Darwinolide, a new diterpene scaffold that inhibits methicillin-resistant *Staphylococcus aureus* biofilm from the Antarctic sponge *Dendrilla membranosa*

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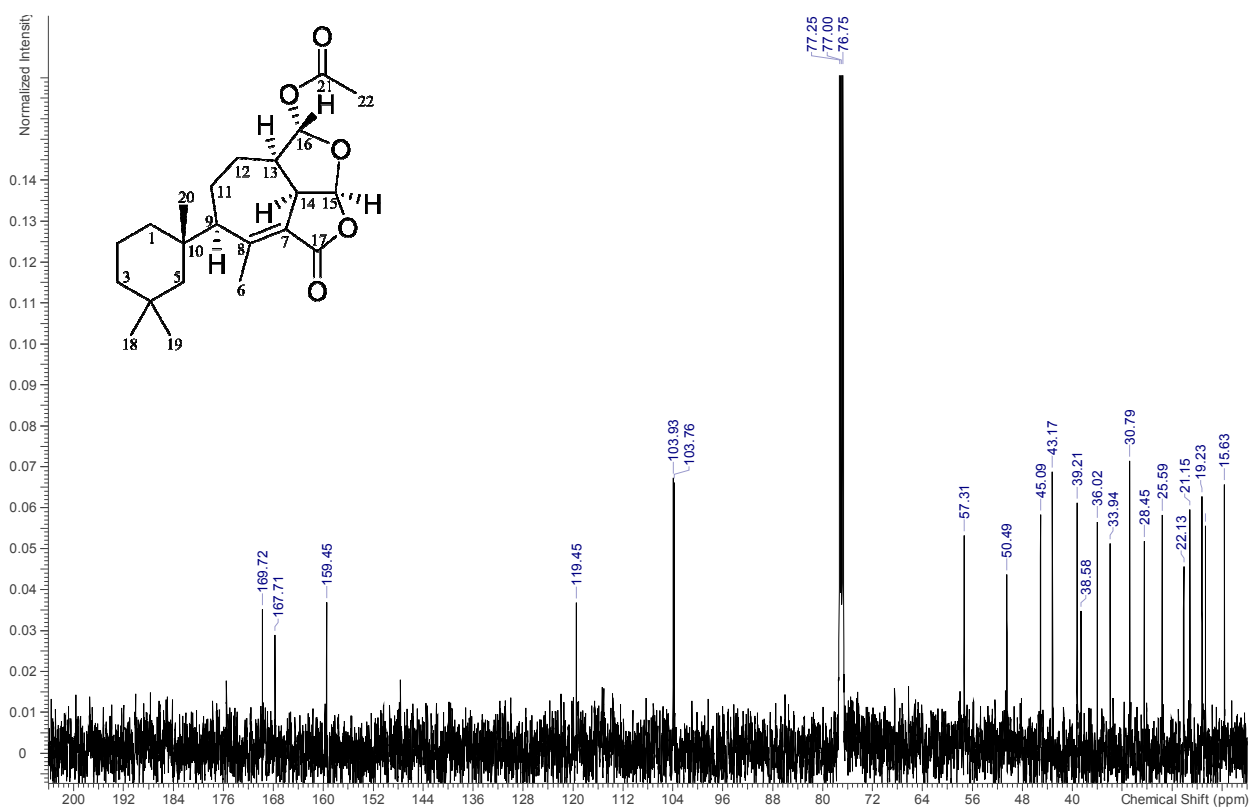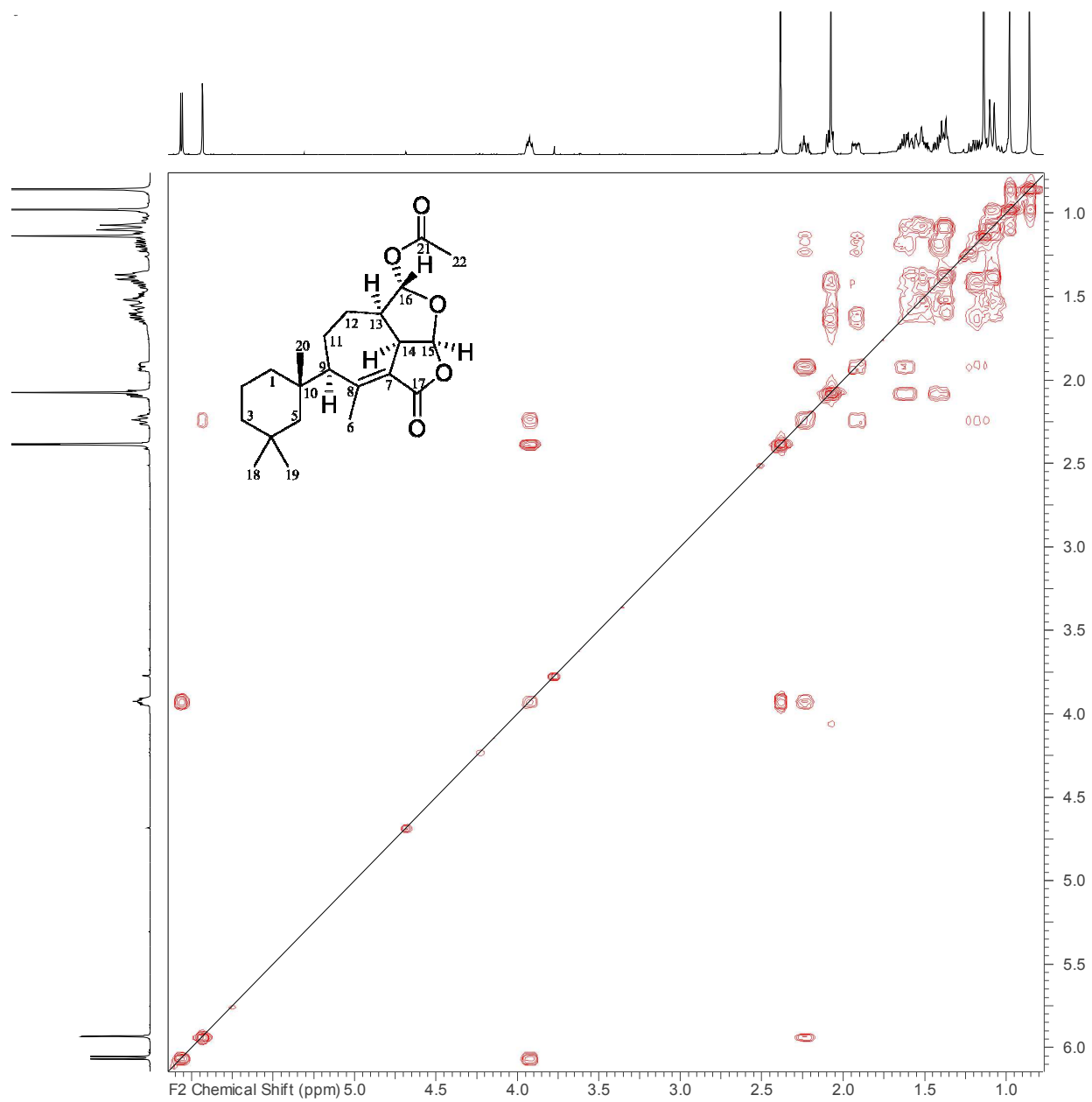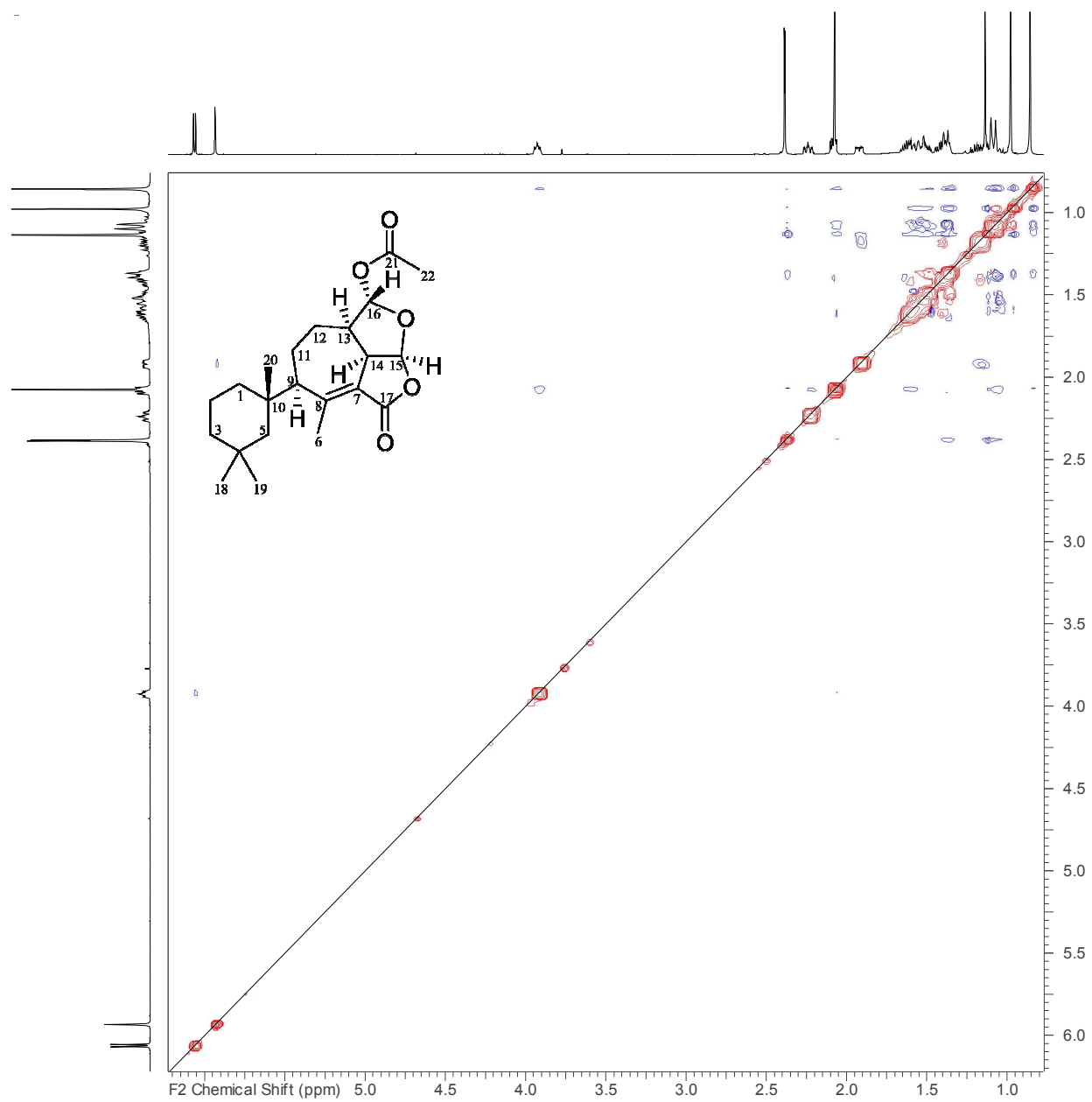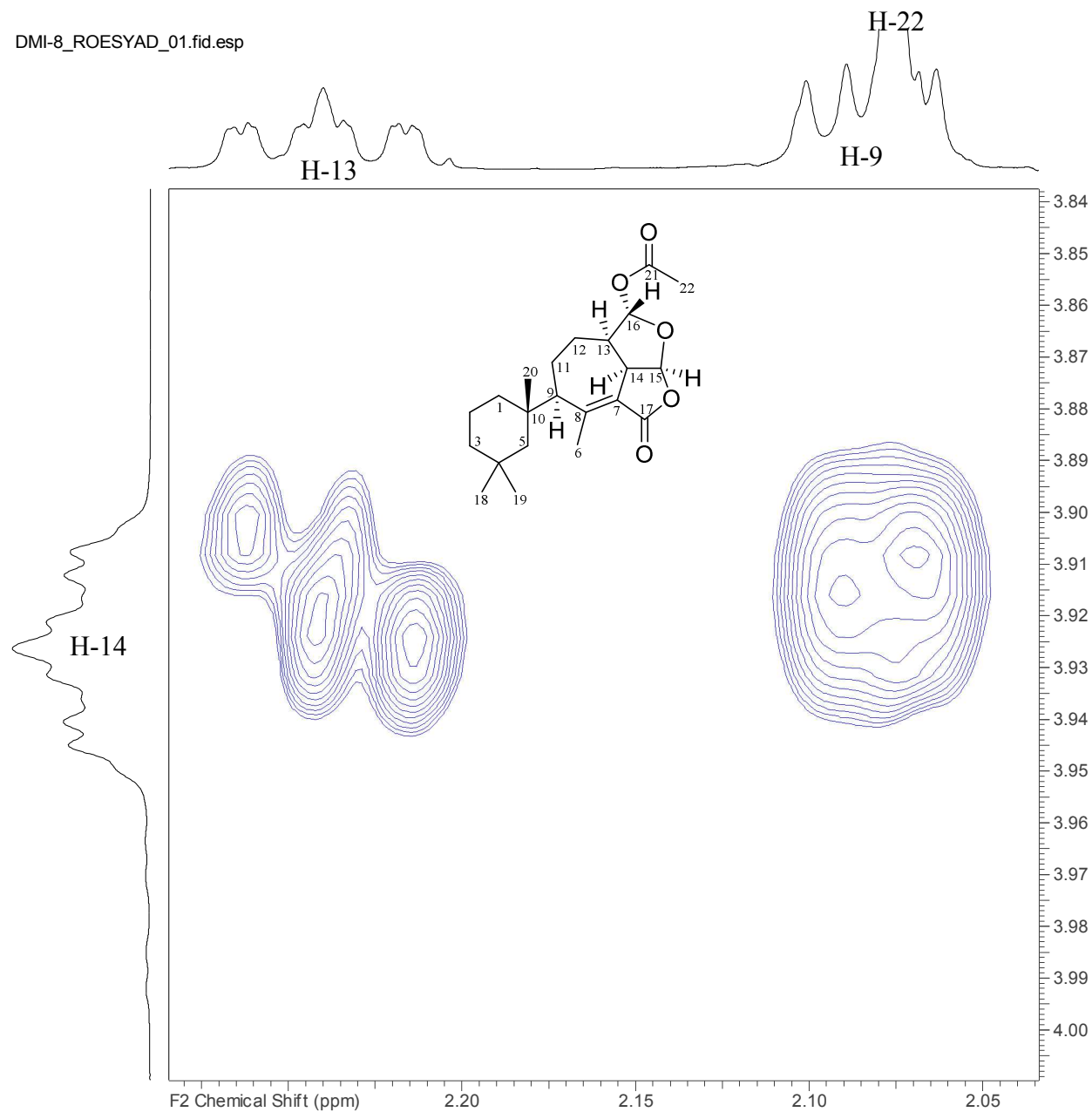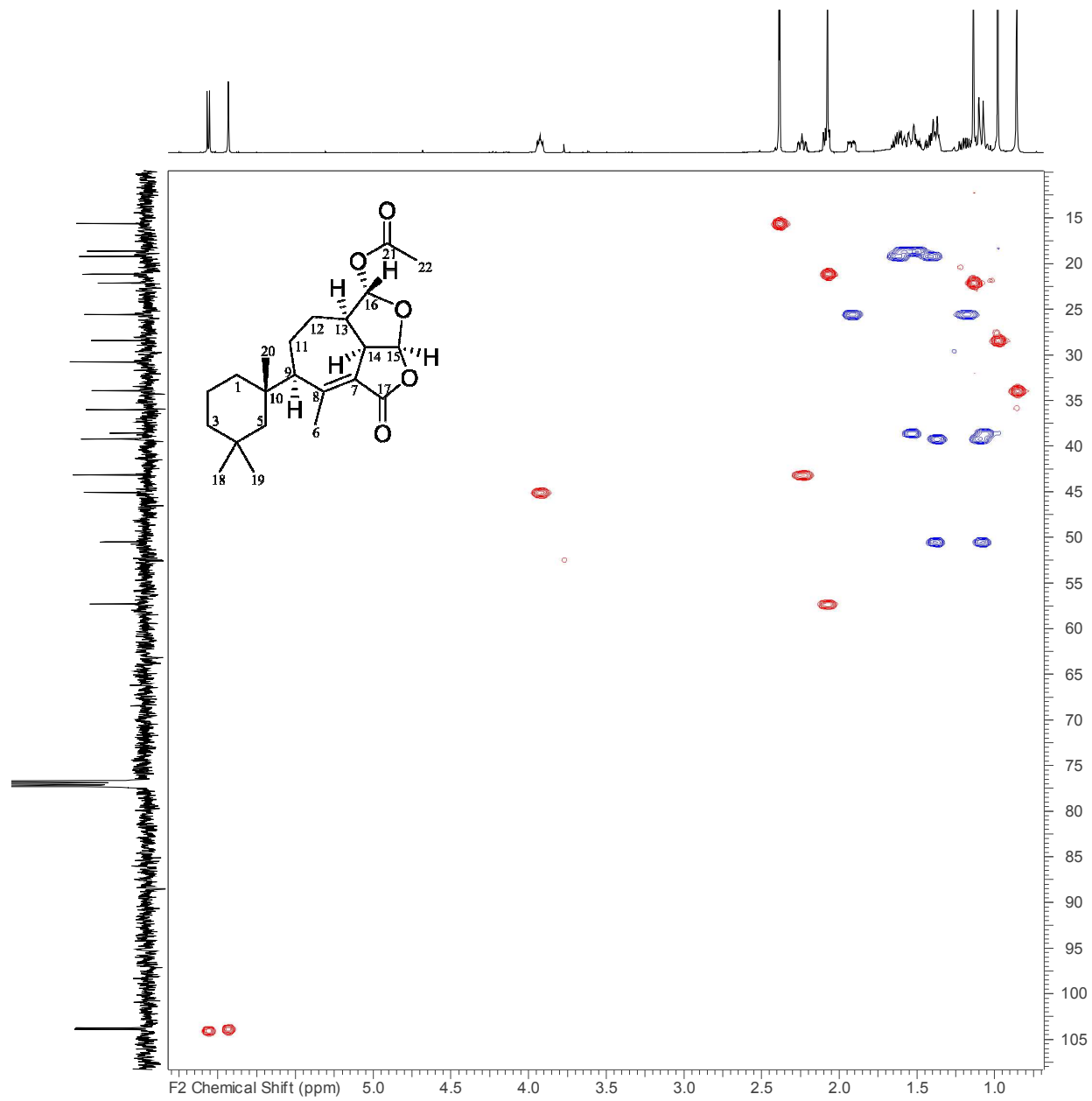
S1: Experimental Procedures for the Isolation of Darwinolide

General Procedures. All solvents were obtained from Fisher Scientific Co. and were HPLC grade (>99% purity) unless otherwise stated. All HPLC analysis was performed on a Shimadzu LC20-AT system equipped with a photodiode array detector (M20A) using semi-preparative [Phenomenex Luna C18 (250 x 10 mm, 5 μm)] or analytical [Phenomenex Luna Silica (250 x 4.6 mm, 5 μm)] conditions. Analytical LCMS was performed on a Phenomenex Kinetex C18 column (50 x 2.1 mm, 2.6 μm) with an Agilent 6540 LC/QToF-MS with electrospray ionization detection. Optical rotations were measured on a Rudolph Research Analytical AUTOPOL IV digital polarimeter. Other spectroscopic data was collected on an Agilent Cary 630 FTIR or Cary 60 UV-Vis spectrometer. All NMR spectra were acquired in $CDCl_3$ with residual solvent referenced as an internal standard (7.26 ppm). All ^1H NMR spectra were recorded on a Varian 500 MHz direct-drive instrument equipped with cold-probe detection and ^{13}C NMR spectra were recorded at 125 MHz.

Collection of *Dendrilla membranosa*. Sponge samples were collected from various sites around Palmer Station, Antarctica in the austral summer of 2011. The collection sites chosen were Norsel Point (64°45.674'S, 64°05.467'W), Bonaparte Point (64°46.748'S, 64°02.542'W), Gamage Point (64°46.345'S 64°02.915'W), and Laggard Island (64°48.568'S, 64 00.984'W) at depths between 5-35 m below sea level. Samples were frozen and transported back to the University of South Florida at -70°C where tissues were lyophilized and stored at -80°C until further processing.

Extraction and Isolation of Natural Products. 25.7 g of freeze-dried *D. membranosa* was extracted with dichloromethane (ACS grade) in triplicate, combined, and concentrated *in vacuo*. The lipophilic extract (994 mg) was absorbed onto Waters Sep-Pak® C18 cartridges and eluted with acetonitrile. The dried eluate (205 mg) was separated by isocratic semi-preparative HPLC using 60% acetonitrile in water for 35 min and ramping up to 100% acetonitrile after 50 min to afford (in retention time order) membranolide (8.7 mg), aplysulphurin (10.2 mg), tetrahydroaplysulphurin (1.5 mg), and darwinolide (2.0 mg). Normal phase chromatography was used to further purify aplysulphurin and tetrahydroaplysulphurin utilizing isocratic conditions (12% ethyl acetate in hexanes).

S2: Biological Assay Protocols

Cytotoxicity Assay. The J774.A-1 cell line was used for cytotoxicity screening via a colorometric method employing a tetrazolium derivative [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] (MTS) and an electron-coupling reagent, penazine methosulphate (PMS). Procedures have been described by us previously.[1]

Assay Against Methicillin-Resistant *Staphylococcus aureus*. A clinical, multi-drug resistant strain of MRSA (CBD-635) was used in these studies for minimum inhibitory concentration (MIC) determination and assessment of anti-biofilm properties, all as described by us previously.[2]

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2. Fleeman, R.M.; LaVoi, T.; Santos, R.G.; Morales, A.; Nefzi, A.; Welmaker, G.S.; Medina-Franco, J.; Houghten, R.A.; Giulianotti, M.A.; Shaw, L.N. *J. Med. Chem*. **2015**, *58*, 3340-3355.

S3: X-ray Diffraction Data for Darwinolide

The X-ray diffraction data for darwinolide were measured on Bruker Smart Apex2 and for remaining crystals, on Bruker D8 Venture PHOTON 100 CMOS system equipped with a Cu K_α INCOATEC ImuS micro-focus source (λ = 1.54178 Å). Indexing was performed using *APEX2*[1] (Difference Vectors method).[1] Data integration and reduction were performed using SaintPlus 6.01.[2] Absorption correction was performed by multi-scan method implemented in SADABS.[3] Space group was determined using XPREP implemented in APEX3 [1]. Structure was solved using SHELXS-97 (direct methods) and refined using SHELXL-2015[4-6] (full-matrix least-squares on F^2) through OLEX2 interface program.[7] All non-hydrogen atoms were refined anisotropically. Hydrogen atoms of –CH, –CH$_2$ and -CH$_3$ groups were placed in geometrically calculated positions and were included in the refinement process using riding model with isotropic thermal parameters: Uiso(H) = 1.2[1.5]Ueq(-CH,-CH$_2$,[-CH3]). Results of Bijvoet-Pair Analysis and Bayesian Statistics[8,9] validating the absolute configuration assignment, are in Table 2. Value of "P2" is a probability that the current model is correct assuming two possibilities only - one of the two possible enantiomers present. Crystal data and refinement conditions are shown in Table 1. The asymmetric unit of darwinolide is shown in Figure 3 of the manuscript.

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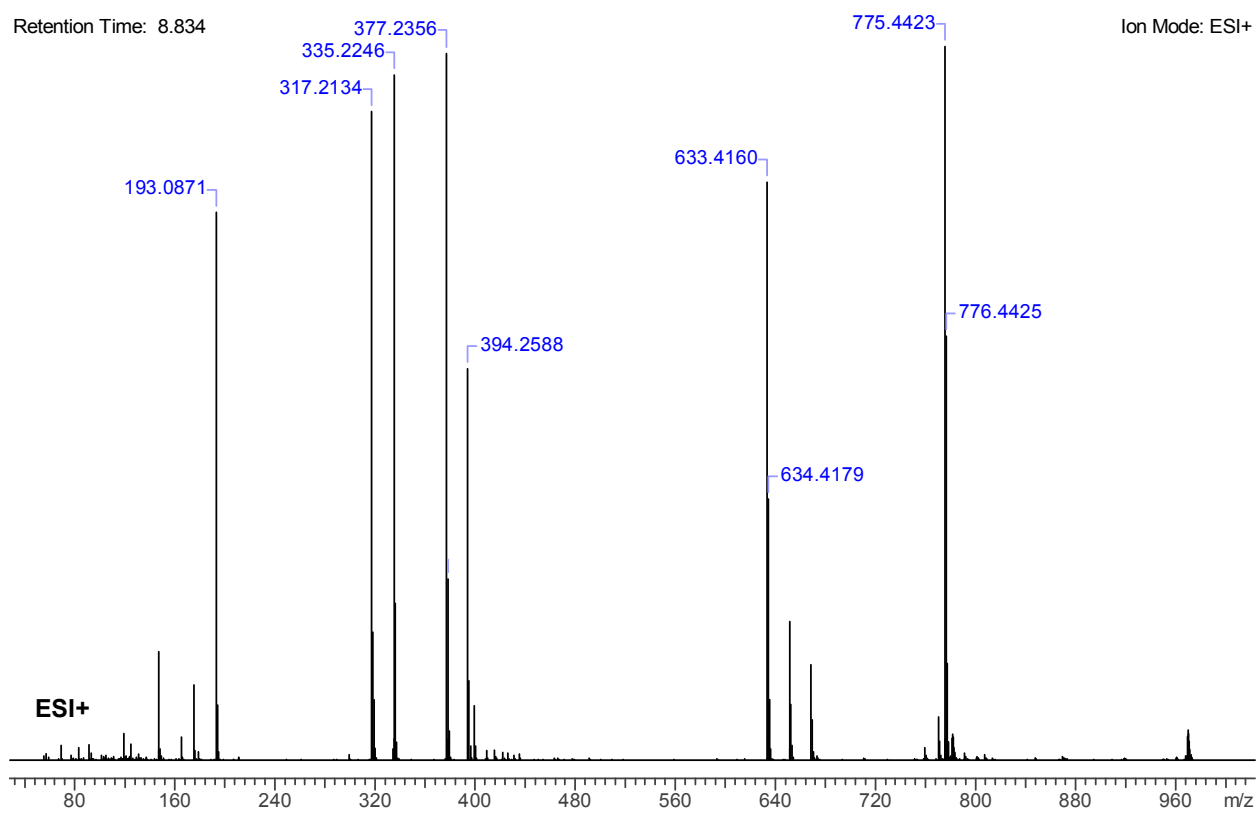
S4: Crystal Data and Structure Refinement

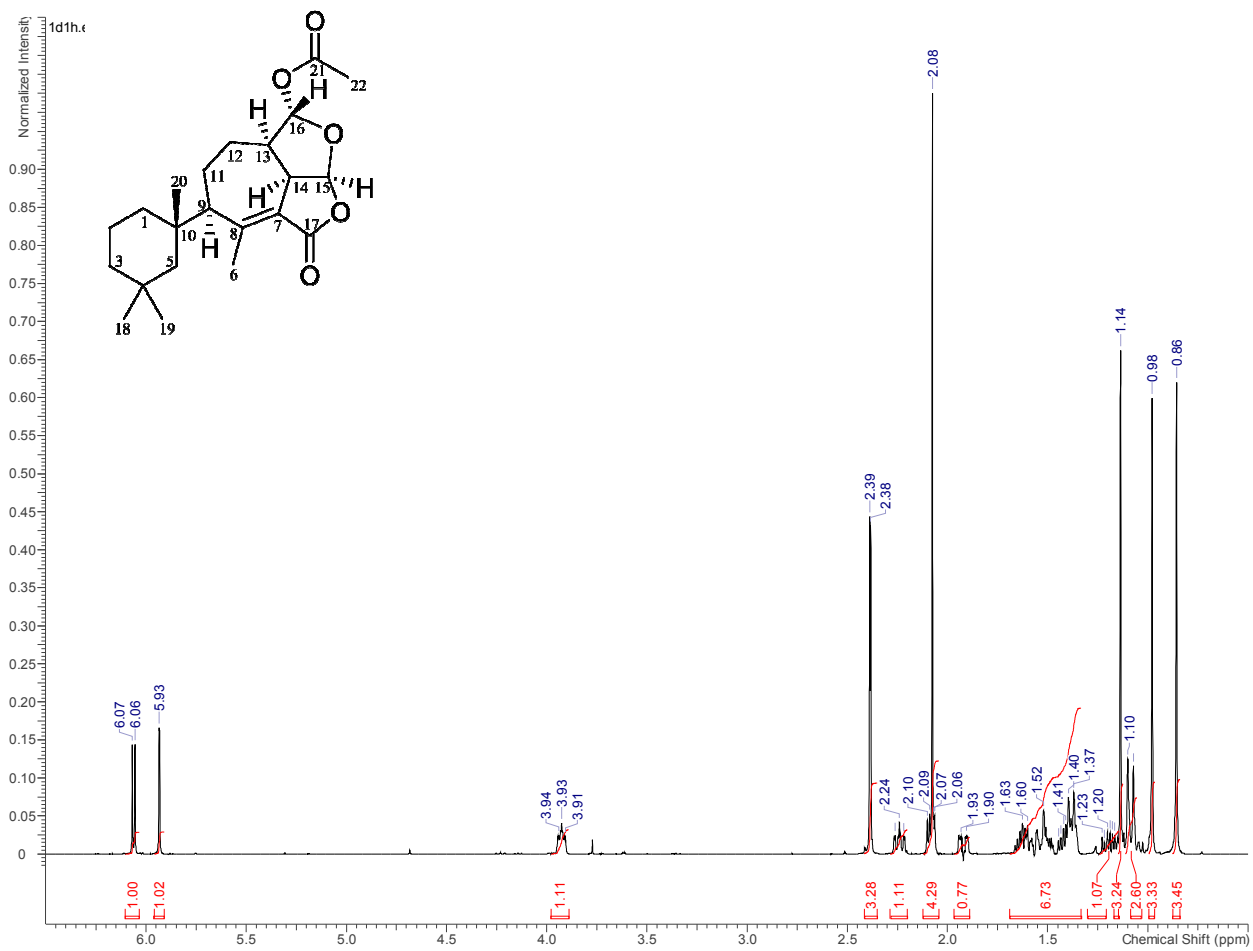| Table 1 Crystal data and structure refinement for darwinolide.	
Identification code	JF_DMI8_om
Empirical formula	$C_{22}H_{32}O_5$
Formula weight	376.47
Temperature/K	99.99
Crystal system	orthorhombic
Space group	$P2_12_12_1$
a/Å	7.6629(6)
b/Å	9.5182(8)
c/Å	27.012(2)
α/°	90
β/°	90
γ/°	90
Volume/Å^3	1970.2(3)
Z	4
ρ_{calc}g/cm^3	1.269
μ/mm^{-1}	0.714
F(000)	816.0
Crystal size/mm^3	0.21 × 0.03 × 0.02
Radiation	CuKα (λ = 1.54178)
2Θ range for data collection/°	6.544 to 136.638
Index ranges	-9 ≤ h ≤ 9, -11 ≤ k ≤ 11, -32 ≤ l ≤ 32
Reflections collected	14341
Independent reflections	3600 [R_{int} = 0.0724, R_{sigma} = 0.0530]
Data/restraints/parameters	3600/0/249
Goodness-of-fit on F^2	1.049
Final R indexes [I>=2σ (I)]	R_1 = 0.0425, wR_2 = 0.0889
Final R indexes [all data]	R_1 = 0.0560, wR_2 = 0.0951
Largest diff. peak/hole / e Å^{-3}	0.21/-0.23
Flack parameter	0.03(16)

S5: Bijvoet-Pair Analysis, Bayesian Statistics and Asymmetric Unit of Darwinolide

Table 2. Results Bijvoet-Pair Analysis and Bayesian Statistics	
Space Group P2₁2₁2₁	Student-T Prob. Plot
Wavelength 1.54178	Sample Size. 1491
Flack x .... 0.03(16)	Corr. Coeff. 0.999
Parsons z .. 0.07(16)	Intercept .. 0.042
	Slope 0.892
Bijvoet Pairs 1501	
Coverage ... 99	Bayesian Statistics
DiffCalcMax. 23.54	Student_T Nu 100
Outlier Crit 47.08	Select Pairs 1501
Scatter Plot	Theta_Min .. 7.60
Sigma Crit.. 0.25	Theta_Max .. 68.32
Select Pairs 15	**P2(true).... 1.000**
Number Plus 12	P3(true).... 0.944
Number Minus 3	P3(rac-twin) 0.056
Slope 1.574	P3(false) .. 0.5E-06
	G 0.8188
	G (su) 0.3353
	Hooft y ... 0.09(17)

Retention Time: 8.834

Ion Mode: ESI+

377.2356

335.2246

317.2134

775.4423

633.4160

193.0871

394.2588

776.4425

634.4179

ESI+

80   160   240   320   400   480   560   640   720   800   880   960   m/z

S7. ^{1}H NMR Spectrum of Darwinolide in CDCl$_3$, 500 MHz