Self-resistance guided genome mining uncovers new topoisomerase inhibitors from myxobacteria

Supplementary information

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1 Molecular biology protocols

1.1 Culture media used in this study

Table S 1 : Comprehensive list of culture medium recipes used for myxobacterial fermentation in this study

CTT medium			
Amount	Ingredient	Concentration	Supplier
10 g/L	Bacto Casiton	-	BD
10 ml/L	Tris solution	1 M	Sigma Aldrich
1 ml/L	KH_2PO_4 solution	0.1 M	Sigma Aldrich
10 ml/L	$MgSO_4$ solution	0.8 M	Grüssing
Dissolved in milliq. water pH adjusted to 7.6 with 1N KOH			
TS-6 medium			
Amount	Ingredient	Concentration	Supplier
6g/L	Bacto Trypton	-	BD
10 ml/L	Tris solution	1 M	Sigma Aldrich
4 g/L	Starch (soluble)	-	Roth
10 ml/L	$MgSO_4$ solution	0.8 M	Grüssing
Dissolved in milliq. water pH adjusted to 7.6 with 1N KOH			
YM – Medium			
Amount	Ingredient	Concentration	Supplier
5 g/L	Bacto Pepton Phyton	-	BD
10 g/L	Glucose	-	Roth
3 g/L	Bacto Malt extract	-	BD
3 g/L	Bacto Yeast Extract	-	BD
1 g/L	$CaCl_2$	-	Sigma Aldrich
1 g/L	$MgSO_4$ x $7H_2O$	-	Grüssing
1 mL/L	$KHPO_4$ solution	0.1 M	Sigma
10 mM	TRIS	-	Sigma Aldrich
Dissolved in milliq. water pH adjusted to 7.5 with 1N KOH			
SC leucine dropout – Medium			
Amount	Ingredient	Concentration	Supplier
6.8 g/L	Yeast Nitrogen base	-	Sigma Aldrich
20 g/L	Glucose	-	Roth
5 g/L	Leucine dropout amino acid mix	-	Sigma Aldrich
Dissolved in milliq. water			
YPAD – Medium			

Amount	Ingredient	Concentration	Supplier
10 g/L	Yeast extract	-	BD
10 g/L	Phytopeptone	-	BD
20 g/L	Glucose	-	Roth
100 mg/L	Adenine hemisulfate	-	Sigma Aldrich
Dissolved in milliq. water			
YMS 20 – Medium			
Amount	Ingredient	Concentration	Supplier
5 g/L	Bacto Pepton Phyton	-	BD
10 g/L	Glucose	-	Roth
20 g/L	Soluble Starch	-	Roth
3 g/L	Bacto Malt extract	-	BD
3 g/L	Bacto Yeast Extract	-	BD
1 g/L	$CaCl_2$	-	Sigma Aldrich
1 g/L	$MgSO_4 \times 7H_2O$	-	Grüssing
1 mL/L	$KHPO_4$ solution	0.1 M	Sigma
10 mM	TRIS	-	Sigma Aldrich
Dissolved in milliq. water pH adjusted to 7.5 with 1N KOH			
LB medium			
Amount	Ingredient	Concentration	Supplier
10 g/L	Tryptone	-	Roth
5 g/L	Yeast extract	-	Roth
5 g/L	NaCl	-	Grüssing
Dissolved in milliq. water pH adjusted to 7.2 with 1N KOH			

For the preparation of Agar, 7.5 g/L agar is added to the medium before autoclaving, Soft agar is prepared by addition of 4 g/L agar to the medium broth.

1.2 Isolation of genomic DNA from myxobacteria

To isolate bigger amounts of higher quality genomic DNA e.g. to amplify the PCR products for TAR cloning, the alkaline lysis gDNA preparation protocol is used.

1) Spin down 50ml of fresh myxobacterial culture 6000 rcf 10 min
2) Discard the supernatant
3) Wash the cells once with SET Buffer, centrifuge at 6000 rcf 10 min
4) Resuspend cell pellet in 5ml SET Buffer

5) Add 100 µL of Lysozym (50 mg/ml in ddH_2O) stock solution as well as 50µL RNAse A (10 mg/ml in ddH_2O) stock solution
6) Add 300 µL Proteinase K solution (10 mg/ml 50 mM Tris 1mM $CaCl_2$) invert several times and add 600µL 10% SDS solution
7) Incubate at 55°C for 2 h, invert every 15 min
8) Add even Volume (6ml) of Phenol/Chloroform/Isoamylalcohol (25:24:1) and swing the tube for 60 min at 5 rpm
9) Centrifuge the mixture at 6000 rcf for 5 min at room temperature
10) Transfer the upper phase into a new tube using a cut end 1ml tip
11) Add even Volume (6ml) of Phenol/Chloroform/Isoamylalcohol (25:24:1) and swing the tube for 60 min at 5 rpm
12) Centrifuge the mixture at 6000 rcf for 5 min at room temperature
13) Transfer the upper phase into a new tube using a cut end 1ml tip
14) Add even Volume (6ml) of Chloroform/Isoamylalcohol (24:1) and swing the tube for 60 min at 5 rpm
15) Centrifuge the mixture at 6000 rcf for 5 min at room temperature
16) Transfer the upper phase into a new tube using a cut end 1ml tip
17) Add 1/10 of the total volume of 3M NaOAc solution pH 5.5 and mix well by inverting several times
18) Add 2.5 Volumes of ice cold ethanol (100% technical purity, -20°C) and invert the tube several times, DNA precipitation should be visible as a cotton like fog in the tube
19) Spool the DNA on a sealed Pasteur pipette
20) Rinse the DNA with 70% Ethanol (cold , -20°C)
21) Air dry the DNA for at least 15 minutes (Dry DNA will become completely translucid)
22) Resuspend dried DNA in 0.5 ml of ddH_2O and keep the Eppendorf tube at room temperature for 24 Hours
23) Check the genomic DNA on an agarose gel

1.3 List of oligonucleotides used in this study

Oligonucleotides used in this study are ordered from Sigma Aldrich. Nucleotides with a length below 40 bp are ordered as desalted primers, while all longer oligonucleotides were ordered as RP-HPLC purified primers.

Table S 2: List of all oligonucleotides used in this study

Primer name	Primer sequence
FP_pSBtn5verif_in	CCTTTGGACAGCAAGCGAAC
FP_pSBtn5verif_out	CACTCATTAGGCACCCCAGG
And48_pcyA_PromEx_verif_fwd	ATTCATTGGAGTGAGGGC
And48_pcyA_PromEx_verif_rev	GATGTACTCGCAGCGGATGA
And48_pcyJ_PromEx_verif_fwd	TGTGATGGATGGACTTCGCG
And48_pcyJ_PromEx_verif_rev	CGAAGATGAGGACGTTGCCT
FP_pFPtettn5verif_in	GCCTCTTCGCTATTACGCCA
FP_pFPtettn5verif_out	CACTCATTAGGCACCCCAGG
FP pCR2.1 in	CCTCTAGATGCATGCTCGAGC
FP pCR2.1 out	GGATCCACTAGTAACGGCCG
FP t2PKS KS1 SCveriv fwd	CGGCAACTACTAACCCCACA
FP t2PKS KS1 SCveriv rev	CATGAAGTCGGTGGGGTTGA
And48 KS1 SC Primer rev	AAGACCTCCCTCGTAGTCCG
And48 KS1 SC Primer fwd	GGACCGGTACATCCAGTTCG
t2PKS_Synthlig_tn5fusion_fwd	AGACAGGATAAGGAGGTACAGCATATGTCCGAAACCGCATCCTTC
t2PKS_Synthlig_tn5fusion_RC	GAAGGATGCGGTTTCGGACATATGCTGTACCTCCTTATCCTGTCT
t2PKS_AMPdepSynth_rev	TCAGAATTCTCATGCTCCTGTGGGGATG
tn5_Promotor_primer_XbaI	AGTCTAGAGTAATACGACTCACTATAGGG
t2PKS_Synthlig_tn5fusion_fwd	AGACAGGATAAGGAGGTACAGCATATGTCCGAAACCGCATCCTTC
t2PKS_Synthlig_tn5fusion_RC	GAAGGATGCGGTTTCGGACATATGCTGTACCTCCTTATCCTGTCT
t2PKS_AMPdepSynth_rev	TCAGAATTCTCATGCTCCTGTGGGGATG
tn5_Promotor_primer_XbaI	AGTCTAGAGTAATACGACTCACTATAGGG
t2PKS_Synthlig_tn5fusion_fwd	AGACAGGATAAGGAGGTACAGCATATGTCCGAAACCGCATCCTTC
p15A_rev	GTGCACCTCTGGCTCACCGAC
p15A_fwd	TCTGTCCCTCCTGTTCAGCT
VanR_pCLY_fwd	GACTCCAACGTCAAAGGGCGATCAGTCGGCGCGAATGCTCCA
VanR_pCLY_RC	TGGAGCATTCGCGCCGACTGATCGCCCTTTGACGTTGGAGTC
pCLY_mx8_RC	GCGTACCCGAAGTTCACCGAAAATTCAGAAGAACTCGTCAAGAAGGCGATAGAAGGCG
pCLY_mx8_fwd	CGCCTTCTATCGCCTTCTTGACGAGTTCTTCTGAATTTTCGGTGAACTTCGGGTACGC
And48_t2PKS_PCR1_rev	CGGGCTGGAAGTAGGTGATG
And48_t2PKS_PCR2_fwd	CGCAACCAGGAGTTCGTGAT
And48_t2PKS_PCR2_rev	GCACGTATGAAATGGCGTCC
And48_t2PKS_PCR3_fwd	CACTCCCTTCACGTACCTGG
And48_t2PKS_PCR4_rev	AACCACTGGGAGATGTGCTG
And48_t2PKS_PCR5_fwd	GGAGGCCTACAAGGAATCCG
And48_PGyrA_fwd	TTCATGCGGAAGTAGCGAGGCGGAAGGGCCCTCATTTTTC
And48_PGyrA_rev	AAGATGGGAGGCCGGGGGACGCCATGAGCTCTGGAAGTGTTCGGA
And48_t2PKS_PCR3_rev	AAAACCCTGGCGTTACCCAACCTCCCCTACCCCGTAGTC
And48_t2PKS_PCR4_fwd	GACTACGGGGTAGGGGAGGTTGGGTAACGCCAGGGTTTT
And48_t2PKS_PCR5_rev	GAAAAATGAGGGCCCTTCCGCCTCGCTACTTCCGCATGAA
And48_t2PKS_PCR6_fwd	TCCGAACACTTCCAGAGCTCATGGCGTCCCCCGGCCTCCCATCTT
And48_t2PKS_PCR6_rev	CGCTCGACAAGCGCGGCCGTCCGGTTCAGCGGCTCTTCC
And48_t2PKS_resOperon_mx8	GGAAGAGCCGCTGAACCGGACGGCCGCGCTTGTCGAGCG

mx8_attB_up	CGAGGAGTACGGGACTTGAAC
mx8_attB_down	CGGATAGCTCAGCGGTAGAG
mx8_attP_up	CATGGTCGACGCCTACACGAC
mx8_attP_down	GGCTTGTGCCAGTCAACTGCG
CmR_Ampdep_fwd	GATGGCGCAGGGGATCAAGATCTGATCAAGAGACAGGATAAGGAGGTACAGCATCTGAACAGG AGGGACAGCTG
CmR_Ampdep_rev	CACCTGCGACGCGGACAGGTGGCGTGGGTCTTCCTGGGTGATGCAGGCAATCAACATTTACGCCC CGCCCTGCCACTCATCGCAGTA
CmR_Mucono_fwd	AGCAGAGCGCGGCGTCCCAGAAGGGCCCGTCCGCCATCCCCACAGGAGCATGAGCCTGAACAGG AGGGACAGCTG
CmR_Mucono_rev	TCGCCCGGAGCGGCCTCGGTCAGTACGGCGAACAGCATGGGTGGGTTCTCCGGTAGGGTTACGC CCCGCCCTGCCACTCATCGCAGTA
CmR_Cyclase_fwd	TCACCCACGCGATGGCCGAGAACGCCTGACCCCCTACCGGAGAACCCACCCCTGAACAGGAGGGA CAGCTG
CmR_Cyclase_rev	TCAGGGGCCGAGAGCGGAACGCCGCGTCGTGTGTCCGGGAAGCTTCCGTCCATTTACGCCCCGCC CTGCCACTCATCGCAGTA
CmR_T7A1_fusion_fwd	TACTGCGATGAGTGGCAGGGCGGGGCGTAAATCCATTACGCCAAGCTTATCA
CmR_T7A1_fusion_fwd	TGATAAGCTTGGCGTAATGGATTTACGCCCCGCCCTGCCACTCATCGCAGTA
T7A1_glycohydr_rev	GCGGCGCTCTTCTGCGGAAGCGCGGTGGGCTTGGACAGGGCTTTGGTCATATGTACACCTCTCGA TGG
TipA_CmR_fwd	CCGATTCACCCGTGACGCCATCATCGCGAACGCGGAGCGGCACGGCGAGTAGCTGAACAGGAGG GACAGCTG

1.4 Ligation protocol used in this study

Ligations performed in this study are done with Thermo scientific T4 ligase.

T4 DNA Ligase mix:

2µL vector DNA (1h dephosphorylated using FastAP (shrimp alkaline phosphatase, Thermo scientific))

2 – 6.5 µL insert DNA (triple the molar amount of the vector DNA)

1 µL T4 DNA ligase buffer

0.5 µL T4 DNA ligase

to 10µL ddH_2O

The ligase mix is incubated at 16°C for 15-24h and 1-5 µL ligation mix are subsequently electroporated into *E. coli* DH10ß using standard *E. coli* electroporation protocol.

1.5 List of polymerase chain reaction protocols used in this study

1.5.1 Thermo scientific Taq Polymerase

Taq Polymerase Protocol:

2µL NH_4SO_4 Buffer

(Alternatively 2µL KCl Buffer)

2µL $MgCl_2$ solution (25mM)

4µL dNTP's (2mM)

4µL 50% Glycerol

1µL Primer fwd and rev (50mM)

0.2 µL Taq DNA Polymerase

1µL gDNA Template (~50 ng/µL)

4.3µL ddH_2O

Taq Polymerase cycler program:

Table S 3: Taq DNA Polymerase PCR program

Step	Time [min:s]	Temperature [°C]
Initial denaturation	4:00	95
	0:30	95
Cycle, repeat 30x	0:15	63
	1:20	72
Final elongation	10	72
Store	forever	8

1.5.2 Thermo scientific Phusion Polymerase

Phusion Polymerase Protocol:

5µL GC Buffer

2.5µL dNTP's (2mM)

1.25µL DMSO

0.5µL Primer fwd and rev (100mM)

0.2 µL Phusion DNA Polymerase

0.5µL gDNA Template (~50 ng/µL)

14.55µL ddH_2O

Phusion Polymerase cycler program:

Table S 4: Phusion DNA Polymerase PCR program

Step	Time [min:s]	Temperature [°C]
Initial denaturation	4:00	95
	0:30	98
Cycle, repeat 30x	0:15	63
	1:20	72
Final elongation	10	72
Store	forever	8

Table S 5: Phusion DNA Polymerase PCR program for long PCR products and long oligonucleotides

Step	Time [min:s]	Temperature [°C]
Initial denaturation	4:00	95
	0:30	98
Cycle, repeat 2x	0:15	61.5
	6:00	68
	0:30	98
Cycle, repeat 30x	3:00	66
	6:00	68
Final elongation	10	72
Store	forever	8

1.6 *Pyxidicoccus fallax* An d48 antibiotics sensitivity tests

In order to determine available resistance markers for genetic manipulation of An d48 the strain's resistance towards different antibiotics was assayed by adding respective antibiotic concentrations to an YM medium agar plate. The strain shows resistance against 12 µg/ml tetracycline and partial resistance against 25 µg/ml zeocin. On the other hand the strain is sensitive to 50 µg/ml kanamycin, 25 µg/ml

chloramphenicol and 12 µg/ml oxytetracycline. For practical reasons kanamycin and oxytetracycline is chosen as a second resistance marker, since the pCR2.1 Topo and pSWU22 vectors will be used. The pCR2.1 Topo is based on kanamycin. The pSWU22 vector harbors a tetracycline resistance cassette as well as a multiple cloning site and has no overlap with the pCR2.1 Topo backbone as overlap could lead to wrong backbone-backbone recombinations.

1.7 Transformation protocols

Transformation protocol *E. coli DH10ß* TOPO TA kit cloning

1) Take competent *E. coli DH10ß* from -80°C on ice
2) Mix competent cells with pCR2.1 TOPO reaction mixture
   (0.5µL salt solution, 0.5µL pCR2.1 TOPO vector (linearized), 1-5 µL PCR product cleaned by gel electrophoresis incubated for 10 minutes)
3) Electroporate the cells at 1250 V, 25 µF, 200 Ohm and 1mm in a standard electroporation cuvette (100 µL).
4) Add 500 µL of fresh LB medium
5) Incubate on a shaker at 37°C for 1h
6) Plate on LB Kan50 Agar and incubate the plates overnight at 37°C
7) Pick around 6 clones and cultivate them for plasmid preparation via alkaline lysis

1.8 Plasmid verification

1.8.1 Plasmid preparation via alkaline lysis

1) Centrifuge 2-4 ml E. coli culture in LB medium in a 2ml Eppendorf tube (13000-15000 rpm table centrifuge 2 min)
2) Resuspend the Pellet in 250 µl Solution I (Store at +4°C after addition of RNAse A)
3) Incubate for 5 min approximately
4) Add 250 µL Solution II, mix well via inverting the Eppendorf tube
5) Add 250 µL Solution III (fast to avoid denaturing the supercoiled plasmid DNA), mix well via inverting the Eppendorf tube
6) Centrifuge for 30 minutes at 13000-15000 rpm with a table centrifuge

7) Transfer the supernatant into fresh 1.5 ml Eppendorf tubes
8) Add 850µL analytical grade Isopropanol
9) Centrifuge the sample for 30 min at 13000-15000 rpm, possibly at 4°C
10) Remove the supernatant and add 350 µL EtOH to wash the plasmid DNA
11) Centrifuge the mixture for 5 min and remove the ethanolic supernatant, this step can be repeated to clean up the DNA
12) Dry the pellet by inverting the tube on a clean piece of paper or at 65°C (make sure to avoid residual EtOH in your plasmid preparations in order to avoid re-precipitation of the vector over time)
13) Add 50 µL of nuclease free H_2O and resuspend the Pellet at 55°C for 1h.

1.8.2 Plasmid verification by restriction digestion

1) Find a restriction Enzyme that cuts one time on the vector backbone and one time on the insert.
2) Mix 8.5µL Plasmid from alkaline lysis with 1µL restriction enzyme buffer and 0.5 µL enzyme.
3) Incubate for 3-4 h
4) Verify the bands on agarose gel (0.8 %) by gel electrophoresis

1.9 Transformation of the myxobacterial strains used in this study

1.9.1 Devising a reliable mutagenesis protocol for the myxobacterium *p. fallax* And48

Electro competence of the myxobacterium *p. fallax* And48 was assayed with the plasmid pMycoMar-Kan that confers kanamycin resistance via the defective transposon *magellan-4* [1]. Following optimization of electroporation parameters, a reliable protocol was created that allowed site directed mutagenesis via single crossover homologous recombination (detailed information in the supplementary information).

1.9.2 Single crossover homologous recombination in *P. fallax* And48

Since Silica membrane prepared plasmid (GeneJET Plasmid Miniprep kit, Thermo scientific) transposon mutagenesis of And48 yields more than 400 clones per 500 µg plasmid while standard alkaline lysis led to clone numbers inferior to 10, all manipulations of *Pyxidicoccus fallax* And48 are done using silica membrane purified plasmid.

Transformation protocol

1) Centrifuge 2ml of an And48 culture in YM Medium at OD_{600} of approx. 0.3 at 8000 rpm for 2 minutes at a table centrifuge.
2) Wash the residual cell pellet 2 times with 500 µL autoclaved ddH_2O and discard the supernatant.
3) Resuspend cells in 50µL of ddH_2O, add 5 µL of plasmid solution (prepared from E. coli with Thermo Scientific Miniprep Kit) at a conc. of 0.3-0.4 ng/µL and transfer the solution into an electroporation cuvette.
4) Electroporation at 675 V, 400 Ohms, 25µF and 1mm cuvette settings for optimum electroporation efficiency.
5) Flush out the cells with 1 mL fresh YM medium, transfer the Cell suspension into a 2ml Eppendorf tube.
6) Incubate the Cells for 5h on a Shaker thermostated to 37°C at 300 rpm.
7) Plating the cells on Kan50 YM Agar after mixing the cell suspension with 3 ml of Kan50 YM Softagar.
8) Slightly red, spherical clones appear after 7-12 Days in the 30°C incubator.

1.9.3 Transformation protocol for *M. xanthus* DK1622

The transformation protocol for *M. fulvus* SBMx132 was elaborated from CTT medium cultures, since CTT medium leads to growth in perfect suspension. This is a prerequisite for efficient electro transformation of myxobacteria.

Transformation protocol

9) Centrifuge 2ml of an Mx132 culture in CTT Medium at OD_{600} of approx. 0.3 at 8000 rpm for 2 minutes at a table centrifuge.
10) Wash the residual cell pellet 2 times with 500 µL autoclaved ddH_2O and discard the supernatant.
11) Resuspend cells in 50µL of ddH_2O, add 5 µL of plasmid solution (prepared from E. coli with Thermo Scientific Miniprep Kit) at a conc. of 0.3-0.4 ng/µL and transfer the solution into an electroporation cuvette.
12) Electroporation at 675 V, 400 Ohms, 25µF and 1mm Cuvette Settings for optimum electroporation efficiency.
13) Flush the cells out with 1 mL fresh CTT medium, transfer the Cell suspension into a 2ml Eppendorf tube.
14) Incubate the Cells for 5h on a Shaker thermostated to 37°C at 300 rpm.

15) Plating the cells on Kan50 CTT Agar after mixing the cell suspension with 3 ml of Kan50 CTT Softagar.
16) Clones appear after 5-7 Days in the 30°C incubator.

1.9.4 Transformation protocol for *S. aurantiaca* DW 4/3-1

The transformation protocol for *S. aurantiaca DW4/3-1* was elaborated from TS-6 medium cultures, since TS-6 medium leads to growth in perfect suspension.

Transformation protocol

17) Centrifuge 2ml of a *S. aurantiaca* DW4/3-1 culture in TS-6 Medium at OD_{600} of approx. 0.3 at 8000 rpm for 2 minutes at a table centrifuge.
18) Wash the residual cell pellet 2 times with 500 µL autoclaved ddH_2O and discard the supernatant.
19) Resuspend cells in 50µL of ddH_2O, add 5 µL of plasmid solution (prepared from E. coli with Thermo Scientific Miniprep Kit) at a conc. of 0.3-0.4 ng/µL and transfer the solution into an electroporation cuvette.
20) Electroporation at 650 V, 400 Ohms, 25µF and 1mm cuvette Settings for optimum electroporation efficiency.
21) Flush the cells out with 1 mL fresh TS-6 medium, transfer the cell suspension into a 2ml Eppendorf tube.
22) Incubate the Cells for 5h on a Shaker thermostated to 37°C at 300 rpm.
23) Plating the cells on Kan50 TS-6 Agar after mixing the cell suspension with 3 ml of Kan50 TS-6 Softagar.
24) Clones appear after 10-14 Days in the 30°C incubator.

1.10 Elaboration of the plasmids used for single crossover integration

1.10.1 Single crossover inactivation plasmids for the pyxidicycline cluster

In order to disrupt the pyxidicycline cluster, ca. 1 kb single crossover homology arms targeting the KSα on the *pcyE* gene and the cyclase on *pcyC* were amplified from An d48 genomic according to the protocol for gDNA isolation from gram-negative bacteria (DNA Puregene Core Kit A, Quiagen) and ligated into pCR2.1-TOPO (TOPO-TA cloning, Thermo scientific TOPO-TA Kit). Single crossover integration was achieved by electro-transformation of An d48 using a protocol elaborated in this work (section S 1.9) and resulting mutants were checked for correct integration by PCR using the TAQ PCR protocol (section S 1.5).

For single crossover inactivation of the pyxidicycline secondary metabolite cluster a pCR 2.1 vector from the TOPO-TA cloning kit (TOPO-TA cloning KIT, Thermo scientific) was used. Amplification of the 1 kb homology fragments was done with TAQ Polymerase (Thermo scientific) from And48 gDNA according to the TAQ polymerase amplification protocol (section S 1.5). The vector containing the KSα encoded on *pcyE* insert was verified by restriction digestion with the restriction Enzyme NcoI that cuts once on the pCR2.1 backbone and once on the KSα homology arm insert and analogously the vector containing homology to the cyclase *pcyC* was verified with NaeI. Two *E. coli* clones whose plasmids show the correct restriction pattern are selected for plasmid isolation with the plasmid isolation kit (GeneJET Plasmid Miniprep kit, Thermo scientific).

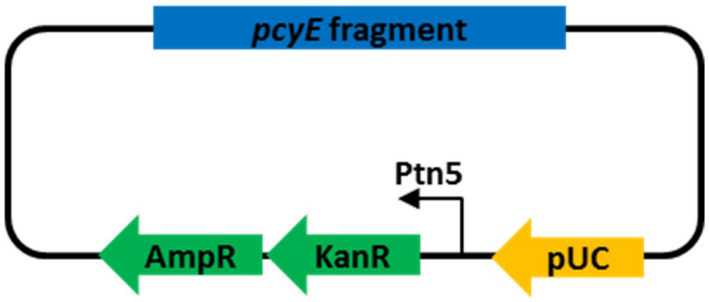

Figure S 1: pCR2.1 vector from the pTOPO Kit carrying a 1kb homology arm for single crossover inactivation of the KSα on the pcyE gene.

1.10.2 Plasmids used for promotor exchange of the *pcyA* gene

The first utilized construct was designed in order to exchange the alleged native promotor against a tn5 promotor. This promotor was chosen as it was known to successfully drive the kanamycin resistance gene expression in *M. xanthus*. The plasmids used for overexpression of the minimal PKS operon are called pSBtn5 and pFPVan, consisting of a pCR2.1 vector backbone from the TOPO-TA cloning kit (Thermo scientific, U.S.A) and a tn5 promotor or a vanillate promotor repressor system. The plasmids created in this section serve as tool to control the transcription and translation of operon 1 of the pyxidicycline cluster via a heterologous promotor.

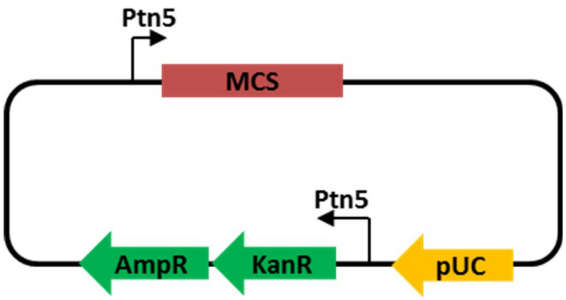

Figure S 2: pSBtn5 vector used for placing the pyxidicycline gene cluster under the control of the tn5 promotor

The first 1-1.5 kb of a biosynthetic assembly line are inserted into the pSBtn5 with the restriction enzymes EcoRI and NdeI that are introduced during PCR with restriction sites on the primers. NdeI is necessary as it contains the start codon and usage of EcoRI as a complementary enzyme facilitates cloning as both enzymes are working in Thermo scientific 1x Orange buffer. In order to get good cloning efficiency and avoid vector relegation clones it is necessary to dephosphorylate the vector backbone thoroughly before ligation. This is done by utilizing the FastAP shrimp phosphatase (Thermo Scientific) which is also compatible with Thermo Scientific 1xO buffer. Electroporation of *Pyxidicoccus fallax* An d48 is done according to the devised protocol (see section S 1.9) with the assembled and purified pSBtn5 plasmid harboring the first gene of the type 2 PKS minimal PKS Operon. As this gene is called *pcyA*, this assembled plasmid will be called pSBtn5 *pcyA*.

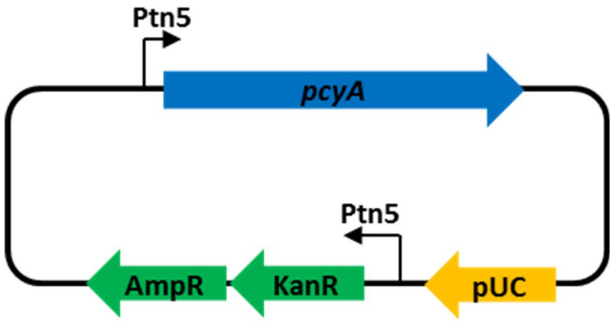

Figure S 3: pSBtn5 pcyA construct used for overexpression of p. fallax And48 pyxidicycline cluster

In order to avoid self-toxicity a second vector for single crossover insertion is created that harbors a promotor-repressor system inducible by vanillate. As the pCR2.1 Vector from the TOPO-TA Kit (Thermo scientific) works well for single crossover insertions of foreign DNA in *P. fallax* An d48, pCR2.1 is taken as a basis for this second vector system. The first gene of the t2PKS assembly line *pcyA* is amplified and the vanillate promotor-repressor system is added via overlap extension PCR using the Phusion PCR protocol

that also introduced NdeI as well as EcoRI restriction sites on the Primers (Table S 3). This PCR fusion product is ligated into the cut vector via TOPO-TA cloning (TOPO TA Kit, Thermo scientific).

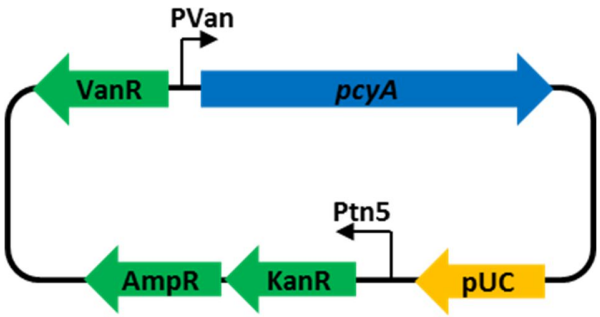

Figure S 4: pFPVan pcyA used for overexpression of the t2PKS cluster in Pyxidicoccus fallax *An d48*

Since both of these vectors are designed to work with the restriction enzymes EcoRI and NdeI, these gene cluster overexpression vectors can be utilized with the same PCR product into which NdeI and EcoRI restriction sites have been introduced.

1.10.3 Creation of an orthogonal tetR based vector for a second single crossover integration

In order to introduce a second promotor exchange into the bacterial chromosome of *Pyxidicoccus fallax* And48 *pcyA* :: pFPVan *pcyA,* a vector with an orthogonal resistance marker had to be created. As preliminary studies showed oxytetracycline to be a usable selection marker for *P. fallax* An d48 (section S 1.6), a vector harboring a p15A origin, the *tetR* gene as well as a fusion of a tn5 promotor to the *pcyJ* gene was created by standard restriction cloning based on the pSWU22 plasmid. This plasmid solely consists of the tetracycline exporter gene as resistance marker, a pUC origin and a multiple cloning site. The plasmid pSWU22 possesses a pUC origin which is not usable in this case as this might lead to single crossover integration of pUC :: pUC which is not desired. Therefore, the origin was exchanged against a p15A origin by restriction ligation using DraI and ApaLI. This also leads to elimination of the NdeI cutting site on the plasmid backbone that would impede efficient use of the created plasmid later. Overlap extension PCR with the Phusion PCR protocol was subsequently used to amplify a fusion PCR product consisting of a tn5 promoter and the *pcyJ* gene harboring XbaI cutting sites on the primer sequences. Standard restriction cloning is subsequently used to insert the tn5-*pcyJ* fusion sequence into the modified pSWU22 plasmid to create the pFPtettn5 *pcyJ* vector.

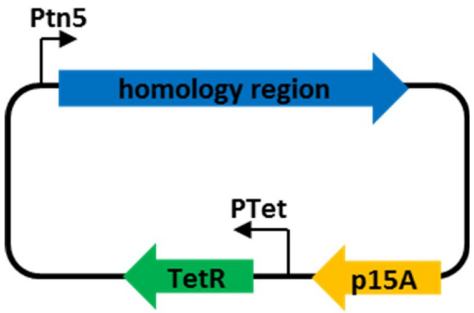

Figure S 5: schematic view of the pFPtettn5 vector used for creation of the Pyxidicoccus fallax *An d48* pcyA *:: pSBtn5* pcyA*;* pcyJ *:: pFPtettn5* pcyJ (*An d48* pcy) *mutant*

This allowed exchange of the native pcyJ promotor against a tn5 promotor via single crossover homologous recombination in *Pyxidicoccus fallax* An d48 *pcyA* :: pFPVan *pcyA* to obtain *Pyxidicoccus fallax* An d48 *pcyA* :: pFPVan *pcyA; pcyJ :: pFPtettn5 pcyJ* (An d48 *pcy*). Single crossover integration of this plasmid into the *Pyxidicoccus fallax* An d48 *pcyA* :: pFPVan *pcyA* strain was achieved by electroporation according to standard protocol (section S 1.9). As mentioned in the results, despite serious efforts we were unable to obtain integration clones of said plasmid into *Pyxidicoccus fallax* An d48 *pcyA* :: pSBtn5 *pcyA.* Plasmid integration verification was done by PCR using the TAQ PCR protocol (section 1.5).

1.10.4 Verification of plasmid integration into the myxobacterial strains

Verification of the myxobacterial mutant clones is done by PCR with TAQ DNA Polymerase (Thermo Scientific). Therefore, genomic DNA from the myxobacterium is isolated with the Puregene Core Kit A (Quiagen) according to the protocol for gDNA isolation from gram-negative bacteria. The Taq DNA Polymerase master mix is prepared according to Table S 3. Primers are in all cases 2 primers that bind on the genomic DNA next to the homologous region if homologous recombination is used or next to the integration site if phage integrase based integration is used as well as two complementary primers that bind on the integrated vector. Therefore the appearance of PCR products of the right size proves homologous recombination to have occurred in the correct position.

1.11 Protocols for transformation assisted recombination (TAR cloning)

1.11.1 Stock solutions needed for TAR cloning of biosynthetic gene clusters

PEG 3500 solution

50 % [w/v] PEG 3500 (Sigma Aldrich) in milliq Water

Single strand carrier DNA stock solution

2 mg/ml salmon sperm DNA (Sigma Aldrich) in autoclaved milliq Water

Boil for 5 min at 95 °C and freeze aliquots of 500 µL, these aliquots can be used several times

LiOAc stock

1M LiOAc in TE Buffer

TE Buffer

1 mM EDTA

10 mM Tris

in milliq water

pH 8.0

1.11.2 TAR cloning protocol adapted from Bylik et al. [2]

Previous day:

1) Inocculate the *S. cerevisiae* strain ATCC4004247 in 10 ml YPAD medium a 50 ml baffled flask over night at 30°C on a rotary shaker at 200 rpm. Simultaneous pre - warm one 300 ml baffled flask containing 50 ml of sterile YPAD medium to 30°C

TAR cloning day:

2) Determine approximate culture titer by measuring OD 600 of 10µL yeast culture in 1ml of milliq H_2O, OD of 0.1 is approximately 10^6 yeast cells.
3) Add ca. 2 x 10^8 yeast cells to the pre warmed YPAD medium and incubate for 4h at 30°C 200 rpm.
4) Denature one aliquot of the SS carrier DNA stock solution for 5 min at 95°C and chill the solution on ice.

5) Harvest the yeast cells at 3000g for 5 min in a 50 ml falcon, wash twice with 25 ml sterile milliq H_2O and re suspend the cells in 1000 µL of sterile milliq H_2O.
6) Pipet aliquots of 200 µL of this solution in 1.5 µL Eppendorf tubes and remove the water by centrifuging at 3000g for 5 min
7) Add 360µL of transformation mix to the pellets and mix by pipetting up and down
8) Place the Eppendorf tubes (closed) in a floating device and heat shock the *S. cerevisiae* cells at 42°C for 30 min
9) Centrifuge at top speed for 30s and remove residual transformation mix by pipetting
10) Re suspend the pellet in 1ml of sterile milliq H_2O
11) Streak the cells on a SC leucine dropout agar plate and incubate the plate for 2-3 days
12) Pick the clones on a replica plate and select for positive clones via colony PCR
13) *S. cerevisiae* clones that give a positive colony PCR signal are grown in 5 mL of sterile SC leucine dropout medium and the plasmid is prepared from 2ml of *S. cerevisiae* culture

1.11.3 Plasmid preparation from yeast strains

Plasmids from TAR cloning for heterologous expression were prepared from the Δ Leu2 leucine auxotrophy strain *S. cerevisiae* ATCC 4004247 [2].

Plasmid preparation follows the following scheme:

1) Centrifuge 1.5 ml *S. cerevisiae* culture in SC dropout medium in a 1.5 ml Eppendorf tube (13000-15000 rpm table centrifuge 2 min)
2) Re suspend the Pellet in 150 µl Solution I
3) Add 2ml of Zymolyase solution (10mg/ml) to each tube
4) Incubate for 30 min approximately
5) Add 150 µL Solution II, mix well via inverting the Eppendorf tube
6) Add 150 µL Solution III (fast to avoid denaturing the supercoiled plasmid DNA), mix well via inverting the Eppendorf tube
7) Centrifuge for 30 minutes at 13000-15000 rpm with a table centrifuge
8) Transfer the supernatant into fresh 1.5 ml Eppendorf tubes
9) Add 700µL analytical grade Isopropanol and mix well by inverting the tube several times
10) Centrifuge the sample for 30 min at 13000-15000 rpm, possibly at 4°C
11) Remove the supernatant and add 500 µL 70% EtOH to wash the plasmid DNA

12) Centrifuge the mixture for 5 min and remove the ethanolic supernatant, this step can be repeated to clean up the DNA
13) Dry the pellet by inverting the tube on a clean piece of paper or at 65°C (make sure to avoid residual EtOH in your plasmid preparation in order to avoid re-precipitation of the vector over time)
14) Add 30 µL of nuclease free H_2O and re suspend the pellet at 55°C for 1h.

1.12 TAR cloning based pyxidicycline gene cluster assembly

Pxyidicoccus fallax An d48 *pcyA* :: pFPVan *pcyA*, *pcyJ* :: pFPtetp15Atn5 *pcyJ* strain's genomic was prepared according to alkaline lysis genomic DNA preparation standard protocol for all further PCR reactions (section S 1.2). For naturally overlapping PCR fragments e.g. PCR2 and PCR3 the PCR product overlap necessary for TAR cloning was introduced by PCR, for non-overlapping fragments as PCR3 and PCR4, the overlap was introduced with the primer sequences (main text Figure 3 and Figure S 6). 25-30 Bp homology introduced through the primers are sufficient for recombination in yeast. Amplification of the cluster sequence was done by using the PCR program for long PCR products (section S 1.5.2). Co-transformation of the PCR products of the pyxidicycline cluster into the TAR yeast *S. cerevisiae* ATCC 4004247 is done at a total concentration of 25 pg x Number of nucleotides per PCR product to get an equimolar PCR product mix in the yeast transformation mix. The capture vector was designed to harbor all genes necessary for replication and auxotrophy selection in S. cerevisiae analogous to the vector described by Bylik et al.[2] The last fragment imperative for the heterologous expression of the pyxidicycline cluster in *Myxococcus xanthus* DK1622 is the Mx8 integrase prophage with the attP site that mediates stable and efficient integration of plasmids into the DK1622 chromosome's attB site [3,1]. This fragment also contains a kanamycin resistance cassette necessary for the latter selection of integration clones in *M. xanthus* DK1622. Furthermore this resistance is helpful during the retransformation of the constructs in *E. coli* as since it is located on a PCR product different of the one harboring the p15A origin - it helps to prevent selecting plasmids consisting only of the pClY backbone as these would not confer kanamycin resistance. TAR cloning was done according to a protocol by Bylik et al.[2] that was slightly adapted (section S 1.11). Yeast clones appearing on leucine deficient medium were checked by PCR for a fragment of the *pcyE* gene by colony PCR. Clones that gave a positive result were prepared from yeast (Zymo Prep Kit, Zymo Research, U.S.A) and transformed into *E. coli* DH10ß by electroporation. Plasmid preparation and plasmid digestion with the enzymes NdeI, BstEII and NotI were used to confirm the plasmid by restriction digestion. The vector pClY pyxidicycline cluster was sequenced by Illumina sequencing at GMAK

(Brunswick, Germany). The sequence was proven correct except for a single nucleotide polymorphism that leads to a silent mutation in the ATP binding protein of the ABC transporter encoded on *pcyQ.* The plasmid is transformed into *M. xanthus* DK1622 and *S. aurantiaca* DW 4/3-1 according to standard protocol (section S 1.9.3 and 1.9.4). Correct integration via the Mx8 integrase was checked by PCR using the TAQ PCR protocol (section 1.5).[1]

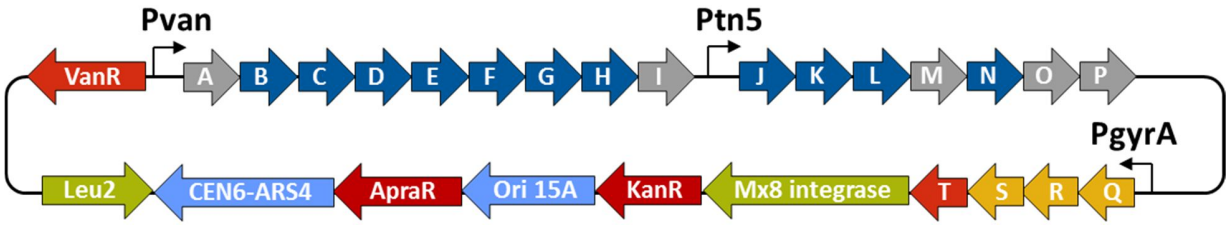

Figure S 6: Schematic view of the pClY Pyxidicycline vector. PKS type-II biosynthesis genes depicted in blue, regulation and resistance genes in red, exporter genes in orange, auxiliary genes for the cloning strategy in light green and orfs of unknown function in gray.

1.13 Protocols for λ-Red prophage recombination (Red E/T)

The strain used for all λ-Red based recombination experiments was *E. coli HS996* carrying the thermosensitive pSC101 Bad red gba plasmid. As the origin of this plasmid stops replicating at 37°C the strain has to be grown at 30°C for cryo cultures.

Incorporation of the target plasmid into the λ-Red recombination strain

1) Inoculate 25 ml LB containing 12 µg/ml oxytetracycline and grow the strain overnight at 30°C 200 rpm on a rotary shaker
2) Inoculate 20 µL of the well grown *E. coli* overnight culture into a 2 ml Eppendorf tube with a punctured lid containing 1.5 ml LB with 12 µg/ml oxytetracycline
3) Incubate the culture for 4h until OD reaches approx. 0.6
4) Mix target vector or Ligation mixture (ca.1-3µL) with competent *E. coli* and pipet the mixture into a Electroporation cuvette (100µL)
5) Electroporate the mixture at 1250V, 25µF, 200 Ohm, 1mm Cuvette
6) Add 1mL fresh sterile LB medium, incubate for 1h at 900 rpm 30°C in a 2ml Eppendorf tube with a punctured lid
7) Plate on LB Kan50 Otc12 Agar (provided the plasmid has kanamycin resistance, if not use appropriate antibiotic) and incubate the plates overnight at 30°C

8) Pick around 6 clones and cultivate them for plasmid preparation via alkaline lysis
9) Confirm the electroporated plasmid via restriction digestion

Modification of plasmids via Red E/T

1) Inoculate 25 ml LB containing 12 µg/ml oxytetracycline and 50 µg/ml Kanamycin and grow the strain overnight at 30°C 200 rpm on a rotary shaker
2) Inoculate 20 µL of the well grown *E. coli* overnight culture into a 2 ml Eppendorf tube with a punctured lid containing 1.5 ml LB with 12 µg/ml oxytetracycline and 50 µg/ml Kanamycin
3) Incubate the culture for 2h until OD reaches approx. 0.2 (sometimes longer incubation times are necessary)
4) Centrifuge the culture at 8000 x g for 5 minutes to pellet the cells
5) Wash the residual cell pellet 2 times with 1000 µL autoclaved milliqH_2O and discard the supernatant.
6) Re suspend the cell pellet in 50 µL autoclaved milliq H_2O and add the PCR product for modification
7) Electroporate the mixture at 1300V, 25µF, 600 Ohm, 1mm Cuvette
8) Add 1mL fresh sterile LB medium, incubate for 1h at 900 rpm 30°C in a 2ml Eppendorf tube with a punctured lid
9) Transfer the mixture into 50 ml LB Kan50 containing appropriate concentration of the antibiotic on the modification PCR product (in my case Chloramphenicol 25µg/ml) and incubate over night at 30°C
10) Prepare plasmid from this mixture via alkaline lysis
11) Electroporate the plasmid mix into *E. coli DH10ß* according to the *E. coli DH10ß* electroporation protocol and plate it on LB Kan50 Cm25
12) Pick around 4 clones and cultivate them for plasmid preparation via alkaline lysis
13) Confirm the electroporated plasmid via restriction digestion

The retransformation is necessary in order to avoid having clones harboring both plasmids (the modified and the unmodified one) in the same *E. coli* clone as well as to get rid of the pSC101 Bad Red gba plasmid

1.14 Elaboration of the Red E/T deletion plasmids utilized in this study

To elucidate functions of the genes in the *pcy* cluster's operon two, genes were subsequently replaced by a chloramphenicol cassette on the heterologous expression construct. Therefore, the plasmid was transformed into the host strain *E. coli* HS996 harboring the pSC101BAD RedγβαA plasmid. Ca. 80 bp

homology arms to the neighboring regions of the genes selected for deletion via Red/ET were put on primers suited to amplify the chloramphenicol resistance cassette (Table S 2). PCR products were obtained using the Phusion PCR protocol (section S 1.5). Subsequent Red/ET replacement of the target genes led to the creation of deletion plasmids for the genes *pcyJ*, *pcyK* and *pcyL.* For overexpression of the *pcyO* gene, a T7A1 promotor was fused to the chloramphenicol resistance cassette equipped with 80 bp homology arms to replace the *pcyO* promotor region. Red/ET recombination was done according to standard linear to circular Red/ET (section S 1.13). The resulting plasmids were transformed into *Myxococcus xanthus* DK1622 via Mx8 mediated integration and the resulting strains termed *M. xanthus* DK1622 *pcy* Δ *pcyJ*, *pcyK* and *pcyL,* depending on the deleted enzyme. The *M. xanthus* mutants were extracted according to standard protocol and analyzed by LC-MS. Pyxidicycline and anthraquinone production titers of M. xanthus DK1622 harboring the pClY pyxidicycline cluster plasmid were compared to the titer of the strains harboring the *pcyJ*, *pcyK* and *pcyL* defective plasmids by LC-MS EIC peak area integration (Table S 21).

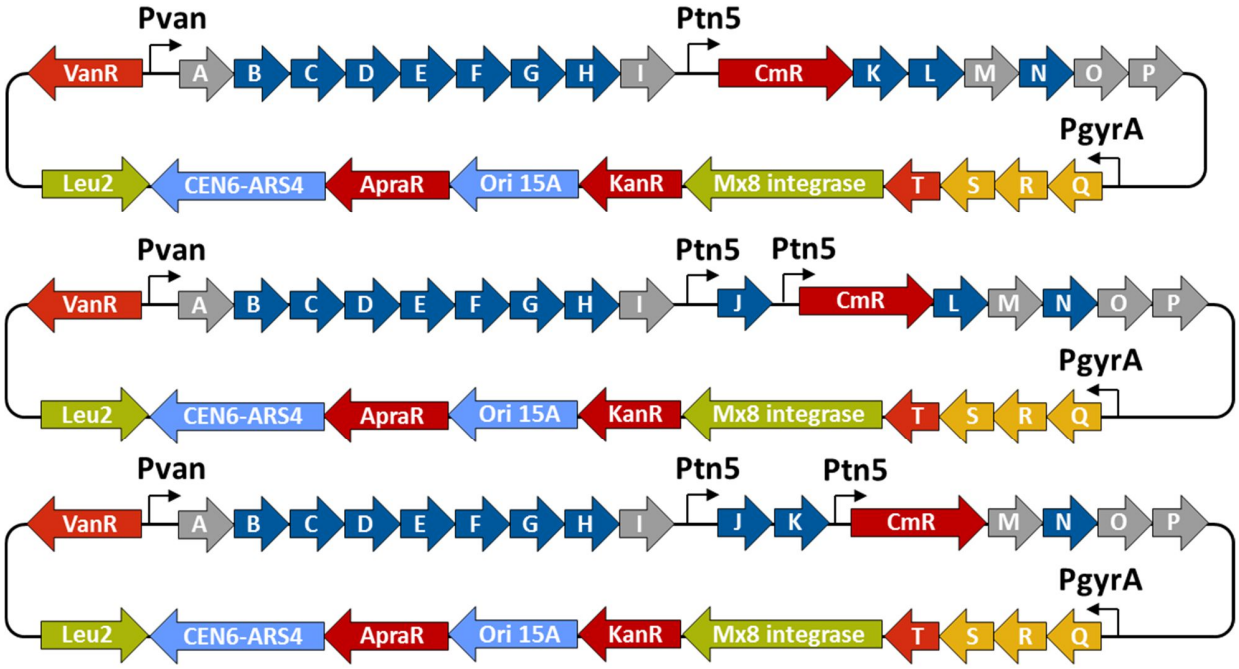

Figure S 7: Schematic view of the pClY Pyxidicycline vector deletion constructs Δ pcyJ, pcyK *and* pcyL*. PKS type-II biosynthesis genes depicted in blue, regulation and resistance genes in red, exporter genes in orange, auxiliary genes for the cloning strategy in light green and orfs of unknown function in gray.*

2 *In silico* analysis of the Pyxidicycline biosynthetic gene cluster

2.1 *In silico* analysis of the pentapeptide repeat protein

In order to analyze similarities of the found putative topoisomerase self-resistance proteins with self-resistance proteins against the gyrase poisons ciprofloxacin, albicidin and cystobactamid the pentapeptide repeat proteins were aligned via MUSCLE alignment. Alignment of the proteins in general shows them to have a rather low overall homology as the proteins are structural proteins with the relaxed consensus motiv [S,T,A,V][D,N][L,F][S,T,R][G] for every repeat. Still the similarity of PcyT to McbG in *E. coli* and CysO in *C. velatus* indicates that this protein might have a similar function.[4]

2.2 3D modelling of the pentapeptide repeat self-resistance protein via the Phyre^2 server

Since the 3D structure of pentapeptide repeat proteins is most likely more important than the primary sequence as these proteins are most likely structural proteins that mimic DNA to protect the hosts topoisomerases an *in silico* 3D structure prediction was done.

Figure S 8: 3D structure homology model of the pentapetide repeat protein putatively conferring topoisomerase self-resistance PcyT modelled by Phyre[2 5]

The best matching proteins found by psi-blast on the Phyre[2] server to obtain a 3D homology model were *Xanthomonas albilineans* AlbG (100.0 confidence scoring; 29 % identity) and the quinolone resistance protein QNR from *Aeromonas Hydrophilia* (99.9 confidence scoring; 17 % identity). Both of these proteins confirm topoisomerase poison resistance and the devised homology model depicted in the picture above looks strikingly similar to described topoisomerase inhibitor resistance proteins.[4]

2.3 *In silico* analysis of the Pyxidicycline biosynthetic gene cluster

Table S 6: Table of all open reading frames belonging to the pyxidicycline (pcy) cluster with proposed function and closest homologue according to a blastp search against the nr (non-redundant protein sequences) protein database at NCBI

Name	Length [bp]	proposed function	closest annotated homologue – organism of origin	Identity [%] and length of alignment [AA]	Accession Nr.
pcyA	1128	precursor release from the acyl carrier protein	acyl-CoA thioester hydrolase – multispecies	27.7 / 361	WP_060519218
pcyB	852	polyketide cyclisation (B-ring cyclisation)	polyketide cyclase - *Streptomyces nogalater*	46.3 / 283	AAF01818
pcyC	963	polyketide cyclisation (A-ring cyclisation)	polyketide cyclase – *Streptomyces atratus*	32.9 / 325	WP_072488808
pcyD	798	C9' type ketoreductase	ketoacyl reductase – *Sorangium cellulosum*	69.1 / 259	WP_061624890
pcyE	1281	ketoacyl synthase KSα subunit	3-oxoacyl-ACP synthase II – *Dendrosporobacter quericolus*	56.0 / 425	SDL92203
pcyF	1242	ketoacyl synthase KSβ subunit (chain length factor)	3-oxoacyl-ACP synthase II – *Pelosinus sp.*	37.7 / 414	WP_038670561
pcyG	237	acyl carrier protein	acyl carrier protein – *Terriglobus saanensis*	33.3 / 78	WP_013566858

pcyH	372	4'-phosphopanthenyl transferase	holo-ACP synthase – *Sorangium cellulosum*	61.5 / 122	WP_061624886
pcyI	249	-	hypothetical protein – *Myxococcus fulvus*	37.9 / 66	WP_052771001
pcyJ	2088	serine incorporation	4-coumarate-CoA ligase family protein – *Modestobater species*	37.3 / 542	WP_082557217
pcyK	303	heterocycle formation	muconolactone delta-isomerase – *Acidovorax valerianellae*	32.5 / 83	SDC81513
pcyL	717	heterocycle formation	polyketide cyclase – *Streptomyces species*	29.3 / 232	WP_053683051
pcyM	378	-	-	-	-
pcyN	762	regulation	MerR family transcriptional regulator – *Myxococcus fulvus*	76.6 / 252	WP_074955963
pcyO	2862	-	glycosyl hydrolase family 31 – *Mucilaginibacter paludis*	60.4 / 934	WP_008504858
pcyP	720	-	hypothetical protein – *Mucilaginibacter paludis*	32.2 / 233	WP_008504859
pcyQ	816	export	ABC transporter, ATP binding protein – *Myxococcus xanthus*	93.0 / 272	ABF90137
pcyR	783	export	ABC transporter permease - *Myxococcus xanthus*	90.7 / 259	WP_011552893
pcyS	1035	export	ABC transporter substrate binding protein - *Myxococcus xanthus*	85.4 / 343	WP_011552892
pcyT	639	putative self-resistance mechanism	pentapeptide repeat containing protein – *Myxococcus fulvus*	70.3 / 209	WP_046715273
orf1	1431	-	serine/threonine protein kinase – *Myxococcus xanthus*	58.5 / 470	WP_011552901
orf2	993	-	hydrolase – *Myxococcus fulvus*	73.4 / 308	WP_013941483
orf3	417	-	elongation factor GreAB – *Myxococcus hansupus*	86.2 / 138	WP_002640736
orf4	714	-	hypothetical protein – *Stigmatella aurantiaca*	30.3 / 238	WP_075011090
orf5	1047	-	hypothetical protein – *Archangium violaceum*	30.8 / 156	WP_043405726
orf6	2163	-	serine/threonine protein kinase – *Archangium gephyra*	38.8 / 707	WP_047858246
orf7	375	-	-	-	-
orf8	1461	-	dihydropyrimidine dehydrogenase subunit A – *Myxococcus xanthus*	86.2 / 486	WP_011553928
orf9	4566	-	glutamate synthase large subunit – *Myxococcus virescens*	90.8 / 1520	SDF16482

The native Pyxidicycline gene cluster will be accessible at GenBank under accession number MH048639 upon publication. All plasmid maps will be provided upon request.

2.4 Identification of KSα and KSβ *in silico*

Both ketosynthase proteins PcyE and PcyF in this assembly line are predicted to be 3-oxoacyl-ACP synthase 2 proteins. Therefore, sequence alignment is done in order to find out which of the sequences acts as the KSα protein and which protein acts as the KSβ. These functions can be identified via NCBI protein blast as, contrary to the KSβ, it possesses all catalytically active residues (see Figure S 9 and Figure S 10). In the PcyF amino acid sequence the catalytically active cysteine residue is mutated to arginine rendering it catalytically inactive.[6]

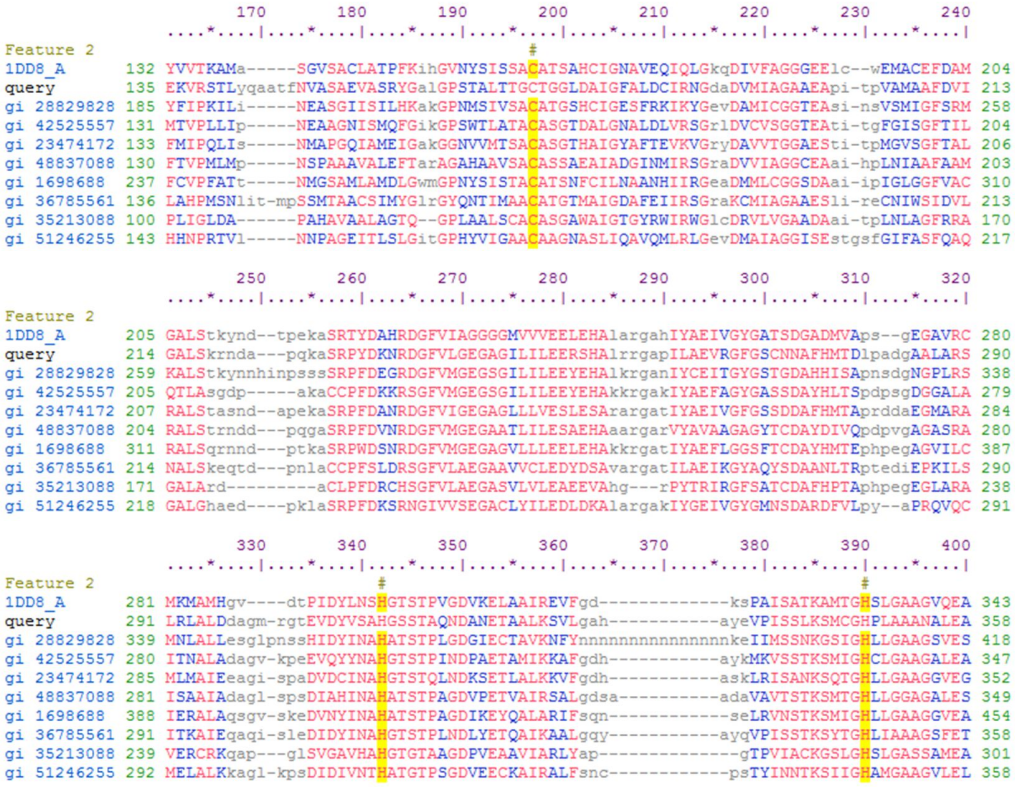

Figure S 9: Identification of the KSα protein on the gene pcyE *via blast alignment against the non-redundant protein sequences collection (nr) on the NCBI protein server*

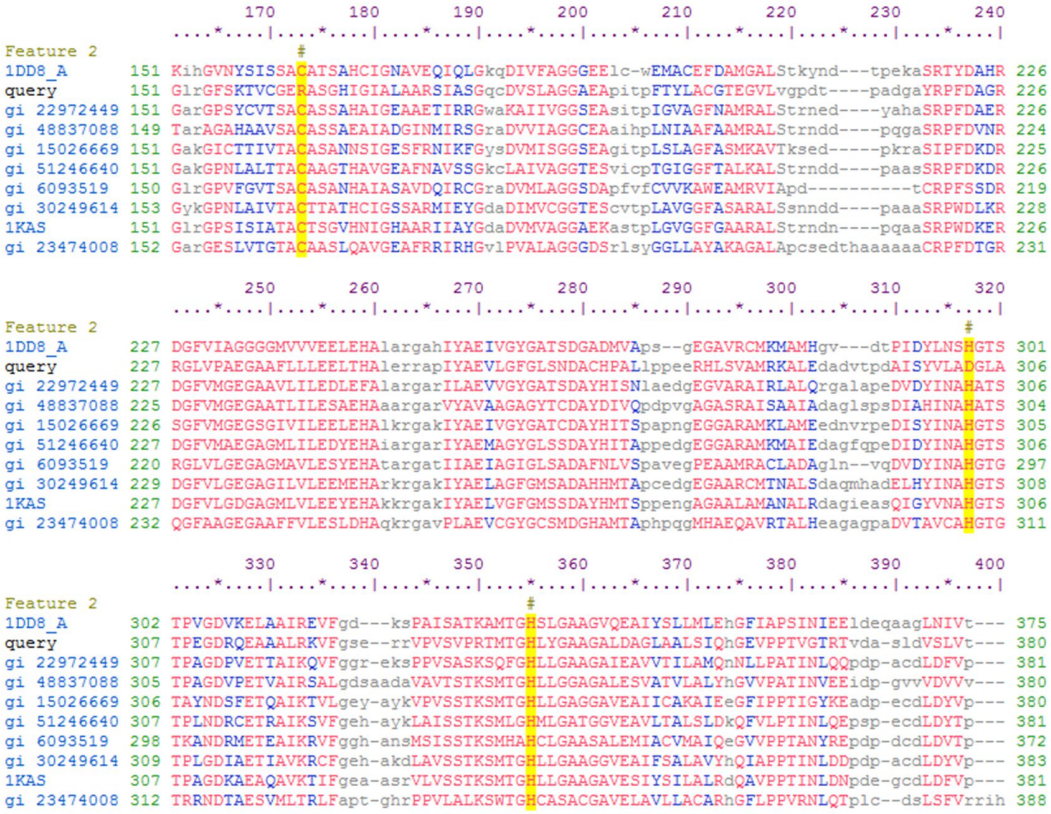

Figure S 10: Identification of the KSδ protein on the gene pcyF *via blast alignment against the non-redundant protein sequences collection (nr) on the NCBI protein server*

2.5 *In silico* analysis of the PcyJ Protein

The PcyJ protein is responsible for amide bond formation between the polyketide intermediate of the pyxidicyline biosynthesis and serine to form the D ring in pyxidicycline A and B. Therefore the protein has to have acyl transfer activity to form an amide bond between serine and the polyketide precursor as well as hetero cyclisation activity.

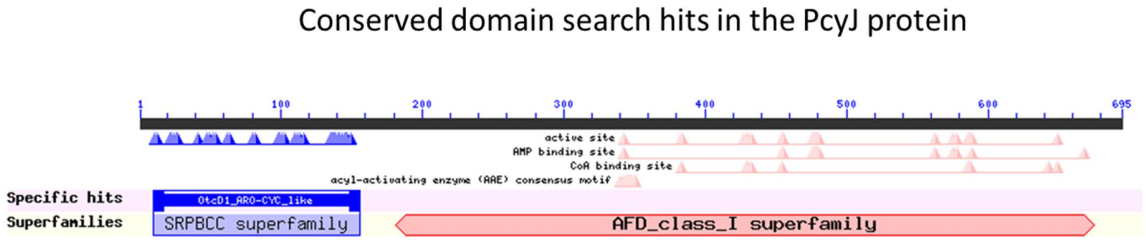

Figure S 11: NCBI conserved domain search hits in the PcyJ protein stating the di-domain nature of the protein as a AMP dependent synthetase/ligase – aromatase/cyclase fusion protein

Analysis of the PcyJ protein by NCBI conserved domain search [7] reveals an aromatase/cyclase domain (blue) putatively responsible for hetero cyclisation and an AMP-dependent synthetase and ligase function putatively responsible for amide bond formation.

2.6 *In silico* assignment of the pyxidicyclines' cyclase proteins

During maturation of the pyxidicyclines there are several cyclase like proteins involved to form the tetracene quinone like core structure. In order to assign the first and second ring cyclases which was not possible by knockout experiments we relied on protein alignment via the MUSCLE [8] algorithm to group the cyclase proteins into groups.

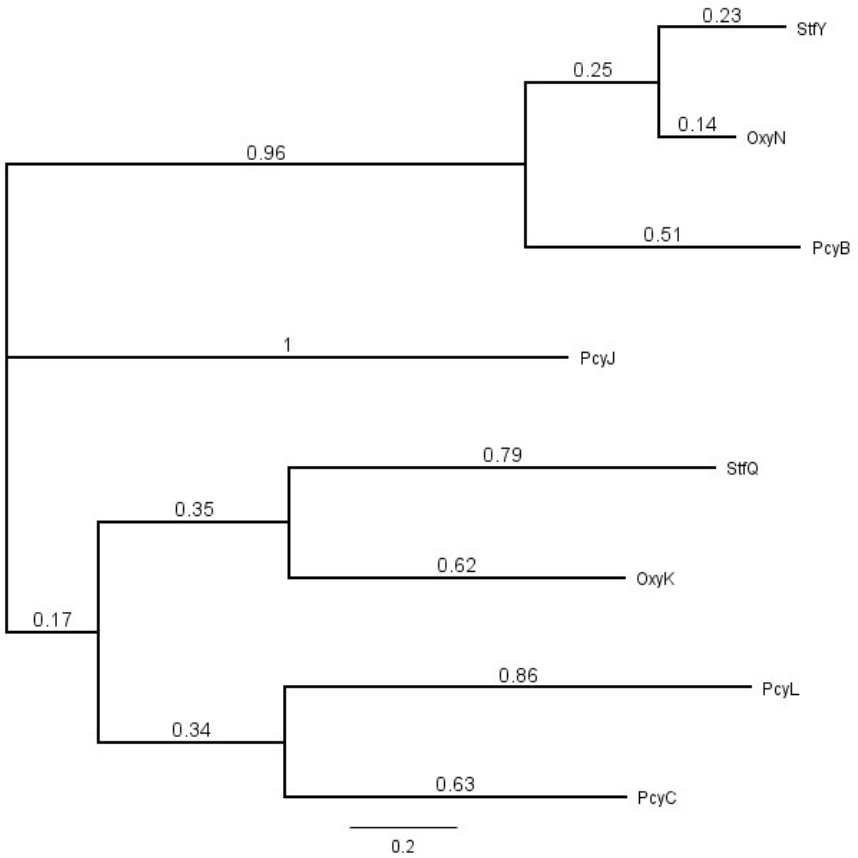

Figure S 12: Neigbour joining tree of the cyclases in the Pyxidicycline, Steffimycin and Oxytetracycline pathways based on MUSCLE alignment of the respective proteins

As one can see, PcyC and PcyL have homology to the first ring cyclase proteins OxyK and StfQ while the PcyB cyclase has homology to the second ring cyclases OxyN and StfY.[9,10] The specialized heterocycle forming protein PcyJ forms an outgroup in this cyclase *in silico* comparison. As we could show by knockout

experiments, PcyL and PcyJ are involved in the cyclisation of the fourth nitrogen containing ring (D ring) and since the third ring closure is spontaneous in these tetracycline like scaffolds,[9] this experiment strongly suggests PcyB to be the second ring (B ring) cyclase while PcyC is the first ring (A ring) cyclase.

3 Isolation and characterization of the pyxidicyclines

3.1 Bacterial fermentation procedures

Cultures for UPLC/high-resolution LC-MS analysis were grown in 300 ml shake flasks containing 50 ml of culture medium inoculated with 1 ml of pre culture. After inoculation the medium was supplemented with 2 % of sterile XAD-16 adsorber resin (Sigma Aldrich) suspension in water. Mutant strains are supplemented with concentrations of the respective antibiotics at concentrations as follows: Kanamycin 50 µg/ml, oxytetracyclin 12 µg/ml, ampicillin 100 µg/ml and apramycin 50 µg/ml. *Pyxidicoccus fallax* strain An d48 was grown in YM medium at 30 °C for 7-9 days using a orbitron shaker (Infors) at 160 rpm, likewise *Myxococcus xanthus* strain DK1622 was grown in CTT medium at 30°C for 5-7 days and *Stigmatella aurantiaca* DW4/3-1 was grown in TS-6 medium at 30°C for 11-14 days. Fermentation cultures of *Pyxidicoccus fallax* strain And48 *pcyA* :: pFPvan *pcyA*, *pcyJ* :: pFPtettn5 *pcyJ* used for isolation of the pyxidicyclines A and B were grown in 5 x 5 L shake flasks containing 2 L of YM medium each. Fermentation duration was 14 days post inoculation; inoculation was done with 50 ml of pre-culture and the medium was supplemented with 2 % of a sterile XAD-16 adsorber resin suspension in water. Fermentation cultures of *Pyxidicoccus fallax* strain And48 *pcyA* :: pSBtn5 *pcyA* used for isolation of the anthraquinone precursors anthraquinone 340, 324 and 296 were grown in 5x 5 L shake flasks containing 2 L of YM medium each. Fermentation duration was 10 days post inoculation; inoculation was done with 50 ml of pre-culture and the medium was supplemented with 2% of sterile XAD-16 adsorber resin suspension in water.

3.2 Preparation of small scale bacterial extracts for UHPLC-HRMS analysis

Pellet and cells were centrifuged at 6500 rcf in a 50 ml falcon centrifuge, the supernatant was decanted and the cell pellets were frozen at -20°C. The frozen cell pellet was transferred into a 100 ml

erlenmeyer flask and a magnetic stirrer added. 50 ml of acetone (fluka analytical grade, redistilled in house) were added onto the pellet and the mixture was stirred for 60 minutes on a magnetic stirrer. The acetone extract was left to settle in order to sediment cell debris and XAD resin before a second extraction step. The supernatant was filtered with a 125 micron folded filter keeping cell pellet and XAD-16 resin in the Erlenmeyer flask for the second extraction step. The residual pellet and XAD-16 resin is extracted again with 30 ml of distilled acetone for 60 min on a magnetic stirrer and filtered through the same folded filter. The combined extracts are transferred into a 100 ml round bottom flask. The acetone was evaporated on a rotary evaporator at 260 mbar and 40 °C water bath temperature. The residual water was evaporated at 20 mbar until the residue in the flask was completely dry. The residue was taken up in 550 µl of methanol (Chromasolv HPLC grade) and transferred into a 1.5 ml Eppendorf tube. This tube was centrifuged at 21500 rcf for 5 minutes to remove residual insolubilities such as salts, cell debris and XAD fragments.

3.3 Standardized UPLC-MS and UPLC-MS^2 Conditions

All measurements were performed on a Dionex Ultimate 3000 RSLC system using a Waters BEH C_{18} column (50 x 2.1 mm, 1.7 µm) equipped with a Waters VanGuard BEH C_{18} 1.7 µm guard column. Separation of 1 µl sample (Crude extract 1/5 dilutions in methanol, section S 3.2) was achieved by a linear gradient from (A) H_2O + 0.1 % FA to (B) ACN + 0.1 % FA at a flow rate of 600 µL/min and a column temperature of 45 °C. Gradient conditions were as follows: 0 – 0.5 min, 5% B; 0.5 – 18.5 min, 5 – 95% B; 18.5 – 20.5 min, 95% B; 20.5 – 21 min, 95 – 5% B; 21-22.5 min, 5% B. UV spectra were recorded by a DAD in the range from 200 to 600 nm. The LC flow was split to 75 µL/min before entering the Bruker Daltonics maXis 4G hr-qToF mass spectrometer using the Apollo II ESI source. Mass spectra were acquired in centroid mode ranging from 150 – 2500 m/z at a 2 Hz full scan rate. Mass spectrometry source parameters were set to 500V as end plate offset; 4000V as capillary voltage; nebulizer gas pressure 1 bar; dry gas flow of 5 l/min and a dry temperature of 200°C. Ion transfer and quadrupole settings were set to Funnel RF 350 Vpp; Multipole RF 400 Vpp as transfer settings and Ion energy of 5eV as well as a low mass cut of 300 m/z as Quadrupole settings. Collision cell was set to 5.0 eV and pre pulse storage time was set to 5 µs. Spectra acquisition rate was set to 2Hz. Calibration of the maXis4G qTOF spectrometer was achieved with sodium formate clusters before every injection to avoid mass drifts. All MS analyses were acquired in the presence of the lock masses $C_{12}H_{19}F_{12}N_3O_6P_3$, $C_{18}H_{19}O_6N_3P_3F_2$ and $C_{24}H_{19}F_{36}N_3O_6P_3$ which generate the $[M+H]^+$ Ions of 622.028960, 922.009798 and 1221.990638. The corresponding MS^2 method operating in automatic precursor selection mode picks up the two most intense precursors per cycle, applies smart exclusion after five spectra and performs CID and MS/MS spectra acquisition time ramping. CID Energy was ramped from

35 eV for 500 m/z to 45 eV for 1000 m/z and 60 eV for 2000 m/z. MS full scan acquisition rate was set to 2Hz and MS/MS spectra acquisition rates were ramped from one to four Hz for precursor Ion intensities of 10kcts to 1000kcts.

3.4 Statistical Data Treatment using Principal Component Analysis

One issue encountered while trying to identify the secondary metabolites produced by the *pcy* biosynthetic gene cluster was that homologous recombination apparently also affected the host strain in ways not necessarily linked to the locus of homologous recombination. While the metabolome detectable with LC-MS changed only minimally upon targeted gene cluster disruption, introduction of the promotor upstream to the pcyA gene lead to diverse changes in the metabolome (main text Figure 4). It was therefore crucial to apply a statistical filter using PCA in order to pinpoint peaks truly unique to the 'activation' mutant and to discriminate those displaying only a fold change in abundance. To search for statistically relevant differences between the two pyxidicycline cluster inactivation mutants and the wild type as well as between the promotor exchange mutants and the inactivation mutants, 6 LC-HRMS measurements from extracts of three independent cultivations per bacterial strain are used for statistical data treatment. For preprocessing of MS data the molecular feature finder implemented in Bruker Compass Data Analysis 4.2 (Bruker, Bremen) was used with the compound detection parameters SN threshold 1; Correlation coefficient 0.9; minimum compound length 10 spectra and smoothing width of 3 spectra. Bucketing was done with Bruker Compass Profile Analysis 2.1 (Bruker, Bremen) with advanced bucketing and window parameters of 30s and 15 ppm. Bucket value was log transformed to avoid under-evaluating low intensity signals in the presence of high intensity signals [11]. The PCA t-Test function was used in order to separate medium derived MS features from the metabolome derived MS features, since the t-Test table that can be exported from Bruker Compass Profile Analysis 2.1 (Bruker, Bremen) contains information upon how many blanks and how many bacterial extracts contain said feature.

3.5 Purification protocols of all pyxidicycline related compounds

3.5.1 Isolation of the anthraquinone precursors 296, 324 and 340

The *Pyxidicoccus fallax* mutant strain *Pyxidicoccus fallax* And48 *pcyA* :: pSBtn5 *pcyA* is fermented in 50 ml YM medium supplemented with Kanamycin 50 µg/ml as a seed culture flasks on an Orbiton shaker at 160 rpm and 30°C. After the culture reaches OD of 0.8 after 5 to 7 days of fermentation it is used to inoculate 6 x 2L YM Kanamycin 50 µg/ml medium supplemented with 2 % XAD-16 resin suspension in

sterilized water in 6 x 5L baffled shake flasks on an Orbiton shaker at 160 rpm and 30°C. Fermentation is complete after 14 days. Cells and XAD-16 resin are harvested by centrifugation on a Beckmann Avanti J-26 XP with the JLA 8.1 rotor at 6000 rcf. Combined resin and cells are extracted with 2 x 500 ml of technical grade methanol (Fluka) and 2 x 500 ml of technical grade acetone (Fluka). The extracts are combined and all solvent is evaporated on a rotary evaporator. Residue is taken up in 10 ml of analytical grade methanol (Fluka) for chromatographical separation. Purification was carried out on a Waters Autopurifier (Eschborn, Germany) high pressure gradient system, equipped with 2545 binary gradient module, SFO system fluidics organizer, 2767 sample manager and a 2998 photodiode array detector coupled to a 3100 single quadrupole mass spectrometer operated in positive ion mode. Source and voltage settings for the MS were as follows: mass range, m/z 300 - 1000; scan duration, 1 s; points per Dalton, 4; capillary voltage, 3.5 kV; cone voltage, 30 V; extractor voltage 3 V; RF lens, 0.1 V; source temperature 120 C, desolvation temperature, 250 C; desolvation gas flow, 400 L/hr; cone gas flow, 50 L/hr; ion counting threshold, 30. Autopurification is done by setting the fraction collection trigger masses 297.1, 325.1 and 340.1 which correspond to the $[M+H]^+$ ions of the anthraquinones. Separation was carried out on Phenomenex Kinetex Biphenyl 5 µm, 250 x 20 mm column using MeOH + 0.1 % FA as B and H2O + 0.1% FA as A with a flow rate of 25 ml/min. Separation is started with a plateau at 95% A for 2 minutes followed by a ramp to 32% A during 4 minutes and a ramp to 2% A during 30 minutes. The A content is kept at 5% A for 2 minutes. The A content is ramped back to starting conditions during 30 seconds and the column is re equilibrated for 5 minutes. Further purification is done using a Dionex Ultimate 3000 SDLC low pressure gradient system on a Phenomenex Luna C-18, 5µm 250x10mm column with the eluents H2O + 0.1 % FA as A and MeOH + 0.1 % FA as B, a flow rate of 5 ml/min and a column thermostated at 30°C. The Anthraquinones are detected by UV absorption at 256 nm and 432 nm. The gradient that starts with a plateau at 95 % A for 2 minutes followed by a ramp to 59 % A during 6 minutes. Then A content is ramped to 30 % during 17 minutes and finally ramped to 5 % A during 1 minute. A content is kept at 5 % for 1 minute and then ramped back to 95 % during 30 seconds. The column is reequilibrated at 95 % A for 5 minutes. The pure compounds are subsequently dried by lyophilization. All three anthraquinones are obtained as dark yellow substances that possess slightly varying fluorescent yellow colors in solution depending on the solvent. Upon deprotonation, the color of all three compounds changes to dark red. The compounds are analyzed by LCUV and LC-HRMS before NMR spectra are acquired.

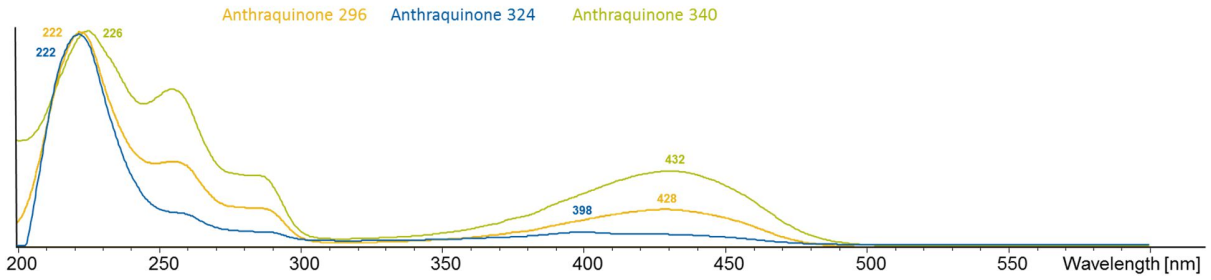

Figure S 13: UV absorption spectra of the anthraquinone metabolites acquired with the Dionex Ultimate 3000 diode array detector during an LC run using H_2O + 0.1% FA and ACN + 0.1% FA as eluents.

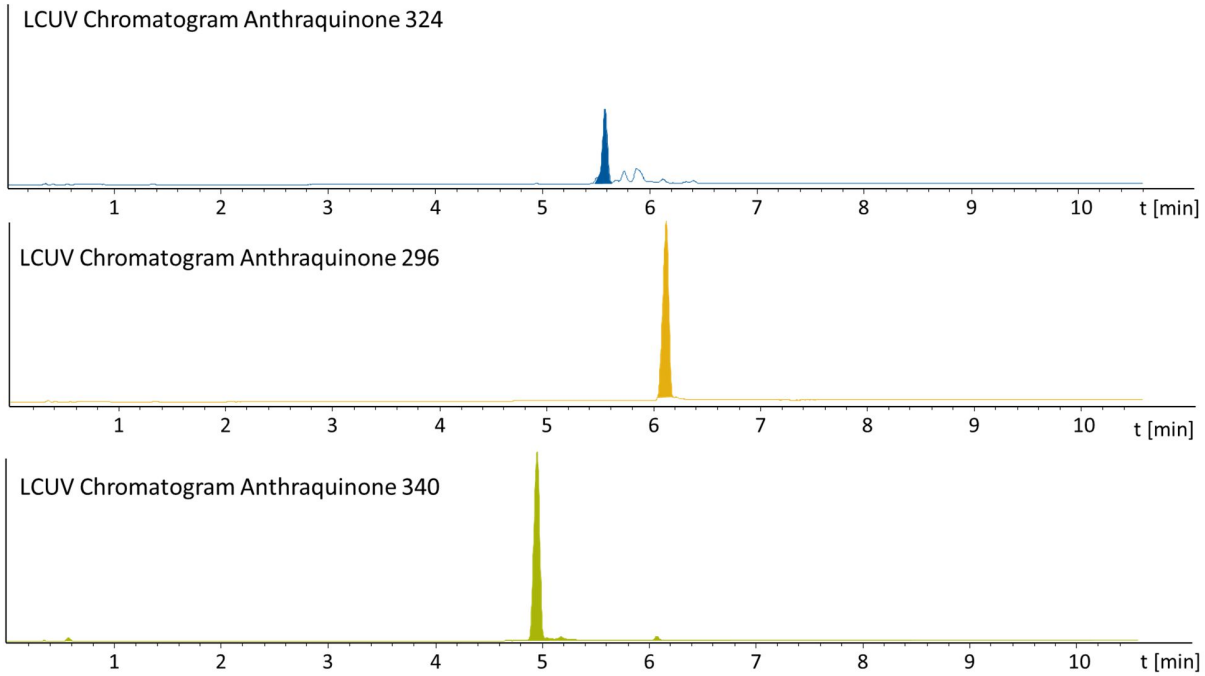

Figure S 14: LC-UV Chromatograms of the three isolated Anthraquinone metabolites for purity analysis by liquid chromatography

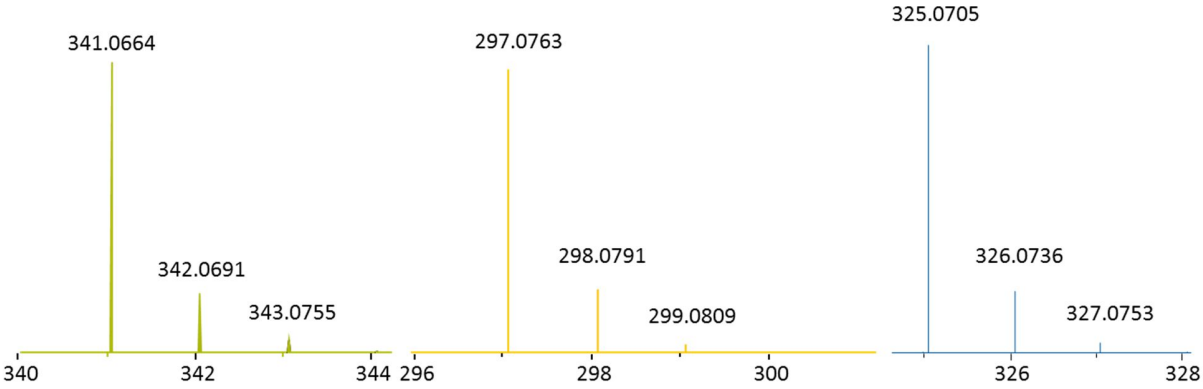

Figure S 15: High resolution masses of anthraquinone 340,296 and 325 $[M+H]^+$ in m/z units acquired on our maXis4G qTOF high resolution mass spectrometer

3.5.2 Isolation of Pyxidicycline A and B

The *Pyxidicoccus fallax* mutant strain *Pyxidicoccus fallax* And48 *pcyA* :: pFPVan *pcyA, pcyJ* :: pFPtettn5 *pcyJ* (An d48 *pcy*) is fermented in 50 ml YM medium supplemented with Kanamycin 50 µg/ml as a seed culture flasks on an Orbiton shaker at 160 rpm and 30°C. After the culture reaches OD of 0.8 after 5 to 7 days of fermentation it is used to inoculate 6 x 2L YM Kanamycin 50 µg/ml medium supplemented with 2 % XAD-16 resin suspension in sterilized water in 6 x 5L baffled shake flasks on an Orbiton shaker at 160 rpm and 30°C. Simultaneously with inoculation the cultures are supplemented with 5mM Sodium vanillate solution to induce pyxidicycline production. Fermentation is complete after 17 days. Cells and XAD-16 resin are harvested by centrifugation on a Beckmann Avanti J-26 XP with the JLA 8.1 rotor at 6000 rcf. Combined resin and cells are extracted with 2 x 500 ml of technical grade methanol (Fluka) and 2 x 500 ml of technical grade acetone (Fluka). The extracts are combined and all solvent is evaporated on a rotary evaporator. The residue is now partitioned between saturated brine and chloroform (2 extractions of 500 ml brine with 400 ml of chloroform) in a 2L separating funnel leading to close to complete transfer of the pyxidicyclines A and B into the chloroform phase. The chloroform phase is subsequently dried on a rotary evaporator. The residue is resuspended in 50 ml of analytical grade Acetonitrile and transferred completely into a 50 ml falcon tube (Sarstedt). After vigorous vortexing of the suspension the falcon tube is centrifuged for 15 min at 7800 rcf to pellet the pyxidicyclines that are close to insoluble in acetonitrile. The dark red residue is taken up in 1 ml of analytical grade formic acid (99 %, Sigma Aldrich) for chromatographic separation of pyxidicycline A and B. Purification is done using a Dionex Ultimate 3000 SDLC low pressure gradient system on an Agilent Zorbax XDB-C8, 5µm 250x10mm column with the eluents H2O + 0.5% FA as A and MeOH + 0.5% FA as B, a flow rate of 5 ml/min and a column thermostated at 30°C.

The pyxidicyclines are detected by UV absorption at 474 nm (pyxidicycline B) and 498 nm (pyxidicycline A). The pyxidicyclines are further purified with the gradient that starts with a plateau at 95% A for 2 minutes followed by a ramp to 74% A during 3 minutes. Then A content is ramped to 56% during 32 minutes and finally ramped to 5% A during 30 seconds. A content is kept stable at 5% for 1 minute and then ramped back to 95% A during 30 seconds. The column is reequilibrated at 95% A for 5 minutes. The pure compounds are subsequently dried by lyophilization and obtaines as the corresponding formate esters. The pyxidicyclines are obtained as dark red substances that possess slightly varying fluorescent orange colors in solution depending on the solvent. Upon deprotonation, the color of both pyxidicycline compounds changes to dark blue. In total 5 mg of pyxidicycline A and 4 mg of pyxidicycline B are obtained from the fermentation culture as formate esters. It is worth noting that the actual theoretical yield of pyxidicyclines seems to be at least 50 % higher as on the one hand elution of the pyxidicyclines from the XAD-16 resin is incomplete and on the other hand peak shape issues with the compounds in reverse phase chromatography due to low insolubility in all tested LC solvents lead to low recovery rates after chromatographic separation. Both compounds are very soluble and stable in formic acid while being insoluble in most common organic solvents and instable in DMSO and DMF.

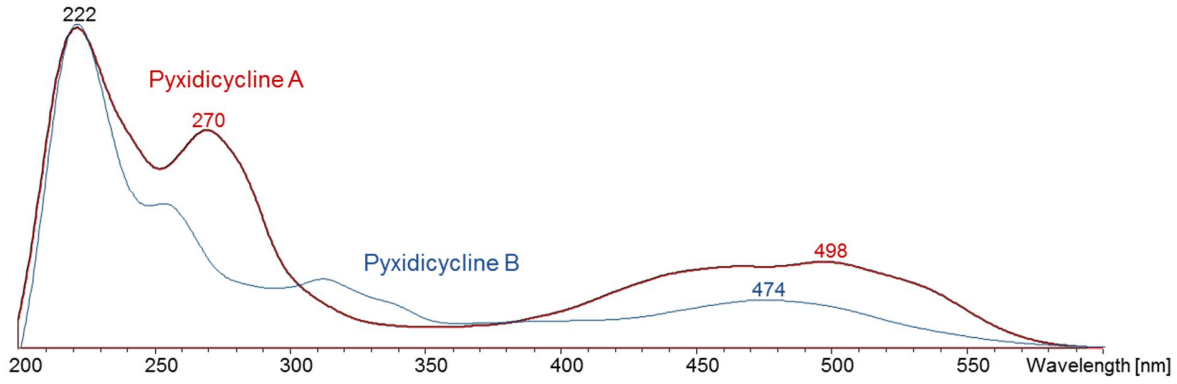

Figure S 16: UV absorption spectra of pyxidicycline A and B acquired with the Dionex Ultimate 3000 diode array detector during an LC run using H_2O + 0.1% FA and ACN + 0.1% FA as eluents.

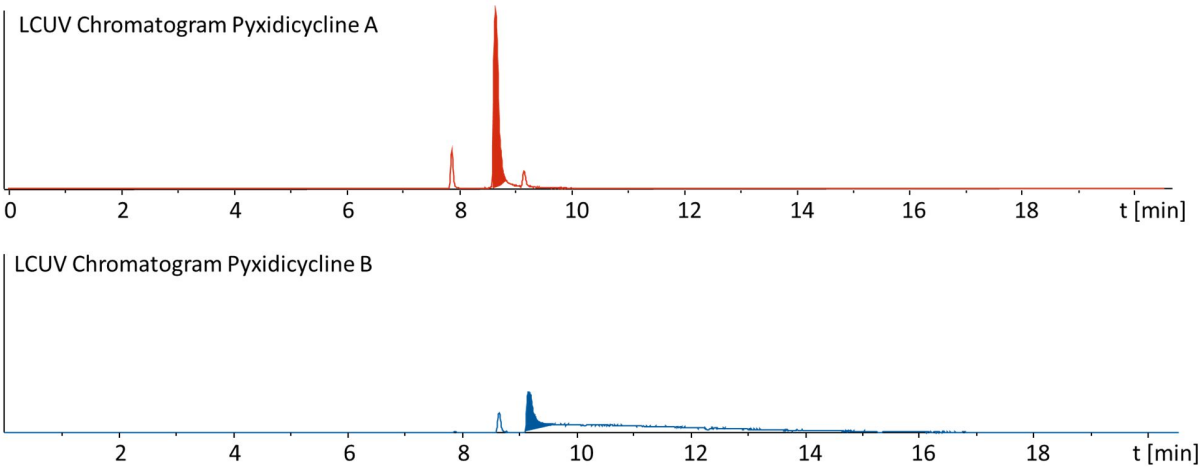

Figure S 17: LCUV Chromatograms of the two Pyxidicyclines for purity analysis by liquid chromatography, the first peak belonging to the formate ester, the second to the free pyxidicyclines

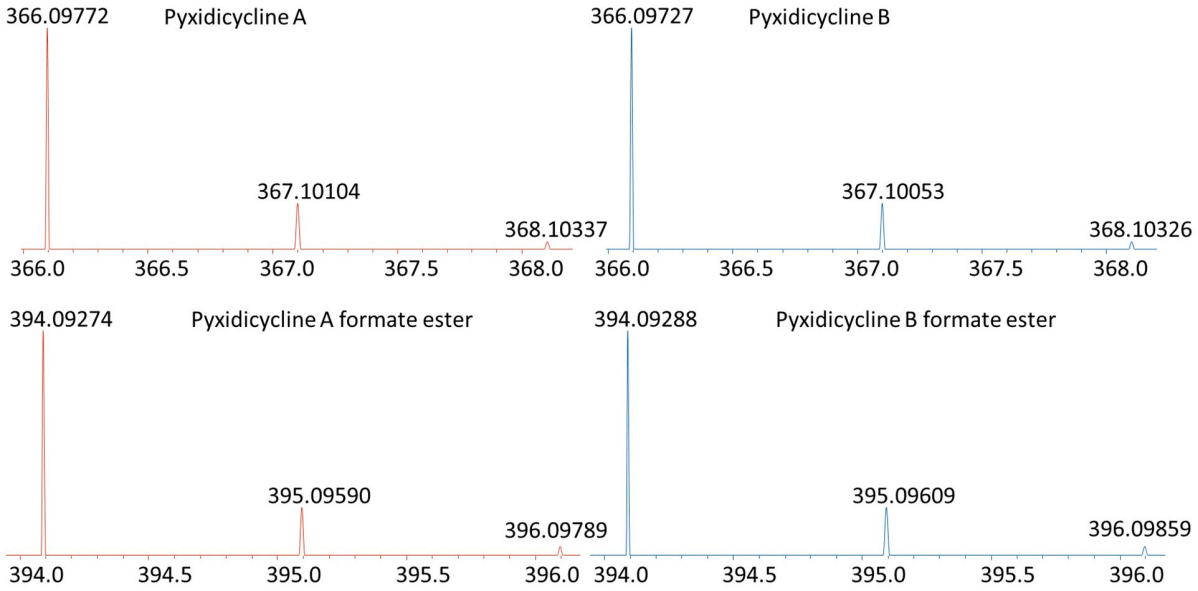

Figure S 18: High resolution masses of pyxidicycline A and B $[M+H]^+$ in m/z units as well as the corresponding formate esters acquired on our maXis4G qTOF high resolution mass spectrometer

3.6 Crystallization parameters for the pyxidicycline A and B

The pure pyxidicyclines A and B are taken up in a minimum of formic acid to an approximate mass concentration of 5 mg/ml and filled into a 2 ml glass vial. The solutions are sonicated for a few minutes in an ultrasound bath until no visible residues remain. The solutions are subsequently placed into the

refrigerator with an unscrewed cap. After two days one can observe crystallization of pyxidicycline B as dark red needle shaped crystals. After one week one starts to observe crystallization of pyxidicycline A as dark red needle shaped crystals.

3.7 Crystal structure parameters obtained through single-crystal X-ray diffraction experiments

The compounds crystallized from formic acid solution as formic acid esters that formed needle shaped dark red crystals. The data were collected at low temperature on a BrukerAXS X8Apex CCD diffractometer operating with graphite-monochromatized Mo Kα radiation. Frames of 0.5° oscillation were exposed; deriving reflections in the θ range of 2 to 27° with a completeness of ~99%. Structure solving and full least-squares refinement with anisotropic thermal parameters of all non-hydrogen atoms were performed using SHELX [12]. Crystal structure parameters for pyxidicycline B: orthorhombic, $Pca2_1$, a=7.0231(8) b=17.534(2) c=14.682(2)Å ; and for pyxidicycline A: triclinic, P-1, a=7.090(1) b=7.910(2) 17.330(3)Å, α=89.56(1) β=84.80(1), γ=74.66(1). All relevant data concerning the crystal structures can be found in this section. Crystallographic data for the structure have been deposited with the Cambridge Crystallographic Data Centre, CCDC, 12 Union Road, Cambridge CB21EZ, UK. Copies of the data can be obtained free of charge on quoting the repository number **CCDC 1831467** and **1831468**. www.ccdc.cam.ac.uk/data_request/cif

3.7.1 Crystallographic data pyxidicycline A

Table S 7: Crystal data and structure refinement for pyxidicycline A formate ester (sh3817)

Identification code	**sh3817**
Empirical formula	C21 H15 N O7, C H2 O2
Formula weight	439.36
Temperature	172(2) K
Wavelength	0.71073 Å
Crystal system	Triclinic
Space group	P-1
Unit cell dimensions	a = 7.0902(12) Å α= 89.561(13)°.
	b = 7.9097(15) Å β= 84.797(11)°.

	c = 17.330(3) Å γ = 74.655(12)°.
Volume	933.2(3) $Å^3$
Z	2
Density (calculated)	1.564 Mg/m^3
Absorption coefficient	0.123 mm^{-1}
F(000)	456
Crystal size	0.311 x 0.048 x 0.030 mm^3
Theta range for data collection	1.180 to 26.505°.
Index ranges	-8<=h<=8, -8<=k<=8, -21<=l<=21
Reflections collected	19696
Independent reflections	3546 [R(int) = 0.1065]
Completeness to theta = 25.242°	93.1 %
Absorption correction	Semi-empirical from equivalents
Max. and min. transmission	0.7454 and 0.6615
Refinement method	Full-matrix least-squares on F^2
Data / restraints / parameters	3546 / 3 / 270
Goodness-of-fit on F^2	1.614
Final R indices [I>2sigma(I)]	R1 = 0.1357, wR2 = 0.3337
R indices (all data)	R1 = 0.2936, wR2 = 0.3784
Extinction coefficient	n/a
Largest diff. peak and hole	0.840 and -0.594 $e.Å^{-3}$

Table S 8: Atomic coordinates (x 10^4) and equivalent isotropic displacement parameters ($Å^2$x 10^3) for pyxidicycline A formate ester (sh3817). U(eq) is defined as one third of the trace of the orthogonalized U^{ij} tensor

	x	y	z	U(eq)
O(1)	1924(9)	1200(8)	1896(3)	66(2)
O(2)	4364(8)	1588(7)	5903(3)	54(2)
O(3)	2204(8)	9208(7)	6772(3)	52(2)
O(4)	1603(7)	8506(7)	5380(3)	37(1)
O(5)	952(7)	7853(7)	4050(3)	42(2)
N(1)	1302(8)	4089(9)	2279(4)	41(2)
C(1)	1909(12)	2248(14)	2430(6)	55(3)
C(2)	2472(11)	1804(11)	3168(5)	44(2)

C(3)	2407(10)	3094(10)	3738(5)	35(2)
C(4)	3025(10)	2570(12)	4475(5)	43(2)
C(5)	2990(9)	3783(11)	5036(4)	32(2)
C(6)	3651(10)	3167(11)	5781(4)	37(2)
C(7)	3500(10)	4478(11)	6402(4)	36(2)
C(8)	4022(11)	3886(12)	7124(5)	44(2)
C(9)	3968(11)	5079(13)	7701(5)	49(3)
C(10)	3345(10)	6815(12)	7571(4)	47(2)
C(11)	2798(10)	7457(11)	6853(4)	37(2)
C(12)	2849(9)	6283(10)	6253(4)	26(2)
C(13)	2217(10)	6858(11)	5500(4)	36(2)
C(14)	2291(9)	5609(10)	4887(4)	30(2)
C(15)	1678(10)	6143(10)	4157(4)	31(2)
C(16)	1755(10)	4910(10)	3545(4)	32(2)
C(17)	1252(10)	5381(10)	2808(4)	33(2)
C(18)	738(11)	7251(10)	2521(4)	48(2)
C(19)	615(12)	4465(13)	1507(5)	69(3)
C(20)	2269(18)	4228(18)	853(7)	114(4)
O(6)	3079(12)	5635(13)	806(5)	125(3)
C(21)	2760(40)	6910(40)	179(15)	271(12)
O(7A)	2560(30)	5770(30)	-324(12)	220(9)
O(7B)	1250(50)	7480(60)	-230(20)	226(11)
O(8A)	5360(20)	11320(20)	-1343(9)	104(5)
C(22)	3920(40)	11070(30)	-1139(11)	148(6)
O(9A)	2430(50)	11450(40)	-1026(16)	210(9)
O(9B)	3890(40)	10020(40)	-719(16)	205(9)
O(8B)	100(20)	9990(20)	-868(9)	127(6)

Table S 9: Bond lengths [Å] and angles [°] for pyxidicycline A formate ester (sh3817)

Bond lengths [Å]	
O(1)-C(1)	1.245(10)
O(2)-C(6)	1.242(8)
O(3)-C(11)	1.347(9)
O(3)-H(3)	0.8400

O(4)-C(13)	1.282(8)
O(5)-C(15)	1.332(8)
O(5)-H(5)	0.8400
N(1)-C(17)	1.369(9)
N(1)-C(1)	1.434(11)
N(1)-C(19)	1.467(9)
C(1)-C(2)	1.389(11)
C(2)-C(3)	1.415(11)
C(2)-H(2)	0.9500
C(3)-C(4)	1.412(10)
C(3)-C(16)	1.434(10)
C(4)-C(5)	1.365(11)
C(4)-H(4)	0.9500
C(5)-C(14)	1.427(10)
C(5)-C(6)	1.450(10)
C(6)-C(7)	1.478(11)
C(7)-C(8)	1.380(9)
C(7)-C(12)	1.409(10)
C(8)-C(9)	1.371(11)
C(8)-H(8)	0.9500
C(9)-C(10)	1.350(11)
C(9)-H(9)	0.9500
C(10)-C(11)	1.388(9)
C(10)-H(10)	0.9500
C(11)-C(12)	1.391(10)
C(12)-C(13)	1.449(10)
C(13)-C(14)	1.445(10)
C(14)-C(15)	1.400(9)
C(15)-C(16)	1.434(10)
C(16)-C(17)	1.379(9)
C(17)-C(18)	1.518(10)
C(18)-H(18A)	0.9800
C(18)-H(18B)	0.9800
C(18)-H(18C)	0.9800
C(19)-C(20)	1.530(13)

C(19)-H(19A)	0.9900
C(19)-H(19B)	0.9900
C(20)-O(6)	1.380(13)
C(20)-H(20A)	0.9900
C(20)-H(20B)	0.9900
O(6)-C(21)	1.47(3)
C(21)-O(7A)	1.30(2)
C(21)-O(7B)	1.31(2)
C(21)-H(21A)	0.9500
C(21)-H(21B)	0.9500
O(8A)-C(22)	1.12(2)
C(22)-O(9A)	1.02(3)
C(22)-O(9B)	1.10(3)
O(9A)-O(9B)	1.45(3)
Bond angles [°]	
C(11)-O(3)-H(3)	109.5
C(15)-O(5)-H(5)	109.5
C(17)-N(1)-C(1)	124.3(7)
C(17)-N(1)-C(19)	122.7(7)
C(1)-N(1)-C(19)	112.9(8)
O(1)-C(1)-C(2)	125.9(9)
O(1)-C(1)-N(1)	118.2(8)
C(2)-C(1)-N(1)	115.8(9)
C(1)-C(2)-C(3)	121.8(8)
C(1)-C(2)-H(2)	119.1
C(3)-C(2)-H(2)	119.1
C(4)-C(3)-C(2)	119.5(7)
C(4)-C(3)-C(16)	121.5(8)
C(2)-C(3)-C(16)	119.0(7)
C(5)-C(4)-C(3)	120.9(8)
C(5)-C(4)-H(4)	119.5
C(3)-C(4)-H(4)	119.5
C(4)-C(5)-C(14)	120.1(7)
C(4)-C(5)-C(6)	118.4(8)
C(14)-C(5)-C(6)	121.5(8)

O(2)-C(6)-C(5)	122.0(8)
O(2)-C(6)-C(7)	119.7(7)
C(5)-C(6)-C(7)	118.3(7)
C(8)-C(7)-C(12)	121.4(8)
C(8)-C(7)-C(6)	118.3(8)
C(12)-C(7)-C(6)	120.3(7)
C(9)-C(8)-C(7)	119.3(8)
C(9)-C(8)-H(8)	120.3
C(7)-C(8)-H(8)	120.3
C(10)-C(9)-C(8)	120.3(8)
C(10)-C(9)-H(9)	119.9
C(8)-C(9)-H(9)	119.9
C(9)-C(10)-C(11)	122.0(8)
C(9)-C(10)-H(10)	119.0
C(11)-C(10)-H(10)	119.0
O(3)-C(11)-C(10)	118.2(8)
O(3)-C(11)-C(12)	122.6(7)
C(10)-C(11)-C(12)	119.3(8)
C(11)-C(12)-C(7)	117.8(7)
C(11)-C(12)-C(13)	122.3(7)
C(7)-C(12)-C(13)	119.9(7)
O(4)-C(13)-C(14)	119.9(7)
O(4)-C(13)-C(12)	119.0(7)
C(14)-C(13)-C(12)	121.1(7)
C(15)-C(14)-C(5)	119.4(7)
C(15)-C(14)-C(13)	121.8(7)
C(5)-C(14)-C(13)	118.8(7)
O(5)-C(15)-C(14)	117.7(7)
O(5)-C(15)-C(16)	120.2(7)
C(14)-C(15)-C(16)	122.1(7)
C(17)-C(16)-C(15)	123.9(7)
C(17)-C(16)-C(3)	120.2(7)
C(15)-C(16)-C(3)	115.9(7)
N(1)-C(17)-C(16)	118.8(7)
N(1)-C(17)-C(18)	116.7(6)

C(16)-C(17)-C(18)	124.4(7)
C(17)-C(18)-H(18A)	109.5
C(17)-C(18)-H(18B)	109.5
H(18A)-C(18)-H(18B)	109.5
C(17)-C(18)-H(18C)	109.5
H(18A)-C(18)-H(18C)	109.5
H(18B)-C(18)-H(18C)	109.5
N(1)-C(19)-C(20)	114.0(7)
N(1)-C(19)-H(19A)	108.7
C(20)-C(19)-H(19A)	108.7
N(1)-C(19)-H(19B)	108.7
C(20)-C(19)-H(19B)	108.7
H(19A)-C(19)-H(19B)	107.6
O(6)-C(20)-C(19)	112.2(11)
O(6)-C(20)-H(20A)	109.2
C(19)-C(20)-H(20A)	109.2
O(6)-C(20)-H(20B)	109.2
C(19)-C(20)-H(20B)	109.2
H(20A)-C(20)-H(20B)	107.9
C(20)-O(6)-C(21)	123.1(15)
O(7A)-C(21)-O(6)	93(2)
O(7B)-C(21)-O(6)	131(3)
O(7A)-C(21)-H(21A)	133.3
O(6)-C(21)-H(21A)	133.3
O(7B)-C(21)-H(21B)	114.7
O(6)-C(21)-H(21B)	114.7
O(9A)-C(22)-O(9B)	86(3)
O(9A)-C(22)-O(8A)	152(3)
O(9B)-C(22)-O(8A)	119(3)
C(22)-O(9A)-O(9B)	49.1(19)
C(22)-O(9B)-O(9A)	44.6(18)

Table S 10: Anisotropic displacement parameters ($Å^2 x\ 10^3$) for pyxidicycline A formate ester (sh3817). The anisotropic displacement factor exponent takes the form: $-2\pi^2[h^2\ a^{*2}U^{11} + ... + 2\ h\ k\ a^*\ b^*\ U^{12}]$

	U^{11}	U^{22}	U^{33}	U^{23}	U^{13}	U^{12}
O(1)	91(5)	46(5)	58(4)	-12(3)	-20(3)	-10(3)
O(2)	77(4)	20(4)	57(4)	1(3)	-17(3)	2(3)
O(3)	70(4)	30(4)	49(4)	-3(3)	-19(3)	2(3)
O(4)	44(3)	20(4)	42(3)	4(2)	-1(2)	0(2)
O(5)	50(4)	27(4)	45(4)	12(3)	-7(3)	-3(3)
N(1)	37(4)	42(6)	36(4)	4(4)	-2(3)	2(3)
C(1)	44(6)	57(8)	59(7)	-1(6)	-2(5)	-5(5)
C(2)	46(5)	32(6)	53(6)	5(4)	-6(4)	-8(4)
C(3)	39(5)	16(6)	47(5)	7(4)	0(4)	-6(4)
C(4)	33(5)	43(7)	50(6)	16(5)	-6(4)	-7(4)
C(5)	18(4)	37(6)	36(5)	3(4)	-4(3)	2(4)
C(6)	34(5)	22(6)	48(6)	12(4)	-1(4)	2(4)
C(7)	33(5)	41(7)	32(5)	0(4)	0(4)	-7(4)
C(8)	45(5)	39(7)	47(6)	7(5)	1(4)	-9(4)
C(9)	48(5)	48(7)	48(6)	16(5)	-11(4)	-4(5)
C(10)	43(5)	65(8)	32(5)	-14(4)	-11(4)	-11(5)
C(11)	38(5)	36(7)	35(5)	0(4)	3(4)	-10(4)
C(12)	22(4)	15(5)	35(5)	4(4)	0(3)	4(3)
C(13)	24(4)	25(6)	57(6)	3(4)	-3(4)	-1(4)
C(14)	27(4)	23(6)	42(5)	2(4)	-8(4)	-6(3)
C(15)	28(4)	21(6)	41(5)	8(4)	2(4)	-2(4)
C(16)	33(4)	25(6)	37(5)	-9(4)	2(4)	-6(4)
C(17)	35(5)	30(6)	34(5)	-3(4)	-7(4)	-7(4)
C(18)	63(6)	37(6)	41(5)	7(4)	-6(4)	-8(4)
C(19)	45(6)	92(9)	63(7)	-21(5)	1(5)	-7(5)

Table S 11: Hydrogen coordinates (x 10^4) and isotropic displacement parameters ($Å^2$x 10^3) for pyxidicycline A formate ester (sh3817).

	x	y	z	U(eq)
H(3)	1821	9436	6329	77
H(5)	1532	8156	3650	63
H(2)	2913	600	3293	53
H(4)	3471	1357	4582	51
H(8)	4414	2665	7220	53
H(9)	4370	4683	8194	59
H(10)	3279	7620	7982	56
H(18A)	747	8057	2946	72
H(18B)	1706	7367	2097	72
H(18C)	-571	7536	2334	72
H(19A)	-214	5687	1505	83
H(19B)	-218	3682	1402	83
H(20A)	3308	3148	941	136
H(20B)	1747	4080	354	136
H(21A)	2717	8123	151	325
H(21B)	3826	7411	46	325

3.7.2 Crystallographic data pyxidicycline B

Table S 12: Crystal data and structure refinement for pyxidicycline B formate ester (sh3811)

Identification code	**sh3811**
Empirical formula	C21 H15 N O7, C H2 O2
Formula weight	439.36
Temperature	152(2) K
Wavelength	0.71073 Å
Crystal system	Orthorhombic
Space group	$Pca2_1$
Unit cell dimensions	a = 7.0231(8) Å α= 90°.

	b = 17.534(2) Å β= 90°.
	c = 14.6817(19) Å γ = 90°.
Volume	1807.9(4) Å^3
Z	4
Density (calculated)	1.614 Mg/m^3
Absorption coefficient	0.127 mm^{-1}
F(000)	912
Crystal size	0.506 x 0.126 x 0.022 mm^3
Theta range for data collection	1.161 to 27.705°.
Index ranges	-8<=h<=9, -22<=k<=22, -17<=l<=19
Reflections collected	15148
Independent reflections	4084 [R(int) = 0.0370]
Completeness to theta = 25.242°	100.0 %
Absorption correction	Semi-empirical from equivalents
Max. and min. transmission	0.7456 and 0.7222
Refinement method	Full-matrix least-squares on F^2
Data / restraints / parameters	4084 / 1 / 346
Goodness-of-fit on F^2	1.054
Final R indices [I>2sigma(I)]	R1 = 0.0452, wR2 = 0.1135
R indices (all data)	R1 = 0.0653, wR2 = 0.1236
Absolute structure parameter	1.8(5)
Extinction coefficient	n/a
Largest diff. peak and hole	0.717 and -0.216 e.Å^{-3}

Table S 13: Atomic coordinates (x 10^4) and equivalent isotropic displacement parameters (Å^{2}x 10^3) for pyxidicycline B formate ester (sh3811). U(eq) is defined as one third of the trace of the orthogonalized U^{ij} tensor

	x	y	z	U(eq)
N(1)	951(4)	1835(2)	5287(2)	20(1)
O(1)	1925(3)	1160(1)	4044(2)	31(1)
O(2)	1993(4)	3824(2)	2963(2)	34(1)
O(3)	117(3)	6865(1)	6376(2)	27(1)
O(4)	-47(3)	5390(1)	6437(2)	25(1)

O(5)	-269(3)	3993(1)	6472(2)	24(1)
O(6)	3284(3)	1336(2)	6796(2)	37(1)
O(7)	6321(4)	960(2)	6715(3)	51(1)
C(1)	1570(5)	1795(2)	4375(3)	23(1)
C(2)	1757(5)	2492(2)	3914(3)	22(1)
C(3)	1381(4)	3165(2)	4327(2)	18(1)
C(4)	1620(5)	3868(2)	3769(2)	20(1)
C(5)	1381(4)	4604(2)	4232(2)	19(1)
C(6)	1716(4)	5262(2)	3746(2)	19(1)
C(7)	1474(4)	5990(2)	4170(3)	19(1)
C(8)	1841(4)	6655(2)	3674(3)	22(1)
C(9)	1629(5)	7353(2)	4092(3)	24(1)
C(10)	1063(5)	7407(2)	4996(3)	24(1)
C(11)	672(4)	6762(2)	5503(3)	21(1)
C(12)	880(4)	6031(2)	5097(3)	18(1)
C(13)	530(4)	5336(2)	5580(3)	18(1)
C(14)	785(4)	4625(2)	5158(2)	17(1)
C(15)	395(4)	3939(2)	5677(2)	18(1)
C(16)	795(4)	3191(2)	5256(2)	18(1)
C(17)	581(4)	2504(2)	5729(2)	18(1)
C(18)	-29(5)	2472(2)	6700(3)	23(1)
C(19)	802(5)	1079(2)	5735(3)	26(1)
C(20)	2705(5)	813(2)	6083(3)	29(1)
C(21)	5108(5)	1344(3)	7039(3)	37(1)
O(8)	6595(5)	759(2)	4422(3)	60(1)
O(9)	4950(5)	692(2)	3115(2)	45(1)
C(22)	6458(6)	580(2)	3644(4)	42(1)

Table S 14: Bond lengths [Å] and angles [°] for pyxidicycline B formate ester (sh3811)

Bond lengths [Å]	
N(1)-C(17)	1.365(4)
N(1)-C(1)	1.410(5)
N(1)-C(19)	1.484(4)
O(1)-C(1)	1.239(4)

O(2)-C(4)	1.215(5)
O(3)-C(11)	1.351(5)
O(3)-H(16)	0.96(6)
O(4)-C(13)	1.326(4)
O(4)-H(17)	0.8400
O(5)-C(15)	1.261(4)
O(6)-C(21)	1.330(5)
O(6)-C(20)	1.449(5)
O(7)-C(21)	1.185(5)
C(1)-C(2)	1.404(5)
C(2)-C(3)	1.353(5)
C(2)-H(1)	0.92(4)
C(3)-C(16)	1.424(5)
C(3)-C(4)	1.490(5)
C(4)-C(5)	1.467(5)
C(5)-C(6)	1.378(5)
C(5)-C(14)	1.424(5)
C(6)-C(7)	1.429(5)
C(6)-H(2)	0.9500
C(7)-C(8)	1.399(5)
C(7)-C(12)	1.425(5)
C(8)-C(9)	1.376(5)
C(8)-H(3)	0.9500
C(9)-C(10)	1.390(6)
C(9)-H(4)	1.01(4)
C(10)-C(11)	1.381(5)
C(10)-H(5)	0.95(4)
C(11)-C(12)	1.421(5)
C(12)-C(13)	1.431(4)
C(13)-C(14)	1.404(4)
C(14)-C(15)	1.449(5)
C(15)-C(16)	1.477(5)
C(16)-C(17)	1.399(5)
C(17)-C(18)	1.489(5)
C(18)-H(7)	0.97(5)

C(18)-H(8)	0.98(5)
C(18)-H(9)	0.85(4)
C(19)-C(20)	1.505(5)
C(19)-H(10)	0.95(4)
C(19)-H(11)	0.94(5)
C(20)-H(12)	0.92(4)
C(20)-H(13)	0.94(4)
C(21)-H(14)	1.06(5)
O(8)-C(22)	1.188(6)
O(9)-C(22)	1.328(6)
O(9)-H(15)	1.03(7)
C(22)-H(18)	1.07(5)
Bond angles [°]	
C(17)-N(1)-C(1)	123.7(3)
C(17)-N(1)-C(19)	122.9(3)
C(1)-N(1)-C(19)	113.4(3)
C(11)-O(3)-H(16)	105(3)
C(13)-O(4)-H(17)	109.5
C(21)-O(6)-C(20)	118.1(3)
O(1)-C(1)-C(2)	125.1(3)
O(1)-C(1)-N(1)	118.7(3)
C(2)-C(1)-N(1)	116.2(3)
C(3)-C(2)-C(1)	121.7(3)
C(3)-C(2)-H(1)	121(2)
C(1)-C(2)-H(1)	117(2)
C(2)-C(3)-C(16)	120.9(3)
C(2)-C(3)-C(4)	116.9(3)
C(16)-C(3)-C(4)	122.2(3)
O(2)-C(4)-C(5)	122.2(3)
O(2)-C(4)-C(3)	120.5(3)
C(5)-C(4)-C(3)	117.4(3)
C(6)-C(5)-C(14)	121.5(3)
C(6)-C(5)-C(4)	118.6(3)
C(14)-C(5)-C(4)	119.9(3)
C(5)-C(6)-C(7)	120.2(3)

C(5)-C(6)-H(2)	119.9
C(7)-C(6)-H(2)	119.9
C(8)-C(7)-C(12)	120.5(3)
C(8)-C(7)-C(6)	119.7(3)
C(12)-C(7)-C(6)	119.8(3)
C(9)-C(8)-C(7)	119.3(4)
C(9)-C(8)-H(3)	120.3
C(7)-C(8)-H(3)	120.3
C(8)-C(9)-C(10)	121.1(3)
C(8)-C(9)-H(4)	116(2)
C(10)-C(9)-H(4)	123(2)
C(11)-C(10)-C(9)	121.1(4)
C(11)-C(10)-H(5)	117(3)
C(9)-C(10)-H(5)	122(3)
O(3)-C(11)-C(10)	117.3(3)
O(3)-C(11)-C(12)	123.2(3)
C(10)-C(11)-C(12)	119.4(3)
C(11)-C(12)-C(7)	118.5(3)
C(11)-C(12)-C(13)	122.8(3)
C(7)-C(12)-C(13)	118.6(3)
O(4)-C(13)-C(14)	121.3(3)
O(4)-C(13)-C(12)	117.6(3)
C(14)-C(13)-C(12)	121.1(3)
C(13)-C(14)-C(5)	118.8(3)
C(13)-C(14)-C(15)	118.8(3)
C(5)-C(14)-C(15)	122.4(3)
O(5)-C(15)-C(14)	119.6(3)
O(5)-C(15)-C(16)	121.6(3)
C(14)-C(15)-C(16)	118.8(3)
C(17)-C(16)-C(3)	118.6(3)
C(17)-C(16)-C(15)	122.5(3)
C(3)-C(16)-C(15)	118.9(3)
N(1)-C(17)-C(16)	118.9(3)
N(1)-C(17)-C(18)	118.6(3)
C(16)-C(17)-C(18)	122.6(3)

C(17)-C(18)-H(7)	107(3)
C(17)-C(18)-H(8)	109(3)
H(7)-C(18)-H(8)	110(4)
C(17)-C(18)-H(9)	111(3)
H(7)-C(18)-H(9)	111(4)
H(8)-C(18)-H(9)	109(4)
N(1)-C(19)-C(20)	111.4(3)
N(1)-C(19)-H(10)	105.3(18)
C(20)-C(19)-H(10)	116(2)
N(1)-C(19)-H(11)	103(3)
C(20)-C(19)-H(11)	112(3)
H(10)-C(19)-H(11)	109(3)
O(6)-C(20)-C(19)	107.4(3)
O(6)-C(20)-H(12)	109(3)
C(19)-C(20)-H(12)	109(2)
O(6)-C(20)-H(13)	112(2)
C(19)-C(20)-H(13)	108(2)
H(12)-C(20)-H(13)	112(3)
O(7)-C(21)-O(6)	125.4(5)
O(7)-C(21)-H(14)	126(2)
O(6)-C(21)-H(14)	108(2)
C(22)-O(9)-H(15)	107(4)
O(8)-C(22)-O(9)	126.0(4)
O(8)-C(22)-H(18)	135(3)
O(9)-C(22)-H(18)	99(3)

Table S 15: Anisotropic displacement parameters ($Å^2 x\ 10^3$) for pyxidicycline B formate ester (sh3811). The anisotropic displacement factor exponent takes the form: $-2\pi^2[h^2\ a^{*2}U^{11} + ... + 2\ h\ k\ a^*\ b^*\ U^{12}]$

	U^{11}	U^{22}	U^{33}	U^{23}	U^{13}	U^{12}
N(1)	19(1)	20(2)	22(2)	1(1)	1(1)	1(1)
O(1)	39(2)	23(1)	32(2)	-4(1)	8(1)	2(1)
O(2)	47(2)	32(1)	24(2)	-1(1)	4(1)	3(1)
O(3)	32(1)	24(1)	25(2)	-5(1)	3(1)	-1(1)
O(4)	35(1)	21(1)	20(1)	-2(1)	5(1)	-1(1)

O(5)	30(1)	23(1)	17(1)	-2(1)	4(1)	-1(1)
O(6)	27(1)	51(2)	34(2)	-7(2)	-1(1)	10(1)
O(7)	32(2)	62(2)	60(2)	-1(2)	-2(2)	16(1)
C(1)	22(2)	25(2)	22(2)	-2(2)	4(2)	2(1)
C(2)	19(2)	29(2)	18(2)	-2(2)	2(2)	2(1)
C(3)	13(1)	21(2)	18(2)	-1(1)	0(1)	0(1)
C(4)	17(2)	27(2)	15(2)	-2(2)	-1(1)	0(1)
C(5)	15(2)	24(2)	17(2)	0(1)	-1(1)	2(1)
C(6)	18(2)	24(2)	16(2)	1(2)	-2(1)	0(1)
C(7)	14(1)	23(2)	21(2)	-1(2)	-3(1)	1(1)
C(8)	22(2)	24(2)	20(2)	2(2)	-2(1)	0(1)
C(9)	21(2)	22(2)	29(2)	5(2)	-4(2)	2(1)
C(10)	21(2)	21(2)	31(2)	-2(2)	-4(2)	2(1)
C(11)	16(2)	22(2)	24(2)	-1(2)	-4(1)	1(1)
C(12)	14(1)	20(2)	21(2)	0(1)	-4(1)	1(1)
C(13)	13(1)	22(2)	18(2)	0(1)	0(1)	-2(1)
C(14)	13(1)	19(2)	18(2)	-2(1)	-4(1)	0(1)
C(15)	15(1)	23(2)	15(2)	0(1)	-4(1)	1(1)
C(16)	15(2)	20(2)	18(2)	-2(1)	-3(1)	1(1)
C(17)	12(1)	21(2)	19(2)	0(1)	-1(1)	1(1)
C(18)	27(2)	23(2)	19(2)	2(2)	2(2)	4(1)
C(19)	26(2)	22(2)	30(2)	2(2)	4(2)	-2(1)
C(20)	34(2)	23(2)	30(2)	6(2)	4(2)	5(2)
C(21)	29(2)	50(3)	33(3)	10(2)	2(2)	8(2)
O(8)	60(2)	46(2)	72(3)	-9(2)	-13(2)	10(2)
O(9)	48(2)	49(2)	39(2)	4(2)	11(2)	5(1)
C(22)	46(3)	27(2)	52(3)	12(2)	11(3)	4(2)

3.8 NMR based structure elucidation of the anthraquinone precursors

The chemical structures of the anthraquinone secondary metabolite precursors were determined via multidimensional NMR analysis. ^{1}H NMR, ^{13}C NMR and 2D spectra were recorded at 700 MHz (^{1}H)/175 MHz (^{13}C) conducting an Ascend 700 spectrometer using a cryogenically cooled triple resonance probe (Bruker Biospin, Rheinstetten, Germany). Samples were dissolved in DMSO-d_6, Acetone-d_6 and

Chrloroform-d. Chemical shifts are reported in ppm relative to TMS, the solvent was used as the internal standard. NMR data are presented in tabulated form by ascending ^{13}C shifts.

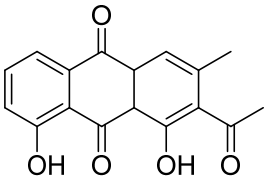

Figure S 19: Structure formula of anthraquinone 296

Table S 16: Tabulated NMR data of the anthraquinone 296 in DMSO-d_6

^{1}H	J, Multiplicity, Nb. of H	^{13}C	H-H Cosy	HMBC	ROESY
2.38	s, 3H	20.5	7.66	114.3; 122.5; 136.4; 105.8; 159.6; 203.2	7.66
2.59	s, 3H	32.1	7.66	203.1; 136.4	-
-	-	114.3	-	-	-
-	-	116.0	-	-	-
7.82	1.15; 7.45, dd, 2H	120.5	7.30; 7.69; 7.66	116.0; 125.2; 181.6	-
7.66	7.45; 8.48, dd, 2H	137.6	2.38, 7.82	114.3; 136.5; 181.6; 203.1; 20.5	2.38
7.3	1.2; 8.48, dd, 2H	125.2	7.69, 7.82	116.0; 120.6; 136.4	-
-	-	133.3	-	-	-
-	-	133.6	-	-	-
-	-	136.4	-	-	-
7.69	s, 1H	122.5	7.30; 7.69; 7.82	162.8; 133.6	-
-	-	145.8	-	-	-
-	-	159.6	-	-	-
-	-	162.8	-	-	-
-	-	181.6	-	-	-
-	-	192.8	-	-	-
-	-	203.1	-	-	-
11.96	s, 1H	-	7.69	116.0; 125.2; 162.8; 120.0; 192.8	-
12.34	s, 1H	-	7.66; 2.59	114.3; 136.4; 159.6; 20.5; 192.8	-

2D NMR data shown is in good agreement with published structure of protetrone [13].

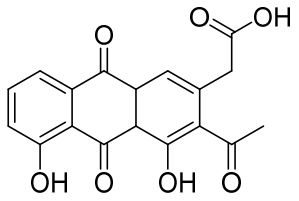

Figure S 20: Structure formula of anthraquinone 340

Table S 17: Tabulated NMR data of the anthraquinone 340 in Acetone-d_6

^{1}H	J, Multiplicity, Nb. of H	^{13}C
2.61	s, 3H	31.7
3.93	s, 2H	38.7
7.82	1.4; 7.0, dd, 1H	120.2
7.79	7.0; 8.0,dd, 1H	122.9
7.4	1.0;8.0, dd, 1H	125.2
7.87	s, 1H	138.4

Acquired NMR data in good agreement with NMR data published by Zhang et al. [14].

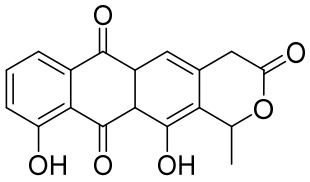

Figure S 21: Structure formula of anthraquinone 324

Table S 18: Tabulated NMR data of the anthraquinone 324 in Chloroform-d

^{1}H	J, Multiplicity, Nb. of H	^{13}C	H-H Cosy	HMBC
1.66	d	20.8	5.98	73.7, 130.2
3.86 + 3.82	2 x s	34.6	7.63	118.8, 130.4, 139.3, 167.9
5.98	6.90, q, 1H	73.7	1.66, 3.82	20.7, 130.4, 139.3, 157.6, 167.9
-		114.6		
-		115.6		
7.63	s, 1H	118.6	3.86, 3.82	34.6, 114.6, 130.2,181.5,197.2
7.83	0.9; 8.5, dd, 1H	120.4	7.69,7.31	115.6, 125,4, 162.8, 181.5,192.7
7.31	1; 8.5, dd, 1H	125.1	7.69, 7.83	115.6, 120.4, 162.8, 192.7
-		130.2		
-		133.2		
7.69	7.4; 8.5, dd, 1H	137.6	7.31, 7.83,11.94	115.6, 133.2, 162.8, 181.5
-	-	139.3	-	-
-	-	157.6	-	-

-	-	167.9	-	-
-	-	162.8	-	-
-	-	181.5	-	-
-	-	192.7	-	-
-	-	197.2	-	-
11.94	s, 1H	-	7.69	115.6, 125.0, 137.7, 162.8, 192.7
12.46	s, 1H	-	-	114.6, 130.2, 139.3, 157.6, 192.7

2D NMR data obtained in from anthraquinone 324 in good agreement with published NMR data for ekatetrone [15].

3.9 Tandem MS based structure assignment of anthraquinone 340

Due to poor stability of anthraquinone 340 in most NMR solvents, which led to decarboxylation of the compound to anthraquinone 296 via decarboxylation, only a ^{1}H and HSQC spectrum were recorded for this compound. To ascertain that the seen anthraquinone possesses the proposed structure, the first fragments of the LC-MS² spectrum recorded on the HPLC coupled maXis 4G qTOF spectrometer (section S 3.3) are interpreted.

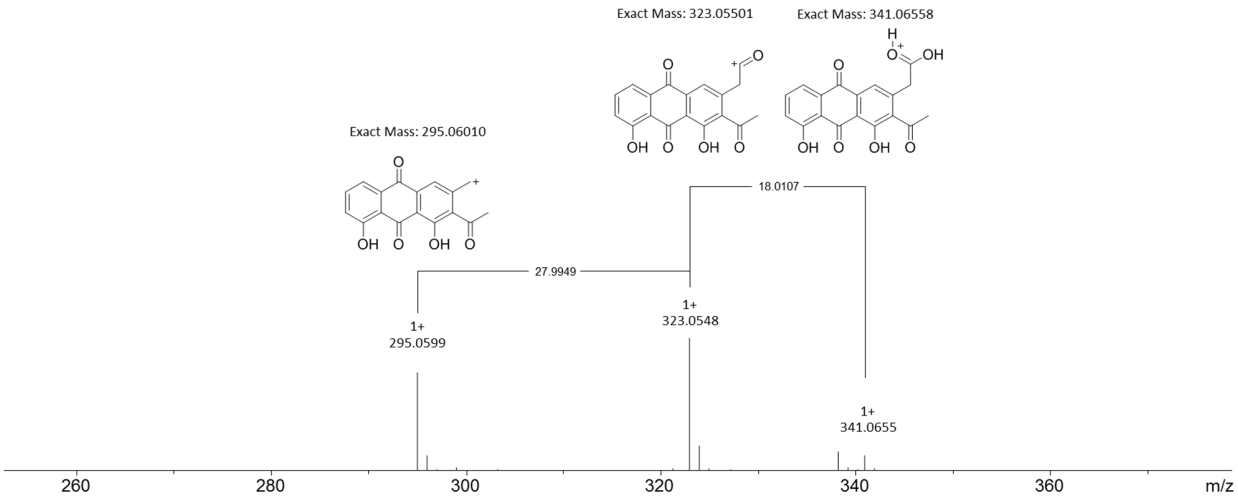

Figure S 22: MS² spectrum of anthraquinone 340 with proposed gas phase dissociation reactions acquired during an LC-MS² run of Pyxidicoccus fallax *And48* pcyA*::pSBtn5* pcyA *extract on the HPLC coupled maXis 4G qTOF spectrometer*

One can clearly see the MS² fragments fitting the proposed structure that is also in accord with the reference NMR data. Furthermore, as this scaffold would indeed favor decarboxylation in solution to anthraquinone 296 as it is observed during longer NMR experiments one can safely assume the anthraquinone 340 to have the presented structure.

3.10 Tandem MS based assignment of the incorporated deuterium atoms into Pyxidicycline A

10 mmol 1,2 Ethanolamine $^{13}C_2$, 2,2-Glycine-d_2 and 2,3,3-serine-d3 are fed into a 25 ml CTT medium culture of *M. xanthus* attB :: attP pClY pyxidicycline cluster in order to observe isotopic labelling of the pyxidicyclines. Extracts were prepared according to standard protocol (section S 3.2) and analyzed via standardized UPLC-MS and UPLC-MS^2 (section S 3.3). Only feeding of 3,3,2-serine-d_3 into *M. xanthus* DK1622 attB::attP pClY pyxidicycline cluster reveals strong labelling of the pyxidicyclines. To prove the incorporation into the side chain of the pyxidicyclines as it was hypothesized in the biosynthesis proposal backed up with labelling experiments the labelled compound is subjected to tandem MS measurements.

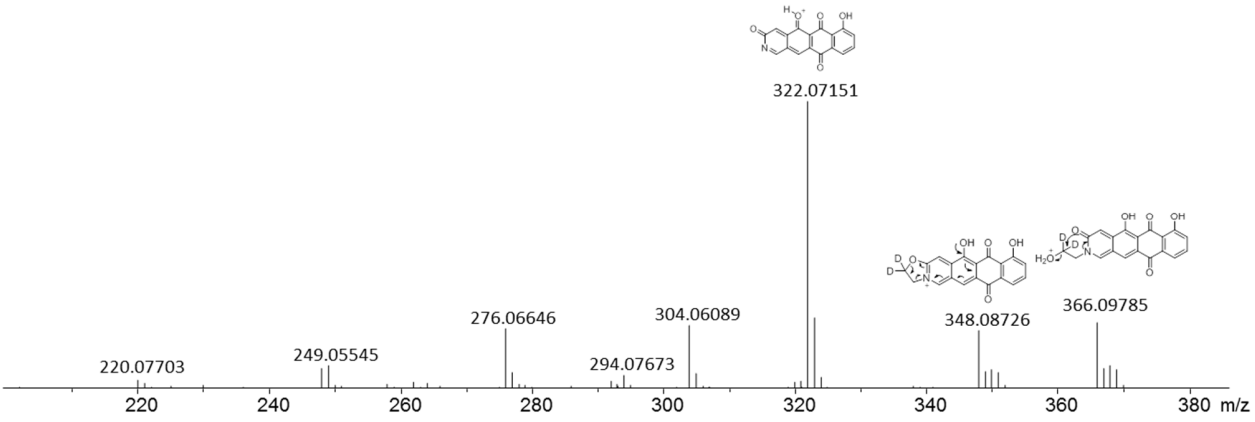

Figure S 23: LC-MS^2 CID-fragmentation pattern of d_2 labelled pyxidicycline A from M. xanthus *DK1622 attB :: attP pClY pyxidicycline with proposed gas phase reaction acquired on the HPLC coupled maXis 4G qTOF spectrometer*

As the molecule fragment with the mass of 322.0715 Daltons lost the deuterium atoms as visible by the isotope pattern one can assume that both deuterium atoms are present on this 2-hydroxyethyl side chain. This fits well with the molecule's structure as one would assume the linear 2-hydroxyethyl side chain to be the most labile part in CID based MS^2 experiments that likely fragments according to the gas phase reactions proposed in Figure S 23. One can therefore experimentally prove the incorporation of serine into the structure.

3.11 NMR based structure elucidation of the pyxidicyclines

As the pyxidicyclines are only soluble in DMSO and DMF that both unfortunately lead to degradation of the compound over time, NMR data is acquired in formic acid-d_2. The pyxidicyclines readily and reversibly form formate esters that are soluble in formic acid. ^{1}H NMR, ^{13}C NMR and 2D spectra were

recorded at 700 MHz (^{1}H)/175 MHz (^{13}C) conducting an Ascend 700 spectrometer using a cryogenically cooled triple resonance probe (Bruker Biospin, Rheinstetten, Germany). Samples were dissolved in formic acid-d_2 Chemical shifts are reported in ppm relative to TMS, the solvent was used as the internal standard. NMR reference information for formic acid is taken from Roy Hoffmann.[16] Carbon numbering for NMR signal assignment purposes is done in a biosynthetic logic meaning that the carbon first integrated into the structure obtains the first number.

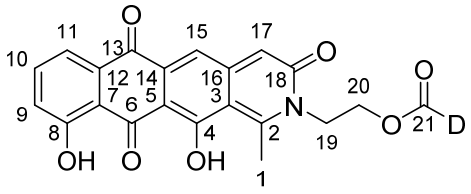

Figure S 24: Carbon numbering for NMR assignment purposes for pyxidicycline A formate ester

Table S 19: Tabulated NMR data of pyxidicycline A formate ester acquired in formic acid-d_2. Carbon numbering is done according to the scheme in Figure S 24.

C Number	1H	J, Multiplicity, Nb of H	13C	H-H Cosy	HMBC
1	3.57	s, 3H	21.4	7.56	108.5, 116.2, 157.5, 168.8
2	-	-	125.5	-	-
3	-	-	116.2	-	-
4	-	-	168.8	-	-
5	-	-	109.3	-	-
6	-	-	190.6	-	-
7	-	-	116	-	-
8	-	-	162.7	-	-
9	7.41	n.d.; 8.3, dd, 1H	125.7	7.81, 7.86	116.0, 120.6, 162.0
10	7.81	7.3; 8.3, dd, 1H	137.8	7.41,7.86	116.0, 133.6, 162.0
11	7.86	n.d.; 7.3, dd, 1H	120.6	7.41, 7.81	116.0, 125.7, 137.8, 182.2, 190.6
12	-	-	133.6	-	-
13	-	-	182.2	-	-
14	-	-	126.6	-	-
15	8.05	s, 1H	118.4	3.57, 7.56	108.5, 109.3, 116.2, 145.5, 182.2, 190.6
16	-	-	145.4	-	-
17	7.56	s, 1H	108.5	8.05	116.2, 125.5, 126.6 157.5, 162.5, 168.8
18	-	-	157.5	-	-
19	5.14	5.30, t, 2H	47.4	4.81	60.8, 157.5, 162.5
20	4.81	5.30, t ,2H	60.8	5.14	47.4,162.5,163.3
FA C21	-	-	163.3	-	-

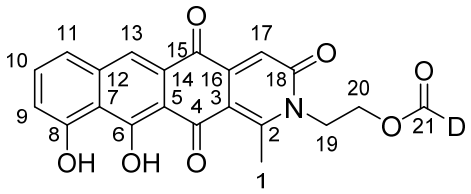

Figure S 25: Carbon numbering for NMR assignment purposes for pyxidicycline B formate ester

Table S 20: Tabulated NMR data of pyxidicycline B formate ester acquired in formic acid-d_2. Carbon numbering is done according to the scheme in Figure S 25.

C Number	1H	J, Multiplicity, Nb of H	13C	H-H Cosy	HMBC	ROESY
1	3.23	s, 3H	19.1	7.38	113.2, 114.8, 142.2, 159.8, 164.5, 186.4	4.74, 4.65
2	-	-	159.8	-	-	-
3	-	-	113.2	-	-	-
4	-	-	186.4	-	-	-
5	-	-	124.1	-	-	-
6	-	-	165.1	-	-	-
7	-	-	108.7	-	-	-
8	-	-	157.2	-	-	-
9	7.1	7.9, d, 1H	115.8	7.54, 7.64	116.0, 122.7, 157.2	-
10	7.64	7.9; 8.0, dd, 1H	133.5	7.10, 7.54	115.8, 122.7, 137.2, 157.2	-
11	7.54	8.0, d, 1H	122.7	7.10, 7.64	115.8, 123.0, 137.2, 157.2, 165.1	-
12	-	-	137.2	-	-	-
13	8.15	s, 1H	123	7.53	108.7, 115.8, 122.7, 124,1, 137.2, 157.2, 165.1, 181.3, 186.4	-
14	-	-	n.d.	-	-	-
15	-	-	181.2	-	-	-
16	-	-	142.2	-	-	-
17	7.38	s, 1H	114.8	3.23	113.2, 142.2, 164.5, 181.6	-
18	-	-	164.5	-	-	-
19	4.74	5.52, t, 2H	44.5	4.65	61.1, 159.8, 164.5	3.23
20	4.65	5.52, t, 2H	61.2	4.74	44.5, 163.0	3.23
FA C21	-	-	163	-	-	-

3.12 Comparison of production levels for pyxidicycline and pyxidicycline precursors

Table S 21 summarizes the relative and absolute peak areas measured on the UHPLC coupled maXis 4G spectrometer system by injection of 0.5 µL of crude extract of the respective species grown and extracted according to standard protocol described in section 3.1 to 3.3. Fold-change values (in blue) show the relative production titres for pyxidicyclines A (**1**) and B (**2**), relative to the base yield from promotor-activated strain named And48 *pcy* in the main text (table row highlighted with blue background). The isolated yields from this producer are 416 µg/L (Pyx A, **1**) and 333 µg/L (Pyx B, **2**), respectively (see section S3). The last five columns contain calculated ratios to illustrate the effect of expression in different hosts or gene deletions upon pyxidicycline expression.

Table S 21: Comparative peak area analysis measured in [cts x min] of the pyxidicyclines and its precursors in all generated producer strains measured by integration the $[M+H]^+$ EIC in the standard LC-MS chromatogram setting (section S 3.3). The two anthraquinone metabolites are measured as LC-MS EIC peak area at 341.06 $[M+H]^+$, 325.07 $[M+H]^+$ and 297.075 $[M+H]^+$, the two pyxidicyclines are measured as LCMS EIC peak areas at 366.10 $[M+H]^+$.

Producer strain	Area Pyx A **(1)**	Pyx A **fold-change**	Area Pyx B **(2)**	PyxB **fold-change**	Area AQ 340 **(3)**	Area AQ 324 **(4)**	Area AQ 296 **(5)**	Area Pyx A / Area AQ 340	Area Pyx B / Area AQ 340	Area Pyx A / Area AQ 296	Area Pyx B / Area AQ 296	Area Pyx A / Area Pyxi B
P. fallax And48 *pcyA* :: pSBtn5 *pcyA*	-		-		1135792	119418	226789	-	-	-	-	-
P. fallax And48 *pcyA* :: pFPVan *pcyA*	-		-		129491	13364	151052	-	-	-	-	-
P. fallax And48 *pcyA* :: pFPVan *pcyA;* *pcyJ* :: pFPtettn5 *pcyJ*	1905806	1	1859927	1	15991	-	1119844	119.18	116.31	1.70	1.66	1.02
M. xanthus DK1622 attB :: attP Pyxidicycline Cluster	1157822	0.61	2240664	1.18	126015	-	540949	9.19	17.78	2.14	4.14	0.52
M. xanthus DK1622 attB :: attP Pyxidicycline Cluster Δ *pcyJ*	-		-		1173697	-	1580399	-	-	-	-	-
M. xanthus DK1622 attB :: attP Pyxidicycline Cluster Δ *pcyK*	170034	0.09	137566	0.07	1156196	-	2065290	0.15	0.12	0.08	0.07	1.24
M. xanthus DK1622 attB :: attP Pyxidicycline Cluster Δ *pcyL*	153049	0.08	132066	0.07	1202235	-	2301855	0.13	0.11	0.07	0.06	1.16
M. xanthus DK1622 attB :: attP Pyxidicycline Cluster T7A1 *pcyO*	1400366	0.74	3180800	1.67	365870	-	399734	3.83	8.69	3.50	7.96	0.44
S. aurantiaca DW 4/3-1 attB :: attP Pyxidicycline Cluster	75069	0.04	290585	0.15	102942	-	75896	0.73	2.82	1.36	3.83	0.26

4 Bioactivity profiling of the pyxidicyclines and its precursors

4.1 Cell based biological assays to valuate MIC values on bacteria and cancer cell lines

4.1.1 Antimicrobial Assay

All microorganisms were handled according to standard procedures and were obtained from the German Collection of Microorganisms and Cell Cultures (*Deutsche Sammlung für Mikroorganismen und Zellkulturen*, DSMZ) or were part of our internal strain collection. For microdilution assays, overnight cultures of Gram-positive bacteria in Müller-Hinton broth (0.2 % (w/v) beef infusion, 0.15 % (w/v) corn starch, 1.75 % (w/v) casein peptone; pH 7.4) were diluted in the growth medium to achieve a final inoculum of ca. 10^6 cfu ml^{-1}. Serial dilutions of pyxidicyclines and the anthraquinone precursors were prepared from freshly prepared DMSO or Methanol stocks in sterile 96-well plates. The cell suspension was added and microorganisms were grown on a microplate shaker (750 rpm, 37°C and 16 h). Growth inhibition was assessed by visual inspection and given MIC (minimum inhibitory concentration) values are the lowest concentration of antibiotic at which no visible growth was observed.

4.1.2 Cytotoxic Activity

Cell lines were obtained from the German Collection of Microorganisms and Cell Cultures (Deutsche Sammlung für Mikroorganismen und Zellkulturen, DSMZ) or were part of our internal collection and were cultured under conditions recommended by the depositor. Cells were seeded at 6 x 10^3 cells per well of 96-well plates in 180 µl complete medium and treated with pyxidicyclines in serial dilution after 2 h of equilibration. Each compound was tested in duplicate as well as the internal solvent control. After 5 d incubation, 20 µl of 5 mg ml^{-1} MTT (thiazolyl blue tetrazolium bromide) in PBS was added per well and it was further incubated for 2 h at 37°C. The medium was discarded and cells were washed with 100 µl PBS before adding 100 µl 2-propanol/10 N HCl (250:1) in order to dissolve formazan granules. The absorbance at 570 nm was measured using a microplate reader (Tecan Infinite M200Pro), and cell viability was expressed as percentage relative to the respective methanol control. IC_{50} values were determined by sigmoidal curve fitting.

4.2 Topoisomerase inhibition assays performed to determine the target of the pyxidicyclines

All topoisomerase related inhibition assays were performed using topoisomerase inhibition evaluation kits for *E. coli* topoisomerase II, *E. coli* topoisomerase IV and human topoisomerase I supplied by Inspiralis (Inspiralis ltd., Norwich, U.K.). The kits were used according to the manuals. All compounds were tested as freshly prepared DMSO solutions. Pyxidicyclines were assayed to be stable in DMSO for about 12 hours. Topoisomerase inhibition assays were evaluated by agarose gel electrophoresis using 0.8 % agarose gels in 1x TAE buffer. Total volume of the assay reaction was 30 µL. All assays are carried out in a 96 well micro titer plate.

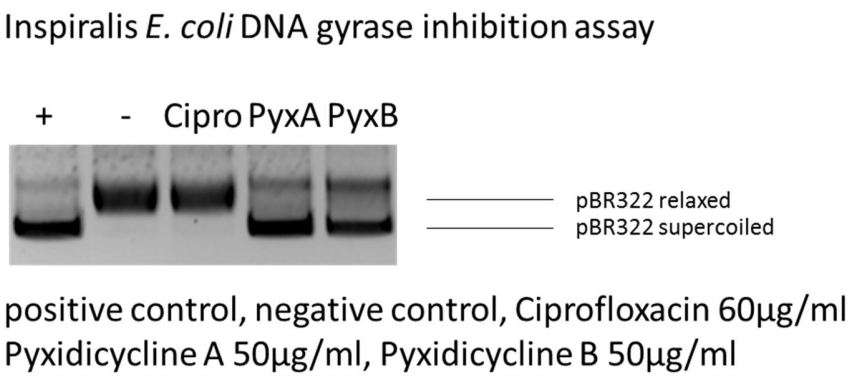

Figure S 26: E. coli *DNA gyrase inhibition assay, positive control is 0.5% DMSO, negative control is assay without enzyme; Ciprofloxacin, pyxidicycline A and pyxidicycline B are supplied in DMSO to a total concentration of 0.5% DMSO and the stated end concentrations in the assay*

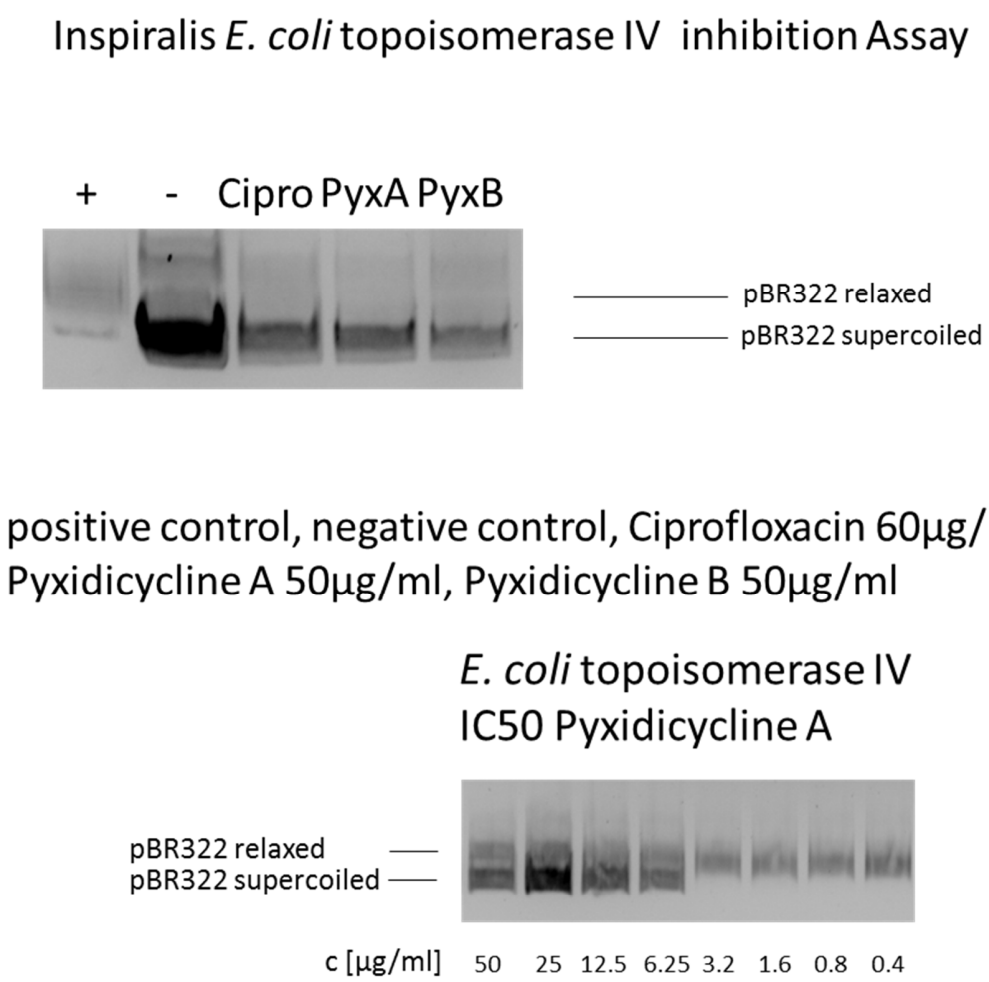

Figure S 27: E. coli *DNA topoisomerase IV inhibition assay, positive control is 0.5% DMSO, negative control is assay without enzyme; Ciprofloxacin, pyxidicycline A and pyxidicycline B are supplied in DMSO to a total concentration of 0.5% DMSO and the stated end concentrations in the assay*

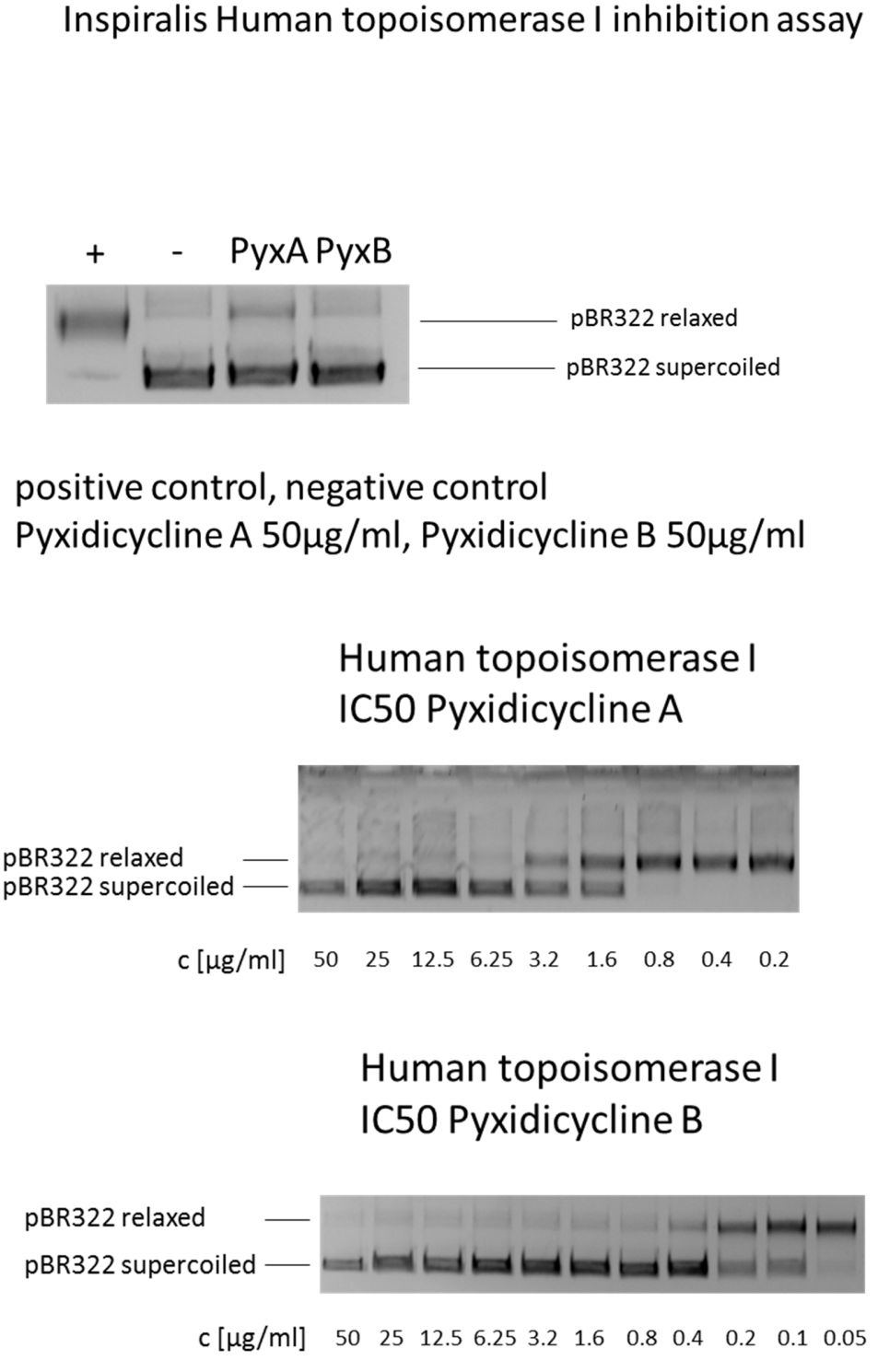

Figure S 28: Human DNA topoisomerase I inhibition assay, positive control is 0.5% DMSO, negative control is assay without enzyme, pyxidicycline A and pyxidicycline B are supplied in DMSO to a total concentration of 0.5% DMSO and the stated end concentrations in the assay

5 NMR spectra of the pyxidicyclines and their precursor molecules

Anthraquinone 296

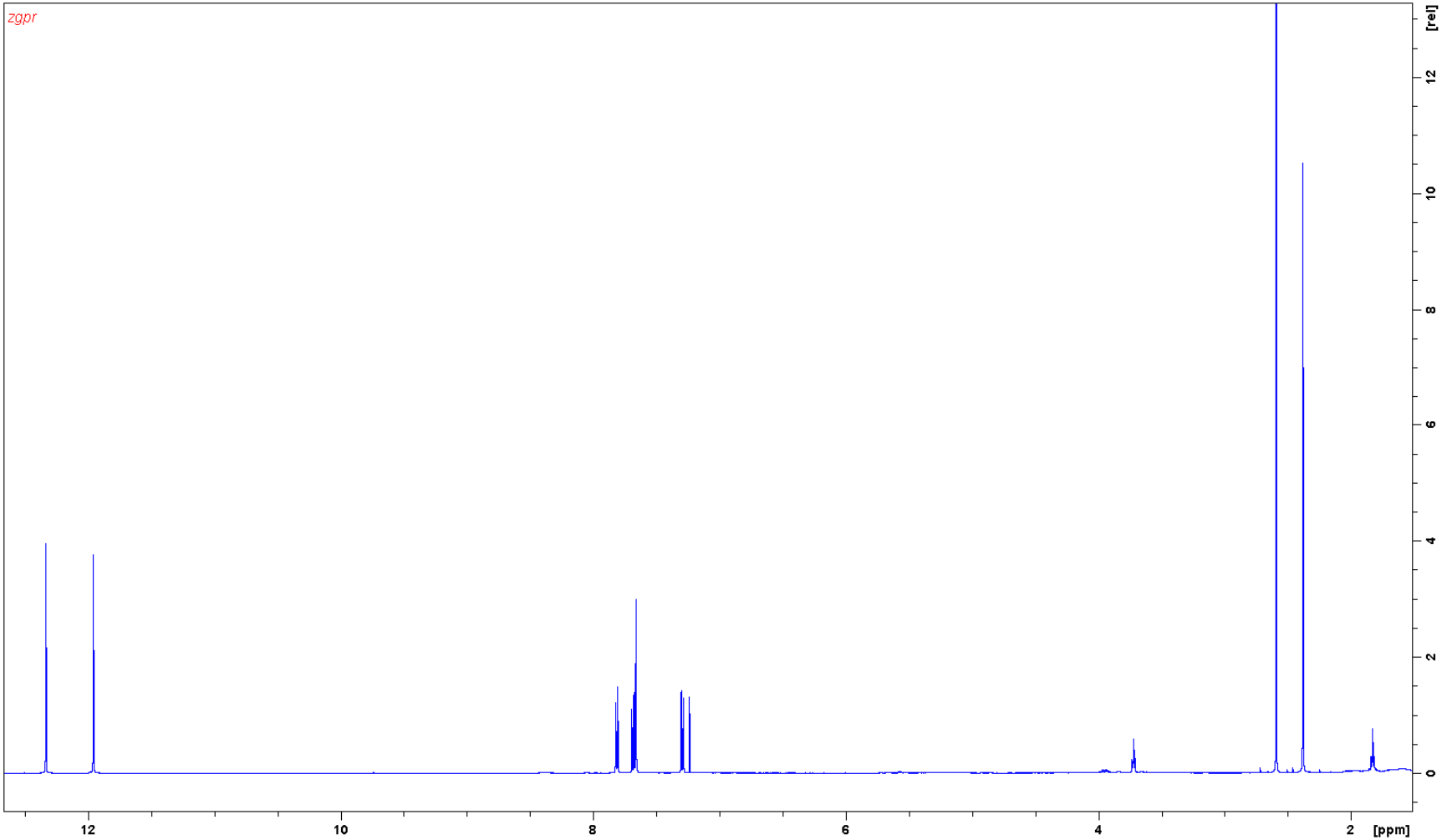

Figure S 29: ^{1}H NMR spectrum of anthraquinone 296 in DMSO-d_6

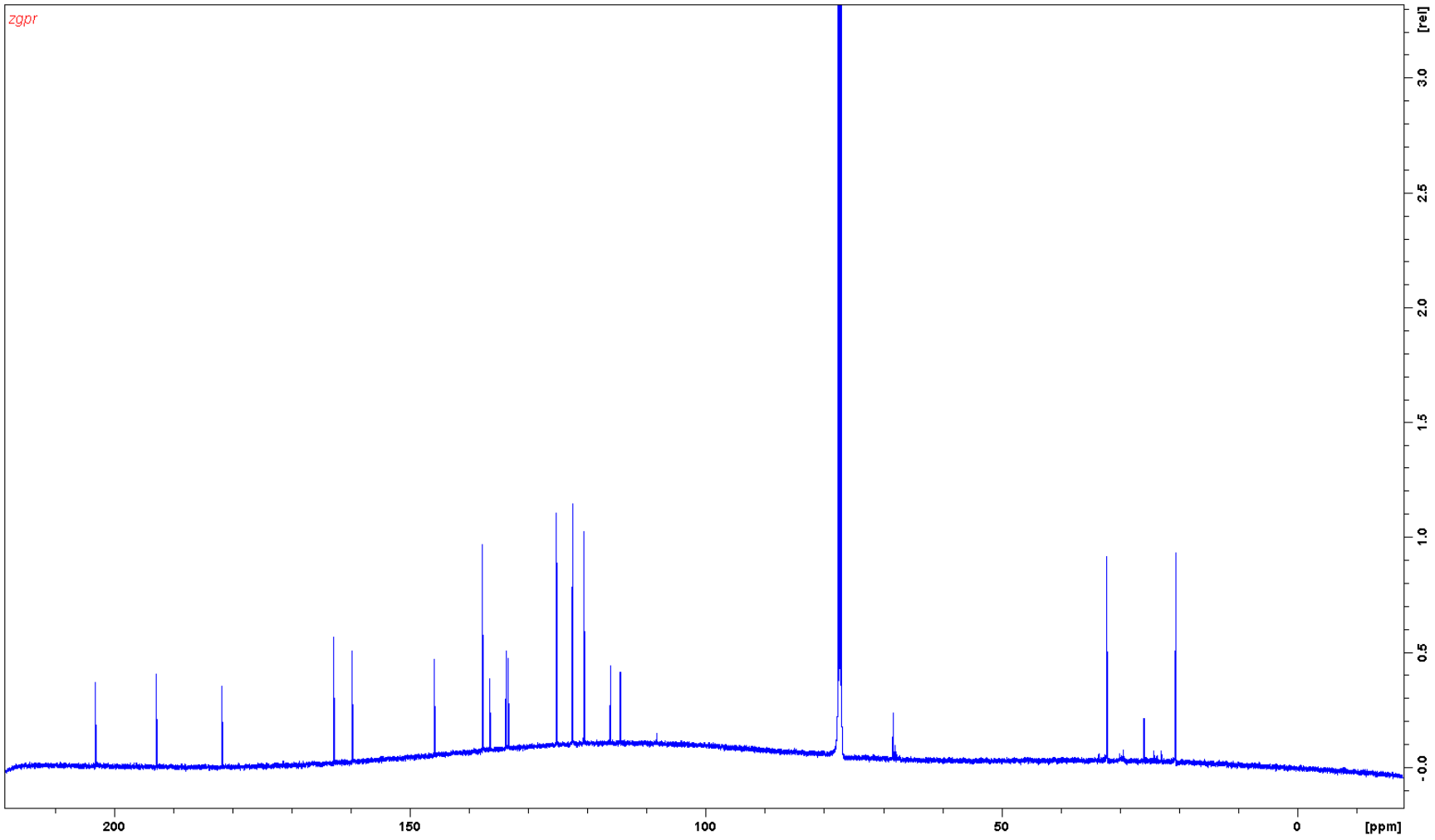

Figure S 30: ^{13}C NMR spectrum of anthraquinone 296 in DMSO-d_6

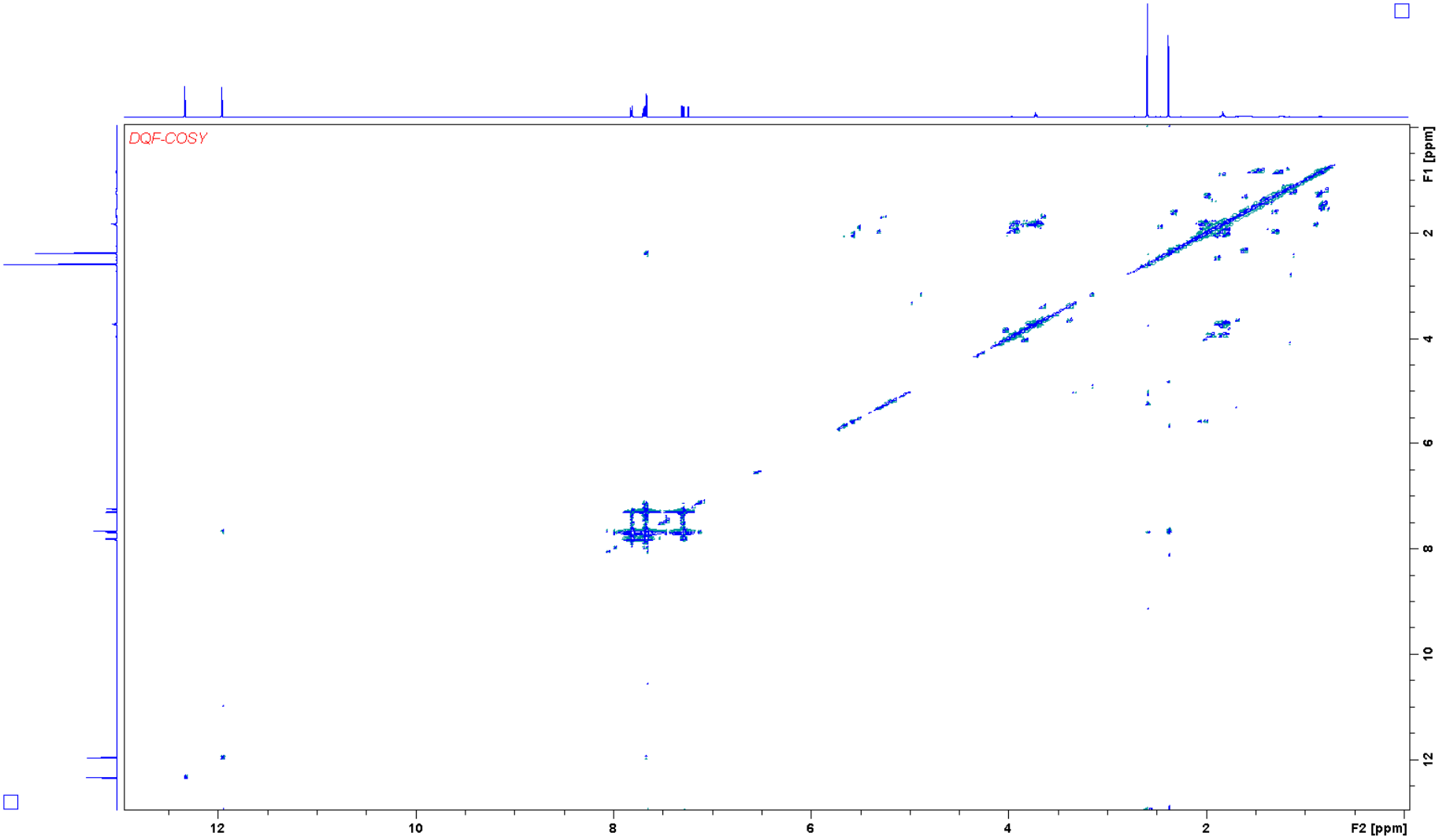

Figure S 31: DQF COSY NMR spectrum of anthraquinone 296 in DMSO-d_6

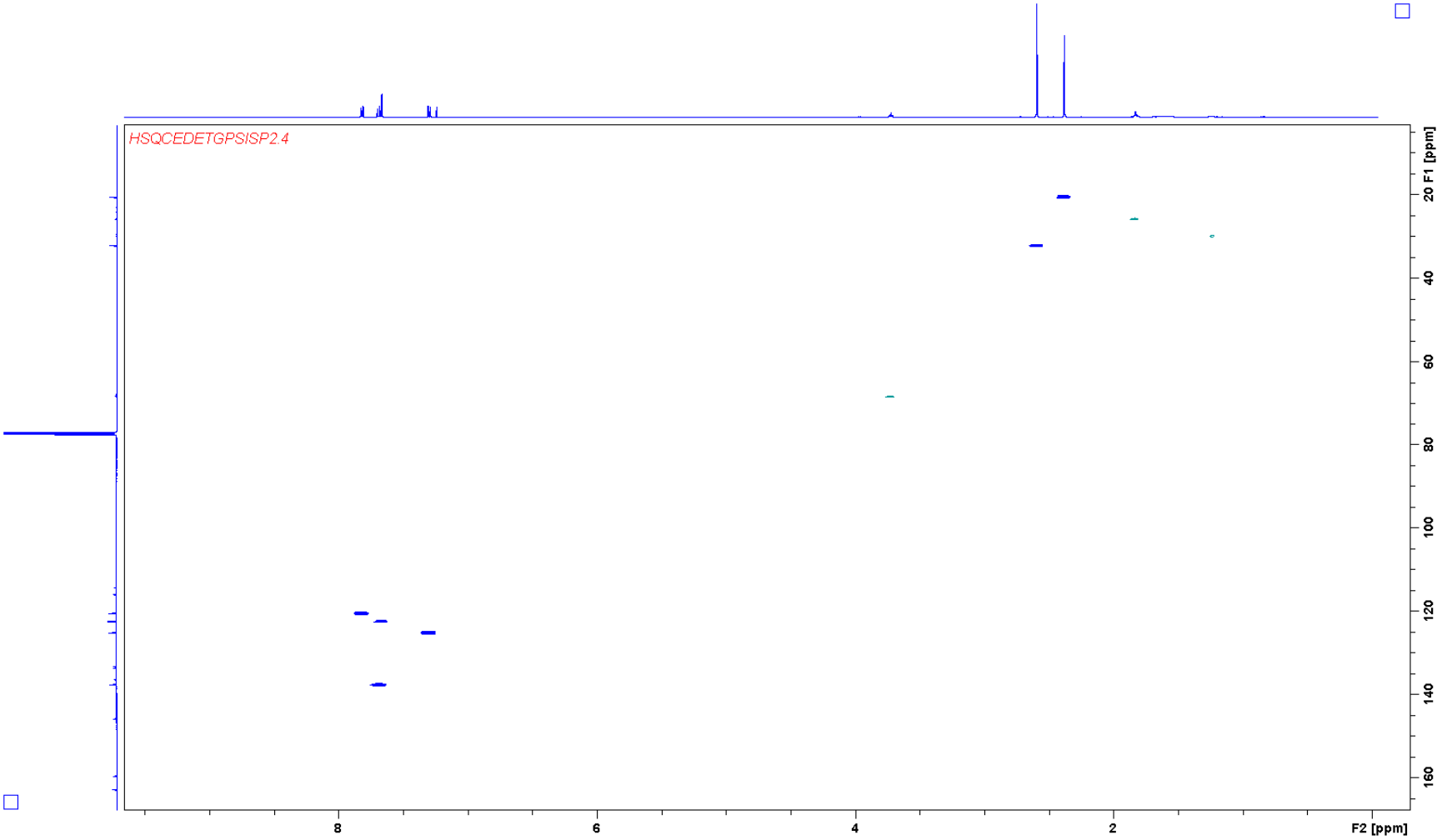

Figure S 32: HSQC NMR spectrum of anthraquinone 296 in DMSO-d_6

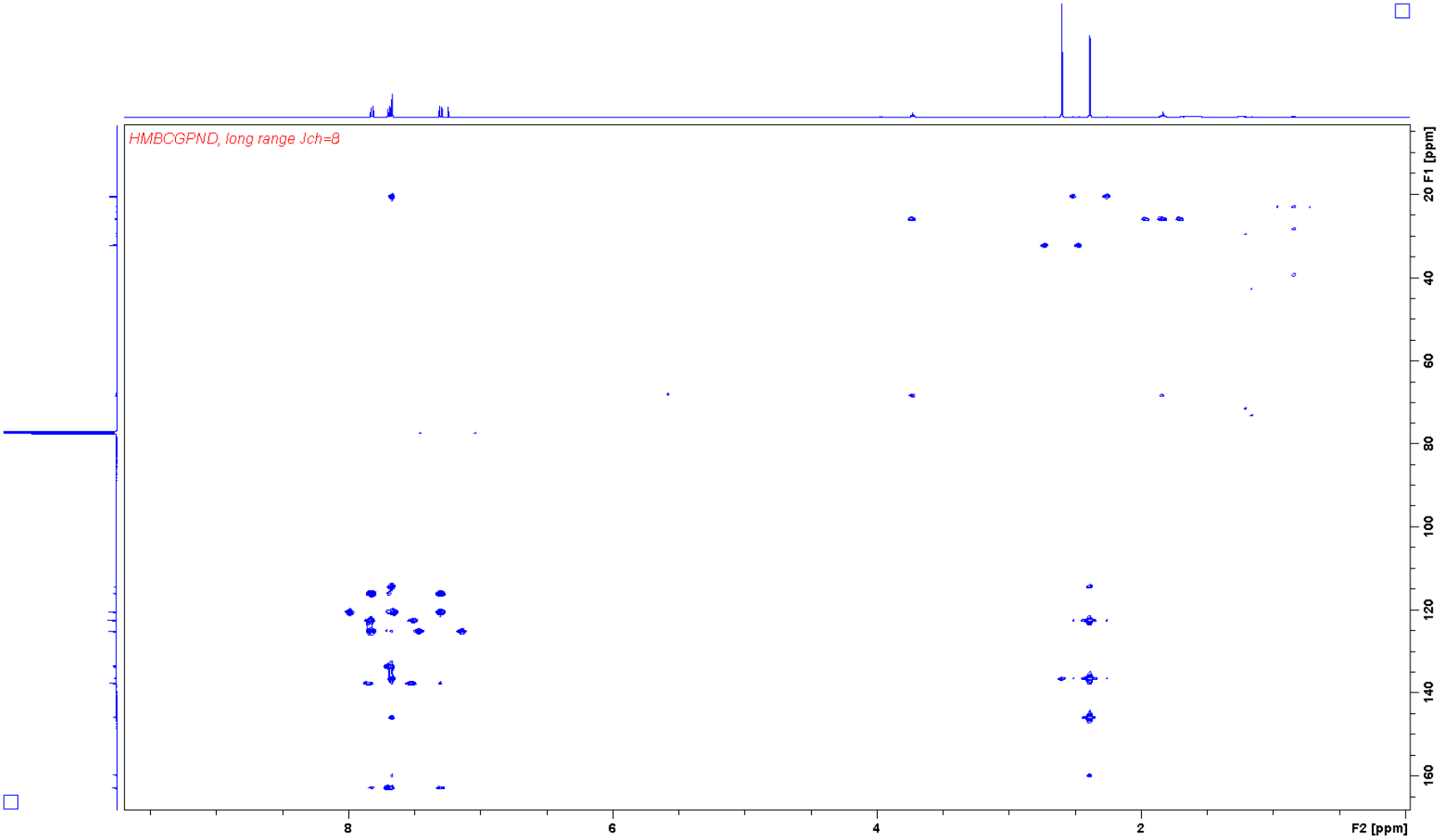

Figure S 33: HMBC NMR spectrum of anthraquinone 296 in DMSO-d_6

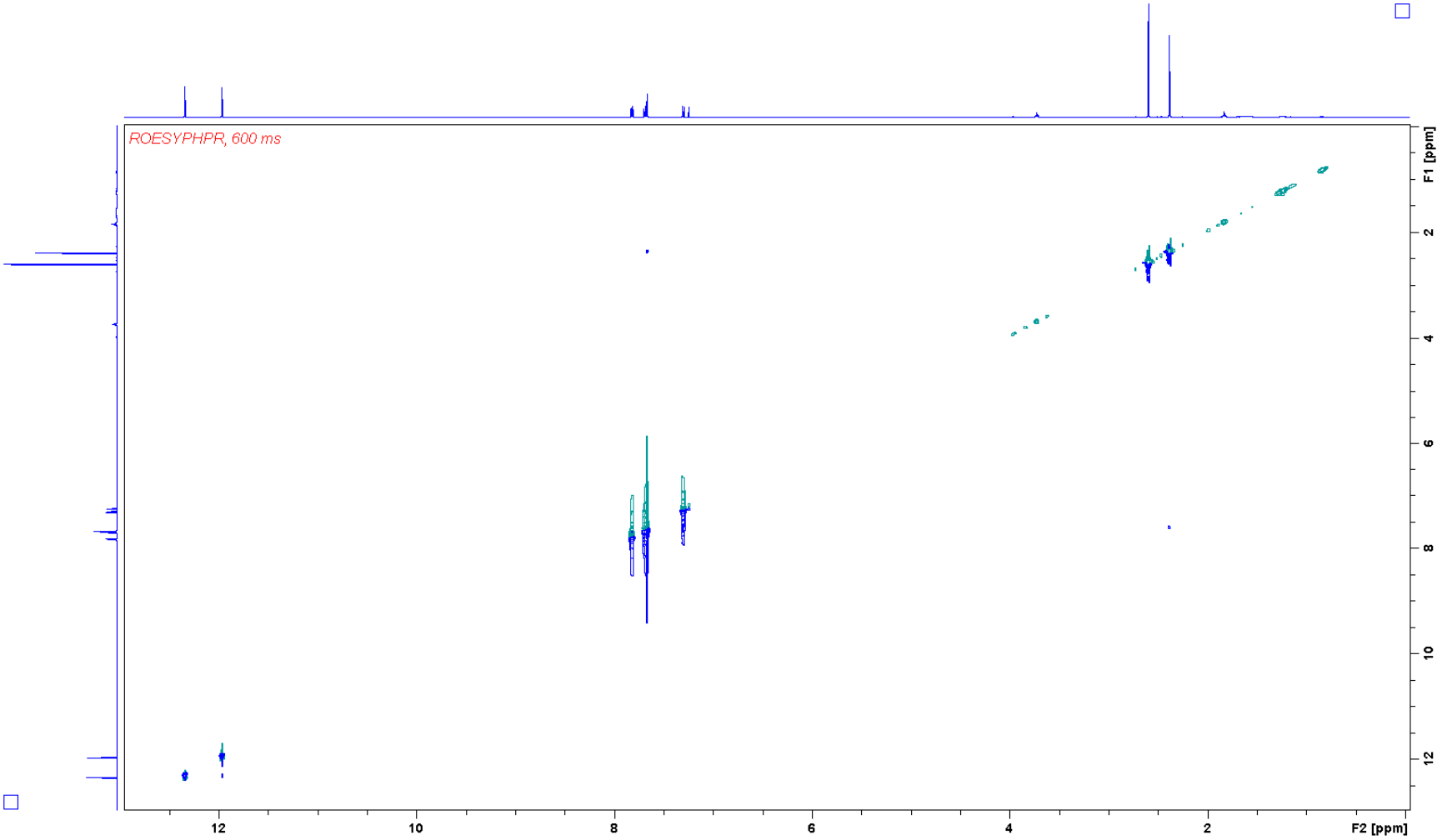

Figure S 34: ROESY NMR spectrum of anthraquinone 296 in DMSO-d_6

Anthraquinone 340

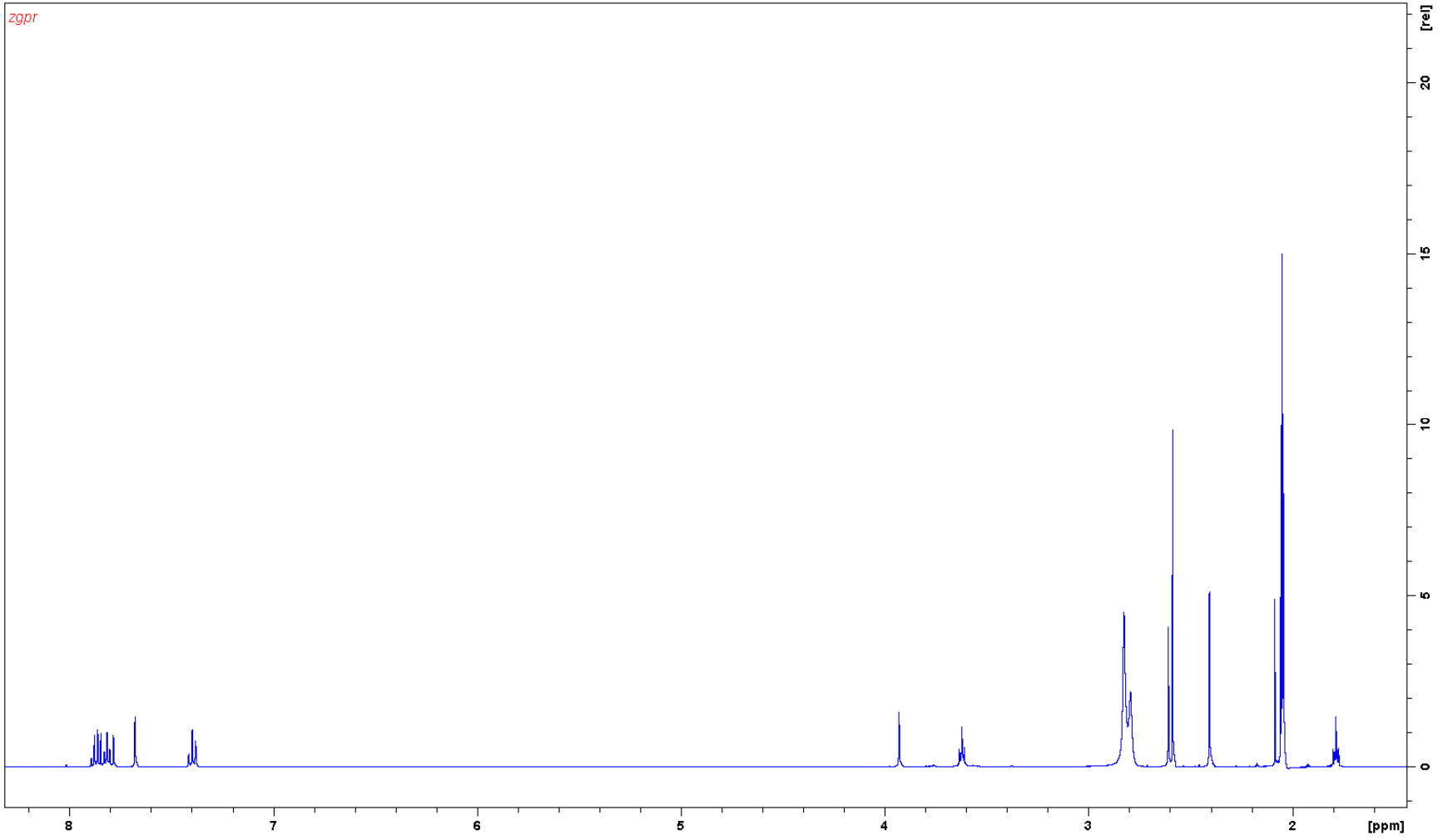

Figure S 35: 1H NMR spectrum of anthraquinone 341 in Acetone-d_6

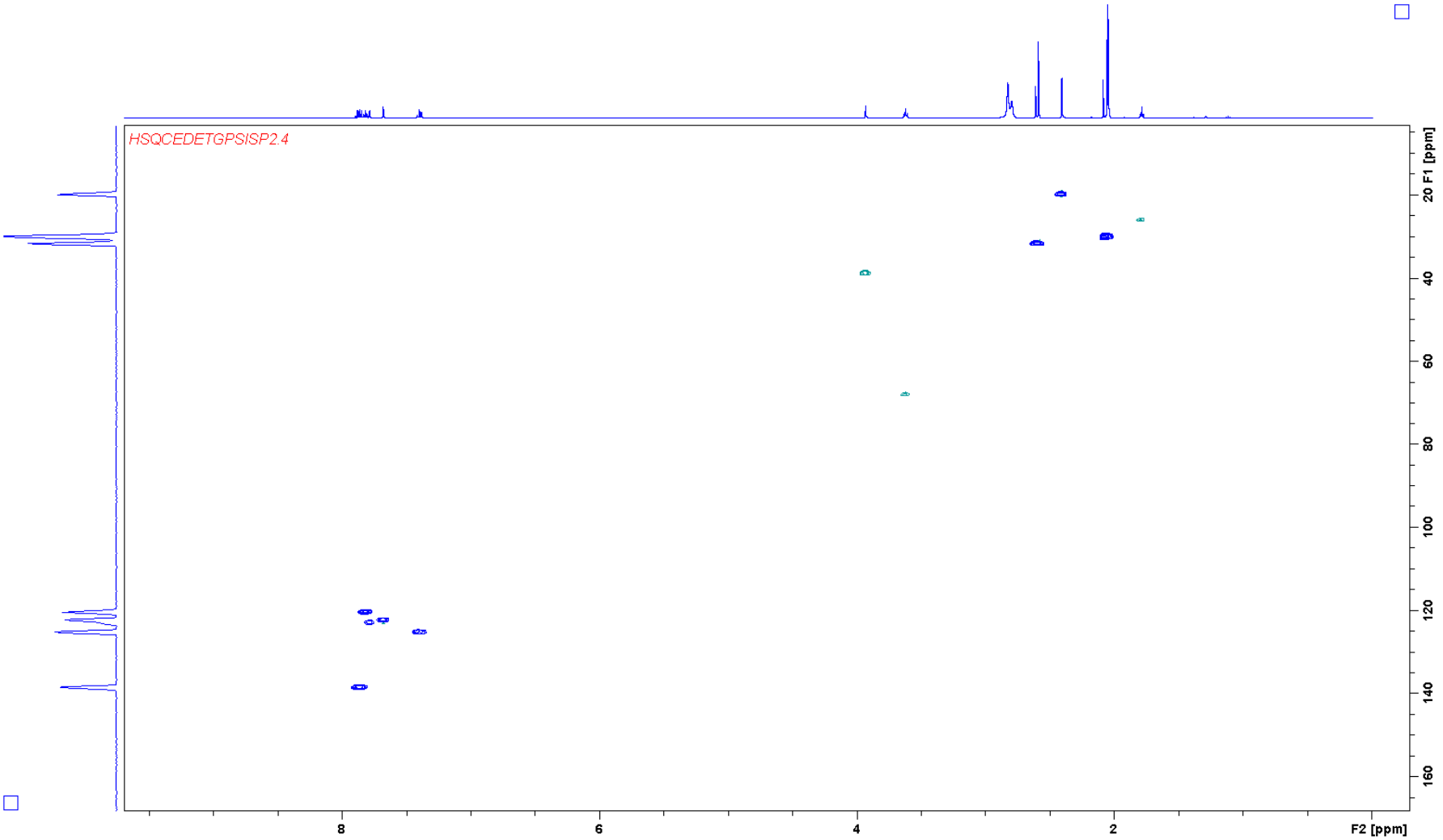

Figure S 36: HSQC NMR spectrum of anthraquinone 341 in Acetone-d_6

Anthraquinone 324

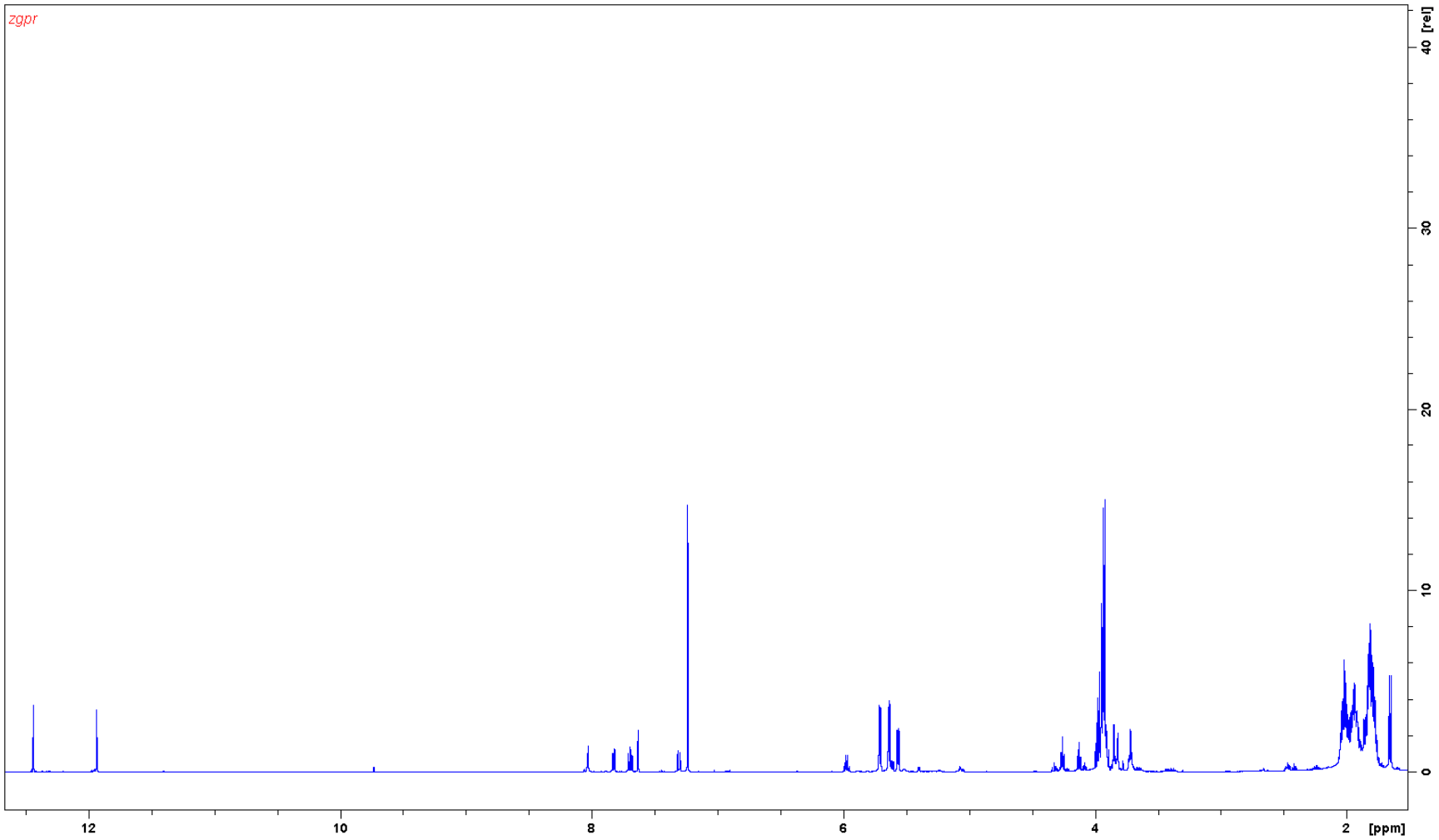

Figure S 37: ^{1}H NMR spectrum of anthraquinone 324 in $CDCl_3$

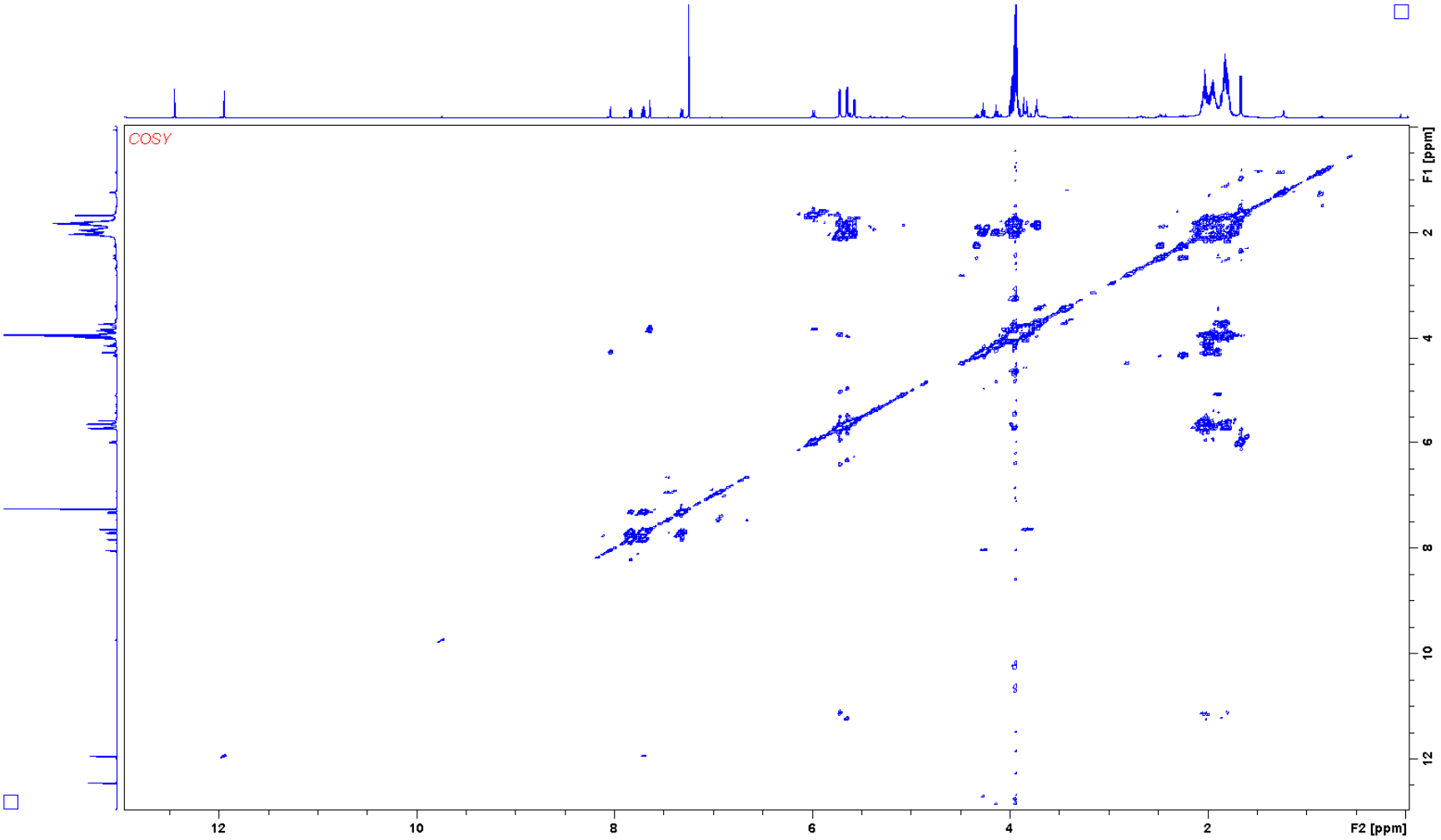

Figure S 38: COSY NMR spectrum of anthraquinone 324 in $CDCl_3$

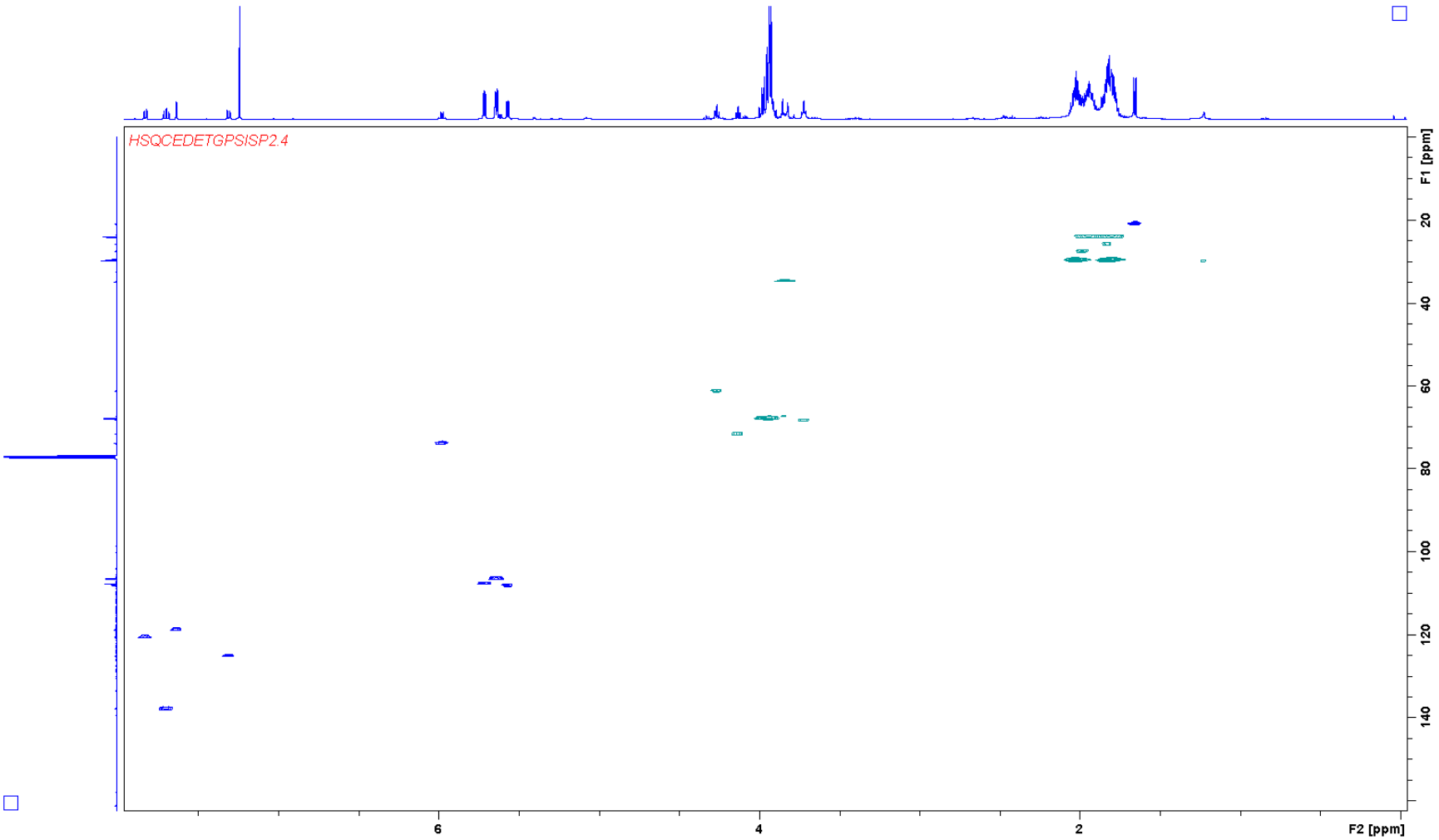

Figure S 39: HSQC NMR spectrum of anthraquinone 324 in $CDCl_3$

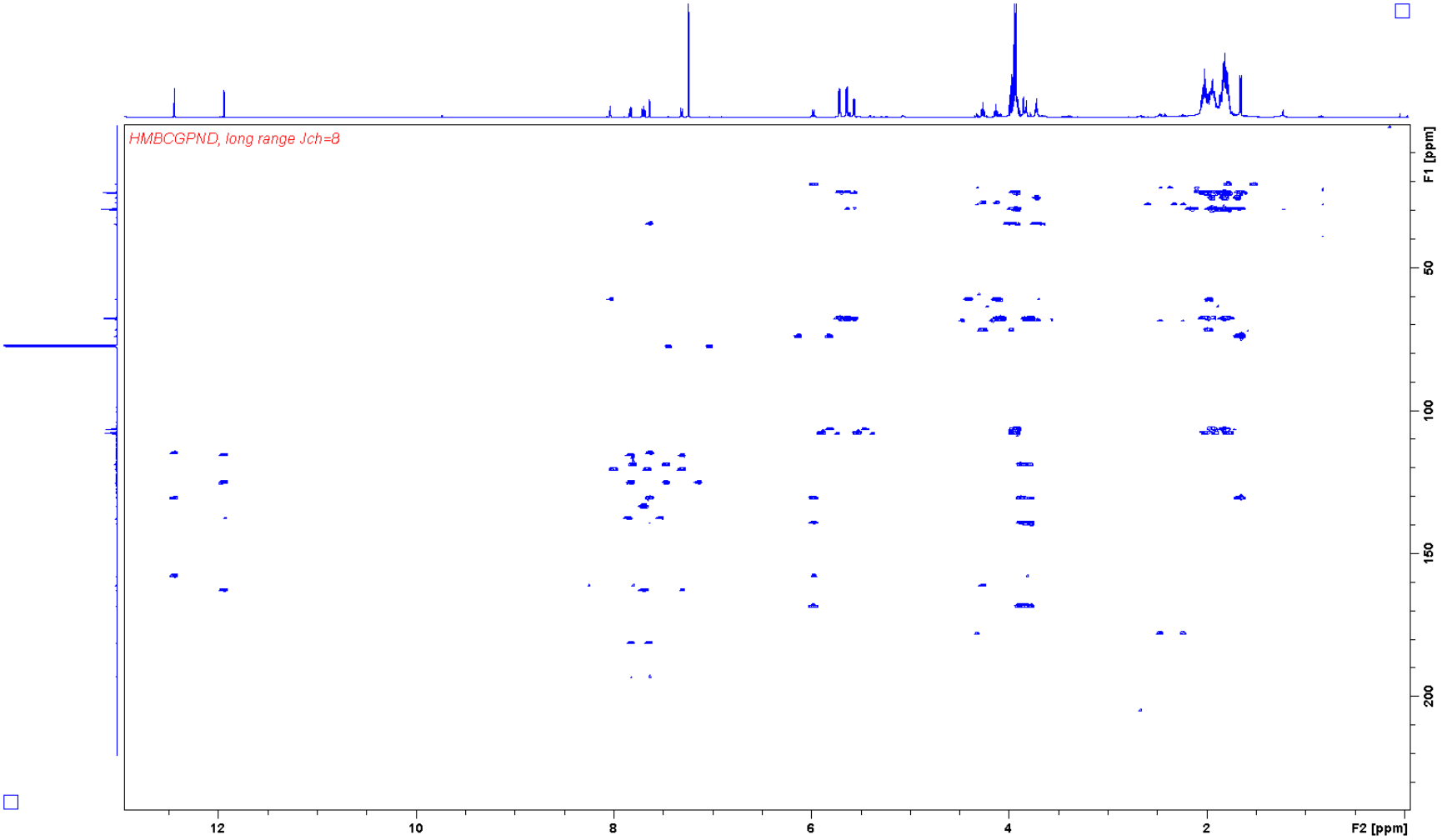

Figure S 40: HMBC NMR spectrum of anthraquinone 324 in $CDCl_3$

Pyxidicycline A

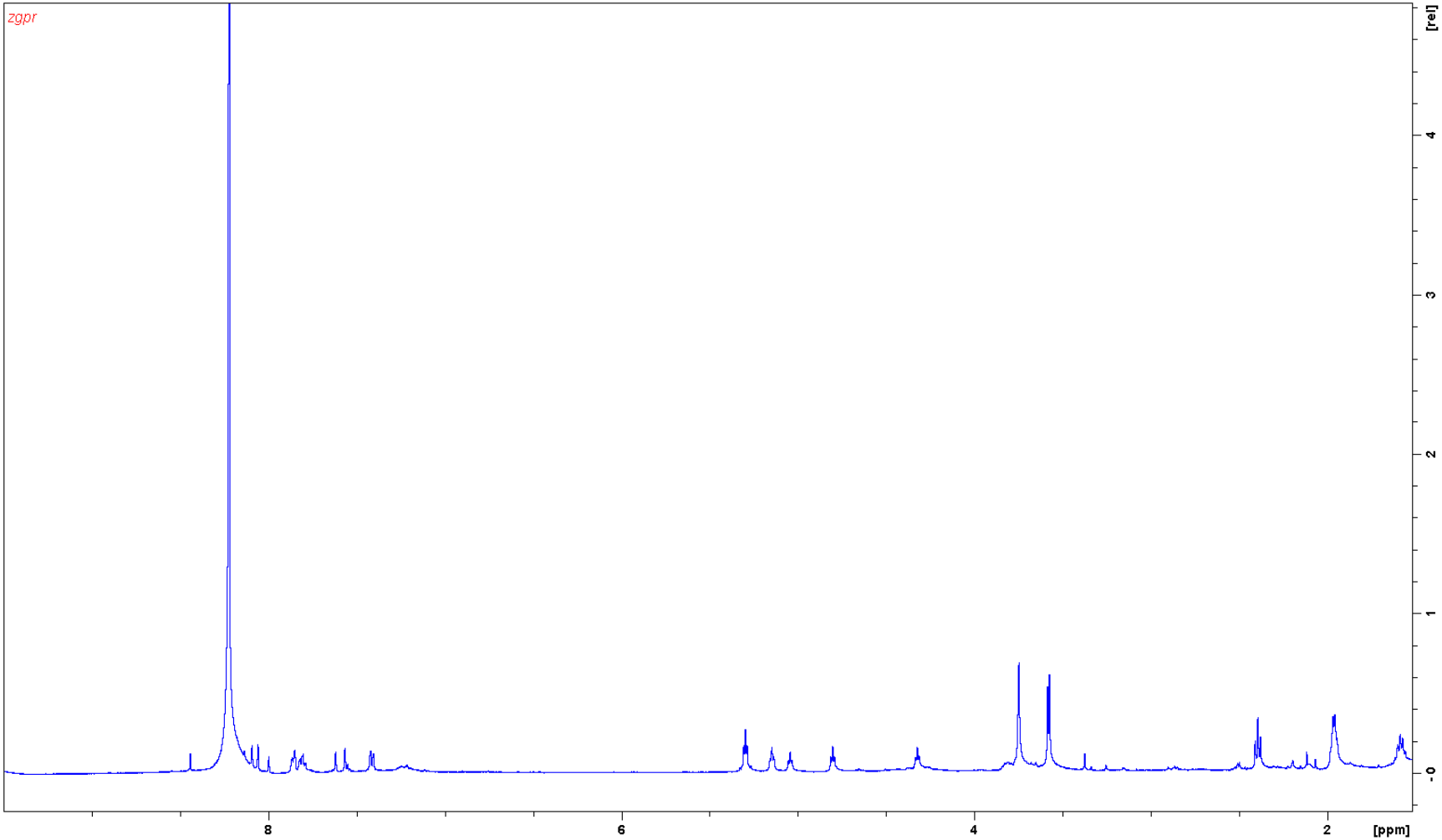

Figure S 41: ^{1}H NMR spectrum of pyxidicycline A in formic acid-d_2

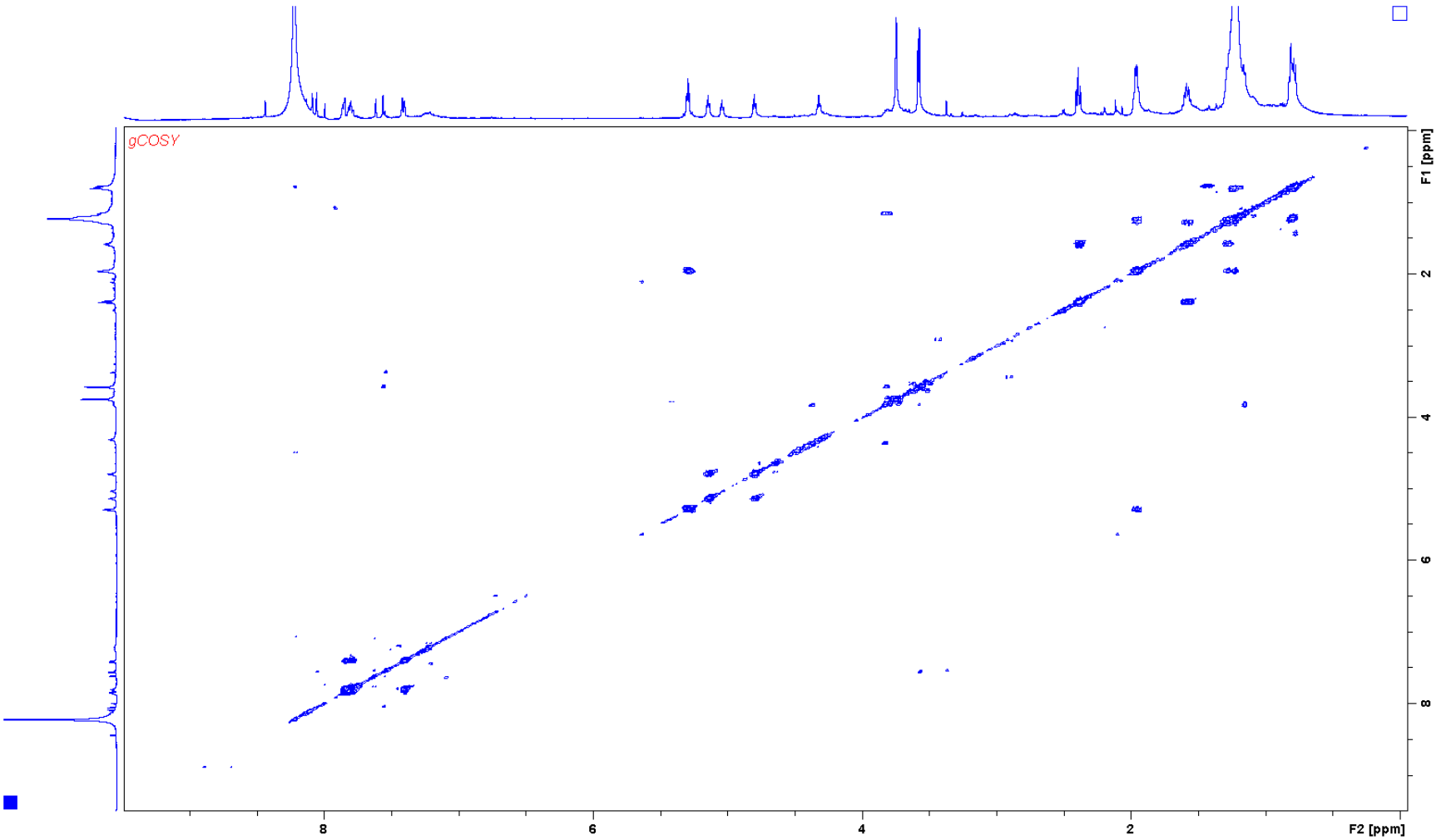

Figure S 42: COSY NMR spectrum of pyxidicycline A in formic acid-d_2

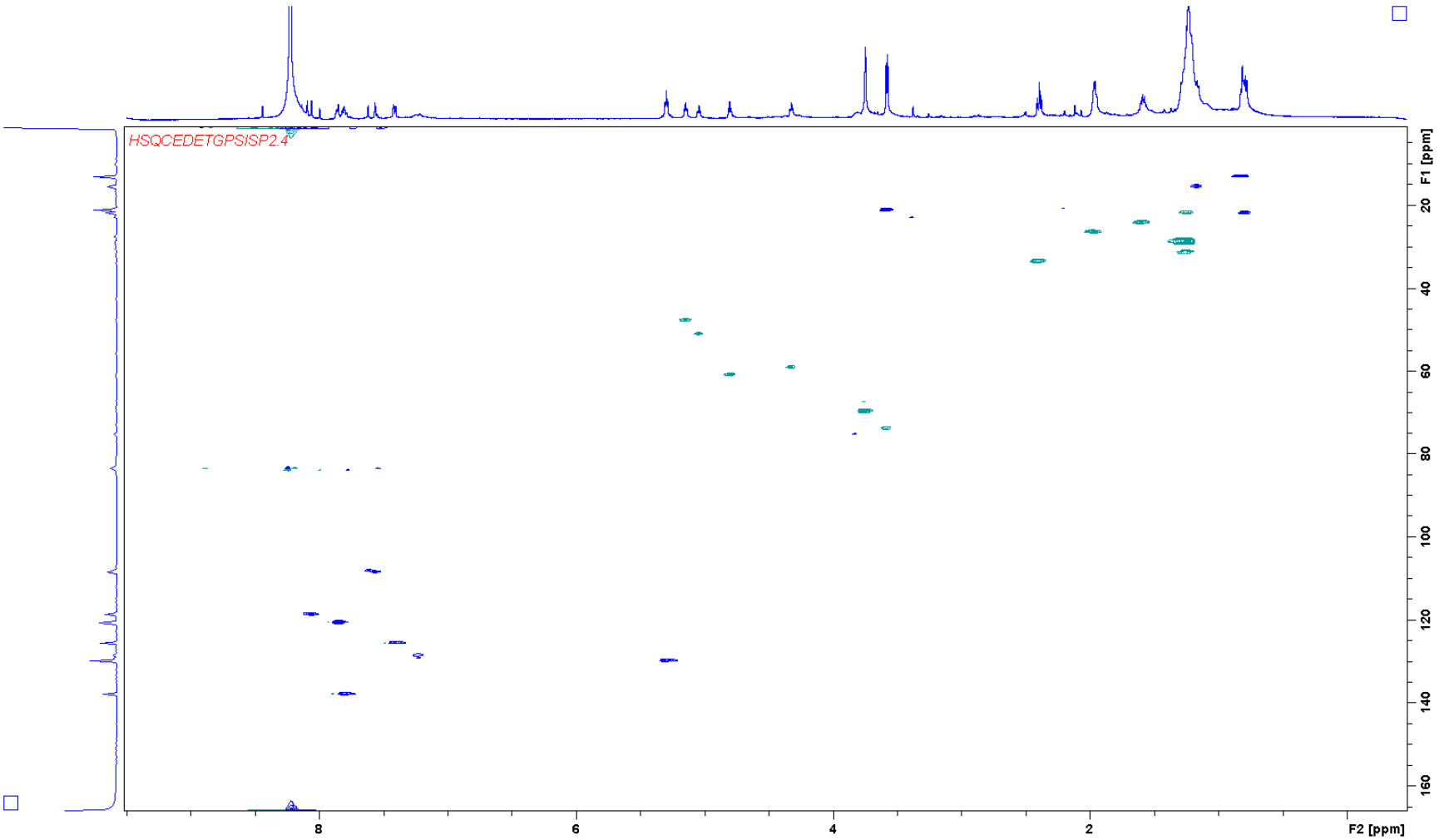

Figure S 43: HSQC NMR spectrum of pyxidicycline A in formic acid-d_2

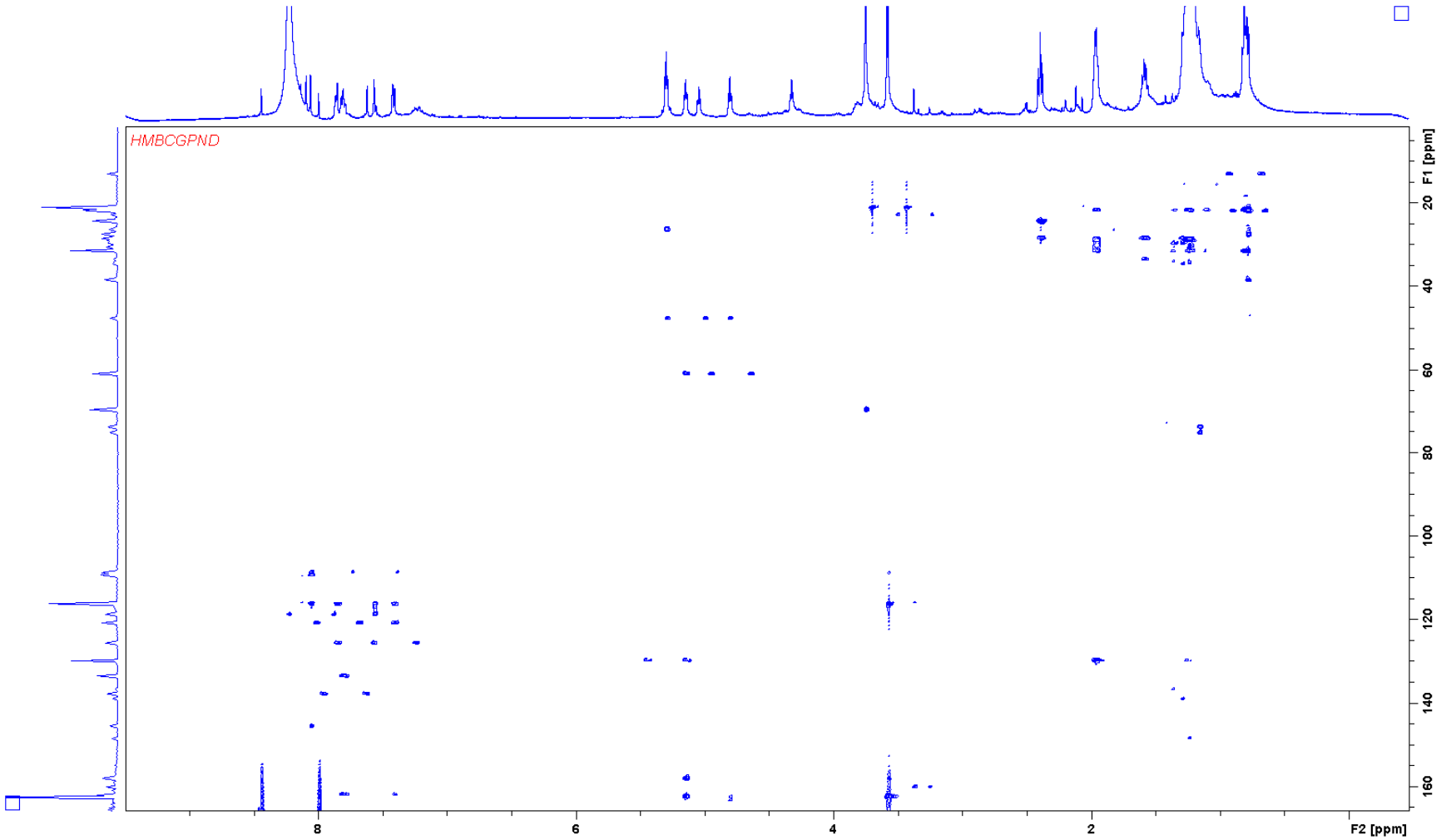

Figure S 44: HMBC NMR spectrum of pyxidicycline A in formic acid-d_2

Pyxidicycline B

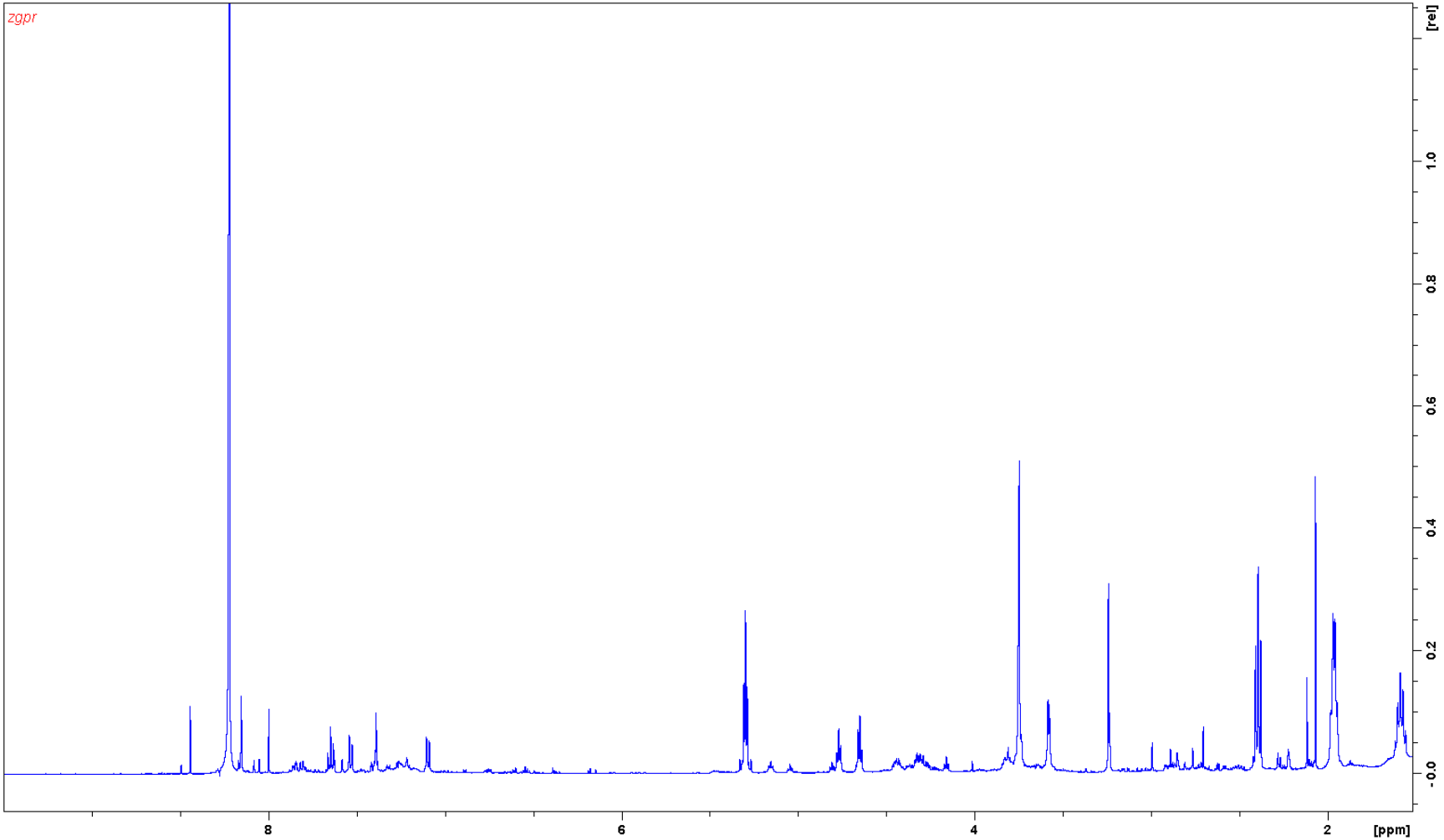

Figure S 45: ^{1}H NMR spectrum of pyxidicycline B in formic acid-d_2

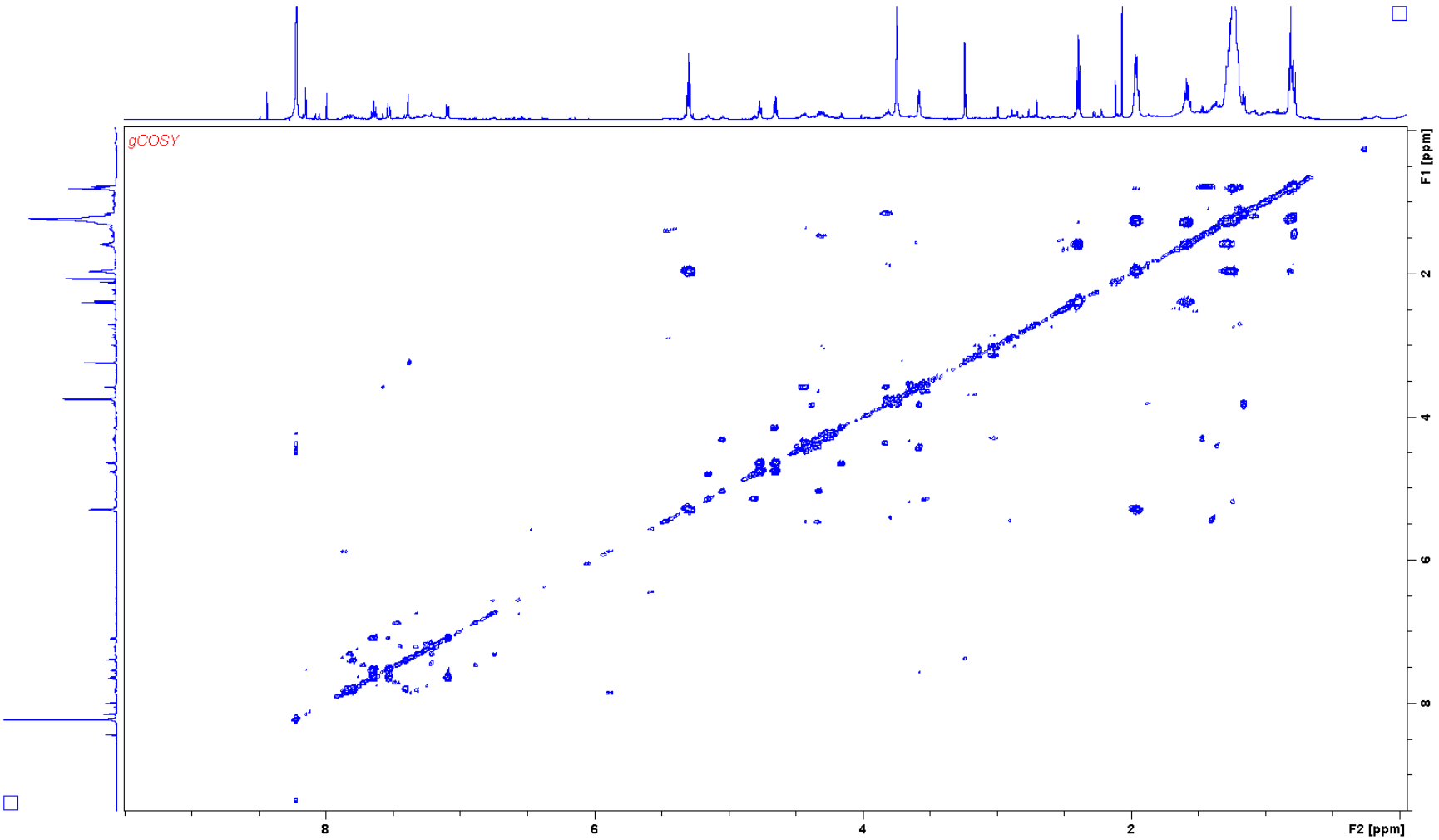

Figure S 46: COSY NMR spectrum of pyxidicycline B in formic acid-d_2

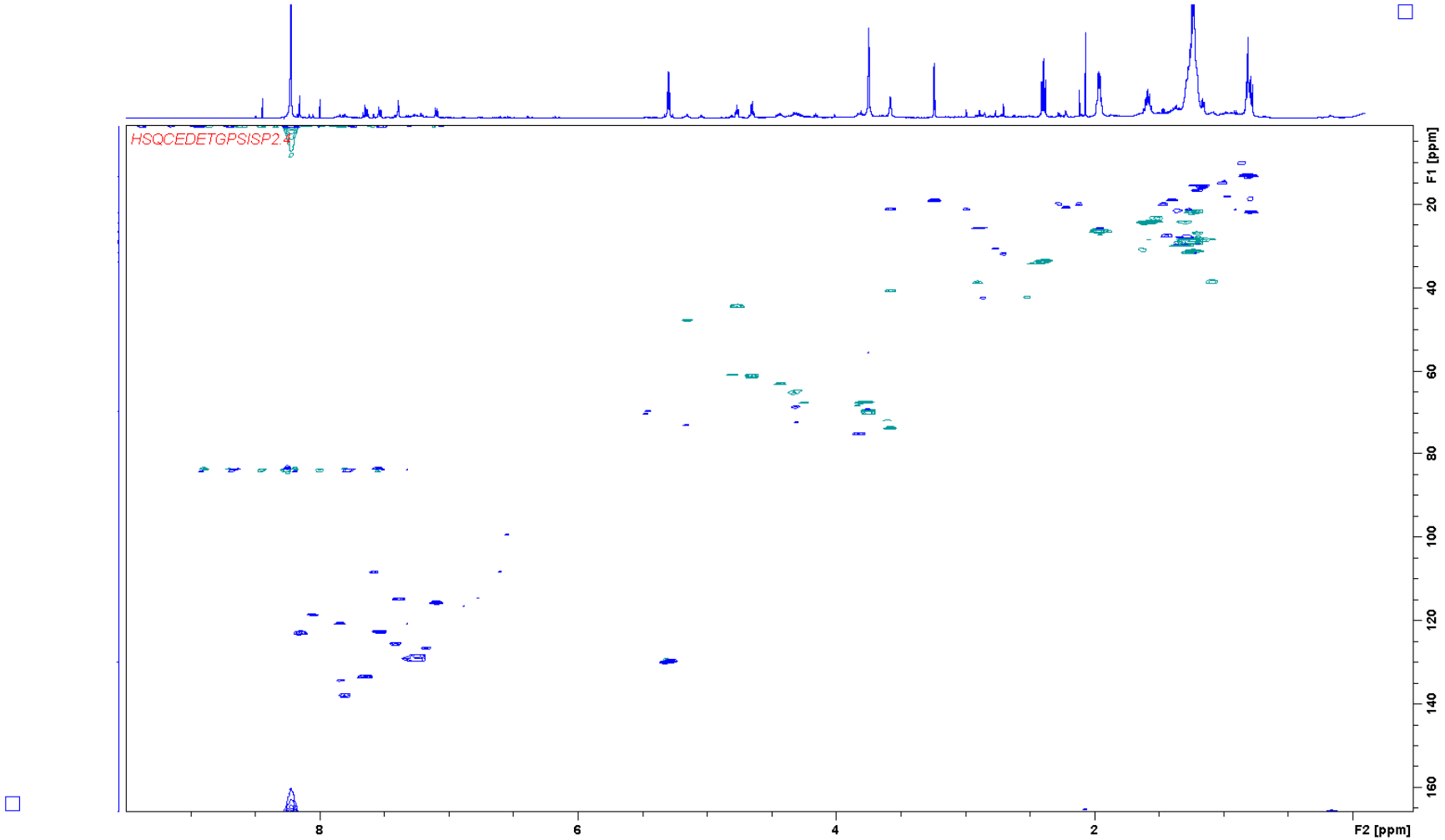

Figure S 47: HSQC NMR spectrum of pyxidicycline B in formic acid-d_2

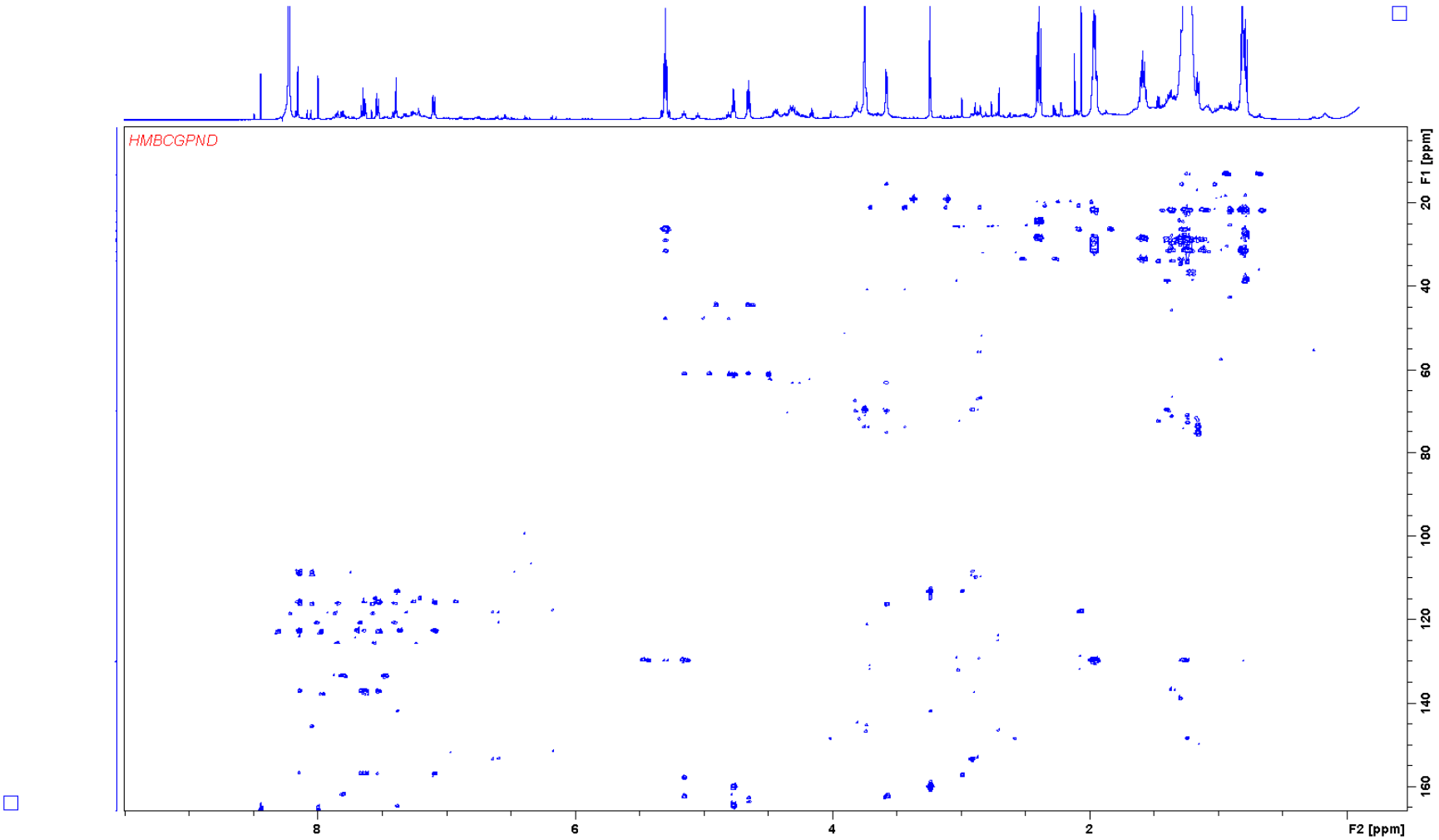

Figure S 48: HMBC NMR spectrum of pyxidicycline B in formic acid-d_2

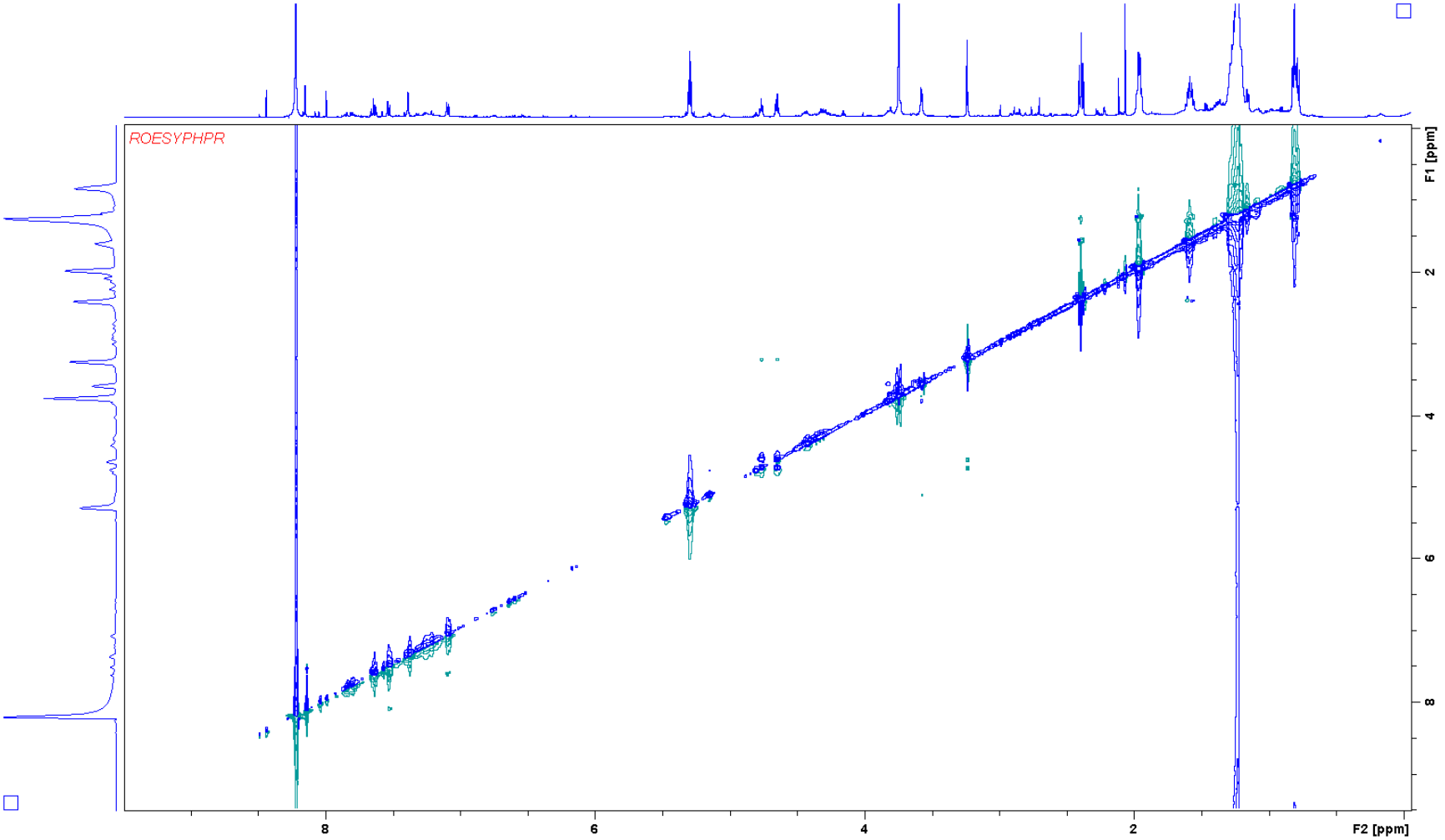

Figure S 49: ROESY NMR spectrum of pyxidicycline B in formic acid-d_2

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