

# Revised structure of anthelvencin A and characterization of the anthelvencin biosynthetic gene cluster

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## Supporting Information

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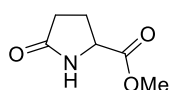
## EXPERIMENTAL PART:

### Description of the synthetic strategy followed to synthesize 5-amino-3,4-dihydro-2H-pyrrole-2-carboxylate (**4**)

#### General remarks

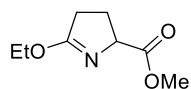
All reactions were carried out under inert atmosphere, in oven-dried glassware, using dry solvents unless otherwise specified. All commercially available compounds were purchased from Aldrich Chemical Co., Acros Organics, or Alfa Aesar and used as received. Analytical thin layer chromatography (TLC) was performed on silica gel plates (Merck 60F<sub>254</sub>) visualised either with a UV lamp (254 nm) or by using solutions of *p*-anisaldehyde/sulfuric acid/acetic acid (AcOH) in ethanol (EtOH) or KMnO<sub>4</sub>/K<sub>2</sub>CO<sub>3</sub>/AcOH in water followed by heating. Flash chromatography was performed on silica gel (60-230 mesh) unless otherwise specified. Organic extracts were dried over anhydrous MgSO<sub>4</sub>. <sup>1</sup>H (250 or 500 MHz), and <sup>13</sup>C (125 MHz) NMR spectra were recorded on a Bruker Nanobay Avance III 250 or a Bruker Avancell 500 in CDCl<sub>3</sub> or DMSO-*d*<sub>6</sub>, and calibrated using residual undeuterated solvent as an internal reference. Chemical shifts are reported in ppm, multiplicities are indicated by s (singlet), d (doublet), t (triplet), q (quartet), p (pentet), and m (multiplet or overlap of nonequivalent resonances), dd (doublet of doublets), td (triplet of doublets), and br (broad signal). Coupling constants, *J*, are reported in hertz (Hz). All NMR spectra were obtained at 300 K unless otherwise specified.

#### Synthesis of 5-oxoproline methyl ester (**8**)



DL-Pyrroglutamic acid (**7**) (20 g, 154 mmol, 1 eq.) was dissolved in dry methanol (70 mL). The solution was cooled to 10 °C using an ice-salt water bath, then thionyl chloride (22 mL, 308 mmol, 2 eq.) was added dropwise via a syringe. Dry N,N-dimethylformamide (DMF) (0.3 mL, 3.5 mmol, 2 mol%) was added. The reaction was allowed to warm up to room temperature and stirring was continued for 24 h. The solvent was finally removed under reduced pressure and the crude product was purified by distillation (130-150 °C, 20 mbar). Pure compound (**8**) was isolated as a colorless oil (19.7 g, 138 mmol, 89% yield). Racemic compound. <sup>1</sup>H NMR (250 MHz, CDCl<sub>3</sub>): δ 7.38 (s br, 1H), 4.25 – 4.19 (m, 1H), 3.70 (s, 3H), 2.45 – 2.27 (m, 3H), 2.20 – 2.12 (m, 1H) ppm. Spectroscopic data were consistent with the literature data for this compound (Drauz et al., 1986).

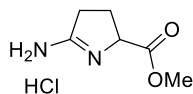
#### Synthesis of methyl 5-ethoxy-3,4-dihydro-2H-pyrrole-2-carboxylate (**9**)



To a stirred solution of ester (**8**) (5.3 g, 37 mmol, 1 eq.) in dry dichloromethane (DCM) (50 mL) was added triethyloxonium tetrafluoroborate (1 M solution in dry DCM, 50 mL, 53 mmol, 1.4 eq.). The resulting mixture was stirred at room temperature for 48 h under an argon atmosphere. The reaction was then quenched with a saturated solution of NaHCO<sub>3</sub> (40 mL). Once the effervescence had subsided, the organic layer was separated and the aqueous phase was extracted with DCM (2 x 30 mL). The combined organic layers were dried over MgSO<sub>4</sub>, filtered and concentrated under reduced pressure to afford product (**9**) as a yellow oil (6.2 g, 36 mmol, 98% yield). This substrate was used in the following synthetic steps without any further purification. Racemic compound. <sup>1</sup>H NMR (250 MHz, CDCl<sub>3</sub>): δ 4.54 – 4.47 (m, 1H), 4.26 – 4.18 (m, 2H), 3.71 (s, 3H), 2.59 – 2.46 (m, 2H), 2.33 – 2.24 (m, 1H), 2.19 – 2.10

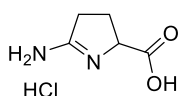
(m, 1H), 128 (t,  $J = 7.1$  Hz, 3H) ppm. Spectroscopic data were consistent with the literature data for this compound (Lee and Lown, 1987).

#### Synthesis of methyl 5-amino-3,4-dihydro-2H-pyrrole-2-carboxylate hydrochloride (10)



A stirred solution of compound (**9**) (5.7 g, 33 mmol, 1 eq.), and anhydrous  $\text{NH}_4\text{Cl}$  (1.9 g, 35 mmol, 1.05 eq.) in dry methanol (30 mL) was heated at reflux for 5 h under an argon atmosphere. The solvent was then removed under reduced pressure. The crude product was purified by recrystallization from DCM/cyclohexane. Pure compound (**10**) was isolated as a white solid (3.6 g, 20.1 mmol, 61% yield). Racemic product.  **$^1\text{H}$  NMR** (250 MHz,  $\text{DMSO}-d_6$ ):  $\delta$  4.59 (dd,  $J = 9.1, 4.8$  Hz, 1H), 3.68 (s, 3H), 2.82 (t,  $J = 8.6$  Hz, 2H), 2.45 – 2.38 (m, 1H), 2.11 – 2.04 (m, 1H) ppm. Spectroscopic data were consistent with the literature data for this compound (Lee and Lown, 1987).

#### Synthesis of 5-amino-3,4-dihydro-2H-pyrrole-2-carboxylate hydrochloride (4)



Derivative (**10**) (1.14 g, 6.38 mmol, 1 eq.) was dissolved in an aqueous solution of hydrochloric acid (10% v/v, 50 mL), and stirred at 50 °C for 3 h. Toluene (15 mL), was then added, and the mixture was concentrated under reduced pressure. The crude product was finally collected, and dried under high vacuum at 65 °C to afford acid (**4**) as a white solid (1.1g, 6.37 mmol, quantitative yield). Racemic product.  **$^1\text{H}$  NMR** (500 MHz,  $\text{DMSO}-d_6$ ):  $\delta$  13.27 (s br, 1H), 9.84 (s br, 1H), 9.53 (s br, 1H), 9.15 (s br, 1H), 4.49 (dd,  $J = 9.1, 4.9$  Hz, 1H), 2.83 – 2.79 (m, 2H), 2.46 – 2.28 (m, 1H), 2.10 – 2.07 (m, 1H) ppm.  **$^{13}\text{C}$  NMR** (125MHz,  $\text{DMSO}-d_6$ ): 172.1 (C), 171.5 (C), 60.1 (CH), 29.4 ( $\text{CH}_2$ ), 24.7 ( $\text{CH}_2$ ) ppm. Spectroscopic data were consistent with the literature data for this compound (Lee and Lown, 1987).

## TABLES

**Table S1:** Strains used in this study

Strain	Description	Resistance gene	Reference
<i>Escherichia coli</i> DH5α	General cloning host	None	Promega
<i>E. coli</i> ET12567 pUZ8002	Host strain for conjugation from <i>E. coli</i> to <i>Streptomyces</i>		(Flett et al., 1997)
<i>Streptomyces venezuelae</i> ATCC 14583	Anthelvencin producer	None	(Probst et al., 1965)
ANT007	<i>S. venezuelae</i> replacement mutant of <i>ant8</i>	<i>aac(3)IV</i>	This study
ANT008	<i>S. venezuelae</i> replacement mutant of <i>ant23</i>	<i>aac(3)IV</i>	This study
ANT009	<i>S. venezuelae</i> replacement mutant of <i>ant24</i>	<i>aac(3)IV</i>	This study
ANT012	<i>S. venezuelae</i> with pANT012 overproducing anthelvencin and methylanthevencin	<i>aac(3)IV</i>	This study
ANT013	ANT008 containing pANT013	<i>aac(3)IV</i> <i>aph(7'')</i>	This study
ANT014	ANT009 containing pANT013	<i>aac(3)IV</i> <i>aph(7'')</i>	This study

**Table S2:** Plasmids used in this study

Plasmid	Description	Reference
pCR®-Blunt	<i>E. coli</i> cloning vector	Invitrogen
pOSV400	Suicide vector for gene disruption in <i>Streptomyces</i> , bearing the <i>aph(7'')</i> gene cassette (hygromycin resistance)	(Boubakri et al., 2015)
pOSV802	Plasmid bearing the <i>aac(3)IV</i> gene cassette (apramycin resistance) and the $\Phi$ C31 integrase gene	(Aubry et al., 2019)
pOSV806	Plasmid bearing the <i>aph(7'')</i> gene cassette (hygromycin resistance) and the $\Phi$ C31 integrase gene	(Aubry et al., 2019)
pW60	Source of the <i>aac(3)IV</i> gene cassette	(Corre et al., 2008)
pANT001	Plasmid pCR®-Blunt containing a 1.8 kb DNA fragment upstream of <i>ant8</i>	This study
pANT002	Plasmid pCR®-Blunt containing a 2.0 kb DNA fragment downstream of <i>ant8</i>	This study
pANT003	Plasmid pCR®-Blunt containing a 1.8 kb DNA fragment upstream of <i>ant23</i>	This study
pANT004	Plasmid pCR®-Blunt containing a 1.8 kb DNA fragment downstream of <i>ant23</i>	This study
pANT005	Plasmid pCR®-Blunt containing a 1.7 kb DNA fragment upstream of <i>ant24</i>	This study
pANT006	Plasmid pCR®-Blunt containing a 1.4 kb DNA fragment downstream of <i>ant24</i>	This study
pANT007	pOSV400 derivative used for the replacement of <i>ant8</i> by the <i>aac(3)IV</i> gene cassette	This study
pANT008	pOSV400 derivative used for the replacement of <i>ant23</i> by the <i>aac(3)IV</i> gene cassette	This study
pANT009	pOSV400 derivative used for the replacement of <i>ant24</i> by the <i>aac(3)IV</i> gene cassette	This study
pANT011	Plasmid pCR®-Blunt containing a 2.0 kb fragment containing <i>ant23</i> and <i>ant24</i>	This study
pCEA005	pOSV802 containing <i>rpsL</i> (TP)p and the <i>tipA</i> RBS	(Aubry et al., 2019)
pANT012	pCEA005 derivative used for the overexpression of <i>ant23</i> and <i>ant24</i>	This study
pANT013	pOSV806 plasmid containing <i>ant23-ant24</i> under the <i>rpsL</i> (TP)p with <i>aph(7'')</i> gene cassette	This study

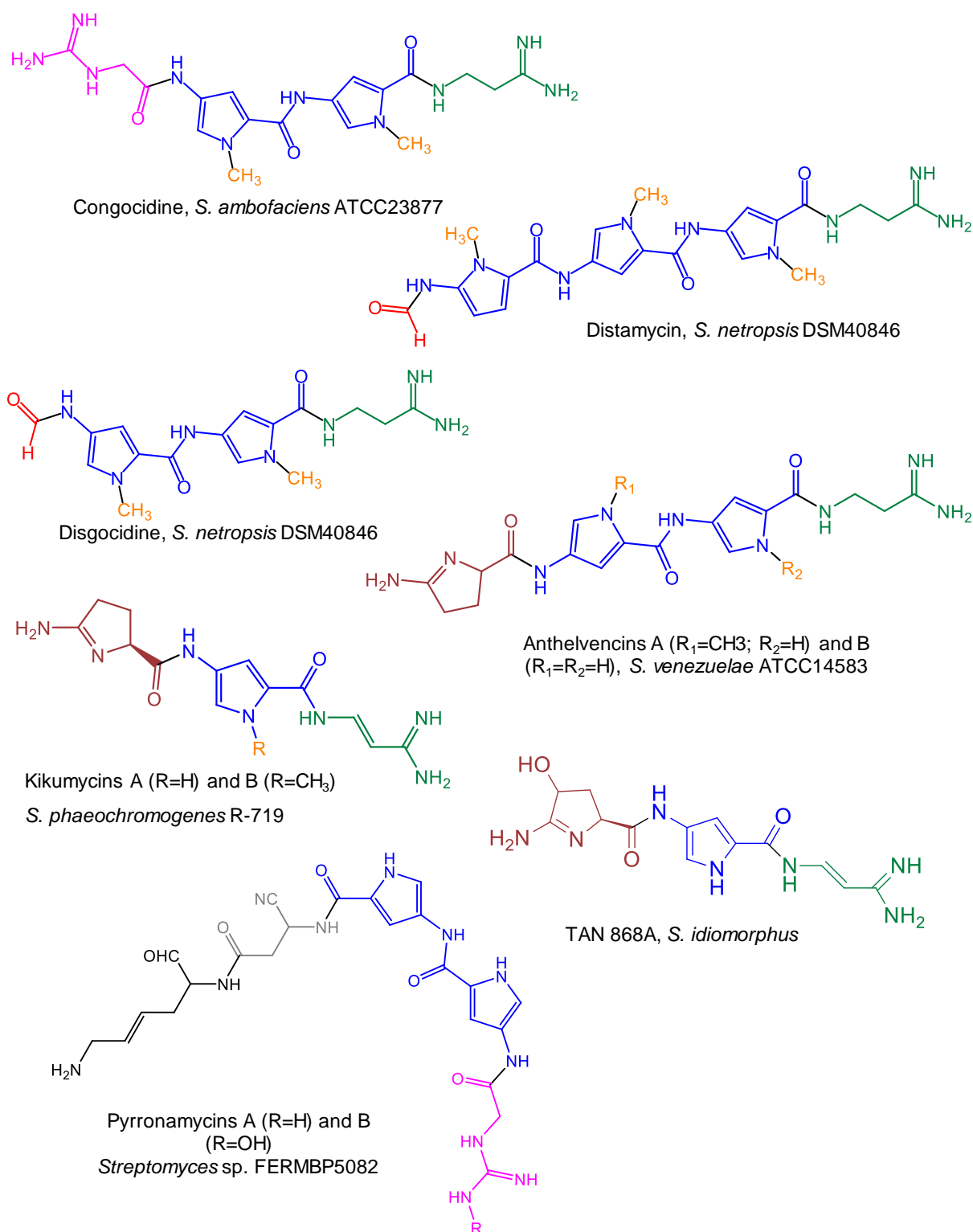
**Table S3:** Oligonucleotides used in this study

Name	Sequence	Description
CEA001	CAGTA <u>AAGCTT</u> CATGCGGTCGCGTACTGATG	Forward primer for region upstream of <i>ant8</i> , <i>HindIII</i> site underlined
CEA002	CAGT <u>CTCGAGT</u> GGGCCAGGAAGCAGTGATG	Reverse primer for region upstream of <i>ant8</i> , <i>XhoI</i> site underlined
CEA003	CAGT <u>ACTAGT</u> CTTGTCGTGGCCGTGTTCTC	Forward primer for region downstream of <i>ant8</i> , <i>SpeI</i> site underlined
CEA004	CAGTA <u>AAGCTT</u> GGCCGTGCGTAAGAAGATCC	Reverse primer for region downstream of <i>ant8</i> , <i>HindIII</i> site underlined
CEA005	CAGT <u>CTCGAG</u> ACCAAGGGAGTCGAGGAATG	Forward primer for region upstream of <i>ant23</i> , <i>XhoI</i> site underlined
CEA006	CAGTA <u>AAGCTT</u> CCCTAGTAGCTCGAATGCAC	Reverse primer for region upstream of <i>ant23</i> , <i>HindIII</i> site underlined
CEA007	CAGTA <u>AAGCTT</u> TCACATGCCGCTGCTCACAC	Forward primer for region downstream of <i>ant23</i> , <i>HindIII</i> site underlined
CEA008	CAGT <u>ACTAGT</u> AACCTGATCGGCGCCTACAC	Reverse primer for region downstream of <i>ant23</i> , <i>SpeI</i> site underlined
CEA009	CAGT <u>CTCGAG</u> CACCGAGATCGGTCTCTACC	Forward primer for region upstream of <i>ant24</i> , <i>XhoI</i> site underlined
CEA010	CAGTA <u>AAGCTT</u> CGCCCGGCTTCTATAAAACC	Reverse primer for region upstream of <i>ant24</i> , <i>HindIII</i> site underlined
CEA011	CAGTA <u>AAGCTT</u> CTCACTCCCGGTGTGCATTTCG	Forward primer for region downstream of <i>ant24</i> , <i>HindIII</i> site underlined
CEA012	CAGT <u>ACTAGT</u> CGGCCGCCCTCTTCTGACC	Reverse primer for region downstream of <i>ant24</i> , <i>XhoI</i> site underlined
A5	CGACGTGGCAGGATCGAACG	Internal to <i>aac(3)IV</i> , used to confirm correct replacement in mutants
A6	GTCAACTGGGCCGAGATCCG	Internal to <i>aac(3)IV</i> , used to confirm correct replacement in mutants
CEA013	GTGAACTGATGCGCACCGAC	Control of the correct replacement of <i>ant8</i>
CEA014	GGGCTTTCTCCGTTTGCTTC	Control of the correct replacement of <i>ant8</i>
CEA015	AGAGCCTGTTCCGGCACCTG	Control of the correct replacement of <i>ant8</i> around <i>aac(3)IV</i> cassette

Name	Sequence	Description
CEA016	CCAGGTGCAGGCCGATGAAG	Control of the correct replacement of <i>ant8</i> around <i>aac(3)IV</i> cassette
CEA018	CACGGCATGACGCTGATGTG	Control of the correct replacement of <i>ant23</i> around <i>aac(3)IV</i> cassette
CEA019	TTCCTCGCGGAGAAGGGCTG	Control of the correct replacement of <i>ant23</i> around <i>aac(3)IV</i> cassette
CEA020	CGGGCACTTCAGTACCGGTC	Control of the correct replacement of <i>ant24</i>
CEA021	ATGCTGCGGAGACTCAGCAC	Control of the correct replacement of <i>ant24</i>
CEA022	GTGTCGGGCATGCTTTCCTG	Control of the correct replacement of <i>ant24</i>
CEA023	GCACGTCTCCCTCTTCATGC	Control of the correct replacement of <i>ant23</i>
CEA034	<u>ATGCATGCGGCCGCTGCTAGCGATGGCGAG</u> GTTTTATAGAAGCC	Amplification of the region <i>ant23-ant24</i> , <i>NsiI</i> site double underlined, <i>NotI</i> and <i>NheI</i> sites underlined
CEA035	<u>CTTAAGGCGGCCGCTACTAGTGTGTGAGCA</u> GCGGCATGTG	Amplification of the region <i>ant23-ant24</i> , <i>AflII</i> site double underlined, <i>NotI</i> and <i>SpeI</i> sites underlined
CEA_vec_seq14	ATTTCAAGTGCAATTTATCTCTTC	Confirmation of the integration of pANT012 in <i>S. venezuelae</i>
CEA_vec_seq15	TTCGATCACGTGGGCGAAGC	Confirmation of the integration of pANT012 in <i>S. venezuelae</i>

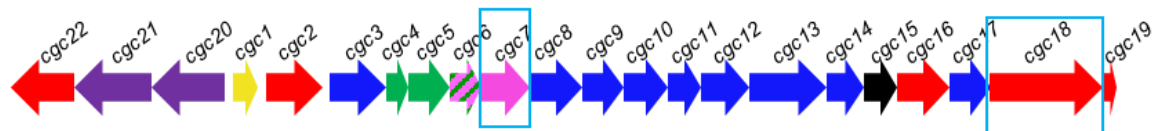


## FIGURES

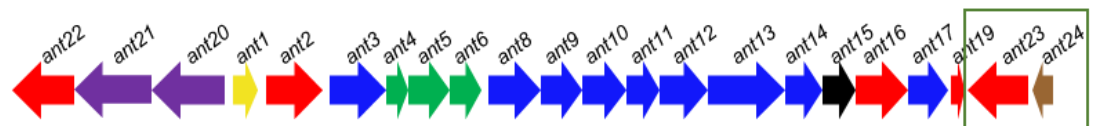


**Figure S1:** Structures of members of the pyrrolamide family and name of the *Streptomyces* producers. In a pyrrolamide molecule, the different colors correspond to the different precursors. Common precursors to several pyrrolamides have the same color.

***S. ambofaciens* ATCC23877 *cgc* cluster**



***S. venezuelae* ATCC14583 *ant* cluster**



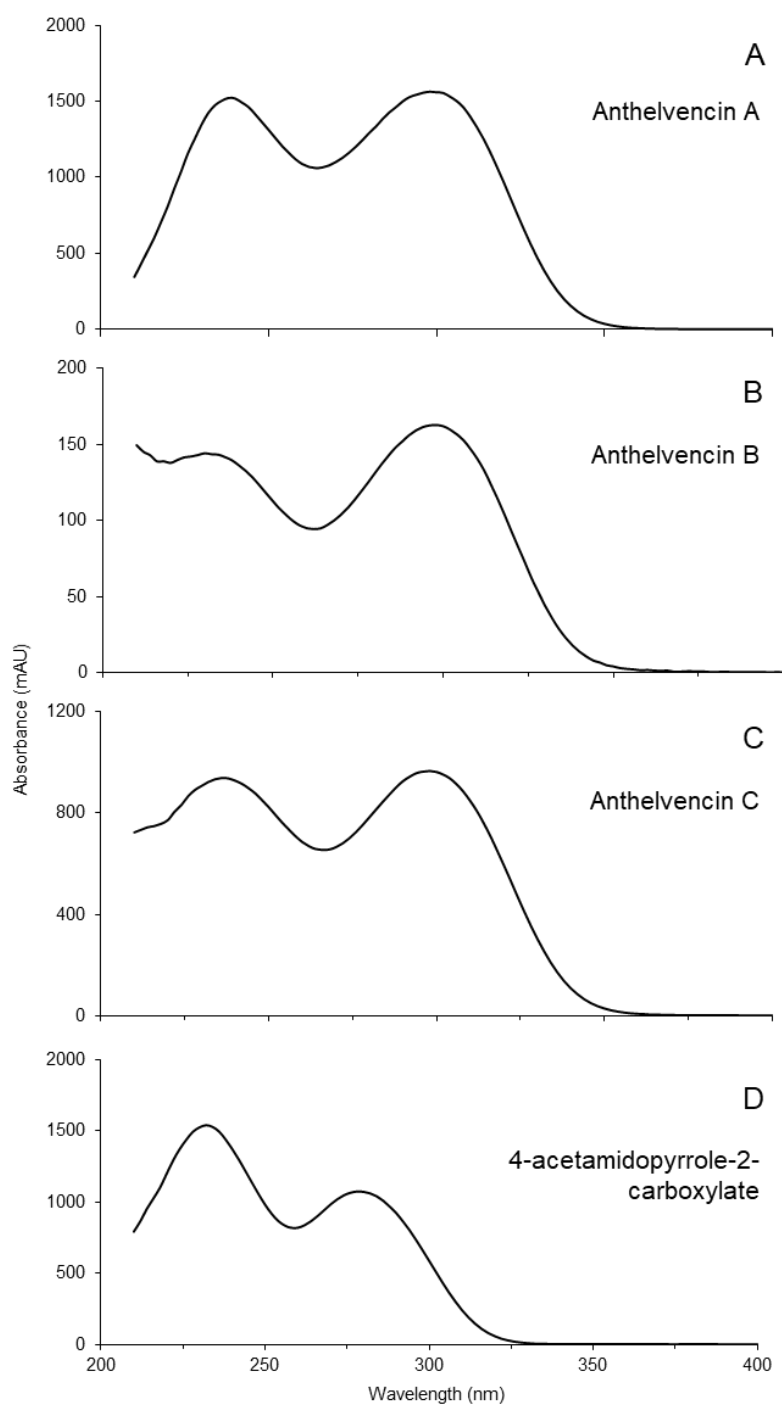
2 kb

- Resistance genes
- Regulatory gene
- Tailoring gene
- Genes involved in assembly

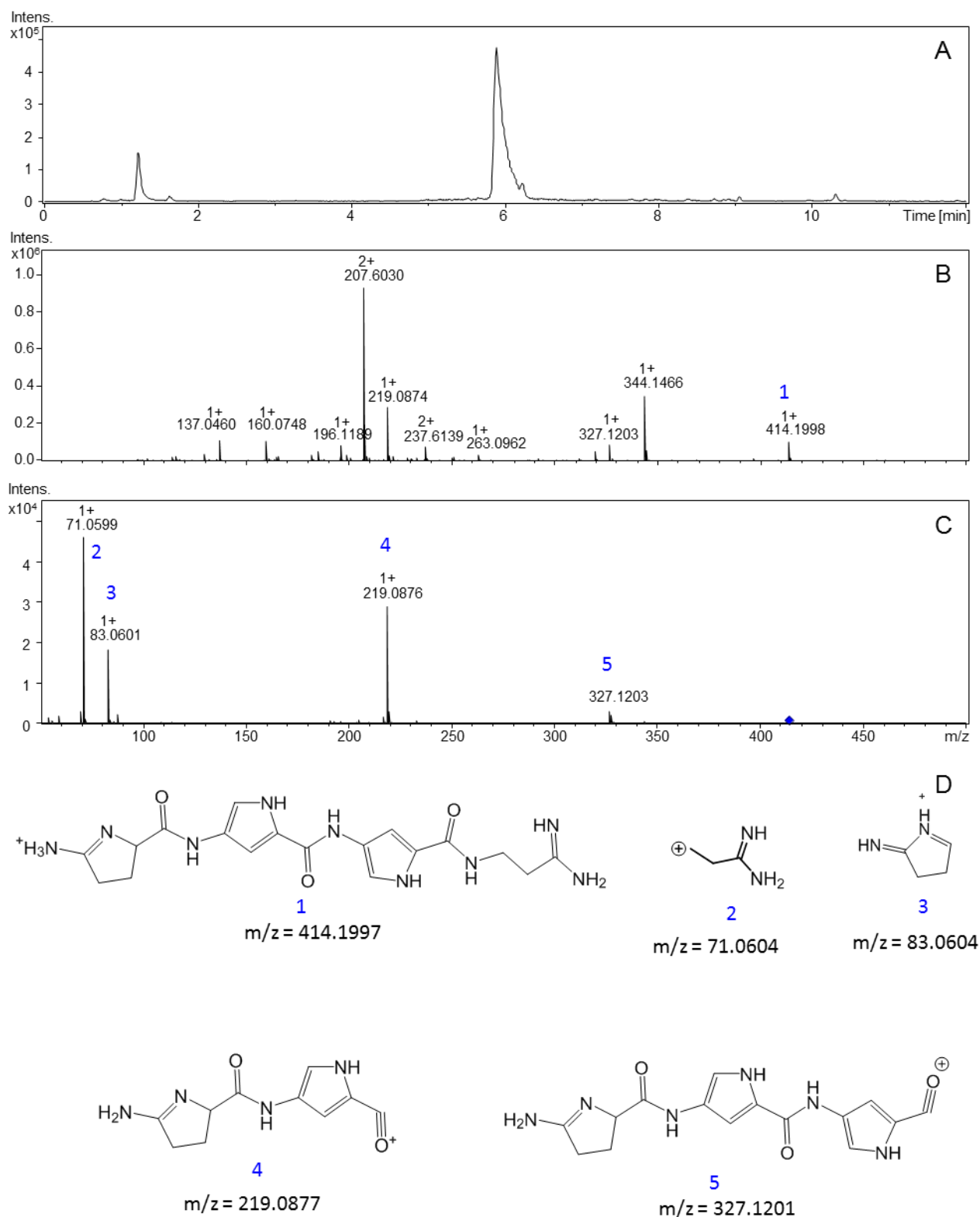
Genes involved in precursor biosynthesis

- 4-acetamidopyrrole-2-carboxylate (**5**)
- 5-amino-3,4-dihydro-2Hpyrrole-2-carboxylate (**4**)
- 3-aminopropionamide (**6**)
- Guanidinoacetate

**Figure S2:** Genetic organization of the congocidine biosynthetic gene cluster in *S. ambofaciens* ATCC 23877, and of the anthelvencin biosynthetic gene cluster in *S. venezuelae* ATCC 14583. Boxed genes do not have homologs in the other biosynthetic gene cluster.



**Figure S3: UV-visible spectra** of (A) anthelvencin A, Rt = 14,3 min, (B) anthelvencin B, Rt = 13,3 min, (C) anthelvencin C, Rt = 15,5 min, and (D) 4-acetamidopyrrole-2-carboxylate, Rt = 11,5 min.



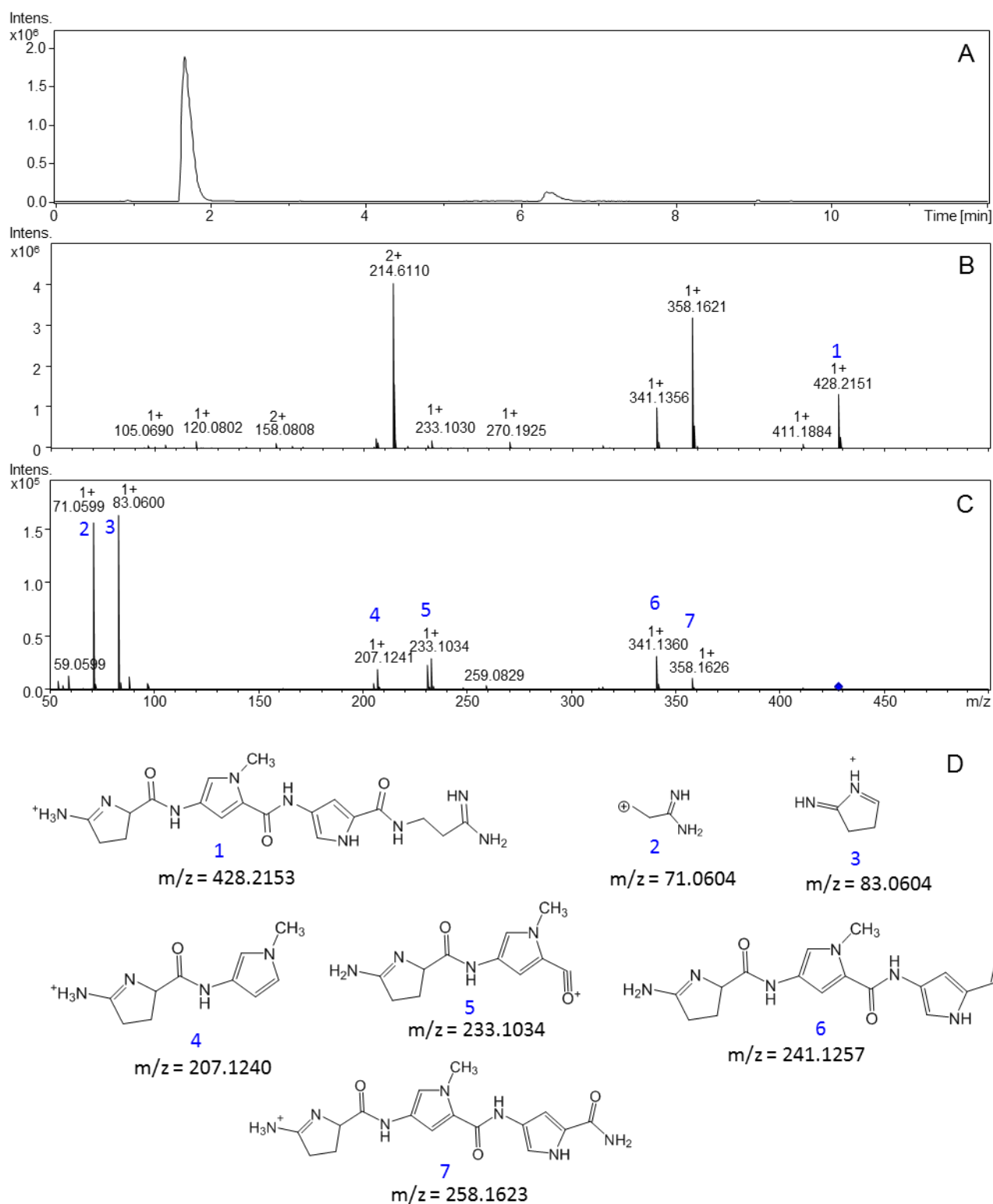
**Figure S4: Identification of anthelvencin B (Peak II) from HR-MS and HR-MS<sup>2</sup>**

(A) Extracted-ion chromatogram 414.2 +All MS

(B) HR-MS spectrum of the peak at 1.3 min in the chromatogram (A)

(C) Fragmentation of peak (1) ( $m/z = 414.1998$ )

(D) Putative structure of the obtained fragments detected in (C).



**Figure S5: Identification of anthelvencin A (peak III) from HR-MS and HR-MS<sup>2</sup>**

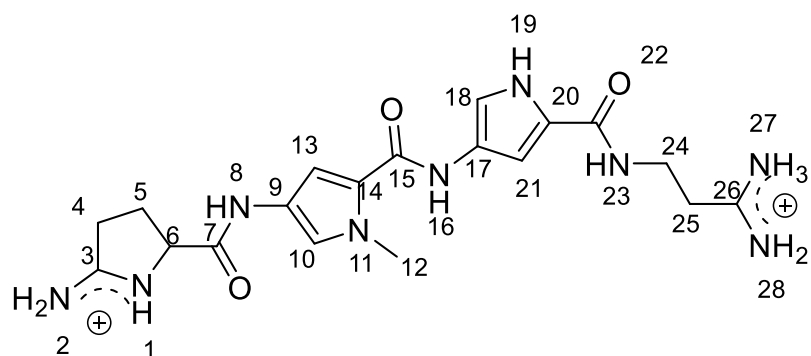
(A) Extracted-ion chromatogram 428.2100 +All MS

(B) HR-MS spectrum of the peak at 1.8 min in the chromatogram (A)

(C) Fragmentation of peak (1) (m/z = 428.2151)

(D) Putative structure of the obtained fragments detected in (C).

**Figure S6: Nuclear Magnetic Resonance (NMR) spectra of anthelvencin A**



$^1\text{H}$  NMR (500 MHz)  $\delta$  (DMSO) : 11.23 (s, 1H, H19); 10.44 (s, 1H, H8); 9.92 (s, 1H, H16); 9.78 (s, 1H); 9.29 (s, 1H); 9.03 (s, 1H), 9.01 (s, 2H); 8.64 (s, 2H); 8.31 (t,  $J=5.5$  Hz, 1H, H23); 7.20 (d,  $J=1.8$  Hz, 1H, H10); 7.14 (dd,  $J = 2.75, 1.45$ , H18); 6.98 (dd,  $J = 2.70, 1.50$ , 1H, H21); 6.94 (d,  $J= 1.80$ , 1H, H13); 4.60 (dd,  $J= 8.6, 4.6$ , 1H, H6); 3.85 (s, 3H, H12); 3.55 (q, dt,  $J = 6.15$ ; 6.25, 2H, H24); 2.85 (t,  $J = 8.35$ , 2H, H4); 2.64 (m, 2H25) ; 2.45 (m, H5); 2.37 (m); 2.04 (m, 1H, H5).

$^{13}\text{C}$  NMR (125 MHz)  $\delta$  (DMSO) : 171.7 (C3); 169.1 (C26); 167.1 (C7); 160.7 (C22); 158.3 (C15); 124.0 (C20); 123.2 (C14); 123.0 (C 9 or C17); 121.1 (C17 or C9) 118.3 (C10); 111.8 (C18) 104.0 (C21); 103.0 (C13); 61.1 (C6); 36.1 (C12); 35.8 (C24); 32.6 (C25); 29.8 (C4); 25.8 (C5)

1H NMR spectrum (400 MHz, CDCl<sub>3</sub>) of compound 10. The spectrum displays several sharp peaks in the aromatic region (6.5-7.5 ppm) and aliphatic region (2.0-4.0 ppm). Integration values are provided below the baseline for each major peak group. Chemical shift values are listed above the peaks.

Chemical Shift (ppm)	Integration
11.23	1.00
10.44	1.00
9.92	1.08
9.78	0.99
9.29	1.08
9.03	3.20
8.64	2.05
8.32	0.99
8.31	
8.30	
7.20	1.03
7.19	
7.14	
7.14	
7.13	
6.99	1.03
6.99	
6.98	
6.94	
6.94	
4.62	0.96
4.61	
4.60	
4.59	
3.96	3.05
3.56	
3.55	
3.53	
2.87	1.96
2.85	
2.84	
2.83	
2.82	
2.81	
2.80	
2.79	
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2.20	
2.19	
2.18	
2.17	
2.16	
2.15	
2.14	
2.13	
2.12	
2.11	
2.10	
2.09	
2.08	
2.07	
2.06	
2.05	
2.04	
2.03	
2.02	
0.89	
0.89	
0.88	
0.88	
0.87	
0.86	
0.85	

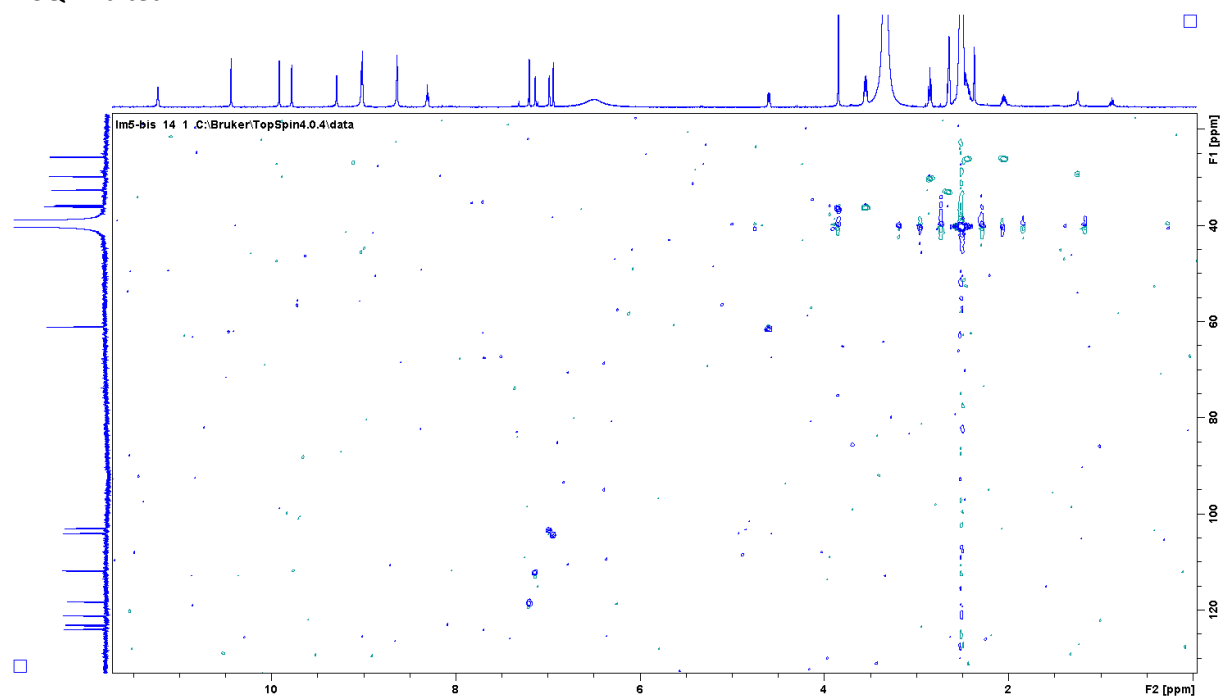
10d-bis 13 1 C:\Bruker\TopSpin6.0.4\data

13C NMR peaks (ppm):

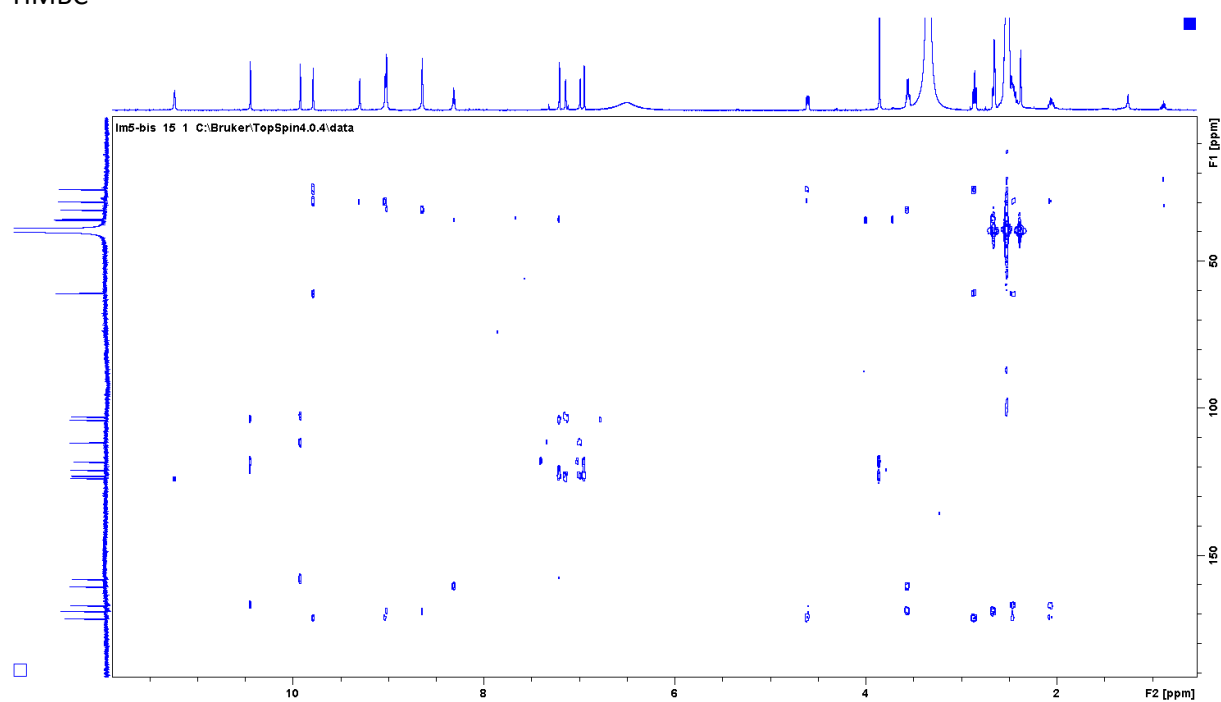
- 171.6397
- 169.0334
- 167.0601
- 160.7206
- 159.2622
- 132.6490
- 132.1647
- 131.9442
- 127.1584
- 119.2644
- 111.3467
- 104.0287
- 102.2975
- 61.0054
- 32.6284
- 32.6226
- 32.6405
- 29.3303
- 25.7679

150 100 50 [ppm]

## HSQD Edited

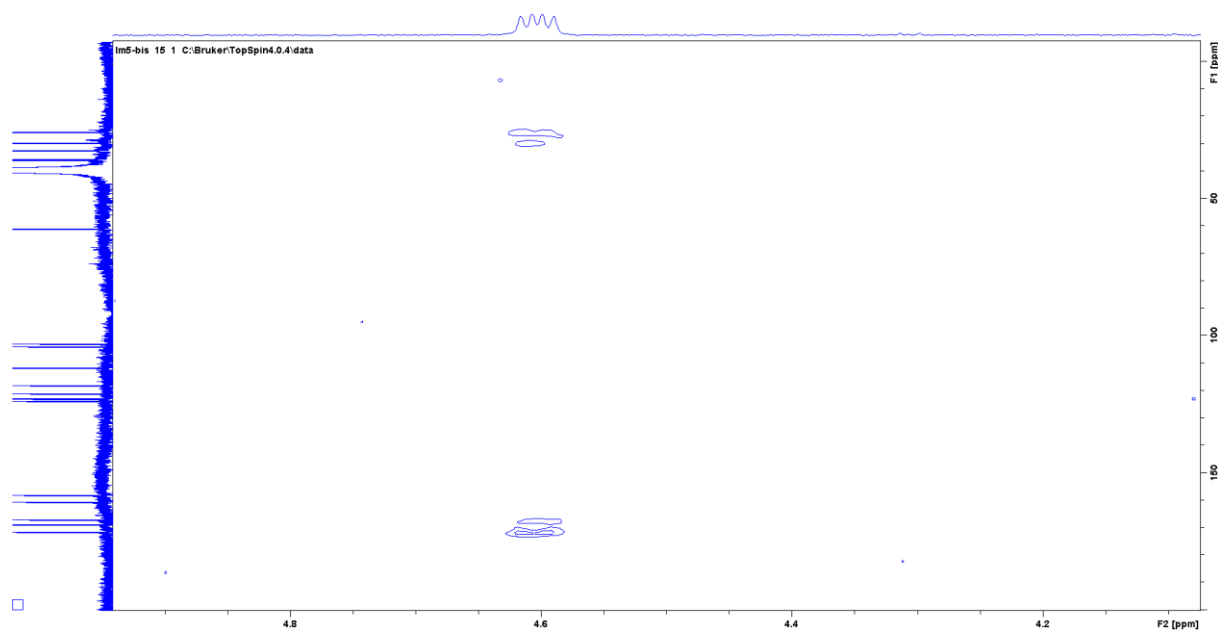


## HMBC

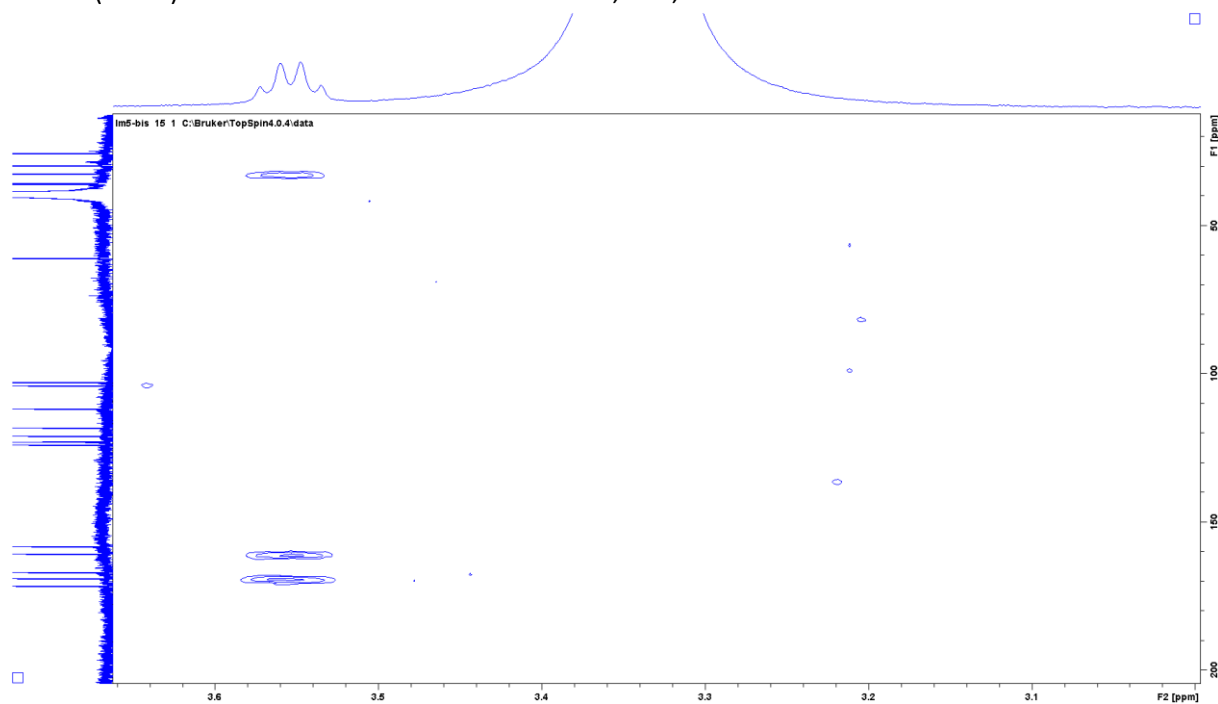




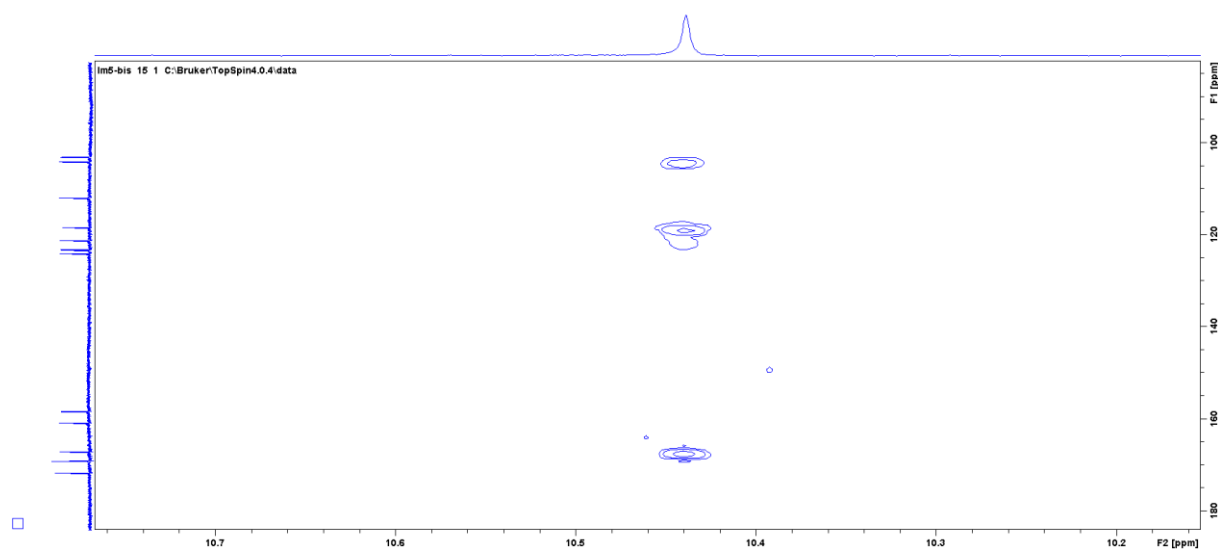
HMBC (detail) : correlations between H6 and C5, C4, C7 and C3



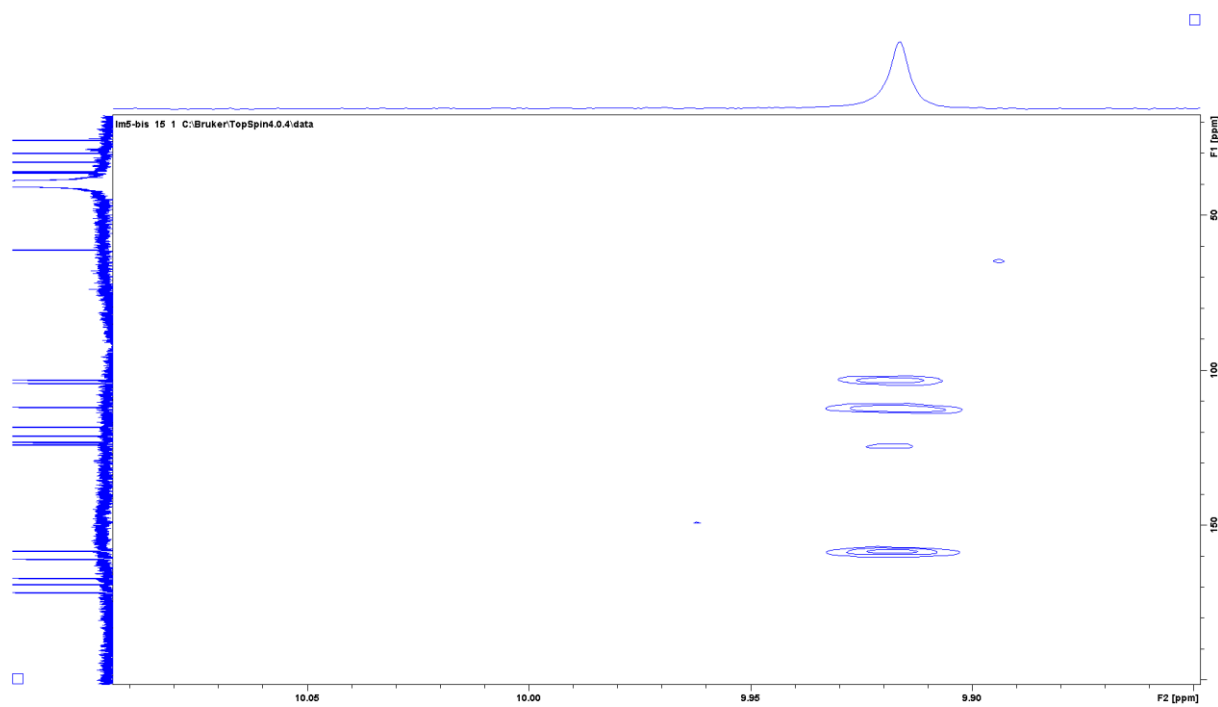
HMBC (detail) : correlations between H24 and C25, C22, C26



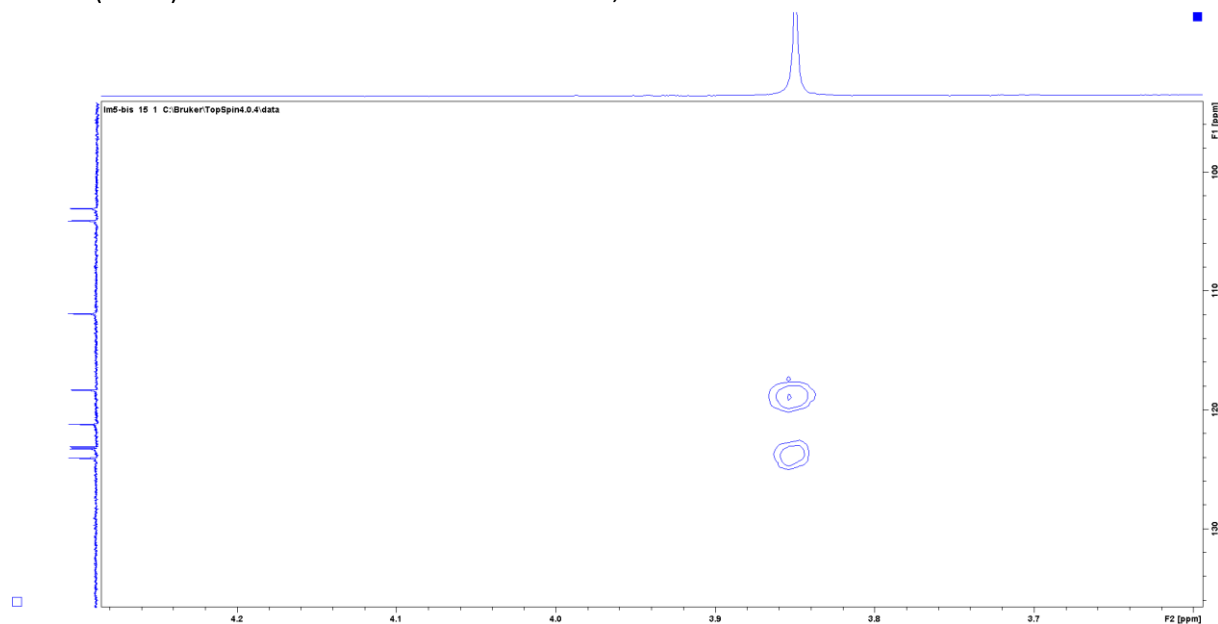
HMBC (detail) : correlations between H8 and C13, C10, C7



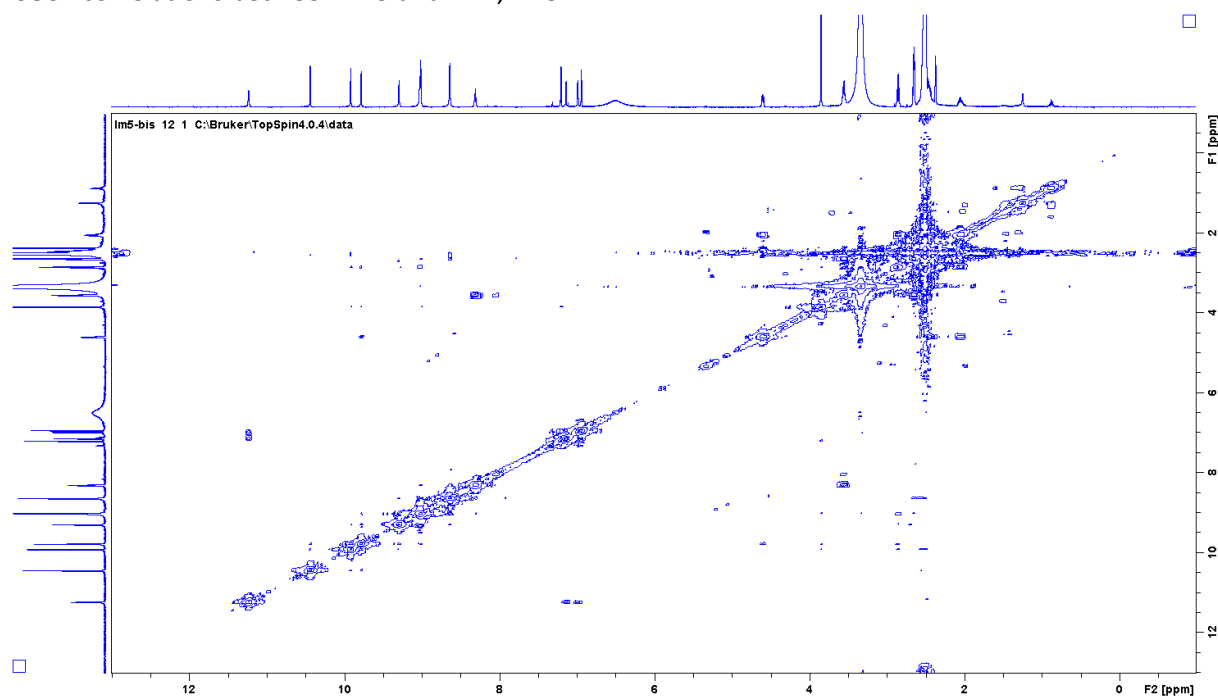
HMBC (detail) : correlations between H16 and C21, C18, C15



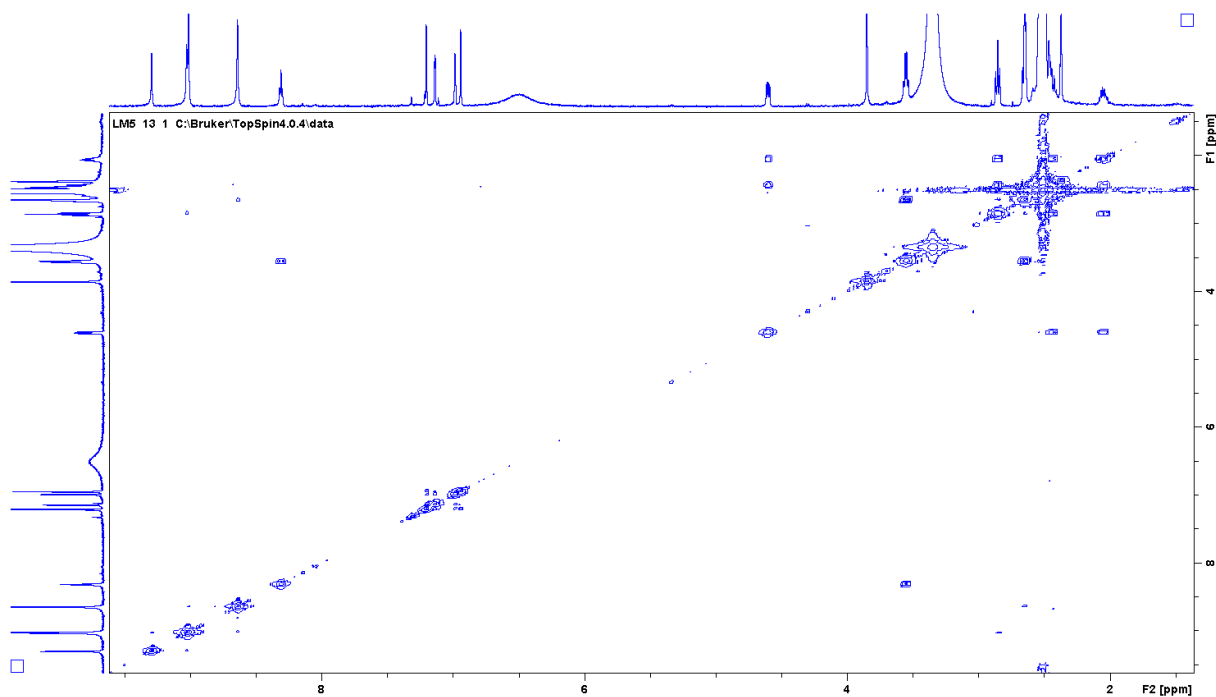
HMBC (detail) : correlations between H12 and C10, C14



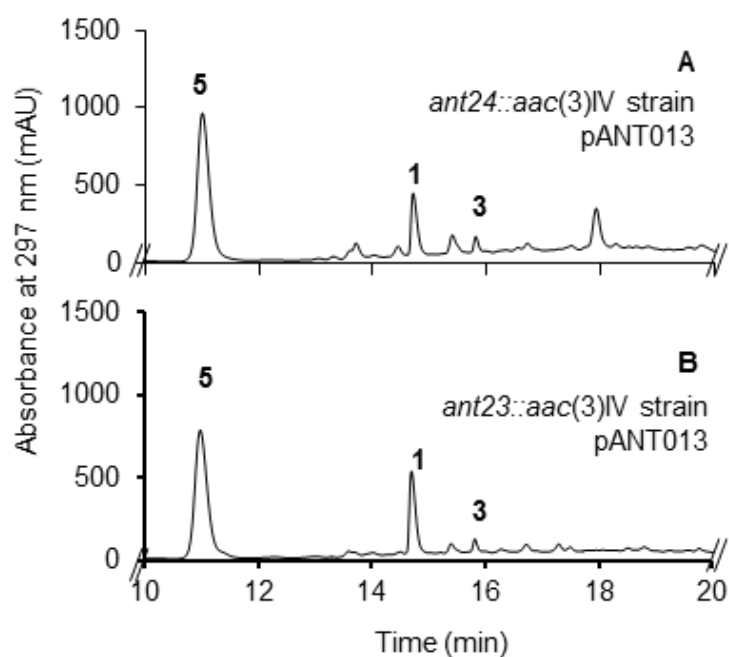
COSY correlations between H19 and H21, H18



COSY (detail) correlations between H23 and H24.





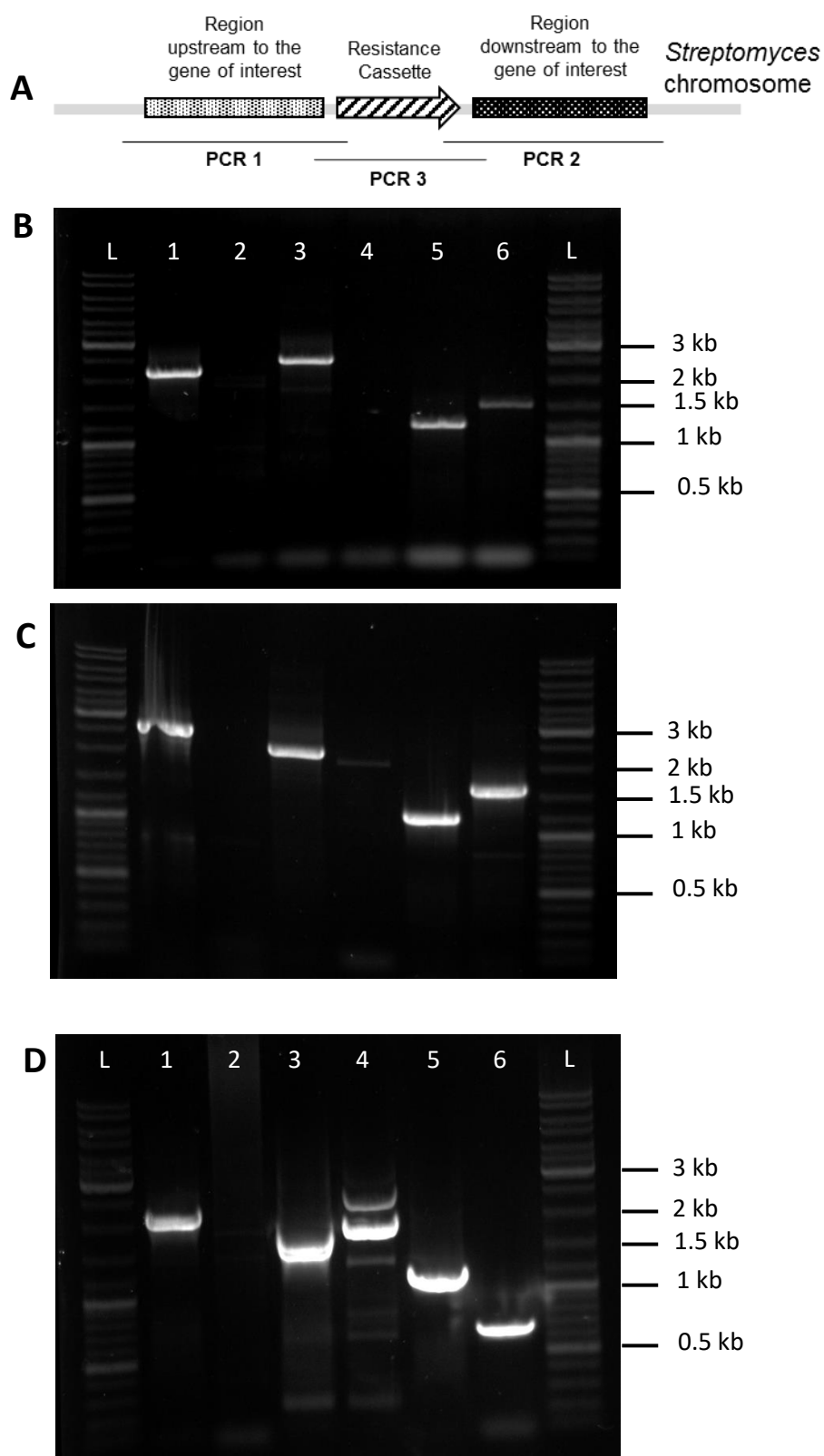


**Figure S8: HPLC analysis of culture supernatants of**

(A) ANT014 (*S. venezuelae* ATCC14583 *ant24::aac(3)IV* harboring pANT013) (genetic complementation of ANT009)

(B) ANT013 (*S. venezuelae* ATCC14583 *ant23::aac(3)IV* harboring pANT013) (genetic complementation of ANT008).

Numbers above peaks correspond to the metabolite numbers in the main text.



**Figure S9: PCR verification of the *S. venezuelae* ATCC 14583 mutant strains ANT007, ANT008 and ANT009**

(A) Principle of the PCR verification in the replacement mutants.

PCR 1, 2 and 3 were carried out to confirm the replacement of the gene of interest by a resistance cassette.

(B) PCR verification 1, 2 and 3 of ANT007.

L : ladder; lane 1: PCR1, ANT007 (PCR fragment expected size 2.2 kb); lane 2: PCR1 *S. venezuelae* ATCC 14583 wild type; lane 3: PCR2, ANT007 (PCR fragment expected size 2.4 kb); lane 4: PCR2 *S. venezuelae* ATCC 14583 wild type; lane 5: PCR3, resistance gene cassette, ANT007 (PCR fragment expected size 1.2 kb); lane 6: PCR3, *ant08* region *S. venezuelae* ATCC 14583 wild type (PCR fragment expected size 1.5 kb)

(C) PCR verification 1, 2 and 3 of ANT008.

L : ladder; lane 1: PCR1, ANT008 (PCR fragment expected size 2.7 kb); lane 2: PCR1 *S. venezuelae* ATCC 14583 wild type; lane 3: PCR2, ANT008 (PCR fragment expected size 2.2 kb); lane 4: PCR2 *S. venezuelae* ATCC 14583 wild type; lane 5: PCR3, resistance gene cassette, ANT008 (PCR fragment expected size 1.2 kb); lane 6: PCR3, *ant08* region *S. venezuelae* ATCC 14583 wild type (PCR fragment expected size 1.6 kb)

(D) PCR verification 1, 2 and 3 of ANT009.

L: ladder; lane 1: PCR1, ANT009 (PCR fragment expected size 2.2 kb); lane 2: PCR1 *S. venezuelae* ATCC 14583 wild type; lane 3: PCR2, ANT009 1.7kb); lane 4: PCR2 *S. venezuelae* ATCC 14583 wild type; lane 5: PCR3, resistance gene cassette, ANT009 (PCR fragment expected size 1.2 kb); lane 6: PCR3, *ant08* region *S. venezuelae* ATCC 14583 wild type (fragment expected size 0.7 kb)

L corresponds to the molecular weight ladder from Thermo Scientific™ GeneRuler™ DNA Ladder Mix



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