SUPPORTING INFORMATION

Cooperative involvement of glycosyltransferases in the transfer of aminosugars in the biosynthesis of the macrolactam sipanmycin by *Streptomyces sp.* CS149

Mónica G. Malmierca,[a,b,c] Ignacio Pérez-Victoria,[d,] , Jesús Martín,[d] Fernando Reyes,[d] Carmen Méndez,[a,b,c] Carlos Olano,[a,b,c] José A. Salas[a,b,c#]

[a] Departamento de Biología Funcional, Universidad de Oviedo, Oviedo (Asturias), Spain.

[b] Instituto Universitario de Oncología del Principado de Asturias (I.U.O.P.A), Universidad de Oviedo, Oviedo (Asturias), Spain.

[c] Instituto de Investigación Sanitaria del Principado de Asturias (IISPA), Oviedo, Spain

[d] Fundación MEDINA, Parque Tecnológico de Ciencias de la Salud, Granada, Spain

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Table S1. Secondary metabolite biosynthesis gene clusters (BGCs) identified in *Streptomyces* sp. CS149 genome sequence (accession number PVZY01000000) using antiSMASH 4.0 analysis platform. Only BGCs with at least **40%** of genes showing similarity are mentioned.

Cluster	Location C6W96_	Type	Most similar known BGC (MIBiG Accession number)
1	00005-00130	Other	-
2	00180-00235	Butyrolactone	-
3	00410-00510	Terpene	-
4	00775-01080	NRPS	Griseobactin (**94%**) BGC0000368_c1
5	01385-01500	Terpene	Isorenieratene (**100%**) BGC0000664_c1
6	01610-01780	Type III PKS	-
7	03395-03570	Type I PKS-NRPS	-
8	04600-04680	Terpene	-
9	06705-06755	Ectoine	Ectoine (**100%**) BGC0000853_c1
10	11240-11310	Lantipeptide	-
11	11635-11685	Siderophore	Desferrioxamine B (**100%**) BGC0000941_c1
12	14475-14540	Butyrolactone-Furan	-
13	18530-18665	Oligosaccharide	-
14	19920-20040	Lassopeptide	SRO15-2005 (**100%**) BGC0000578_c1
15	23490-23695	Type I PKS-NRPS	-
16	23975-24065	Lantipeptide	AmfS (**100%**) BGC0000496_c1
17	25205-25485	NRPS	-
18	25790-25885	Terpene	-
19	27530-27580	Siderophore	-
20	27710-28050	Oligosaccharide-Type I PKS	Incednine (**41%**) BGC0000078_c1
21	28720-28940	Type I PKS-NRPS	Collismycin A (**81%**) BGC0000973_c1
22	29155-29215	Bacteriocin	-
23	29250-29520	NRPS	Holomycin (**92%**) BGC0000373_c1
24	31085-31315	NRPS-Other KS	-
25	31985-32105	Terpene	Hopene (**69%**) BGC0000663_c1
26	32370-32665	Bacteriocin-Type I PKS-NRPS	SGR PTM (**100%**) BGC0001043_c1
27	33040-33240	Type I PKS-NRPS	-
28	33375-33420	Melanin	-
29	33475-33590	Thiopeptide	-
30	33615-33820	Type III PKS	-
31	34195-34380	Type I PKS-NRPS	-

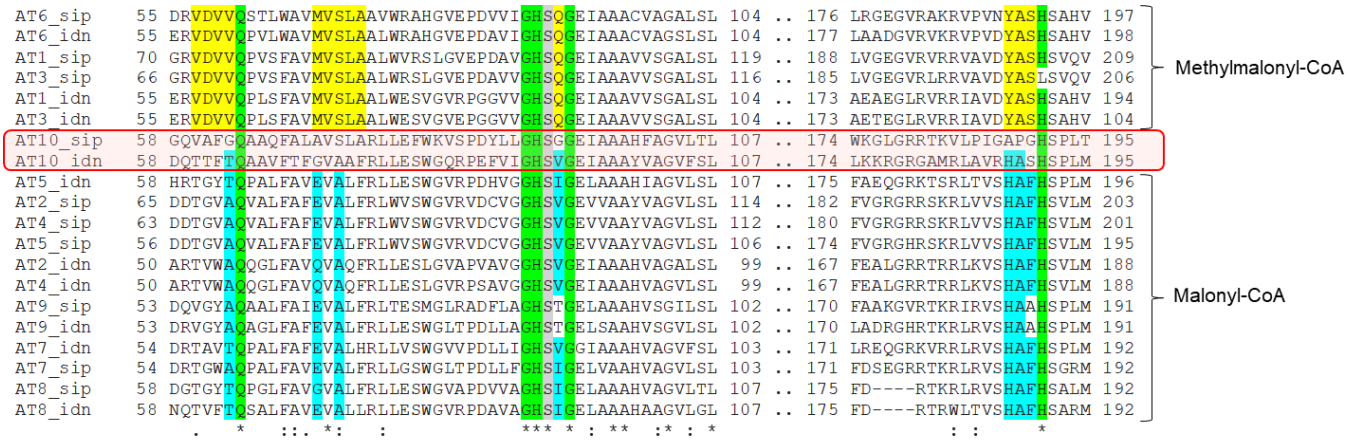


Fig. S1. Amino acid alignment of AT domains of PKS involved in sipanmycin and incednine biosynthesis. Conserved residues in AT domains are highlighted in gray (active site serine) and green. Consensus sequences for methylmalonyl-CoA and malonyl-CoA recognition are shown in yellow and blue, respectively. AT domains that introduce different units in sipanmycin and incednine polyketide chain are highlighted by a red box.

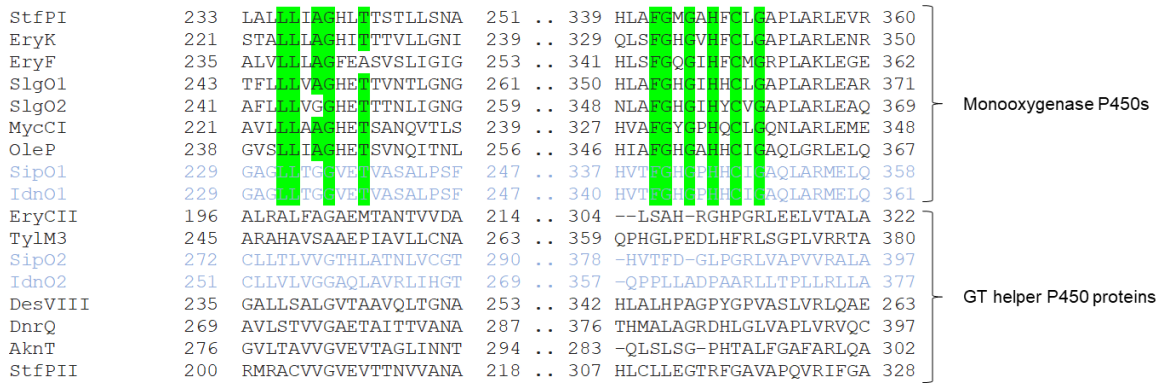


Fig. S2. Amino acid alignment of P450s involved in sipanmycin and incednine biosynthesis, together with several P450s functionally characterized in other actinomycetes. Conserved residues are highlighted in green. StfPI and StfII: Steffimycin biosynthesis (*Streptomyces steffisburgensis*, CAJ42333.1 and CAJ42339.1); EryK, EryF and EryCII: Erythromycin A biosynthesis (*Saccharopolyspora erythraea* NRRL 2338, P48635.3, AAA26496.1 and A4F7P2.1); SlgO1 and SlgO2: Streptolydigin biosynthesis (*Streptomyces lydicus*, CBA11578.1 and CBA11565.1); MycCI: Mycinamicin biosynthesis (*Micromonospora griseorubida*, Q83WF5.3); OleP: Oleandomycin biosynthesis (*Streptomyces antibioticus*, AAA92553.1); IdnO1 and IdnO2: Incednine biosynthesis (*Streptomyces* sp. ML694-90F3, BAP34719.1 and BAP34747.1); TylM3: tylosin biosynthesis (*Streptomyces fradiae*, P95746.2); DesVIII: methymycin/pikromycin biosynthesis (*Streptomyces venezuelae*, Q9ZGH8.1); DnrQ: daunorubicin biosynthesis (*Streptomyces peucetius*, Q54823.1); AknT: aclacinomycin A biosynthesis (*Streptomyces galilaeus*, Q9L4U5.1).

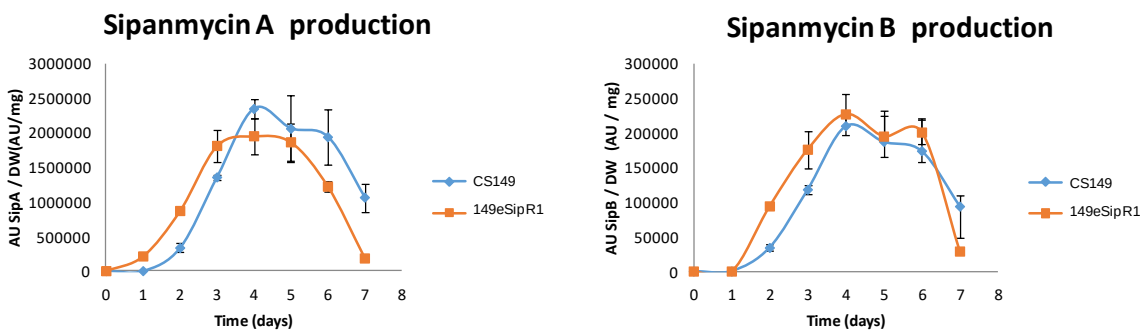


Fig. S3. Sipanmycin production in CS149 and 149eSipR1. Production is referred as absorbance units measured in UPLC per mg dry weight.

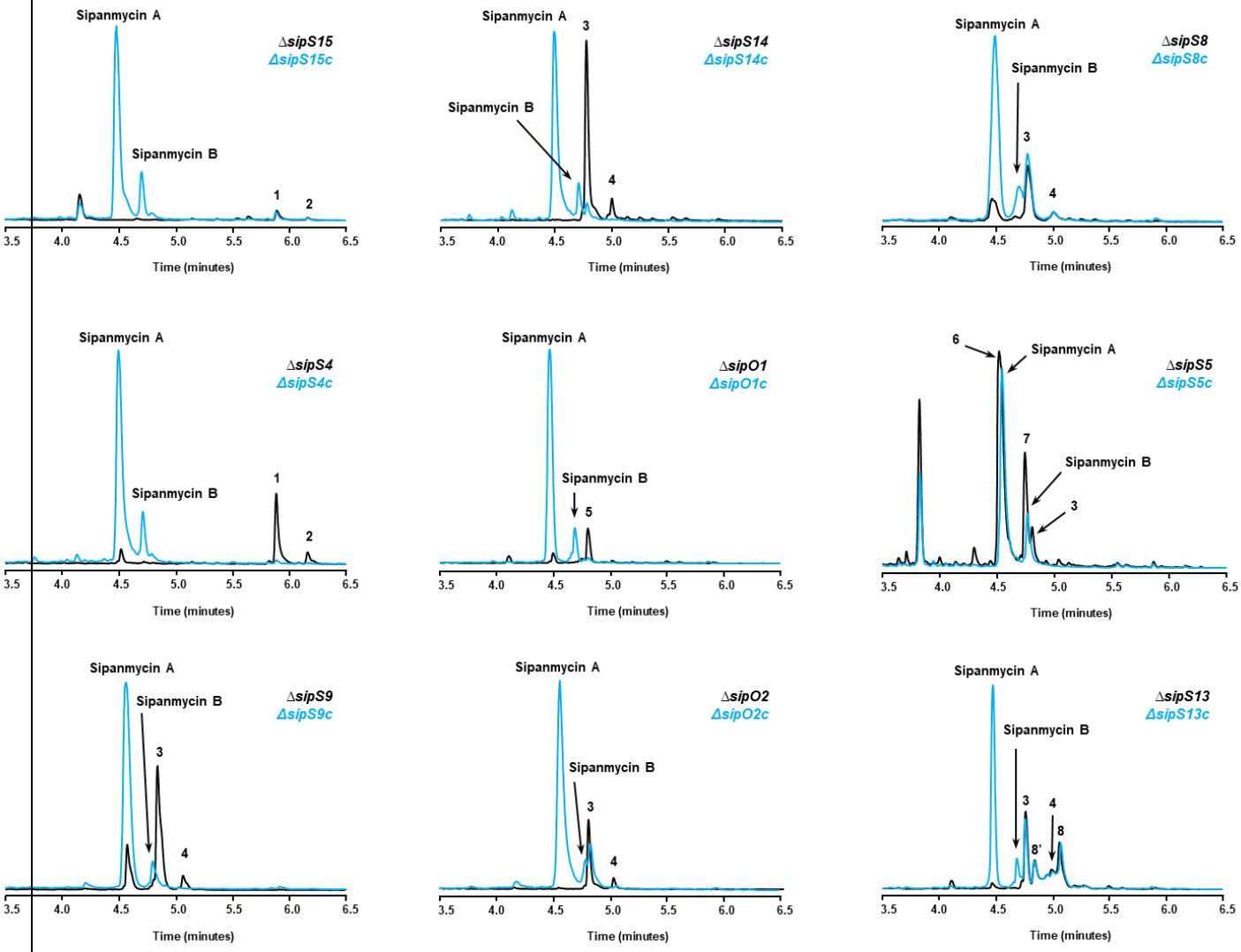
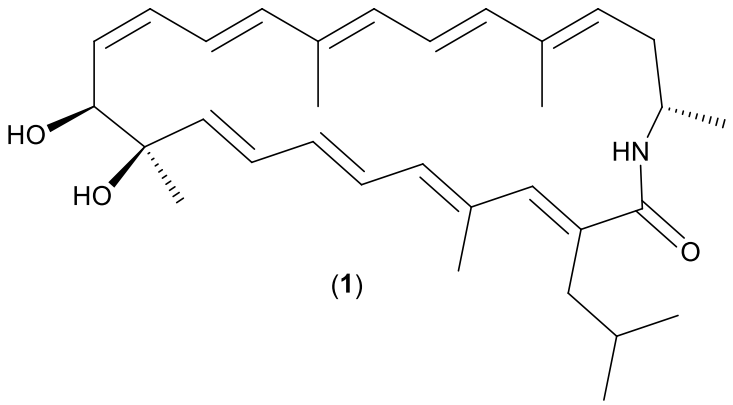


Fig. S4. UPLC analysis of genetic complementation experiments. Chromatograms at 320 nm comparing each mutant strain (black) obtained during this work with its complemented partner (blue).

Structure elucidation of sipanmycin A aglycon (1).

The molecular formula of **1** was established as $C_{32}H_{45}NO_3$ based on the observed ion $[M+H]^+$ at *m/z* 492.3474 (calcd. for $C_{32}H_{46}NO_3^+$ = 492.3472, Δm = 0.4 ppm). As expected, this molecular formula matches that of sipanmycin A aglycon. Likewise, its UV (DAD) spectrum is identical to that of sipanmycins A and B, showing a maximum at 320 nm and ashoulder at 360 nm, due to the presence of the polyene moieties. Comparison of the NMR data of **1** and sipanmycin A (1) immediately revealed that compound **1** corresponded to sipanmycin A aglycon. The absence of carbohydrate residues was evident due to the lack of any observable anomeric signals. On the other hand, most of the observed signals in **1** are very similar in resonance frequency to those of the macrolactam aglycon moiety of sipanmycin A. The main difference was observed for the ^{13}C chemical shift of C-11, 75.7 ppm compared to 83.3 ppm in sipamycin A,due to the lack of glycosylation at this position **1**. Analysis of the full set of 2D spectra including COSY, TOCSY, NOESY, HSQC and HMBC spectra further confirmed the connectivity of **1**. The absolute configuration of the chiral centers at positions C-10, C-11 and C-23 was assumed to be identical to that of sipanmycin A since the cyclic macrolactam polyketide skeleton is obviously biosynthesized via the same enzymatic machinery in both cases. As expected, the same key NOESY correlations are observed in the spectra of both, sipanmycin A and **1**.



(**1**)

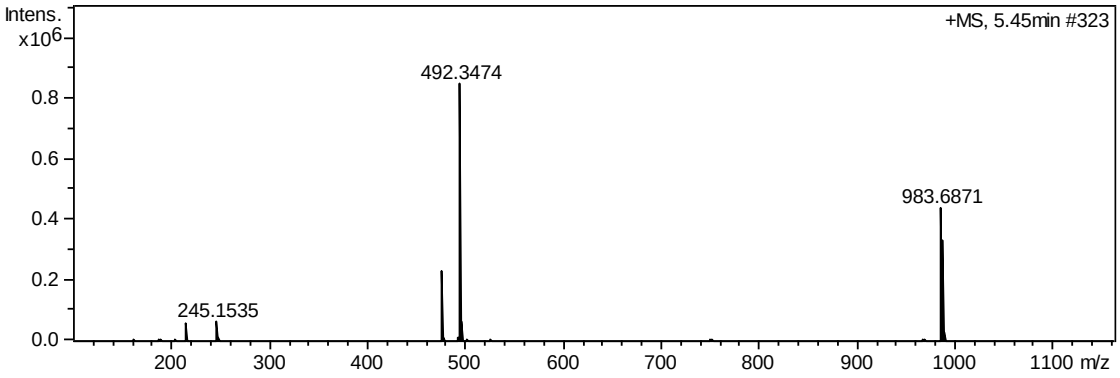


Fig. S5. HRMS spectrum of sipanmycin A aglycon (**1**).

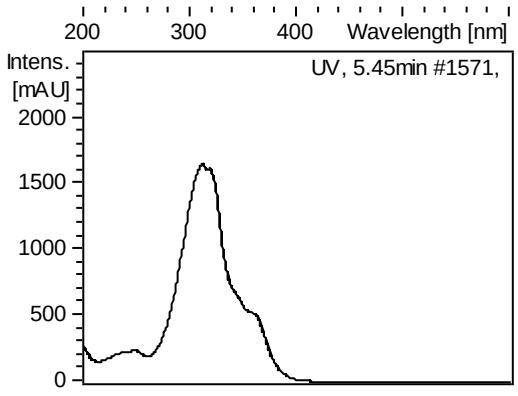
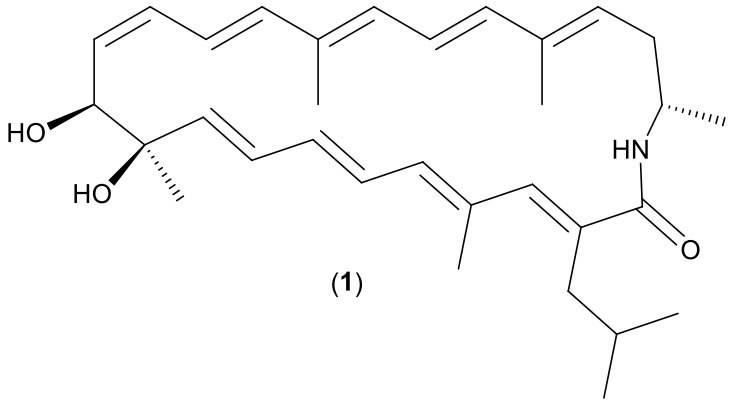


Fig. S6. UV-vis (DAD) spectrum of sipanmycin A aglycon (**1**).



(**1**)

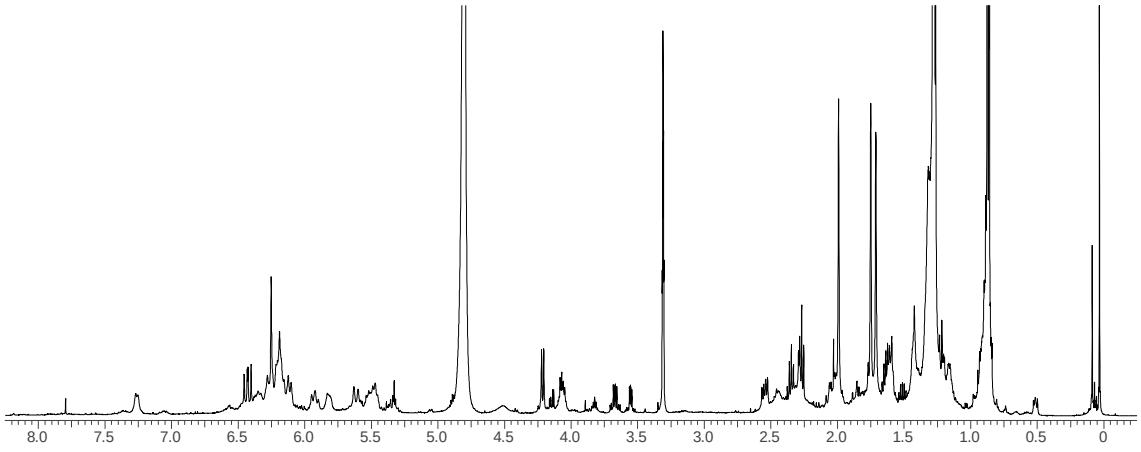


Fig. S7. ^{1}H NMR spectrum (CD_3OD, 500 MHz) of sipanmycin A aglycon (**1**).

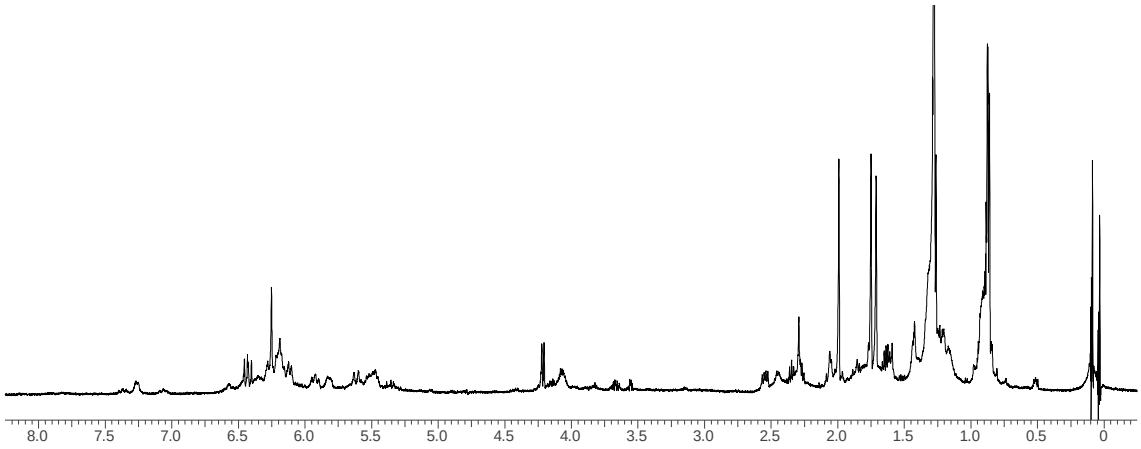
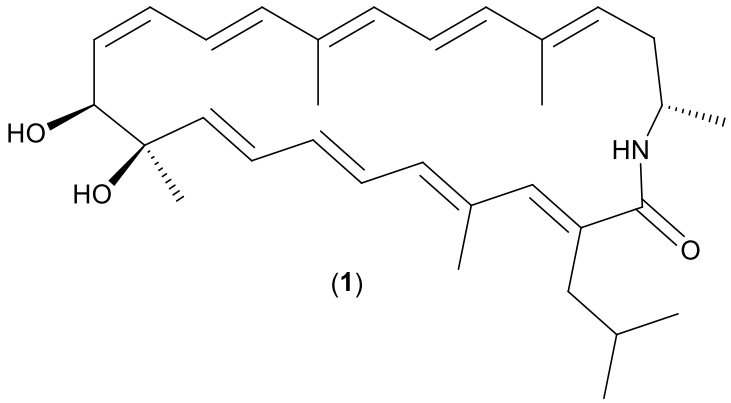


Fig. S8. Diffusion-filtered ^{1}H NMR spectrum of sipanmycin A aglycon (**1**).



(**1**)

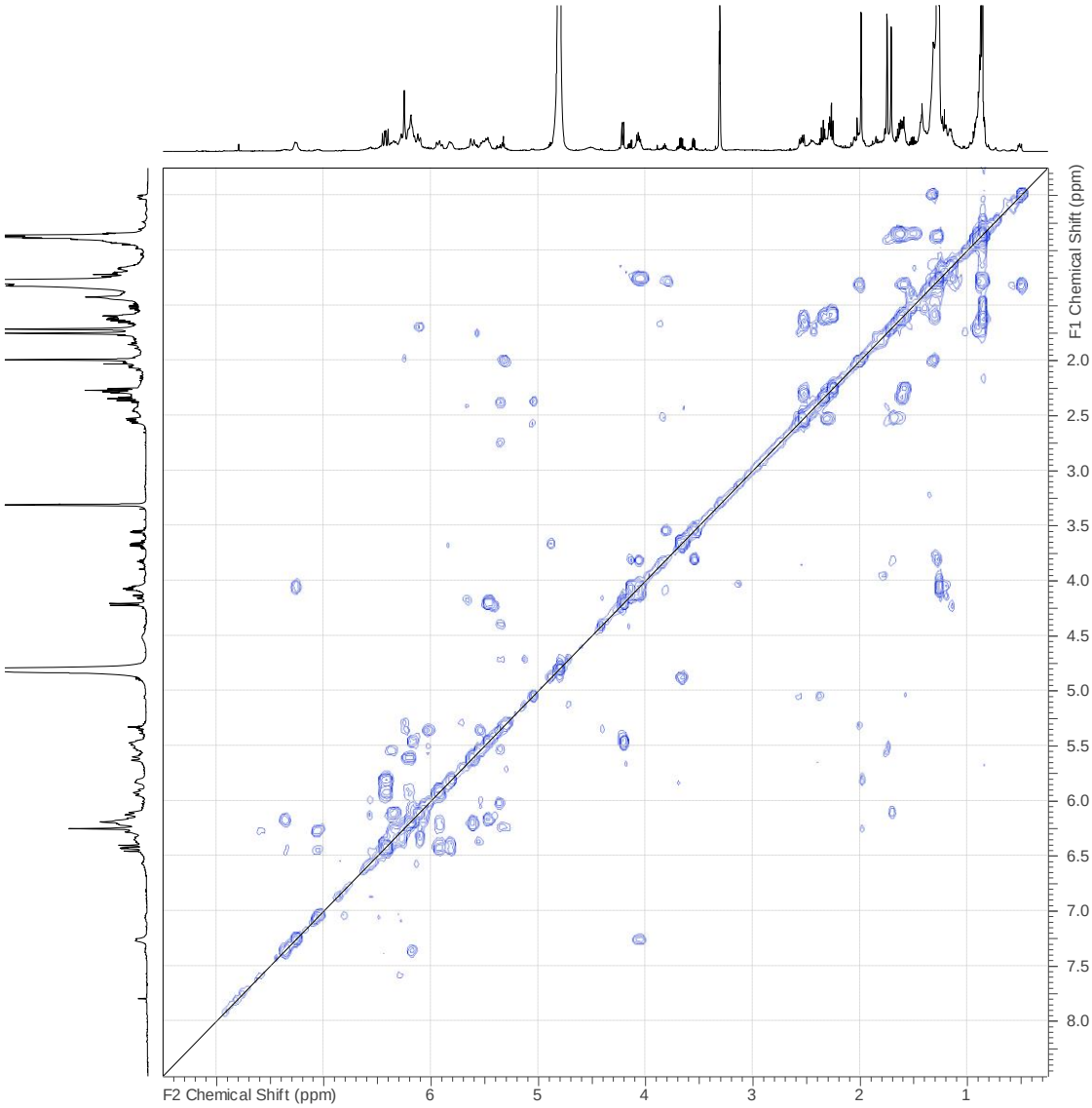


Fig. S9. COSY spectrum of sipanmycin A aglycon (**1**).

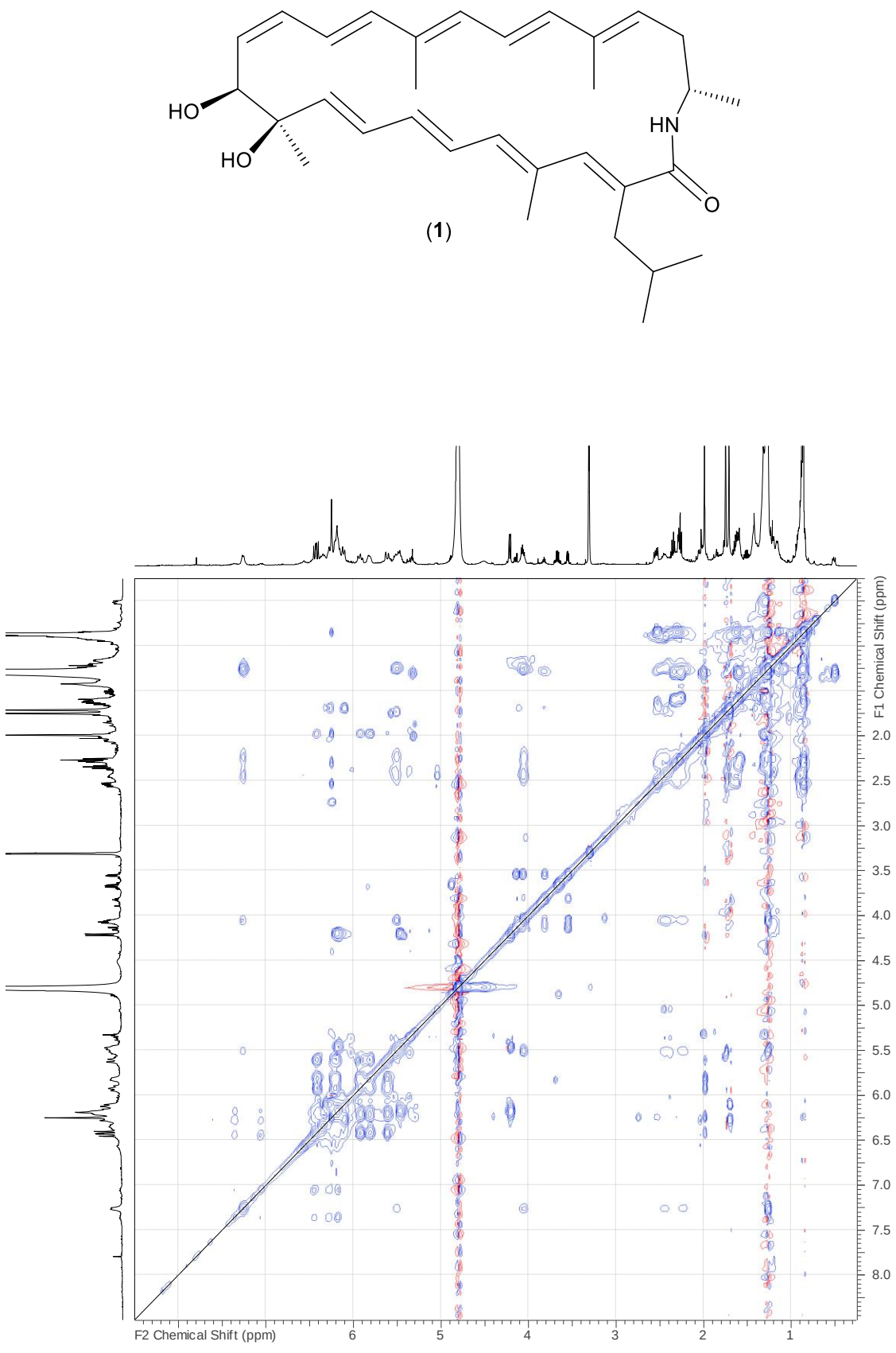
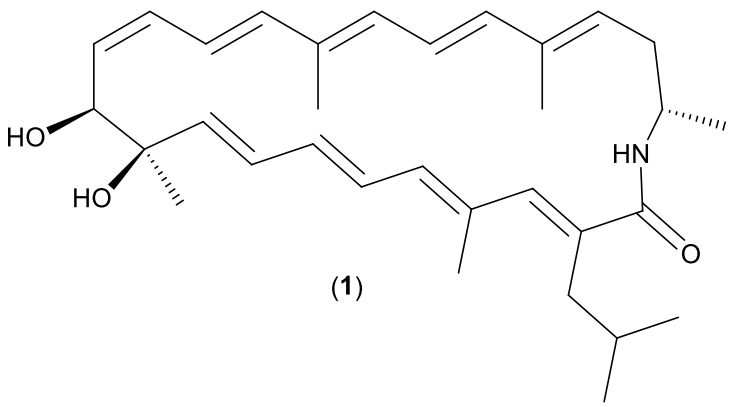


Fig. S10. TOCSY spectrum of sipanmycin A aglycon (**1**).



(**1**)

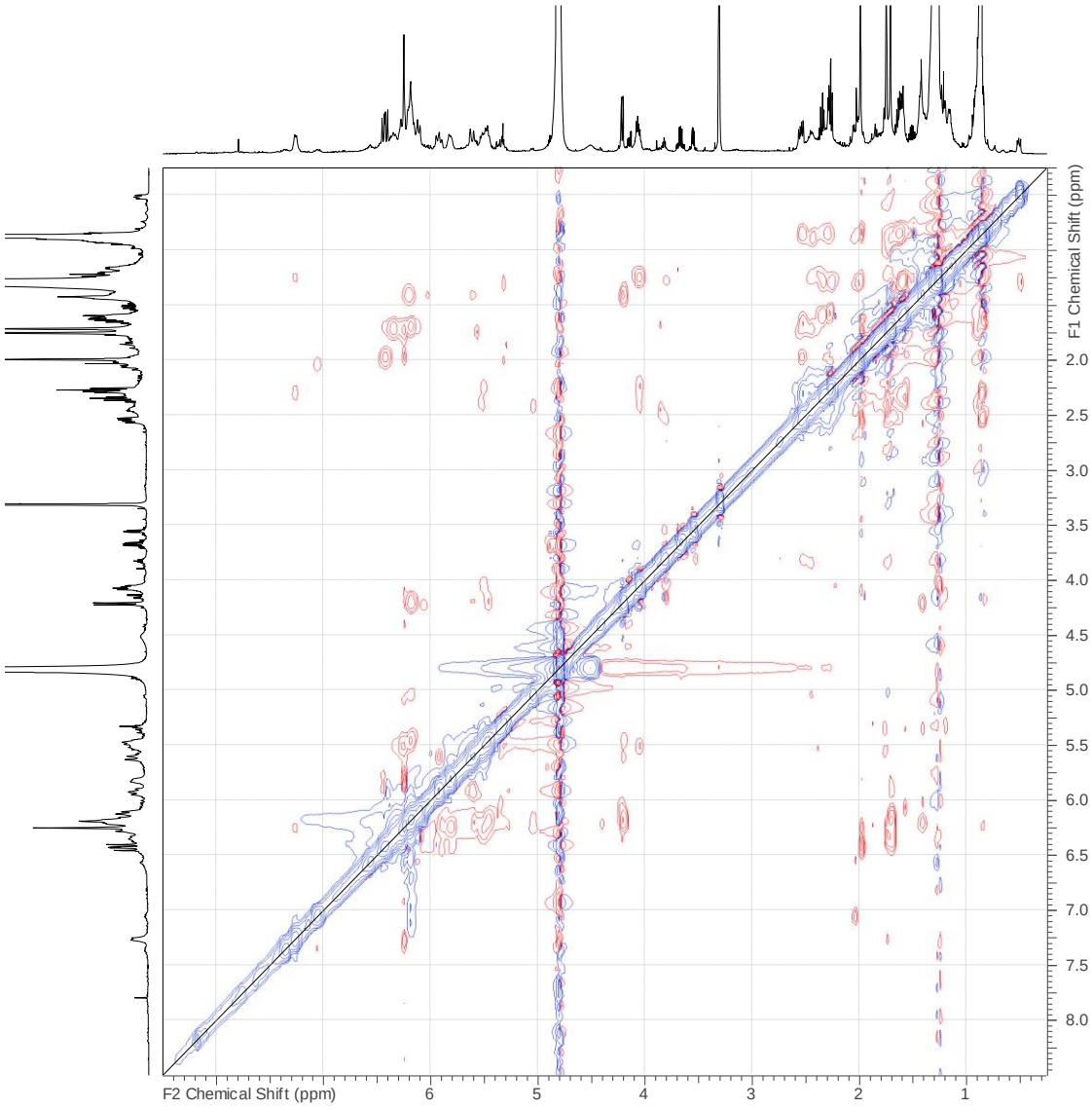


Fig. S101NOESY spectrum of sipanmycin A aglycon (**1**).

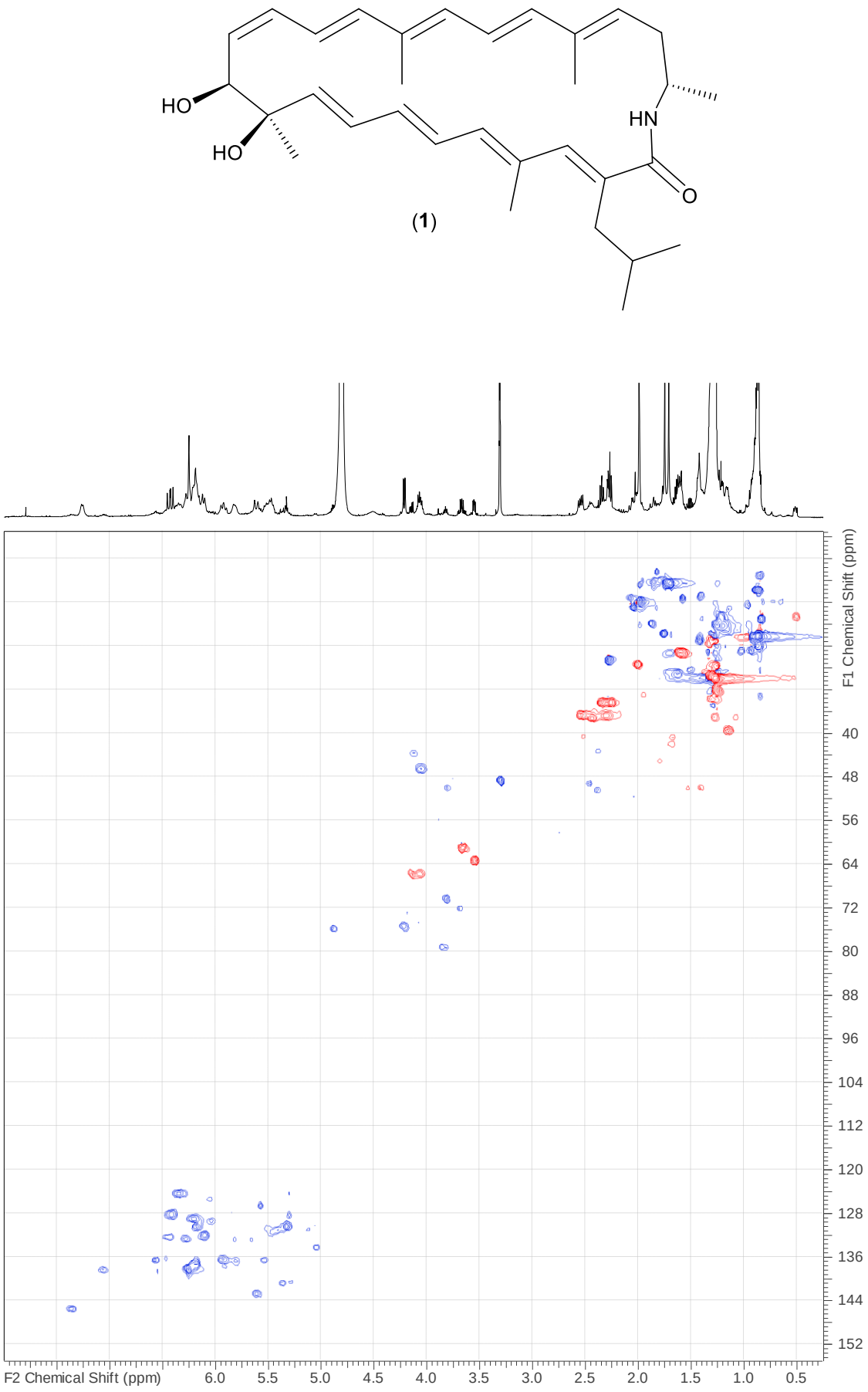


Fig. S12. Edited HSQC spectrum of sipanmycin A aglycon (**1**).

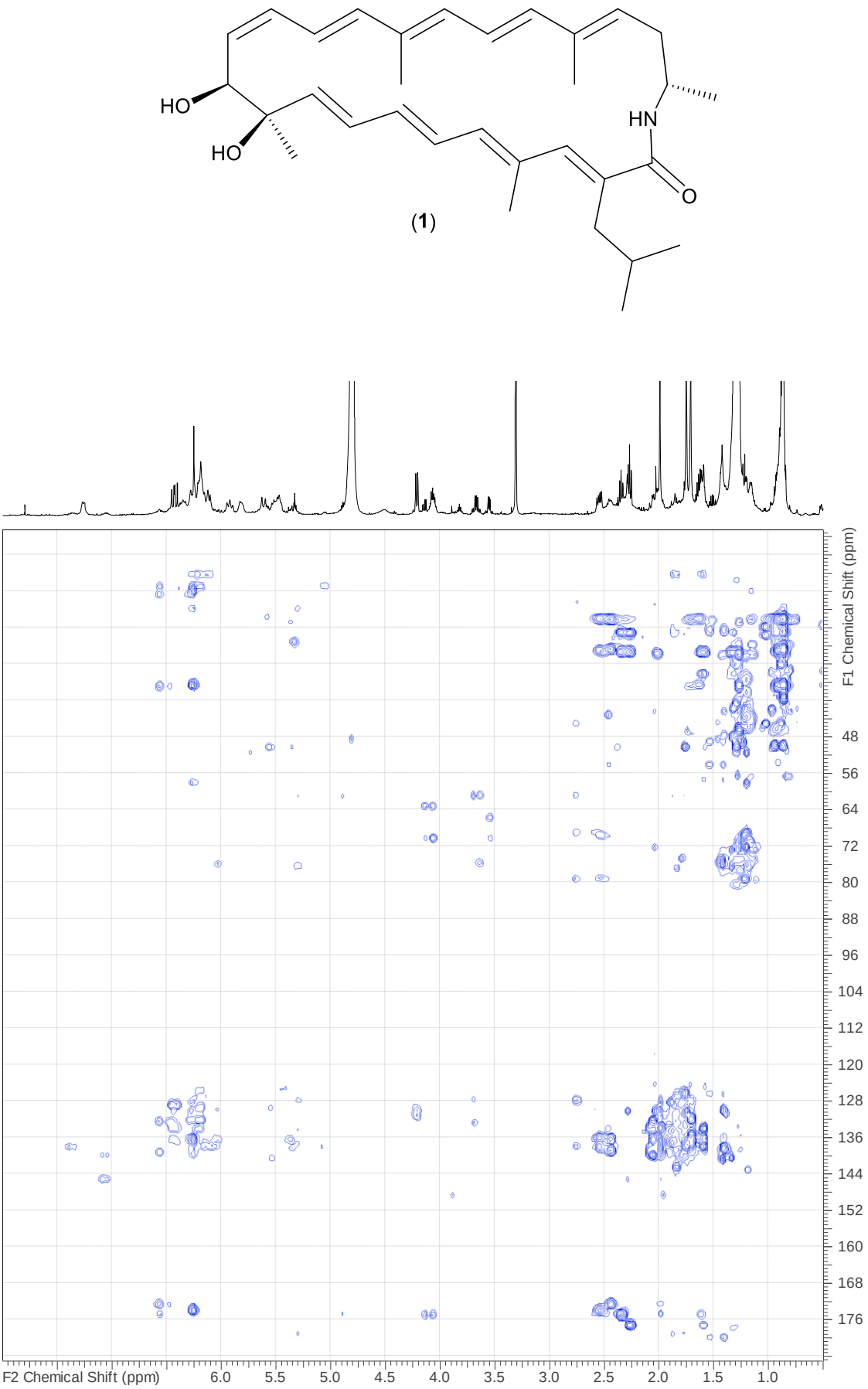


Fig. S13. HMBC spectrum of sipanmycin A aglycon (**1**).

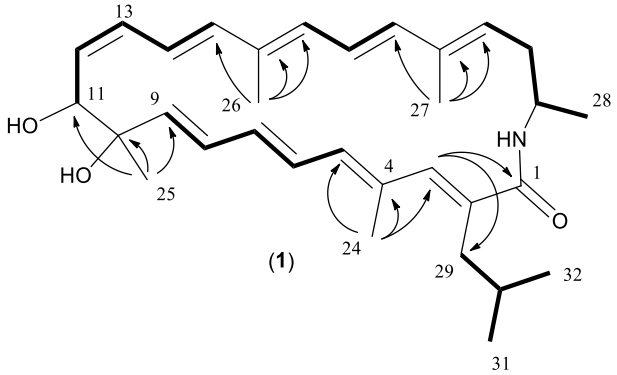


Fig. S14. Connectivity of sipanmycin A aglycon (**1**) confirmed by 2D-NMR. COSY correlations (further corroborated by the spin systems observed in the TOCSY spectrum) are indicated as bold bonds. Key HMBC correlations connecting independent spin systems are indicated by arrows.

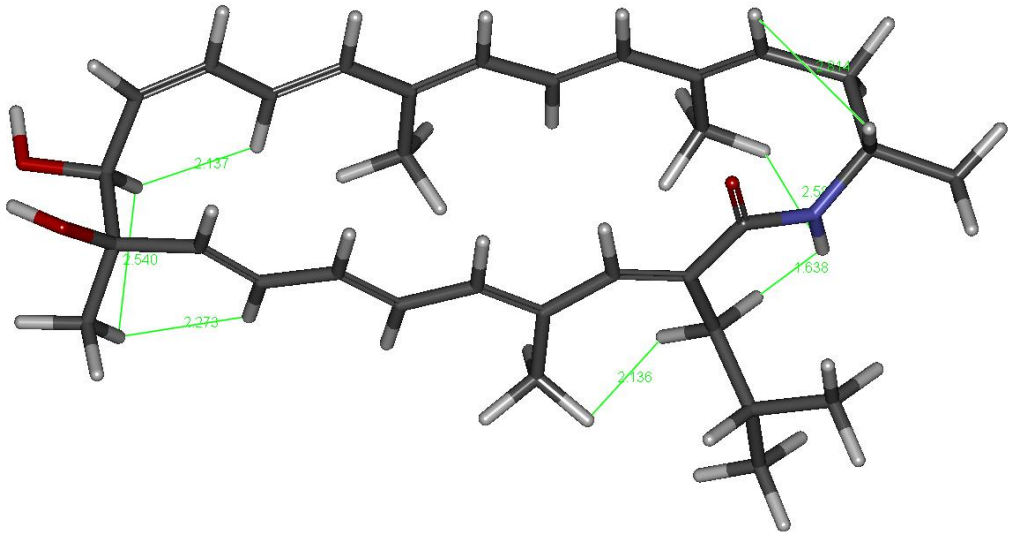


Fig. S15. Molecular model of sipanmycin A aglycon (**1**) showing the same key NOEs (highlighted in green) observed for the macrocyclic moiety in sipanmycin A which confirm the identical absolute configuration of both compounds.

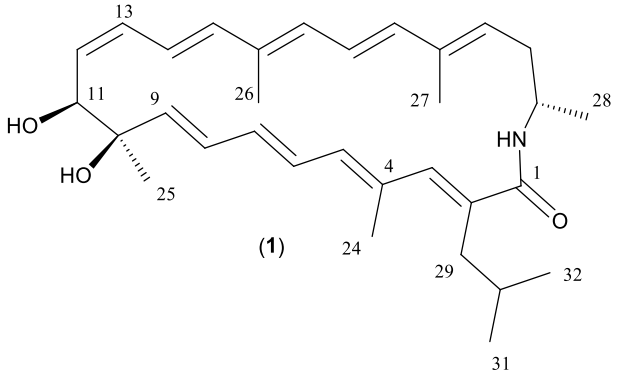


Fig. S16. Structure of sipanmycin A aglycon (**1**).

Table S2. ^{1}H and ^{13}C NMR data for sipanmycin A aglycon (**1**) in CD_3OD at 24 C°.

Position	δ_C, type	δ_H (*J* in Hz)
1	174.0, C	-
2	136.2, C	-
3	138.3, CH	6.26, s
4	133.9, C	-
5	136.8, CH	5.82, m
6	128.3, CH	6.43, dd (14.8, 11.4)
7	136.5, CH	5.93, br t (13.4)
8	129.0, CH	6.21, m
9	142.8, CH	5.62, br d (15.6)
10	76.0, C	-
11	75.7, CH	4.21, d (8.2)
12	131.5, CH	5.47, m
13	130.5, CH	6.18, m
14	125.6, CH	6.08, m
15	137.3, CH	6.20, m
16	135.8, C	-
17	132.0, CH	6.12, br d (11.1)
18	124.5, CH	6.35, m
19	138.1, CH	6.27, m
20	137.4, C	-
21	130.7, CH	5.51, m
22	37.4, CH_2	2.45, m
23	46.8, CH	4.06, m
24	16.2, CH_3	1.99, br s
25	23.2, CH_3	1.43, br s
26	12.8, CH_3	1.71, br s
27	12.8, CH_3	1.75, br s
28	20.4, CH_3	1.27, d (6.9)
29	37.0, CH_2	2.55, dd (13.8, 7.0) 2.32, m
30	29.4, CH	1.64, m
31	22.4, CH_3	0.87, d (6.4)
32	22.4, CH_3	0.87, d (6.4)
1-NH	-	7.26 br d (7.5)*

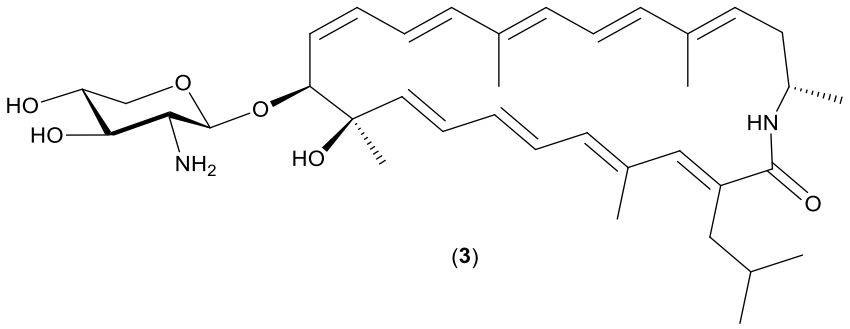
^{13}C chemical shifts obtained from HSQC and HMBC spectra.

* The amide proton exchanges very slowly and is clearly observed.

Structure elucidation of 3'-*O*-demethylsilvalactam (3).

The molecular formula of **3** was established as $C_{37}H_{54}N_2O_6$ based on the observed ion $[M+H]^+$ at *m/z* 623.4052 (calcd. for $C_{37}H_{55}N_2O_6^+$ = 623.4055, Δm = 0.5 ppm). As expected, this molecular formula matches that of sipanmycin A aglycon with just the first aminosugar of sipanmycins (D-xylosamine) attached. Likewise, its UV (DAD) spectrum is identical to that of sipanmycin A and **1**, showing maxima at 320 and 360 nm (sh), due to the presence of the polyene moieties. Due to its low solubility in deuterated methanol, the NMR sample concentration just allowed to acquire ^{1}H, COSY, TOCSY and HSQC spectra. Comparison of the NMR data of **3** and sipanmycin A (1) clearly indicated that both compounds shared an identical macrolactam aglycon. The observed signals for the aglycon moiety of **3** resonated very close in frequency to those of the macrolactam aglycon of sipanmycin A. This feature alongside obvious biosynthetic reasons also imply the same absolute configuration for the chiral centers in the macrolactam ring of **3** and sipanmycin A. The presence of just one carbohydrate residue was evident since only one anomeric signal was observed. The spin system of such monosaccharide residue, determined by analysis of the COSY and TOCSY spectra, was identical to that of the first aminosugar residue in sipanmycin A and the corresponding resonance frequencies were likewise very similar, the main difference being observed on the ^{13}C chemical shift of C-4, 71.0 ppm (compared to 78.3 ppm in sipamycin A),because this position is not glycosylated in **3**. The expected relative configuration of this monosaccharide was confirmed by the TOCSY spectrum using the methodology proposed by Martins and co-workers (2). Briefly, in the TOCSY spectrum of **3**, acquired with a mixing time of 90 ms, it can be clearly seen that starting magnetization in the anomeric proton (position 1') is transferred stepwise reaching methylene protons at 5' without any difficulty since all the involved couplings in this pathway are large because the corresponding protons are in a

trans-diaxial configurational relationship with respect to each other (as in the case of the glucopyranose configuration) thus confirming the pentopyranose unit corresponds, as expected, to 2-deoxy-2-amino-*β*-xylopyranose (the *β*-pyranose form of the monosaccharide xylosamine). Once again, for obvious biosynthetic reasons, the absolute configuration of the aminosugar in **3** must be identical to that of the same sugar in sipanmycin A, which corresponds to D-xylosamine. Compound **3** was thus elucidated as 3'-*O*-demethylsilvalactam, a molecule identical to silvalactam (3) just lacking methylation at the 3'-OH of the aminosugar.



(**3**)

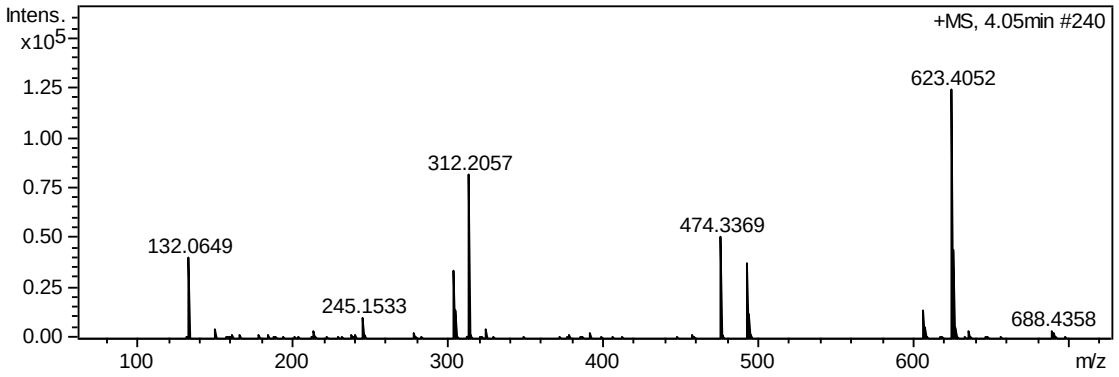


Fig. S17. HRMS spectrum of 3'-*O*-demethylsilvalactam (**3**).

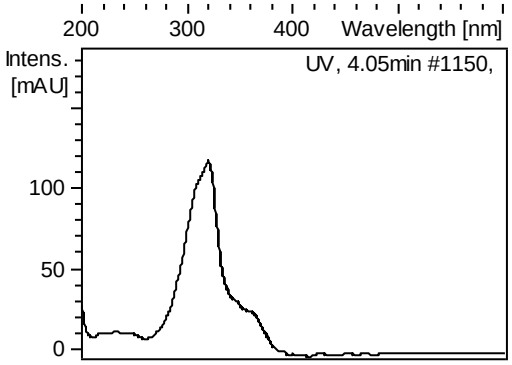
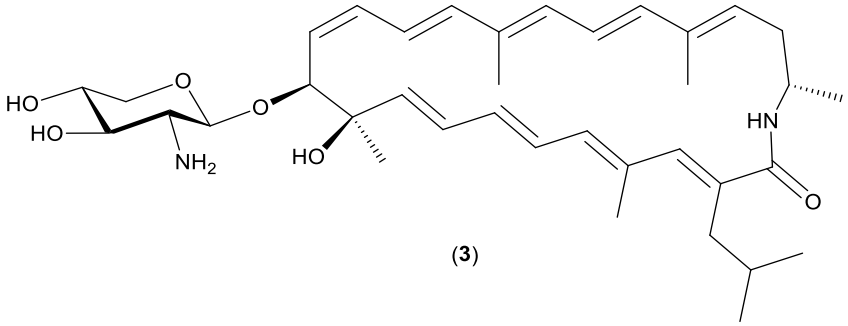


Fig. S18. UV-vis (DAD) spectrum of 3'-*O*-demethylsilvalactam (**3**).



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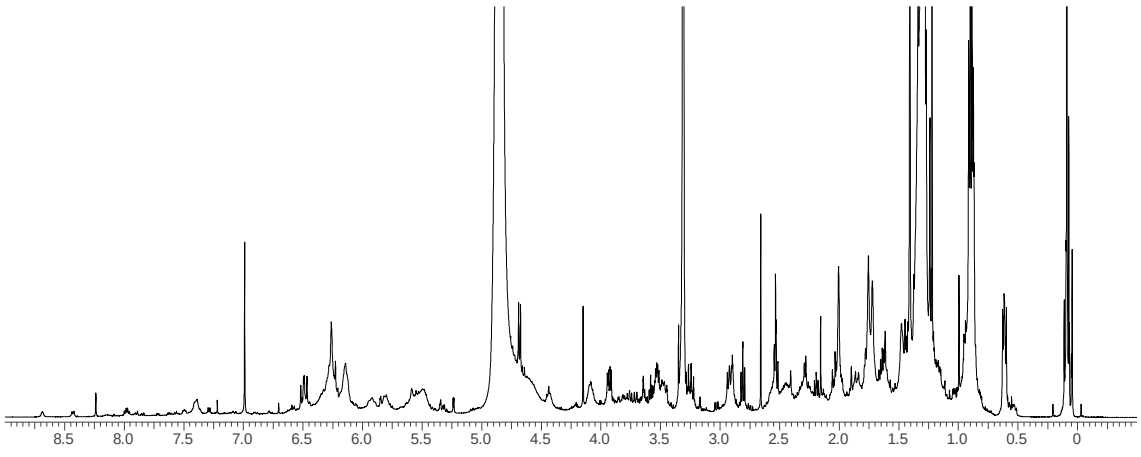
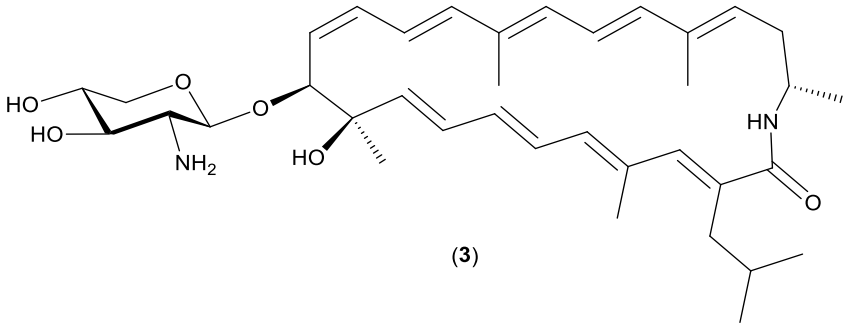


Fig. S19. ^{1}H NMR spectrum (CD_3OD, 500 MHz) of 3'-*O*-demethylsilvalactam (**3**).



(**3**)

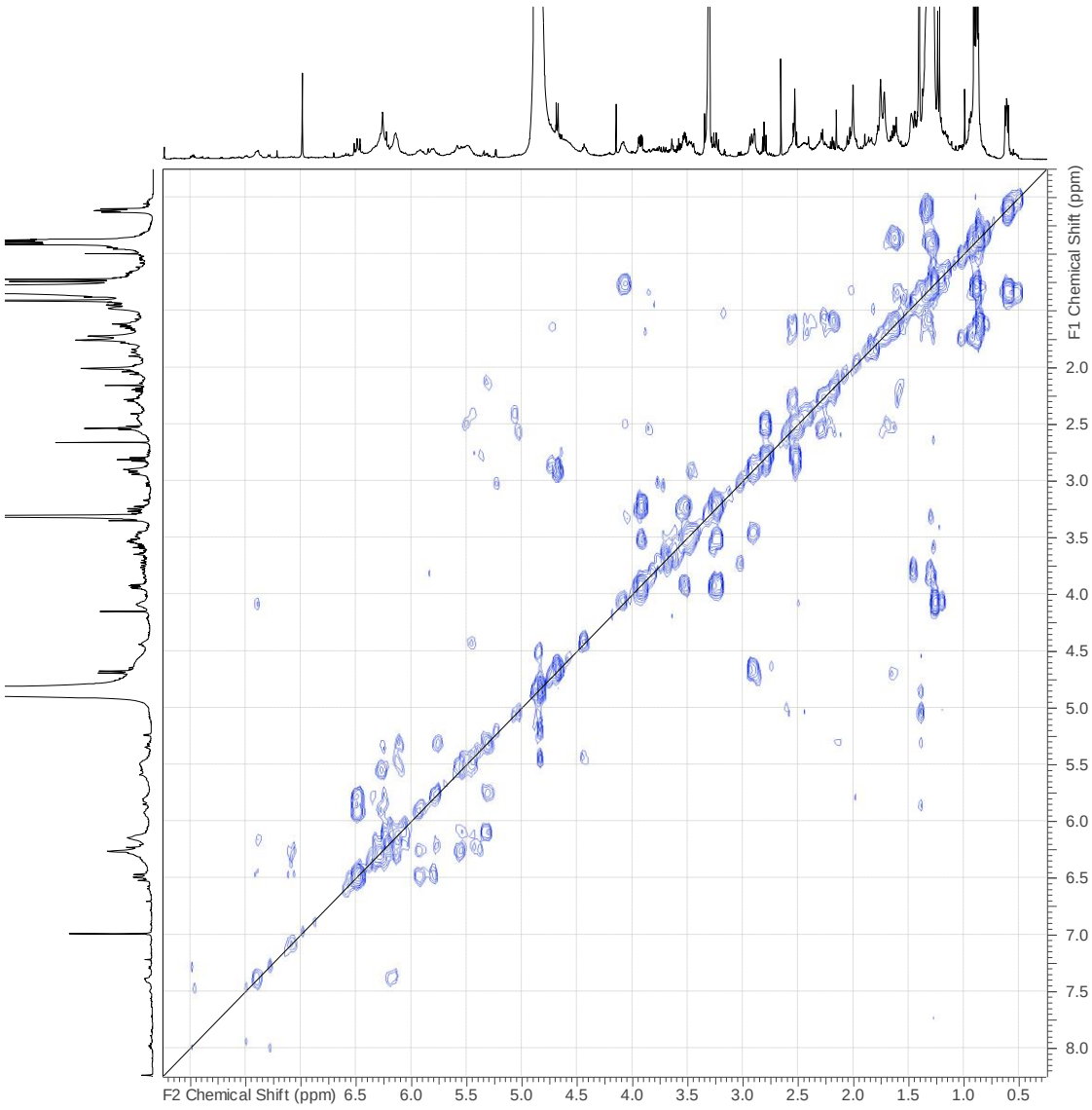
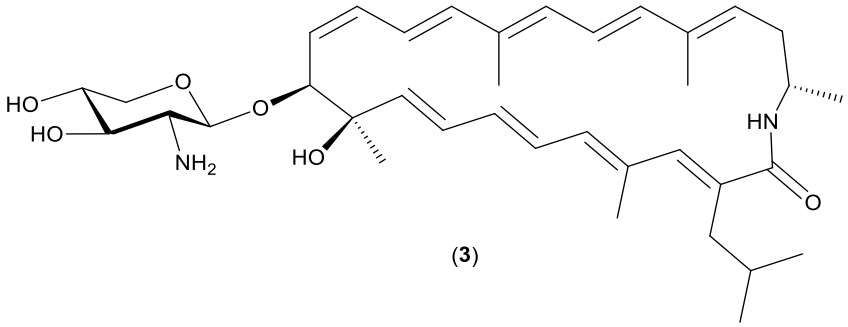


Fig. S20. COSY spectrum of 3'-*O*-demethylsilvalactam (**3**).



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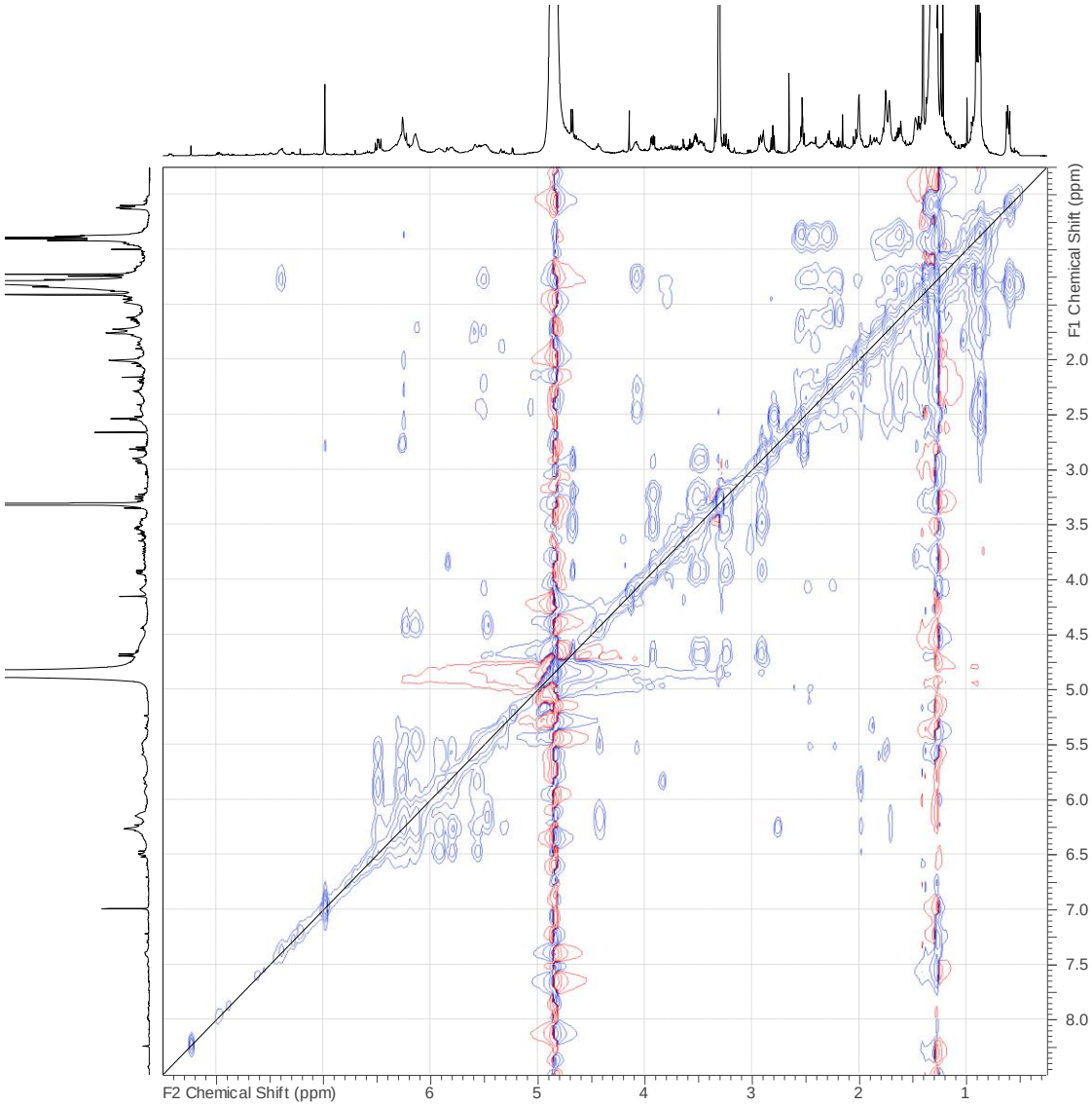
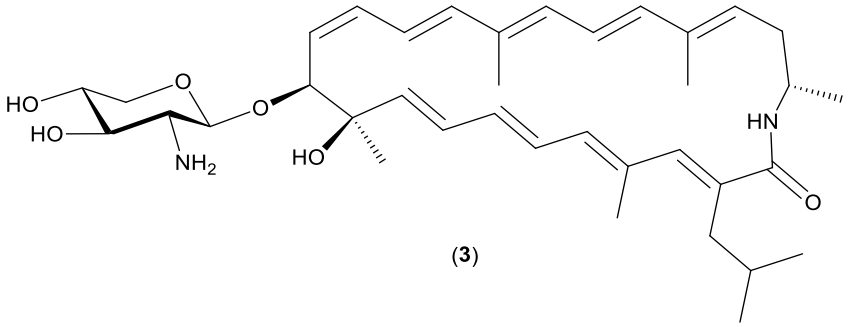


Fig. S21. TOCSY spectrum of of 3'-*O*-demethylsilvalactam (**3**).



(**3**)

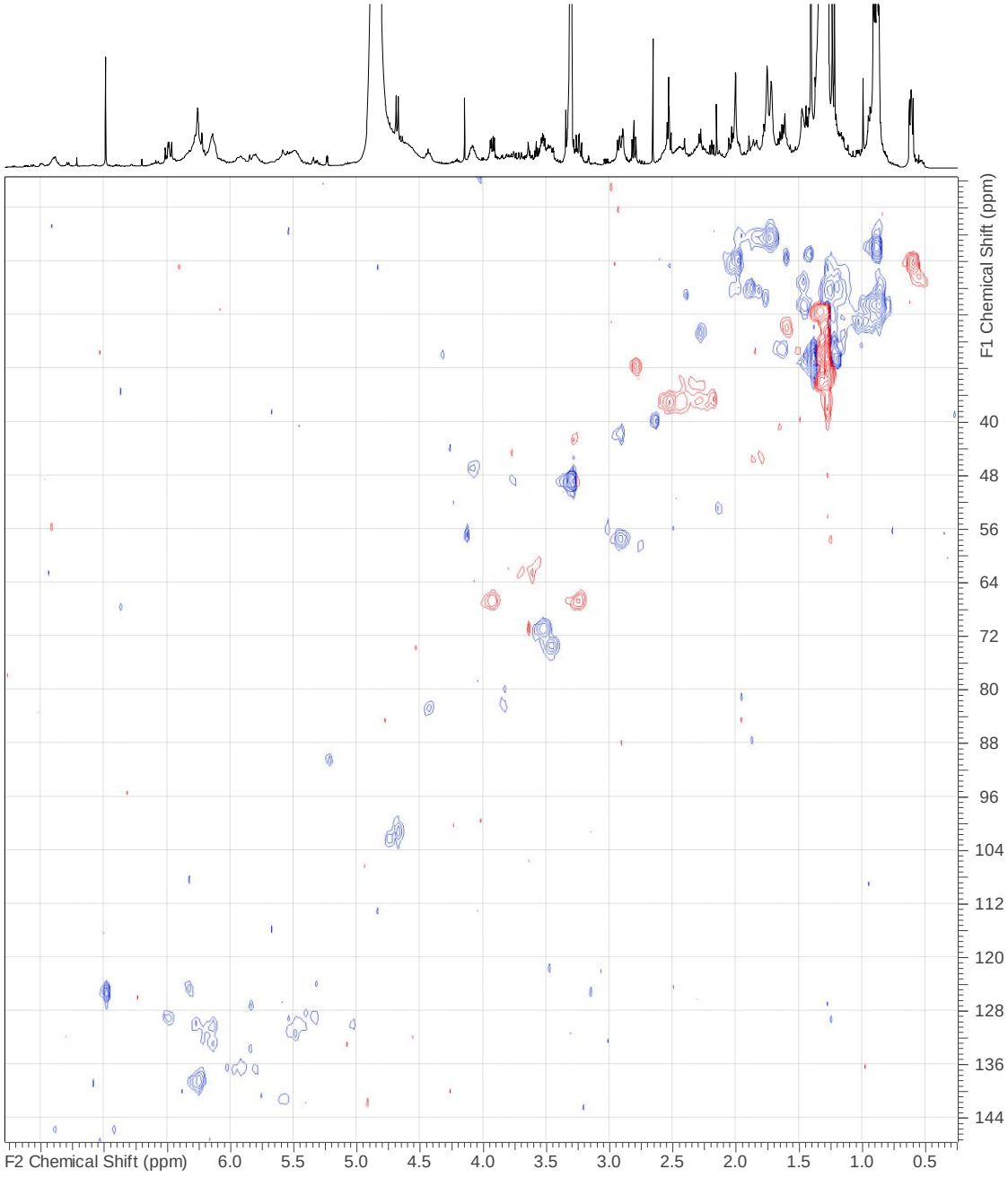
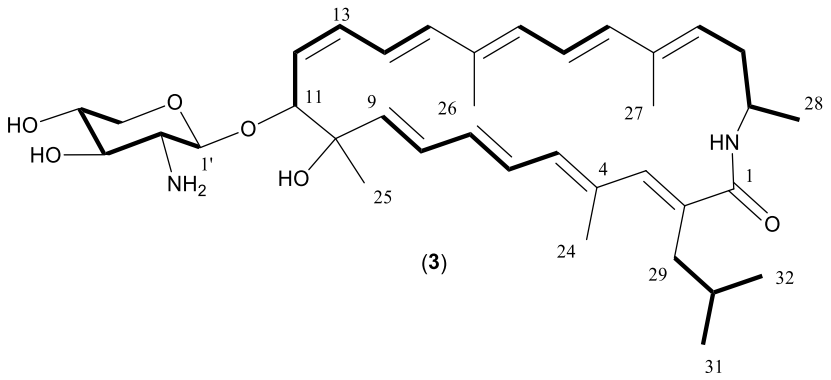
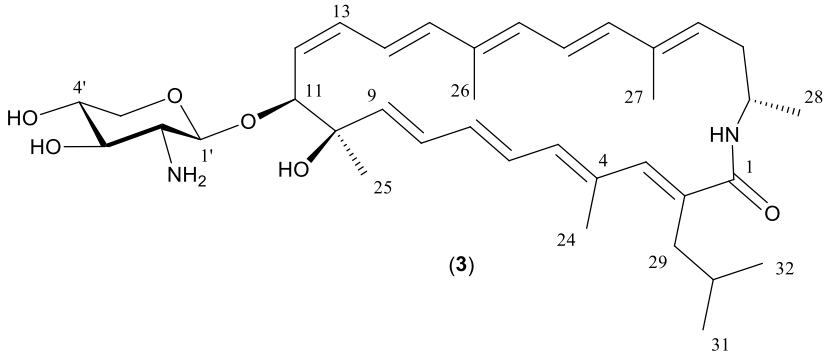


Fig. S22. Edited HSQC spectrum of 3'-*O*-demethylsilvalactam (**3**).



(**3**)

Fig. S23. Gross structure of 3'-*O*-demethylsilvalactam (**3**) determined by 2D-NMR (COSY, TOCSY and HSQC) and comparison with the data of sipanmycin A. COSY correlations (further corroborated by the spin systems observed in the TOCSY spectrum) are indicated as bold bonds.



(**3**)

Fig. S24. Structure of 3'-*O*-demethylsilvalactam (**3**)

Table S3. ^{1}H and ^{13}C NMR data for 3'-*O*-demethylsilvalactam (**3**) in CD_3OD at 24 C°.

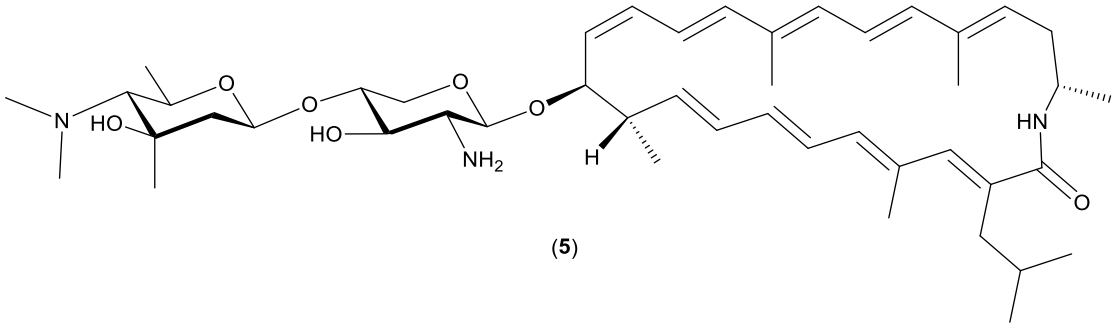
Position	δ_C, type	δ_H (*J* in Hz)	Position	δ_C, type	δ_H (*J* in Hz)
1	n.d., C	-	1'	101.3, CH	4.68, d (7.8)
2	n.d., C	-	2'	57.7, CH	2.92, dd (9.4, 7.9)
3	138.6, CH	6.26, m	3'	73.5, CH	3.47, m
4	n. d., C	-	4'	71.0,CH	3.54, m
5	136.6, CH	5.80, m	5'	66.7, CH_2	3.93, dd (11.6, 5.1) 3.26, dd (11.5, 9.7)
6	129.2, CH	6.49, dd (14.7, 11.4)			
7	136.2, CH	5.91, m			
8	130.1, CH	6.27, m			
9	141.2, CH	5.57, m			
10	n. d., C	-			
11	83.0, CH	4.43, m			
12	129.9, CH	5.47, m			
13	130.3, CH	6.14, m			
14	125.9, CH	6.14, m			
15	138.1, CH	6.25, m			
16	n. d. , C	-			
17	132.8, CH	6.14, m			
18	124.5, CH	6.34, m			
19	138.6, CH	6.27, m			
20	n.d., C	-			
21	131.3, CH	5.51, m			
22	37.4, CH_2	2.50, m 2.26, m			
23	47.2, CH	4.07, m			
24	16.2, CH_3	2.00, br s			
25	22.9, CH_3	1.49, br s			
26	12.8, CH_3	1.72, br s			
27	12.8, CH_3	1.76, br s			
28	20.6, CH_3	1.28, m			
29	37.2, CH_2	2.56, m 2.31, m			
30	29.2, CH	1.65, m			
31	22.5, CH_3	0.88, d (6.4)			
32	22.5, CH_3	0.88, d (6.4)			
1-NH	-	7.39 br s*			

^{13}C chemical shifts obtained from HSQC spectrum. Chemical shifts of quaternary carbons were not determined.

* The amide proton exchanges very slowly and is clearly observed in the spectra.

Structure elucidation of 10-deoxysipanmycin A (5).

The molecular formula of **5** was established as $C_{46}H_{71}N_3O_7$ based on the observed ion $[M+H]^+$ at *m/z* 778.5366 (calcd. for $C_{46}H_{72}N_3O_7^+$ = 778.5365, Δm = 0.12 ppm). As expected, this molecular formula matches that of sipanmycin A lacking an oxygen atom. Likewise, its UV (DAD) spectrum is identical to that of sipanmycin A, **1** and **3**. Comparison of the NMR data of **5** and sipanmycin A (1) immediately revealed that compound **5** was identical to sipanmycin A just lacking the hydroxyl group at C-10. The carbohydrate signals of **5** are very similar in resonance frequency to those observed for sipanmycin A indicating as expected that the glycosylating disaccharide moiety is identical for both molecules. Likewise, most of the observed signals for the macrocycle in **5** are very similar in resonance frequency to those of the macrolactam moiety of sipanmycin A. A very remarkable difference is observed however at C-10 which resonates at 41.5 ppm (compared to 76.5 ppm in sipanmycin A) because this position now lacks the hydroxyl substituent and a new proton corresponding to this methine appears in the ^{1}H spectrum at 2.46 ppm. Not surprisingly some differences are also observed for the chemical shifts at positions surrounding C-10. Analysis of the full set of 2D spectra, including COSY, TOCSY, NOESY, HSQC and HMBC,further confirmed the connectivity of **5**. The absolute configuration of the chiral centers at the carbohydrate residues and positions C-11 and C-23 must be identical to that of sipanmycin A for obvious biosynthetic reasons. Interestingly, the absolute configuration at C-10 was also determined to be equivalent (methyl C-25 pointing towards the same direction) to that of sipanmycin A. Molecular modelling shows the almost *trans* periplanar relationship of H-11 with both H-10 and H-12 explaining its appearance as a triplet (double doublet with two equal coupling constants $J_{11\text{-}10} = J_{11\text{-}12} = 8.9$ Hz), unambiguously determining the configuration at C-10. Compound **5** was thus elucidated as 10-deoxysipanmycin A.



(**5**)

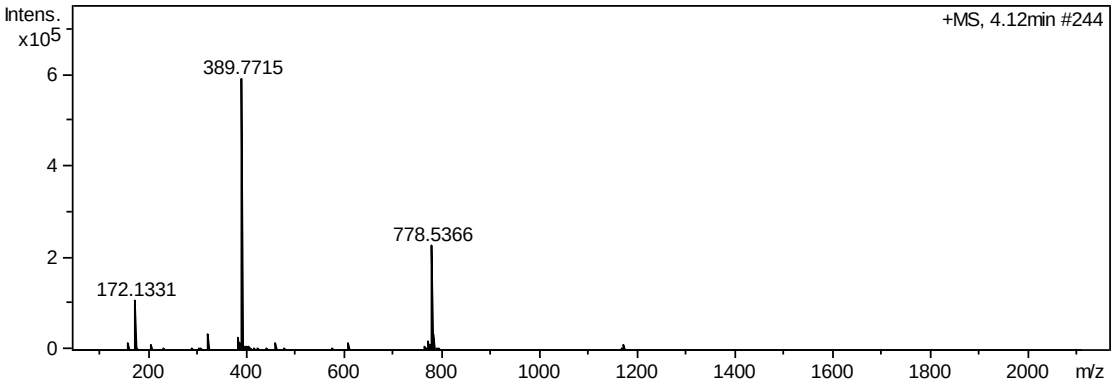


Fig. S25. HRMS spectrum of 10-deoxysipanmycin A (**5**).

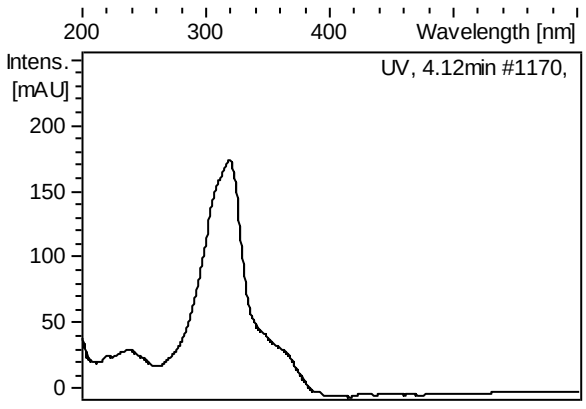
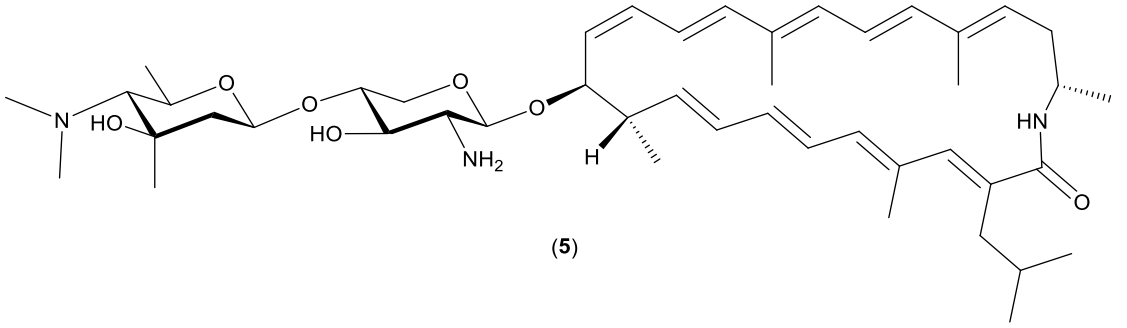


Fig. S26. UV-vis (DAD) spectrum of 10-deoxysipanmycin A (**5**).



(**5**)

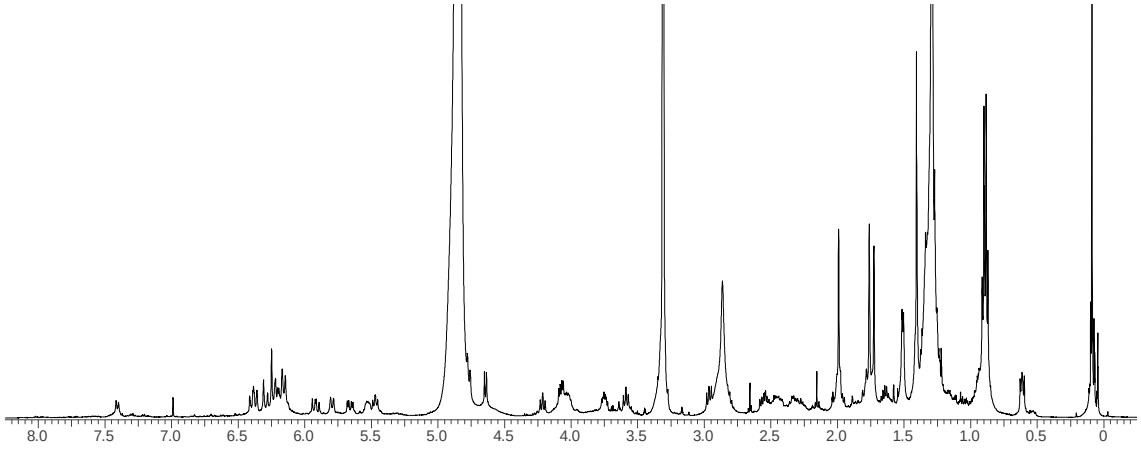


Fig. S27. ^{1}H NMR spectrum (CD_3OD, 500 MHz) of 10-deoxysipanmycin A (**5**).

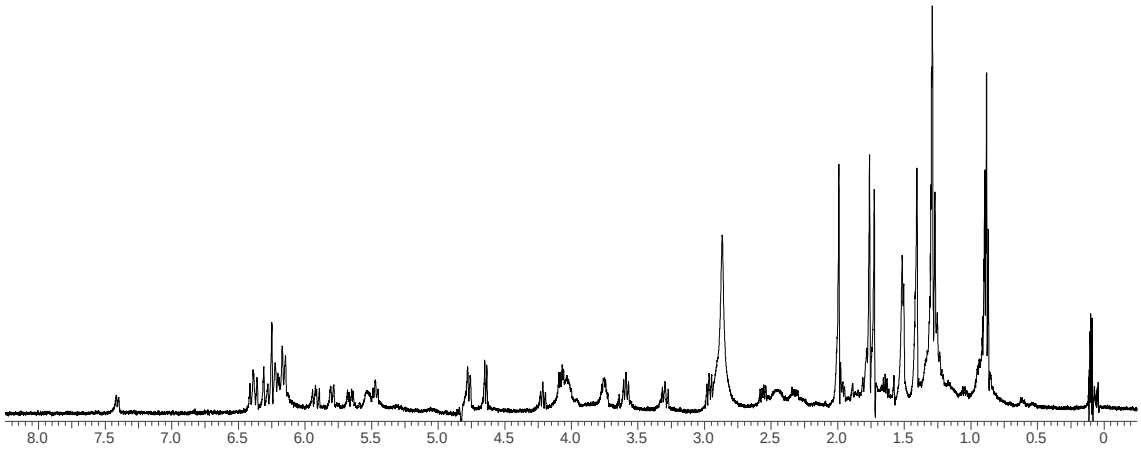
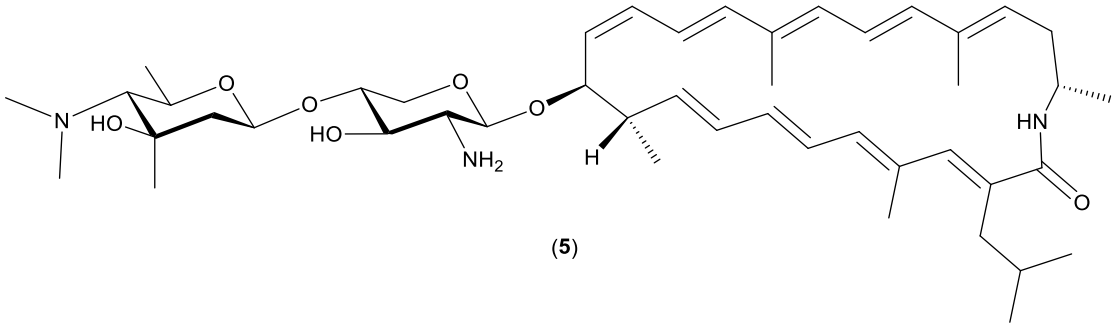


Fig. S28. Diffusion-filtered ^{1}H NMR spectrum of 10-deoxysipanmycin A (**5**).



(**5**)

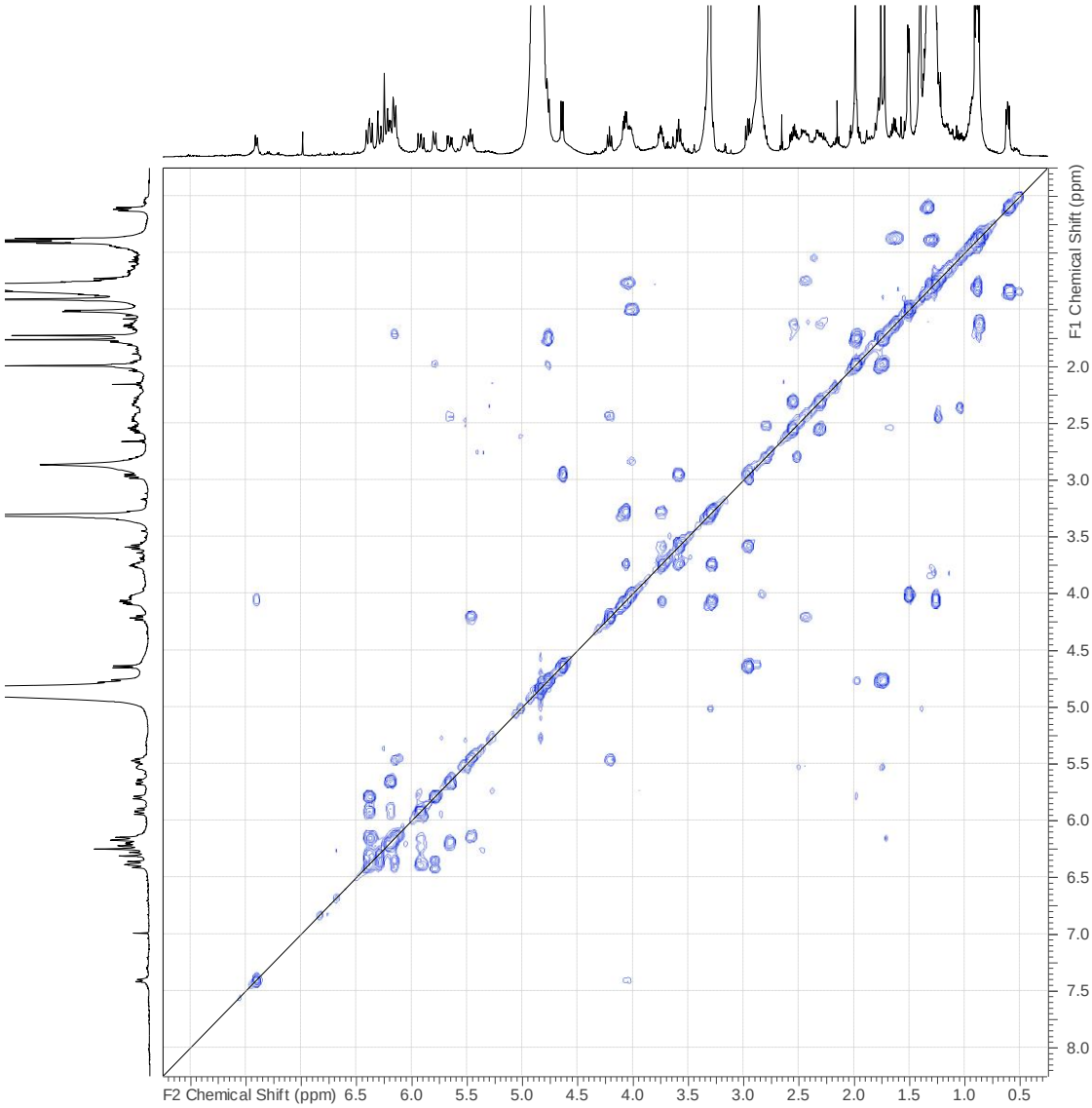


Fig. S29. COSY spectrum of 10-deoxysipanmycin A (**5**).

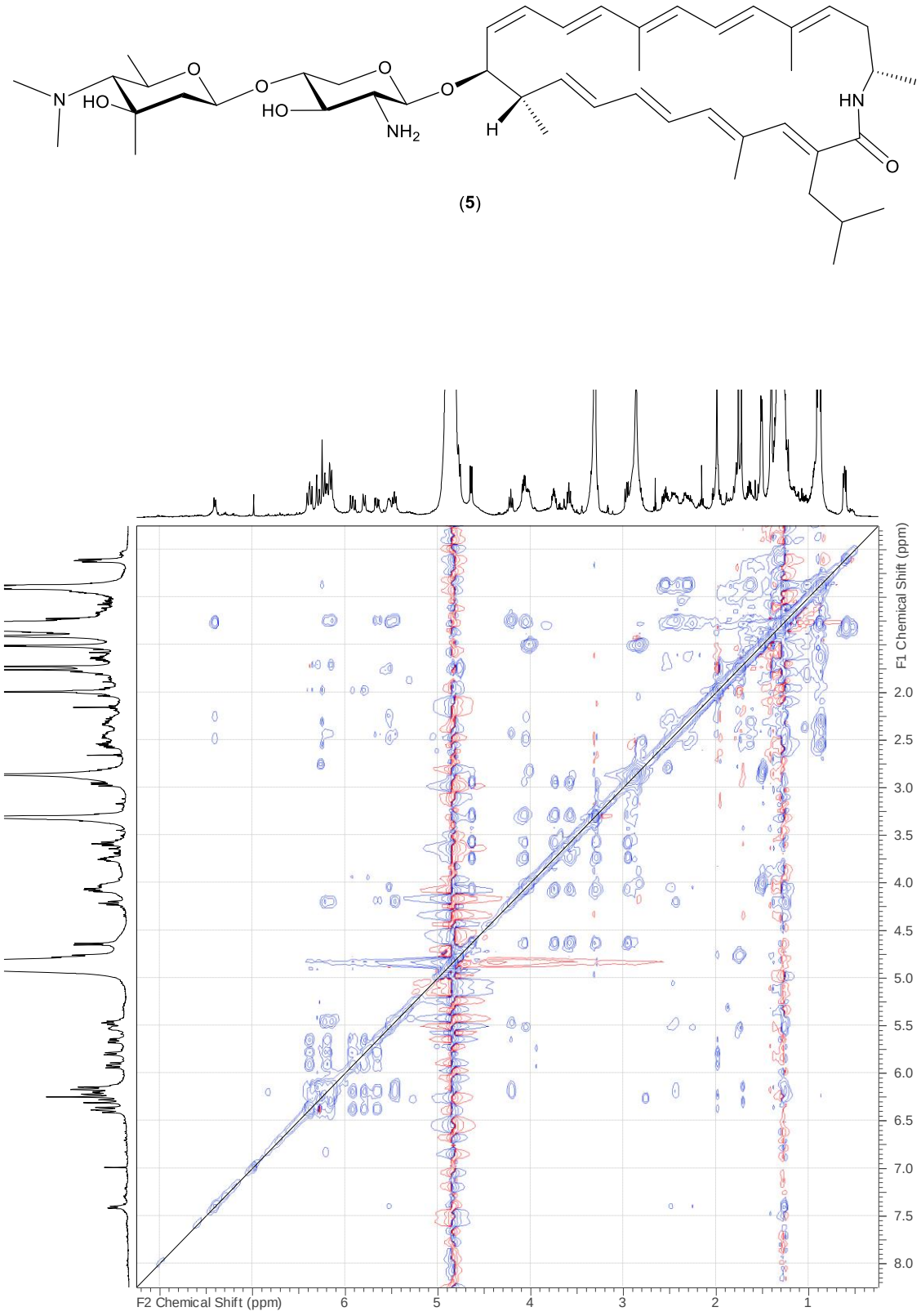


Fig. S30. TOCSY spectrum of 10-deoxysipanmycin A (**5**).

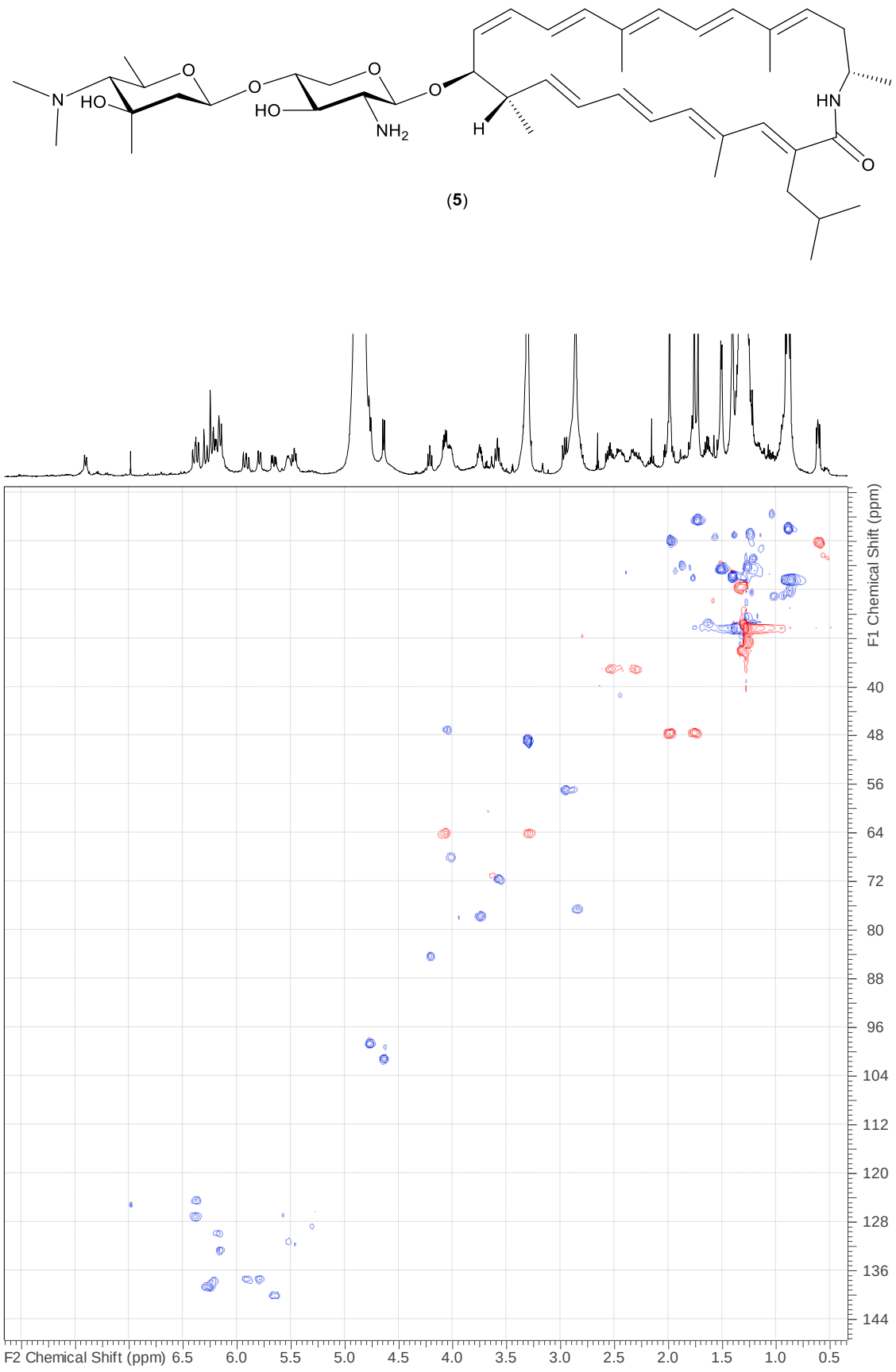
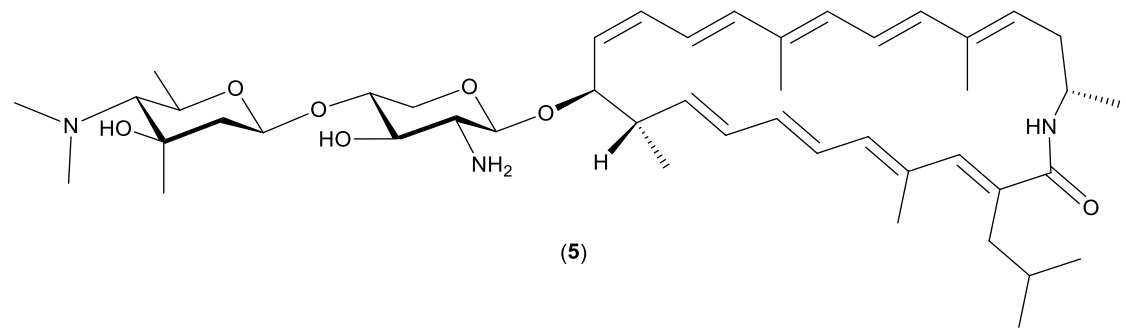


Fig. S31. Edited HSQC spectrum of 10-deoxysipanmycin A (**5**).



(**5**)

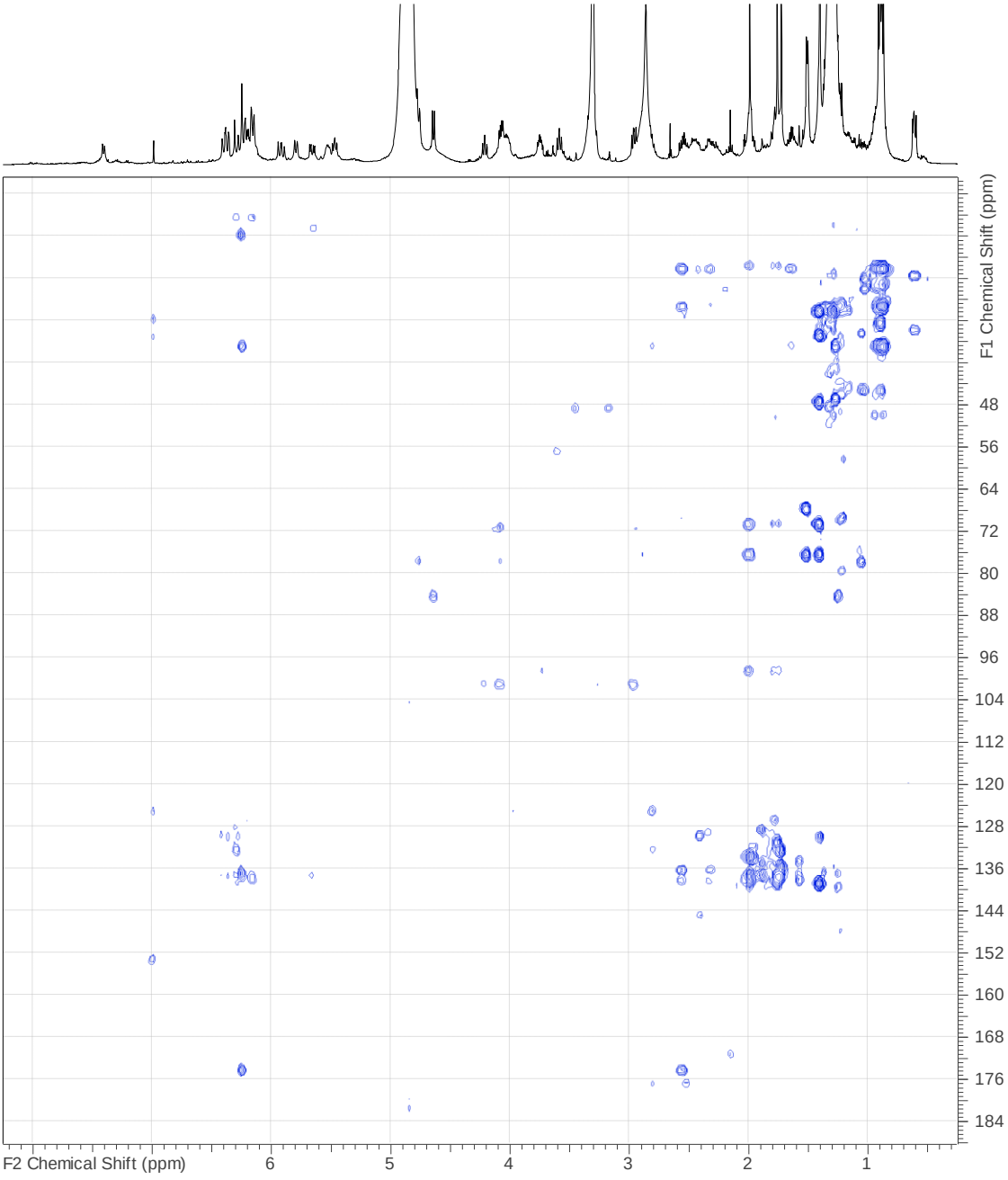


Fig. S32. HMBC spectrum of 10-deoxysipanmycin A (**5**).

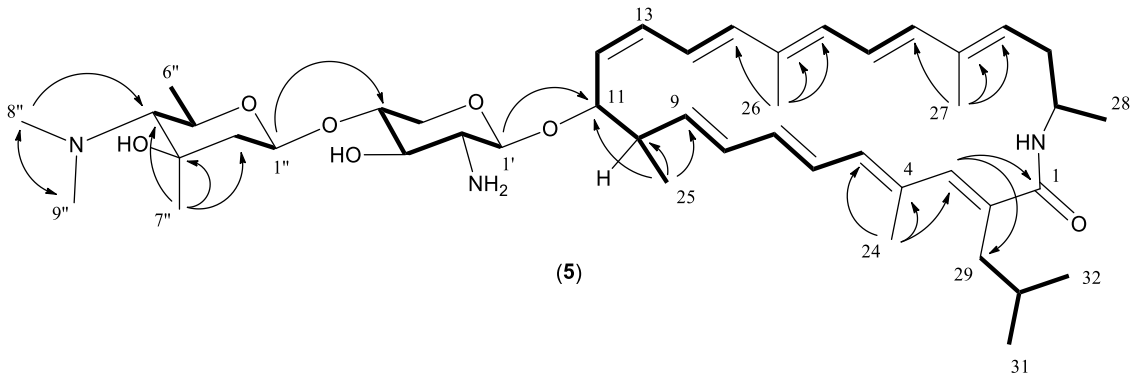


Fig. S33. Gross structure of compound **5** determined by 2D-NMR. COSY correlations (further corroborated by the spin systems observed in the TOCSY spectrum) are indicated as bold bonds. Key HMBC correlations are indicated by arrows.

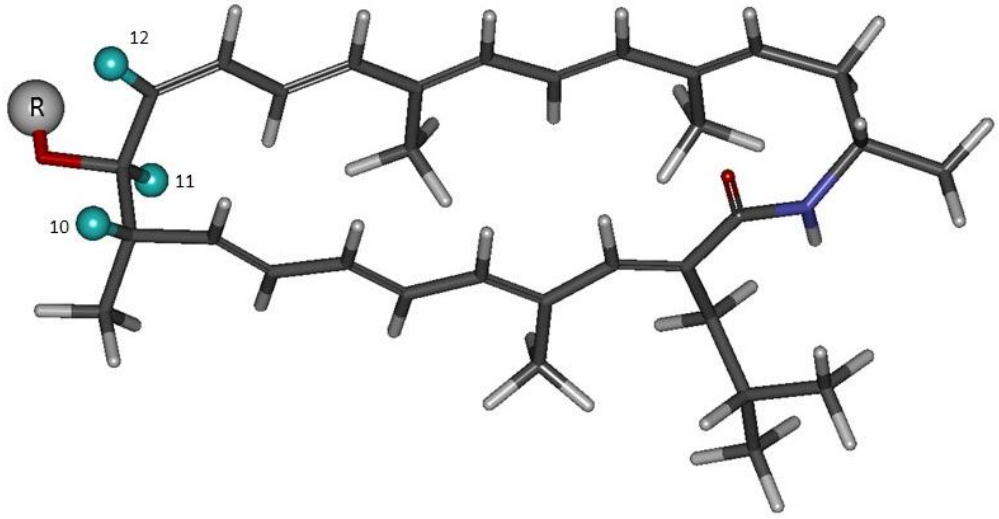


Fig. S34. Molecular model of 10-deoxysipanmycin A (**5**) showing the almost *trans* periplanar relationship of H-11 with both H-10 and H-12 explaining its appearance as a triplet (double doublet with two equal coupling constants $J_{11\text{-}10} = J_{11\text{-}12} = 8.9$ Hz). These key protons are highlighted as cyan colored spheres, R represents the disaccharide substituent of **5**.

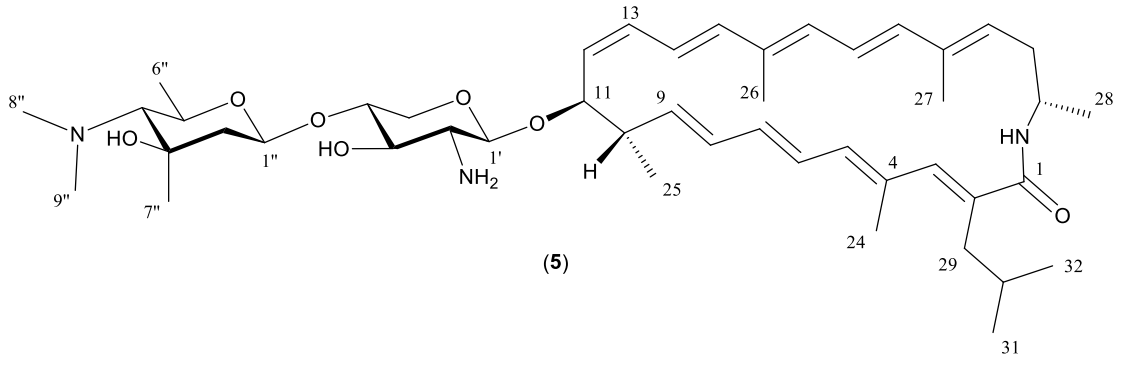


Fig. S35. Structure of 10-deoxysipanmycin A (**5**).

Table S4. ^{1}H and ^{13}C NMR data for 10-deoxysipanmycin A (**5**) in CD_3OD at 24 C°.

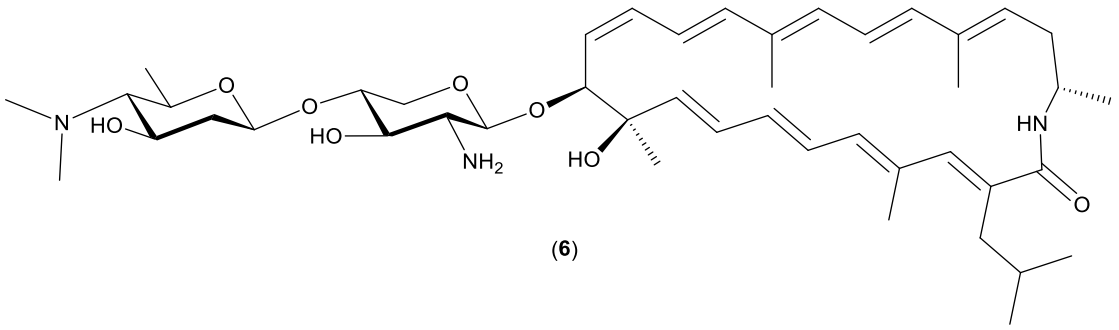
Position	δ_C, type	δ_H (*J* in Hz)	Position	δ_C, type	δ_H (*J* in Hz)
1	174.3, C	-	1'	101.3, CH	4.68, d (7.8)
2	136.4., C	-	2'	57.5, CH	2.96, dd (9.2, 8.0)
3	138.6, CH	6.26, m	3'	72.0, CH	3.58, m
4	133.9, C	-	4'	78.3,CH	3.72, ddd (9.0, 8.5, 5.4)
5	137.6, CH	5.80, m	5'	64.4, CH_2	4.06, dd (12.4, 5.0) 3.32, dd (12.4, 10.0)
6	127.3, CH	6.38, dd (14.8, 11.3)	1"	99.3, CH	4.72,br d (9.7)
7	137.5, CH	5.92, m	2"	47.4, CH_2	1.91, br d (12.4) 1.67, m
8	129.9, CH	6.20, m	3"	72.3, C	-
9	140.1, CH	5.66, dd (15.5, 5.9)	4"	75.7, CH	2.49, m
10	41.5, CH	2.46, m	5"	69.9, CH	3.90, dq (9.7, 6.0)
11	84.5, CH	4.22, dd (8.9, 8.9)	6"	20.9, CH_3	1.39, d (5.9)
12	131.8, CH	5.48, dd (10.1, 8.7)	7"	22.5, CH_3	1.34, s
13	130.0, CH	6.17, m	8''	43.8, CH_3	2.64, s
14	n. d., CH	n.d., m	9''	43.8, CH_3	2.64, s
15	137.8, CH	6.22, m			
16	135.9, C	-			
17	132.7, CH	6.16, m			
18	124.6, CH	6.38, m			
19	138.6, CH	6.29, m			
20	137.4, C	-			
21	131.2, C	5.53, m			
22	37.3, CH_2	2.55, m 2.32, m			
23	47.3, CH	4.06, m			
24	16.2, CH_3	1.99, br s			
25	15.0, CH_3	1.26, d (overlap)			
26	12.8, CH_3	1.72, br s			
27	12.8, CH_3	1.76, br s			
28	20.5, CH_3	1.28, d (overlap)			
29	37.3, CH_2	2.55, m 2.32, m			
30	29.6, CH	1.65, m			
31	22.6, CH_3	0.88, d (6.4)			
32	22.6, CH_3	0.88, d (6.4)			
1-NH	-	7.40 br s*			

^{13}C chemical shifts obtained from HSQC and HMBC spectra.

* The amide proton exchanges very slowly and is clearly observed in the spectra.

Structure elucidation of 3''-demethylsipanmycin A (6).

The molecular formula of **6** was established as $C_{45}H_{69}N_3O_8$ based on the observed ion $[M+H]^+$ at *m/z* 780.5167 (calcd. for $C_{45}H_{70}N_3O_8^+$ = 780.5157, Δm = 1.2 ppm). As expected, this molecular formula matches that of sipanmycin A formally lacking a "CH_2" group. Likewise, its UV (DAD) spectrum is identical to that of sipanmycins A, **1**, **3** and **5**. Comparison of the NMR data of **6** and sipanmycin A (1) immediately revealed that the signals of the aglycon and its directly attached aminosugar are identical for both compound **6** and sipanmycin A. However, important differences are observed regarding the NMR signals of the second aminosugar. In compound **6**, this sugar has lost one aliphatic methyl signal and has gained a new aliphatic methine signal which, according to the proton and carbon chemical shifts corresponds to an oxygenated position. The relative configuration of this monosaccharide was determined by the TOCSY spectrum using the methodology proposed by Martins and co-workers (2) as previously described for compound **3**. This relative configuration determination was further supported by coupling constants analysis. Briefly, H-4'' appears as a triplet (double doublet with two identical couplings equal to 9.8 Hz) indicating its *trans* diaxial relationship with both H-3'' and H-5'', unambiguously establishing that the hydroxyl substituent at C-3'' is equatorial. Thus, this monosaccharide residue is identical in connectivity and configuration to sipanose (the corresponding monosaccharide found in sipanmycin A), just displaying a proton instead of a methyl substituent at position C-3''. Once again, for obvious biosynthetic reasons, the absolute configuration of this aminosugar in **6** must be identical to that of the corresponding sugar in sipanmycin A, belonging to the D- series. This monosaccharide should be named *N*,*N*-dimethyl-D-pyrrolosamine, the enantiomer of the aminosugar found as L- form in the antitumor antibiotics lomaiviticins A and B (4). Compound **6** was thus elucidated as 3''-demethylsipanmycin A.



(**6**)

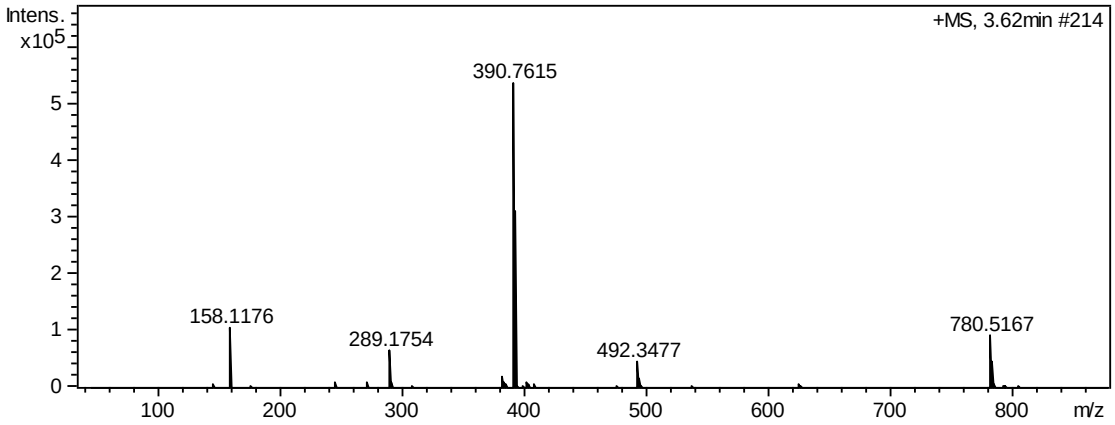


Fig. S36. HRMS spectrum of 3’’-demethylsipanmycin A (**6**).

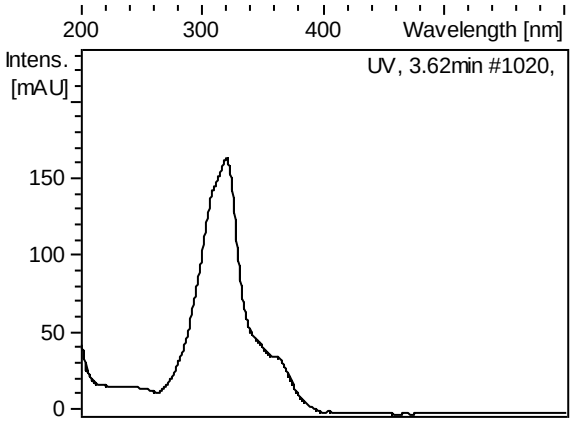
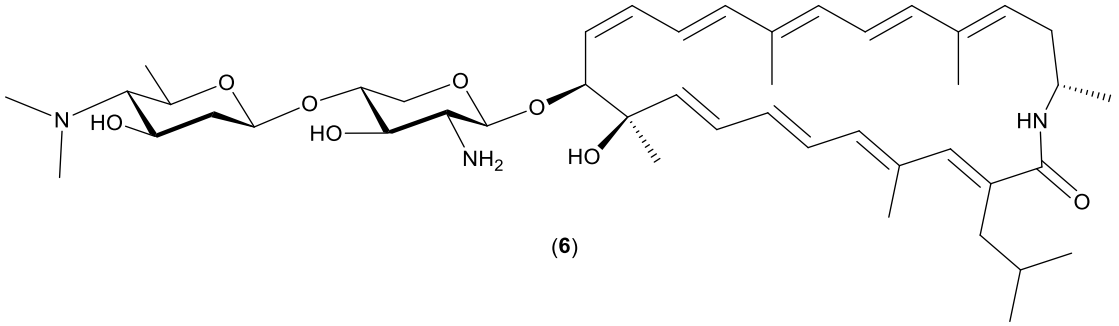


Fig. S37. UV-vis (DAD) spectrum of 3’’-demethylsipanmycin A (**6**).



(**6**)

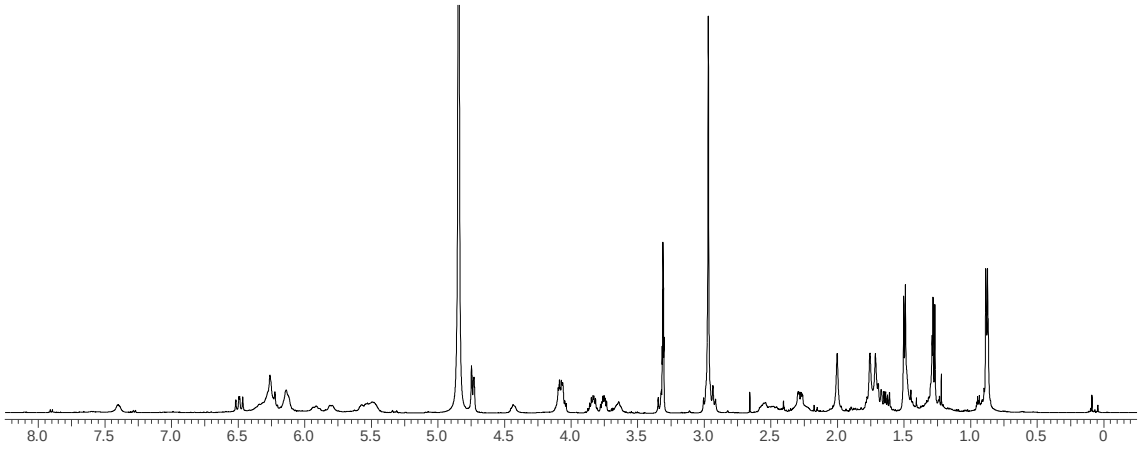


Fig. S38. ^{1}H NMR spectrum (CD_3OD, 500 MHz) of 3’’-demethylsipanmycin A (**6**).

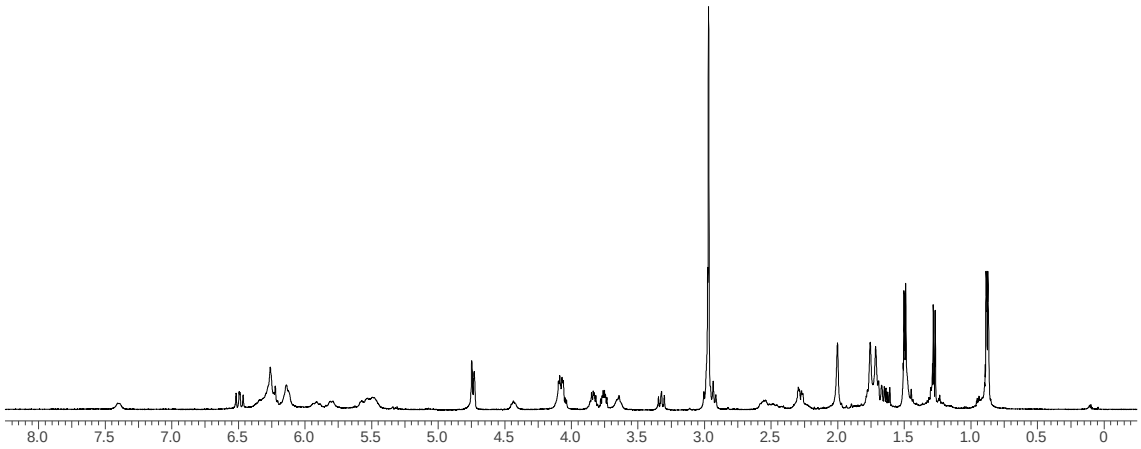
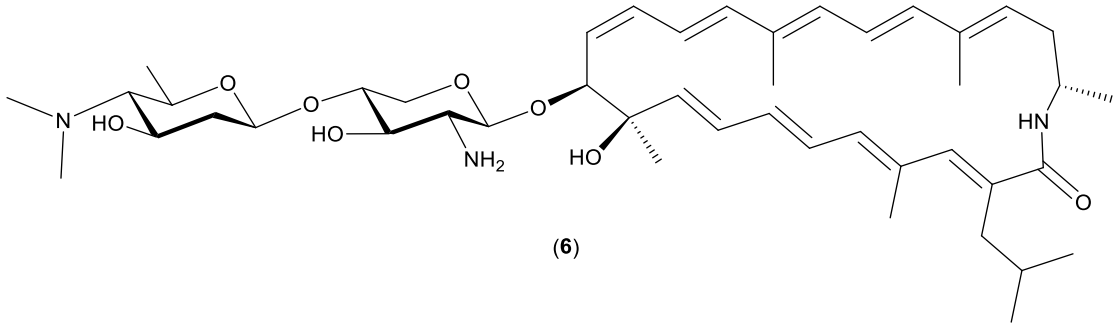


Fig. S39. Diffusion-filtered ^{1}H NMR spectrum of 3’’-demethylsipanmycin A (**6**).



(**6**)

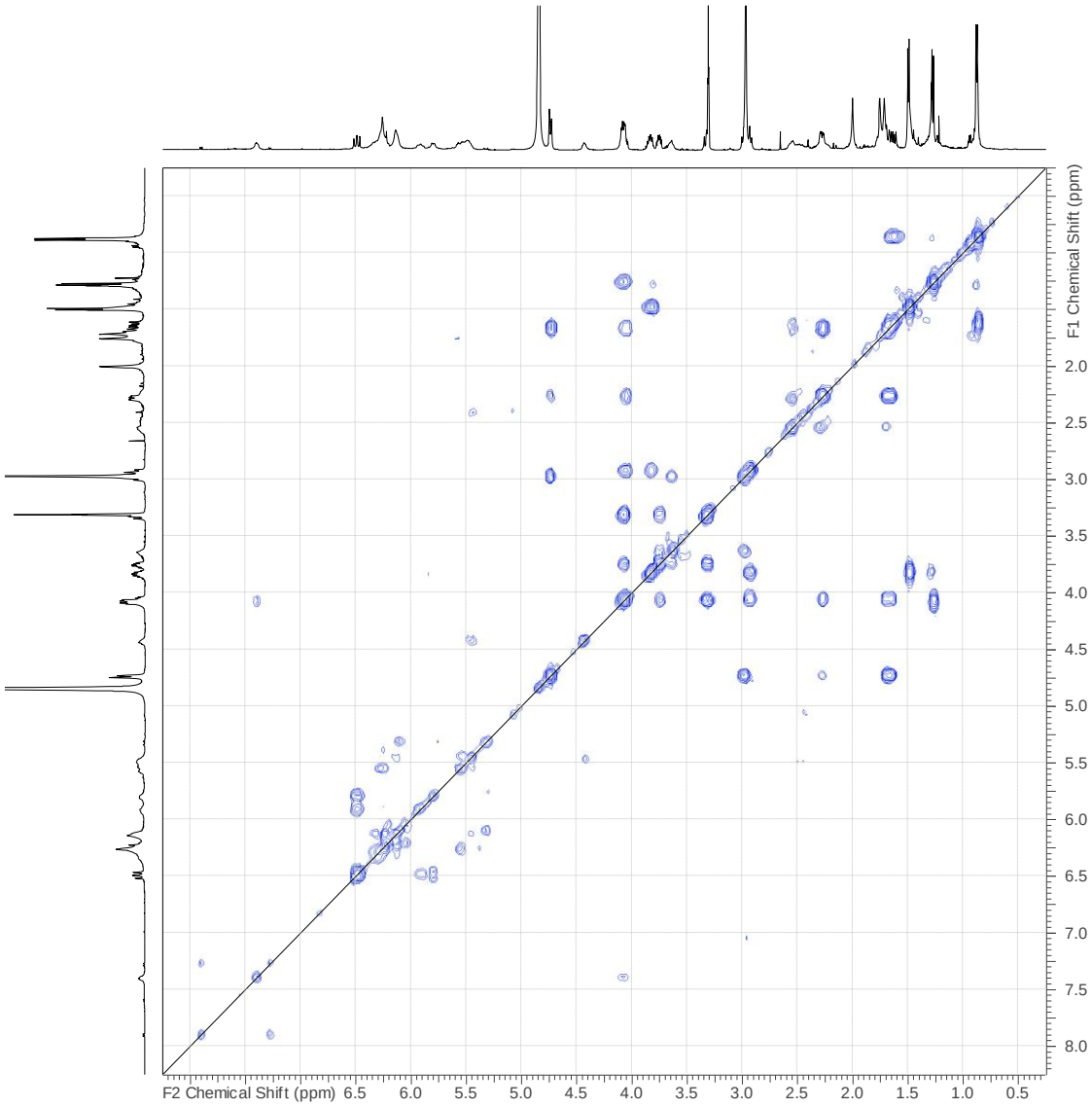
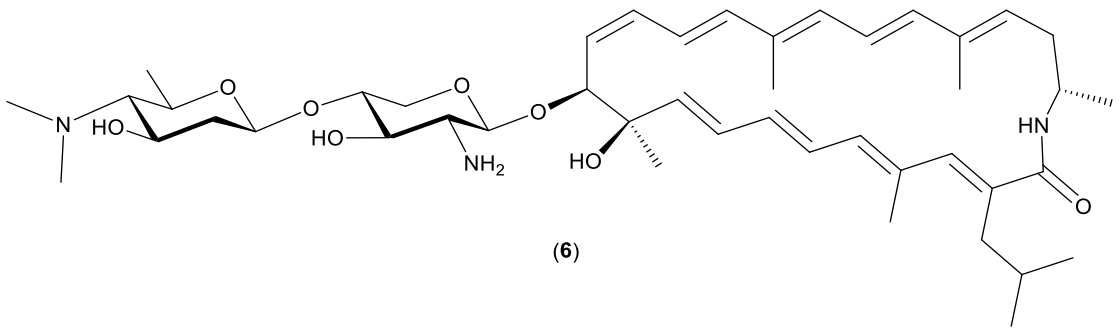


Fig. S40. COSY spectrum of 3’’-demethylsipanmycin A (**6**).



(**6**)

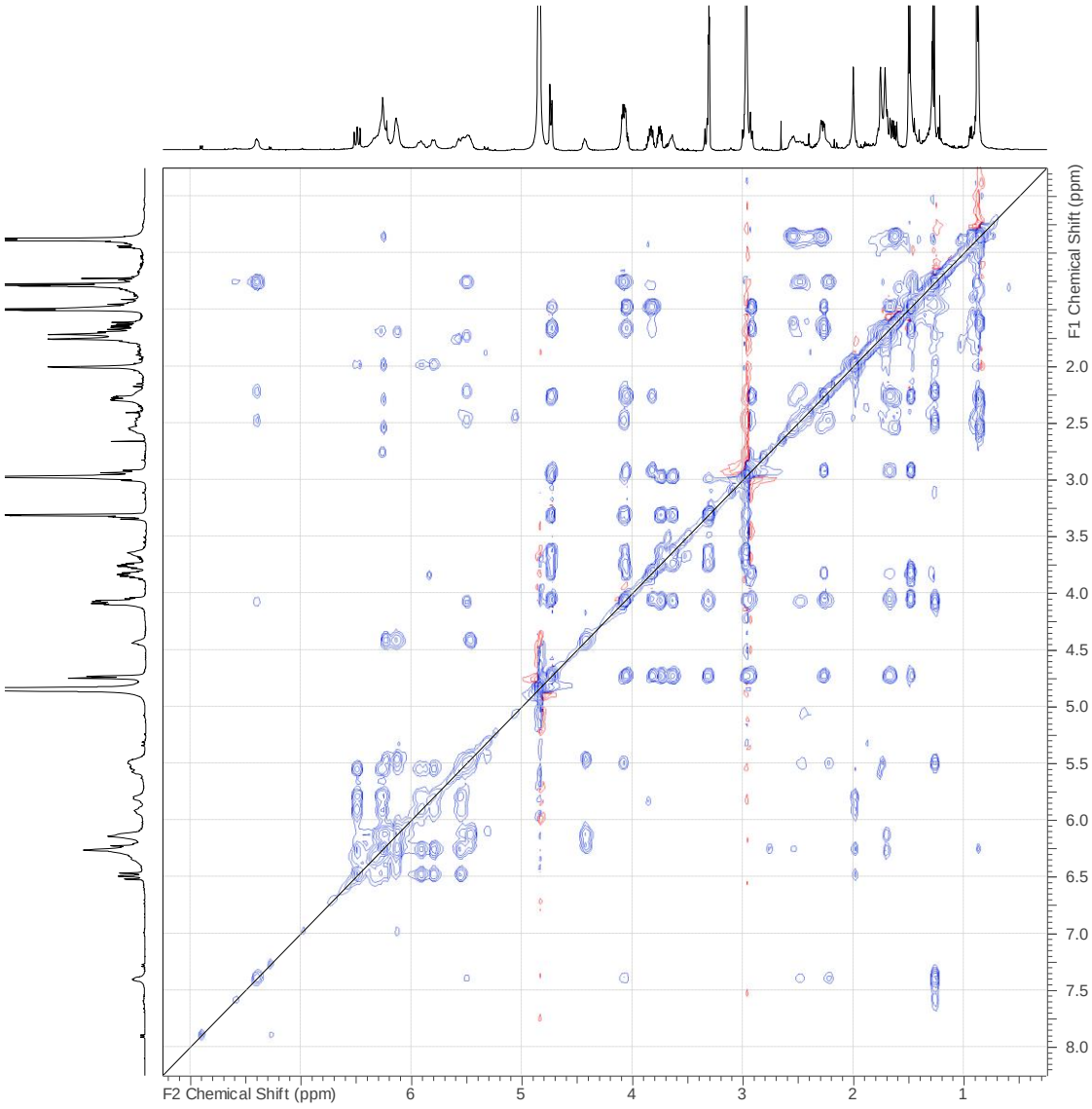
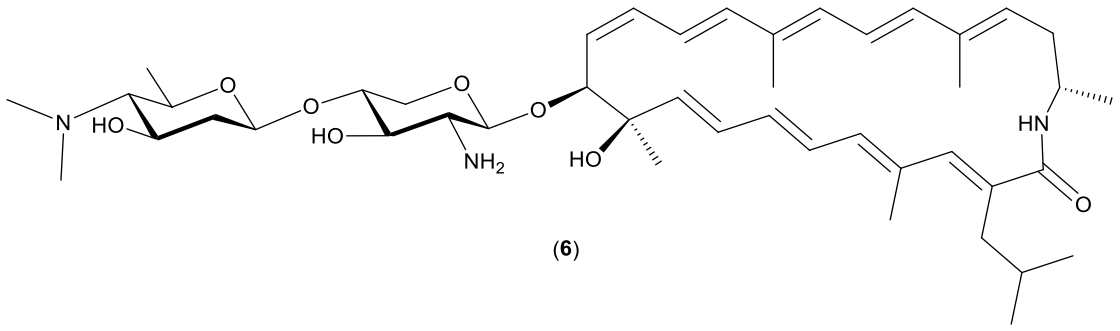


Fig. S41. TOCSY spectrum of 3’’-demethylsipanmycin A (**6**).



(**6**)

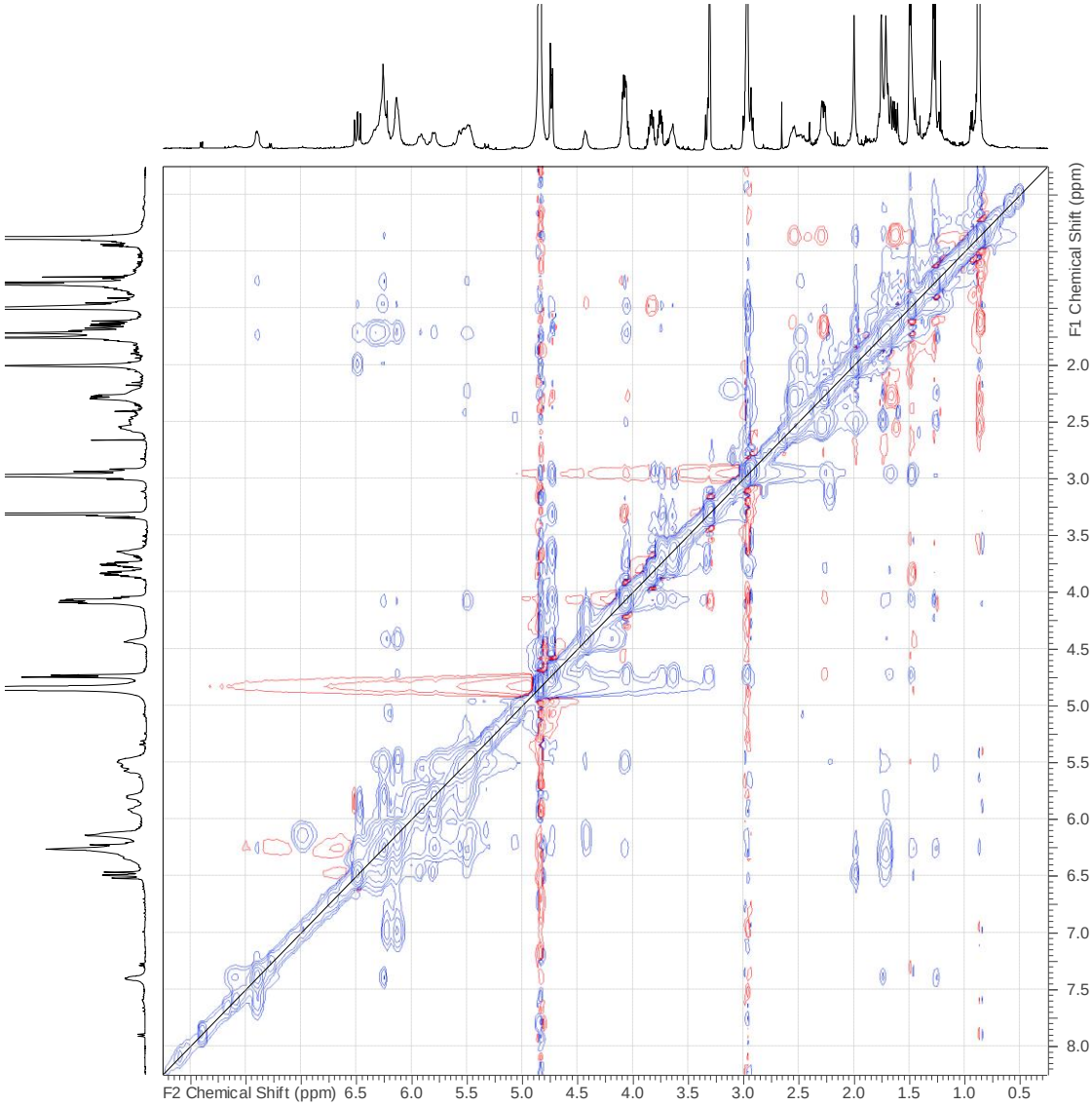
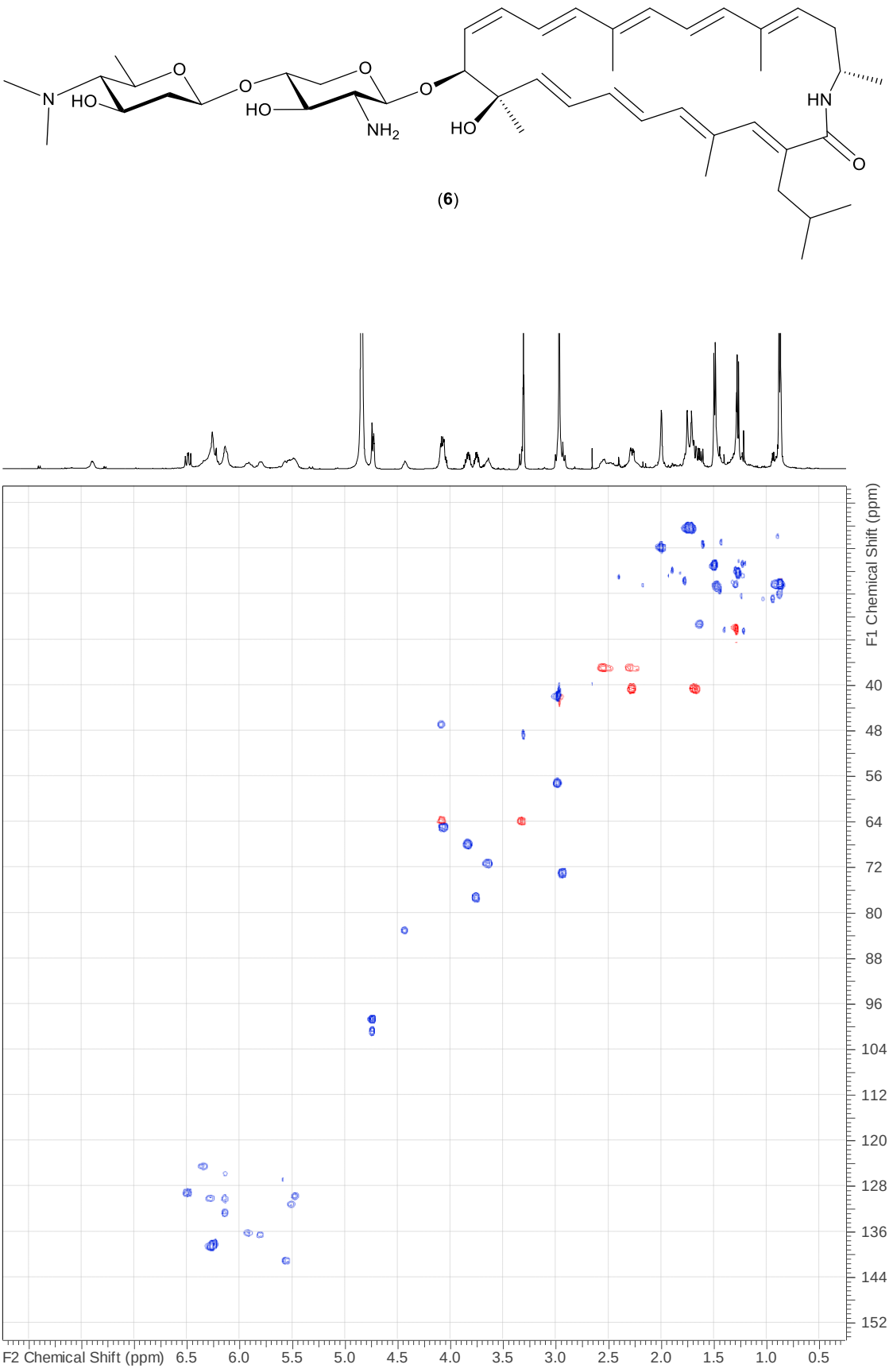
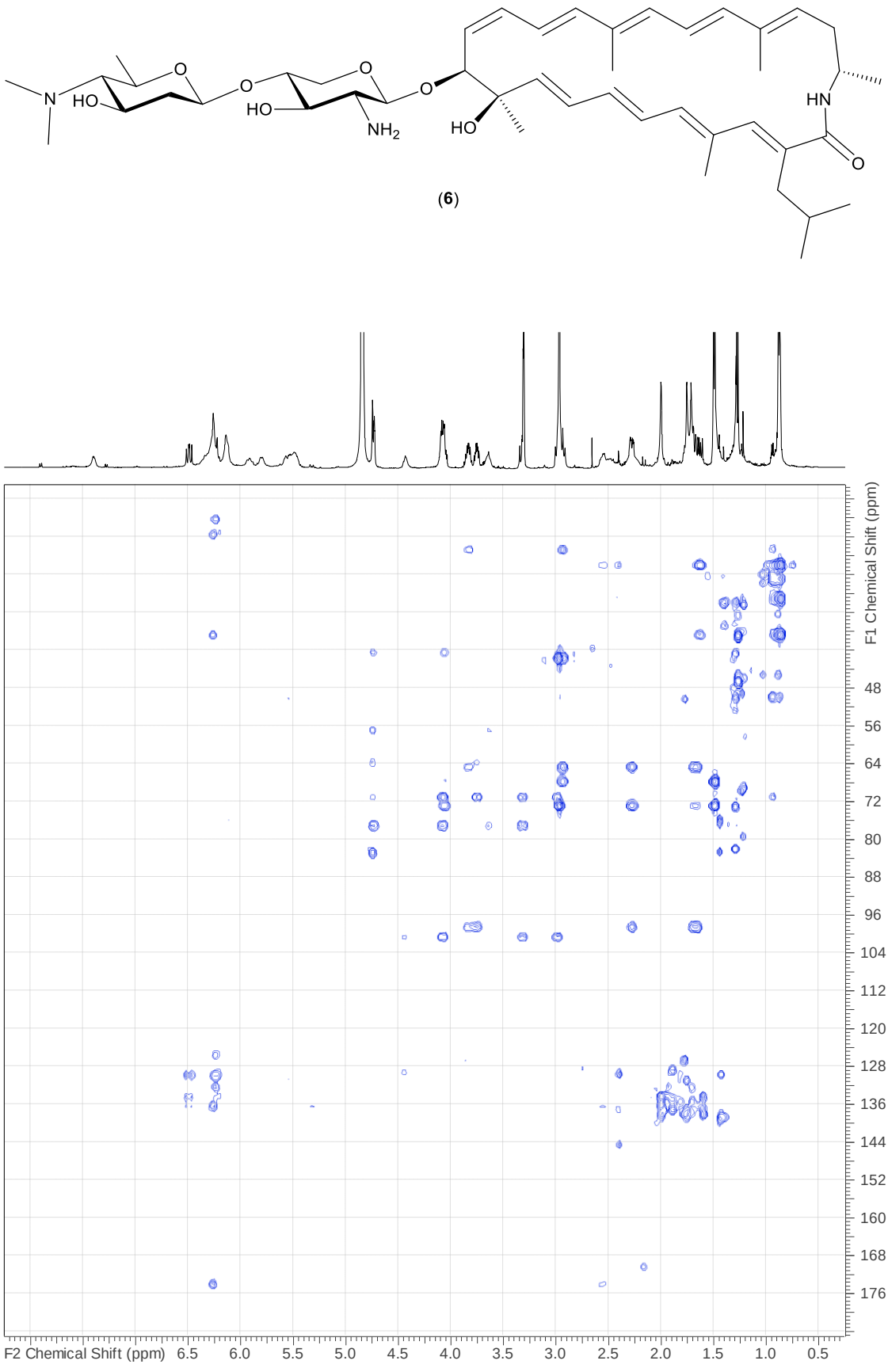


Fig. S42. NOESY spectrum of 3''-demethylsipanmycin A (**6**).



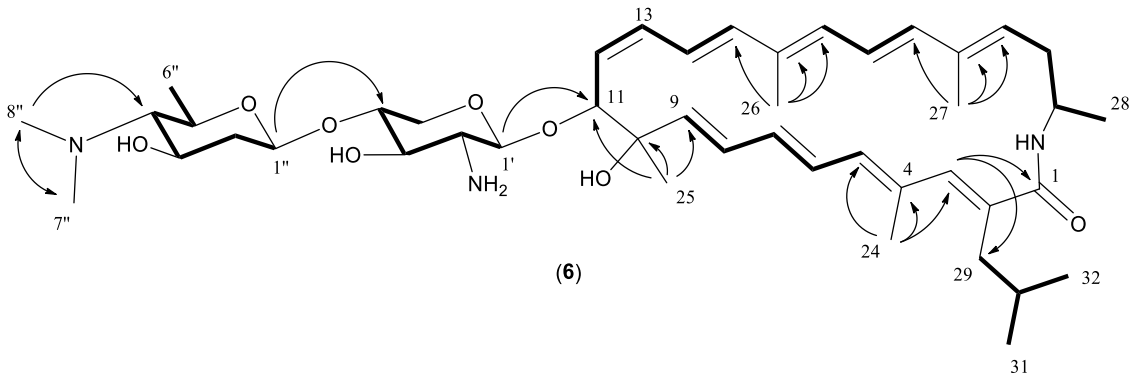
(**6**)

Fig. S43. Edited HSQC spectrum of 3''-demethylsipanmycin A (**6**).



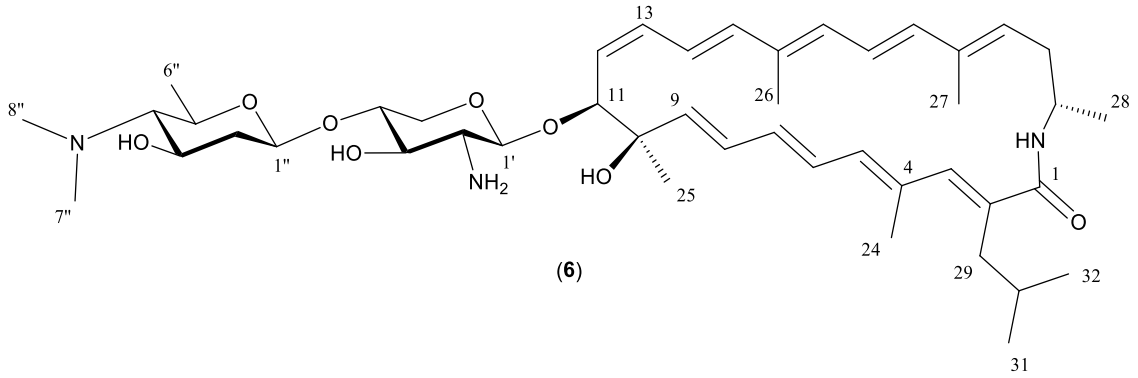
(**6**)

Fig. S44. HMBC spectrum of 3’’-demethylsipanmycin A (**6**).



(**6**)

Fig. S45. Gross structure of 3’’-demethylsipanmycin A (**6**) determined by 2D-NMR. COSY correlations (further corroborated by the spin systems observed in the TOCSY spectrum) are indicated as bold bonds. Key HMBC correlations connecting independent spin systems are indicated by arrows.



(**6**)

Fig. S46. Structure of 3’’-demethylsipanmycin A (**6**).

Table S5. ^{1}H and ^{13}C NMR data for 3''-demethylsipanmycin A (**6**) in CD_3OD at 24 C°.

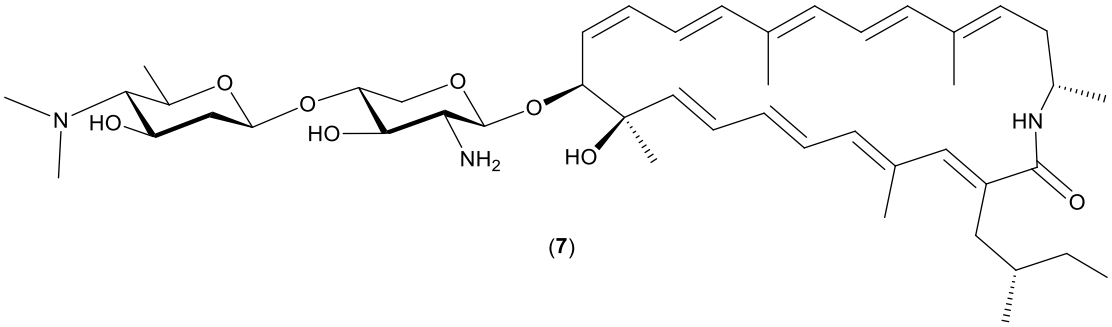
Position	δ_C, type	δ_H (*J* in Hz)	Position	δ_C, type	δ_H (*J* in Hz)
1	174.1, C	-	1'	100.9, CH	4.74, d (ca. 7.8)
2	136.5, C	-	2'	57.4, CH	2.99, dd (ca. 9.2, 8.0)
3	138.6, CH	6.26, m	3'	71.4, CH	3.65, m
4	134.8, C	-	4'	77.5,CH	3.76 ddd (9.0, 8.5, 5.4)
5	136.6, CH	5.80, m	5'	64.1, CH_2	4.08, m 3.33, dd (12.4, 10.0)
6	129.2, CH	6.49, dd (14.5, 11.4)	1"	99.3, CH	4.76,br d (ca. 9.7)
7	136.2, CH	5.92, m	2"	40.9, CH_2	2.28, m 1.68, m
8	130.1, CH	6.27, m	3"	65.2, CH	4.06, m
9	141.2, CH	5.56, m	4"	73.2, CH	2.94, dd (9.8, 9.8)
10	76.5, C	-	5"	68.1, CH	3.83, dq (9.7, 6.0)
11	83.3, CH	4.43, m	6"	19.2, CH_3	1.49, d (6.0)
12	129.9, CH	5.47, m	7"	42.1, CH_3	2.97, s
13	130.3, CH	6.14, m	8"	42.1, CH_3	2.97, s
14	125.9, CH	6.13, m			
15	138.1, CH	6.24, m			
16	135.8, C	-			
17	132.8, CH	6.14, m			
18	124.5, CH	6.34, m			
19	138.6, CH	6.27, m			
20	137.5, C	-			
21	131.3, C	5.51, m			
22	37.4, CH_2	2.50, m 2.26, m			
23	47.2, CH	4.08, m			
24	16.2, CH_3	2.00, br s			
25	22.9, CH_3	1.49, br s			
26	12.8, CH_3	1.72, br s			
27	12.8, CH_3	1.76, br s			
28	20.6, CH_3	1.28, d (6.9)			
29	37.2, CH_2	2.56, m 2.31, m			
30	29.5, CH	1.65, m			
31	22.5, CH_3	0.88, d (6.4)			
32	22.5, CH_3	0.88, d (6.4)			
1-NH	-	7.40 br s*			

^{13}C chemical shifts obtained from HSQC and HMBC spectra.

* The amide proton exchanges very slowly and is clearly observed in the spectra.

Structure elucidation of 3''-demethylsipanmycin B (7).

The molecular formula of **7** was established as $C_{46}H_{71}N_3O_8$ based on the observed ion $[M+H]^+$ at *m/z* 794.5323 (calcd. for $C_{46}H_{72}N_3O_8^+$ = 794.5314, Δm = 1.1 ppm). As expected, this molecular formula, which is identical to that of sipanmycin A, can be viewed as equal to that of sipanmycin B formally lacking a "CH_2" group. Its UV (DAD) spectrum was identical to that of sipanmycin A, **1**, **3**, **5** and **6**. Comparison of the NMR data of **7** and sipanmycin B (1) immediately revealed the same differences already found when comparing **6** with sipanmycin A. That is, the signals of the aglycon and its directly attached aminosugar are identical for both compound **7** and sipanmycin B, but the differences are found in the second sugar which is identical to that displayed by **6**. On the other hand, comparing the signals of **6** and **7**, there are the same differences already found when comparing sipanmycin A with sipanmycin B. Compound **6** was thus elucidated as 3''-demethylsipanmycin B. Both compounds **6** and **7** share identical absolute configuration for obvious biosynthetic reasons. As mentioned in the main text of this article, the extra chiral center at C-30 has an *S* configuration since this side chain is originated from L-isoleucine.



(**7**)

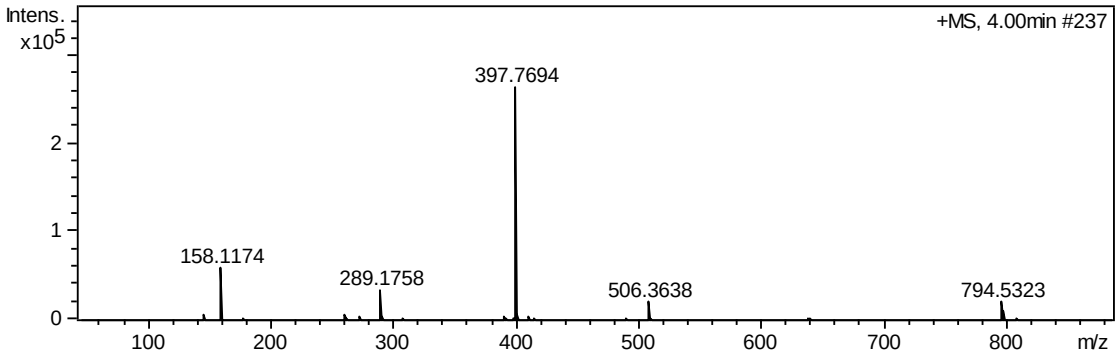


Fig. S47. HRMS spectrum of 3’’-demethylsipanmycin B (**7**)

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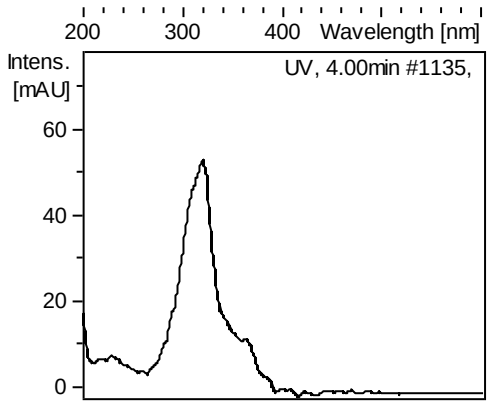
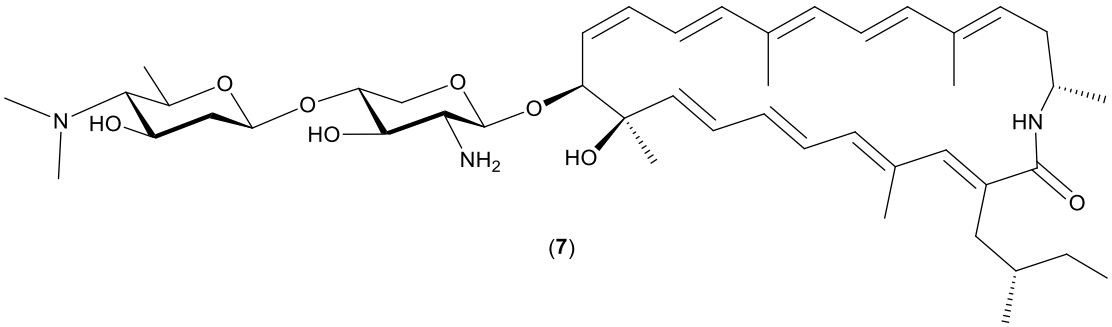


Fig. S48. UV-vis (DAD) spectrum of 3’’-demethylsipanmycin B (**7**)



(**7**)

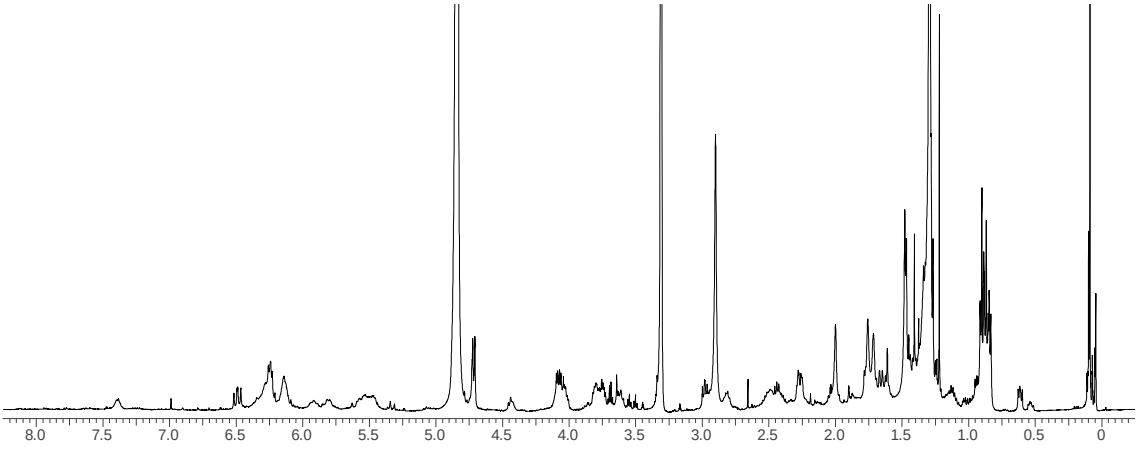


Fig. S49. ^{1}H NMR spectrum (CD_3OD, 500 MHz) of 3’’-demethylsipanmycin B (**7**)

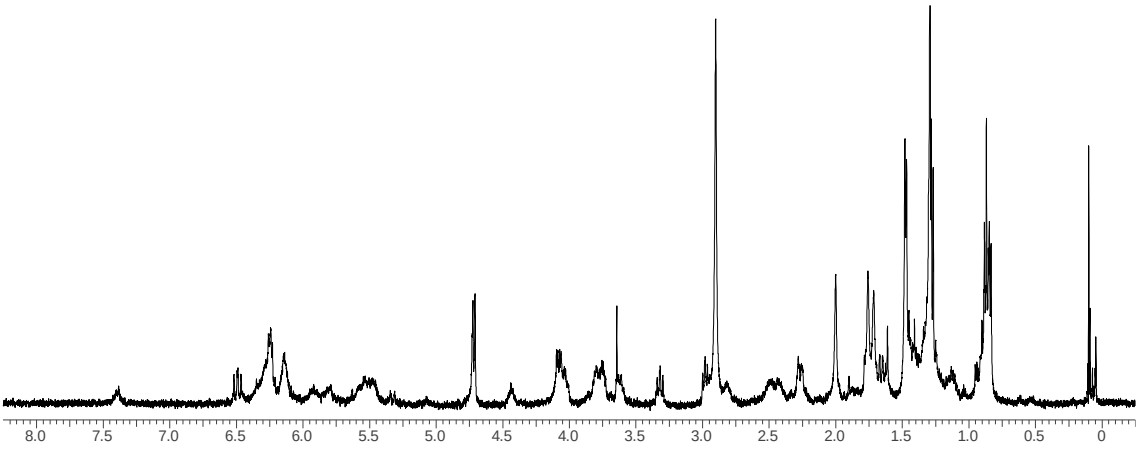


Fig. S50. Diffusion-filtered ^{1}H NMR spectrum of 3’’-demethylsipanmycin B (**7**)

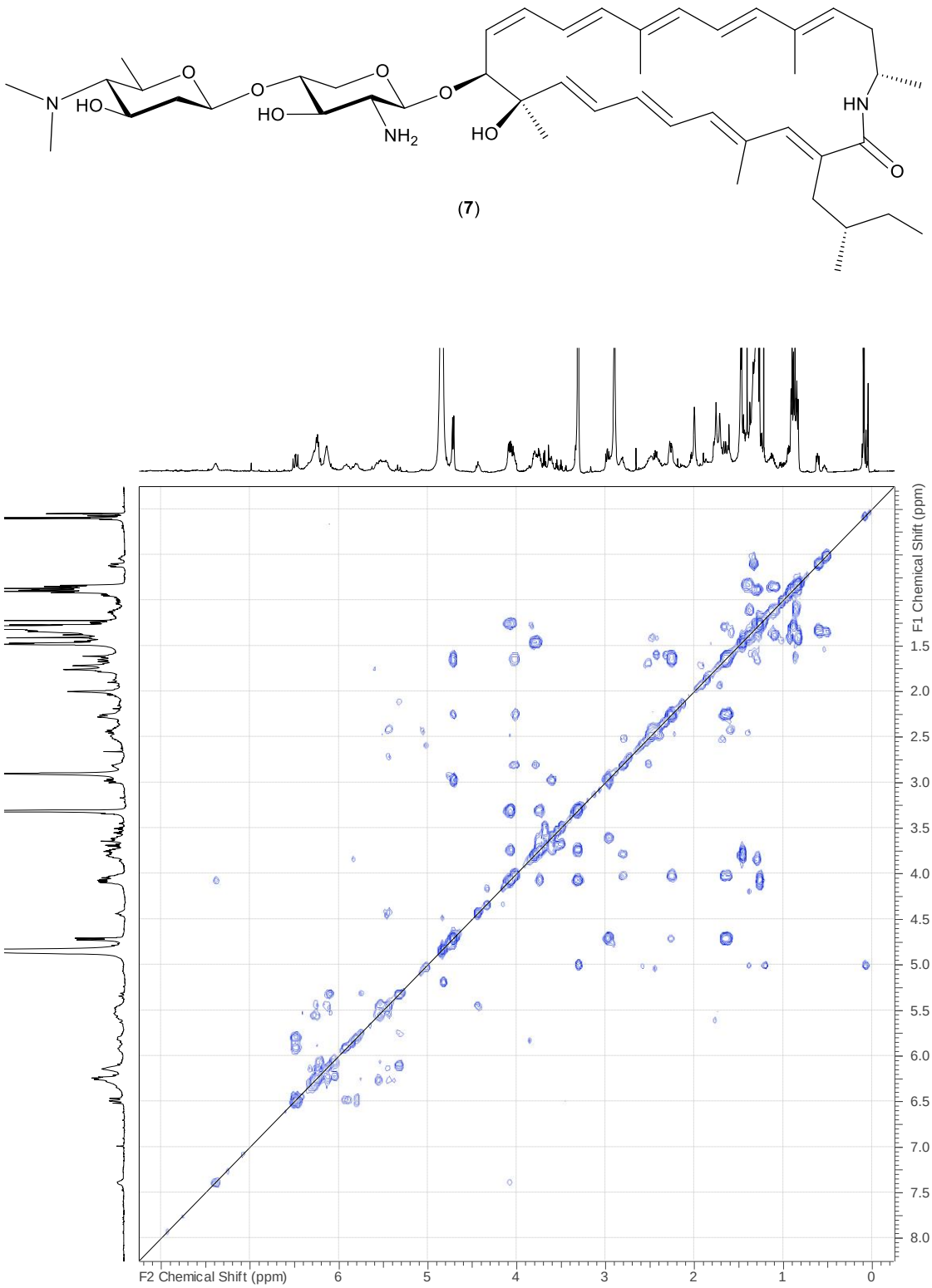
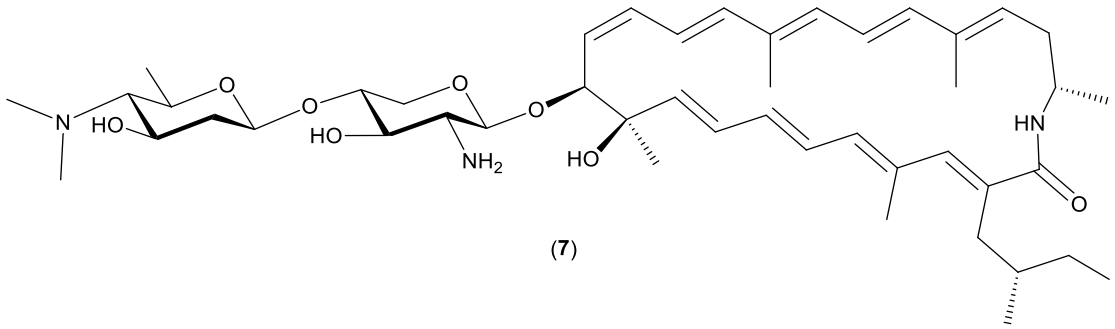


Fig. S51. COSY spectrum of 3’’-demethylsipanmycin B (**7**)



(**7**)

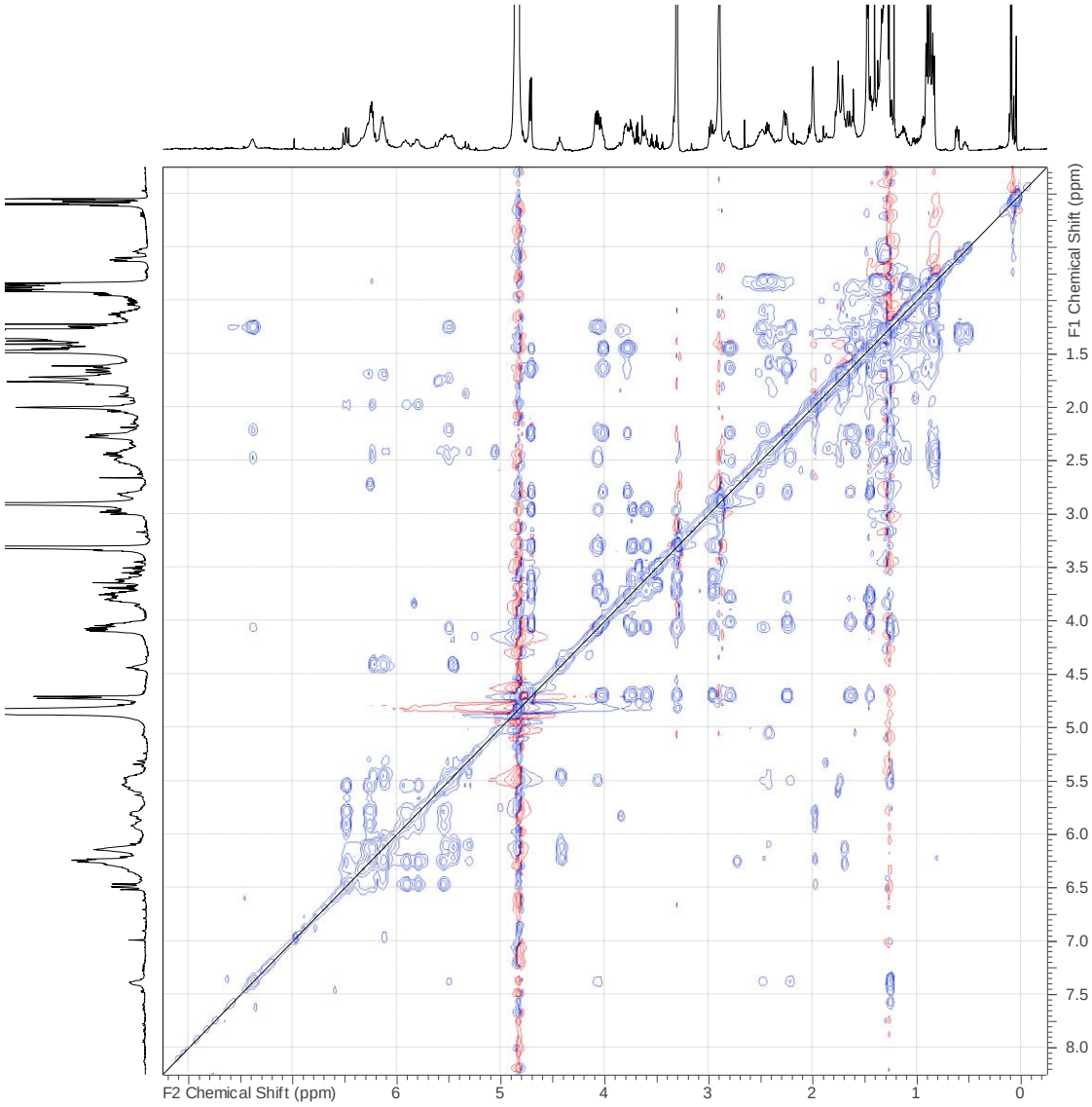
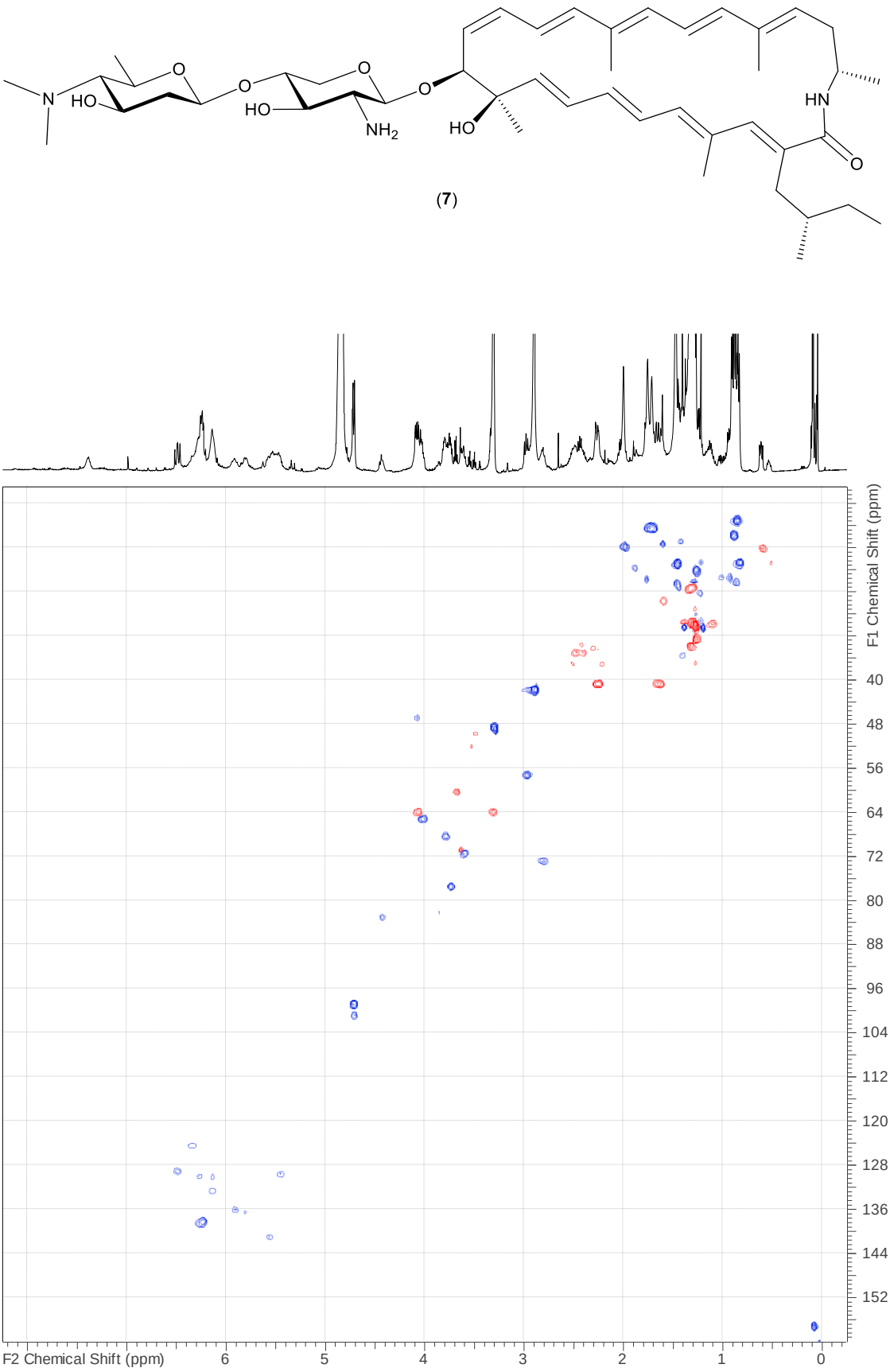
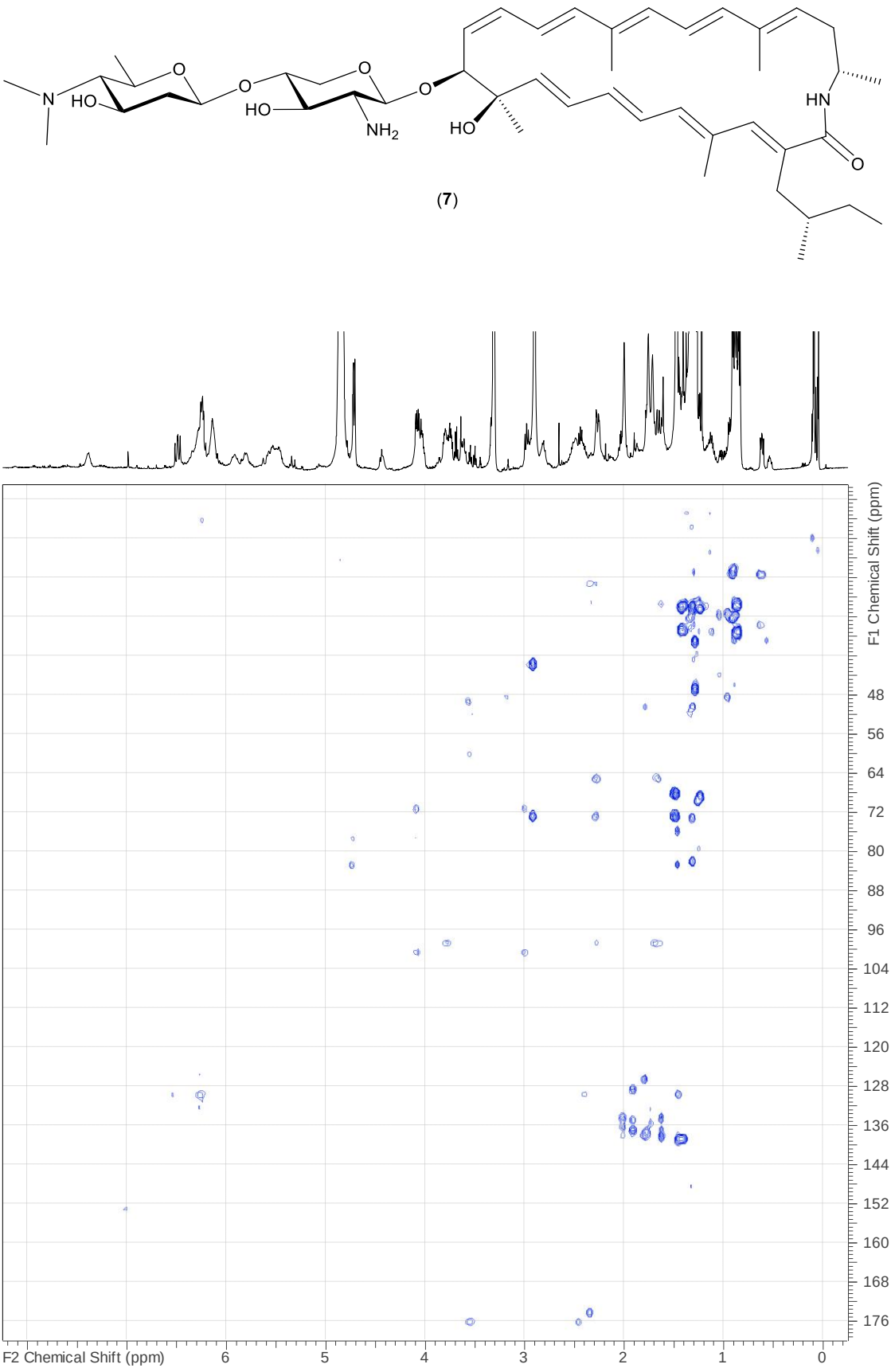


Fig. S52. TOCSY spectrum of 3’’-demethylsipanmycin B (**7**)



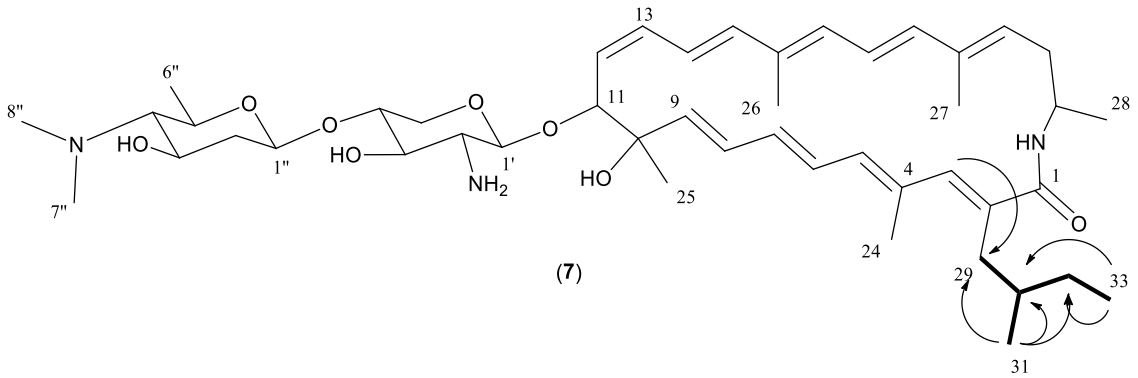
(**7**)

Fig. S53. Edited HSQC spectrum of 3’’-demethylsipanmycin B (**7**)



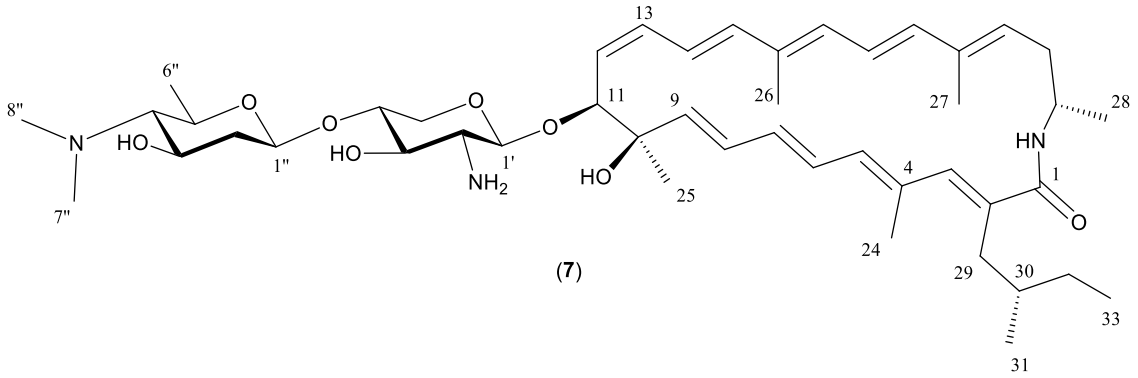
(**7**)

Fig. S54. HMBC spectrum of 3''-demethylsipanmycin B (**7**)



(**7**)

Fig. S55. Gross structure of 3’’-demethylsipanmycin B (**7**) determined by 2D-NMR. Only the COSY correlations (further corroborated by the spin systems observed in the TOCSY spectrum) of the side chain at C-2 are shown as bold bonds. Key HMBC correlations of this side chain are indicated by arrows. The COSY and key HMBC correlations of the rest of the structure are identical to those of 3’’-demethylsipanmycin A (**6**) and are not shown.



(**7**)

Fig. S56. Structure of 3’’-demethylsipanmycin B (**7**).

Table S6. ^{1}H and ^{13}C NMR data for 3''-demethylsipanmycin B (**7**) in CD_3OD at 24 C°.

Position	δ_C, type	δ_H (*J* in Hz)	Position	δ_C, type	δ_H (*J* in Hz)
1	n. d., C	-	1'	100.9, CH	4.71, d (ca. 7.8)
2	n.d., C	-	2'	57.4, CH	2.98, dd (ca. 9.4, 7.9)
3	138.6, CH	6.26, m	3'	71.7, CH	3.61, m
4	134.7, C	-	4'	77.5,CH	3.75 ddd (9.1, 8.3, 5.2)
5	136.7, CH	5.81, m	5'	64.1, CH_2	4.08, dd (12.3, 5.2) 3.33, m
6	129.2, CH	6.49, dd (14.5, 11.5)	1"	99.1, CH	4.72,br d (ca. 9.7)
7	136.2, CH	5.91, m	2"	40.9, CH_2	2.27, m 1.66, m
8	130.1, CH	6.27, m	3"	65.4, CH	4.03, m
9	141.2, CH	5.56, m	4"	73.2, CH	2.82, m
10	n. d., C	-	5"	68.6, CH	3.80, m
11	83.3, CH	4.42, m	6"	19.2, CH_3	1.48, d (6.0)
12	129.8, CH	5.47, m	7"	42.0, CH_3	2.90, s
13	130.2, CH	6.14, m	8"	42.0, CH_3	2.90, s
14	125.9, CH	6.13, m			
15	138.1, CH	6.24, m			
16	135.8, C	-			
17	132.8, CH	6.14, m			
18	124.5, CH	6.34, m			
19	138.6, CH	6.27, m			
20	137.5, C	-			
21	131.3, CH	5.51, m			
22	37.4, CH_2	2.52, m 2.24, m			
23	47.2, CH	4.07, m			
24	16.2, CH_3	2.00, br s			
25	22.9, CH_3	1.49, br s			
26	12.8, CH_3	1.71, br s			
27	12.8, CH_3	1.76, br s			
28	20.6, CH_3	1.28, d (6.9)			
29	35.3, CH_2	2.49, m 2.42, m			
30	35.8, CH	1.42, m			
31	19.0, CH_3	0.84, d (6.4)			
32	30.0, CH_2	1.12, m			
33	11.4, CH_3	0.87, m			
1-NH	-	7.39, br s*			

^{13}C chemical shifts obtained from HSQC and HMBC spectra.

* The amide proton exchanges very slowly and is clearly observed in the proton and the homonuclear 2D spectra.

Structure elucidation of 4''-deamino-4''-oxosipanmycin A (8) and its ketal derivative artifact (8') formed under acidic chromatographic conditions.

After isolation of compound **8**, it was observed that its analytical chromatographic analysis rendered two peaks (**8** and **8'**) rather than the expected single peak. This immediately suggested a probable interconversion of both compounds under the acidic chromatographic conditions used. The molecular formula of **8** was established as $C_{44}H_{64}N_2O_9$ based on the observed ion $[M+H]^+$ at *m/z* 765.4676 (calcd. for $C_{44}H_{65}N_2O_9^+$ = 765.4685, Δm = 1.2 ppm). This molecular formula remarkably contains one nitrogen less than the formula of sipanmycin A indicating the possible absence of one of amino groups in the carbohydrate residues. As expected, the UV (DAD) spectrum of **8** was identical to that of sipanmycin A, **1**, **3**, **5, 6** and **7**. The NMR spectra clearly show a main component in the sample which corresponds to compound **8**. Comparison of the NMR data of **8** and sipanmycin A (1) immediately revealed that the signals of the aglycon and its directly attached aminosugar were identical for both, compound **8** and sipanmycin A. However, important differences were observed regarding the NMR signals of the second carbohydrate residue. Detailed analysis of the COSY, TOCSY, HSQC and HMBC spectra unambiguously confirmed its identity as4-keto-β-D-olivomycose, confirming the absence of one of the amino functionalities in this residue with respect to the other compounds in the series. Though the NOESY spectrum was not acquired, the configuration of this sugar residue can safely be established based on biosynthetic arguments since *N*, *N*-dimethyl-D-sipanose and 4-keto-β-D-olivomycose are biosynthesized with a common enzymatic machinery. Compound **8** was thus elucidated as 4''-deamino-4''-oxosipanmycin A.

The molecular formula of the minor component **8'**, which appear under acidic chromatographic conditions when the sample of isolated **8** is analyzed, was established as $C_{44}H_{66}N_2O_{10}$ based on the observed ion $[M+H]^+$ at *m/z* 783.4780 (calcd. for

$C_{44}H_{67}N_2O_{10}^+$ = 783.4790, Δm = 1.3 ppm). This molecular formula formally corresponds to a water addition to the formula of compound **8**. Based on the structure of **8** and the fact that apparently **8** and **8'** interconvert under the acidic conditions employed in the chromatography it can be proposed that **8**' is formed by the acid catalyzed addition of water (hydration to render a ketal) over the ketone at position 4'' in **8**. Not surprisingly, the expected signals of **8**' are hardly visible in the NMR spectra of the analyzed sample (isolated compound **8**) since this was prepared in conditions (neutral deuterated methanol) drastically opposed to the acidic aqueous chromatographic conditions. The structure ofrtifact **8'** was thus elucidated as 4''-deamino-4''-dihydroxysipanmycin A.

(**8**)

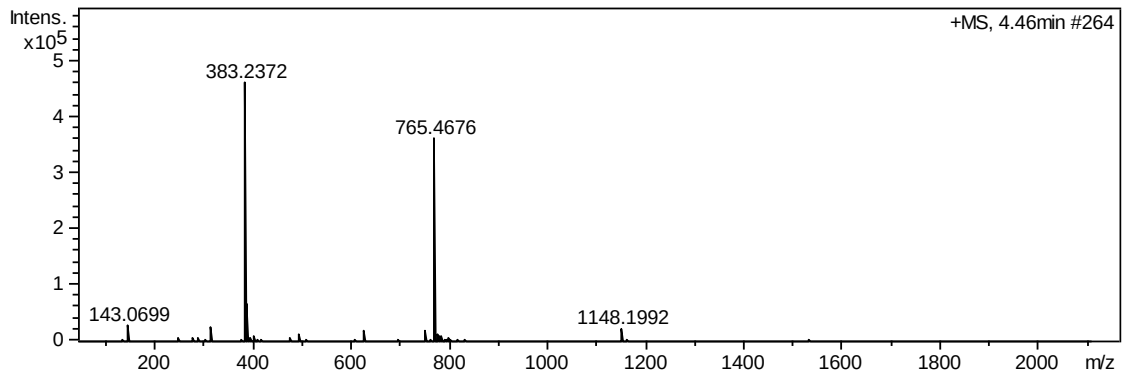


Fig. S57. HRMS spectrum of 4’’-deamino-4’’-oxosipanmycin A (**8**).

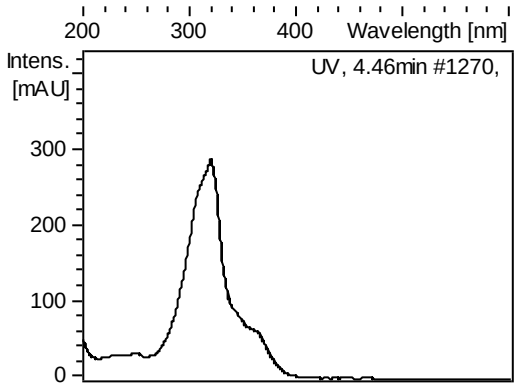
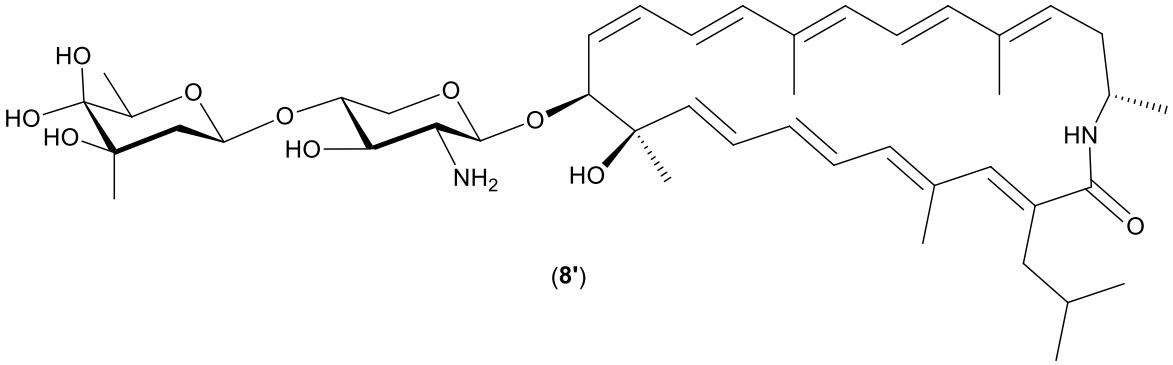


Fig. S58. UV-vis (DAD) spectrum of 4’’-deamino-4’’-oxosipanmycin A (**8**).



(**8'**)

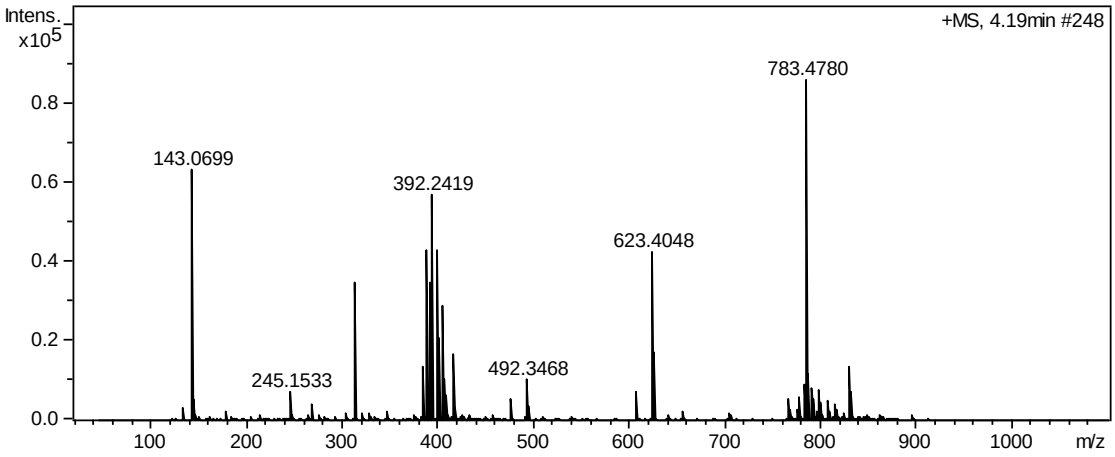


Fig. S59. HRMS spectrum of artifact 4''-deamino-4''-dihydroxysipanmycin A (**8'**).

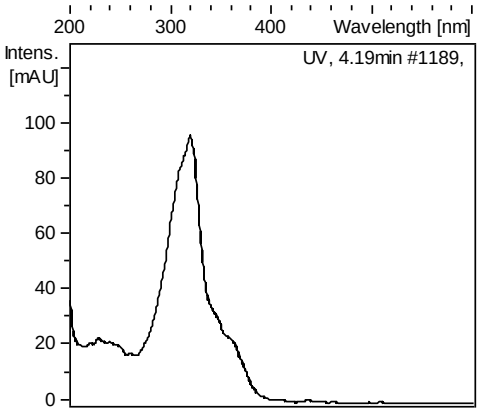
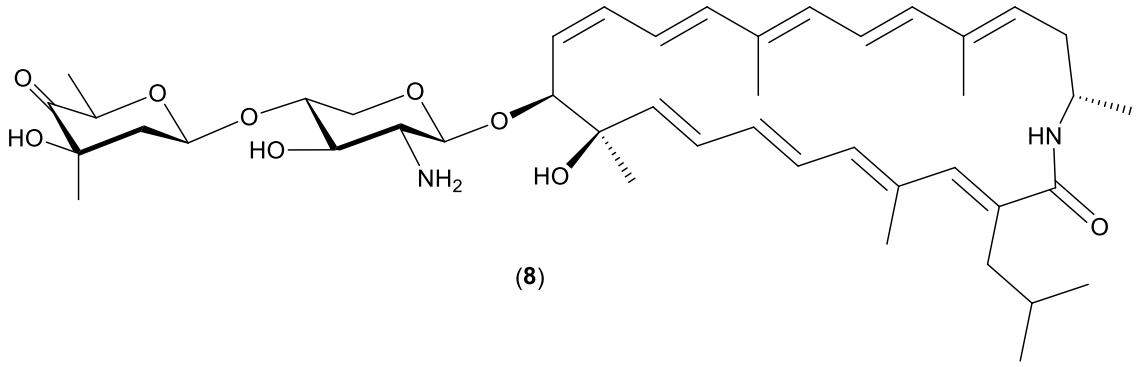


Fig. S60. UV-vis (DAD) spectrum of artifact 4''-deamino-4''-dihydroxysipanmycin A (**8'**).



(**8**)

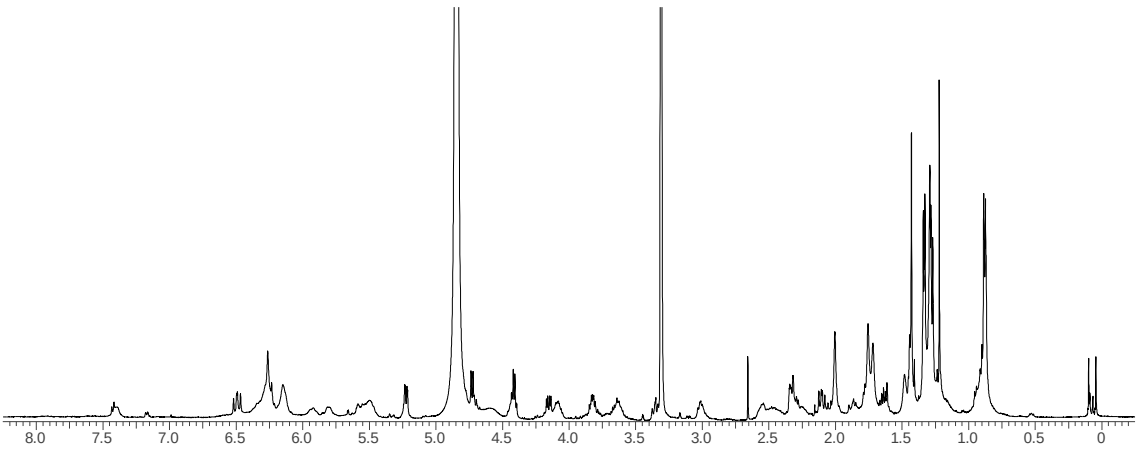
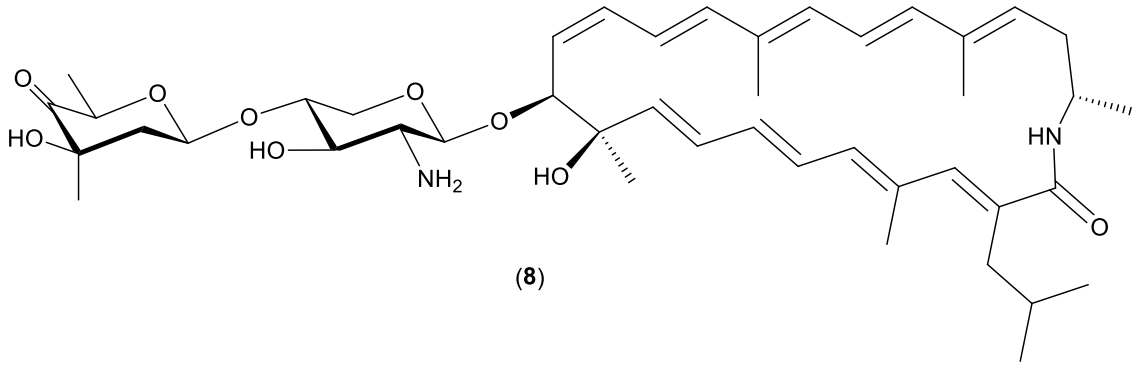


Fig. S61. ^{1}H NMR spectrum (CD_3OD, 500 MHz) of 4’’-deamino-4’’-oxosipanmycin A (**8**).



(**8**)

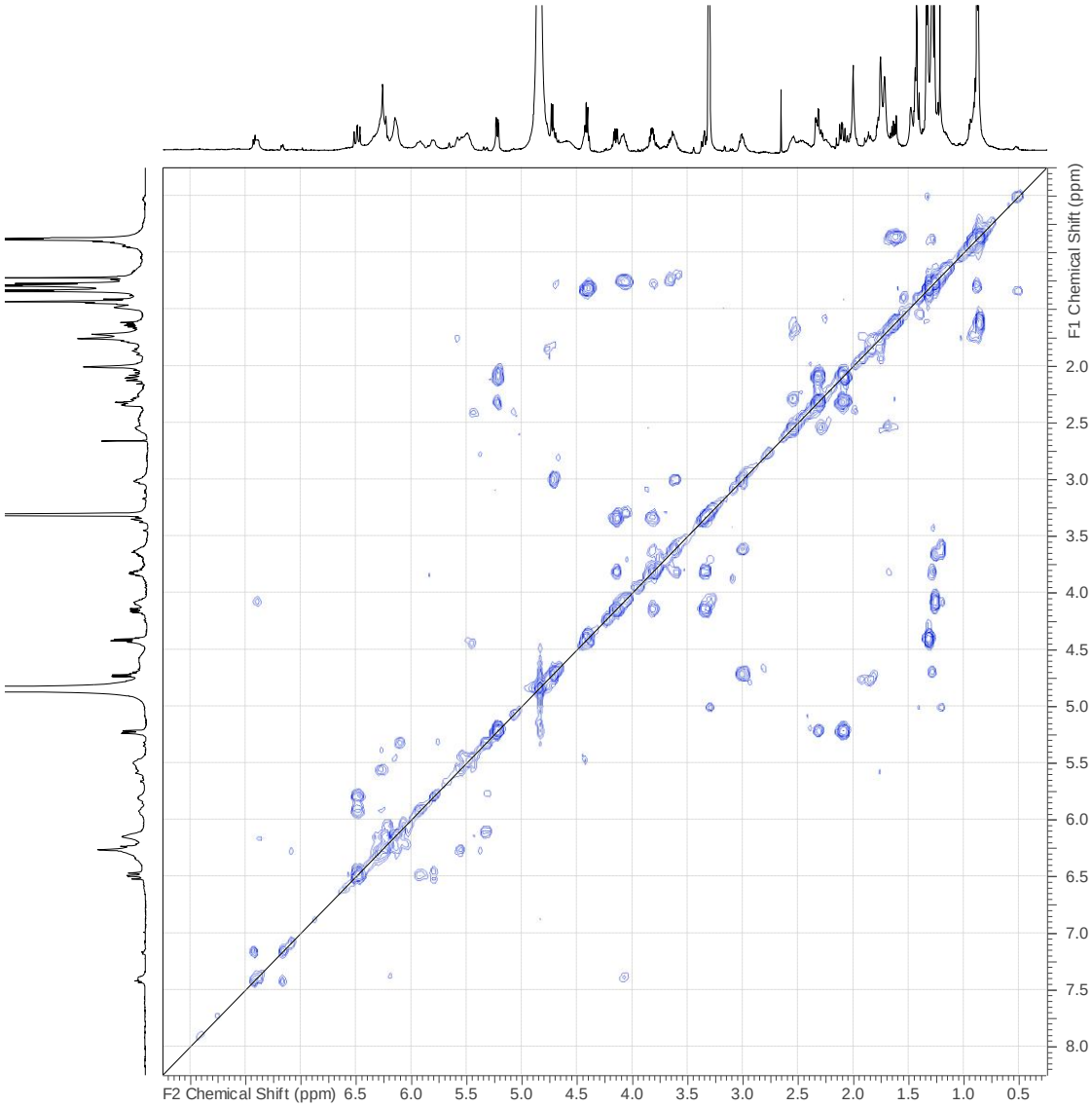
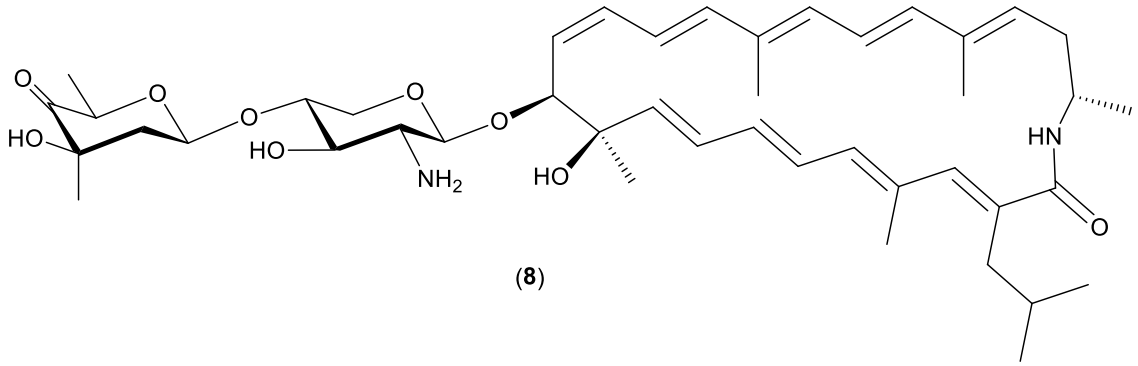


Fig. S62. COSY spectrum of 4’’-deamino-4’’-oxosipanmycin A (**8**).



(**8**)

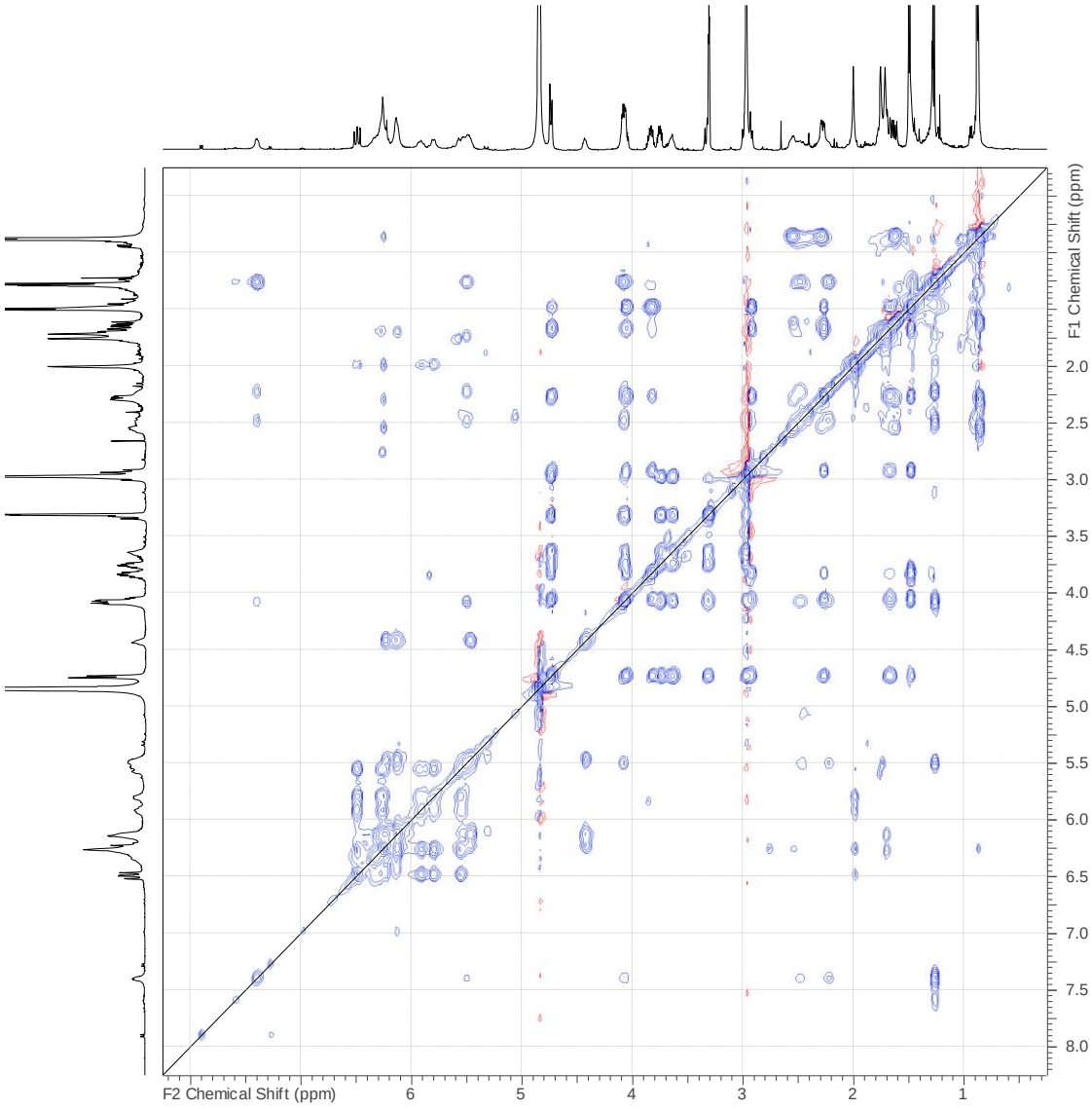
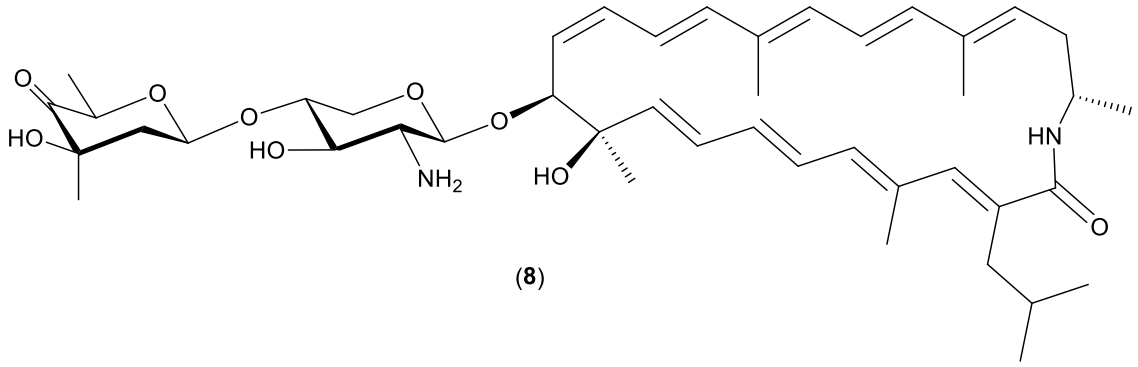


Fig. S63. TOCSY spectrum of 4’’-deamino-4’’-oxosipanmycin A (**8**).



(**8**)

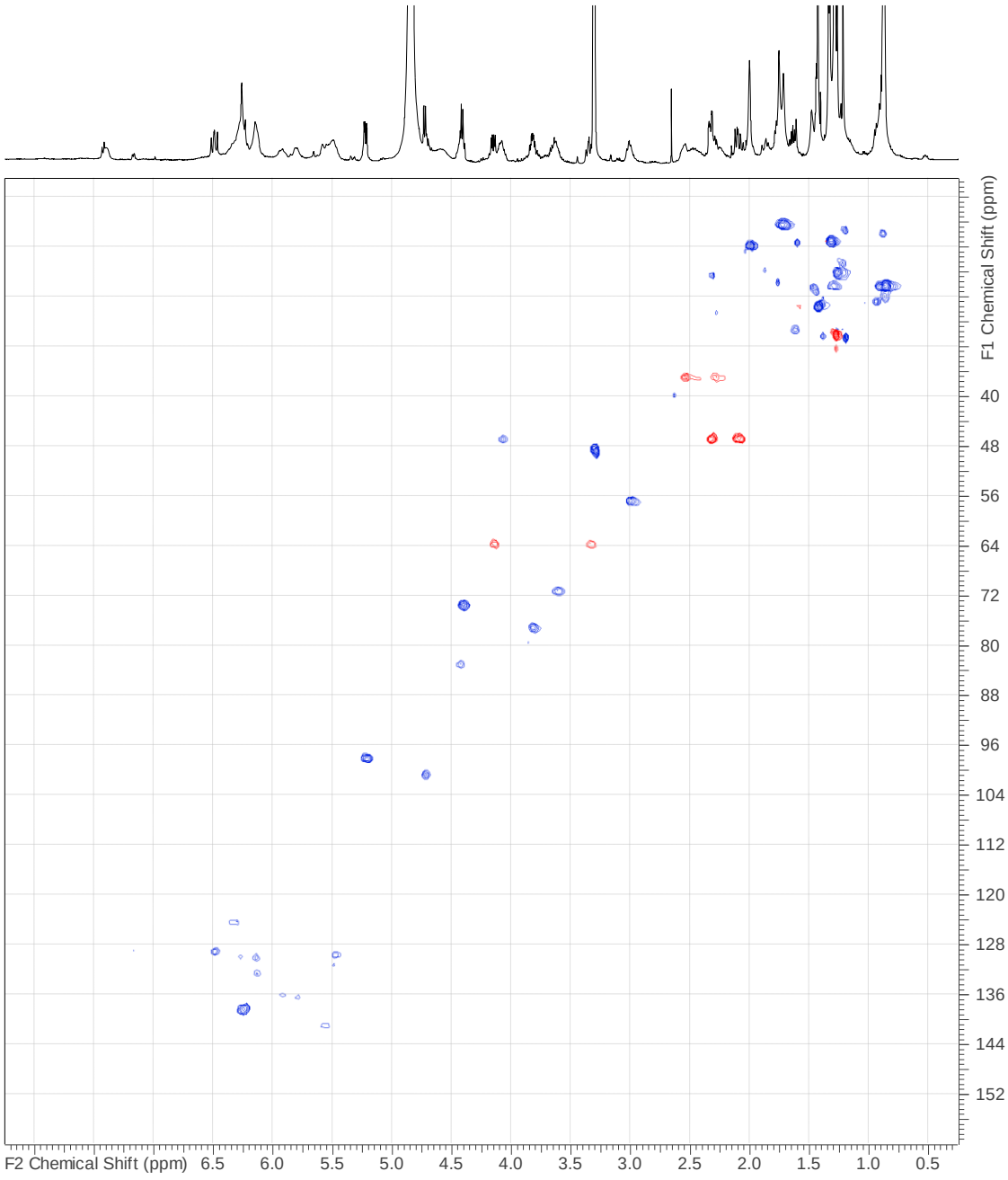
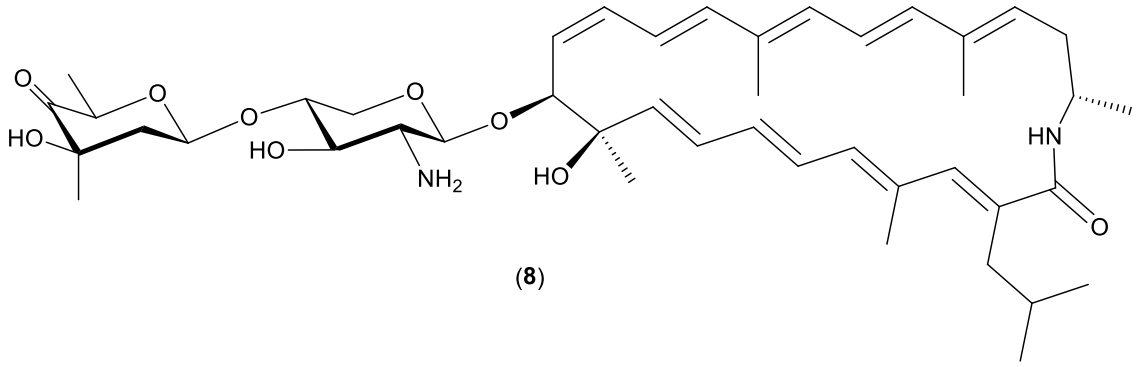


Fig. S64. Edited HSQC spectrum of 4’’-deamino-4’’-oxosipanmycin A (**8**).



(**8**)

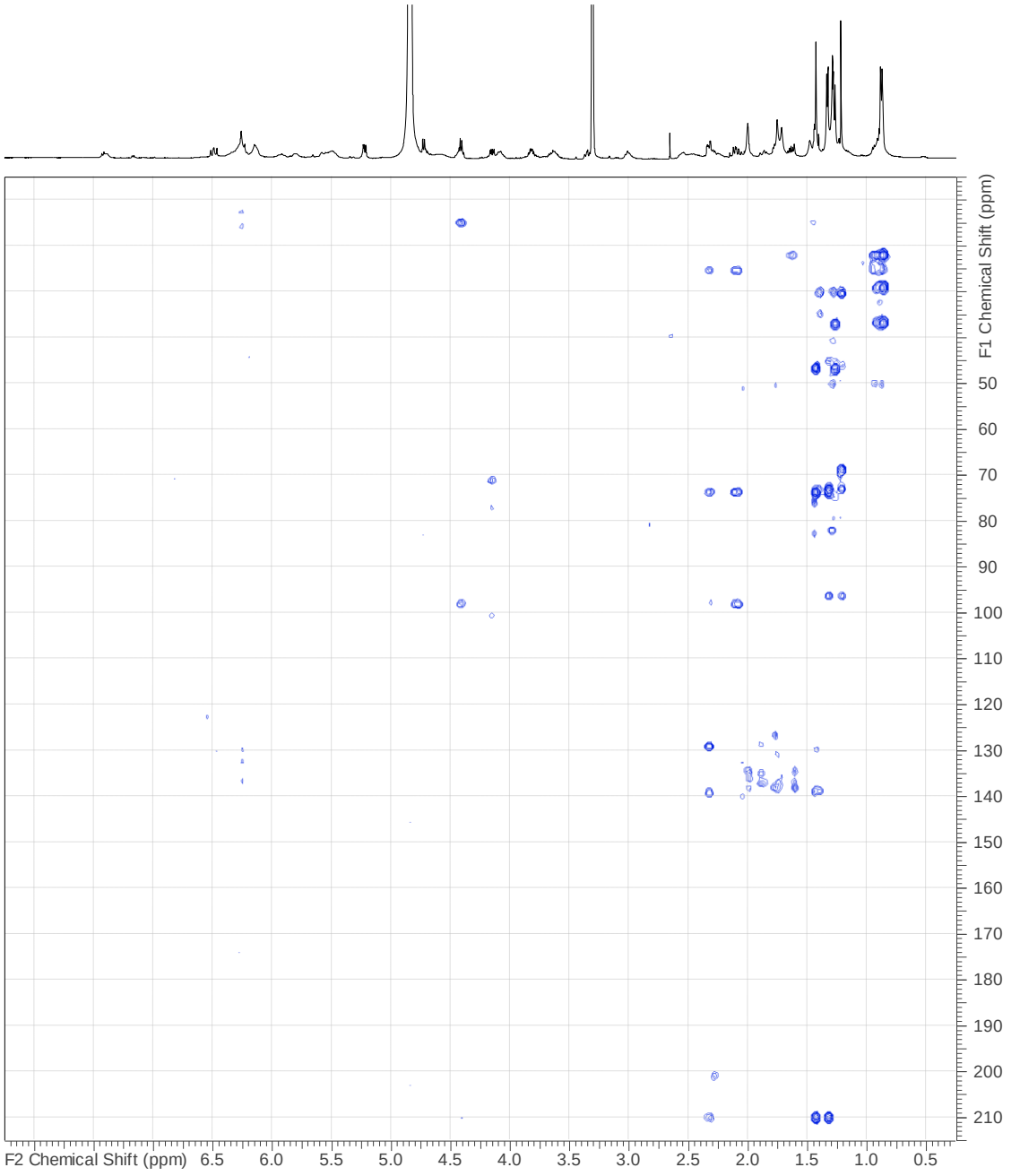


Fig. S65. HMBC spectrum of 4’’-deamino-4’’-oxosipanmycin A (**8**).

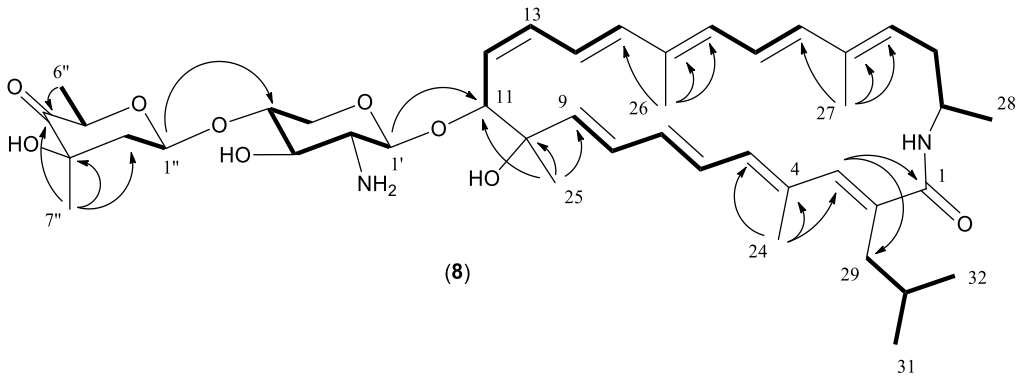


Fig. S66. Gross structure of 4’’-deamino-4’’-oxosipanmycin A (**8**).determined by 2D-NMR. COSY correlations (further corroborated by the spin systems observed in the TOCSY spectrum) are indicated as bold bonds. Key HMBC correlations connecting independent spin systems are indicated by arrows.

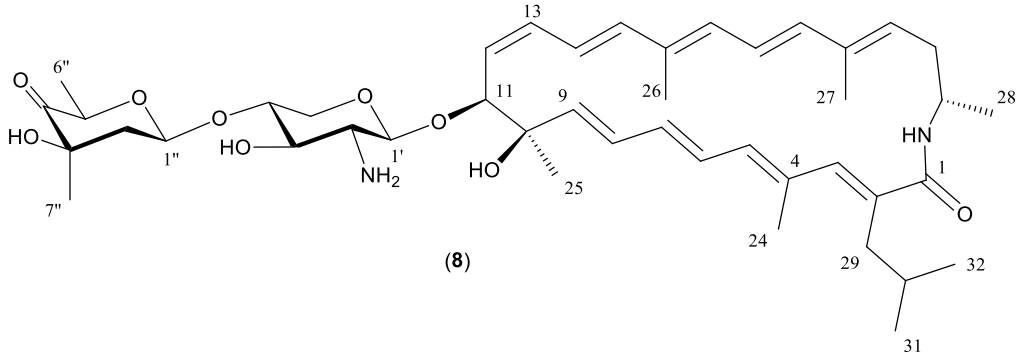


Fig. S67. Structure of 4’’-deamino-4’’-oxosipanmycin A (**8**).

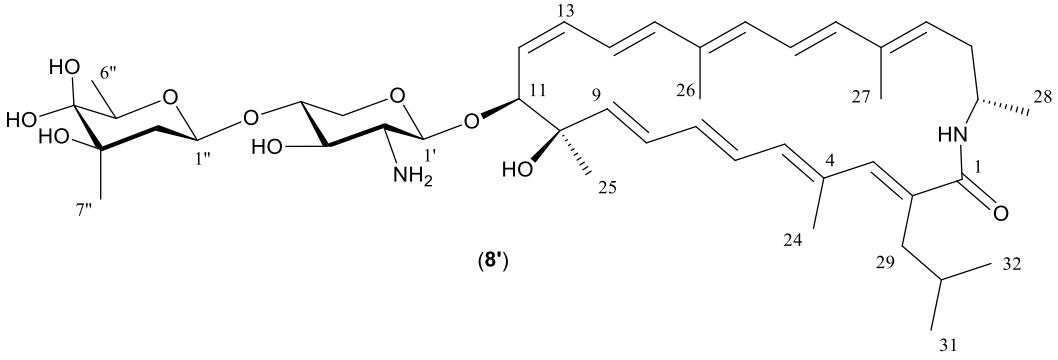


Fig. S68. Structure of artifact 4’’-deamino-4’’-dihydroxysipanmycin A (**8’**) produced by hydration of the ketone functional group in **8** under acidic chromatographic conditions.

Table S7. ^{1}H and ^{13}C NMR data for 4''-deamino-4''-oxosipanmycin A (**8**) in CD_3OD at 24 C°.

Position	δ_C, type	δ_H (*J* in Hz)	Position	δ_C, type	δ_H (*J* in Hz)
1	174.2, C	-	1'	100.9, CH	4.73, d (7.5)
2	n. d., C	-	2'	57.1, CH	3.01, m
3	138.6, CH	6.26, m	3'	71.4, CH	3.62, m
4	n. d., C	-	4'	77.4, CH	3.82, m
5	136.6, CH	5.80, m	5'	64.0, CH_2	4.15, dd (11.9, 4.8) 3.35, dd (11.8, 10.9)
6	129.2, CH	6.49, dd (14.5, 11.4)	1"	98.3, CH	5.22, dd (9.0, 2.8)
7	136.2, CH	5.92, m	2"	47.1, CH_2	2.33, dd (13.3, 2.6 2.10, dd (13.2, 9.1)
8	130.1, CH	6.27, m	3"	74.0, C	-
9	141.2, CH	5.56, m	4"	210.1, C	-
10	76.2, C	-	5"	73.7, CH	4.41, q (6.4)
11	83.2, CH	4.43, m	6"	15.4, CH_3	1.34, d (6.4)
12	129.9, CH	5.47, m	7"	25.9, CH_3	1.43, s
13	130.3, CH	6.14, m			
14	125.9, CH	6.13, m			
15	138.1, CH	6.24, m			
16	n. d., C	-			
17	132.8, CH	6.14, m			
18	124.5, CH	6.34, m			
19	138.6, CH	6.27, m			
20	n. d., C	-			
21	131.3, CH	5.51, m			
22	37.4, CH_2	2.50, m 2.26, m			
23	47.2, CH	4.08, m			
24	16.2, CH_3	2.00, br s			
25	22.9, CH_3	1.49, br s			
26	12.8, CH_3	1.72, br s			
27	12.8, CH_3	1.76, br s			
28	20.6, CH_3	1.28, d (6.9)			
29	37.2, CH_2	2.56, m 2.31, m			
30	29.5, CH	1.65, m			
31	22.5, CH_3	0.88, d (6.4)			
32	22.5, CH_3	0.88, d (6.4)			
1-NH	-	7.40 br s*			

^{13}C chemical shifts obtained from HSQC and HMBC spectra.

* The amide proton exchanges very slowly and is clearly observed in the spectra.

Table S8. Antibiotic activity of sipanmycins and derivatives.

	MIC (μM)			
	Micrococcus luteus	*Staphylococcus aureus*	*Escherichia coli*	*Candida albicans*
SipanmycinA	1.56	25	-	-
SipanmycinB	1.56	25	-	-
AgA (1)	3.12	50	-	-
AgB (2)	1.56	25	-	-
AgA+S1 (3)	6.25	35	-	-
dOHSipA (5)	1.56	25	-	-
dSipA (6)	1.56	17.5	-	-
dSipB (7)	1.56	17.5	-	-

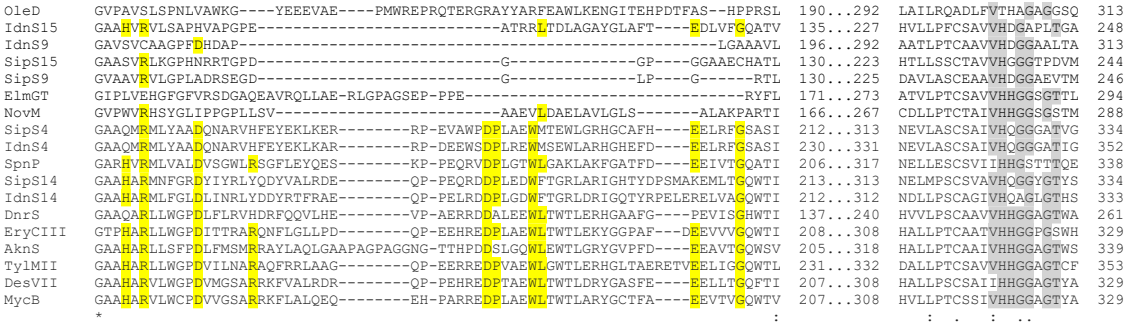


Fig. S68. Amino acid alignment of GTs involved in sipanmycin and incednine biosynthesis, together with several GTs funcionally characterized in other actinomycetes. Conserved residues of the active site and putative motif involved in GT-auxiliary protein interaction are highlighted in gray and yellow, respectively. ElmGT: elloramycin biosynthesis (*Streptomyces olivaceus*, Q9F2F9.2); IdnS15, IdnS9, IdnS4 and IdnS14: incednine biosynthesis (*Streptomyces* ML694-90F3, BAP34748.1, BAP34741.1, BAP34712.1 and BAP34746.1); NovM: novobiocin biosynthesis (*Streptomyces niveus*, Q9L9F5.1); AknS: aclacinomycin biosynthesis (*Streptomyces galilaeus*, Q9L4U6.1); EryCIII: erythromycin biosynthesis (*Saccharopolyspora erythraea*, O33939.1); DnrS: daunorubicin biosynthesis (*Streptomyces peucetius*, Q54824.1); DesVII: methymycin biosynthesis (*Streptomyces venezuelae*, Q9ZGH7.1); TylMII: tylosin biosynthesis (*Streptomyces fradiae*, P95747.3); OleD: oleandomycin biosynthesis (*Streptomyces antibioticus*, ABA42119.2); SpnP: spinosyn biosynthesis (*Saccharopolyspora spinosa*, 4LEI_A); MycB: mycinamicin biosynthesis (*Micromonospora griseorubida*, BAC57037.1).

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