Combined Chemical and Biosynthetic Route to Access a New Apoptolidin Congener

*Victor P. Ghidu, Ioanna Ntai, Jingqi Wang, Aaron T. Jacobs, Lawrence J. Marnett, Brian O. Bachmann and Gary A. Sulikowski**

Departments of Chemistry and Biochemistry, Institute of Chemical Biology, Vanderbilt University, Nashville, Tennessee 37235–1822

Supporting Information

1. General experimental procedures S2

2. Fermentation and feeding experiments S2-S3

3. Apoptolidin and apoptolidin D disaccharide purification S3

4. Mass spectrometry S3-S4

5. Isolation and characterization of apoptolidin disaccharide S4-S5

6. Cytotoxicity assay S5

7. ^{1}H and ^{13}C NMR spectra S6

8. Spectral comparison of apoptoldin D to apoptolidin D disaccharide S7

9. References and notes S7

General Experimental Procedures: Proton nuclear magnetic resonance (^{1}H NMR) spectra and carbon-13 (^{13}C NMR) spectra were recorded on a 600 MHz spectrometer at ambient temperature. ^{1}H and ^{13}C NMR data are reported as δ values relative to residual non-deuterated solvent δ 3.31 ppm from CH_3OD. For ^{13}C spectra, chemical shifts are reported relative to the δ 49.00 ppm resonance of CD_3OD. High-resolution mass spectra were obtained at Texas A&M University Mass Spectrometry Service Center by Dr. Shane Tichy on an API QSTAR Pulsar.

Fermentation: One hundred microliters of frozen glycerol stock of *Nocardiopsis sp.* was plated onto a Petri dish containing Bennett's medium and incubated at 30 °C for 3 days. Bennett's medium consisted of yeast extract, 1g/L; beef extract, 1 g/L; NZ amine A (casein digest), 2 g/L; glucose, 10 g/L; and agar, 20 g/L dissolved in distilled water and was adjusted to pH 7.0 before autoclaving. It was then poured into sterile Petri dishes (~25 mL/dish). The production protocol suggested by Hayakawa and coworkers[1] was followed. Specifically, fermentation was initiated by aseptically inoculating one loopful of mycelia grown on Bennett's agar plates into a sterile 50-mL Falcon tube containing 5 mL seed medium. The seed cultures were incubated at 30 °C for 3 days in a shaker incubator. The seed medium consisted of soluble starch, 10 g/L; molasses, 10 g/L; peptone, 10 g/L; and beef extract, 10 g/L dissolved in deionized water and was adjusted to pH 7.2 before autoclaving.

The 5 mL seed culture was transferred into a 2800-mL Fernbach flask containing 250 mL production medium. The flask was incubated for 5 days at 30 °C in a shaker incubator. The production medium consisted of glycerol, 20 g/L; molasses, 10 g/L; casamino acids, 5 g/L; peptone, 1 g/L; and calcium carbonate ($CaCO_3$), 4 g/L dissolved in deionized water and was adjusted to pH 7.2 before autoclaving.

Feeding experiments: To determine the appropriate cerulenin concentration, 0.2 mM (4 mg/100 mL production culture) and 0.1 mM (2 mg/100 mL production culture) were dissolved in 1 mL DMSO and administered separately to 100 mL production culture through a sterile syringe filter every 24 hours starting at the time of inoculation for a total of 5 times. To the control culture 1 mL DMSO (no cerulenin) was added in the same manner daily.

For the aglycone feeding studies, aglycones were added at the time of inoculation while cerulenin was pulse fed daily. Specifically, 7 mg apoptolidinone A were dissolved in 1 mL DMSO and added through a sterile syringe filter to a 100 mL production medium at the time of inoculation while 0.2 mM amount of cerulenin (4 mg/100 mL culture) was dissolved in 0.8 mL

DMSO and added through a sterile syringe filter every 24 hours for 5 days. To the control culture 1 mL DMSO was added at the time of inoculation and 0.8 mL DMSO in the same manner daily. 15 mg apoptolidinone D were dissolved in 1 mL DMSO and added through a sterile syringe filter to a 200 mL production medium at the time of inoculation while 0.2 mM amount of cerulenin (4 mg/100 mL culture) was dissolved in 1 mL DMSO and added through a sterile syringe filter every 24 hours for 5 days. To the control culture 1 mL DMSO was added at the time of inoculation and 0.8 mL DMSO in the same manner daily.

Apoptolidin and apoptolidin D disaccharide purification: The production culture was centrifuged at 4000 rpm for 15 min. The supernatant was extracted with ethyl acetate (3 x 100 mL) followed by TLC analysis. The mycelia were stirred with acetone (100 mL) for one hour and centrifuged at 4000 rpm for 15 minutes. The acetone supernatant was extracted with ethyl acetate (3 x 50 mL). The ethyl acetate extracts were combined, concentrated to ca. 1 mL by rotary evaporation and analyzed by mass spectrometry

Mass spectrometry: Mass spectrometry was performed using ThermoFinnigan (San Jose, CA) TSQ® Quantum triple quadrupole mass spectrometer equipped with a standard electrospray ionization source outfitted with a 100-μm I.D. deactivated fused Si capillary. Data acquisition and spectral analysis were conducted with Xcalibur™ Software, version 1.3, from ThermoFinnigan (San Jose, CA), on a Dell Optiplex GX240 computer running the Microsoft® Windows 2000 operating system. The source spray head was oriented at an angle of 90° to the ion-transfer tube. Nitrogen was used for both the sheath and auxiliary gas. The sheath and auxiliary gases were set to 33 and 14 (arbitrary units) respectively. Samples were introduced by HPLC. A Surveyor® Autosampler and a Surveyor® MS Pump from ThermoFinnigan (San Jose, CA) were used. The injection volume was 10μL. Crude apoptolidin extracts were separated using a Jupiter™ minibore 5μm C18 column with an isocratic mobile phase consisting of 65% water, 35% acetonitrile and 10 mM ammonium acetate. The flow rate was 0.2 mL/min. The mass spectrometer was operated in the positive ion mode and the electrospray needle was maintained at 4200V. The ion transfer tube was operated at 35V and 342 $^{\circ}$C. The tube lens voltage was set to 85V. Source CID (offset voltage between skimmer and the first ion guide, Q00) was used at 15V. The mass spectrometer was operated in full scan mode using Quad 1. The mass spectral resolution was set to a peak width of 0.70u (full width at half maximum, FWHM). Full scan spectra were acquired from m/z 600.0 to 1200.0 over 1.0 second. Data were

acquired in profile mode. The electron multiplier gain was set to 3×10^5. Apoptolidin and derivatives formed ammonium adducts and using the Xcalibur™ Software, data was digitally filtered for the *m/z* of interest.

Isolation and Characterization of Apoptolidin D Disaccharide HPLC was carried out on a Varian system equipped with Prostar 210 pumps and a photodiode array (PDA) detector. The mobile phase was comprised of 10% methanol in dichloromethane. Preparative HPLC was conducted using a Rainin Instrument Company's Dynamax column (300 Å 250 x 10 mm, Woburn, MA) eluted with a mobile phase was comprised of 10% methanol in dichloromethane at a flow rate of 3.0 mL/min with UV detection at 254 nm. Following the above procedures 15 mg of apoptolidinone D afforded 3.7 mg (ca. 18% isolated yield) of apoptolidin D disaccharide (**9**).

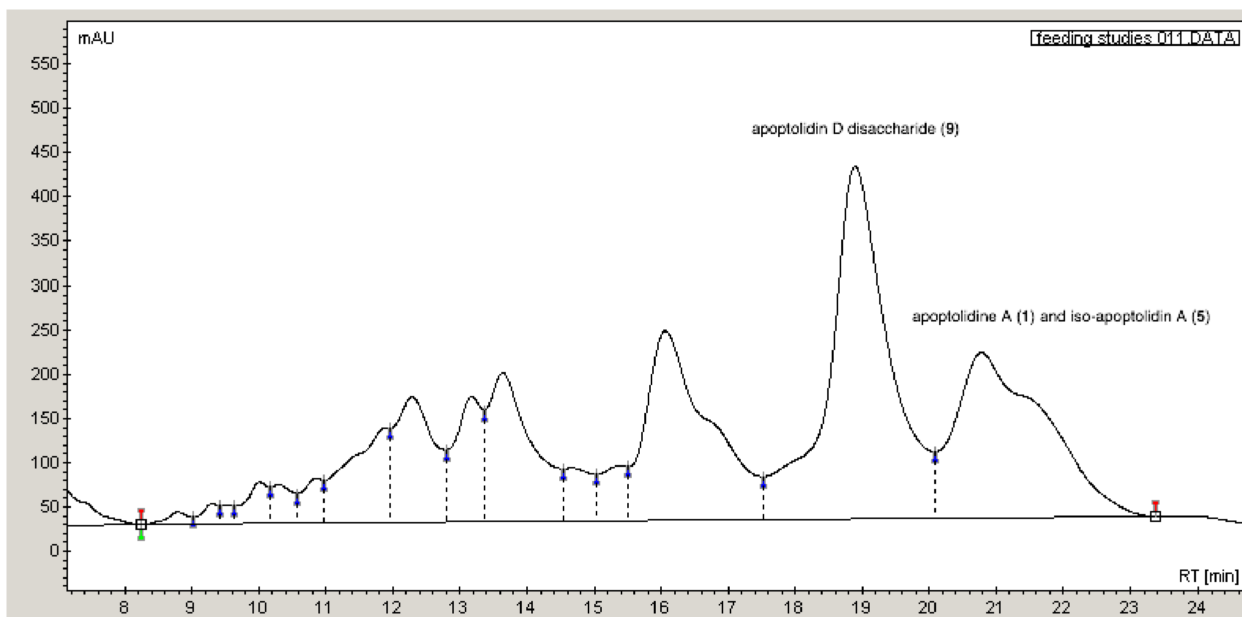
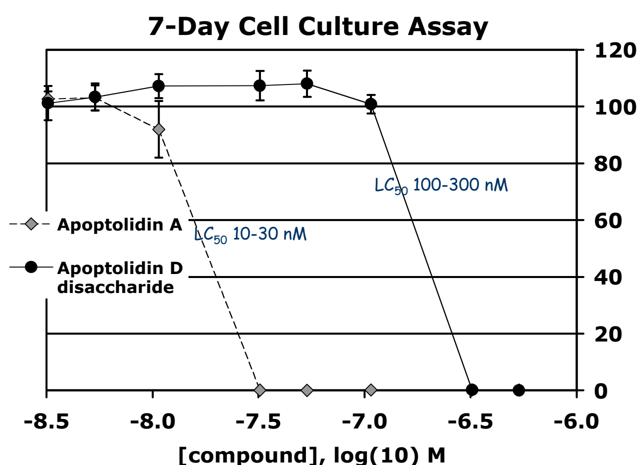


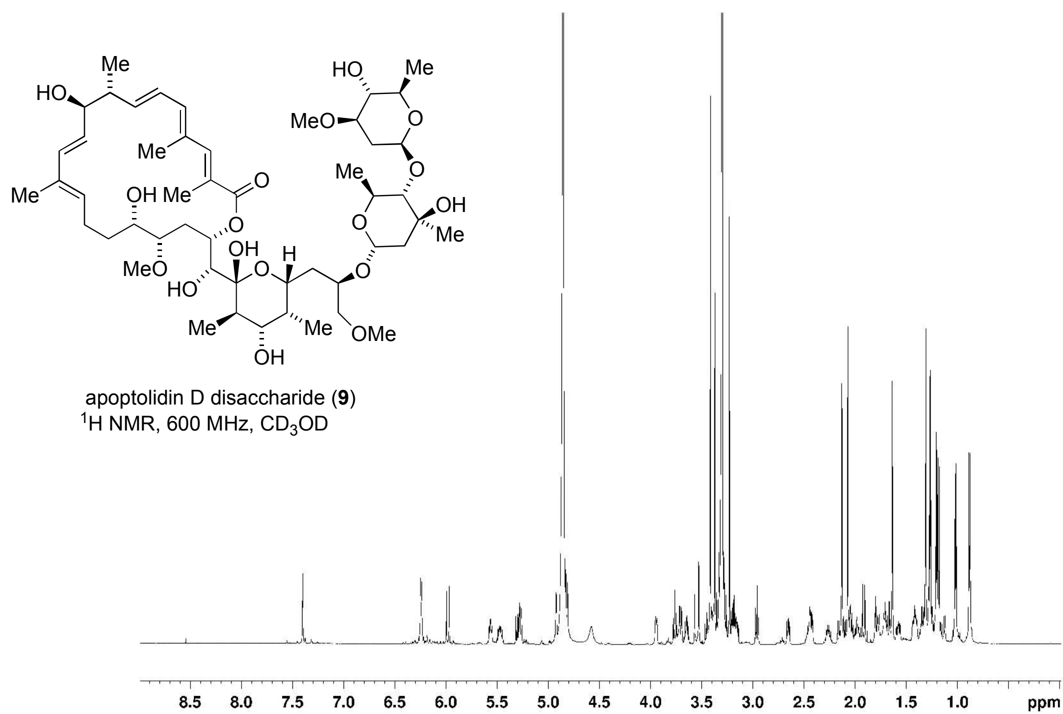
Figure S1. HPLC trace of extract from apoptolidinone D feeding experiment. Spectroscopic analysis of the unlabeled peaks in the above chromatogram provided no correlation to the apoptolidins.

Apotolidin D disaccharide (9): ^{1}H NMR (600 MHz, CD_3OD) δ 7.40 (s, 1H), 6.24-6.23 (m, 2H), 5.98 (d, J = 15.6 Hz, 1H), 5.56 (t, J = 7.8 Hz, 1H), 5.47 (m, 1H), 5.29 (dd, J = 15.6, 8.4 Hz, 1H), 5.27 (d, J = 11.4 Hz, 1H), 4.92 (d, J = 4.2 Hz, 1H), 4.82 (dd, J = 10.2, 1.8 Hz, 1H), 3.94 (dd, J = 5.4, 1.8 Hz, 1H), 3.76 (t, J = 9.0 Hz, 1H), 3.71 (dd, J = 10.8, 4.2 Hz, 1H), 3.65 (dq, J = 9.6, 6.0 Hz, 1H), 3.53 (d, J = 1.8 Hz, 1H), 3.42 (m, 1H), 3.41 (s, 3H), 3.38 (m, 1H), 3.37 (s, 3H),

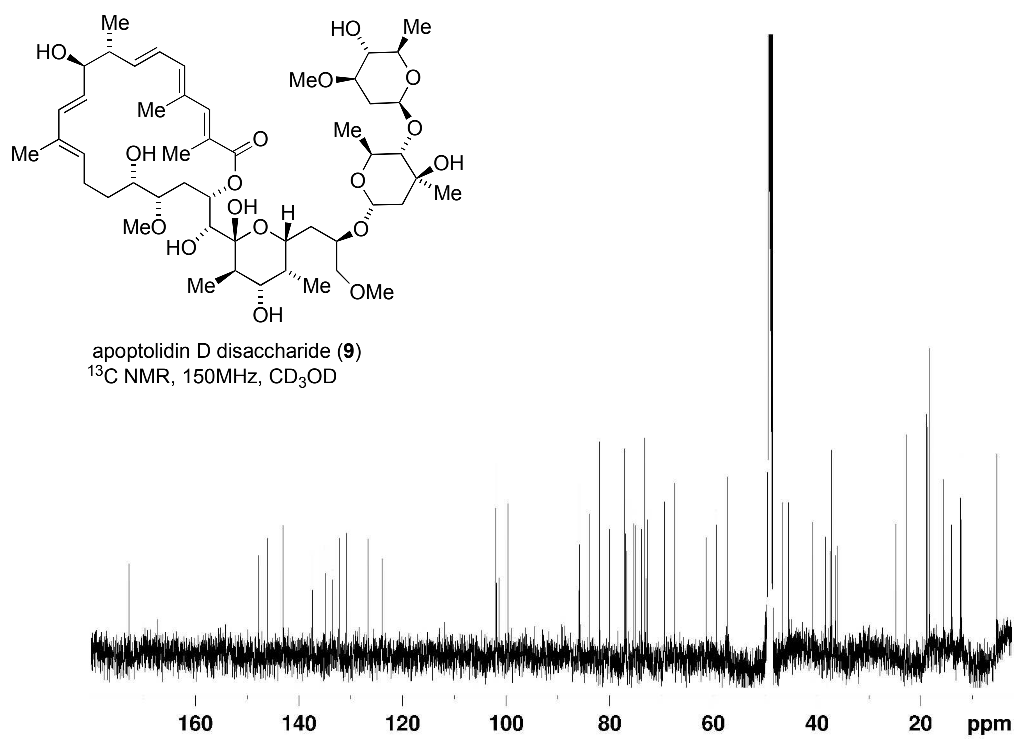
3.33 (m, 1H), 3.28 (m, 1H), 3.23 (s, 3H), 3.20 (dd, J = 9.2, 6.2 Hz), 3.16 (m, 1H), 2.96 (t, J = 9.3 Hz, 1H), 2.65 (dd, J = 9.6, 5.4 Hz, 1H), 2.46 (m, 1H), 2.43 (m, 1H), 2.26 (m, 1H), 2.14 (m, 1H), 2.13 (s, 3H), 2.07 (s, 3H), 2.05 (m, 1H), 1.97 (m, 1H), 1.91 (m, 1H), 1.79 (m, 1H), 1.71 (m, 1H), 1.67 (m, 1H), 1.64 (s, 3H), 1.58 (ddd, J = 14.5, 8.5, 2.6 Hz, 1H), 1.42 (m, 1H), 1.39 (m, 1H), 1.31 (s, 3H), 1.28 (m, 1H), 1.27 (m, 2H), 1.27 (d, J = 6.6 Hz, 3H), 1.20 (d, J = 6.6 Hz, 3H), 1.18 (d, J = 6.6 Hz, 3H), 1.02 (d, J = 6.6 Hz, 3H), 0.88 (d, J = 7.2 Hz, 3H); ^{13}C NMR (150 MHz, CD_3OD) δ 172.8, 147.7, 146.1, 143.1, 137.4, 135.0, 133.5, 132.2, 130.9, 126.7, 124.0, 101.9, 101.3, 99.6, 85.9, 84.0, 82.0, 80.0, 77.2, 77.0, 76.7, 75.4, 74.9, 73.9, 73.2, 73.0, 72.7, 69.4, 67.4, 61.3, 59.5, 57.3, 46.7, 45.5, 40.8, 38.3, 37.4, 37.2, 36.4, 36.1, 24.8, 22.8, 18.9, 18.6, 18.3, 15.6, 14.0, 12.2, 12.1, 5.2; ; HRMS (TOF MS) m/z 961.5723 [(M+Li)+ calculated for $C_{50}H_{82}LiO_{17}$: 961.5712].

Cytotoxicity Assay: H292 lung carcinoma cells were plated at a density of 500/well in 96-well plates in RPMI 1640 medium containing 10% fetal bovine serum. Compounds were dissolved in DMSO, and cells were treated with DMSO alone (control); apoptolidin A and apoptolidinone D disaccharide from 3 nM - 10 mM (0.5% DMSO final concentration in culture medium). Viability was measured after 7 days by adding 2 μM Calcein-AM, and measuring fluorescence on a Spectramax (Molecular Dynamics) plate reader, λ_{abs} = 494; λ_{em} = 517. Effective concentration 50 (EC_{50}) values for apoptolidin A and apoptolidin D disaccharide are defined as the concentration of compound at which Calcein-AM fluorescence is inhibited by 50%.





apoptolidin D disaccharide (**9**)
^{1}H NMR, 600 MHz, CD_3OD



apoptolidin D disaccharide (**9**)
^{13}C NMR, 150MHz, CD_3OD

Table S1. ^{1}H (600 MHz) and ^{13}C (150 MHz) NMR spectral data of apoptolidin D disaccharide (**9**) and apoptolidin D (**4**) in CD_3OD

apoptolidin D disaccharide (**9**)			apoptolidin D (**4**)		apoptolidin D disaccharide (**9**)			apoptolidin D (**4**)	
Number	δ_C	δ_H (J=Hz)	δ_C	δ_H	Number	δ_C	δ_H (J=Hz)	δ_C	δ_H
1	172.8	--	172.3	--	2-Me	14.0	2.13	13.9	2.14
2	124.0	--	123.7	--	4-Me	15.6	2.07	15.4	2.08
3	147.7	7.40	147.2	7.41	8-Me	18.6	1.20 (6.6)	18.6	1.20
4	133.5	--	132.9	--	12-Me	12.2	1.64	12.2	1.65
5	143.1	6.23	142.5	6.26	22-Me	12.1	1.02 (6.6)	12.0	1.03
6	126.7	6.24	127.0	6.30	24-Me	5.2	0.88 (7.2)	5.0	0.90
7	146.1	5.47	144.9	5.48	17-OMe	61.3	3.37	61.1	3.38
8	46.7	2.26	44.5	2.52	28-OMe	59.5	3.23	59.3	3.24
9	80.0	3.76 (9.0)	83.3	3.84	1’	--	--	96.0	4.81
10	130.9	5.29 (15.6, 8.4)	127.5	5.21	2’	--	--	61.1	3.39
11	137.4	5.98 (15.6)	140.5	6.08	3’	--	--	74.7	3.71
12	135.0	--	134.6	--	4’	--	--	87.2	2.72
13	132.2	5.56 (7.8)	132.7	5.62	5’	--	--	67.9	3.74
14	24.8	2.46	24.4	2.48	6’	--	--	17.9	1.26
		1.97		1.98	4’-OMe	--	--	60.9	3.58
15	36.4	1.42	35.8	1.43	1”	99.6	4.92 (4.2)	99.4	4.93
		1.39		1.33	2”	45.5	1.91	45.2	1.93
16	74.9	3.38	74.1	3.40			1.79		1.80
17	84.0	2.65 (9.6, 5.4)	83.6	2.66	3”	73.0	--	72.9	--
18	38.3	2.14	37.8	2.14	4”	85.9	3.33	85.5	3.33
		1.67		1.66	5”	67.4	3.65 (9.6, 6.0)	67.2	3.66
19	72.7	5.27 (11.4)	72.3	5.28	6”	18.9	1.18 (6.6)	18.6	1.21
20	75.4	3.53 (1.8)	75.1	3.53	3”-Me	22.8	1.31	22.5	1.32
21	101.3	--	100.8	--	1’”	101.9	4.82 (10.2, 1.8)	101.7	4.83
22	36.1	2.05	36.2	2.05	2’”	37.4	2.43	37.0	2.43
23	73.9	3.71 (10.8, 4.2)	74.0	3.72			1.28		1.28
24	40.8	1.71	40.6	1.71	3’”	82.0	3.16	81.7	3.16
25	69.4	3.94 (5.4, 1.8)	69.2	3.95	4’”	77.2	2.96	76.9	2.97
26	37.2	1.58 (14.5, 8.5, 2.6)	37.1	1.59	5’”	73.2	3.20 (9.2, 6.2)	73.0	3.20
		1.27		1.43	6’”	18.3	1.27 (6.6)	17.9	1.27
27	77.0	3.42	76.6	3.42	3’”-OMe	57.3	3.41	57.1	3.42
28	76.7	3.28	76.5	3.28					

References and Notes

1. Kim, J. W.; Adachi, H.; Shin-Ya, K.; Hayakawa, Y.; Seto, H. *J. Antibiot.*, **1997,** *50(7)*, 628