity was assessed against *Mycobacterium tuberculosis* strain $H_{37}Rv$ (ATCC 27294) using the alamar blue susceptibility test (MABA) as described previously.[4] Compounds **1**-**2** were tested at a maximum concentration of 100 μM.

Antimalarial activity was determined with a SYBR Green based parasite proliferation assay, adapted from Smilkstein[5] and Bennett.[6] Briefly, *Plasmodium falciparum* parasites (3D7 strain MR4/ATCC, Manassas, VA) were cultured in human O+ erythrocytes as previously described.[7] Compounds were diluted in complete medium and 40 μL transferred to 96-well assay plates. To this 80 μL of complete media with 3D7 infected erythrocytes were dispensed in order to obtain a 2.5% hematocrit and 0.5% parasitemia in the assay. Uninfected erythrocytes were dispensed into the background wells at the same final hematocrit. Plates were incubated for 72 hours in a low oxygen environment (96% N_2, 3% CO_2, 1% O_2) in a modular incubation chamber. The plates were sealed and placed in a -80 ^oC freezer overnight then thawed, and 120 μL of lysis buffer (20 mM Tris-HCl, pH 7.5, 5mM EDTA, 0.08% Triton X-100, 0.008% saponin with 0.2 μL/mL Sybr Green I) was dispensed into each well and incubated at 37 ^oC in the dark for 6 hours. The plates were read with a Molecular Devices SpectraMAX Gemini EM at ex: 495 nm, em: 525 nm with 515 nm cut-off.

Table S1: COSY correlations for neurymenolides A-B (**1-2**). For diastereotopic protons with dissimilar chemical shifts, the proton whose chemical shift is listed first in Table 1 of the main article is termed "a" and the other is "b". "NA" (not applicable) indicates that no proton signal exists for that position.

^{1}H at position	COSY correlations observed between protons listed on far left and those below:	
#:	1	2
4	-	-
6a	6b, 7a, 7b	6b, 7a, 7b
6b	6a, 7a, 7b	6a, 7a, 7b
7a	6a, 6b, 7b, 8a	6a, 6b, 7b, 8a, 8b
7b	6a, 6b, 7a, 8b	6a, 6b, 7a, 8b
8a	7a	7a
8b	7b, 9a	7a, 7b, 9a
9a	8b, 9b	8b, 9b, 10a, 10b
9b	9a, 10a, 10b	9a
10a	9b	9a
10b	9b, 11a, 11b	9a
11a	10b	-
11b	10b, 12	-
12a	11b	13a, 13b
12b	NA	13a, 13b
13a	14a, 14b	12a, 12b, 13b, 14
13b	NA	12a, 12b, 13a, 14
14a	13, 14b, 15	13a, 13b
14b	13, 14a, 15	NA
15	14a, 14b	16a, 16b
16a	17	15, 16b, 17
16b	NA	15, 16a, 17
17	16, 18	16a, 16b
18	17	19
19	20	18, 20
20	19, 21	19
21	20, 22	22
22	21, 23	21, 23
23	22, 24	22, 24
24	23	23, 25
25	NA	24, 26
26	NA	25

Table S2: HMBC correlations for neurymenolides A-B (**1-2**). For diastereotopic protons with dissimilar chemical shifts, the proton whose chemical shift is listed first in Table 1 of the main article is termed "a" and the other is "b". "NA" (not applicable) indicates that no proton signal exists for that position.

^{1}H at position #:	HMBC correlations observed between protons listed on far left and those below:	
	1	**2**
4	2, 5, 6	2, 5, 6
6a	4, 5, 7	-
6b	4, 5, 7	5
7	-	-
8	-	-
9a	8, 10	-
9b	8, 10	-
10a	9	9
10b	9	9
11a	10	-
11b	-	10
12a	14	-
12b	NA	13
13a	14	14
13b	NA	14
14a	-	13
14b	15	NA
15	14, 17	16
16a	17	-
16b	NA	15
17	2, 18	-
18	17, 20	-
19	17, 20	-
20	19, 21	19,22
21	-	19, 22
22	-	21
23	22, 24	-
24	22, 23	-
25	NA	23, 24, 26
26	NA	24, 25
-OH	3, 4	2, 3, 4

Table S3: Observed NOEs from ROESY NMR experiments, for neurymenolides A-B (**1-2**). For diastereotopic protons with dissimilar chemical shifts, the proton whose chemical shift is listed first in Table 1 of the main article is termed "a" and the other is "b". Only NOEs important to determinations of stereochemistry are listed.

^{1}H at position #:	NOEs observed between protons listed on far left and protons at positions listed below:	
	1	**2**
4	9a	
9a	4	
11a	-	
11b	14a, 14b	
13a		16a
13b		16b
14a	11b, 17	
14b	11b, 17	
16a		13a, 19
16b		13b, 19
17	14a, 14b	
19		16a, 16b
20	23	
22		25
23	20	
25		22

Figure S1. ^{1}H NMR spectrum of neurymenolide A (**1**) (500 MHz; $CDCl_3$)

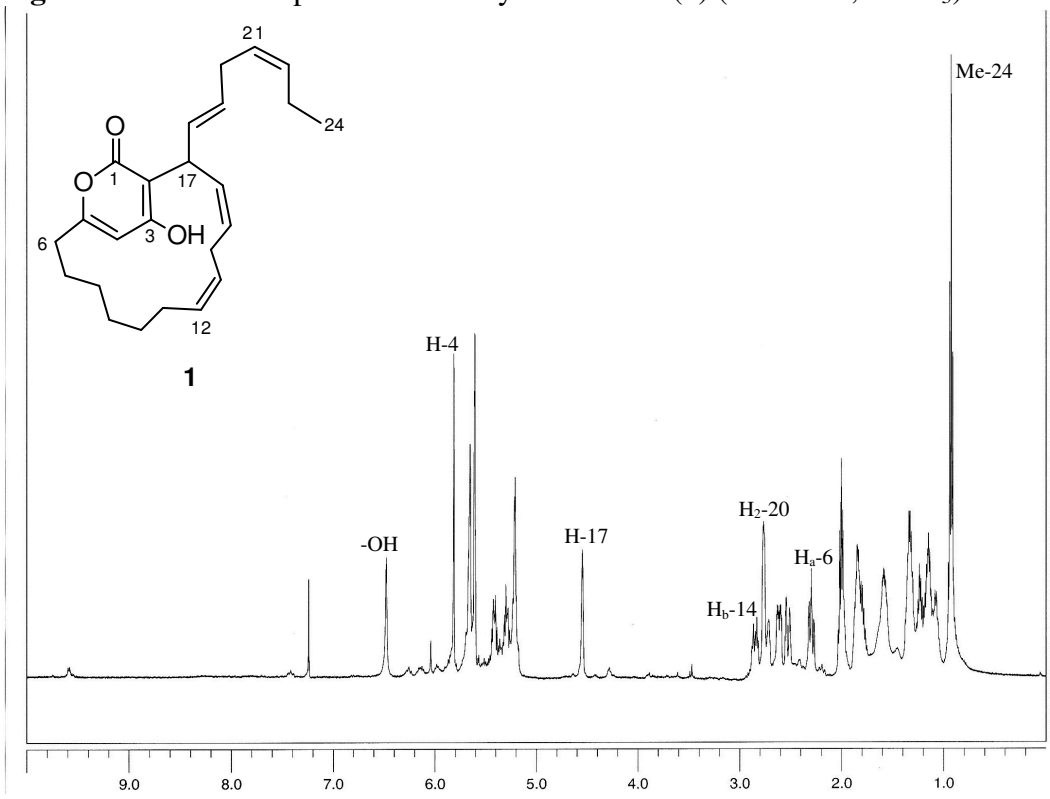


Figure S2. ^{1}H NMR spectrum of neurymenolide B (**2**) (500 MHz; $CDCl_3$)

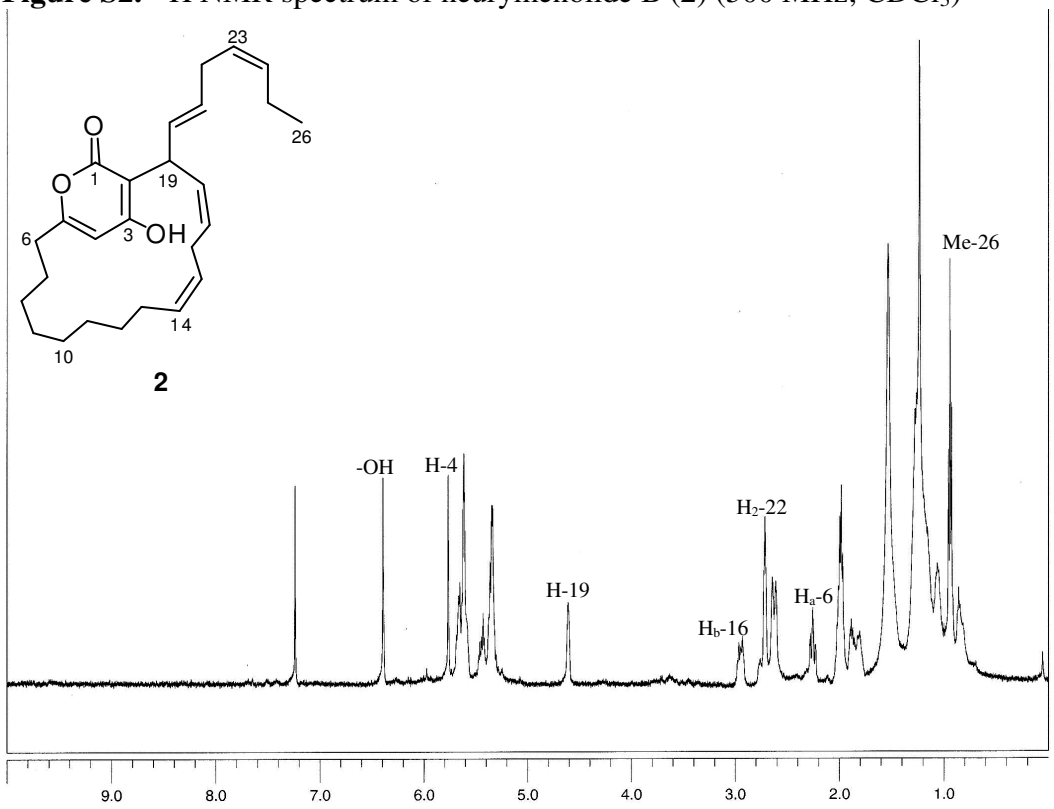


Figure S3. Comparison of ^{1}H NMR spectra of neurymenolide A (**1**; red spectrum) with 3:2 mixture of atropisomers of 3-*O*-acetylneurymenolide A (blue spectrum) (500 MHz; $CDCl_3$)

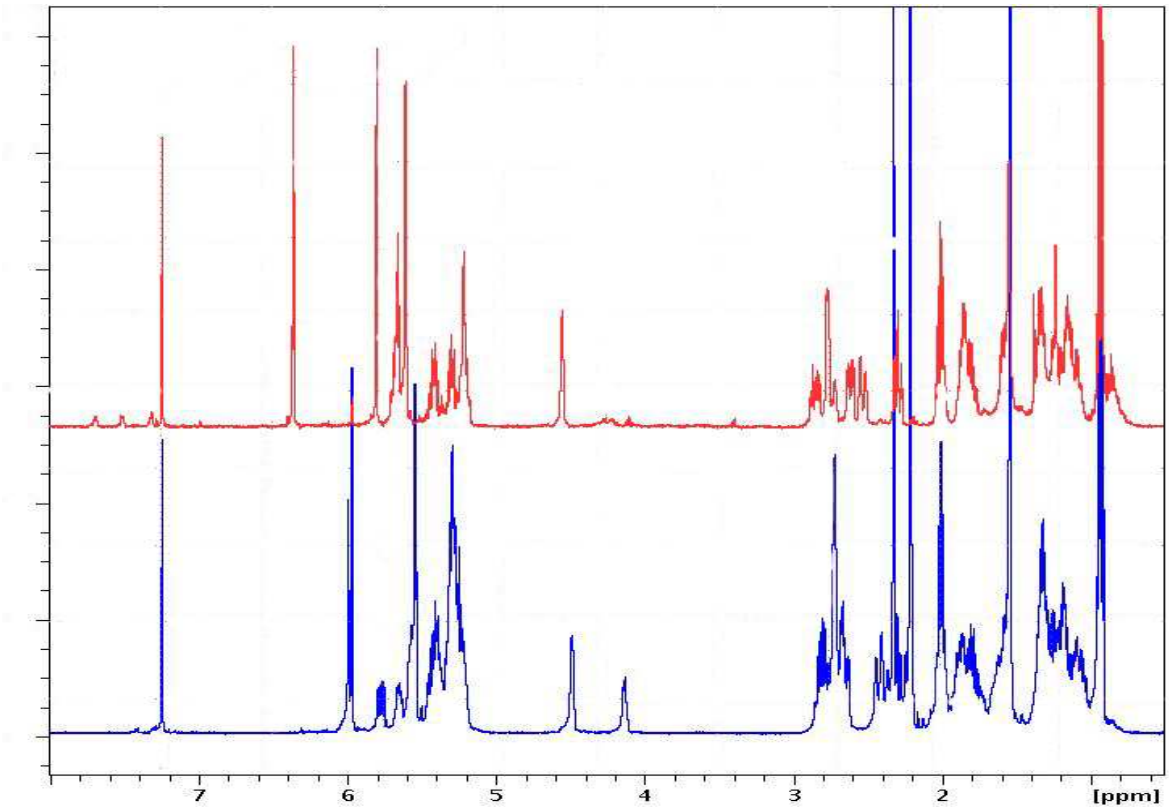


Figure S4. ^{13}C NMR spectrum of neurymenolide A (**1**) (125 MHz; $CDCl_3$)

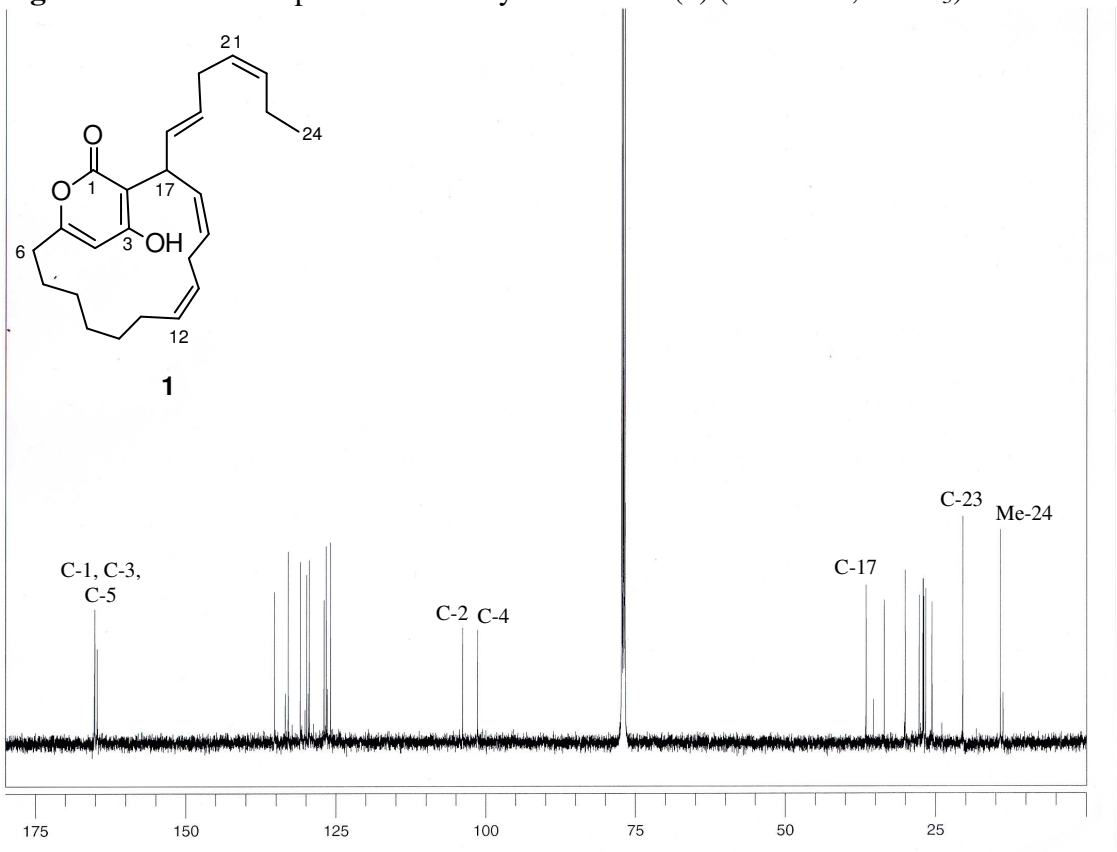
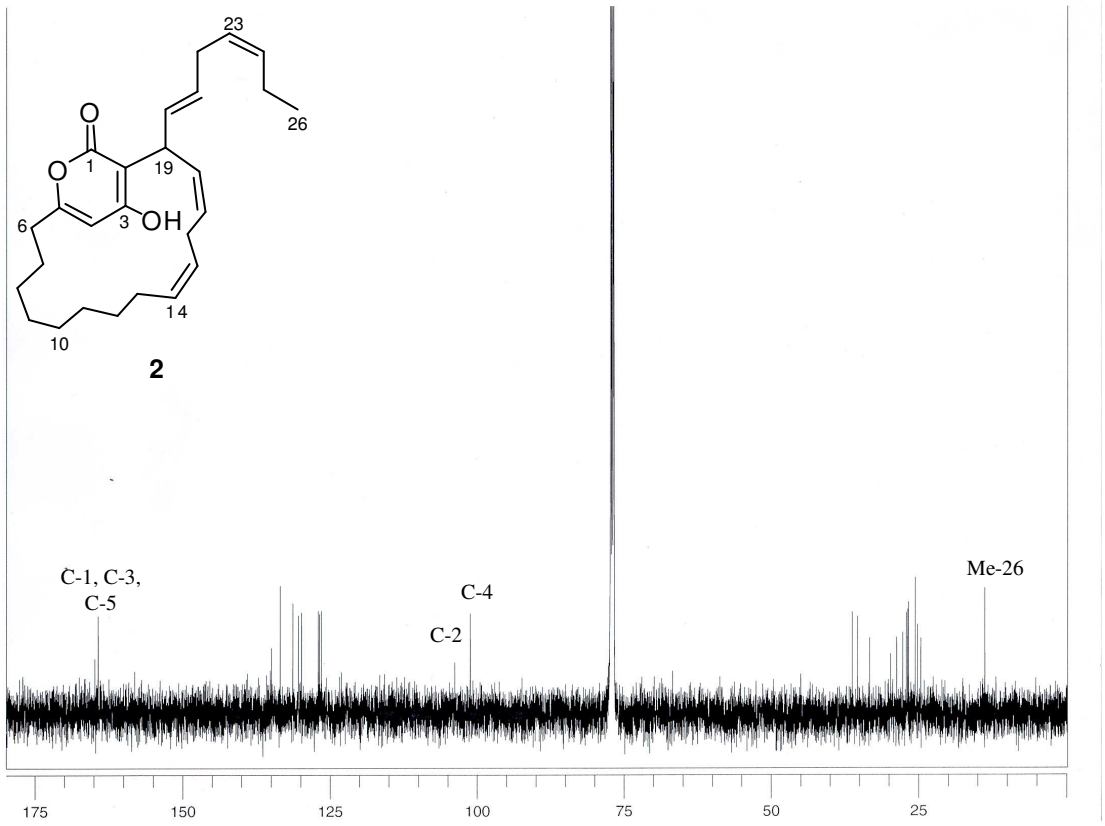


Figure S5. ^{13}C NMR spectrum of neurymenolide B (**2**) (125 MHz; $CDCl_3$)



References

[1] Kubanek, J.; Prusak, A. C.; Snell, T. W.; Giese, R. A.; Hardcastle, K. I.; Fairchild, C. R.; Aalbersberg, W.; Raventos-Suarez, C.; Hay, M. E. *Org. Lett.* **2005**, *7*, 5261-5264.

[2] Lane, A. L.; Stout, E. P.; Hay, M. E.; Hardcastle, K.; Fairchild, C. R.; Franzblau, S. G.; Le Roch, K.; Prudhomme, J.; Aalbersberg, W.; Kubanek, J. *J. Org. Chem.* **2007,** *72,* 7343-7351.

[3] Lee, F. Y. F.; Borzilleri, R.; Fairchild, C. R.; Kim, S. H.; Long, B. H.; Reventos-Suarez, C.; Vite, G. D.; Rose, W. C.; Kramer, R. A. *Clinical Cancer Research* **2001**, *7*, 1429-1437.

[4] Collins, L. A.; Franzblau, S. G. *Antimicrobial Agents and Chemotherapy* **1997**, *41*, 1004-1009.

[5] Smilkstein, M.; Sriwilaijaroen, N.; Kelly, J. X.; Wilairat, P.; Riscoe, M. *Antimicrobial Agents and Chemotherapy* **2004**, *48*, 1803-1806.

[6] Bennett, T. N.; Paguio, M.; Gligorijevic, B.; Seudieu, C.; Kosar, A. D.; Davidson, E.; Roepe, P. D. *Antimicrobial Agents and Chemotherapy* **2004**, *48*, 1807-1810.

[7] Trager, W.; Jensen, J. B. *Science* **1976**, *193*, 673-675.