Supporting Information

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Characterization of an Anthracene Intermediate in Dynemicin Biosynthesis

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Abstract: *Despite the identification of a β-hydroxyhexaene produced by the enediyne polyketide synthases (PKSs), the post-PKS biosynthetic steps to the individual members of this antitumor antibiotic family remain largely unknown. The massive biosynthetic gene clusters (BGCs) that direct the formation of each product caution that many steps could be required. It was recently demonstrated that the enediyne PKS in the dynemicin A BGC from* Micromonospora chersina *gives rise to both the anthraquinone and enediyne halves of the molecule. We now present the first evidence for a mid-pathway intermediate in dynemicin A biosynthesis, an iodoanthracene bearing a fused thiolactone, which was shown to be incorporated selectively into the final product. This unusual precursor reflects just how little is understood about these biosynthetic pathways, yet constrains the mechanisms that can act to achieve the key heterodimerization to the anthraquinone-containing subclass of enediynes.*

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Experimental Procedures

General

All reagents were purchased from Millipore Sigma unless otherwise specified.

Small-scale fermentation and HPLC analysis of dynemicin A and anthracene derivatives 4 and 5

Spores of wild-type *M. chersina* (10 µL) or mycelia of the ΔDynE8 mutant (100 µL) were plated on medium 53[1] with 2% agar and grown at 28 °C for 5 days. Mycelia on plates were then inoculated into 50 mL medium 53 in 125 mL flasks and shaken at 250 rpm and 28 °C for 7 days. 2 mL each starter culture was inoculated into 50 mL H881[1] and shaken again at 250 rpm and 28 °C. Depending on the experiment, the medium composition of H881 was modified by either excluding or increasing the amount of sodium iodide, or replacing it with sodium chloride or sodium bromide. After 7 days, 5 mL each culture was extracted with an equal volume of ethyl acetate (EtOAc) by vortexing for 1 min. Layers were separated by centrifugation at 4000 x *g* and 4 °C, following which 4 mL of each EtOAc layer was dried by SpeedVac without heating. Samples were resuspended in 200 µL dimethyl sulfoxide (DMSO), sterile filtered through 0.2 µm polytetrafluoroethylene (PTFE) filters, and analyzed on an Agilent 1200 HPLC equipped with a Prodigy ODS3 100 Å, 5 µm, 250 x 4.6 mm column (Phenomenex). Injections of 20 µL were separated with a 1 mL min^{-1} flow rate over a gradient of 5 – 95% ACN + 0.1% (v/v) formic acid over 40 min, followed by a 10-min hold at 95% ACN + 0.1% (v/v) formic acid before column re-equilibration.

Large-scale fermentation and purification of unlabeled and ^{13}C-enriched 4 and 5 for mass analysis, NMR, and small-scale incorporation experiments

Spores of wild-type *M. chersina* (10 µL) were plated on medium 53 with 2% agar and grown at 28 °C for 5 days. Mycelia on plates were subsequently inoculated into 3 x 50 mL medium 53 and shaken at 250 rpm and 28 °C. After 7 days, cultures were combined and 6 x 20 mL inoculated into 6 x 1 L H881 in 2.8 L flasks. For the initial fermentation from which unlabeled **4** and **5** were isolated, 0.5 mg L^{-1} sodium iodide was included in the H881 according to the reported recipe;[1] for subsequent fermentations, 2.5 mg L^{-1} was used. To enhance carbon signals for NMR analysis, 1 mM [1-^{13}C]- and [2-^{13}C]sodium acetate were added to the fermentations at 0 h and 24 h to achieve a final concentration of 2 mM each. Labeled sodium acetate solutions were prepared as 1 M stocks, adjusted to pH 7.0 with 1 M HCl, and sterile filtered through 0.2 µm polyethersulfone (PES) filters. Cultures were shaken for three days at 28 °C and 250 rpm, after which 1% (w/v) Diaion HP-20 (sterilized by autoclave) was added to each flask, and shaking resumed.

On the tenth day of fermentation, cultures were combined and extracted with 2 L EtOAc by stirring for 2 h on a magnetic stir plate. The extract was filtered through a bed of Celite in a fritted funnel (to which brine was added to assist layer separation), and the resulting EtOAc layer was dried over anhydrous sodium sulfate (Na_2SO_4) and concentrated by rotary evaporation. The extract was then adsorbed onto Celite and dry-loaded on a silica gel flash chromatography column pre-equilibrated with chloroform ($CHCl_3$). The column was run with a gradient of 0 – 2 – 10% methanol (MeOH) in $CHCl_3$, with **4** and **5** co-eluting in 100% $CHCl_3$ and dynemicin A in 2% MeOH. The $CHCl_3$ elution fractions were concentrated by rotary evaporation, and **4** and **5** then separated by HPLC or silica gel flash chromatography. HPLC was used for the initial purification of unlabeled **4** and **5**. The partially purified material was dissolved in 1.5 mL 1:1 DMSO:MeOH, sterile filtered through a 0.2 µm PTFE filter, and analyzed on an Agilent 1100 equipped with a Kinetex 100

Å, 5 µm, 250 x 10.0 mm C18 LC column (Phenomenex). Injections of 250-300 µL were separated with a 4 mL min^{-1} flow rate over a gradient of 50 – 95% ACN + 1% (v/v) formic acid over 20 min, followed by a 5-min hold at 95% ACN before column re-equilibration. Peaks containing **4** and **5** were manually collected, frozen in liq. N_2, and lyophilized for purification on Sephadex LH-20 (GE Healthcare Life Sciences), as described below. For all subsequent purifications, **4** and **5** were separated by silica gel flash chromatography, since it afforded greater yields of the final compounds with comparable purity to HPLC. The material for silica gel flash chromatography was dissolved in 99:1 hexanes:diethyl ether (Et_2O), and loaded on a column pre-equilibrated with 99:1 hexanes:Et_2O. The eluent was immediately ramped up to 95:5 hexanes:Et_2O and held for the duration of the chromatography, following which fractions containing **4** and **5** were concentrated by rotary evaporation. Regardless of the separation method, **4** and **5** were finally purified by Sephadex LH-20 with MeOH as the mobile phase. Neon-yellow elutions were combined and concentrated by rotary evaporation, yielding pure **4** and **5** to be used for mass or NMR analysis and incorporation experiments.

Compound 4: ^{1}**H NMR** (400 MHz, $CDCl_3$): δ 8.47 (s, 1H), 8.35 (d, *J* = 8.4 Hz, 1H), 8.28 (d, *J* = 6.8 Hz, 1H), 8.09 (br d, *J* = 8.4 Hz, 1H), 7.93 (br d, *J* = 8.4 Hz, 1H), 7.79 (dd, *J* = 6.8, 8.4 Hz, 2H), 7.59 (m, 2H); ^{13}**C NMR** (101 MHz, $CDCl_3$): δ 192.98, 134.02, 133.92, 132.87, 130.75, 129.95, 129.06, 128.95, 127.65, 127.59, 126.97, 126.63, 126.56, 125.19, 124.68; **ESI-MS** $[M+H]^+$ calcd for $C_{15}H_9OS$, 237.0374; found, 237.0370.

Compound 5: ^{1}**H NMR** (400 MHz, $CDCl_3$): δ 8.45 (s, 1H), 8.33 (d, *J* = 7.2 Hz, 1H), 8.13 (br d, *J* = 8.4 Hz, 1H), 7.89 (br d, *J* = 8.0 Hz, 1H), 7.87 (d, *J* = 7.2 Hz, 1H), 7.62 (m, 2H); ^{13}**C NMR** (101 MHz, $CDCl_3$): δ 192.14, 138.10, 134.59, 133.47, 131.63, 131.16, 130.19, 129.16, 128.33 (2 carbons), 127.85, 127.25, 126.21, 125.06, 108.19; **EI-MS** $[M]^+$ calcd for $C_{15}H_7IOS$, 361.9262; found, 361.9268.

Mass analysis using UPLC-ESI-MS

Samples for UPLC-ESI-MS mass analysis were prepared in MeOH or 5% DMSO in MeOH and analyzed on a Waters ACQUITY/Xevo G-2 UPLC-ESI-MS with positive mode ESI ionization. Injections of 5 – 10 µL were separated on an Agilent Eclipse 1.8 µm, 50 x 2.1 mm C18 column with a 0.3 mL min^{-1} flow rate and the following gradient method: a 1-min hold at 100% H_2O + 0.1% (v/v) formic acid, followed by 0 – 90% ACN + 0.1% (v/v) formic acid over 6.5 min, and finally a 1-min hold at 90% ACN before column re-equilibration.

Mass analysis of 5 using EI-MS

The high resolution EI mass spectrum of **5** was obtained using a VG70S double-focusing magnetic sector mass spectrometer (VG Analytical, Manchester, UK, now Micromass/Waters) equipped with an MSS data acquisition system (MasCom, Bremen, Germany). The resolution of the instrument was set at 10,000 (100 ppm peak width). The sample was introduced into the source (block temperature = 200 ^{o}C) using a heated direct insertion probe fitted with a deep quartz cup, with a heating rate of 1 ^{o}C s^{-1}. Spectra were acquired under control of the computer data system. The electron energy was 70 eV. Nominal mass scan spectra were acquired for a scan mass range of 1000-10 amu using a magnet scan rate of 20 s $decade^{-1}$. An accurate mass measurement was then obtained across a narrower mass range with high boiling perfluorokerosene (PFK) as the reference.

Crystallization of 5

Crystals of **5** were obtained as red needles in Et_2O, and reflection intensities of a crystal were then measured at 110(2) K using a SuperNova diffractometer (Rigaku OD, equipped with an Atlas detector) with Mo *Kα* radiation (λ = 0.71073 Å) under the program CrysAlisPro (Version CrysAlisPro 1.171.39.29c, Rigaku OD, 2017). This program was also used for refining the cell dimensions and for data reduction. The structure was solved with the program SHELXS-2014/7[2] and refined on F^2 with SHELXL-

2014/7.[2] Numerical absorption correction based on Gaussian integration over a multifaceted crystal model was applied using CrysAlisPro. The temperature of the data collection was controlled using the Cryojet system (manufactured by Oxford Instruments). The H atoms were placed at calculated positions using the instructions AFIX 43 with isotropic displacement parameters having values 1.2 *U*eq of the attached C atoms. The molecule was slightly disordered over two orientations. The occupancy factor of the major component of the disorder refined to 0.9600(11).

Small-scale supplementation of 4 and 5 and analysis of dynemicin A production

Spores of wild-type *M. chersina* (10 µL) were plated on medium 53 with 2% agar and grown at 28 °C for 5 days. Mycelia on the plate were then inoculated into 50 mL medium 53 in a 125 mL flask and shaken at 250 rpm and 28 °C. After 7 days, 2 mL of the starter culture was inoculated into several 250 mL flasks containing 50 mL H881 prepared with or without sodium iodide depending on the experiment. A culture with 0.5 mg L^{-1} sodium iodide and a culture without sodium iodide were always included as controls. After shaking at 250 rpm and 28 °C for 24 h, 250 µL of ~1 mM stock solutions of **4** and **5** in DMSO (sterile filtered through 0.2 µm PTFE filters) was added to the appropriate fermentation flasks with or without sodium iodide to achieve ~5 µM final concentrations. Sterile DMSO (250 µL) was added to the control flasks. Fermentations proceeded at 28 °C and 250 rpm for an additional 3 days, following which extracts were prepared and analyzed by HPLC or UPLC-ESI-MS as described above.

Fermentation and purification of unlabeled triacetyldynemicin A

Unlabeled triacetyldynemicin A was prepared and purified from the 6 L wild-type fermentation (described above) from which unlabeled **4** and **5** were isolated. Partially purified dynemicin A from the silica gel flash chromatography column (eluting at 2% MeOH in $CHCl_3$) was acetylated and then purified by silica gel flash chromatography as previously described.[3] One-third of the acetylated material was taken forward for the final two Sephadex LH-20 purification steps,[3] and then ^1H and ^{13}C NMR spectra recorded in DMSO-d_6.

Large-scale fermentation and purification of 5 labeled from [1-^{13}C]sodium acetate

Spores of wild-type *M. chersina* (10 µL) or mycelia of *M. chersina* ΔE10[3] were plated on medium 53 with 2% agar and grown at 28 °C for 5 days. Mycelia on plates were subsequently inoculated into 3 x 50 mL medium 53 and shaken at 250 rpm and 37 °C. After 5 days, cultures were combined and 6 x 20 mL inoculated into 6 x 1 L H881 in 2.8 L flasks. Cultures were shaken for 24 h at 37 °C and 250 rpm, following which 2 mM [1-^{13}C]sodium acetate (prepared as a 1 M stock, adjusted to pH 7.0 with 1 M HCl, and sterile filtered through 0.2 µm PES filters) and 1% (w/v) Supelite DAX-8 (sterilized by autoclave) were added to each flask. Shaking resumed at 250 rpm and 37 °C for an additional 3 days, and purification of **5** labeled from incorporation of [1-^{13}C]sodium acetate then proceeded as described above.

Fermentation and purification of triacetyldynemicin A from feeding of 5 labeled from [1-^{13}C]sodium acetate

To obtain appreciable amounts of triacetyldynemicin A for comparison to the unlabeled standard, administration of **5** labeled from incorporation of [1-^{13}C]sodium acetate was repeated twice. For the first experiment, spores of *M. chersina* ΔPKS5 (10 µL) were plated on medium 53 with 2% agar and grown at 28 °C for 5 days. Mycelia on the plate were subsequently inoculated into 50 mL medium 53 and shaken at 250 rpm and 37 °C. After 5 days, 4 x 10 mL were inoculated into 4 x 500 mL H881 in 2.8 L flasks with 250 µL of a 1 mg/mL stock of **5** labeled from [1-^{13}C]sodium acetate (purified from 9 L *M. chersina* ΔE10; Figures S15-S16) in DMSO (sterile filtered through a 0.2 µm PTFE filter). Cultures were shaken for 24 h at 37 °C and 250 rpm, following which 1% (w/v) Supelite DAX-8 (sterilized by autoclave) was added to each flask. After an additional 3 days at 250 rpm and 37 °C, the cultures were

combined and extracted with 800 mL EtOAc by stirring for 2 h on a magnetic stir plate. The resulting extract was filtered through a bed of Celite in a fritted funnel (to which brine was added to assist layer separation), and the resulting EtOAc layer was dried over anhyd. Na_2SO_4 and concentrated by rotary evaporation. The extract was then adsorbed onto Celite and dry-loaded on a silica gel flash chromatography column pre-equilibrated with $CHCl_3$. This column was run with a gradient of 0 – 2 – 10% MeOH in $CHCl_3$, with dynemicin A eluting in 2% MeOH.

The partially purified dynemicin A was dissolved in 6 mL acetic anhydride and 8 mL pyridine and stirred at room temperature overnight. The acetylated material was then diluted in EtOAc and washed with ~200 mL H_2O and 2 x ~150 mL saturated ammonium chloride (NH_4Cl). The EtOAc layer was dried over anhyd. Na_2SO_4 and concentrated by rotary evaporation, following which triacetyldynemicin A was purified by silica gel flash chromatography with a gradient of 80%:20% hexanes:EtOAc to 100% EtOAc to 95%:5% EtOAc:MeOH. Orange fractions were combined, concentrated by rotary evaporation, and then purified twice by Sephadex LH-20 with MeOH as the mobile phase. Orange material was again concentrated by rotary evaporation, and then purified by silica gel flash chromatography with a gradient from 70%:30% hexanes:EtOAc to 30%:70% hexanes:EtOAc with 0.5% acetic acid. The purified orange material was concentrated by rotary evaporation, and the remaining acetic acid was removed by azeotropic distillation with cyclohexane. Finally, the material was purified once more on Sephadex LH-20 as described above.

For the second experiment, spores of *M. chersina* ΔPKS5 (10 µL) were plated on medium 53 with 2% agar and grown at 28 ^{o}C for 5 days. Mycelia on the plate were subsequently inoculated into 50 mL medium 53 and shaken at 250 rpm and 37 ^{o}C. After 5 days, 3 x 10 mL were inoculated into 3 x 500 mL H881 in 2.8 L flasks. Cultures were shaken for 24 h at 37 ^{o}C and 250 rpm, following which 250 µL of a ~2.4 mg/mL stock of **5** labeled from [1-^{13}C]sodium acetate (purified from 12 L wild-type *M. chersina*; Figures S17-S18) in DMSO (sterile filtered through a 0.2 µm PTFE filter) was added to each flask. After an additional 24 h, 1% (w/v) Supelite DAX-8 (sterilized by autoclave) was added to each flask. Shaking finally resumed for an additional 3 days at 250 rpm and 37 ^{o}C, and triacetyldynemicin A was then purified as above, without the final two silica gel flash chromatography and Sephadex LH-20 steps.

Samples were combined and acetylated again with 0.75 mL acetic anhydride and 1.0 mL pyridine by stirring for 1 h, owing to the slow deprotection of the acetates over time (the experiments were repeated ~1 month apart). This acetylation reaction was diluted in EtOAc, and then washed with ~30 mL H_2O and 2 x ~30 mL sat. NH_4Cl. Subsequently, triacetyldynemicin A was dried over anhyd. Na_2SO_4, concentrated by rotary evaporation, and purified by silica gel flash chromatography with 9:1 dichloromethane:acetone + 0.1% acetic acid. The purified orange material was combined and concentrated by rotary evaporation, and the remaining acetic acid was removed by azeotropic distillation with cyclohexane. The sample was then dissolved in MeOH and purified by Sephadex LH-20 with MeOH as the mobile phase. Orange fractions were collected and again concentrated by rotary evaporation, and ^{1}H and ^{13}C NMR spectra were finally recorded in DMSO-d_6.

Supporting Information Tables

Table S1: Experimental details of X-ray crystallography of 5

Crystal data	
Chemical formula	$C_{15}H_7IOS$
M_r	362.17
Crystal system, space group	Monoclinic, $P2_1/c$
Temperature (K)	110
a, *b*, *c* (Å)	3.99143 (15), 20.0252 (9), 14.8789 (6)
b (°)	96.410 (4)
V (Å^3)	1181.82 (8)
Z	4
Radiation type	Mo *Ka*
m (mm^{-1})	2.87
Crystal size (mm)	0.28 × 0.02 × 0.02
Data collection	
Diffractometer	SuperNova, Dual, Cu at zero, Atlas
Absorption correction	Gaussian *CrysAlis PRO* 1.171.39.29c (Rigaku Oxford Diffraction, 2017) Numerical absorption correction based on Gaussian integration over a multifaceted crystal model Empirical absorption correction using spherical harmonics, implemented in SCALE3 ABSPACK scaling algorithm.
T_{min}, T_{max}	0.665, 1.000
No. of measured, independent and observed [I > 2s(I)] reflections	11329, 2713, 2256
R_{int}	0.045
(sin q/l)$_{max}$ (Å^{-1})	0.650
Refinement	
$R[F^2 > 2s(F^2)]$, $wR(F^2)$, S	0.031, 0.060, 1.05
No. of reflections	2713
No. of parameters	218
No. of restraints	638
H-atom treatment	H-atom parameters constrained
$D\rho_{max}$, $D\rho_{min}$ (e Å^{-3})	0.87, -0.55

Computer programs: *CrysAlis PRO* 1.171.39.29c (Rigaku OD, 2017), *SHELXS2014*/7,[2] *SHELXL2014*/7,[2] *SHELXTL* v6.10[4]

Supporting Information Figures

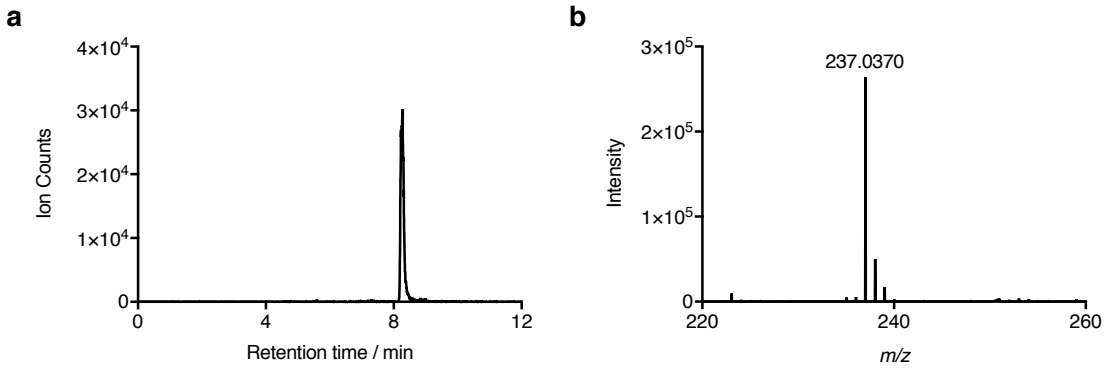

Figure S1: UPLC-ESI-MS analysis of 4. (a) Extracted ion chromatogram for $[M+H]^+$ = 237.0374, and (b) zoom-in of mass spectrum.

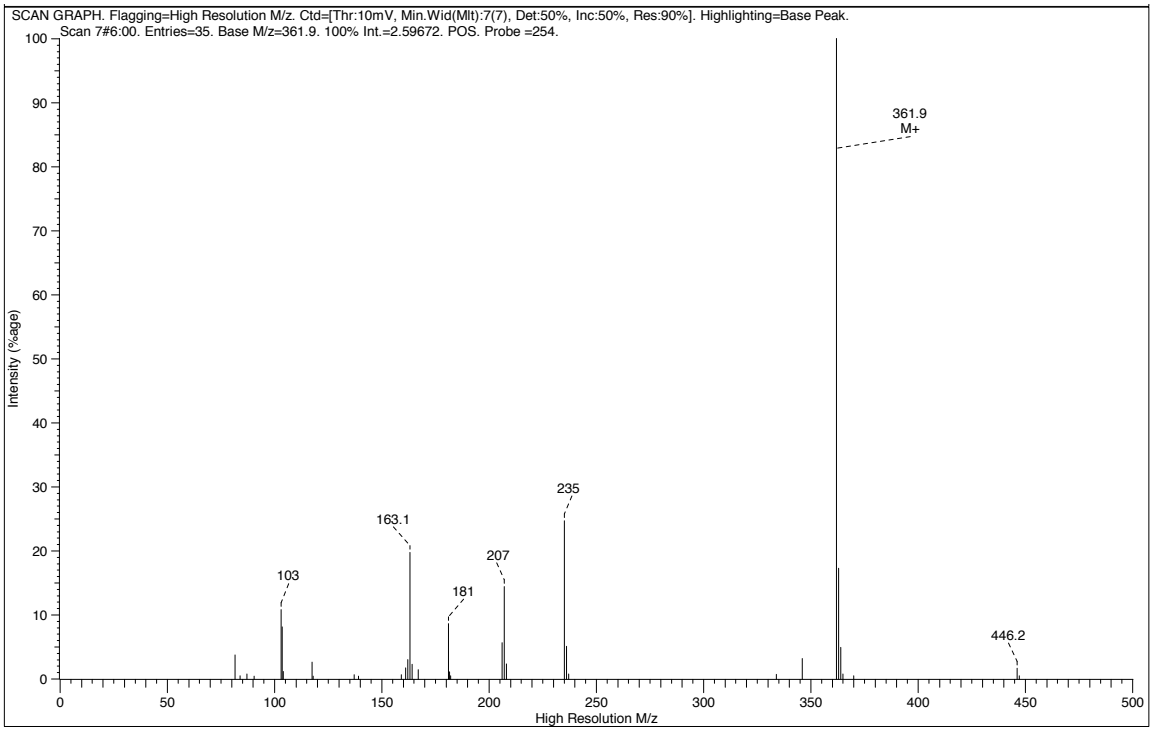

Figure S2: Nominal mass spectrum of 5 acquired by EI-MS

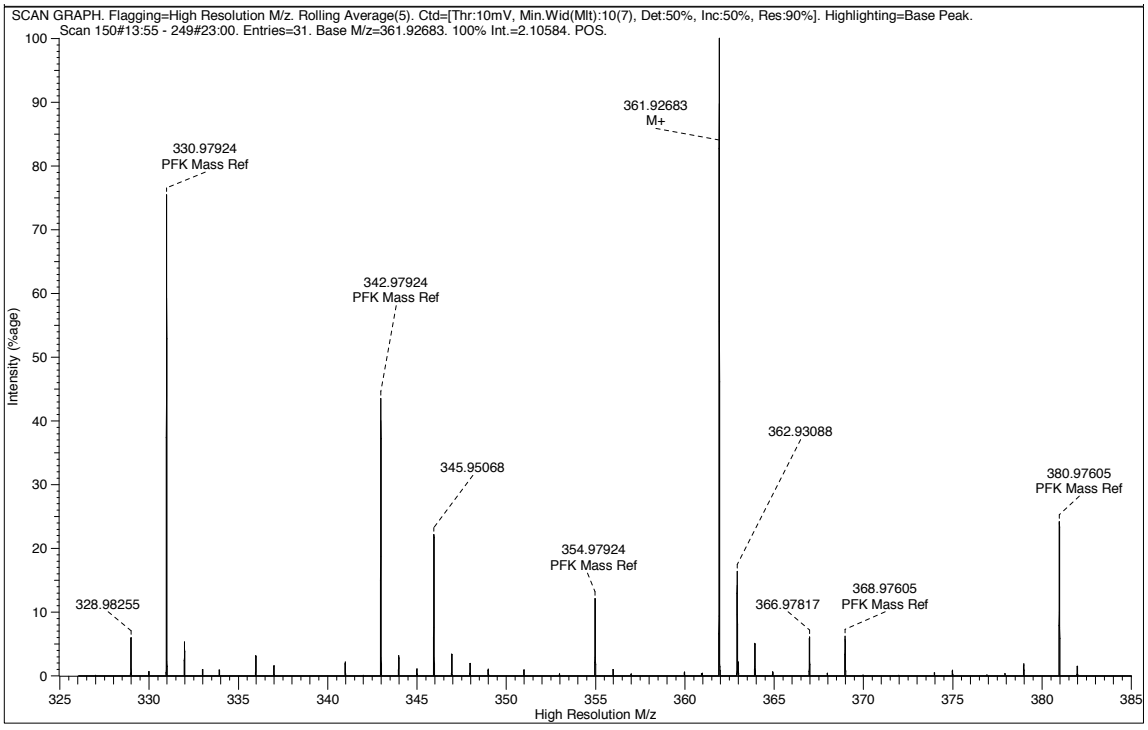

Figure S3: Exact mass spectrum of 5 acquired by EI-MS and peak matching to perfluorokerosene (PFK)

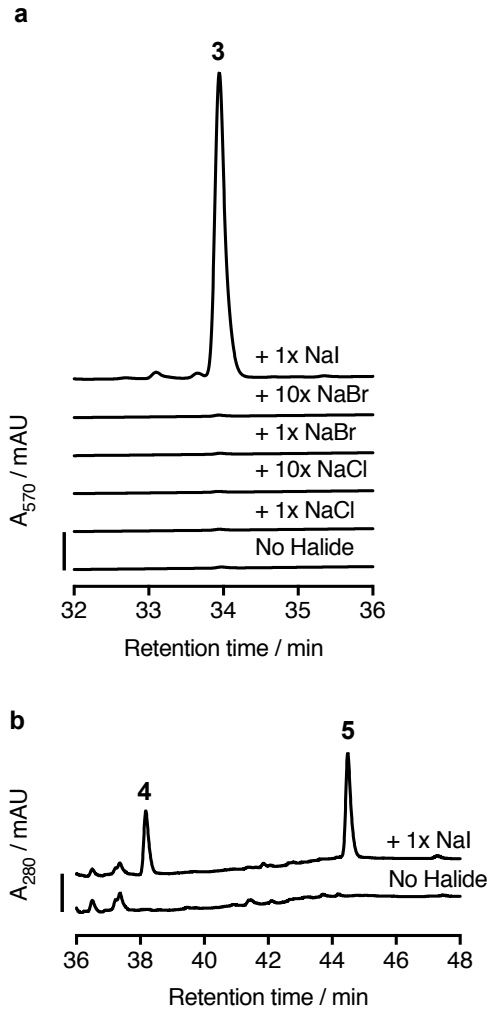

Figure S4: a) HPLC traces comparing the effect of various sodium halides on the production of dynemicin A in wild-type *M. chersina*. "1x" and "10x" represent the concentration of sodium halide used in comparison to the 0.5 mg L^{-1} (3.3 µM) sodium iodide normally added to the dynemicin fermentation medium (1x is ~ equimolar, 10x is 10-fold larger molar concentration). The y-axis scale bar represents 10 milliabsorbance units. b) HPLC traces comparing the effect of sodium iodide on the production of anthracene derivatives 4 and 5 in wild-type *M. chersina*. The y-axis scale bar represents 10 milliabsorbance units.

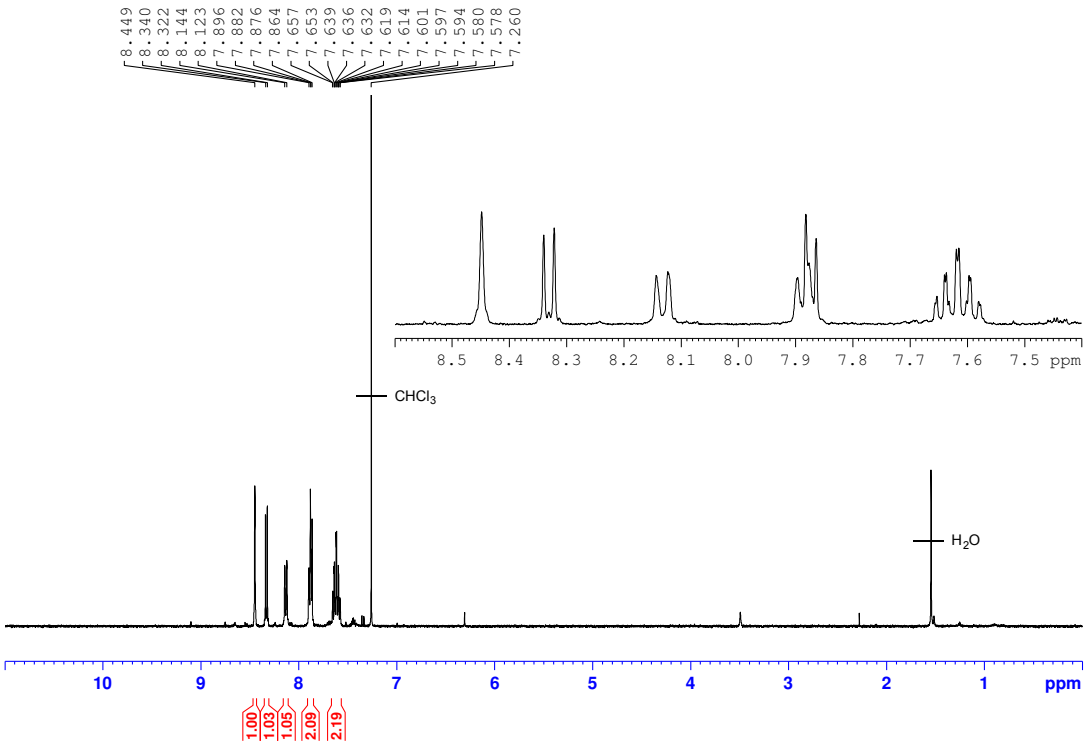

Figure S5: ^{1}H NMR (400 MHz, $CDCl_3$) of 5 labeled from [1-^{13}C]- and [2-^{13}C]sodium acetate

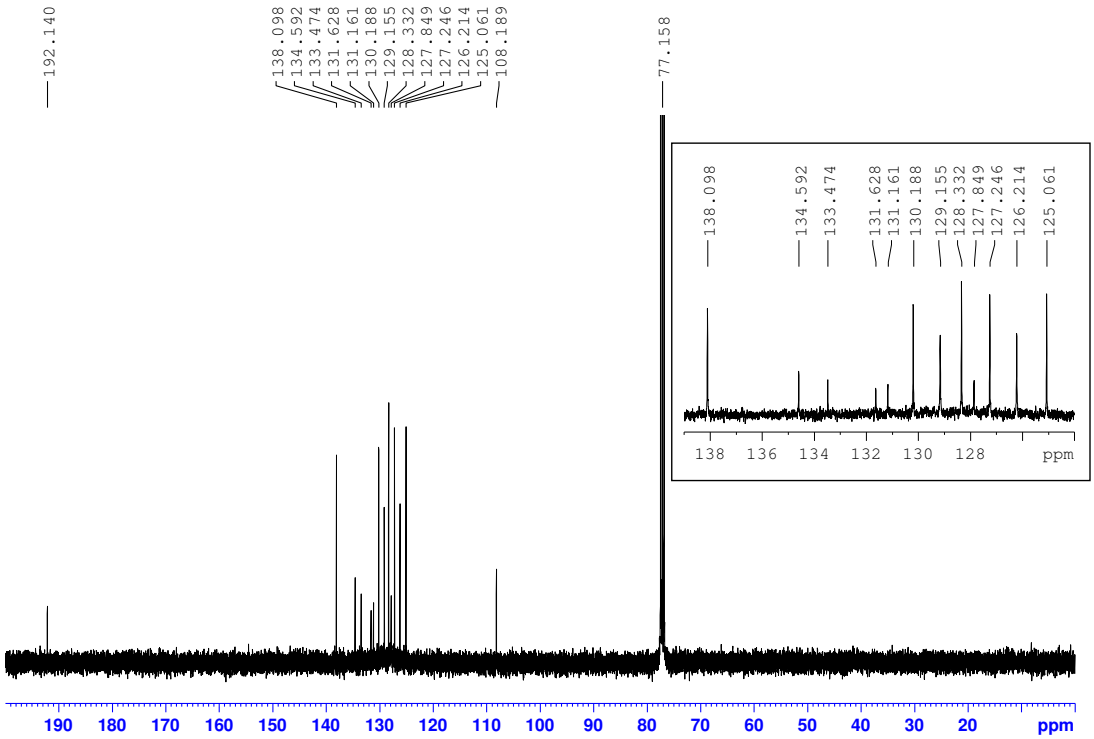

Figure S6: ^{13}C NMR (101 MHz, $CDCl_3$) of 5 labeled from [1-^{13}C]- and [2-^{13}C]sodium acetate

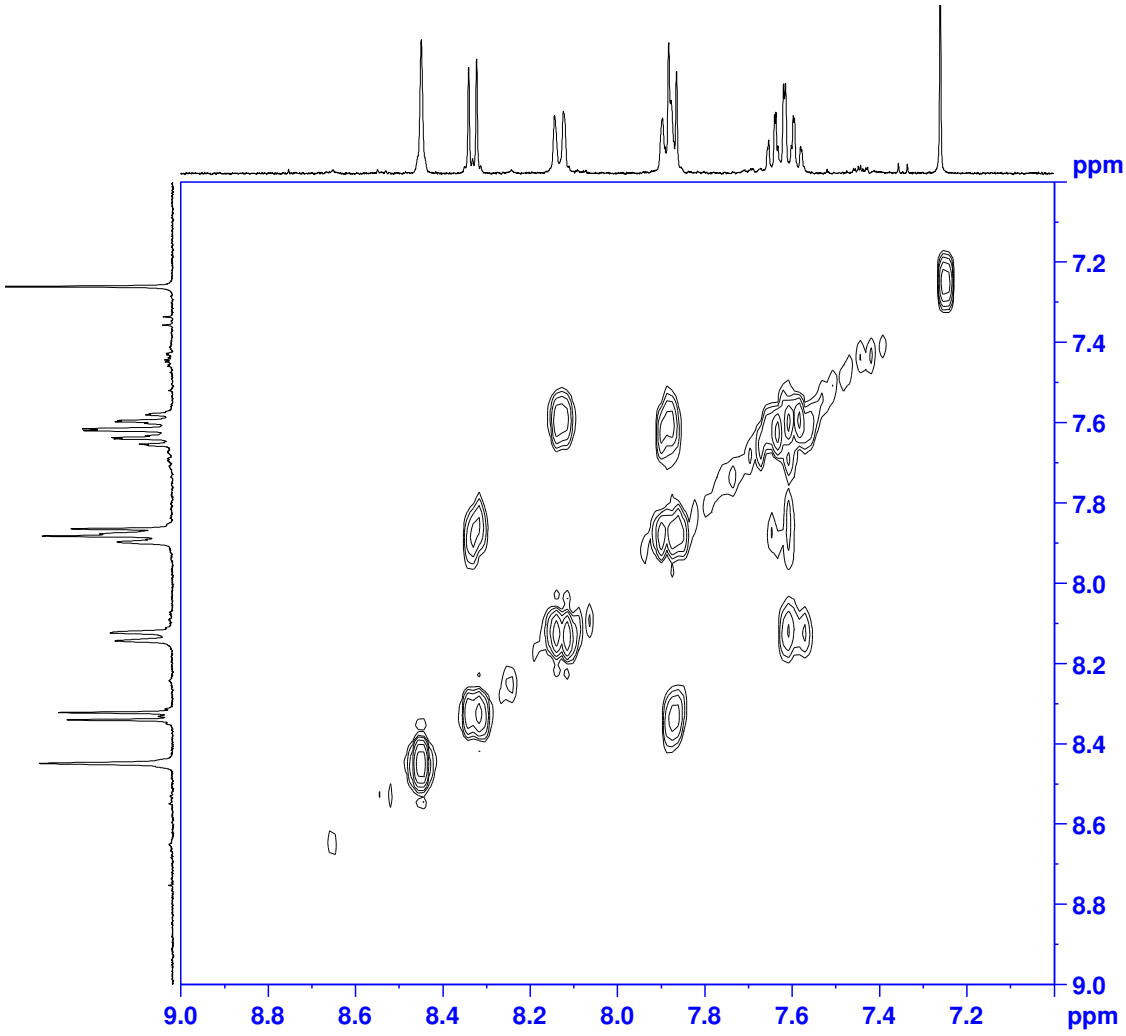

Figure S7: COSY (400 MHz, $CDCl_3$) of 5 labeled from [1-^{13}C]- and [2-^{13}C]sodium acetate

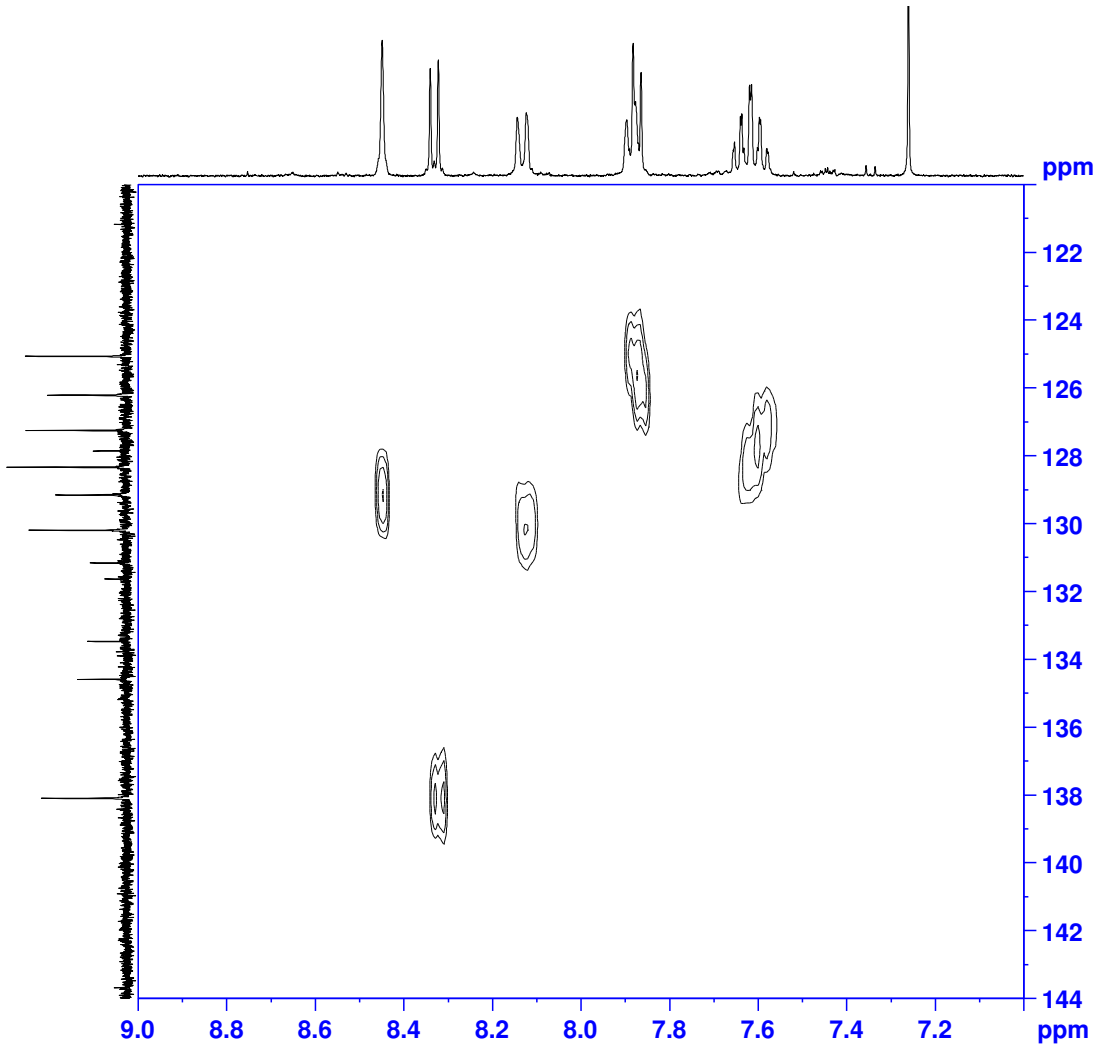

Figure S8: HSQC (400 MHz, $CDCl_3$) of 5 labeled from [1-^{13}C]- and [2-^{13}C]sodium acetate

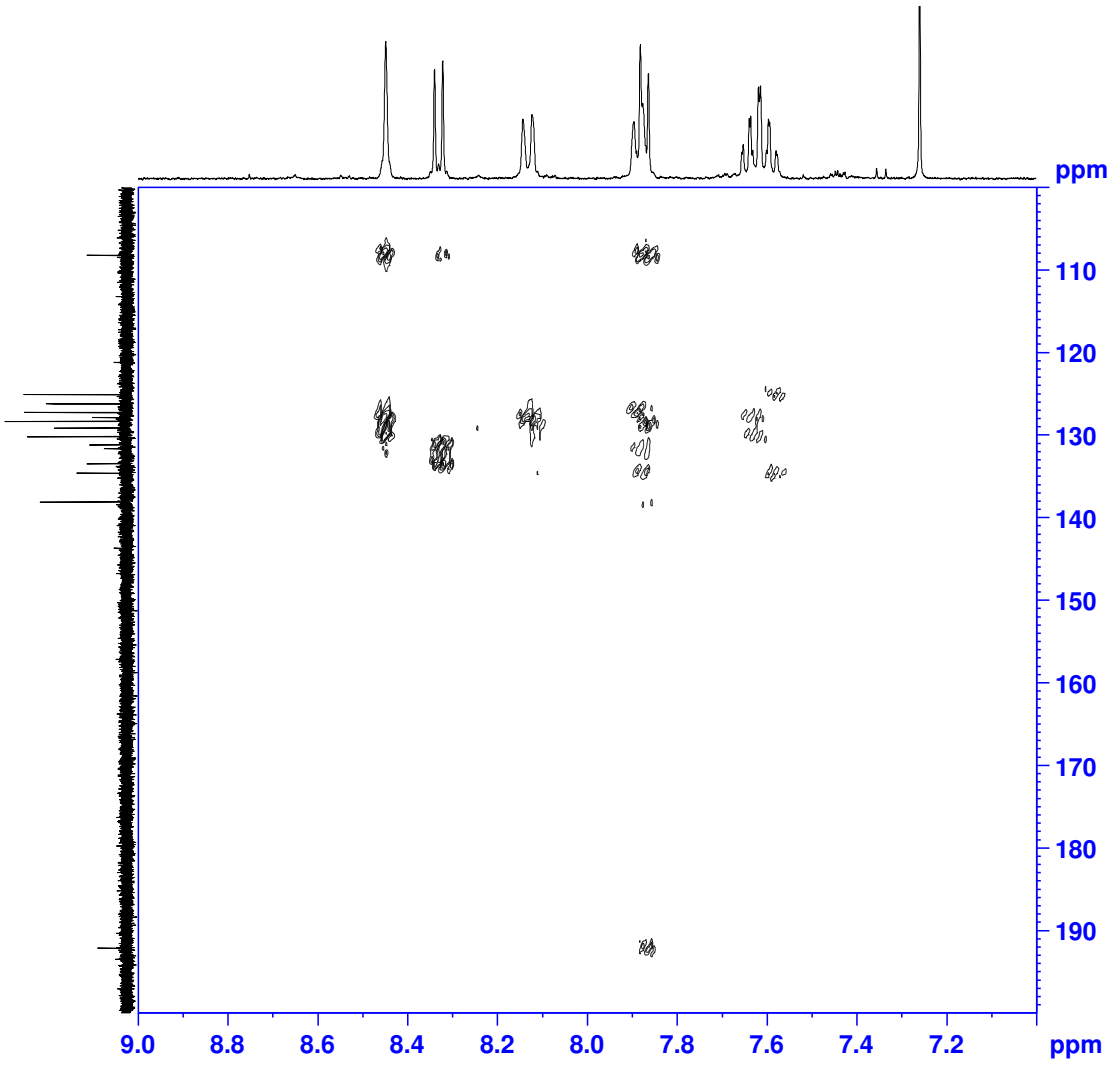

Figure S9: HMBC (400 MHz, $CDCl_3$) of 5 labeled from [1-^{13}C]- and [2-^{13}C]sodium acetate

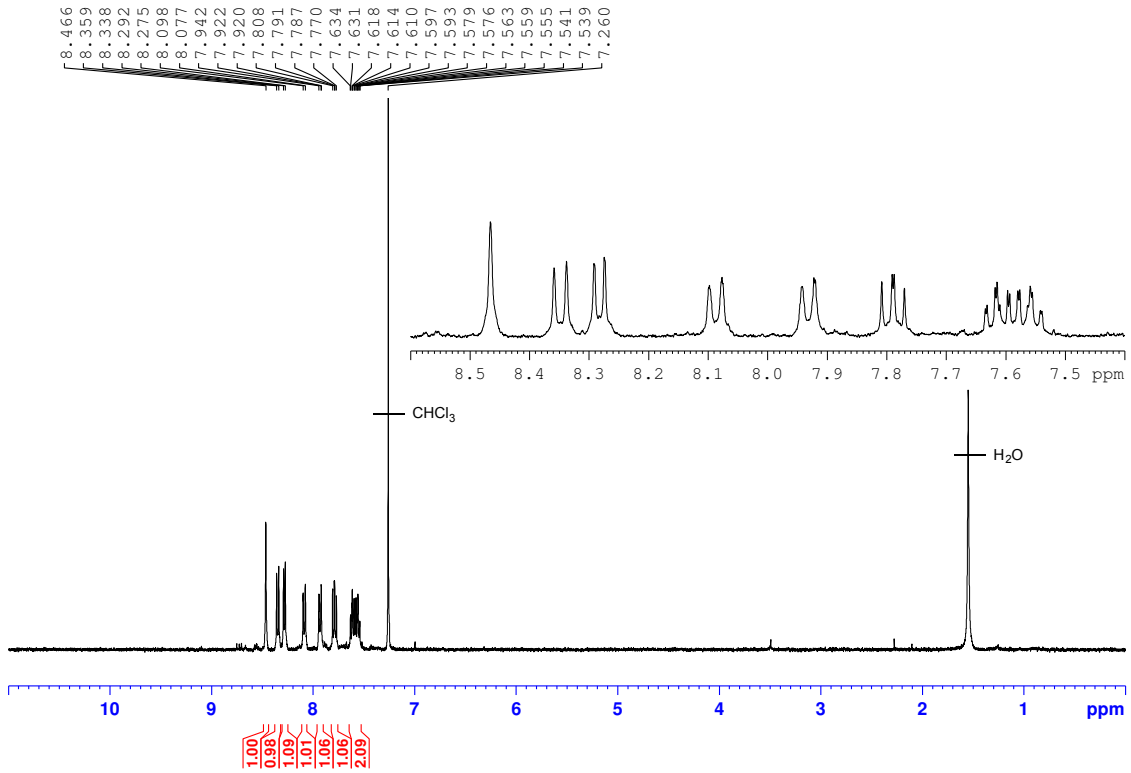

Figure S10: ^{1}H NMR (400 MHz, $CDCl_3$) of 4 labeled from [1-^{13}C]- and [2-^{13}C]sodium acetate

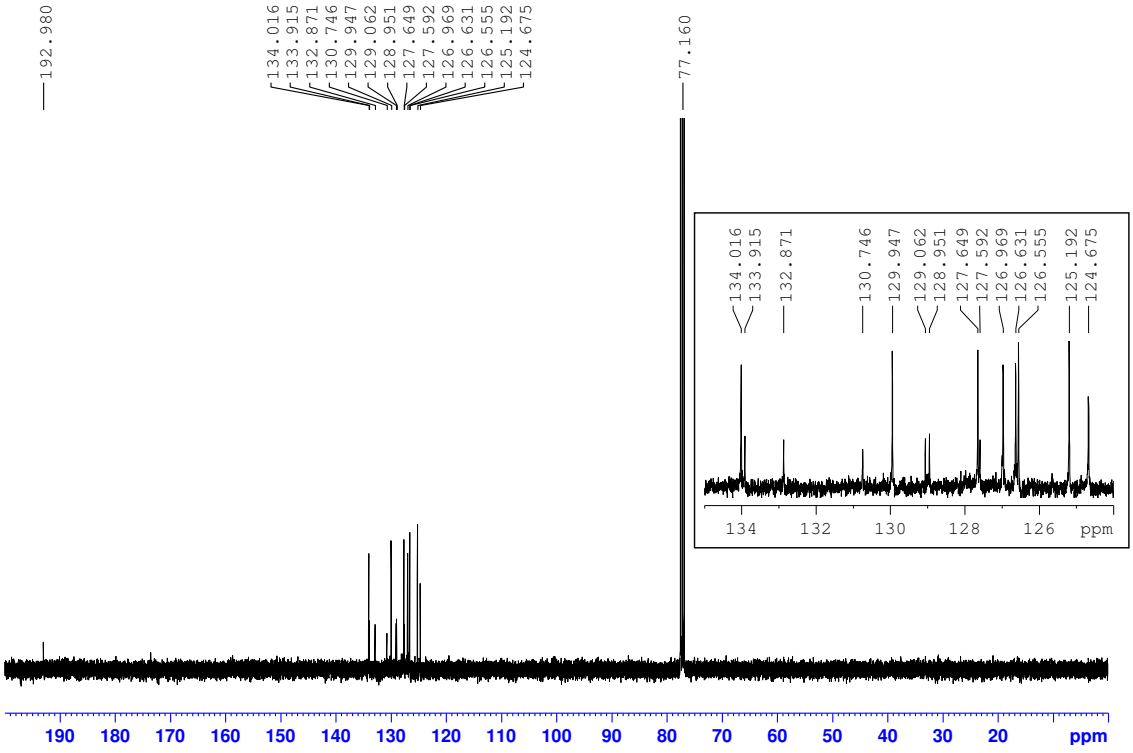

Figure S11: ^{13}C NMR (101 MHz, $CDCl_3$) of 4 labeled from [1-^{13}C]- and [2-^{13}C]sodium acetate

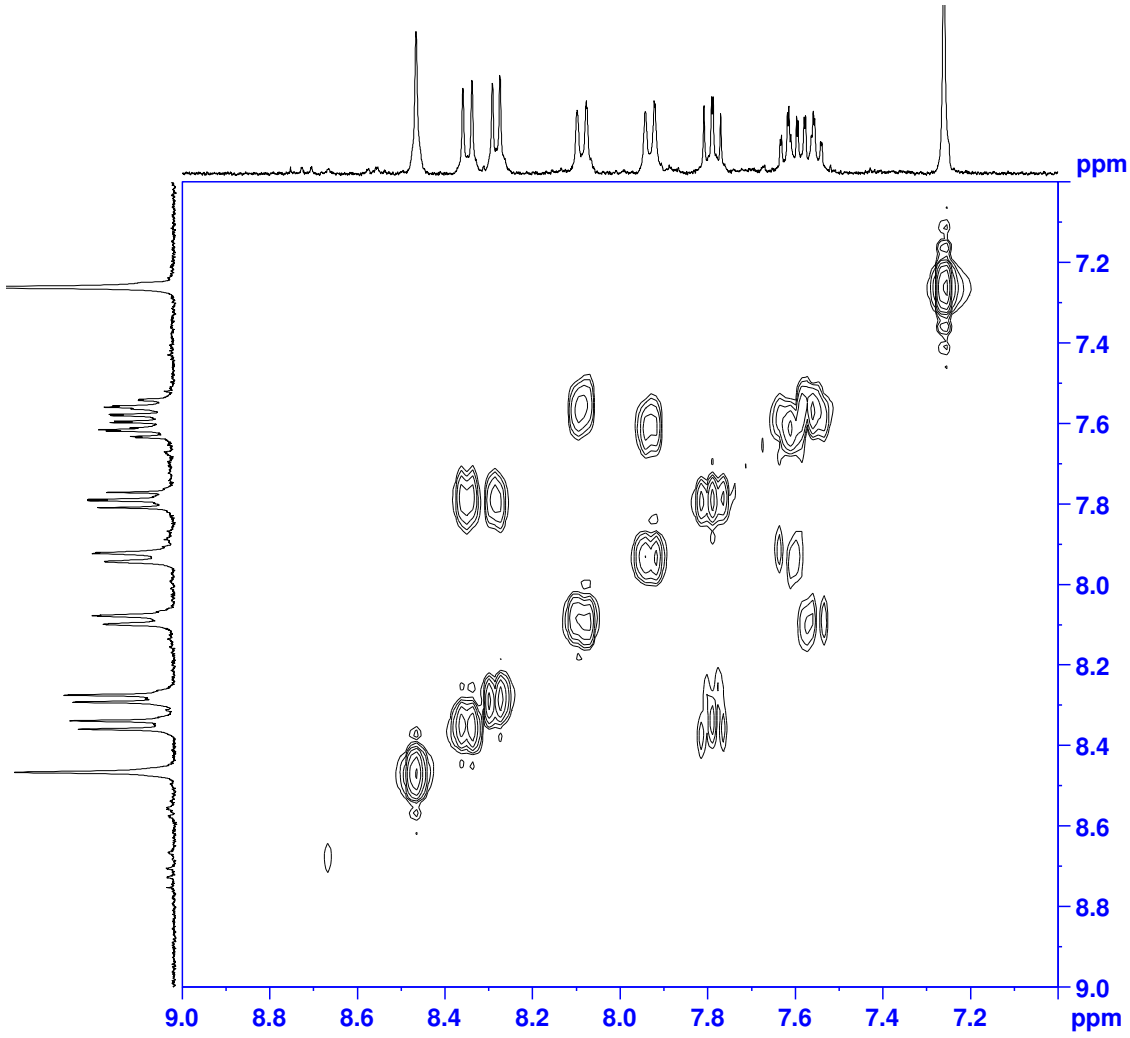

Figure S12: COSY (400 MHz, $CDCl_3$) of 4 labeled from [1-^{13}C]- and [2-^{13}C]sodium acetate

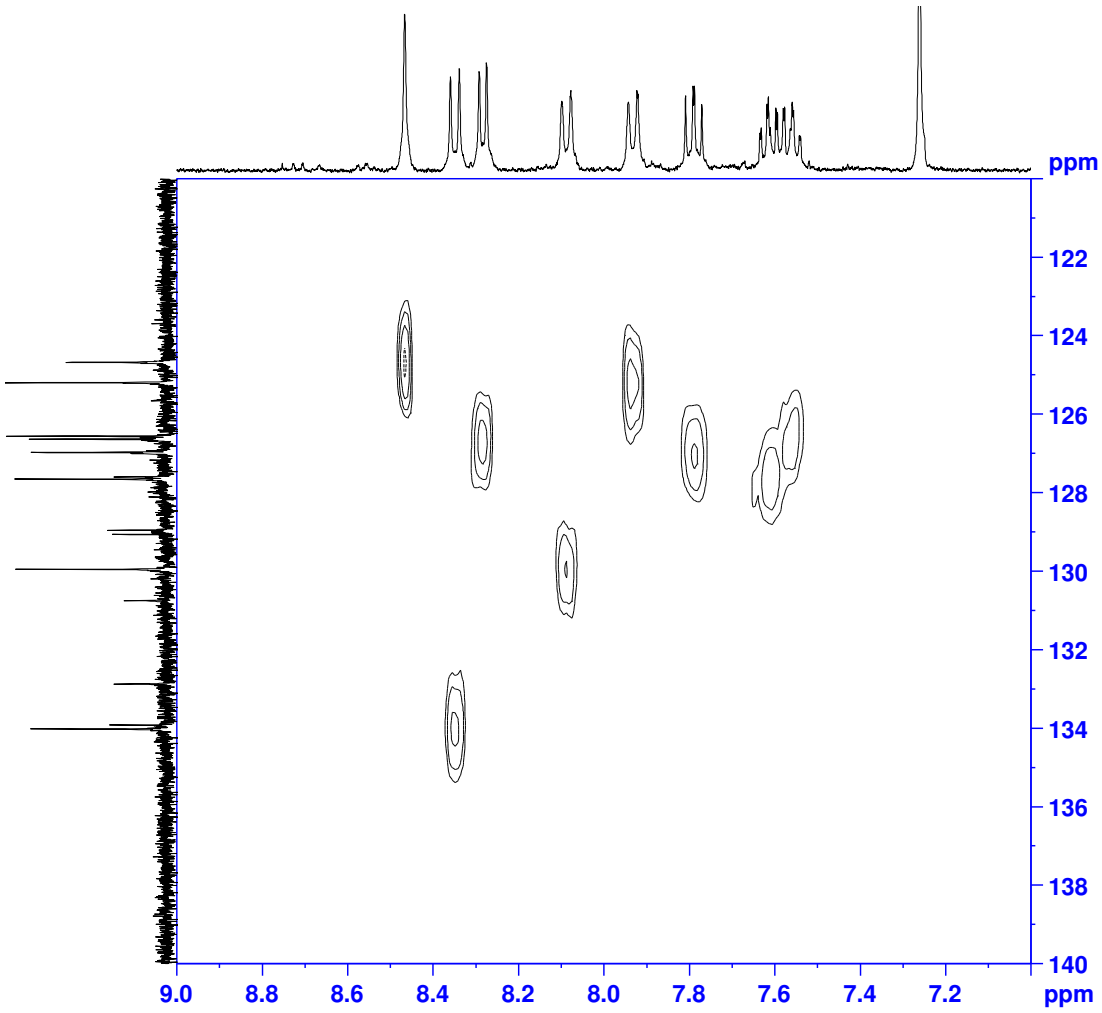

Figure S13: HSQC (400 MHz, $CDCl_3$) of 4 labeled from [1-^{13}C]- and [2-^{13}C]sodium acetate

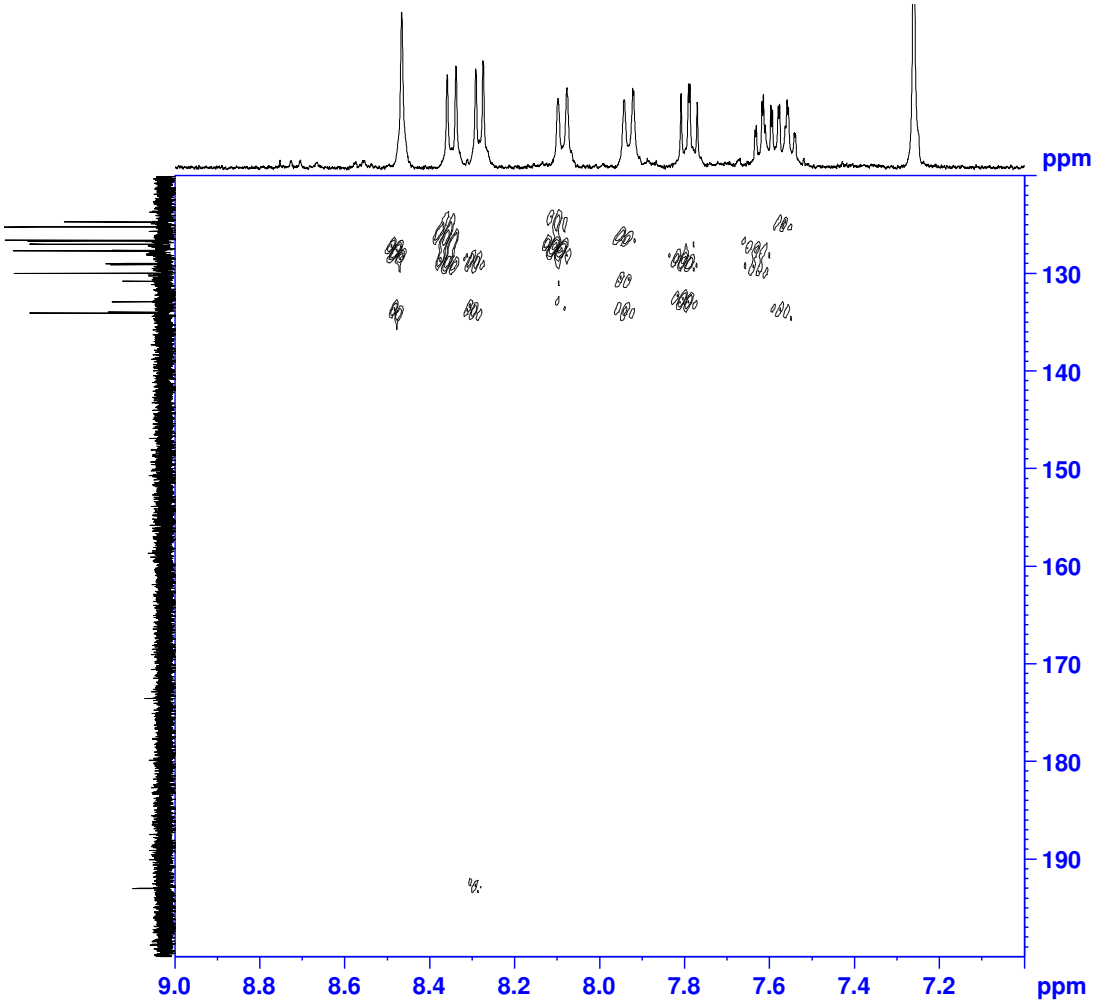

Figure S14: HMBC (400 MHz, $CDCl_3$) of 4 labeled from [1-^{13}C]- and [2-^{13}C]sodium acetate

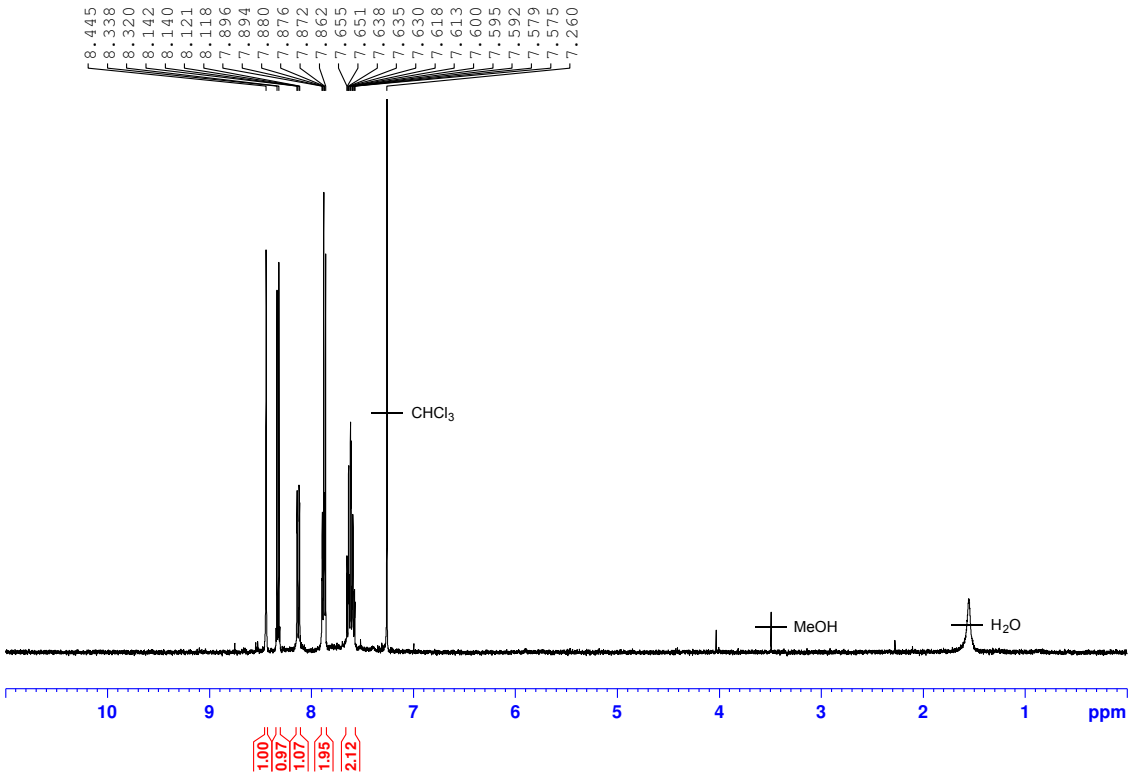

Figure S15: ^{1}H NMR (400 MHz, $CDCl_3$) of 5 isolated from *M. chersina* ΔE10 and labeled from [1-^{13}C]sodium acetate

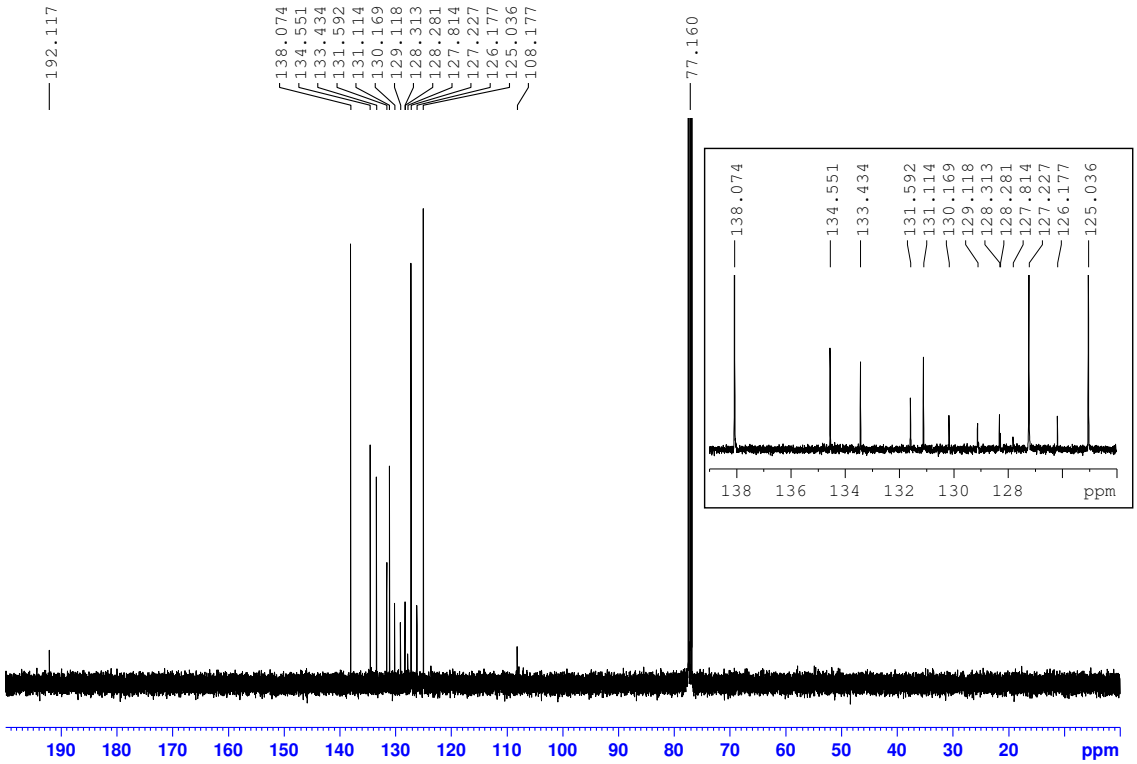

Figure S16: ^{13}C NMR (101 MHz, $CDCl_3$) of 5 isolated from *M. chersina* ΔE10 and labeled from [1-^{13}C]sodium acetate

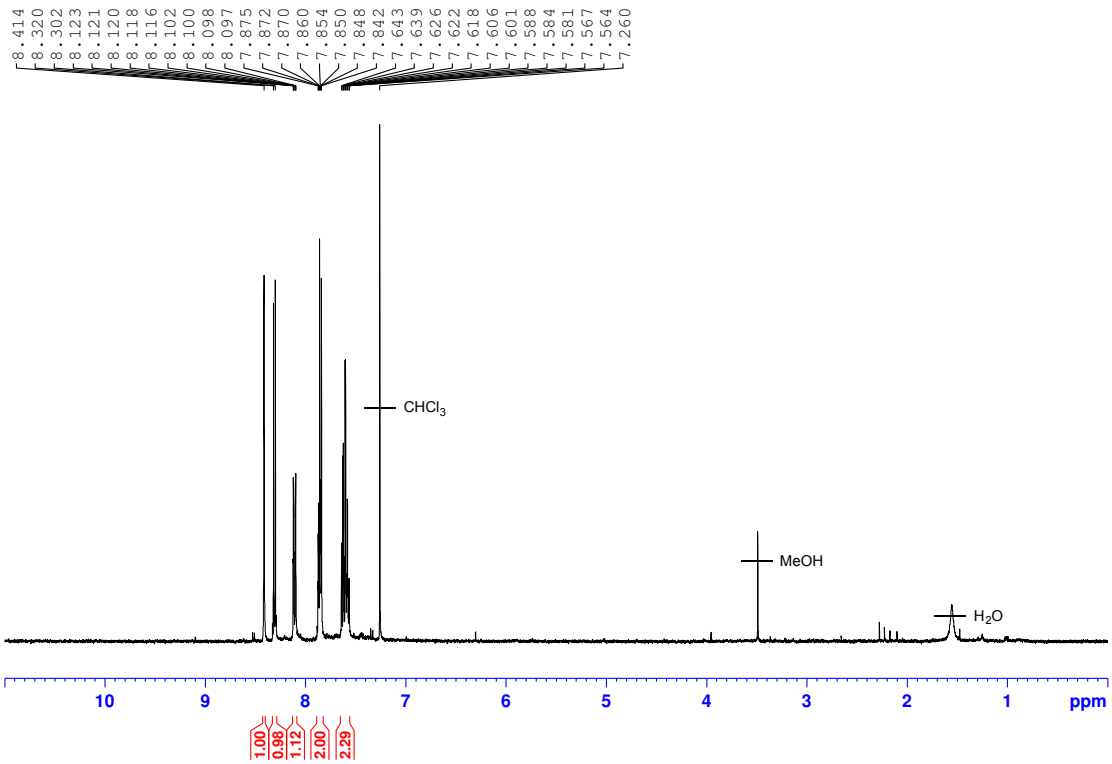

Figure S17: ^{1}H NMR (400 MHz, $CDCl_3$) of 5 isolated from wild-type *M. chersina* and labeled from [1-^{13}C]sodium acetate

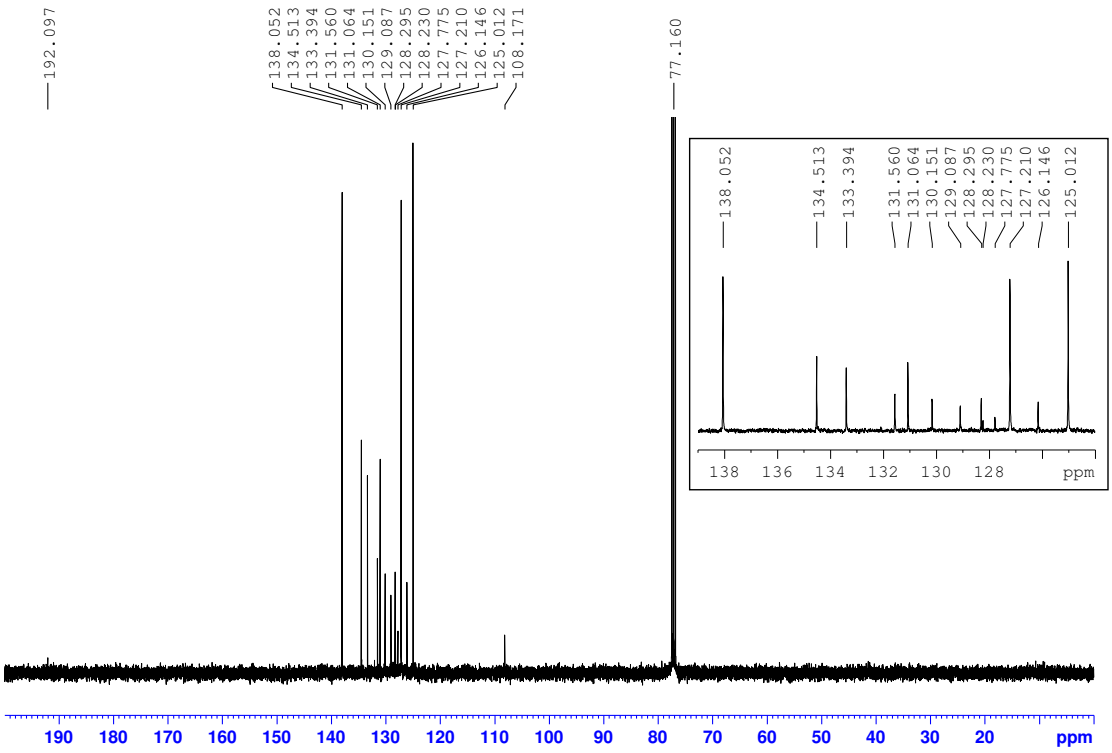

Figure S18: ^{13}C NMR (101 MHz, $CDCl_3$) of 5 isolated from wild-type *M. chersina* and labeled from [1-^{13}C]sodium acetate

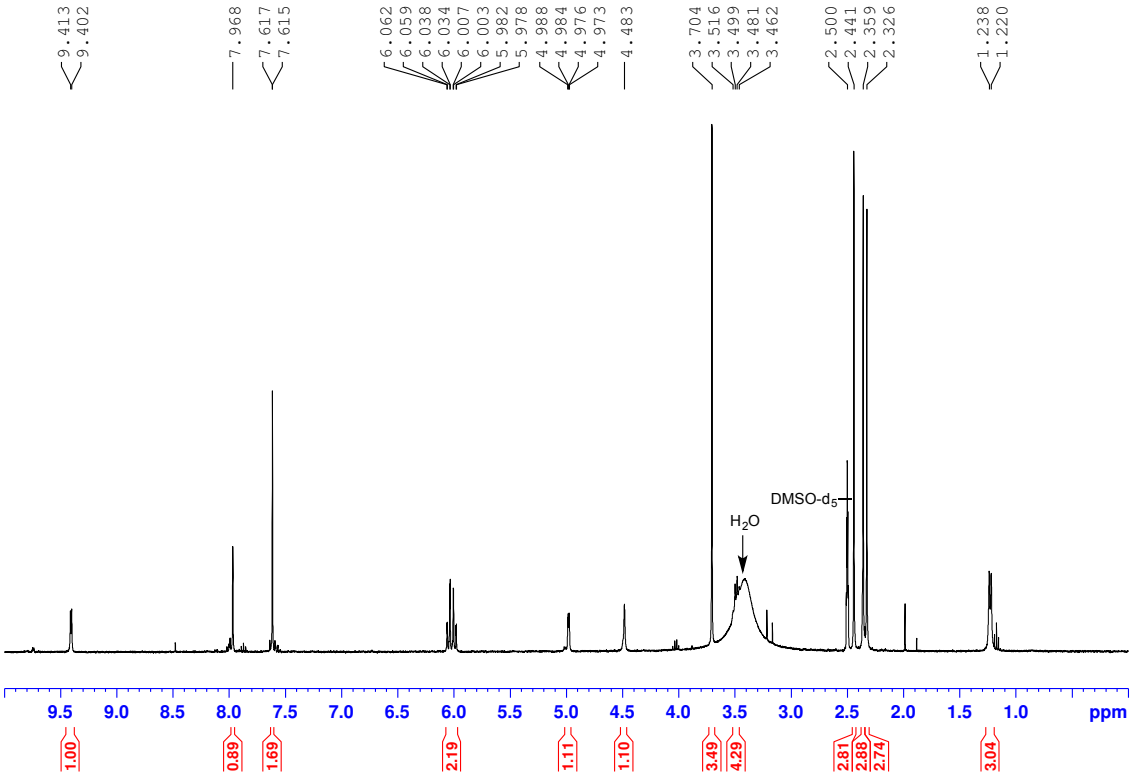

Figure S19: ^{1}H NMR (400 MHz, DMSO-d_6) of unlabeled triacetyldynemicin A

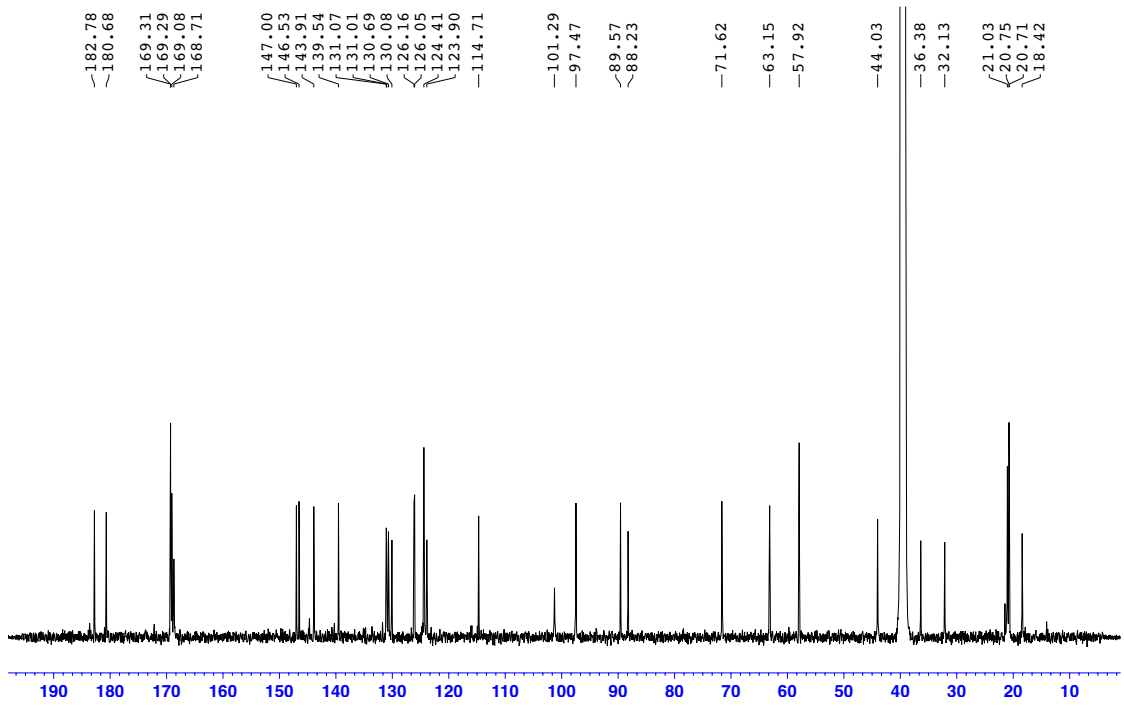

Figure S20: ^{13}C NMR (151 MHz, DMSO-d_6) of unlabeled triacetyldynemicin A

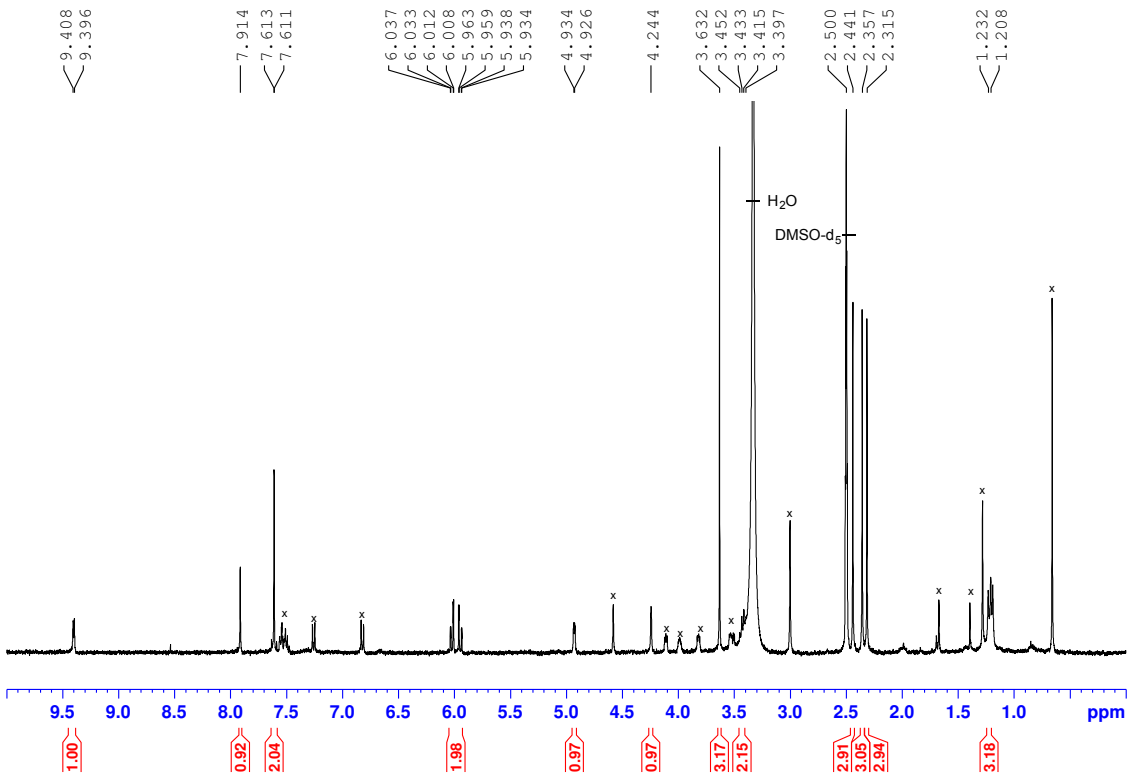

Figure S21: ^{1}H NMR (400 MHz, DMSO-d_6) of triacetyldynemicin A from supplementation of 5 labeled from incorporation of [1-^{13}C]sodium acetate. Peaks marked with an "x" are small impurities.

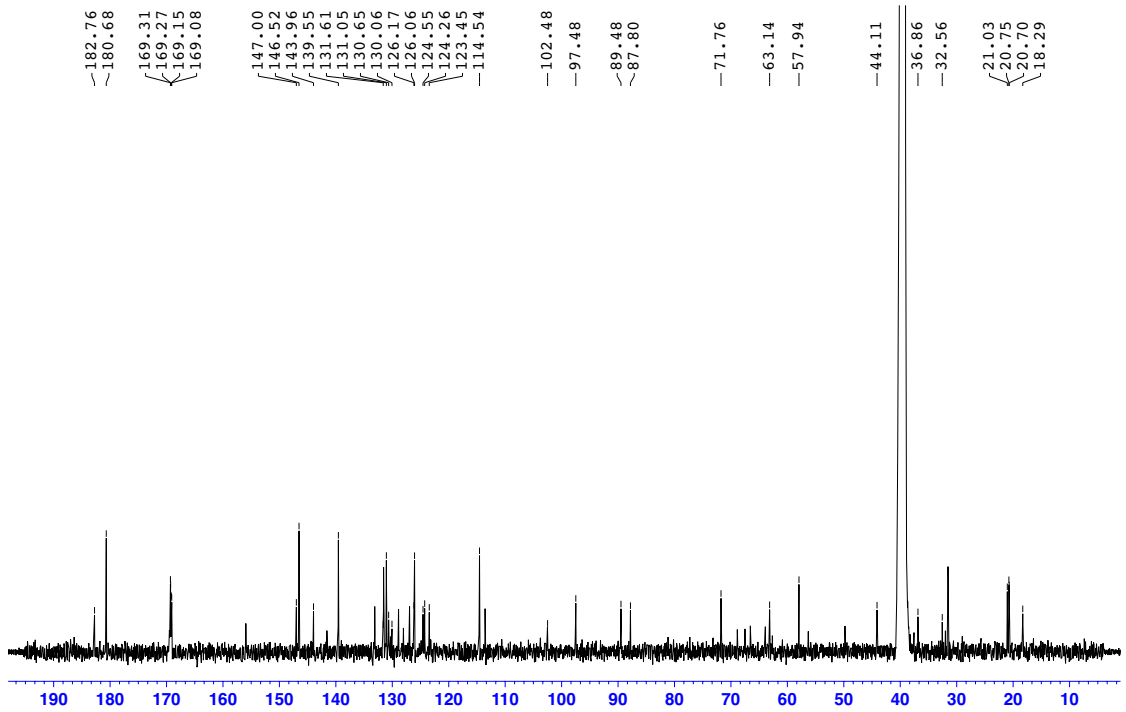

Figure S22: ^{13}C NMR (151 MHz, DMSO-d_6) of triacetyldynemicin A from supplementation of 5 labeled from incorporation of [1-^{13}C]sodium acetate. Unlabeled peaks are small impurities.

References

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Author Contributions

DRC performed experiments, CAT did the crystallization, and DRC and CAT designed experiments, analyzed the data, and wrote the manuscript.