

SUPPORTING INFORMATION

Natural Products, Stylissadines A and B, Specific Antagonists of the P2X₇ Receptor, an Important Inflammatory Target

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General Experimental Procedures. Water was Milli-Q PF filtered, while all other solvents used were HPLC grade. A Betasil C₁₈ 5μm (50 mm x 150 mm i.d.) was used for preparative HPLC, while a Betasil C₁₈ 5μm (21.2 mm x 150 mm i.d.) was used for semi-preparative HPLC. C₁₈ was 04K-4348 Septra C₁₈ End-Capped Silica. NMR spectra were recorded at 30°C on 500 and 600 MHz NMR spectrometers. Samples were dissolved in DMSO-*d*₆ (residual ¹H δ 2.50 and ¹³C δ 39.5 ppm). Multiplicity determined by DEPT (s = C, d = CH, t = CH₂, q = CH₃). Standard parameters were used for the 2D experiments, which included gradient COSY, HSQC (¹J_{CH} = 140 Hz) and HMBC (ⁿJ_{CH} = 8.3 Hz).

General Haemolysin Assay Principle: THP-1 cells were incubated with haemolysin a non-specific pore forming compound and a cell membrane impermeable nucleic acid stain, Sytox® Orange. Once the pore is open, Sytox® Orange passes through the pore into the cell and binds nuclei acids which results in an increase in fluorescence.

General Haemolysin Assay Procedure: The test substances were added to microtitre plates. Then the three reagents, haemolysin, THP-1 cells and Sytox® Orange were multidispensed to the 384 plate. The plate was incubated for 90 min at 37°C and read on a fluorescence plate reader using a 530 nm excitation and 620 nm emission wavelengths. The percentage inhibition was then calculated.

Haemolysin Assay Method: The THP-1 cells were centrifuged twice and resuspended into HKS (consisting of 0.125 M KCl, 1 mM EDTA, 0.02 M HEPES free acid, 5 mM glucose made up to pH 7.4 with KOH) at a concentration of 5×10^5 cells/mL (15000/well). Sytox® Orange was added to the cell mix to give a 10 μ M final solution (1:500 dilution) just before addition to the microtitre plate. Haemolysin in HKS was then added to each well to give a final concentration of 1.1 μ g/mL. The assay was performed in 384 microtiter plates and consisted of the following: 0.5 μ L of compound (stock 10 mM in DMSO), 4.5 μ L water, 10 μ L haemolysin, 30 μ L cells with sytox orange. In-controls consisted of 100 % activity (0.5 μ L DMSO plus 10 μ L of haemolysin) and 0 % activity (0.5 μ L DMSO plus 10 μ L of HKS in place of haemolysin). All plates were incubated at 37°C for 90 min and read at 530 nm excitation and 620 nm emission on a fluorescence plate reader. Compound activity was expressed as percentage inhibition compared to the 100% activity control.

General Alamar Blue Cytotoxicity Assay Principle: Cell viability was assessed via the innate metabolic activity of proliferating cells. Proliferating cells reduce Alamar Blue giving increased fluorescence. Decreased fluorescence was observed when there was inhibition of cell growth as a result of decreased metabolic activity.

General Alamar Blue Cytotoxicity Assay Procedure: The test substances were added to microtitre plates. Then the cells in growth media were multidispensed to the 384 plate. Viability was assessed after 90 min and overnight incubation by the addition of Alamar Blue to each well. The plate was then incubated at 37°C with 5% carbon dioxide for a further 1 hour and then left at room temperature overnight before reading on a fluorescence plate reader using 535 nm excitation and 590 nm emission. The percentage cytotoxicity was calculated.

Alamar Blue Cytotoxicity Assay Method: The THP-1 cells were centrifuged twice and resuspended into growth media (RPMI with glutamax plus 10% FBS) at a concentration of 5×10^5 cells/mL (15000/well). The assay was performed in 384 microtiter plates and consisted of the following additions: 0.5 μ L of compound (stock 10 mM in DMSO), 4.5 μ L water, 30 μ L cells. Plates were incubated for 90 min and overnight at 37°C with 5% carbon dioxide. In-plate controls consisted of

100% activity (0.5 μ L DMSO) and 0% activity (0.5 μ L DMSO plus 30 μ L of growth media without cells). Following incubation, 35 μ l of 20% Alamar blue in growth media was added and incubated for a further 1 hour at 37°C with 5% carbon dioxide. Plates were left overnight at room temperature before being read on a fluorescence plate reader with an excitation of 535 nm and emission of 590 nm. Compound cytotoxicity was expressed as percentage inhibition compared to the 100% activity control.

Table S1. ¹H (600 MHz), ¹³C (125 MHz), gCOSY and gHMBC NMR data for 4,5-Dibromopalau'amine (4) in DMSO-*d*₆

position	δ_C	δ_H (mult, <i>J</i> Hz)	COSY (H no.)	^{2,3} <i>J</i> _{CH} HMBC (C no.)
2	124.8 s			
3	115.0 d	7.02 (s)		2, 5, 15
4	102.0 s			
5	106.0 s			
6	68.2 d	6.47 (brs)	7	2, 5, 8, 10, 11
7(N)		9.92 (brs)	6	6, 8, 10
8	156.8 s			
8-NH ₂		8.11 (brm, 2H) ^b		
9		10.04 (brs)		
10	79.5 s			
11	55.6 d	3.13 (d, 15.0)	12	6, 10, 12, 13, 16, 18, 20
12	40.5 d	2.55 (m)	11, 13 α , 13 β , 18	10, 11, 18, 19
13	44.7 t	4.10 (dd, 10.0, 7.5) α 3.09 (t, 10.0) β	12, 13 β 12, 13 α	10, 11, 12 12, 15, 18
15	154.1 s			
16	70.6 s			
17	73.4 d	4.42 (d, 8.4)	18	11, 16, 18, 19, 20
18	47.2 d	2.28 (m)	12, 17, 19a, 19b	12, 17
19	40.1 t ^a	3.15 (m)a 2.98 (m)b	18, 19b, 24 18, 19a, 24	
20	82.2 d	5.74 (brd, 4.8)	21, 20-OH	11, 17, 22
20-OH		7.90 (d, 4.8)	20	16, 20
21(N)		9.45 (brs)	20	16, 20, 22
22	157.1 s			
22-NH ₂		8.27 (brm, 2H) ^b		
23(N)		9.13 (brs)		20, 22
24(N)		8.11 (brm, 2H)	19a, 19b	

^aChemical shift determined from DEPT experiment as the ¹³C signal was obscured by DMSO.

^bInterchangeable ¹H signals.

Table S2. ¹H (600 MHz), ¹³C (125 MHz) and gHMBC NMR data for 4,5-Dibromopalau'amine (4) in D₂O

position	δ_C^a	δ_H (mult, <i>J</i> Hz) ^b	^{2,3} <i>J</i> _{CH} HMBC correlations (C no.)
2	124.5 s		
3	118.0 d	7.01 (s)	2, 5
4	104.1 s		
5	109.2 s		
6	69.9 d	6.54 (s)	2, 8, 10, 11
8	156.7 s		
10	80.8 s		
11	56.8 d	3.13 (d, 13.8)	10, 16, 18
12	41.8 d	2.52 (m) ^c	
13	46.0 t	4.03 (dd, 10.0, 7.0) α 3.30 (m) β^d	11
15	157.9 s		
16	72.3 s		
17	73.9 d	4.37 (d, 7.8)	16, 18
18	48.6 d	2.52 (m) ^c	
19	41.9 t	3.31 (m, 2H) ^d	
20	83.8 d	6.00 (s)	17, 22
22	158.0 s		

^a¹³C NMR referenced to C-20 (δ 83.8) in 4,5-dibromopalau'amine.¹² ^b¹H NMR referenced to residual solvent (δ 4.63). ^{c-d}Overlapping ¹H signals.

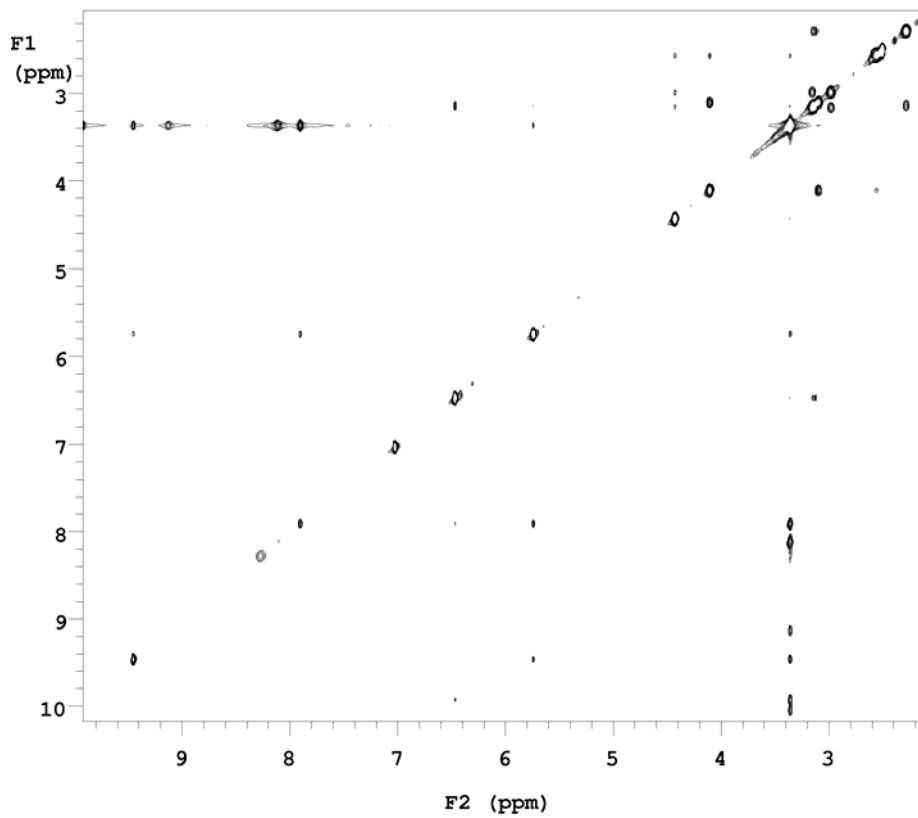
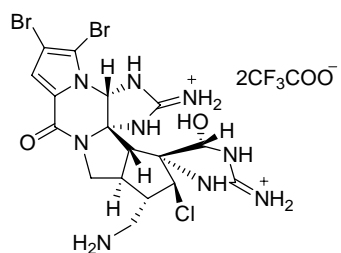


Figure S1. ROESY Spectrum of 4,5-Dibromopalau'amine (4) in DMSO-*d*₆

Table S3. ^1H (600MHz), ^{13}C (125 MHz), gCOSY and gHMBC NMR data for Massadine (5) in $\text{DMSO-}d_6$

position	δ_{C}^a	δ_{H} (mult, J in Hz)	COSY (H no.)	$^{2,3}J_{\text{CH}}$ HMBC (C no.)
1	82.0 d	5.50 (s)		3, 5, 13
2 (N)		9.17 (s)		3, 14
3	157.0 s			
4 (N)		8.69 (brs)		1, 3, 14
5	89.9 d	5.30 (d, 2.4)	6	1, 7, 9
6 (N)		8.85 (brs)	5	9
7	156.8 s			
8 (N)		9.21 (s)		5, 7, 9
9	87.2 s			
10	42.4 d	2.25 (d, 12.0)	11	9, 14, 23
11	n.o.	2.00 (brdq, 12.0, 7.0)	10	
12	50.0 d	1.94 (brq, 7.0)	15a, 15b	
13	77.4 d	3.53 (d, 5.4) ^b	13-OH	10
14	70.0 s			
15	41.3 t	3.44 (m)a ^c 3.35 (m)b ^c	12, 16 12, 16	
16 (N)		8.31 (brt, 5.0)	15a, 15b	17
17	159.2 s			
18	128.0 s			
19 (N)		12.68 (d, 2.4)	22	21, 22
20	104.8 s			
21	98.0 s			
22	112.5 d	6.96 (d, 2.4)	19	18, 20
23	40.7 t	3.67 (dt, 14.4, 5.4)a 3.55 (m)b ^{b,c}	23b, 24 23a, 24	25
24 (N)		8.00 (brt, 6.0)	23a, 23b	25
25	159.5 s			
26	127.7 s			
27 (N)		12.75 (d, 2.4)	30	29, 30
28	105.0 s			
29	98.0 s			
30	112.5 d	6.94 (d, 2.4)	27	26, 28
9-OH		7.40 (s)		9, 10
13-OH		5.74 (d, 5.4)	13	12, 14

^aChemical shifts estimated from 2D NMR experiments. ^bOverlapping ^1H signals. ^cChemical shift determined from 2D experiments as ^1H signal obscured by H_2O . n.o. = not observed.

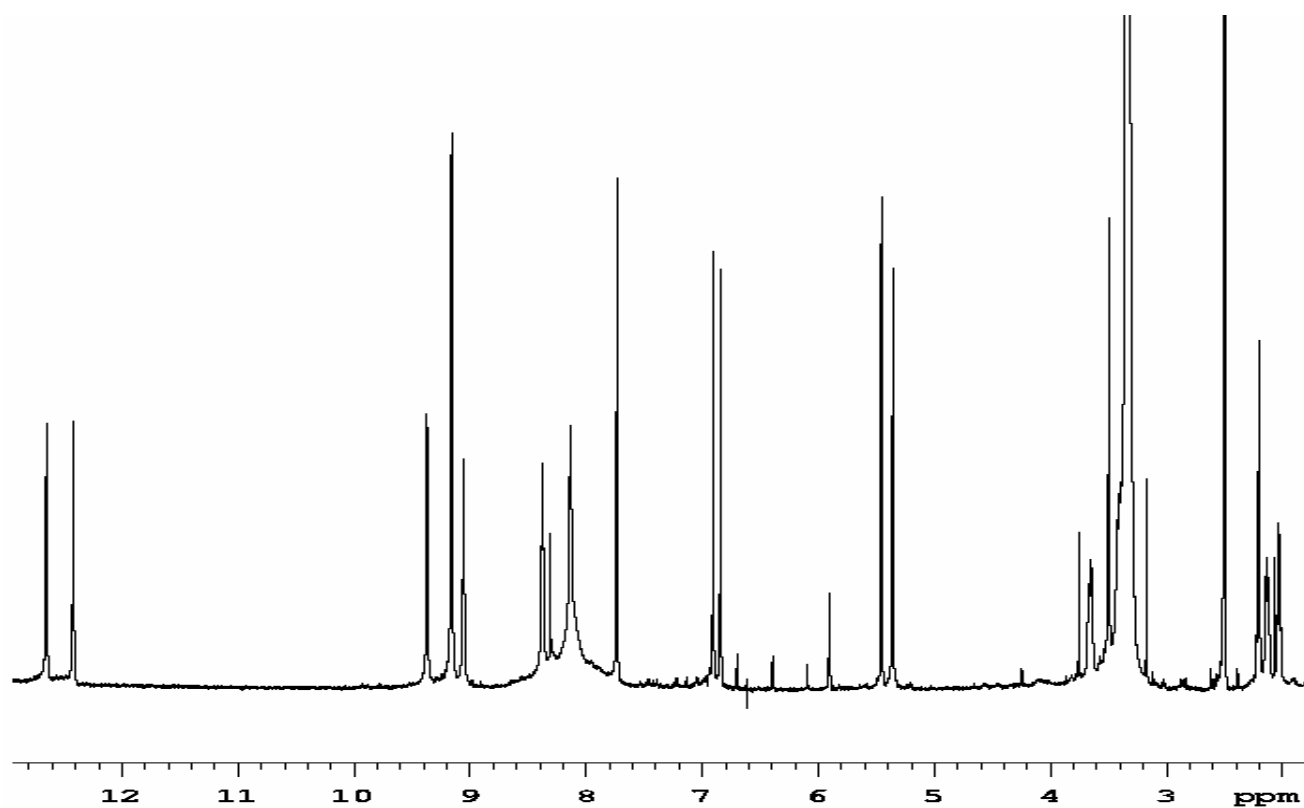
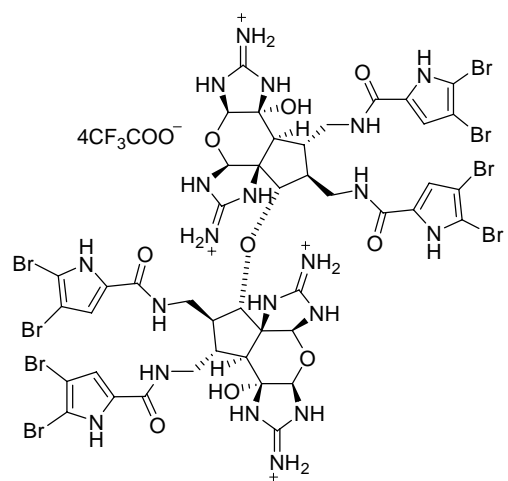


Figure S8. ¹H NMR (600 MHz) spectrum of Stylistadine A (1) in DMSO-*d*₆

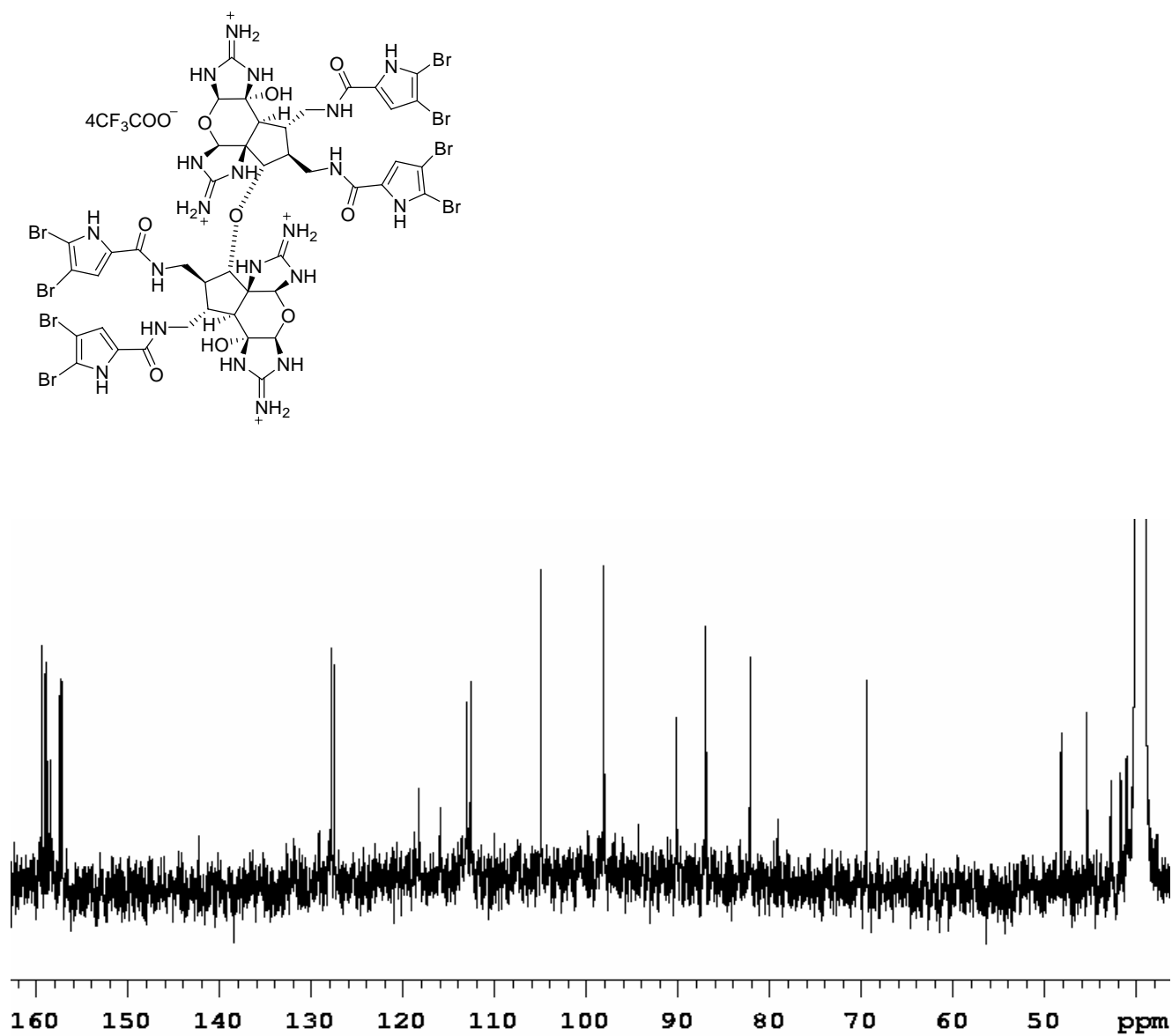


Figure S9. ¹³C NMR (125 MHz) spectrum of Styliissadine A (1) in DMSO-*d*₆

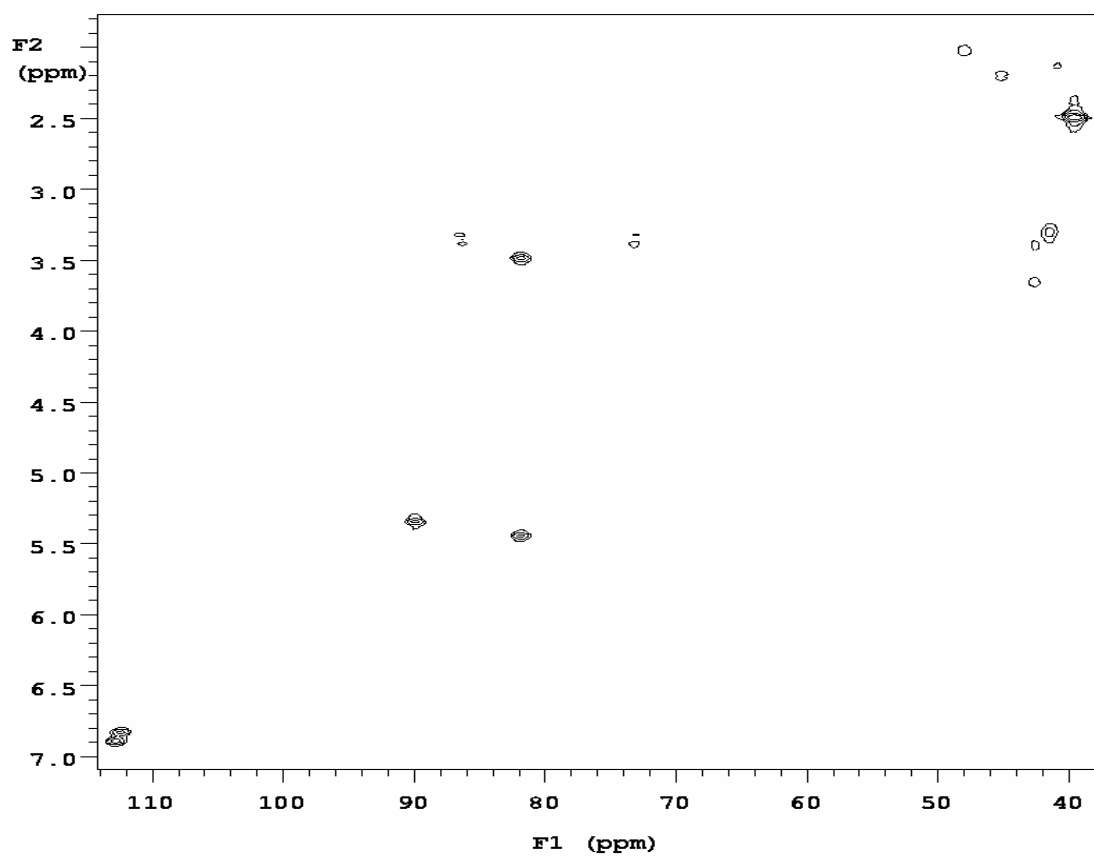
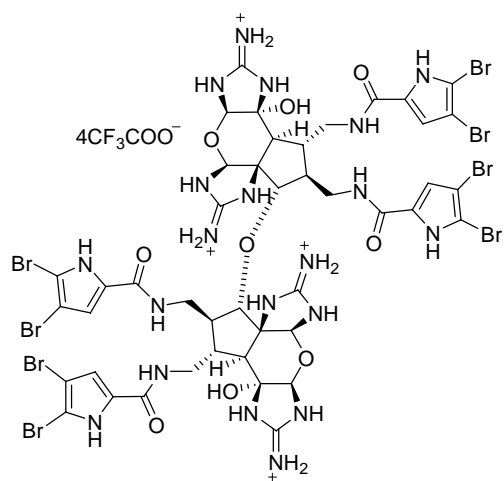


Figure S11. gHSQC Spectrum of Styliissadine A (1) in DMSO- d_6

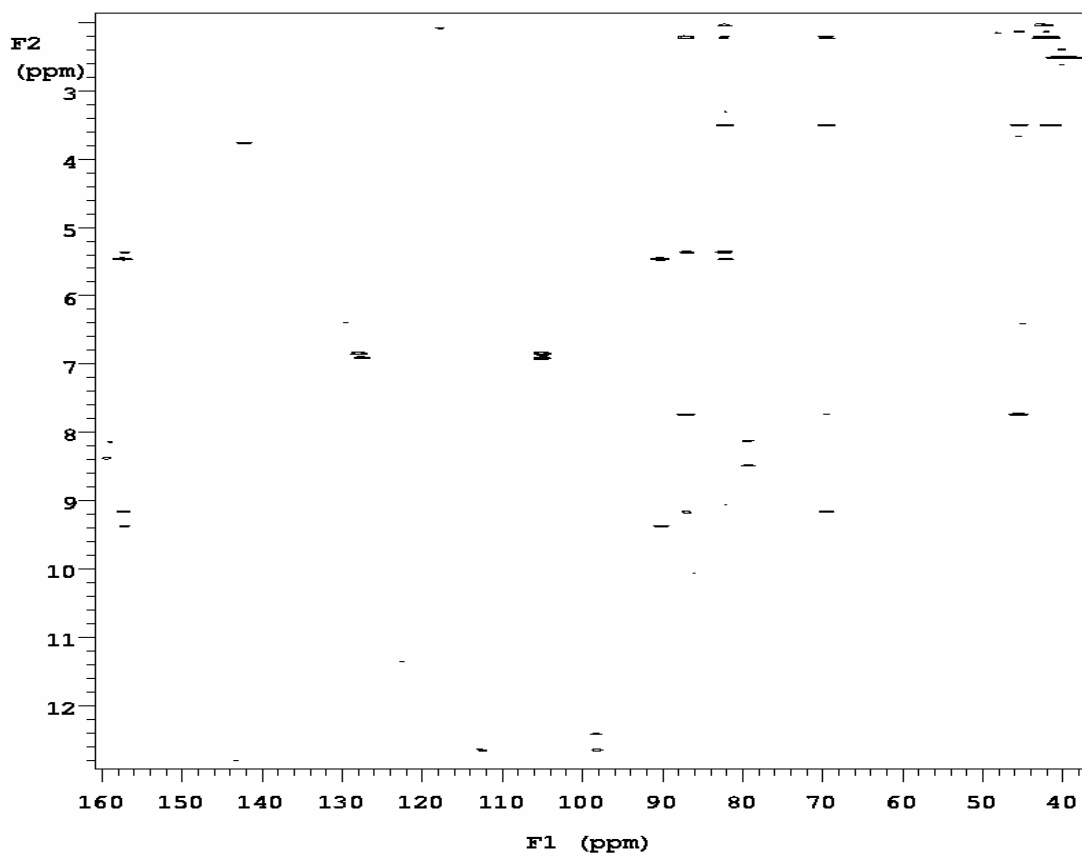
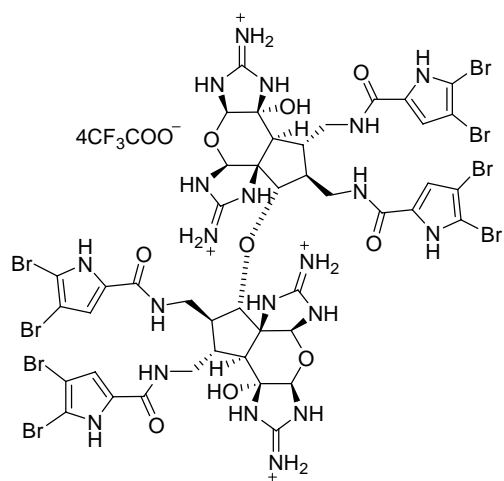


Figure S12. gHMBC Spectrum of Stylissadine A (1) in DMSO- d_6

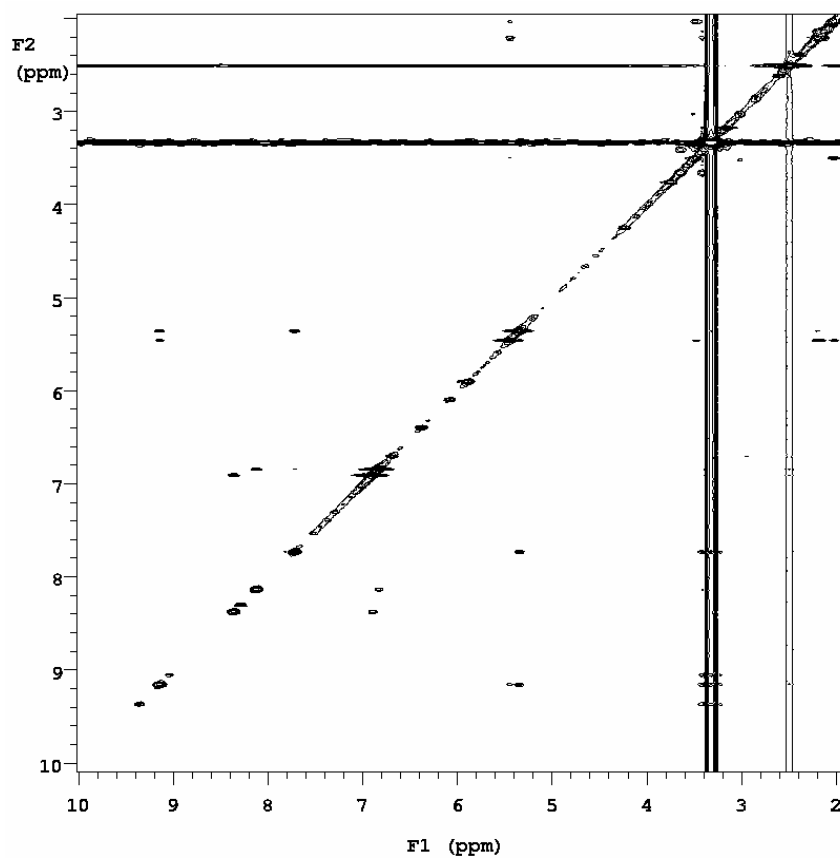
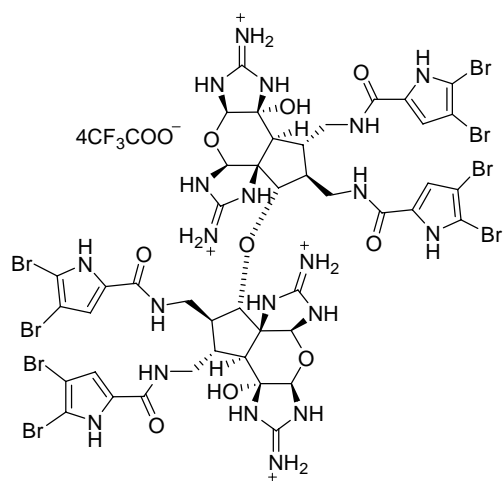


Figure S13. ROESY Spectrum of Stylyssadine A (1) in DMSO- d_6

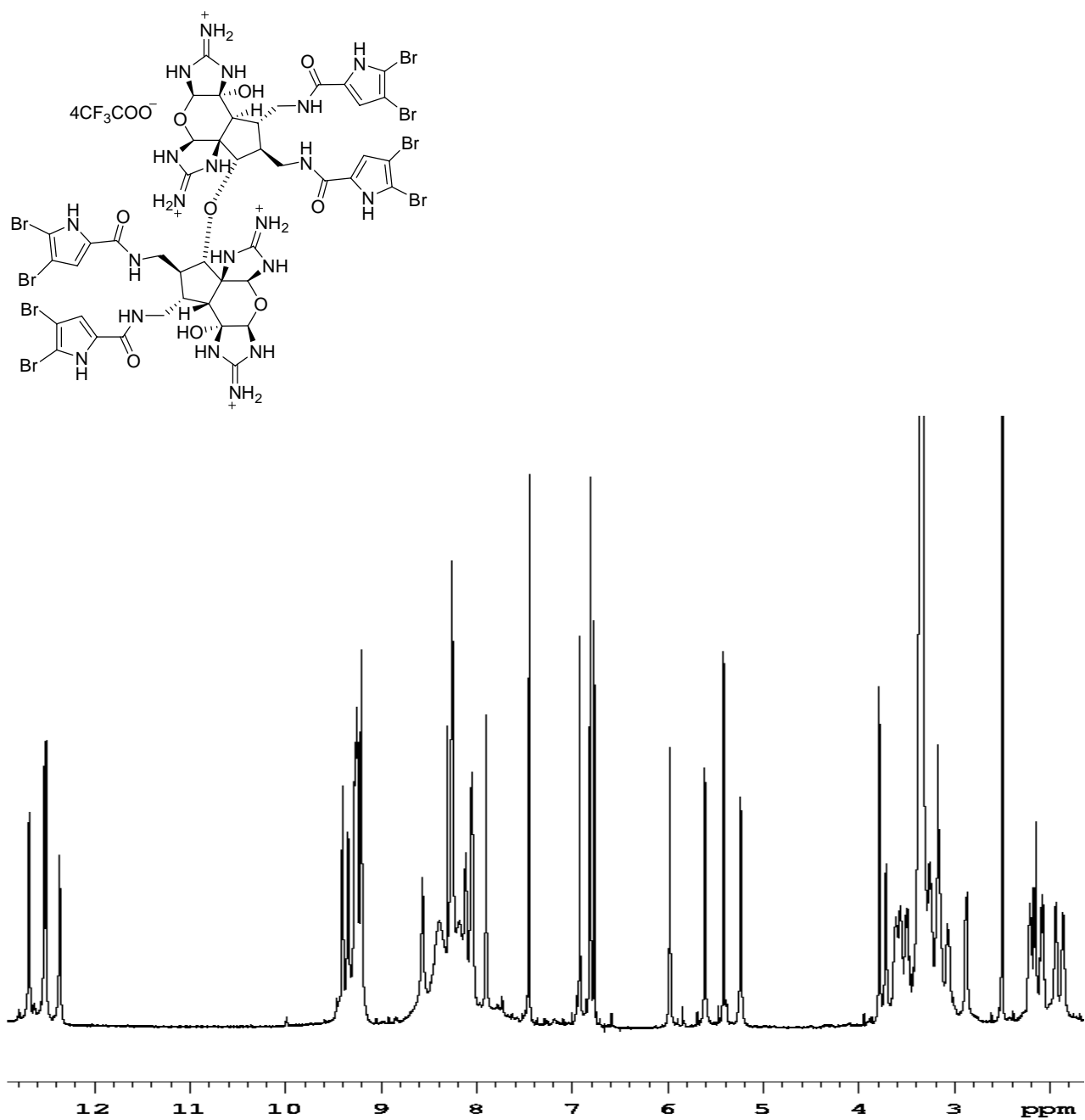


Figure S2. ¹H NMR (600 MHz) spectrum of Stylistadine B (2) in DMSO-*d*₆

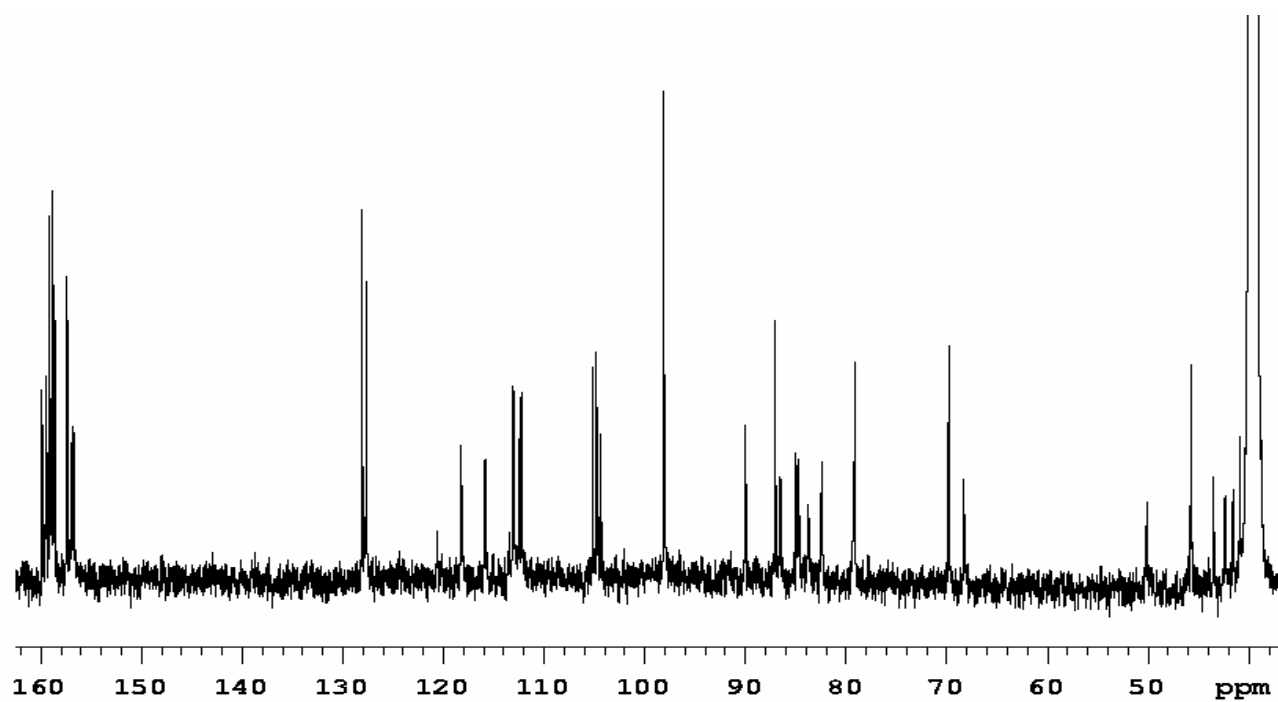
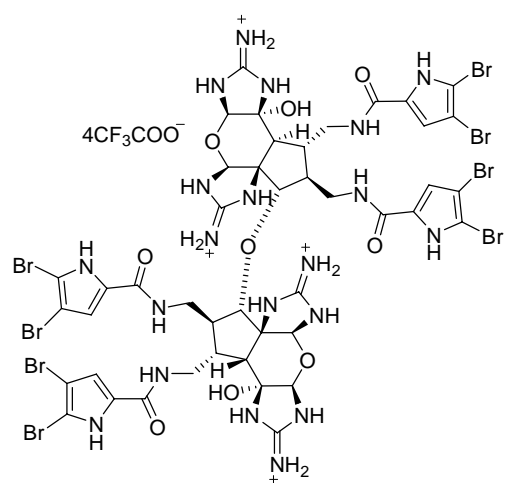


Figure S3. ¹³C NMR (125 MHz) spectrum of Stylyssadine B (2) in DMSO-*d*₆

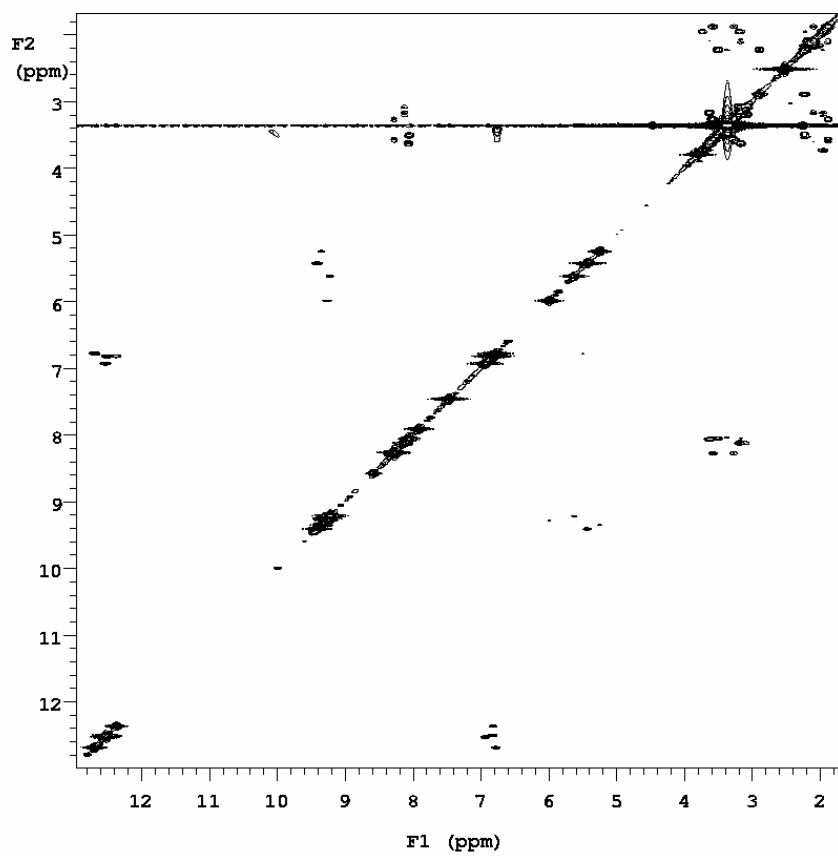
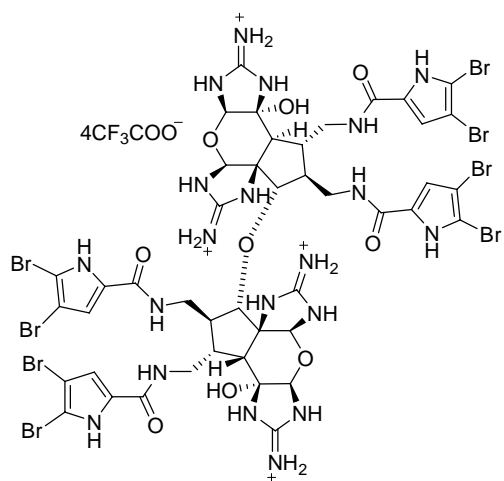


Figure S4. gCOSY Spectrum of Stylissadine B (2) in DMSO-*d*₆

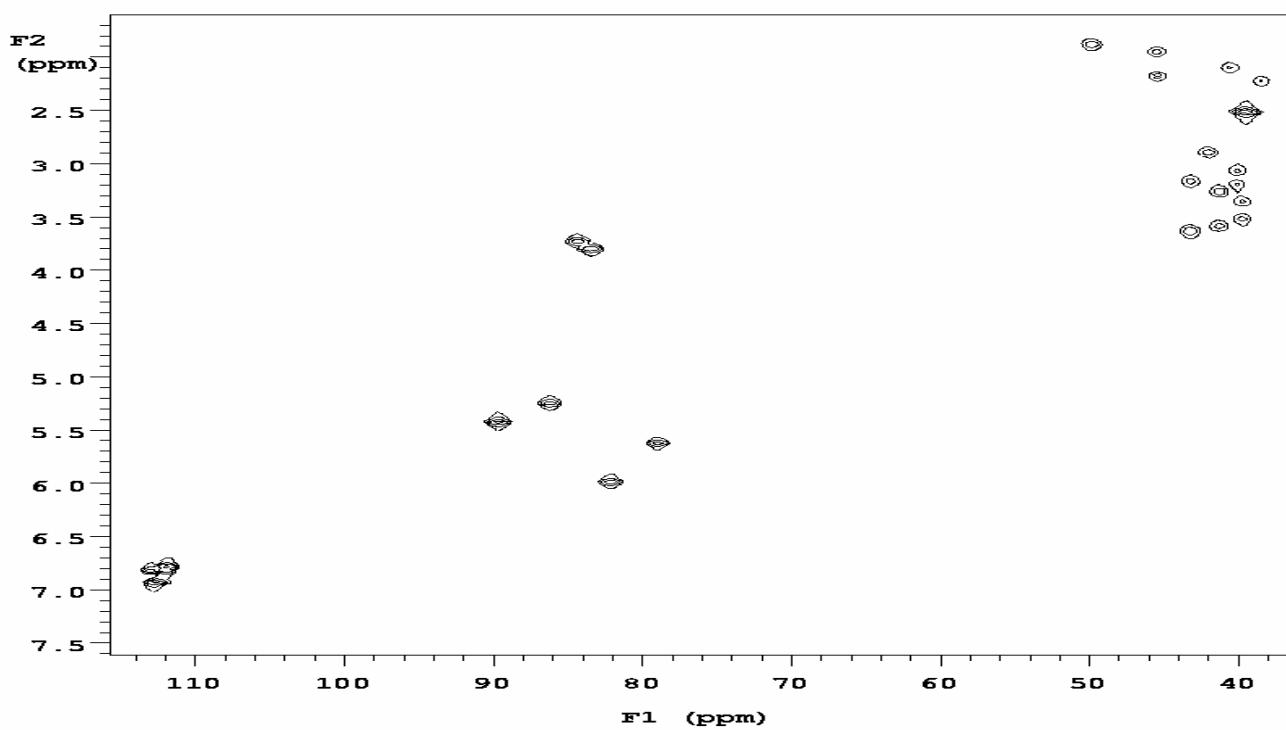
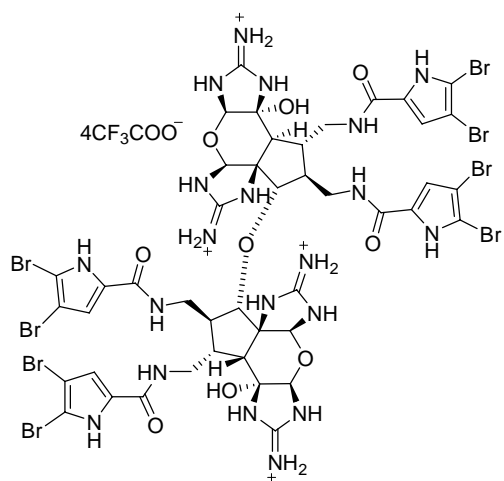


Figure S5. gHSQC Spectrum of Stylistadine B (2) in DMSO- d_6

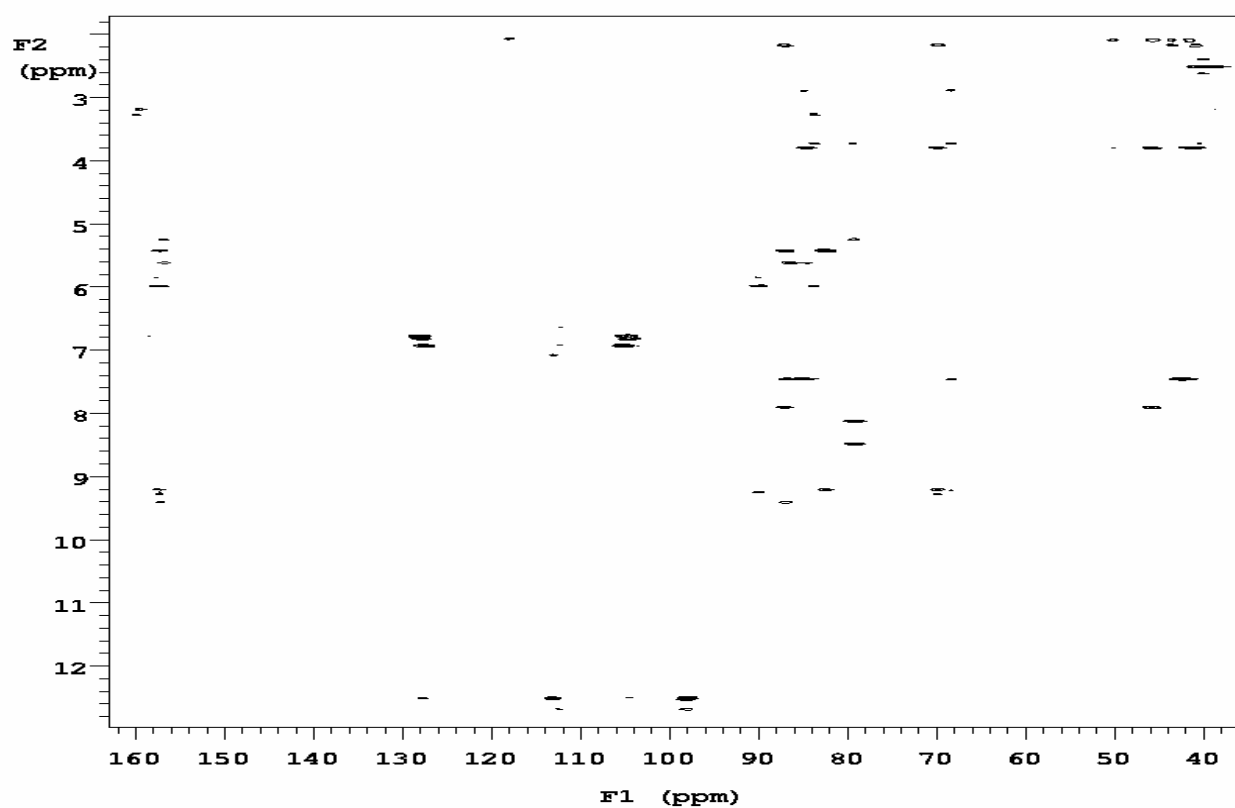
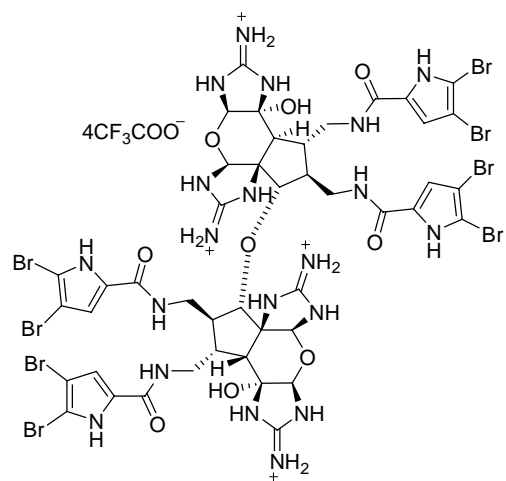


Figure S6. gHMBC Spectrum of Stylistadine B (2) in DMSO-*d*₆

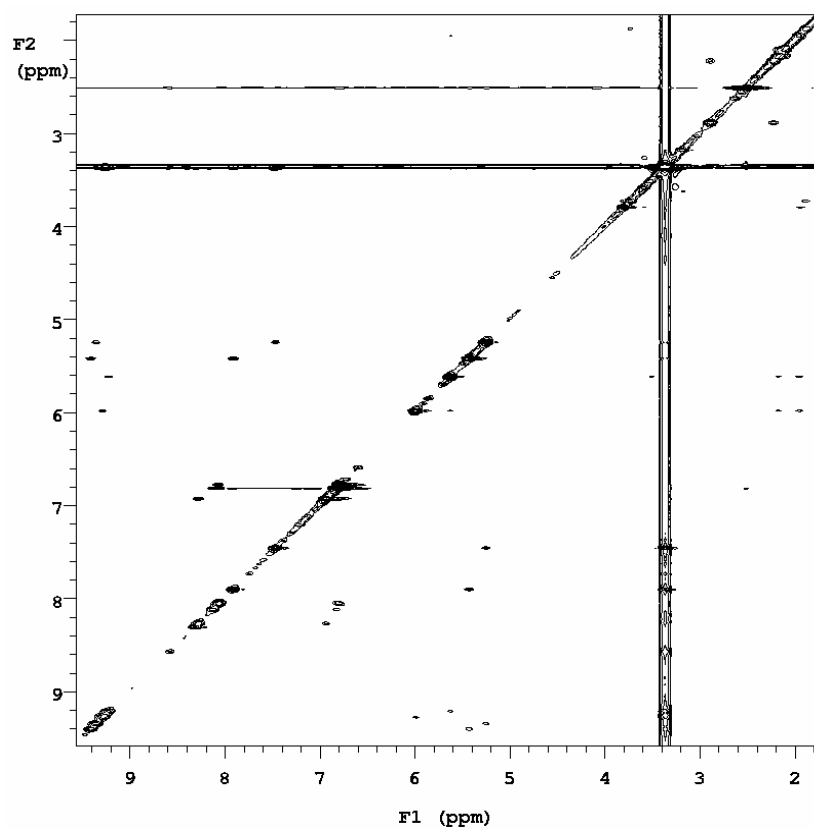
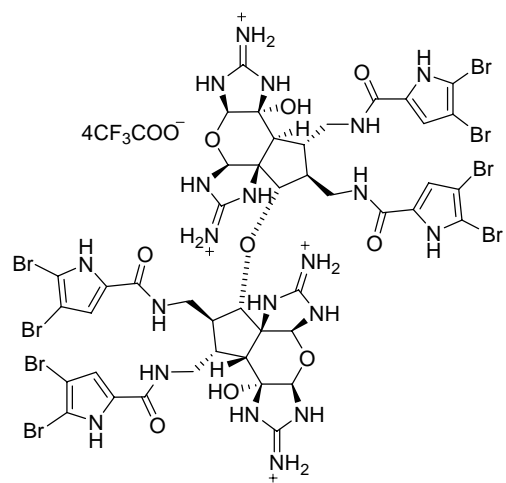


Figure S7. ROESY Spectrum of Stylyssadine B (2) in DMSO-*d*₆

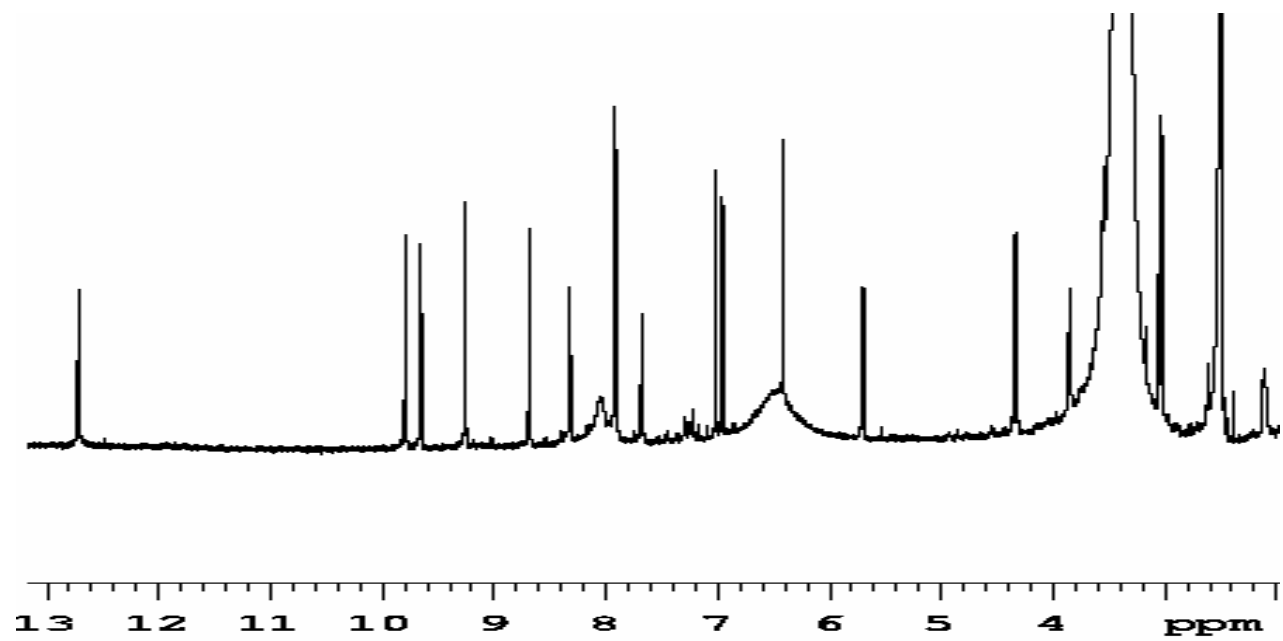
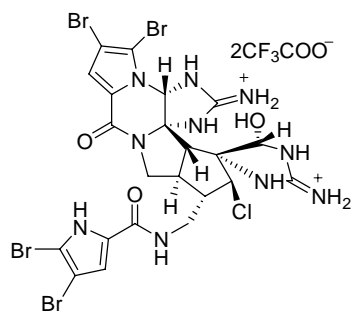


Figure S14. ¹H NMR (600 MHz) Spectrum of Konbu'acidin B (3) in DMSO-*d*₆

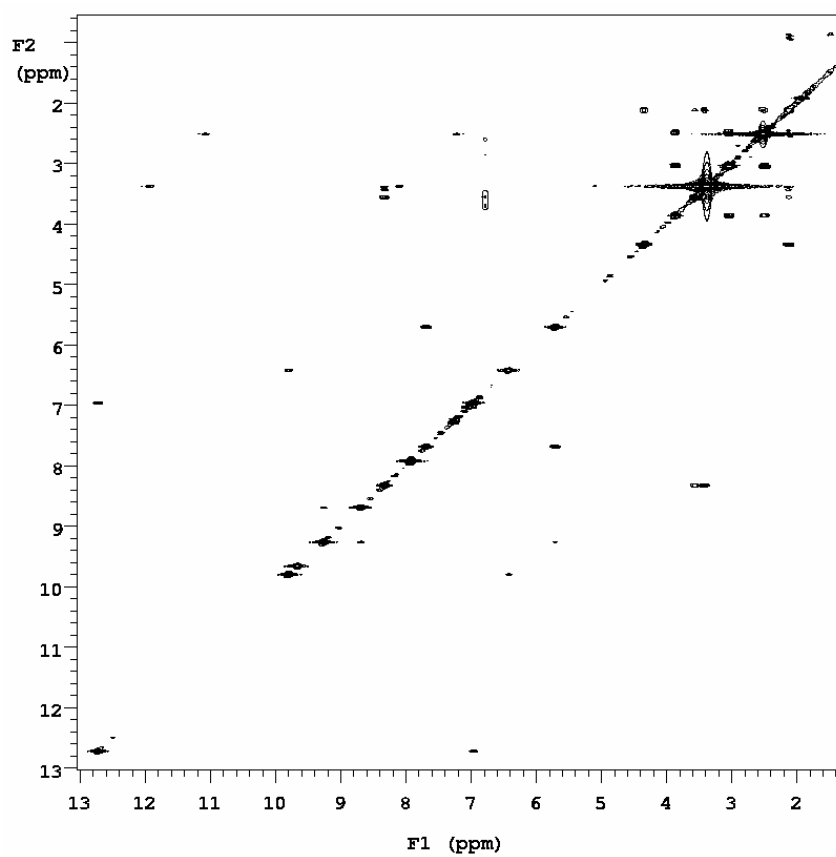
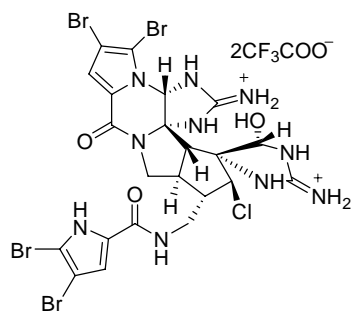


Figure S15. gCOSY Spectrum of Konbu'acidin B (3) in DMSO- d_6

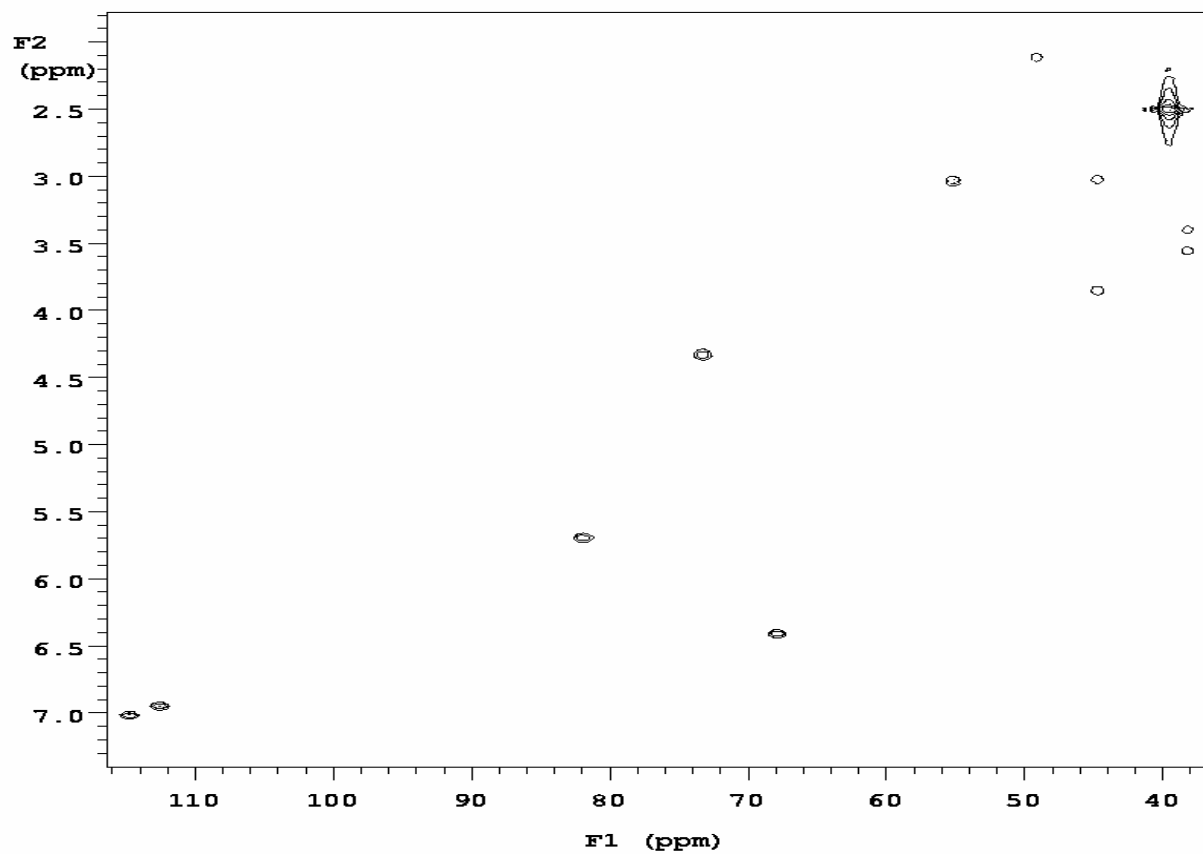
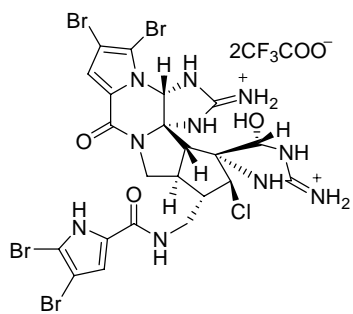


Figure S16. gHSQC Spectrum of Konbu'acidin B (3) in DMSO-*d*₆

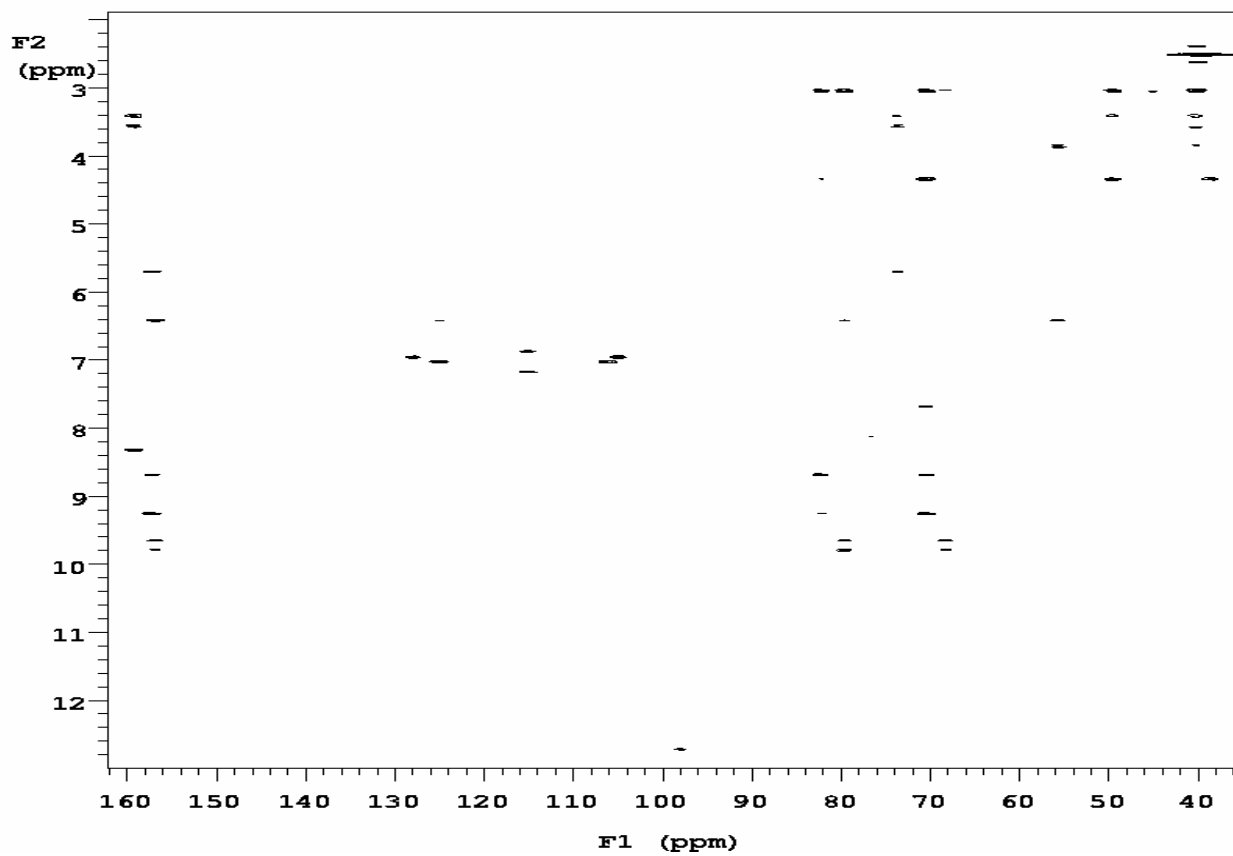
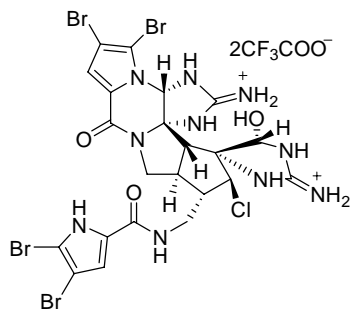


Figure S17. gHMBC Spectrum of Konbu'acidin B (3) in DMSO-*d*₆

