Supporting Information

Elucidation of the Herbicidin Tailoring Pathway Offers New Insights into Its Structural Diversity

Hai-Xue Pan,[†] Zhang Chen,[‖] Tianfang Zeng,[‡] Wen-Bing Jin,[†] Yujie Geng,[⊥] Geng-Min Lin,[‖] Juan Zhao,[†] Wei-Tao Li,[†] Zijun Xiong,[§] Sheng-Xiong Huang,[§] Xin Zhai,[‡] Hung-wen Liu,*[,‖,⊥] and Gong-Li Tang*[,†]

[†]State Key Laboratory of Bio-organic and Natural Products Chemistry, Center for Excellence in Molecular Synthesis, Shanghai Institute of Organic Chemistry, University of Chinese Academy of Sciences (CAS), CAS, Shanghai, 200032, China

[‖]Department of Chemistry; [⊥]Division of Chemical Biology and Medicinal Chemistry, College of Pharmacy, University of Texas at Austin, Austin, TX 78712, USA

[‡]Key Laboratory of Structure-Based Drug Design & Discovery, Ministry of Education, Shenyang Pharmaceutical University, Shenyang 110016, China

[§]State Key Laboratory of Phytochemistry and Plant Resources in West China, Center for Excellence in Molecular Plant Sciences, Kunming Institute of Botany, CAS, Kunming, 650201, China

Table of contents

Experimental Procedures

1. Materials

General chemicals, media, enzymes and other molecular biological reagents were purchased from commercial sources. Specific bacterial strains and plasmids used in this study were summarized in Table S8, PCR primers were listed in Table S9.

2. Production and analysis of herbicidins and aureonuclemycin

Streptomyces sp. KIB-027 wild type and the mutants, as well as *S. lividans-anmBCDE* were cultured on MS agar plate (add 25 μg/mL apramycin for *S. lividans-anmBCDE*) at 30°C for 7 days. For fermentation, a piece of spore-containing medium of about 1 cm^2 were seeded into a 250 mL flask containing 50 mL of the fermentation medium (dextrin 4%, tomato paste 0.75%, NZ amine A 0.25%, yeast extract 0.5%, pH = 7.0) and incubated at 30°C, 220 rpm for 4 days. The fermentation broth was centrifuged to remove the precipitate and the supernatant was used for HPLC and HPLC-MS analysis. HPLC was performed using a reverse-phase column (Grac e Alltima, C18, 5 μm, 4.6 × 250 mm) with UV detection at 260 nm under the following program: gradient elution of mobile phase A (H_2O supplemented with 0.1% formic acid) and mobile phase B (CH_3OH supplemented with 0.1% formic acid) using a flow rate of 0.8 mL/min: 0-4 min, 5% phase B; 4-10 min, 5%-20% phase B; 10-20 min, 20%-80% phase B; 20-25 min, 80% phase B; 25-30 min, 80%-5% phase B.

Streptomyces sp. L-9-10 wild type and *Δher10* mutant were cultured on ISP4 agar at 30 °C plate for 5 days. For fermentation, a piece of colony-containing medium about 1 cm^2 was seeded into a 125 mL Erlenmeyer flask containing 20 mL of TSB (tryptic soy broth) medium and this seeding culture was incubated at 28 °C, 220 rpm for 3 days. Then 1 mL of the resulting culture was seeded into 20 mL of the fermentation medium (0.5% yeast extract, 0.5% malt extract, 1.5% soytone, 1% glucose, 0.3% sodium chloride, 1.5% glycerol, and 2% 3-morpholinopropane-1-sulfonic acid (MOPS) buffer at pH 7). This production culture was incubated at 28 °C, 220 rpm for 4 days. For HPLC sample preparation, 1 mL of the fermentation broth was centrifuged (15,000 *g*, 10 min) to pellet the cellular material and 0.5 mL of the supernatant was extracted with 0.5 mL *n*-butanol.

The *n*-butanol extract was evaporated under reduced pressure. The dried material was resuspended in 0.1 mL H_2O and filtered by Nanosep (Pall, 10K) spin filter before analysis. HPLC was performed using a C18 column (Agilent, C18, 5 μm, 4.6 × 250 mm) with UV detection at 260 nm and the samples were eluted with mobile phase A (0.1% formic acid in water), and mobile phase B (0.1% formic acid in acetonitrile) using a flow rate of 1 mL/min: 0-5 min, 12.5% phase B; 5-20 min, 12.5-50% phase B; 20-22 min, 50%-80% phase B, 27-30 min, 80-12.5% phase B.

3. Identifications of the gene clusters and sequence analyses

Genomic sequencing of *Streptomyces* sp. KIB-027 and *S. aureus var. suzhoueusis* was performed by BGI-Shenzhen. The gene cluster for herbicidins (*hbc*) was found from the genomic sequence of *Streptomyces* sp. KIB-027 using O-methyltransferase and cytochrome P450 monooxygenase as query sequences for local BLAST search. The gene cluster for aureonuclemycin (*anm*) was found from the genomic sequence of *S. aureus var. suzhoueusis* using *hbcE* as a query sequence for local BLAST search. The functions of the genes in *hbc* and *anm* were predicted by BLAST analyses in NCBI database.

4. Heterologous expression of anm cluster in *S. lividans*

The *anmB-C-D-E* gene locus was amplified by PCR with genomic DNA of *S. aureus var. suzhoueusis* as a template using the primers anmBE-For and anmBE-Rev (Table S9). The PCR product was purified and ligated to pMD19-T simple vector by T-A ligation. The *Hind*III/*Xba*I fragment of the gene cassette was cloned into the *EcoR*I/*Xba*I sites of pSET152 with an *EcoR*I/*Hind*III fragment of the *ermE*p* promoter to yield the heterologous expression plasmid pSET-anmBCDE. This vector can be specifically integrated at the *attP* attachment site of *S. lividans*. Plasmid pSET-anmBCDE and the control plasmid pSET152 were transferred into *E. coli* S17-1 and then introduced into *S. lividans* through conjugation respectively. Apramycin resistant exconjugants were selected as the heterologous expression stain *S. lividans-anmBCDE* and the control strain *S. lividans-pSET152*, and the genotype of *S. lividans-anmBCDE* was verified by PCR with primers anmBE-For and anmBE-Rev.

5. Construction of gene deletion mutants of *S.* sp. KIB-027

For *hbcA* inactivation, a 1.8 kb *Hind*III/*Xba*I fragment (primers hbcA-L-For and hbcA-L-Rev) and a 1.8 kb *Xba*I/*EcoR*I fragment (primers hbcA-R-For and hbcA-R-Rev) were cloned into the *Hind*III/*EcoR*I sites of the plasmid pKC1139 to generate the *hbcA* deletion plasmid, pKC1139-hbcA.

For *hbcF* inactivation, a 1.8 kb *Hind*III/*Xba*I fragment (primers hbcF-L-For and hbcF-L-Rev) and a 1.8 kb *Xba*I/*EcoR*I fragment (primers hbcF-R-For and hbcF-R-Rev) were cloned into the *Hind*III/*EcoR*I sites of the plasmid pKC1139 to generate the *hbcF* deletion plasmid, pKC1139-hbcF.

For *hbcG* inactivation, a 1.8 kb *Hind*III/*Xho*I fragment (primers hbcG-L-For and hbcG-L-Rev) and a 1.8 kb *Xho*I/*EcoR*I fragment (primers hbcG-R-For and hbcG-R-Rev) were cloned into the *Hind*III/*EcoR*I sites of the plasmid pKC1139 to generate the *hbcG* deletion plasmid, pKC1139-hbcG.

For *hbcH* inactivation, a 1.8 kb *Hind*III/*Xba*I fragment (primers hbcH-L-For and hbcH-L-Rev) and a 1.8 kb *Xba*I/*EcoR*I fragment (primers hbcH-R-For and hbcH-R-Rev) were cloned into the *Hind*III/*EcoR*I sites of the plasmid pKC1139 to generate the *hbcH* deletion plasmid, pKC1139-hbcH.

For *hbcI* inactivation, a 1.6 kb *Hind*III/*Xho*I fragment (primers hbcI-L-For and hbcI-L-Rev) and a 1.6 kb *Xho*I/*Xba*I fragment (primers hbcI-R-For and hbcI-R-Rev) were cloned into the *Hind*III/*Xba*I sites of the plasmid pKC1139 to generate the *hbcI* deletion plasmid, pKC1139-hbcI.

For *hbcG* and *hbcI* double inactivation, the 1.6 kb *Hind*III/*Xho*I fragment (primers hbcI-L-For and hbcI-L-Rev) and the 1.8 kb *Xho*I/*EcoR*I fragment (primers hbcG-R-For and hbcG-R-Rev) were cloned into the *Hind*III/*EcoR*I sites of the plasmid pKC1139 to generate the *hbcGI* deletion plasmid, pKC1139-hbcGI.

The resulting plasmids were introduced into *Streptomyces* sp. KIB-027 through conjugation respectively, and apramycin-resistant exconjugants were selected at 30°C. These exconjugants were first grown in MS plates with apramycin at 37 ℃ to obtain single-crossover mutants, which were further inoculated at 30 ℃ in TSB media without apramycin to generate the apramycin-sensitive clones. The double-crossover mutants were selected by PCR analyses using the primers listed in Table S9 (hbcA-v-For and hbcA-v-Rev for hbcA mutants, hbcF-v-For and hbcF-v-Rev for hbcF mutants, hbcG-v-For and hbcG-v-Rev for hbcG mutants, hbcH-v-For and

hbcH-v-Rev for hbcH mutants, hbcI-v-For and hbcI-v-Rev for hbcI mutants, hbcGI-v-For and hbcGI-v-Rev for hbcGI mutants). The resulting gene deletion mutants for each gene are shown in Table S8.

6. The *her10* deletion and complementation of *S.* sp. L-9-10

For *her10* in-frame deletion, a 2.1 kb *Nde*I/*Bgl*II fragment (primers her10-L-For and her10-L-Rev) and a 2.1 kb *Bgl*II/*Hind*III fragment (primers her10-R-For and her10-R-Rev) were cloned into the *Nde*I/*Hind*III sites of the plasmid pYH7 to generate the *her10* deletion plasmid, pYH7-her10.

The resulting plasmid was introduced into *S.* sp. L-9-10 through conjugation on ISP4 plate, and apramycin-resistant conjugants were selected at 30 ^{o}C. These conjugants were first grown on ISP4 plate with 50 μg/mL apramycin to obtain single-crossover mutants, which were further incubated on ISP4 plate without apramycin to generate apramycin-sensitive clones. The double-crossover mutants were selected by PCR analysis using the primers listed in Table S9 (her10-v-For and her10-v-Rev).

For *Δher10* mutant complementation, *her10* gene of *S.* sp. L-9-10 was amplified by PCR using the primer listed in Table S9 (her10-139-For and her10-139-Rev). The PCR product was ligated into pIB139 digested with *Nde*I using Gibson assembly. The resulting plasmid was introduced into *S.* sp. L-9-10 through conjugation to generate *Δher10: her10*.

7. Isolation of herbicidins and aureonuclemycin

S. sp. KIB-027 wild type and the gene deletion mutants, as well as the *anm* expression strain *S. lividans-anmBCDE* were fermented on a larger scale using the same culture procedure as described above.

To isolate compounds **1**, **2**, **7** and **10** from *S.* sp. KIB-027 wild type, a total of 3 L of culture was prepared for fermentation. The fermentation broth was centrifuged at 5000 rpm for 20 min. Then the supernatant was collected and 300 g of macro resin HP20 was added, followed by shaking at room temperature for 1 hr. The HP20 was applied to a column and eluted with H_2O/CH_3OH (90/10, 50/50, 10/90, 0/100). Four compounds were included in the eluate of H_2O/CH_3OH (10/90), which was evaporated to 30 mL and subjected to a new HP20 column eluted with H_2O/CH_3OH

(50/50, 10/9) to yield corresponding fractions. Fractions containing target components were evaporated to about 5 mL and performed HPLC semi-preparation over a 30 min gradient program. The program for compound **10**: T = 0 min, 20% B; T = 4 min, 20% B; T = 25 min, 70% B; T = 30 min, 20% B; (A = H_2O with 0.1 % TFA, B = CH_3OH, Flow rate = 3 mL/min). The program for compound **1**, **2**, **7**: T = 0 min, 35% B; T = 4 min, 35% B; T = 25 min, 85% B; T = 30 min, 35% B; (A = H_2O with 0.1 % TFA, B = CH_3OH, Flow rate = 3 mL/min). Finally, 11 mg of **1**, 18 mg of **2**, 25 mg of **7** and 8 mg of **10** were obtained.

To isolate compound **9** from *S. lividans-anmBCDE*, a total of 2 L of culture was prepared for fermentation. The fermentation broth was centrifuged at 5000 rpm for 20 min. An equal volume of methanol was added to the supernatant, and a large amount of precipitate was removed by centrifugation. The supernatant was concentrated to half volume and an equal volume of methanol was added again. The above experiments were repeated until the supernatant volume was concentrated to 50 mL in H_2O/CH_3OH (50/50). The crude concentrate was subjected to silica gel column (100-200 mesh) and eluted with H_2O/CH_3OH (50/50, 0/100). Fractions containing **9** were evaporated to about 3 mL and performed HPLC semi-preparation over a 20 min gradient program: T = 0 min, 20% B; T = 6 min, 20% B; T = 15 min, 30% B; T = 17 min, 20% B; T = 20 min, 20% B; (A = H_2O with 0.1 % TFA, B = CH_3OH, Flow rate = 3 mL/min). Finally, 12 mg of **9** was obtained.

To isolate compound **6** from *S.* sp. KIB-027-*ΔhbcF*, a total of 2 L of culture was prepared for fermentation. To isolate compounds **8** and **11** from *ΔhbcG*, a total of 3 L of culture was prepared for fermentation. To isolate compound **3** from *ΔhbcH*, a total of 2 L of culture was prepared for fermentation. To isolate compound **5** from *ΔhbcI*, a total of 2 L of culture was prepared for fermentation. The separation of these compounds is similar to that of herbicidins in the wild type. The program for HPLC semi-preparation for these compounds: T = 0 min, 20% B; T = 4 min, 20% B; T =20 min, 80% B; T = 25 min, 20% B; T = 30 min, 20% B; (A = H_2O with 0.1 % TFA, B = CH_3OH, Flow rate = 3 mL/min). Finally, about 10 mg of **6**, **8**, **3**, **5** and 3 mg of **11** were obtained.

Isolation of compounds **1** and **2** from *S.* sp. L-9-10 wild type was carried out as previously described.[4]

To isolate compounds **8** from *S.* sp. L-9-10 *Δher10* mutant, a 200 mL culture was prepared as

described above for fermentation. The fermentation broth was centrifuged at 4,000 *g* for 10 min. Then the supernatant was extracted by the same volume of EtOAc three times. The organic layers were combined and the solvent was evaporated to about 5 mL. HPLC semi-preparation was performed over a 30 min gradient program: T = 0 min, 10% B; T = 2 min, 10% B; T = 15 min 35% B; T = 17 min, 65 % B, T = 21 min, 65% B, T = 22 min, 10% B; (A = H_2O with 0.1% formic acid, B = acetonitrile with 0.1% formic acid). Finally, 10 mg of **8** was obtained.

To isolate compound **5** from *Streptomyces scopuliridis* RB72 wild type, a 200 mL culture was similarly prepared, except that the production culture was harvested after two days of fermentation. Compound **5** was isolated using the same extraction and HPLC methods described above. Finally, about 0.3 mg of compound **5** was obtained.

8. Hydrolysis of herbicidin K

An aqueous reaction mixture of 1 mM herbicidin K (**8**) and 100 mM LiOH was incubated at room temperature for 3 hrs. The reaction was then neutralized with formic acid and the mixture was then purified using HPLC to yield compounds **6** and **9**.

9. Protein expression and purification

Protein expression

The genes encoding individual proteins HbcF, HbcG, HbcH and HbcI were amplified by PCR from genomic DNA of *S.* sp. KIB-027 respectively. The primers for the amplification are listed in Table S9. The PCR products were confirmed by sequencing. Each gene was digested with *Nde*I/*Hind*III and ligated with pET28a to provide the plasmids for protein expression. For HbcF, HbcG and HbcH expression, plasmids pET28a-HbcF, pET28a-HbcG and pET28a-HbcH were transformed into *E. coli* BL21 (DE3), respectively. The cells were cultured in 1000 mL LB with 100 μg/mL kanamycin to an OD_{600} of 0.6 and induced with 0.1 mM IPTG. Then the cells were cultured for 24 hrs at 16°C. For HbcI expression, expression plasmid pET28a-HbcI was transformed into *E. coli* BL21 (DE3). The cells were cultured in 500 mL LB with 100 μg/mL kanamycin to an OD_{600} of 0.6 and followed by addition of 0.1 mM IPTG, 1 mM 5-aminolevulinic acid and 0.5 mM $FeSO_4$. Then the cells were cultured for 24 hrs at 16°C.

Her8 and Her10 proteins encoded by *her8* and *her10* genes of *S.* sp. L-9-10 were obtained as

previously described.[4] The gene encoding Her9 was amplified by PCR from genomic DNA of *S.* sp. L-9-10. The primers for amplification are listed in Table S9 (her9-pro-For and her9-pro-Rev). The PCR product was digested with *Nde*I/*Hind*III and ligated with pET28b digested with the same restriction enzymes. The resulting plasmid was confirmed by sequencing. For Her9 expression, plasmid pET28b-Her9 was transformed into *E. coli* BL21 (DE3). The cells were cultured in 2,000 mL LB with 50 μg/mL kanamycin to an OD_{600} of 0.5, followed by addition of 0.1 mM IPTG to induce protein expression. Then the cells were cultured for 24 hrs at 18 °C before purification.

Purification:

After 24 hours of incubation, cells were harvested by centrifugation (4, 000 rpm, 10 min) and resuspended in 30 mL of lysis buffer (50 mM Tris, pH 8.0, 10 mM imidazole, 500 mM NaCl). The cells are then lysed by ultra-sonication on ice. After centrifugation at 12, 000 rpm for 60 min, 3 mL of Ni-NTA resin was added to the supernatant and incubated at 4°C for 2 hrs. The Ni-NTA resin was collected by centrifugation (2, 000 rpm, 2 min) and eluted with 10 mL lysis buffer (50 mM Tris, pH 8.0, 10 mM imidazole, 500 mM NaCl). The resin was then eluted with elution buffers containing different concentrations of imidazole. The target protein was collected and concentrated to 2.5 mL and then exchanged into storage buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 10% glycerol).

For Her9 purification, cells after 24 hrs of incubation were harvested by centrifugation (4,000 *g*, 10 min) and resuspended in 60 mL of lysis buffer (50 mM HEPES, pH 7.5, 10 mM imidazole, 150 mM NaCl, 20% glycerol). The cells are then lysed by ultra-sonication on ice. After centrifugation at 13,000 *g* for 60 min, 2 mL of Ni-NTA resin was added to the supernatant and incubated at 4 °C for 2 hrs. The mixture was packed into a column. The flowthrough was discarded, and the resin was washed by 10 mL of wash buffer (50 mM HEPES, pH 7.5, 20 mM imidazole, 150 mM NaCl, 20% glycerol) twice. Her9 protein was then eluted by 10 mL of elution buffer (50 mM HEPES, pH 7.5, 250 mM imidazole, 150 mM NaCl, 20% glycerol), dialyzed against storage buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 20% glycerol), and concentrated to 3 mL.

10. Chemical synthesis of tiglyl-CoA and angelyl-CoA

The reactions were conducted under strictly anaerobic conditions. 8.0 mg of Tiglic acid (1 eq) was dissolved in 8 mL of oxygen-free THF and was transferred into the round-bottom flask

containing 31.2 mg of PyBOP (Benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate) (1 eq) and 8.28 mg of K_2CO_3 (1 eq), then 46 mg of CoASH (1 eq) was dissolved in 4 mL of oxygen-free H_2O and was added into the mixture described above. After stirring at room temperature for 3.5 hours, the reaction solvent was removed by rotary evaporation and the residue was dissolved in 4 mL of H_2O, Tiglic-CoA was detected when this aqueous solution was subjected to LC-MS with reversed-phase column (Syncronis aQ Dim, 250×4.6 mm). The column was equilibrated with 97% solvent A (H_2O + 1‰ TFA)/3% solvent B (CH_3CN + 1‰ TFA) for 5 min, the gradient of elution was as follows: 0-5 min: 97% A/3% B, 5-20 min: a linear gradient increase to 80% A/20% B, 20-22 min: a linear gradient increase to 40% A/60% B, 22-24 min: a linear gradient increase to 10% A/90% B, 24-26 min: maintain at the ratio of 10% A/90% B, 26-28 min: a linear gradient decrease to 97% A/3% B, 28-30 min: 97% A/3% B. Flow rate was of 1 mL•min^{-1} and detection wavelength was 210 nm. LC-MS of $C_{26}H_{43}N_7O_{17}P_3S^+$ ($[M]^+$), calculated 402.1633, found 402.1633. The produced Tiglic-CoA dissolved in H_2O was then subjected to reverse silica gel column chromatography for purification by elution with the mixture of H_2O and MeOH in different ratios, the radio of H_2O: MeOH gradient changes as 8:2→6:4→4:6→2:8→100% MeOH, the eluent was detected by LC-MS and Tiglic-CoA was eluted during ratio of 6:4→4:6→2:8→100% MeOH. The eluent was then subjected to lyophilization to get 42 mg of Tiglic-CoA in white powder.

Synthesis and purification of Angelic-CoA was conducted in the similar procedure.

11.Biochemical assays

For HbcF and HbcG-catalyzed methylation reactions, assays were performed in 50 μL reaction mixtures containing 50 mM PBS (pH 7.0), 2 mM SAM, 0.5 mM substrates. The reactions were initiated by adding 10 μM HbcF or HbcG. The enzymatic reactions were carried out at 30 °C for 8 hrs and quenched with 100 μL MeOH and stored at -80 °C until they would be subjected to analyses. After centrifugation at 12, 000 rpm for 10 min, the protein precipitate was removed and the supernatant was subjected to HPLC analysis using an analytic C18 column (Acclaim 120 C18, 5 μm, 4.6 × 250 mm) with UV detection at 260 nm under the following program: gradient elution of mobile phase A (H_2O supplemented with 0.1% formic acid) and mobile phase B (CH_3OH) using a flow rate of 0.8 mL/min: 0-4 min, 4% phase B; 4-25 min, 4%-80% phase B; 25-28 min, 80%

phase B; 28-30 min, 4% phase B. For HPLC-ESI-MS analysis, conditions are the same as HPLC analysis, and the extracted ion chromatograms (EICs) of molecular weight for the compounds were shown in the results.

For HbcH-catalyzed acylation reactions, assays were performed in 50 μL reaction mixtures containing 50 mM PBS (pH 7.0), 3 mM acyl donors, 0.5 mM substrates. The reactions were initiated by adding 10 μM HbcH. The enzymatic reactions were carried out at 30 °C for 8 hrs and quenched with 100 μL MeOH and stored at -80 °C until they would be subjected to analyses. After centrifugation at 12, 000 rpm for 10 min, the protein precipitate was removed and the supernatant was subjected to HPLC and LC-MS analysis using the same conditions as above.

For HbcH-catalyzed hydrolytic reactions, assays were performed in 50 μL reaction mixtures containing 50 mM PBS (pH 7.0), 0.5 mM substrates. The reactions were initiated by adding 10 μM HbcH, or 10 μM HbcH and 2 mM HSCoA, or protein storage buffer. The enzymatic reactions were carried out at 30 °C for 8 hrs and quenched with 100 μL MeOH and stored at -80 °C until they would be subjected to analyses. After centrifugation at 12, 000 rpm for 10 min, the protein precipitate was removed and the supernatant was subjected to HPLC and LC-MS analysis using the same conditions as above.

For Her8 and Her10-catalyzed methylation reactions, assays were performed in 100 μL reaction mixture containing 50 mM Tris-HCl (pH 7.5), 0.5 mM SAM, 0.1 mM substrates, and 1 μM enzyme. The reaction was carried out at room temperature (about 24 °C) for 24 hrs, and the enzyme was subsequently removed using a 10 kD MW cut off spin filter. The filtrate were then subjected to HPLC analysis using a reverse-phase column (Agilent, C18, 5 μm, 4.6 × 250 mm) with a two-phase elution system of mobile phase A (0.1% formic acid in water), and mobile phase B (0.1% formic acid in acetonitrile) using a flow rate of 1 mL/min: 0-5 min, 12.5% phase B; 5-20 min, 12.5-50% phase B; 20-22 min, 50-80% phase B; 27-30 min, 80-12.5% phase B. When compound **9** was used as the substrate, HPLC analysis was carried out using a flow rate of 1 mL/min: 0-5 min, 5% phase B; 5-20 min, 5-50% phase B; 20-22 min, 50-80% phase B; 27-30 min, 80-5% phase B.

For Her9-catalyzed acylation reactions, assays were performed in 100 μL reaction mixture containing 50 mM Tris-HCl (pH 7.5), 1 mM tiglyl-CoA, 0.1 mM substrates, and 1 μM Her9. The

reactions were carried out at 30 ºC for 16 hrs. Workup and analysis were the same as described above.

12.Feeding study analysis of Her11 activity

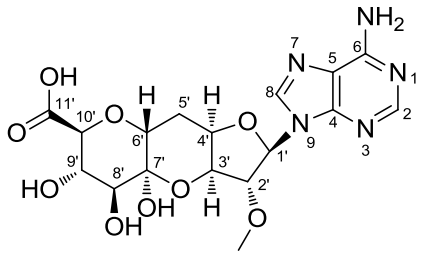
For pIB139-her11 construction, *her11* gene of *S.* sp. L-9-10 was amplified by PCR using the primer listed in Table S9 (her11-139-For and her11-139-Rev). The PCR product was ligated into pIB139 digested with *Nde*I using Gibson assembly. pIB139 empty vector and pIB139-her11 was introduced into *Streptomyces albus* J1074 by conjugation to generate J1074-pIB139 and J1074-pIB139-her11, respectively. For feeding study, 100 μL J1074-pIB139-her11 mycelia cell stock was seeded into 20 mL of TSB medium, and the culture was incubated at 28 ºC, 220 rpm for 3 days. Then 0.3 mL of the seeding culture was used to inoculate 5 mL of the fermentation medium (0.5% yeast extract, 0.5% malt extract, 1.5% soytone, 1% glucose, 0.3% sodium chloride, 1.5% glycerol, and 2% 3-morpholinopropane-1-sulfonic acid (MOPS) buffer at pH 7), and cultured at 28 ºC, 220 rpm for 3 days. Herbicidin F was subsequently added to the cultures to a final concentration of 0.05 mM, and the cultures were incubated at 28 ºC, 220 rpm for additional 2 days. The resulting cultures were prepared for HPLC analysis using the same method as described for *S.* sp. L-9-10 wild type production analysis. Control with J1074-pIB139 was performed in parallel.

References

1. Sambrook, J.; Fritsch, E. F.; Maniatis, T. Molecular cloning: a laboratory manual, 2nd edn. Cold Spring Harbor Laboratory, New York., 1989.
2. Bierman, M.; Logan, R.; O'Brien, K.; E. Seno, T.; R. Rao, N.; Schoner, B. E. *Gene*, **1992**, 116, 43–49.
3. Kieser, T.; Bibb, M. J.; Buttner, M. J.; Chater, K. F.; Hopwood, D. A. Practical Streptomyces Genetics, The John Innes Foundation, Norwich, UK, 2001.
4. Lin, G.-M.; Romo, A. J.; Liem, P. H.; Chen, Z.; Liu, H.-w. *J. Am. Chem. Soc.*, **2017**, 139, 16450–16453

Supplementary Tables:

Table S1 NMR data of 10 in CD_3OD (500 MHz for ^1H NMR and 125 MHz for ^{13}C NMR)



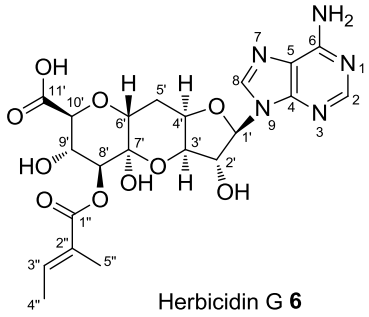
Herbicidin M **10**

No.	δ_C [ppm]	δ_H [ppm]	HSQC	COSY	HMBC	NOESY
2	146.65	8.38 (s)	C-2	-	C-4, C-6	
4	149.77	-	-	-	-	
5	119.37	-	-	-	-	
6	152.53	-	-	-	-	
8	144.84	8.88 (s)	C-8	-	C-4, C-5	H-1', H-2', H-6'
1'	89.34	6.21 (d)	C-1'	H-2'	C-4, C-8, C-2'	H-2', H-4', H-8, 2'-OC$\underline{H}_3$
2'	91.96	4.02 (s)	C-2'	H-1'	2'-O$\underline{C}H_3$, C-3', C-4'	H-1', H-8, H-3', 2'-OC$\underline{H}_3$
3'	74.08	4.46 (d)	C-3'	H-4'	C-1', C-4'	H-2', H-4', 2'-OC$\underline{H}_3$
4'	80.19	4.45 (m)	C-4'	H-3', H-5'	C-6'	H-5', H-3', H-1'
5'	26.32	2.25-2.20 (m)	C-5'	H-4', H-6'	C-4', C-6', C-7'	H-4', H-6',
6'	65.23	4.66 (dd)	C-6'	H-5'		H-5', H-8
7'	94.75	-	-	-	-	-
8'	71.42	3.71 (d)	C-8'	H-9'	C-7', C-10'	H-9'
9'	73.74	4.38 (dd)	C-9'	H-8'	C-8', C-7'	H-8', H-10'
10'	78.01	4.31 (br s)	C-10'		C-6', C-8', C-9', C-11'	H-9'
11'	173.08	-	-	-	-	
2'-OCH_3	58.42	3.47 (s)	2'-O$\underline{C}H_3$	-	C-2'	H-1', H-2', H-3',

Table S2 Predicted functions of genes in herbicidin (*hbc*) biosynthetic gene cluster and the comparison with aureonuclemycin (*anm*) cluster

Gene in *hbc*	Amino acids	Protein homolog by BLAST	Predicted function	*Gene in anm* (size, identity)
hbcR1	215	TetR family transcriptional regulator	Regulator	
hbcR2	43	XRE family transcriptional regulator	Regulator	
hbcR3	103	XRE family transcriptional regulator	Regulator	
hbcJ	123	short-chain dehydrogenase (incomplete)	Unknown	
hbcA	**328**	**ketoacyl-ACP synthase III**	**acyltransferase**	
hbcB	**552**	**hypothetical protein / S-adenosyl-L-homocysteine (SAH) hydrolase**	**oxidoreductase**	***anmB* (556, 56%)**
hbcC	**365**	**ABC transporter ATP-binding protein / oxidoreductase, NAD-binding domain protein**	**oxidoreductase**	***anmC* (366, 48%)**
hbcE	**435**	**B12-dependent radical SAM enzyme**	**skeleton formation**	***anmE* (421, 56%)**
hbcD	**375**	**gfo/Idh/MocA family oxidoreductase**	**oxidoreductase**	***anmD* (372, 62%)**
hbcF	**285**	**SAM-dependent methyltransferases**	**methyltransferase**	
hbcH	**544**	**serine hydrolase**	**hydrolase**	
hbcG	**368**	**SAM-dependent methyltransferases**	**methyltransferase**	
hbcI	**375**	**cytochrome P450**	**hydroxylase**	
hbcR4	940	transcriptional regulator, LuxR family	Regulator	
hbcR5	420	silent information regulator protein Sir2	Regulator	

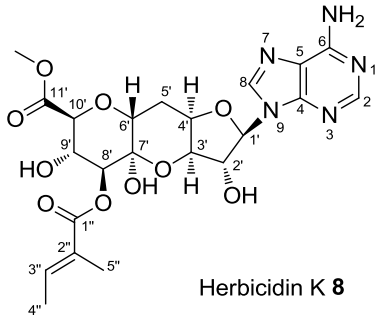
Table S3 NMR data of 6 in CD_3OD (500 MHz for ^1H NMR and 125 MHz for ^{13}C NMR)



Herbicidin G **6**

No.	δ_C [ppm]	δ_H [ppm]	HSQC	COSY	HMBC	NOESY
2	153.90	8.19 (s)	C-2		C-4, C-6	
4	150.64	-				
5	119.80	-				
6	157.37	-				
8	140.66	7.96 (s)	C-8		C-4, C-5	H-3'', H-5'', H-1', H-2', H-6'
1'	91.13	5.97 (d)	C-1'	H-2'	C-4, C-8, C-2'	H-2', H-4', H-8
2'	82.61	4.33 (br d)	C-2'	H-1'	C-3', C-1'	H-1', H-8
3'	77.86	4.33 (br d)	C-3'	H-4'	C-2', C-4'	H-5'$_b$, H-4'
4'	78.94	4.46 (m)	C-4'	H-3', H-5'$_a$, H-5'$_b$	C-6'	H-5'a, H-5'$_b$, H-3', H-1'
5'$_a$	26.66	2.34 (m)	C-5'	H-4', H-6' , H-5'$_b$	C-3', C-4', C-6', C-7'	H-5'$_b$, H-4', H-6'
5'$_b$	26.66	2.19 (m)	C-5'	H-4', H-6' , H-5'$_a$	C-6'	H-5'$_a$, H-4'
6'	66.12	4.63 (dd)	C-6'	H-5'$_a$, H-5'$_b$	C-5'	H-5'$_a$, H-3'', H-8
7'	93.57					
8'	72.04	4.99 (d)	C-8'	H-9'	C-6', C-7', C-9', C-10', C-1''	H-9'
9'	70.94	4.42 (dd)	C-9'	H-8', H-10',	C-8', C-7'	H-8', H-10'
10'	79.35	4.25 (br s)	C-10'	H-9'	C-6', C-8', C-9', C-11'	H-9'
11'	173.75					
1''	168.04					
2''	128.33					
3''	141.98	6.84 (q)	C-3''	H-4''	C-4'', C-5'', C-1''	H-4'', H-6', H-8
4''	15.13	1.87 (m)	C-4''	H-3''	C-2'', C-3''	H-3''
5''	12.38	1.85 (m)	C-5''		C-1'', C-2'', C-3''	H-8

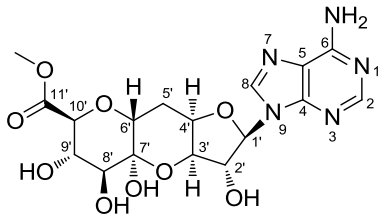
Table S4 NMR data of 8 in CD_3OD (500 MHz for ^1H NMR and 125 MHz for ^{13}C NMR)



Herbicidin K **8**

No.	δ_C [ppm]	δ_H [ppm]	HSQC	COSY	HMBC	NOESY
2	154.05	8.20 (s)	C-2		C-4, C-6	
4	150.68	-	-	-	-	
5	119.79	-	-	-	-	
6	157.41	-	-	-	-	
8	140.56	7.95 (s)	C-8		C-4, C-5	H-3'', H-5'', H-1', H-2', H-6'
1'	91.05	5.98 (d)	C-1'	H-2'	C-4, C-8, C-2'	H-2', H-4', H-8
2'	82.40	4.38 (d)	C-2'	H-1'	C-3', C-4'	H-1', H-8
3'	77.86	4.35 (d)	C-3'	H-4'	C-1', C-2', C-4'	H-5', H-4'
4'	78.90	4.49 (m)	C-4'	H-3', H-5'	C-3', C-5', C-6'	H-5', H-3', H-1'
5'	26.69	2.26 (m)	C-5'	H-4', H-6'	C-3', C-4', C-6', C-7'	H-4', H-3', H-6',
6'	66.64	4.51 (dd)	C-6'	H-5'	C-5'	H-5', H-3'', H-8
7'	93.23	-	-	-	-	-
8'	71.90	4.98 (d)	C-8'	H-9'	C-7' C-9', C-10', C-1''	H-9'
9'	70.45	4.31 (dd)	C-9'	H-8', H-10'	C-7', C-8', C-10'	H-8', H-10', 11'-OC$\underline{H}_3$
10'	78.26	4.44 (br s)	C-10'	H-9'	C-6', C-8', C-9', C-11'	H-9'
11'	171.21	-	-	-	-	-
1''	167.19	-	-	-	-	-
2''	128.37	-	-	-	-	-
3''	141.69	6.65 (q)	C-3''	H-4''	C-1'', C-4'', C-5''	H-4'', H-6', H-8, 11'-OC$\underline{H}_3$
4''	15.01	1.86 (d)	C-4''	H-3''	C-2'', C-3''	H-3''
5''	12.29	1.84 (s)	C-5''	-	C-1'', C-2'', C-3''	H-8, 11'-OC$\underline{H}_3$
11'-OCH_3	52.65	3.59 (s)	11'-O$\underline{C}H_3$	-	C-11'	H-9', H-3'', H-5''

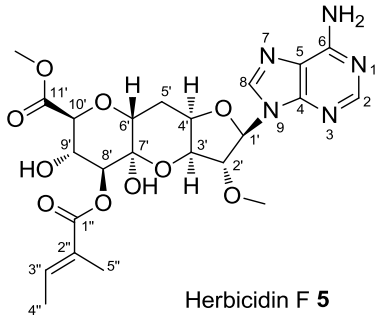
Table S5 NMR data of 3 in CD_3OD (500 MHz for ^1H NMR and 125 MHz for ^{13}C NMR)



Herbicidin C **3**

No.	δ_C [ppm]	δ_H [ppm]	HSQC	COSY	HMBC	NOESY
2	153.87	8.20 (s)	H-2		C-4, C-5, C-6	
4	150.37	-				
5	119.38	-				
6	157.15	-				
8	142.56	8.68 (s)	H-8		C-4, C-5	H-2', H-6'
1'	91.63	6.09 (d)	H-1'	H-2'	C-4, C-8, C-2'	H-2', H-4', H-8
2'	82.78	4.32 (br d)	H-2'	H-1'	C-3', C-4'	H-1', H-8
3'	77.32	4.30 (d)	H-3'	H-4'	C-1', C-2', C-4'	H-5', H-4'
4'	79.72	4.53 (m)	H-4'	H-3', H-5'	C-6'	H-5', H-3', H-1'
5'	26.52	2.24-2.21 (dd)	H-5'	H-4', H-6'	C-3', C-4', C-6', C-7'	H-4', H-3', H-6',
6'	65.57	4.65 (dd)	H-6'	H-5'	C-5'	H-5', H-8
7'	94.51	-				-
8'	71.21	3.69 (d)	H-8'	H-9'	C-6', C-7', C-9', C-10'	H-9'
9'	73.81	4.33 (dd)	H-9'	H-8'	C-7', C-8'	H-8', 11'-OC<u>H</u>$_3$
10'	78.01	4.35 (br, s)	H-10'		C-6', C-8', C-9', C-11'	
11'	171.81	-				
11'-OCH_3	52.32	3.70 (s)	2'-O<u>C</u>H_3		C-11'	H-9'

Table S6 NMR data of 5 in CD_3OD (500 MHz for ^1H NMR and 125 MHz for ^{13}C NMR)

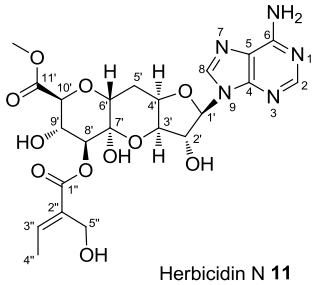


Herbicidin F **5**

No.	δ_C [ppm]	δ_H [ppm]	HSQC	COSY	HMBC	NOESY
2	154.04	8.22 (s)	C-2		C-4, C-6	
4	150.59	-	-	-	-	
5	119.89	-	-	-	-	
6	157.40	-	-	-	-	
8	140.64	7.95 (s)	C-8		C-4, C-5	H-5'', H-1', H-2', H-3', H-3'', H-6'
1'	88.87	6.05 (d)	C-1'	H-2'	C-4, C-8, C-2'	H-2', H-4', H-8, 2'-OC<u>H</u>$_3$
2'	91.83	4.07 (d)	C-2'	H-1'	C-3', C-4', 2'-O<u>C</u>H$_3$	H-1', H-8, H-3', 2'-OC<u>H</u>$_3$
3'	74.77	4.50 (d)	C-3'	H-4'	C-1', C-2', C-4'	H-5', H-2', H-4', 2'-OC<u>H</u>$_3$
4'	79.06	4.40 (m)	C-4'	H-3', H-5'	C-6'	H-5', H-3', H-1'
5'	26.68	2.25-2.27 (m)	C-5'	H-4', H-6'	C-3', C-4', C-6', C-7'	H-4', H-3', H-6'
6'	66.72	4.52 (dd)	C-6'	H-5'	C-5'	H-5', H-3'', H-8
7'	93.47	-	-	-	-	-
8'	72.00	5.00 (d)	C-8'	H-9'	C-6', C-7', C-9', C-10', C-1''	H-9'
9'	70.60	4.33 (dd)	C-9'	H-8', H-10'	C-7', C-8'	H-8', H-10'
10'	78.40	4.47 (br s)	C-10'	H-9'	C-6', C-8', C-9', C-11'	H-9'
11'	171.41	-	-	-	-	-
1''	167.35	-	-	-	-	-
2''	128.58	-	-	-	-	-
3''	141.88	6.70 (q)	C-3''	H-4''	C-1'', C-4'', C-5''	H-4'', H-6', H-8, 11'-OC<u>H</u>$_3$

4''	15.19	1.89 (br d)	C-4''	H-3''	C-2'', C-3''	H-3''
5''	12.44	1.86 (m)	C-5''	-	C-1'', C-2'', C-3''	H-8, 11'-OC<u>H</u>$_3$
2'-OCH$_3$	58.52	3.41 (s)	2'-O<u>C</u>H$_3$	-	C-2'	H-1', H-2', H-3'
11'-OCH$_3$	52.87	3.62 (s)	11'-O<u>C</u>H$_3$	-	C-11'	H-5'', H-3''
HCOOH (Contam.)	165.36	8.13	H<u>C</u>OOH			
CH$_3$OH (Contam.)	50.00	3.35	<u>C</u>H$_3$OH			

Table S7 NMR data of 11 in CD_3OD (500 MHz for ^1H NMR and 125 MHz for ^{13}C NMR)



Herbicidin N **11**

No.	δ_C [ppm]	δ_H [ppm]	HSQC	COSY	HMBC	NOESY
2	154.16	8.22 (s)	C-2		C-4, C-6	
4	150.68	-	-	-	-	
5	119.50 (by HMBC)	-	-	-	-	
6	157.37	-	-	-	-	
8	141.04	8.01 (s)	C-8		C-4, C-5	H-3'', H-6', H-2'
1'	90.82	6.01 (d)	C-1'	H-2'	C-8, C-2'	H-2', H-4'
2'	82.32	4.38 (br d)	C-2'	H-1'	C-3', C-4'	H-8, H-1'
3'	77.70	4.36 (d)	C-3'	H-4'	C-1', C-2', C-4'	H-4'
4'	79.06	4.52 (m)	C-4'	H-3', H-5'	C-6'	H-1', H-5', H-3'
5'	26.62	2.25 (m)	C-5'	H-4', H-6'	C-4', C-6'	H-4'
6'	66.59	4.53 (m)	C-6'	H-5'		H-8, H-3''
7'	93.27	-	-	-		
8'	71.94	5.06 (d)	C-8'	H-9'	C-7'	H-9'
9'	70.62	4.33 (dd)	C-9'	H-8', H-10'		H-8', H-10'
10'	78.31	4.46 (s)	C-10'	H-9'	C-6', C-8', C-9', C-11'	H-9'
11'	171.39	-	-	-	-	
1''	166.14	-	-	-	-	
2''	132.44	-	-	-	-	
3''	145.69	6.74 (q)	C-3''	H-4''	C-1'', C-2'', C-5'', C-4''	H-8, H-6', H-4'', H-11'-OC$\underline{H}_3$
4''	14.99	1.96 (d)	C-4''	H-3''	C-2'', C-3''	H-3'', H-5'', H-11'-OC$\underline{H}_3$
5''	56.34	4.41 (d)	C-5''	-	C-1'', C-2'', C-3''	H-4''
11'-OCH_3	52.82	3.62 (s)	11'-O$\underline{C}H_3$	-	C-11'	H-3'', H-4''

Table S8 Strains and plasmids used in this study

Strains/Plasmids	Characteristics*	Reference/source
Strains		
E. coli DH5α	Host for general cloning	Invitrogen
E. coli BL21 (DE3)	Host for protein expression	Invitrogen
E. coli S17-1	Donor strain for conjugation	1
E. coli ET12567/pUZ8002	Donor strain for conjugation	Gift from Dr. Wenjun Zhang
Streptomyces lividans 1326	Host for heterologous expression of *anm* cluster	1
Streptomyces sp. KIB-027	Herbicidins-producing strain, wild type	This work
Streptomyces aureus var. suzhoueusis	Aureonuclemycin-producing strain, wild type	2
Streptomyces sp. L-9-10	Herbicidin producing strain, wild type	3
Streptomyces. scopuliridis RB72	Herbicidin producing strain, wild type	4
S. lividans-anmBCDE	Heterologous expression strain of *anm* cluster	This work
S. sp. *ΔhbcA*	*hbcA* gene deletion mutant	This work
S. sp. *ΔhbcF*	*hbcF* gene deletion mutant	This work
S. sp. *ΔhbcG*	*hbcG* gene deletion mutant	This work
S. sp. *ΔhbcH*	*hbcH* gene deletion mutant	This work
S. sp. *ΔhbcI*	*hbcI* gene deletion mutant	This work
S. sp. *ΔhbcGI*	*hbcG* and *hbcI* double deletion mutant	This work
S. sp. *Δher10*	*her10* gene deletion mutant	This work
Δher10: her10	*her10* gene deletion mutant transformed with pIB139-her10	This work
Streptomyces albus J1074	Host for *her11* expression	Gift from Dr. Ben Shen
J1074-pIB139	*Streptomyces albus* J1074 with empty pIB139 vector	This work
J1074-pIB139-her11	*Streptomyces albus* J1074 with pIB139-her11 plasmid	This work
Plasmids		
pMD19-T simple	Ap^R, *E. coli* TA cloning vector	TaKaRa
pMD19-T	Ap^R, *E. coli* TA cloning vector	TaKaRa
pET28a	Km^R, protein expression vector in *E. coli*	Invitrogen
pET37b	Km^R, protein expression vector in *E. coli*	Invitrogen
pET28b	Km^R, protein expression vector in *E. coli*	Invitrogen
pIB139	Am^R, *E. coli-Streptomyces* shuttle vector for gene complementation and heterologous expression	Gift from Dr. Yuhui Sun
pYH7	Am^R, Ap^R, *E. coli-Streptomyces* shuttle vector for gene inactivation	5
pSET152	Am^R, *E. coli-Streptomyces* shuttle vector for gene complementation and heterologous expression	1
pKC1139	Am^R, *E. coli-Streptomyces* shuttle vector for gene inactivation	1
pSET-anmBCDE	pSET152 derivative for heterologous expression of *anmB-E*	This work
pKC1139-hbcA	pKC1139 derivative for in-frame gene deletion of *hbcA*	This work
pKC1139-hbcF	pKC1139 derivative for in-frame gene deletion of *hbcF*	This work
pKC1139-hbcG	pKC1139 derivative for in-frame gene deletion of *hbcG*	This work

pKC1139-hbcH	pKC1139 derivative for in-frame gene deletion of *hbcH*	This work
pKC1139-hbcI	pKC1139 derivative for in-frame gene deletion of *hbcI*	This work
pKC1139-hbcGI	pKC1139 derivative for gene deletion of *hbcG* and *hbcI*	This work
pET28a-HbcF	pET28a derivative containing *hbcF* gene for protein expression	This work
pET28a-HbcG	pET28a derivative containing *hbcG* gene for protein expression	This work
pET28a-HbcH	pET28a derivative containing *hbcH* gene for protein expression	This work
pET28a-HbcI	pET28a derivative containing *hbcI* gene for protein expression	This work
pYH7-her10	pYH7 derivative for in-frame gene deletion of *her10*	This work
pIB139-her10	pIB139 derivative for *S.* sp. L-9-10 *Δher10* complementation	This work
pIB139-her11	pIB139 derivative for heterologous expression of Her11	This work
pET28b-Her9	pET28b derivative containing *her9* gene for protein expression	This work

Abbreviations: Ap^R, ampicillin resistance; Km^R, kanamycin resistance; Am^R, apramycin resistance.

[1] T. Kieser, M. J. Bibb, M. J. Buttner, K. F Chater, D. A. Hopwood, *Practical Streptomyces Genetics*, The John Innes Foundation, Norwich, UK, 2001.

[2] X. Dai, G. Li, Z. Wu, D. Lu, H. Wang, Z. Li, L. Zhou, X. Chen, W., Chen, *Chem. Abstr.* **1989**, 111, 230661f.

[3] X. Chai, U. J. Youn, D. Sun, J. Dai, P. Williams, T. P. Kondratyuk, R. P. Borris, J. Davies, I. G. Villanueva, J. M. Pezzuto, L. C. Chang, *J. Nat. Prod.* **2014**, 77, 227–233

[4] M. H. Farris, C. Duffy, R. H. Findlay, J. B. Olson, *Int. J. Syst. Evol. Microbiol.* **2011**, 61, 2112–2116

[5] Y. Sun, X. He, J. Liang, X. Zhou, Z. Deng, *Appl. Microbiol. Biotechnol.* **2009**, 82, 303–310.

Table S9 Primers used in this study

Primer	**Sequence**
anmBE-For	TAAAAGCTTATCGGGTCAATGCCAAGTCG (*Hind*III)
anmBE-Rev	TAATCTAGAGCCCAGTAGAGGAGGGAGAAGG (*Xba*I)
hbcA-L-For	AAGCTTGACATGCGCGCTGTCACAG (*Hind*III)
hbcA-L-Rev	TCTAGACCATTCCGACGTGGTGAGC (*Xba*I)
hbcA-R-For	TCTAGAAGCCACCCAGTCTTCCGGAG (*Xba*I)
hbcA-R-Rev	GAATTCGCTGGACCTCGTGATGGAC (*EcoR*I)
hbcA-v-For	TGATGACATCGTGGAGTTC
hbcA-v-Rev	GGGCTCATAGGGGTTGTAG
hbcF-L-For	AAGCTTTTCGACTGCGCTCAGCAAG (*Hind*III)
hbcF-L-Rev	TCTAGAGAACTGGACTCCCTCAAG (*Xba*I)
hbcF-R-For	TCTAGACCCGACCCGTTCGTAGGTC (*Xba*I)
hbcF-R-Rev	GAATTCGGGTGATCGCCGAAGTGGAG (*EcoR*I)
hbcF-v-For	TCAGGATGTCGAGCGCGTC
hbcF-v-Rev	GGACCGCATCGGCCTGCTG
hbcG-L-For	AAGCTTCTGTCTTGAGTGCGCAGAG (*Hind*III)
hbcG-L-Rev	CTCGAGCCCGAATACATGGTCGTC (*Xho*I)
hbcG-R-For	CTCGAGGATCGCGTGTGGAGATCC (*Xho*I)
hbcG-R-Rev	GAATTCGGAACTGGACTCCCTCAAG (*EcoR*I)
hbcG-v-For	CGGTCGCCGGTCGGCGCGAGCACA
hbcG-v-Rev	ACACGGCCTTCAGCCAATACGCCT
hbcH-L-For	AAGCTTCCAGTACCTGGCAGATCGTC (*Hind*III)
hbcH-L-Rev	TCTAGATACGGACTATGGACGTCCGTG (*Xba*I)
hbcH-R-For	TCTAGACGGCGAGAACAGTGCGTAC (*Xba*I)

hbcH-R-Rev	GAATTCGACGCCGTCCTGCTGTGCAC (*EcoR*I)
hbcH-v-For	TCCGCGACTGAGTCATTC
hbcH-v-Rev	TCACCATGACCTCGAAAG
hbcI-L-For	AAGCTTAGCTCCCTCATCTCCATG (*Hind*III)
hbcI-L-Rev	CTCGAGGCACTGGAAGAACTGGTC (*Xho*I)
hbcI-R-For	CTCGAGGTGACGAGCCAGAGACGGT (*Xho*I)
hbcI-R-Rev	TCTAGACCTCGGCAAGGAGTACAC (*Xba*I)
hbcI-v-For	GAGAAATTACAGGCCAAAT
hbcI-v-Rev	GAAATCAGGCGGTTGCCGC
hbcGI-v-For	AACAGACGTCGCCCGCCGCGAAGA
hbcGI-v-Rev	ACACGGCCTTCAGCCAATACGCCT
hbcF-pro-For	CATATGAGCGACGTCATGCACTAC (*Nde*I)
hbcF-pro-Rev	AAGCTTTTACTCGAGGGACCTCTTGGCCAGAGTG (*Xho*I *Hind*III)
hbcG-pro-For	CATATGACTCAGTCGCGGAATG (*Nde*I)
hbcG-pro-Rev	AAGCTTTTACTCGAGTTTGACGCCGACGACCATG (*Xho*I *Hind*III)
hbcH-pro-For	CATATGGGGACGGCTCCCGCCAGGA (*Nde*I)
hbcH-pro-Rev	AAGCTTTTACTCGAGCACAAGGCCGAGATCGTGA (*Xho*I *Hind*III)
hbcI-pro-For	CATATGCTCCCCTACCTCCCAC (*Nde*I)
hbcI-pro-Rev	AAGCTTTTACTCGAGCCAGGACACCGGGAGAGTG (*Xho*I *Hind*III)
her10-L-For	GGGCATATGAGGCCGGTTCGGGGTGTCCTGGTGG (*Nde*I)
her10-L-Rev	GGGAGATCTGTCGATGATGGCGTGTGGAGATCCG (*Bgl*II)
her10-R-For	GGGAGATCTGCCCAGTGCCGGGACTGGCTGACCG (*Bgl*II)
her10-R-Rev	GGGAAGCTTCACCGCAATCCGAACCTCGTGGTTA (*Hind*III)
her10-v-For	CGGCAAGGAGTACACAGCATT
her10-v-Rev	ACACGCACCACGTCCGGTAC
her9-pro-For	CACCATATGATGGGGACGGCTCCCGCCAG (*Nde*I)
her9-pro-Rev	CACAAGCTTTCACGCAAGGCCGAGATCGT (*Hind*III)
her10-139-For	TTGGTAGGATCCACATATGACTCAGCCGCGGAATGACGCCG
her10-139-Rev	AGGATCCCCAACATACTACTTGACGCCGACGACCATGTAC
her11-139-For	TTGGTAGGATCCACATATGACGACCGACGAAGGCGAG
her11-139-Rev	AGGATCCCCAACATATCACCATGACACGGGGAGAGT

Supplementary Figures

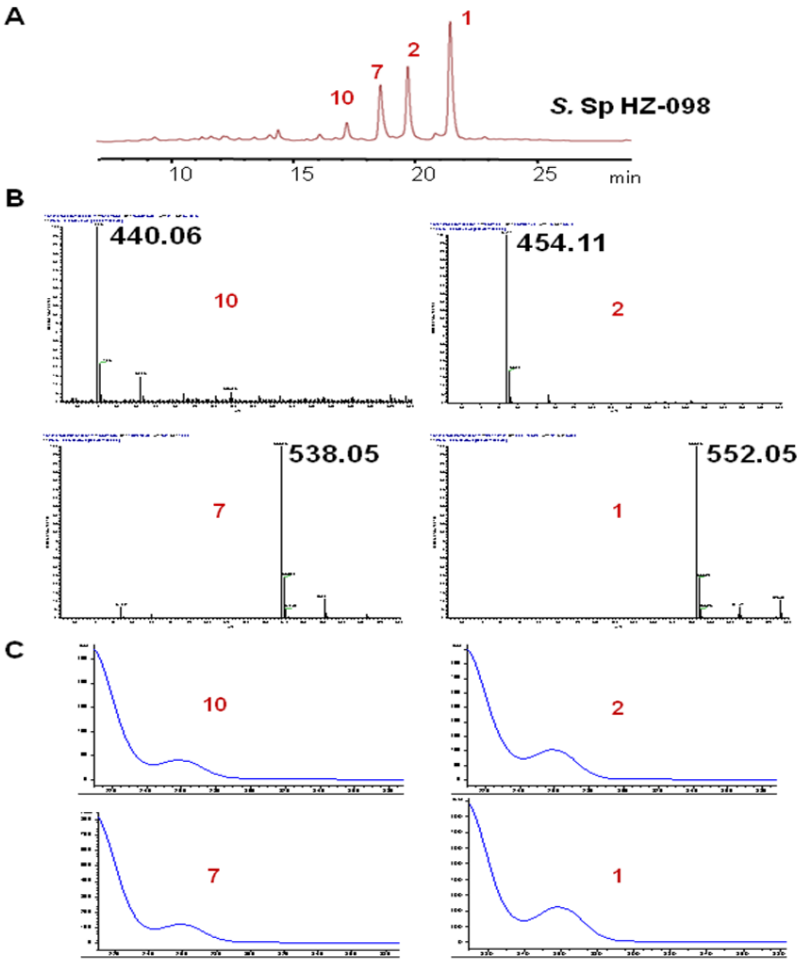


Figure S1 LC-MS analysis of *S.* sp KIB-027 fermentation broth.

A) HPLC profile; B) Mass spectra for compounds 1, 2, 7 and 10; C) UV spectra for compounds 1, 2, 7 and 10.

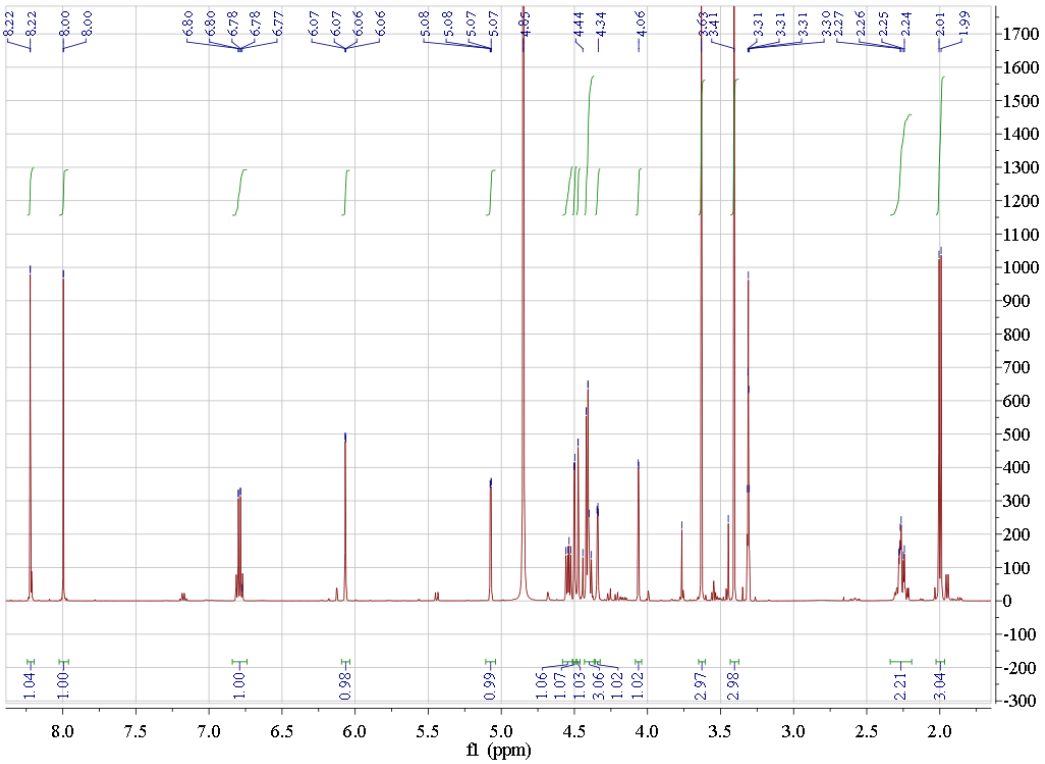


Figure S2 ^{1}H NMR spectrum of 1 in CD_3OD

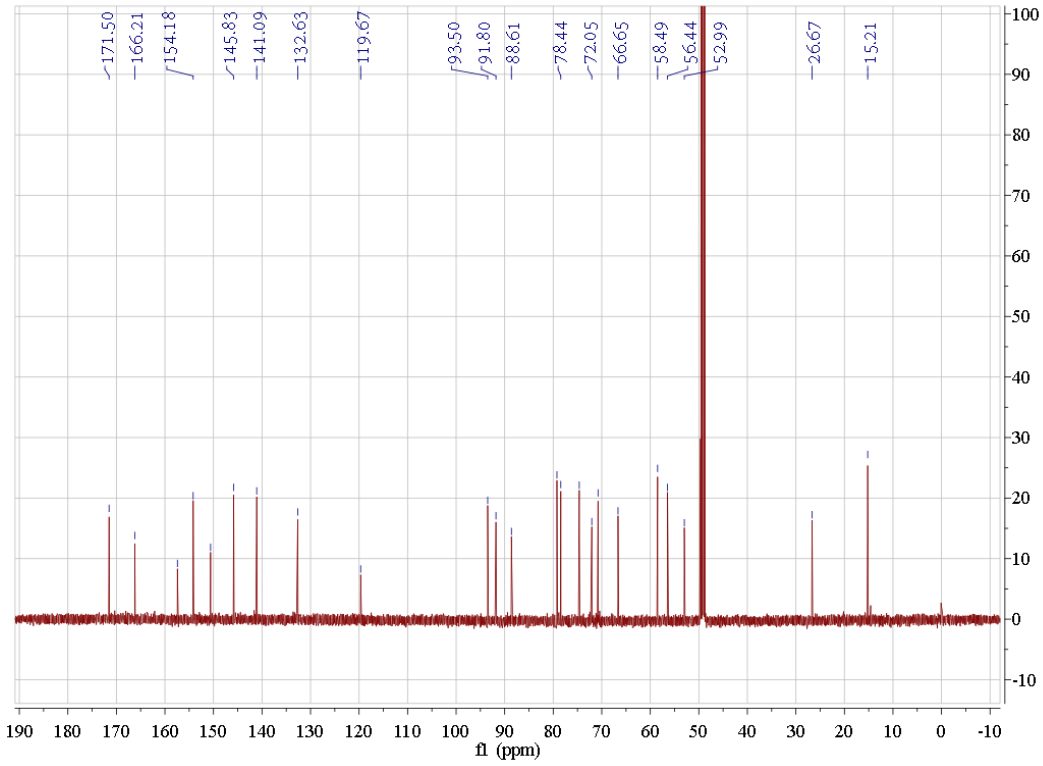
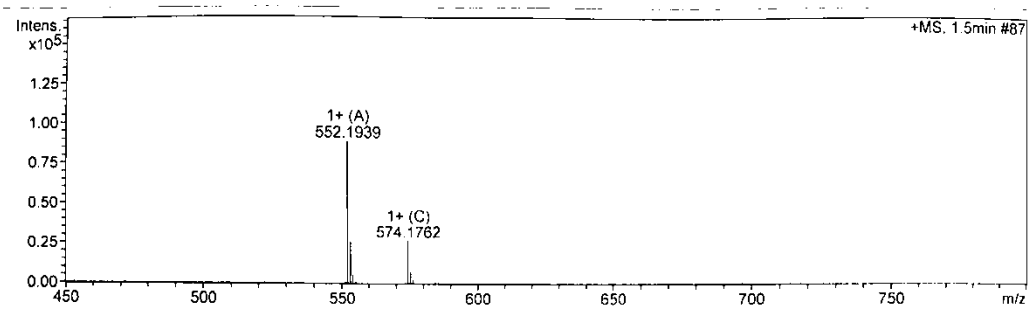


Figure S3 ^{13}C NMR spectrum of 1 in CD_3OD



Meas. m/z	#	Formula	Score	m/z	err [ppm]	Mean err [ppm]	mSig ma	rdb	e⁻ Conf	N-R ule
552.1939	1	C 23 H 30 N 5 O 11	100.00	552.1936	-0.5	-0.1	12.0	11.5	even	ok
574.1762	1	C 23 H 29 N 5 Na O 11	100.00	574.1756	-1.0	-0.6	13.2	11.5	even	ok

Figure S4 HRMS data of 1

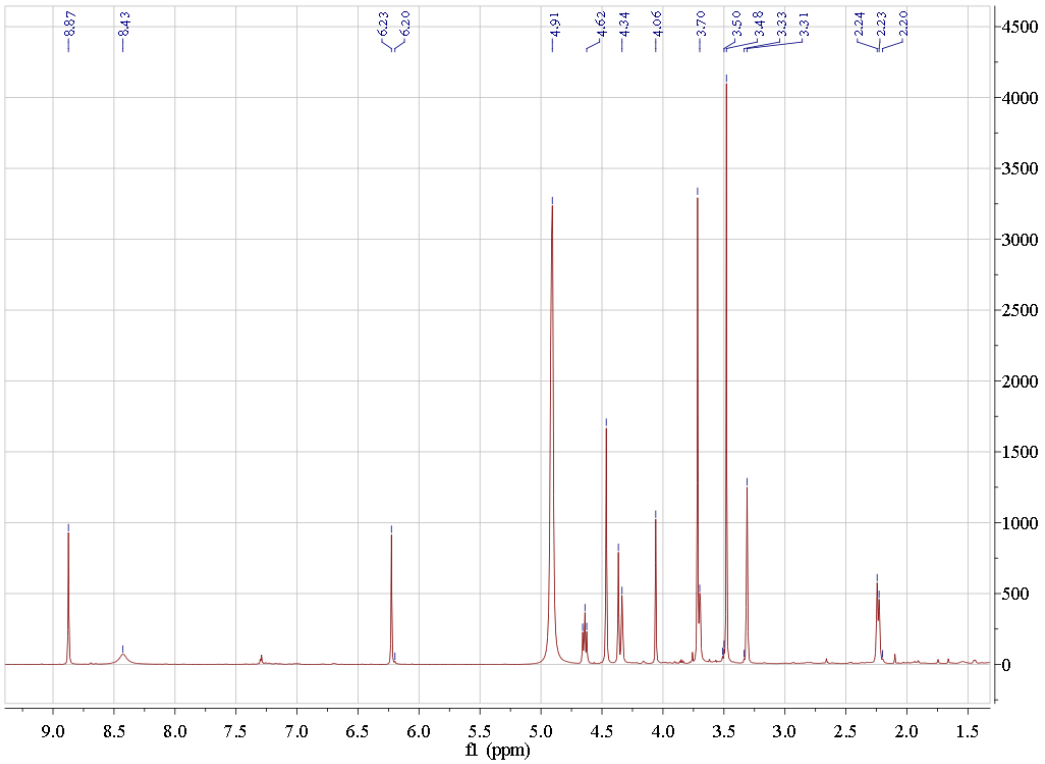


Figure S5 ^{1}H NMR spectrum of 2 in CD_3OD

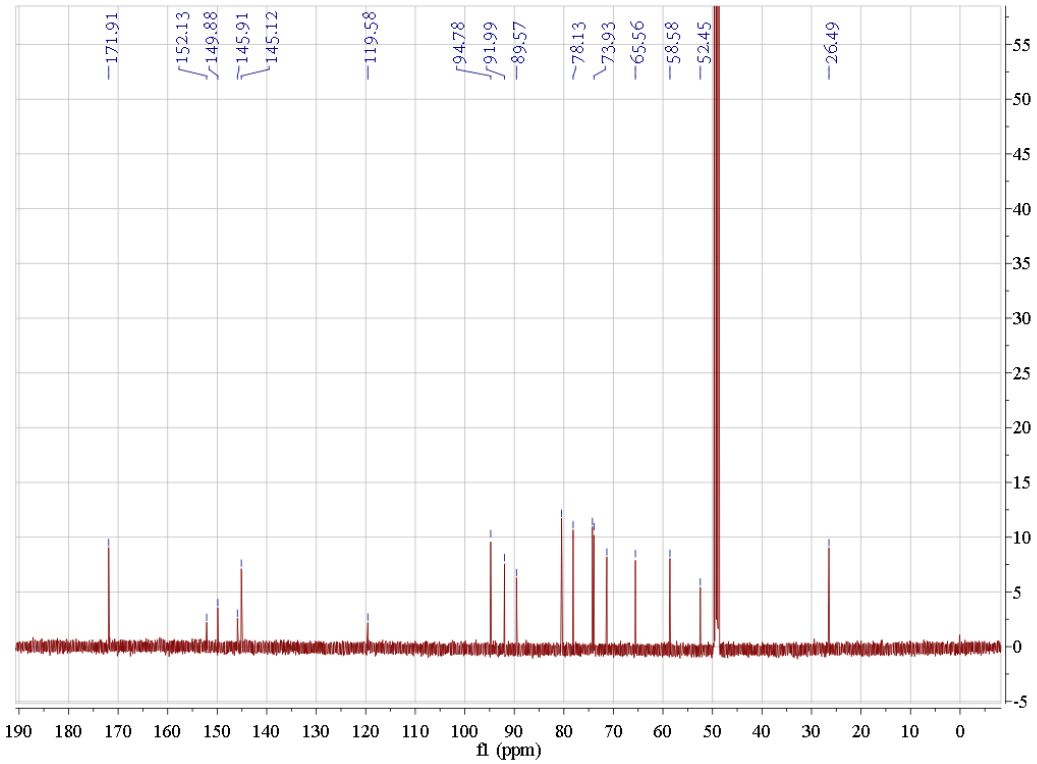
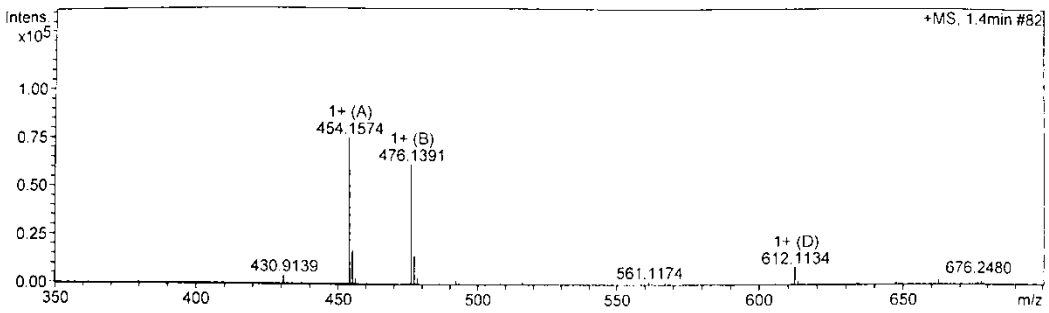


Figure S6 ^{13}C NMR spectrum of 2 in CD_3OD



Meas. m/z	#	Formula	Score	m/z	err [ppm]	Mean err [ppm]	mSigma	rdb	e⁻ Conf	N-Rule
454.1574	1	C 18 H 24 N 5 O 9	100.00	454.1569	-1.2	-1.0	7.3	9.5	even	ok
476.1391	1	C 18 H 23 N 5 Na O 9	100.00	476.1388	-0.6	-0.5	12.1	9.5	even	ok

Figure S7 HRMS data of 2

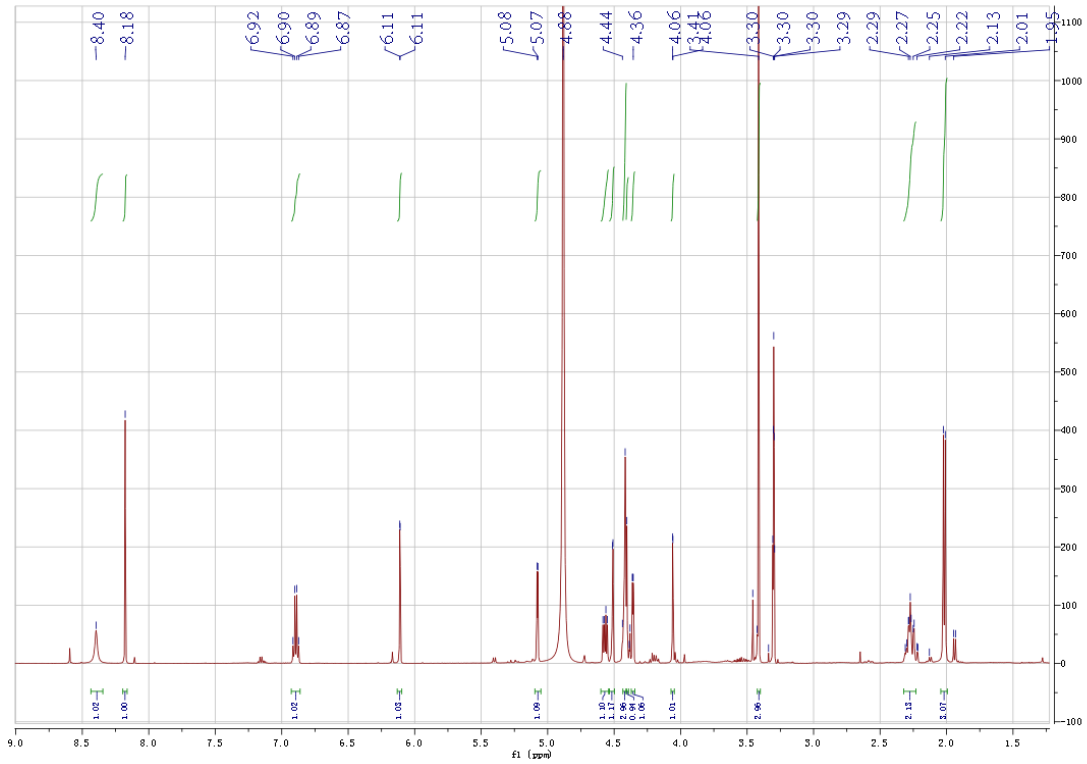


Figure S8 ^{1}H NMR spectrum of 7 in CD_3OD

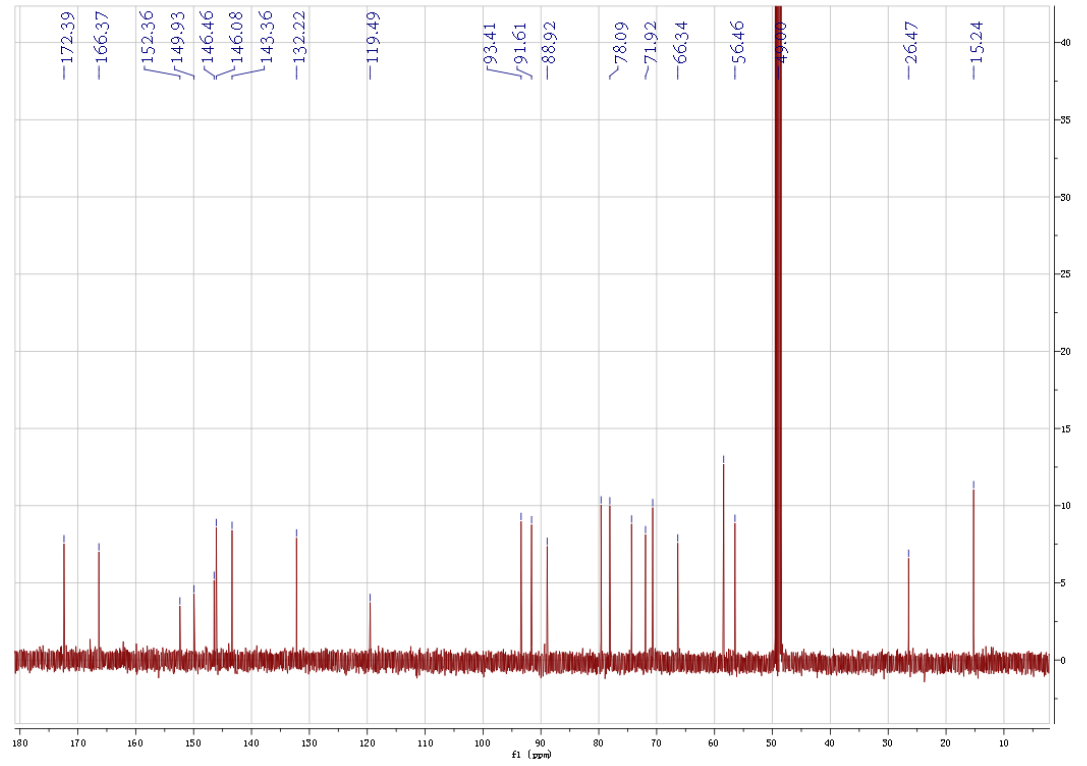


Figure S9 ^{13}C NMR spectrum of 7 in CD_3OD

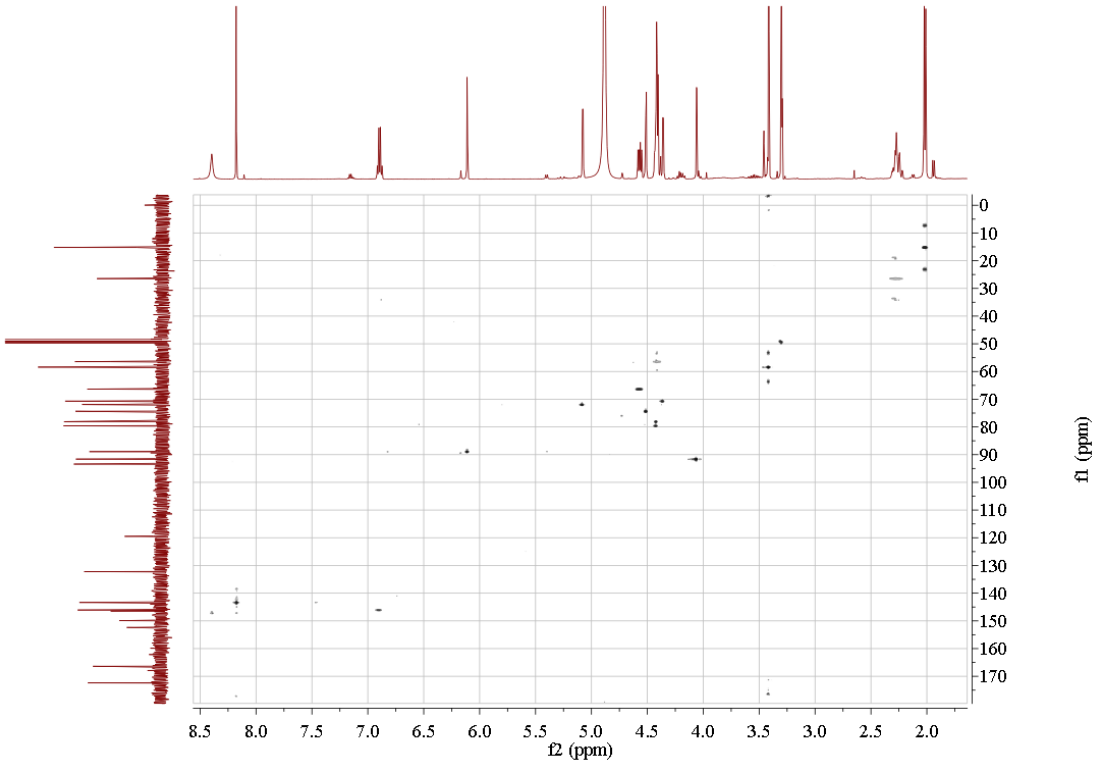


Figure S10 HSQC spectrum of 7 in CD_3OD

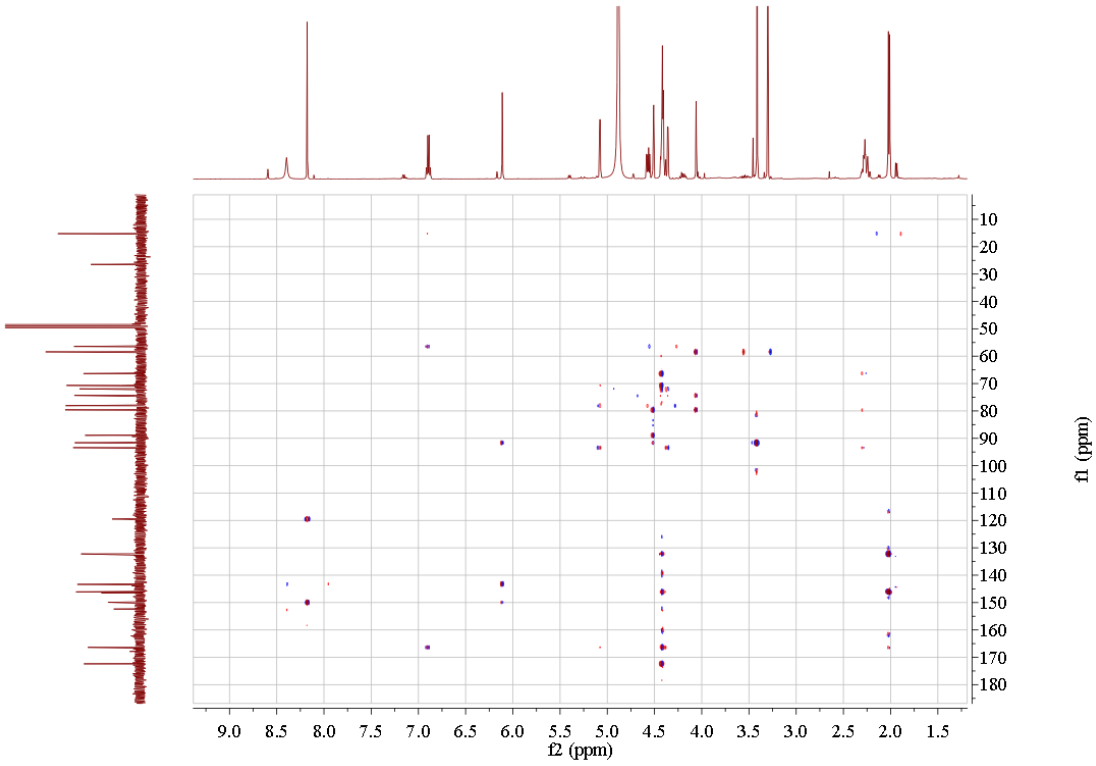


Figure S11 HMBC spectrum of 7 in CD_3OD

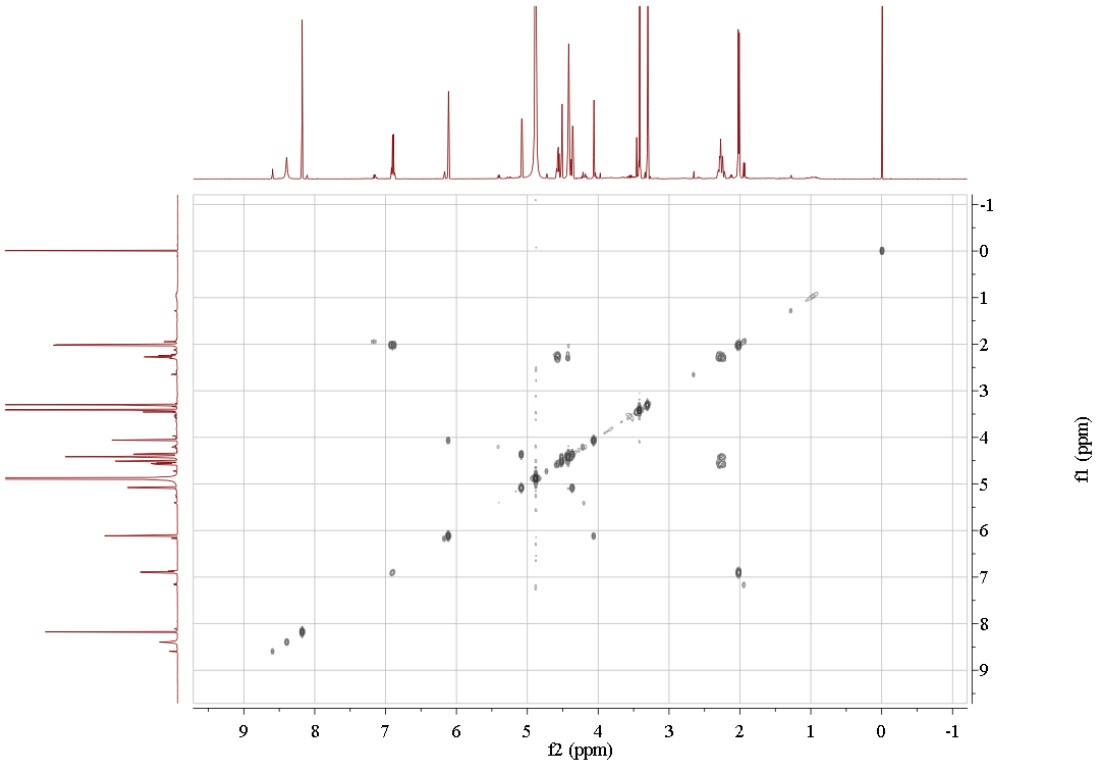
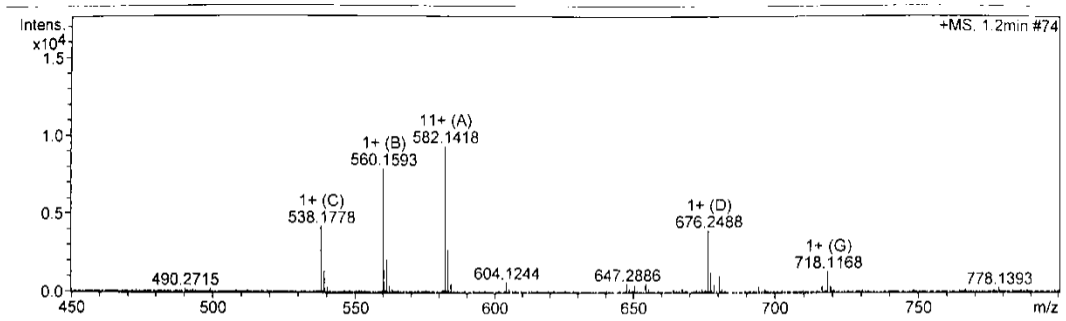


Figure S12 ^{1}H-^{1}H COSY spectrum of 7 in CD_3OD



Meas. m/z	#	Formula	Score	m/z	err [ppm]	Mean err [ppm]	mSigma	rdb	e⁻ Conf	N-Rule
538.1778	1	C 22 H 28 N 5 O 11	100.00	538.1780	0.3	1.1	36.4	11.5	even	ok
560.1593	1	C 22 H 27 N 5 Na O 11	100.00	560.1599	1.1	1.0	5.6	11.5	even	ok

Figure S13 HRMS data of 7

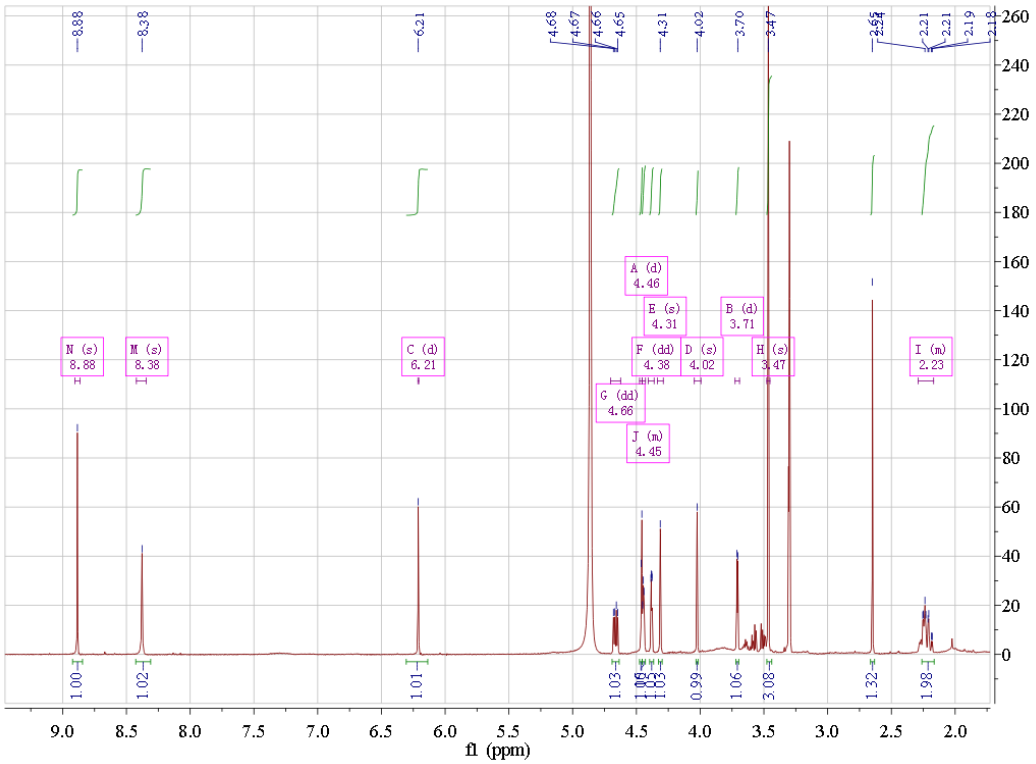


Figure S14 ^{1}H NMR spectrum of 10 in CD_3OD

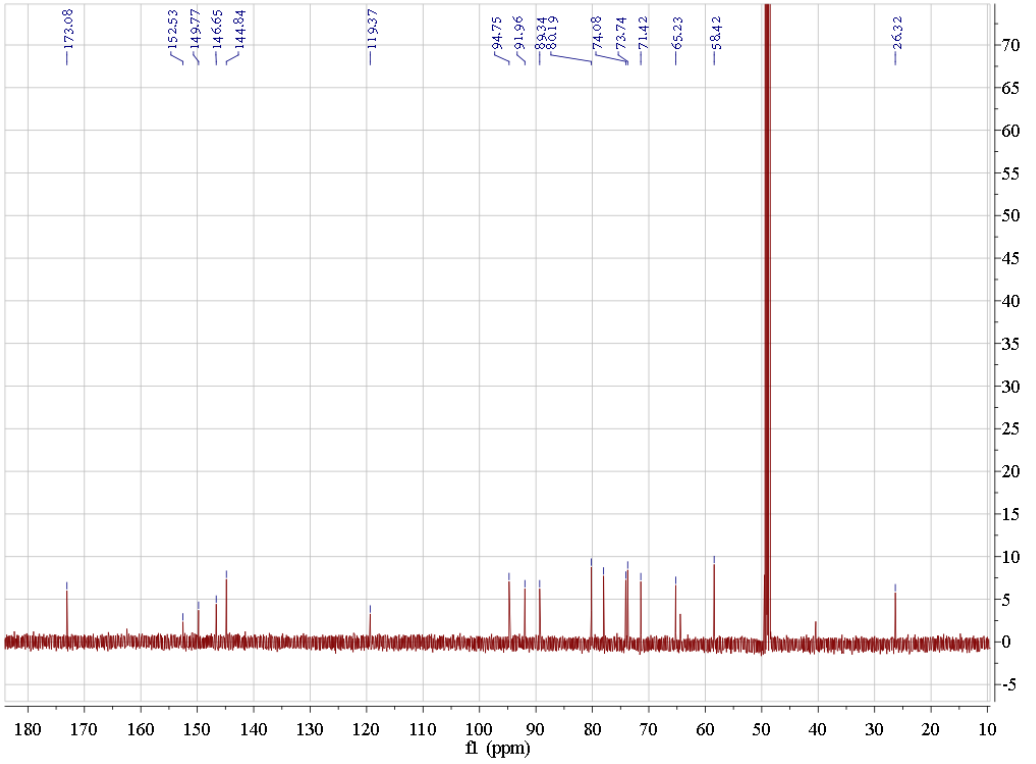


Figure S15 ^{13}C NMR spectrum of 10 in CD_3OD

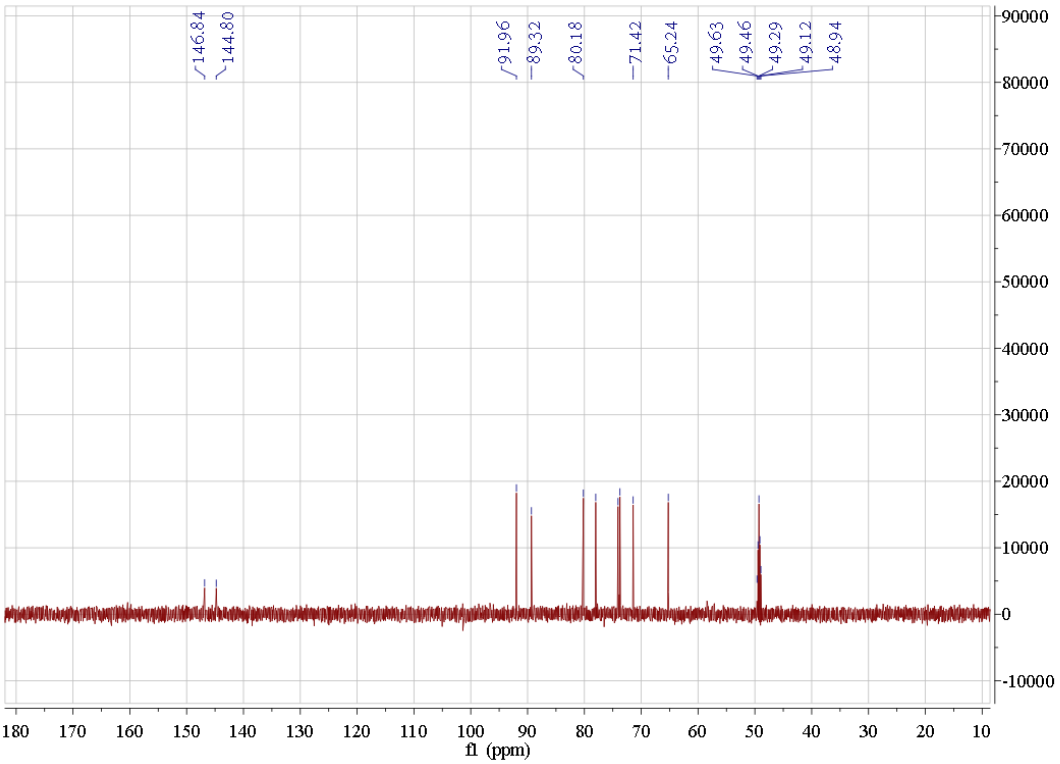


Figure S16 DEPT90 spectrum of 10 in CD_3OD

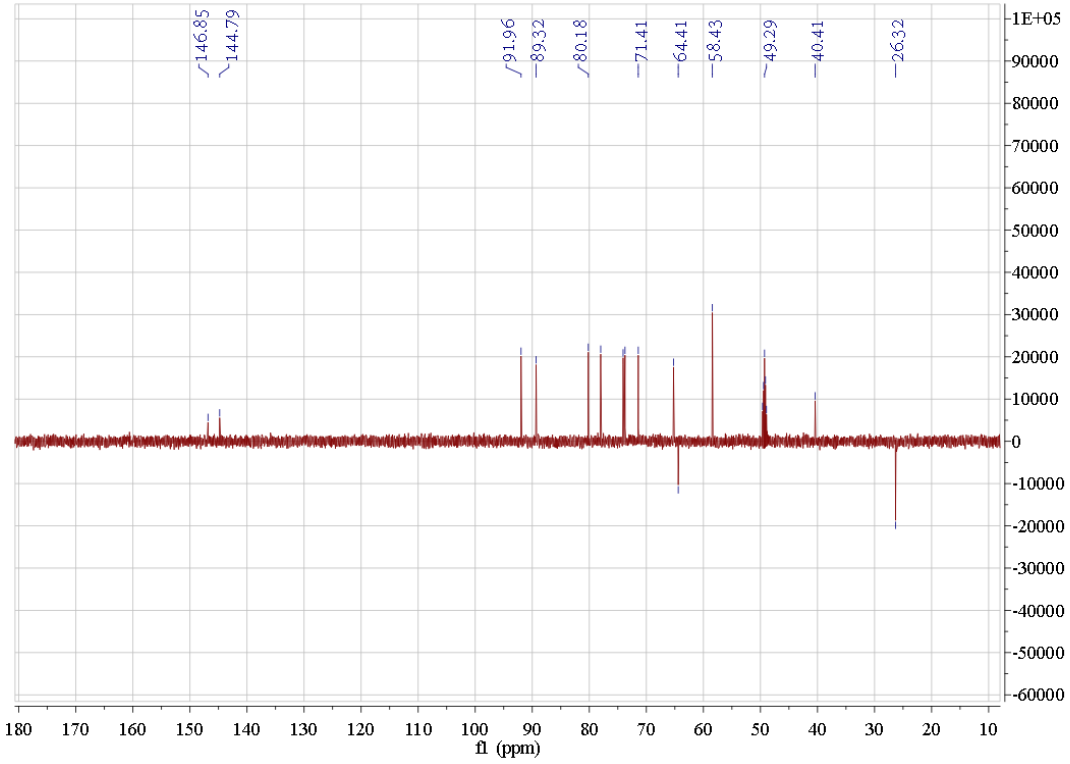


Figure S17 DEPT135 spectrum of 10 in CD_3OD

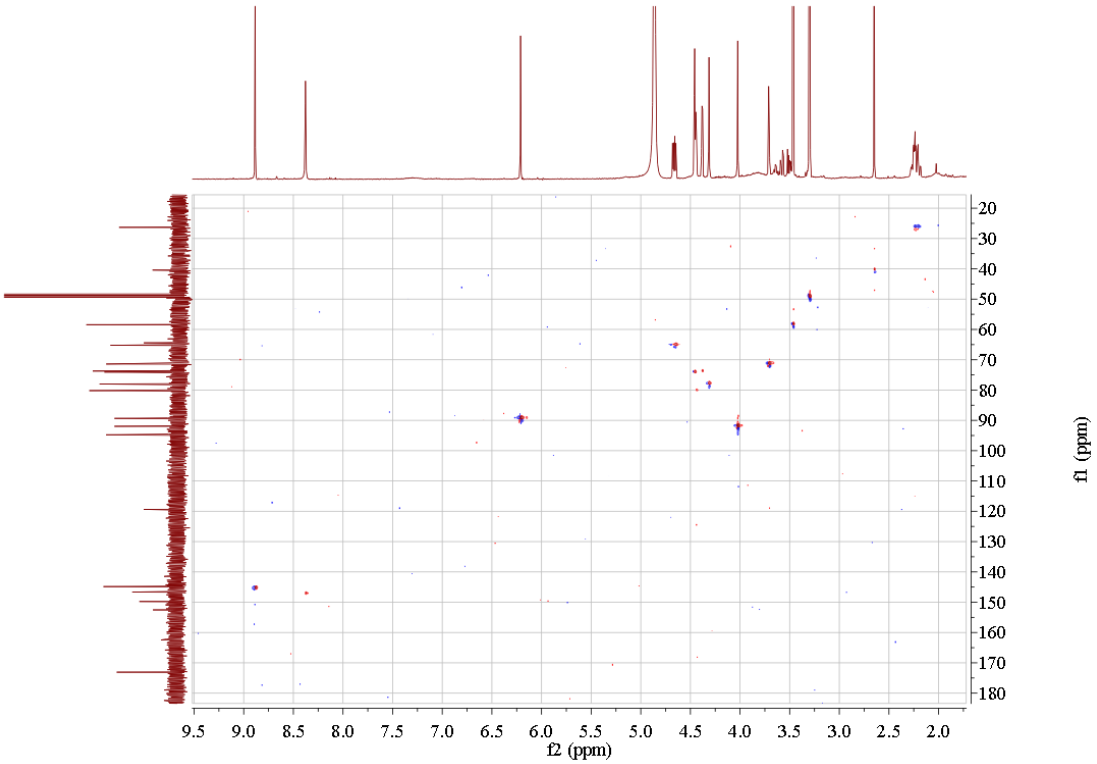


Figure S18 HSQC spectrum of 10 in CD_3OD

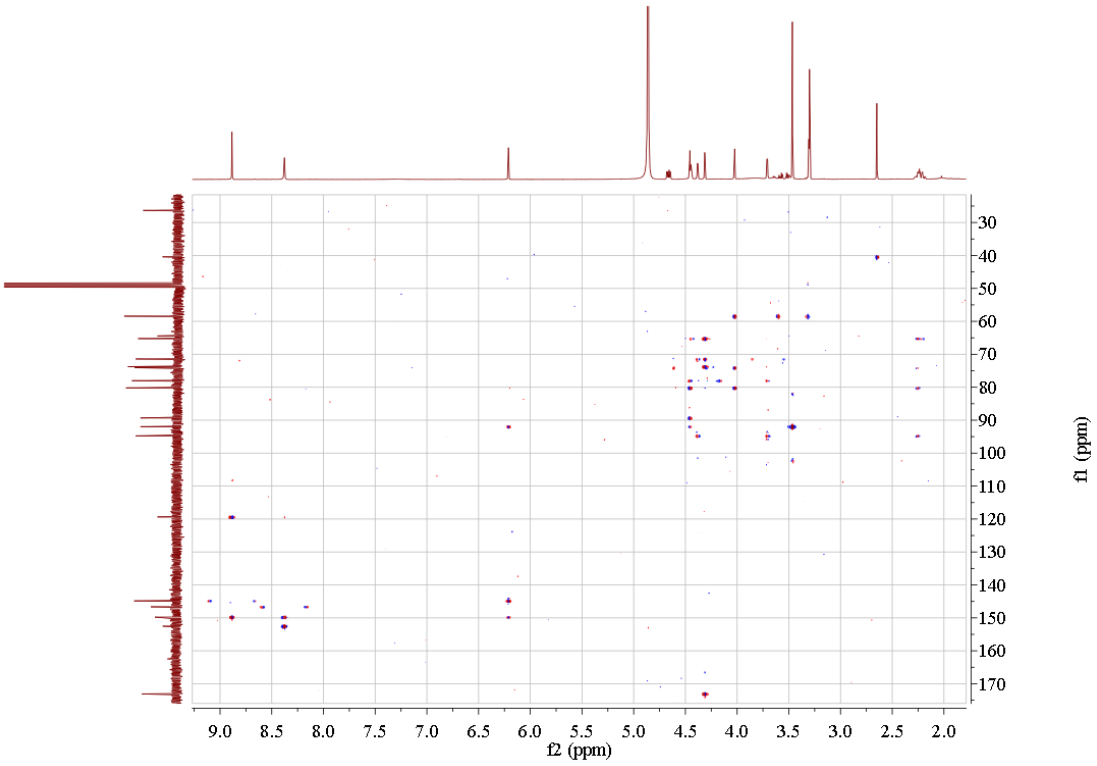


Figure S19 HMBC spectrum of 10 in CD_3OD

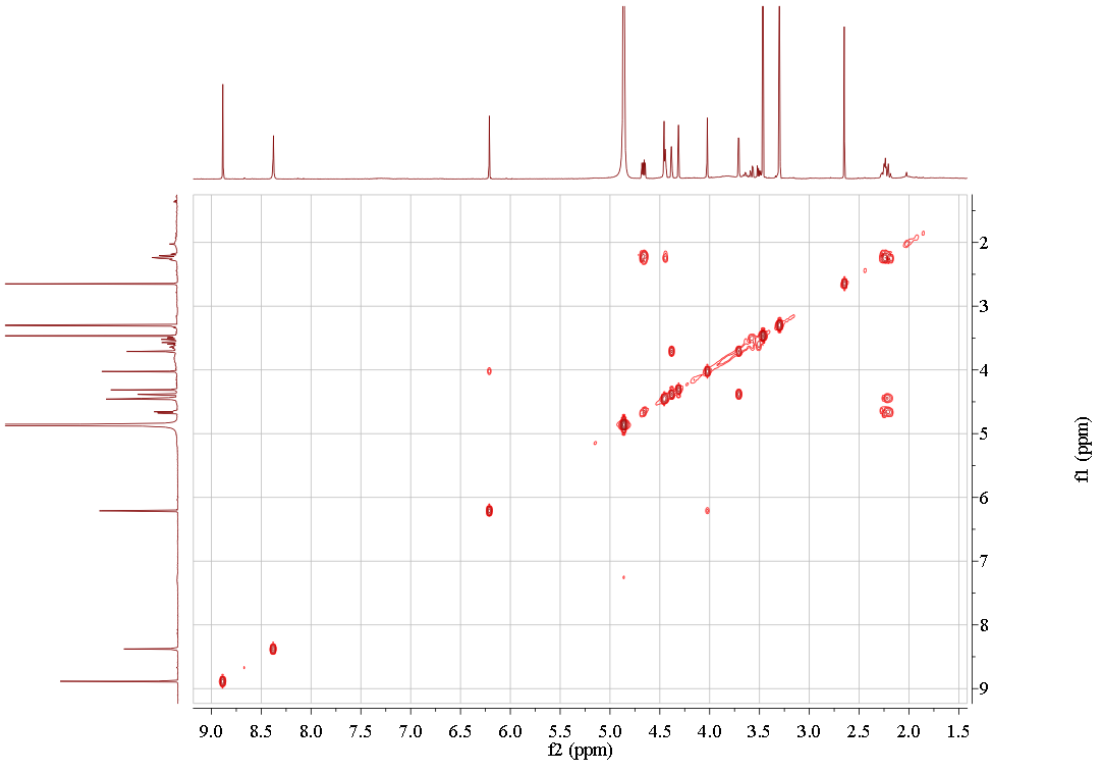


Figure S20 ^{1}H-^{1}H COSY spectrum of 10 in CD_3OD

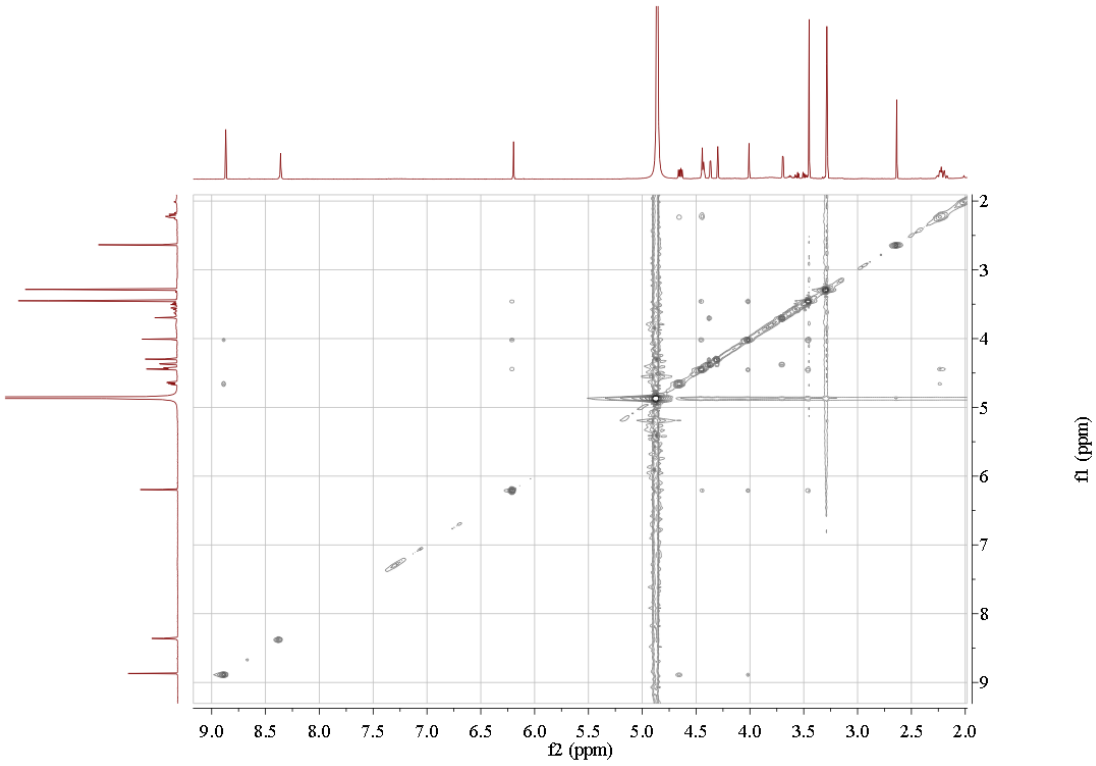


Figure S21 NOESY spectrum of 10 in CD_3OD

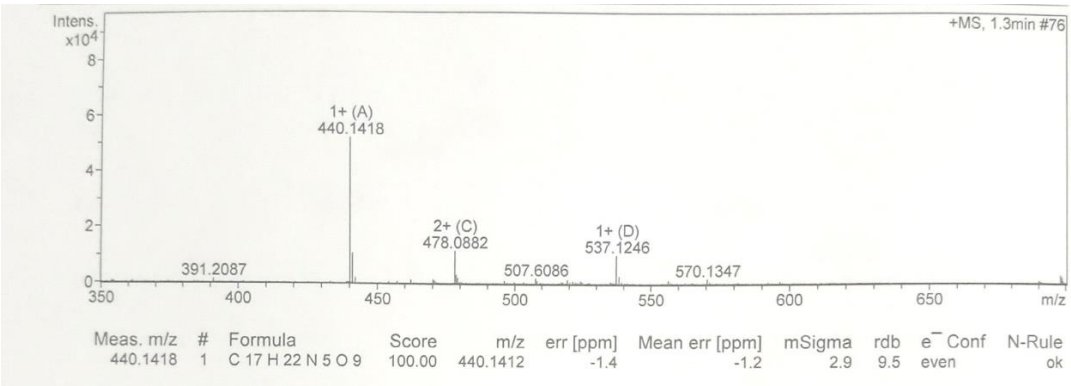


Figure S22 HRMS data of 10

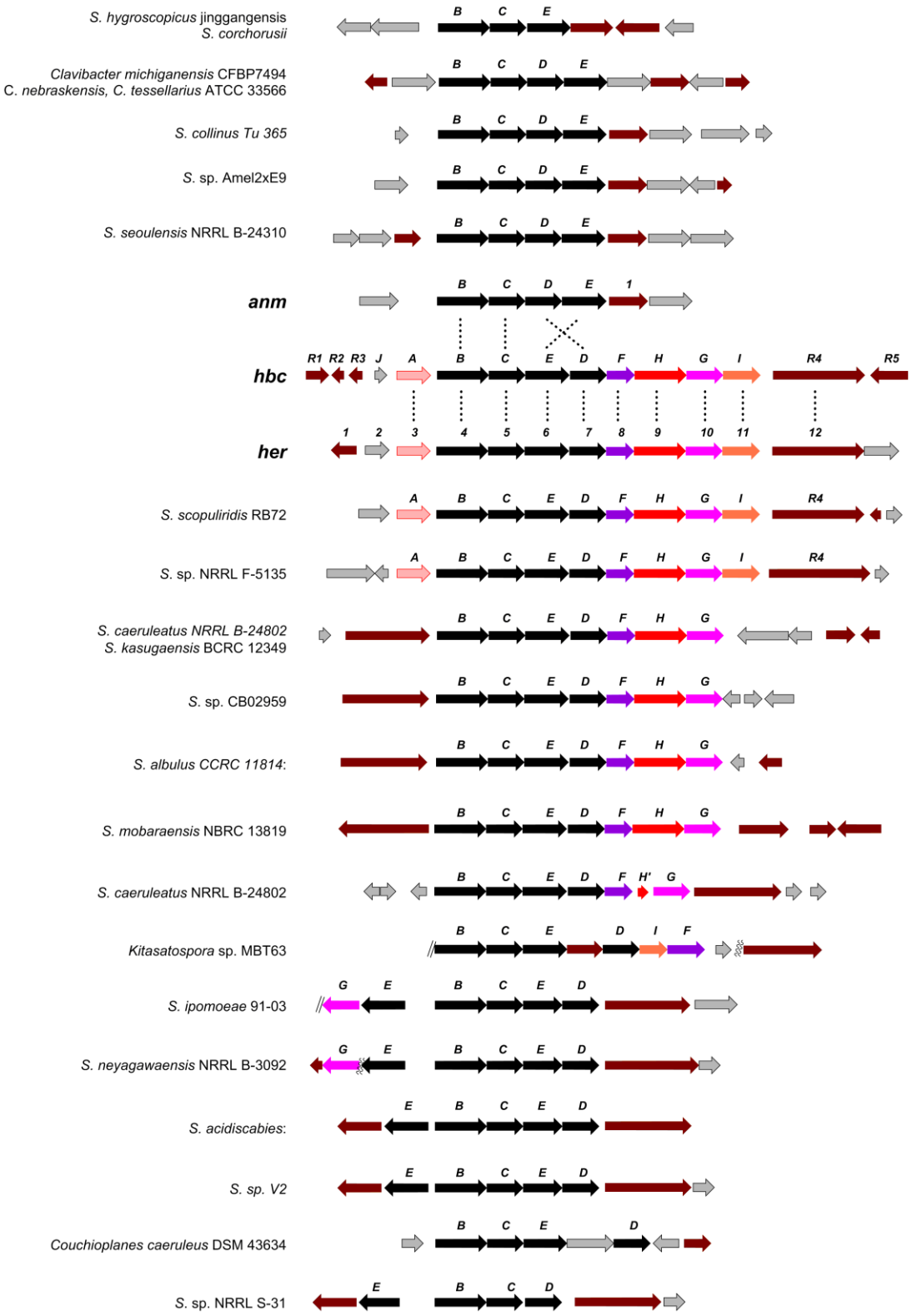


Figure S23 Genetic organization of biosynthetic gene clusters for herbicidins (*hbc*, *her*), aureonuclemycin (*anm*), and some homologous BGCs found in NCBI Genbank

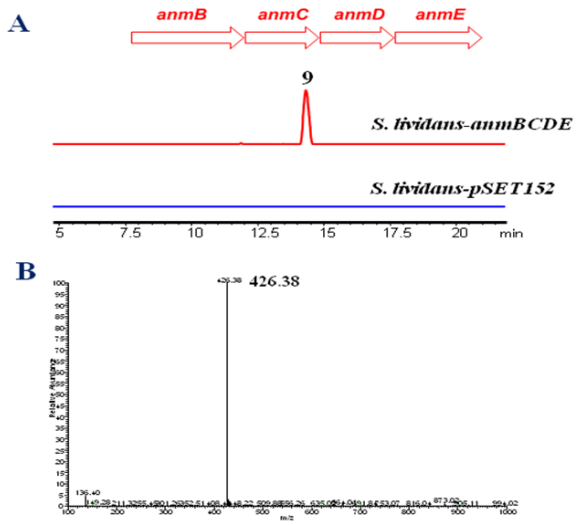


Figure S24 Heterologous expression of the *anm* cluster in *S. lividans*.
A) HPLC analysis of the *anm* expression strain *S. lividans-anmBCDE* and the control strain *S. lividans-pSET152*, B) Mass spectrum for compound 9.

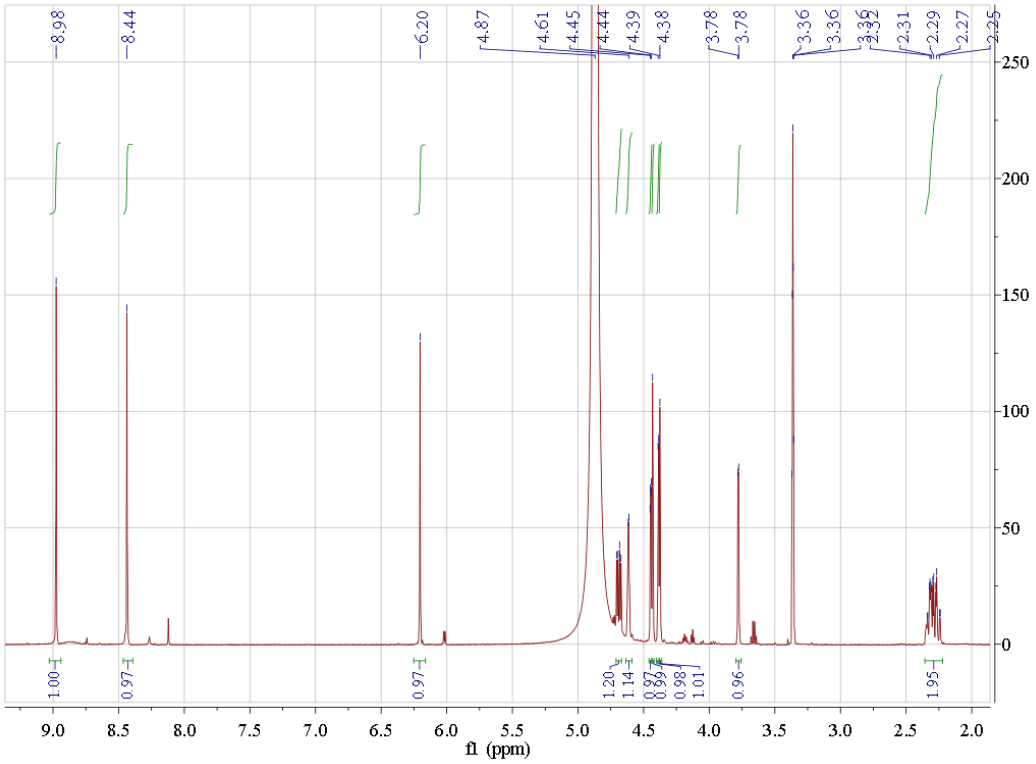


Figure S25 ^{1}H NMR spectrum of 9 in CD_3OD

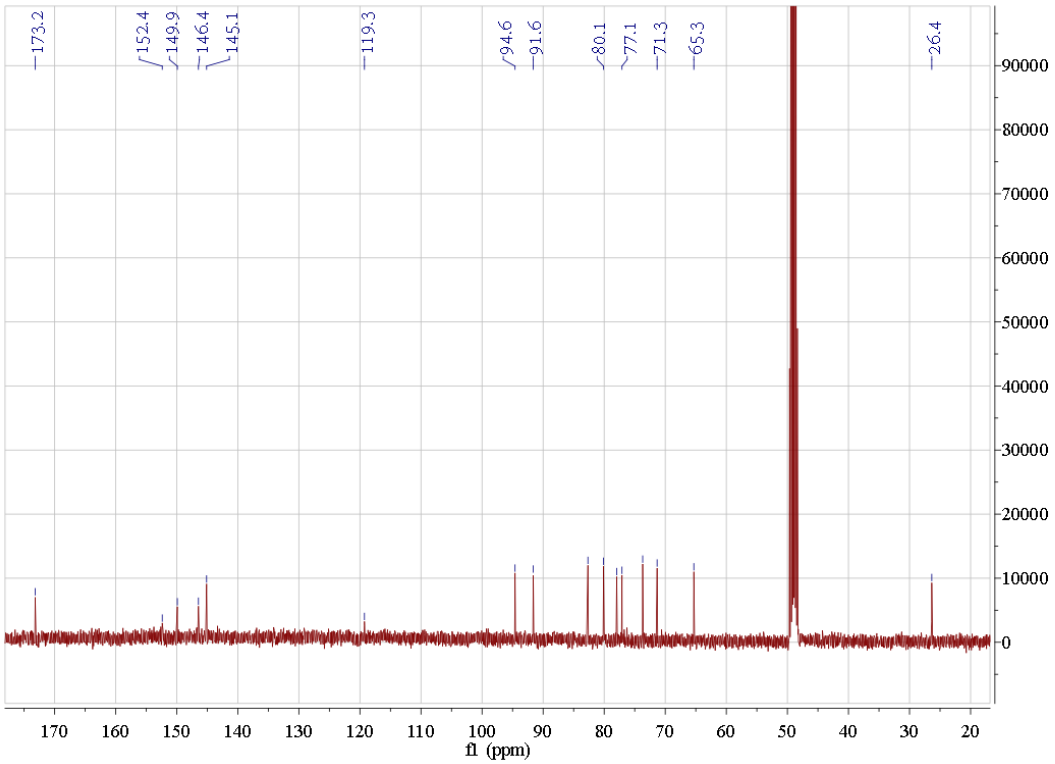


Figure S26 ^{13}C NMR spectrum of 9 in CD_3OD

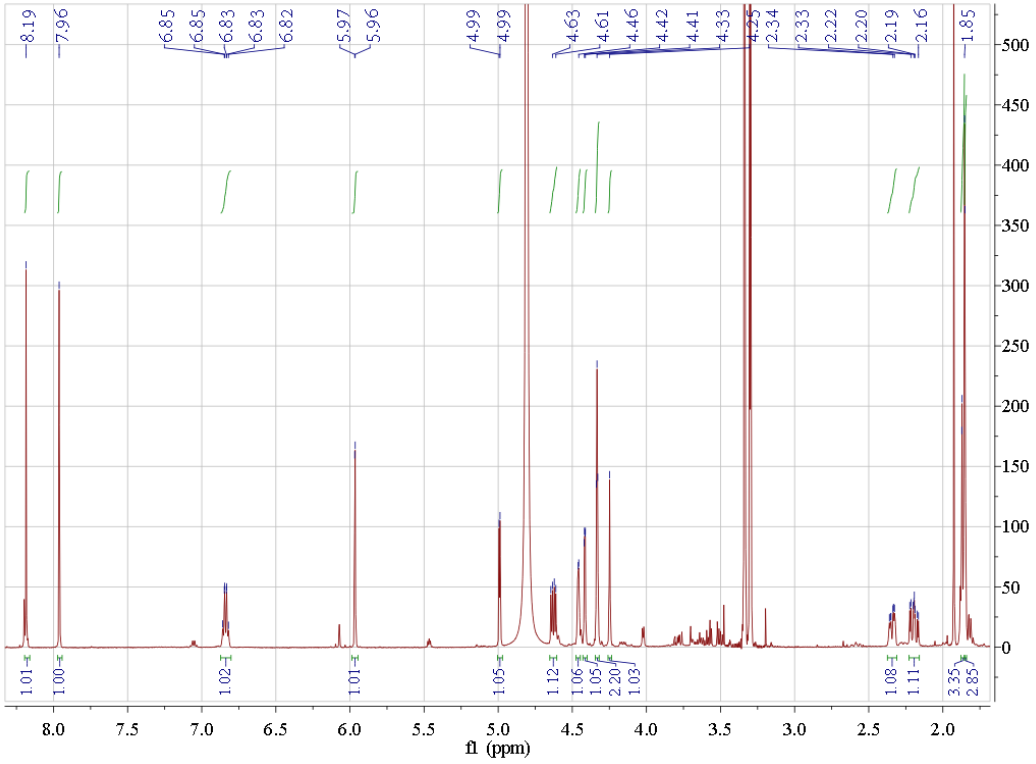


Figure S27 ^{1}H NMR spectrum of 6 in CD_3OD

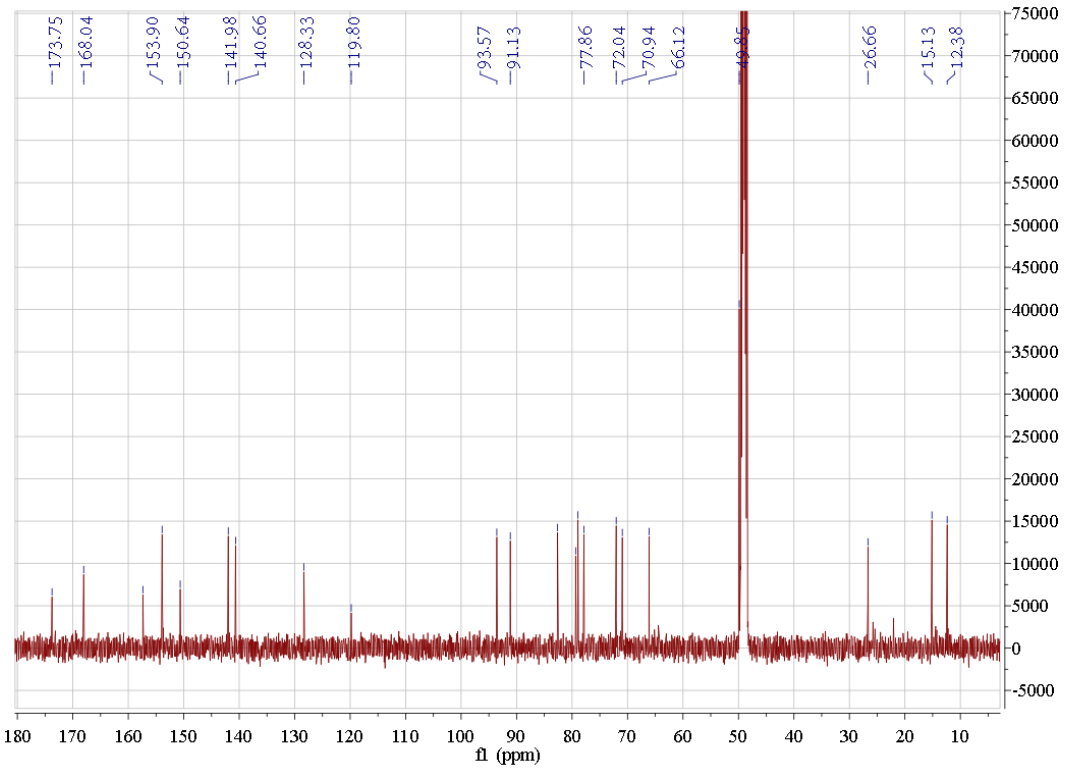


Figure S28 ^{13}C NMR spectrum of 6 in CD_3OD

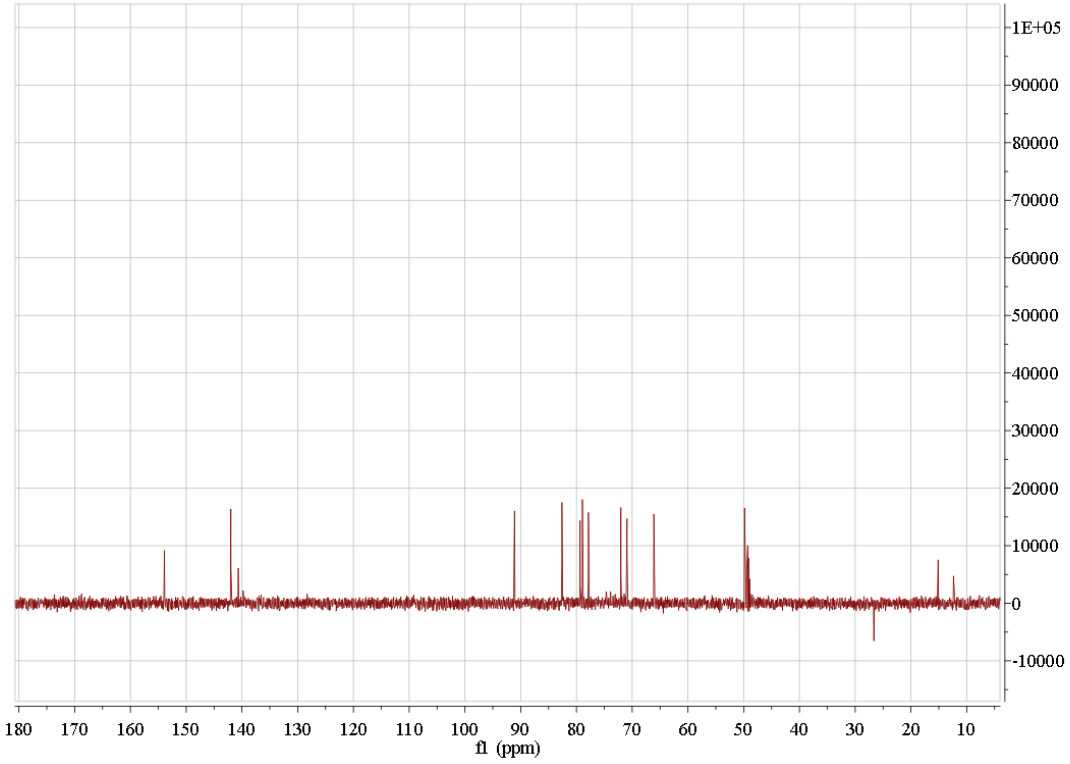


Figure S29 DEPT135 spectrum of 6 in CD_3OD

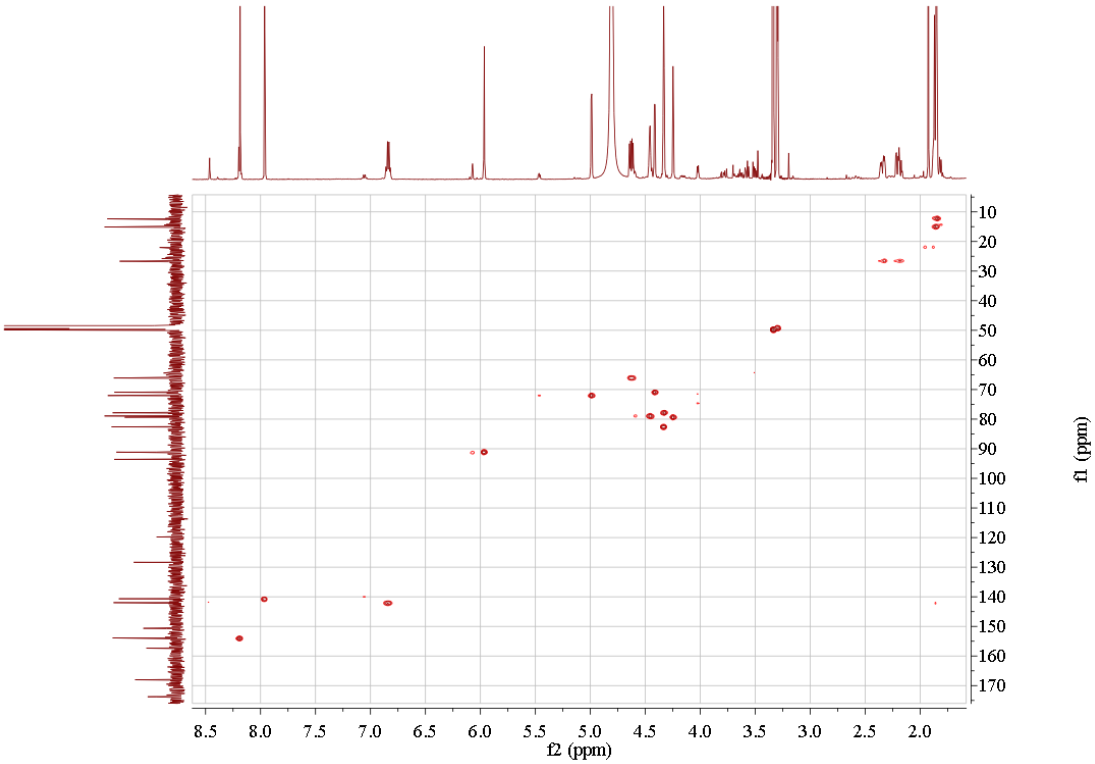


Figure S30 HSQC spectrum of 6 in CD_3OD

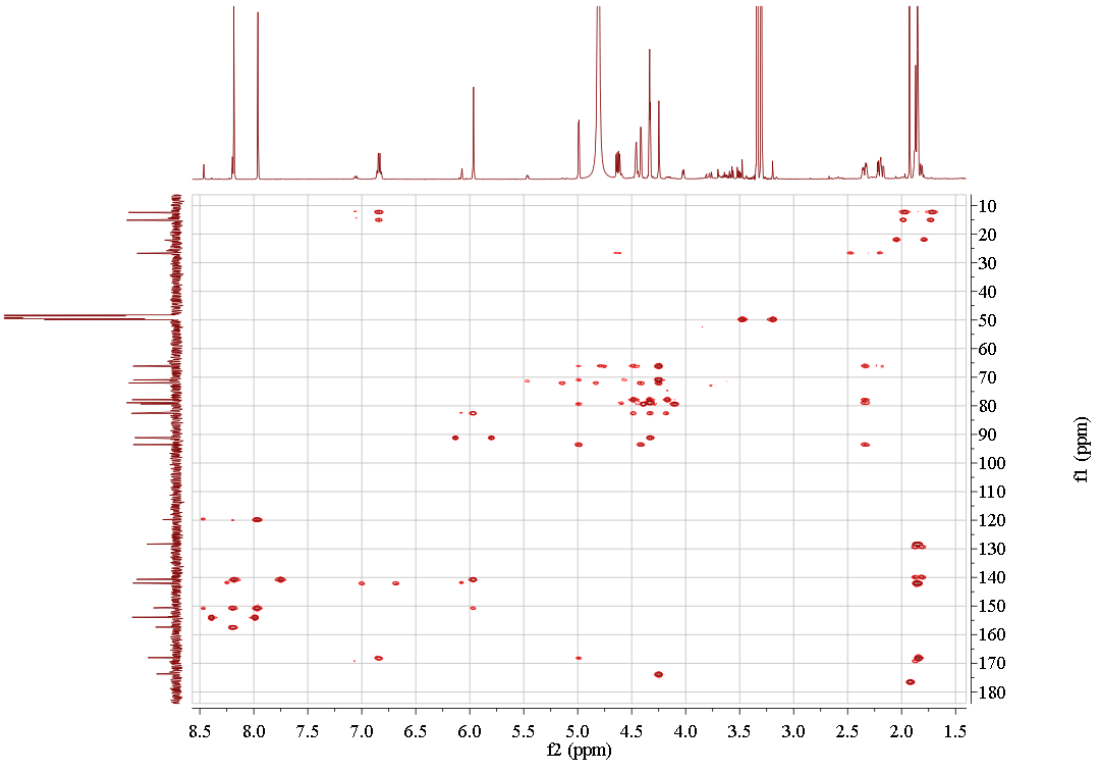


Figure S31 HMBC spectrum of 6 in CD_3OD

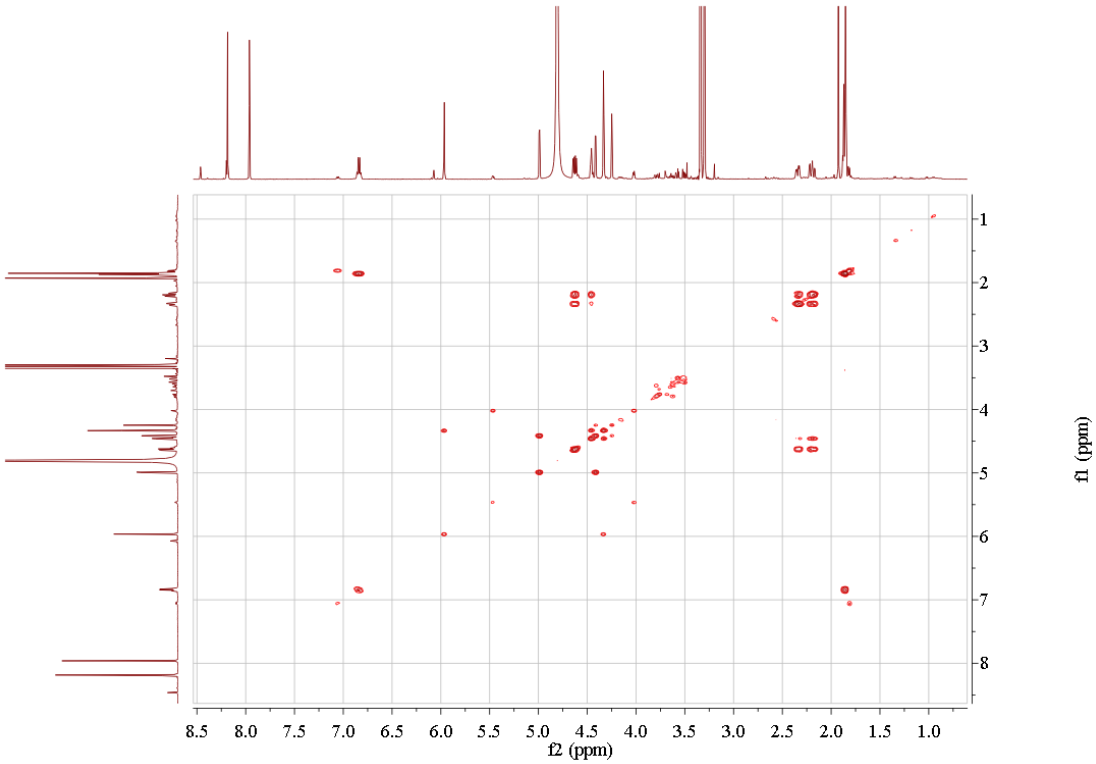


Figure S32 ^{1}H-^{1}H COSY spectrum of 6 in CD_3OD

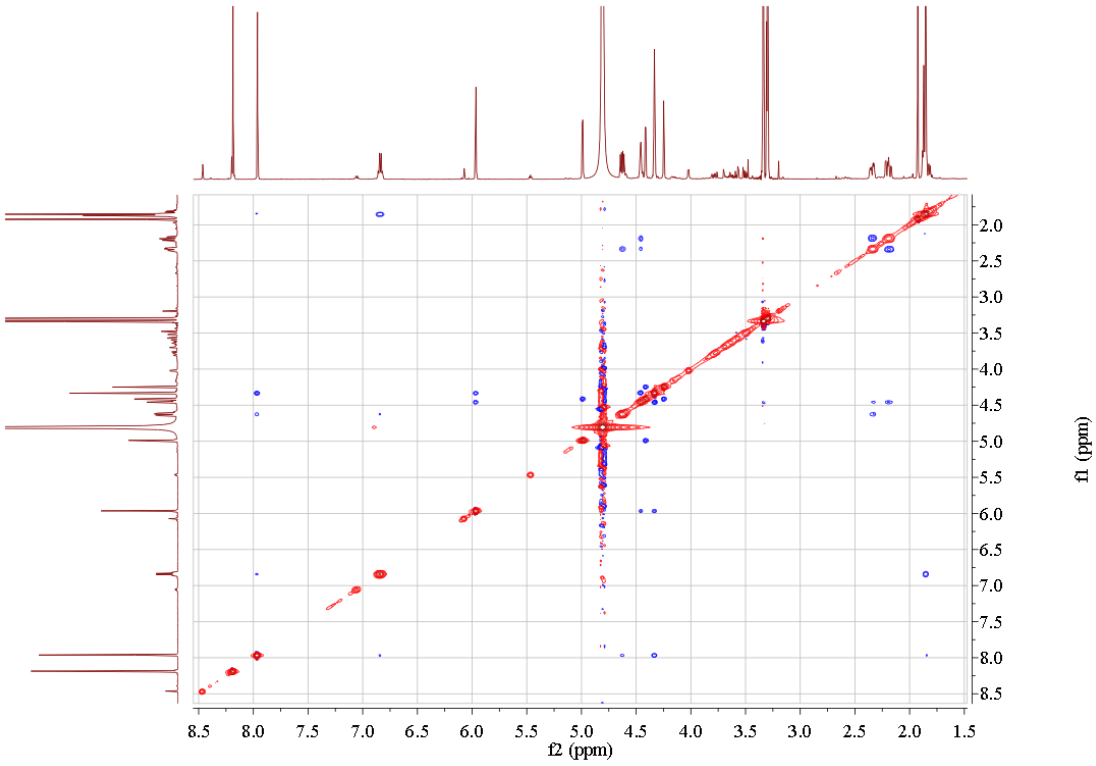


Figure S33 NOESY spectrum of 6 in CD_3OD

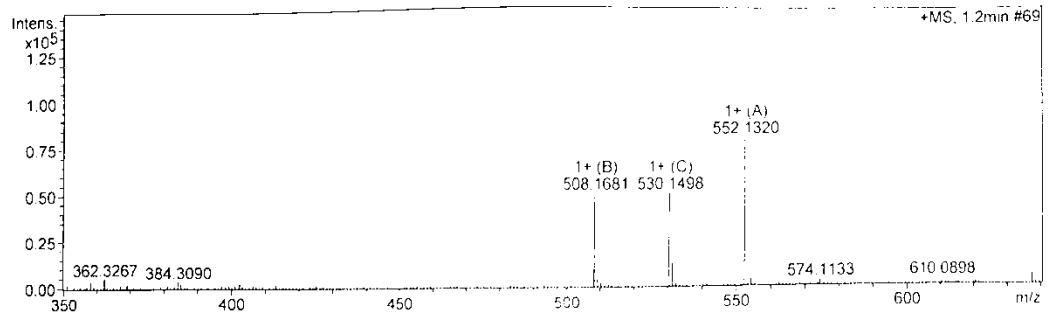
Mass Spectrum SmartFormula Report

Analysis Info

Analysis Name	D:\Data\SHUJVFENXI\tanggongli-group\2005015Her_RB4_01_7280.d	Acquisition Date	12/21/2016 4:13:31 AM
Method	20150915.m	Operator	BDAL@DE
Sample Name	2005015Her	Instrument / Ser#	maXis 4G 21240
Comment			

Acquisition Parameter

Source Type	ESI	Ion Polarity	Positive	Set Nebulizer	1.0 Bar
Focus	Not active	Set Capillary	4000 V	Set Dry Heater	220 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	6.0 l/min
Scan End	1500 m/z	Set Collision Cell RF	600.0 Vpp	Set Divert Valve	Waste



Meas. m/z	#	Formula	Score	m/z	err [ppm]	Mean err [ppm]	mSig ma	rdb	e⁻ Conf	N R ule
508.1681	1	C 21 H 26 N 5 O 10	100.00	508.1674	-1.3	-1.1	3.9	11.5	even	ok
	2	C 20 H 23 N 9 Na O 6	49.90	508.1664	-3.4	-3.2	6.5	13.5	even	ok
	3	C 24 H 27 N 3 Na O 8	81.12	508.1690	1.9	2.1	7.1	12.5	even	ok
	4	C 22 H 22 N 9 O 6	91.99	508.1688	1.3	1.6	8.5	16.5	even	ok
	5	C 21 H 19 N 13 Na O 2	98.02	508.1677	-0.8	-0.6	12.3	18.5	even	ok
	6	C 19 H 14 N 19	82.25	508.1674	-1.3	-1.2	14.3	22.5	even	ok
	7	C 36 H 23 N Na O	15.67	508.1672	-1.8	-1.5	69.2	25.5	even	ok
	8	C 38 H 22 N O	6.94	508.1696	3.0	3.3	80.7	28.5	even	ok
530.1498	1	C 19 H 20 N 11 O 8	88.75	530.1491	-1.3	-1.2	2.4	15.5	even	ok
	2	C 21 H 25 N 5 Na O 10	100.00	530.1494	-0.8	-0.6	3.5	11.5	even	ok
	3	C 18 H 17 N 15 Na O 4	44.84	530.1480	-3.3	-3.2	4.0	17.5	even	ok
	4	C 22 H 28 N O 14	83.41	530.1504	1.2	1.3	7.3	9.5	even	ok
	5	C 23 H 24 N 5 O 10	32.78	530.1518	3.8	4.0	12.1	14.5	even	ok
	6	C 22 H 21 N 9 Na O 6	65.79	530.1507	1.7	1.9	12.3	16.5	even	ok
	7	C 20 H 16 N 15 O 4	76.09	530.1504	1.2	1.3	12.5	20.5	even	ok
	8	C 20 H 29 N Na O 14	38.18	530.1480	-3.3	-3.2	13.2	6.5	even	ok
	9	C 19 H 13 N 19 Na	79.32	530.1494	-0.8	-0.7	15.7	22.5	even	ok
	10	C 21 H 12 N 19	23.89	530.1518	3.7	3.8	27.3	25.5	even	ok
	11	C 35 H 20 N 3 O 3	17.87	530.1499	0.3	0.5	72.1	27.5	even	ok
	12	C 38 H 21 N Na O	4.34	530.1515	3.3	3.6	84.7	28.5	even	ok

Figure S34 HRMS data of 6

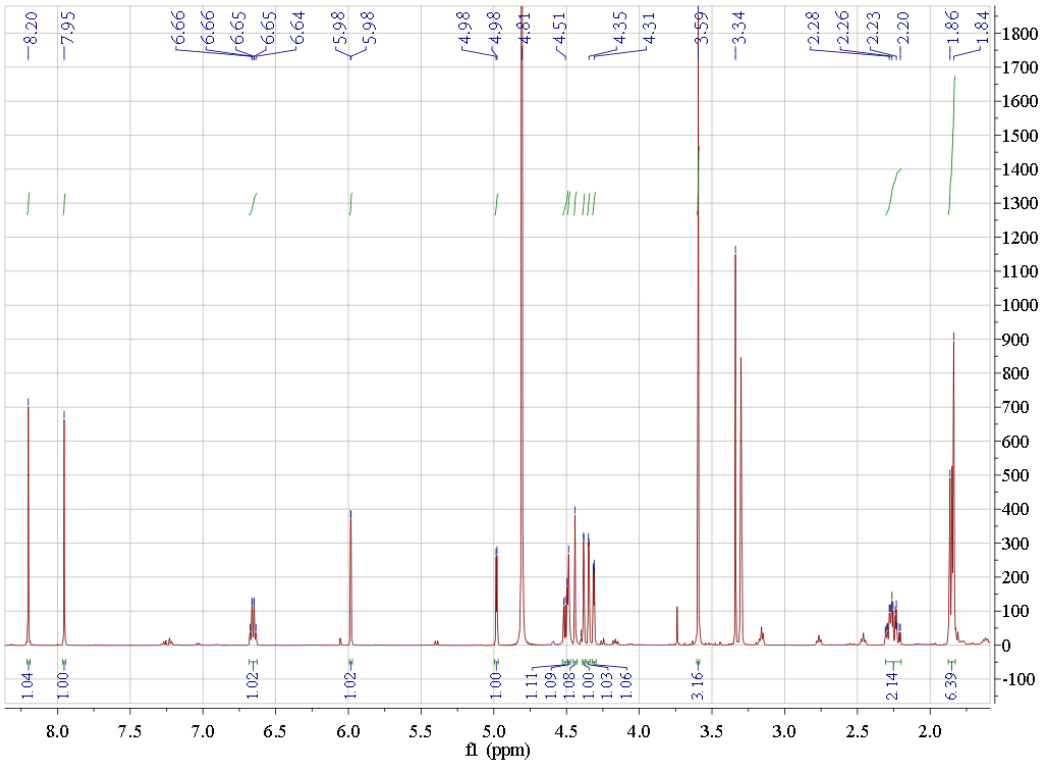


Figure S35 ^{1}H NMR spectrum of 8 in CD_3OD

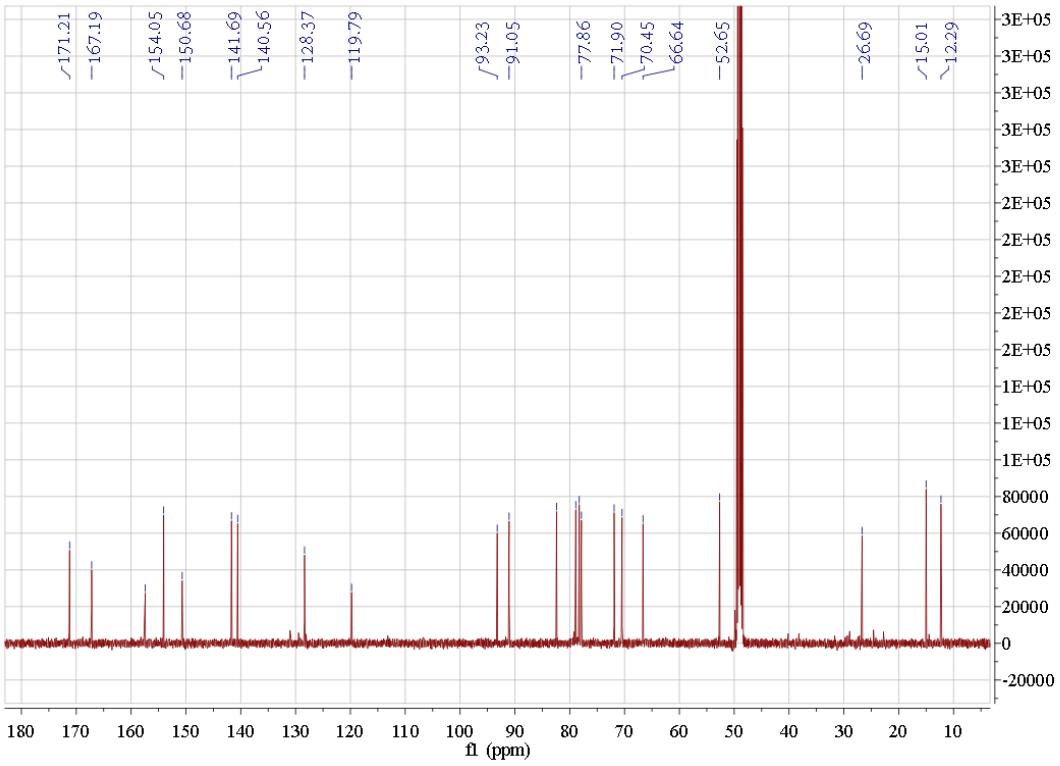


Figure S36 ^{13}C NMR spectrum of 8 in CD_3OD

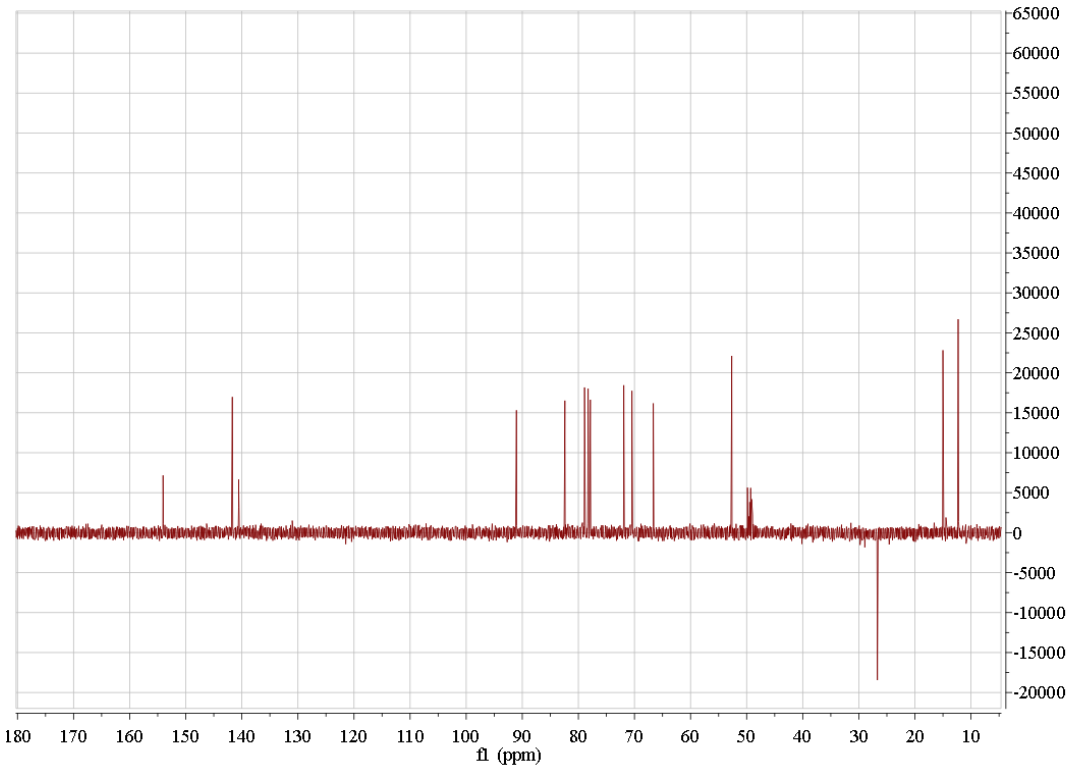


Figure S37 DEPT135 spectrum of 8 in CD_3OD

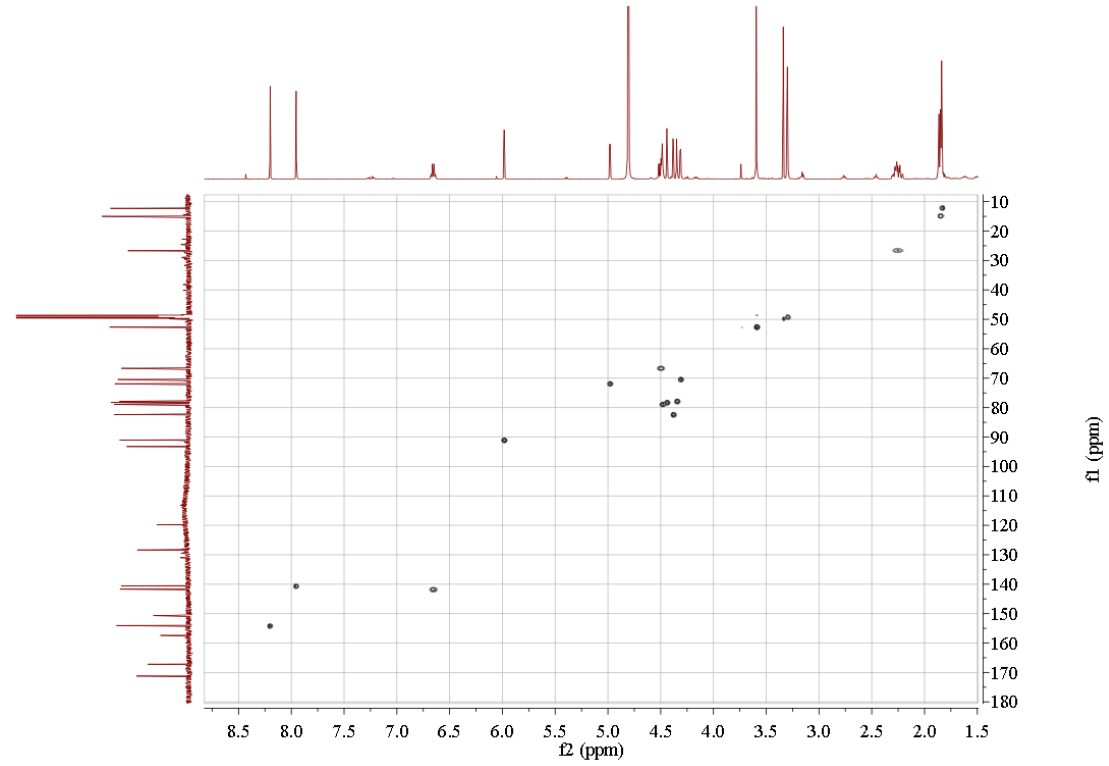


Figure S38 HSQC spectrum of 8 in CD_3OD

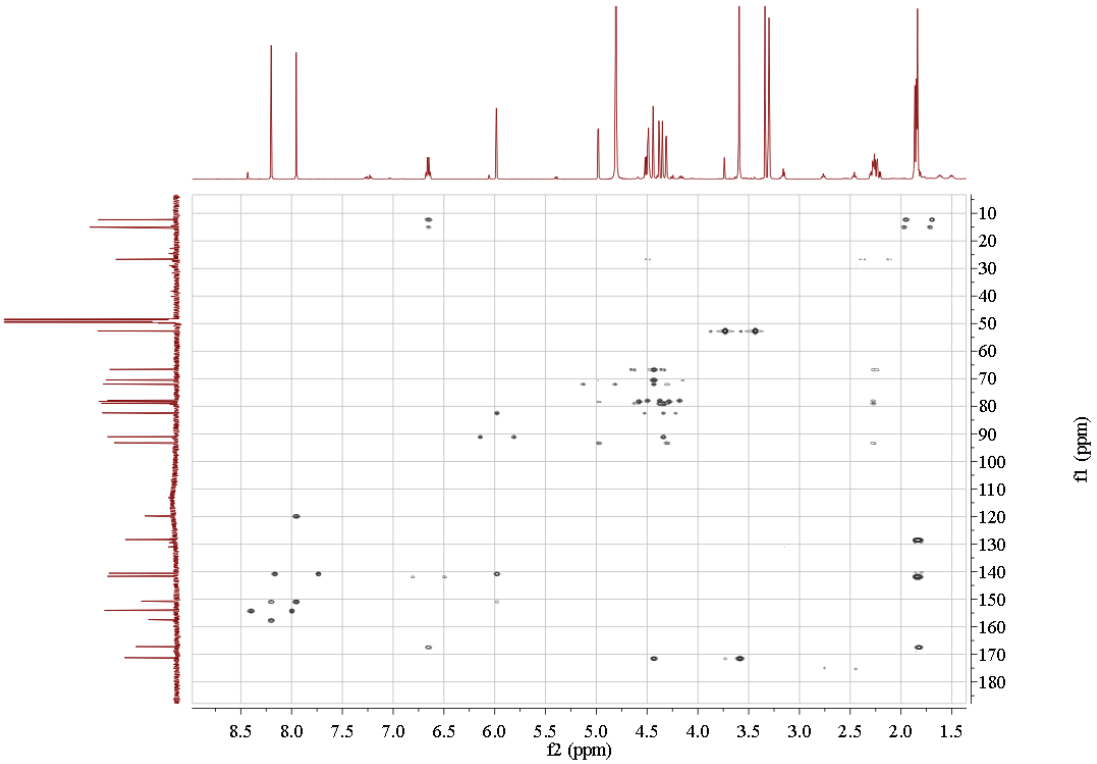


Figure S39 HMBC spectrum of 8 in CD_3OD

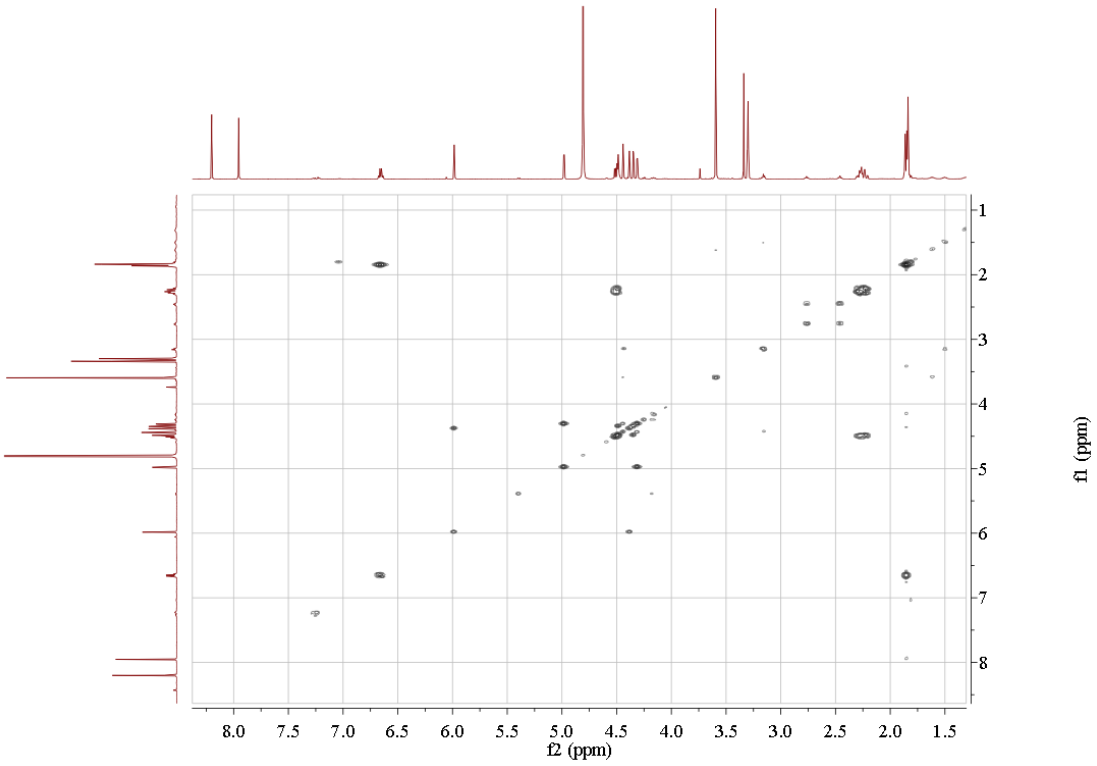


Figure S40 ^{1}H-^{1}H COSY spectrum of 8 in CD_3OD

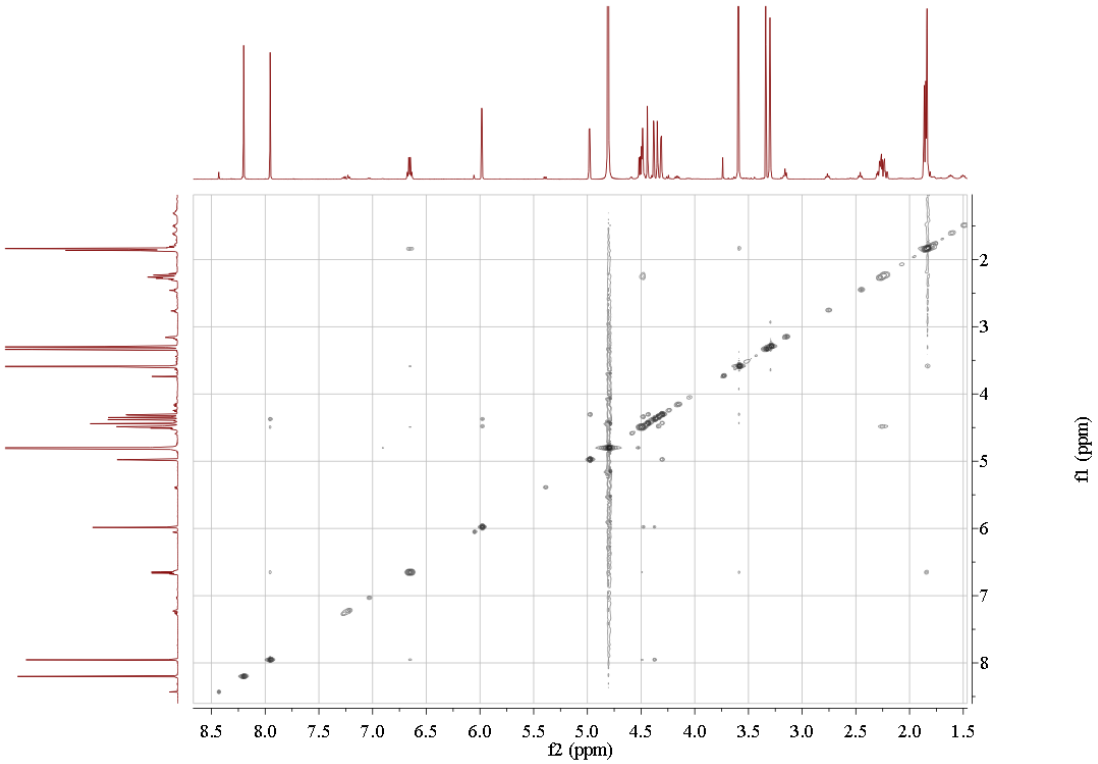


Figure S41 NOESY spectrum of 8 in CD_3OD

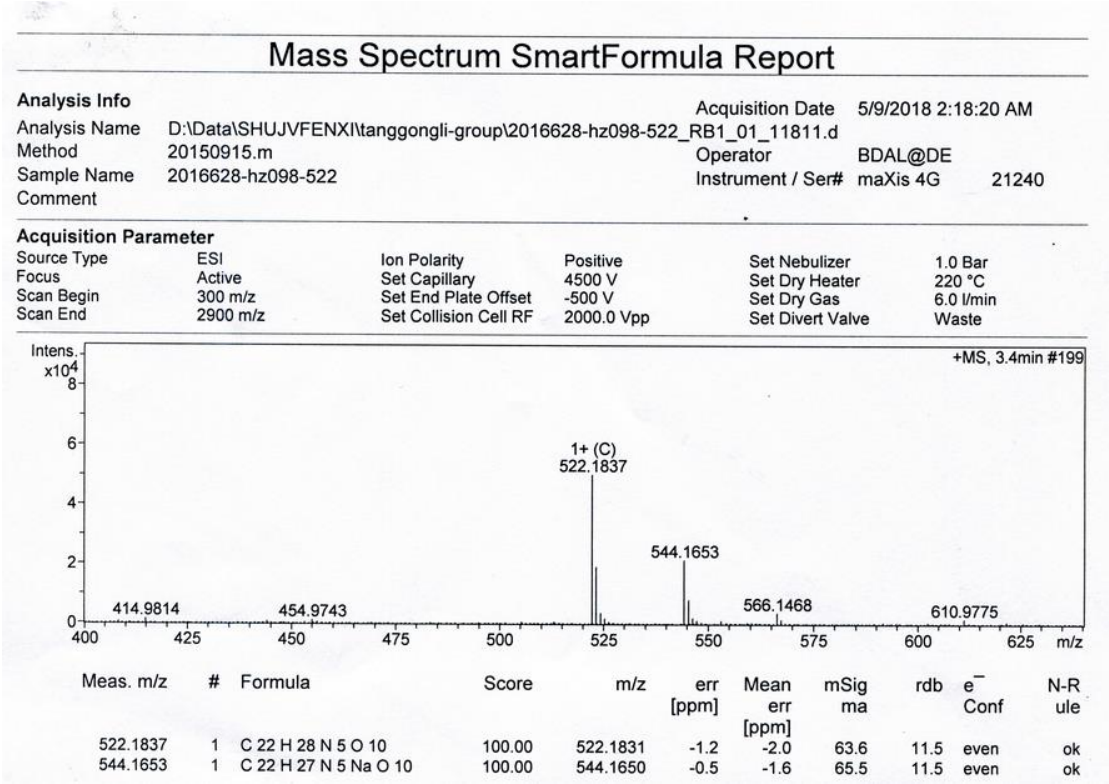


Figure S42 HRMS data of 8

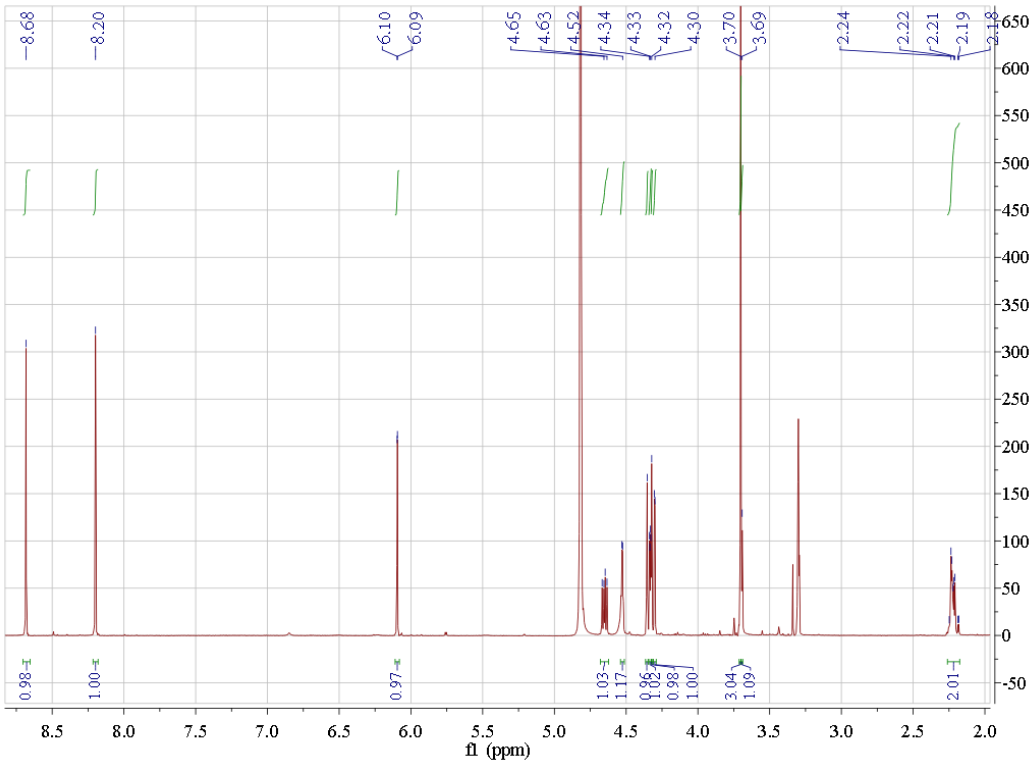


Figure S43 ^{1}H NMR spectrum of 3 in CD_3OD

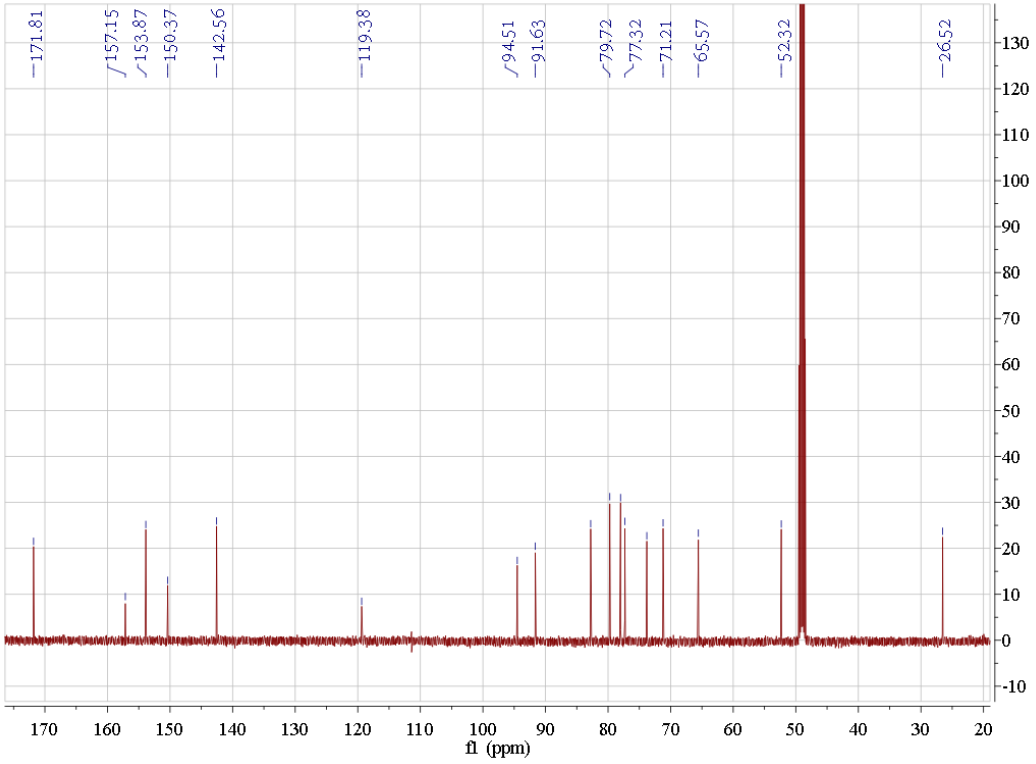


Figure S44 ^{13}C NMR spectrum of 3 in CD_3OD

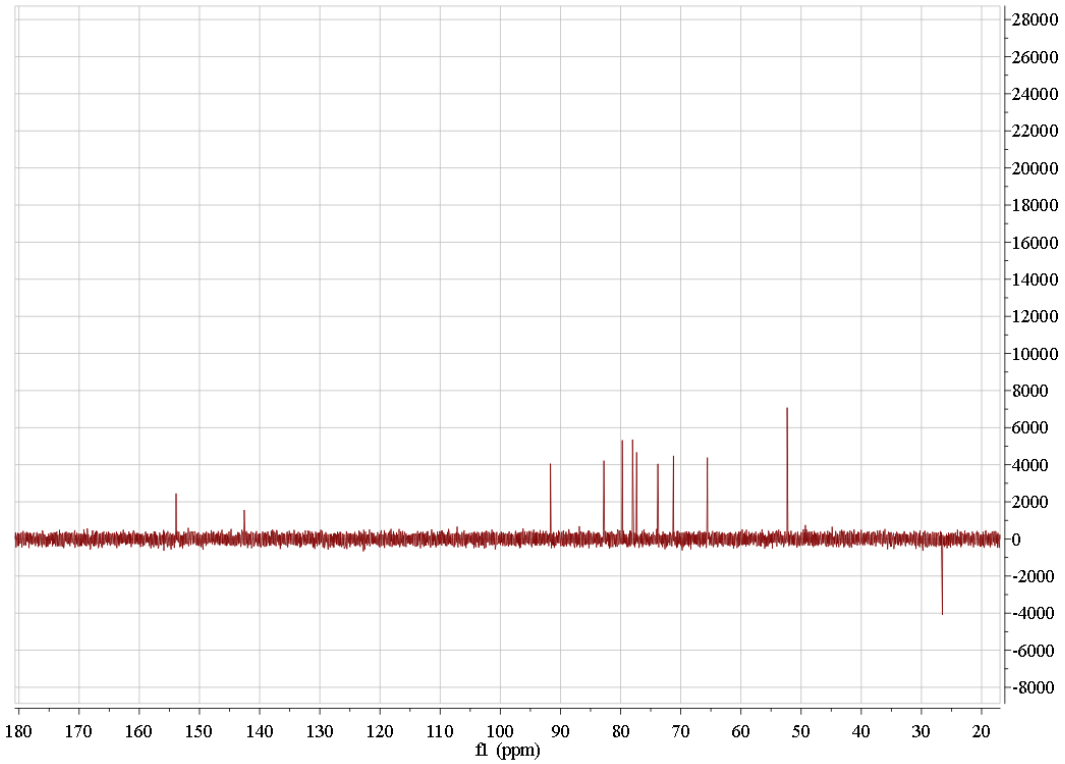


Figure S45 DEPT135 spectrum of 3 in CD_3OD

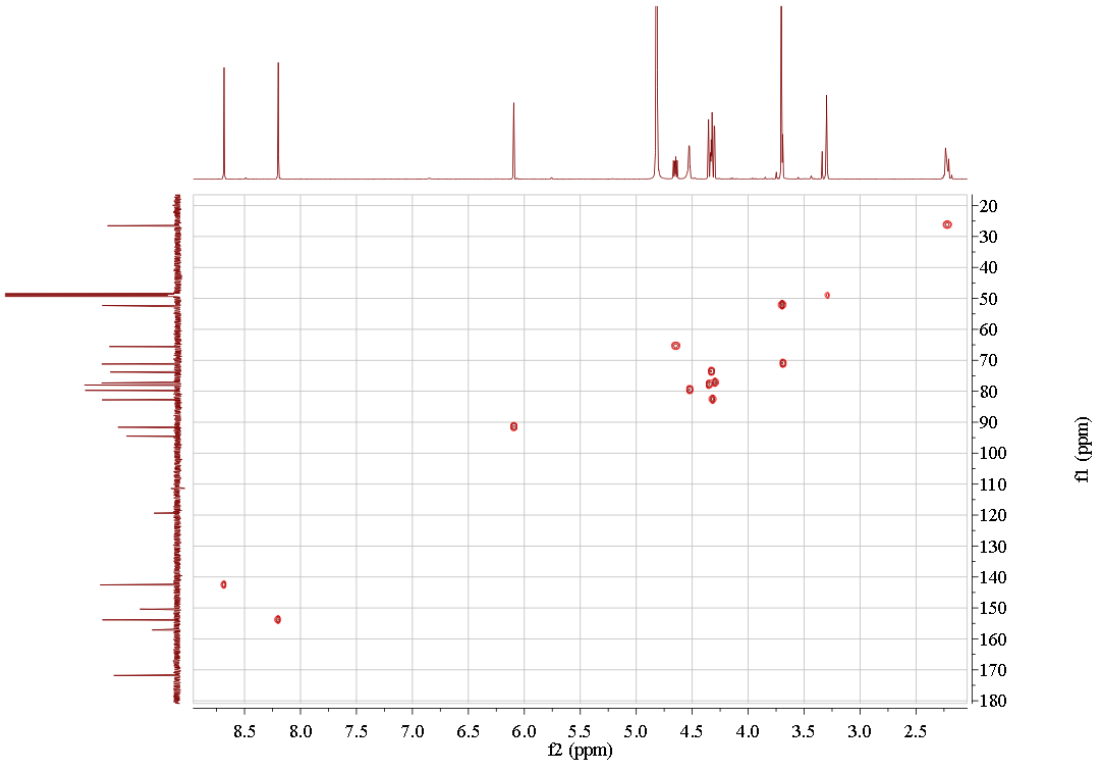


Figure S46 HSQC spectrum of 3 in CD_3OD

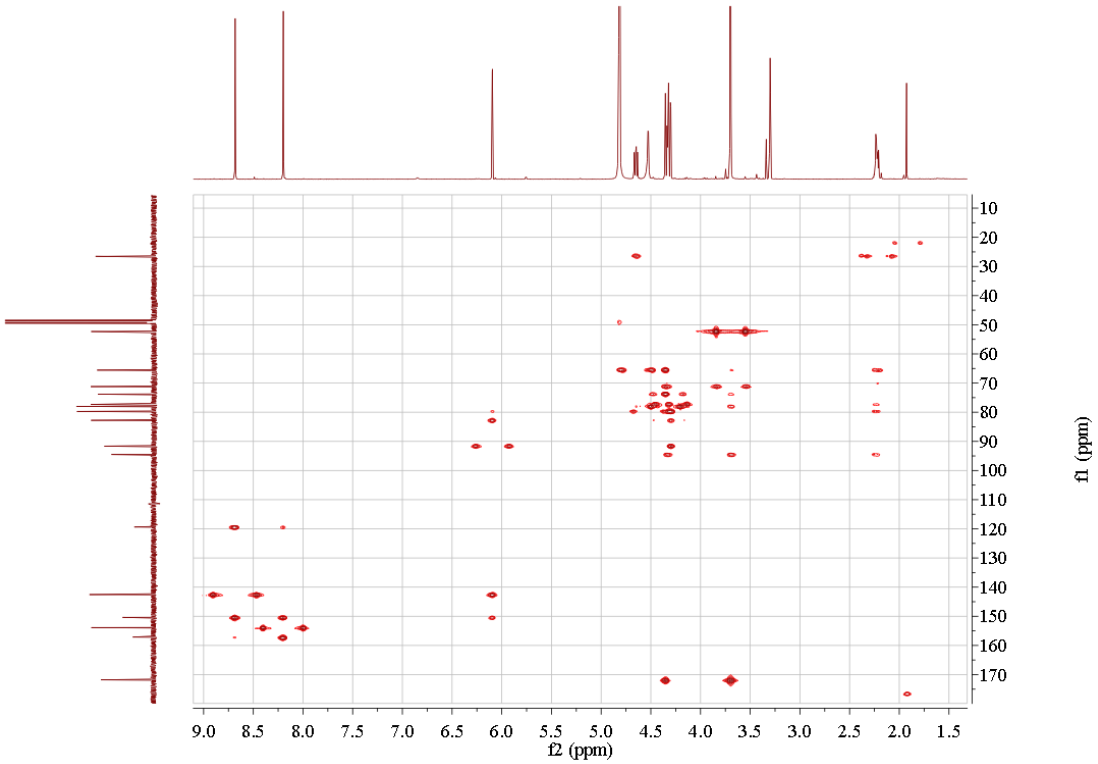


Figure S47 HMBC spectrum of 3 in CD_3OD

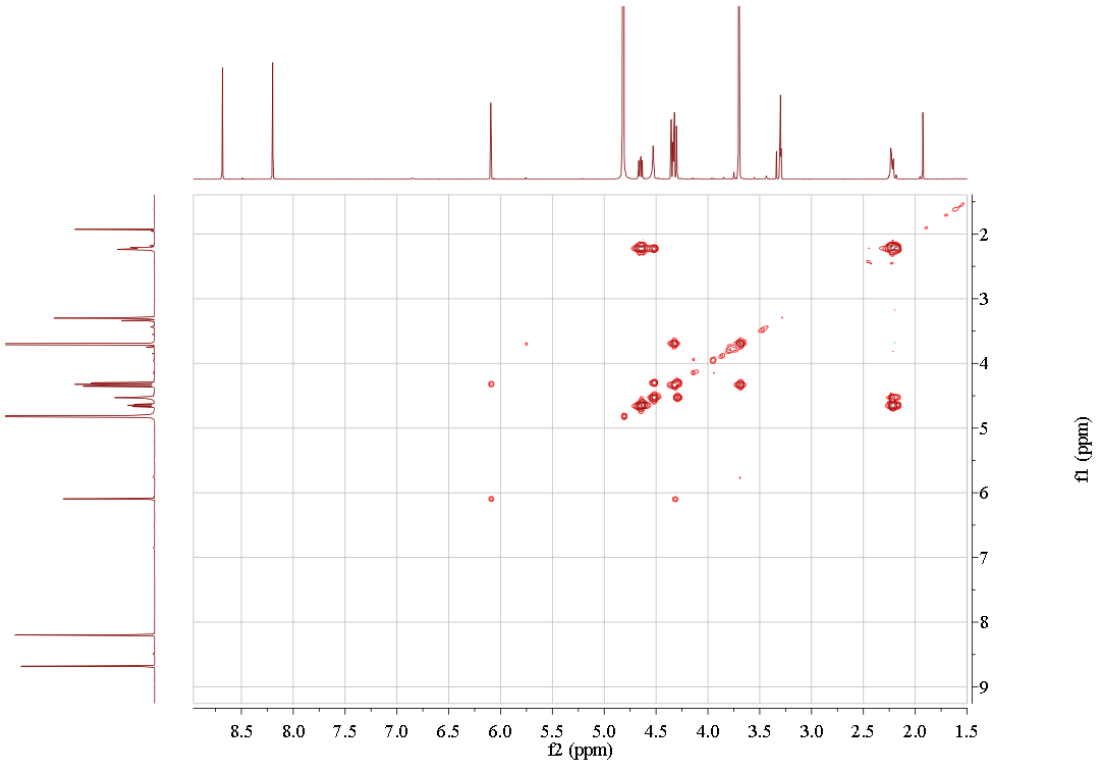


Figure S48 ^{1}H-^{1}H COSY spectrum of 3 in CD_3OD

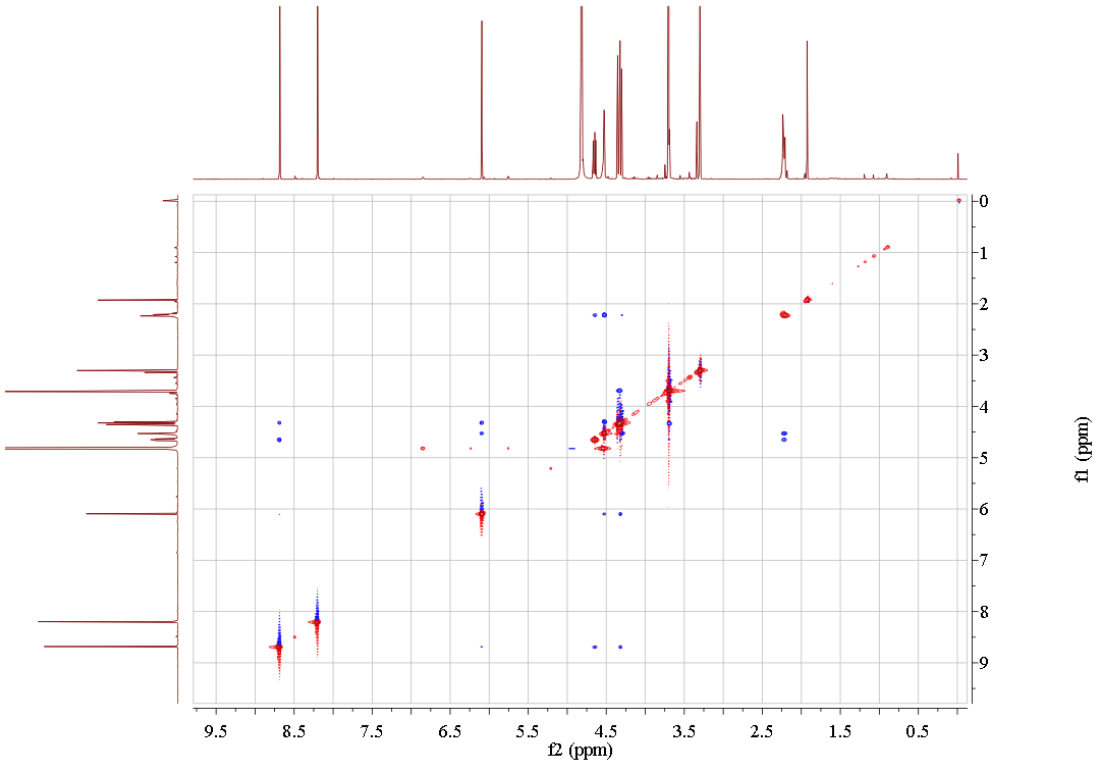


Figure S49 NOESY spectrum of 3 in CD_3OD

National Center for Organic Mass Spectrometry in Shanghai
Shanghai Institute of Organic Chemistry
Chinese Academic of Sciences
High Resolution MS DATA REPORT

Instrument: Thermo Fisher Scientific LTQ FT Ultra

Card Serial Number : E170045

Sample Serial Number: ZTF440b

Operator :zhufj Date: 2016/12/16

Operation Mode: DART Positive

Elemental composition search on mass 440.14

m/z= 435.14-445.14

m/z	Theo. Mass	Delta (ppm)	RDB equiv.	Composition
440.1417	440.1412	1.06	9.5	$C_{17}H_{22}O_9N_5$

Figure S50 HRMS data of 3

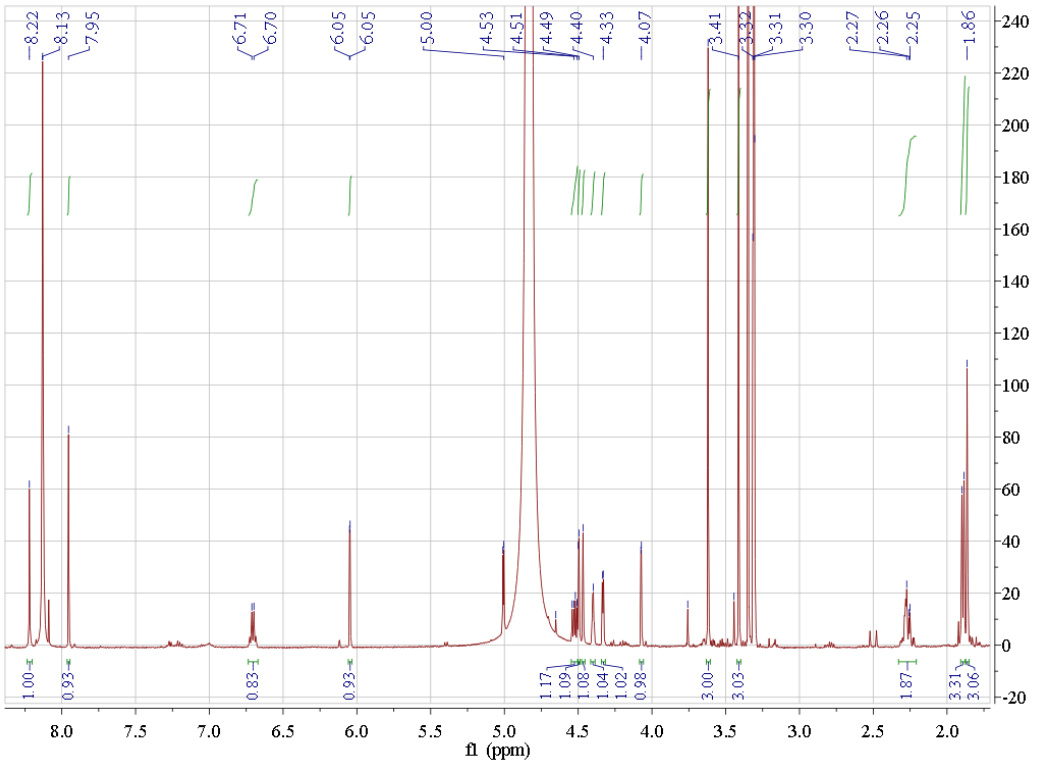


Figure S51 ^{1}H NMR spectrum of 5 in CD_3OD

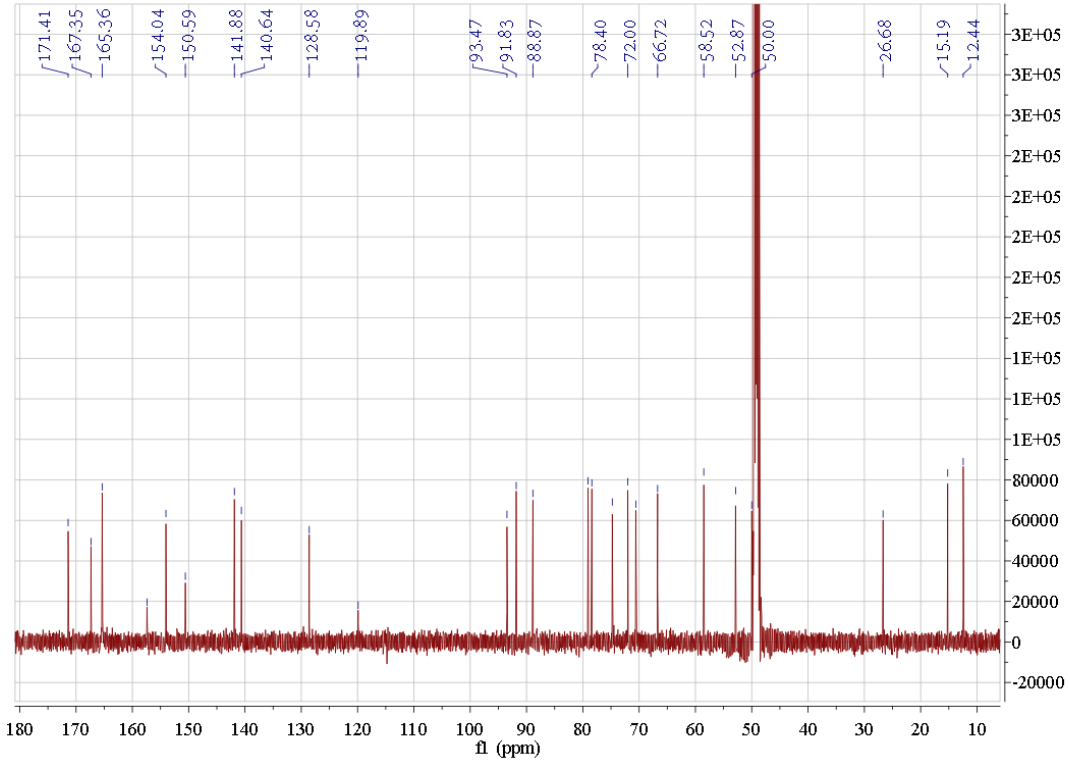


Figure S52 ^{13}C NMR spectrum of 5 in CD_3OD

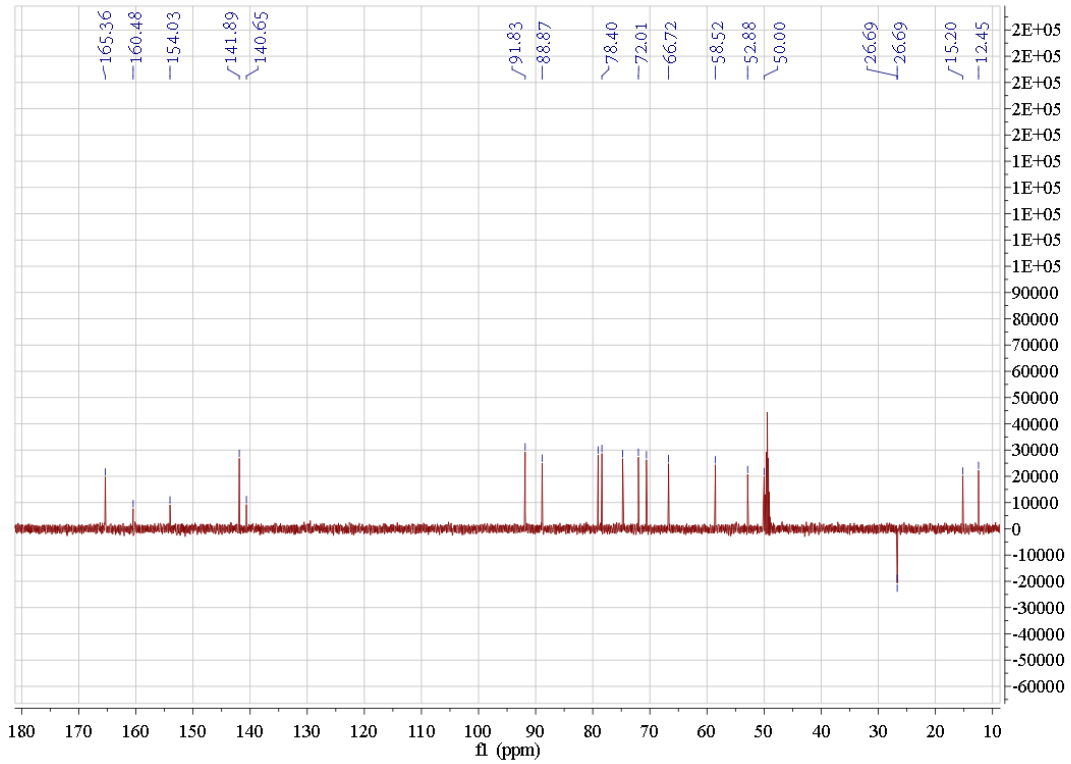


Figure S53 DEPT135 spectrum of 5 in CD_3OD

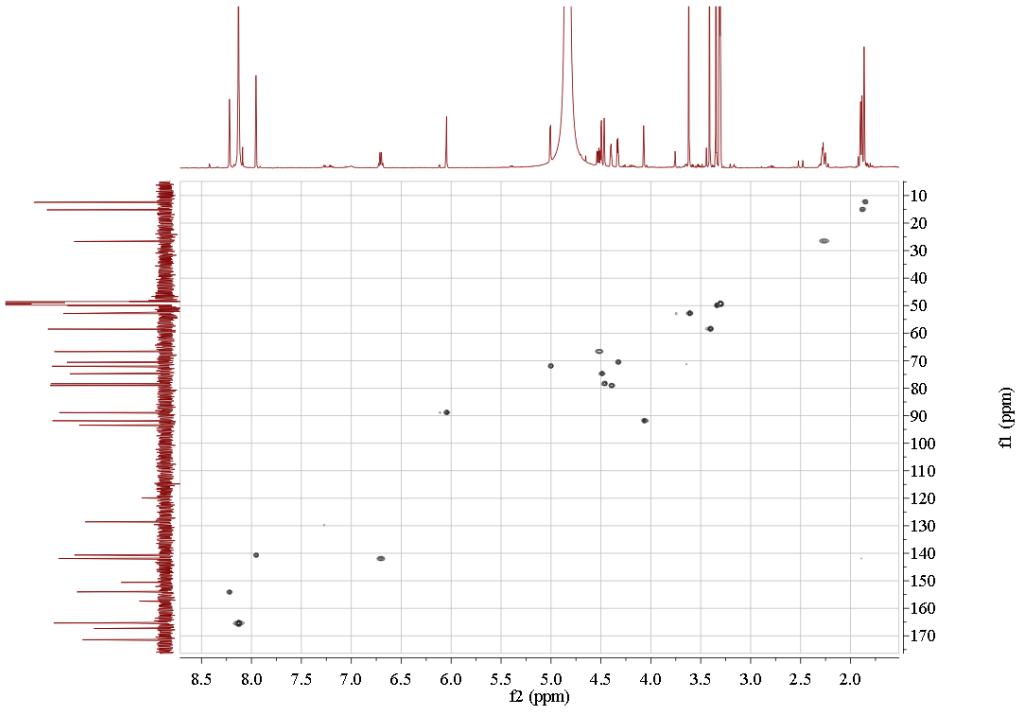


Figure S54 HSQC spectrum of 5 in CD_3OD

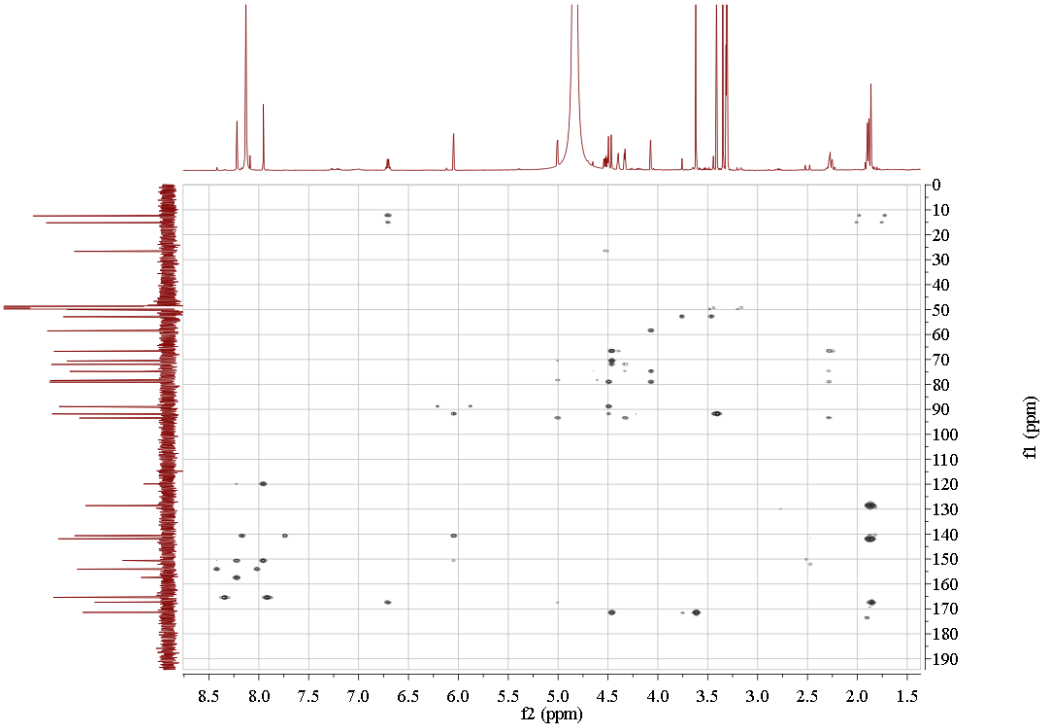


Figure S55 HMBC spectrum of 5 in CD_3OD

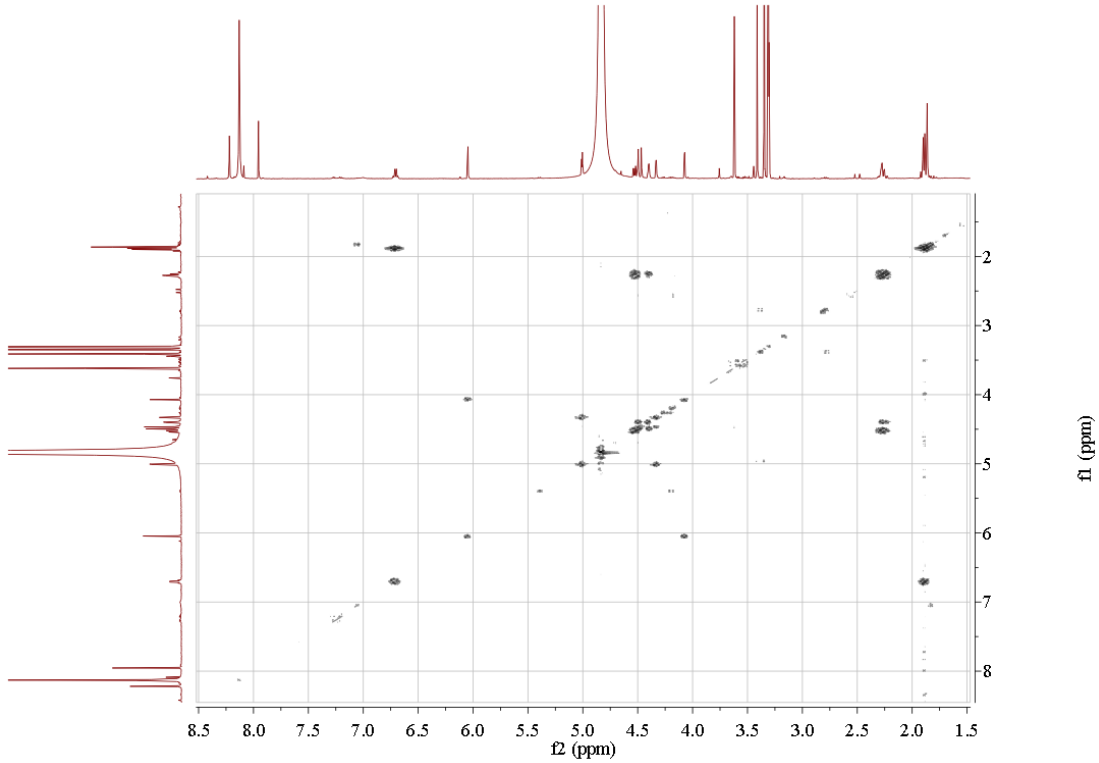


Figure S56 ^1H-^1H COSY spectrum of 5 in CD_3OD

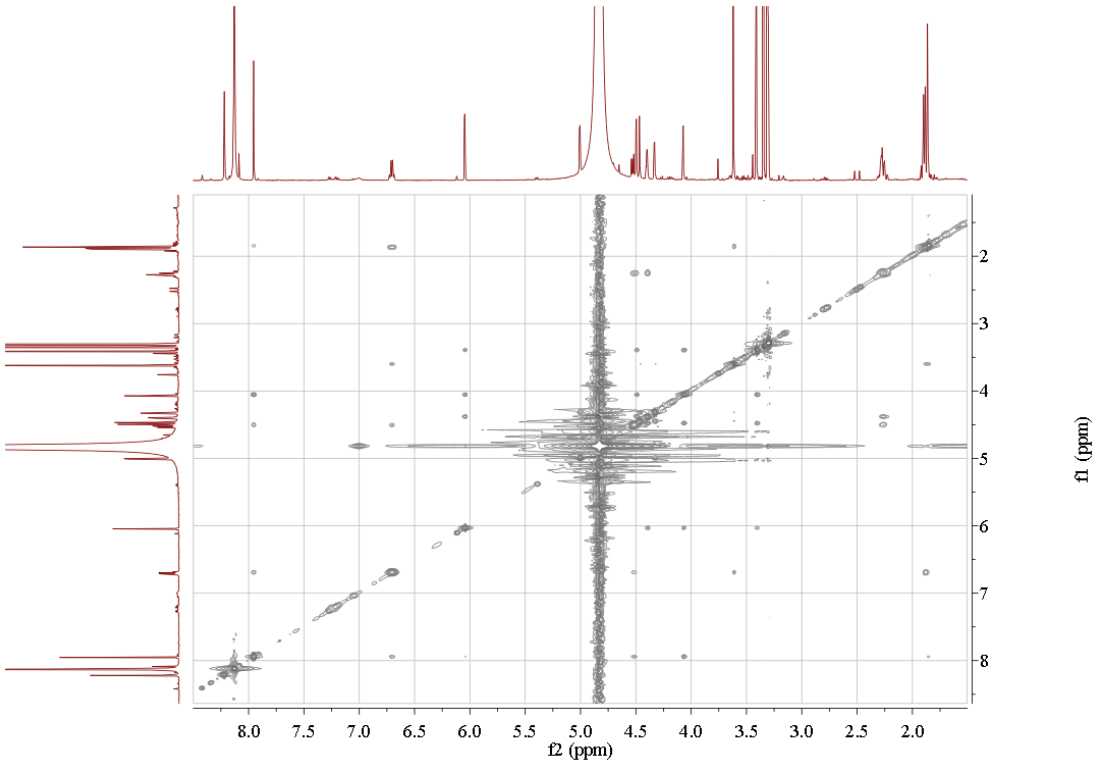


Figure S57 NOESY spectrum of 5 in CD_3OD

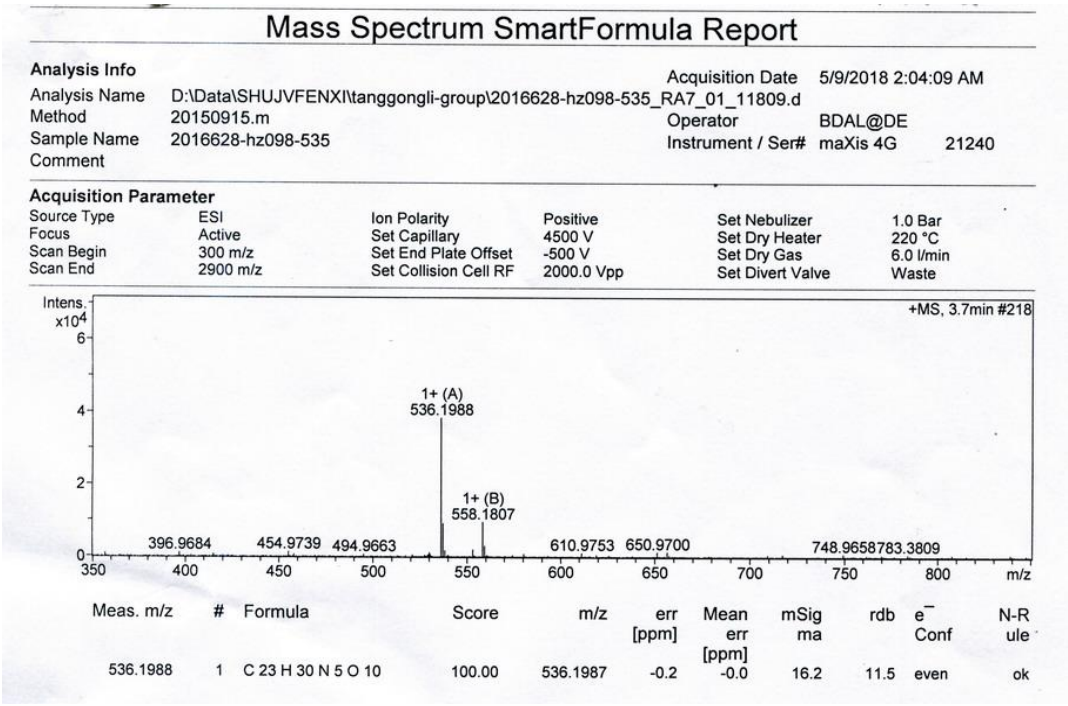


Figure S58 HRMS data of 5

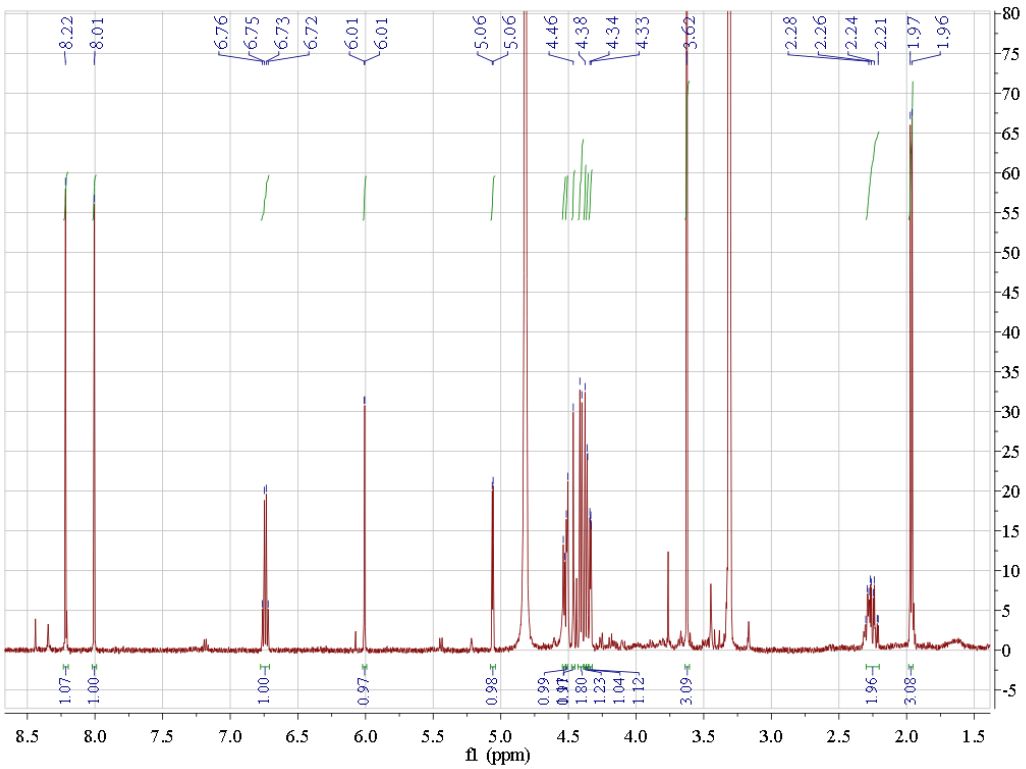


Figure S59 ^{1}H NMR spectrum of 11 in CD_3OD

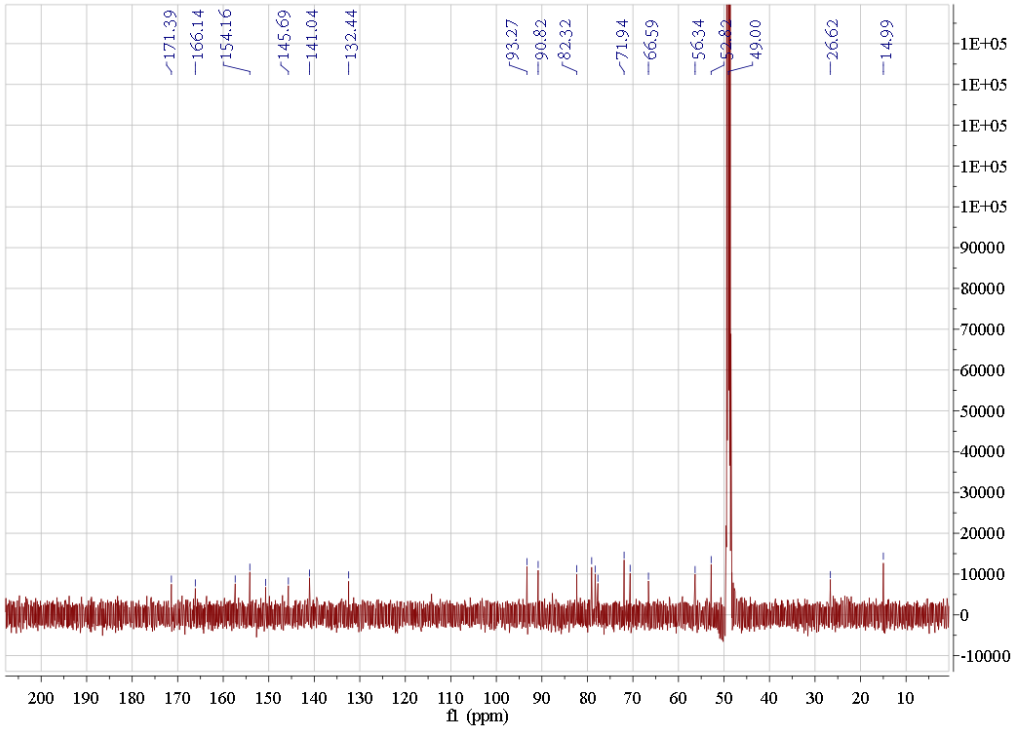


Figure S60 ^{13}C NMR spectrum of 11 in CD_3OD

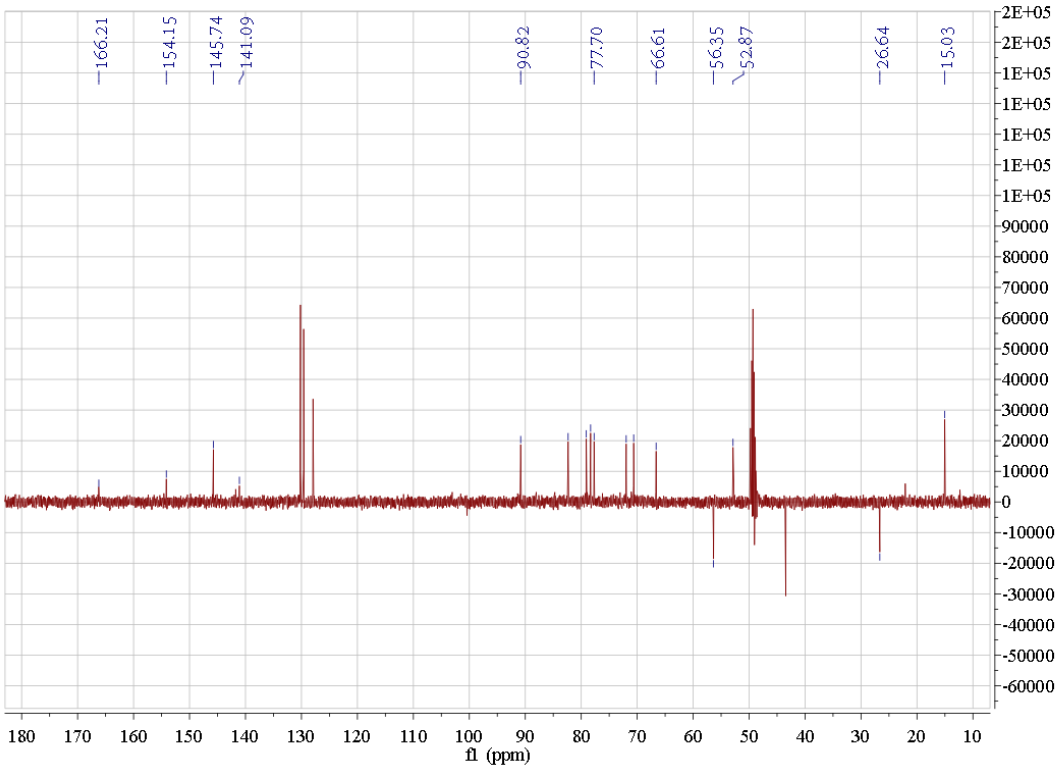


Figure S61 DEPT135 spectrum of 11 in CD_3OD

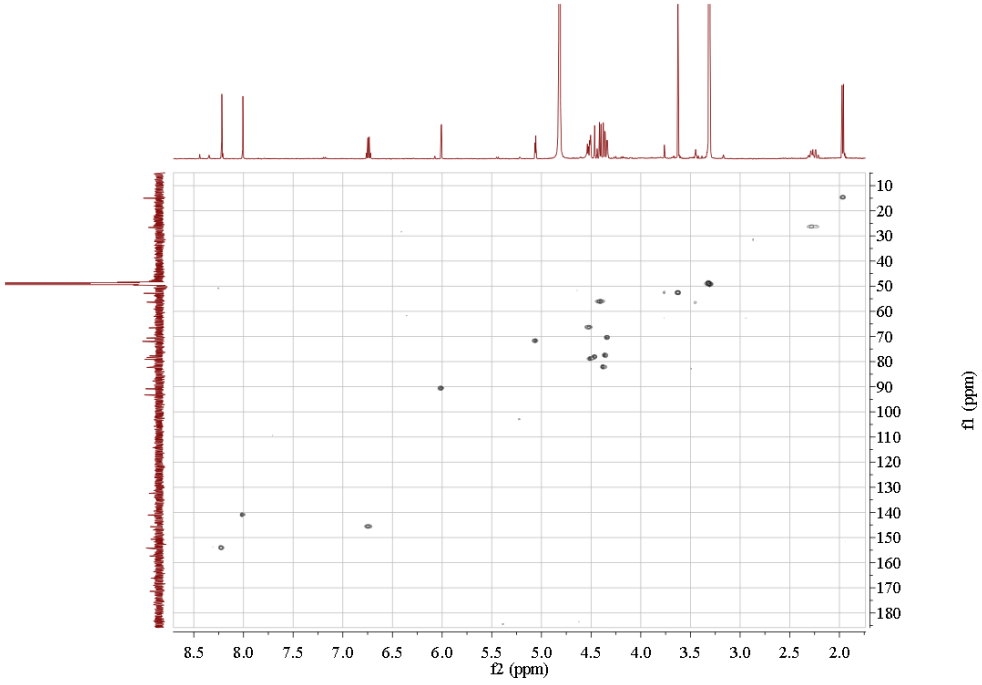


Figure S62 HSQC spectrum of 11 in CD_3OD

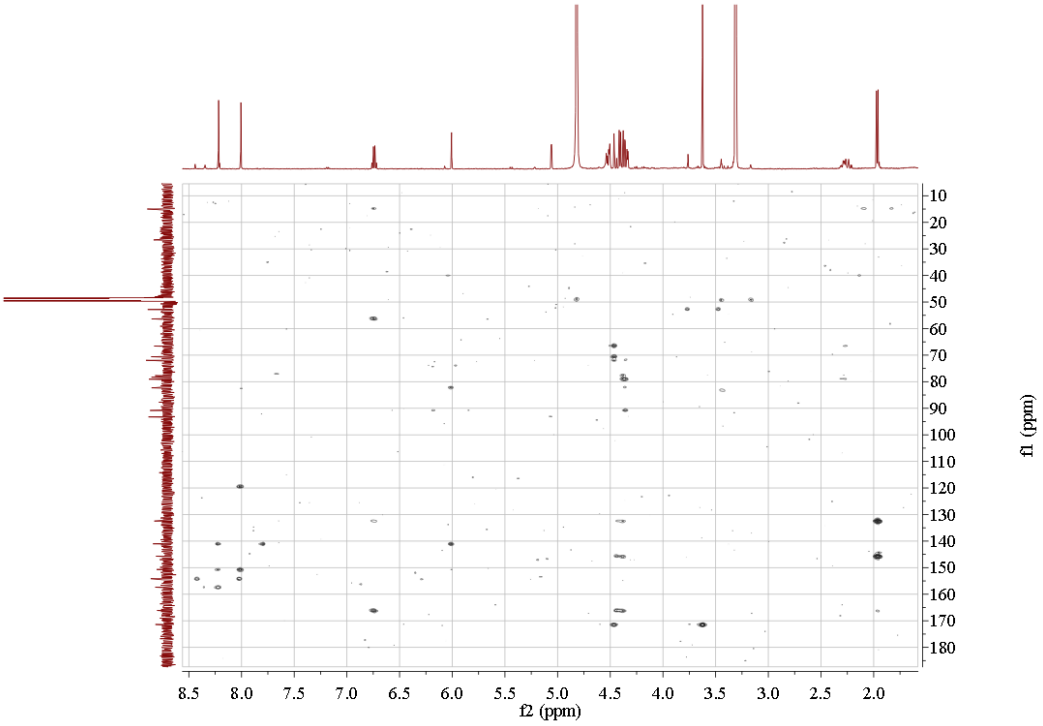


Figure S63 HMBC spectrum of 11 in CD_3OD

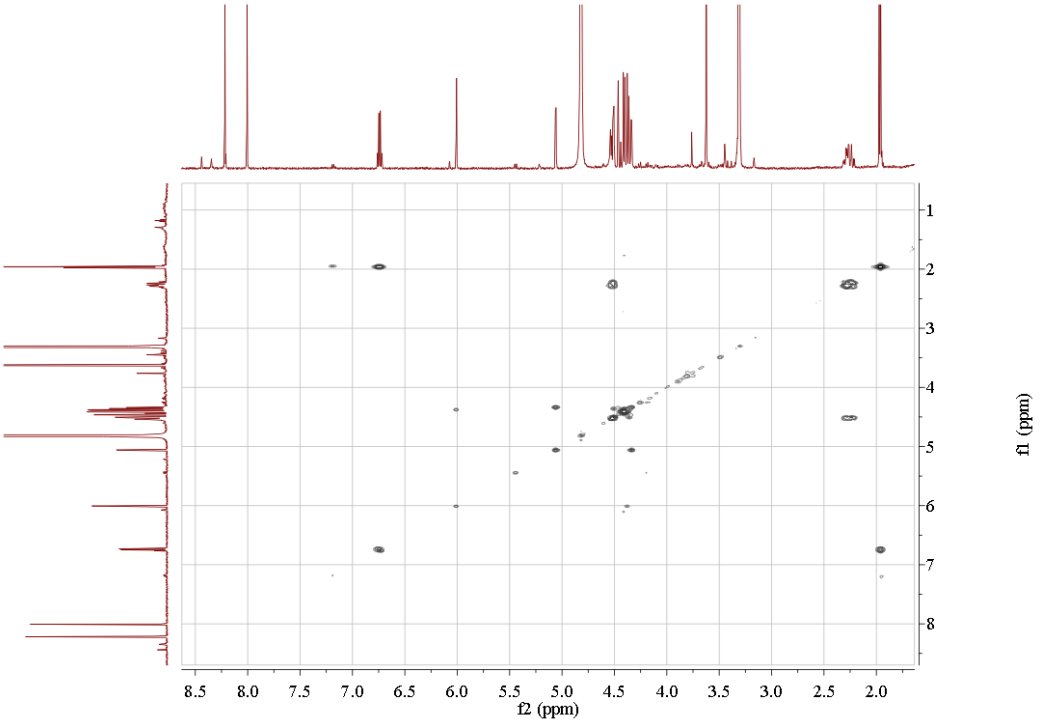


Figure S64 ^1H-^1H COSY spectrum of 11 in CD_3OD

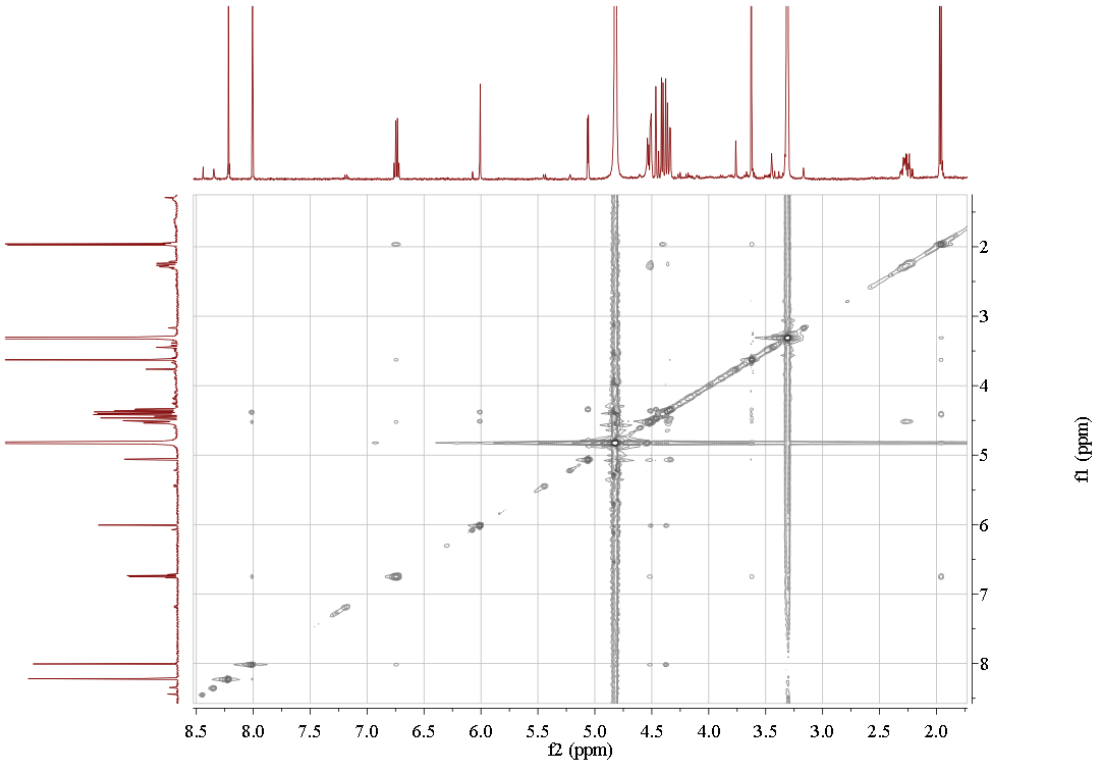


Figure S65 NOESY spectrum of 11 in CD_3OD

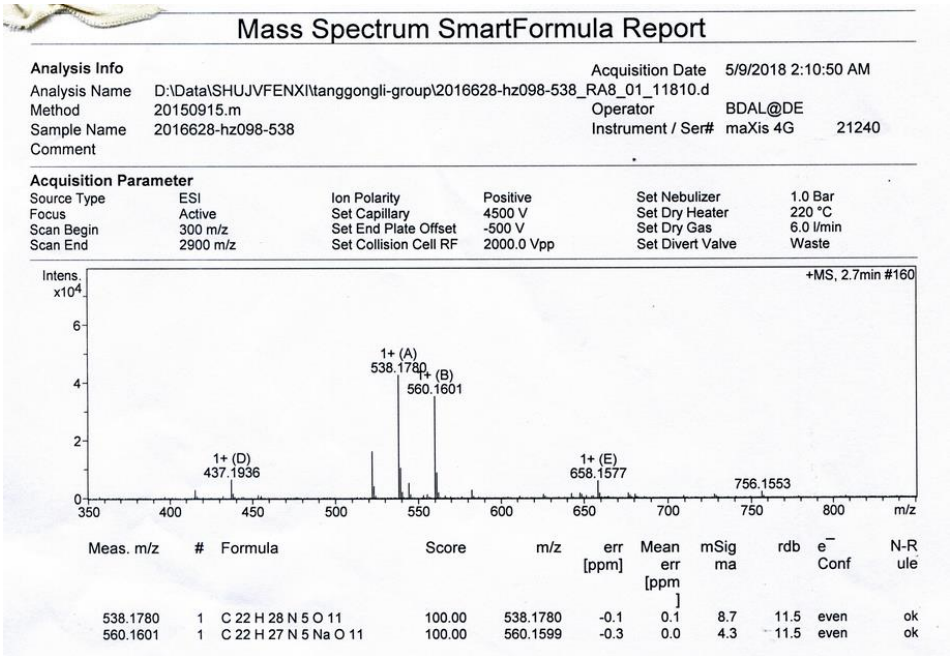


Figure S66 HRMS data of 11

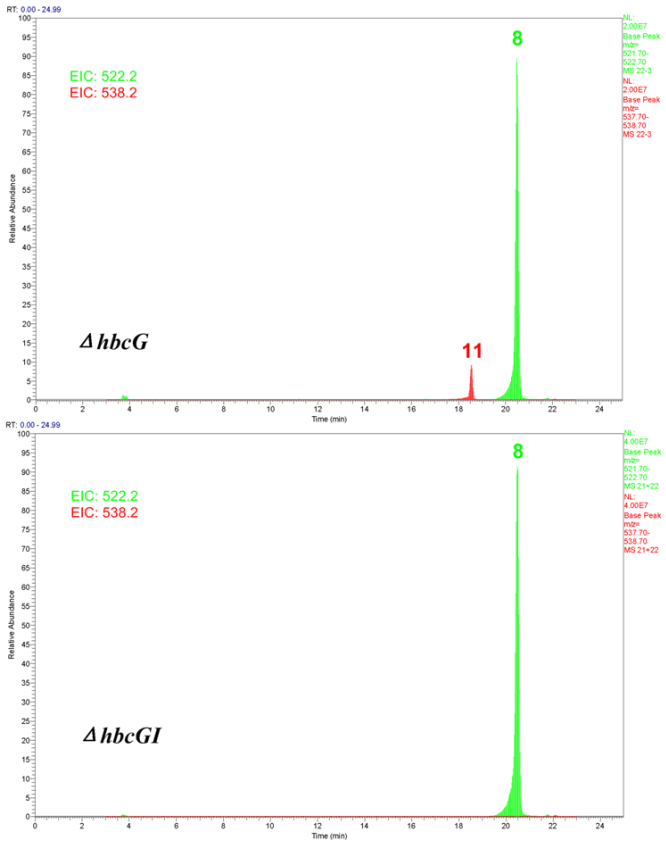


Figure S67 LC-MS analysis of *S.* sp KIB-027 derived gene knock-out mutants, *ΔhbcG* and *ΔhbcGI*.

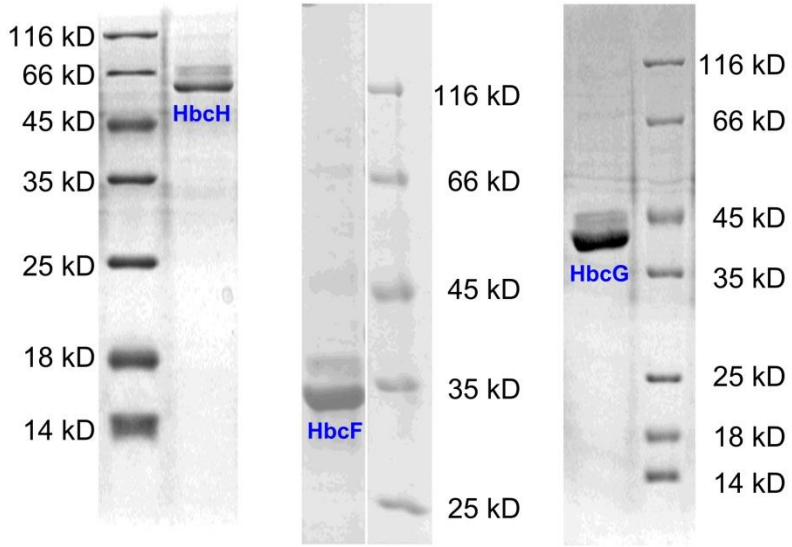


Figure S68 SDS-PAGE analysis of the purified proteins HbcH (61.4 kDa), HbcF (33.0 kDa) and HbcG (41.4 kDa).

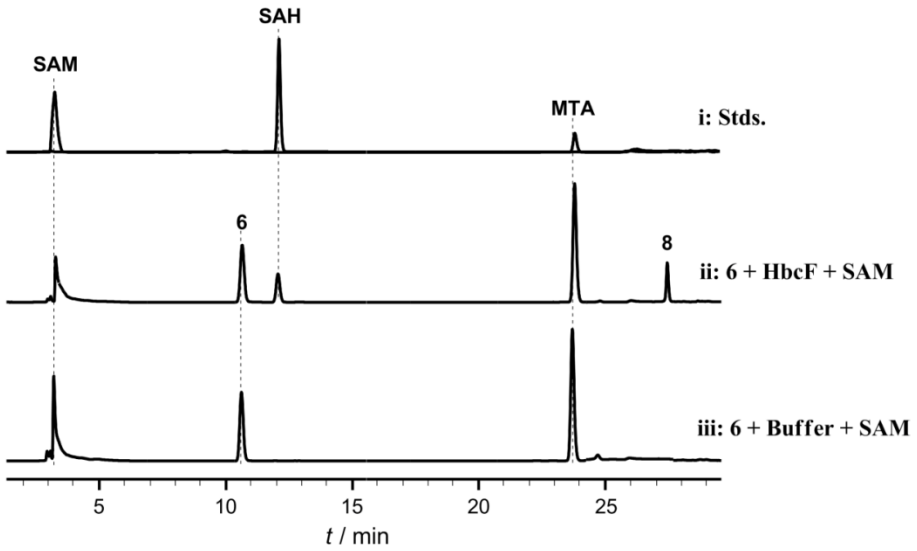


Figure S69 *In vitro* assay of HbcF with 6 as substrate using HPLC method 2 (0.8 mL/min, 260 nm, T = 0 min, 4% B; T = 4 min, 4 % B; T = 25 min, 80% B; T = 28 min, 80% B; T = 30 min, 4% B; (A = H2O, B = CH3OH).

(Note: Using the common HPLC method for *in vitro* experiments, 6 was co-eluted with 5'-deoxy-5'-methylthioadenosine (MTA), see Figure 3, i)

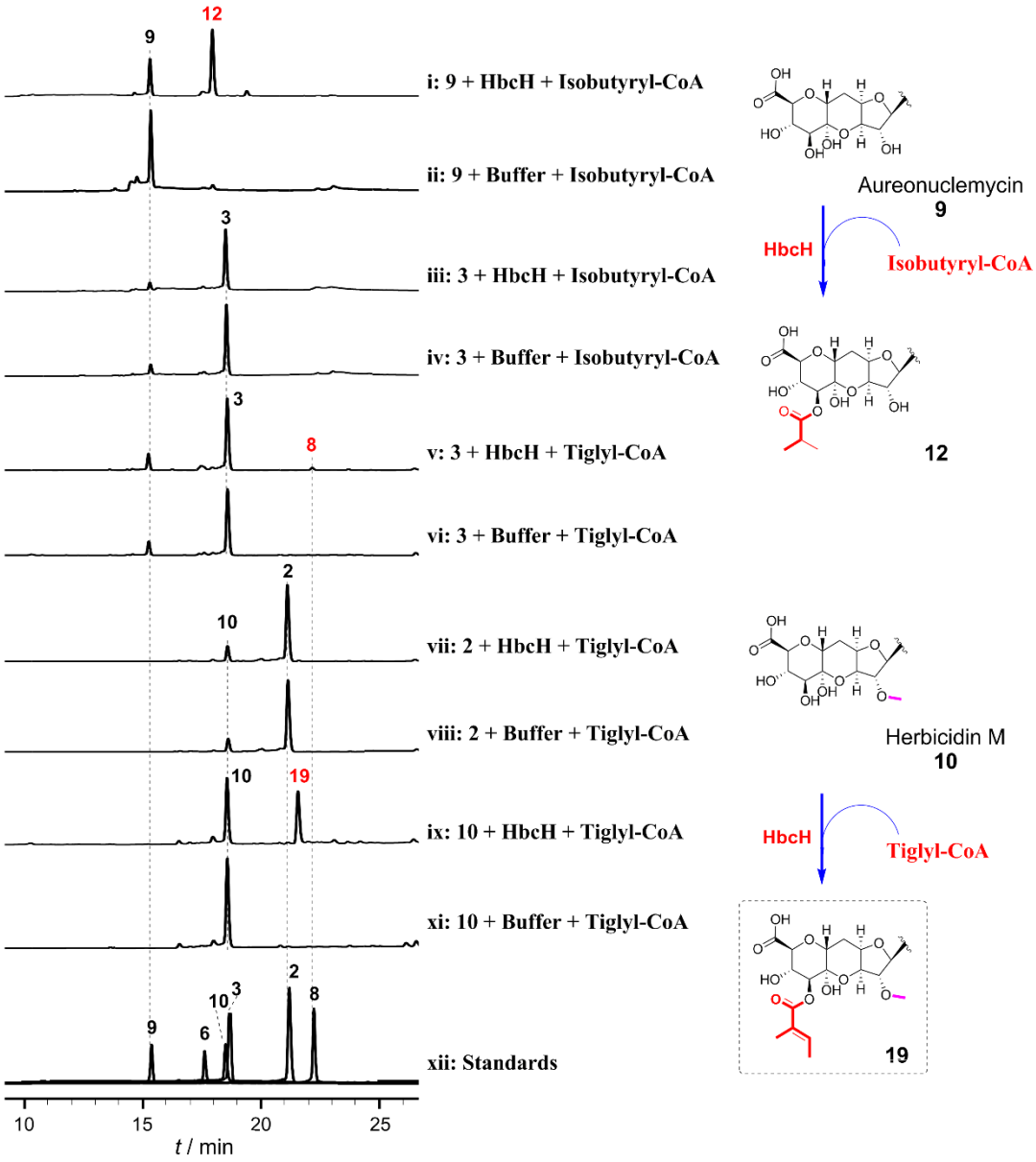


Figure S70 *In vitro* assay of HbcH with 9, 3, 2 and 10 as substrates.

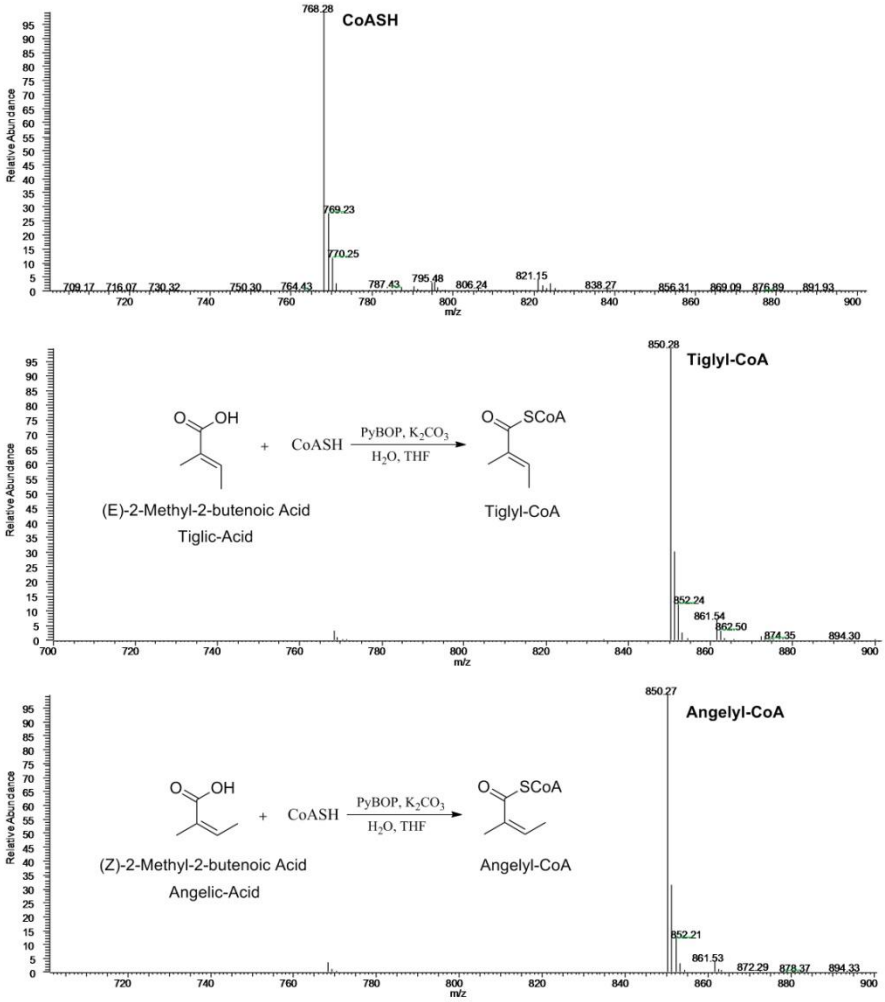


Figure S71 Chemical synthesis of tiglyl-CoA and angelyl-CoA.

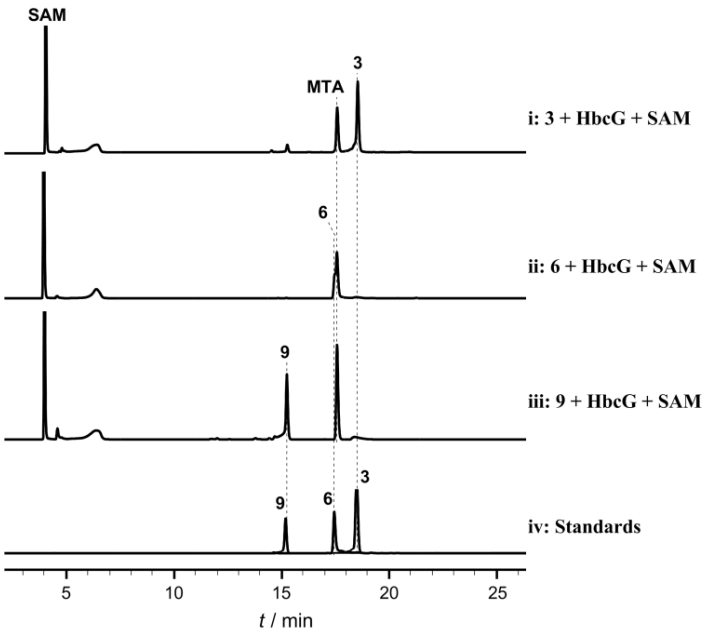


Figure S72 *In vitro* assay of HbcG with 3, 6 and 9 as substrates.

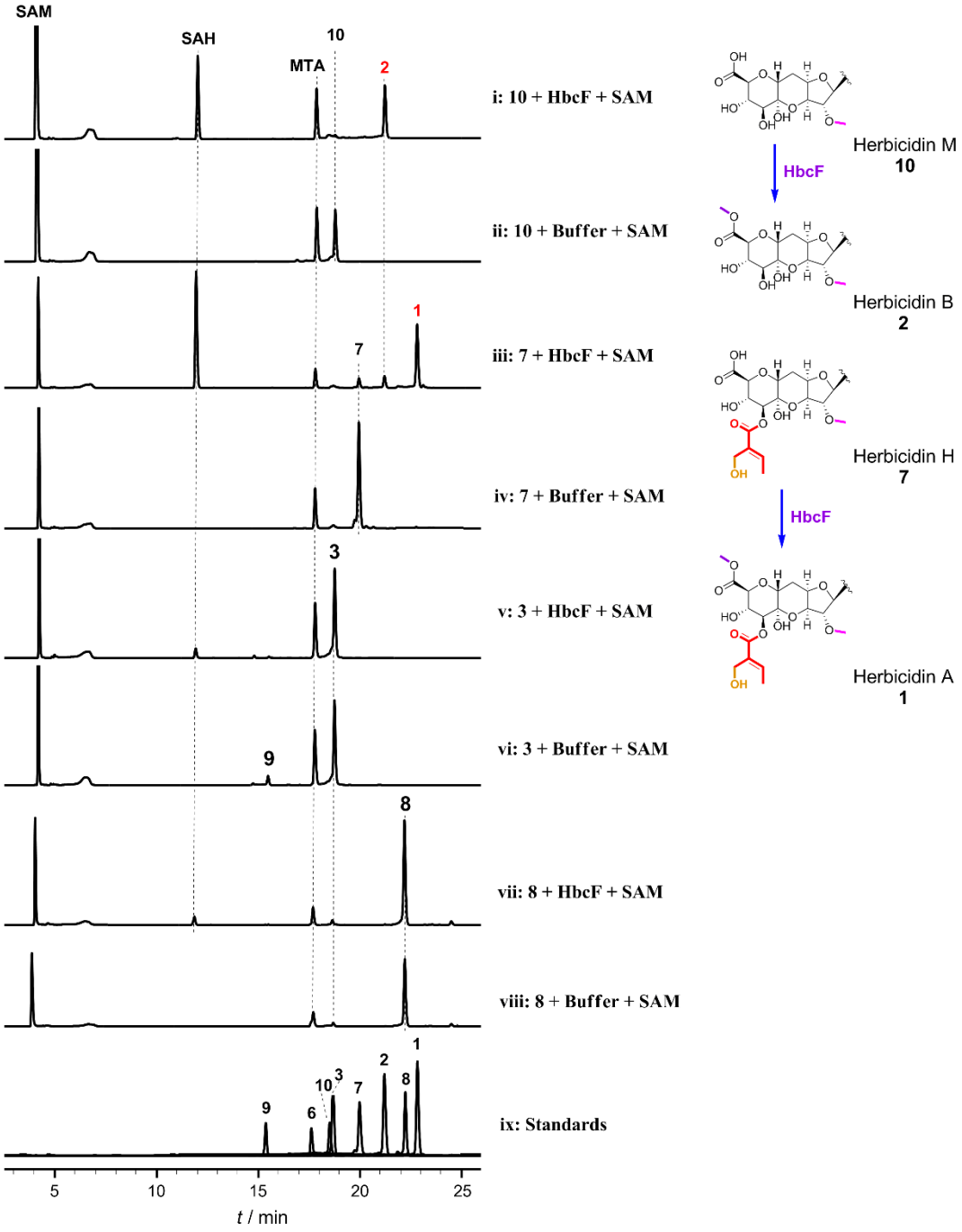


Figure S73 *In vitro* assay of HbcF with 10, 7, 3 and 8 as substrates.

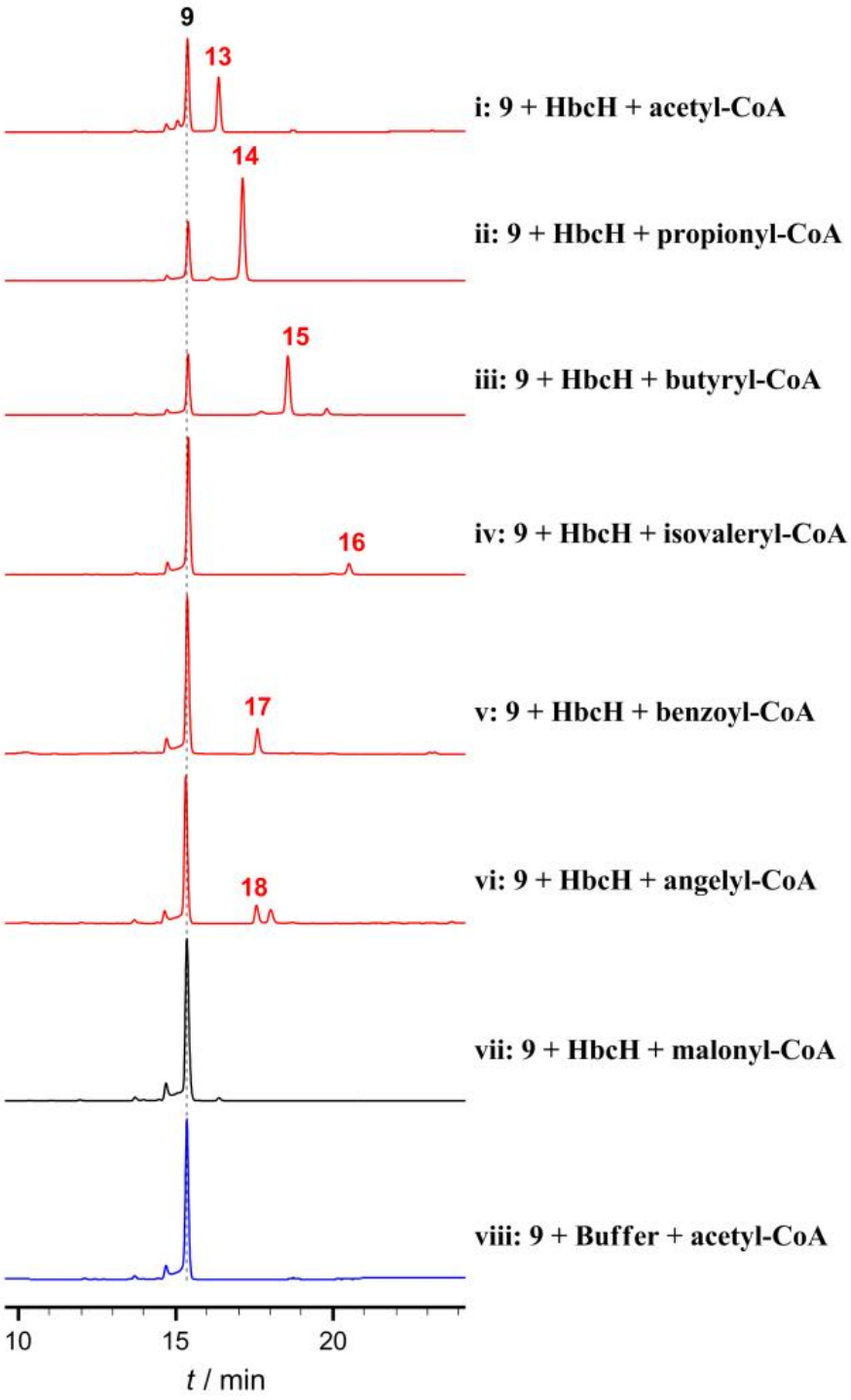


Figure S74 ***In vitro*** **assay of HbcH with 9 as substrate and various acyl donors including: i) acetyl-CoA, ii) propionyl-CoA, iii) butyryl-CoA, iv) isovaleryl-CoA, v) benzoyl-CoA, vi) angelyl-CoA, and vii) malonyl-CoA. viii) control reaction.**

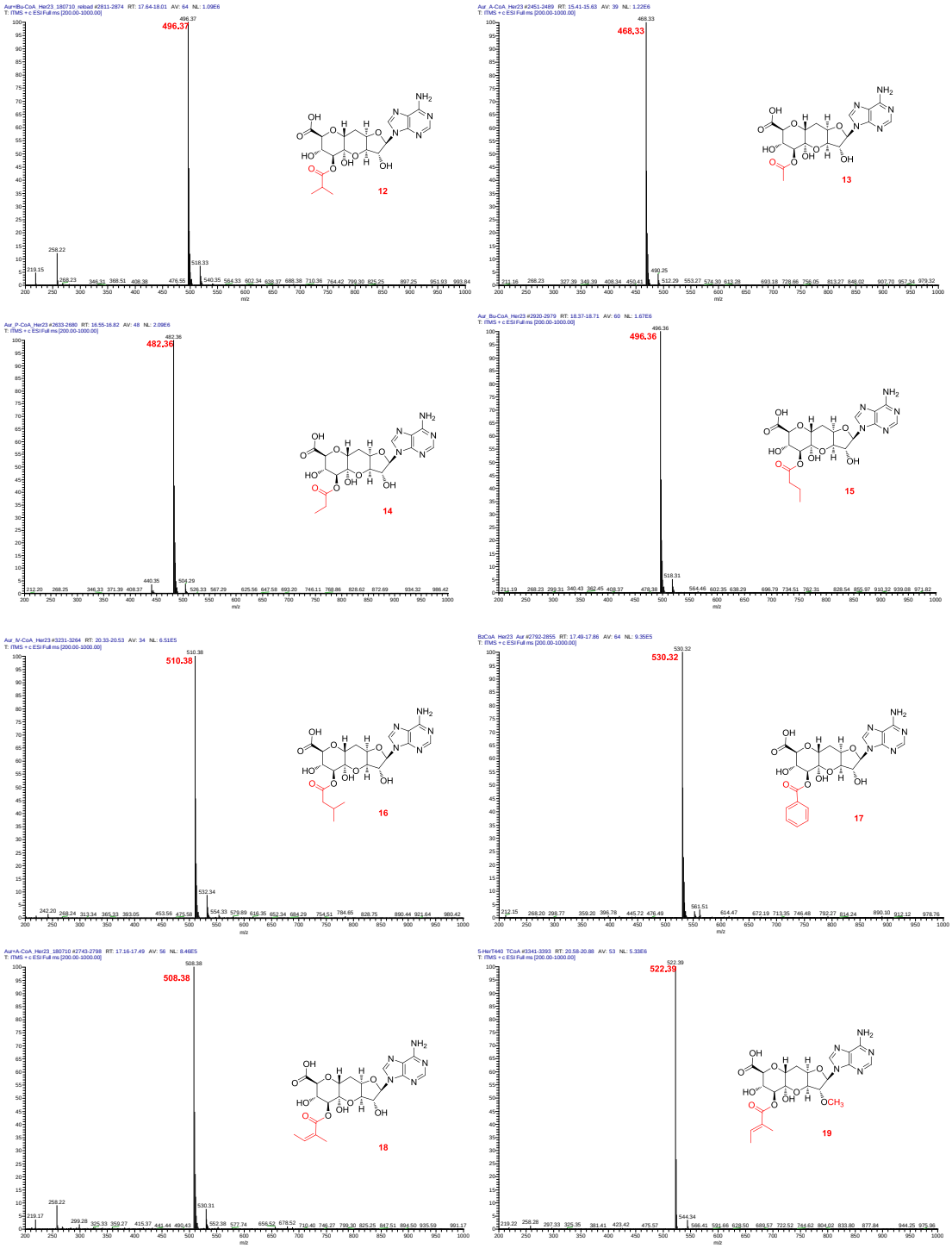


Figure S75 Mass spectra for 12-19 and the putative structures of these compounds.

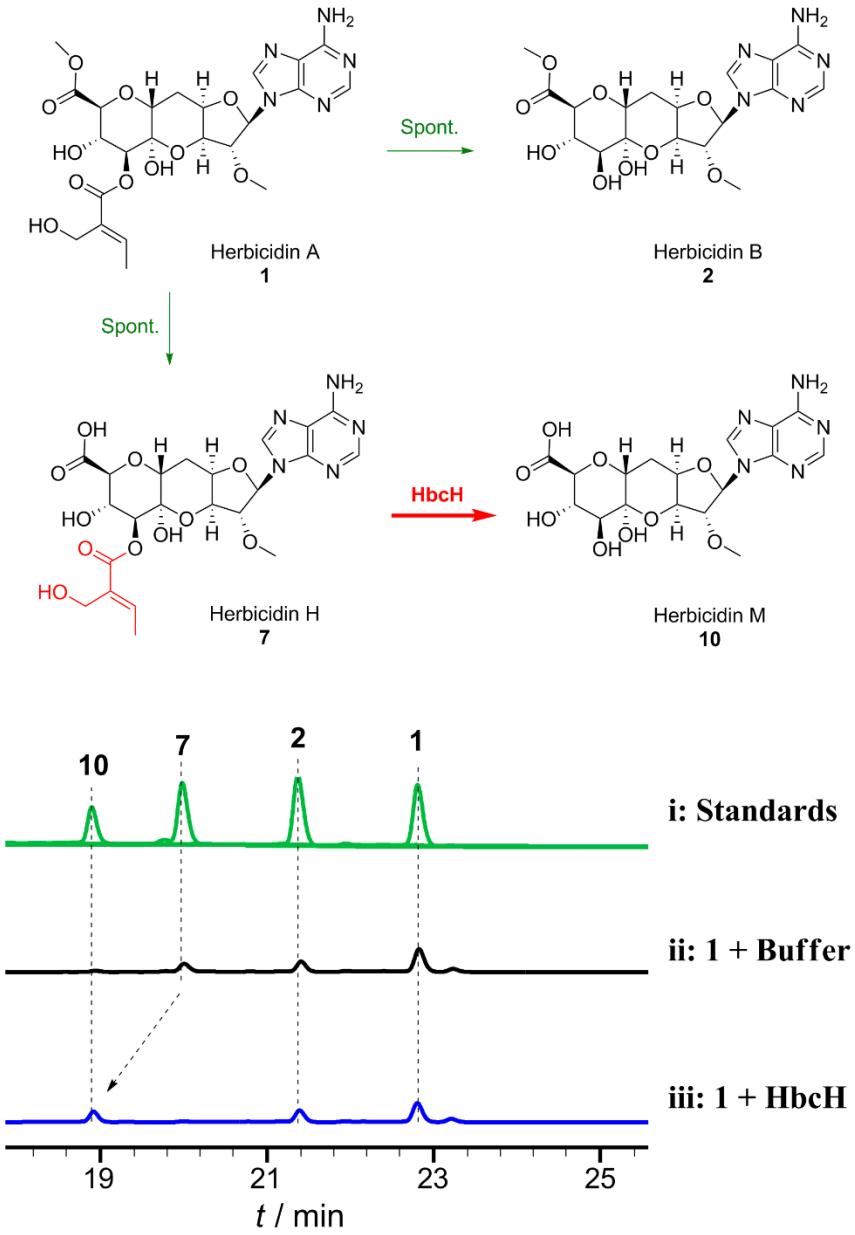


Figure S76 ***In vitro*** **assay of the hydrolytic activity of HbcH with 1 as substrate.**

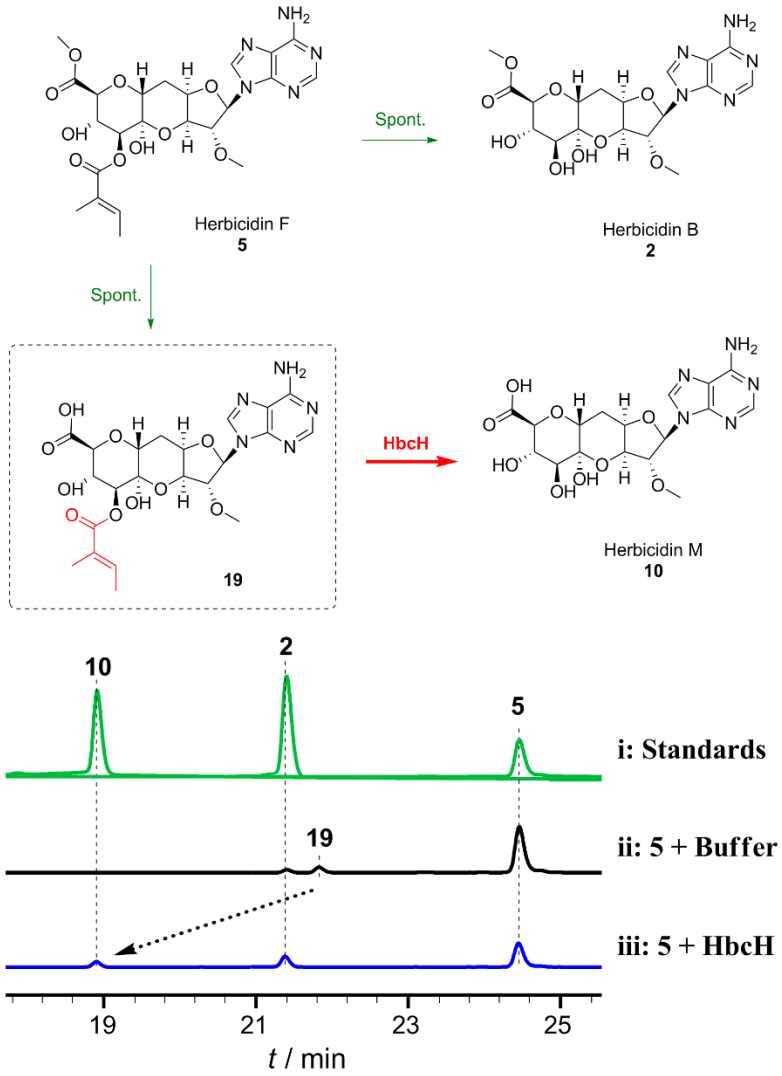


Figure S77 ***In vitro*** **assay of the hydrolytic activity of HbcH with 5 as substrate.**

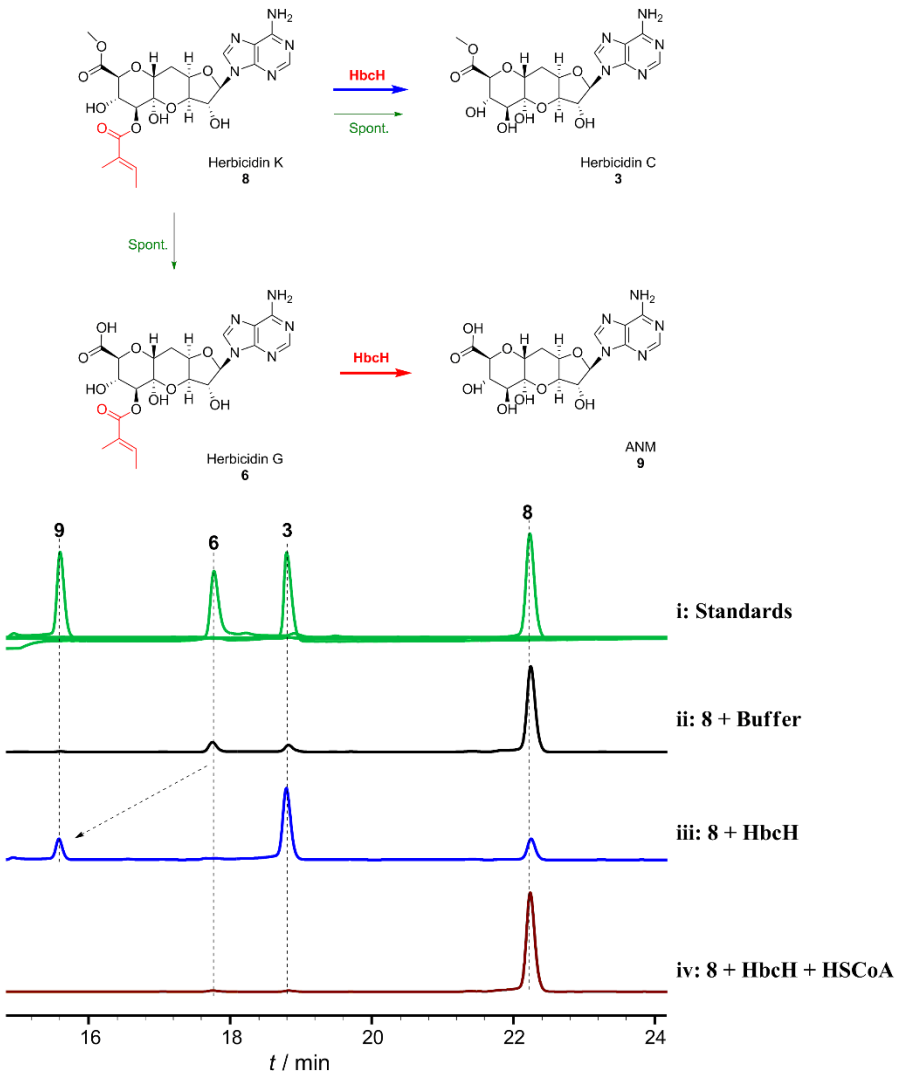


Figure S78 *In vitro* assay of the hydrolytic activity of HbcH with 8 as substrate.

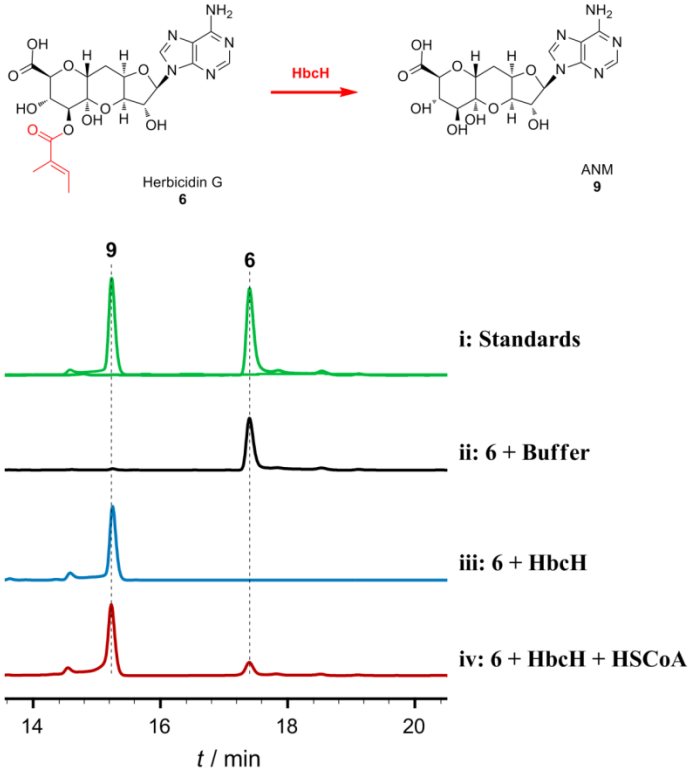


Figure S79 *In vitro* assay of the hydrolytic activity of HbcH with 6 as substrate.

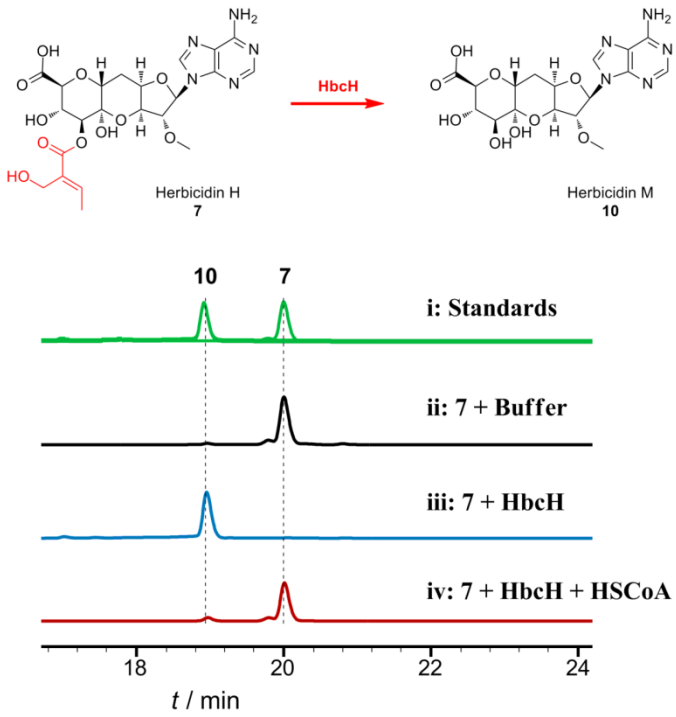


Figure S80 *In vitro* assay of the hydrolytic activity of HbcH with 7 as substrate.

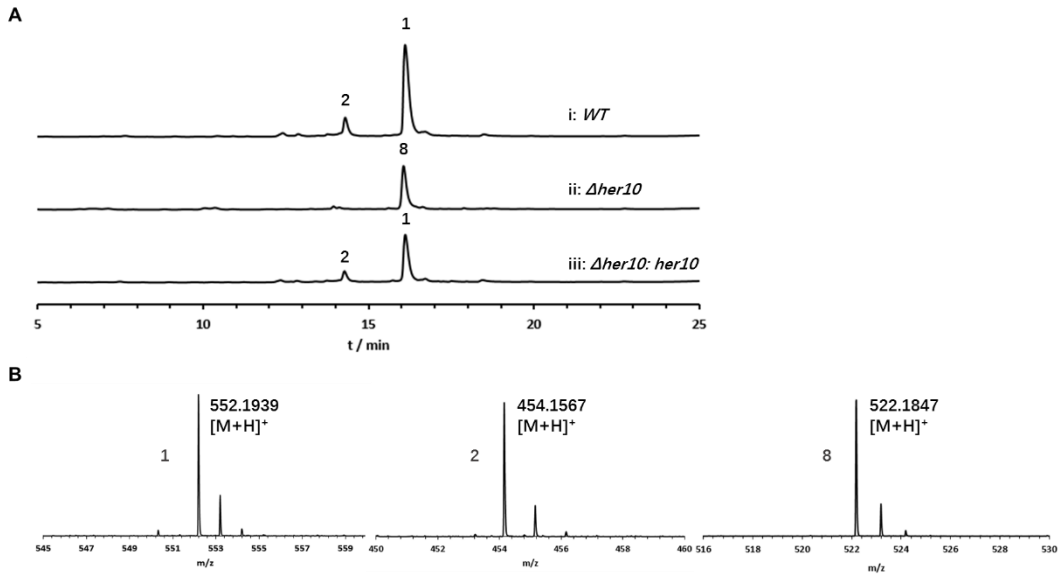


Figure S81. HPLC analysis method 3 and MS data of *S*. sp. L-9-10 wild type, *Δher10* mutant, *Δher10* mutant complementation fermentation broth

A) HPLC profile; B) MS data for compounds 1, 2 and 8.

(HPLC method 3: 1 mL/min: T = 0 min, 12.5% B; T = 5 min, 12.5% phase B; T = 20 min, 50% B; T = 22 min, 80% phase B, T = 27 min, 80% B; T = 30 min, 12.5% phase B; A = H_2O with 0.1% formic acid, B = acetonitrile with 0.1% formic acid).

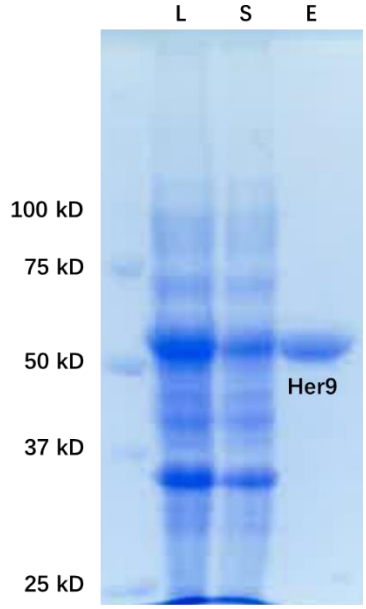


Figure S82. SDS-PAGE analysis of the purified protein Her9 (61.0 kDa)

L: whole-cell lysate, S: soluble fraction, E: purified proteins

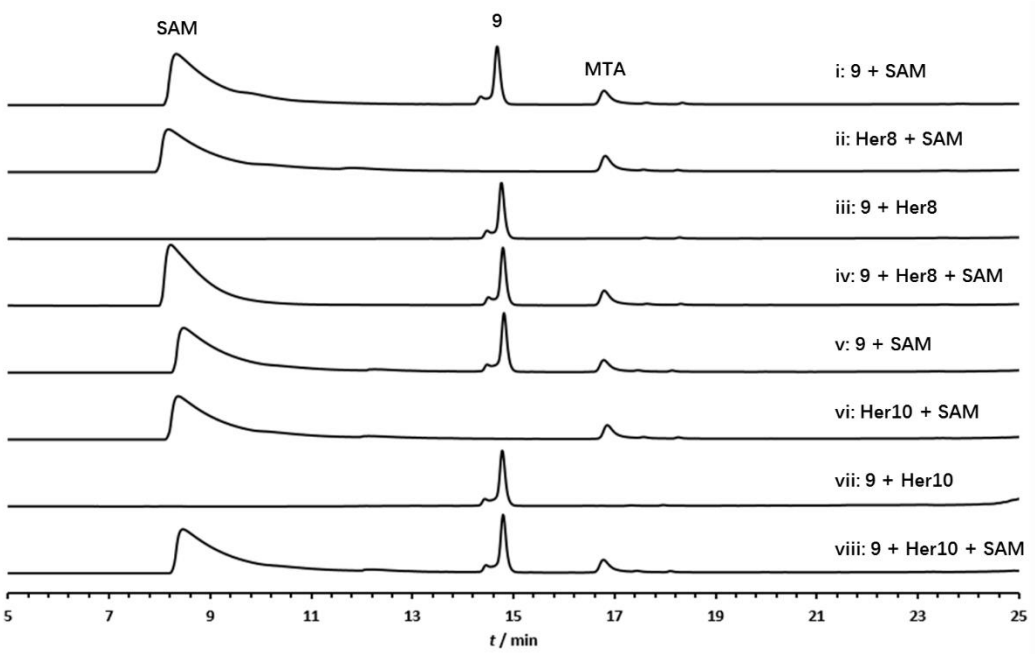


Figure S83. *In vitro* assay of Her8, Her10 with compound 9 as substrate using HPLC analysis method 4

(HPLC Method 4: 1 mL/min: T = 0 min, 5% B; T = 5 min, 5% phase B; T = 20 min, 50% B; T = 22 min, 80% phase B, T = 27 min, 80% B; T = 30 min, 5% phase B; A = H_2O with 0.1% formic acid, B = acetonitrile with 0.1% formic acid).

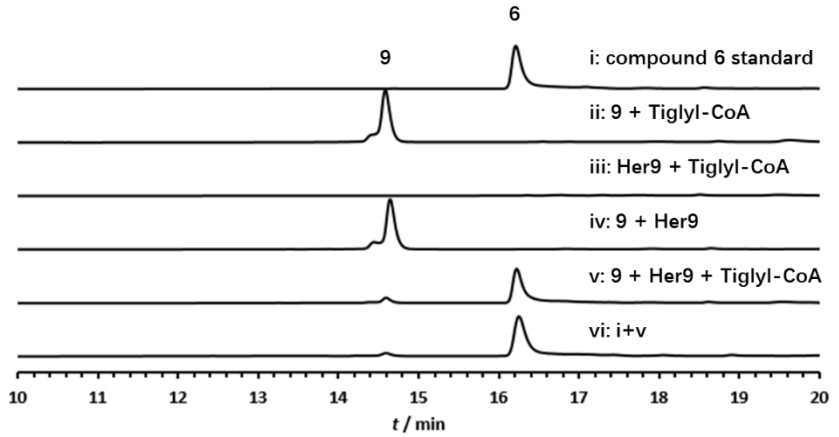


Figure S84. *In vitro* assay of Her9 with compound 9 as substrate using HPLC analysis method 4

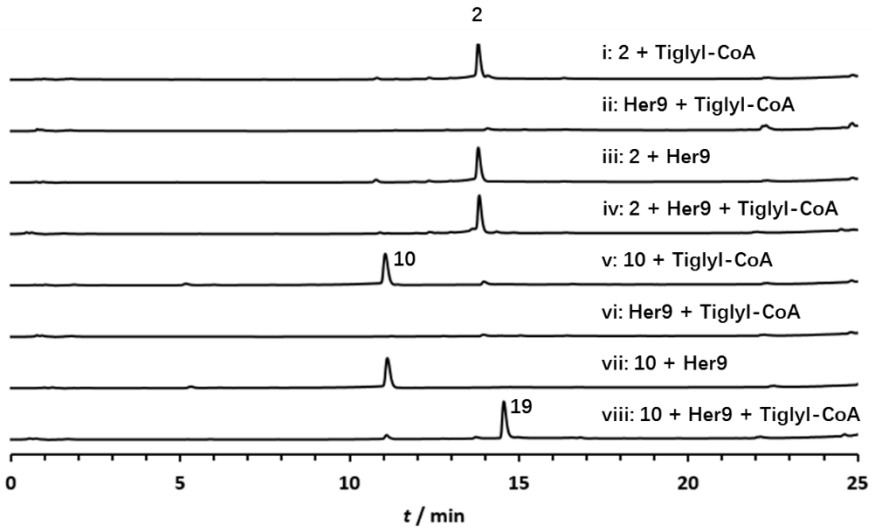


Figure S85. ***In vitro*** **assay of Her9 with compounds 2 and 10 as substrate using HPLC analysis method 3**

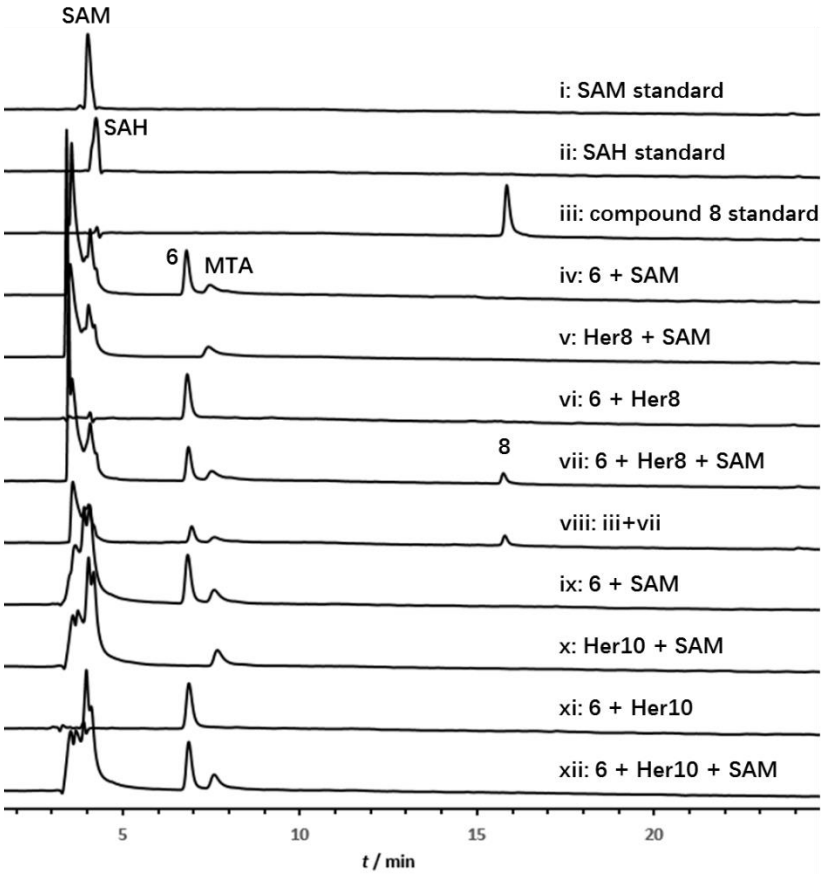


Figure S86. ***In vitro*** **assay of Her8, Her10 with compound 6 as substrate using HPLC analysis method 3**

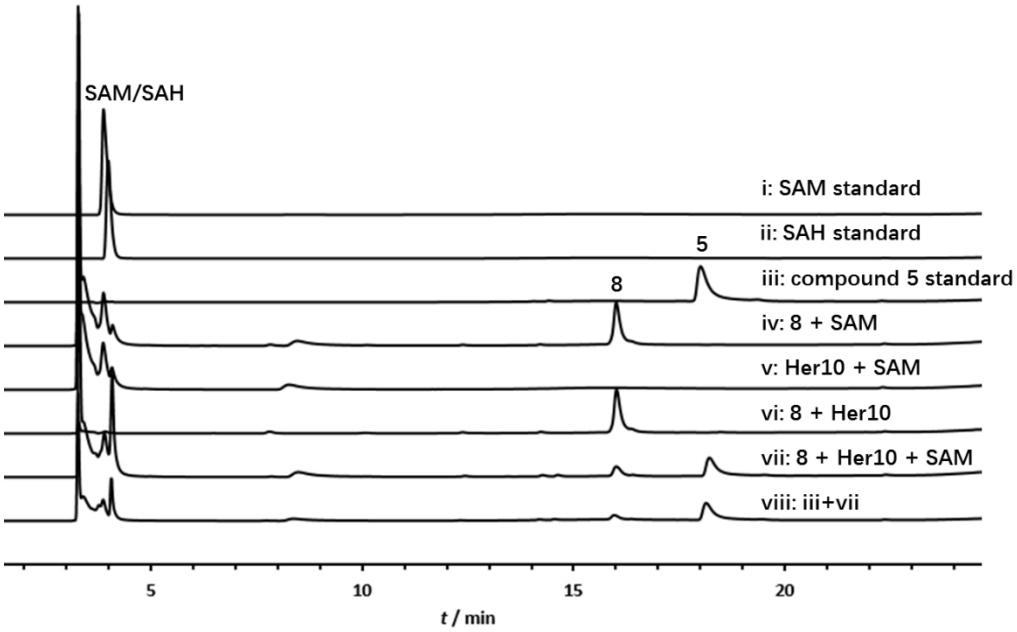


Figure S87. *In vitro* assay of Her10 with compound 8 as substrate using HPLC analysis method 3

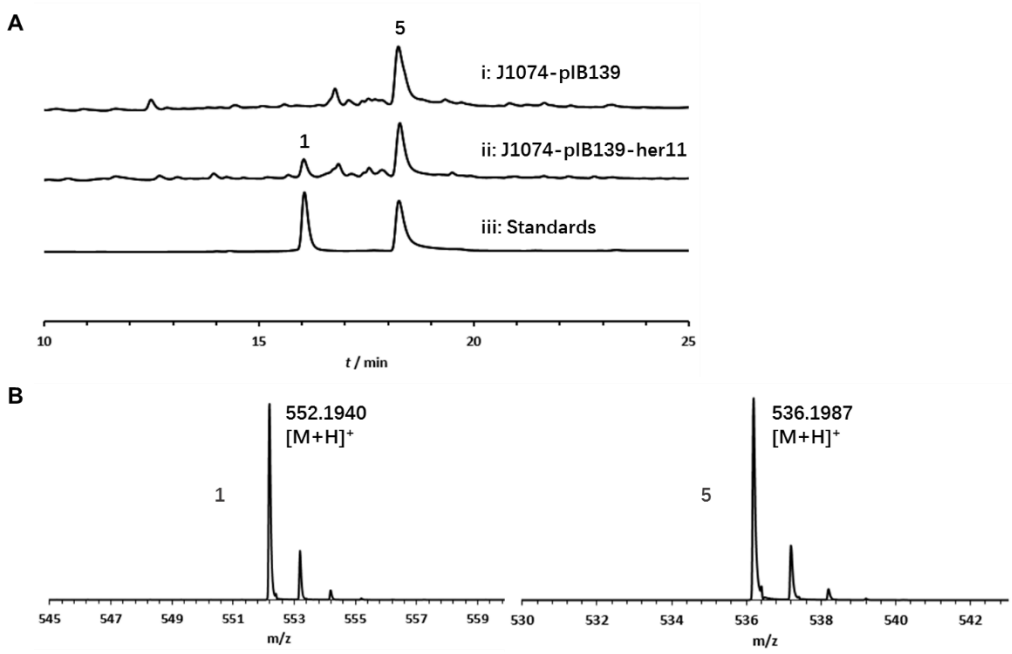


Figure S88. HPLC and MS analysis of feeding result for *in vivo* Her11 activity
A) HPLC profile; B) MS data for compounds 1 and 5