Supporting Information

Tryptorubin A, a polycyclic peptide from a fungus-derived Streptomycete

Thomas P. Wyche,[†] Antonio C. Ruzzini,[†] Laura Schwab,[‡] Cameron R. Currie,[‡] and Jon Clardy[†]

[†]*Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, 240 Longwood Avenue, Boston, MA, 02115, United States*

[‡]*Department of Bacteriology, University of Wisconsin-Madison, 1550 Linden Drive, Madison, WI, 53706, United States*

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EXPERIMENTAL SECTION

General Experimental Procedures

Optical rotation was measured with a Jasco P-2000 polarimeter. UV spectra were recorded on an Amersham Biosciences Ultrospec 5300 Pro Spectrophotometer. IR spectrum was measured with a Bruker Alpha-P FTIR spectrometer. NMR spectra were obtained in CD_3OD and DMSO-d_6 with a Bruker AVANCE II 600 MHz spectrometer equipped with a $^{1}H\{^{13}C/^{15}N\}$ cryoprobe and a Bruker AVANCE 500 MHz spectrometer equipped with a $^{13}C/^{15}N\{^{1}H\}$ cryoprobe. HRMS data were acquired with an Agilent 6530B qTOF mass spectrometer. RP HPLC was performed using an Agilent 1100 HPLC system and a Phenomenex Luna C18 column (250 × 10 mm, 5 µm).

Biological Material. Fungal specimens were collected and bacteria were isolated in July 2008, in Madison, WI.

Fermentation, Extraction, and Isolation. Two 5 mL seed cultures (17 × 100 mm tubes) in medium ISP2 (10 g malt extract, 4 g glucose, 4 g yeast extract per liter of H_2O) were inoculated with strain CLI2509 and shaken (200 RPM, 30 °C) for seven days. Two hundred fifty mL baffled flasks (2 × 50 mL) containing ISP2 were inoculated with 1 mL seed culture and were incubated (200 RPM, 28 °C) for seven days. Four-liter flasks (8 × 1000 mL) containing medium ISP2 with Diaion HP20 (4% by weight) were inoculated with 10 mL from the 50 mL culture and shaken (200 RPM, 30 °C) for seven days. Filtered HP20 and cells were washed with H_2O and extracted with acetone. The acetone extract was subjected to RP HPLC (10/90% to 100/0% acetonitrile-H_2O containing 0.1% acetic acid, 25 min) using a Phenomenex Luna C18 column (250 × 21.2 mm, 5 µm). Fractions containing **1** were subjected to additional RP HPLC (10/90% to 60/40% MeOH-H_2O containing 0.1% acetic acid, 20 min) using a Phenomenex Luna C18 column (250 × 10 mm, 5 µm). A final step of RP HPLC (10/90% to 35/65% acetonitrile-H_2O

containing 0.1% acetic acid, 16 min) using a Phenomenex Kinetex biphenyl column (250 × 10, 5 µm) yielded **1** (2.0 mg, t_R 16.0 min). For ^{13}C incorporation, the same procedure was used (6 × 500 mL) with labeled medium ISP2 (10 g malt extract, 4 g U^{13}C-glucose, 4 g yeast extract per liter of H_2O). For ^{15}N incorporation, the same procedure was used with four-liter flasks (2 × 1000 mL) containing labeled medium ISP2 (10 g malt extract, 2 g ^{15}N ammonium chloride, 2 g yeast extract, 4 g glucose per liter of H_2O).

16S Sequencing. 16S rDNA sequencing was conducted according to standard DNA extraction and PCR protocols procedures.[1] CLI2509 was identified as a *Streptomyces* sp. and demonstrated 99% sequence similarity to *Streptomyces* sp. SPB 171 (accession number EU798708.1). The 16S sequence for CLI2509 was deposited in GenBank (accession number KX839264).

Molecular Modeling Calculations. Molecular modeling calculations were performed using Gaussian 09 Revision A.02[2] on the Odyssey cluster maintained by Research Computing at Harvard University and Schrödinger Release 2014-2 MacroModel 9.8. Low energy conformers were obtained using Schrödinger (MMFF, 10000 conformers examined). The low energy conformer for each compound was analyzed using Gaussian 09 for geometry optimization (B3LYP/6-31G(d,p)).[3] Molecules were modeled in the gas phase.

Determination of Amino Acid Configurations. L- and DL-FDLA were synthesized as previously reported.[4] Tryptorubin A (**1**) (0.2 mg) was hydrolyzed with 6 N HCl (0.6 mL) for 4 h at 110 °C and dried under vacuum. The acid hydrolysate was dissolved in 100 µL H_2O and split into two equal portions. Each portion was mixed with 1 N $NaHCO_3$ (20 µL), acetone (110 µL), and 20 µL of L- or DL-FDLA (10 mg/mL in acetone). Each solution was stirred for 1 h at 40 °C. The reaction was quenched with 1 N HCl (20 µL) and dried under vacuum. A portion of each product was dissolved in MeOH:H_2O (1:1) for LCMS analysis.[5] Separation of the derivatives

was achieved with a Phenomenex Kinetex EVO C18 reversed-phase column (2.6 µm, 100 x 2.1 mm) at a flow rate of 0.3 mL/min and with a linear gradient of H_2O (containing 0.1% formic acid) and acetonitrile (90:10 to 100:0 over 11 min). The absolute configuration of the amino acids was determined by comparing the retention times of the L- and DL-FDLA derivatives, which were identified by MS. Retention times of the DL-FDLA amino acid derivatives were 6.8 and 7.2 min (Ala), 7.5 and 8.4 min (Ile), and 5.4 and 5.5 min (Tyr). The retention times of the L-FDLA amino acid derivatives were 6.8 (L-Ala), 7.5 (L-Ile) and 5.4 min (L-Tyr). The absolute configuration of the L-Ile was assigned based on a comparison of retention time of amino acid standards (L-Ile, L-*allo*-Ile) derivatized with L-FDLA.

Isotopically-labeled amino acid studies. Small-scale cultures (25 mL) of CLI2509 with HP-20 were grown using the aforementioned fermentation conditions and contained the following labeled amino acids (one labeled amino acid per culture; 15 mg/each): ^{15}N L-alanine, ^{15}N L-isoleucine, $^{15}N_2$ L-tryptophan, and ^{15}N L-tyrosine. A control culture with no labeled amino acids was also grown. LCMS analysis of each crude extract showed an increase in *m/z* compared to unlabeled tryptorubin A (**1**), making evident the presence of one alanine, one isoleucine, two tryptophans, and two tyrosines in tryptorubin A (**1**) (Figure S2). Fermentation of CLI2509 in ISP2 (500 mL) with 300 mg L-tryptophan-(indole-d_5) and containing HP-20 using the aforementioned fermentation conditions and extraction/purification protocol led to the production of tryptorubin A (**1**) with *m/z* 906.4487. The resulting ^{1}H NMR showed that nine hydrogen signals were missing, corresponding to the two tryptophan moieties (See Figure S3). Fermentation of CLI2509 in ISP2 (500 mL) with 300 mg L-tyrosine (ring-3,5-d_2) and containing HP-20 using the aforementioned fermentation conditions and extraction/purification protocol led

to the production of tryptorubin A (**1**) with *m/z* 900.4106. The resulting ^{1}H NMR showed that three hydrogen signals were missing, corresponding to the two tyrosine moieties (Figure S4).

Synthetic modification of tryptorubin A. For acetylation,[6] 150 µL acetic anhydride was added to tryptorubin A (1.0 mg in 150 µL pyridine), stirred for 24 h at rt, and dried by rotovapor. The product was purified by reversed-phase HPLC (Phenomenex C18 Luna column; 250 × 10 mm, 5 µm) with MeOH:H_2O containing 0.1% acetic acid (10:90 to 50:50 in 25 min) and analyzed by NMR and HRMS ($[M+H]^+$ *m/z* 1023.4226). The HRMS data demonstrated that three acetyl groups were added to tryptorubin A (**1**). For methylation,[7] (trimethylsilyl)diazomethane (2 M in hexanes) was added to tryptorubin A (0.5 mg in 400 µL MeOH), stirred at rt for 1 h, and dried by rotovapor. The product was purified by reversed-phase HPLC (Phenomenex C18 Luna column; 250 × 10 mm, 5 µm) with acetonitrile:H_2O containing 0.1% acetic acid (60:40 to 100:0 in 25 min) and analyzed by NMR and HRMS. The major product contained one methyl group, for the *O*-methyl at C-1, as evidenced by 1D and 2D NMR (Figure S5).

DNA isolation, sequencing and analysis. DNA was isolated from a culture of *Streptomyces* sp. CLI2509 grown in ISP2 for 5 days at 30 °C and shaking at 200 rpm. Briefly, cells were harvested by centrifugation at 4000 rpm, washed once with PBS, and then frozen at -80 °C until the cell pellet was physically disrupted in liquid N_2 using a mortar and pestle. The disrupted cells were then treated with lysozyme (2.5 mg/mL), protease (1 mg/ml), and 1% SDS in a solution of 10 mM Tris:HCl and 1 mM EDTA, adjusted to pH 8.0 (TE buffer), for 30 minutes at 50 °C. The resulting lysate was clarified by centrifugation at 10,000 *g* for 15 minutes before the nucleic acids were precipitated using an equal volume of cold *iso*-propanol. The precipitate was washed twice with a solution of 70% ethanol, resuspended in 10 mM Tris:HCl, pH 8.0 containing 0.1 mg/mL RNase A, and incubated at 37 °C for 1 h. Finally, a solution of

phenol:chloroform:isoamyl acohol (25:24:1) saturated with TE buffer was used to further purify the DNA, which was then precipitated using *iso*-propanol, dried, and resuspended in water for library preparation and sequencing. Sequencing was performed using PacBio SMRT sequencing technology at the Duke University Center for Genomic and Computational Biology. A 15-20 kb library was prepared and data were collected from 3 SMRT cells using P6-C4 sequencing chemistry. Assembly was performed using the hierarchical genome assembly process (HGAP), and unique contigs were manually curated to remove the low quality positions located at the sequence termini. The final assembly and raw sequence reads are available for download from the NCBI [Genbank accession no. CP021118 and CP021119; SRA accession no. SRP106654]. Putative biosynthetic gene clusters were identified using antiSMASH3.[8] The Genome-to-Genome Distance Calculator 2.1 was used to compare the chromosomes of CLI2509, *Streptomyces* sp. Tü6071, and the *Streptomyces* sp. SPB84 scaffold. The *in silico* DNA-DNA hybridization values are reported for Formula 2; a range is reported for SPB84 due to the incomplete nature of the assembly, which is missing an estimated 0.66 Mb.

Tryptorubin A (1): colorless solid; $[\alpha]^{26}_{D}$ +18 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ε) 214 (4.04), 271 (3.44) nm; IR (ATR) υ_{max} 3360, 1651, 1569, 1408, 1247 cm^{-1}; ^{1}H and ^{13}C NMR (Table S1); HRMS $[M+H]^{+}$ *m/z* 897.3928 (calcd for $C_{49}H_{52}N_8O_9$, 897.3930).

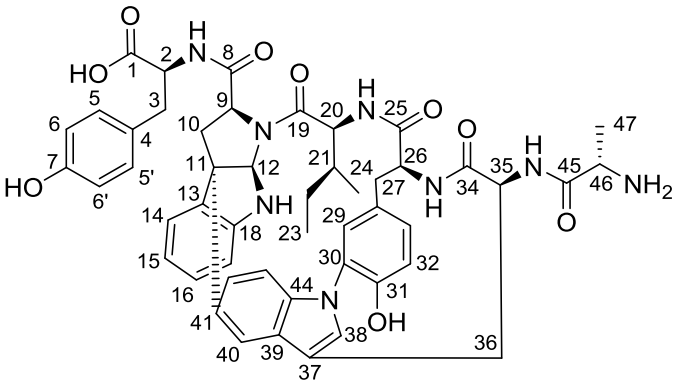


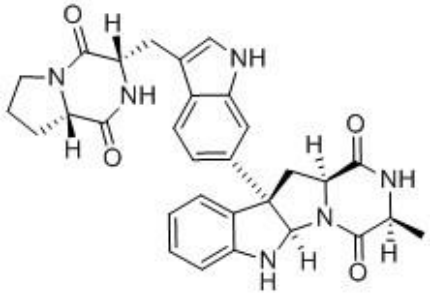
Table S1. ^{1}H and ^{13}C NMR data for **1** (600 MHz for ^{1}H, 125 MHz for ^{13}C, in DMSO-d_6)

Position	δ_C, mult.	δ_H (J in Hz)	COSY	HMBC
1	174.0, C			
2	56.6, CH	3.93, q (6.8)	2-NH, 3	1, 3, 4, 8
2-NH		7.27, d (6.8)	2	1, 8
3	37.9, CH_2	2.64, m	2	1, 2, 5, 5'
		2.84, m		
4	129.8, C			
5, 5'	130.0, CH	6.88, d (8.3)	6, 6'	5, 5', 6, 6', 7
6, 6'	114.9, CH	6.42, d (8.3)	5, 5'	5, 5', 6, 6', 7
7	155.8, C			
8	170.1, C			
9	63.1, CH	4.52, m	10	8, 10
10	39.1, CH_2	3.13, m	9	8, 9, 11, 12, 13
		2.10, t (13.2)		
11	60.9, C			
12	92.3, CH	6.78, m	12-NH	9, 10, 11, 13, 18
12- NH		6.50, d (3.4)	12	11, 13
13	136.3, C			
14	123.4, CH	6.77, m	15	16, 18
15	118.4, CH	6.64, m	14, 16	13, 17
16	128.2, CH	7.09, t (7.8)	15, 17	14, 18
17	110.0, CH	6.71, m	16	13, 15
18	149.6, C			
19	174.8, C			
20	58.2, CH	3.67, m	20-NH, 21	
20-NH		7.79, m	20	
21	36.5, CH	1.59, m	20, 22, 24	23, 24
22	25.3, CH_2	1.22, m	21, 23	
23	11.5, CH_3	0.86, t (6.8)	22	21, 22
24	15.6, CH_3	0.95, d (6.4)	21	20, 21, 22
25	170.2, C			
26	52.0 CH	4.07, m	26-NH, 27	28
26-NH		6.14, d (3.4)	26	
27	32.4, CH_2	2.71, m	26	26, 28
		2.66, m		
28	125.9, C			
29	131.8, CH	5.76, s		30, 31, 33
30	132.5, C			
31	149.6, C			
31-OH		7.28, s		
32	117.5, CH	6.75, m	33	28, 30
33	128.3, CH	6.66, m	32	30, 31
34	169.0, C			
35	54.0, CH	4.52, m	35-NH, 36	
35-NH		7.97, d (8.3)	35	45
36	27.5, CH_2	2.67, m	35	37
37	120.9, C			
38	146.4, CH	6.89, s		37, 39, 44
39	139.1, C			
40	123.6, CH	7.17, s		11, 37, 42, 44
41	136.1, C			
42	123.3, CH	7.44, d (8.3)	43	11, 40, 44
43	115.7, CH	7.06, d (8.3)	42	39, 41
44	153.1, C			
45	175.2, C			
46	50.7, CH	3.18, m	47	
47	22.0, CH_3	1.07, d (6.8)	46	45, 46

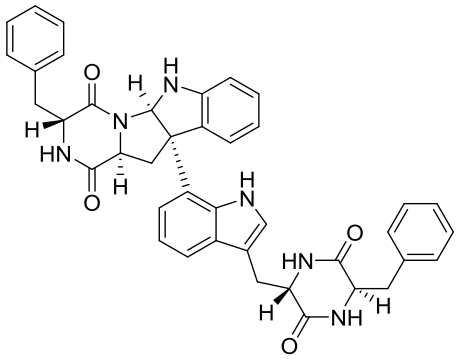
Table S2. ^{1}H and ^{13}C NMR data for **1** (600 MHz for ^{1}H, 125 MHz for ^{13}C, in CD_3OD)

Position	δ_C, mult.	δ_H (J in Hz)
1	173.9, C	
2	57.3, CH	4.39, m
3	38.1, CH_2	3.05, m
		2.86, m
4	129.8, C	
5, 5'	130.0, CH	6.92, d (7.8)
6, 6'	115.9, CH	6.40, d (7.8)
7	155.8, C	
8	170.6, C	
9	63.1, CH	4.70, m
10	38.9, CH_2	3.20, m
		2.16, t (12.7)
11	59.9, C	
12	94.1, CH	6.97, m
13	137.0, C	
14	123.4, CH	6.86, m
15	118.5, CH	6.77, m
16	128.2, CH	7.18, m
17	112.0, CH	6.89, m
18	150.3, C	
19	173.9, C	
20	57.7, CH	3.87, m
21	36.5, CH	1.71, m
22	24.3, CH_2	1.30, m
23	11.5, CH_3	0.95, t (7.6)
24	16.3, CH_3	1.00, d (6.4)
25	170.2, C	
26	51.6 CH	4.13, m
27	33.4, CH_2	3.00, m
		2.77, m
28	125.9, C	
29	131.8, CH	5.94, s
30	132.6, C	
31	150.2, C	
32	117.2, CH	6.76, m
33	128.3, CH	6.74, m
34	169.0, C	
35	53.7, CH	4.38, m
36	27.5, CH_2	2.97, m
		2.85, m
37	120.8, C	
38	147.7, CH	6.83, s
39	139.1, C	
40	123.6, CH	7.44, s
41	136.1, C	
42	123.3, CH	7.44, m
43	116.0, CH	7.17, m
44	154.7, C	
45	176.4, C	
46	51.0, CH	3.49, m
47	20.6, CH_3	1.35, d (6.5)

Figure S1. Natural products with similar structural features[9-16]



Naseseazine A



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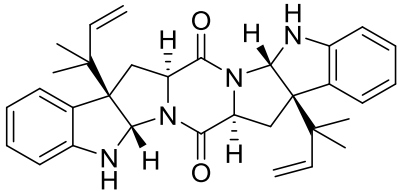
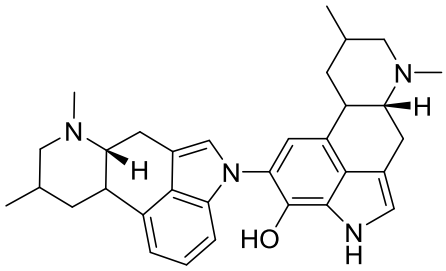
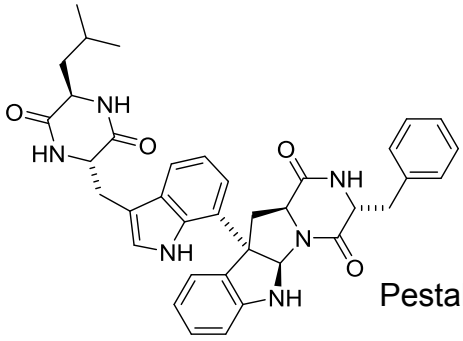


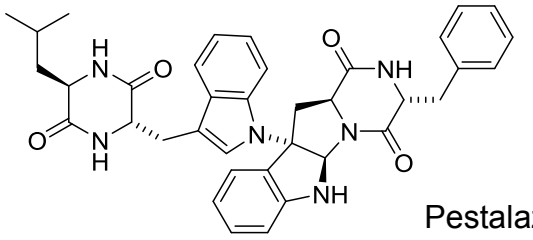
Amauromine



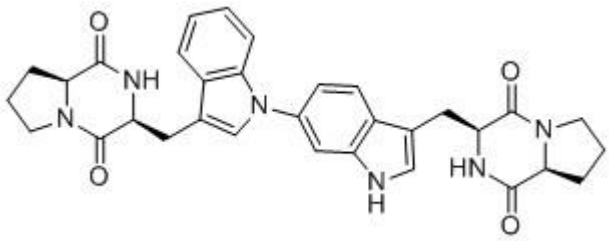
alkaloid



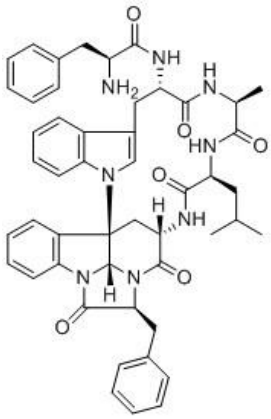
Pestalazine A



Pestalazine B



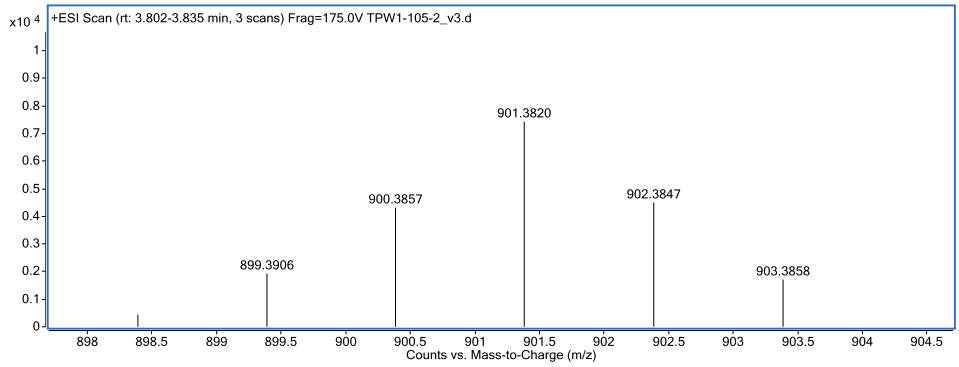
Aspergilazine A



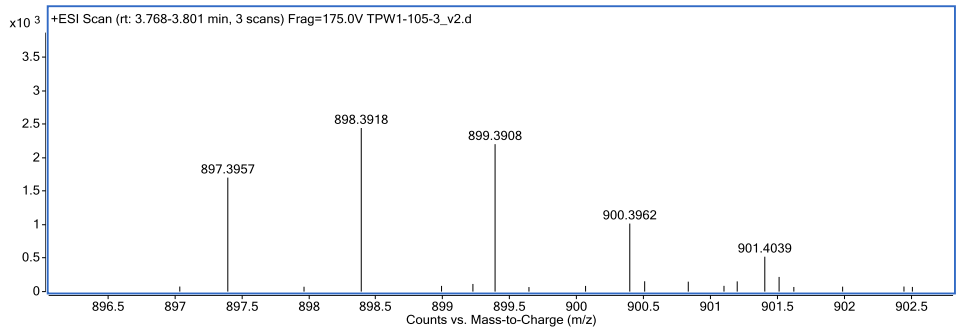
Kapakahine B

Figure S2. Incorporation of ^{15}N-labeled amino acids in tryptorubin A

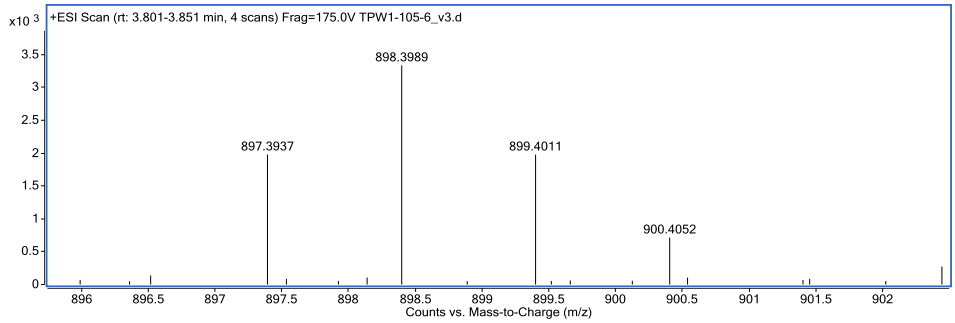
$^{15}N_2$ tryptophan



^{15}N tyrosine



^{15}N alanine



^{15}N isoleucine

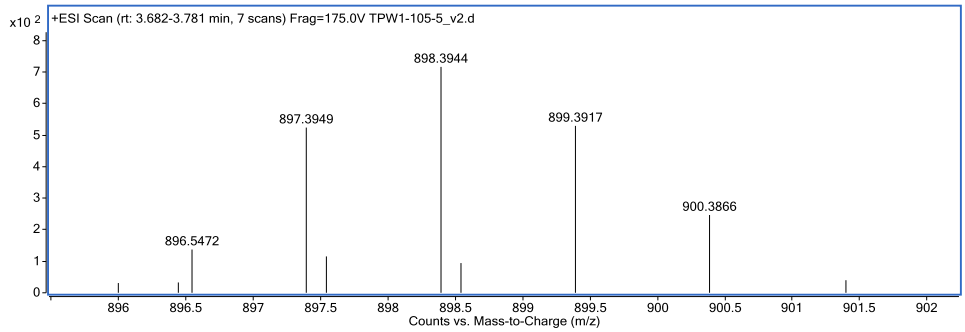
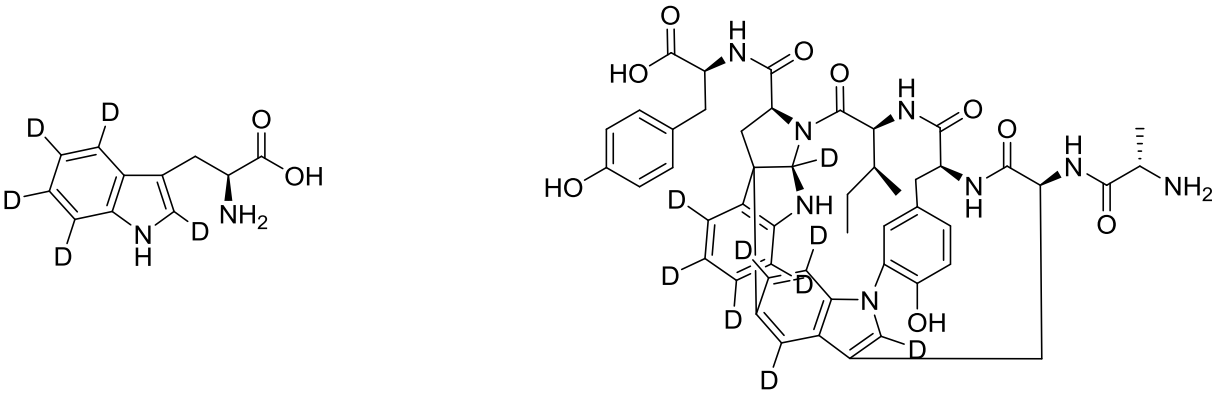
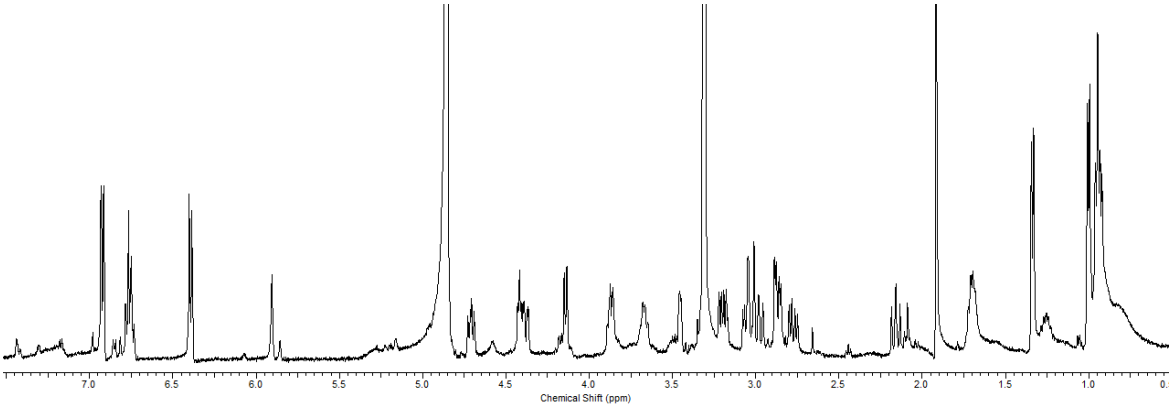


Figure S3. Incorporation of L-tryptophan-(indole-d_5) in tryptorubin A



^{1}H NMR in CD_3OD (500 MHz)



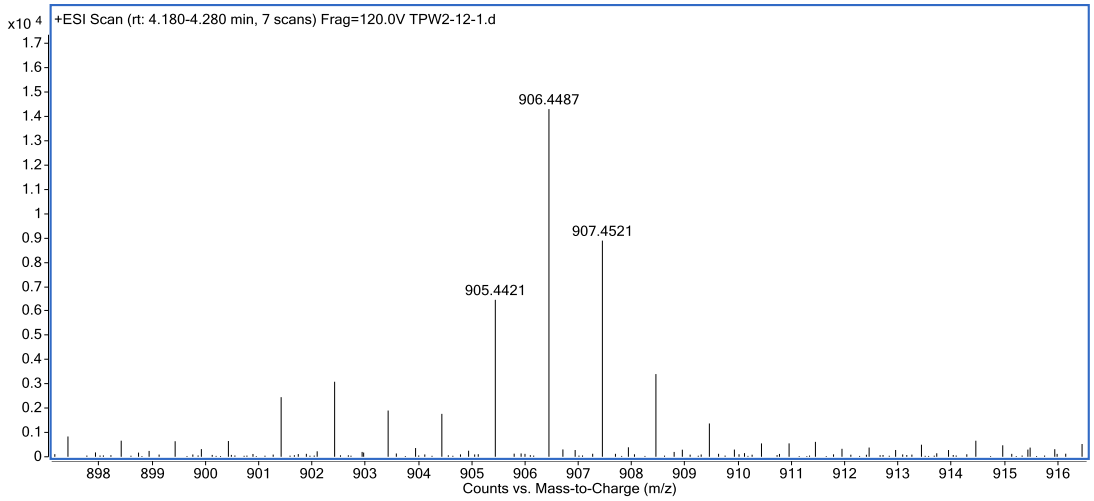
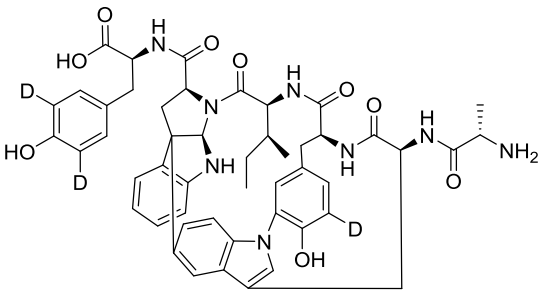
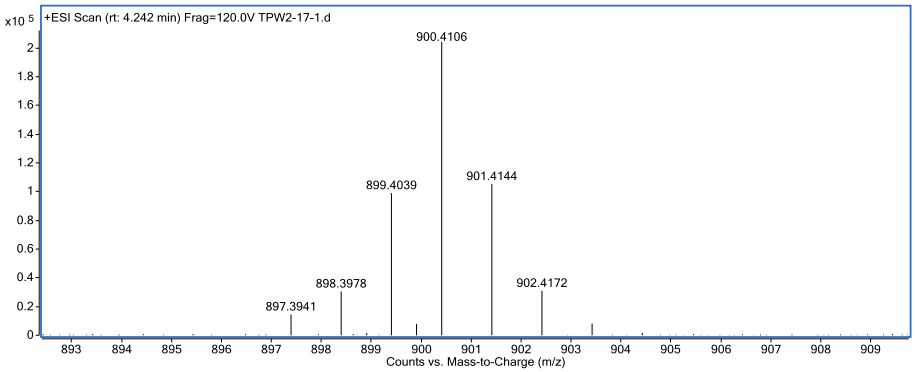
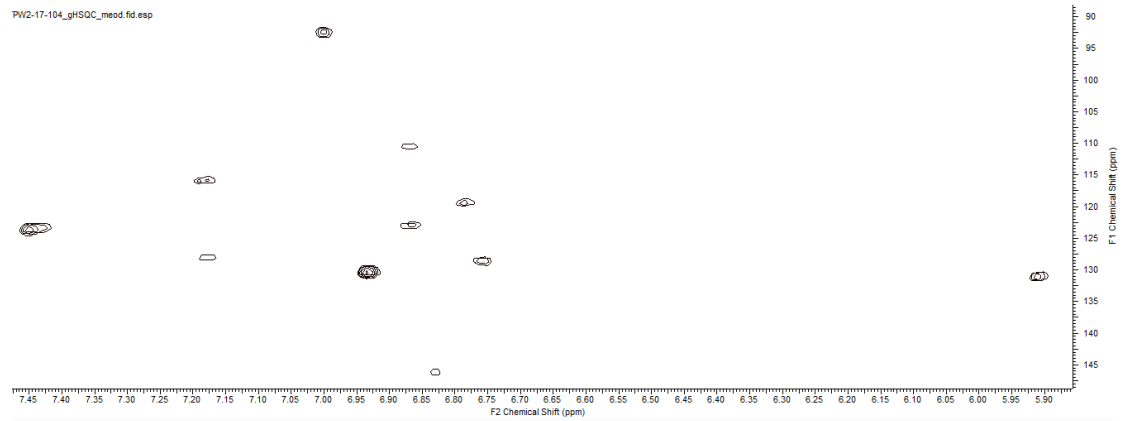


Figure S4. Incorporation of L-tyrosine-(ring-3,5-d_2) in tryptorubin A





^{1}H and gHSQC NMR (CD_3OD) of tryptorubin A (deuterium-labeled)



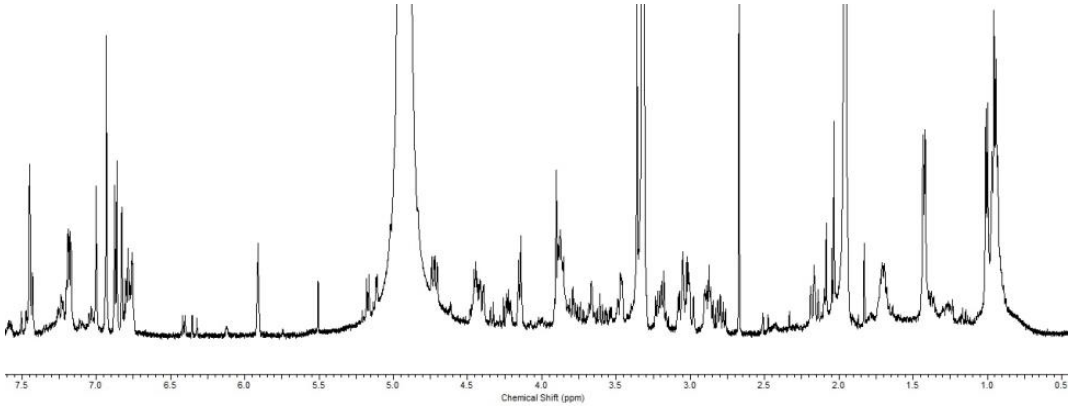
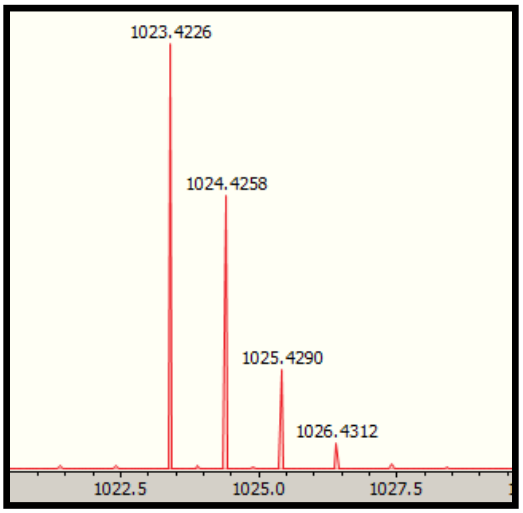
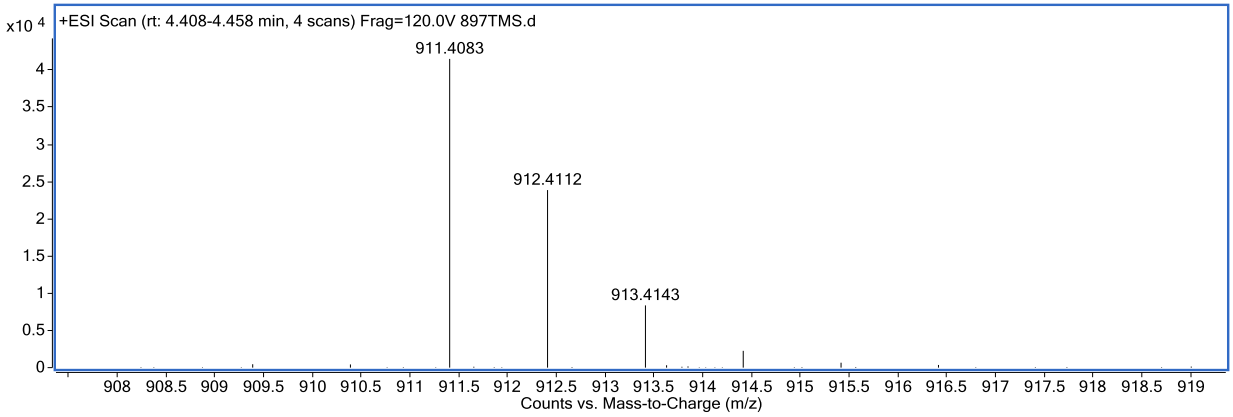


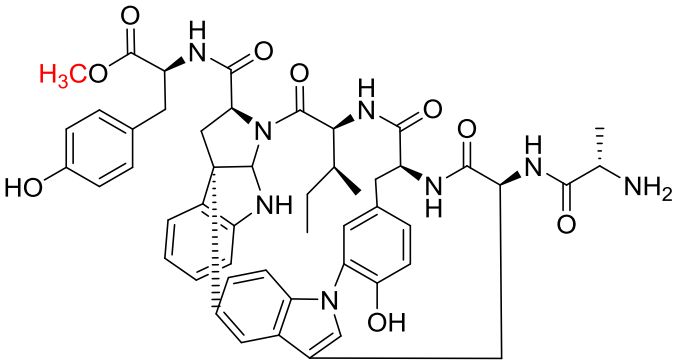
Figure S5. Synthetic modifications of tryptorubin A

Acetylated tryptorubin A



Methylated tryptorubin A

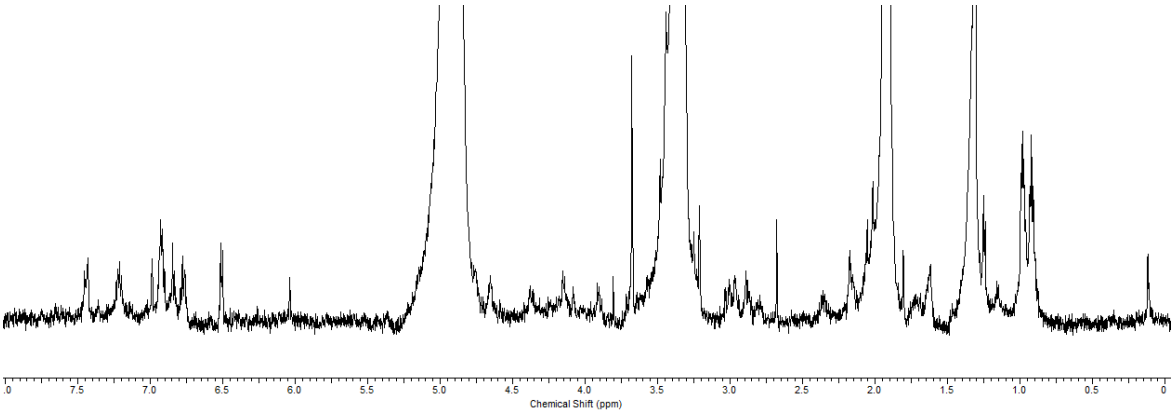




Major product

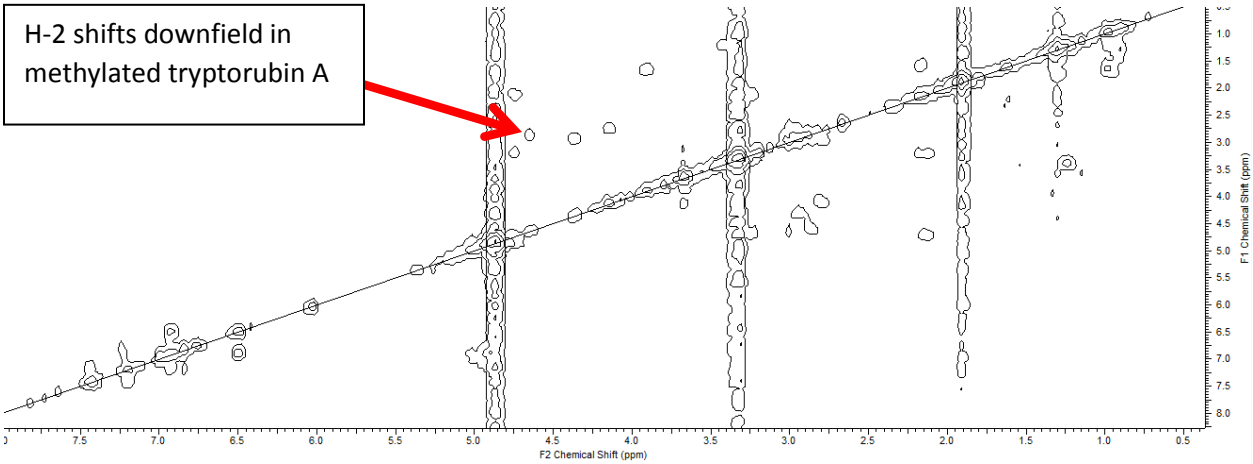
Methylated tryptorubin A

1H NMR (600 MHz, CD_3OD)



Methylated tryptorubin A

gCOSY NMR (600 MHz, CD_3OD)



Tryptorubin A (not methylated)

gCOSY NMR (600 MHz, CD_3OD)

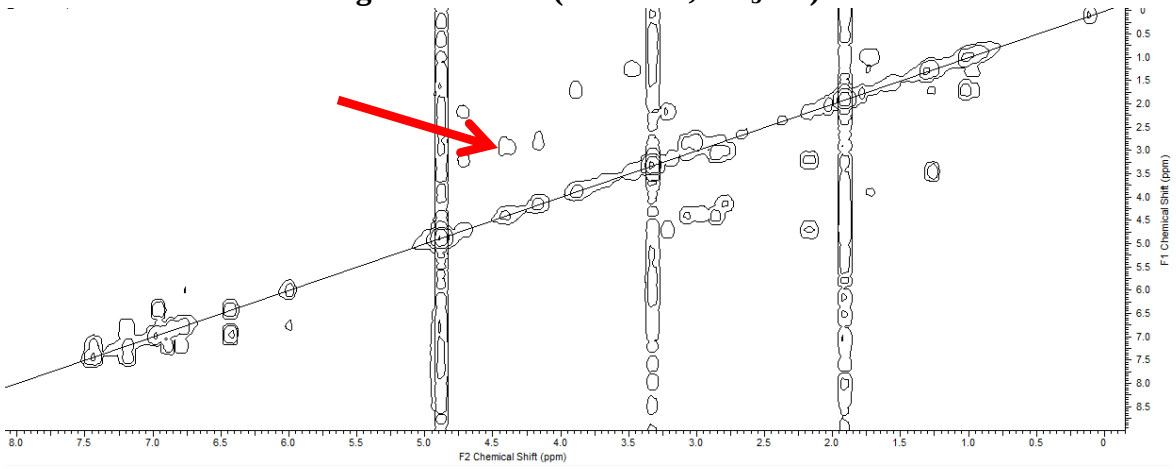
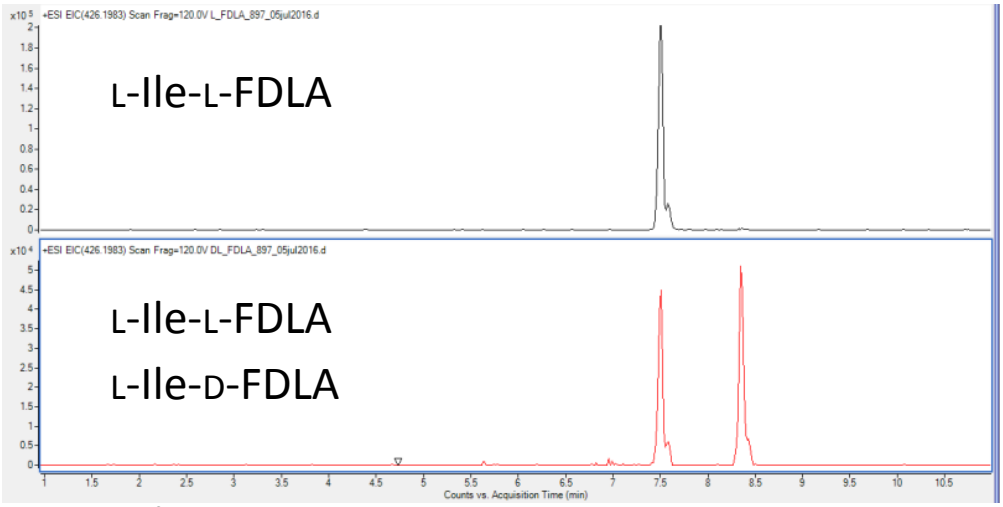
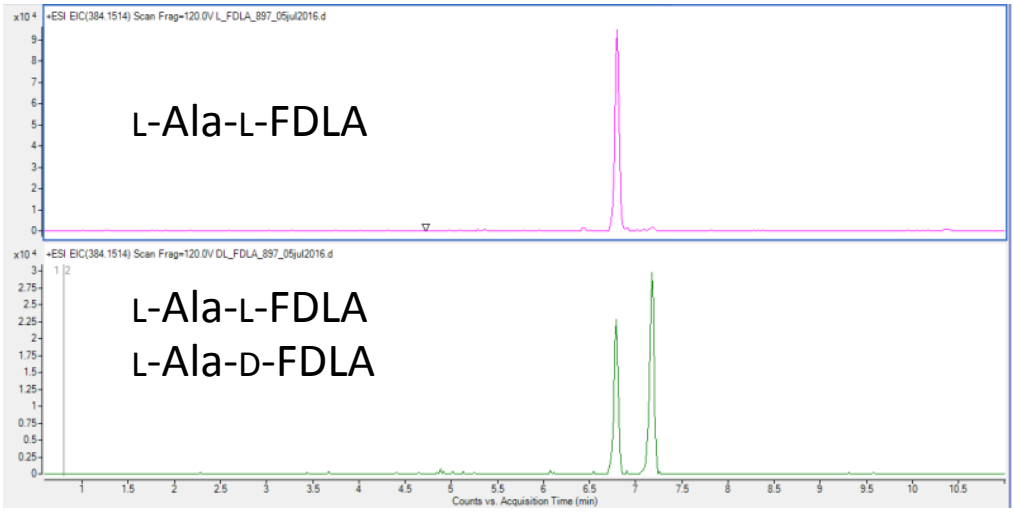


Figure S6. Marfey's Method – amino acid analysis



Standards of L-Ile and L-*allo*-Ile, each derivatized with L-FDLA, were also analyzed by LCMS.



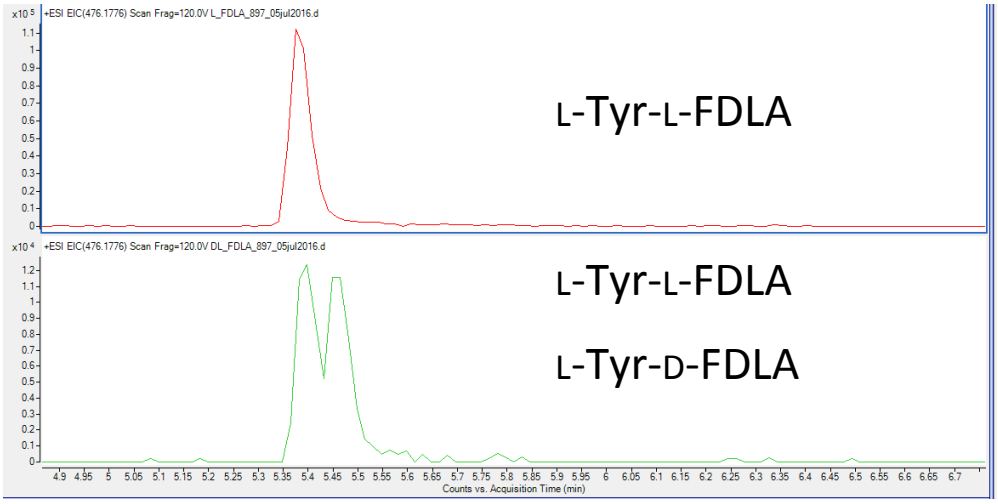


Figure S7. Molecular modeling analysis

Low energy conformers were obtained using Schrödinger (MMFF, 10000 conformers examined). The low energy conformer for each compound was analyzed using Gaussian 09 for geometry optimization (B3LYP/6-31G(d,p)). Below are the four stereoisomers that were modeled. Only structure 1A fit the ROESY NMR data (^{1}H-^{1}H distance < 5 Å).

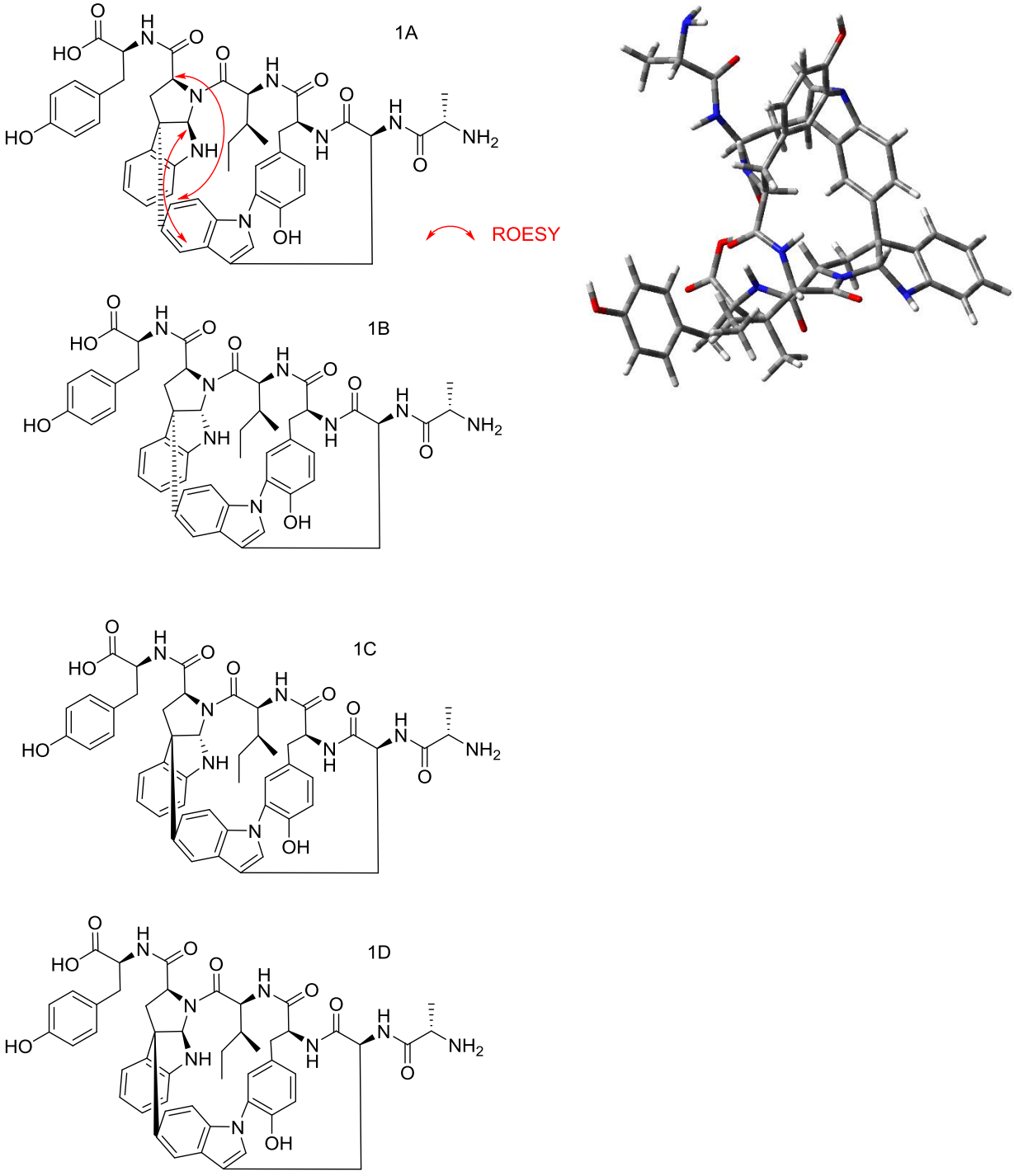


Table S3. Summary of *Streptomyces* sp. CLI2509 replicons

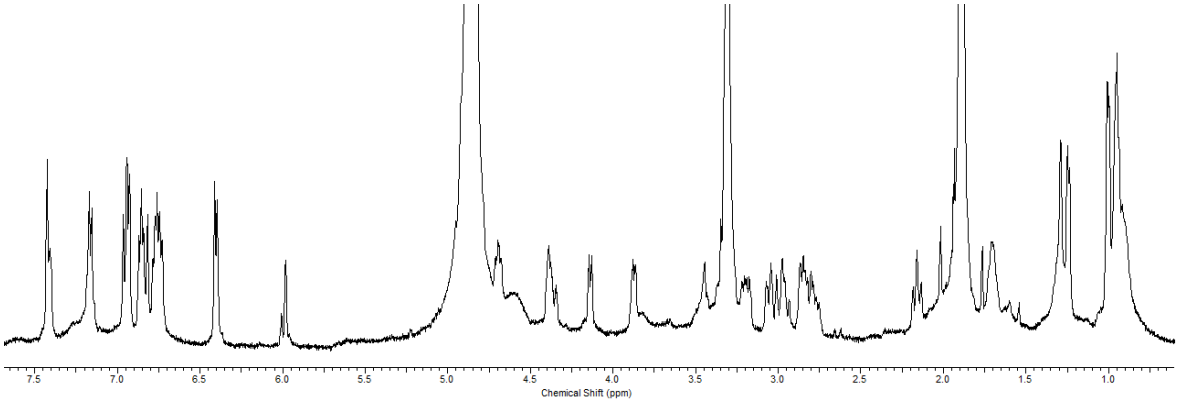
	Genbank accession no.	length	G/C content	coverage	mean QV (expected errors)	antiSMASH predicted BGCs
chromosome	CP021118	7.09 Mb	73.3%	253x	48.5 (4885)	18
plasmid	CP021118	147 kb	71.2 %	335x	48.0 (190)	0

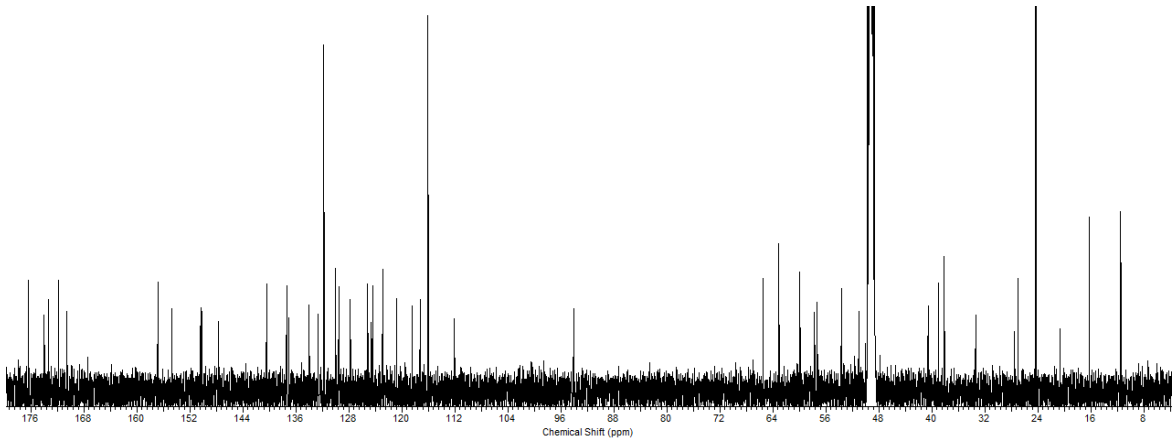
Table S4. Gene annotations near NRPSs assigned to linear hexapeptide biosynthesis

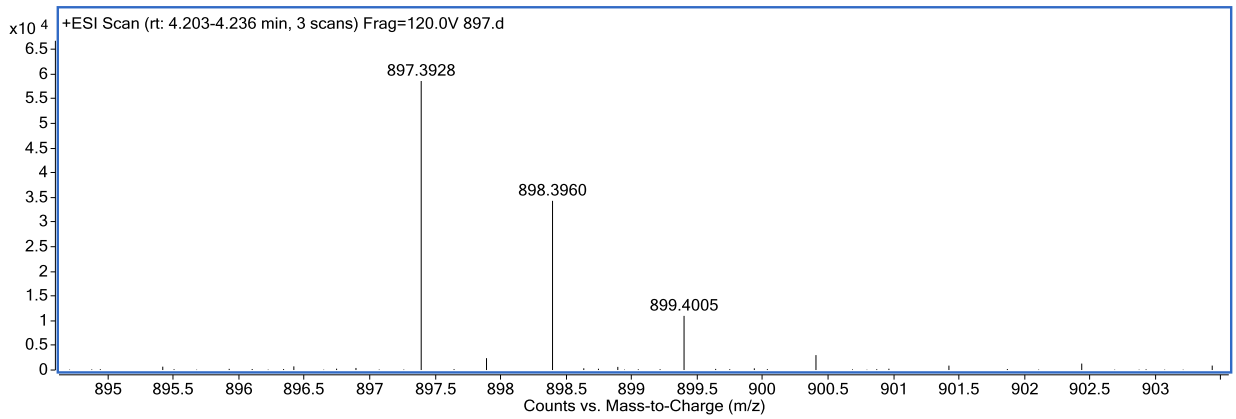
locus tag	**NCBI PGAP Annotation**	**RAST annotation**[17]
04995	hypothetical protein	hypothetical protein SC6C5.04c
05000	GntR family transcriptional regulator	GntR-family transcriptional regulator
05005	iron-sulfur protein	putative iron-sulfur protein
05010	hypothetical protein	putative transmembrane protein
05015	hypothetical protein	Cys-tRNA(Pro) deacylase YbaK
05020	pyruvate oxidase	Pyruvate oxidase [ubiquinone, cytochrome] (EC 1.2.2.2)
05025	hypothetical protein	FIG01124407: hypothetical protein
05030	hypothetical protein	Cys-tRNA(Pro) deacylase YbaK
05035	hypothetical protein	hypothetical protein
05040	hypothetical protein	small hydrophobic protein (putative membrane protein)
05045	flavodoxin	Multimeric flavodoxin WrbA
05050	ribosome small subunit-dependent GTPase A	Probable GTPase related to EngC
05055	MFS transporter	major facilitator superfamily MFS 1
05060	enoyl-CoA hydratase	Enoyl-CoA hydratase (EC 4.2.1.17)
05065	hypothetical	nonribosomal peptide synthetase
05070	methyltransferase type 11	Glutamate synthase [NADPH] large chain (EC 1.4.1.13)
05075	hypothetical	regulatory protein
05080	enoyl-CoA hydratase	enoyl-CoA hydratase (EC 4.2.1.17)
05085	enoyl-CoA hydratase	enoyl-CoA hydratase/isomerase
05090	type III synthase	chalcone and stilbene synthases-like
05095	peptide synthase	CDA peptide synthetase III
05100	hypothetical	nonribosomal peptide synthetase
05105	GntR family transcriptional regulator	putative aminotransferase
05110	MbtH family protein	putative MbtH family protein
05115	hypothetical protein	putative secreted protein
05120	hydrophobic protein	hydrophobic protein
05125	allophanate hydrolase (subunit 2)	allophanate hydrolase 2 subunit 2 (EC 3.5.1.54)
05130	allophanate hydrolase (subunit 1)	allophanate hydrolase 2 subunit 1 (EC 3.5.1.54)
05135	hypothetical protein	lactam utilization protein LamB
05140	FAD-binding protein	xylitol oxidase (EC:1.1.3.41)
05145	hypothetical protein	FIG00761799: membrane protein
05150	DNA alkylation response protein	acyl-CoA dehydrogenase (EC 1.3.8.7)
05155	diguanylate cyclase	transcriptional regulator
05160	N-acetyltransferase	hypothetical protein

*The antiSMASH-predicted gene cluster spans nucleotide 1 204 680 to 1 272 231 on the chromosome, however, the last ~8.7 kb encodes for 11 genes involved in sulfate and adenylsulfate metabolism and have been omitted from this annotation table.

^{1}H (600 MHz) and ^{13}C NMR (125 MHz) for **1** (CD_3OD) and HRMS data

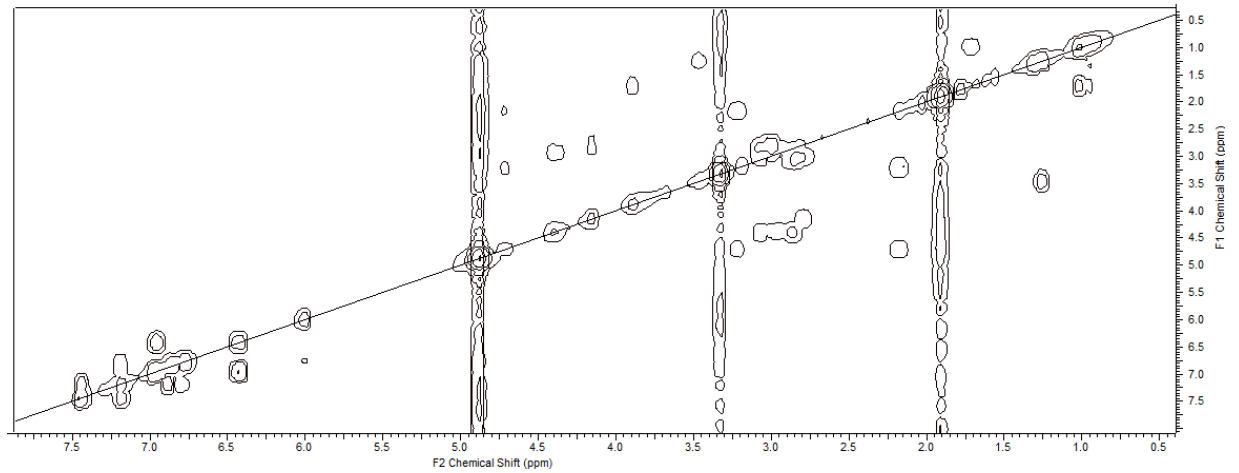




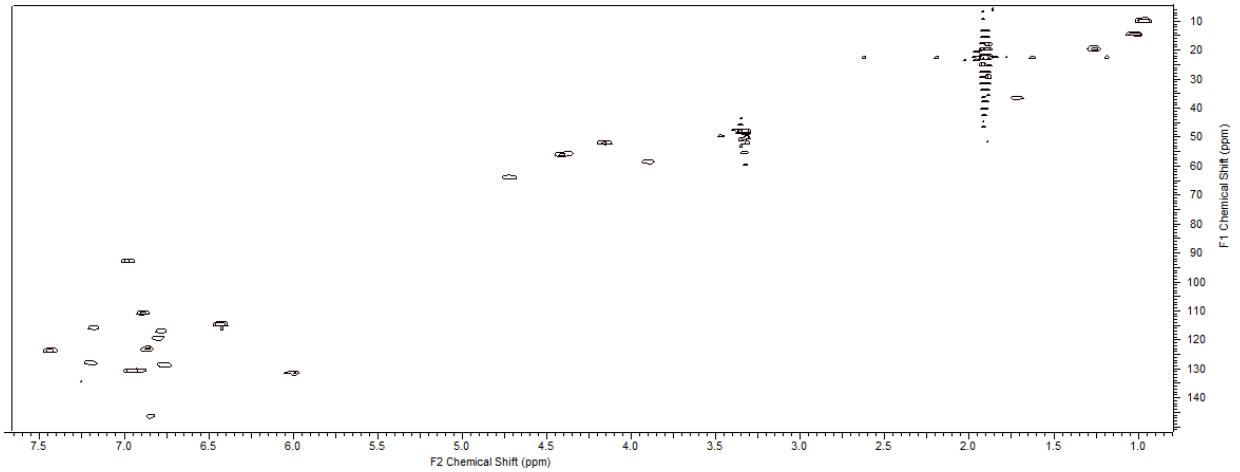


Additional NMR Data of tryptorubin A (**1**)

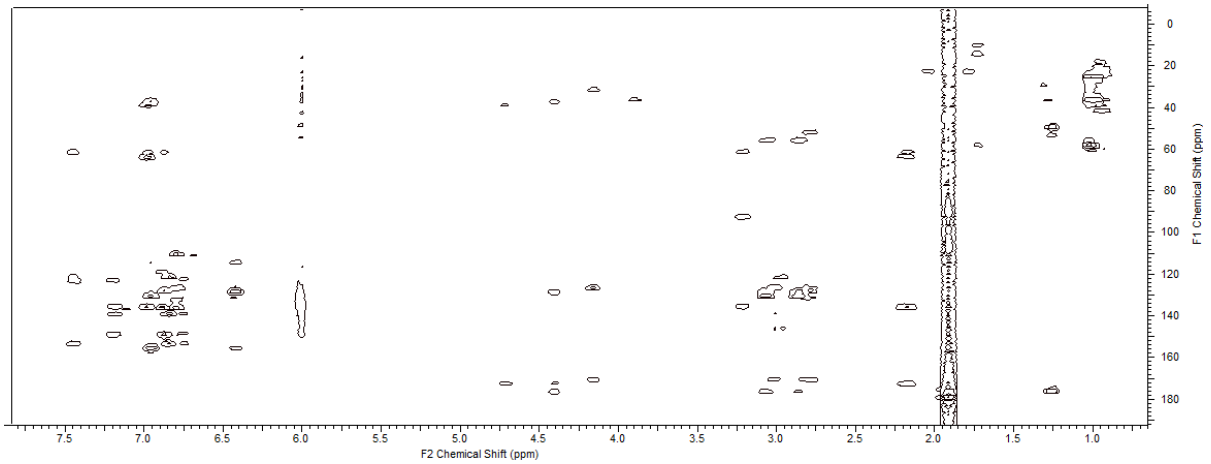
gCOSY of **1** (CD_3OD, 600 MHz)



gHSQC of **1** (CD_3OD, 600 MHz)

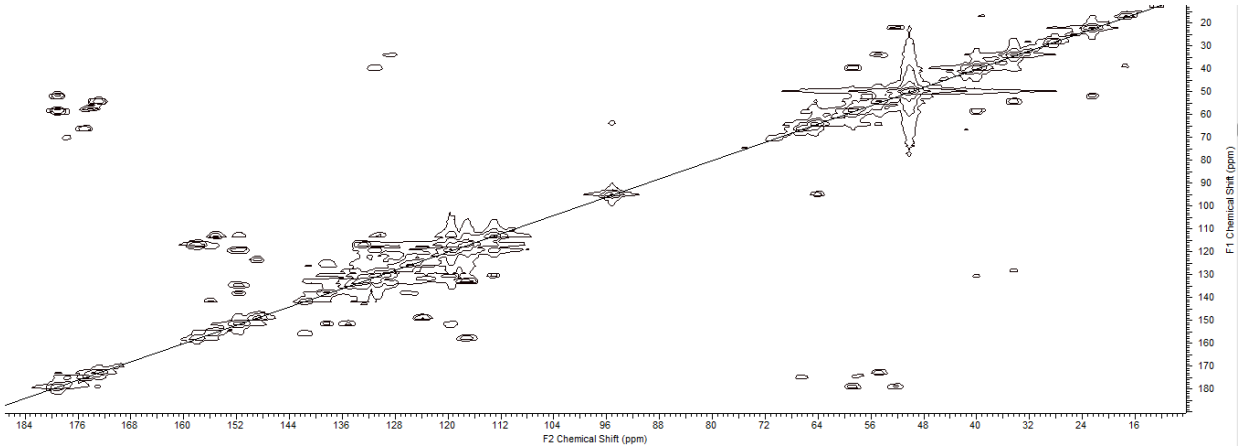


HMBC of **1** (CD_3OD, 600 MHz)

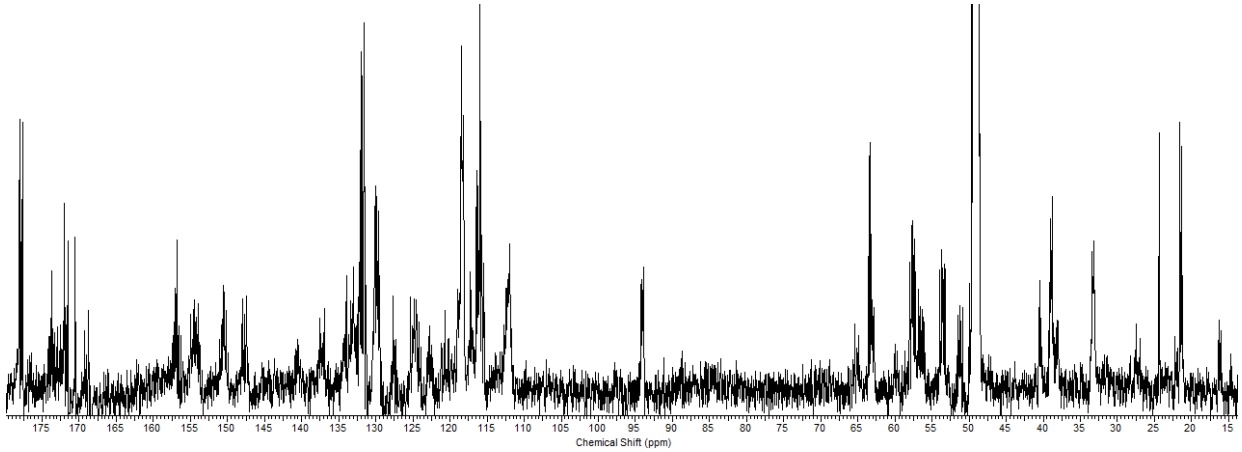


NMR data of ^{13}C-labeled tryptorubin A (**1**)

^{13}C-^{13}C COSY NMR (CD_3OD, 125 MHz)

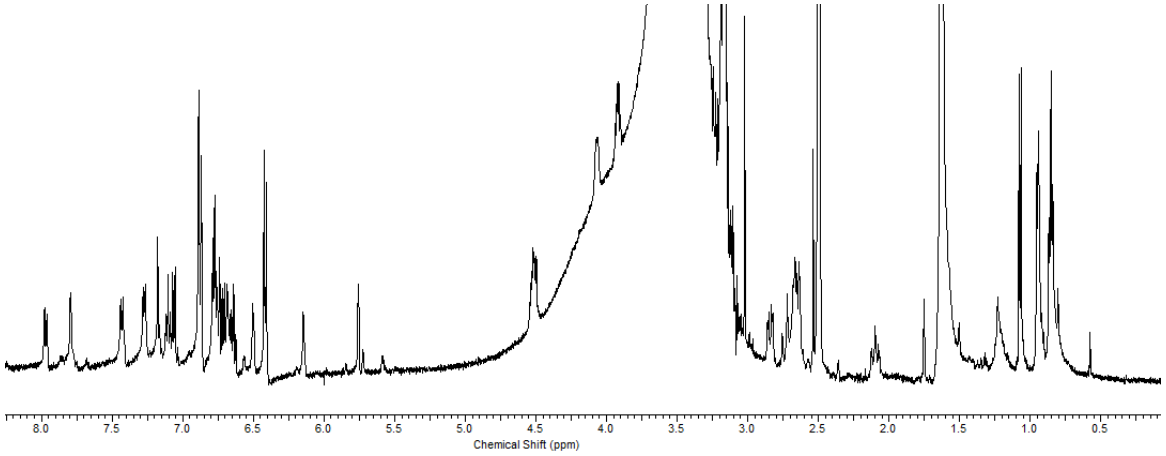


^{13}C NMR of ^{13}C-labeled **1** (CD_3OD, 125 MHz)

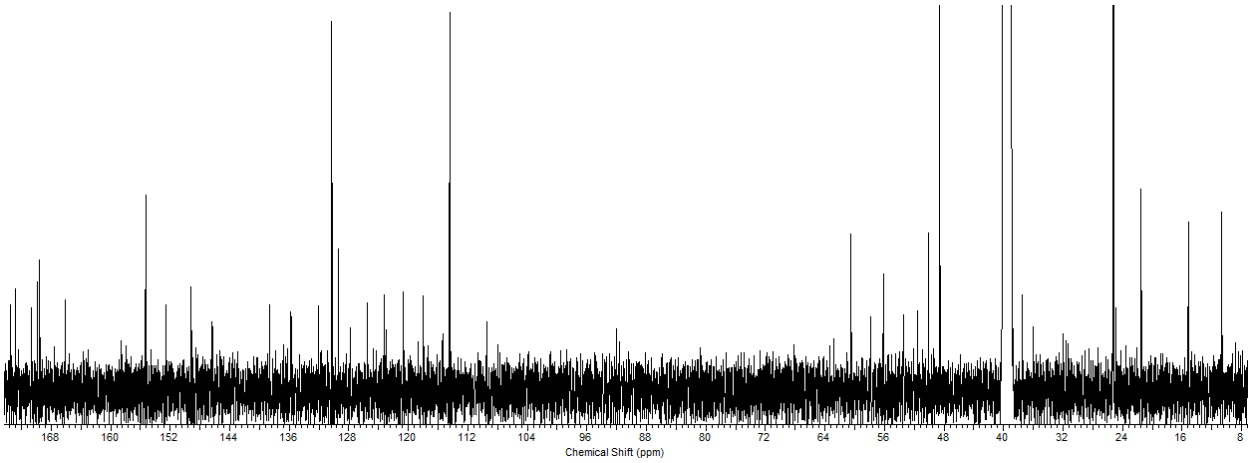


Additional NMR data of tryptorubin A (**1**)

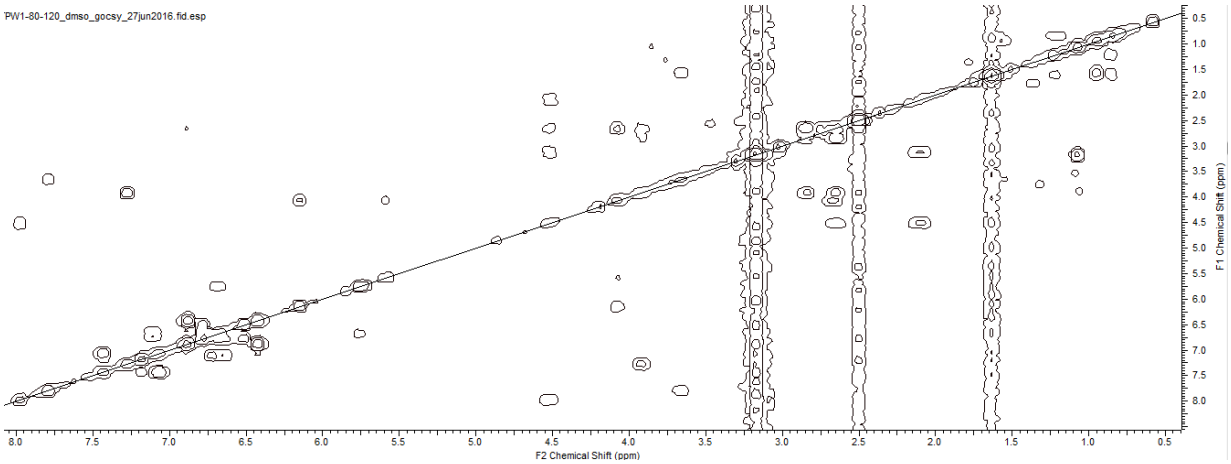
^{1}H NMR of **1** (DMSO-d_6, 600 MHz)



^{13}C NMR of **1** (DMSO-d_6, 125 MHz)

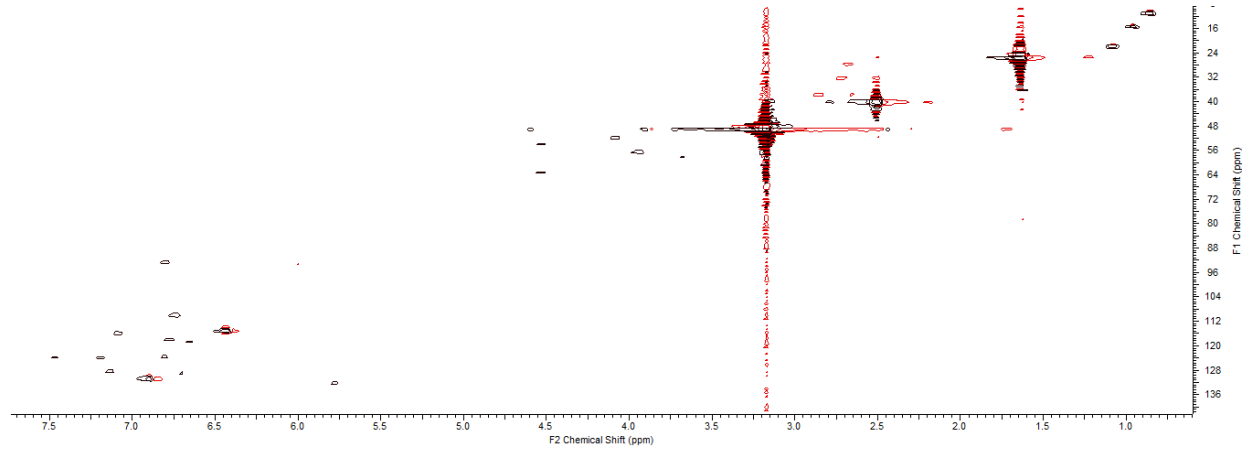


gCOSY of **1** (DMSO-d_6, 600 MHz)

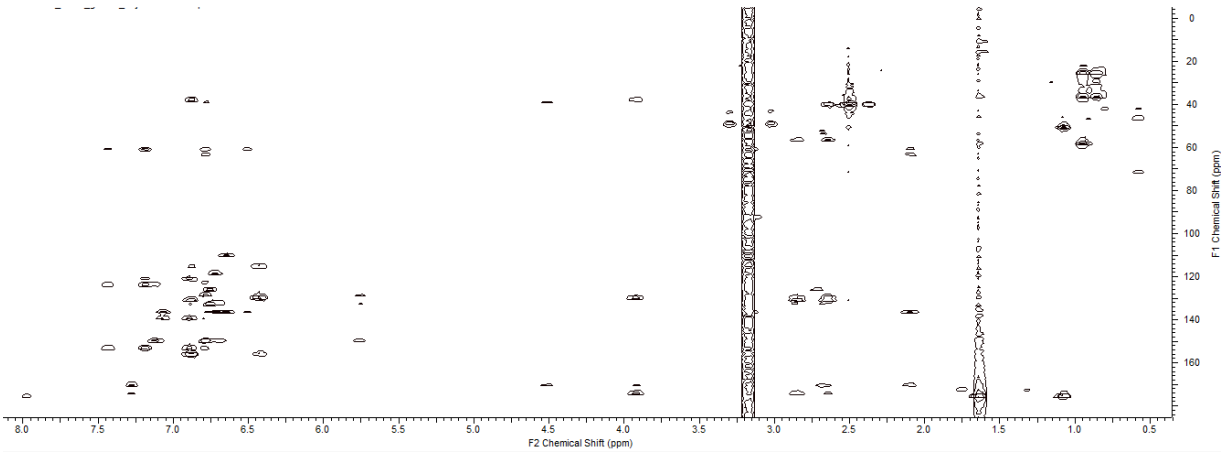


Additional NMR data of tryptorubin A (**1**)

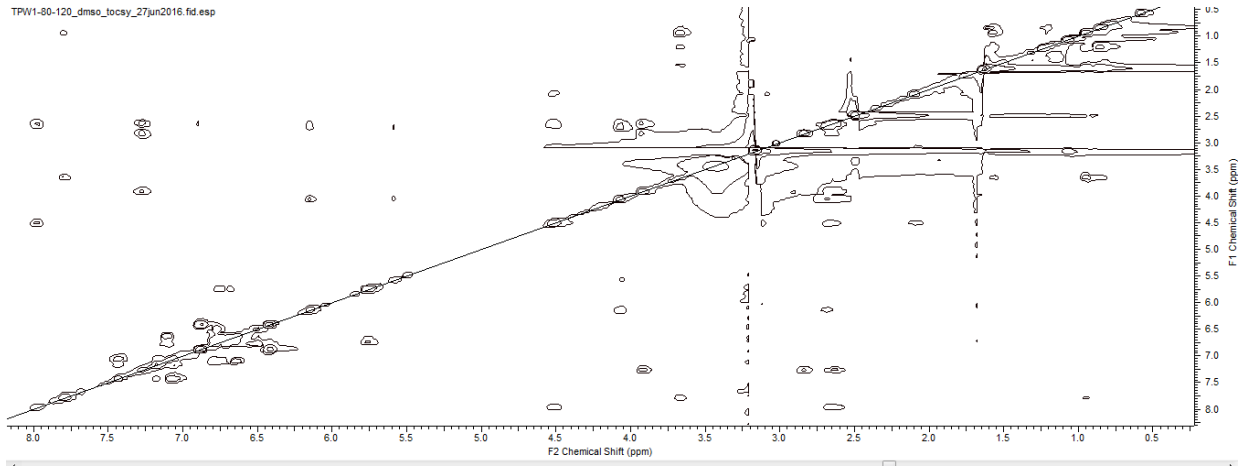
gHSQC of **1** (DMSO-d_6, 600 MHz)



gHMBC of **1** (DMSO-d_6, 600 MHz)

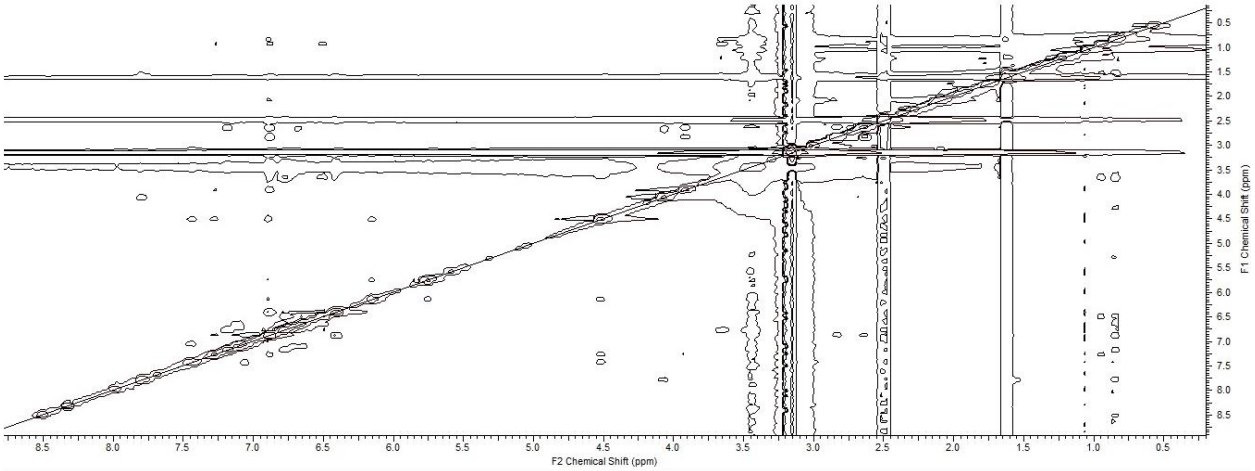


TOCSY of **1** (DMSO-d_6, 600 MHz)

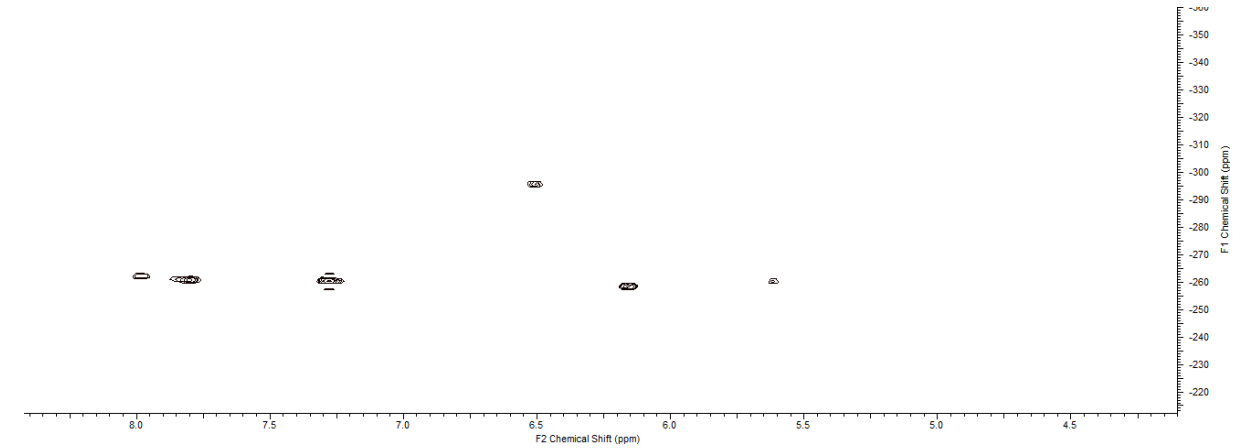


Additional NMR Data of tryptorubin A (**1**)

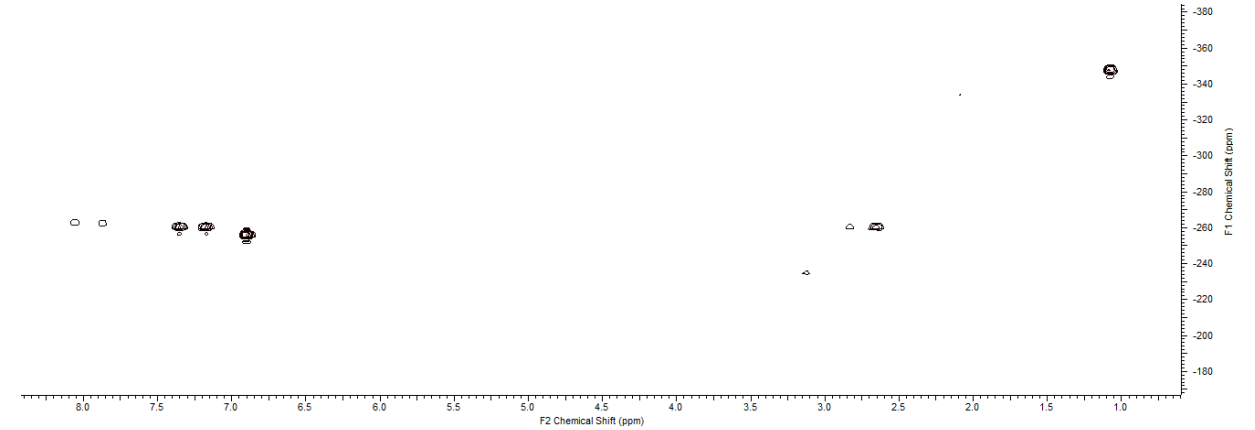
ROESY of **1** (DMSO-d_6, 600 MHz)



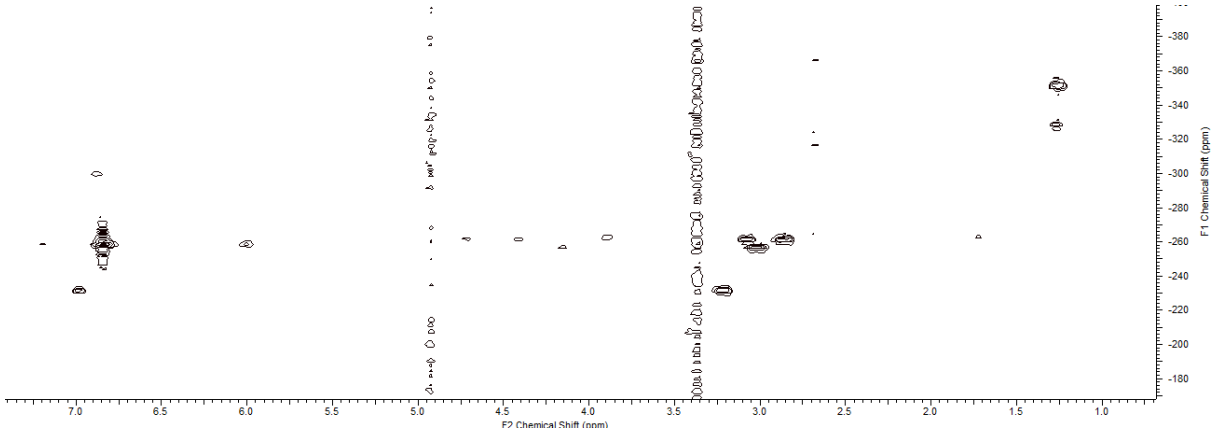
^{15}N HSQC of **1** (DMSO-d_6, 600 MHz)



^{15}N HMBC of **1** (DMSO-d_6, 600 MHz)



^{15}N HMBC of **1** (CD_3OD, 600 MHz)



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