Contents

Supporting Methods and Compound Characterizations

General experimental procedures. Reactions were performed under an argon atmosphere using freshly dried solvents. Methylene chloride (DCM) was dried by passing through an activated alumina column. Solvents used for HPLC chromatography were HPLC grade and were used without further purification. Optical rotations were measured on a Jasco P-2000 polarimeter using a 10 mm path length cell at 589 nm. NMR spectra were acquired on a Varian Inova 600 MHz spectrometer equipped with a 5 mm HCN triple resonance cryoprobe, and referenced to residual solvent proton and carbon signals (δH 2.500, δC 39.520 for DMSO-d_6 and δH 3.310, δC 49.00 for Methanol-d_4). High resolution mass spectrometer data were acquired using an Agilent 6230 electrospay ionization (ESI) accurate-mass time-of-flight (TOF) liquid chromatograph/mass spectrometer.

Prefractionated natural product library generation. Purified bacterial colonies were grown in 1 L of modified SYP [1] broth (1L MilliQ water, 32.1 g Instant Ocean™, 10 g starch, 4 g peptone, 2 g yeast) with 20 g of Amberlite XAD-16 resin for 10 days at 27°C. Culture broth and resin slurries were filtered through glass microfiber filters, washed with water (3×200 mL) and the cells, resin, and filter paper extracted with 1:1 MeOH/DCM (250 mL). Organic fractions were dried *in vacuo* and subjected to solid phase extraction (SPE) using a Supelco-Discovery C_{18} cartridges (5 g) eluting with a step gradient of 40 mL of MeOH/H_2O solvent mixtures (20% MeOH, 40% MeOH, 60% MeOH, 80% MeOH, 100% MeOH) and finally with EtOAc to afford six fractions. The resulting fractions were dried *in vacuo*, resolubilized in 1 mL of DMSO, and transferred to deep well 96-well plates for screening.

Skyllamycin isolation. *Streptomyces sp.* 1675 was grown in 1 L of modified SYP broth with 20 g of Amberlite XAD-16 resin for 7 days at 27°C and was submitted the organic extraction described above. The active 80% MeOH fraction was subjected to C18 RP-HPLC Phenomenex Synergi C_{18} (4.6×250 mm, 10 µm), [60% MeOH/40% H_2O to 70%MeOH/30%H_2O (acidified with 0.002% formic acid) over 26 minutes at 2 mL/min, monitored at 254 nm, to give skyllamycins A-C.

Marfey's analysis experimental.

General amino acid derivatization: Using a modified procedure by Bhushan and Brückner, [2] aqueous solutions (50 mM) of proteinogenic amino acids (D- and L-isomers) were prepared as starting materials for synthesis. Solutions of each amino acid (50 µL, 2.5 micromoles) were placed in separate 5 mL vials with microstir bars. To each was added 100 µL of 1% acetone solution of fluorodinitrophenyl-5-L-valine amide (FDVA, 1 mg, 3.6 micromoles), the molar ratio of FDVA to amino acid 1.4:1, followed by $NaHCO_3$ (1 M, 20 µL, 20 micromoles) and 10 µL of DMSO. The contents were stirred in an oil bath at 40°C for 1 hr. After cooling to room temperature, HCl (2 M, 10 µL, 20 micromoles) was added to each reaction mixture. The contents were dried *in vacuo*, taken up into 1 mL of 50:50 MeOH:H_2O, and centrifuged. A 1:10 dilution into 50:50 MeOH:H_2O was then injected into the LC-ESI-MS-TOF for analysis.

General skyllamycin analog derivatization: Each of the skyllamycins (0.2 mg) were placed into separate 5 mL vials with stir bars. 1 mL of 6 N HCl was added to each vial and stirred at 80°C for 6 hours, dried *in vacuo,* and resuspended in 100 µL of H_2O. To each vial, 500 µL of 1% solution of fluorodinitrophenyl-5-L-valine amide (FDVA, 5 mg, 18.0 micromoles) in acetone was added, the molar ratio of FDVA to amino acid equivalence is approximately 12:1, followed by $NaHCO_3$ (1 M, 100 µL, 100 micromoles), and 50 µL of DMSO.

The contents were stirred in an oil bath at 40°C for 1 hr. After cooling to room temperature, HCl (2 M, 50 µL, 100 micromoles) was added to each reaction mixture. The contents were dried *in vacuo,* taken up into 1 mL of 50:50 MeOH:H_2O, and centrifuged. A 1:10 dilution into 50:50 MeOH:H_2O was then injected into the LC-ESI-MS-TOF for analysis.

Chemical Chracterization of Compounds.

Skyllamycin C: $[\alpha]^{D}_{24}$ - 22.7 (c 0.033, MeOH); UV (MeOH) λ_{max} (log ε) 230 nm (4.90); ^{1}H NMR (600 MHz, Methanol-d_4) δ 7.62 (d, *J* = 8.1 Hz, 1H), 7.48 (d, *J* = 7.6 Hz, 2H), 7.35 (t, *J* = 7.5 Hz, 2H), 7.33 (d, *J* = 7.8 Hz, 1H), 7.31 (d, *J* = 8.3 Hz, 1H), 7.26 (t, *J* = 7.4 Hz, 1H), 7.19 (s, 1H), 7.17 (m, 1H), 7.13 (m, 1H), 7.12 (m, 1H), 7.09 (t, *J* = 7.6 Hz, 1H), 6.98 (t, *J* = 7.5 Hz, 1H), 6.71 (d, *J* = 7.2 Hz, 2H), 6.61 (d, *J* = 8.3 Hz, 2H), 6.48 (d, *J* = 11.3 Hz, 1H), 5.60 (s, 1H), 5.55 (dq, *J* = 11.3, 6.9 Hz, 1H), 5.47 (q, *J* = 6.8 Hz, 1H), 4.99 (s, 1H), 4.90 (m, 1H), 4.81 (d, *J* = 6.8 Hz, 1H), 4.66 (d, *J* = 10.1 Hz, 1H), 4.63 (dd, *J* = 9.5, 4.6 Hz, 1H), 4.59 (d, *J* = 6.8 Hz, 1H), 4.55 (d, *J* = 9.1 Hz, 1H), 4.50 (s, 1H), 4.38 (dd, *J* = 8.8, 2.9 Hz, 1H), 4.36 (d, *J* = 6.6 , 1H), 4.09 (q, *J* = 7.3 Hz, 1H), 4.05 (d, *J* = 17.9 Hz, 1H), 3.80 (d, *J* = 9.4 Hz, 1H), 3.73 (m, 2H), 3.71 (s, 3H), 3.46 (d, *J* = 17.9, 1H), 3.30 (dd, *J* = 14.9, 4.6 Hz, 1H), 3.11 (m, 1H), 3.11 (m, 1H), 3.05 (t, *J* = 8.4 Hz, 2H), 2.95 (dt, *J* = 14.8, 8.9 Hz, 1H), 2.80 (dt, *J* = 15.7, 8.4 Hz, 1H), 2.74 (m, 1H), 1.98 (m, 1H), 1.819 (m, 1H), 1.79 (m, 1H), 1.78 (m, 1H), 1.77 (m, 1H), 1.72 (m, 1H), 1.66 (m, 1H), 1.51 (d, *J* = 7.1 Hz, 3H), 1.50 (m, 1H), 1.47 (d, *J* = 7.5 Hz, 3H), 1.25 (d, *J* = 6.8 Hz, 3H), 1.04 (d, *J* = 6.7 Hz, 3H), 1.00 (d, *J* = 6.1 Hz, 3H), 0.92 (d, *J* = 6.2 Hz, 3H), 0.86 (d, *J* = 6.7 Hz, 3H). ^{13}C NMR** (151 MHz, Methanol-d_4) 177.66, 175.04, 174.86, 173.30, 173.07, 172.93, 172.26, 172.17, 171.25, 170.94, 170.43, 170.03, 169.76, 159.37, 141.70, 139.20, 136.72, 136.59, 136.31, 131.48, 129.13, 128.29, 128.16, 127.77, 127.64, 127.37, 126.94, 126.62, 126.23, 125.24, 123.72, 120.93, 118.61, 118.20, 113.05, 110.91, 109.38, 75.73, 73.36, 72.65, 72.47, 69.42, 60.42, 59.61, 57.57, 57.00, 55.08, 54.89, 54.23, 54.00, 51.24, 50.59, 47.00, 41.95, 39.86, 38.40, 35.49, 31.08, 28.74, 28.58, 27.84, 24.68, 23.56, 21.64, 20.68, 18.49, 17.85, 15.86, 15.33, 12.89; HR-ESI-TOF-MS m/z $[M+Na]^+$ 1493.6616 (calcd for $C_{74}H_{94}N_{12}O_{20}Na^+$, 1493.6600).

** Obtained indirectly with HSQC and HMBC data.

Supporting Tables

Supporting Table S1. EC_{50} values of antibiotics for macrolides, aminoglycosides, and tetracyclines. Data based on one biological replicate with four technical replicates.

Antibiotic	Inhibition EC_{50} (μM)	Dispersal EC_{50} (μM)	Resistance Increase
Doxycycline	142	> 1567	> 11
Demeclocycline	17	1330	78
Oxytetracycline	> 2500	> 2500	-
Spiramycin	> 1450	> 1450	-
Erythromycin	489	> 1810	> 3
Azithromycin	48	> 2430	> 50
Kanomycin	> 700	> 700	-
Amikacin	13	114	8
Tobramycin	12	>2010	167

Supporting Table S2. ^{1}H and ^{13}C** NMR data for skyllamycin C. (** Inferred by HMBC and HSQC data)

Unit	Position	δ C	δ H	Integration, J (Hz)
Thr	C=O	174.9		
	α	59.6	4.99	1 s
	β	69.4	5.47	1 q, *J* = 6.8 Hz
	Methyl	15.9	1.25	3 d, *J* = 6.8 Hz
Ala	C=O	173.3		
	α	51.2	4.09	1 q, *J* = 7.3 Hz
	Methyl	15.3	1.47	3 d, *J* = 7.5 Hz
Glu	C=O	170.4		
	C=O	172.9		
	α	50.6	4.90	1 m
	β	38.4	3.11	1 m
	β'		2.74	1 m
Gly	C=O	170.9		
	α	42.0	4.05	1 d, *J* = 17.9 Hz
	α'		3.46	1 d, *J* = 17.9
oxy-Phe	C=O	172.3		
	1	141.7		
	3/5	127.8	7.35	2 t, *J* = 7.5 Hz
	4	127.4	7.26	1 t, *J* = 7.4 Hz
	2/6	126.2	7.48	2 d, *J* = 7.6 Hz
	α	57.0	4.55	1 d, *J* = 9.1 Hz
	β	72.6	4.66	1 d, *J* = 10.1 Hz
Pro	C=O	172.2		
	α	60.4	4.38	1 dd, *J* = 8.8, 2.9 Hz
	β	23.6	1.82	1 m
	β'		1.50	1 m
	γ	28.7	1.98	1 m
	γ'		1.77	1 m
	δ	47.0	3.73	2 m
oxy-Tyr-Me	C=O	170.0		
	4	159.4		
	1	131.5		
	2/6	127.6	6.71	2 d, *J* = 7.2 Hz
	3/5	113.1	6.61	2 d, *J* = 8.3 Hz
	OMe	54.2	3.71	3 s
	α	57.6	4.81	1 d, *J* = 6.8 Hz
	β	73.4	4.59	1 d, *J* = 6.8 Hz

Unit	Position	δ C	δ H	Integration, J (Hz)
Trp	C=O	173.1		
	7a	136.7		
	3a	136.6		
	2	123.7	7.19	1 s
	6	120.9	7.09	1 t, *J* = 7.6 Hz
	5	118.6	6.98	1 t, *J* = 7.5 Hz
	4	118.2	7.62	1 d, *J* = 8.1 Hz
	7	110.9	7.31	1 d, *J* = 8.3 Hz
	3	109.4		
	α	54.0	4.63	1 dd, *J* = 9.5, 4.6 Hz
	β	27.8	3.30	1 dd, *J* = 14.9, 4.6 Hz
	β'		3.11	1 m
oxy-Gly	C=O	171.3		
	α	72.5	5.61	1 s
Leu	C=O	175.0		
	α	54.9	4.36	1 d, *J* = 6.6
	β	39.9	1.78	1 m
	β'		1.66	1 m
	γ	24.7	1.79	1 m
	Methyl	21.6	1.00	3 d, *J* = 6.1 Hz
	Methyl	20.7	0.92	3 d, *J* = 6.2 Hz
oxy-Leu	C=O	169.8		
	α	55.1	4.50	1 s
	β	75.7	3.80	1 d, *J* = 9.4 Hz
	γ	31.1	1.72	1 m
	Methyl	18.5	1.04	3 d, *J* = 6.7 Hz
	Methyl	17.8	0.86	3 d, *J* = 6.7 Hz
alkyl-PKS	C=O	177.7		
	ben-1	139.2		
	ben-2	136.3		
	ben-3	129.1	7.12	1 m
	sp^2-1	128.3	6.48	1 d, *J* = 11.3 Hz
	ben-6	128.2	7.33	1 d, *J* = 7.8 Hz
	sp^2-2	126.9	5.55	1 dq, *J* = 11.3, 6.9 Hz
	ben-5	126.6	7.17	1 m
	ben-4	125.2	7.13	1 m
	β	35.5	2.95	1 dt, *J* = 14.8, 8.9 Hz
			2.80	1 dt, *J* = 15.7, 8.4 Hz
	α	28.6	3.05	2 t, *J* = 8.4 Hz
	Me	12.9	1.51	3 d, *J* = 7.1 Hz

Supporting Table S3. BDM assay co-dose experiments.

Replicate 1

BDM
Percent Biofilm Coverage
(Normalized)

Skyllamycin B [µM] \ Azithromycin [µM]	1580	397	99	0
100.00	0.63	0.67	0.65	0.69
50.00	1.00	0.96	0.92	0.95
25.00	1.16	1.11	1.14	1.01
12.50	0.97	1.02	1.16	1.23
6.25	1.06	1.14	0.98	1.10
3.13	1.01	1.05	1.14	0.96
1.56	1.04	1.06	1.05	1.14
0.78	1.03	1.07	1.06	1.07
0.00	1.03	1.05	1.07	1.05

BDM
Bacterial Cellular Activity
(Normalized)

Skyllamycin B [µM] \ Azithromycin [µM]	1580	397	99	0
100.00	0.24	0.33	0.49	1.08
50.00	0.21	0.29	0.43	0.92
25.00	0.24	0.28	0.49	1.03
12.50	0.21	0.39	0.50	0.89
6.25	0.23	0.36	0.51	1.08
3.13	0.24	0.31	0.53	1.01
1.56	0.26	0.41	0.56	1.17
0.78	0.25	0.32	0.51	1.15
0.00	0.21	0.35	0.51	1.19

Replicate 2

BDM
Percent Biofilm Coverage
(Normalized)

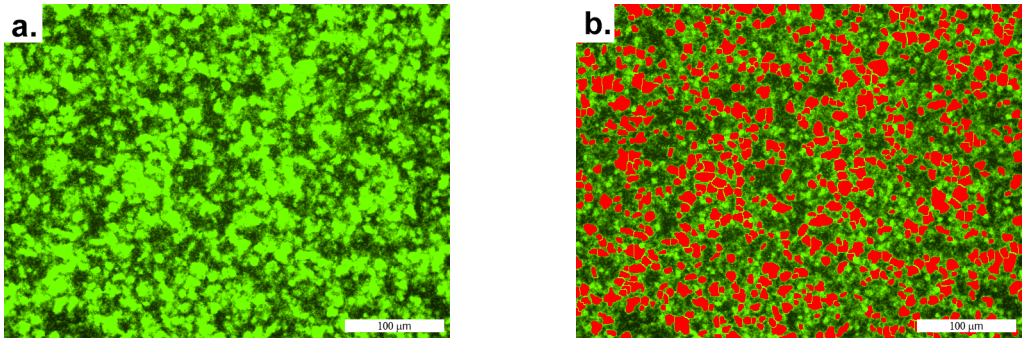
Skyllamycin B [µM] \ Azithromycin [µM]	1580	397	99	0
100.00	0.65	0.58	0.64	0.64
50.00	1.16	1.01	0.83	0.92
25.00	1.17	1.23	1.24	1.24
12.50	1.21	1.21	1.14	1.23
6.25	1.19	1.21	1.19	1.19
3.13	1.20	1.19	1.20	1.20
1.56	1.19	1.20	1.20	1.20
0.78	1.20	1.19	1.20	1.20
0.00	1.19	1.19	1.20	1.20

BDM
Bacterial Cellular Activity
(Normalized)

Skyllamycin B [µM] \ Azithromycin [µM]	1580	397	99	0
100.00	0.19	0.19	0.36	0.93
50.00	0.21	0.30	0.43	0.71
25.00	0.12	0.22	0.37	0.87
12.50	0.19	0.28	0.44	0.73
6.25	0.17	0.30	0.47	1.09
3.13	0.20	0.36	0.56	0.69
1.56	0.15	0.25	0.42	1.03
0.78	0.20	0.29	0.51	0.75
0.00	0.22	0.30	0.36	1.23

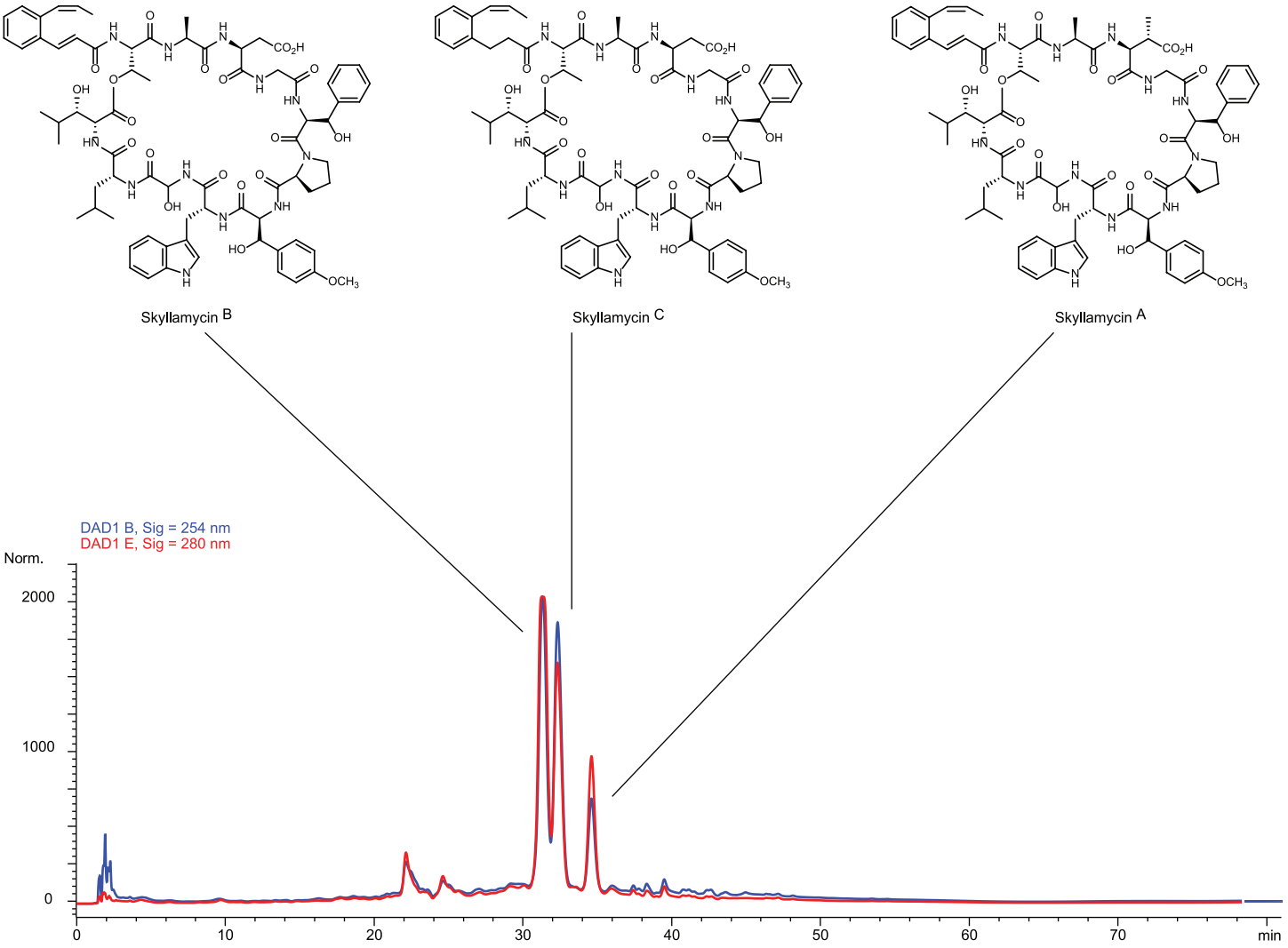
Supporting Figures

Supporting Fig. S1. Biofilm image pixel mask.



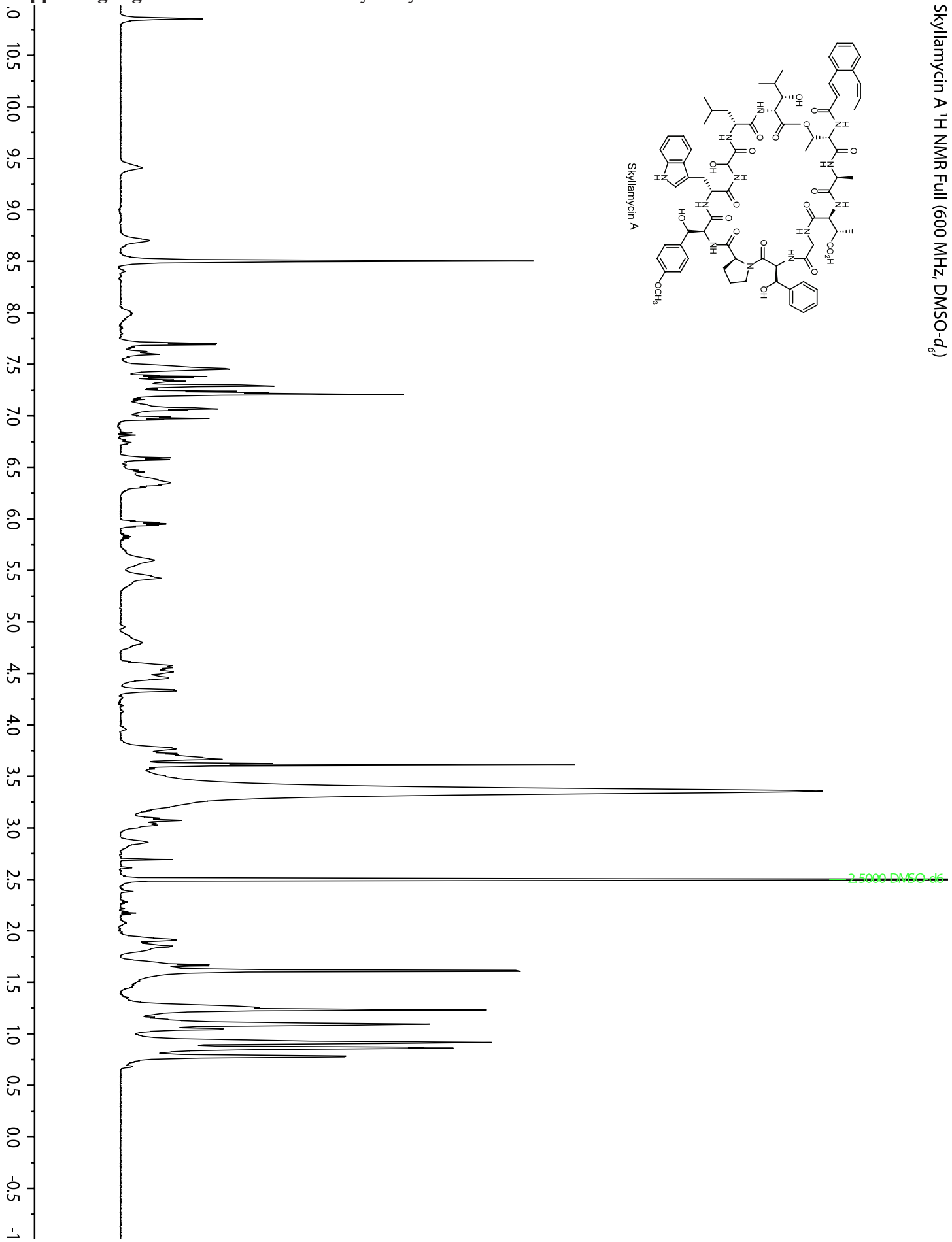
False colored images of *Pseudomonas aeruginosa* biofilms. A 20x image of a single site within a well. White bars represent 100 micron. a) DMSO treated biofilm well site image. b) Biofilm quantifying pixel mask. Total pixel count on mask quantifies biofilm coverage.

Supporting Fig. S2. 1675D LC-MS analysis.

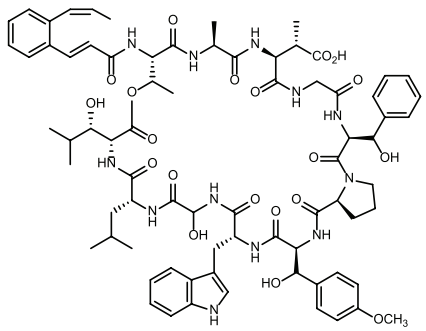


LC-MS analysis of extract 1675D showing three major constituents. Major constituents are annotated. LCMS run conditions were 60:40 to 100:0 MeOH:H_2O (with 0.02% formic acid) over 80 minutes on RP-HPLC Phenomenex Synergi C_{18} column (4.6 x 250 mm, 10 µM) 2 mL/min.

Supporting Fig. S3. NMR data set for skyllamycins A - C.

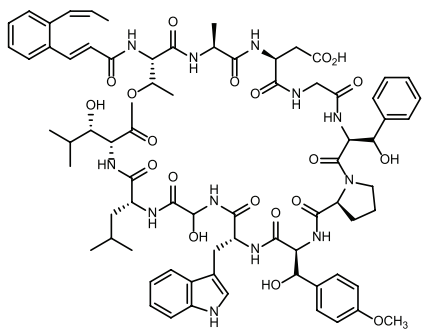


Skyllamycin A ^{1}H NMR Full (600 MHz, Methanol-d_4)



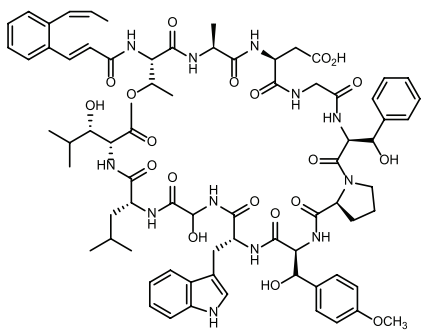
Skyllamycin A

Skyllamycin B ^{1}H NMR Full (600 MHz, DMSO-d_6)



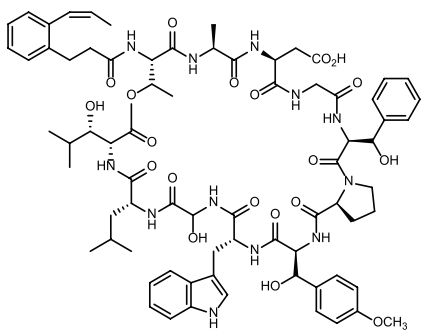
Skyllamycin B

Skyllamycin B ^{1}H NMR Full (600 MHz, Methanol-d_4)



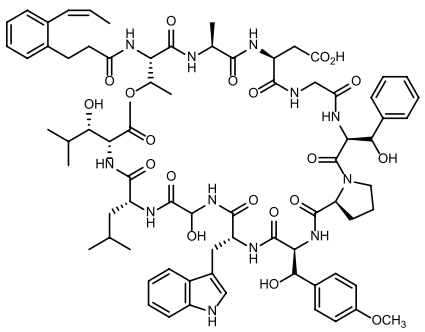
Skyllamycin B

Skyllamycin C ^{1}H NMR Full (600 MHz, Methanol-d_4)



Skyllamycin C

Skyllamycin C ^{1}H NMR Zoomed (600 MHz, Methanol-d_4)



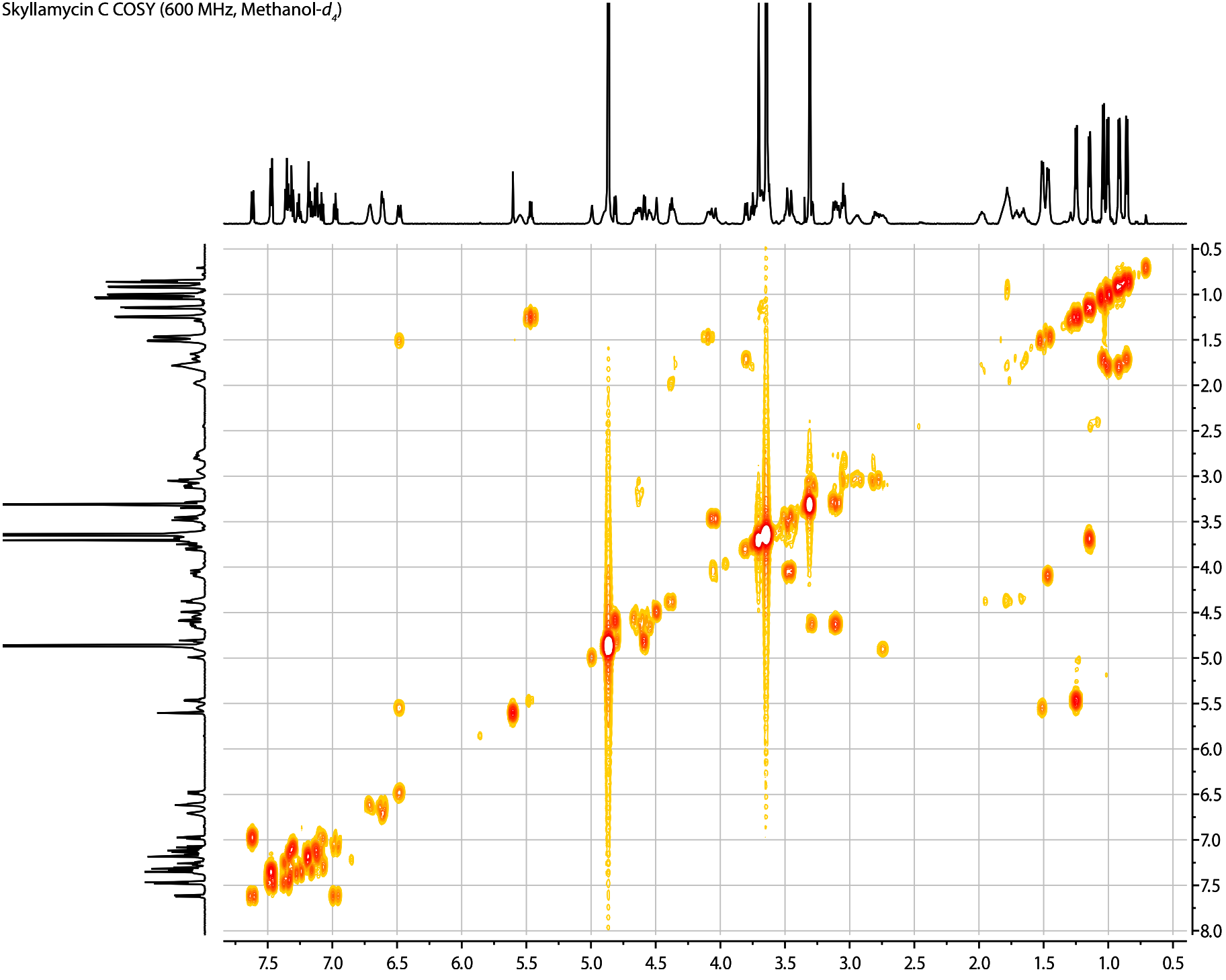
Skyllamycin C

3.3100 CD3OD

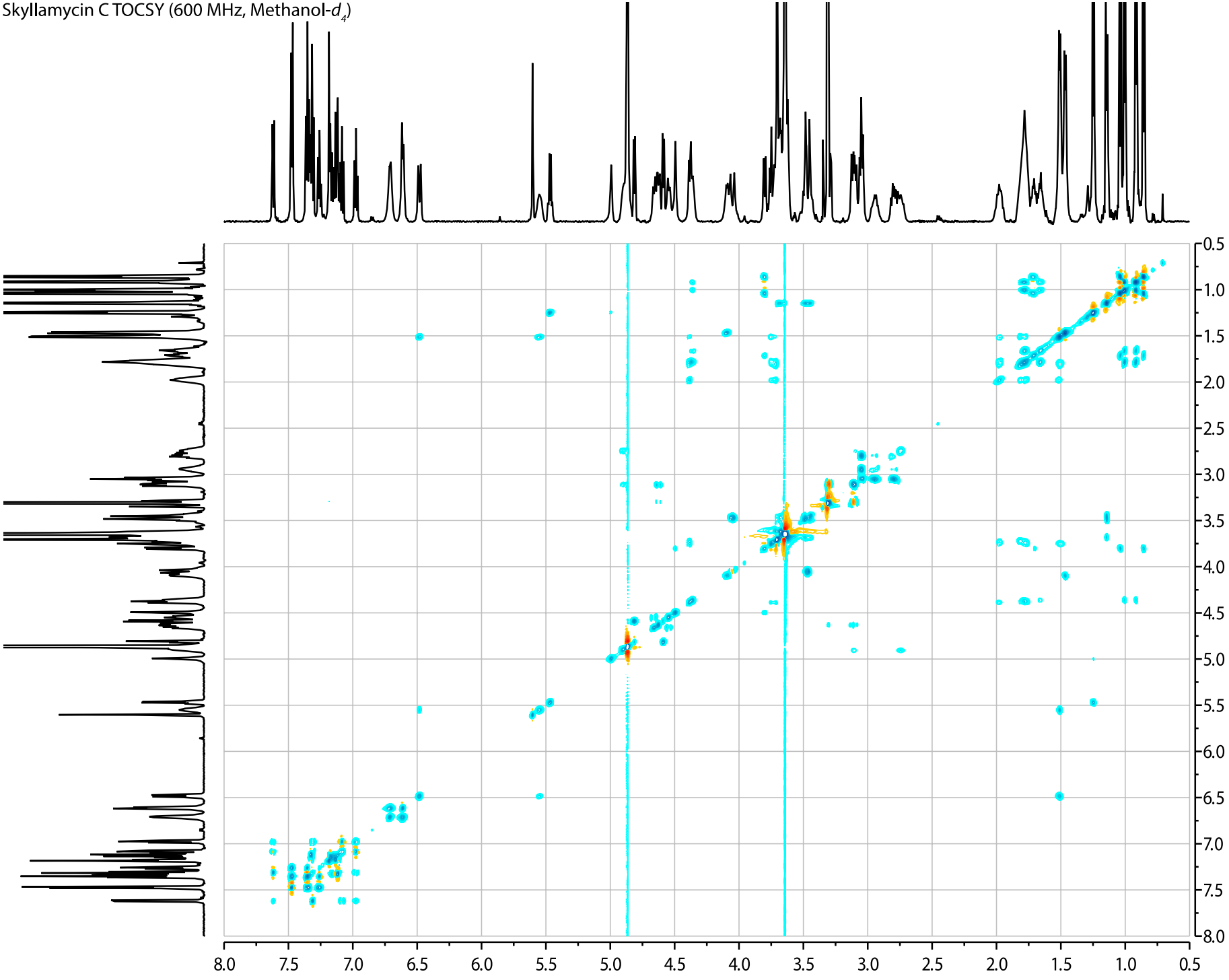
1.00 2.21 2.35 1.12 4.17 0.91 0.96 1.34 1.44 1.09 0.85 0.86 1.01 0.76 0.95 1.94 1.11 1.05 1.09 2.17 2.25 1.05 1.06 3.76 1.19 3.12 0.74 2.15 2.31 1.05 1.13 1.03 1.39 4.50 2.44 3.69 3.44 3.50 3.02 3.39 3.31 3.52 3.24

0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0

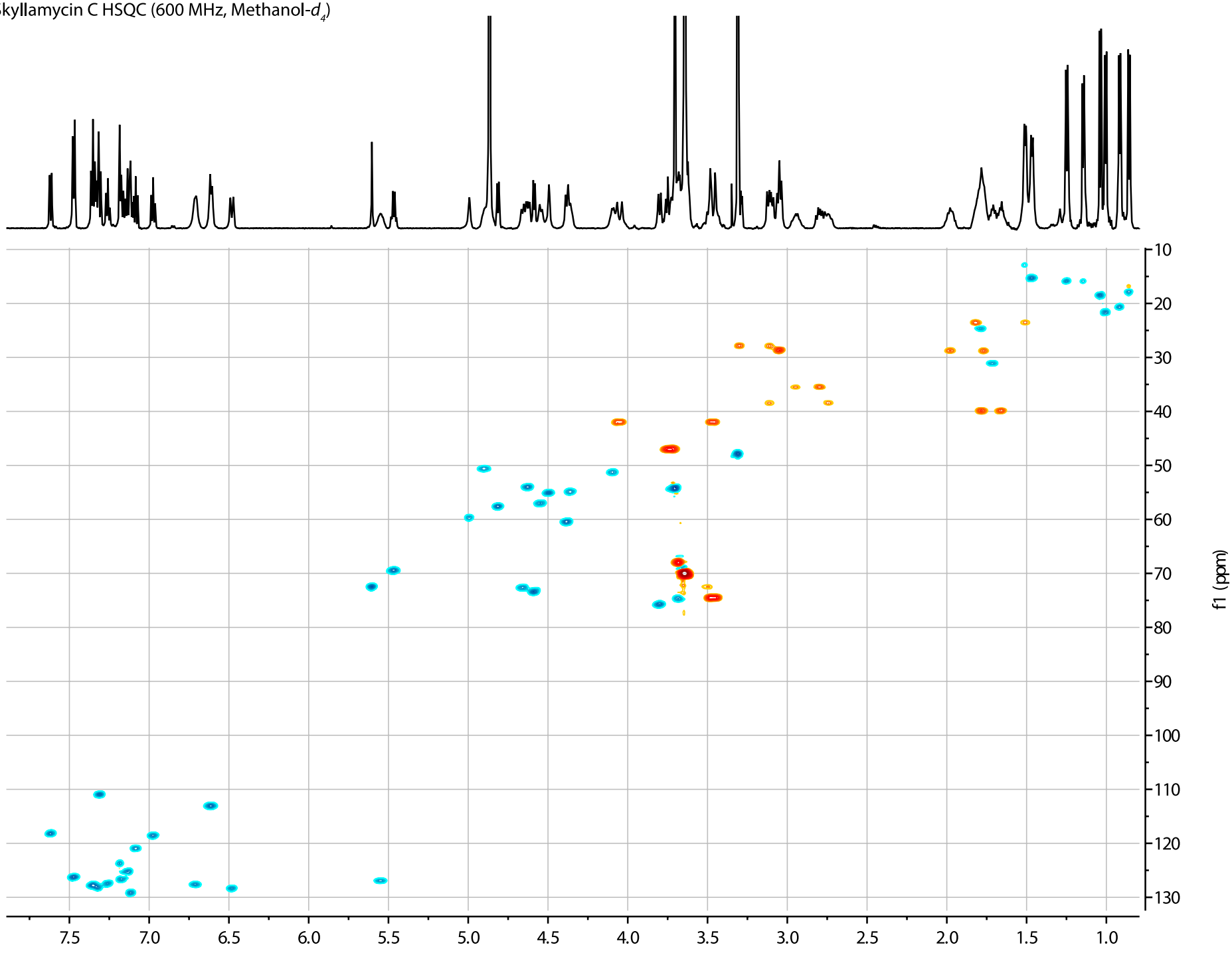
Skyllamycin C COSY (600 MHz, Methanol-d_4)



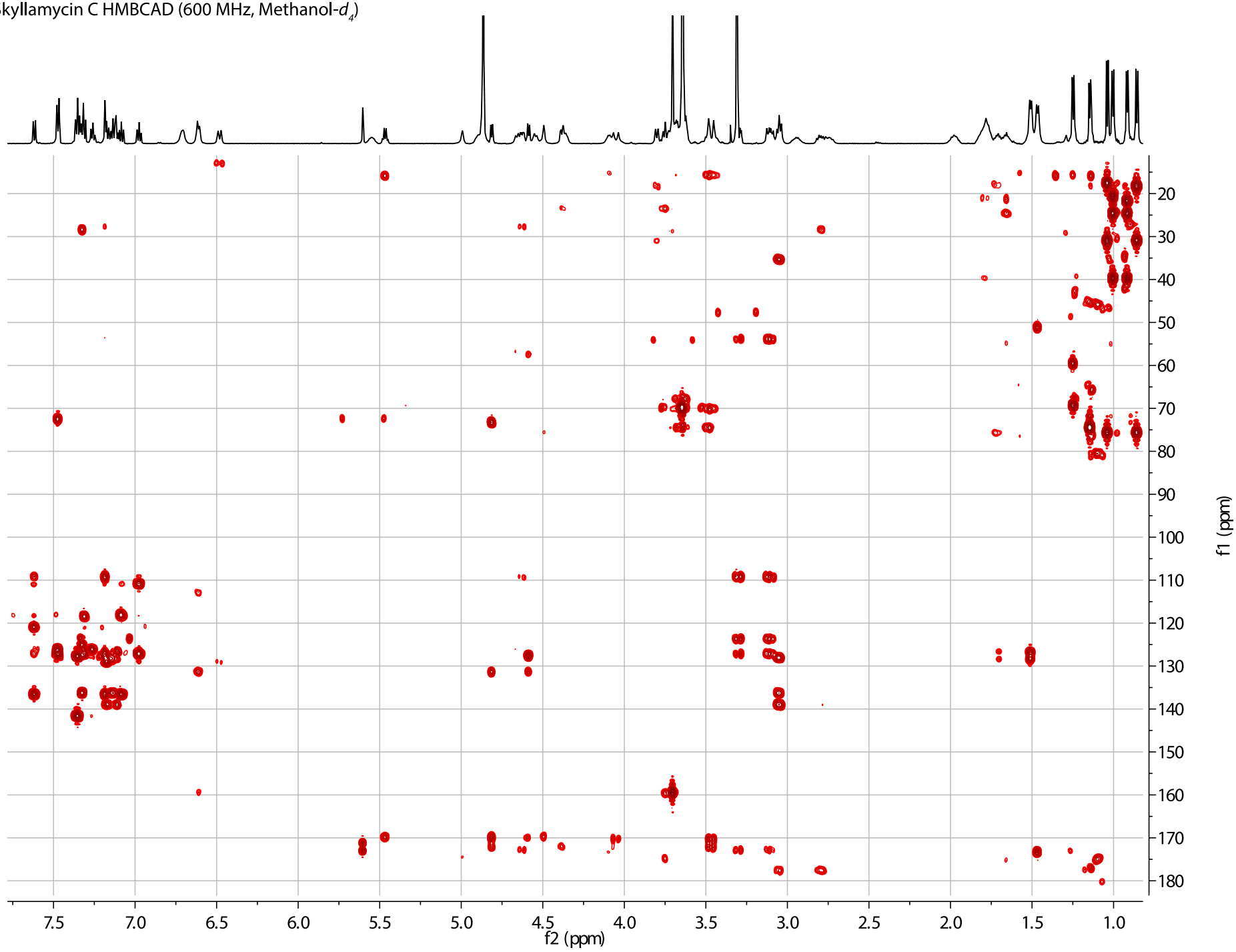
Skyllamycin C TOCSY (600 MHz, Methanol-d_4)



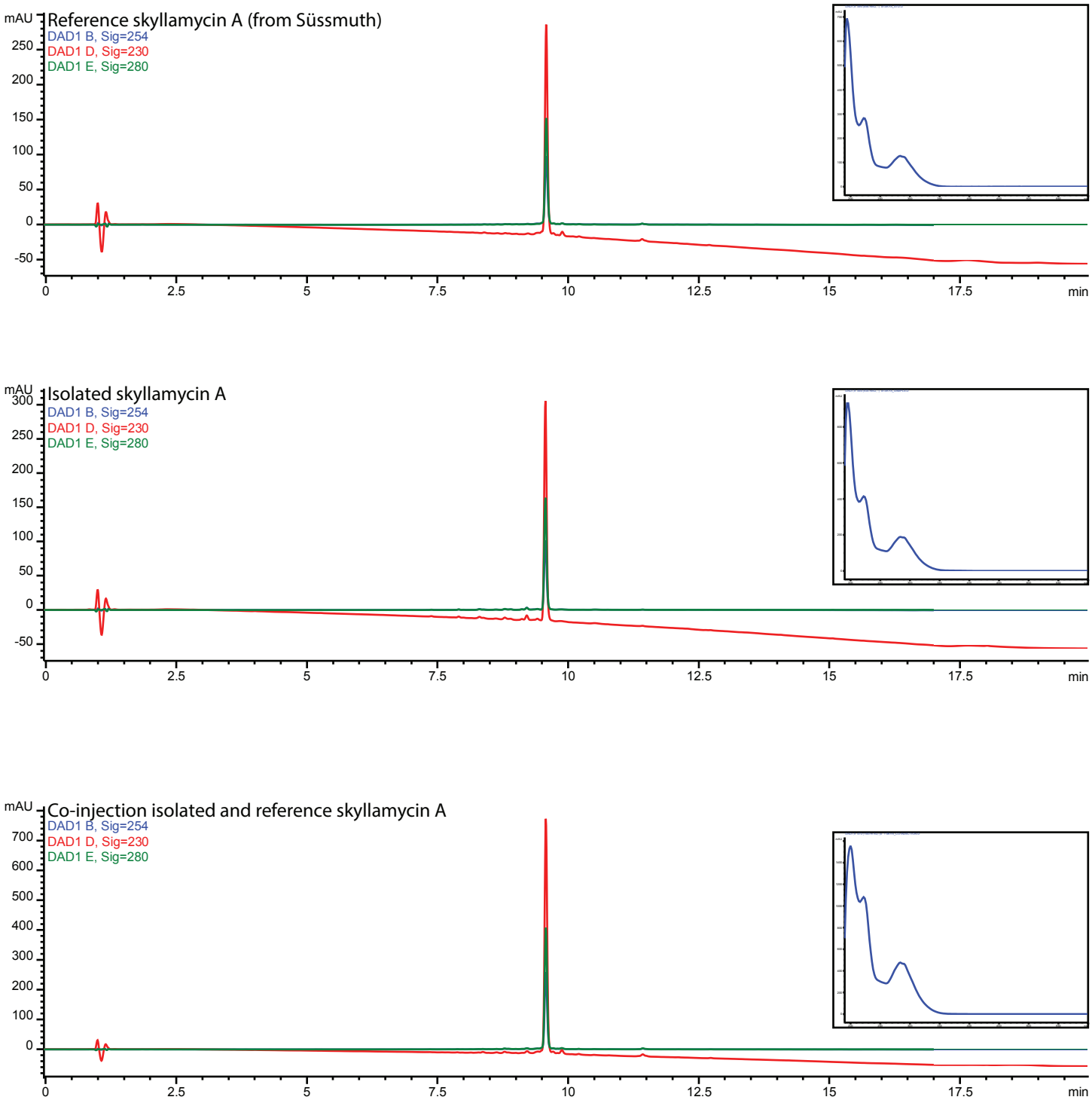
Skyllamycin C HSQC (600 MHz, Methanol-d_4)



Skyllamycin C HMBCAD (600 MHz, Methanol-d_4)



Supporting Fig. S4. LC-MS co-injection analysis for skyllamycin A.



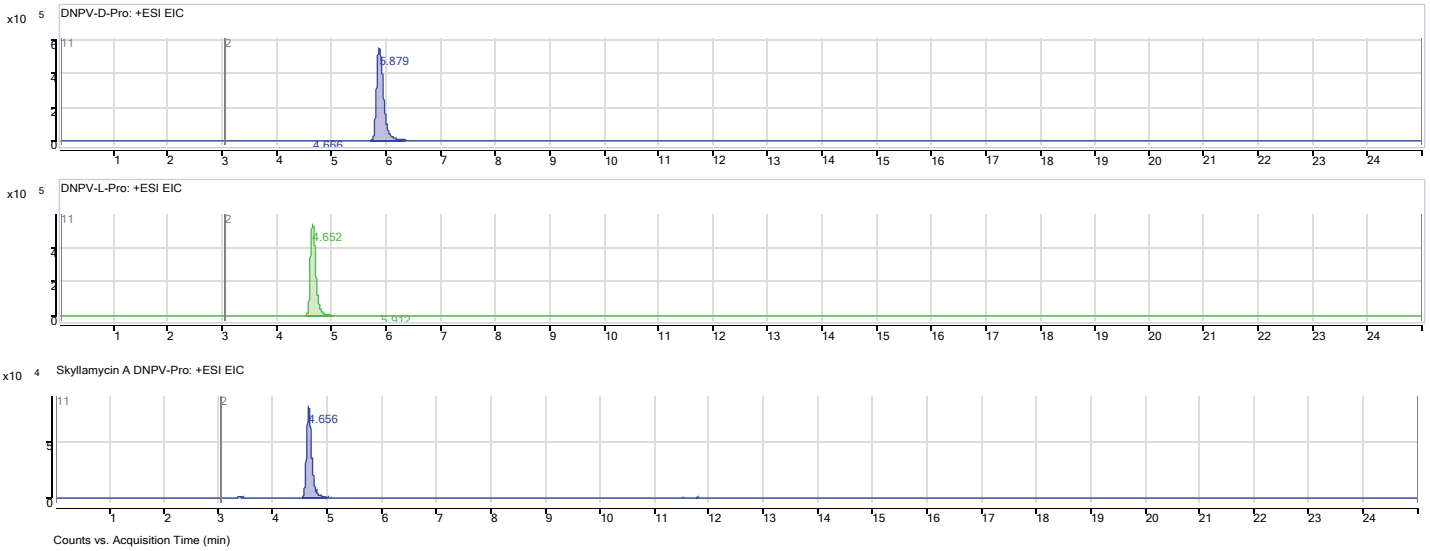
LC-MS run of authentic skyllamycin A, isolated skyllamycin A, and co-injection of both. LCMS run conditions were 0:100 to 100:0 MeOH:H_2O (with 0.02% formic acid) over 20 minutes on RP-HPLC Phenomenex Kinetex C_{18} column (4.6 x 100 mm, 2.6 µM) 1mL/min. UV profiles of each of the peaks are presented to the right.

Supporting Fig. S5. Marfey's analysis for skyllamycins A - C.

Pure skyllamycin A-C were individually subjected to conditions described above and injected into LC-HR-ESI-TOF-MS. The expected 2,4-dinitrophenyl-5-L-valine amino acid (DNPV-aa) ions were extracted within 1 ppm of expected value with a symmetric expansion of 5 ppm. The resulting extracted ion chromatrograms (EIC) were compared to enantiomeric pure standards.

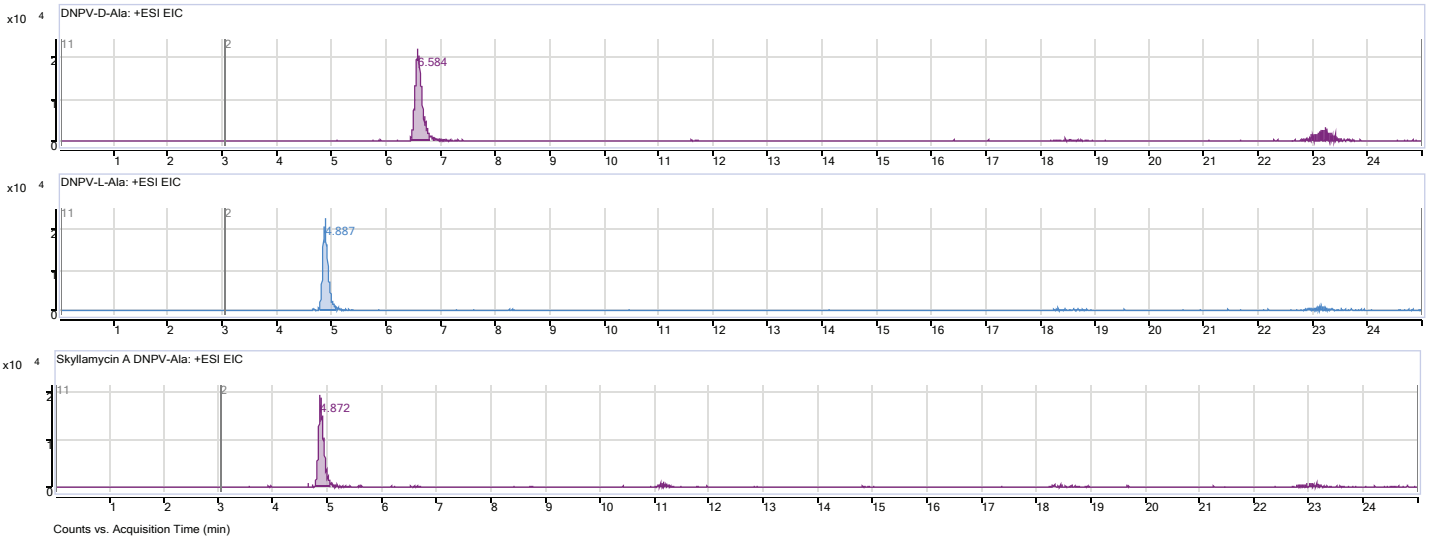
Marfey's analysis of skyllamycin A.

Marfey's analysis of skyllamycin A, EIC for Pro-FDVA:



By ion extraction of DNPV-proline conjugate (within 1 ppm difference), skyllamycin A proline is designated as **L-proline**.

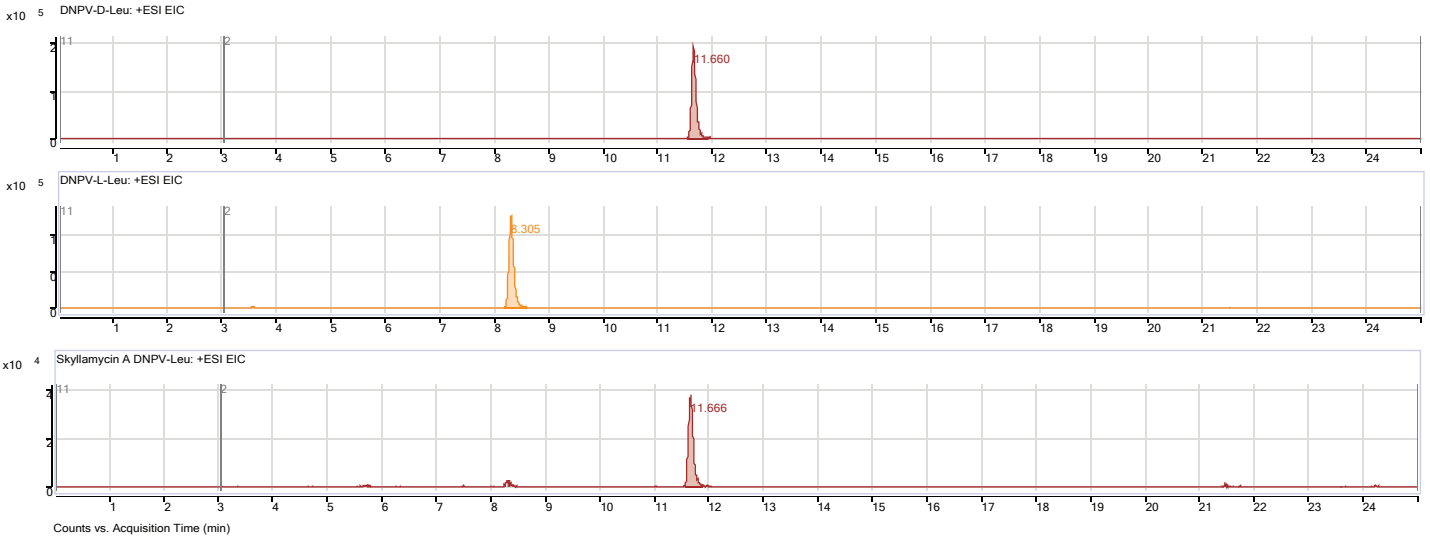
Marfey's analysis of skyllamycin A, EIC for Ala-FDVA:



By ion extraction of DNPV-alanine conjugate (within 1 ppm difference), skyllamycin A alanine is designated as **L-alanine**.

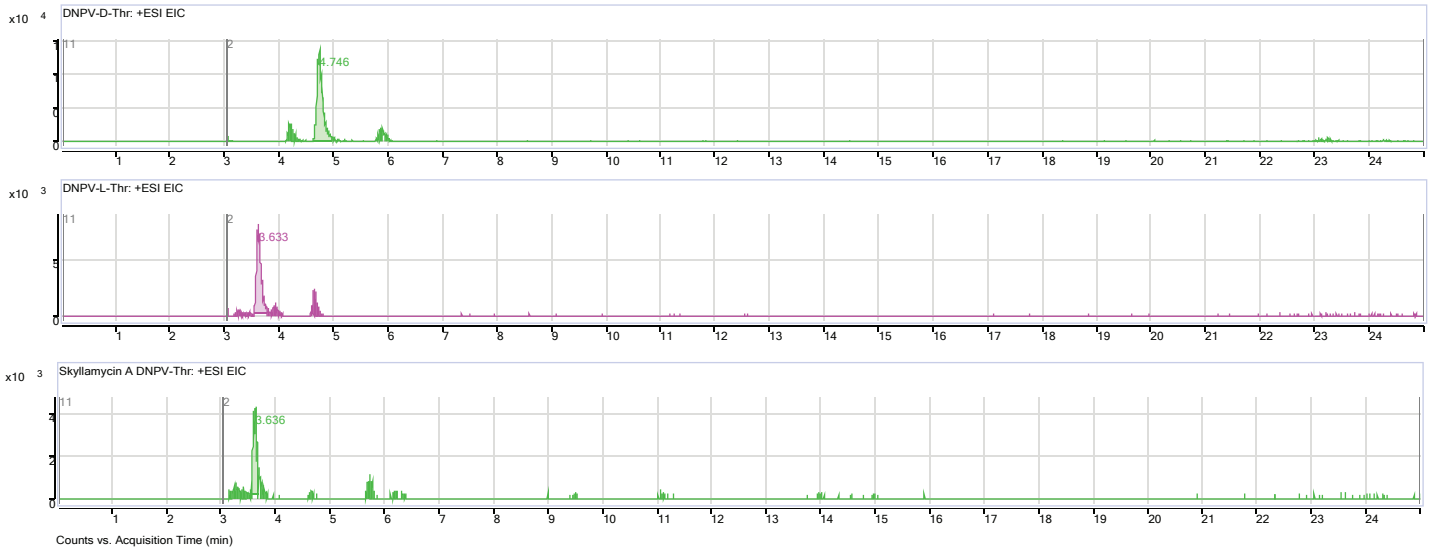
Marfey's analysis of skyllamycin A continued.

Marfey's analysis of skyllamycin A, EIC for Leu-FDVA:



By ion extraction of DNPV-leucine conjugate (within 1 ppm difference), skyllamycin A leucine is designated as **D-leucine**.

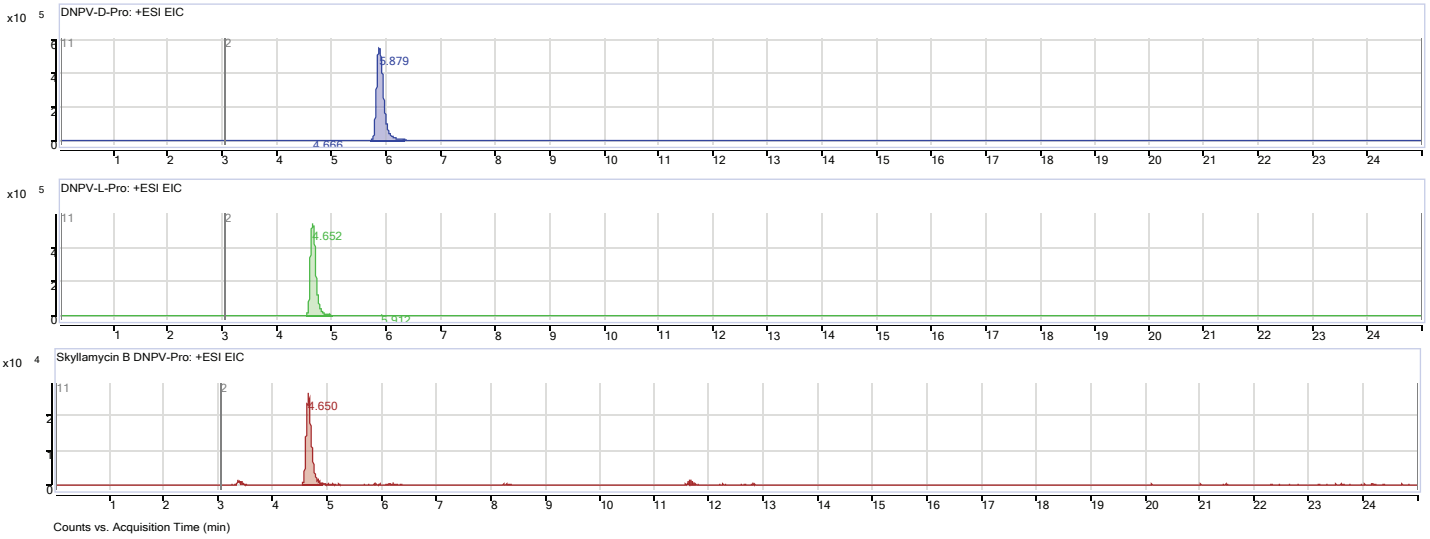
Marfey's analysis of skyllamycin A, EIC for Thr-FDVA:



By ion extraction of DNPV-threonine conjugate (within 1 ppm difference), skyllamycin A threonine is designated as **L-threonine**.

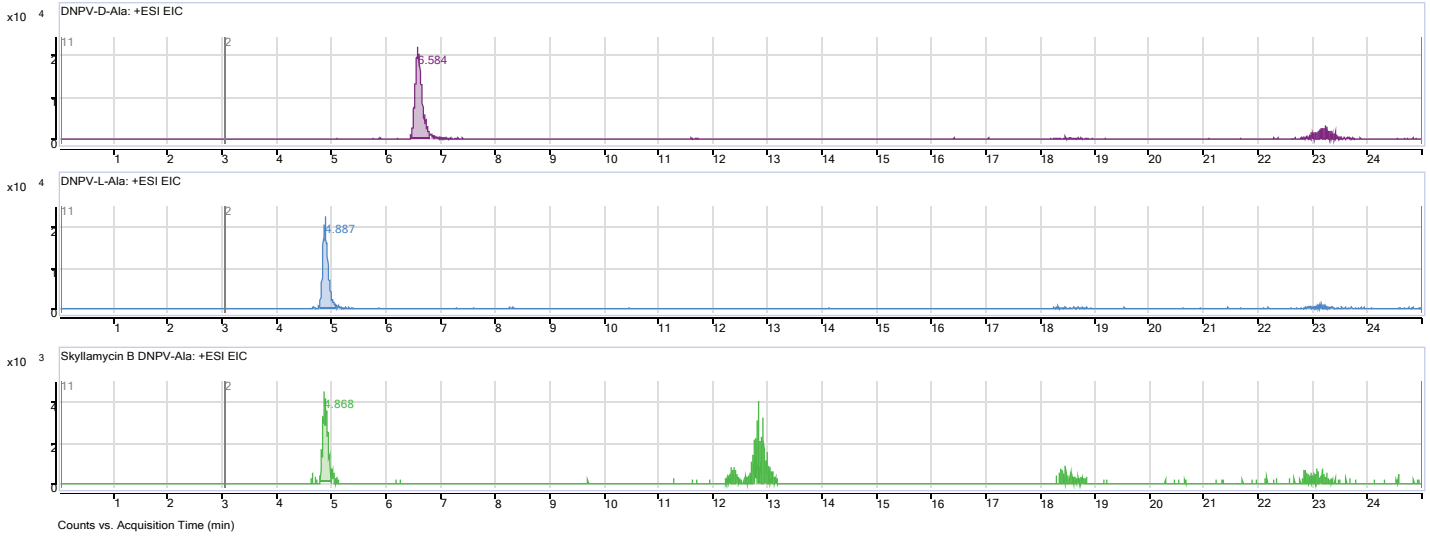
Marfey's analysis of skyllamycin B.

Marfey's analysis of skyllamycin B, EIC for Pro-FDVA:



By ion extraction of DNPV-proline conjugate (within 1 ppm difference), skyllamycin B proline is designated as **L-proline**.

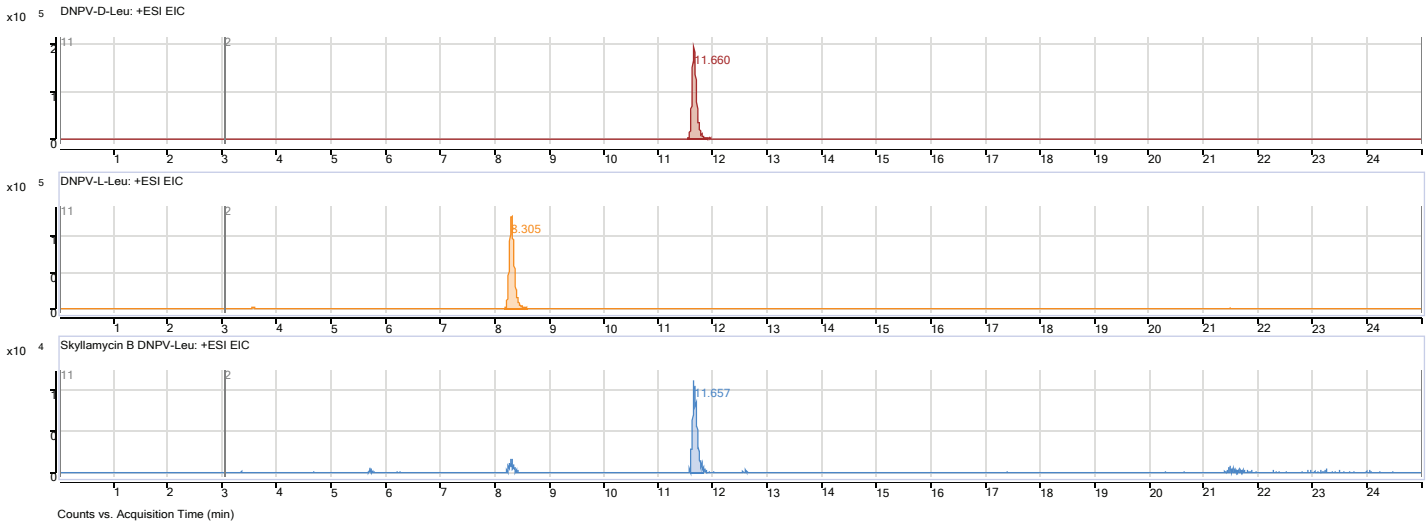
Marfey's analysis of skyllamycin B, EIC for Ala-FDVA:



By ion extraction of DNPV-alanine conjugate (within 1 ppm difference), skyllamycin B leucine is designated as **L-alanine**.

Marfey's analysis of skyllamycin B continued.

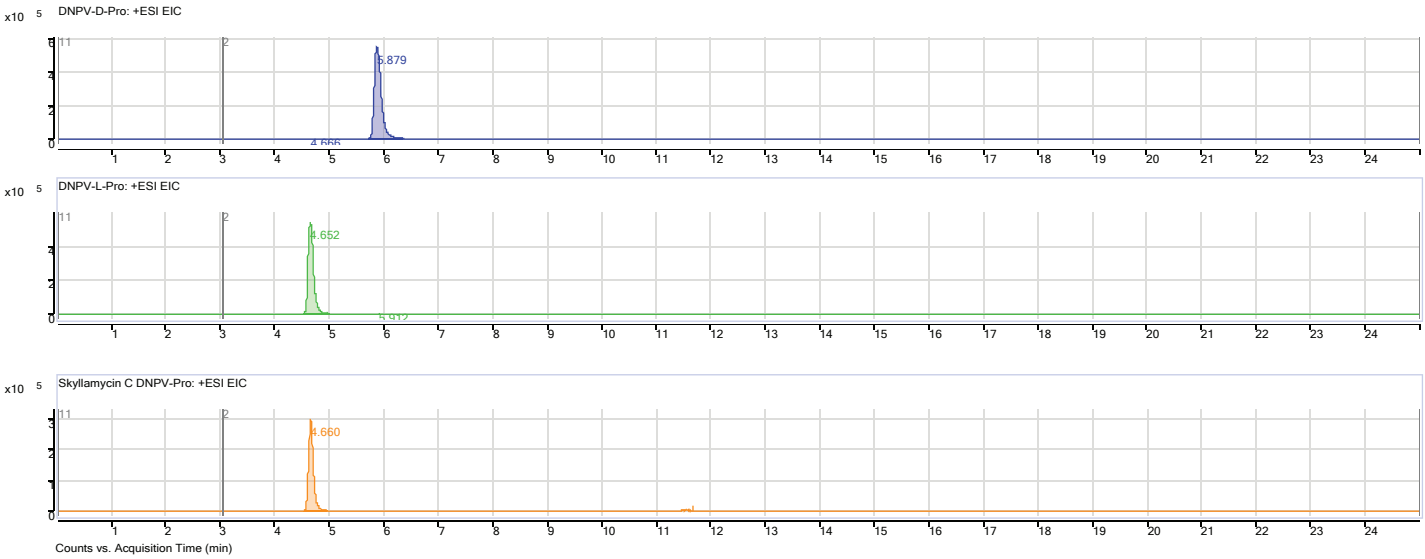
Marfey's analysis of skyllamycin B, EIC for Leu-FDVA:



By ion extraction of DNPV-leucine conjugate (within 1 ppm difference), skyllamycin B alanine is designated as **D-leucine**.

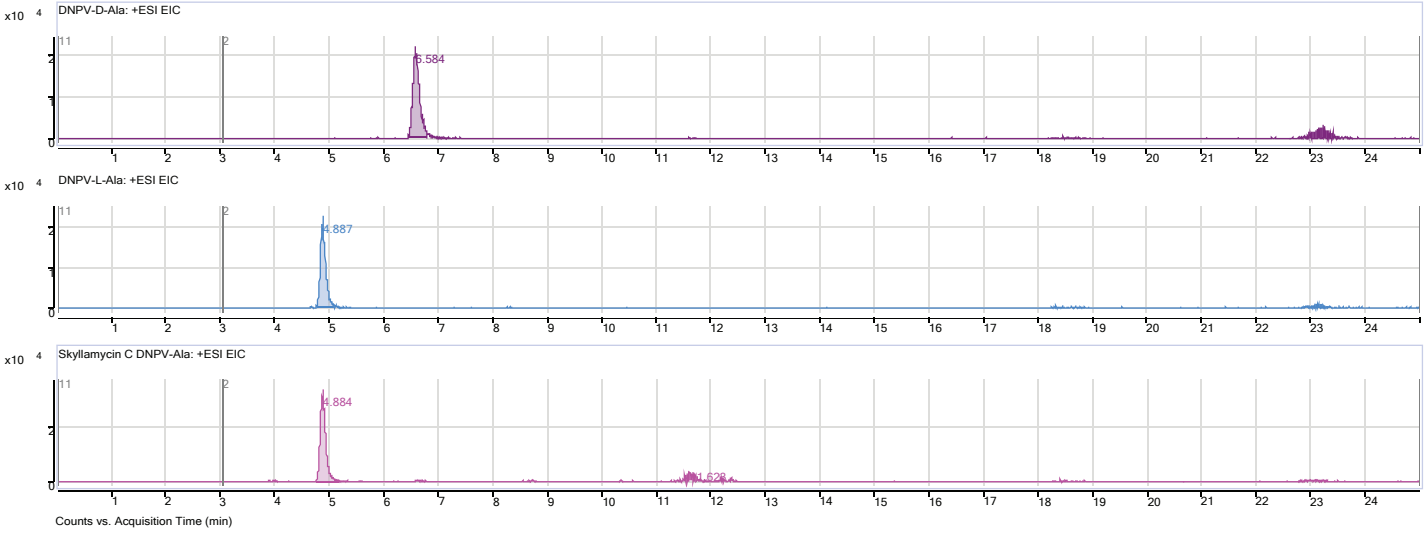
Marfey's analysis of skyllamycin C.

Marfey's analysis of skyllamycin C, EIC for Pro-FDVA:



By ion extraction of DNPV-proline conjugate (within 1 ppm difference), skyllamycin C proline is designated as **L-proline**.

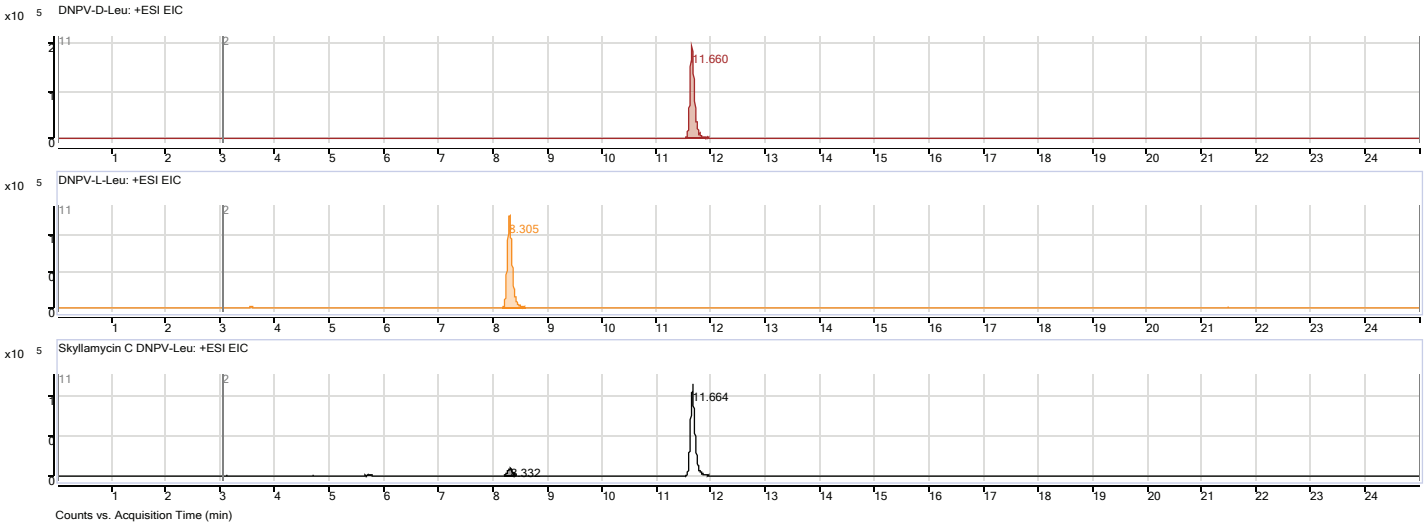
Marfey's analysis of skyllamycin C, EIC for Ala-FDVA:



By ion extraction of DNPV-alanine conjugate (within 1 ppm difference), skyllamycin C alanine is designated as **L-alanine**.

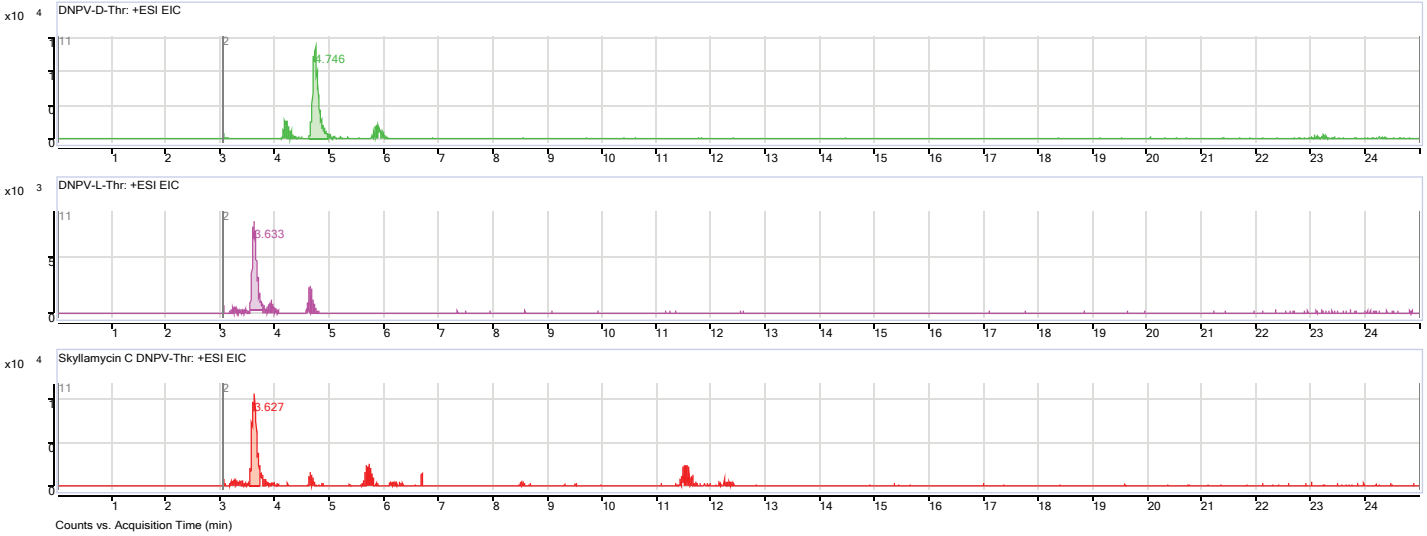
Marfey's analysis of skyllamycin C continued.

Marfey's analysis of skyllamycin C, EIC for Leu-FDVA:



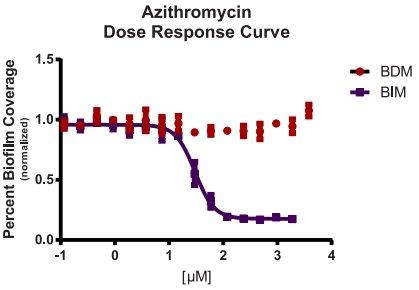
By ion extraction of DNPV-leucine conjugate (within 1 ppm difference), skyllamycin C leucine is designated as **D-leucine**.

Marfey's analysis of skyllamycin C, EIC for Thr-FDVA:

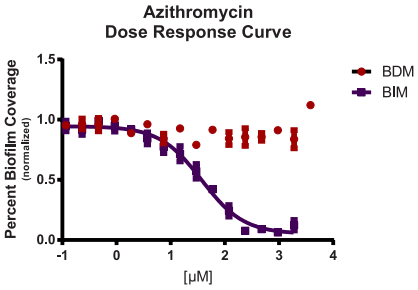


By ion extraction of DNPV-threonine conjugate (within 1 ppm difference), skyllamycin C threonine is designated as **L-threonine**.

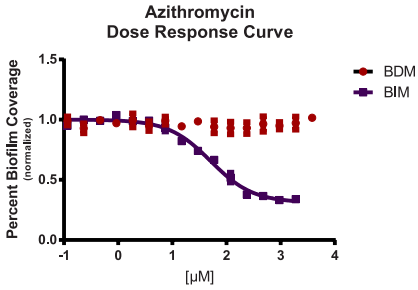
Supporting Fig. S6. Azithromycin, tobramycin, and demeclocycline BIM/BDM dose response curve replicates.



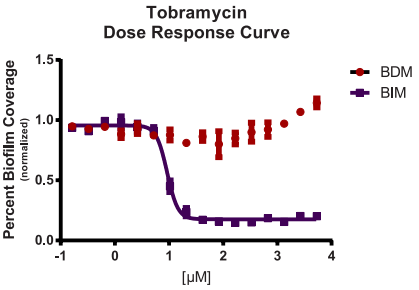
BIM_{EC50} = 30.0 μM
BDM_{EC50} > 3810 μM



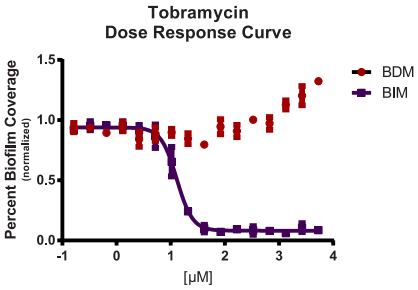
BIM_{EC50} = 37.4 μM
BDM_{EC50} > 3810 μM



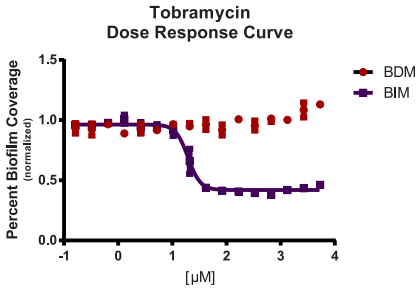
BIM_{EC50} = 51.1 μM
BDM_{EC50} > 3810 μM



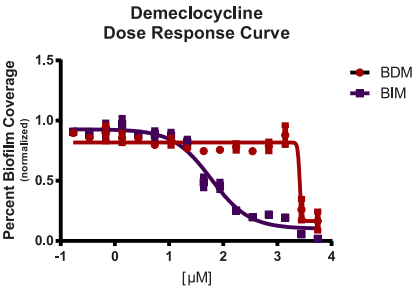
BIM_{EC50} = 9.2 μM
BDM_{EC50} > 5350 μM



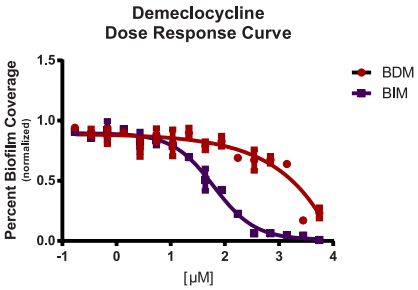
BIM_{EC50} = 13.o μM
BDM_{EC50} > 5350 μM



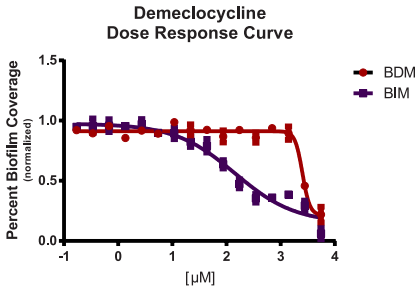
BIM_{EC50} = 19.5 μM
BDM_{EC50} > 5350 μM



BIM_{EC50} = 60.1 μM
BDM_{EC50} ~ 2590 μM



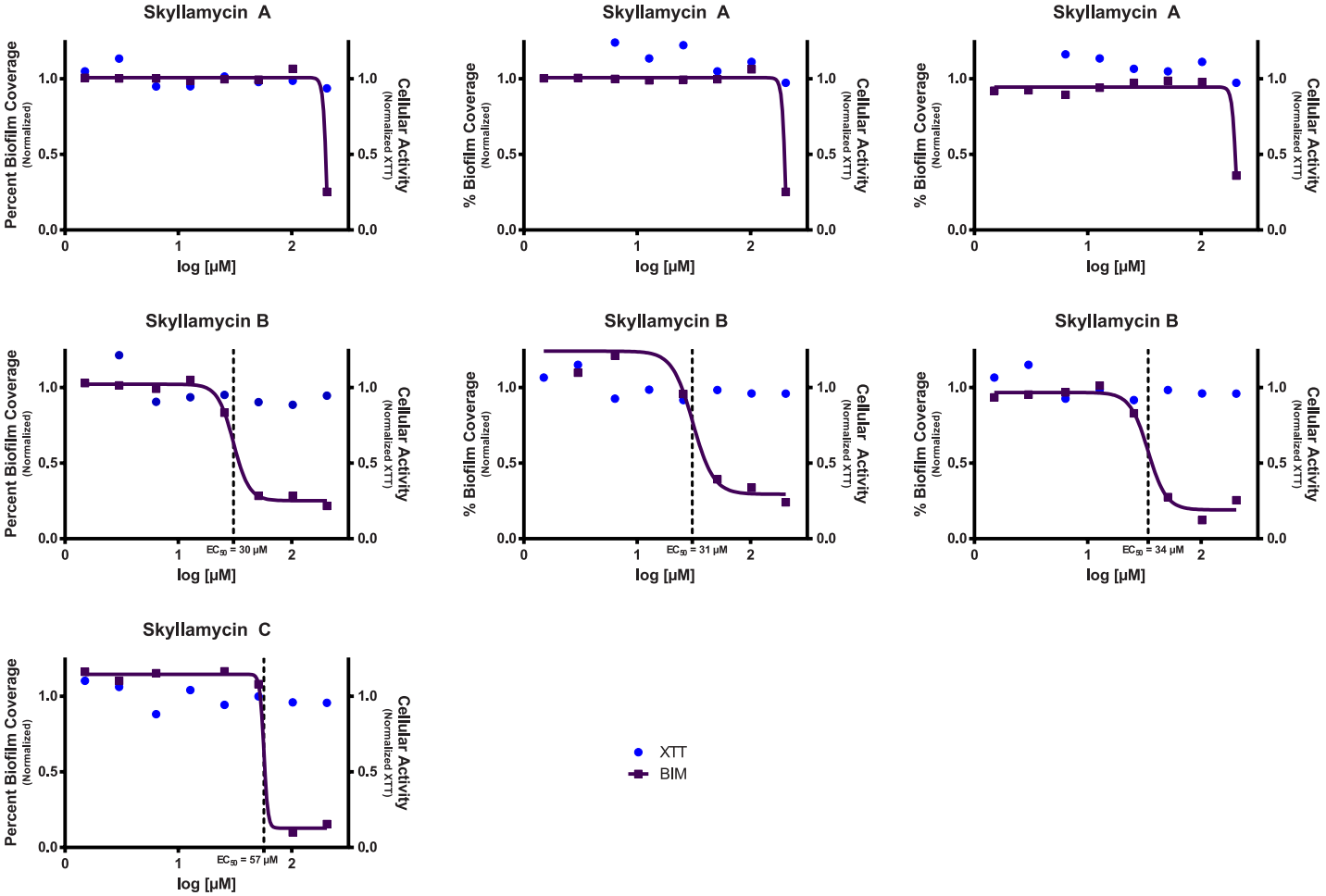
BIM_{EC50} = 62.8 μM
BDM_{EC50} ~ 2790 μM



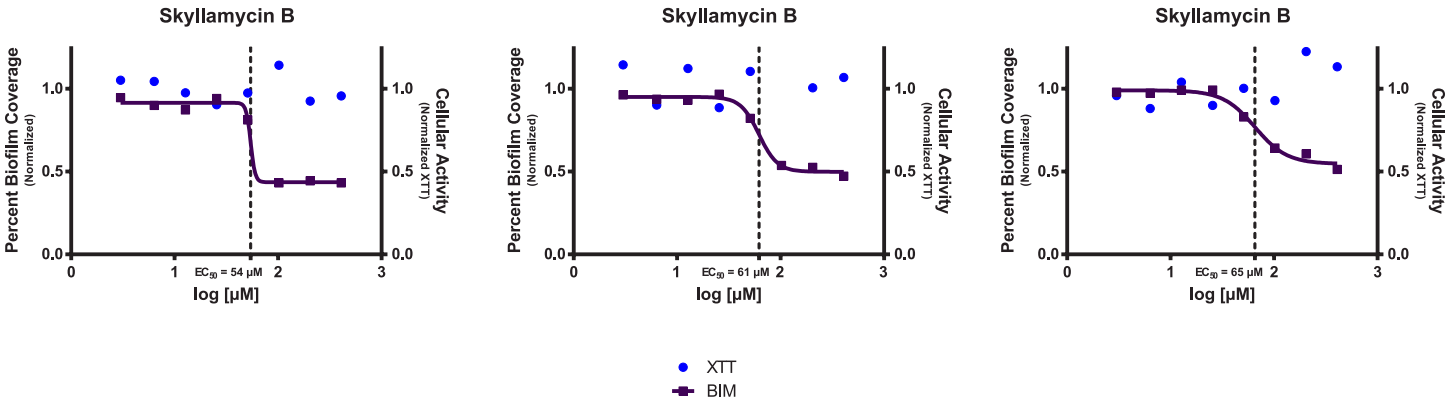
BIM_{EC50} = 144 μM
BDM_{EC50} ~ 2530 μM

Supporting Fig. S7. Skyllamycins A - C BIM/BDM dose response curve replicates.

Biofilm inhibition model assay dose response curve.



Biofilm dispersion model assay dose response curve.



References.

[1] Kim, T. K.; Garson, M. J.; Fuerst, J. A. Environmental Microbiology 2005, 7, 509–18.

[2] Bhushan, R.; Brückner, H. Amino Acids 2004, 27, 231–47.