Supporting Information

A fungal P450 deconstructs the 2,5-diazabicyclo[2.2.2]octane ring *en route* to the complete biosynthesis of 21*R*-citrinadin A

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Experimental procedures

1. Strains and culture conditions.

Penicillium citrinum ATCC 9849 was obtained from ATCC (https://www.atcc.org/). *P. citrinum* ATCC 9849 and the mutants were cultured in YM (5 g/L yeast extract, 10 g/L peptone, 10 g/L maltose) at 28°C for secondary metabolite production. *Aspergillus nidulans* A1145 was used as the host for heterologous expression of the *ctd* gene cluster. *Saccharomyces cerevisiae* RC01[1] was used for in vivo homologous recombination to construct the *Aspergillus nidulans* A1145 overexpression plasmids. Yeast extract peptone dextrose (YPD) medium (20 g/L peptone, 10 g/L yeast extract, 20 g/L dextrose) was used for routine growth, while uracil-dropout semisynthetic medium was used for selection of plasmids transformed into *S. cerevisiae*. *Escherichia coli* BL21 (DE3) and *E. coli* stbl3 were grown in LB media and used for standard DNA manipulations.

2. Preparation of protoplast of *Penicillium citrinum* ATCC 9849 and *Aspergillus nidulans* A1145.

Protoplasts of *P. citrinum* ATCC 9849 and *A. nidulans* A1145 were prepared in a similar manner. Fresh spores were inoculated into 25 mL liquid CD medium 10 g/L glucose, 50 mL 20 × nitrate salts, 1 mL trace elements, pH 6.5) in 250 mL flask and germinated at 30°C and 250 rpm for approximately 9 h. For the preparation of 20 × nitrate salts, 120 g $NaNO_3$, 10.4 g KCl, 10.4 g $MgSO_4 \cdot 7H_2O$, 30.4 g KH_2PO_4 were dissolved in 1 L double distilled water. The 100 mL trace elements with pH 6.5 contained 2.20 g $ZnSO_4 \cdot 7H_2O$, 1.10 g H_3BO_3, 0.50 g $MnCl_2 \cdot 4H_2O$, 0.16 g $FeSO_4 \cdot 7H_2O$, 0.16 g $CoCl_2 \cdot 5H_2O$, 0.16 g $CuSO_4 \cdot 5H_2O$, and 0.11 g $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$. Mycelia were harvested by centrifugation at 3500 rpm for 10 min, and washed with 10 mL of Osmotic buffer (1.2 M $MgSO_4$, 10 mM sodium phosphate, pH 5.8). Then the mycelia were transferred to 10 mL of Osmotic buffer containing 30 mg of lysing enzymes from *Trichoderma* and 20 mg of Yatalase in a 125 mL flask. The flask was shaken at 80 rpm overnight at 30°C. Cells were collected in a 30 mL Corex tube and overlaid gently by 10 mL of Trapping buffer (0.6 M sorbitol, 0.1 M Tris-HCl, pH 7.0). After centrifugation at 3500 rpm for 15 min at 4°C, the protoplasts were collected at the interface of the two buffers. The protoplasts were then transferred to a sterile 15 mL Falcon tube and washed with 10 mL STC buffer (1.2 M sorbitol, 10 mM $CaCl_2$, 10 mM Tris-HCl, pH 7.5). The protoplasts were resuspended in 1 mL STC buffer for transformation.[2]

3. Gene knock-out of *Penicillium citrinum* ATCC 9849.

The hygromycin-resistance split-marker approach was used for targeted gene knockout by homologous recombination. The homologous regions (~2 kb) were amplified by PCR from the *P. citrinum* ATCC 9849 genome. Deletion cassettes were generated by a fusion PCR technique. The two overlapping hygromycin resistance gene fragments were transformed into *P. citrinum* ATCC 9849 by polyethylene glycol (PEG)-mediated protoplast transformation. The lyophilized DNA fragments were incubated with 100 μL of the protoplasts then 50 μL of filtered PEG solution (25 % PEG average molecular weight 3350, 0.6 M KCl, 50 mM $CaCl_2$, 10 mM Tris-HCl, pH 7.5) was added and gently mixed. Place in an ice water bath for 25 min. Add 1 mL of room temperature, filtered PEG solution. Mix the PEG solution with the protoplast suspension by gently aspirating and ejecting the solution into the tip of the micropipette at least ten times. Leave at room temperature for 25 min. Plate transformation mixture onto selective plates.[3]

4. Heterologous expression in *A. nidulans* A1145.

The genes in the *ctd* cluster were amplified by PCR and cloned into vectors by recombination in yeast. The genomic DNA of *P. citrinum* ATCC 9849 were used as the templates of PCR. pYTU, pYTR and pYTP were used for amplification of *glaA*, *gpdA*, and *amyB* promoters, respectively. The overlapping DNA fragments and *PacI*/*SwaI*-digested expression vectors pYTP and pYTR were co-transformed into yeast. The primers used for the heterologous expression are listed in Table S3.

The selection markers of the three expression vectors were uridine, pyridoxine and riboflavin in *A. nidulans* A1145.[4] CDST media (20 g/L starch, 20 g/L tryptone, 50 mL 20 × nitrate salts, 1 mL trace elements, pH 6.5) was used to induce gene expression. The constructed plasmids for heterologous expression were transformed into protoplasts of *A. nidulans* A1145 by polyethylene glycol (PEG) mediated protoplast transformation as described in above.

5. gDNA extraction, RNA extraction, and RT-PCR.

The Zymo ZR Fungal/Bacterial DNA Microprep™ kit was used to extract gDNA from *P. citrinum* ATCC 9849. The Zymo ZR Fungal/Bacterial RNA Microprep™ kit was used to extract RNA from *P. citrinum* ATCC 9849. RevertAid RT Reverse Transcription Kit from Thermo Scientific was used to synthesize cDNA from the RNA extracted from *P. citrinum* ATCC 9849.

6. Expression and preparation of CtdY-containing microsome for in vitro assays.

The intron-free *ctdY* was amplified from *P. citrinum* cDNA using the primer pair xw55-ctdY-F/ xw55-ctdY-R. The PCR product and *NdeI*/*PmlI*-linearized 2μ expression plasmid which containing the *ura* marker were co-transformed into *S. cerevisiae* RC01 using a ZYMO Frozen-EZ Yeast Transformation II Kit, yielding the pXW55-CtdY[5] by in vivo recombination. The resulting pXW55-CtdY plasmid was recovered using Zymoprep™ Yeast Plasmid Miniprep (Zymo Research) for propagation in *E. coli* stbl3 and verified by sequencing. *ctdY* was expressed under the control of the *ADH2* promoter.

The constructed plasmids pXW55-CtdY were transferred into the *S. cerevisiae* RC01. Selected cells were grown in 5 mL of SC medium without uracil at 30°C for 48 h at 250 rpm. The culture was transferred into 100 mL YPD medium at 30°C for 72 h at 250 rpm. Cells were harvested by centrifugation at 3000 g. All the subsequent procedures were performed at 4°C or on ice. Harvested cells were resuspended in 100 mL of TEK buffer (0.1 M KCl, 50 mM Tris-HCl (pH 7.4) and 1 mM EDTA). Cells were incubated at 4°C or on ice and collected by centrifugation at 3000 g. Cells were resuspended in 5 mL TES buffer (0.6 M sorbitol, 50 mM Tris-HCl (pH 7.4) and 1 mM EDTA) supplemented with 0.2 mM phenylmethylsulfonyl fluoride. Approximately 2.5 mL of zirconia/silica beads (0.5 mm in diameter, Biospec Products) were added. Cell walls were manually disrupted by hand shaking in a cold room for 20 min at 30 s intervals separated by 30 s intervals on ice. The lysate was clarified by centrifugation at 8000 g. Finally, microsomes were obtained by centrifugation at 100000 g for 70 min at 4°C. The pellet was resuspended in 1 mL of TEG buffer (20% (v/v) glycerol, 50 mM Tris-HCl (pH 7.4) and 1 mM EDTA) to obtain a microsomal fraction.[4]

7. Chemical complementation assays.

For precursor feeding in *A. nidulans* A1145, 1 mg precursors were dissolved in DMSO and then added to 20 mL of liquid CDST cultures of each strain. After further cultivation at 30°C for 5 days, the secondary metabolites were extracted with ethyl acetate, dissolved in MeOH, and analyzed by LC-MS.

8. Expression and purification of CtdR, CtdV, and CtdJ from *E. coli*.

To construct the plasmid for expression of CtdR, CtdV, and CtdJ in *E. coli*, the ORF was amplified by PCR using the *P. citrinum* ATCC 9849 cDNA as the template. The recovered DNA fragment was ligated into the vector pET by Gibson assembly. After confirmation by sequencing, the plasmids were transferred into *E. coli* BL21 (DE3) for protein expression and purification.

Overexpression and subsequent protein purification were performed as follows: *E. coli* harboring the plasmid was grown overnight in 5 mL of LB with 50 µg/mL ampicillin at 37°C. 500 mL of fresh LB with 50 µg/mL ampicillin was inoculated with 5 mL of the overnight culture and incubated at 37°C until the optical density at 600 nm reached 0.6. The protein was overexpressed with 0.1 mM IPTG for 16 h at 16°C. Cells were harvested by centrifugation (3000 g, 15 min). All subsequent procedures were performed at 4°C or on ice. Harvested cells were resuspended in disruption buffer (50 mM Tris-HCl (pH 7.8), 200 mM NaCl). After sonication and centrifugation (17000 g, 60 min, 4°C), the supernatant was subjected to His-tag affinity purification.

9. In vitro assays of CtdY.

The 100 µL CtdY microsomal fractions containing 0.5 mM compound **20** and 2 mM NADPH were incubated overnight at 28 °C and extracted twice with 200 µL ethyl acetate. The organic phase was dried and dissolved in 50 µL MeOH for LC-MS analysis. Samples were analyzed on an Agilent 6120B Single Quadrupole LC-MS using an Agilent Poroshell 120 EC-C18 column (3.0 × 150 mm) with the following time program: 5%-95% acetonitrile for 15 min, 95% acetonitrile for 5 min, 95%-5% acetonitrile for 5 min, and 5% acetonitrile for 5 min. 0.1% of formic acid was added to H_2O. The flow rate was 0.5 mL/min.

10. In vitro assays of CtdR.

Assays of CtdR were performed in 100 µL volumes containing 50 mM Tris-HCl (pH 7.8), 0.2 mM compound **19**, 20 µM CtdR, 2.5 mM NADPH. The mixture was incubated at 30°C for 12 h and extracted twice with ethyl acetate. The organic phases were dried and dissolved in 50 µL MeOH and subjected to LC-MS analysis. Samples were analyzed on an Agilent 6120B Single Quadrupole LC-MS using an Agilent Poroshell 120 EC-C18 column (3.0×150 mm) with the following time program: 5% acetonitrile over 5 min, 5%-60% acetonitrile for 25 min, 95% acetonitrile for 5 min, 95%-5% acetonitrile for 1 min, and 5% acetonitrile for 4 min. 0.1% of formic acid was added to H_2O. The flow rate was 0.5 mL/min, and the reactions were monitored at 280 nm.

11. In vitro assays of CtdV.

Assays of CtdV were performed in 100 µL volumes containing 50 mM Tris-HCl (pH 7.8), 0.2 mM compound **15** or **16**, 20 µM CtdV, 2 mM ascorbic acid, 2 mM *α*-ketoglutarate, 0.5 mM $FeSO_4 \cdot 7H_2O$. The mixture was incubated at 30°C for 12 h and extracted twice with ethyl acetate. The organic phases were dried and dissolved in 50 µL MeOH and subjected to LC-MS analysis as described for CtdR assays.

12. In vitro assays of CtdJ.

Assays of CtdJ were performed in 100 μL volumes containing 50 mM Tris-HCl (pH 7.8), 0.2 mM compound **17**, 20 μM CtdJ, 2 mM *α*-ketoglutarate. The mixture was incubated at 30°C for 12 h and extracted twice with ethyl acetate. The organic phases were dried and dissolved in 20 μL MeOH and subjected to LC-MS analysis as described for CtdR assays.

Supplementary Tables

Table S1. Strains in this study.

Strain	**Characteristics**	**source**
P. citrinum ATCC 9849	Wild type *P. citrinum* used in this study	ATCC
Δ*ctdT*	*ctdT* knockout mutant of *P. citrinum* ATCC 9849	This study
Δ*ctdF*	*ctdF* knockout mutant of *P. citrinum* ATCC 9849	This study
Δ*ctdY*	*ctdY* knockout mutant of *P. citrinum* ATCC 9849	This study
Δ*ctdS*	*ctdS* knockout mutant of *P. citrinum* ATCC 9849	This study
Δ*ctdG*	*ctdG* knockout mutant of *P. citrinum* ATCC 9849	This study
Δ*ctdR*	*ctdR* knockout mutant of *P. citrinum* ATCC 9849	This study
Δ*ctdV*	*ctdV* knockout mutant of *P. citrinum* ATCC 9849	This study
Δ*ctdJ*	*ctdJ* knockout mutant of *P. citrinum* ATCC 9849	This study
Δ*ctdD*	*ctdD* knockout mutant of *P. citrinum* ATCC 9849	This study
Δ*ctdC*	*ctdC* knockout mutant of *P. citrinum* ATCC 9849	This study

Table S2. Plasmids used in this study.

Plasmids	**Description of Plasmid**
pXW55-CtdY	2μ yeast expression plasmid CtdY gene under the *ADH2* promoter
pXW55-CtdY(L102A)	2μ yeast expression plasmid CtdY(L102A) gene under the *ADH2* promoter
pXW55-CtdY(N106A)	2μ yeast expression plasmid CtdY(N106A) gene under the *ADH2* promoter
pXW55-CtdY(F110A)	2μ yeast expression plasmid CtdY(F110A) gene under the *ADH2* promoter
pXW55-CtdY(F119A)	2μ yeast expression plasmid CtdY(F119A) gene under the *ADH2* promoter
pXW55-CtdY(P218A)	2μ yeast expression plasmid CtdY(P218A) gene under the *ADH2* promoter
pXW55-CtdY(F300A)	2μ yeast expression plasmid CtdY(F300A) gene under the *ADH2* promoter
pXW55-CtdY(H304A)	2μ yeast expression plasmid CtdY(H304A) gene under the *ADH2* promoter
pXW55-CtdY(S305A)	2μ yeast expression plasmid CtdY(S305A) gene under the *ADH2* promoter
pXW55-CtdY(M308A)	2μ yeast expression plasmid CtdY(M308A) gene under the *ADH2* promoter
pXW55-CtdY(I365A)	2μ yeast expression plasmid CtdY(I365A) gene under the *ADH2* promoter
pXW55-CtdY(F486A)	2μ yeast expression plasmid CtdY(F486A) gene under the *ADH2* promoter
pXW55-CtdY(S305Y)	2μ yeast expression plasmid CtdY(S305Y) gene under the *ADH2* promoter
pXW55-CtdY(S305D)	2μ yeast expression plasmid CtdY(S305D) gene under the *ADH2* promoter
pXW55-CtdY(S305T)	2μ yeast expression plasmid CtdY(S305T) gene under the *ADH2* promoter
pXW55-CtdY(S305V)	2μ yeast expression plasmid CtdY(S305V) gene under the *ADH2* promoter
pXW55-CtdY(S305C)	2μ yeast expression plasmid CtdY(S305C) gene under the *ADH2* promoter
pYTP-CtdY	*Aspergillus nidulans* expression vector containing gene *ctdY* under the *amyB* promoter
pYTR-CtdS	*Aspergillus nidulans* expression vector containing gene *ctdS* under the *gpdA* promoter
pYTP-CtdG	*Aspergillus nidulans* expression vector containing gene *ctdG* under the *amyB* promoter
pYTP-CtdGY	*Aspergillus nidulans* expression vector containing gene *ctdG* under the *amyB* promoter and *ctdY* under the *gpdA* promoter
pYTP-CtdYRGJ	*Aspergillus nidulans* expression vector containing gene *ctdY* under the *amyB* promoter and *ctdR* under the *gpdA* promoter

	ctdG under the *glaA* promoter and *ctdJ* under the *trpC* promoter
pYTR-CtdCSDV	*Aspergillus nidulans* expression vector containing gene *ctdC* under the *gpdA* promoter and *ctdS* under the *amyB* promoter *ctdD* under the *glaA* promoter and *ctdV* under the *trpC* promoter
pET-CtdR	vector for CtdR expression in *E. coli* BL(DE3)
pET-CtdV	vector for CtdV expression in *E. coli* BL(DE3)
pET-CtdJ	vector for CtdJ expression in *E. coli* BL(DE3)

Table S3. Primers used in this study.

Primer	**Sequence (5'→3')**
KO-CtdT-1F	CGAGGTCCGATGTATAACTCG
KO-CtdT-1R	ACTCCAGAATCAGCTAGGTCAACGCAACGCGCTAAGATGTATCCACAT
KO-CtdT-2F	ATGTGGATACATCTTAGCGCGTTGCGTTGACCTAGCTGATTCTGGAGT
KO-CtdT-2R	CACATGCCCTCATCATCCCAGTCACCGTTGATCTGCTTGATCTCGT
KO-CtdT-3F	ACGAGATCAAGCAGATCAACGGTGACTGGGATGATGAGGGCATGTG
KO-CtdT-3R	CTTGCATAGCAGCTTCTCTG
YZ-KO-CtdT-F	TCATCTAGGCGCTCAGGGTG
YZ-KO-CtdT-R	CTATCTTCAATAGATTGACAG
KO-CtdF-1F	CTCGAAGCCTACGGTTCCA
KO-CtdF-1R	ACTCCAGAATCAGCTAGGTCAACGTACCCCAAGAAAGAGGACATTGTT
KO-CtdF-2F	AACAATGTCCTCTTTCTTGGGGTACGTTGACCTAGCTGATTCTGGAGT
KO-CtdF-2R	GGAATGGCACAAAGTATATGGTCCACCGTTGATCTGCTTGATCTCGTC
KO-CtdF-3F	GACGAGATCAAGCAGATCAACGGTGGACCATATACTTTGTGCCATTCC
KO-CtdF-3R	AGAGTACAGTTGGAGGTTGG
YZ-KO-CtdF-F	CGAGGAGTACTTTCAAACAGC
YZ-KO-CtdF-R	ACCGTCTGTGCAATCAGAGA
KO-CtdY-1F	CTGATATTGGATTTCTGCGTG
KO-CtdY-1R	ACTCCAGAATCAGCTAGGTCAACGCGAAACCAAAGATGACATTGT
KO-CtdY-2F	ACAATGTCATCTTTGGTTTCGCGTTGACCTAGCTGATTCTGGAGT
KO-CtdY-2R	CAAGATGAAGAAGAATGAGCACACCGTTGATCTGCTTGATCTCGT
KO-CtdY-3F	ACGAGATCAAGCAGATCAACGGTGTGCTCATTCTTCTTCATCTTG
KO-CtdY-3R	TGTCGAGAACAAGATGTCCA
YZ-KO-CtdY-F	AAATACAGGTCGGTGCTTAG
YZ-KO-CtdY-R	CCAAGAGTTGTGAAAGTTTCA
KO-CtdS-1F	TCCAGCTGCTGAGATCTTGG
KO-CtdS-1R	ACTCCAGAATCAGCTAGGTCAACGCGCCAGACTTTCTACACCATA
KO-CtdS-2F	TATGGTGTAGAAAGTCTGGCGCGTTGACCTAGCTGATTCTGGAGT
KO-CtdS-2R	GTAGCCAACCAACCTCTCGATTTACCGTTGATCTGCTTGATCTCGT
KO-CtdS-3F	ACGAGATCAAGCAGATCAACGGTAAATCGAGAGGTTGGTTGGCTAC
KO-CtdS-3R	CTCGTACGCAAAAGGAACAC
YZ-KO-CtdS-F	AATAATACCAAACAGCGAAGT
YZ-KO-CtdS-R	GCCCTCATCATCCCAGTCC
KO-CtdG-1F	GAATCGGTTGTCTTTGGCAC
KO-CtdG-1R	ACTCCAGAATCAGCTAGGTCAACGTGTCACCCTGCACCTGCATTACT
KO-CtdG-2F	AGTAATGCAGGTGCAGGGTGACACGTTGACCTAGCTGATTCTGGAGT
KO-CtdG-2R	CAGAGAATCGACCCATTCTAGGCACCGTTGATCTGCTTGATCTCGT
KO-CtdG-3F	ACGAGATCAAGCAGATCAACGGTGCCTAGAATGGGTCGATTCTCTG
KO-CtdG-3R	CCAAGGCCAATAGCATATGG
YZ-KO-CtdG-F	CAAGCACTATTCGCGTCCGA
YZ-KO-CtdG-R	TGGCATGTAAAGTGGAGACAG
KO-CtdR-1F	AGGGTAAACAACACCCAAAC
KO-CtdR-1R	ACTCCAGAATCAGCTAGGTCAACGGGCGTCGTGTTTTCTTTGATGACC
KO-CtdR-2F	GGTCATCAAAGAAAACACGACGCCCGTTGACCTAGCTGATTCTGGAGT
KO-CtdR-2R	CATTAGTGGATGCCTTTGCTCGAACCGTTGATCTGCTTGATCTCGT
KO-CtdR-3F	ACGAGATCAAGCAGATCAACGGTTCGAGCAAAGGCATCCACTAATG
KO-CtdR-3R	CCCTGAAAGAGGTTCTCTGT
YZ-KO-CtdR-F	CGACCGTGGCCTACATCCGG
YZ-KO-CtdR-R	GAACGCGGTCGAATGAACAG
KO-CtdV-1F	GTGGAGCAGCAGTATCTCTAC
KO-CtdV-1R	ACTCCAGAATCAGCTAGGTCAACGCAATGGGACCTTCTTCCTCGAGTC
KO-CtdV-2F	GACTCGAGGAAGAAGGTCCCATTGCGTTGACCTAGCTGATTCTGGAGT
KO-CtdV-2R	TTCCAAAGTTGTGACTATCAGATACCGTTGATCTGCTTGATCTCGT
KO-CtdV-3F	ACGAGATCAAGCAGATCAACGGTATCTGATAGTCACAACTTTGGAA

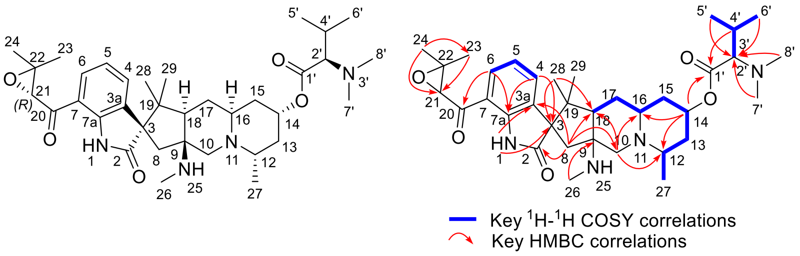
KO-CtdV-3R	AGTACACGTGTCGTGGTCTG
YZ-KO-CtdV-F	CCACTGCTTGCACGGGTCTT
YZ-KO-CtdV-R	TCTAAGGAGTTCTAGCCTAGG
KO-CtdJ-1F	CCCTATGTCAAGTTAGCTAGT
KO-CtdJ-1R	ACTCCAGAATCAGCTAGGTCAACGATGCTGGGCTTTCAACATTTTG
KO-CtdJ-2F	CAAAATGTTGAAAGCCCAGCATCGTTGACCTAGCTGATTCTGGAGT
KO-CtdJ-2R	TCAAAGTGCATACCTTGTGAGCTACCGTTGATCTGCTTGATCTCGT
KO-CtdJ-3F	ACGAGATCAAGCAGATCAACGGTAGCTCACAAGGTATGCACTTTGA
KO-CtdJ-3R	GCAGACCCATACGATGGGCC
YZ-KO-CtdJ-F	AATCCATGAAATTGCTGACT
YZ-KO-CtdJ-R	GCTTCAGTCGGGCAAACATA
KO-CtdD-1F	GCACTTGGAATATCCTGAGAG
KO-CtdD-1R	ACTCCAGAATCAGCTAGGTCAACGAGCTATTTCAATTTTGCACGCCAT
KO-CtdD-2F	ATGGCGTGCAAAATTGAAATAGCTCGTTGACCTAGCTGATTCTGGAGT
KO-CtdD-2R	GAATGAAATACTTACGTATGATAGACCGTTGATCTGCTTGATCTCGT
KO-CtdD-3F	ACGAGATCAAGCAGATCAACGGTCTATCATACGTAAGTATTTCATTC
KO-CtdD-3R	GTTCTTGAATCTTGGCCACT
YZ-KO-CtdD-F	ATCGATGATACTTTCAACTCC
YZ-KO-CtdD-R	AAGACGCTCCAACAATGATAA
KO-CtdC-1F	CTACCTAGACTGCTTCAAGG
KO-CtdC-1R	ACTCCAGAATCAGCTAGGTCAACGGAGGACGTGGCTTCAATGGGTGGA
KO-CtdC-2F	TCCACCCATTGAAGCCACGTCCTCCGTTGACCTAGCTGATTCTGGAGT
KO-CtdC-2R	TGACTTCCTGAATGAGGATGCGAAACCGTTGATCTGCTTGATCTCGT
KO-CtdC-3F	ACGAGATCAAGCAGATCAACGGTTTCGCATCCTCATTCAGGAAGTCA
KO-CtdC-3R	AAGTTCCTTCCAGTCCAGAG
YZ-KO-CtdC-F	ACTGGATACGGGAGATACCG
YZ-KO-CtdC-R	TCGTGCACACACATATCTACT
Hyg-F	GTGTCACGTTGCAAGACCTG
Hyg-R	AATTGCCGTCAACCAAGCTC
pXW55-CtdY-F	CTATATCGTAATACCATATGTCATCTTTGGTTTCGTTCTGTG
pXW55-CtdY-R	TAGTGATGGTGATGGTGATGCACGTGTTACTTCTTTTGGCCTGACT
CtdY-F110A-R	GGGGATGTATAAGCTGTTTGGGCGTACTCCTCGTTGAAAGCGT
CtdY-F110A-F	GCCCAAACAGCTTATACATCCCC
CtdY-F119A-R	ACCGTAGGCTTCGAGTCGACTGCTCCGGGGGATGTATAAGCTG
CtdY-F119A-F	GCAGTCGACTCGAAGCCTACGGT
CtdY-F300A-R	TGCGATGACGGAAACCATCATCT
CtdY-F300A-F	AGATGATGGTTTCCGTCATCGCAGGCTCCGTACATTCTGCAGG
CtdY-F486A-R	GACGCGAATAGTGCTTGAGGCACTAAATCCTTTCGAGGTCT
CtdY-F486A-F	GCCTCAAGCACTATTCGCGTC
CtdY-H304A-R	GCCAAGACCATGCCTGCAGAGGCTACGGAGCCGAAGATGACGG
CtdY-H304A-F	GCCTCTGCAGGCATGGTCTTGGC
CtdY-I365A-R	GGCAGGGTTATGACGGTGACTTT
CtdY-I365A-F	AAAGTCACCGTCATAACCCTGCCGGAGCAGCTTCGTTGTTTCG
CtdY-L102A-F	TGCCGAAAATATACTATCGGGTA
CtdY-L102A-R	TACCCGATAGTATATTTTCGGCAAACGCTTTCAACGAGGAGTA
CtdY-M308A-R	TAAATAGAACTGGCCAAGACTGCGCCTGCAGAATGTACGGAGC
CtdY-M308A-F	GCAGTCTTGGCCAGTTCTATTTA
CtdY-P218A-R	TGCGACTGTGGCACCGTAGCGAG
CtdY-P218A-F	CTCGCTACGGTGCCACAGTCGCACGAGACGGCCAACGGATTGC
CtdY-S305A-R	GGCATGTACGGAGCCGAAGATGA
CtdY-S305A-F	TCATCTTCGGCTCCGTACATGCCGCAGGCATGGTCTTGGCCAG
CtdY-S305Y-R	GTAATGTACGGAGCCGAAGATGA
CtdY-S305Y-F	TCATCTTCGGCTCCGTACATTACGCAGGCATGGTCTTGGCCAG
CtdY-S305C-R	ACAATGTACGGAGCCGAAGATGA
CtdY-S305C-F	TCATCTTCGGCTCCGTACATTGTGCAGGCATGGTCTTGGCCAG
CtdY-S305V-R	CACATGTACGGAGCCGAAGATGA
CtdY-S305V-F	TCATCTTCGGCTCCGTACATGTGGCAGGCATGGTCTTGGCCAG
CtdY-S305D-R	ATCATGTACGGAGCCGAAGATGA
CtdY-S305D-F	TCATCTTCGGCTCCGTACATGATGCAGGCATGGTCTTGGCCAG
CtdY-S305T-R	GGTATGTACGGAGCCGAAGATGA
CtdY-S305T-F	TCATCTTCGGCTCCGTACATACCGCAGGCATGGTCTTGGCCAG
pYTP-CtdY-F	CCTTCTCTGAACAATAAACCCCACAGAAGGCATTTATGTCATCTTTGGTTTCGTT
pYTP-CtdY-R	ATGAGACCCAACAACCATGATACCAGGGGATTTAAATTTTGAATTCCTGAA

	AATGTA
pYTR-CtdS-F	GCCACATAGACACATCTAAACAATGGCCTCTGTAGCCAACCAA
pYTR-CtdS-R	CATCGAAAGGGAGTCATCCAATTTAAATCTGAATATGTCTCCCATCTG
pYTP-CtdG-F	CCTTCTCTGAACAATAAACCCCACAGAAGGCATTTATGCAGGTGCAGGGTG ACAC
pYTP-CtdG-R	ATGAGACCCAACAACCATGATACCAGGGGATTTAAATCTTGTACTGAGACT TTGAAT
pYTP-CtdGY-1F	CCTTCTCTGAACAATAAACCCCACAGAAGGCATTTATGCAGGTGCAGGGTG ACAC
pYTP-CtdGY-1R	TCACCCAAATCAATTCACCGGAGTCTTGTACTGAGACTTTGAATTGAA
pYTP-CtdGY-2F	TTCAATTCAAAGTCTCAGTACAAGACTCCGGTGAATTGATTTGGGTGA
pYTP-CtdGY-2R	ACAGAACGAAACCAAAGATGACATTGTTTAGATGTGTCTATGTGGCGG
pYTP-CtdGY-3F	CCGCCACATAGACACATCTAAACAATGTCATCTTTGGTTTCGTTCTGT
pYTP-CtdGY-3R	ATGAGACCCAACAACCATGATACCAGGGGATTTAAATTTTGAATTCCTGAA AATGTAT
pYTP-CtdYRGJ-1F	CCTTCTCTGAACAATAAACCCCACAGAAGGCATTTATGTCATCTTTGGTTTC GTT
pYTP-CtdYRGJ-1R	CACCCAAATCAATTCACCGGAGTTTTGAATTCCTGAAAATGTATTA
pYTP-CtdYRGJ-2F	TAATACATTTTCAGGAATTCAAAACTCCGGTGAATTGATTTGGGTG
pYTP-CtdYRGJ-2R	ATCTTTCTTTCAACAGTCATTGTTTAGATGTGTCTATGTGGC
pYTP-CtdYRGJ-3F	GCCACATAGACACATCTAAACAATGACTGTTGAAAGAAAGAT
pYTP-CtdYRGJ-3R	TACGACCAGTTCGGAAGATCAGGCTGGTGCCCCTGGAAAGGATGTG
pYTP-CtdYRGJ-4F	CACATCCTTTCCAGGGGCACCAGCCTGATCTTCCGAACTGGTCGTA
pYTP-CtdYRGJ-4R	ACTGTGTCACCCTGCACCTGCATTGCTGAGGTGTAATGATGCTGGG
pYTP-CtdYRGJ-5F	CCCAGCATCATTACACCTCAGCAATGCAGGTGCAGGGTGACACAGT
pYTP-CtdYRGJ-5R	AGCCAAGCCCAAAAAGTGCTCCTCTTGTACTGAGACTTTGAATTGA
pYTP-CtdYRGJ-6F	TCAATTCAAAGTCTCAGTACAAGAGGAGCACTTTTTGGGCTTGGCT
pYTP-CtdYRGJ-6R	AGTTGATGCTGGGCTTTCAACATGGTTACTTCCTAATCGAAGCTT
pYTP-CtdYRGJ-7F	AAGCTTCGATTAGGAAGTAACCATGTTGAAAGCCCAGCATCAACT
pYTP-CtdYRGJ-7R	GACCCAACAACCATGATACCAGGGGATTTAAATGCCTTTCATAGGCGACCA AGAA
pYTP-CtdCSDV-1F	GCCACATAGACACATCTAAACAATGAAAGACGGATACCACTCACC
pYTP-CtdCSDV-1R	TAGCTCGTTCGGCACCTTTAATCCAGGATACGGTATATCGTACCCT
pYTP-CtdCSDV-2F	AGGGTACGATATACCGTATCCTGGATTAAAGGTGCCGAACGAGCTA
pYTP-CtdCSDV-2R	AGGTTGGTTGGCTACAGAGGCCATAAATGCCTTCTGTGGGGTTTATT
pYTP-CtdCSDV-3F	AATAAACCCCACAGAAGGCATTTATGGCCTCTGTAGCCAACCAACCT
pYTP-CtdCSDV-3R	GTACGACCAGTTCGGAAGATCAGGCTGAATATGTCTCCCATCTGACA
pYTP-CtdCSDV-4F	TGTCAGATGGGAGACATATTCAGCCTGATCTTCCGAACTGGTCGTAC
pYTP-CtdCSDV-4R	CTTTTCTGGACCGTGACAGTCATTGCTGAGGTGTAATGATGCTGGG
pYTP-CtdCSDV-5F	CCCAGCATCATTACACCTCAGCAATGACTGTCACGGTCCAGAAAAG
pYTP-CtdCSDV-5R	AGCCAAGCCCAAAAAGTGCTCCTTCCTGCAACGATGGCCGCGAGCTT
pYTP-CtdCSDV-6F	AAGCTCGCGGCCATCGTTGCAGGAAGGAGCACTTTTTGGGCTTGGCT
pYTP-CtdCSDV-6R	GGCTGATTGAGGATTACAGTCATGGTTACTTCCTAATCGAAGCTTT
pYTP-CtdCSDV-7F	AAAGCTTCGATTAGGAAGTAACCATGACTGTAATCCTCAATCAGCC
pYTP-CtdCSDV-7R	ATCATCGAAAGGGAGTCATCCAATTTAAATTTGTCTTATGGCGCTGTCAGGG T
pET-CtdR-F	CTTTAAGAAGGAGATATACCATGACTGTTGAAAGAAAGAT
pET-CtdR-R	TTAGTGATGGTGGTGGTGATGGGAAGCATTGAGACGCTTAAA
pET-CtdV-F	CTTTAAGAAGGAGATATACCATGACTGTAATCCTCAATCAGCC
pET-CtdV-R	TTAGTGATGGTGGTGGTGATGGATCGCCCGCTCAGAACAGACA
pET-CtdJ-F	CTTTAAGAAGGAGATATACCATGTTGAAAGCCCAGCATCAA
pET-CtdJ-R	TTAGTGATGGTGGTGGTGATGTGTTGTCCTGGCTTCATGGAA

Table S4. Proteins encoded in the ctd gene cluster.

Gene	Size(bp)	Putative function	Protein homologue	Coverage/Identify (%)	Organism
ctdC	867	Methyltransferase	EPS25854.1	99/52.54	*Penicillium oxalicum* 114-2
ctdD	4157	Nonribosomal peptide synthetase	KAF3403516.1	93/44.15	*Penicillium rolfsii*
ctdE	1421	FAD-dependent monooxygenase	XP_040756164.1	96/58.02	*Aspergillus ochraceoroseus* IBT 24754

ctdY	1916	Cytochrome P450 monooxygenase	EPS25875.1	89/47.05	*Penicillium oxalicum* 114-2
ctdF	1547	Cytochrome P450 monooxygenase	RHZ66411.1	87/44.24	*Aspergillus turcosus*
ctdG	1990	Cytochrome P450 monooxygenase	RLL97463.1	99/44.07	*Aspergillus turcosus*
ctdH	1371	Prenyltransferase	OQE41216.1	98/70.77	*Penicillium coprophilum*
ctdI	1383	Transporter	KAF3403517.1	92/49.37	*Penicillium rolfsii*
ctdJ	1066	Dioxygenase	KAF3403672.1	96/59.09	*Penicillium rolfsii*
ctdK	946	Hydroxymethylglutaryl-CoA lyase	XP_026615404.1	97/62.76	*Aspergillus thermomutatus*
ctdL	1918	Cystathionine gamma-synthase	GFF48424.1	94/65.62	*Penicillium arizonense*
ctdM	1043	Imine reductase	XP_031920864.1	99/64.26	*Aspergillus caelatus*
ctdN	801	SDR family oxidoreductase	XP_040756165.1	97/57.92	*Aspergillus ochraceoroseus* IBT 24754
ctdO	1253	NmrA	XP_040756166.1	97/60.18	*Aspergillus ochraceoroseus* IBT 24754
ctdP	1252	NmrA	XP_040756163.1	92/62.77	*Aspergillus ochraceoroseus* IBT 24754
ctdQ	7215	Nonribosomal peptide synthetase	KKK13836.1	98/49.49	*Aspergillus rambellii*
ctdR	1163	NmrA	KKK12906.1	98/50.74	*Aspergillus ochraceoroseus*
ctdS	885	Methyltransferase	KKK13831.1	91/46.18	*Aspergillus rambellii*
ctdT	1848	FAD-dependent monooxygenase	EPS25873.1	96/55.07	*Penicillium oxalicum* 114-2
ctdU	1367	Prenyltransferase	XP_040756152.1	96/62.17	*Aspergillus ochraceoroseus* IBT 24754
ctdV	1074	Dioxygenase	KKK13834.1	94/45.79	*Aspergillus rambellii*



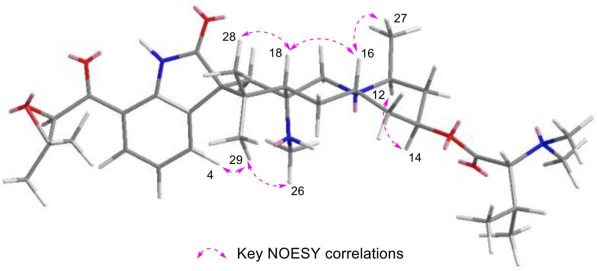
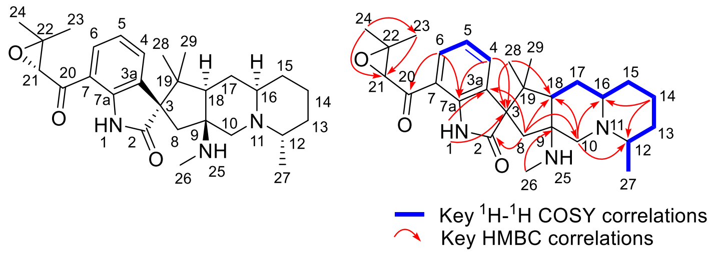


Table S5. NMR data of **15** (*δ* in ppm, *J* in Hz).

Position	¹H	¹³C	
1	9.41, s		NH
2		184.0	C
3		60.6	C
3a		136.7	C
4	7.67, d (8.0)	133.4	CH
5	7.08, t (7.7)	121.2	CH
6	7.76, d (7.4)	126.8	CH
7		116.7	C
7a		143.6	C
8	2.08, ov. 1.97, ov.	42.9	CH_2
9		68.2	C
10	2.98, m 2.28, ov.	57.6	CH_2
12	3.07, br s	54.0	CH
13	2.04, ov. 1.76, m	36.2	CH_2
14	5.20, brs	68.1	CH
15	1.87, m 1.53, ov.	38.8	CH_2
16	2.64, m	47.5	CH
17	1.55, ov. 1.16, ov.	32.1	CH_2
18	1.87, ov.	56.6	CH
19		48.0	C
20		194.8	C
21	4.04, s	64.4	CH
22		61.5	C
23	1.60, s	24.4	CH_3
24	1.25, s	18.7	CH_3
26	2.32, s	29.8	CH_3
27	1.16, ov.	13.5	CH_3
28	1.03, s	26.6	CH_3
29	1.24, s	29.3	CH_3

1'		171.0	C
2'	2.70, d (10.5)	75.0	CH
4'	2.04, ov.	27.6	CH
5'	0.90, d (6.6)	19.4	CH_3
6'	0.99, d (6.6)	20.1	CH_3
7'	2.32, s	41.7	CH_3
8'	2.32, s	41.7	CH_3

[a]Measured in $CDCl_3$, 600 MHz for ^1H and 150 MHz for ^{13}C NMR. Overlapped signals are reported without designating multiplicity.



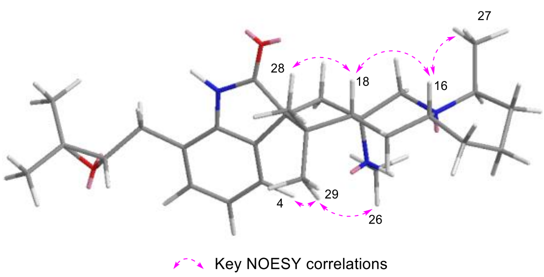
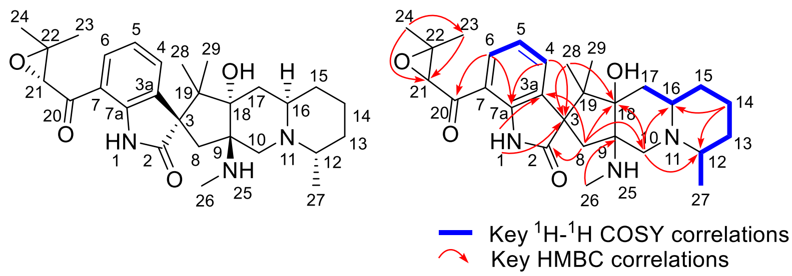


Table S6. NMR data of **16** (*δ* in ppm, *J* in Hz).

Position	^{1}H	^{13}C	
1	9.41, s		NH
2		184.1	C
3		60.3	C
3a		136.9	C
4	7.66, d (8.0)	133.6	CH
5	7.07, t (7.1)	121.2	CH
6	7.78, d (7.4)	126.7	CH
7		116.6	C
7a		143.6	C
8	2.07, ov. 1.95, ov.	42.8	CH_2
9		62.6	C
10	2.94, d (11.1) 2.17, d (11.1)	57.9	CH_2
12	3.01, m	55.4	CH
13	1.66, m 1.23, ov.	34.5	CH_2
14	1.43, m 1.14, m	29.9	CH_2
15	1.77, ov. 1.52, ov.	32.8	CH_2
16	2.36, m	52.6	CH
17	1.77, m 1.53, ov.	32.8	CH_2

18	1.79, ov.	57.0	CH
19		48.0	C
20		194.8	C
21	4.05, s	64.4	CH
22		61.7	C
23	1.60, s	24.5	CH_3
24	1.25, s	18.7	CH_3
26	2.27, s	29.3	CH_3
27	0.99, d (6.7)	10.2	CH_3
28	1.25, ov.	29.6	CH_3
29	1.01, ov.	26.7	CH_3

[a]Measured in $CDCl_3$, 600 MHz for ^1H and 150 MHz for ^{13}C NMR. Overlapped signals are reported without designating multiplicity.



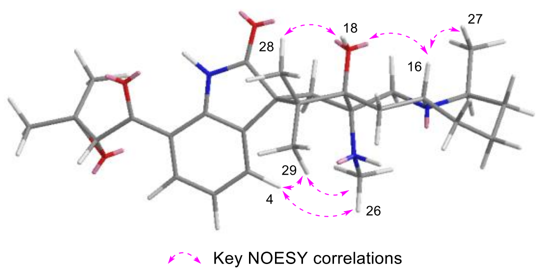
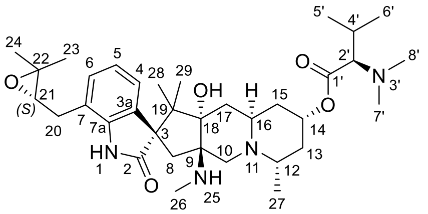


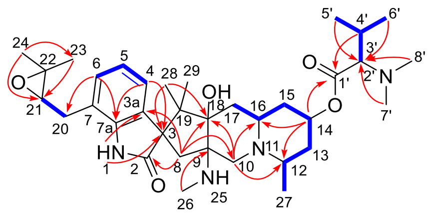
Table S7. NMR data of **17** (δ in ppm, J in Hz).

Position	**^1H**	**^{13}C**	
1	9.65, s		NH
2		185.6	C
3		60.7	C
3a		134.9	C
4	7.66, d (7.4)	133.5	CH
5	7.19, t (7.8)	122.7	CH
6	7.74, d (8.0)	127.6	CH
7		117.6	C
7a		142.8	C
8	2.21, d (14.0)	41.9	CH_2
	2.13, d (14.0)		
9		68.1	C
10	3.65, d (11.5)	51.0	CH_2
	3.24, d (11.5)		
12	3.73, ov.	52.5	CH
13	2.80, m	29.0	CH_2
	1.64, ov.		
14	2.36, br s	29.4	CH_2
	1.70, ov.		

15	1.72, ov.	17.5	CH_2
16	3.70, ov.	58.2	CH
17	2.25, m	31.7	CH_2
	1.71, ov.		
18		82.7	C
18-OH	5.25, s		OH
19		51.3	C
20		195.0	C
21	4.07, s	64.3	CH
22		61.9	C
23	1.61, s	24.4	CH_3
24	1.25, s	18.7	CH_3
26	2.46, s	30.4	CH_3
27	1.41, d (6.9)	12.2	CH_3
28	1.38, s	28.2	CH_3
29	1.00, s	21.9	CH_3

[a]Measured in $CDCl_3$, 600 MHz for ^1H and 150 MHz for ^{13}C NMR. Overlapped signals are reported without designating multiplicity.





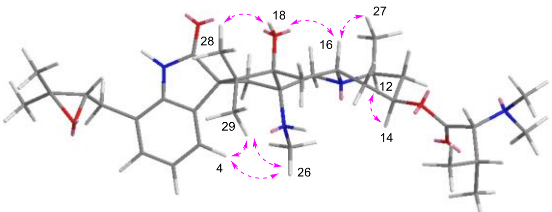
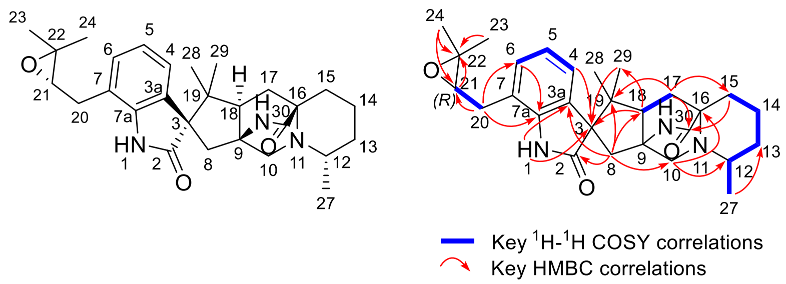


Table S8. NMR data of **18** (δ in ppm, J in Hz).

Position	^1H	^{13}C	
1	8.63, s		NH
2		185.6	C
3		62.3	C
3a		133.9	C
4	7.02, ov.	128.7	CH
5	6.99, ov.	122.5	CH
6	7.33, d (7.3)	126.6	CH
7		119.8	C
7a		140.2	C
8	2.08, ov.	41.1	CH_2
	2.12, m		
9		68.3	C
10	3.34, ov.	50.3	CH_2
	2.80, ov.		
12	3.29, ov.	54.5	CH

13	2.26, ov.	35.1	CH_2
	1.82, m		
14	5.21, s	67.4	CH
15	1.80, ov.	37.5	CH_2
	1.58, ov.		
16	3.44, ov.	43.7	CH
17	1.69, ov.	34.2	CH_2
	1.57, ov.		
18		83.3	C
19		51.3	C
20	3.02, m	32.6	CH_2
	2.60, m		
21	2.97, m	63.7	CH
22		59.8	C
23	1.41, s	19.3	CH_3
24	1.36, s	24.7	CH_3
26	2.32, s	29.8	CH_3
27	1.31, ov.	13.9	CH_3
28	1.40, s	28.1	CH_3
29	1.00, s	22.1	CH_3
1'		170.8	C
2'	2.71, d (10.2)	74.5	CH
4'	2.05, ov.	27.5	CH
5'	1.00, d (6.4)	18.9	CH_3
6'	0.89, d (6.4)	19.3	CH_3
7'	2.30, s	41.5	CH_3
8'	2.30, s	41.5	CH_3

[a]Measured in $CDCl_3$, 600 MHz for ^1H and 150 MHz for ^{13}C NMR. Overlapped signals are reported without designating multiplicity.



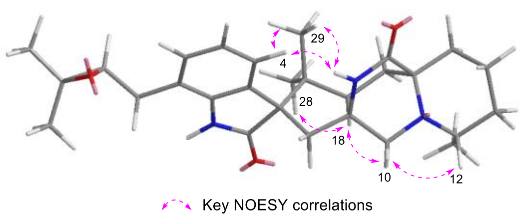
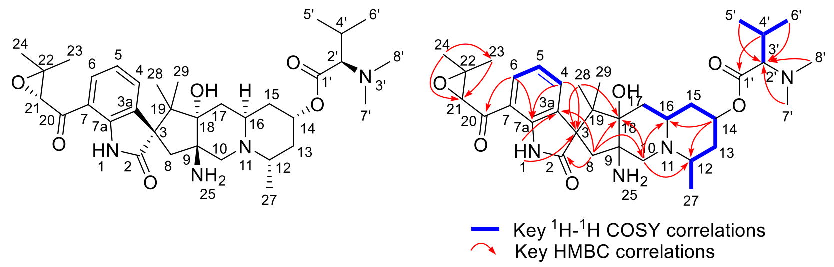


Table S9. NMR data of **20** (δ in ppm, J in Hz).

Position	^1H	^{13}C	
1	9.21, s		NH
2		183.8	C
3		63.0	C
3a		130.5	C
4	7.38, d (7.4)	124.8	CH

5	6.98, t (7.6)	121.8	CH
6	7.08, d (7.6)	128.9	CH
7		119.5	C
7a		140.7	C
8	2.38, d (15.3)	40.5	CH_2
	2.25, d (15.3)		
9		61.6	C
10	3.55, d (9.1)	64.5	CH_2
	2.44, d (9.1)		
12	2.22, m	59.4	CH
13	1.61, ov.	34.4	CH_2
	1.26, m		
14	1.84, m	20.9	CH_2
	1.61, ov.		
15	2.29, ov.	31.9	CH_2
	1.40, m		
16	3.12, ov.	46.8	CH
17	1.93, dd (13.5, 10.2)	35.4	CH_2
	1.52, dd (13.5, 8.0)		
18	2.22, ov.	59.5	CH
19		47.5	C
20	2.94, dd (15.4, 3.9)	31.4	CH_2
	2.75, dd (15.4, 8.0)		
21	2.99, dd (7.9, 4.0)	63.7	CH
22		59.6	C
23	1.43, s	18.8	CH_3
24	1.37, s	24.7	CH_3
25	8.50, s		NH
27	1.08, d (6.0)	21.2	CH_3
28	0.97, s	20.7	CH_3
29	0.80, s	23.1	CH_3
30		174.9	C

[a]Measured in $CDCl_3$, 600 MHz for ^1H and 150 MHz for ^{13}C NMR. Overlapped signals are reported without designating multiplicity.



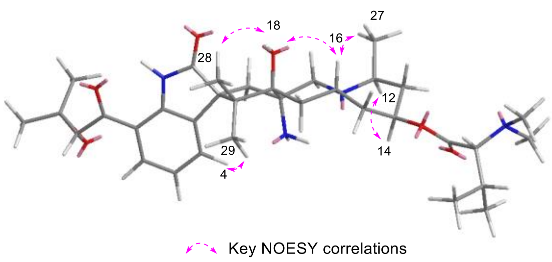
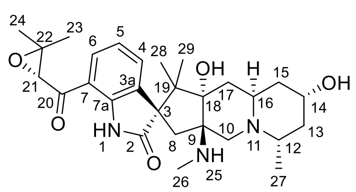
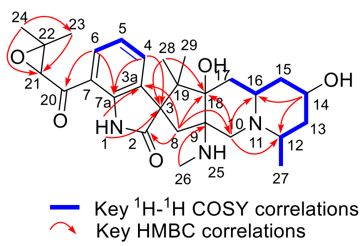


Table S10. NMR data of **25** (δ in ppm, J in Hz).

Position	^{1}H	^{13}C	
1	9.70, s		NH
2		185.5	C
3		60.4	C
3a		134.8	C
4	7.75, d (7.9)	133.6	CH
5	7.18, t (7.8)	122.5	CH
6	7.90, d (7.4)	127.5	CH
7		117.4	C
7a		142.7	C
8	2.45, ov,	48.2	CH_2
	1.85, ov.		
9		63.6	C
10	3.99, m	58.9	CH_2
	3.09, m		
12	3.85, m	55.7	CH
13	2.63, m	33.1	CH_2
	1.92, ov.		
14	5.28, br s	65.7	CH
15	2.48, m	34.6	CH_2
	1.97, ov.		
16	3.99, ov.	47.0	CH
17	2.13, m	31.9	CH_2
	1.70, m		
18		81.7	C
19		51.7	C
20		194.9	C
21	4.07, s	64.2	CH
22		61.7	C
23	1.61, s	24.3	CH_3
24	1.26, s	18.5	CH_3
27	1.52, m	14.6	CH_3
28	1.40, s	27.9	CH_3
29	1.03, ov.	21.9	CH_3
1'		170.2	C
2'	2.81, m	74.2	CH
4'	2.06, m	27.6	CH
5'	1.01, ov.	19.3	CH_3
6'	0.91, d (6.6)	19.9	CH_3
7'	2.35, s	41.3	CH_3
8'	2.35, s	41.3	CH_3

[a]Measured in $CDCl_3$, 600 MHz for ^{1}H and 150 MHz for ^{13}C NMR. Overlapped signals are reported without designating multiplicity.





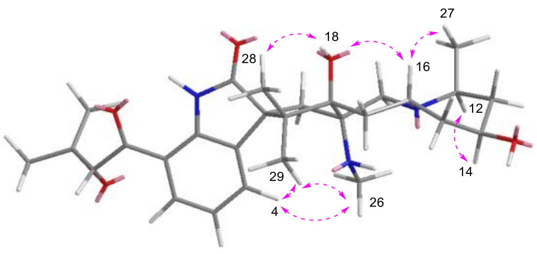
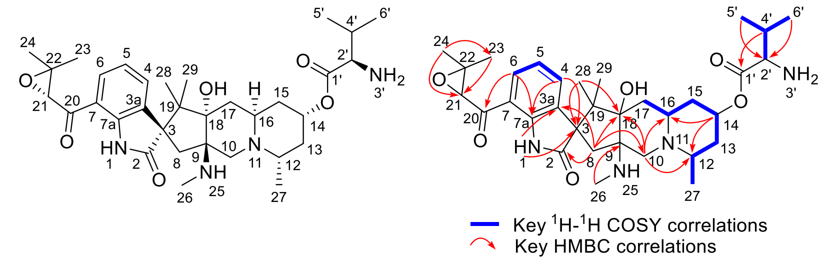


Table S11. NMR data of **23** (δ in ppm, J in Hz).

Position	**^{1}H**	**^{13}C**	
1	9.68, s		NH
2		185.6	C
3		60.8	C
3a		135.1	C
4	7.68, d (7.1)	133.5	CH
5	7.20, t (7.7)	122.6	CH
6	7.76, d (7.9)	127.5	CH
7		117.6	C
7a		142.9	C
8	2.22, d (14.0)	41.8	CH_2
	2.16, d (14.0)		
9		68.0	C
10	3.70, ov.	50.2	CH_2
	3.19, d (11.0)		
12	3.65, ov.	56.7	CH
13	2.50, m	37.3	CH_2
	1.82, m		
14	4.27, brs	63.6	CH
15	2.24, m	31.4	CH_2
	1.81, m		
16	4.10, ov.	46.3	CH
17	2.70, m	35.5	CH_2
	1.86, m		
18		82.8	C
18-OH	5.17, s		OH
19		51.5	C
20		195.0	C
21	4.08, s	64.3	CH
22		61.8	C
23	1.63, s	24.4	CH_3
24	1.27, s	18.7	CH_3
26	2.43 s	29.9	CH_3
27	1.60, d (7.0)	15.3	CH_3
28	1.40, s	28.3	CH_3
29	1.03, s	22.0	CH_3

[a]Measured in $CDCl_3$, 600 MHz for ^{1}H and 150 MHz for ^{13}C NMR. Overlapped signals are reported without designating multiplicity.



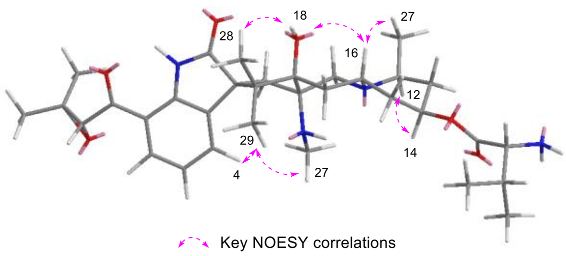


Table S12. NMR data of **24** (δ in ppm, J in Hz).

Position	¹H	¹³C	
1	9.72, s		NH
2		185.4	C
3		60.6	C
3a		134.7	C
4	7.67, brs	133.3	CH
5	7.22, m	122.6	CH
6	7.77, brs	127.7	CH
7		117.5	C
7a		142.7	C
8	2.25, m 2.17, m	41.6	CH_2
9		67.9	C
10	3.76, m 3.33, m	50.6	CH_2
12	3.85, br s	56.1	CH
13	2.66, m 1.96, m	33.5	CH_2
14	5.32, m	67.9	CH
15	2.94, ov. 1.94, ov.	33.0	CH_2
16	4.03, m	47.3	CH
17	2.67, m 1.96, ov.	33.6	CH_2
18		82.5	C
19		51.2	C
20		194.9	C
21	4.09, s	64.2	CH
22		61.8	C
23	1.64, s	24.3	CH_3
24	1.27, s	18.5	CH_3
26	2.45, s	30.0	CH_3
27	1.27, m	18.6	CH_3
28	1.04, ov.	19.4	CH_3
29	1.40, s	28.1	CH_3

1'		172.2	C
2'	2.88, ov.	74.0	CH
4'	2.21, ov.	31.0	CH
5'	0.95, ov.	17.7	CH_3
6'	1.01, ov.	21.7	CH_3

[a]Measured in $CDCl_3$, 600 MHz for ^1H and 150 MHz for ^{13}C NMR. Overlapped signals are reported without designating multiplicity.

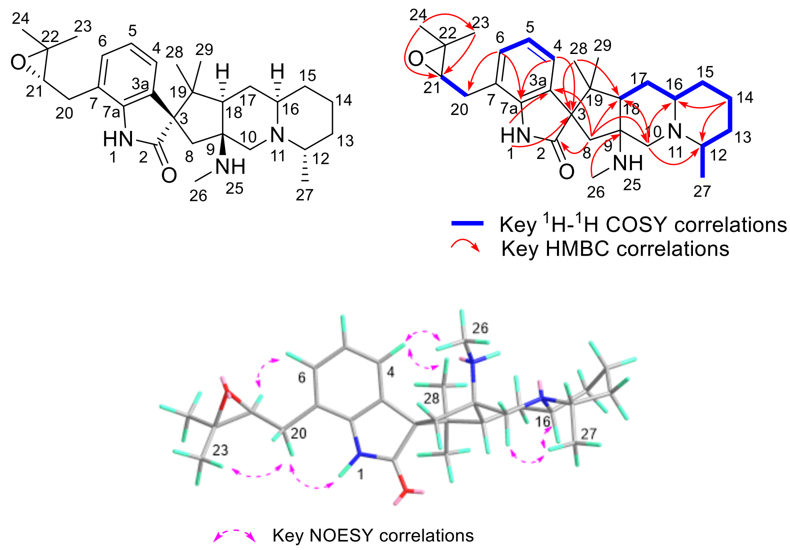


Table S13. NMR data of **22R** (δ in ppm, J in Hz).

Position	**^1H**	**^{13}C**	
1	8.31, s		NH
2		183.3	C
3		61.5	C
3a		132.9	C
4	7.39, d (7.0)	126.4	CH
5	6.97, t (7.5)	122.1	CH
6	7.01, d (6.9)	128.8	CH
7		119.2	C
7a		140.2	C
8	2.02, d (13.6)	43.0	CH_2
	2.29, d (13.6)		
9		62.5	C
10	2.78, d (11.9)	57.5	CH_2
	3.49, d (11.9)		
12	3.61, m	58.1	CH
13	1.61, ov.	28.9	CH_2
14	1.25, m	29.8	CH_2
	1.77, ov.		
15	1.65, ov.	17.2	CH_2
	1.75, ov.		
16	3.01, ov.	58.4	CH
17	1.67, ov.	26.5	CH_2
	2.06, ov.		
18	2.19, dd (13.5, 2.6)	55.5	CH
19		47.1	C

20	2.63, ov.	32.6	CH_2
	2.99, ov.		
21	2.97, ov.	63.7	CH
22		59.6	C
23	1.42, s	18.9	CH_3
24	1.37, s	24.7	CH_3
26	2.50, s	29.6	CH_3
27	1.36, d (6.8)	12.1	CH_3
28	1.17, s	25.5	CH_3
29	1.05, s	26.1	CH_3

[a]Measured in $CDCl_3$, 600 MHz for ^1H and 150 MHz for ^{13}C NMR. Overlapped signals are reported without designating multiplicity.

Supplementary Figures

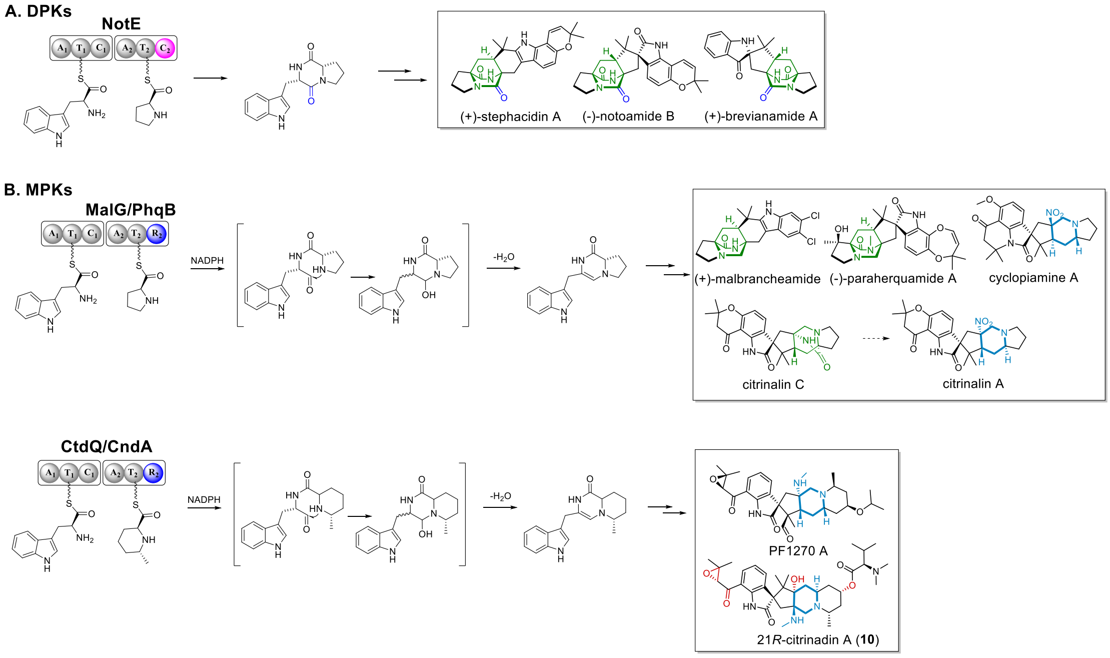


Figure S1. Biosynthesis of the precursor of DKPs and MKPs.

A) Proposed early steps of DPKs biosynthesis involve the NRPS NotE. B) Proposed early steps of MPKs biosynthesis involve the NRPS MalG, PhqB, CtdQ, and CndA.[6-8]

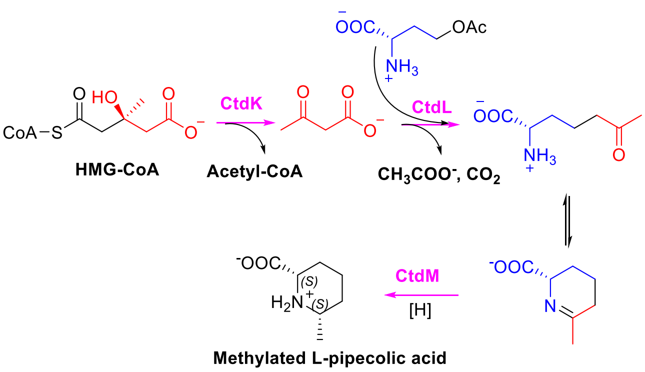


Figure S2. Proposed biosynthesis pathway of methylated L-pipecolic acid.[9]

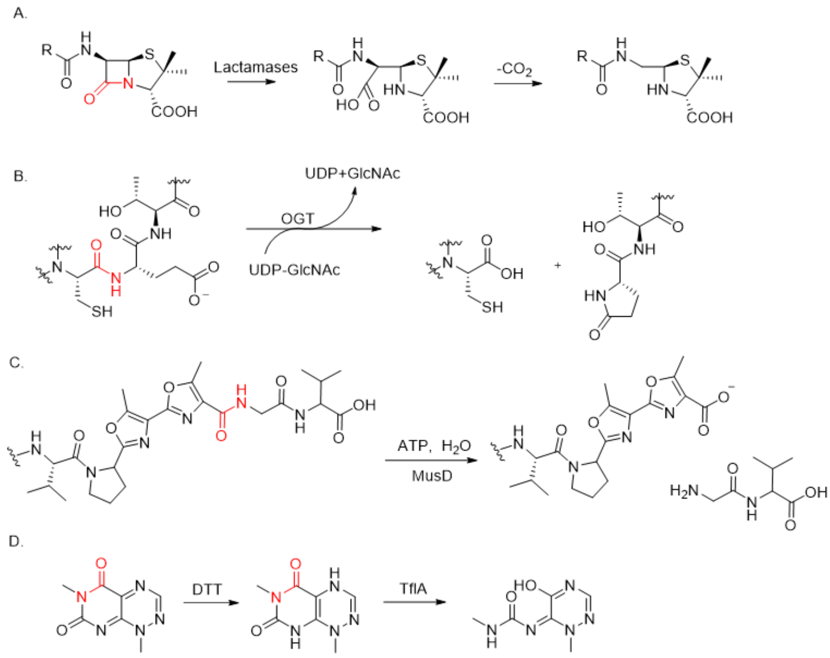


Figure S3. Cleavage of the amide bonds by different enzymes.[10-13]

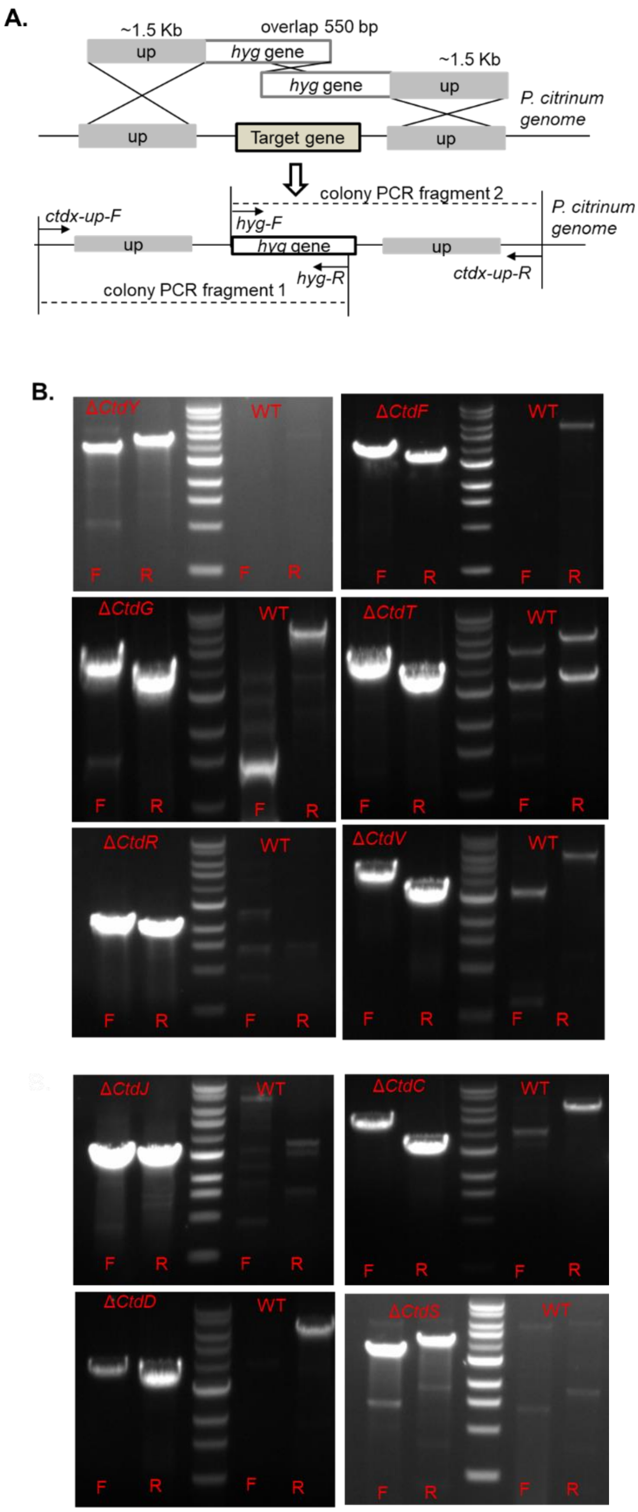


Figure S4. PCR verification of *ctd* mutants.

A) Scheme of hygromycin-resistance split-marker approach for gene knockout of genes in *ctd*. B) Verification of mutants by PCR.

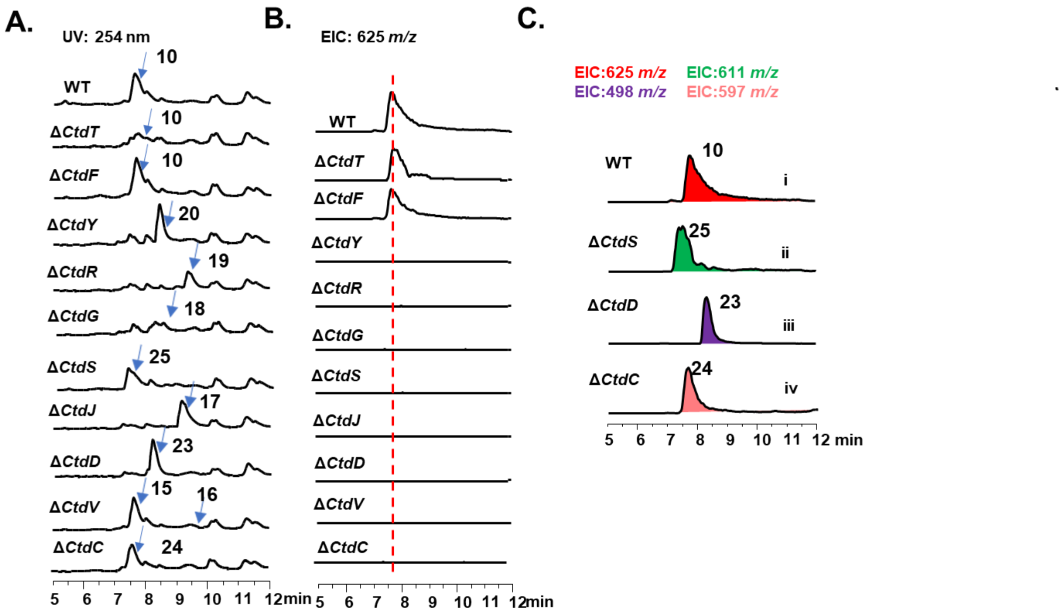
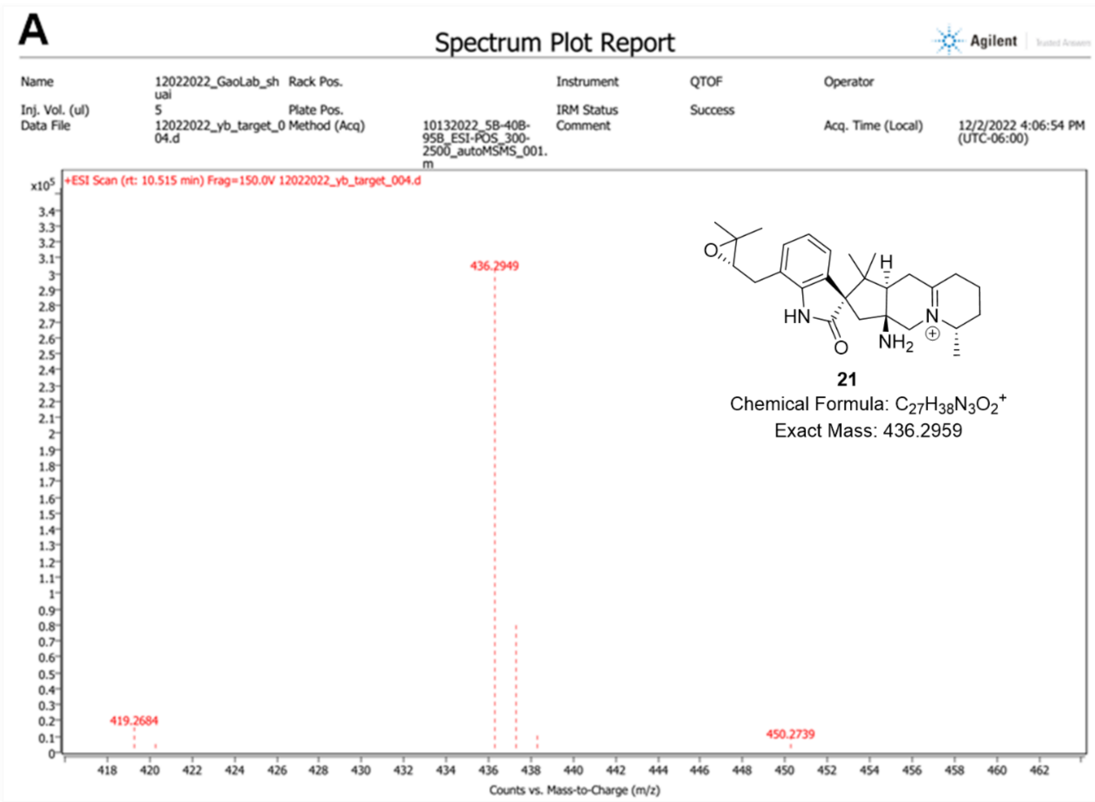
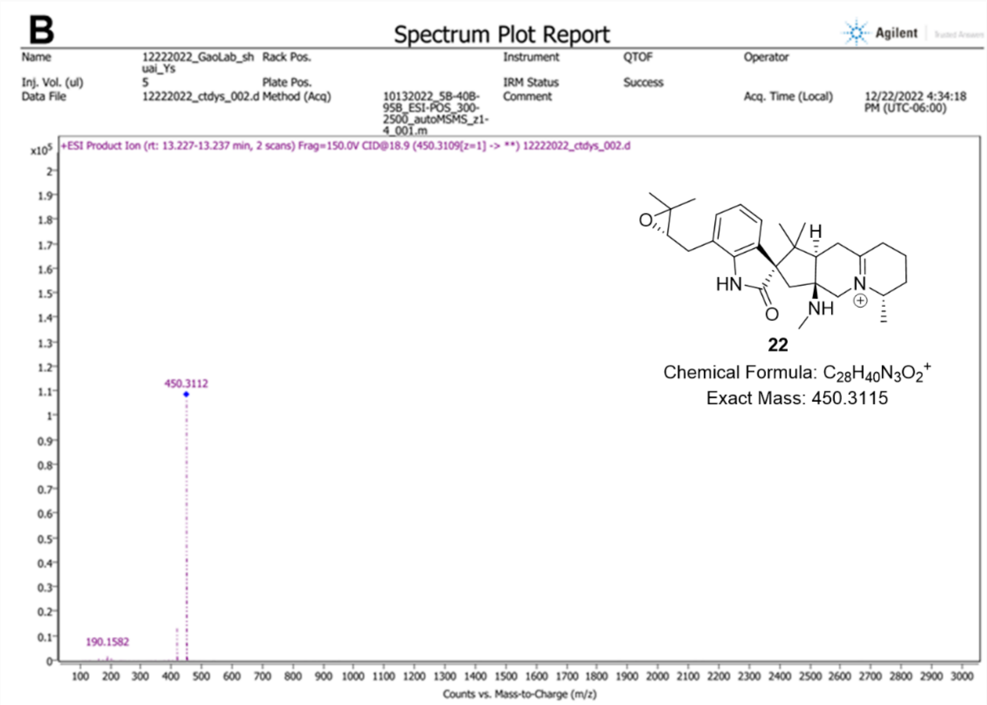


Figure S5. LC-MS analysis of *ctd* mutants.

A) HPLC traces of metabolic extracts from *ctd* mutants. B) LC-MS analysis (extracted ion chromatogram, EIC) of *P. citrinum* wild-type (WT) and *ctd* mutants. Δ*ctdF* and Δ*ctdT* mutants all retained the production of compound **10**. C) LC-MS analysis (EIC) of *P. citrinum* WT and mutants Δ*ctdS*, Δ*ctdD* and Δ*ctdC*. i) *P. citrinum* wild-type, ii) Δ*ctdS* mutant produces **25**, iii) Δ*ctdD* mutant produces **23**, iv) Δ*ctdC* mutant produces **24**.



B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Agilent
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Inj. Vol. (ul) 5 Plate Pos. IRM Status Success
Data File 12222022_ctdys_002.d Method (Acq) 10132022_5B-40B-95B_ESI-POS_300-2500_autoMSMS_z1-4_001.m Comment Acq. Time (Local) 12/22/2022 4:34:18 PM (UTC-06:00)
+ESI Product Ion (rt: 13.227-13.237 min, 2 scans) Frag=150.0V CID@18.9 (450.3109[z=1] -> **) 12222022_ctdys_002.d
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190.1582
22
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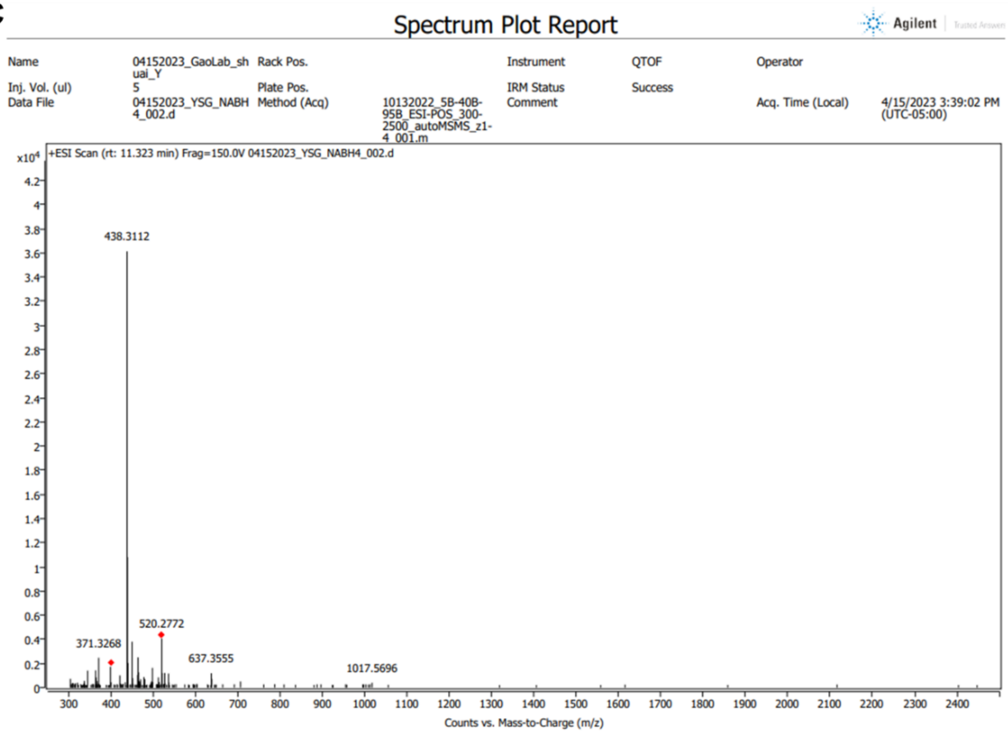
Exact Mass: 450.3115
Counts vs. Mass-to-Charge (m/z)

C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Agilent
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520.2772
637.3555
1017.5696
Counts vs. Mass-to-Charge (m/z)

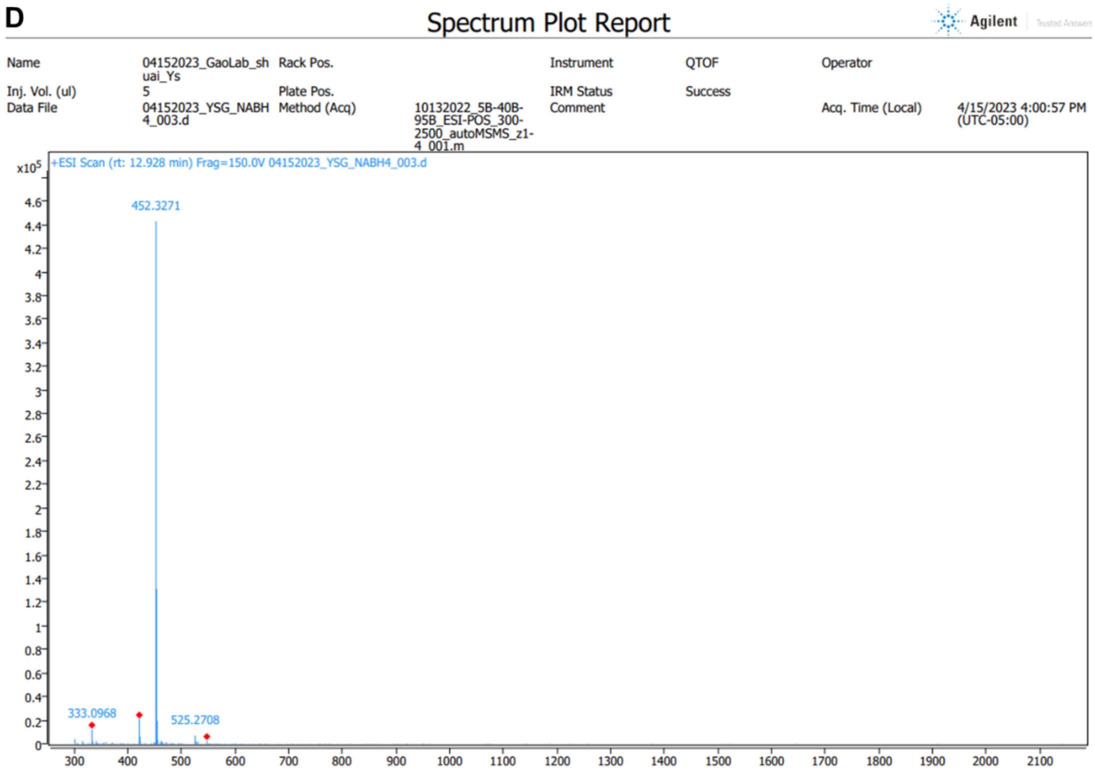

Figure S6. HR-ESI-MS data of compounds **21** (A), **22** (B), **21R** (C) and **22R** (D).

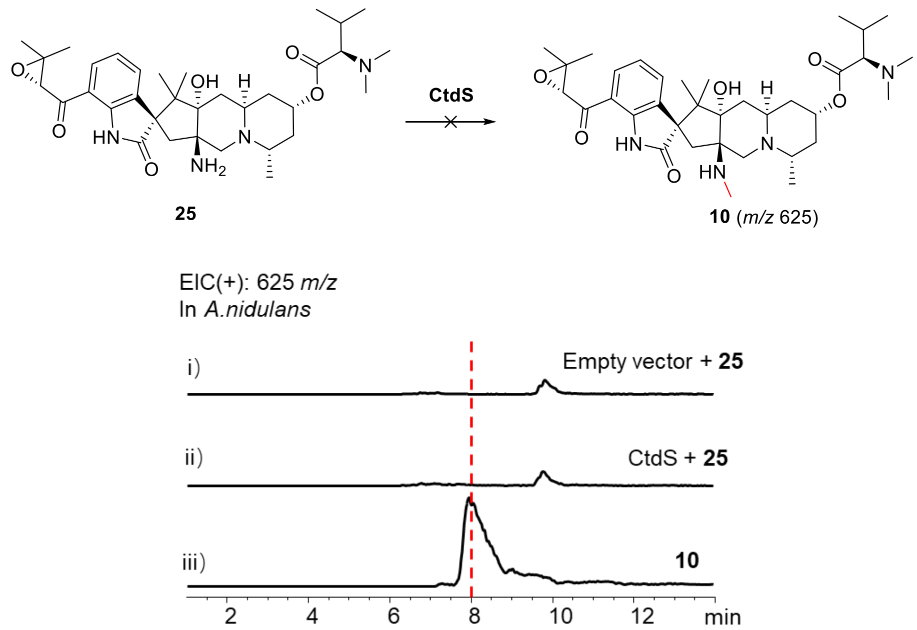

Figure S7. Feeding of **25** to *A. nidulans* expressing *ctdS.*

LC-MS analysis of feeding experiments of **25** to *A. nidulans* with i) empty vector, ii) with pYTR-CtdS, iii) standard compound **10**.

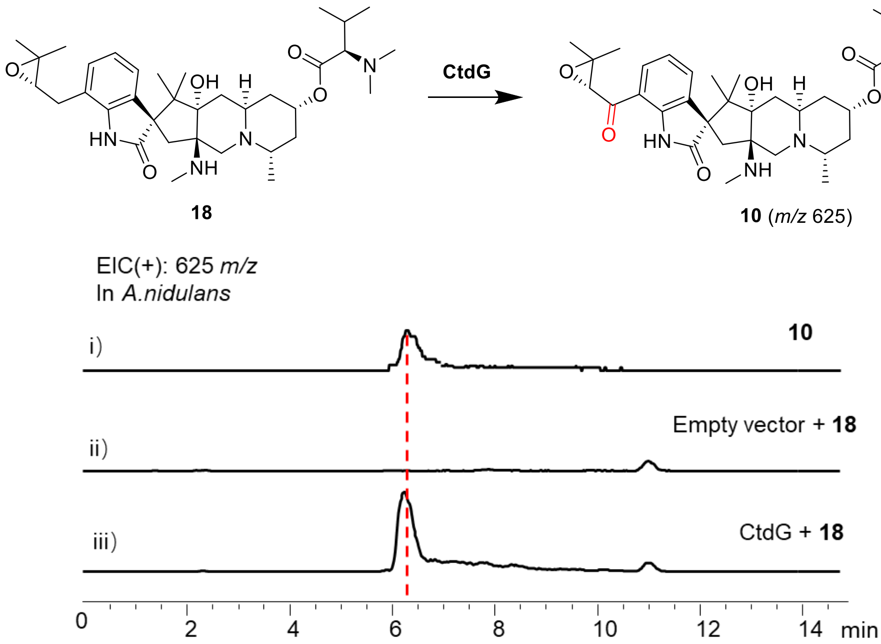

Figure S8. Feeding of **18** to *A. nidulans* expressing *ctdG*.
LC-MS analysis of feeding experiments of **18** to *A. nidulans.* i) standard compound **10**, ii) pYTP, iii) pYTP-CtdG.

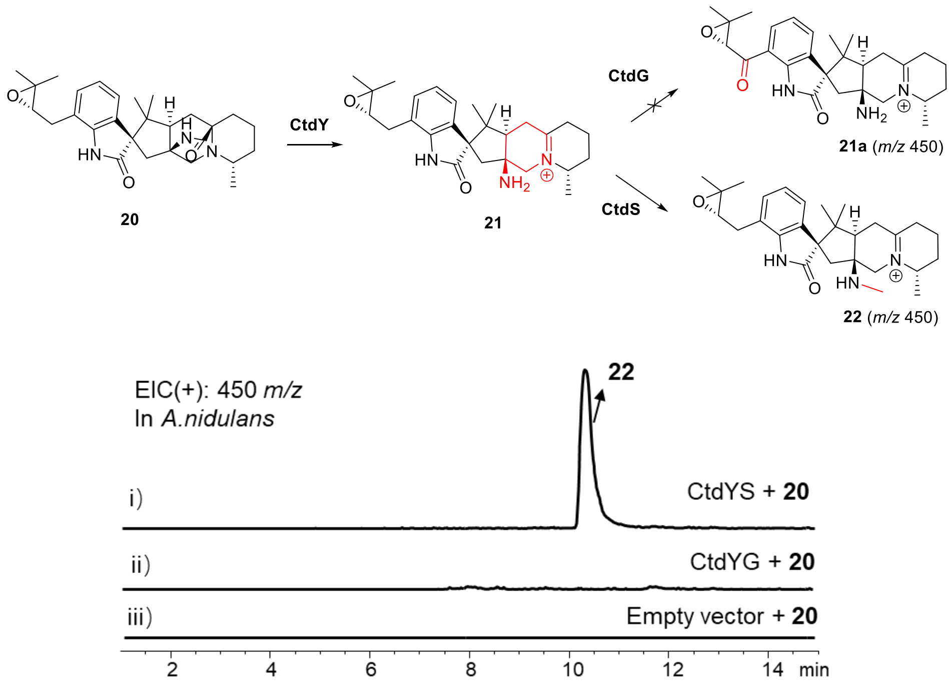

Figure S9. Feeding of **20** to *A. nidulans* expressing *ctdYG* and *ctdYS*.
LC-MS analysis of feeding experiments of **20** to *A. nidulans* containing i) pYTP-CtdY and pYTR-CtdS, ii) pYTP-CtdYG, iii) pYTP and pYTR.

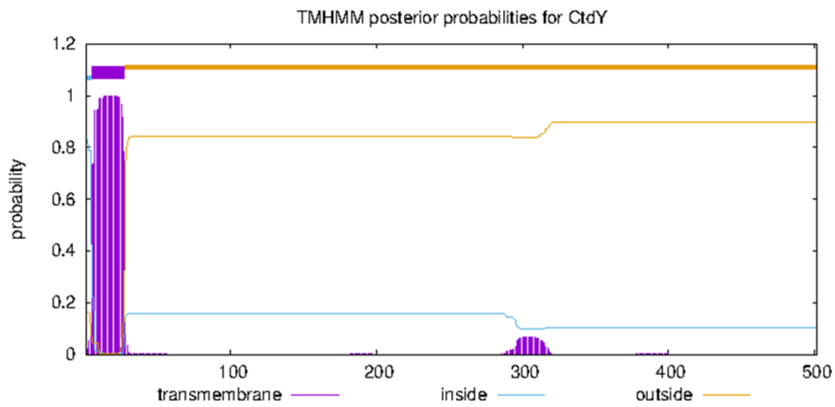

Figure S10. TMHMM analysis of the transmembrane domain of CtdY.[14]

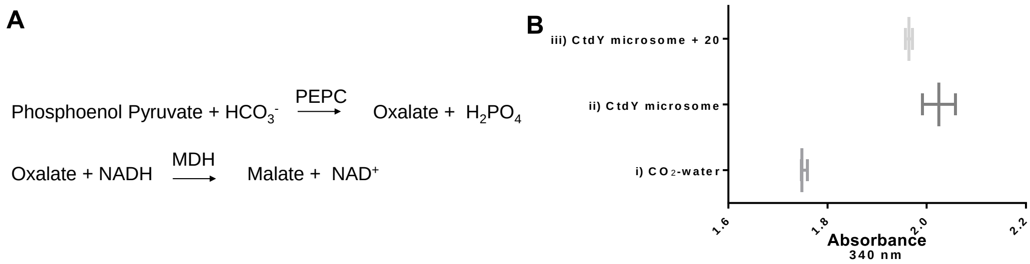

Figure S11. Detection of carbon dioxide produced in the CtdY assay with a kit. (A) The mechanism of CO_2 detection kit (Attogene, Austin, USA). The assay uses a coupled enzyme assay to detect CO_2 (as HCO_3^-) as follows. In the first step, the bicarbonate condenses with phosphoenol pyruvate to form oxalate (and phosphoric acid); this reaction is catalyzed by the enzyme Phosphoenolpyruvate Decarboxylase, PEPC. The oxalate is then enzymatically reduced by the enzyme Malate Dehydrogenase (using an NADH cofactor) to form malate and NAD^+. Since the NADH molecule absorbs light at 340 nm but the NAD^+ does not, the decrease in absorbance is dependent on the presence of CO_2 in the reaction. Therefore, the CO_2 analyte causes a decrease in absorbance at 340 nm which is directly proportional to the CO_2 concentration in the samples. The kit comes with a CO_2 standard that contains 30 mM of sodium bicarbonate in an aqueous solution. (B) The absorbance at 340 nm of i) positive control, ii) CtdY microsome (negative control), iii) CtdY microsome + **20**.

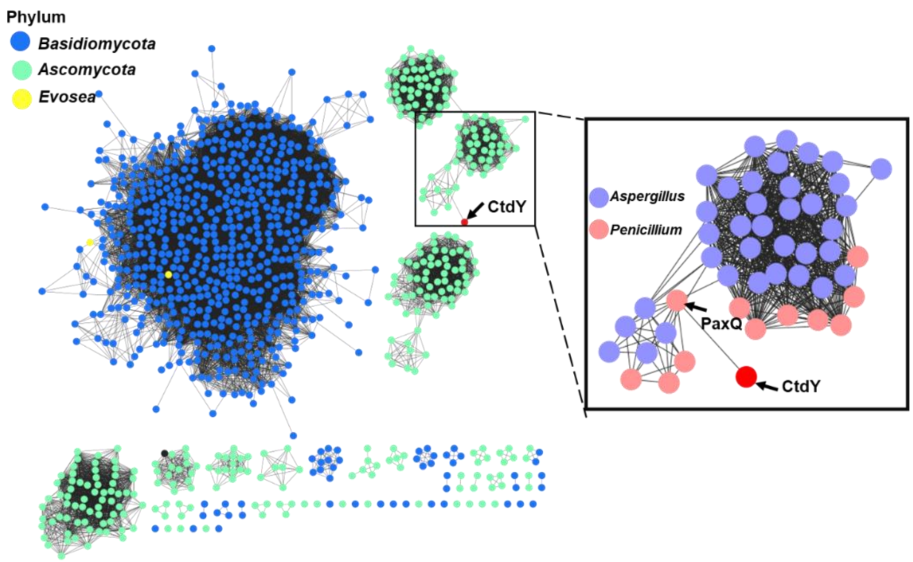

Figure S12. Sequence similarity network (SSN) of CtdY.

The SSN was generated with the Enzyme Similarity Tool (EFI-EST) web tool using the UniRef databases. A total of 1000 nodes are included in this SSN. The alignment score is 129 and SSN colored by Phylum and Genus.[15]

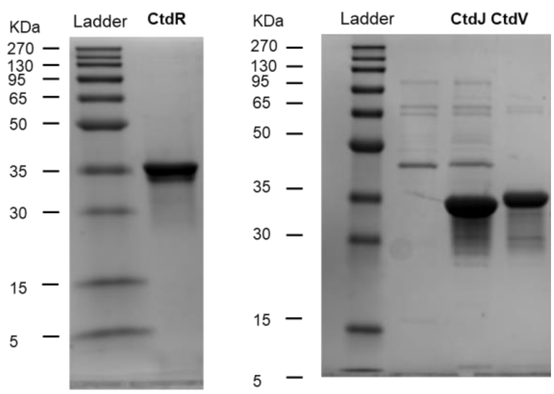

Figure S13. SDS-PAGE gels of CtdR, CtdV and CtdJ.

CtdR. CtdV and CtdJ contain the C-terminal His_6-Tag.

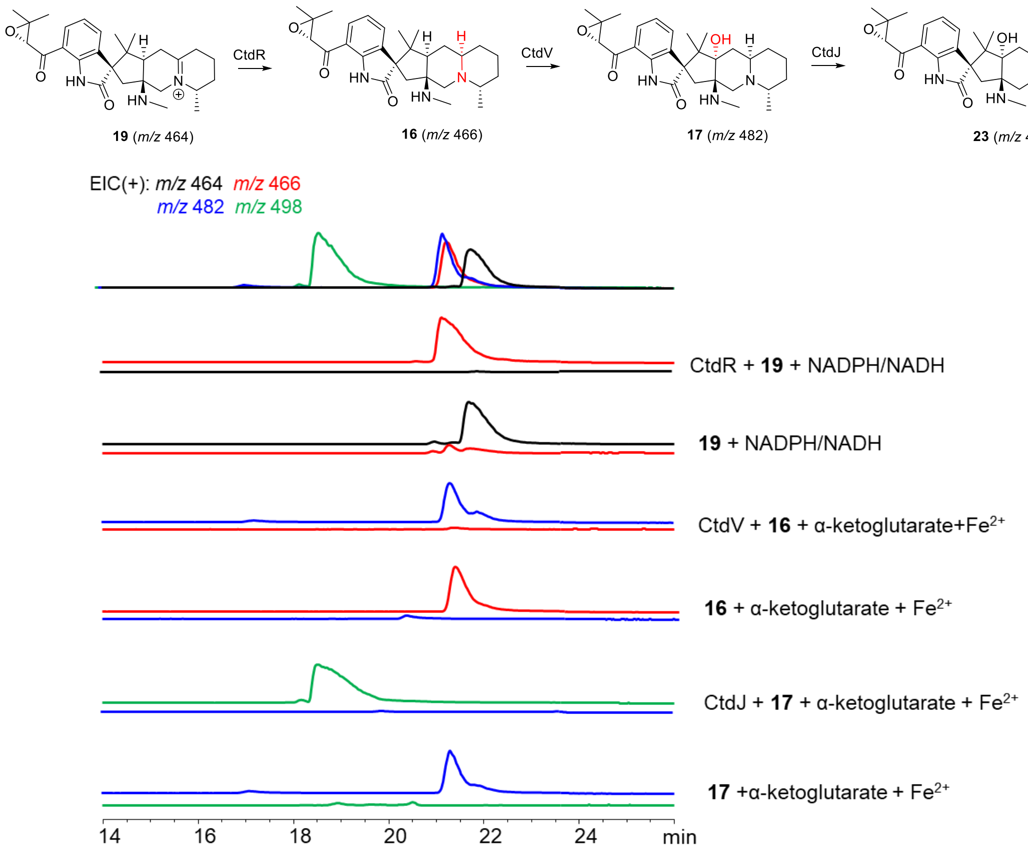

Figure S14. LC-MS analysis of the in vitro assays of CtdV, CtdV and CtdJ.

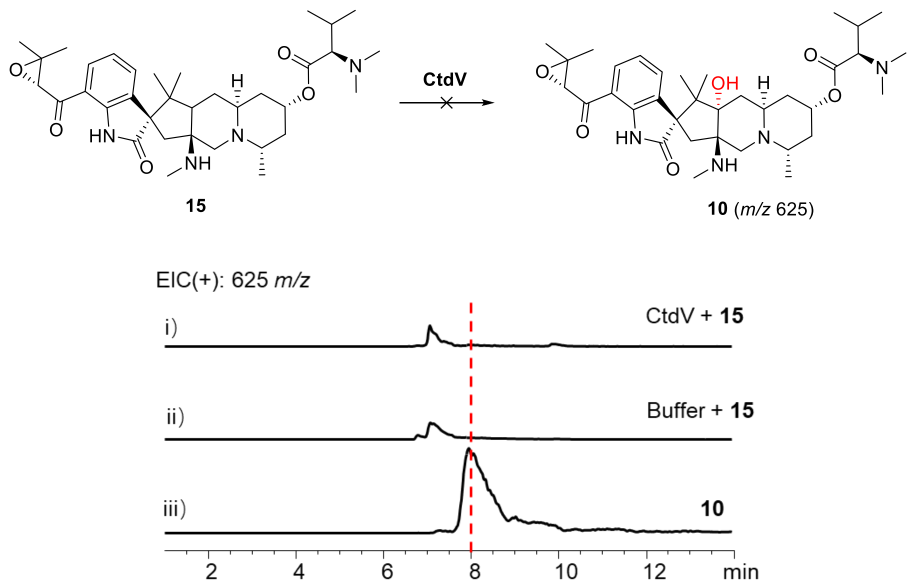

Figure S15. In vitro assays of CtdV with **15**.

The initial concentration of **15** was 0.2 mM, CtdV was 40 μM, the reactions were carried out at 28°C. Tris-HCl (pH 7.5) buffer contained 2 mM ascorbic acid, 2 mM *α*-ketoglutarate, 0.5 mM $FeSO_4 \cdot 7H_2O$.

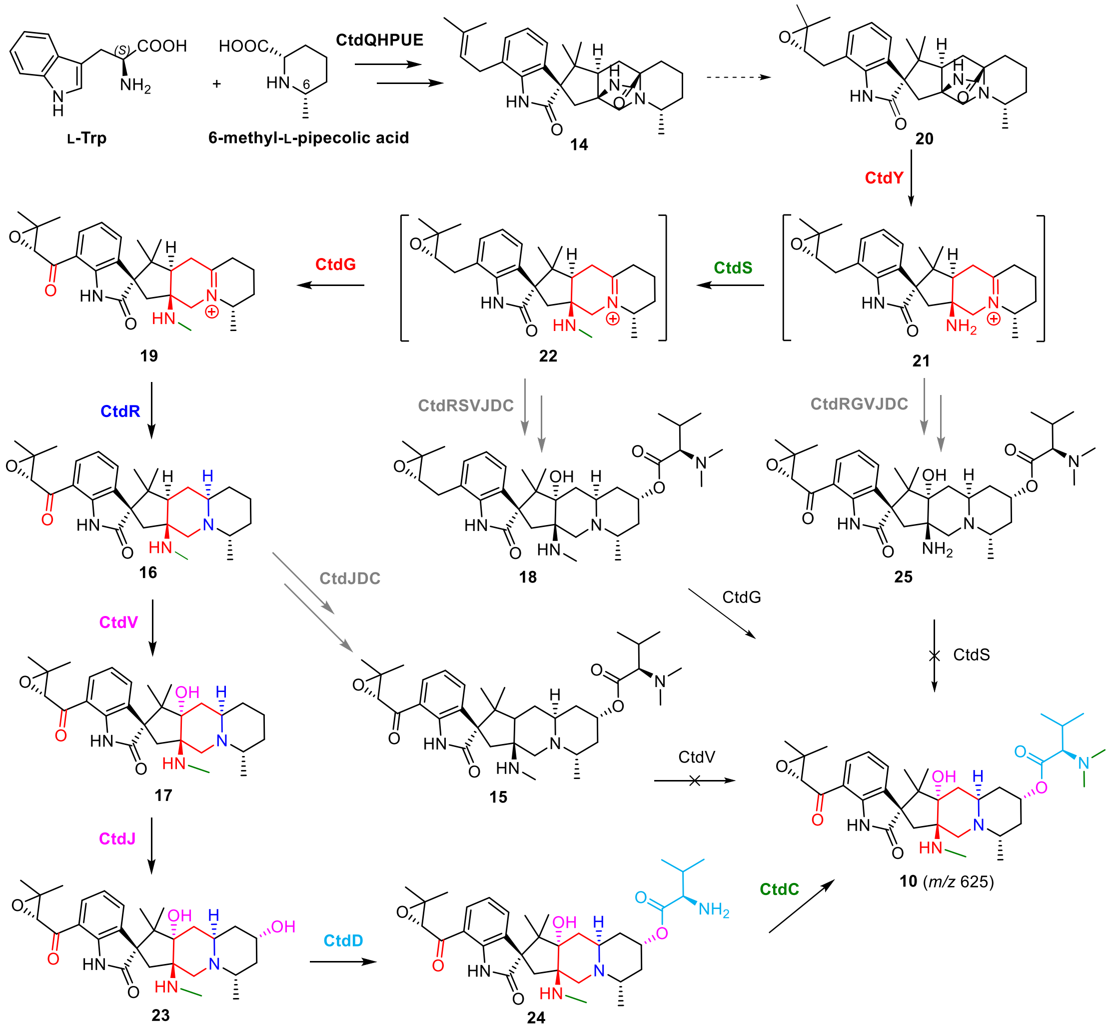

Figure S16. Biosynthetic pathway of 21*R*-citrinadin A.

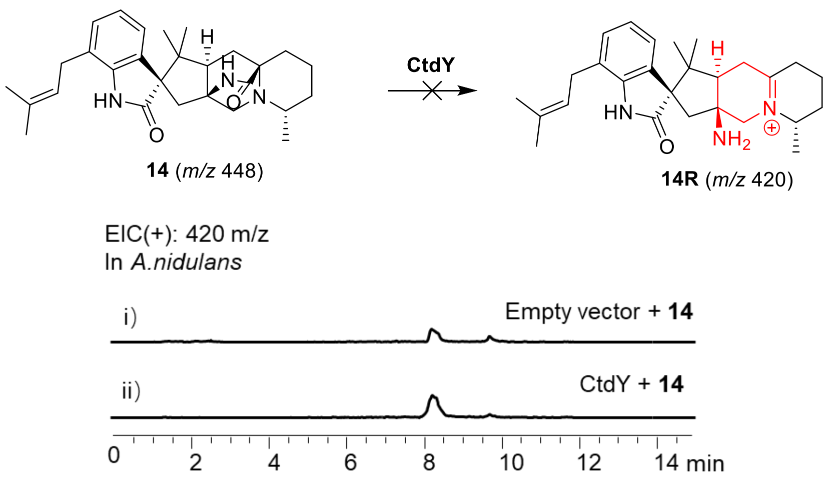

Figure S17. Feeding of **14** to *A. nidulans* expressing *ctdY*.

LC-MS analysis of feeding experiments of **14** to *A. nidulans* containing i) pYTP-CtdG ii) pYTP.

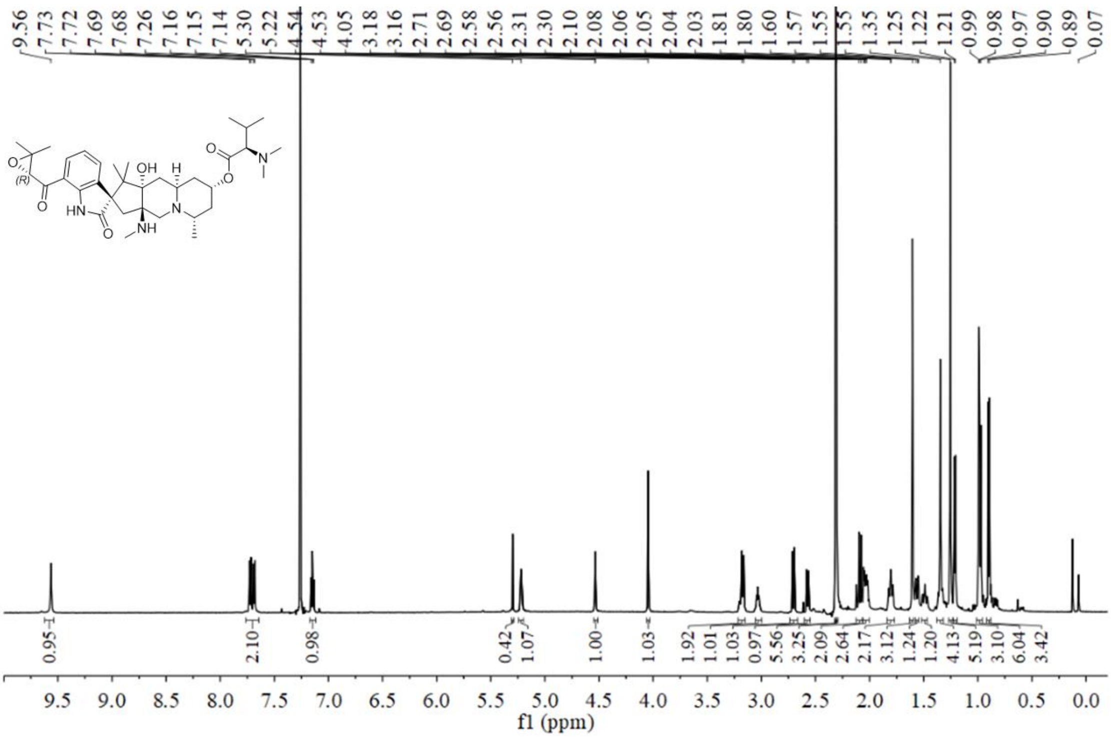

Figure S18-1. ^{1}H NMR (600 Hz) spectrum of **10** in $CDCl_3$.

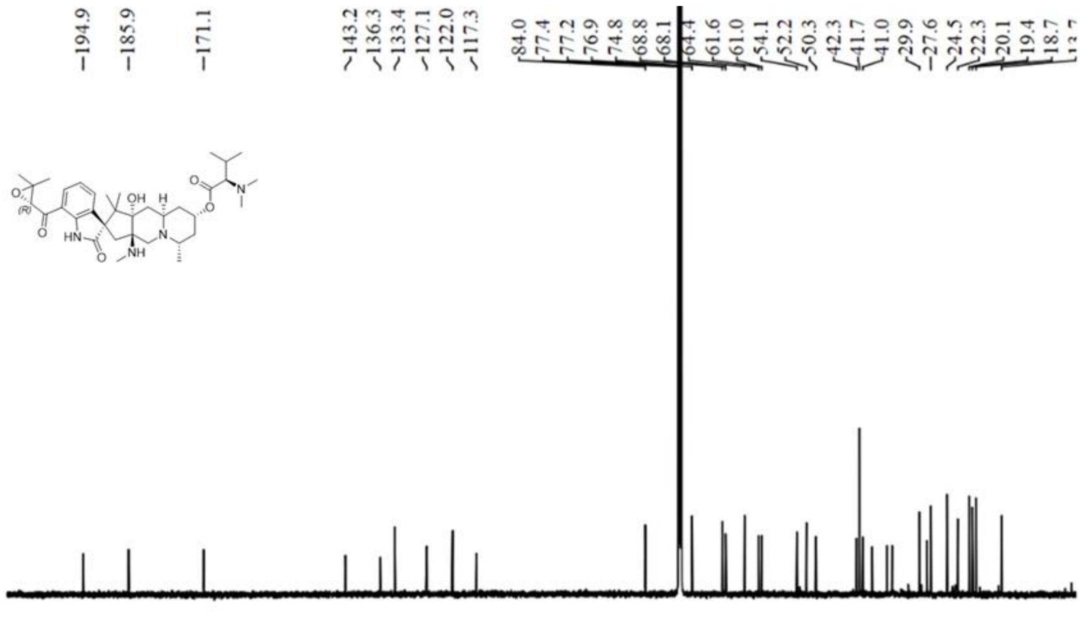

Figure S18-2. ^{13}C NMR (150 Hz) spectrum of **10** in $CDCl_3$.

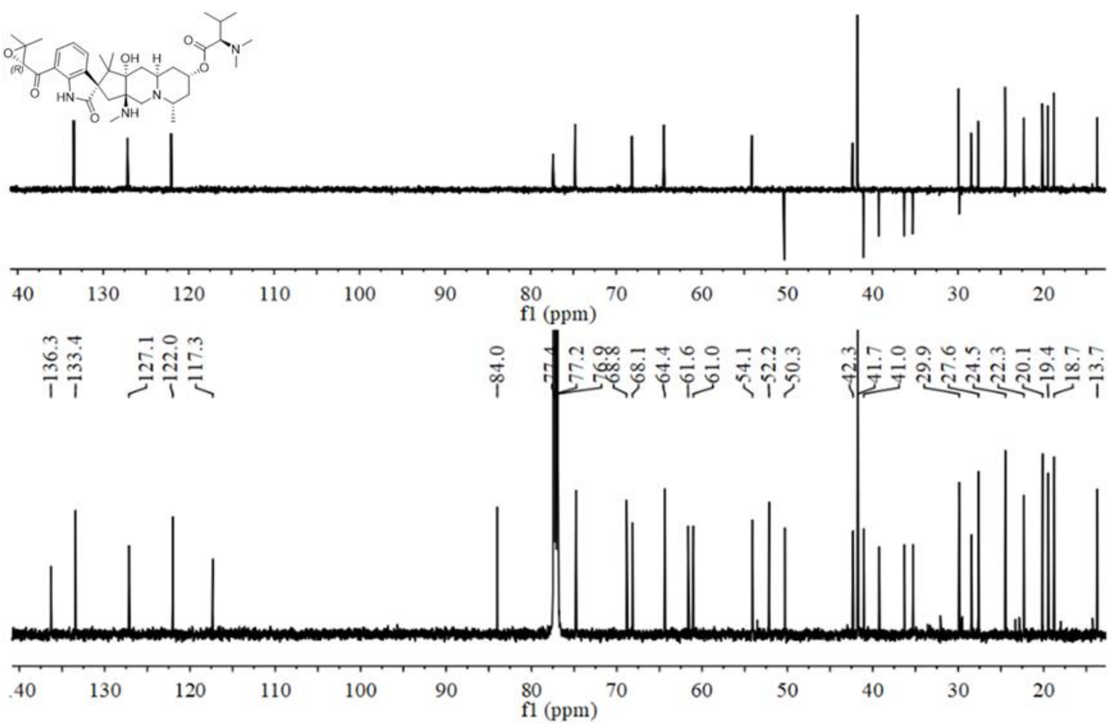

Figure S18-3. DEPT spectrum of **10** in $CDCl_3$.

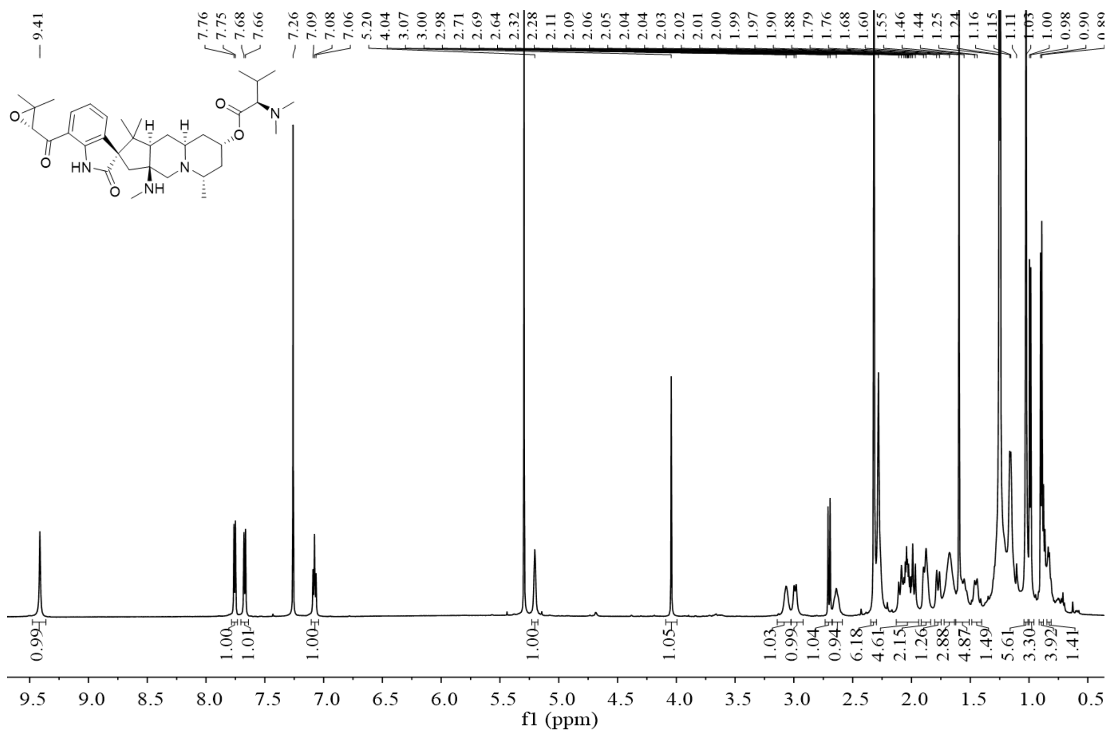

Figure S19-1. ^1H NMR (600 Hz) spectrum of **15** in $CDCl_3$.

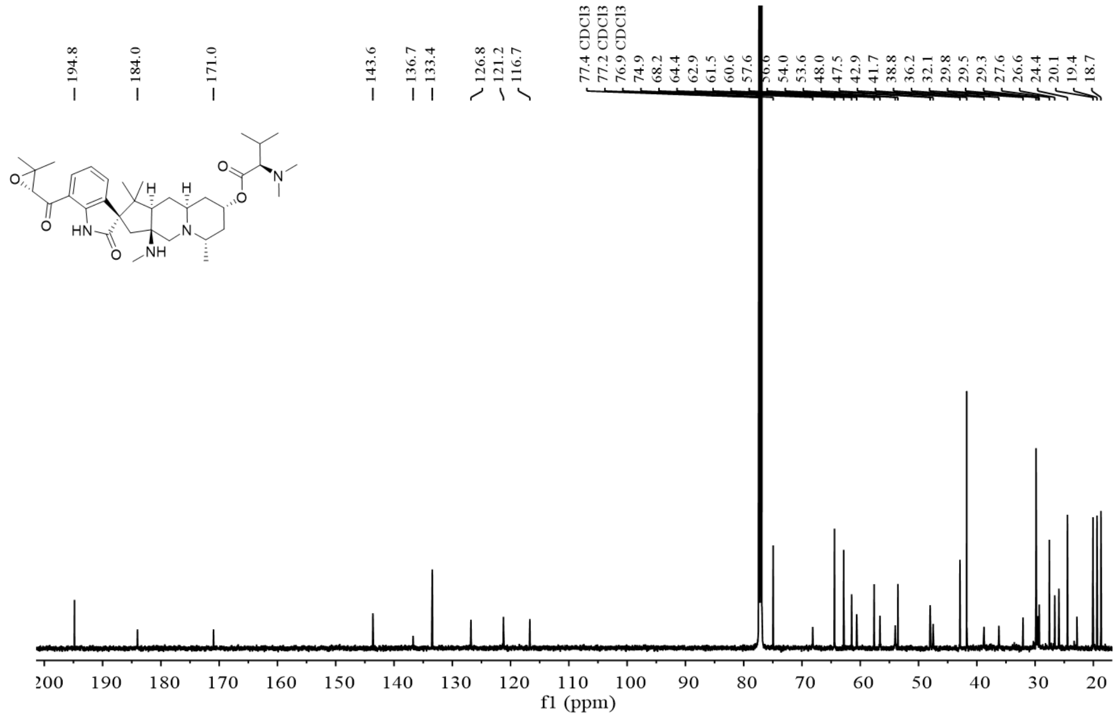

Figure S19-2. ^{13}C NMR (150 Hz) spectrum of **15** in $CDCl_3$.

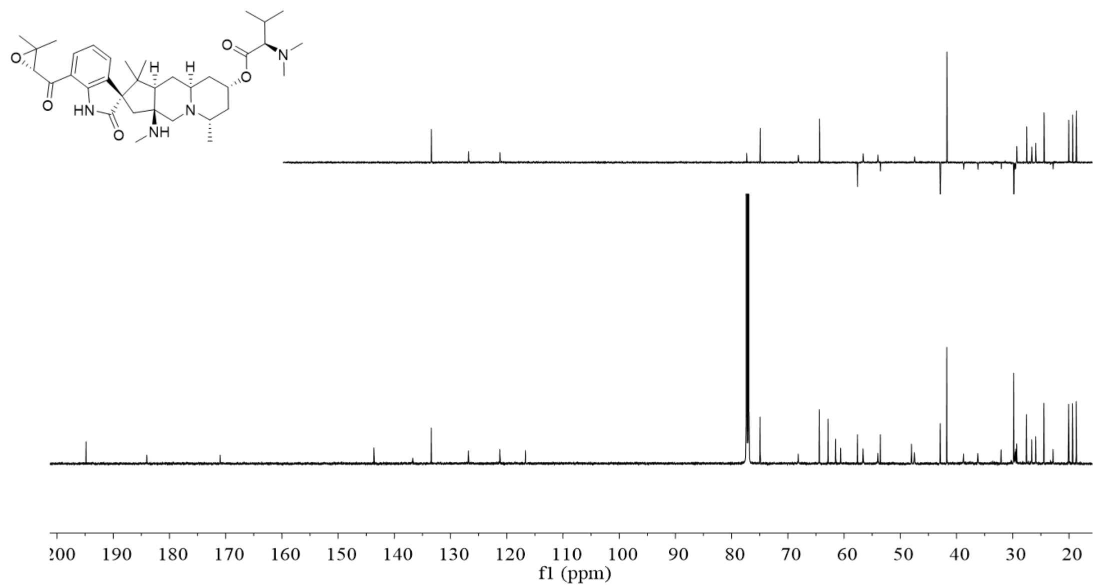

Figure S19-3. DEPT spectrum of **15** in $CDCl_3$.

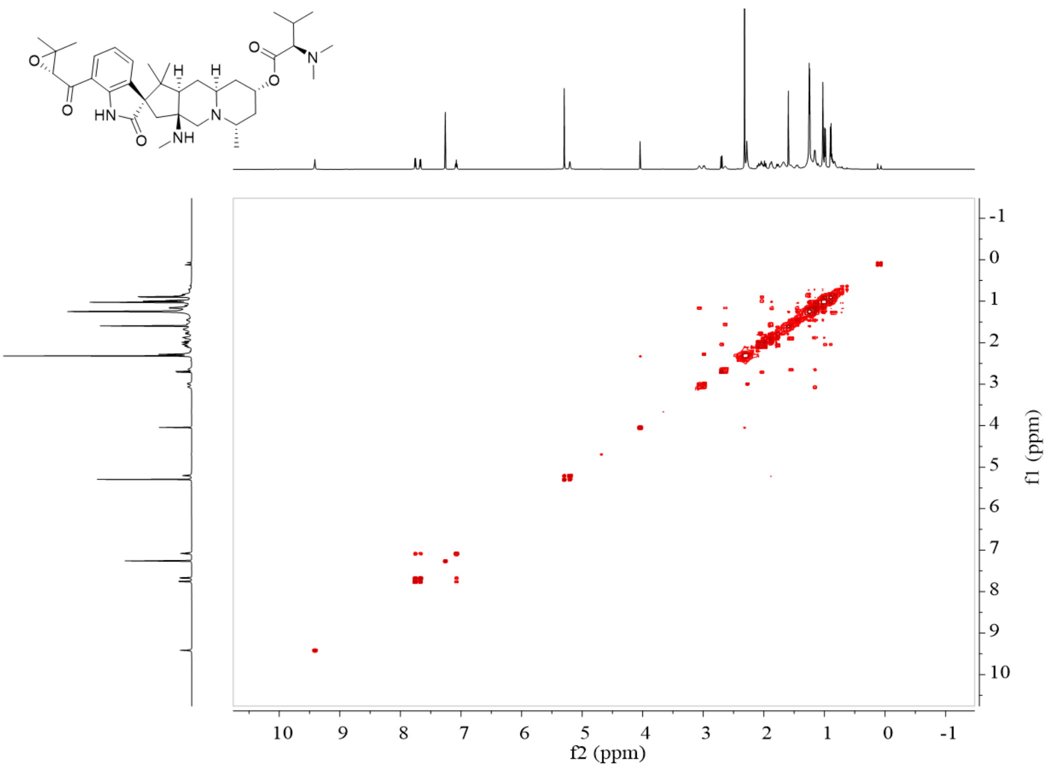

Figure S19-4. ${}^{1}H$-${}^{1}H$ COSY spectrum of **15** in $CDCl_3$.

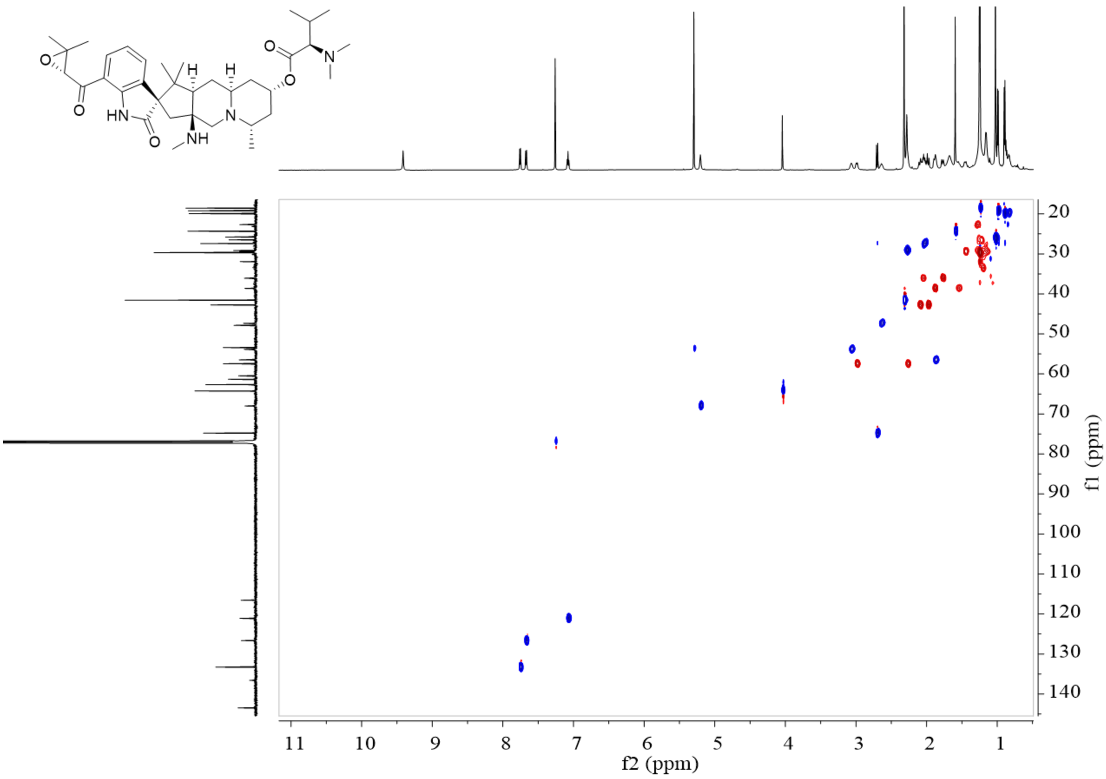

Figure S19-5. HSQC spectrum of **15** in $CDCl_3$.

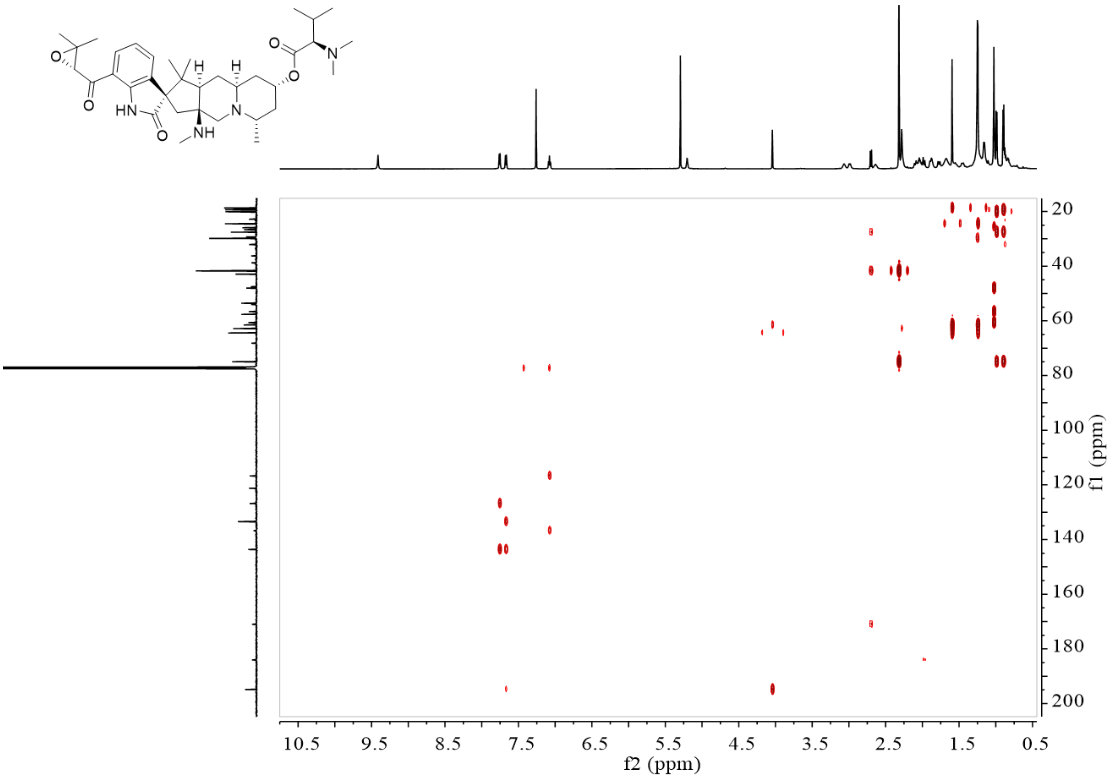

Figure S19-6. HMBC spectrum of **15** in $CDCl_3$.

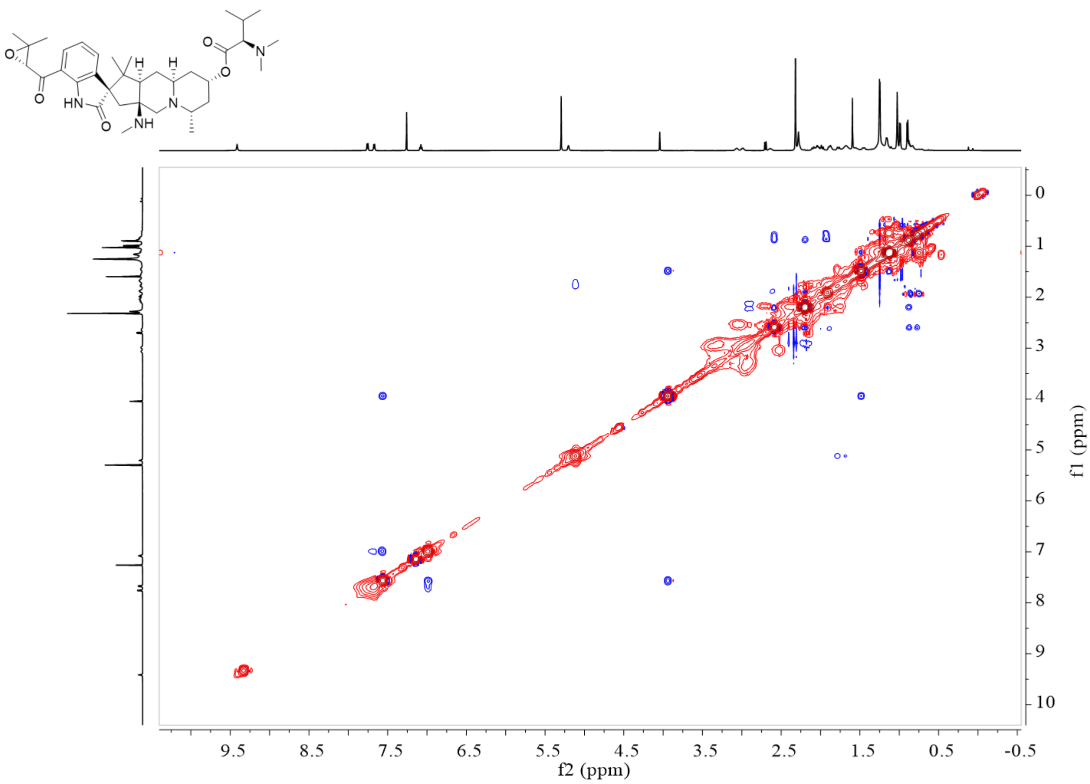

Figure S19-7. NOESY spectrum of **15** in $CDCl_3$.

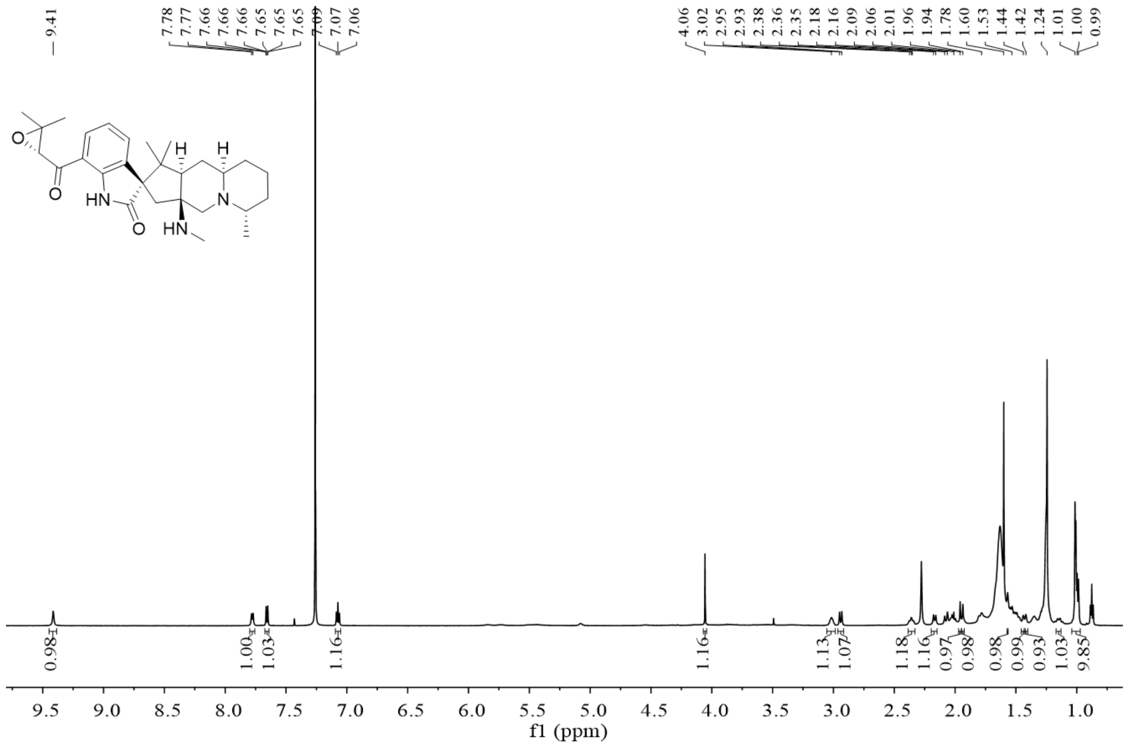

Figure S20-1. ^{1}H NMR (600 Hz) spectrum of **16** in $CDCl_3$.

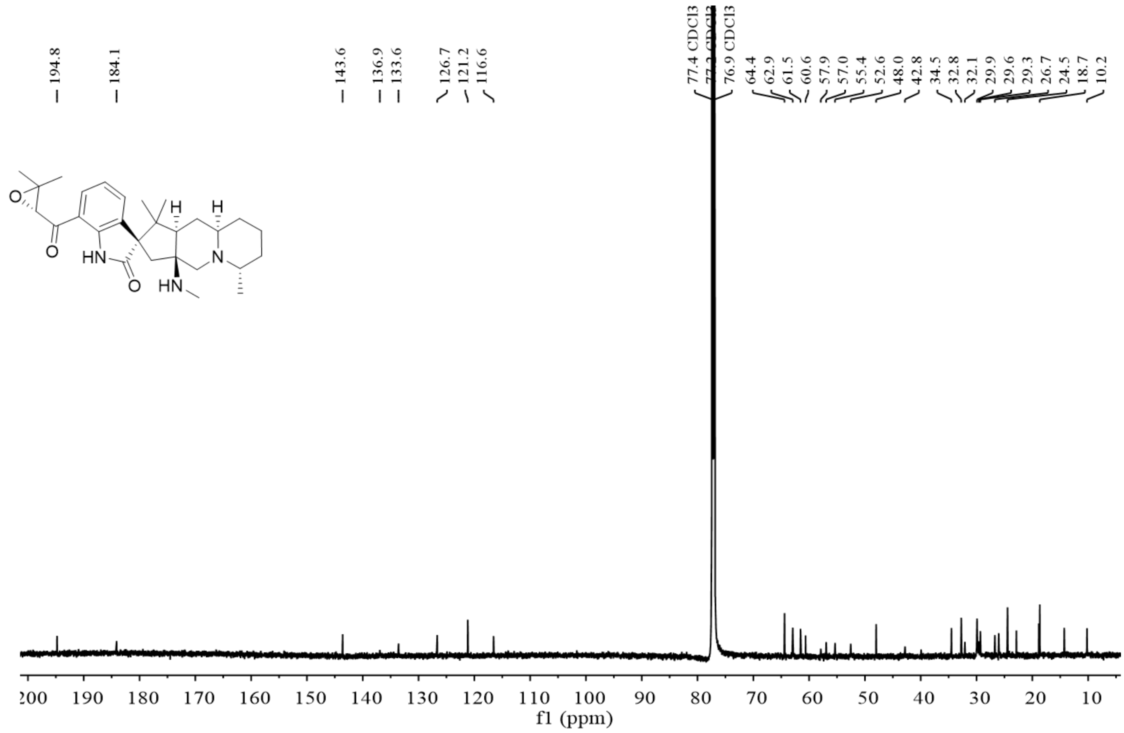

Figure S20-2. ^{13}C NMR (150 Hz) spectrum of **16** in $CDCl_3$.

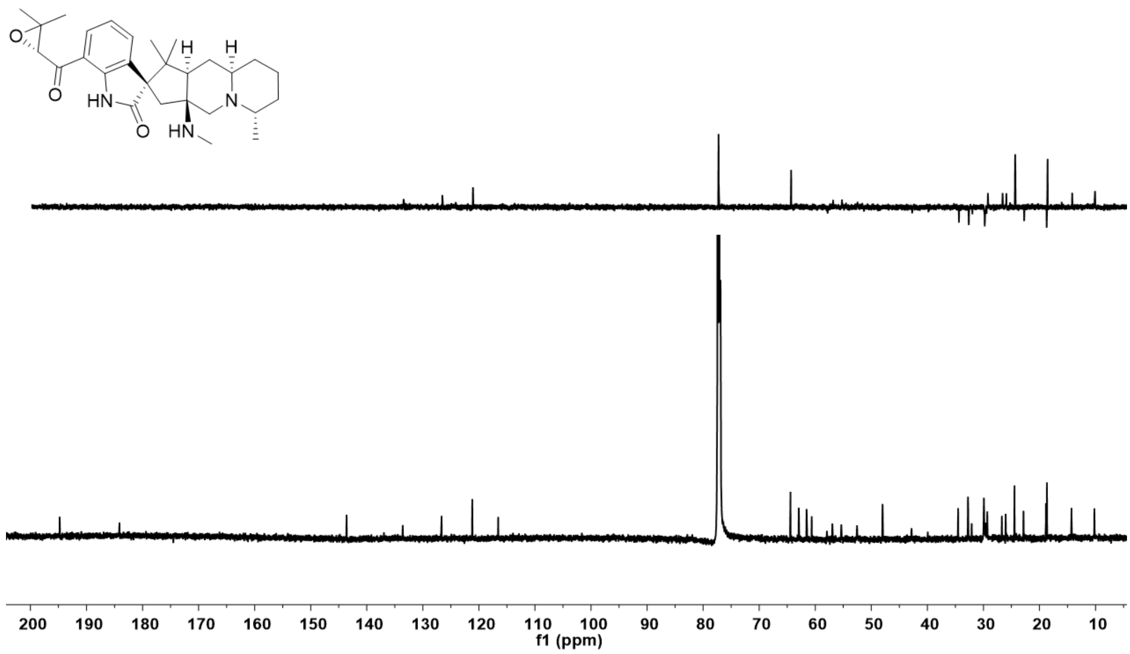

Figure S20-3. DEPT spectrum of **16** in $CDCl_3$.

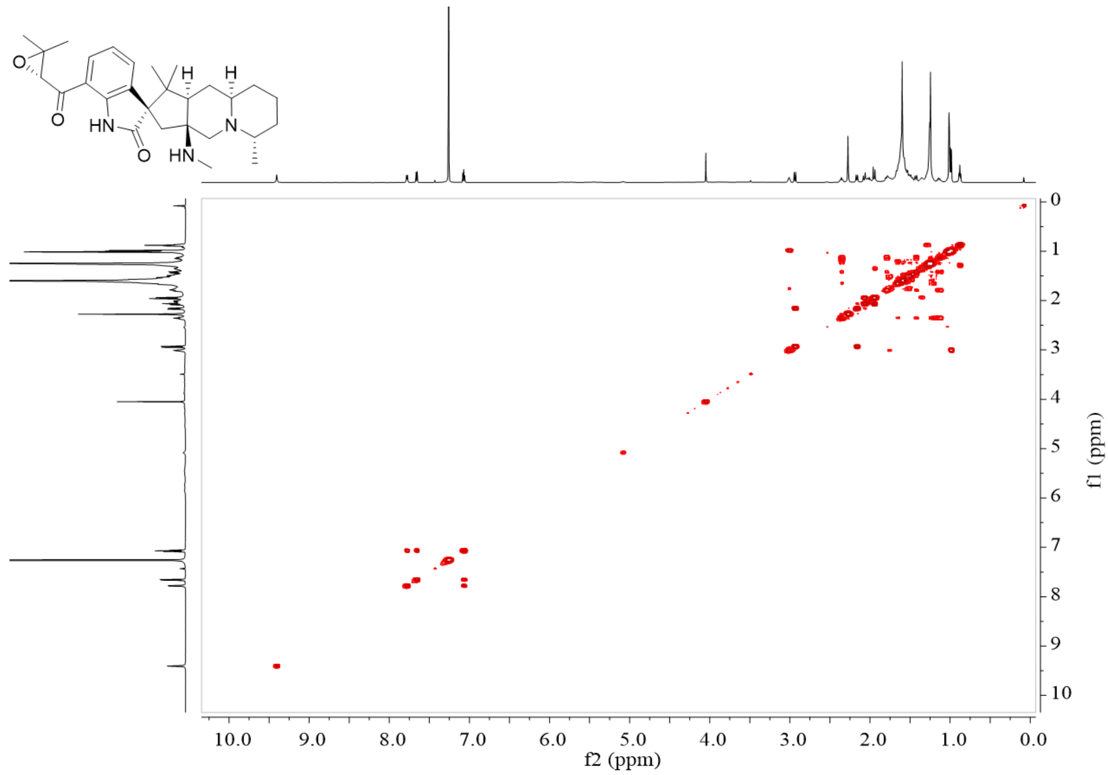

Figure S20-4. ^{1}H-^{1}H COSY spectrum of **16** in $CDCl_3$.

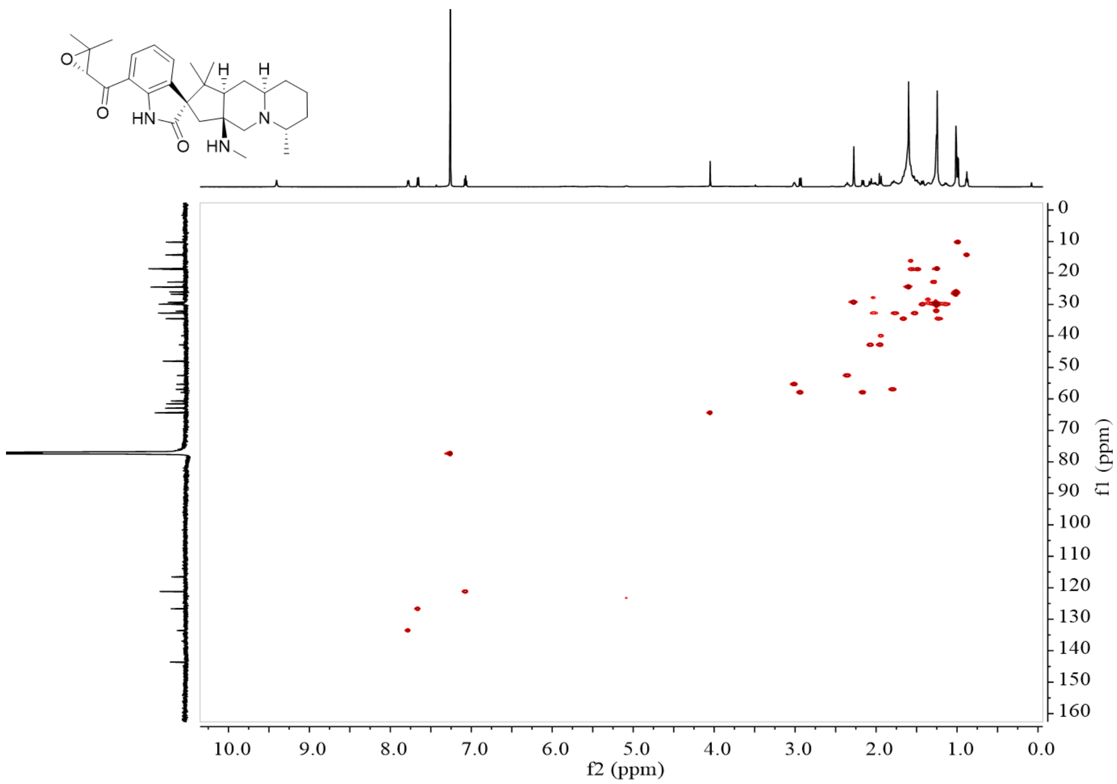

Figure S20-5. HSQC spectrum of **16** in $CDCl_3$.

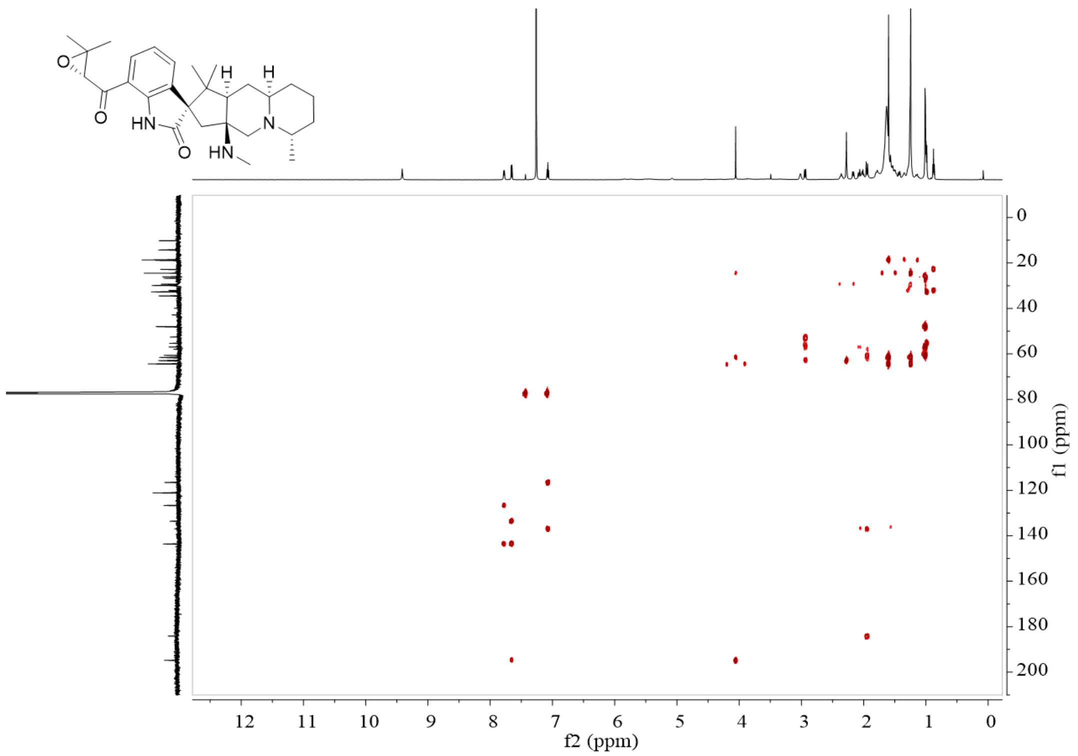

Figure S20-6. HMBC spectrum of **16** in $CDCl_3$.

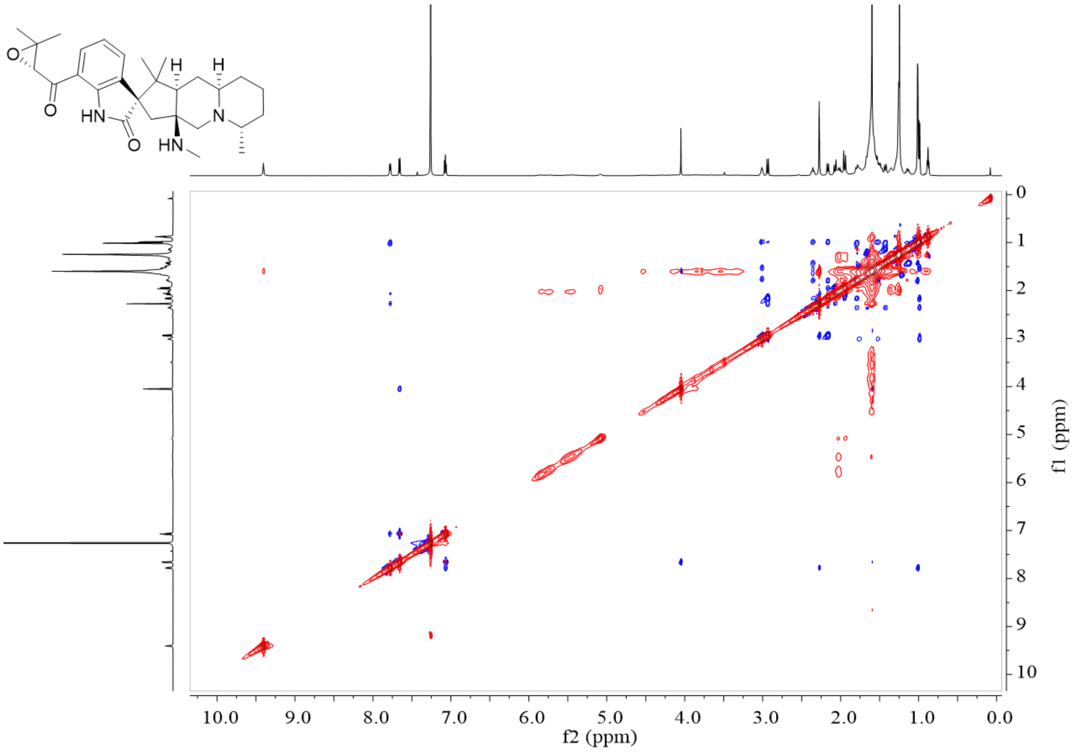

Figure S20-7. NOESY spectrum of **16** in $CDCl_3$.

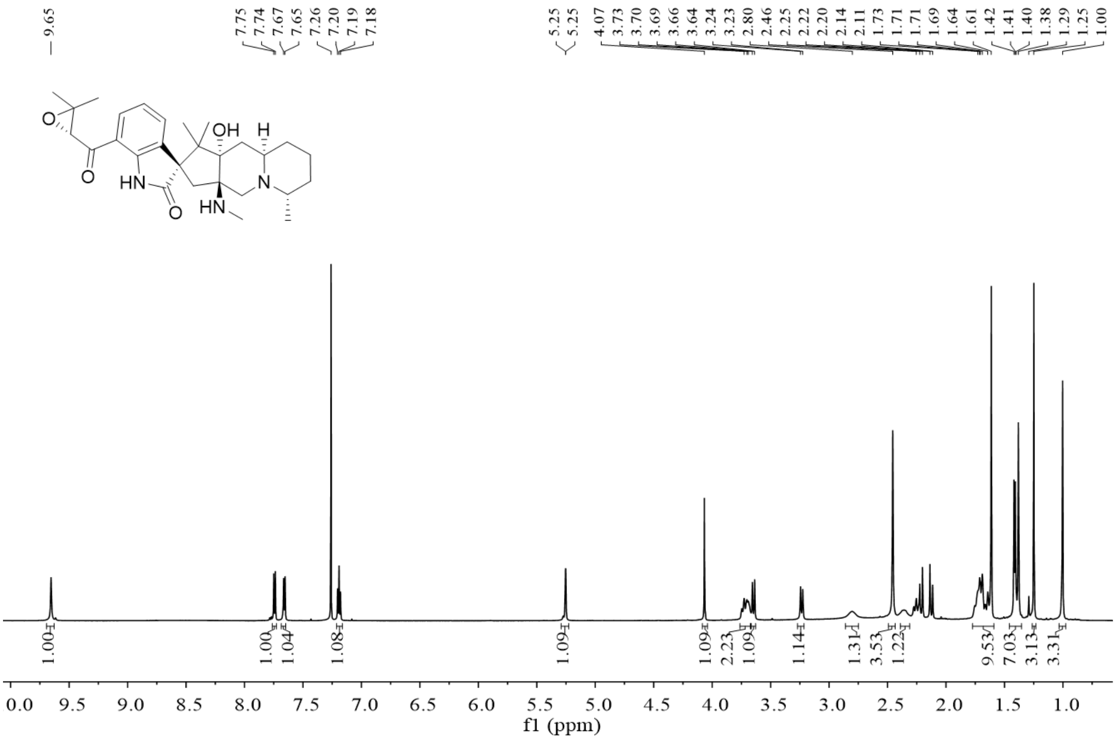

Figure S21-1. ^{1}H NMR (600 Hz) spectrum of **17** in $CDCl_3$.

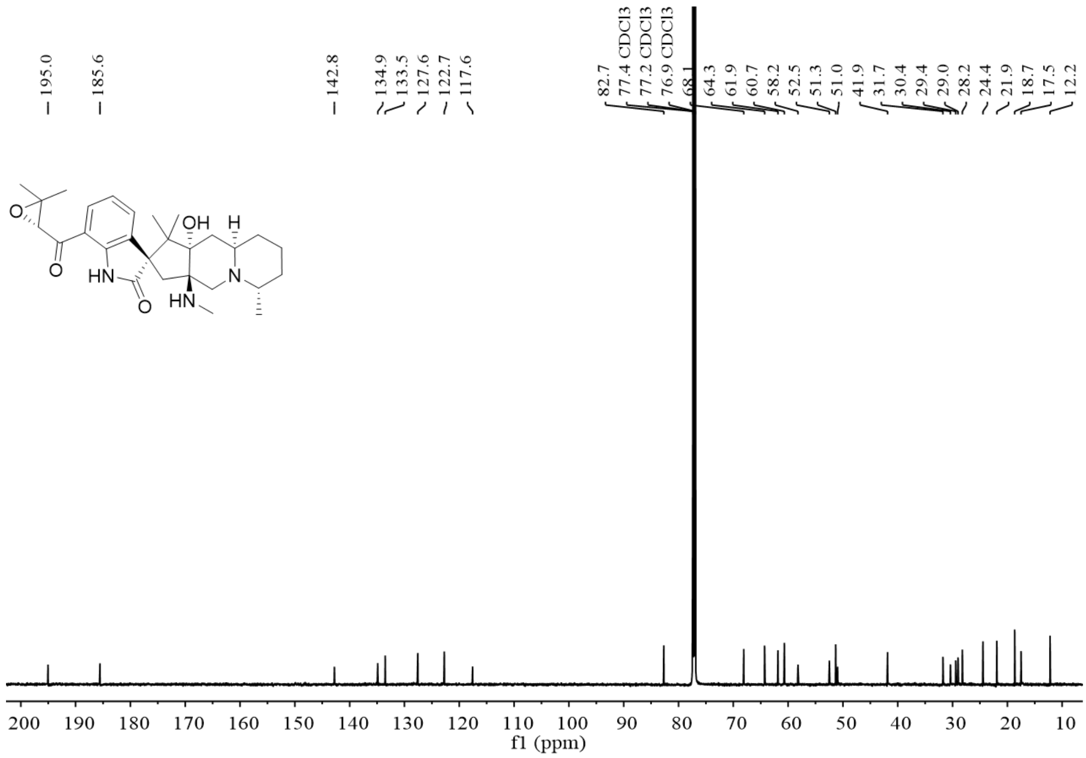

Figure S21-2. ^{13}C NMR (150 Hz) spectrum of **17** in $CDCl_3$.

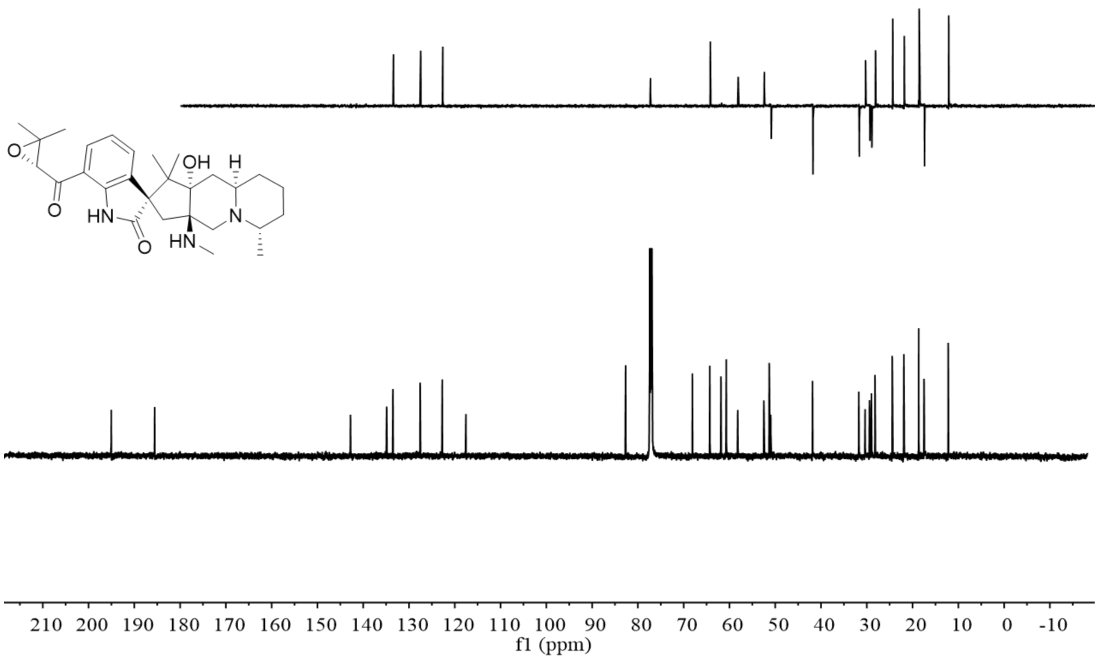

Figure S21-3. DEPT spectrum of **17** in $CDCl_3$.

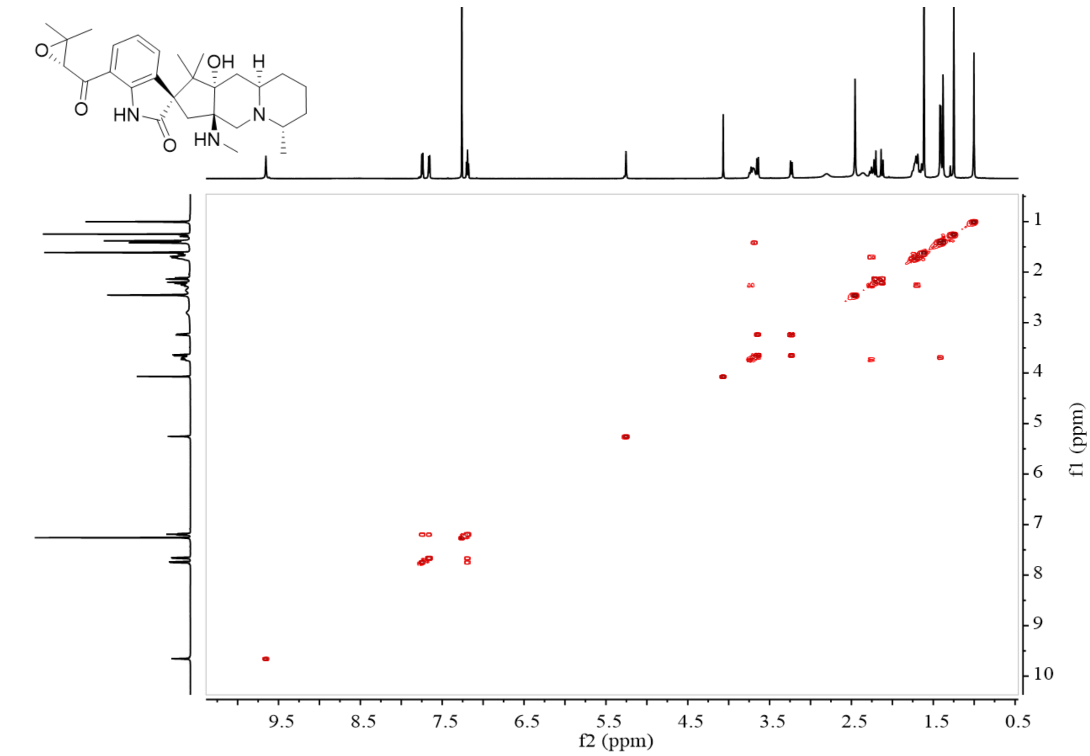

Figure S21-4. [1]H-[1]H COSY spectrum of **17** in $CDCl_3$.

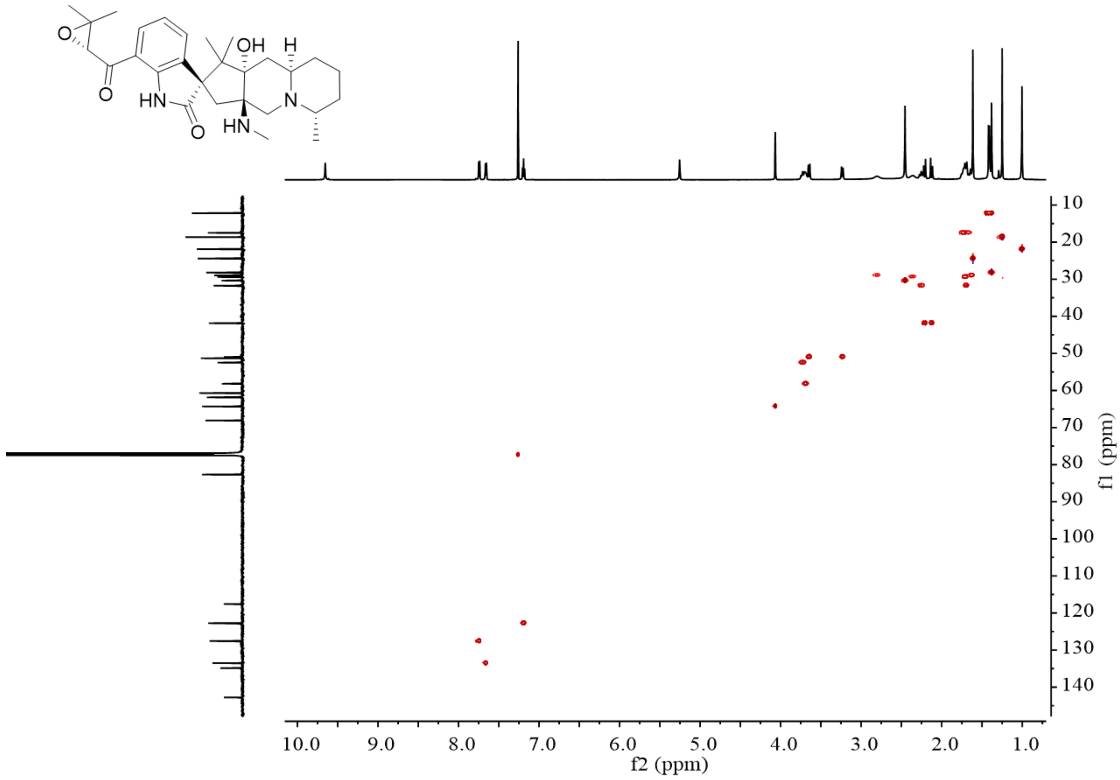

Figure S21-5. HSQC spectrum of **17** in $CDCl_3$.

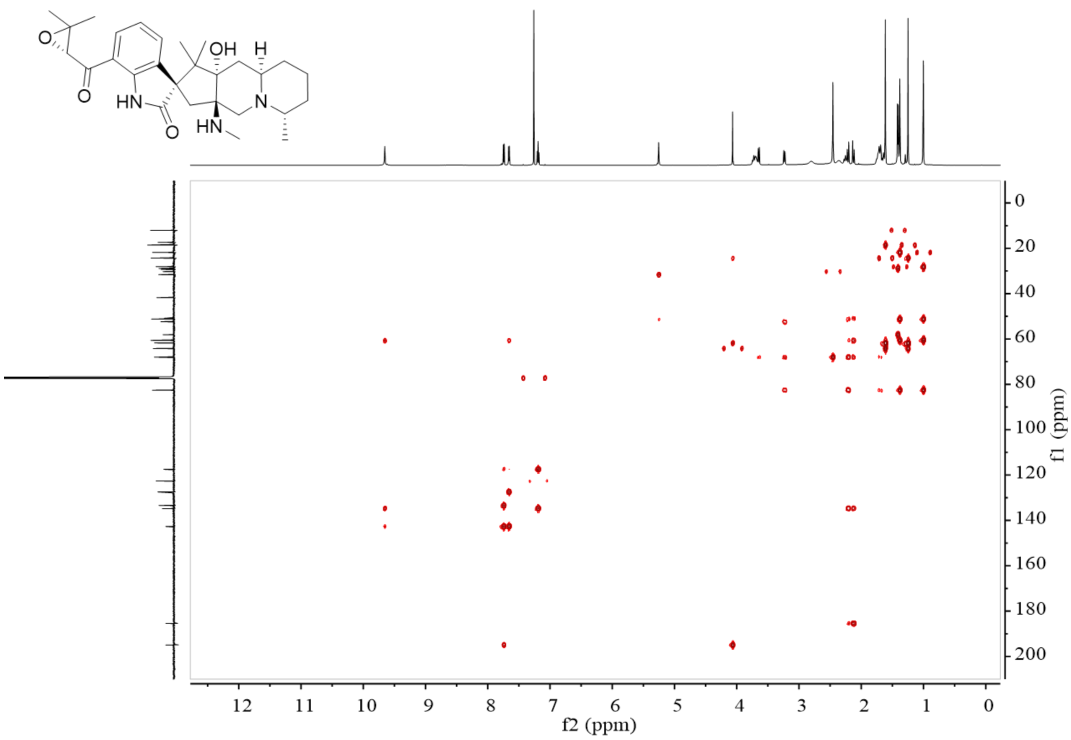

Figure S21-6. HMBC spectrum of **17** in $CDCl_3$.

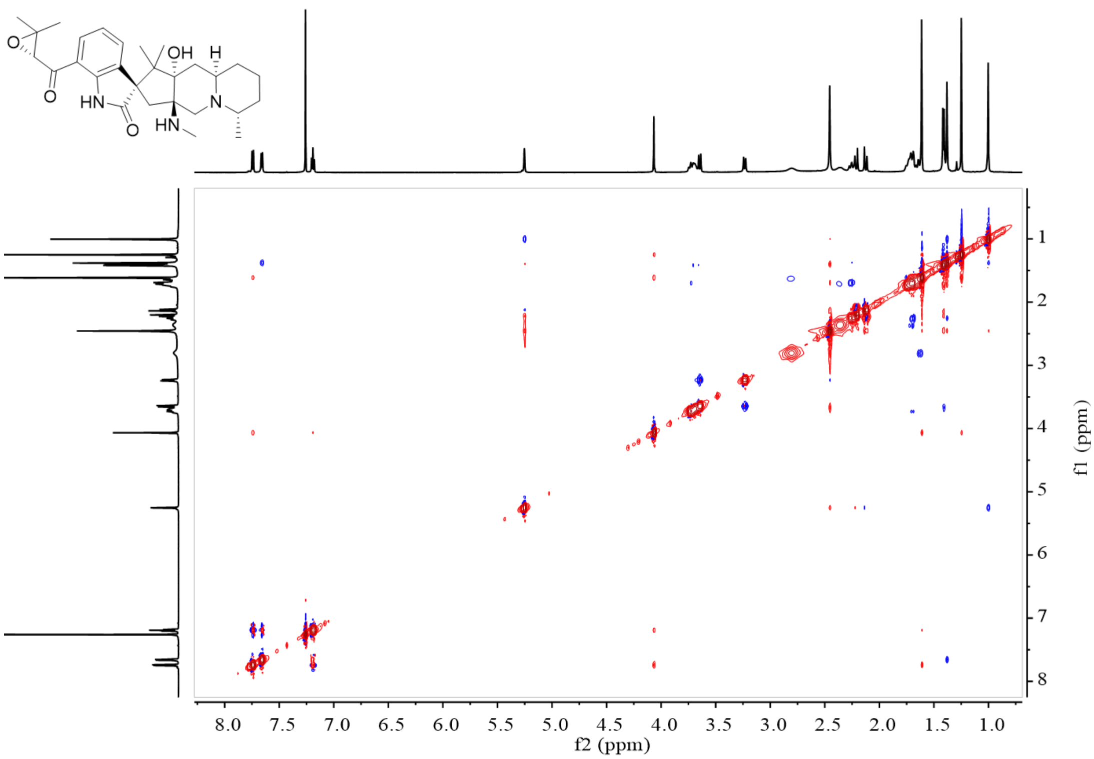

Figure S21-7. NOESY spectrum of **17** in $CDCl_3$.

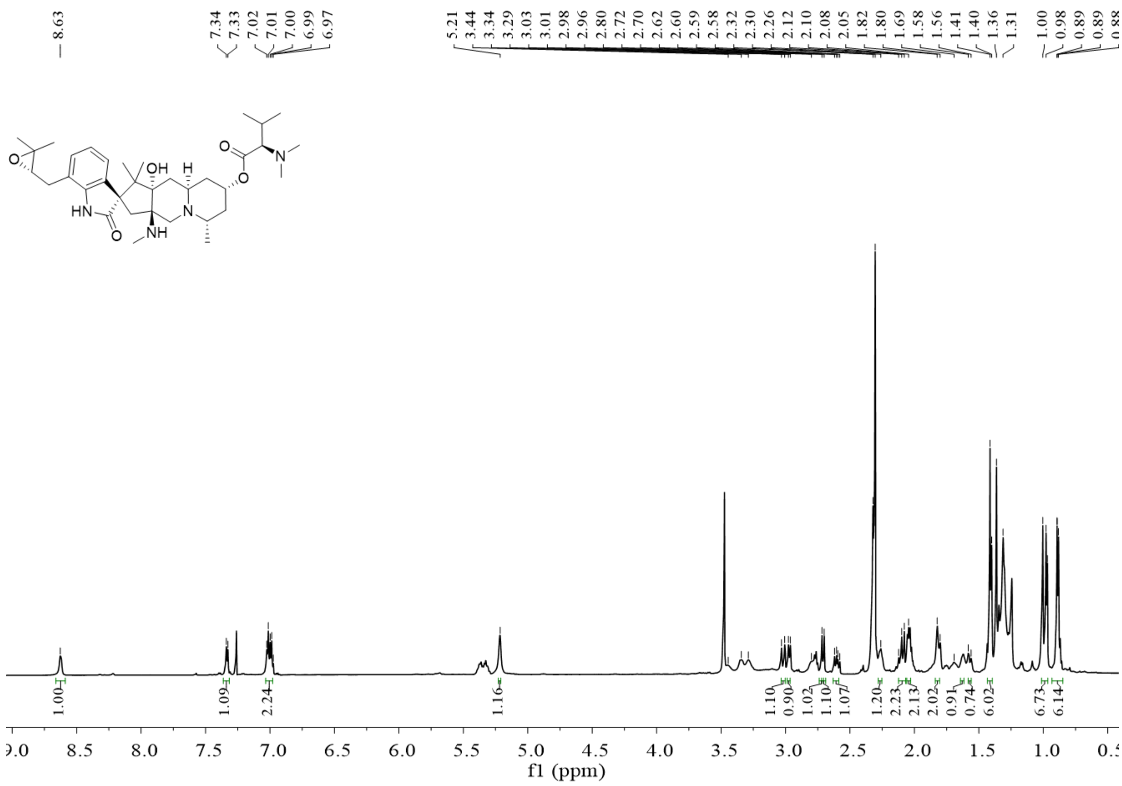

Figure S22-1. ^{1}H NMR (600 Hz) spectrum of **18** in $CDCl_3$.

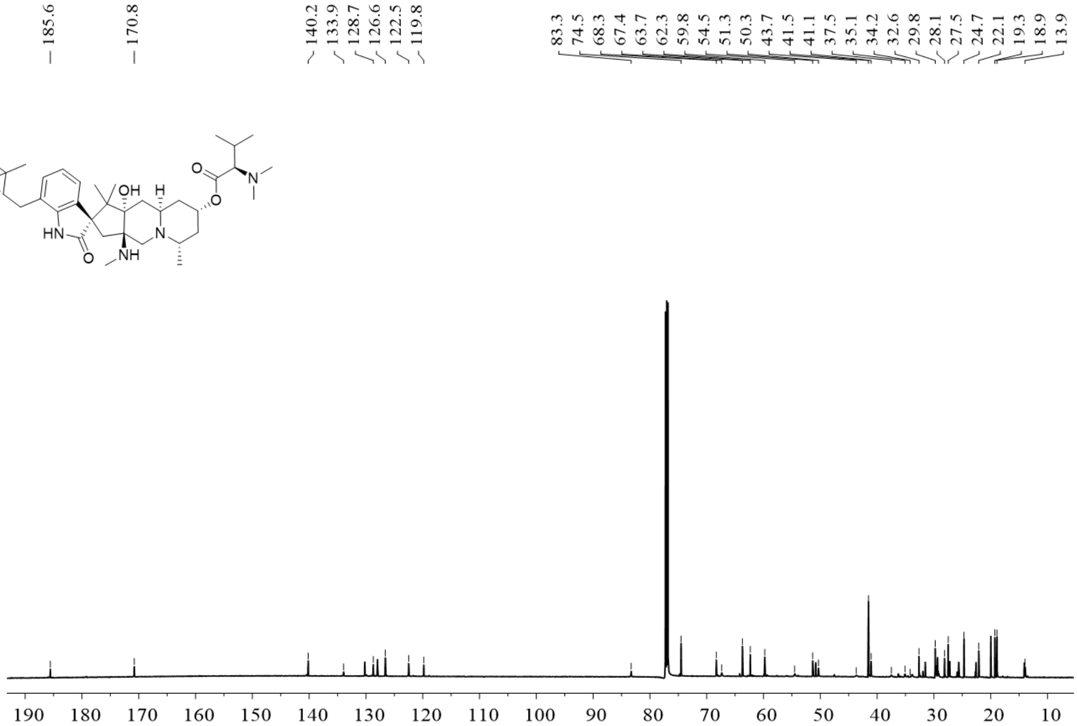

Figure S22-2. ^{13}C NMR (150 Hz) spectrum of **18** in $CDCl_3$.

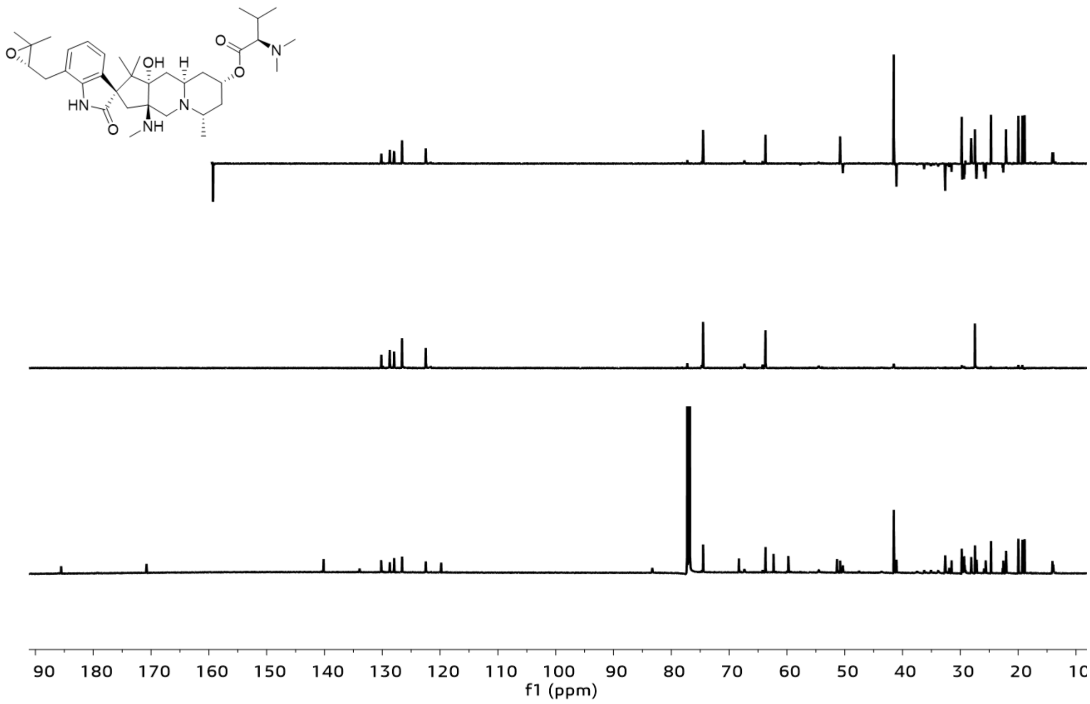

Figure S22-3. DEPT spectrum of **18** in $CDCl_3$.

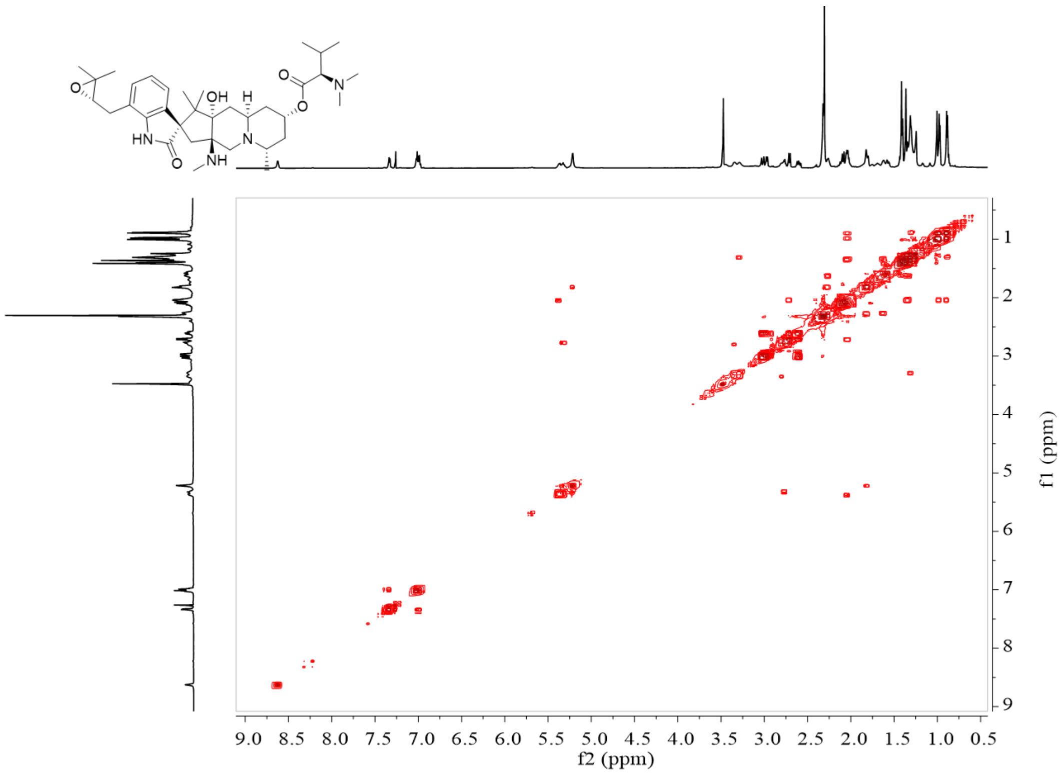

Figure S22-4. ^1H-^1H COSY spectrum of **18** in $CDCl_3$.

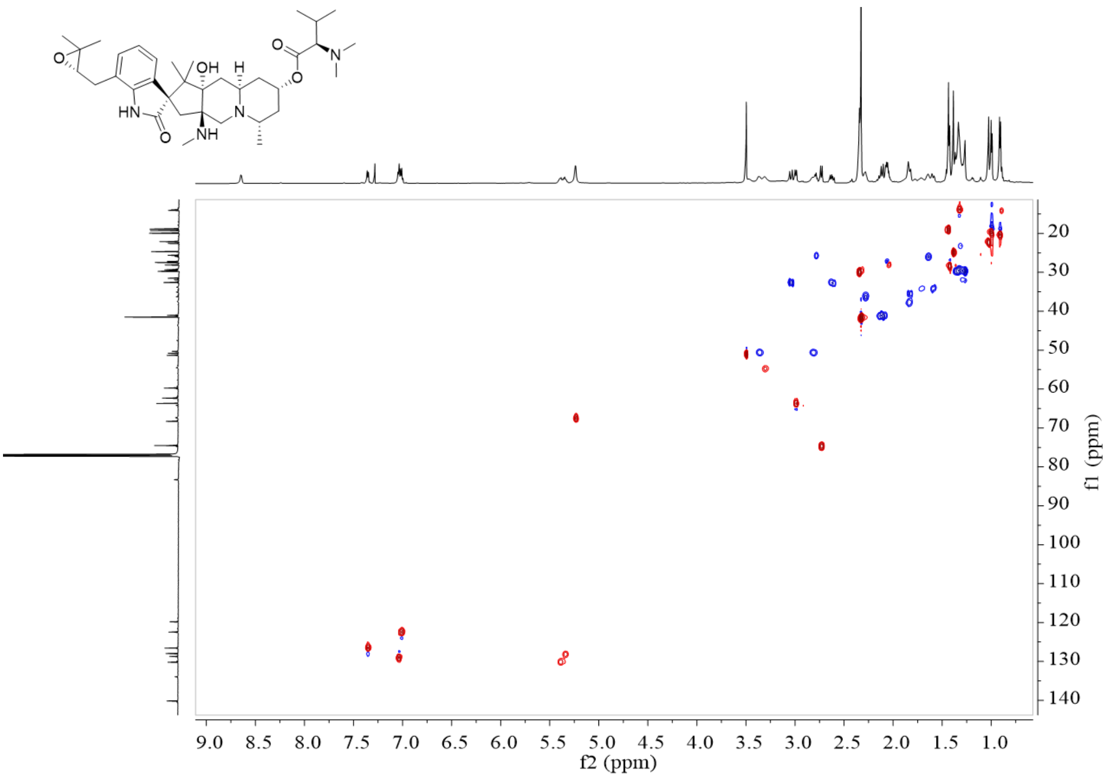

Figure S22-5. HSQC spectrum of **18** in $CDCl_3$.

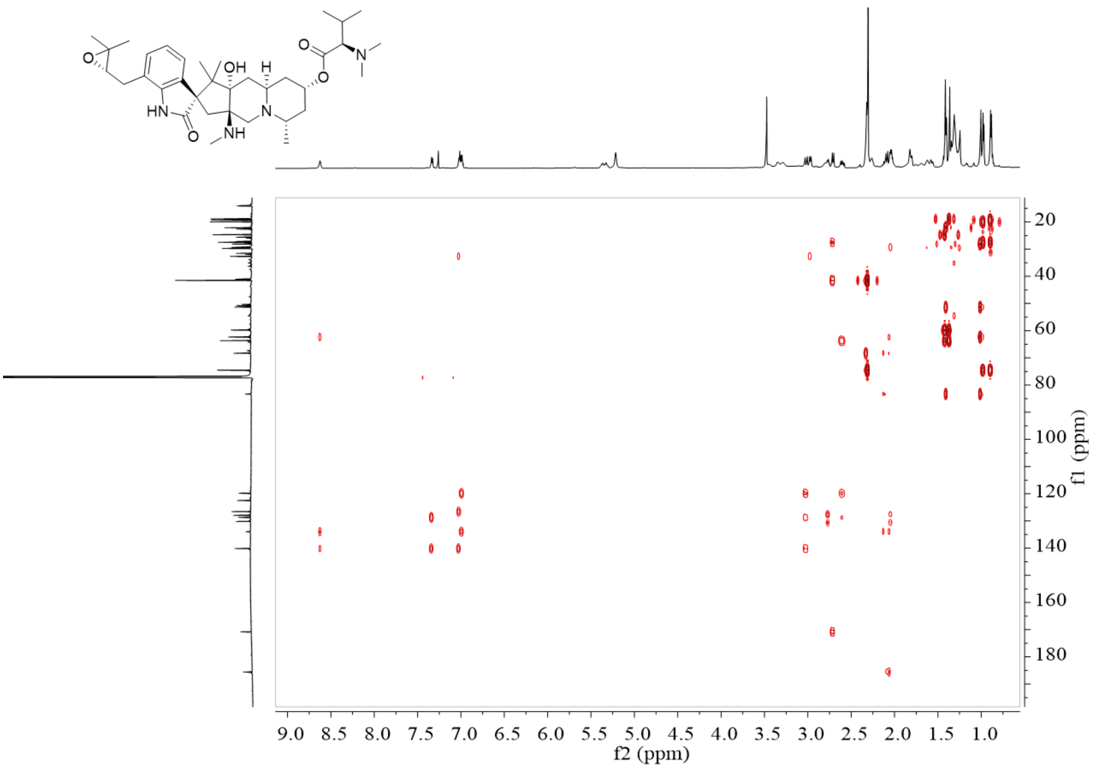

Figure S22-6. HMBC spectrum of **18** in $CDCl_3$.

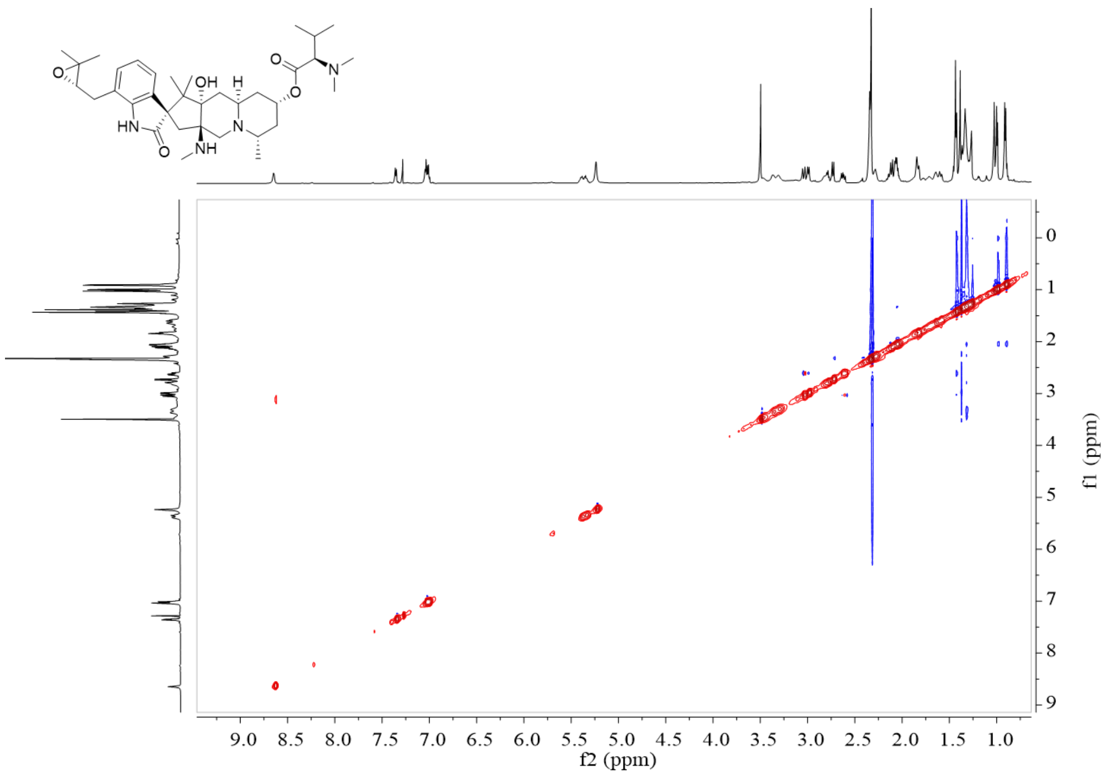

Figure S22-7. NOESY spectrum of **18** in $CDCl_3$.

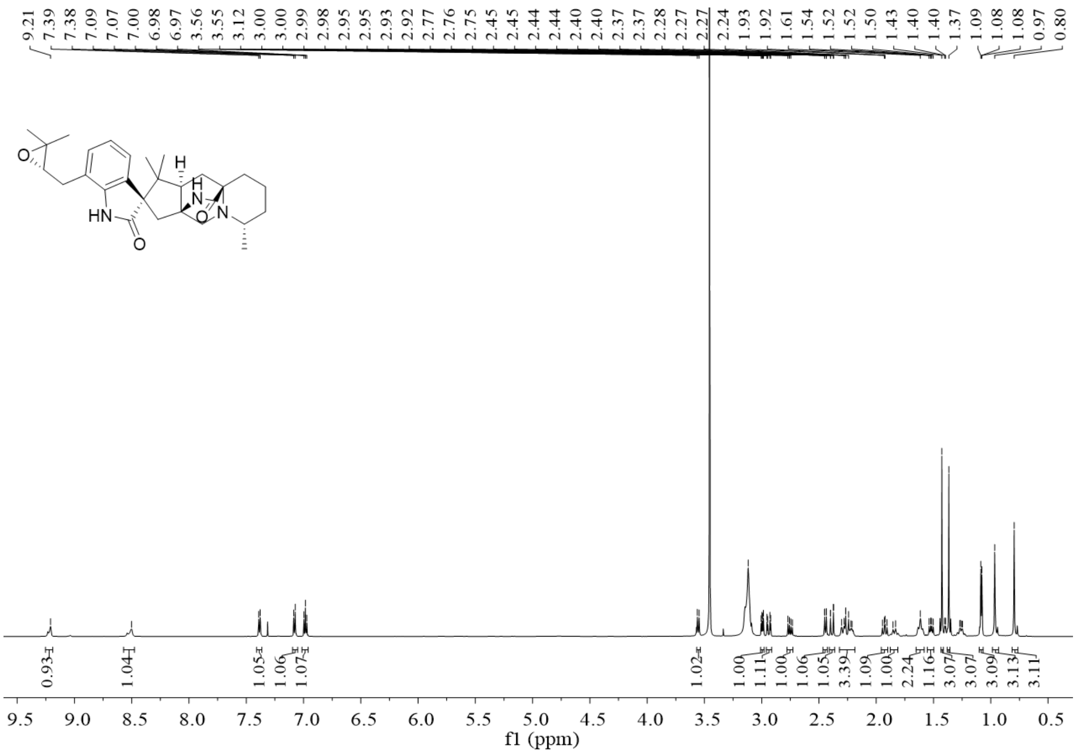

Figure S23-1. ^1H NMR (600 Hz) spectrum of **20** in $CDCl_3$.

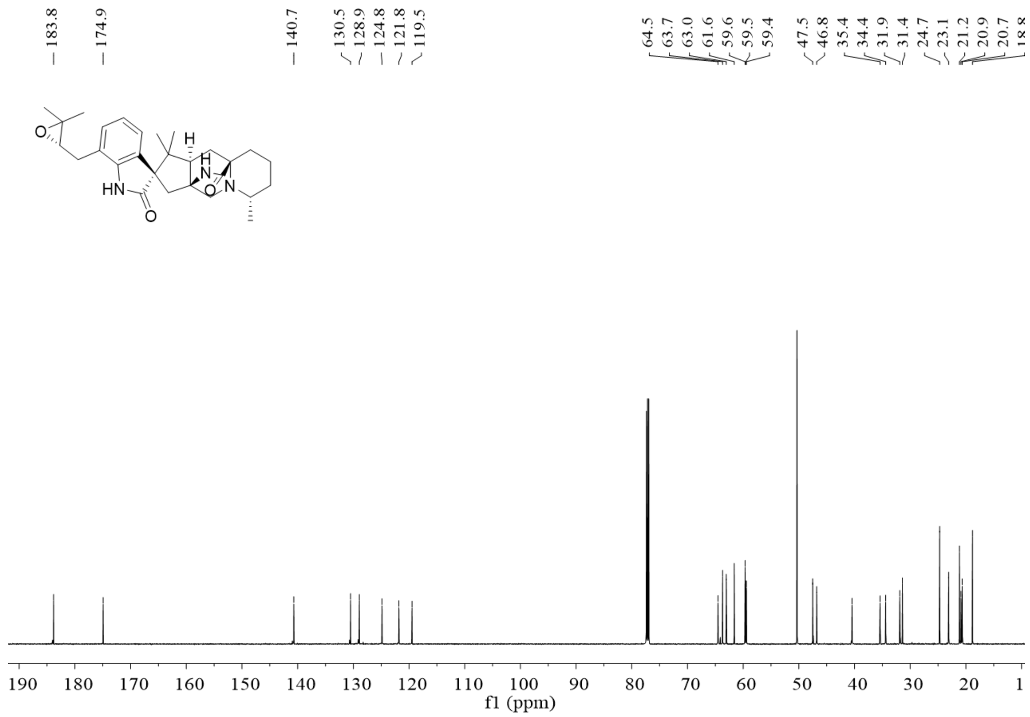

Figure S23-2. ^{13}C NMR (150 Hz) spectrum of **20** in $CDCl_3$.

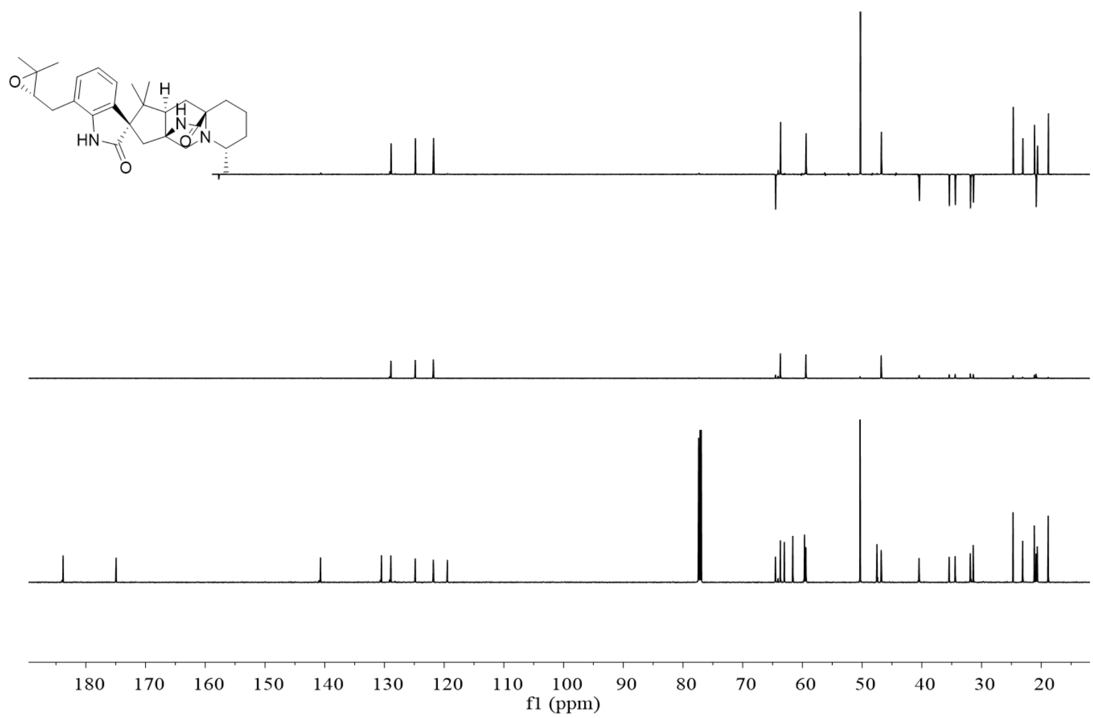

Figure S23-3. DEPT spectrum of **20** in $CDCl_3$.

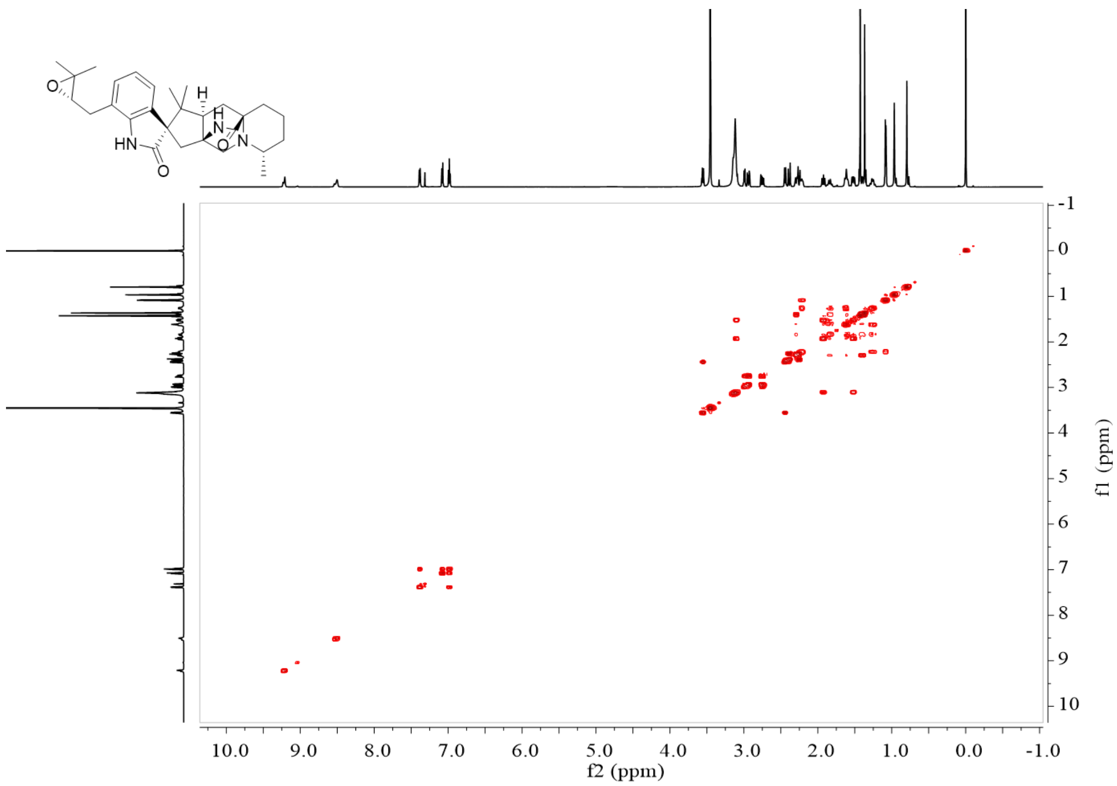

Figure S23-4. ^{1}H-^{1}H COSY spectrum of **20** in $CDCl_3$.

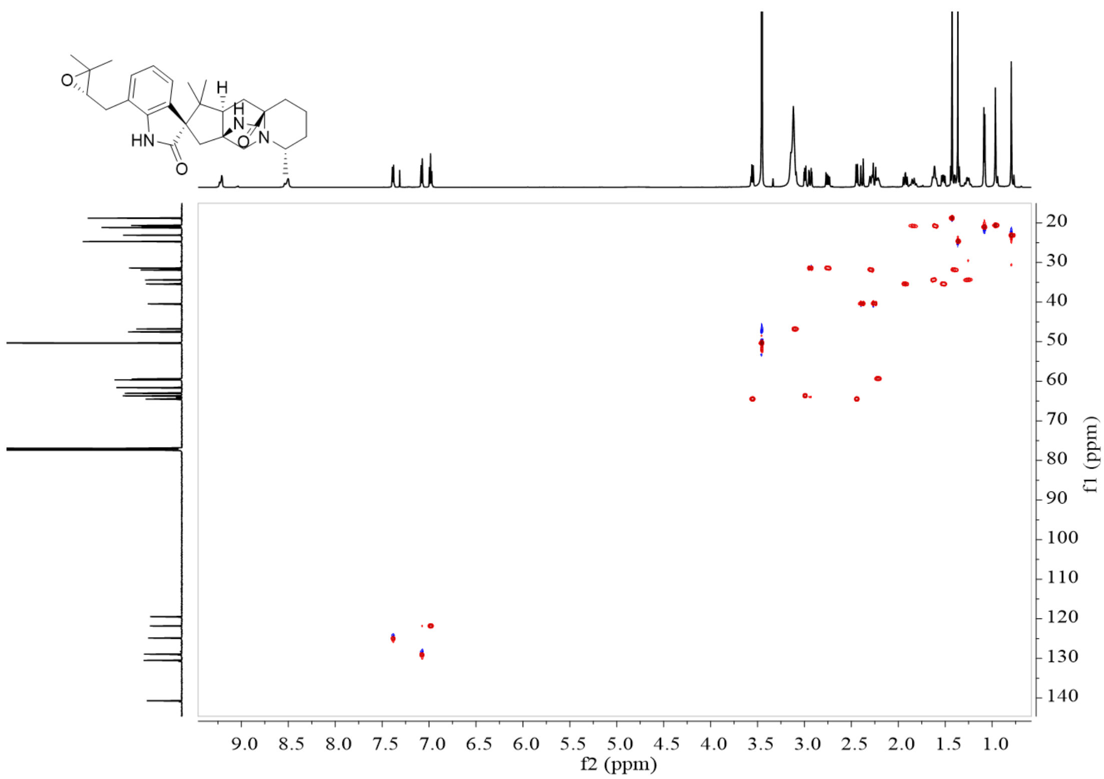

Figure S23-5. HSQC spectrum of **20** in $CDCl_3$.

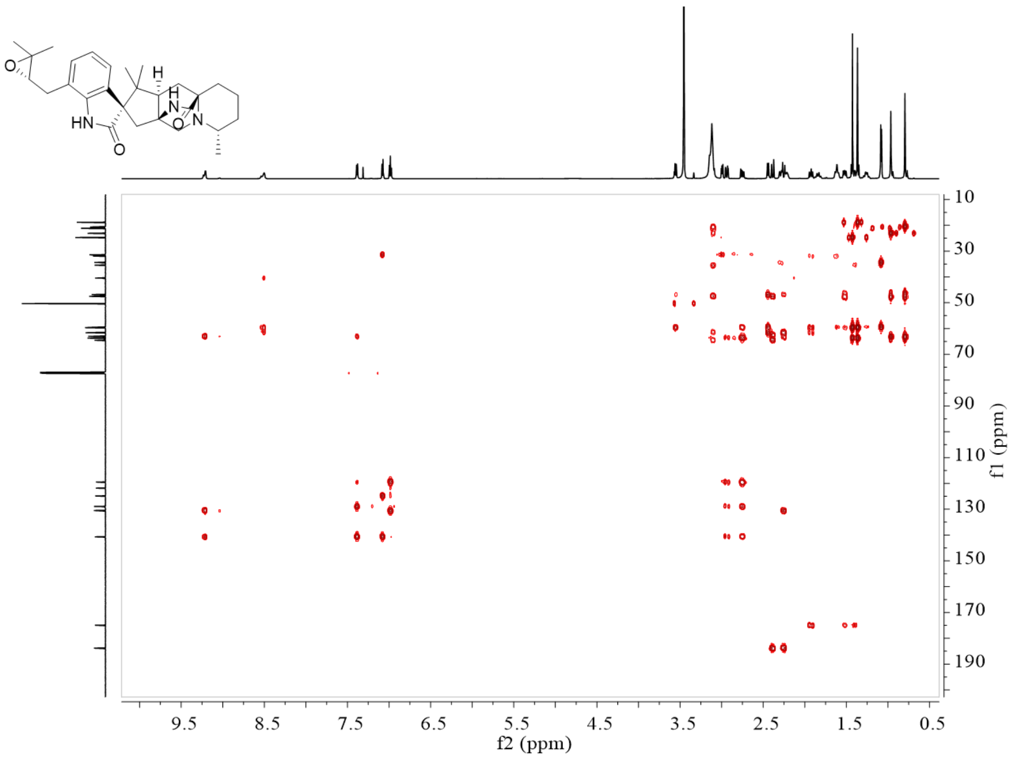

Figure S23-6. HMBC spectrum of **20** in $CDCl_3$.

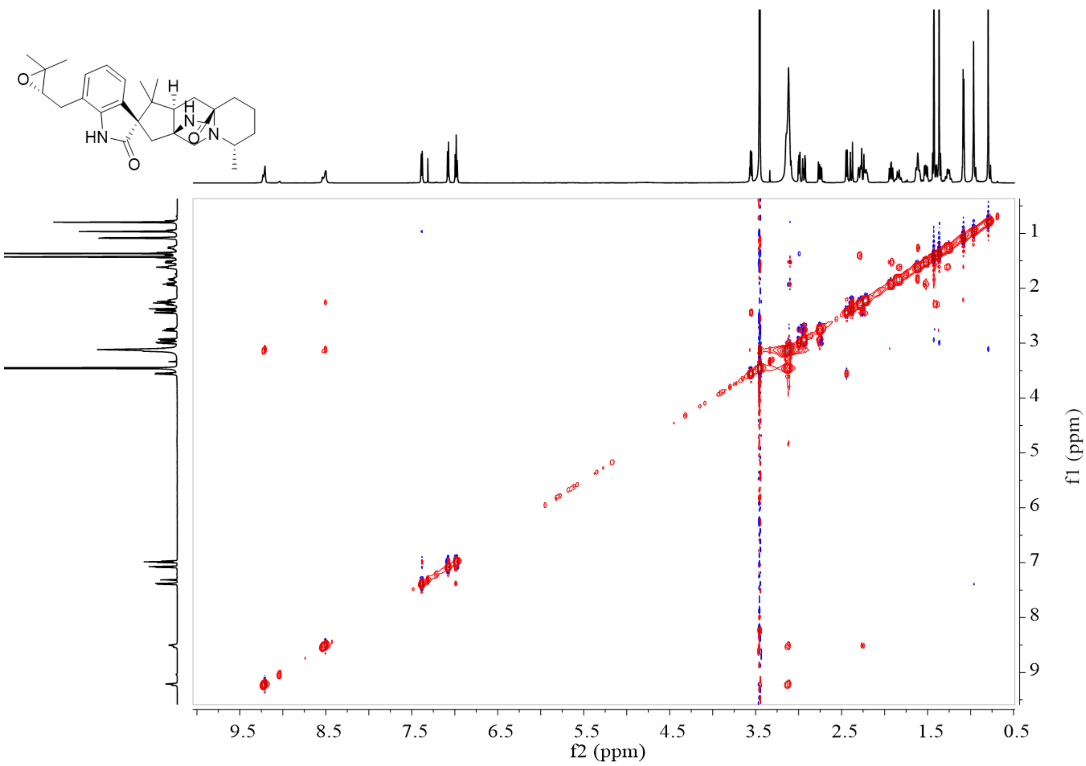

Figure S23-7. NOESY spectrum of **20** in $CDCl_3$.

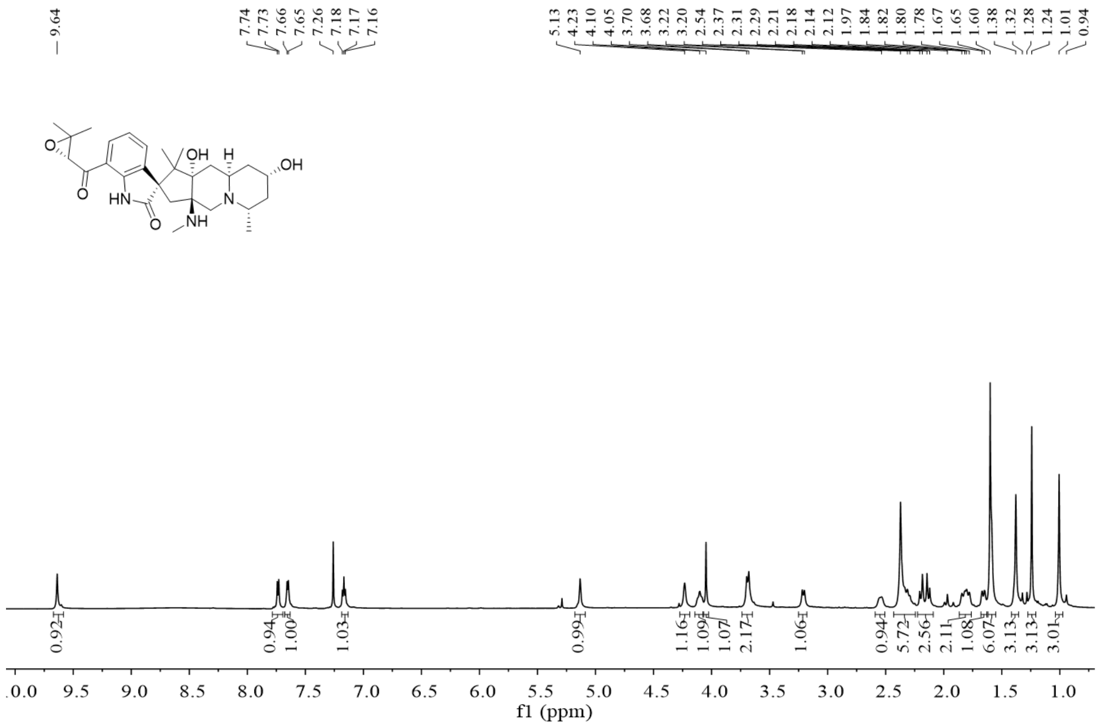

Figure S24-1. ^{1}H NMR (600 Hz) spectrum of **23** in $CDCl_3$.

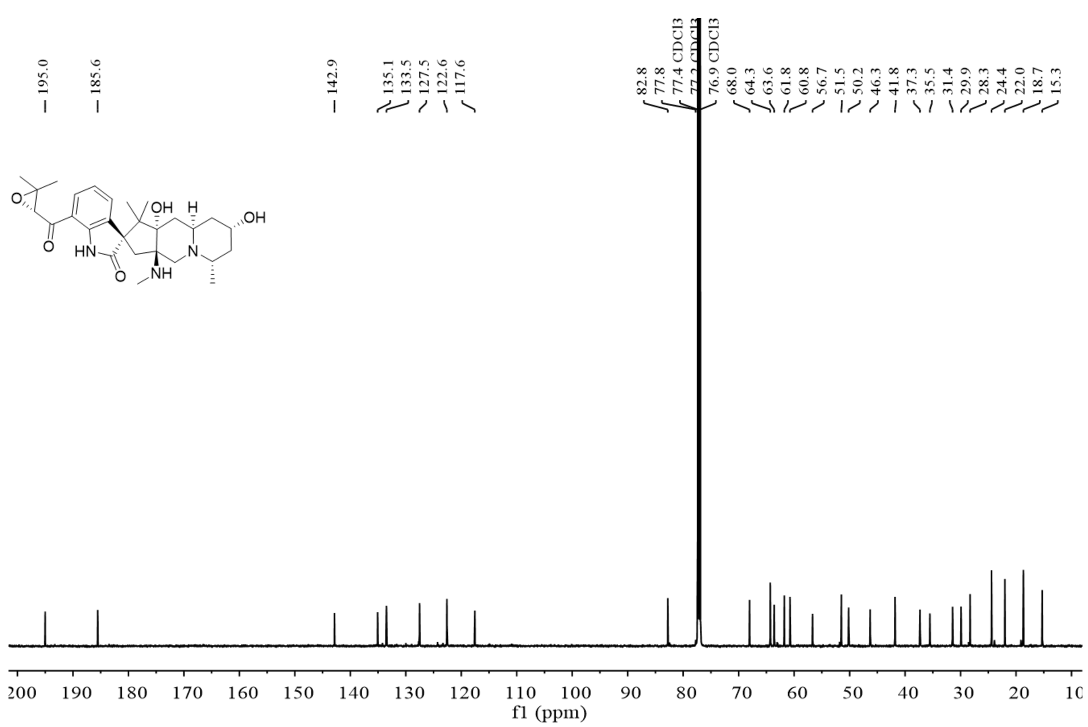

Figure S24-2. ^{13}C NMR (150 Hz) spectrum of **23** in $CDCl_3$.

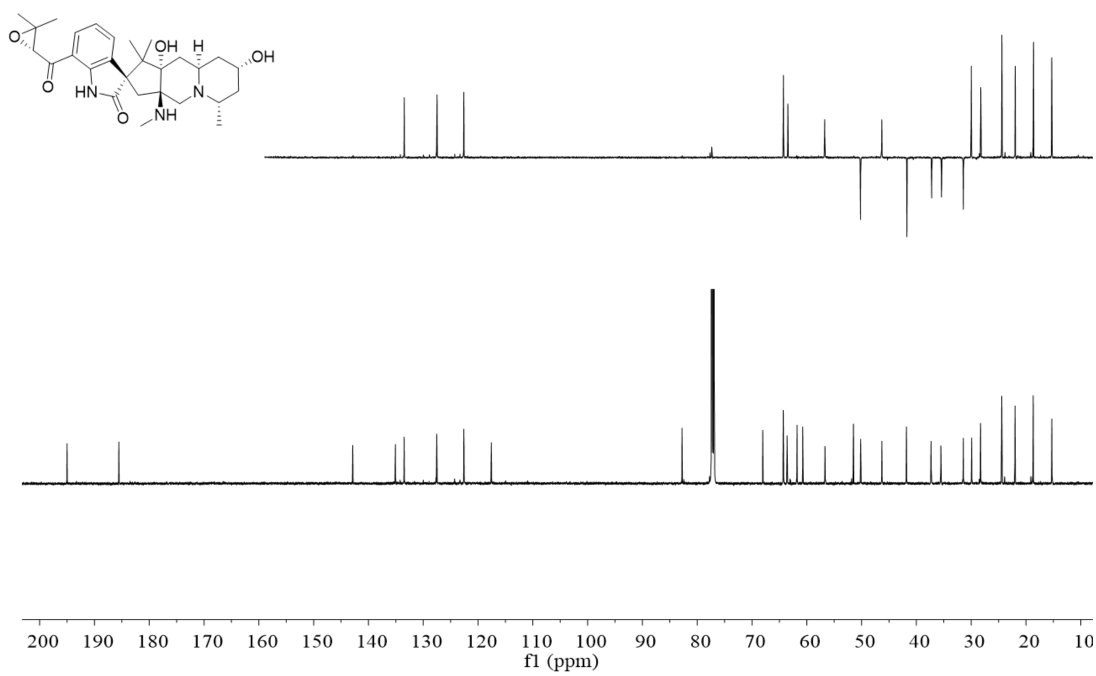

Figure S24-3. DEPT spectrum of **23** in $CDCl_3$.

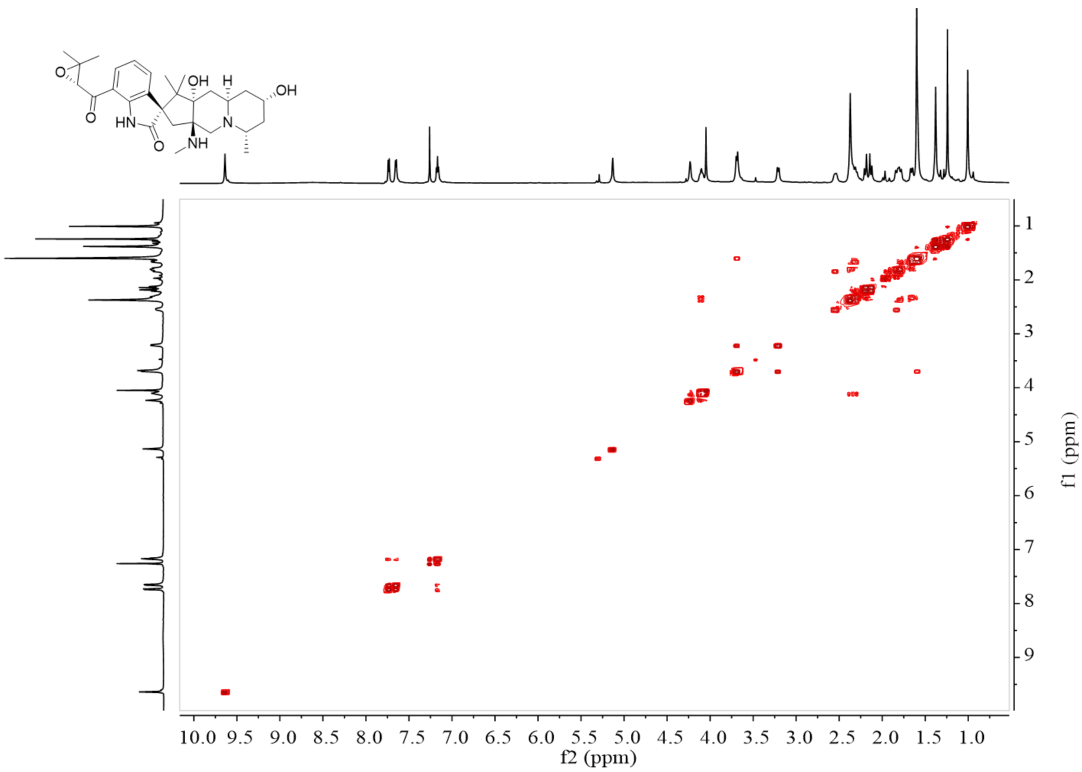

Figure S24-4. ^{1}H-^{1}H COSY spectrum of **23** in $CDCl_3$.

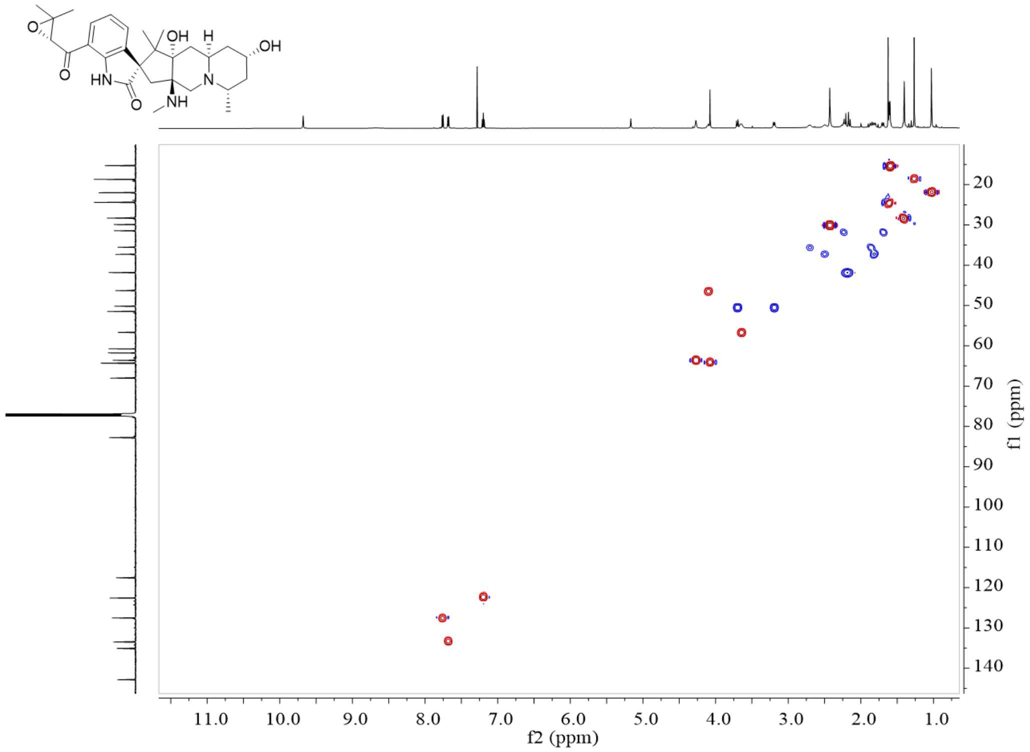

Figure S24-5. HSQC spectrum of **23** in $CDCl_3$.

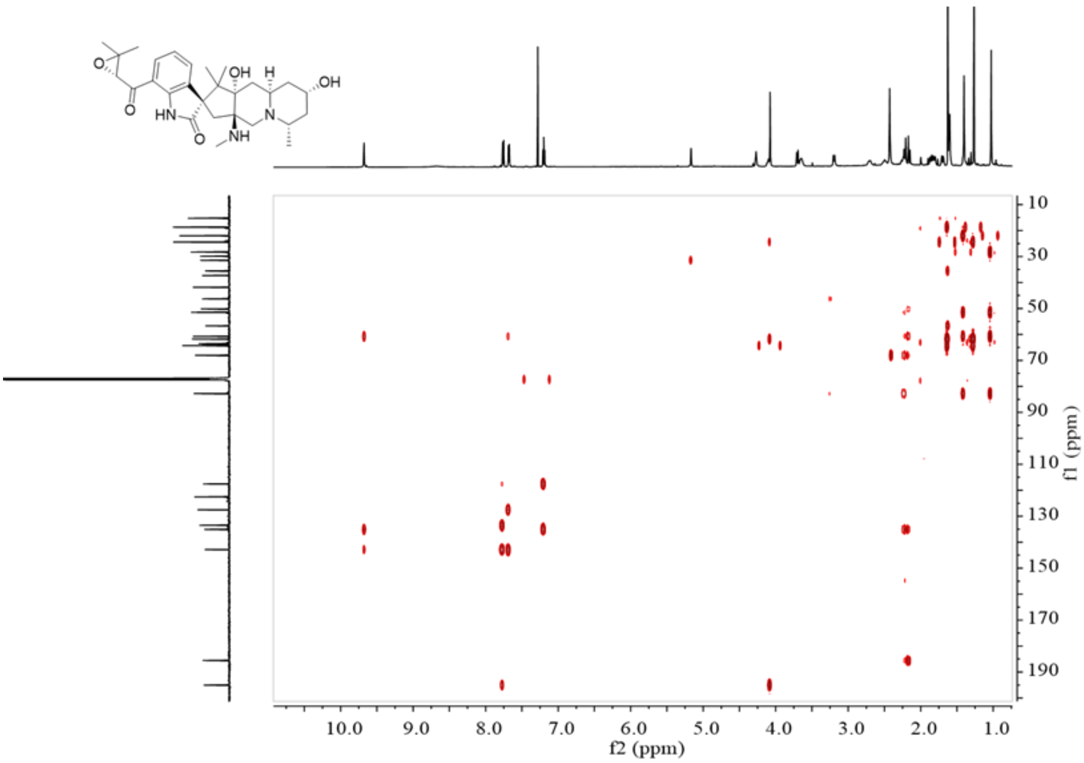

Figure S24-6. HMBC spectrum of **23** in $CDCl_3$.

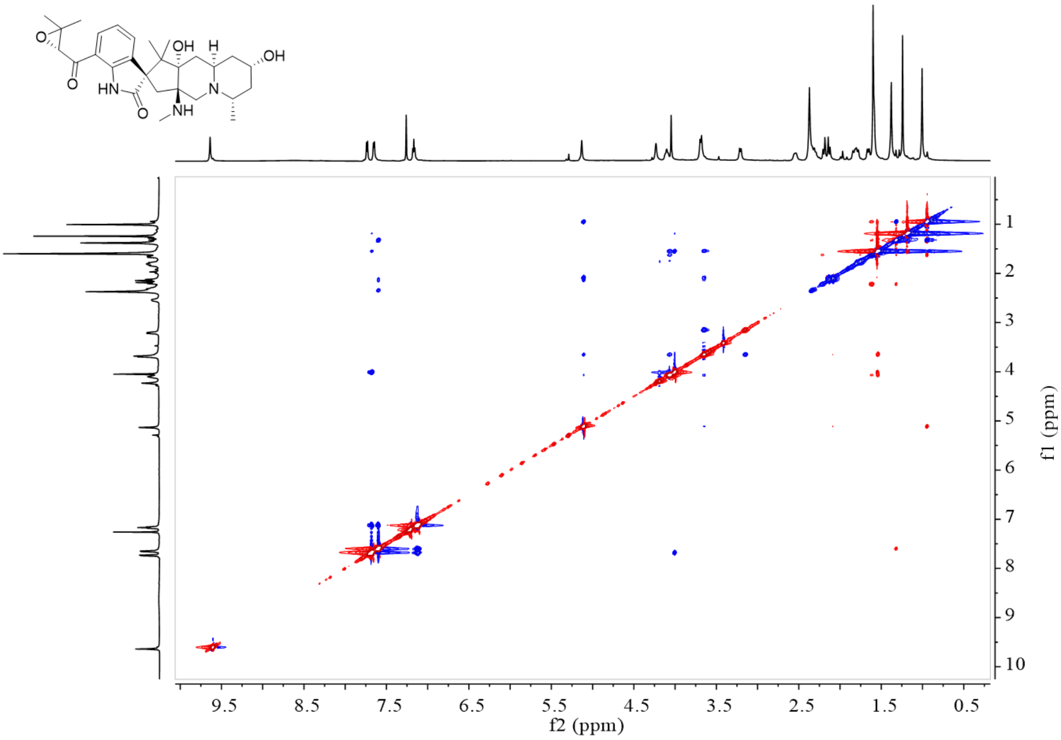

Figure S24-7. NOESY spectrum of **23** in $CDCl_3$**.**

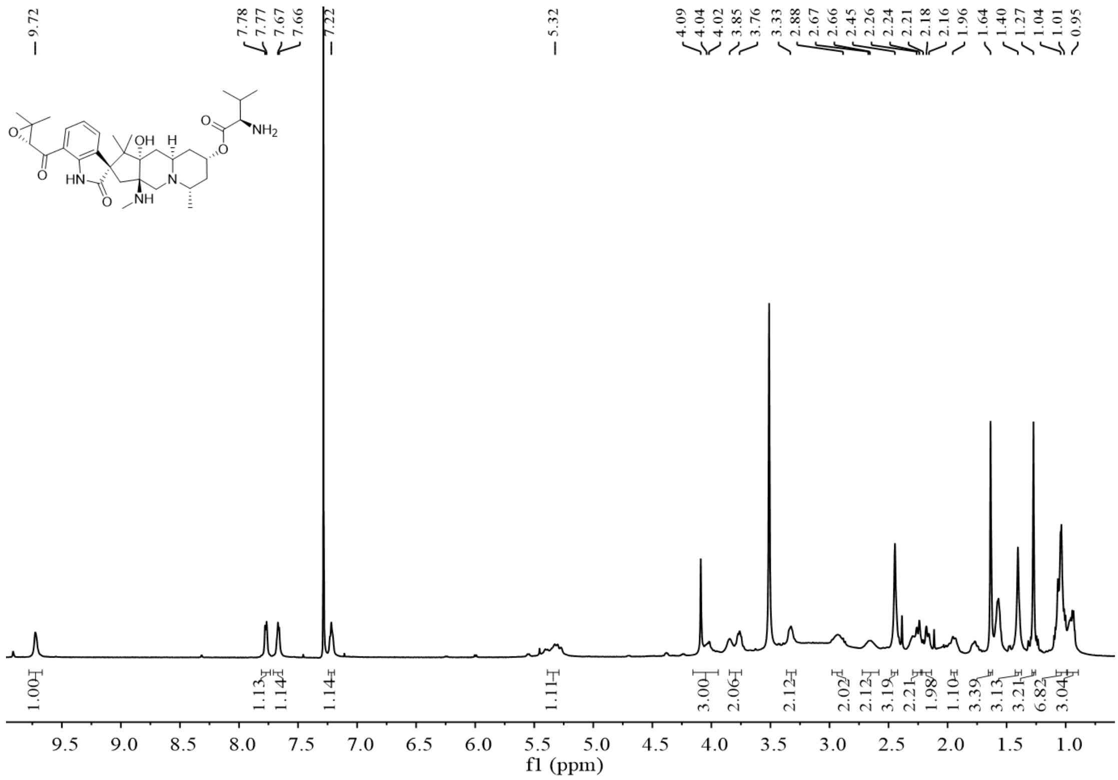

Figure S25-1. ^{1}H NMR (600 Hz) spectrum of **24** in $CDCl_3$.

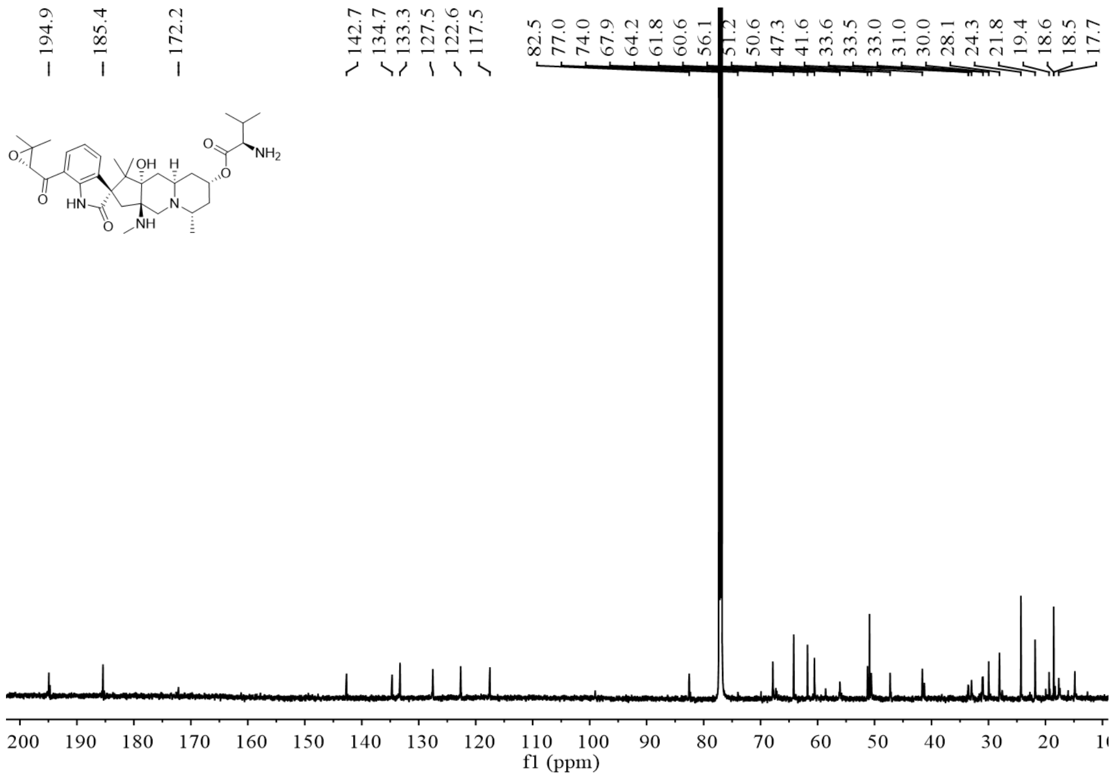

Figure S25-2. ^{13}C NMR (150 Hz) spectrum of **24** in $CDCl_3$.

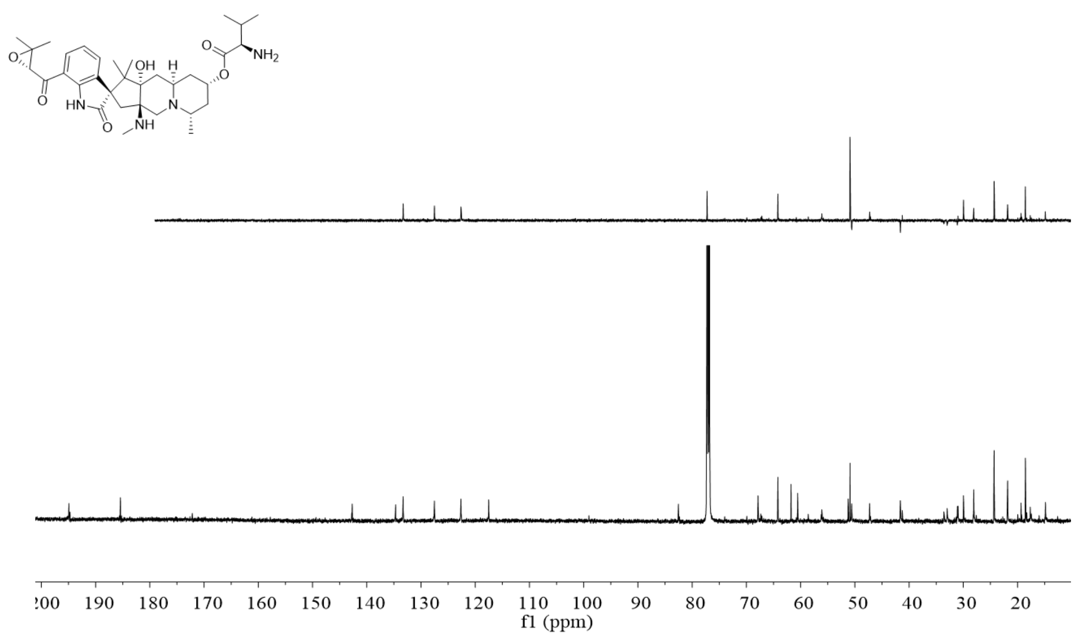

Figure S25-3. DEPT spectrum of **24** in $CDCl_3$.

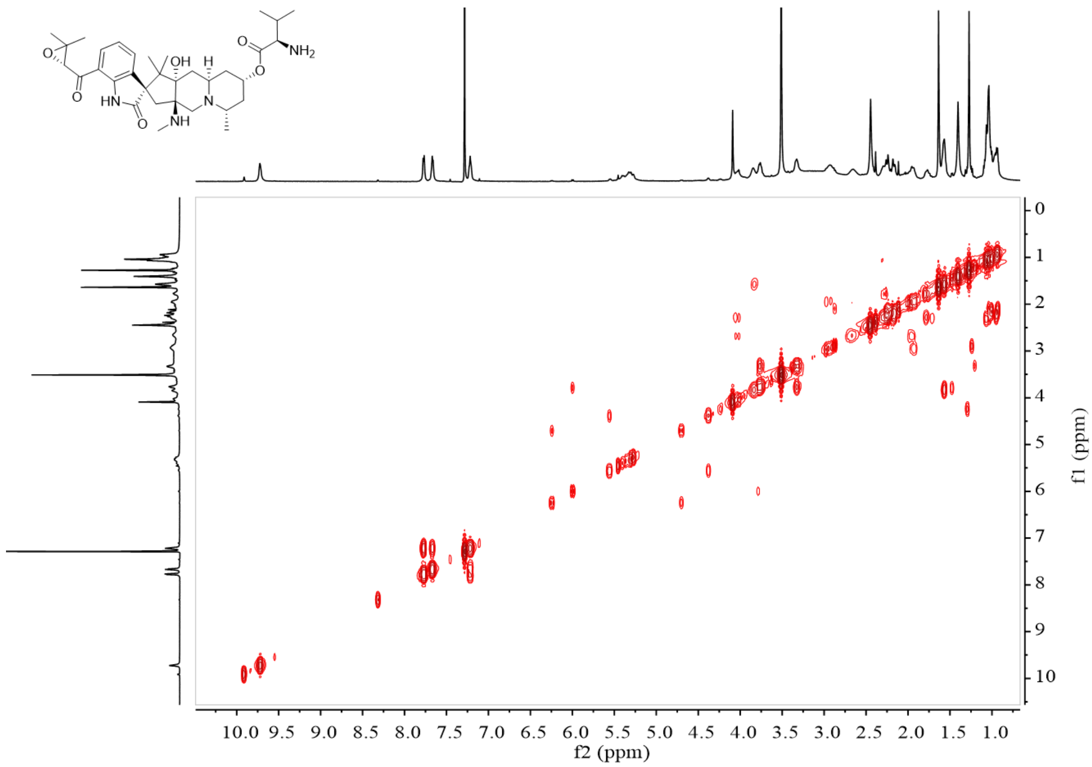

Figure S25-4. ^{1}H-^{1}H COSY spectrum of **24** in $CDCl_3$.

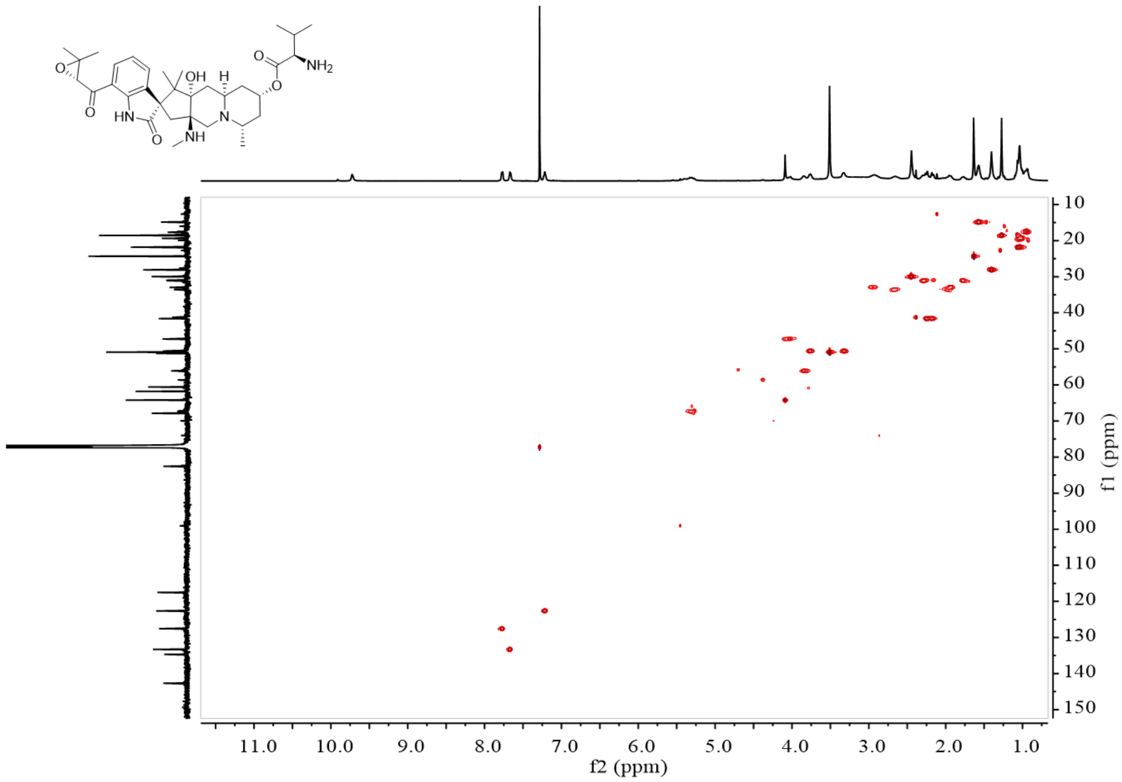

Figure S25-5. HSQC spectrum of **24** in $CDCl_3$.

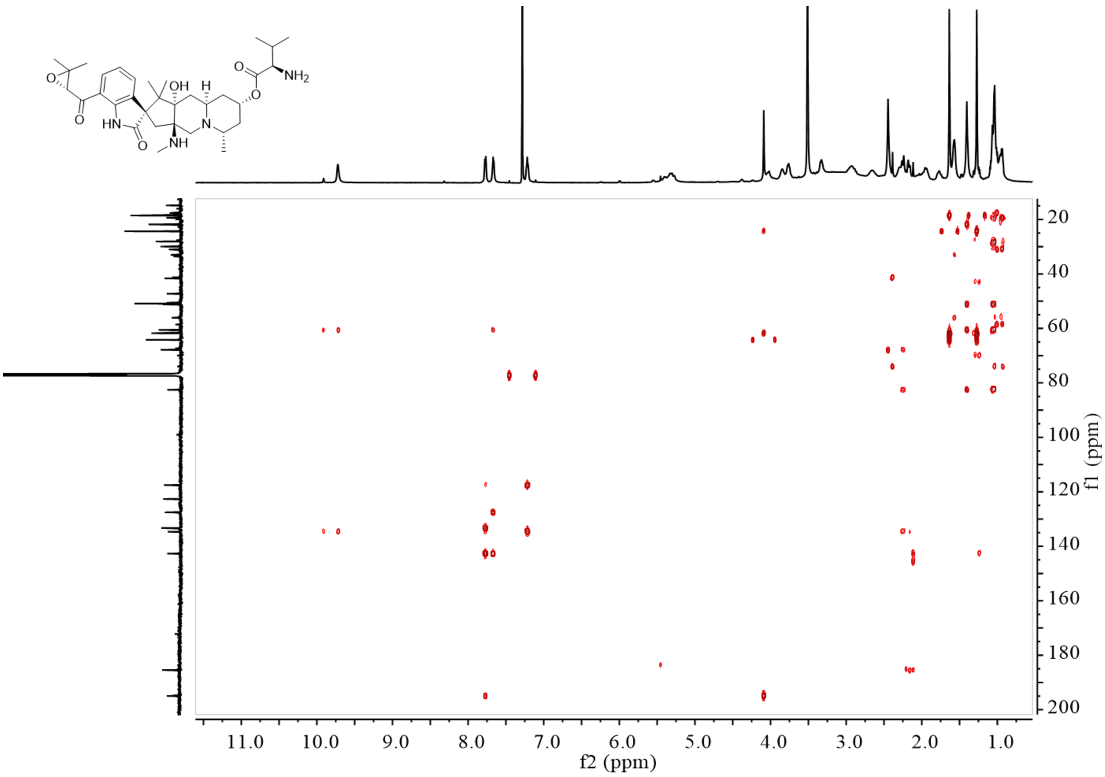

Figure S25-6. HMBC spectrum of **24** in $CDCl_3$.

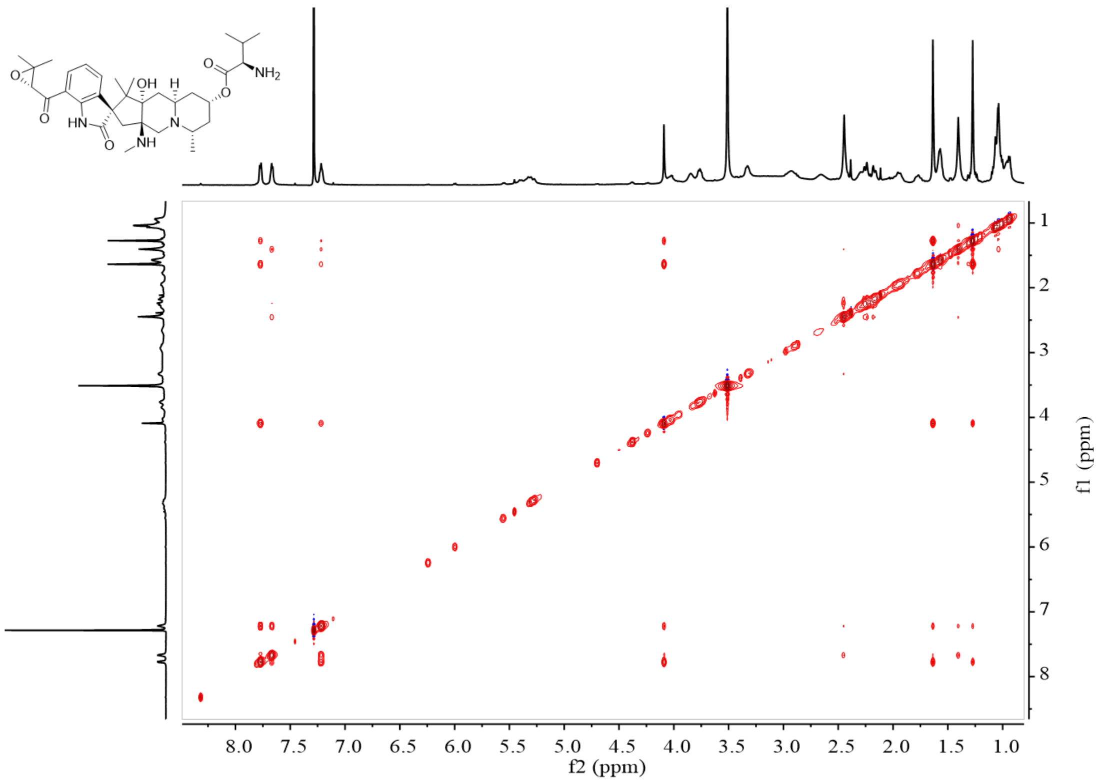

Figure S25-7. NOESY spectrum of **24** in $CDCl_3$.

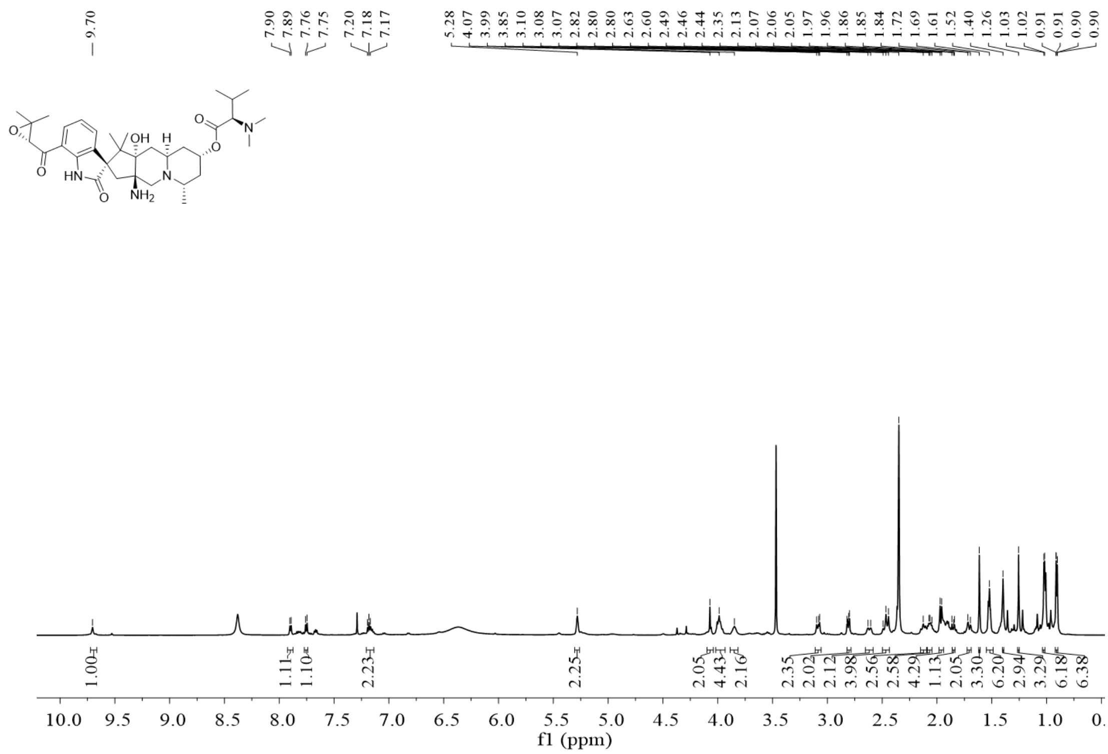

Figure S26-1. ^{1}H NMR (600 Hz) spectrum of **25** in $CDCl_3$.

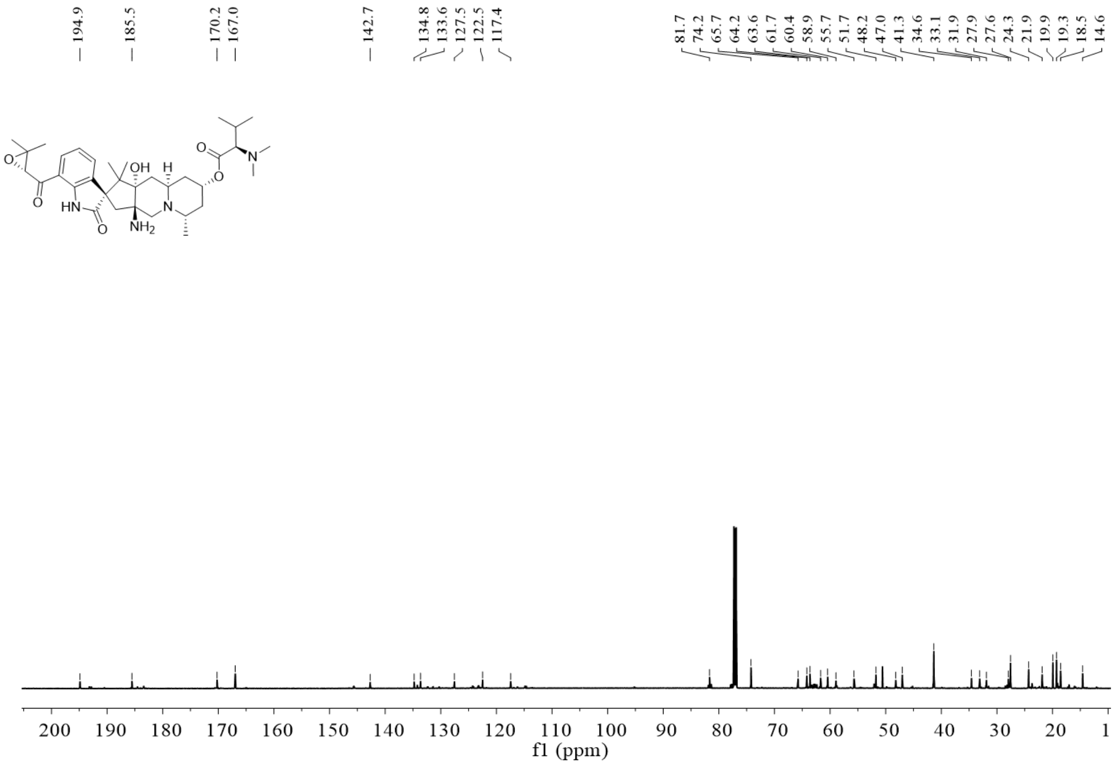

Figure S26-2. ^{13}C NMR (150 Hz) spectrum of **25** in $CDCl_3$.

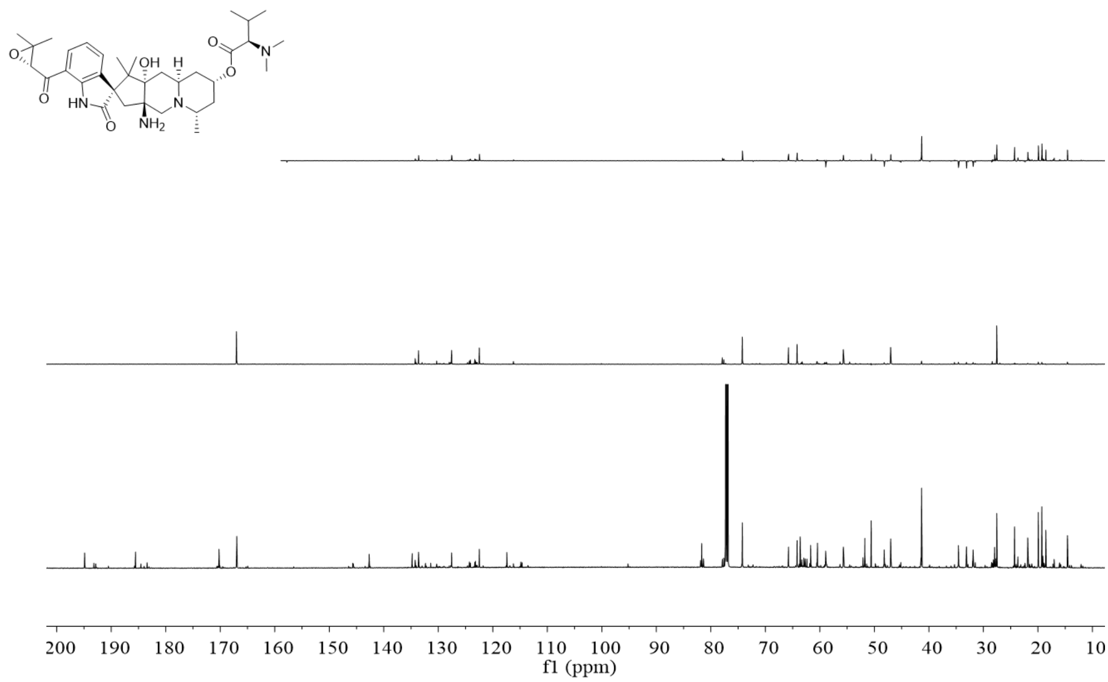

Figure S26-3. DEPT spectrum of **25** in $CDCl_3$.

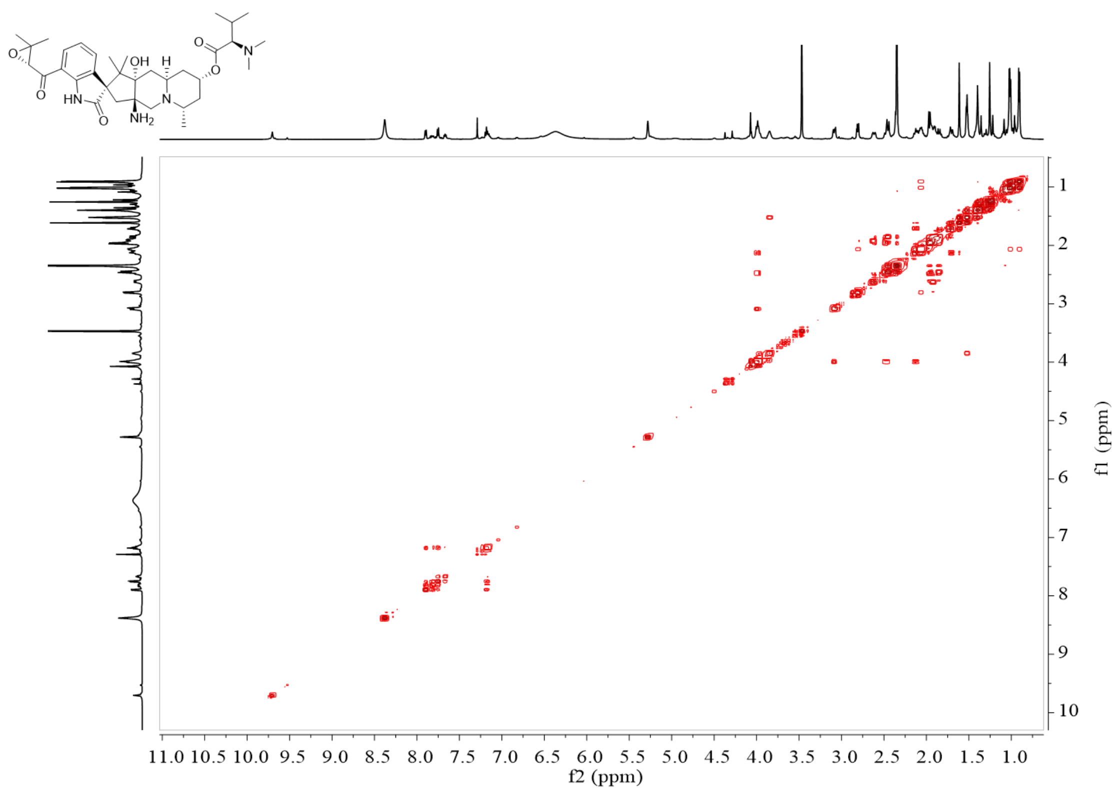

Figure S26-4. ^1H-^1H COSY spectrum of **25** in $CDCl_3$.

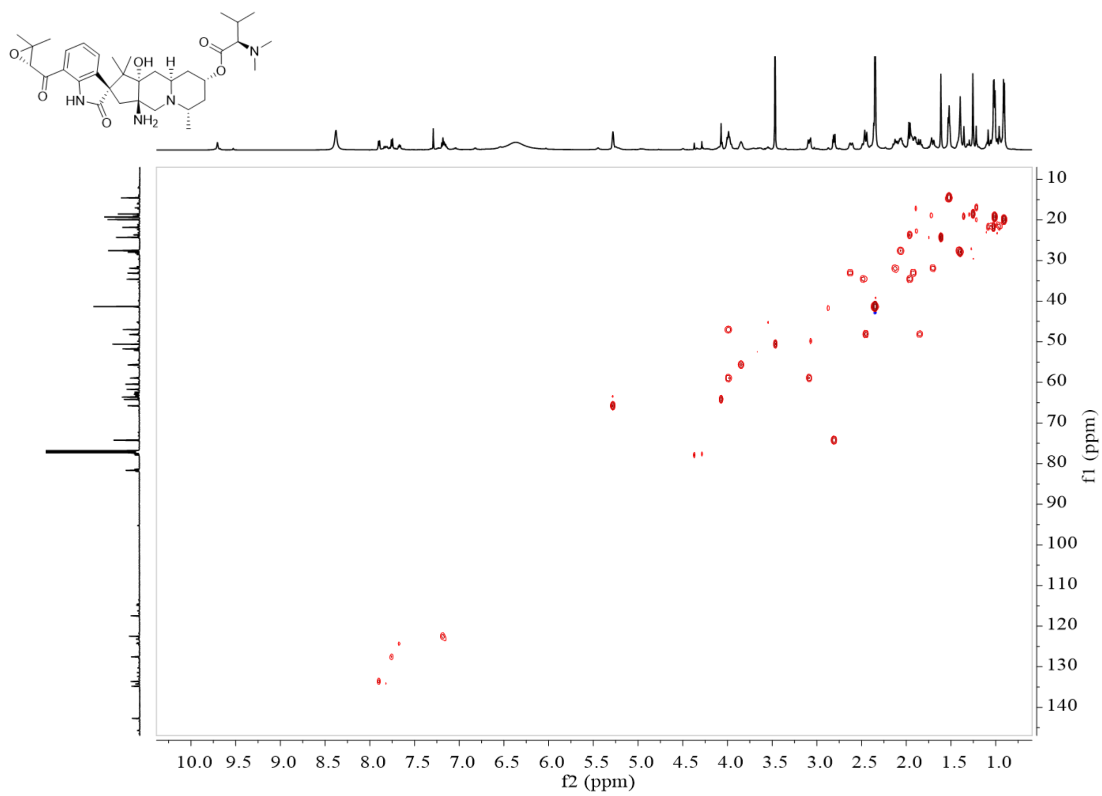

Figure S26-5. HSQC spectrum of **25** in $CDCl_3$.

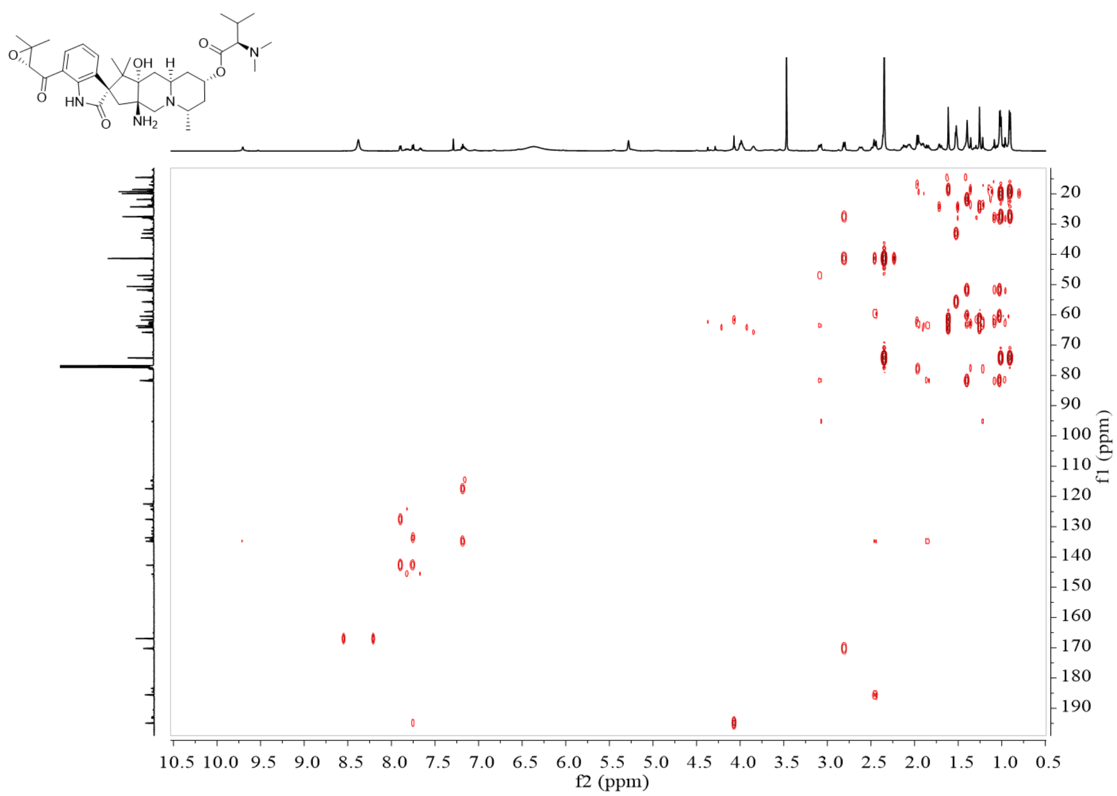

Figure S26-6. HMBC spectrum of **25** in $CDCl_3$.

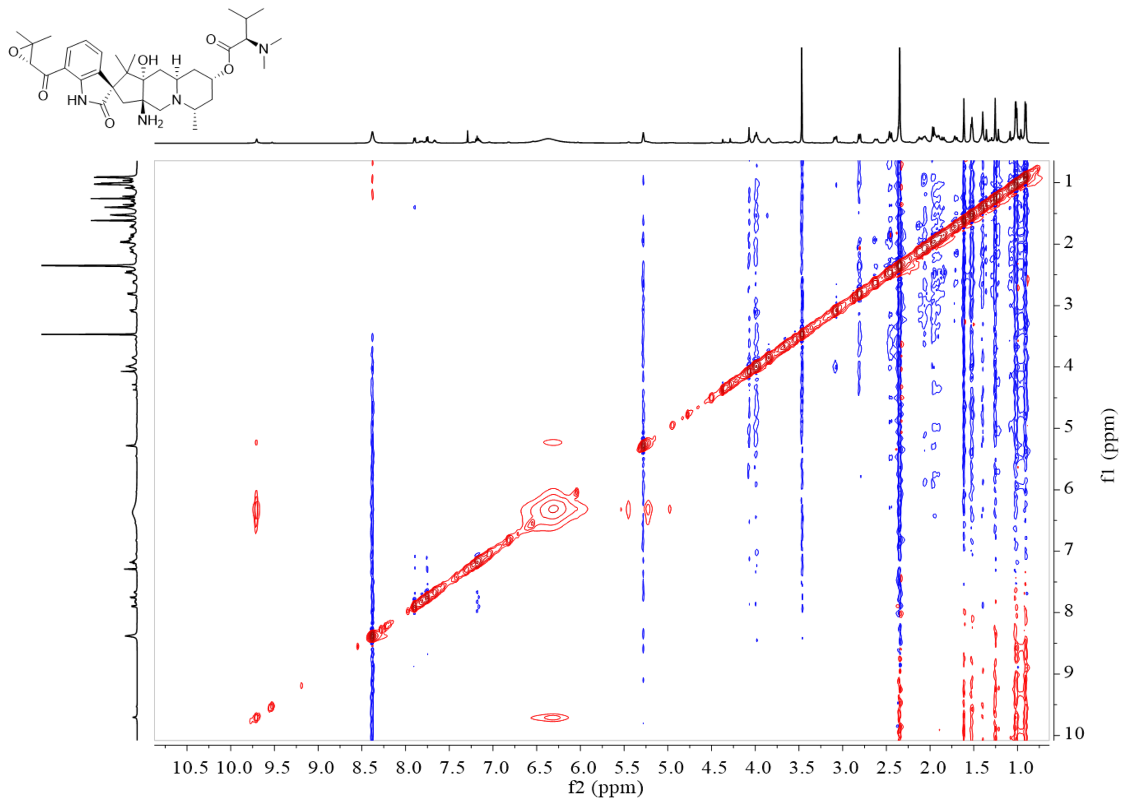

Figure S26-7. NOESY spectrum of **25** in $CDCl_3$.

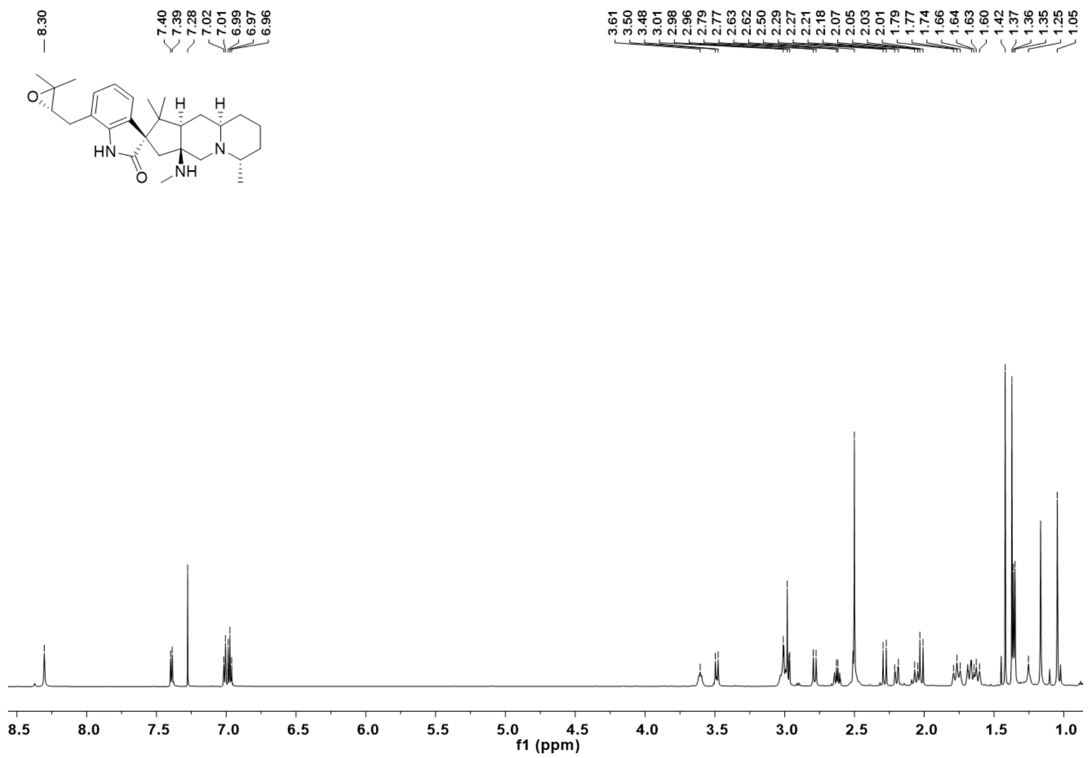

Figure S27-1. ^{1}H NMR (600 Hz) spectrum of **22R** in $CDCl_3$.

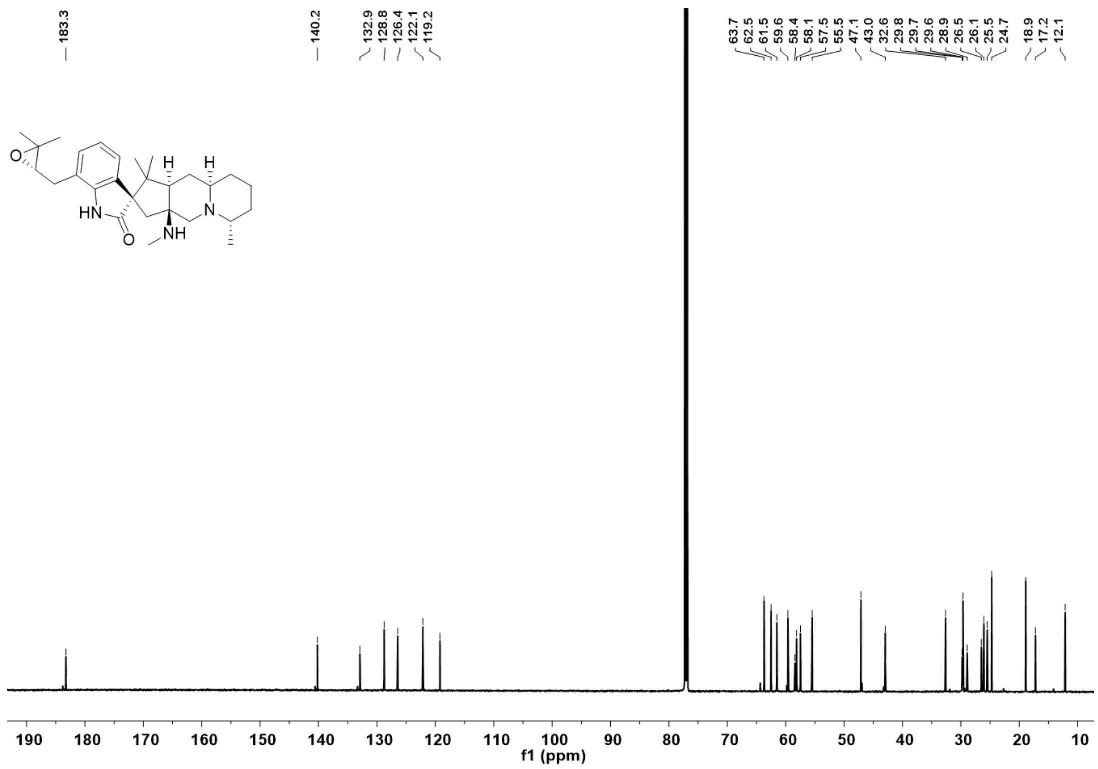

Figure S27-2. ^{13}C NMR (150 Hz) spectrum of **22R** in $CDCl_3$.

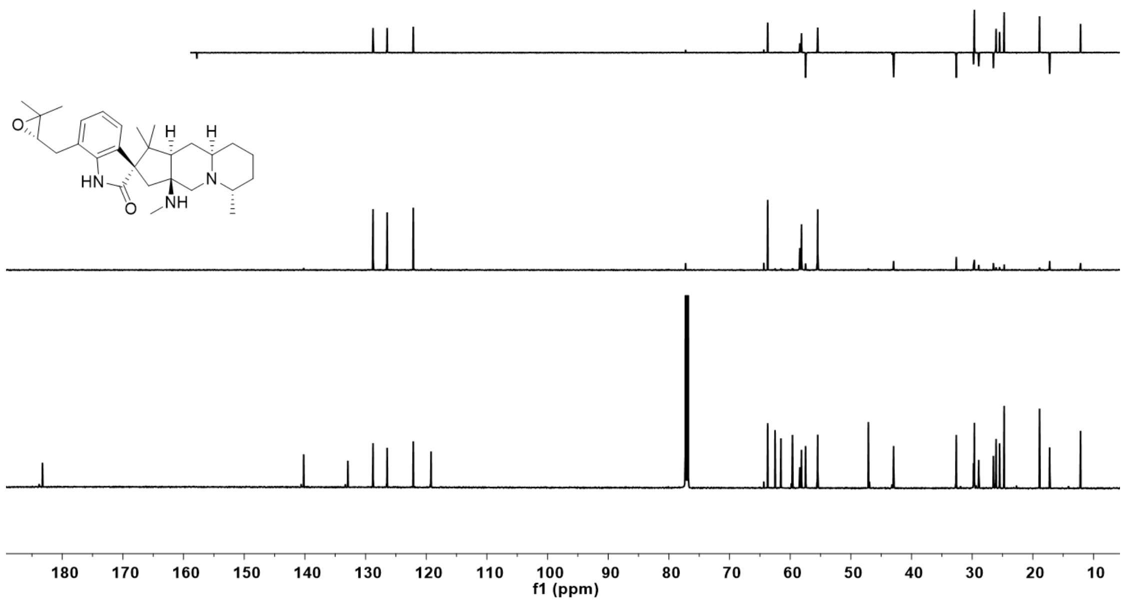

Figure S27-3. DEPT spectrum of **22R** in $CDCl_3$.

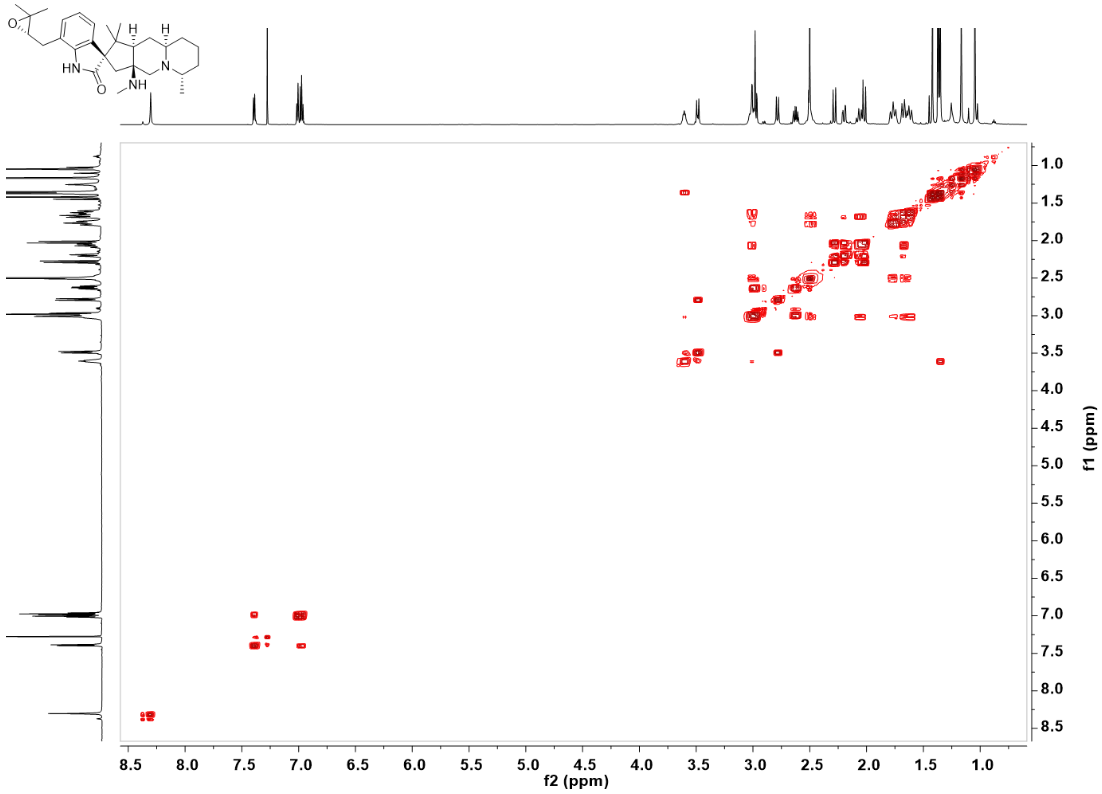

Figure S27-4. ^{1}H-^{1}H COSY spectrum of **22R** in $CDCl_3$.

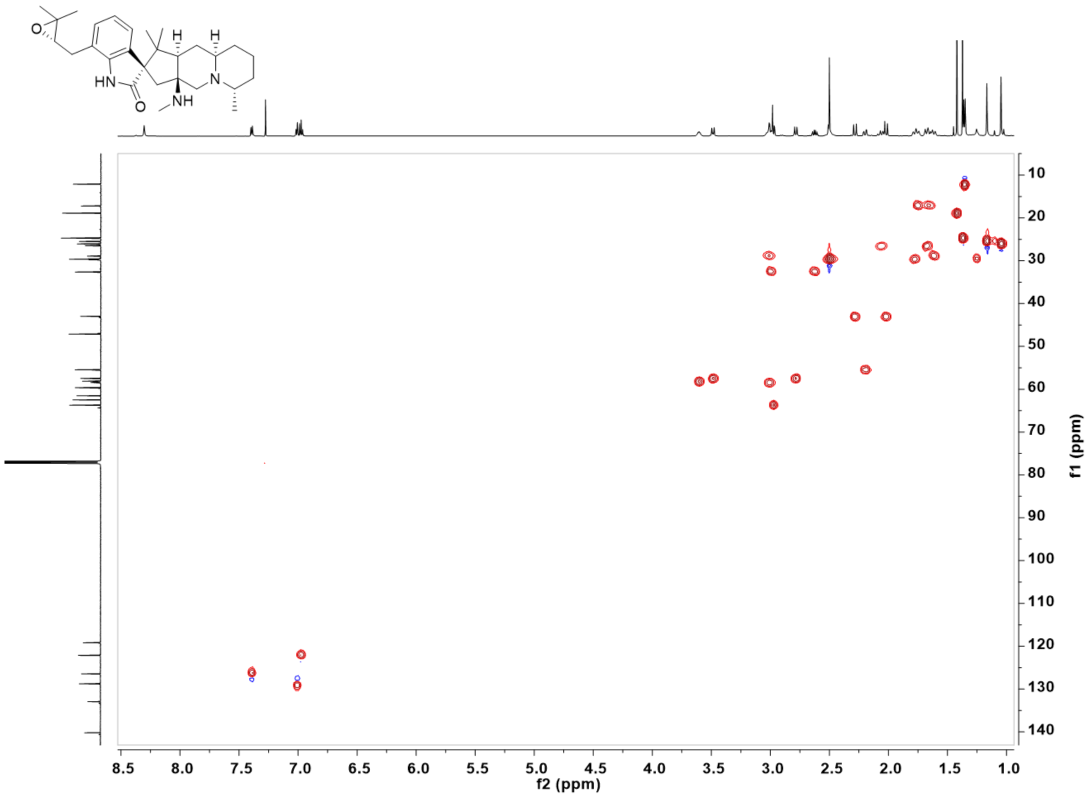

Figure S27-5. HSQC spectrum of **22R** in $CDCl_3$.

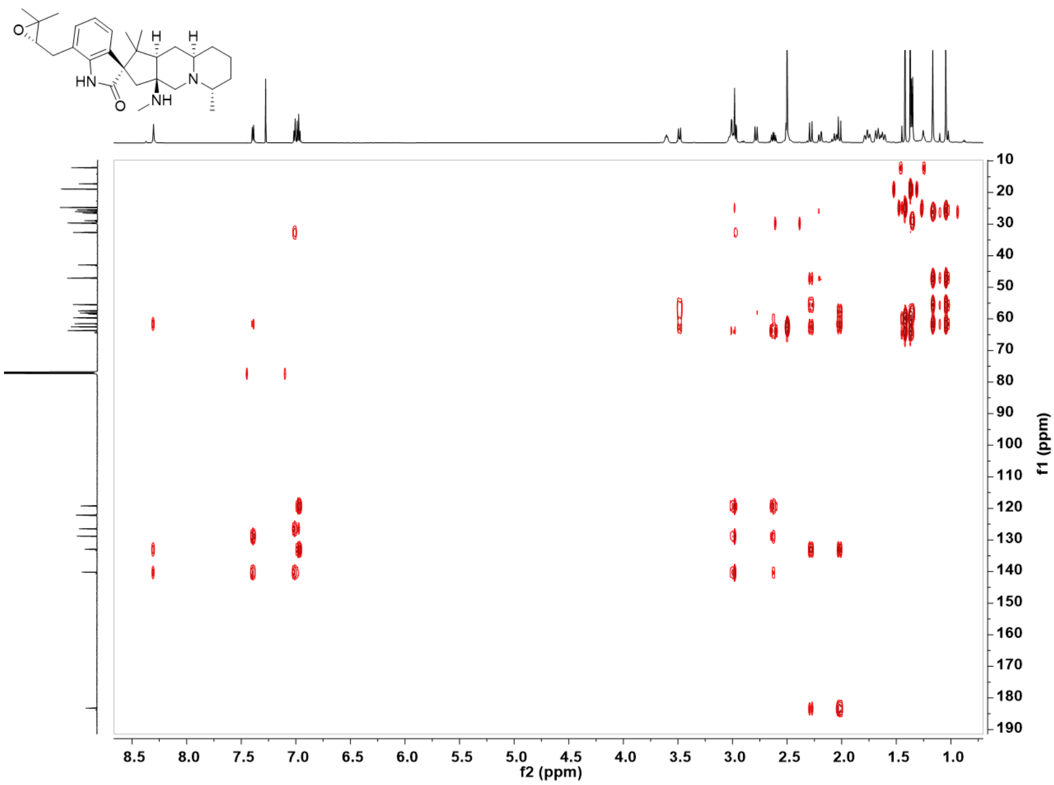

Figure S27-6. HMBC spectrum of **22R** in $CDCl_3$.

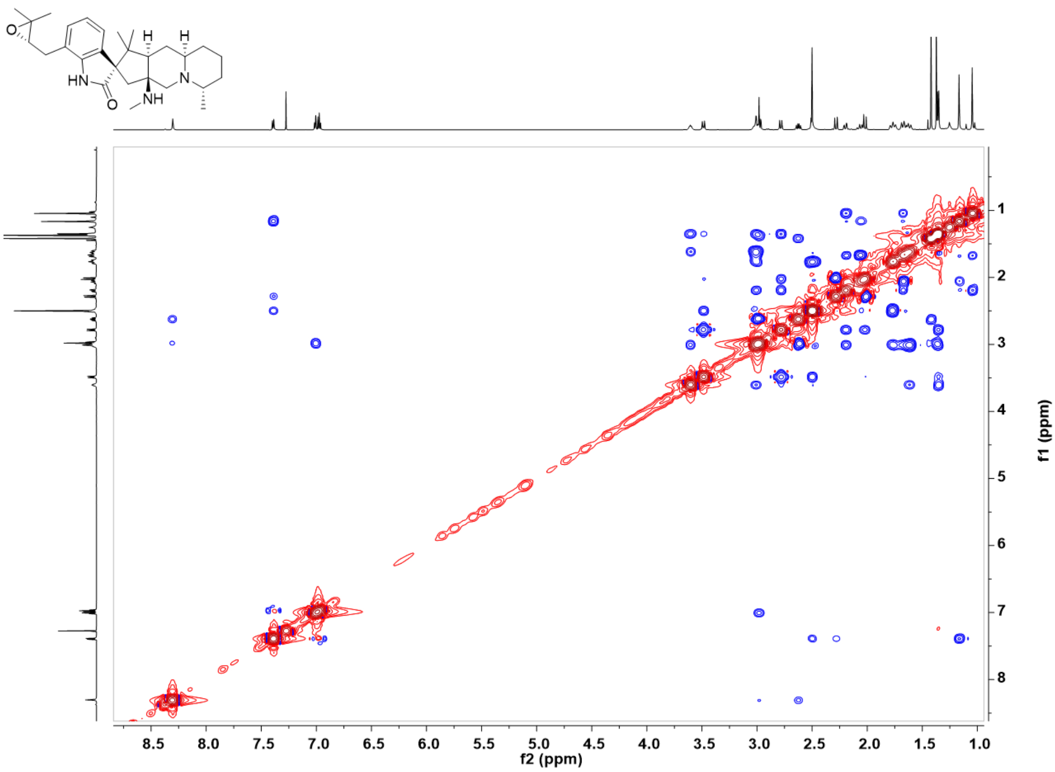

Figure S27-7. NOESY spectrum of **22R** in $CDCl_3$.

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