Laxaphycins B5 and B6 from the cultured cyanobacterium UIC 10484

Supplementary Information

Peter Sullivan[1], Aleksej Krunic[1], Joanna E. Burdette[1], Jimmy Orjala[1]

[1]Department of Medicinal Chemistry and Pharmacognosy, College of Pharmacy, University of Illinois at Chicago, Chicago, Illinois, 60612, United States

Correspondence: Jimmy Orjala – orjala@uic.edu

Table of Contents

S1. Photomicrographs and morphological description of strain UIC 10484 – cf. *Phormidium* sp.
S2. MS/MS comparison of the novel laxaphycins and trichormamides B and C
S3. Laxaphycin B5 (**1**) ^{1}H NMR spectrum (900 MHz, T=300 K, CD_3OH +TFA)
S4. Laxaphycin B5 (**1**) ^{13}C DEPTQ spectrum (226 MHz, T=300 K, CD_3OD + TFA)
S5. Laxaphycin B5 (**1**) HSQC spectrum (900 MHz, T=300 K, CD_3OD + TFA)
S6. Laxaphycin B5 (**1**) COSY spectrum (900 MHz, T=300 K, CD_3OD + TFA)
S7. Laxaphycin B5 (**1**) COSY spectrum (900 MHz, T=300 K, CD_3OH + TFA)
S8. Laxaphycin B5 (**1**) HMBC spectrum (900 MHz, T=300 K, CD_3OD + TFA)
S9. Laxaphycin B5 (**1**) HMBC spectrum (900 MHz, T=300 K, CD_3OH + TFA)
S10. Laxaphycin B5 (**1**) Selective HMBC spectrum (900 MHz, T=300 K, CD_3OH + TFA)
S11. Laxaphycin B5 (**1**) ROESY (900 MHz, T=300 K, CD_3OH + TFA)
S12. Laxaphycin B5 (**1**) TOCSY (900 MHz, T=300 K, CD_3OH + TFA)
S13. Laxaphycin B6 **(2)** ^{1}H NMR spectrum (900 MHz, T=300 K, CD_3OD + TFA)
S14. Laxaphycin B6 **(2)** ^{13}C DEPTQ NMR spectrum (226 MHz, T=300 K, CD_3OD + TFA)
S15. Laxaphycin B6 **(2)** HSQC NMR spectrum (900 MHz, T=300 K, CD_3OD + TFA)
S16. Laxaphycin B6 (**2**) COSY NMR spectrum (900 MHz, T=300 K, CD_3OD + TFA)
S17. Laxaphycin B6 (**2**) HMBC NMR spectrum (900 MHz, T=300 K, CD_3OD + TFA)
S18. Laxaphycin B6 (**2**) TOCSY NMR spectrum (900 MHz, T=300 K, CD_3OD + TFA)
S19. MS/MS comparison of laxaphycin B5 and B6 – fragmentation pattern 1
S20. MS/MS comparison of laxaphycin B5 and B6 – fragmentation pattern 2
S21. MS/MS comparison of laxaphycin B5 and B6 – fragmentation pattern 3
S22. Map of laxaphycin producers
S23. Structure dendrogram of laxaphycin type natural products
S24: Laxaphycin type-B metabolites
S25. Taxonomic tree
S26. Advanced Marfey's HPLC parameters
S27. Laxaphycin B5 advanced Marfey's data
S28. Laxaphycin B6 advanced Marfey's data

S1. Photomicrographs and morphological description of strain UIC 10484 – cf. *Phormidium* sp.

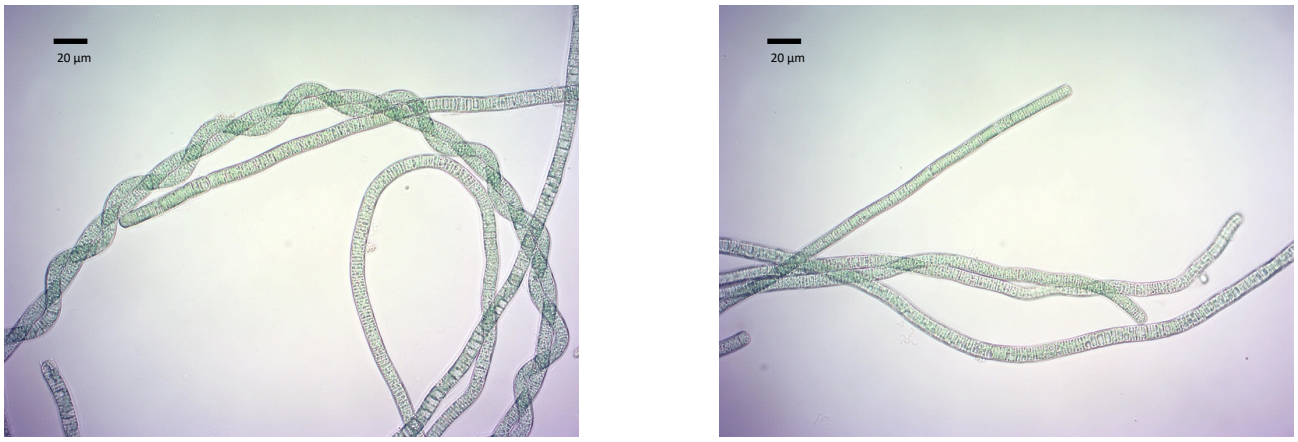


Figure: UIC 10484; 400x, brightfield

Morphological description – Macroscopically this strain grows as tangled clusters of dark green cell mass in BG12 media. The trichomes lack heterocysts, are unbranched, and isopolar. They are cylindrical-like in shape with smooth, round ends. These filaments are roughly 8 µm in width and uniseriate with each cell about 1-2 µm in length. There is no sheath or envelope surrounding the filaments. It is relatively common to find one filament coiled around another.

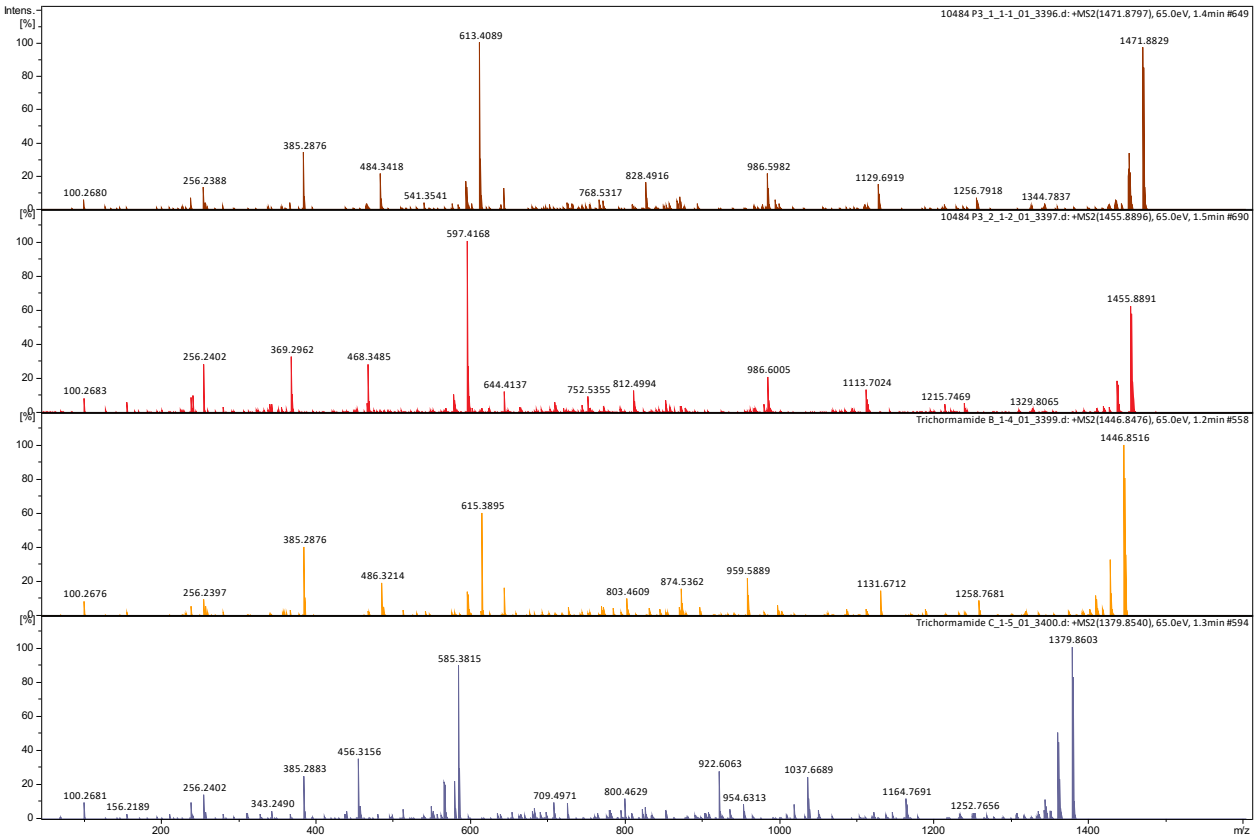
S2. MS/MS comparison of the novel laxaphycins and trichormamides B and C

Laxaphycin B5

Laxaphycin B6

Trichormamide B

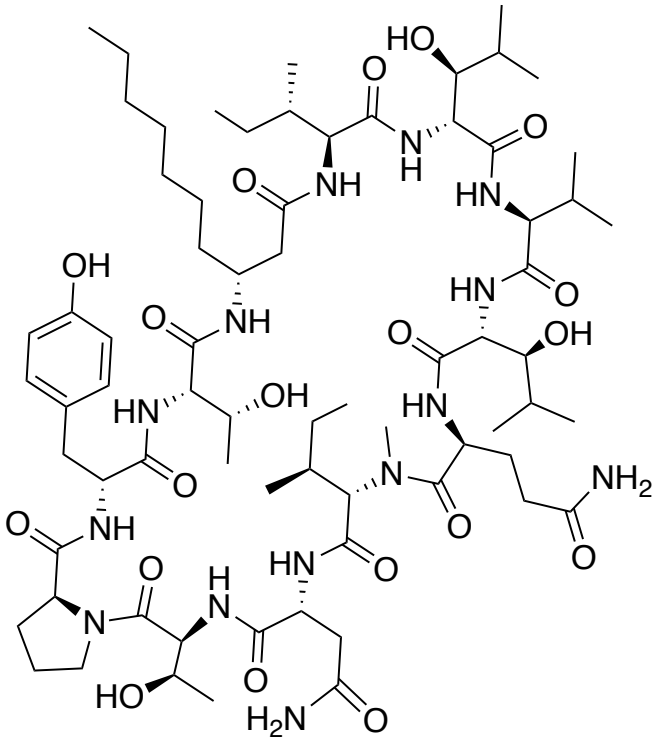
Trichormamide C



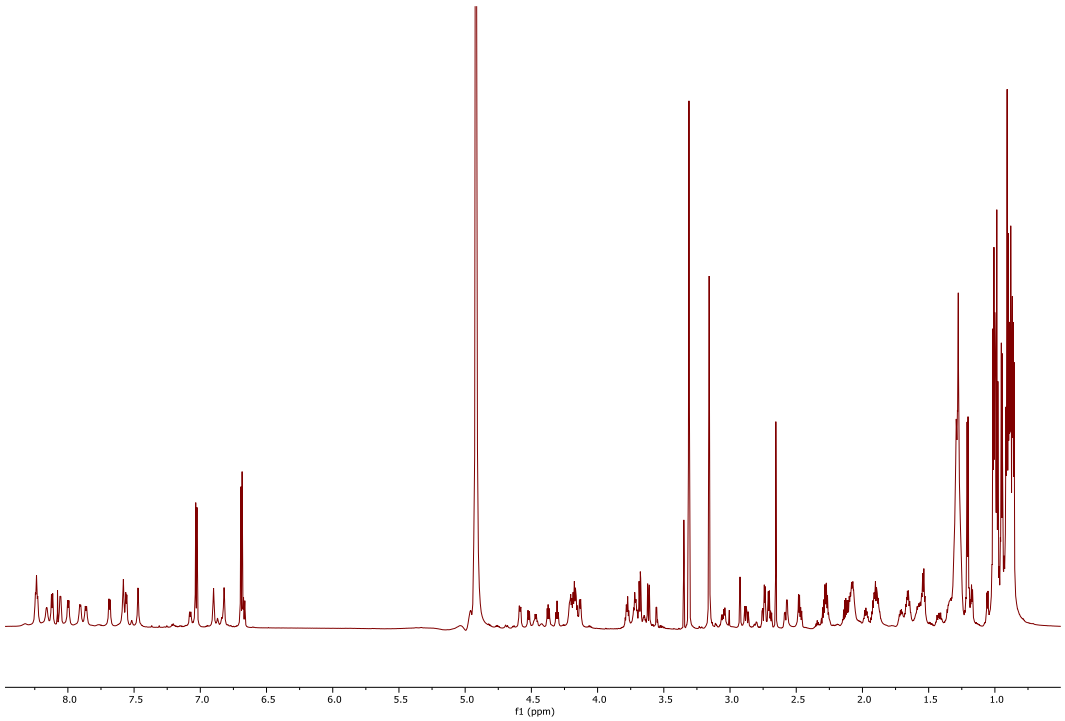
Ionization energy = 65 eV

Laxaphycin B5

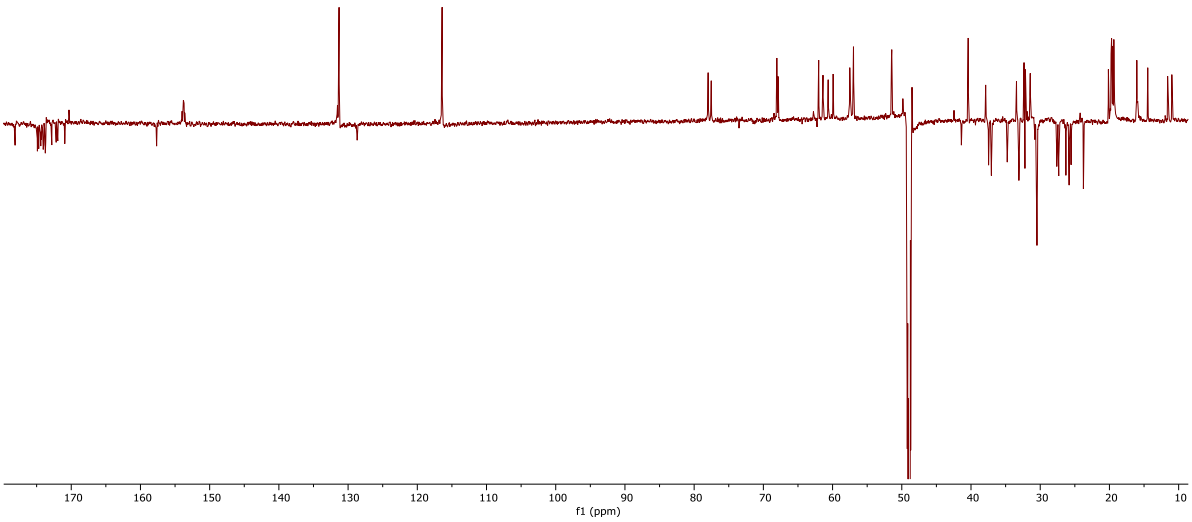
NMR Spectra



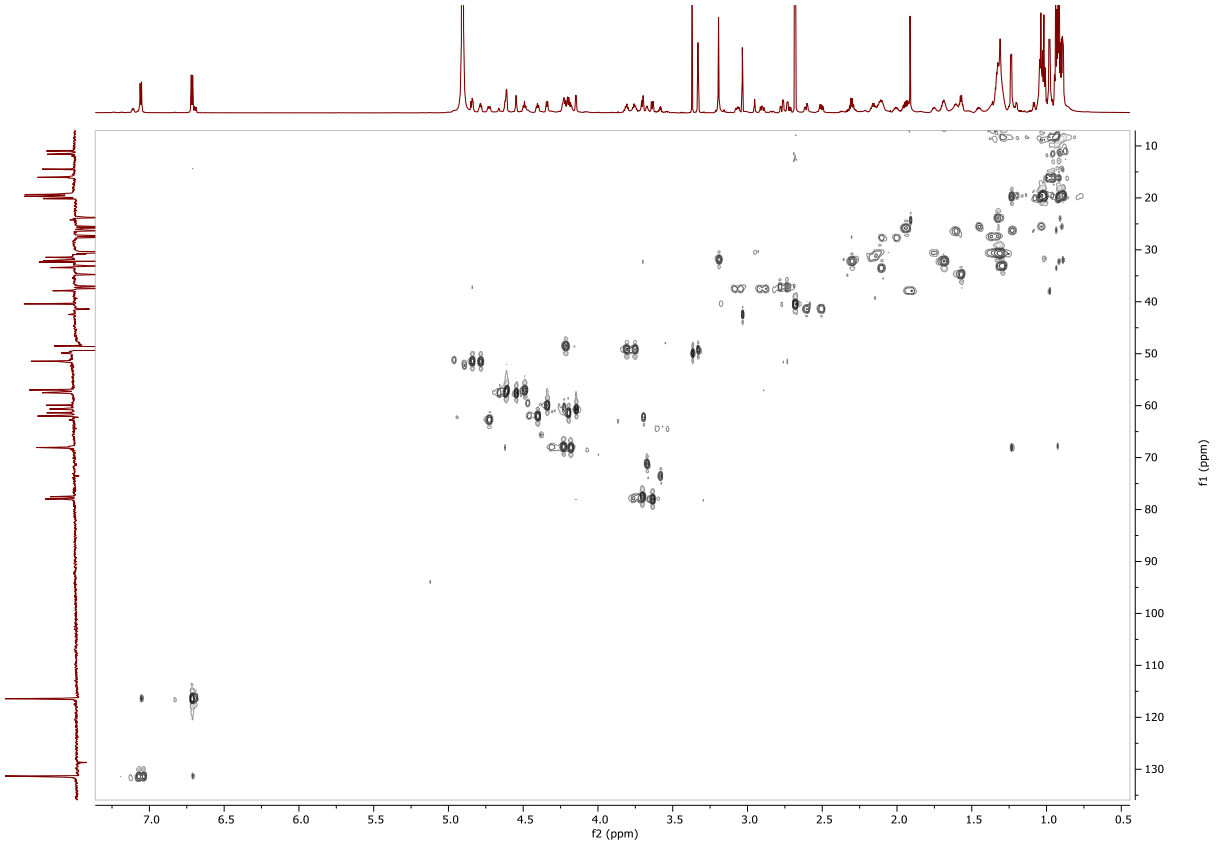
S3. ^{1}H NMR spectrum (900 MHz, T=300 K, CD_3OH +TFA)



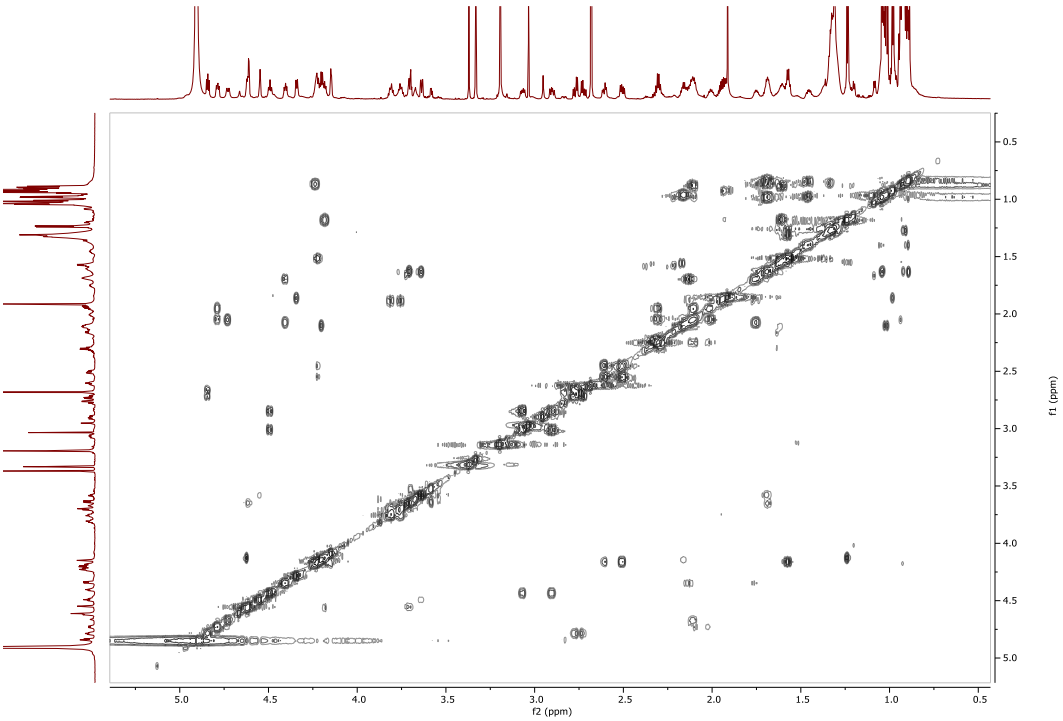
S4. DEPTQ spectrum (226 MHz, T=300 K, CD_3OD + TFA)



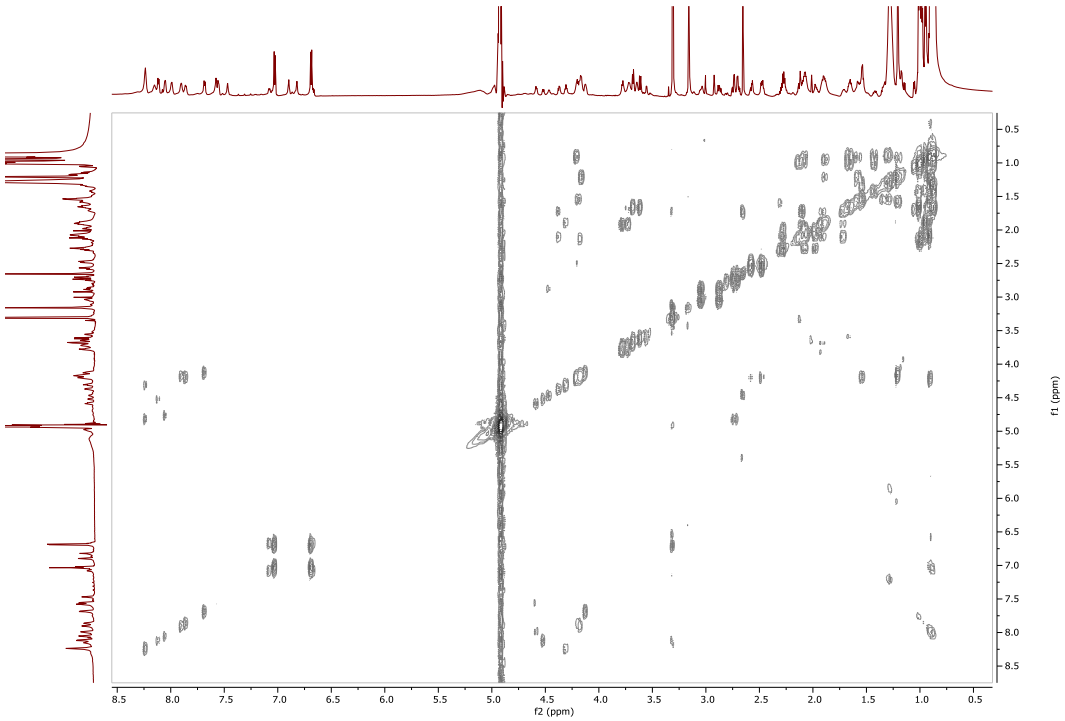
S5. HSQC spectrum (900 MHz, T=300 K, CD_3OD + TFA)



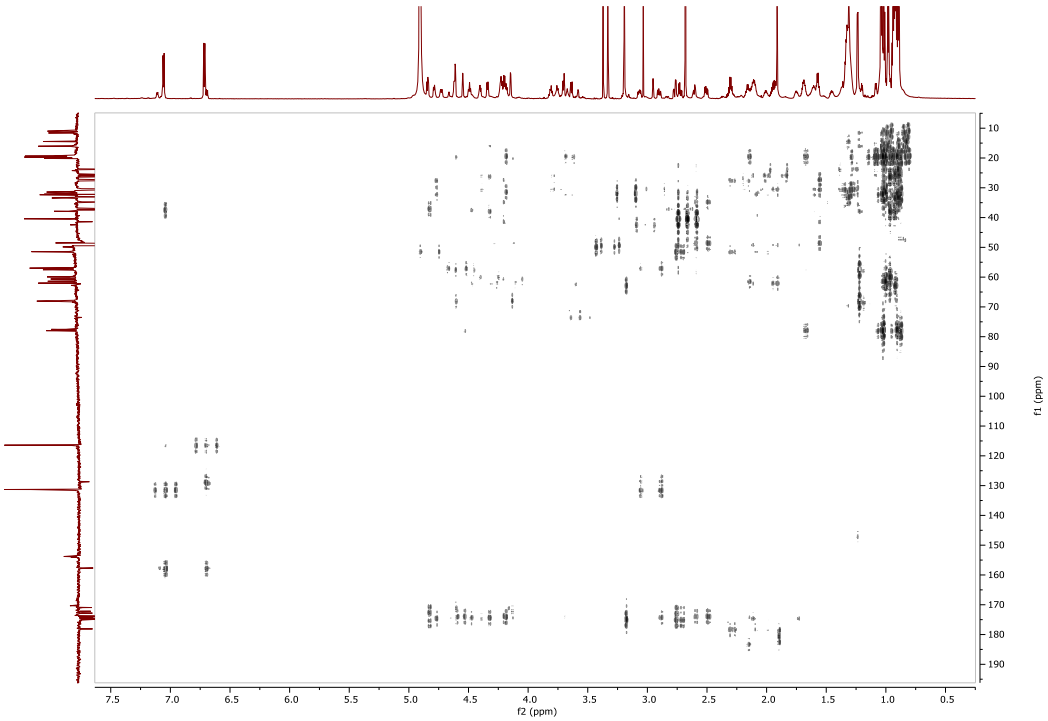
S6. COSY spectrum (900 MHz, T=300 K, CD_3OD + TFA)



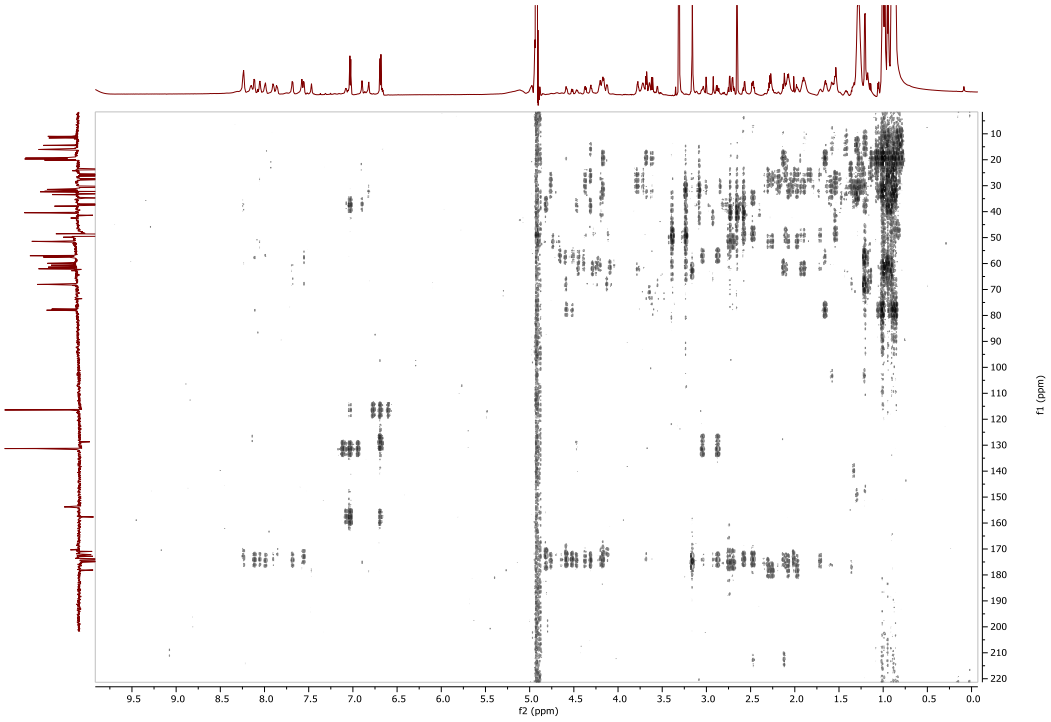
S7. COSY spectrum (900 MHz, T=300 K, CD_3OH + TFA)



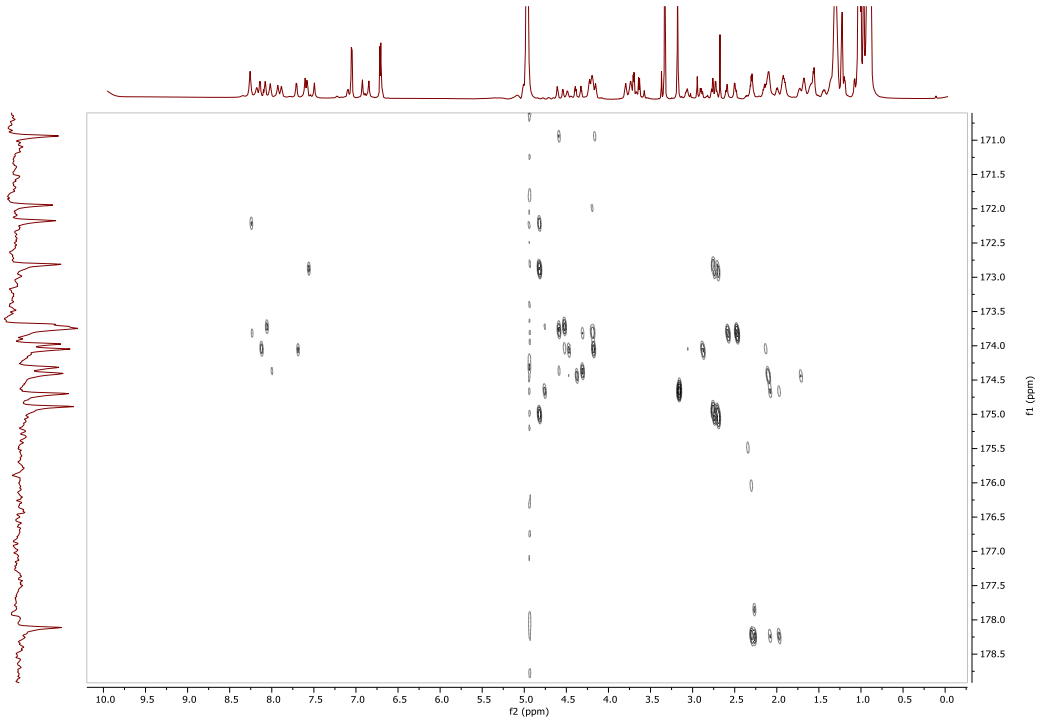
S8. HMBC spectrum (900 MHz, T=300 K, CD_3OD + TFA)



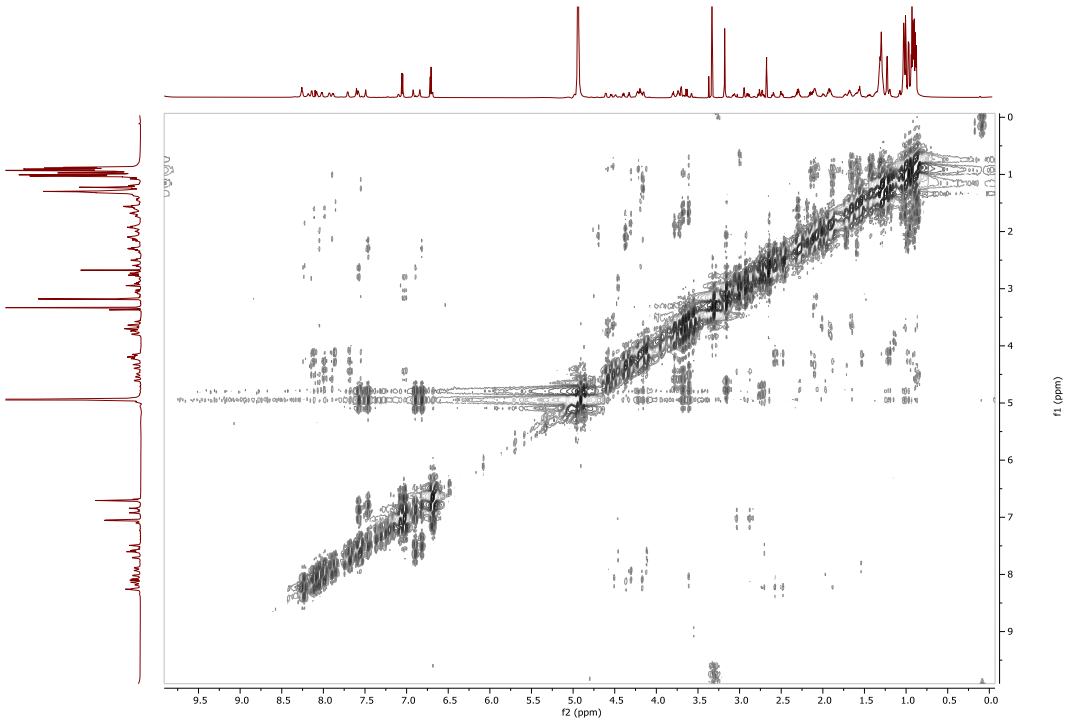
S9. HMBC spectrum (900 MHz, T=300 K, CD_3OH + TFA)



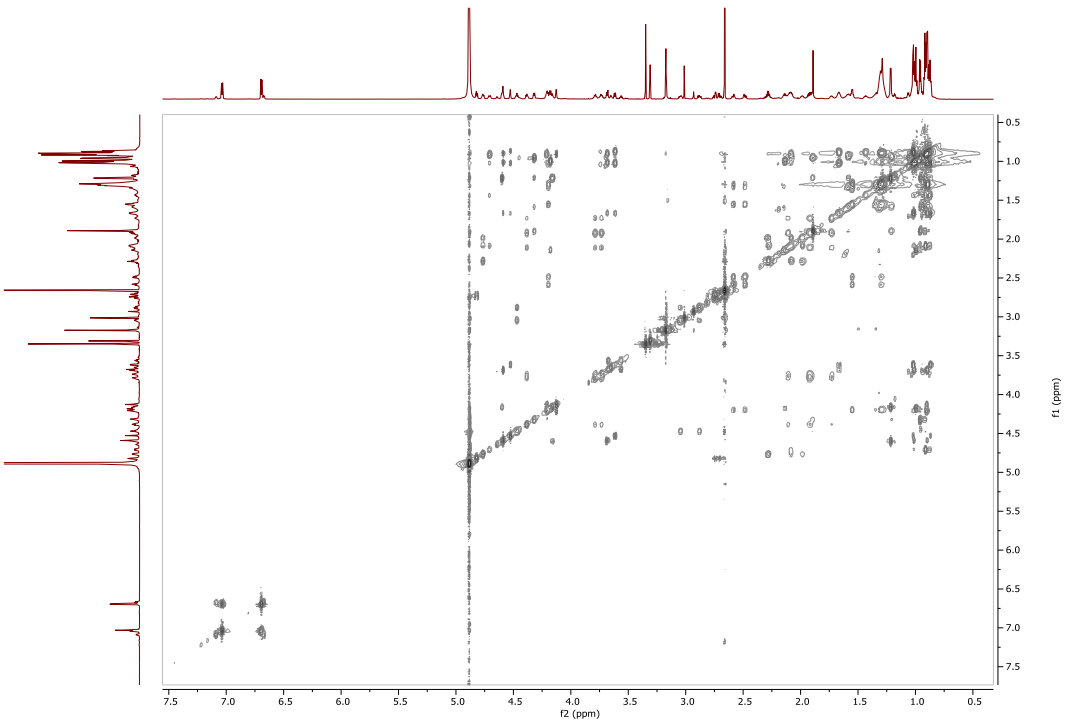
S10. Band-selective HMBC spectrum (900 MHz, T=300 K, CD_3OH + TFA)



S11. ROESY (900 MHz, T=300 K, CD_3OH + TFA)

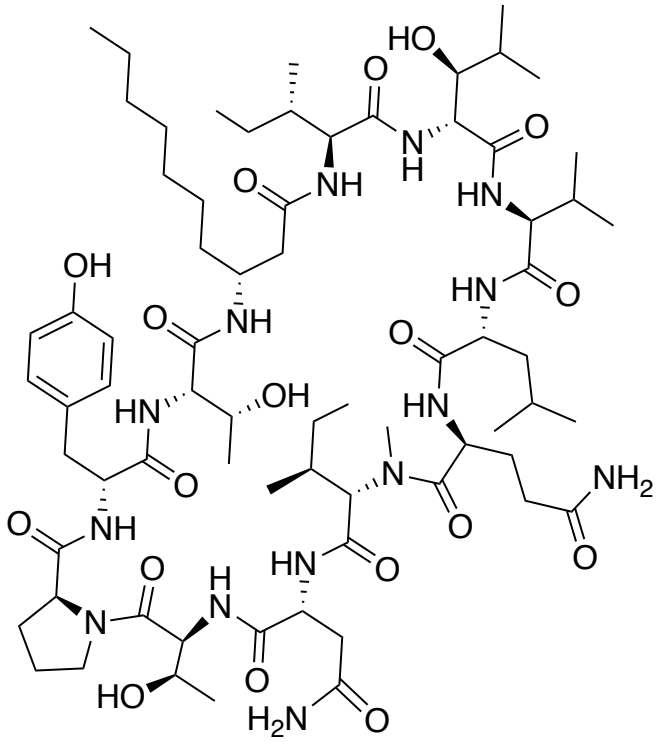


S12. TOCSY (900 MHz, T=300 K, CD_3OH + TFA)

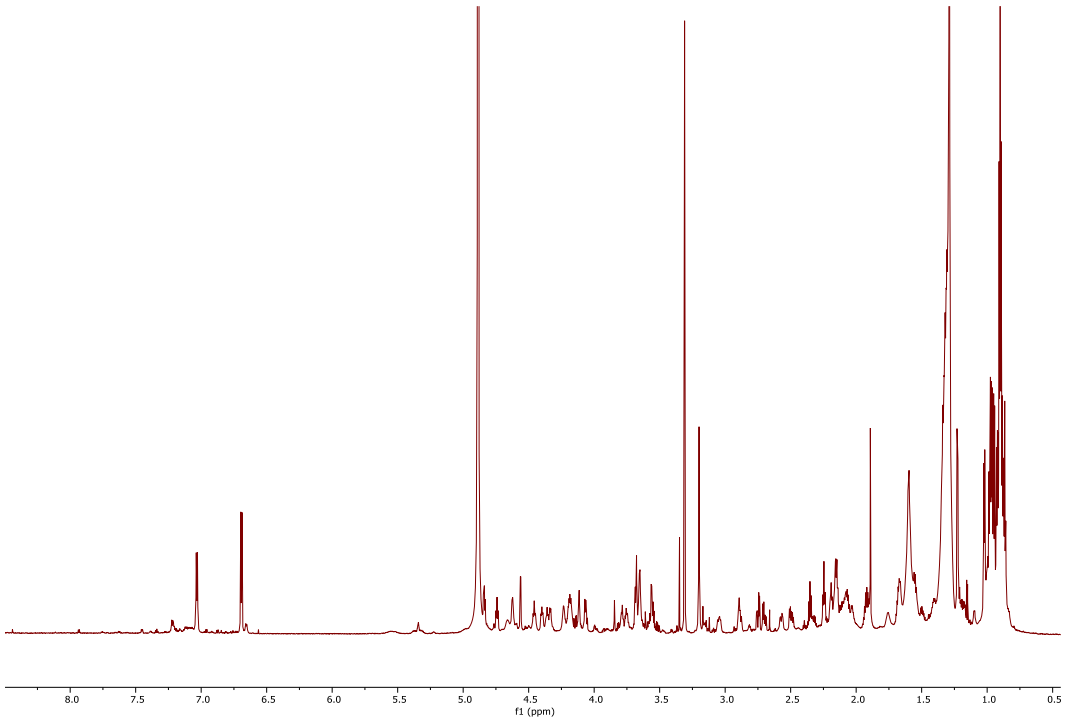


Laxaphycin B6

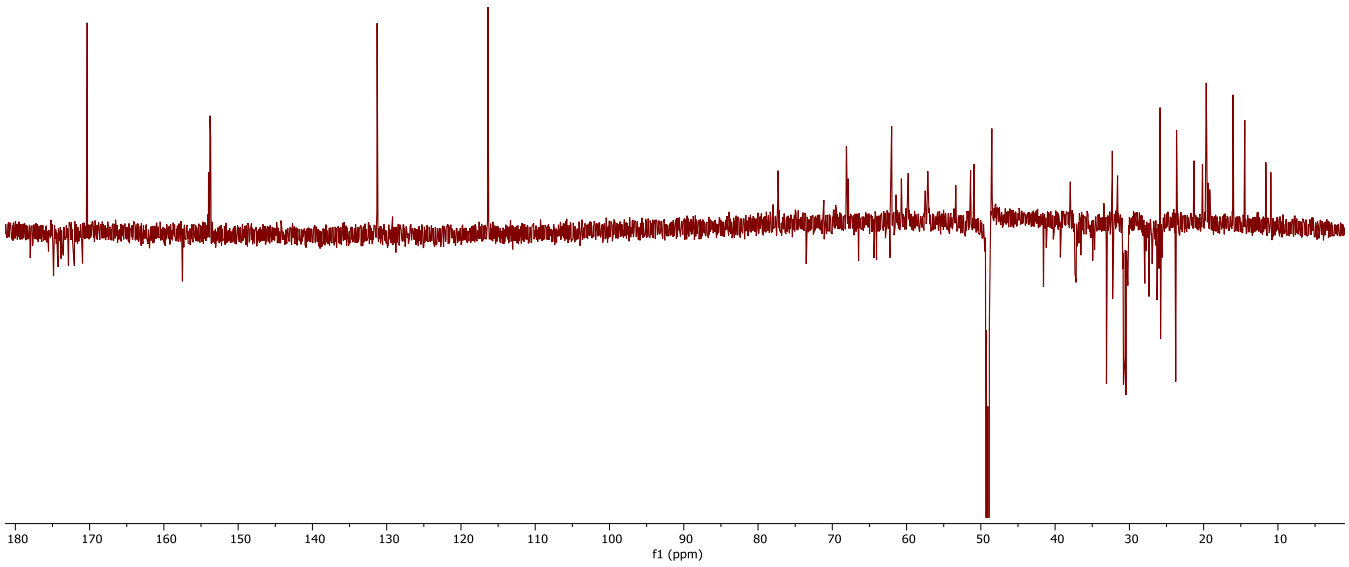
NMR Spectra



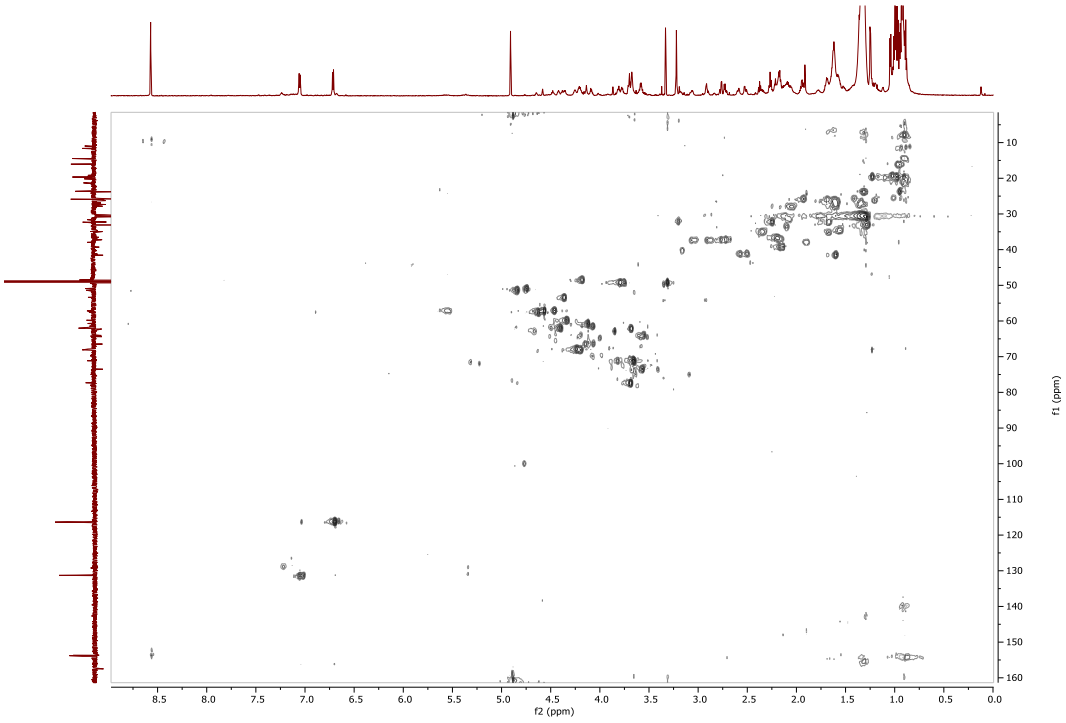
S13. ^{1}H NMR spectrum (900 MHz, T=300 K, CD_3OD + TFA)



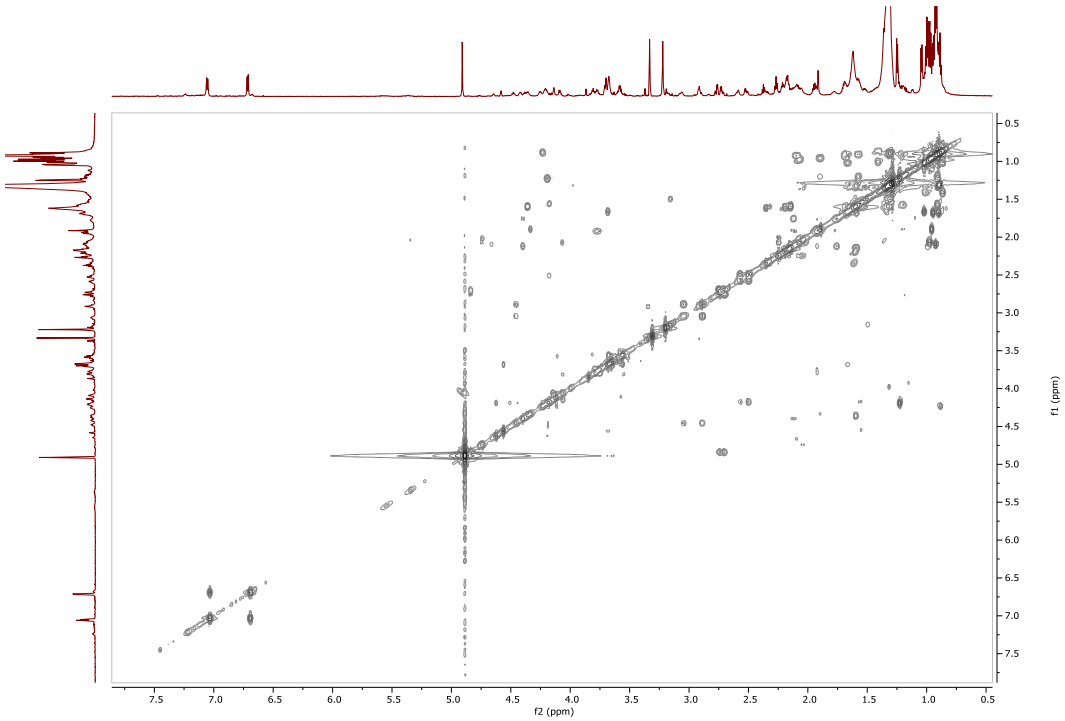
S14. ^{13}C DEPTQ NMR spectrum (226 MHz, T=300 K, CD_3OD + TFA)



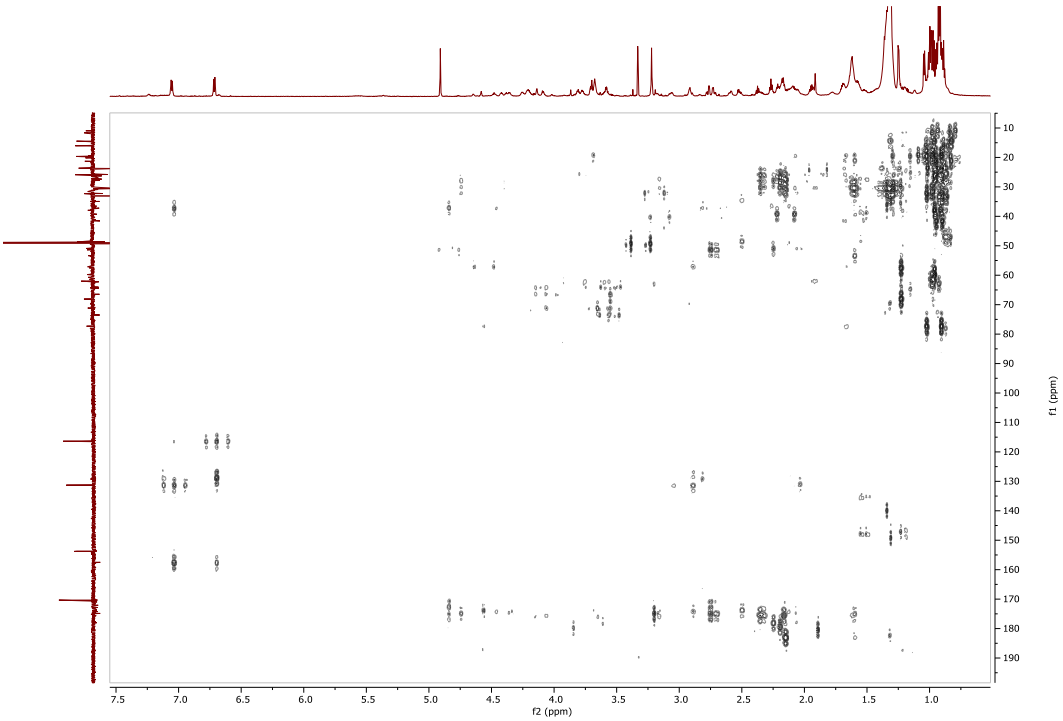
S15. HSQC NMR spectrum (900 MHz, T=300 K, CD_3OD + TFA)



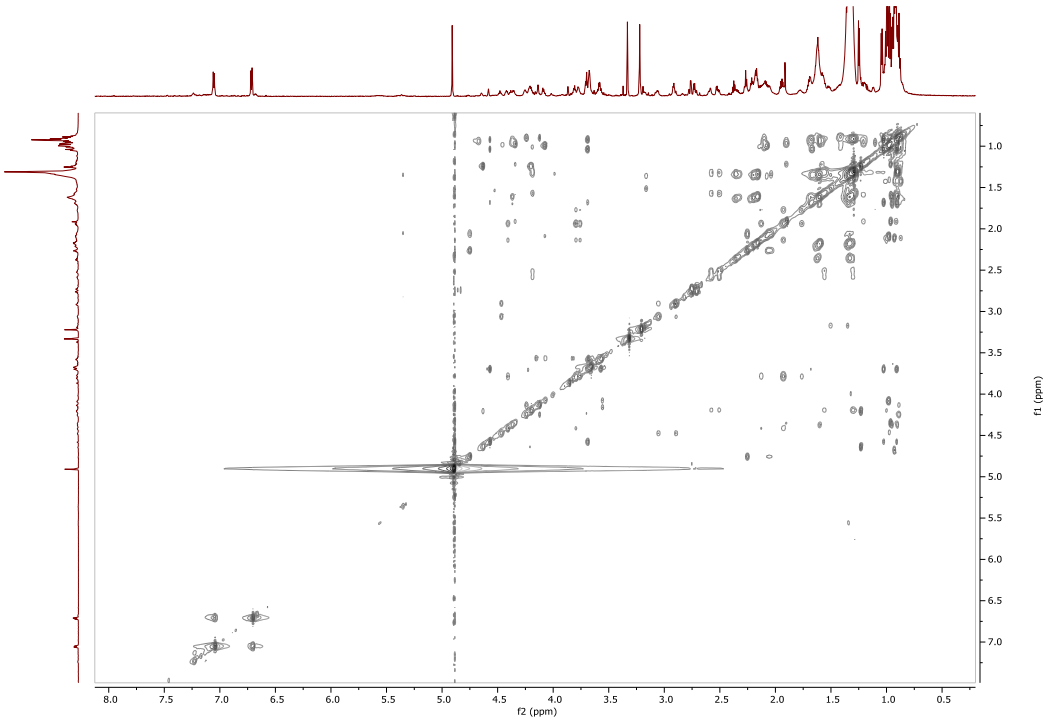
S16. COSY NMR spectrum (900 MHz, T=300 K, CD_3OD + TFA)



S17. HMBC NMR spectrum (900 MHz, T=300 K, CD_3OD + TFA)



S18. TOCSY NMR spectrum (900 MHz, T=300 K, CD_3OD + TFA)



S19. MS/MS comparison of laxaphycin B5 and B6 – fragmentation pattern 1
(Corresponds to figure 4 in main text)

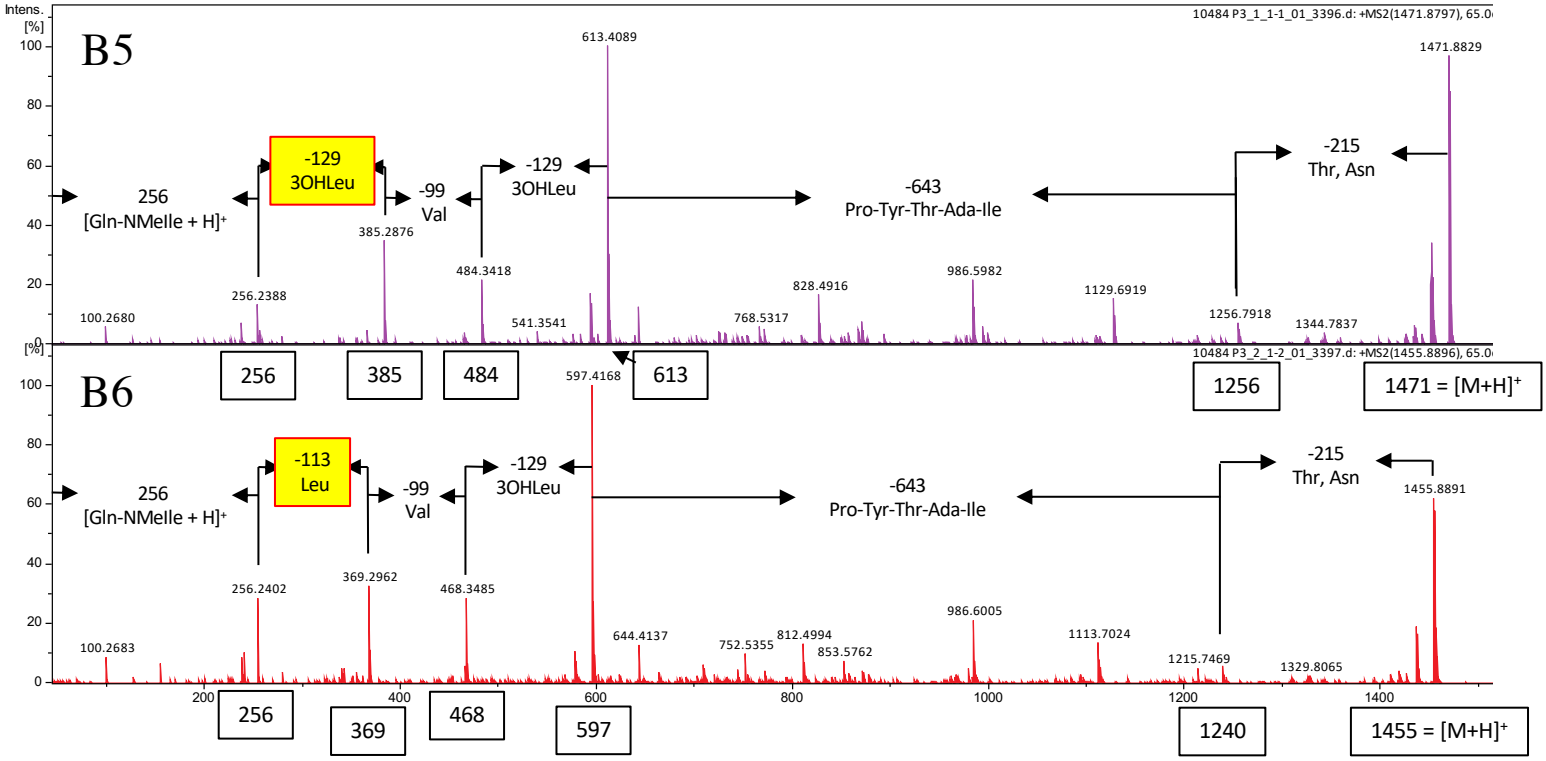
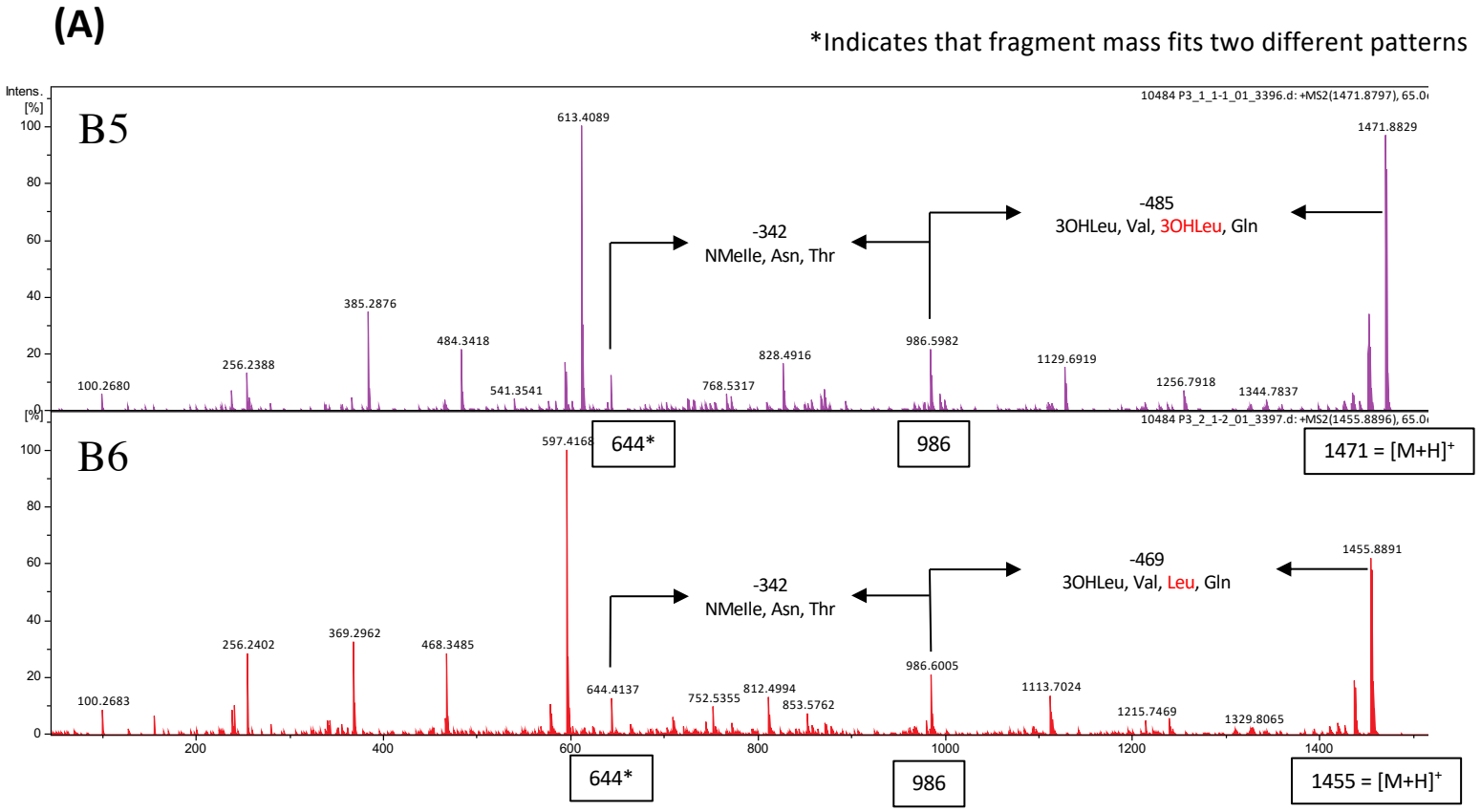


Figure S19: MS/MS comparison between laxaphycins B5 and B6 with ionization energy = 65 eV. Spectra correspond to figure 4 structures in the main text.

S20. MS/MS comparison of laxaphycin B5 and B6 – fragmentation pattern 2



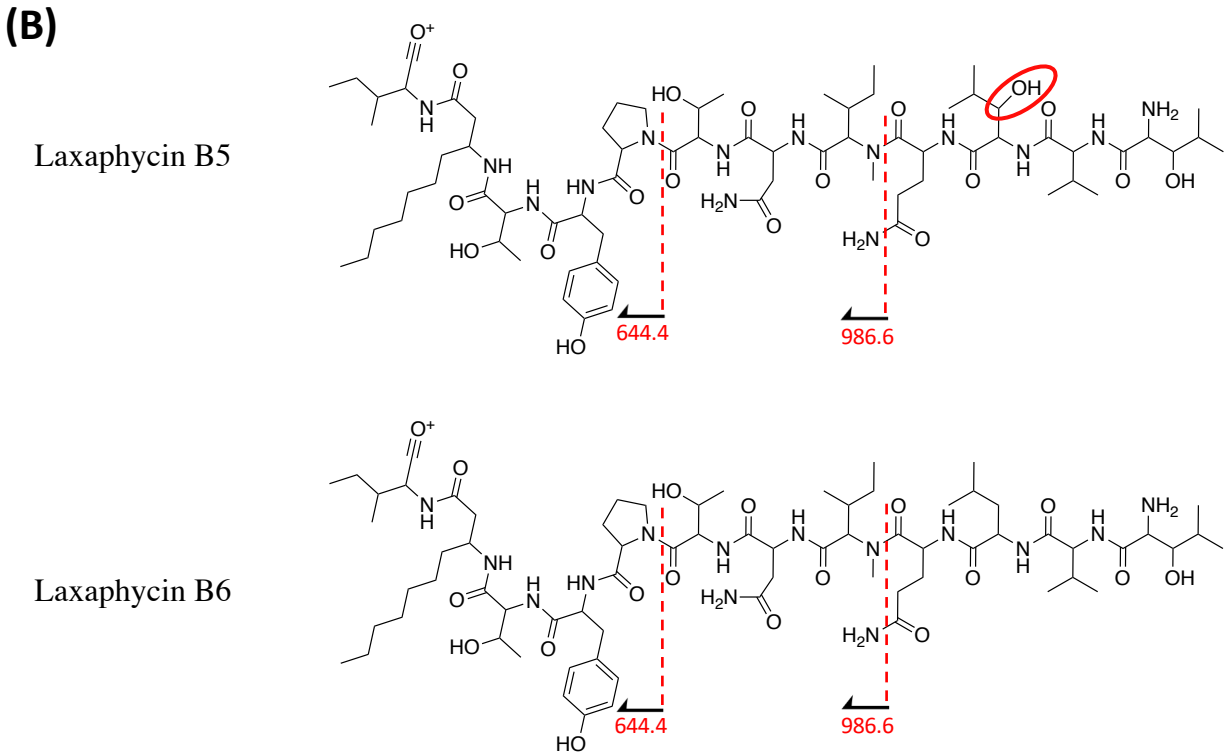


Figure S20: (A) There is a 16 *m/z* difference between the two $[M+H]^+$ values as laxaphycin B6 has a Leu rather than a second 3OHLeu. In pattern 2, there is no mass difference after the first fragmentation because 3OHLeu2 and Leu are no longer part of the smaller fragmented ions (*m/z*=986). (B) Linear versions of B5 and B6 highlighting fragmentation pattern 2.

S21. MS/MS comparison of laxaphycin B5 and B6 – fragmentation pattern 3

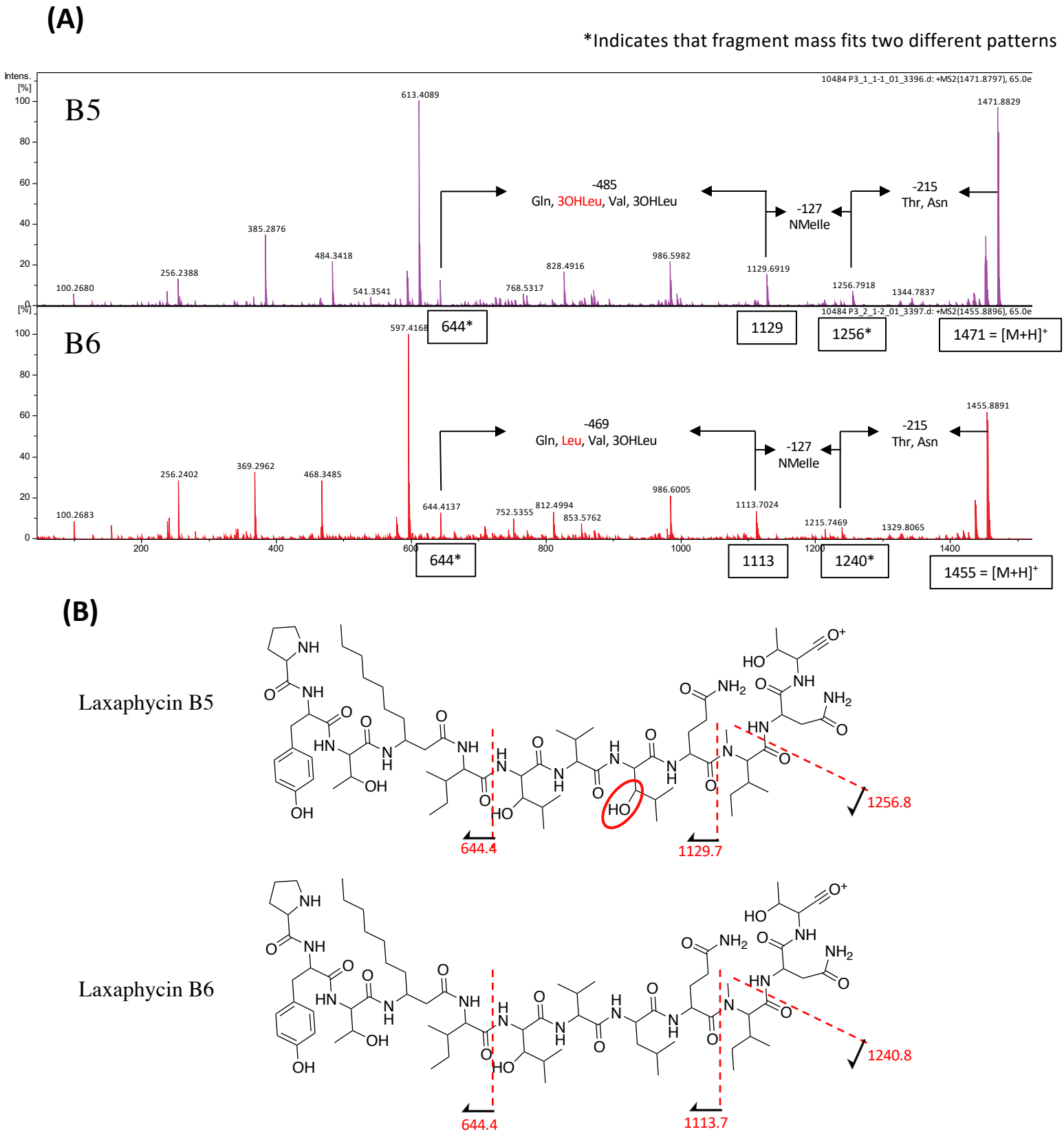


Figure S21: (A) There is a 16 *m/z* difference between corresponding fragment peaks until the losses of 3OHLeu2 from laxaphycin B5 and Leu from laxaphycin B6. (B) Linear versions of B5 and B6 highlighting fragmentation pattern 3.

S22. Map of laxaphycin producers

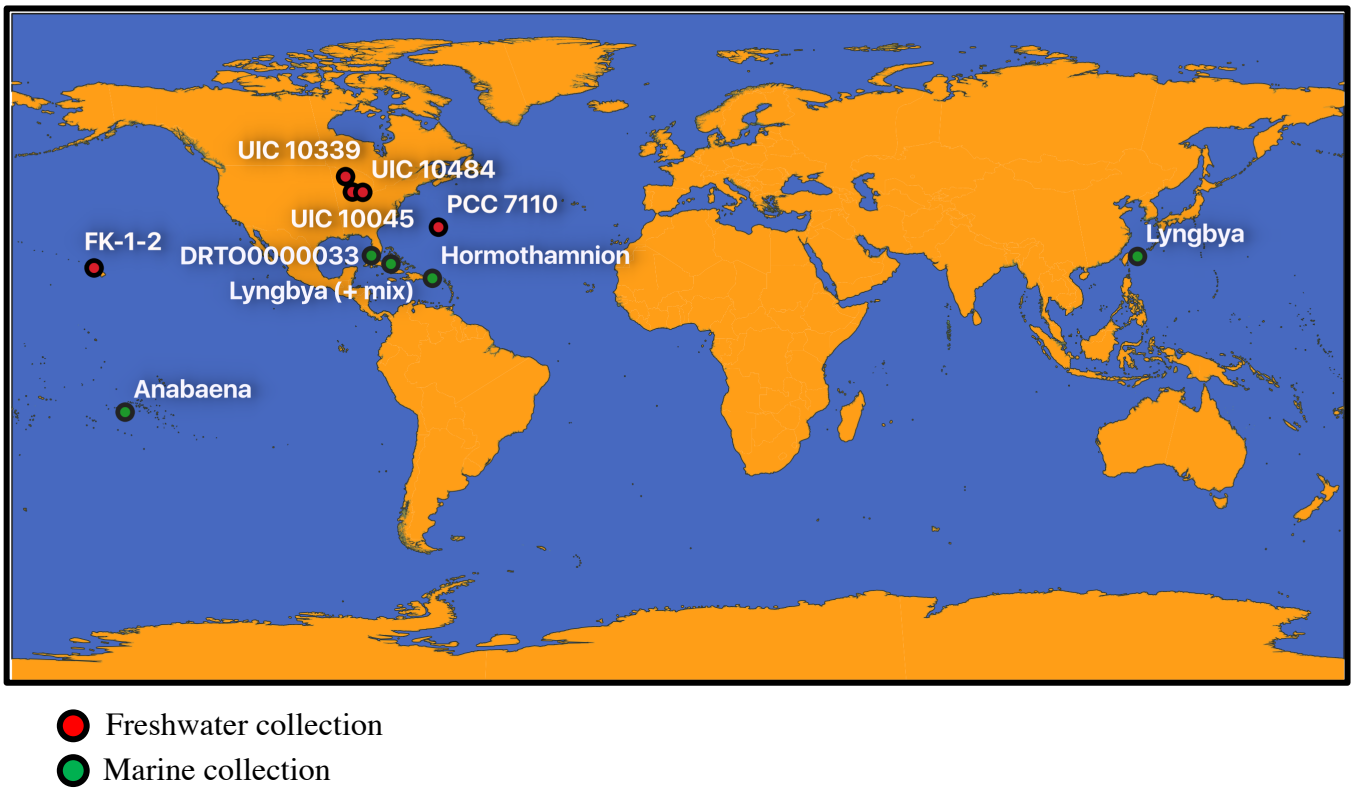


Figure S22: Collection locations and environments (marine or freshwater) of known laxaphycin producers. While strains have generally been found to biosynthesize both type-A and -B laxaphycins, the cyanobacteria reported to produce only type-A or only type-B metabolites are included in this map. As is shown in the map, cyanobacterial strains that produce laxaphycins have been collected in several regions of Earth. The map was generated using QGIS 2.18.2.[1]

S23. Structure dendrogram of laxaphycin type natural products

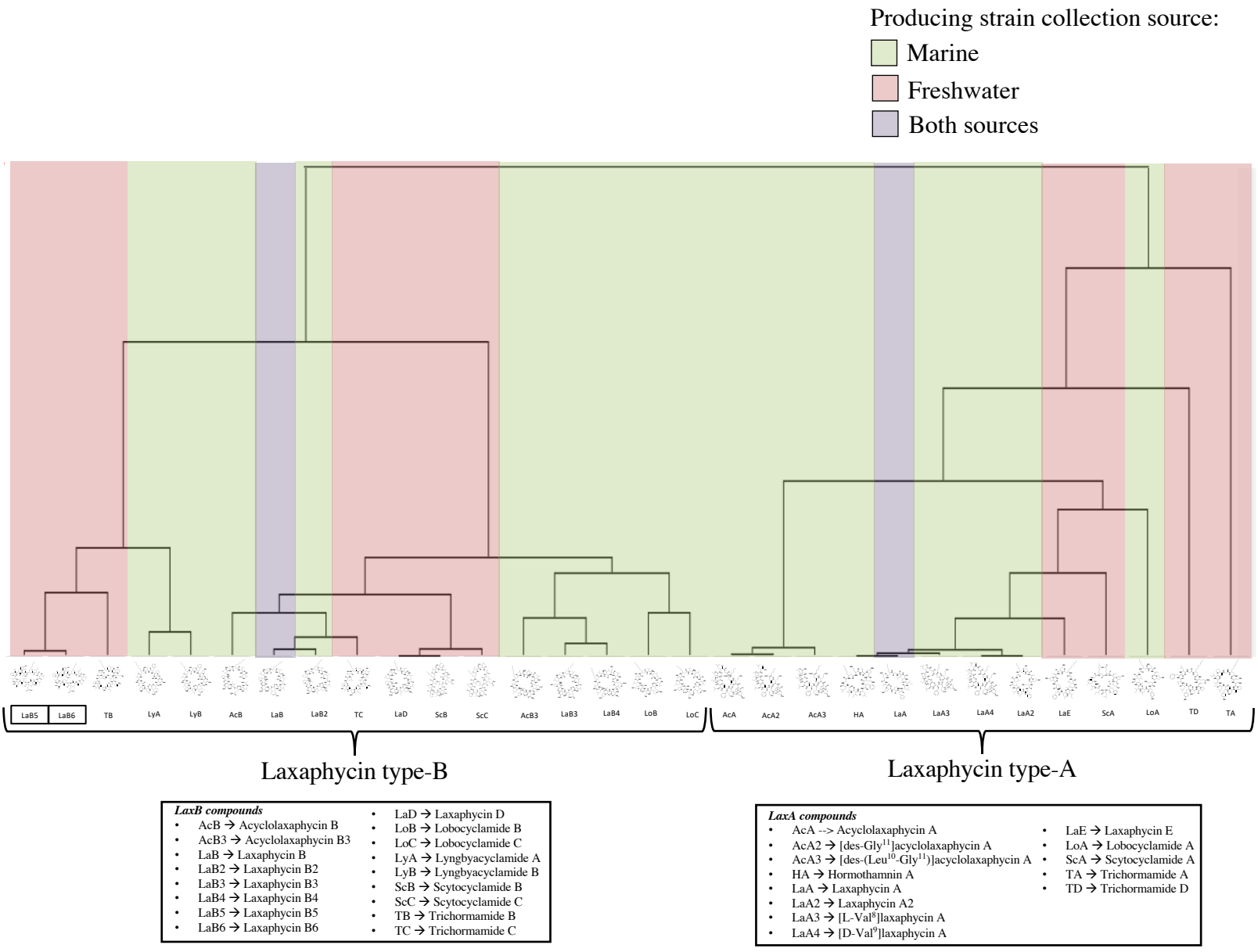
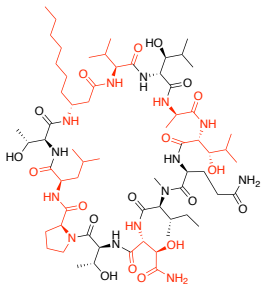
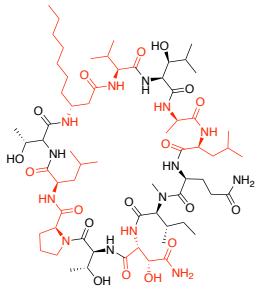


Figure S23: To analyze the structural similarity amongst the laxaphycins, a structure dendrogram was built using Scaffold Hunter.[2] Each line leading to a compound represents a secondary metabolite. Compounds whose lines branch closer to the bottom of the figure are more structurally related. As can be seen, there is a clear distinction between laxaphycin type-A and -B secondary metabolites, however, no distinction between secondary metabolites produced by cyanobacteria collected from freshwater and marine sources. While both types are generally cyclic and contain an aliphatic β-amino acid residue, there is little overlap in amino acid sequence between the two. The structure similarity analysis was performed using daylight bit fingerprinting with a path length of 16 and fingerprint size of 8,000 bit. The group average linkage method with a Tanimoto bit distance function was used to build the dendrogram.

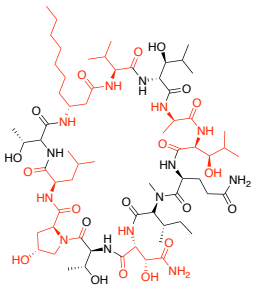
S24. Laxaphycin type-B metabolites



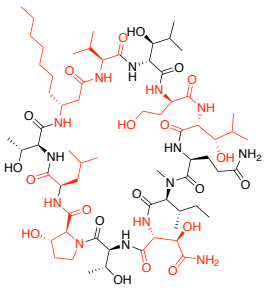
Laxaphycin B



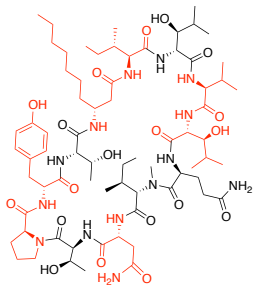
Laxaphycin B2



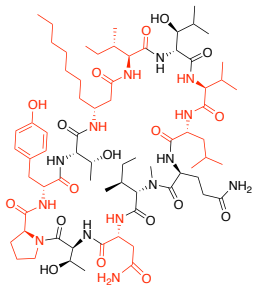
Laxaphycin B3



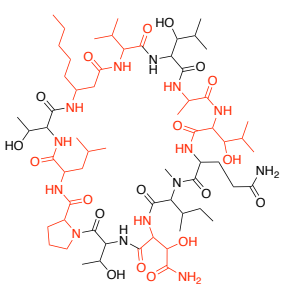
Laxaphycin B4



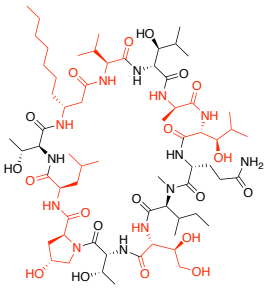
Laxaphycin B5



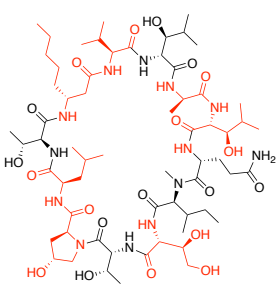
Laxaphycin B6



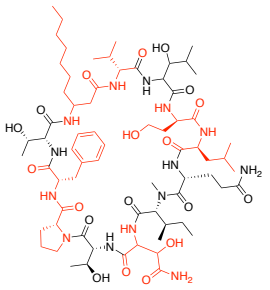
Laxaphycin D



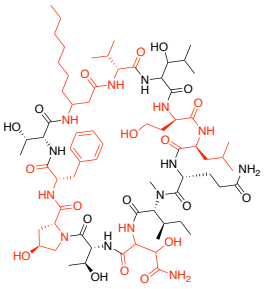
Lobocyclamide B



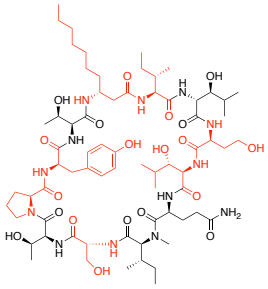
Lobocyclamide C



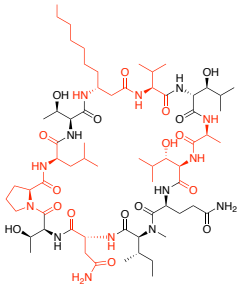
Lyngbyacyclamide A



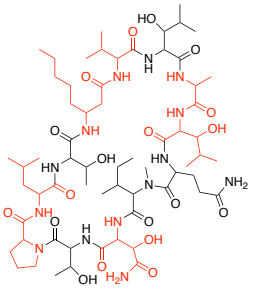
Lyngbyacyclamide B



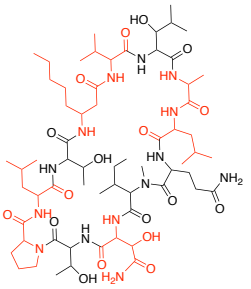
Trichormamide B



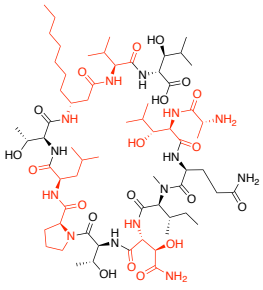
Trichormamide C



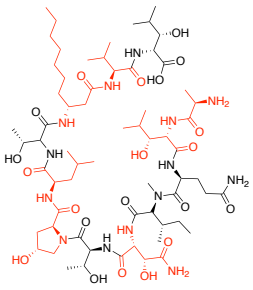
Scytocyclamide B



Scytocyclamide C



Acyclolaxaphycin B



Acyclolaxaphycin B3

Figure S24: The 17 laxaphycin type-B structures, including the two acyclic forms. A black residue indicates a conserved amino acid at that position throughout all 17 of the secondary metabolites while a red residue indicates the position's amino acid variability.

S25. Taxonomic tree

T – accepted type strain by CyanoDB or CyanoDB *and* CyanoType
T* – provisional type strain ONLY acknowledged by CyanoType
R – reference strain from Bergey's Manual of Systematic Bacteriology: 2nd Edition
(Genus) – NCBI taxonomic classification

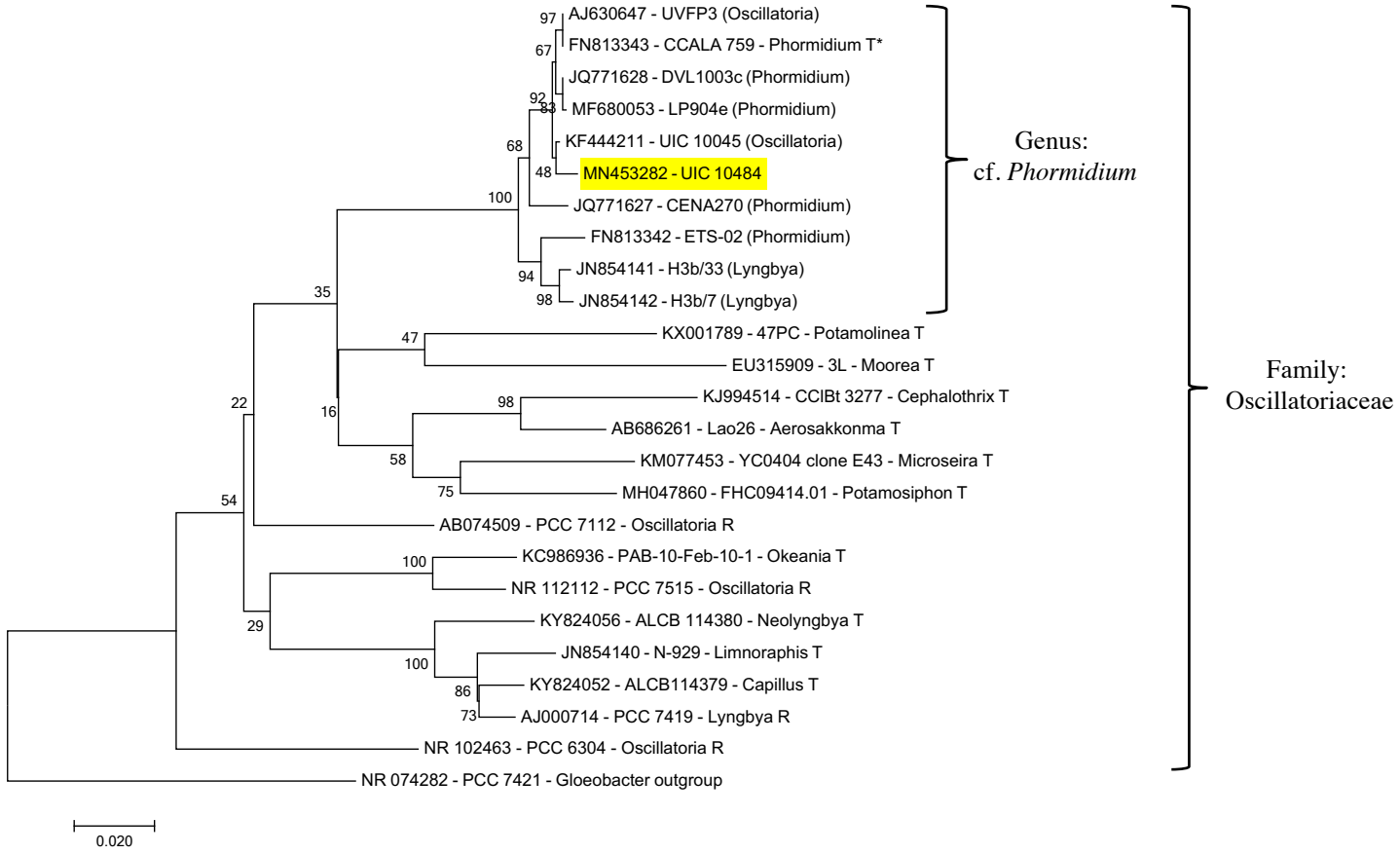


Figure S25: Using a partial SSU 16S rRNA gene sequence (1150 bp), phylogenetic relatedness of the strain was evaluated amongst Bergey's Manual of Systematic Bacteriology[3] reference strains (R), type strains (T) confirmed by Komarek et al,[4] strains provisionally defined as type strains (T*) from the CyanoType database,[5] and eight strains with high sequence similarity (≥98%) to UIC 10484 using NCBI's BLAST tool.[6] A phylogenetic tree was built using MEGA7.[7] UIC 10484 was found to clade with CCALA 759^{T}* (>99% 16S sequence similarity) along with UIC 10045 (>99% 16S sequence similarity). Previously, UIC 10045 was classified as a cf. *Oscillatoria* sp., however, its taxonomic analysis did not include the provisional type strain CCALA 759. In 2012, CCALA 759 was originally proposed to be the designated *Phormidium* epitype after performing a polyphasic analysis to taxonomically classify it.[8] Since, it has been described as a reference strain, suggested to be the *Phormidium sensu stricto*, and referred to as the epitype for the genus based on the polyphasic analysis.[9–13] However, because CCALA 759^{T}* has not been recognized as a type strain in the CyanoDB database, UIC 10484 was assigned as a cf. *Phormidium* sp.

S26. Advanced Marfey's HPLC parameters

Instrument: Bruker Impact II Qq-TOF coupled to Shimadzu Nexera X2 UHPLC system
- Column: Phenomenex C18 (50x2.1mm)
- Flow: 0.5 ml/min
- Oven temp: 37 °C

- *Method a*

Gradient (with 0.1% FA):
- 0-9 min → 25-65% ACN

- *Method b*

Gradient (with 0.1% FA):
- 0-9 min → 25-45% ACN

- *Method c*

Gradient (with 0.1% FA):
- 0-7 min → 10-35% ACN

Instrument 1: Agilent 1100 HPLC
Instrument 2: Agilent 6545 LC-Q-TOF (to confirm the derivatized hydrolysate masses)
- Column: Phenomenex C18 (250x4.6mm)
- Flow: 1.0 ml/min
- Oven temp: 32 °C

- *Method d*

Gradient (with 0.1% FA)
- 0-5 min → 30% ACN
- 5-65 min → 30-43% ACN

*Amino acid absolute configurations evaluated using *method d* were initially established based on peak overlap when co-injecting standard amino acids with laxaphycin B5 and B6 FDLA derivatized hydrolysates. $[M+H]^+$ values and elution orders of the L-FDLA and D-FDLA derivatized hydrolysates from the LC-Q-TOF experiments confirmed the HPLC co-injection results. The mass spectrometer retention times were two minutes delayed compared to the Agilent 1100 HPLC retention times.

S27. Laxaphycin B5 advanced Marfey's data

Advanced Marfey's – Laxaphycin B5

Amino acid	Retention time (min)			Assignment
	L	D	Measured	
Ada[a]	6.8 (*S*)	8.3 (*R*)	8.3	*R*
Val[a]	3.4	4.9	3.4	L
Gln (Glu)[b]	2.2	2.5	2.3	L
Asn (Asp)[b]	6.0	6.3	6.0	D
Pro[a]	2.7	3.3	2.7	L
Tyr[a]	3.1	3.6	3.6	D

Amino acid	Retention time (min)					Assignment
	L	D	L-allo	D-allo	Measured	
Ile[d]	34.4	58.4	33.6	58.0	34.4	L
NMeIle[c]	7.0	8.8	7.2	8.9	7.0	L
Thr (x2)[b]	5.8	7.1	6.0	6.5	5.8	L

Amino acid	Retention time (min)					Assignment
	2S,3R	2R,3S	2S,3S	2R,3R	Measured	
3OHLeu (x2)[b]	3.3	6.7	3.4	5.7	6.7	2R, 3S

Table S27: Method *d* initially evaluated the FDLA derivatized laxaphycin B5 Ile on an Agilent 100 HPLC system by running experiments with co-injected amino acid standards. Retention times for Ile listed above are for each co-injected standard. To evaluate amino acids with two different stereocenters, the enantiomer 'trick' was performed.[14]

Method used to determine amino acid absolute configuration

- [a] – Method *a*
- [b] – Method *b*
- [c] – Method *c*
- [d] – Method *d*

S28. Laxaphycin B6 advanced Marfey's data

Advanced Marfey's – Laxaphycin B6

Amino acid	Retention time (min)			Assignment
	L	D	Measured	
Ada[a]	6.9 (*S*)	8.4 (*R*)	8.4	*R*
Val[d]	26.3	45.7	26.3	L
Gln (Glu)[d]	12.9	16.1	12.9	L
Asn (Asp)[d]	12.2	13.7	13.7	D
Pro[a]	2.8	3.4	2.8	L
Tyr[d]	21.5	28.1	28.1	D
Leu[d]	35.9	60.0	60.0	D

Amino acid	Retention time (min)					Assignment
	L	D	L-allo	D-allo	Measured	
Ile[d]	34.4	58.4	34.0	57.9	34.4	L
NMeIle[d]	43.4	57.6	44.7	58.9	43.4	L
Thr (x2)[d]	9.4	17.8	11.3	14.3	9.4	L

Amino acid	Retention time (min)					Assignment
	2S,3R	2R,3S	2S,3S	2R,3R	Measured	
3OHLeu[d]	16.7	39.6	17.3	32.4	39.6	2R, 3S

Table S28: Method *d* initially evaluated the FDLA derivatized laxaphycin B6 amino acids on an Agilent 100 HPLC system by running experiments with co-injected amino acid standards. Retention times for the residues listed above using method *d* are for each co-injected standard. To evaluate amino acids with two different stereocenters, the enantiomer 'trick' was performed.[14]

Method used to determine amino acid absolute configuration

- [a] – Method *a*
- [d] – Method *d*

Citations

1. QGIS Geographic Information System. Open Source Geospatial Foundation Project. https://qgis.org/en/site/. Published 2018.

2. Schäfer T, Kriege N, Humbeck L, Klein K, Koch O, Mutzel P. Scaffold Hunter: a comprehensive visual analytics framework for drug discovery. J Cheminform. 2017;9(1):1-18.

3. Garrity GM. Boone DR, Castenholz RW. Bergey's Manual of Systematic Bacteriology. 2nd ed. New York: Springer; 2001.

4. Hauer T, Komarek J. CyanoDB.cz 2.0 - On-line database of cyanobacterial genera. Univ. of South Bohemia & Inst. of Botany AS CR. http://www.cyanodb.cz/. Published 2019. Accessed April 3, 2019.

5. Ramos V, Morais J, Vasconcelos VM. A curated database of cyanobacterial strains relevant for modern taxonomy and phylogenetic studies. Sci Data. 2017;4:1-8.

6. Kumar S, Stecher G, Tamura K. MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. Mol Biol Evol. 2016;33(7):1870-1874.

7. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic Local Alignment Search Tool. J Mol Biol. 1990;215(3):403-410.

8. Sciuto K, Andreoli C, Rascio N, Rocca N La, Moro I. Cladistics Polyphasic approach and typification of selected *Phormidium*. Cladistics. 2012;1:1-18.

9. Malone CFS, et al. *Cephalothrix* gen. nov. (Cyanobacteria): Towards an intraspecific phylogenetic evaluation by multilocus analyses. Int J Syst Evol Microbiol. 2015;65(9):2993-3007.

10. Martins MD, Machado-de-Lima NM, Branco LHZ. Polyphasic approach using multilocus analyses supports the establishment of the new aerophytic cyanobacterial genus *Pycnacronema* (Coleofasciculaceae, Oscillatoriales). J Phycol. 2018:1-14.

11. Engene N, Tronholm A, Salvador-Reyes LA, Luesch H, Paul VJ. *Caldora penicillata* gen. nov., comb. nov. (Cyanobacteria), a pantropical marine species with biomedical relevance. J Phycol. 2015;51(4):670-681.

12. Brito Â, et al. Description of new genera and species of marine cyanobacteria from the Portuguese Atlantic coast. Mol Phylogenet Evol. 2017;111:18-34.

13. Buch B, Martins MD, Branco LHZ. A widespread cyanobacterium supported by polyphasic approach: proposition of *Koinonema pervagatum* gen. & sp. nov. (Oscillatoriales)1. J Phycol. 2017;53(5):1097-1105.

14. Luo S, et al. Trichormamides A and B with antiproliferative activity from the cultured freshwater cyanobacterium *Trichormus* sp. UIC 10339. J Nat Prod. 2014;77(8):1871-1880.