Electronic Supplementary Material (ESI) for Organic & Biomolecular Chemistry.

Supporting information

Biosynthesis of a new skyllamycin analogue in *Streptomyces nodosus*: a cytochrome P450 forms an epoxide in the cinnamoyl chain

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Fig. S19. Expansion 1 (6.3-8.0 ppm region) of ^{1}H $^{-1}$H-TOCSY NMR spectrum.

Fig. S20. Expansion 2 (0.4-5.6 ppm region) of ^{1}H $^{-1}$H-TOCSY NMR spectrum.

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Fig. S23. Alignment of P450sky2 with Avm43, EpcF and DmlF.

Table S1. Biological activities of cinnamate-containing non-ribosomal peptide natural products.

CCNP	Biological activity	Reference
Skyllamycin	Inhibit PDGF pathway, antibacterial, biofilm inhibition and dispersal	13
WS9326A	Tachykinin receptor agonist, inhibits asparaginyl tRNA synthetase from parasite *Brugia malayi*, inhibits transcription in *Clostridium perfringens*	6
Mohangamide A	Inhibits *Candida albicans* isocitrate lyase	7
Coprisimides A and B	Inducers of quinone reductase, a phase II detoxification enzyme that protects against cancer	4
Pepticinnamin E	Inhibits farnesyltransferase	2
Atrovimycin	Antitubercular, antifungal	1
Atratumycin	Antitubercular	3
Kitacinnamycins	Activates stimulator of interferon gene (STING) protein	20
Nyuzenamides A and B	Antifungal, cytotoxic	8
Nyuzenamide C	Anti-angiogenic, induces quinone reductase in murine Hepa 1c1c7 cells	30

Table S2. Orphan P450s with > 85% sequence identity to P450sky2.

Microorganism	Genome accession number	P450sky2 homologue, % identity	NRPS
Streptomyces sp SID2888	WWKB01000082	99%	Incomplete
Streptomyces CB01373	NNBK01000012	88%	Skyllamycin
Streptomyces sp 6-11-2	BJOR01000001	88%	Skyllamycin
Streptomyces scopuliridis	NZ_JOEI01000022.1	85%	Skyllamycin
Streptomyces sp. CoT10	NZ_JAIQYX010000027.1	88%	10-module NRPS, skyllamycin-related

Table S3. Comparison between NMR spectral data for synthetic skyllamycin A (11) and isolated oxy-skyllamycin A.

Residue	Position	^{1}H-NMR (500 MHz, CD_3OD, 25 °C) δ_H ppm (mult., *J* = Hz)[a]	^{13}C-NMR δ_C ppm {from HSQC}	Synthetic skyllamycin A ^{1}H-NMR (600 MHz, CD_3OD) δ_H ppm
Thr	C=O	-	-	-
	α-CH	5.08-5.12 (m)	60.5	5.08
	β-CH	5.46-5.53 (m)	68.9	5.48
	CH_3	1.37 (d, *J* = 6.5 Hz)	15.9	1.35
Ala	C=O	-	-	-
	α–CH	4.06-4.10 (m)	51.3	4.06
	CH_3	1.49 (d, *J* = 7.0 Hz)	15.5	1.48
β-Me-Asp	C=O	-	-	
	α-CH	5.15-5.21 (m)	52.9	5.21
	β-CH	3.25-3.32 (m)	47.8	3.26
	CH_3	1.27 (d(br), *J* = 5.0 Hz)	13.1	1.30
	C=O	-	-	-
Gly	C=O	-	-	-
	α-CH_2	3.65-3.74 (m)	46.7	3.66/3.74
β-**OH-Phe**	C=O	-	-	-
	β-CH	4.56-4.60 (m)	71.5	4.54
	α-CH	4.61-4.66 (m)	53.9	4.63
	C-1	-	-	-
	CH-2/6	7.45	-	7.43
	CH-3/5	7.37	-	7.36
	CH-4	7.25	-	7.24
Pro	C=O	-	-	-
	α-CH	4.32-4.38 (m)	59.8	4.35
	β-CH_2	1.57-1.62 (m)/1.85-1.92 (m)	39.9	1.64/1.96
	γ-CH_2	1.25-1.35 (m)/1.72-1.77 (m)	29.0/22.6	1.37/1.76
	δ-CH_2	3.46-3.51 (m)/4.07-4.13 (m)	42.3	3.47/4.14
β-**OH-*O*-Me-Tyr**	C=O	-	-	-
	α-CH	4.81-4.85 (m)	57.8/57.6[b]	4.76
	β-CH	4.52 (d, *J* = 6.0 Hz)	73.4	4.50
	C-1	-	-	-
	CH-2/6	6.46-6.54 (m)	113.0/127.6	6.71/6.59
	CH-3/5	6.46-6.54 (m)	127.6/113.0	6.59/6.71
	C-4	-	-	-
	OCH_3	3.67 (s)	54.3	3.67
D-Trp	C=O	-	-	-
	α-CH	4.81-4.85 (m)	57.6/57.8[b]	4.72
	β-CH_2	3.10-3.16 (m) 3.37-3.42 (m)	27.8	3.16
	CH-2	7.15 (s)	123.5	7.18

	C-3	-	-	-
	C-3a	-	-	-
	CH-4	7.46-7.49 (m)	118.2	7.60
	CH-5	6.85 (t(br), *J* = 7.5 Hz)	118.8	6.94
	CH-6	7.05 (t, *J* = 7.5 Hz)	121.0	7.07
	CH-7	7.25-7.30 (m)	-	7.30
	C-7a	-	-	-
α-OH-Gly	C=O	-	-	-
	α-CH	5.34-5.38 (m)	73.6	5.44
D-Leu	C=O	-	-	-
	α-CH	4.28-4.34 (m)	55.1	4.35
	β-CH_2	1.57-1.62 (m)/1.80-1.85 (m)	23.5	1.63/1.82
	γ-CH	1.85-1.89 (m)	24.8	1.86
	CH_3	0.91 (d, *J* = 6.5 Hz)	20.3	0.93
	CH_3	1.01 (d, *J* = 6.5 Hz)	21.8	0.99
β-OH-D-Leu	C=O	-	-	-
	α-CH	4.45-4.48 (m)	54.8	4.46
	β-CH	3.81 (d, *J* = 9.0 Hz)	75.5	3.81
	γ-CH	1.70-1.75 (m)	31.1	1.72
	CH_3	0.85 (d, *J* = 7.0 Hz)	17.6	0.85
	CH_3	1.06 (d, *J* = 6.5 Hz)	18.2	1.04
Cinnamoyl	C=O	-	-	-
	CH-2	7.30 (d, *J* = 15.0 Hz)	123.0	7.19
	CH-3	7.81 (d, *J* = 15.0 Hz)	136.0	7.85
	C-4	-	-	-
	CH-5	7.95 (d, *J* = 7.5 Hz)	126.6	7.90
	CH-6	7.25-7.30 (m)		7.29
	CH-7	7.25-7.30 (m)		7.29
	CH-8	7.25-7.30 (m)		7.15
	C-9	-	-	-
	CH-10	3.95 (s(br))	55.5	6.39
	CH-11	2.76-2.79 (m)	54.6	5.77
	CH_3-12	0.49 (m(br))	11.9	1.51

[a]mult. = signal multiplicity (m = multiplet, s = singlet (br = broad), d = doublet, t = triplet, *J* = coupling constant, value given in Hertz); [b]Signals coincident and under HDO signal in ^{1}H-NMR spectrum.

Table S4 P450Sky2 crystallographic data collection and refinement statistics

Parameter	Value
PDB entry	8FZ8
Wavelength (Å)	0.97946
Resolution range (Å)	35.84 - 1.43 (1.481 - 1.43)
Space group	P 1 2_1 1
Unit cell (Å and degrees)	35.98 97.8501 52.5 90 95.12 90
Total reflections	253782 (25702)
Unique reflections	64328 (6466)
Multiplicity	3.9 (4.0)
Completeness (%)	96.44 (97.29)
Mean I/sigma(I)	11.94 (2.90)
Wilson B-factor	15.55
R-merge	0.05438 (0.366)
R-meas	0.06255 (0.4233)
R-pim	0.0303 (0.209)
CC1/2	0.998 (0.896)
CC*	1 (0.972)
Reflections used in refinement	64284 (6465)
Reflections used for R-free	2000 (201)
R-work	0.1750 (0.2198)
R-free	0.1889 (0.2505)
CC(work)	0.966 (0.900)
CC(free)	0.970 (0.855)
Number of non-hydrogen atoms	3503
macromolecules	3162
ligands	54
solvent	287
Protein residues	392
RMS(bonds)	0.006
RMS(angles)	0.93
Ramachandran favored (%)	96.38
Ramachandran allowed (%)	2.84
Ramachandran outliers (%)	0.78
Rotamer outliers (%)	1.45
Clashscore	10.19
Average B-factor	21.88
macromolecules	21.31
ligands	16.53
solvent	29.16

Values in () are for statistics in the highest resolution shell. Statistics generated using by the “Table 1” utility in Phenix.

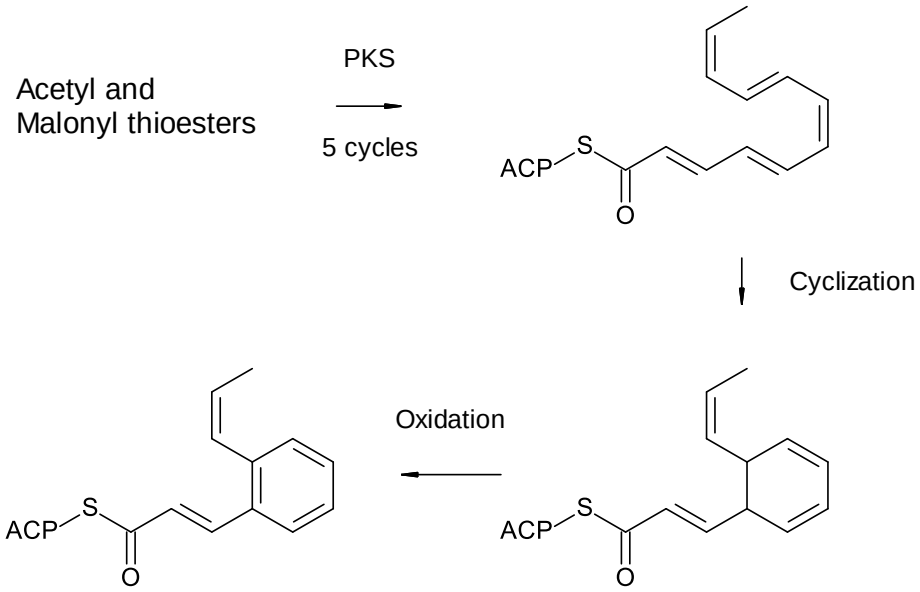


Fig. S1. Biosynthesis of the propenyl-cinnamoyl chain of skyllamycin A. An all-trans polyene polyketide intermediate undergoes trans-cis isomerization of double bonds, followed by electrocyclization and oxidation to give the aromatic ring. The cyclase involved in skyllamycin biosynthesis has not been identified but at least two classes of electrocyclase have been identified in biosynthetic pathways to other cinnamoyl-containing natural products (20).

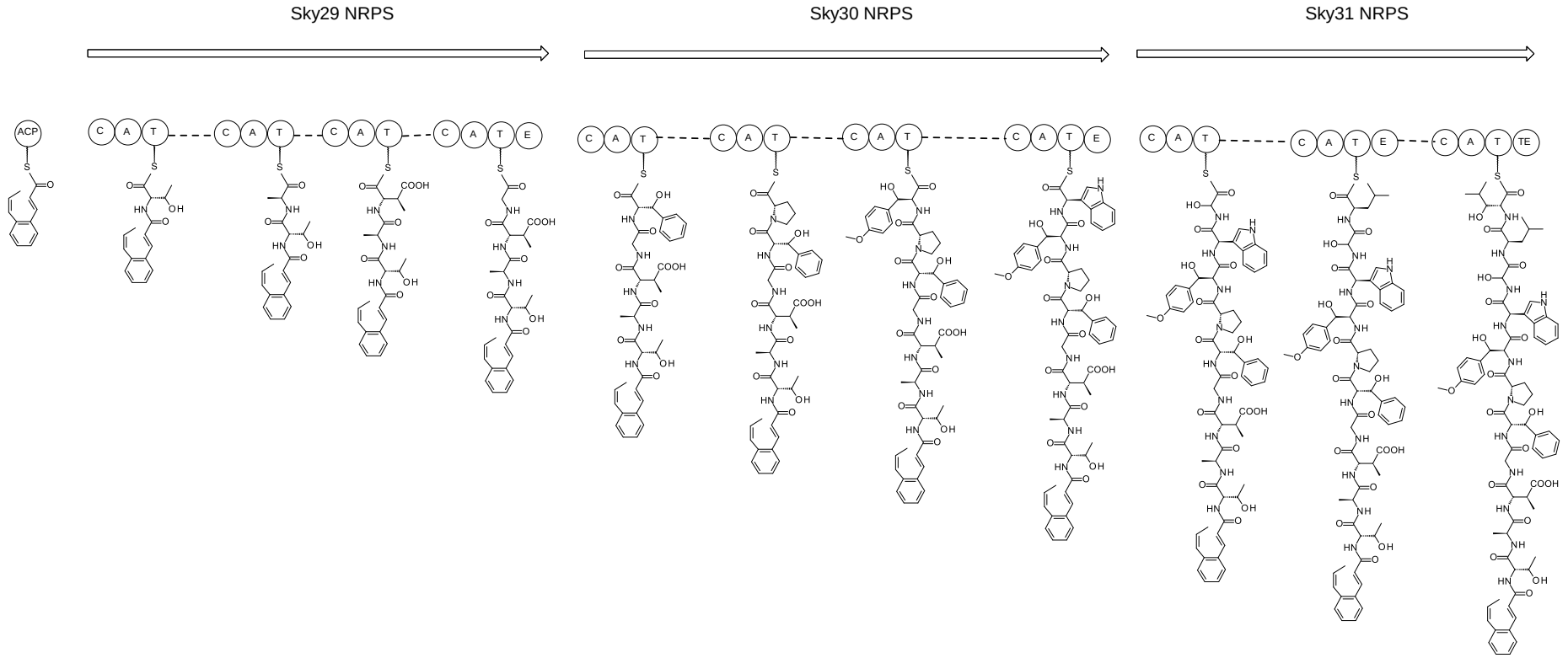
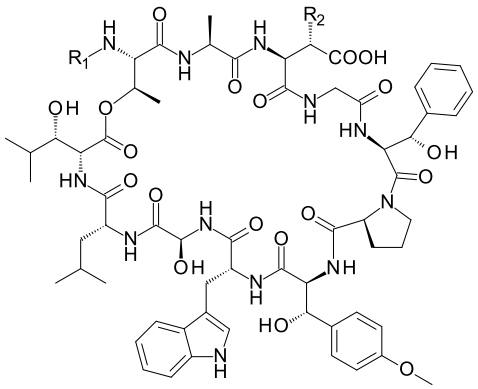
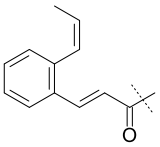


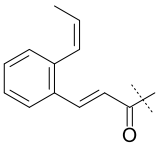
Fig. S2 Lipopeptide intermediates synthesized by the skyllamycin A NRPS.



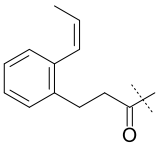
Skyllamycin A $R_2 = CH_3$, R_1 =



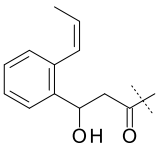
Skyllamycin B $R_2 = H$, R_1 =



Skyllamycin C $R_2 = H$, R_1 =



Skyllamycin D $R_2 = CH_3$, R_1 =



Skyllamycin E $R_2 = CH_3$, R_1 =

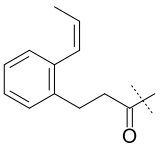


Fig. S3 Structures of skyllamycins A to E (10, 13, 14, 15).

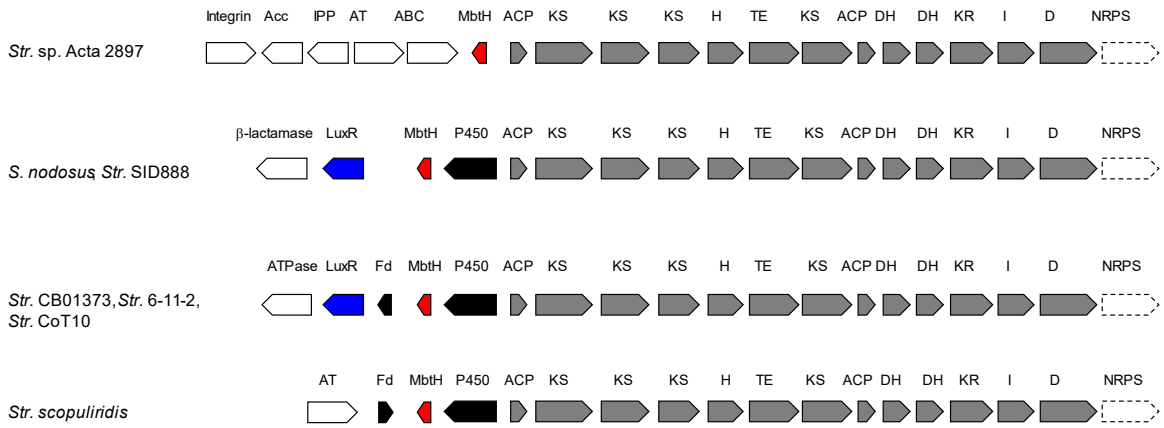
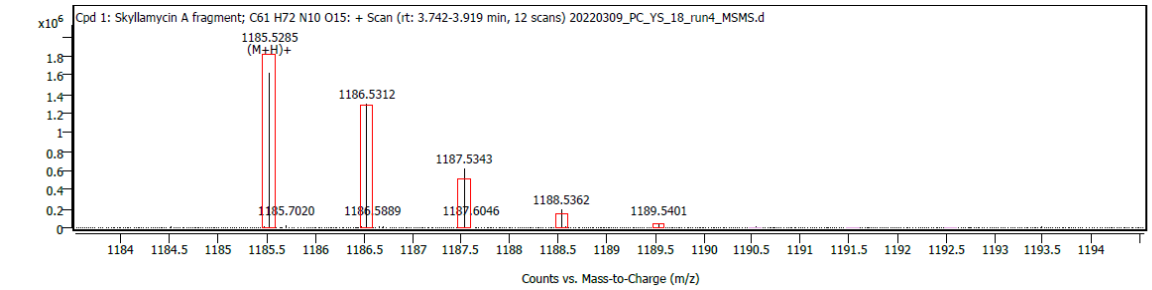


Fig. S4 Organization of propenyl-cinnamoyl biosynthetic genes in different skyllamycin BGCs (See also Table S2). *Streptomyces* sp Acta 2897 produces skyllamycins A and B and does not have the gene for P450Sky2 (black box). Abbreviations are as follows: ACP, acyl carrier protein; KS, ketosynthase; H, hydrolase; TE, thioesterase; DH, dehydratase; KR, ketoreductase; I, isomerase; D, dehydrogenase; Fd, ferredoxin. MbtH proteins stimulate the A domains of non-ribosomal peptide synthetases. The product of the LuxR gene (blue) activates the silent skyllamycin cluster in *S. nodosus*.

A



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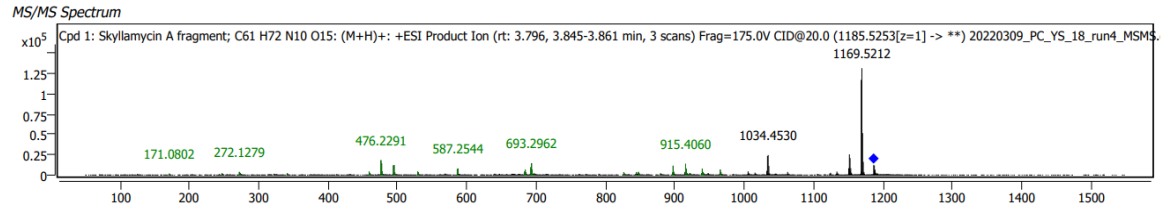


C

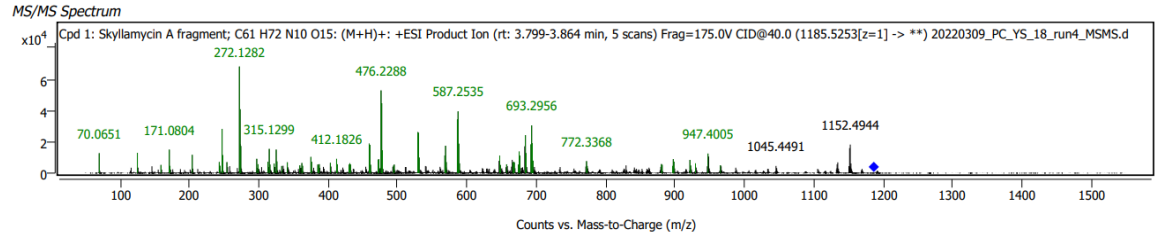
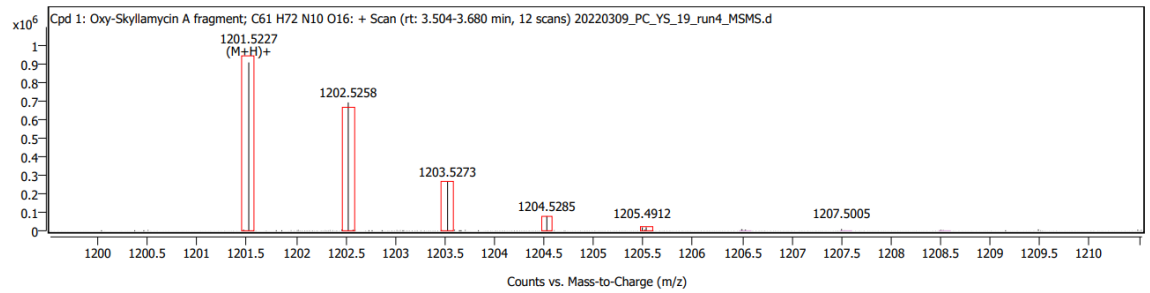
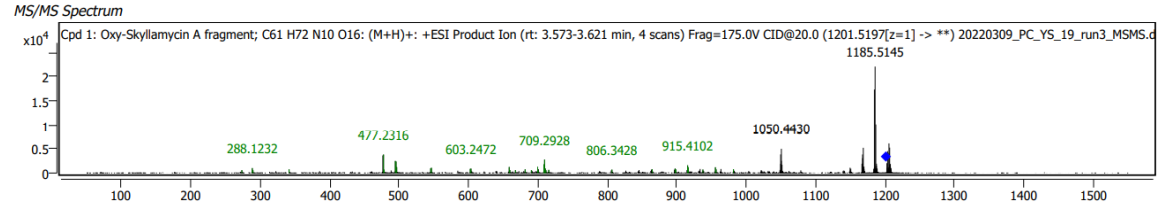


Fig. S5 Mass spectrometry of skyllamycin A fragment generated by ammonia cleavage of the intact cyclodepsipeptide. (A) Detection of acyl-octapeptide fragment, $[M + H]^+$ = 1185.5285. (B) MS-MS spectrum obtained with collision energy of 20 eV. (C) MS-MS spectrum obtained with collision energy of 40 eV.

A



B



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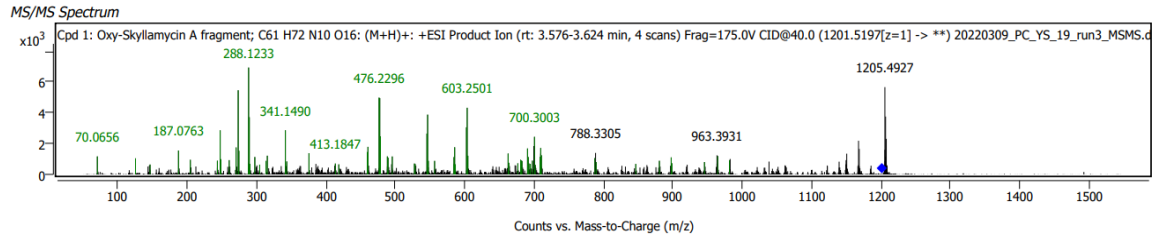
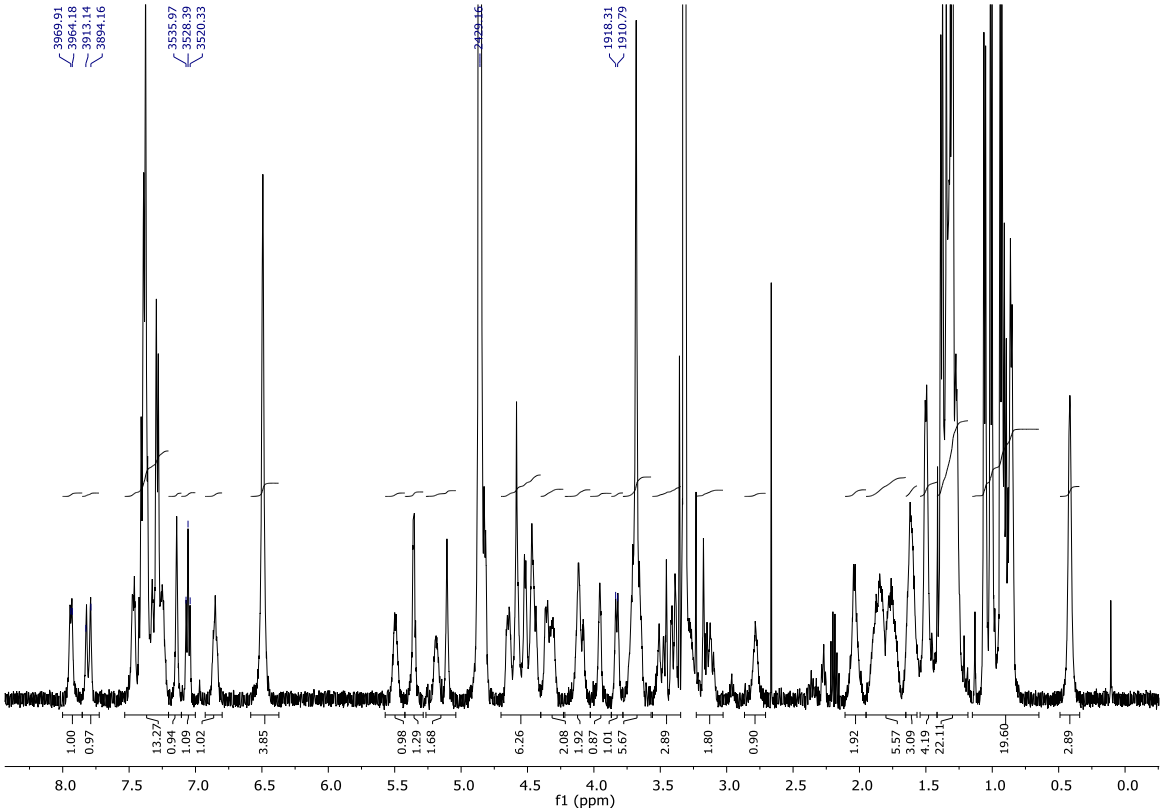


Fig. S6 Mass spectrometry of oxy-skyllamycin A fragment generated by ammonia cleavage of the intact cyclodepsipeptide. (A) Detection of acyl-octapeptide fragment, $[M + H]^+$ = 1201.5227. (B) MS-MS spectrum obtained with collision energy of 20 eV. (C) MS-MS spectrum obtained with collision energy of 40 eV.



	Parameter	Value
1	Title	YS1_PROTON_20220512_01
2	Solvent	CD_3OD
3	Temperature	25.0 °C
4	Experiment	1D
5	Number of Scans	128
6	Relaxation Delay	2.0000 sec
7	Acquisition Time	3.6000 sec
8	Spectrometer Frequency	500.03 MHz
9	Acquired Size	28846
10	Spectral Size	65536

Fig. S7 ^{1}H-NMR spectrum of oxy-skyllamycin (500 MHz, CD_3OD, 25 °C).

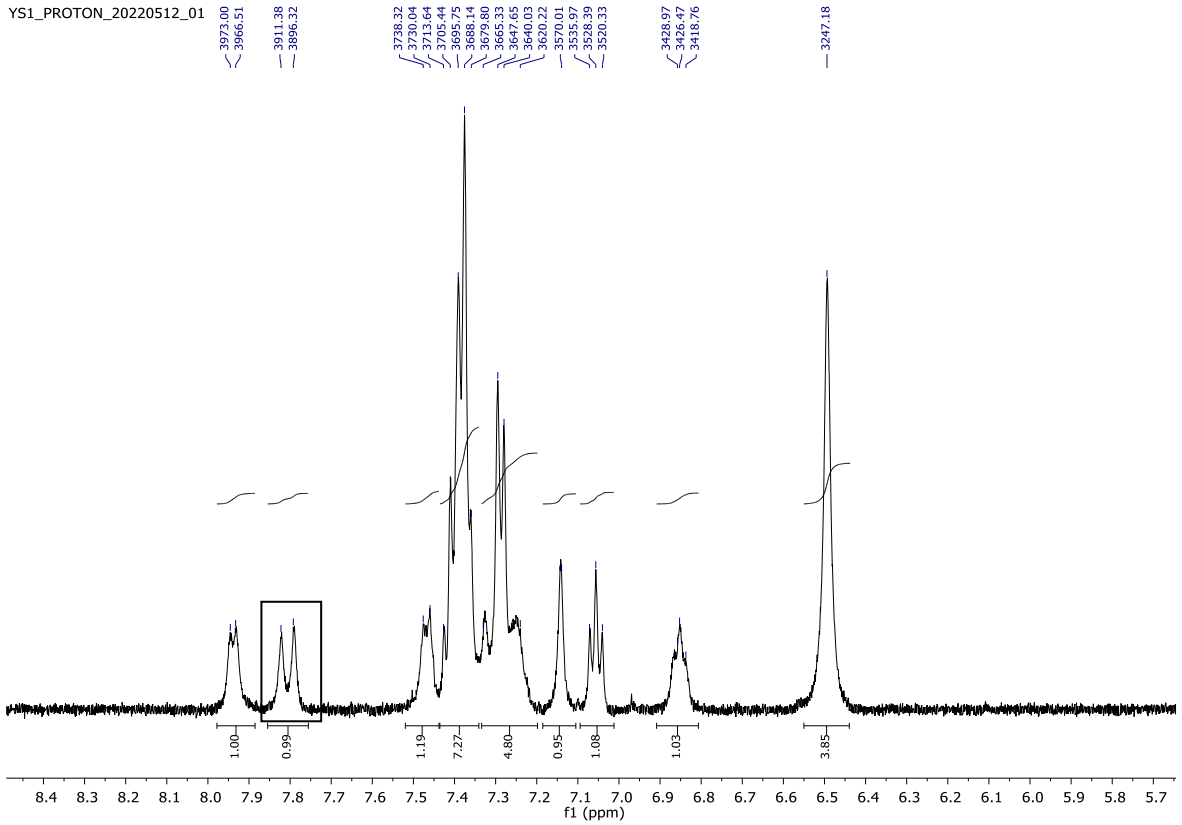


Fig. S8 Expansion 1 (5.7 to 8.4 ppm region) of the ^{1}H-NMR spectrum of oxy-skyllamycin (500 MHz, CD_3OD, 25 °C). The cinnamoyl β-CH is in the box.

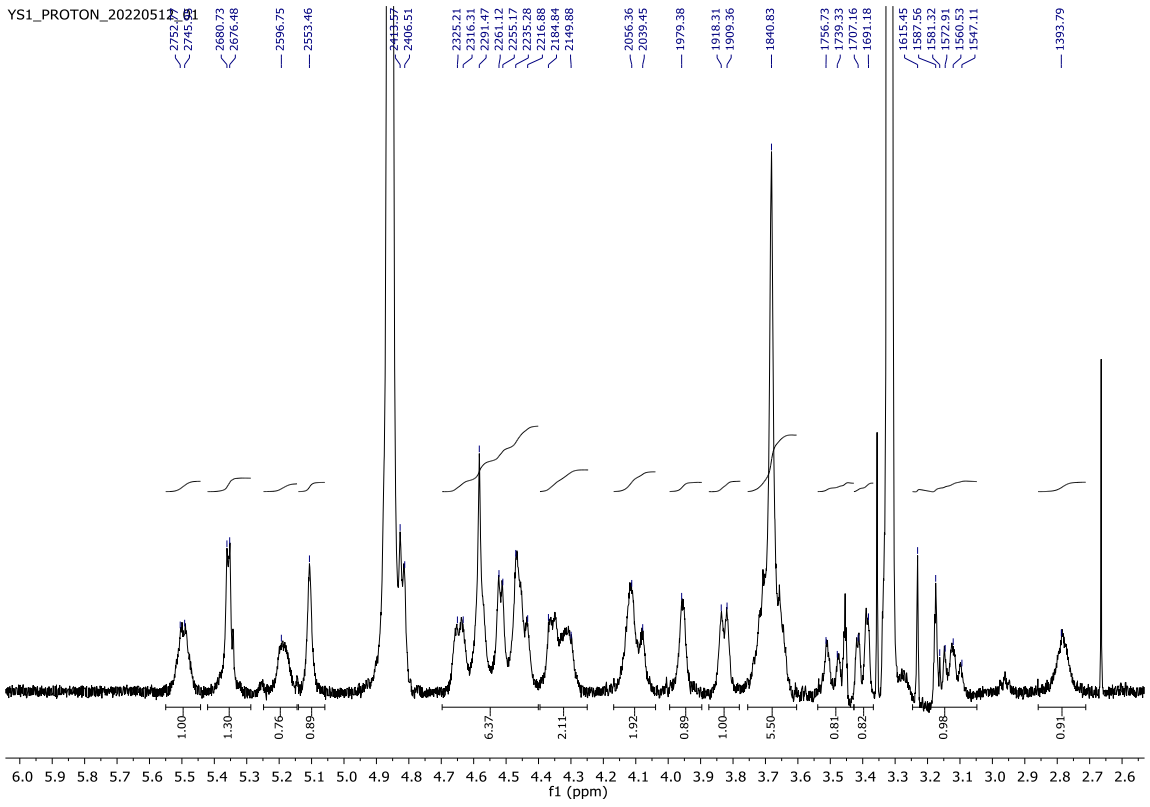


Fig. S9 Expansion 2 (2.6 to 6.0 ppm region) of the ^{1}H-NMR spectrum of oxy-skyllamycin (500 MHz, CD_3OD, 25 °C).

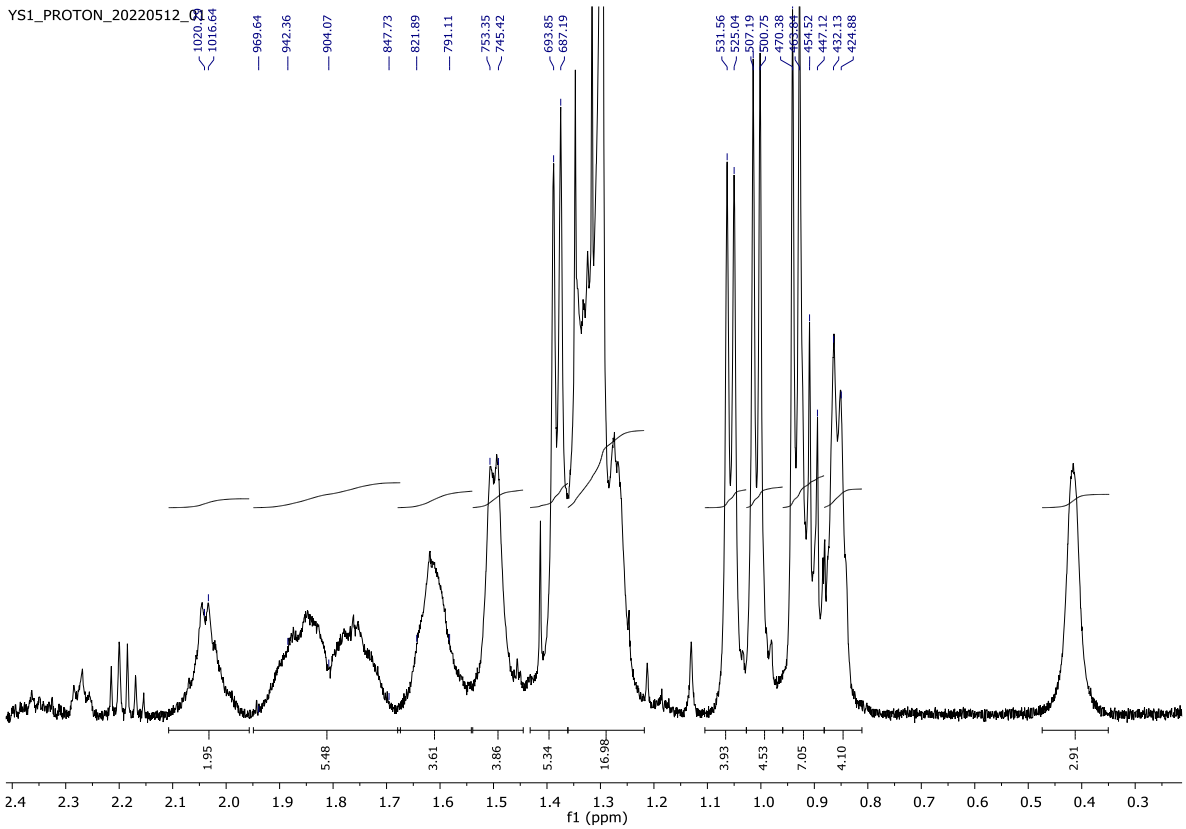
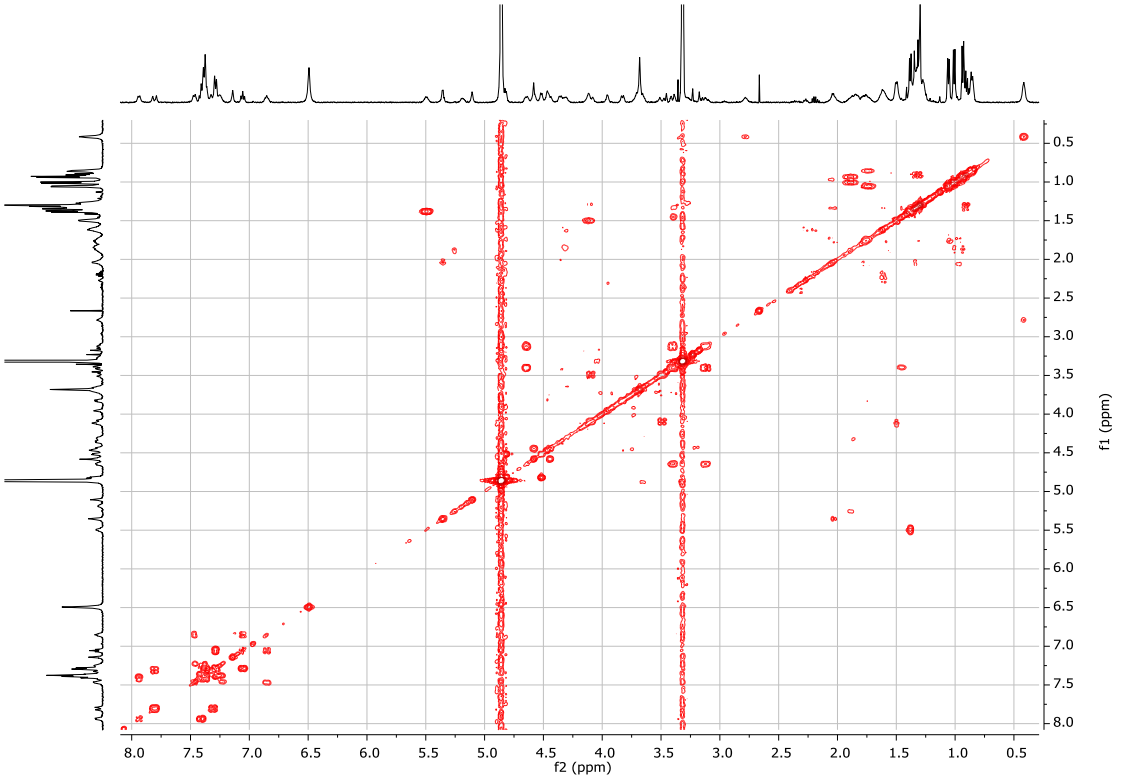


Fig. S10 Expansion 3 (0.3 to 2.4 ppm region) of the ^{1}H-NMR spectrum of oxy-skyllamycin (500 MHz, CD_3OD, 25 °C).



	Parameter	Value
1	Title	YS1_gCOSY_20220512_01
2	Solvent	CD_3OD
3	Temperature	25.0 °C
4	Experiment	COSY
5	Number of Scans	16
6	Relaxation Delay	1.0000 sec
7	Acquisition Time	0.1500 sec
8	Spectrometer Frequency	(500.03 MHz, 500.03 MHz)
9	Acquired Size	(597, 400)
10	Spectral Size	(1024, 1024)

Fig. S11 ^{1}H-^{1}H-gCOSY NMR spectrum for oxy-skyllamycin (500 MHz, CD_3OD, 25 °C).

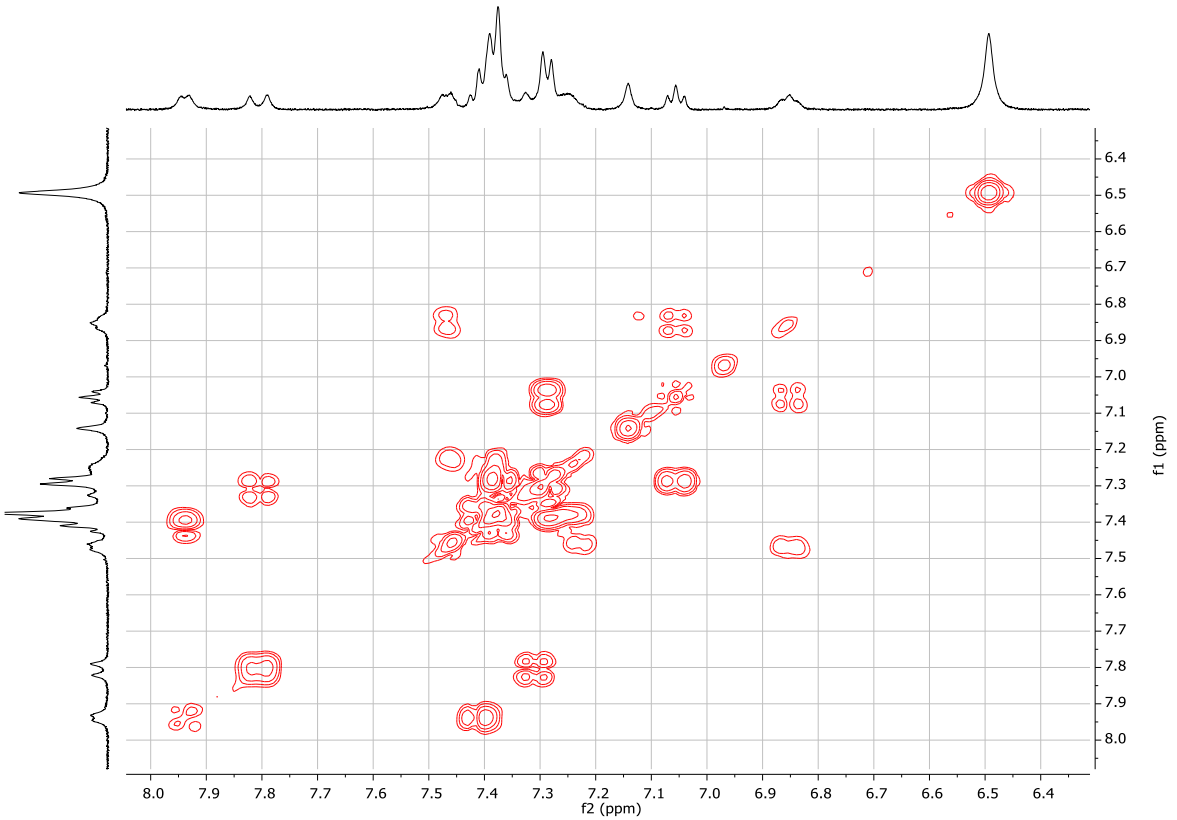


Fig. S12 Expansion 1 (6.4 to 8.0 ppm region) of ^{1}H-^{1}H-gCOSY NMR spectrum for oxyskyllamycin (500 MHz, CD_3OD, 25 °C).

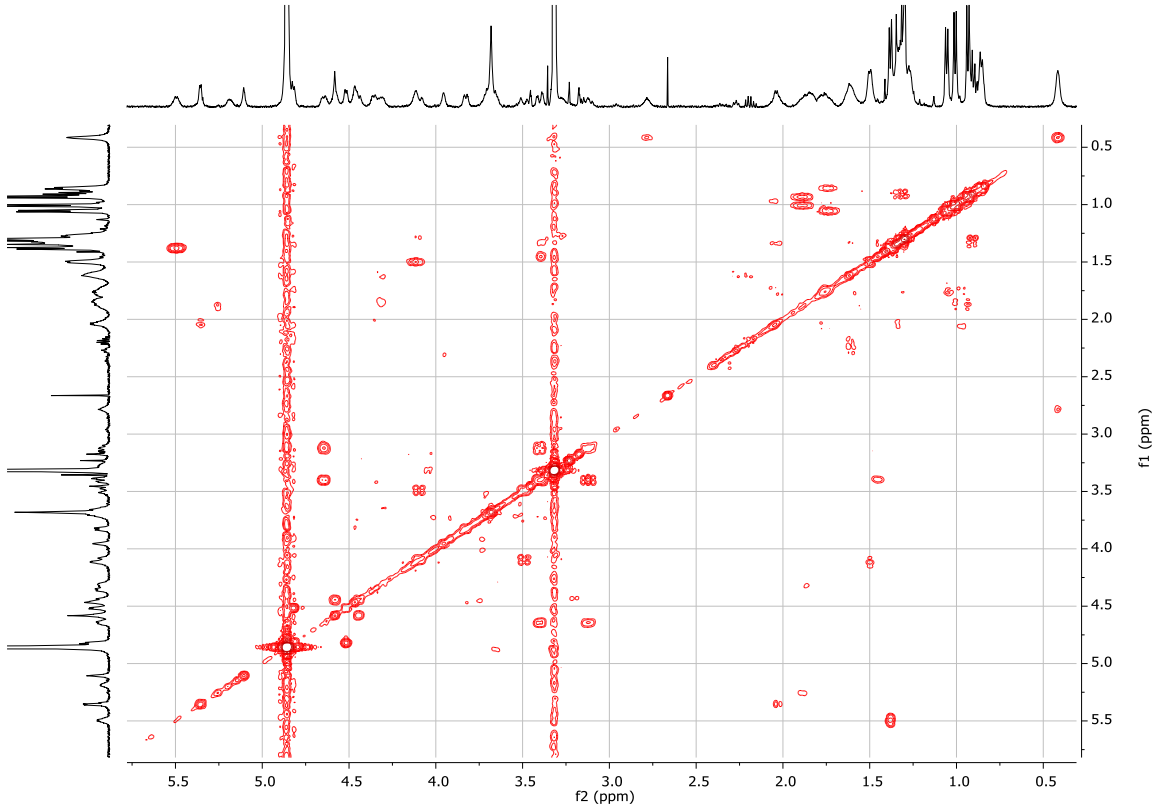
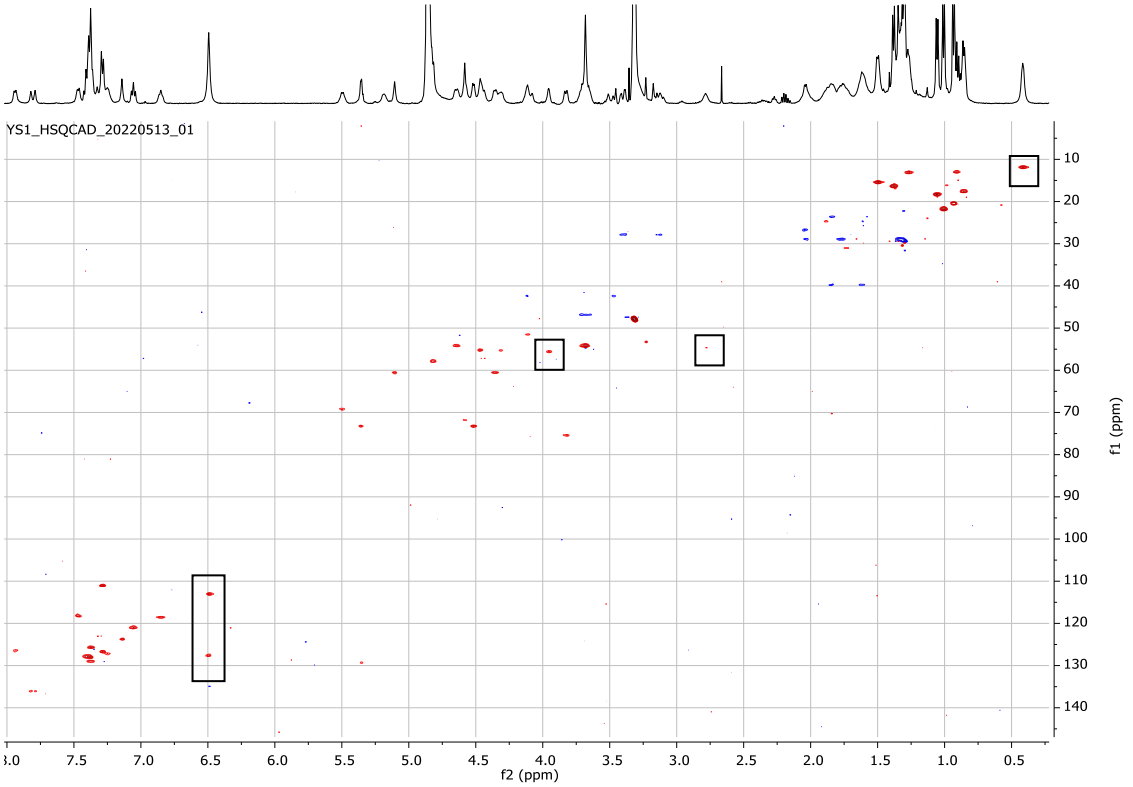


Fig. S13 Expansion 2 (0.5 to 5.5 ppm region) of ^{1}H-^{1}H-gCOSY NMR spectrum for oxyskyllamycin (500 MHz, CD_3OD, 25 °C).



	Parameter	Value
1	Title	YS1_HSQCAD_20220513_01
2	Solvent	CD_3OD
3	Temperature	25.0 °C
4	Experiment	HSQC-EDITED
5	Number of Scans	32
6	Relaxation Delay	1.0000 sec
7	Acquisition Time	0.1500 sec
8	Spectrometer Frequency	(500.03 MHz, 125.74 MHz)
9	Acquired Size	(597, 400)
10	Spectral Size	(1024, 1024)

Fig. S14 ^{1}H-^{13}C-HSQC NMR spectrum for oxy-skyllamycin (500 MHz, CD_3OD, 25 °C) – blue correlations represent CH_2 groups, red correlations represent CH, or CH_3 groups. Significant ^{1}H-^{13}C connections are indicated in boxes.

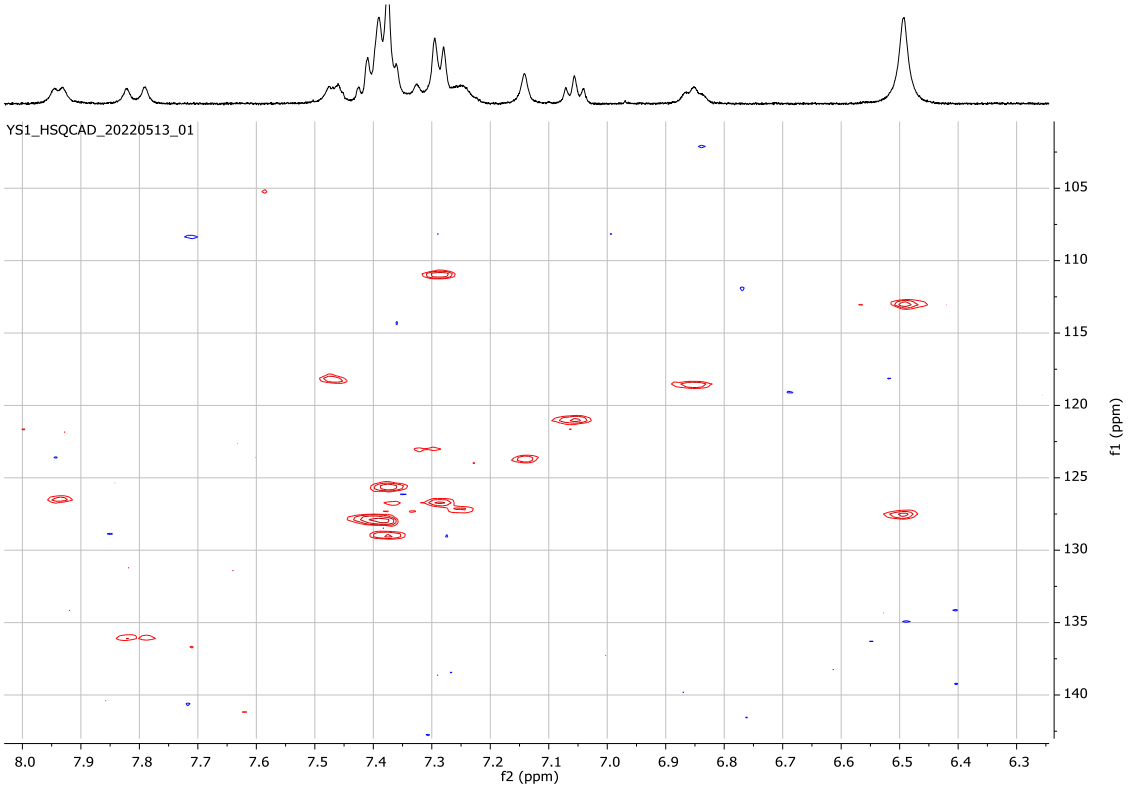


Fig. S15 Expansion 1 (6.3-8.0 ppm/100-145 ppm region) of ^{1}H-^{13}C-HSQC NMR spectrum for oxy-skyllamycin (500 MHz, CD_3OD, 25 °C).

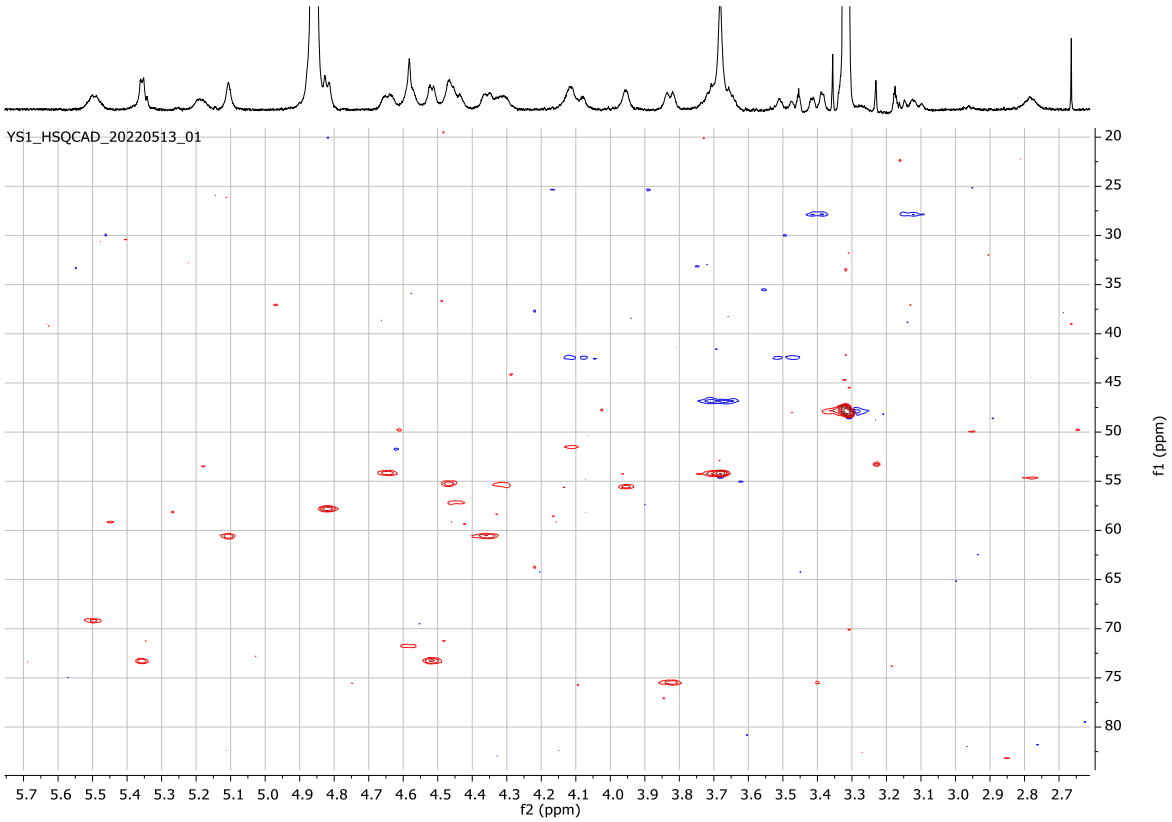


Fig. S16 Expansion 2 (2.7-5.7 ppm/20-80 ppm region) of ^{1}H-^{13}C-HSQC NMR spectrum for oxy-skyllamycin (500 MHz, CD_3OD).

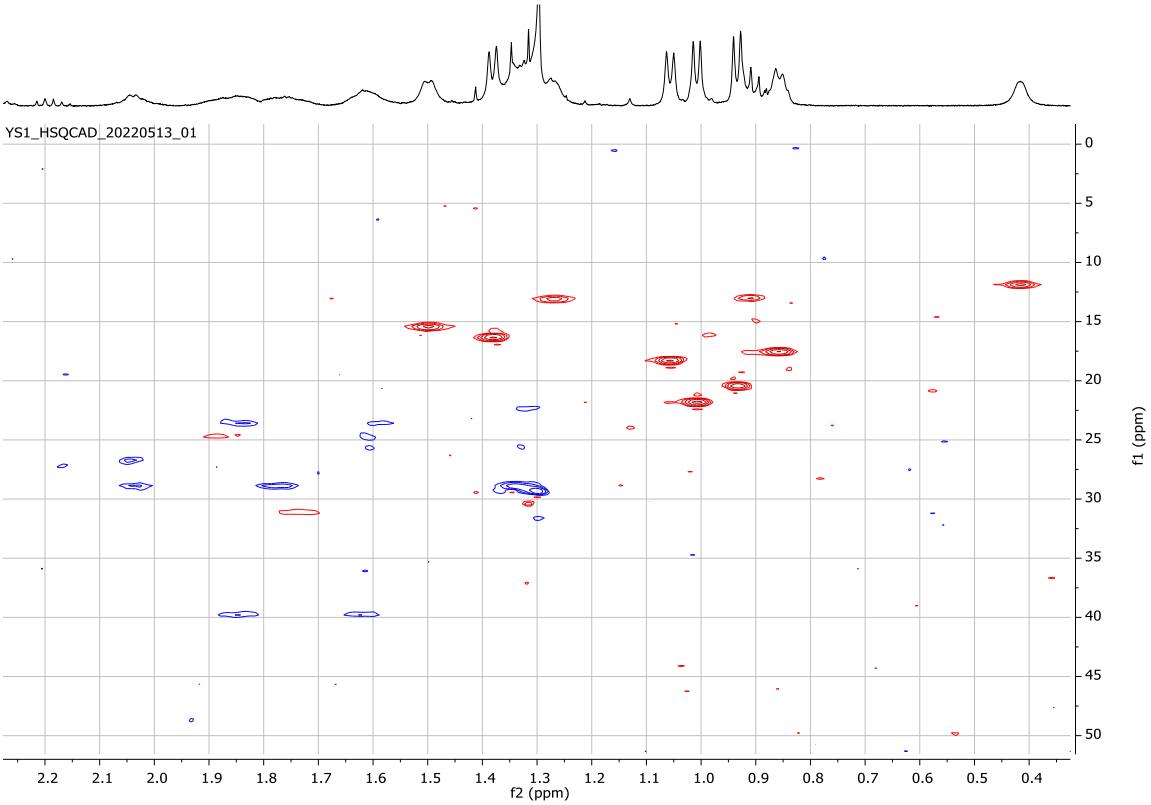
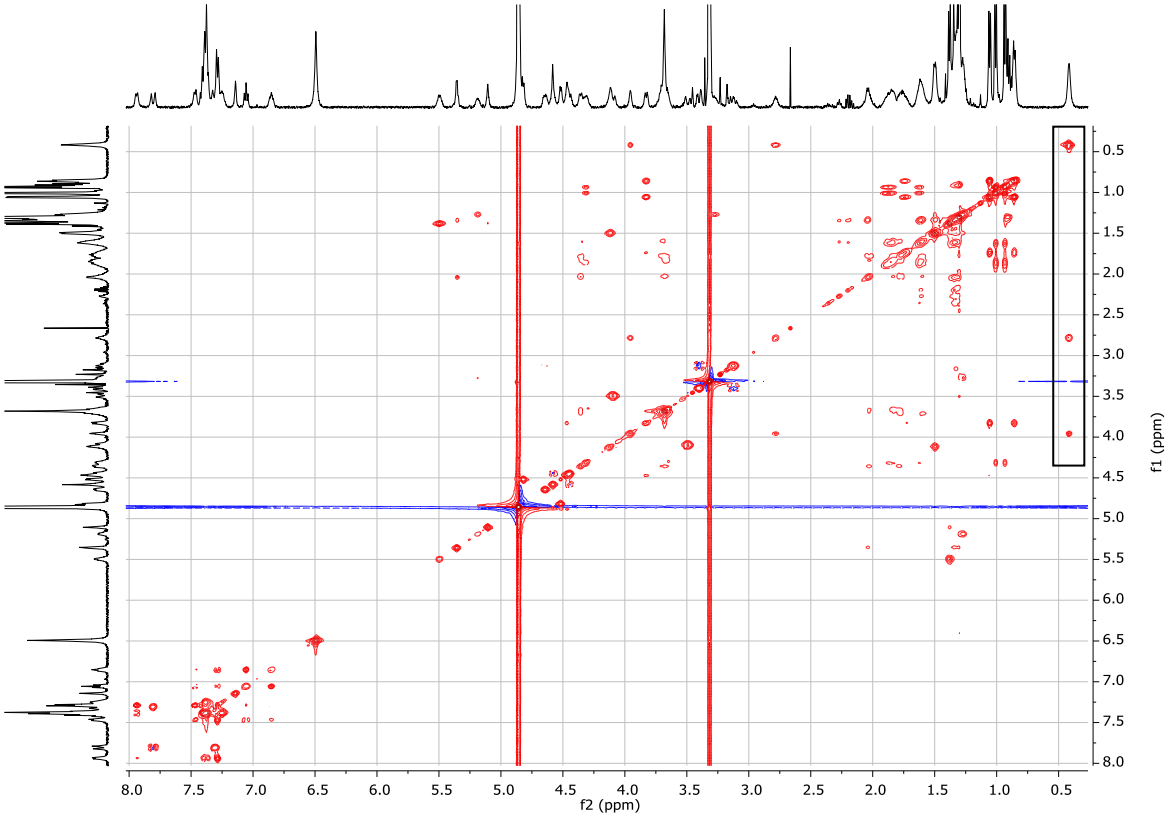


Fig. S17 Expansion 3 (0.4-2.2 ppm/0-50 ppm region) of ^{1}H-^{13}C-HSQC NMR spectrum for oxyskyllamycin (500 MHz, CD_3OD, 25 °C).



	Parameter	Value
1	Title	YS1_TOCSY_20220512_01
2	Solvent	CD_3OD
3	Temperature	25.0 °C
4	Experiment	TOCSY
5	Number of Scans	16
6	Relaxation Delay	1.0000 sec
7	Acquisition Time	0.1500 sec
8	Spectrometer Frequency	(500.03, 500.03)
9	Acquired Size	(597, 400)
10	Spectral Size	(1024, 1024)

Fig. S18 ^{1}H-^{1}H-TOCSY NMR spectrum for oxy-skyllamycin (500 MHz, CD_3OD, 25 °C) – epoxide spin system indicated in the box.

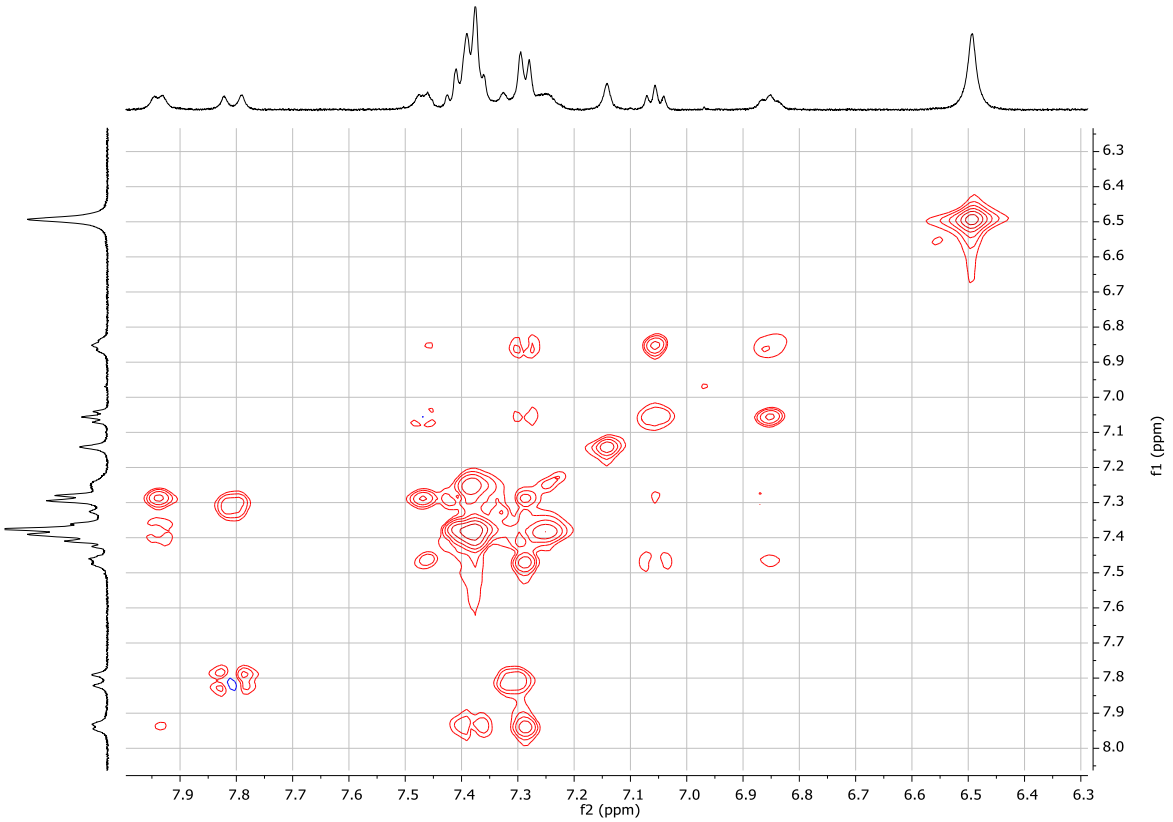


Fig. S19 Expansion 1 (6.3-8.0 ppm region) of ^{1}H ^{-1}H-TOCSY NMR spectrum.

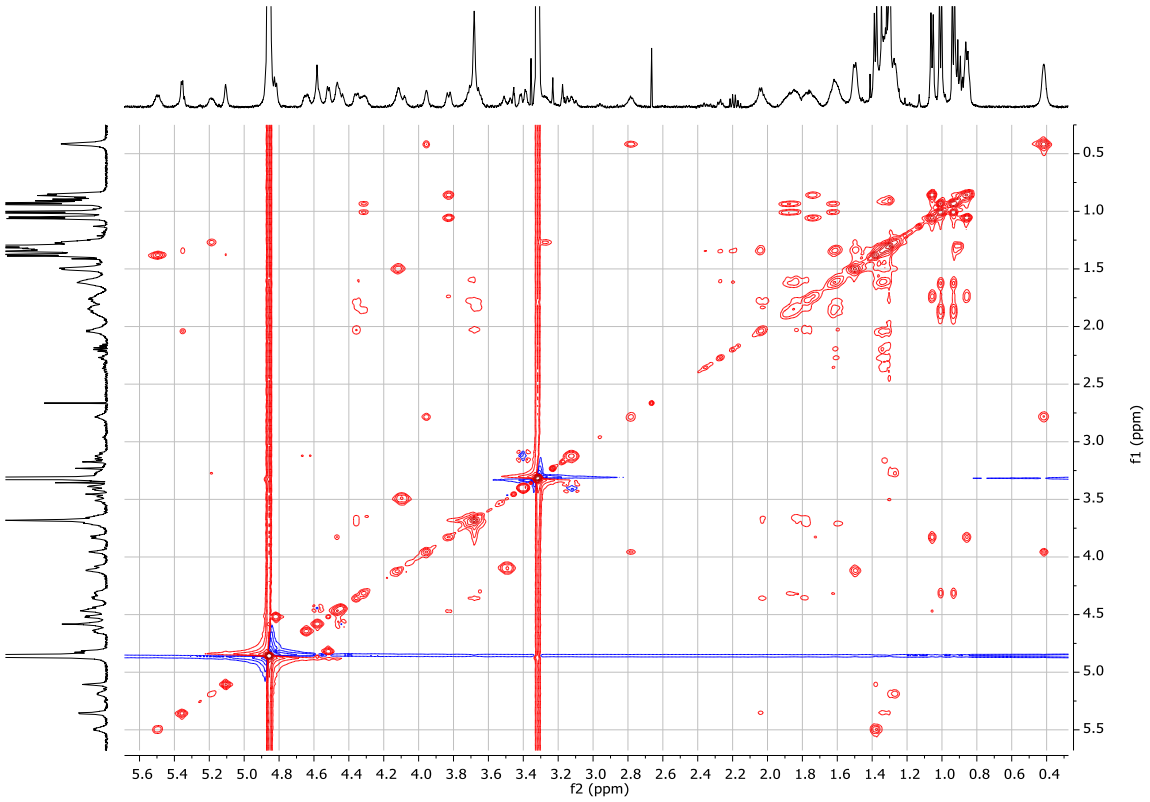
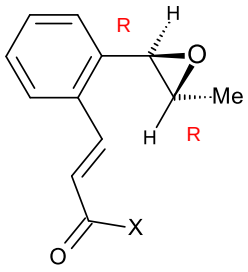
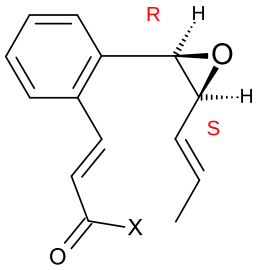


Fig. S20 Expansion 2 (0.4-5.6 ppm region) of ^{1}H ^{-1}H-TOCSY NMR spectrum.

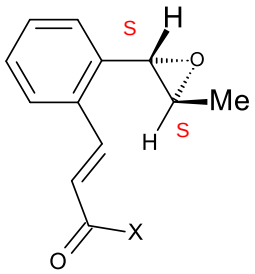
A



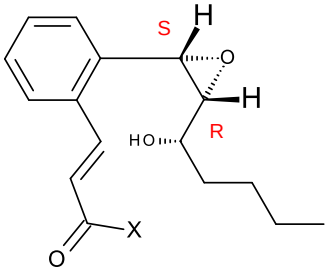
Nyuzenamide C
trans epoxide
DmlF P450



Atrovimycin
cis epoxide
Avm43 P450

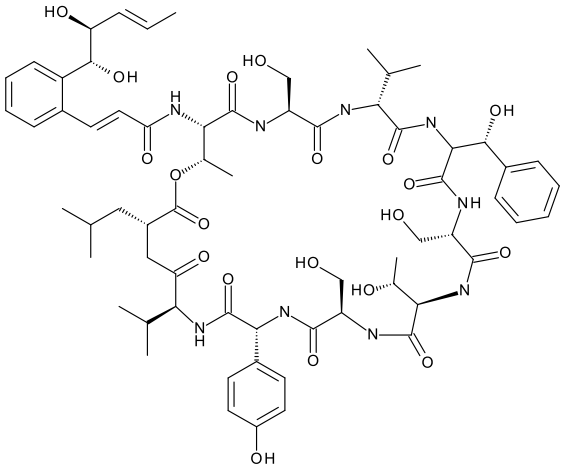


Epoxinnamide
trans epoxide
EpcF P450

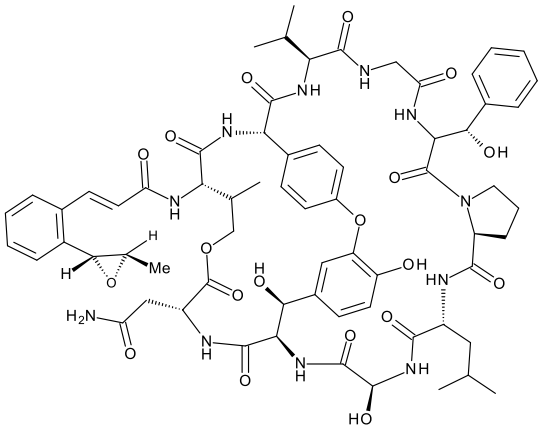


NC1
cis epoxide
Unknown P450

B



Atrovimycin



Nyuzenamide C

Fig. S21 A. Chiral epoxides formed during biosynthesis of cinnamoyl chains of CCNPs. X represents the remainder of the peptide macrocycle. The reported structures of nyuzenamide C, the epoxide-containing atrovimycin intermediate, epoxinnamide and NC1 suggest that the epoxide-forming P450s give all four possible stereochemical outcomes. B. Complete structures of atrovimycin and nyuzenamide C. The epoxide in the atrovimycin cinnamate is hydrolyzed in the final peptide.

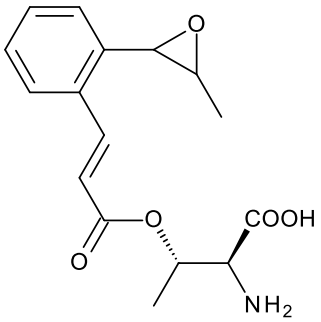


Fig. S22 Epoxy-cinnamoyl-threonine made by *Streptomyces* HS-NF-1222A

```
Avm43          MRGENTMEQRKISKYWMLTDDFTQNPYPVLERVREEQPVCELSLPDGGRAWVVTRHEDAK   60
P450Sky2       -----MAAGGALSKYWMFSDEYTQNPYPIFSTLRSEQPVTMVQTPDGARAWVITRHEDVR   55
DmlF           -----MTHTGTLSRYWMFSDEYTQDPYPFLAQLREEQPVCRVETPDGVRAWVVSRYDDVR   55
EpcF           -----MTNTGTLSRYWMFSDEYTQDPYPFLAQLRAEQPVCRVETPDGVRAWVVSRYDDVR   55
                         :*:***::*::**:***.:  :* ****  :. *** ****::*::*.:

Avm43          AALSDPRLSRDINVHFDLVSRLTGTTLTPPPEHANHLANLEPPRHTPLRKAISSAFTPRR   120
P450Sky2       NALADPRLSRDIGNLYQALGRQIGKELKPTDEITHHLANSDPPRHTRLRKALVFAFTPKR   115
DmlF           DALSDPRLGRDIGKLYAALGRQLGQDIKPADEISNHLANSDPPRHTPLRKALTFAFTPKR   115
EpcF           DALSDPRLGRDIGKLYAALGKQLGQEIKPADEISNHLANSDPPRHTPLRKALTFAFTPKR   115
                ***:****.***.  :  :.:  *  :.*  * ::**** :***** ****:  ****:*

Avm43          ADALRPQIETVADELLDRMAGAGGADLIAAYADPLPVIVIATLMGVPAAAWPDFLRWSTQ   180
P450Sky2       VANMRPRLEQVVEGLLDELAAQHQPDLLEGLAEPLPIIAIAQLLGVPDSDWRQFKIWSNT   175
DmlF           VRGLRDGWGKVVDDLLDEMVRTGNRDLVSGLNEPLPIITIAQLMGVPDTDWPRFLVWTNT   175
EpcF           VRGLRDGWEKVVDDLLDEMVRTGNRDLVSGLNEPLPIITIAQLMGVPDADWPRFLVWTNT   175
               .  :*     *.: ***.:.     **: .  :***:*.** *:*** : *  *  *:.

Avm43          LRAVQATDPAA--DGTVKELSSYMSALIAEKEREPGDDLISALIHADPDRRLTGTEILST   238
P450Sky2       MRSTDAADPTGLLAEHTRELSAYMADLIAEKERHPTDDLISAMVHAEGDKQLTPKEILST   235
DmlF           LRRVDASDPTGIIAEHTRQLSDYLKALIAAKQRDPQDDLISALVHADEDRRLTAAEALST   235
EpcF           LRRVDASDPTGIIAEHTRQLSDYLKALIATKQRTPQDDLISALVHADEDKRLTAAEALST   235
               :* .:*:**:.     .::** *:  *** *:* * ******::**: *::**  * ***

Avm43          SFALMTGGNDTTASLLGGVLHALLTHPGERAELLAAPGRWVAEMDELIRYVSPITNTLQR   298
P450Sky2       AFALMTGGNETTTALVTGCFAALLTHPEQAKRLKADLDRLPQVVDELIRFSSPMLYTLQR   295
DmlF           AFALMTGGNDTTTSLLNGSFAALLTHTGEADKIRADWSLLPNAVEELLRYTSPLINSLQR   295
EpcF           AFALMTGGNDTTTSLLNGSFAALLTHTGEADKIRADWSLLPNAVEELLRFTNPLINSLQR   295
               :********:**::*: * : *****  :  .: *  .     ::**:*: .*:  :***

Avm43          VTLEPVEIGGVTIPADEVVIISVISTNRDRCPFPERPDELDLKRPKPAHLSFGHGIHYCS   358
P450Sky2       LTLEDVEIAGTTIPAGEILMLSPASANHDPEALPDRPDELDIDRPRPVHLTFGHGIHYCI   355
DmlF           VTLEPVELCGVKIPKDEIIIISLAGANHDPHAFPDRPAELDITRPRPAHMSFGHGIHYCL   355
EpcF           VTLEPVELCGVKIPKDEIIIISLAGANHDPHAFADRAAELDITRPKPAHVSFGYGIHYCL   355
               :*** **: *..** .*::::*  .:*:*   : :*  ***: **:*.*::**:*****

Avm43          GAHLAKLMTEVAVRRFFERFPDARLAVDPSALRHTQSMVVRPWESLPVVW*   408
P450Sky2       GSHLARAQAEISIRRVLERFPDVRLAVHPSELRYRPALLARAPVALPVRL-   405
DmlF           GSHMSKALTELAIRRVFERFPDIRLAVHPSEVRYRPGLMVRPMIDLPVRF-   405
EpcF           GSHMSKALTELAIRRVYERFPGIRLAVHPSEVRYRPGLMVRPMIDLPVRF-   405
               *:*:::  :*:::**. ****. ****.** :*:  .::.*    ***
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Fig. S23 Alignment of P450Sky2 with Avm43, DmlF and EpcF epoxidases. P450Sky2 shows 63% sequence identity with EpcF and DmlF and 53% sequence identity with Avm43 P450. Active site residues identified from the P450Sky2 structure are coloured red. All are conserved or conservatively substituted in the other epoxidases except Tyr-291.