Supplementary Materials for

Phylogeny-directed discovery and mutagenesis of a tricyclic gersemiane synthase

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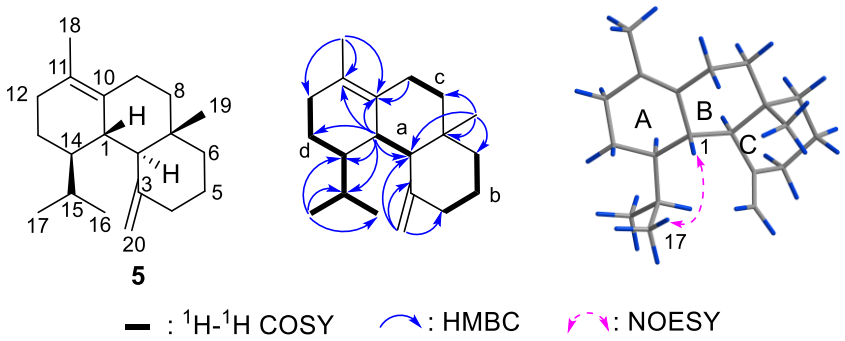
Compound structure elucidation

Compound **4** was structurally characterized as identical to the eunicellane derivative produced by the MicA^{V220A} mutant, by comparing its ^{1}H and ^{13}C NMR spectroscopic data (Figs. S4 and S5) to those reported in literature[1] and was further confirmed through 2D NMR analysis (Figs. S7-S10). For ^{1}H NMR (600 MHz) and ^{13}C NMR (150 MHz) spectral data, see Table S3.

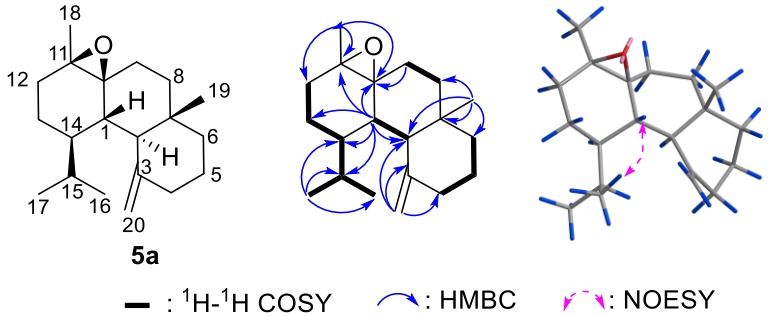


5

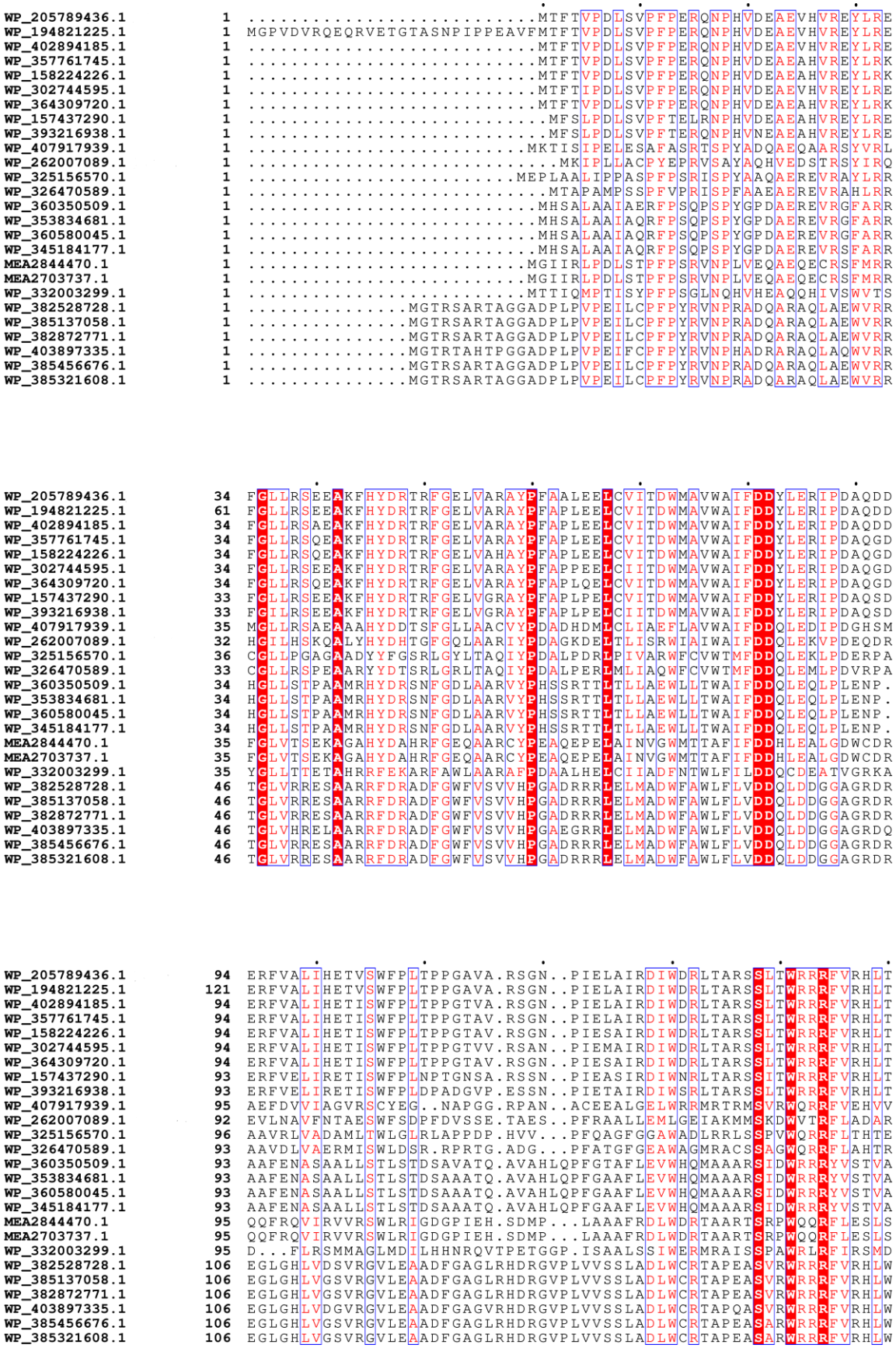
: ^{1}H-^{1}H COSY : HMBC : NOESY

Compound **5** was obtained as a colorless oil, with its molecular formula ($C_{20}H_{32}$) determined by the GC-MS ion peak at *m/z* 272.2, indicating five degrees of unsaturation. The ^{13}C NMR and DEPT^{135} spectra revealed 20 carbon signals, including four methyls, four methylenes, seven methines, and four quaternary carbons, accounting for two degrees of unsaturation and suggesting a tricyclic structure. Key ^{1}H–^{1}H COSY correlations established four structural fragments: a) C-1 to C-2, b) C-4 to C-6, c) C-8 to C-9, and d) C-12 to C-17. HMBC correlations linked these fragments, with CH_3-20 (δ_H 4.91/4.48) connecting C-2 (δ_C 57.1), C-3 (δ_C 149.2), and C-4 (δ_C 39.2) to merge fragments a and b, while CH_3-18 (δ_H 1.62, s) with C-10 (δ_C 133.5), C-11 (δ_C 123.1) and C-12 (δ_C 27.9), combined with HMBC cross-peaks of H-9a (δ_H 2.48 dt, J = 12.9, 3.5 Hz) with C-10 allowed the connection of fragments c and d through C-10. Further HMBC correlations from H-1 (δ_H 2.17) to C-2, C-10, C-11, C-13 (δ_C 21.3), C-14 (δ_C 37.5), and C-15 (δ_C 27.9) confirmed the 6,6,6-tricyclic gersemiane scaffold and planar structure. NOESY analysis revealed a *trans* A/B ring junction (CH_3-17/H-1 interaction), but the near-identical ^{1}H NMR shifts of CH_3-16 (δ_H 0.90) and CH_3-19 (δ_H 0.91) obscured absolute configuration determination. To resolve this, *m*CPBA-mediated epoxidation of **5** afforded derivative **5a**, which subsequently formed a eutectic mixture with Ag_3Pz_3. Single-crystal X-ray diffraction analysis of this complex unequivocally established the absolute configuration as 1*S*,2*R*,7*S*,10*S*,11*R*,14*R*. Compound **5** was identified as identical to both the gersemiane diterpene product formed through spontaneous conversion of MicA^{V220A} during silica gel chromatography[1] and the catalytic product generated by the non-canonical terpene synthase PeuTPS[1].

Compound **5**: colorless oil; $[\alpha]_D^{20}$ -29.6 (*c* 0.18, $CHCl_3$); For ^{1}H NMR (600 MHz) and ^{13}C NMR (150 MHz) spectral data, see Table S3.



Compound **5a** was identified as an epoxy derivative of **5**, with the epoxide moiety located at C10 and C11. This structural assignment was supported by HMBC correlations from CH_3-18 (δ_H 1.38) to C-10 (δ_C 67.8), C-11 (δ_C 61.7), and C-12 (δ_C 27.3). Further 2D NMR analysis (Figs. S22–S25) not only corroborated this hypothesis but also confirmed the planar structure of **5a** as a 10,11-epoxy derivative of **5**. The absolute configuration of **5a** was unambiguously determined by X-ray crystallography (Fig. S2) using Cu Kα radiation.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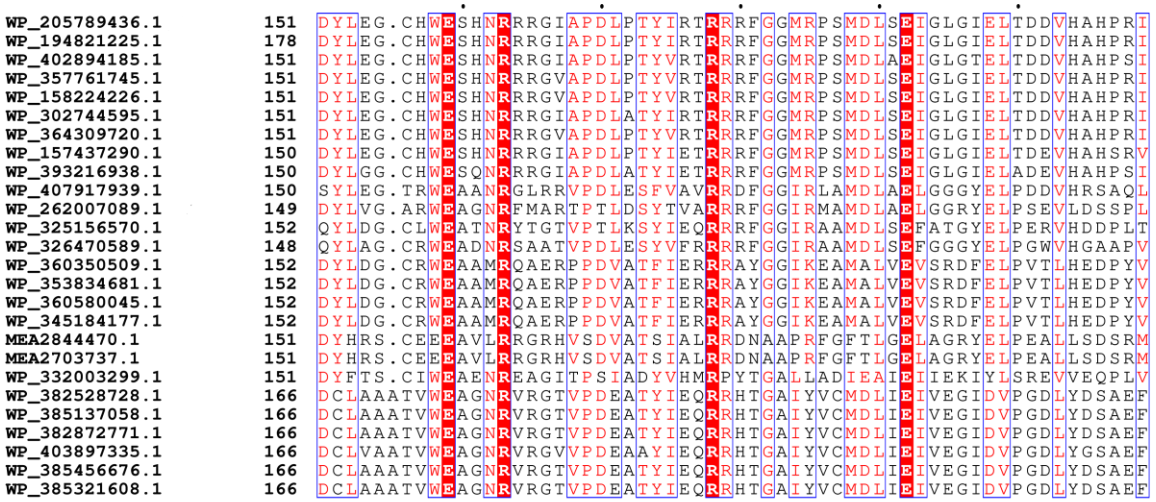
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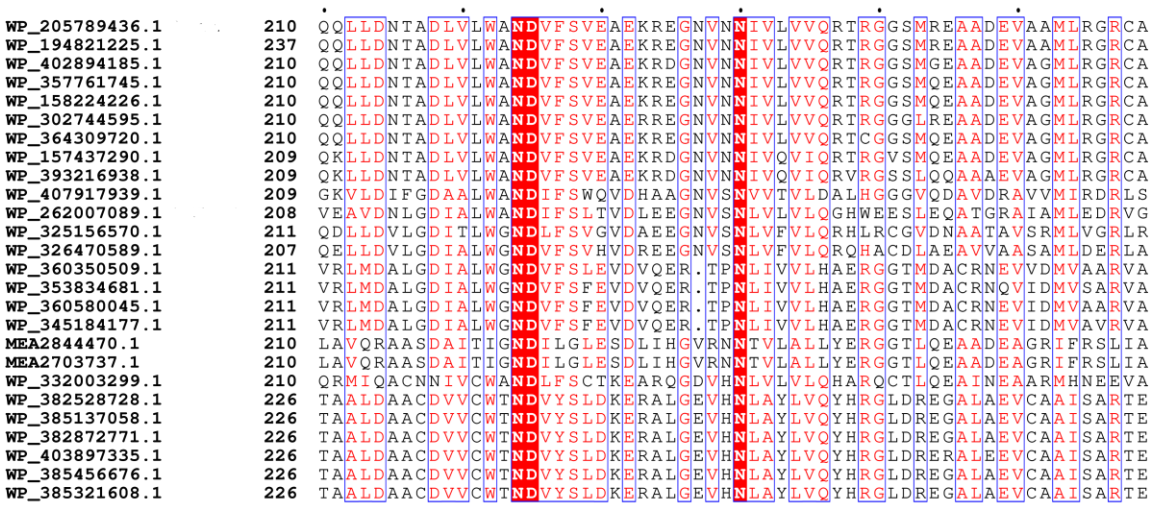
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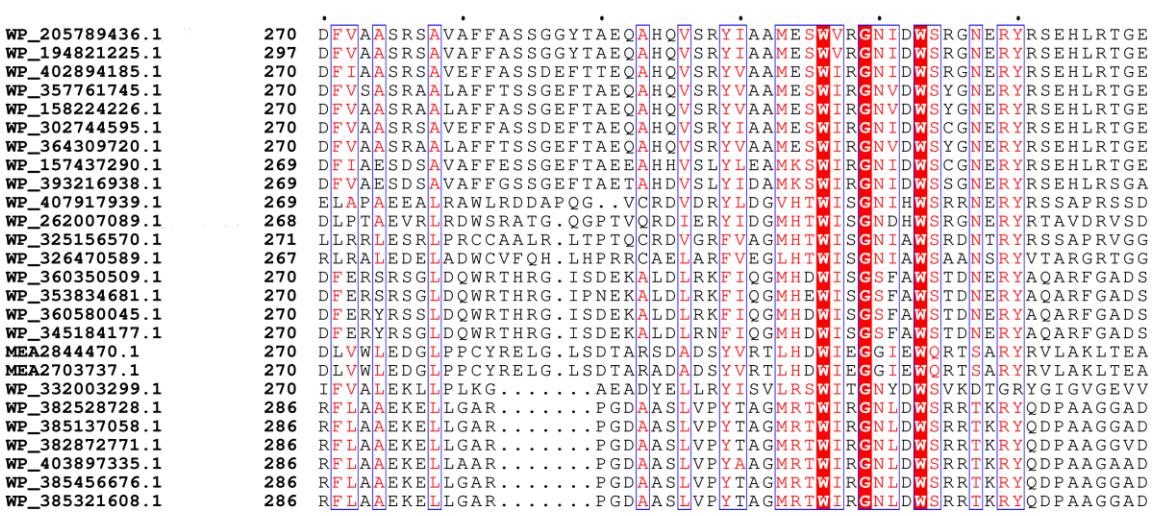
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MEA2844470.1 270 DLVWLEDGLPPCYRELG.LSDTARSDADSYVRTLHDWIEGGIEWQRTSARYRVLAKLTEA
MEA2703737.1 270 DLVWLEDGLPPCYRELG.LSDTARADADSYVRTLHDWIEGGIEWQRTSARYRVLAKLTEA
WP_332003299.1 270 IFVALEKLLPLKG.....AEADYELLRYISVLRSWITGNYDWSVKDTGRYGIGVGEVV
WP_382528728.1 286 RFLAAEKELLGAR.....PGDAASLVPYTAGMRTWIRGNLDWSRRTKRYQDPAAGGAD
WP_385137058.1 286 RFLAAEKELLGAR.....PGDAASLVPYTAGMRTWIRGNLDWSRRTKRYQDPAAGGAD
WP_382872771.1 286 RFLAAEKELLGAR.....PGDAASLVPYTAGMRTWIRGNLDWSRRTKRYQDPAAGGVD
WP_403897335.1 286 RFLAAEKELLAAR.....PGDAASLVPYAAGMRTWIRGNLDWSRRTKRYQDPAAGAAD
WP_385456676.1 286 RFLAAEKELLGAR.....PGDAASLVPYTAGMRTWIRGNLDWSRRTKRYQDPAAGGAD
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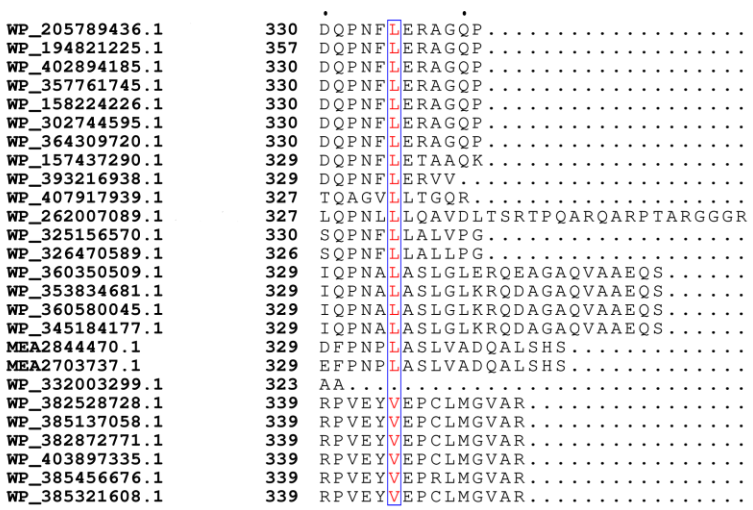


Fig. S1. Sequence alignment of MicA homologs sharing >35% identity.

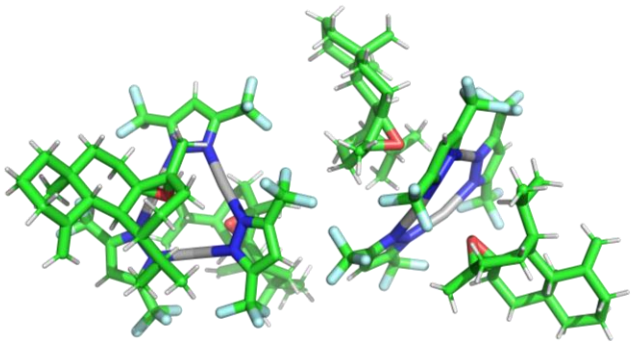


Fig. S2. Crystal structures of Ag_3Pz_3 and compound 5a highlighting the single-site binding mode.

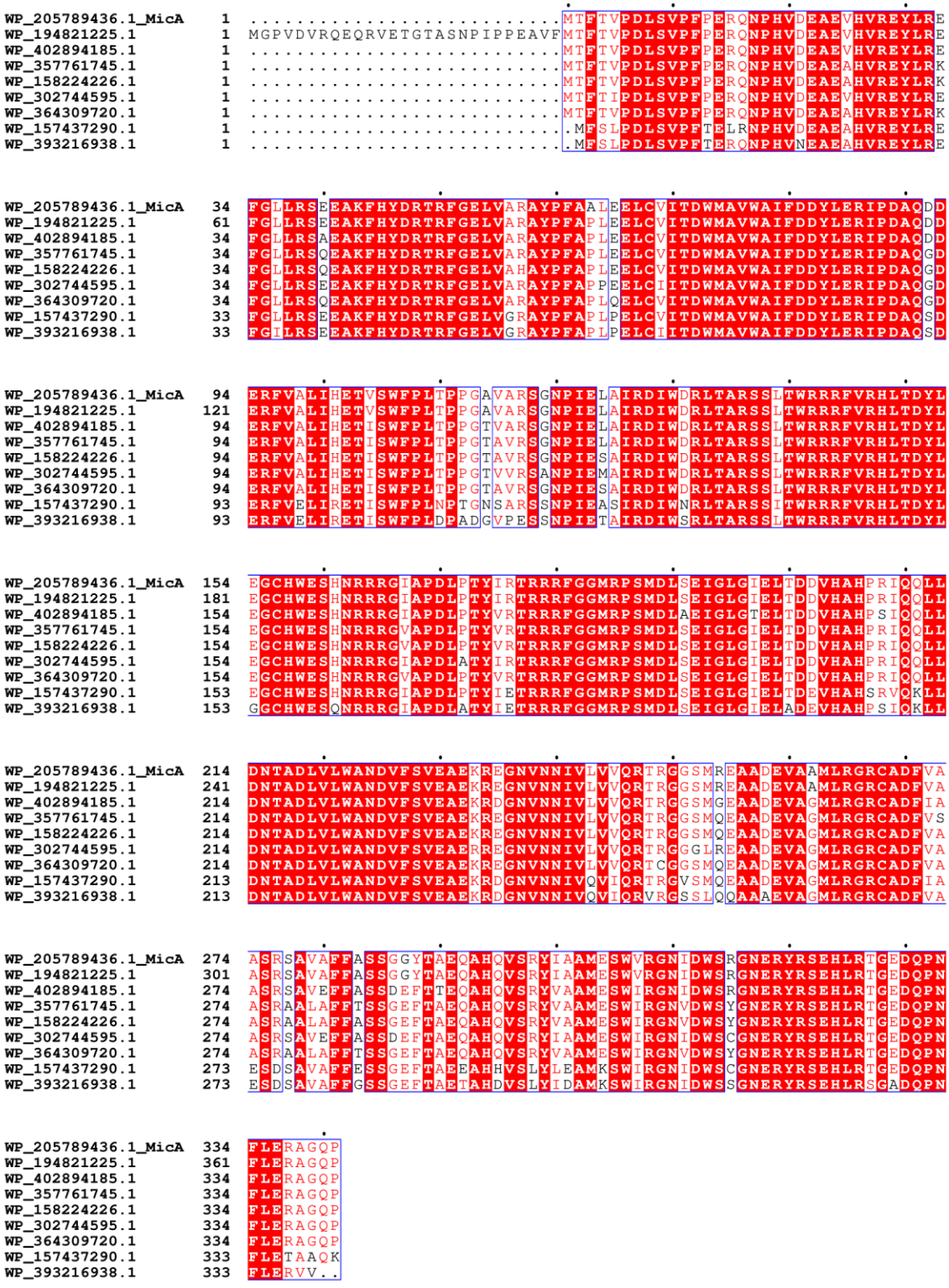


Fig. S3. Sequence alignment of TSs sharing >85% identities with MicA.

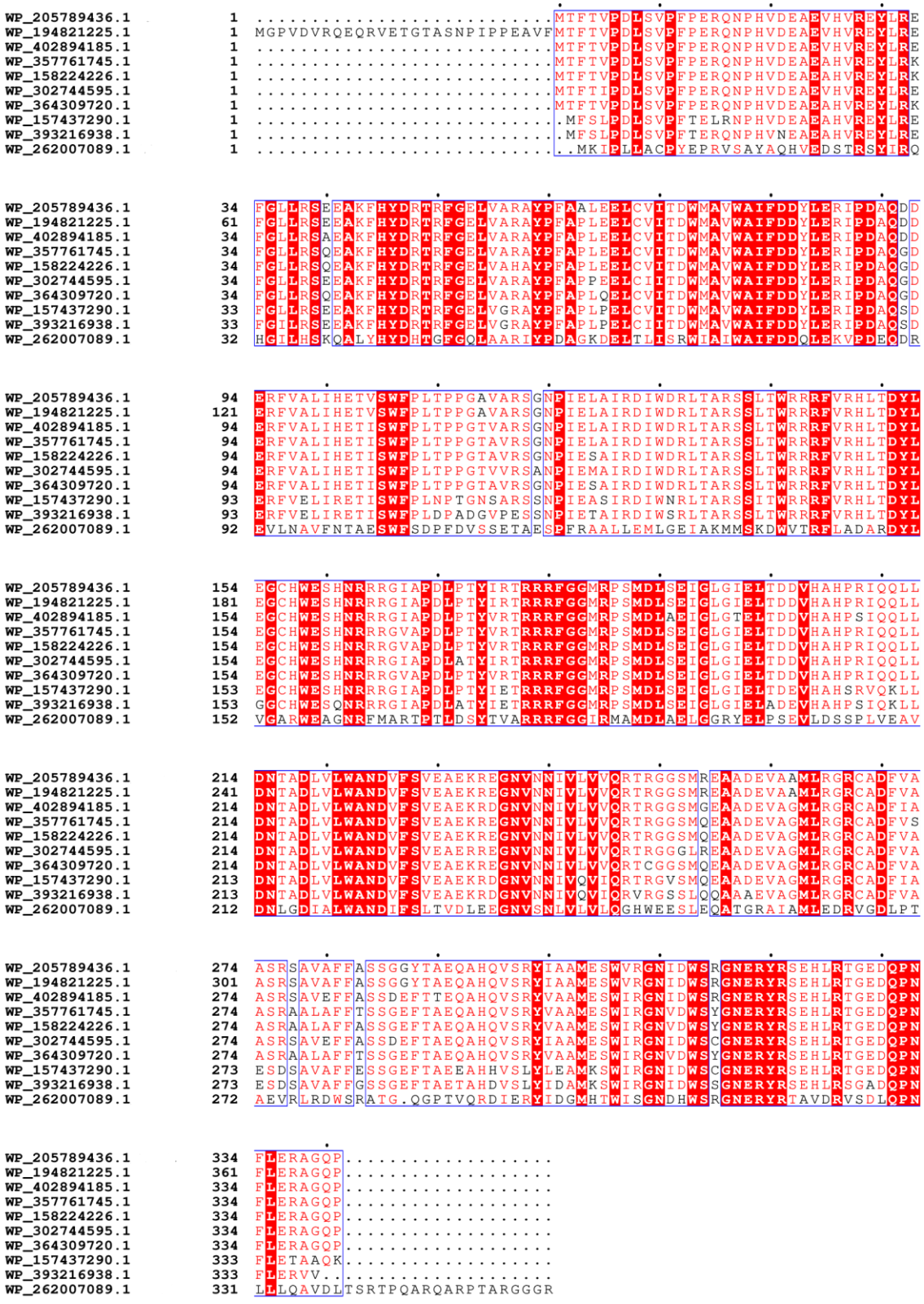


Fig. S4. Sequence alignment of SsFitTS and TSs sharing >85% identity with MicA.

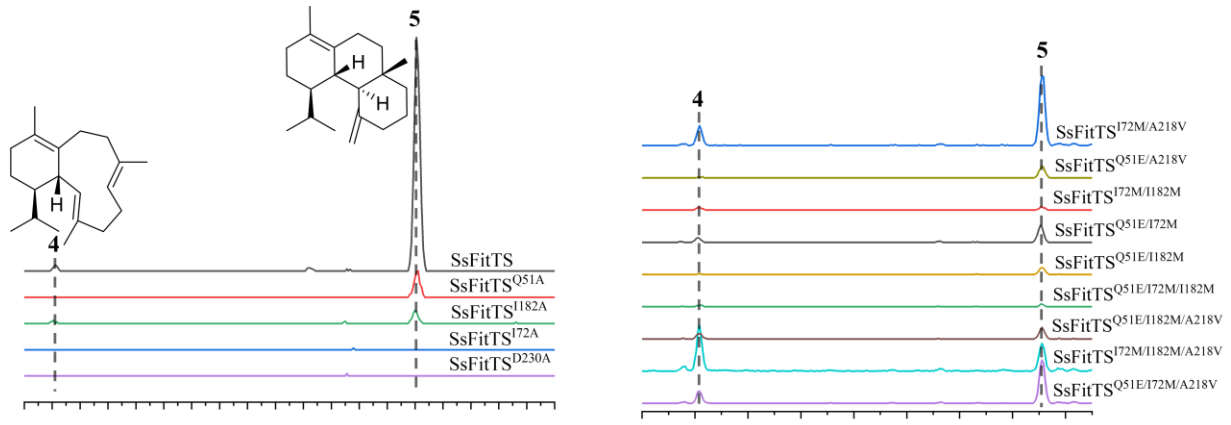


Fig. S5. GC-MS chromatography (EIC, *m/z* 272) of SsFitTS mutants' activities.

Alanine scanning mutagenesis of the selected key amino acid residues revealed that the SsFitTS^{I72A} and SsFitTS^{D230A} mutants lost their ability to synthesize compound **5**, while the SsFitTS^{Q51A} and SsFitTS^{I182A} mutants exhibited severely impaired enzymatic activity. Double and triple mutants of residues (Q51E, I72M, I182M, and A218V) associated with enhanced production of the bicyclic eunicellane compound **4** were also constructed and compared to the wild-type SsFitTS. Although none of the mutants exclusively produced compound **4**, the triple mutant SsFitTS$^{I72M/I182M/A218V}$ showed a higher yield of compound **4** relative to the tricyclic gersemiane compound **5**. These results further indicate that these residues play a critical role in the re-protonation process catalyzed by SsFitTS.

Original spectra for compound 4.

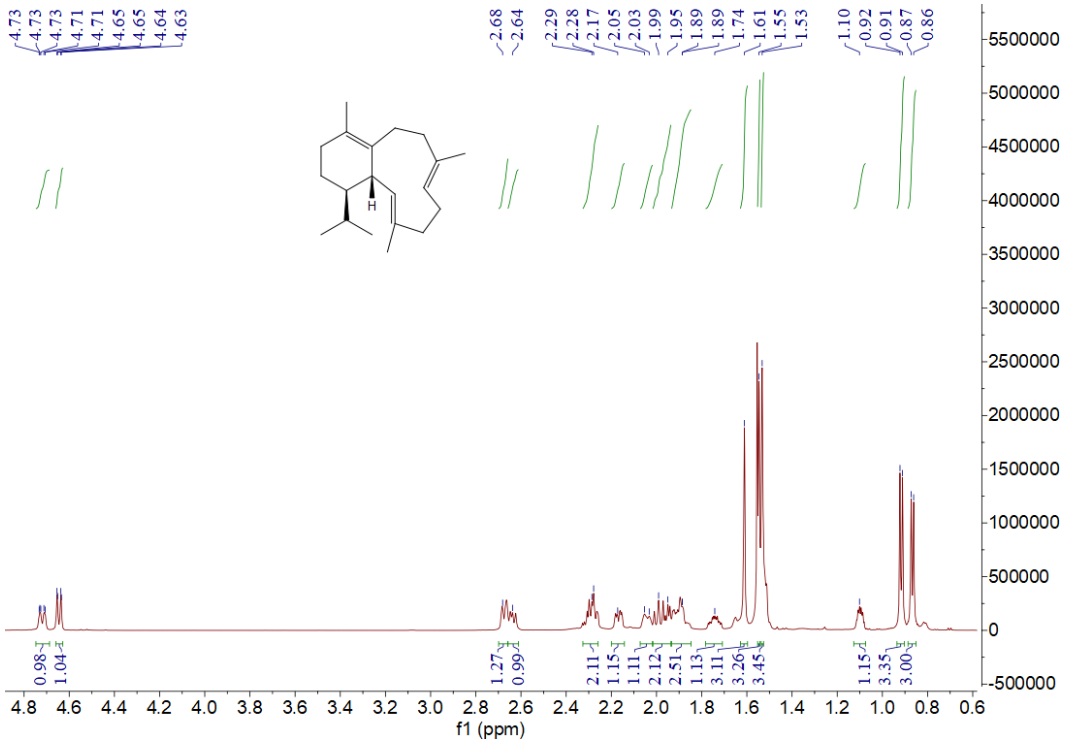


Fig. S6. ^{1}H NMR spectrum (600 MHz) of compound **4** in $CDCl_3$.

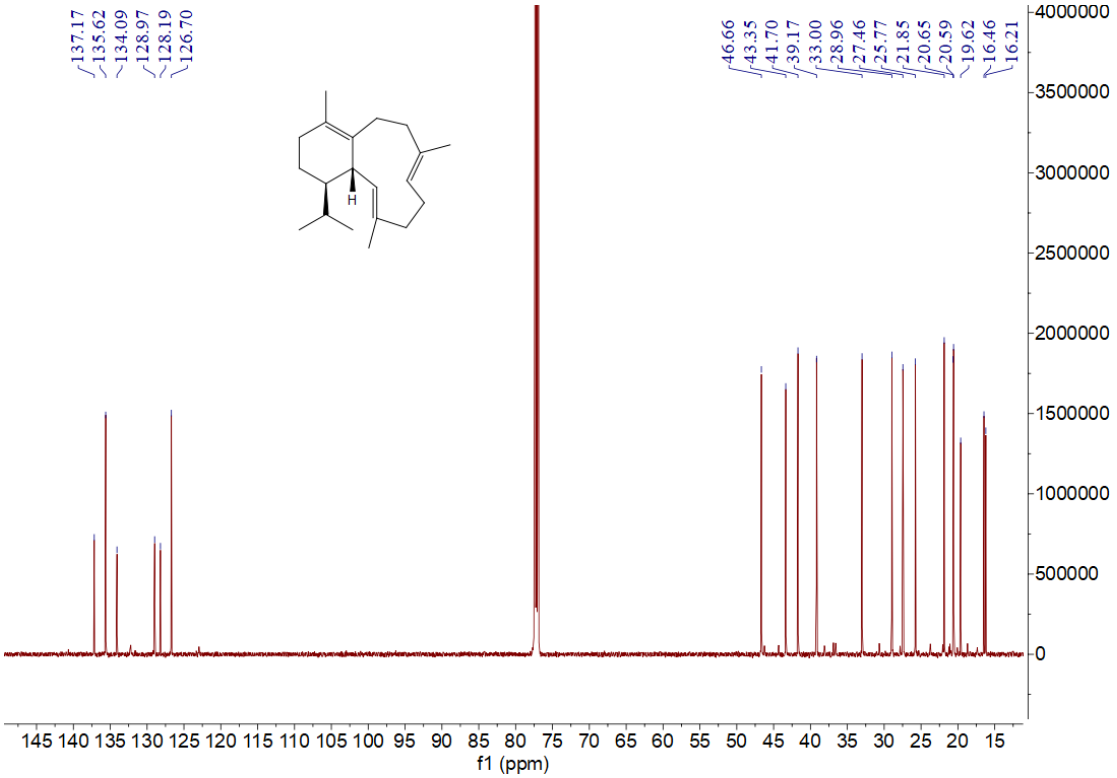


Fig. S7. ^{13}C NMR spectrum (150 MHz) of compound **4** in $CDCl_3$.

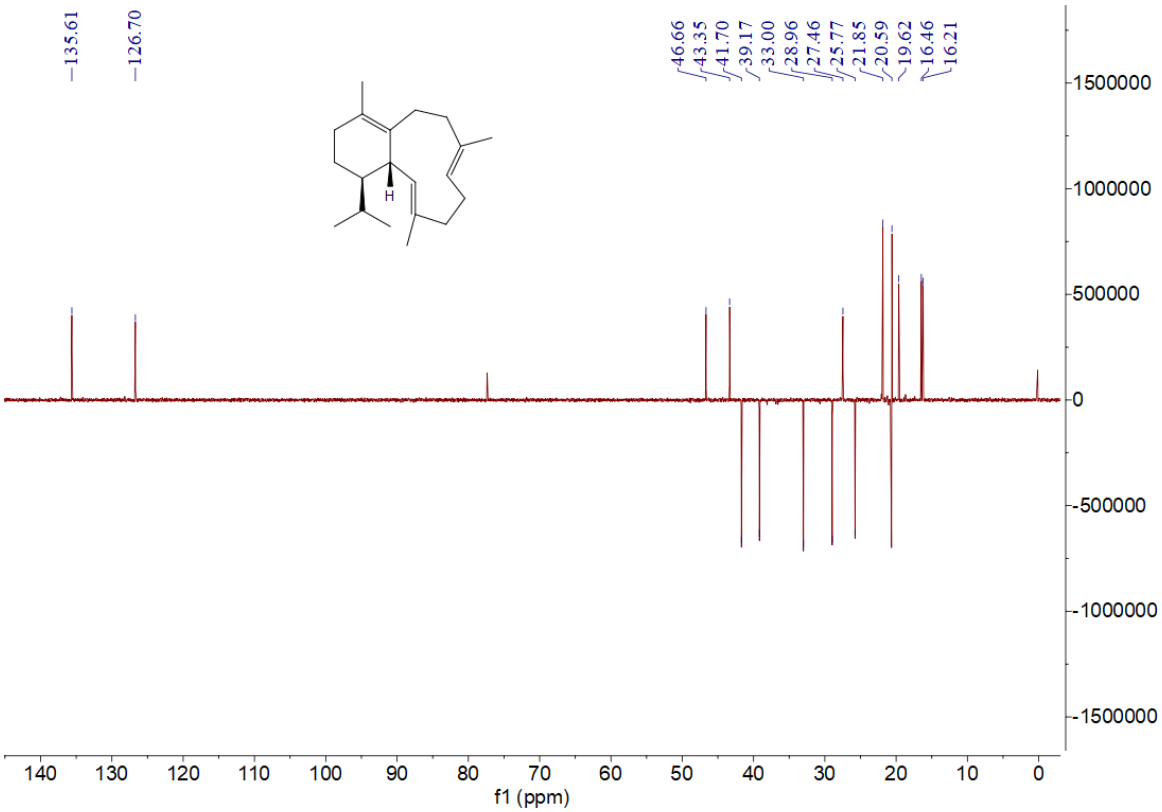


Fig. S8. DEPT^{135} spectrum (150 MHz) of compound **4** in $CDCl_3$.

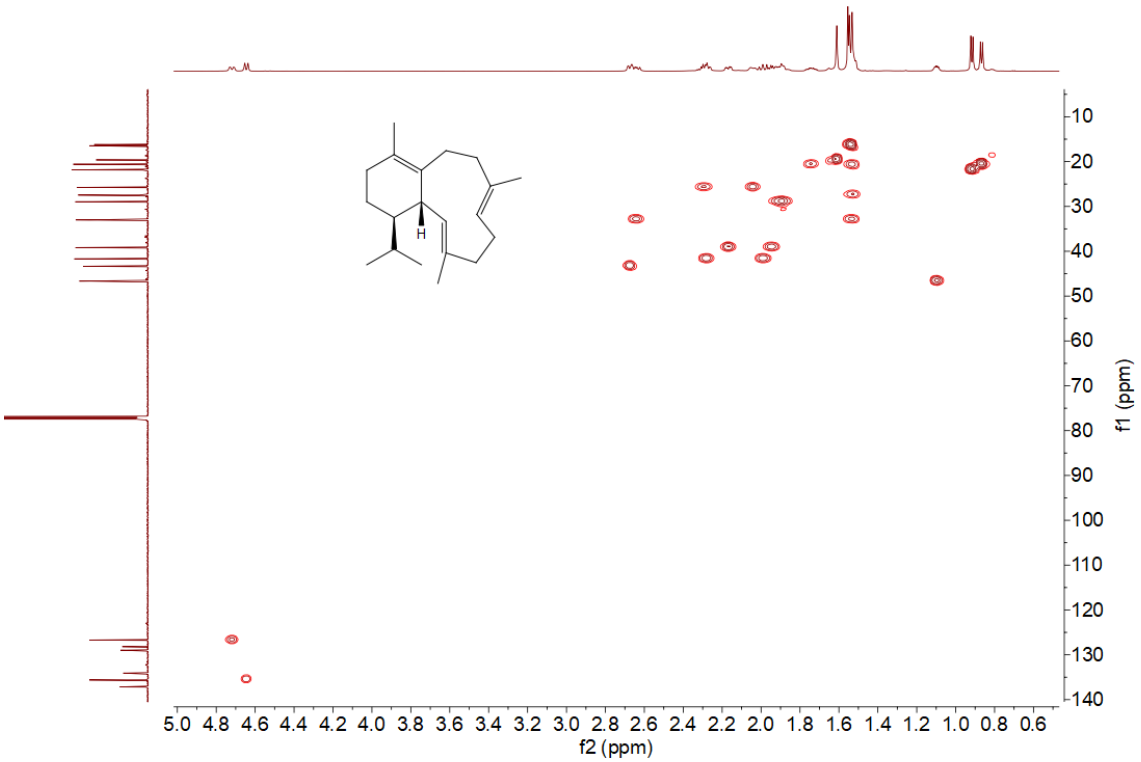


Fig. S9. HSQC NMR spectrum of compound **4** in $CDCl_3$.

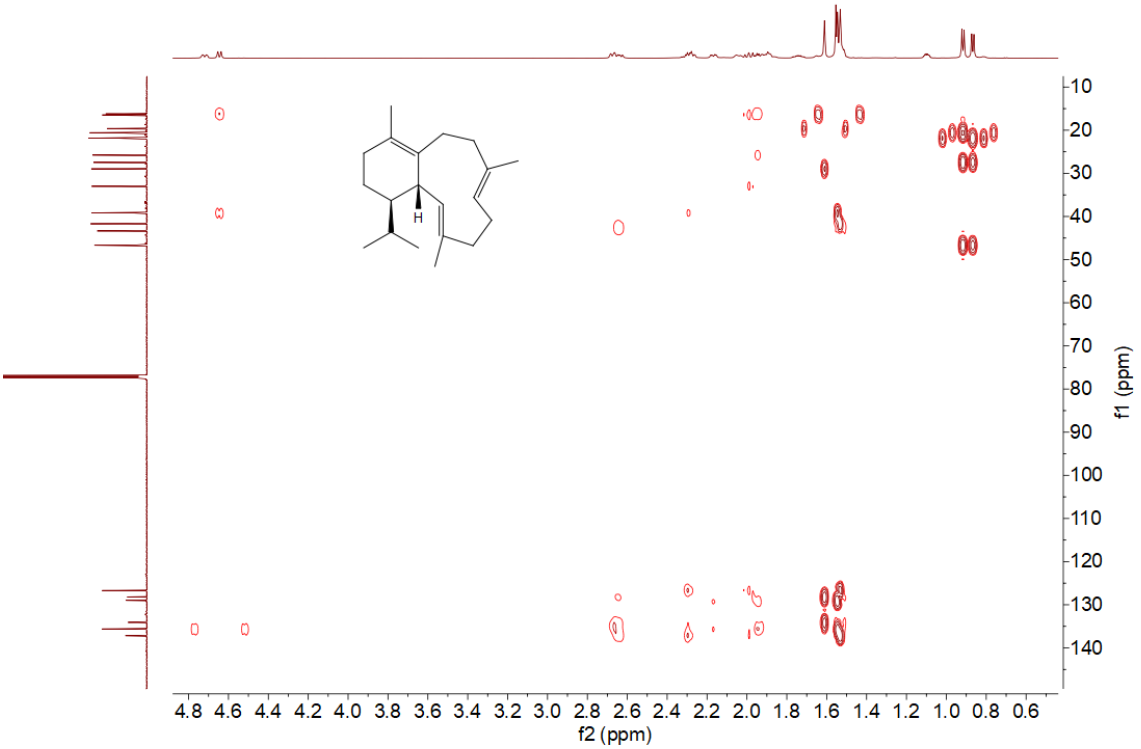


Fig. S10. HMBC NMR spectrum of compound **4** in $CDCl_3$.

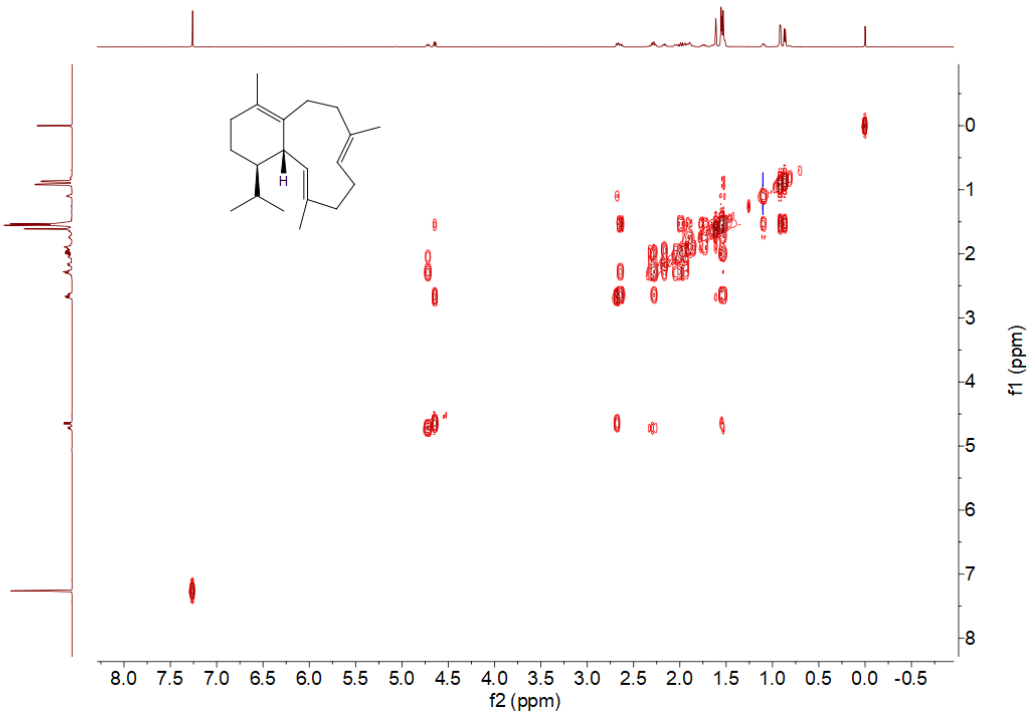


Fig. S11. ^{1}H-^{1}H COSY NMR spectrum of compound **4** in $CDCl_3$.

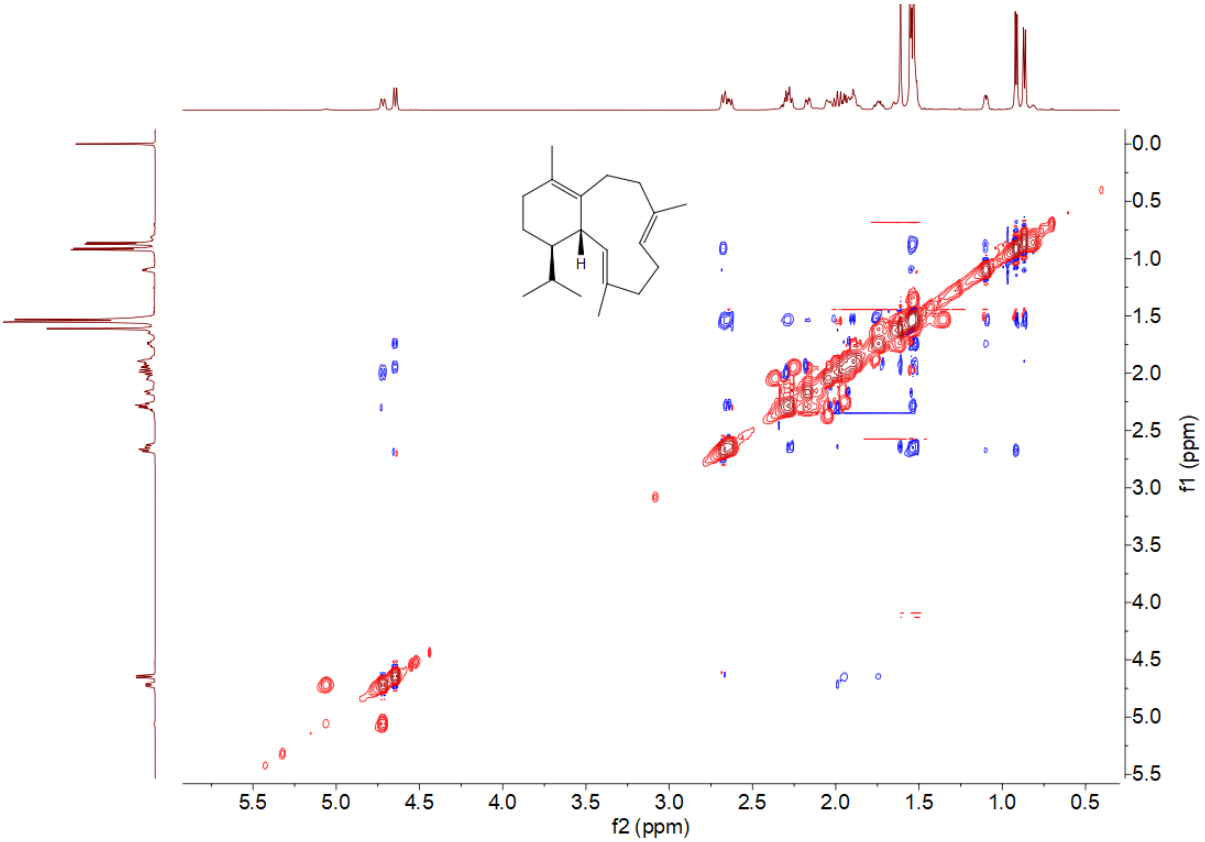


Fig. S12. NOESY NMR spectrum of compound **4** in $CDCl_3$.

Original spectra for compound 5.

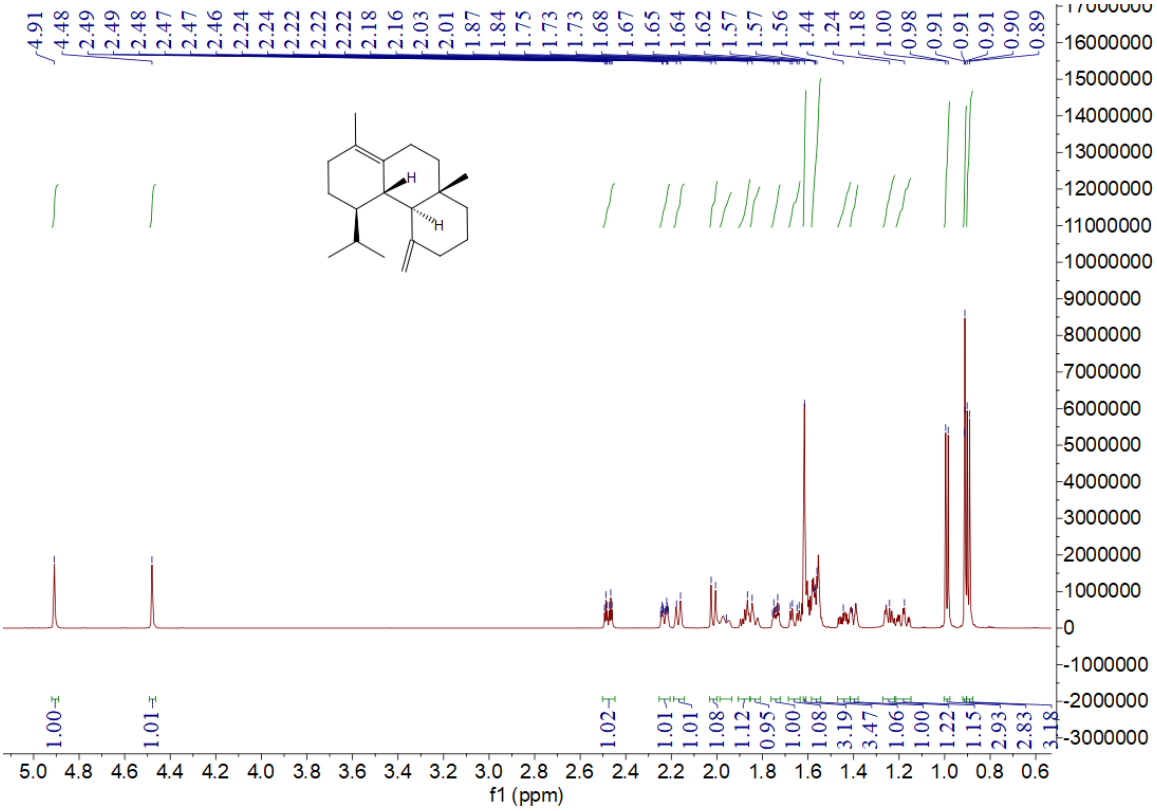


Fig. S13. ^{1}H NMR spectrum (600 MHz) of compound **5** in $CDCl_3$.

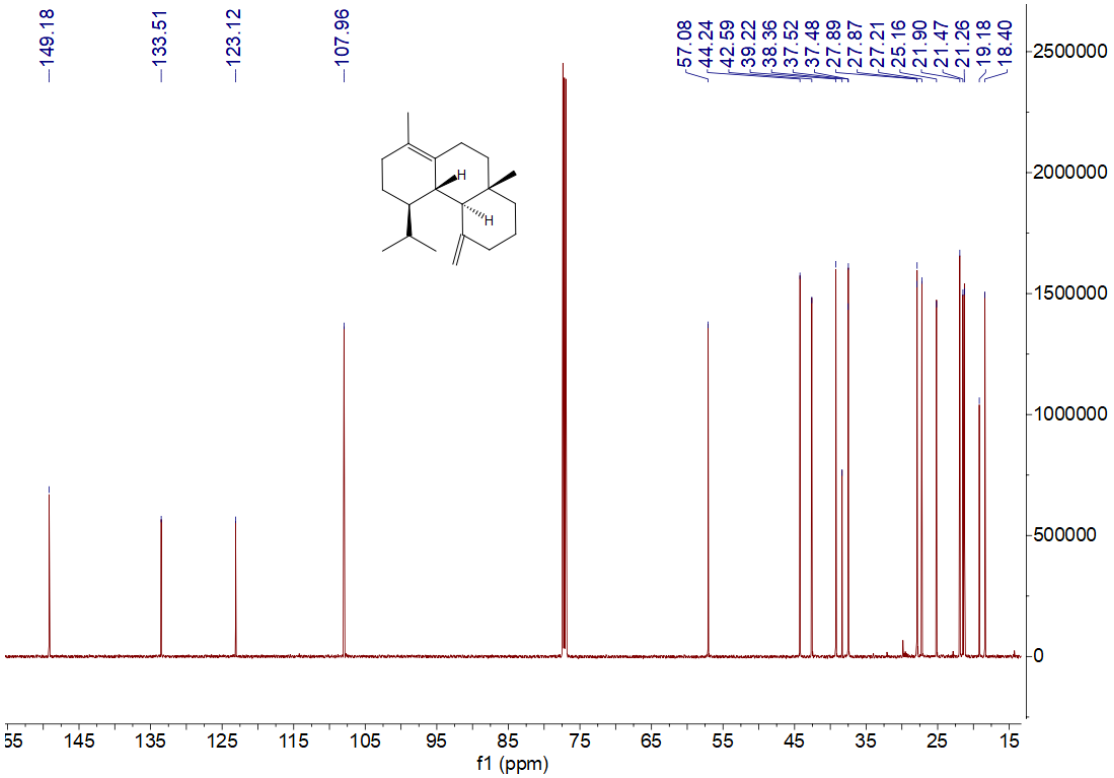


Fig. S14. ^{13}C NMR spectrum (150 MHz) of compound **5** in $CDCl_3$.

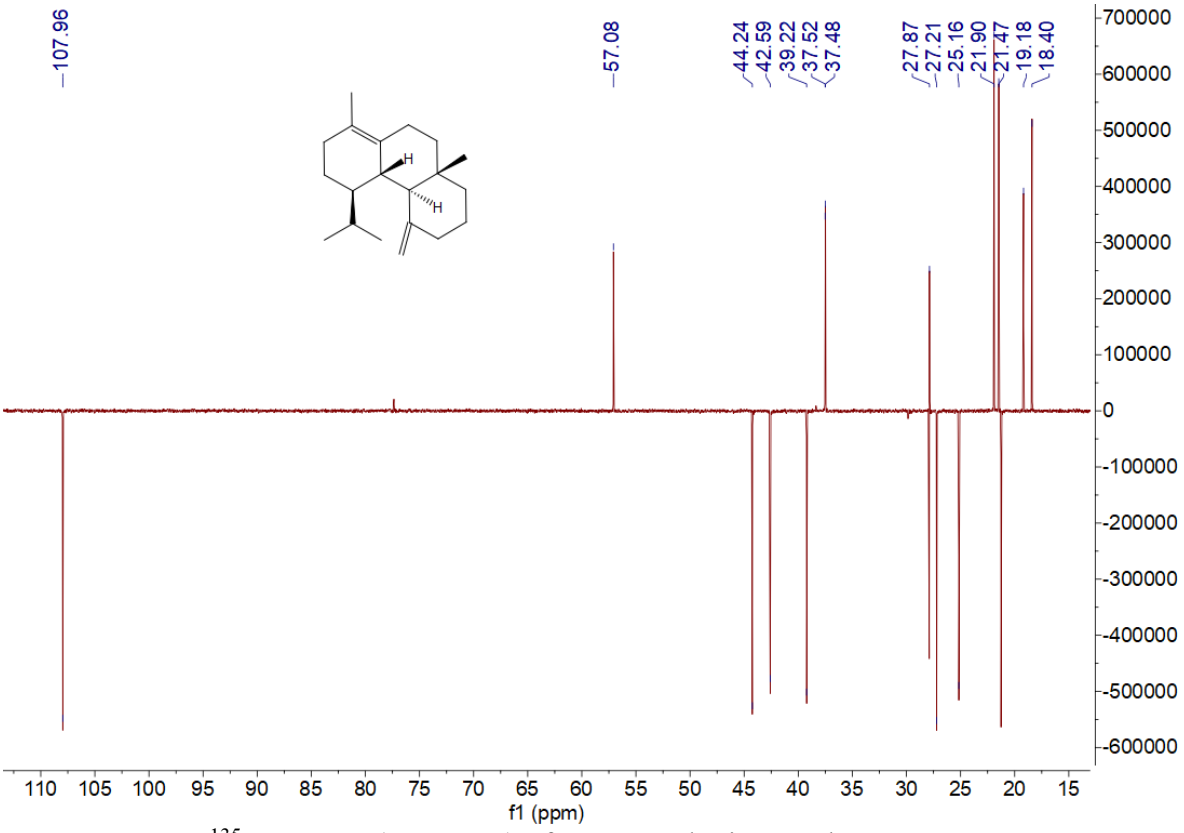


Fig. S15. DEPT^{135} spectrum (150 MHz) of compound **5** in $CDCl_3$.

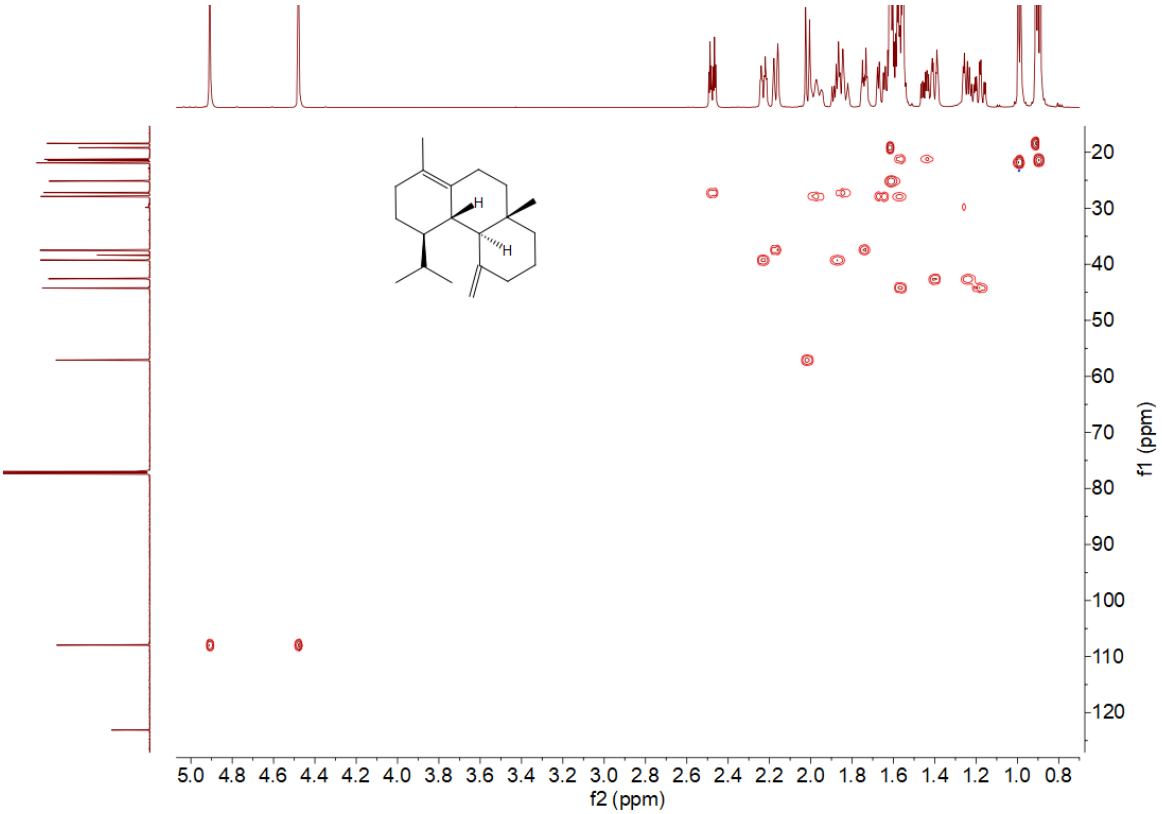


Fig. S16. HSQC NMR spectrum of compound **5** in $CDCl_3$.

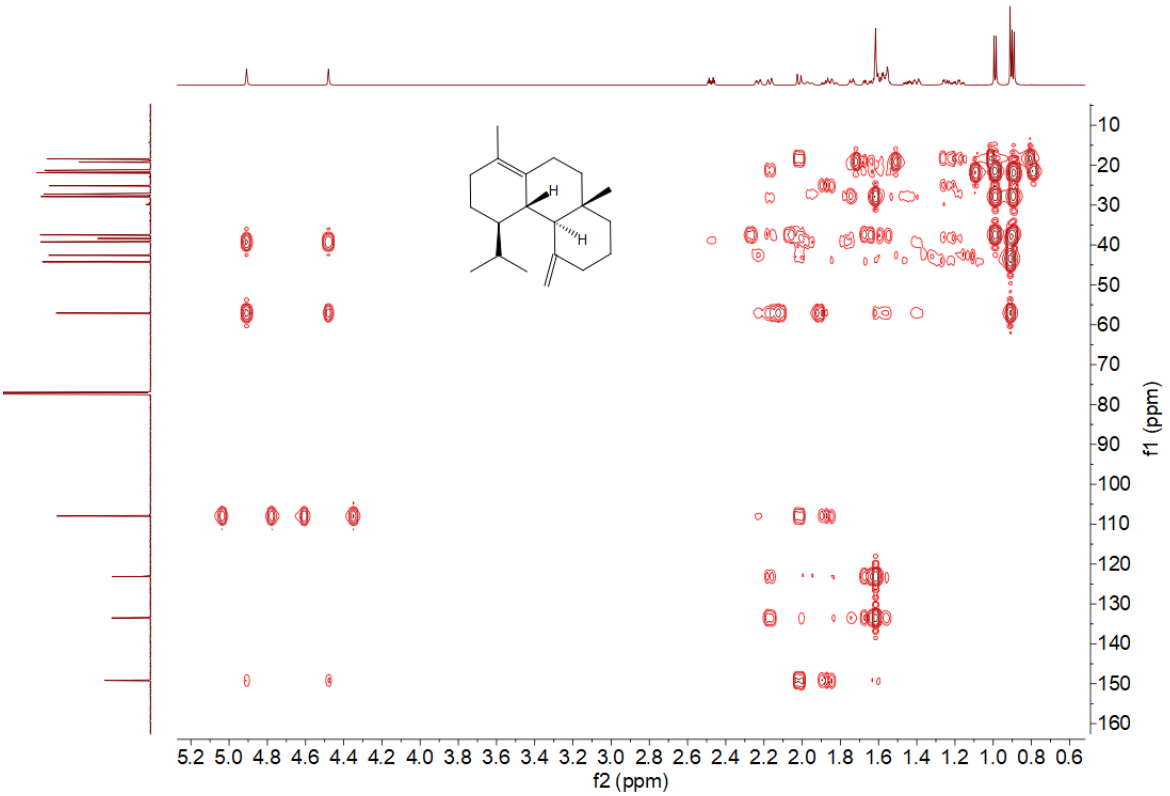


Fig. S17. HMBC NMR spectrum of compound **5** in $CDCl_3$.

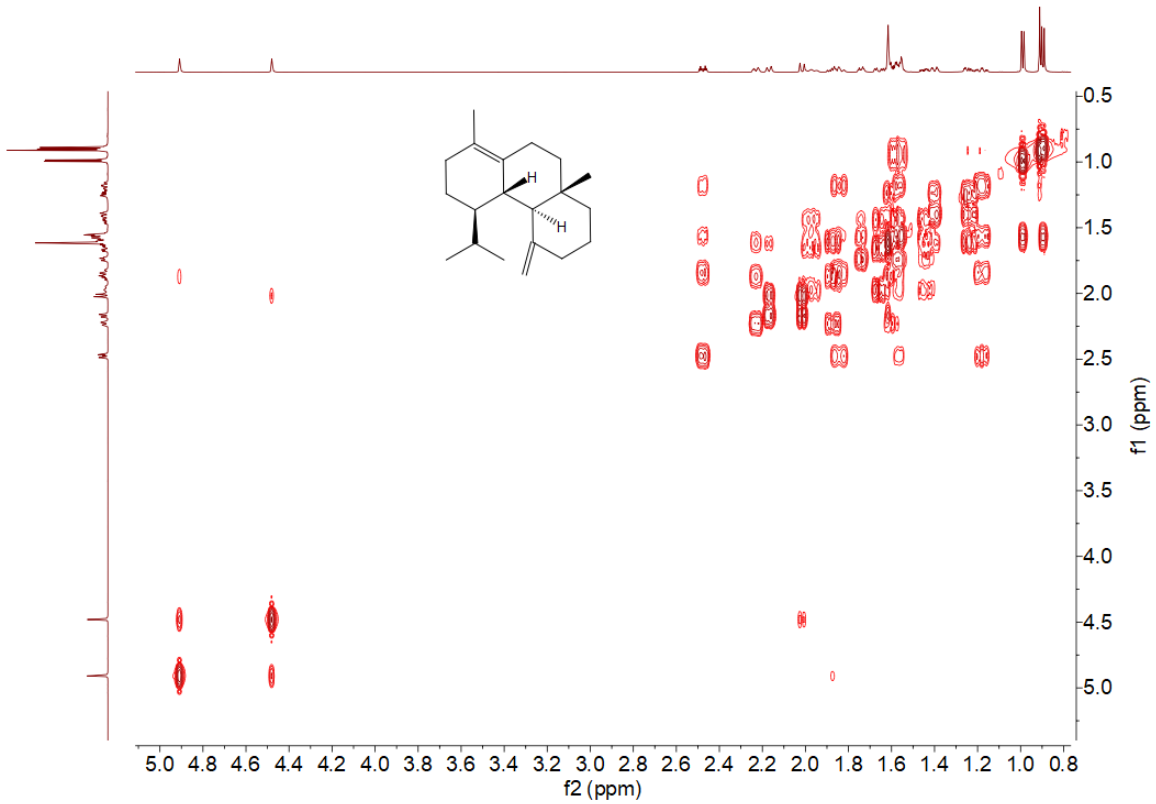


Fig. S18. ^{1}H-^{1}H COSY NMR spectrum of compound **5** in $CDCl_3$.

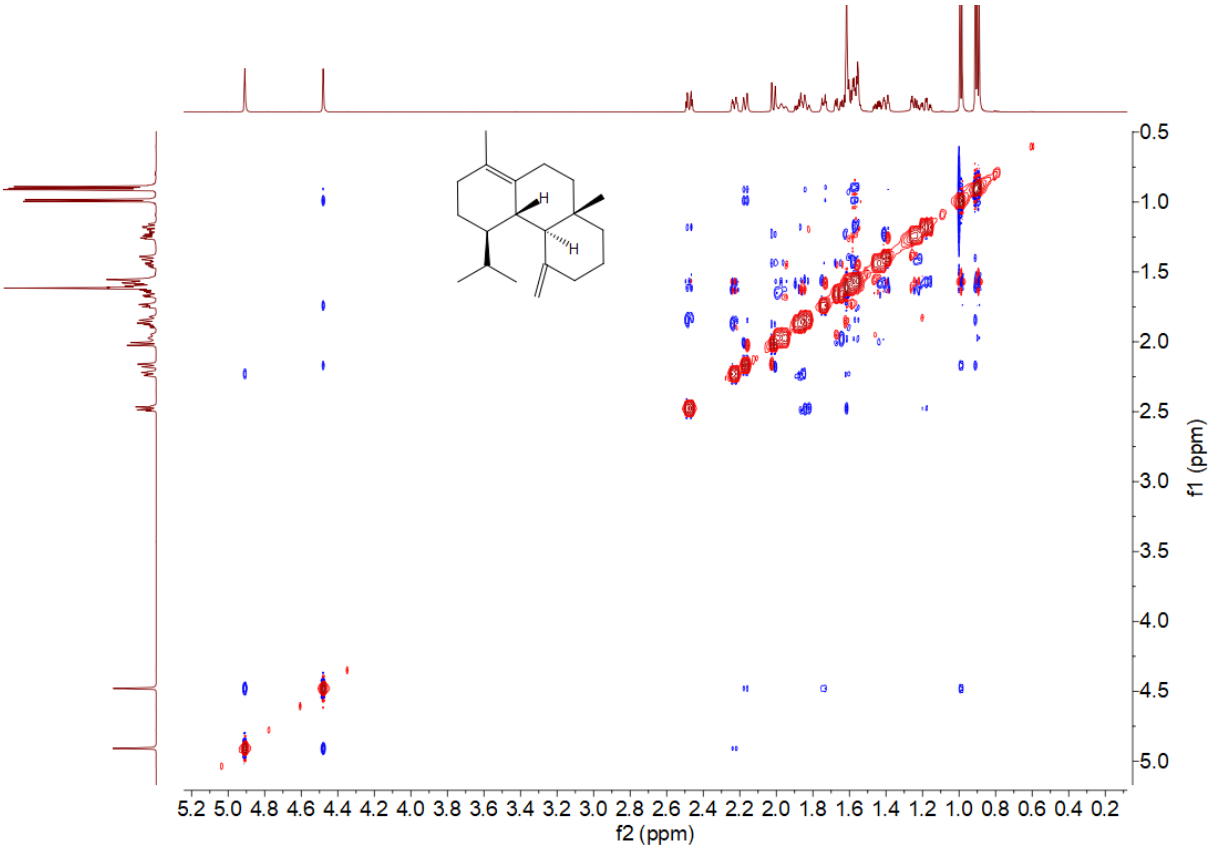


Fig. 19. NOESY NMR spectrum of compound **5** in $CDCl_3$.

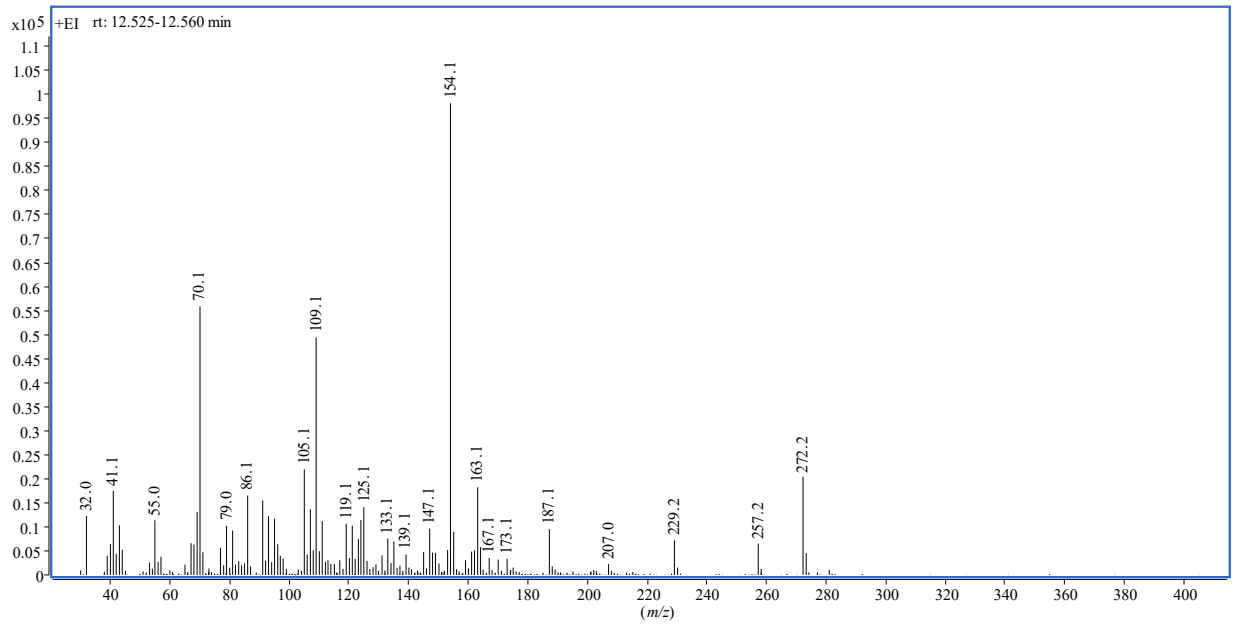


Fig. S20. GC-MS spectrum of compound **5**.

Original spectra for compound 5a.

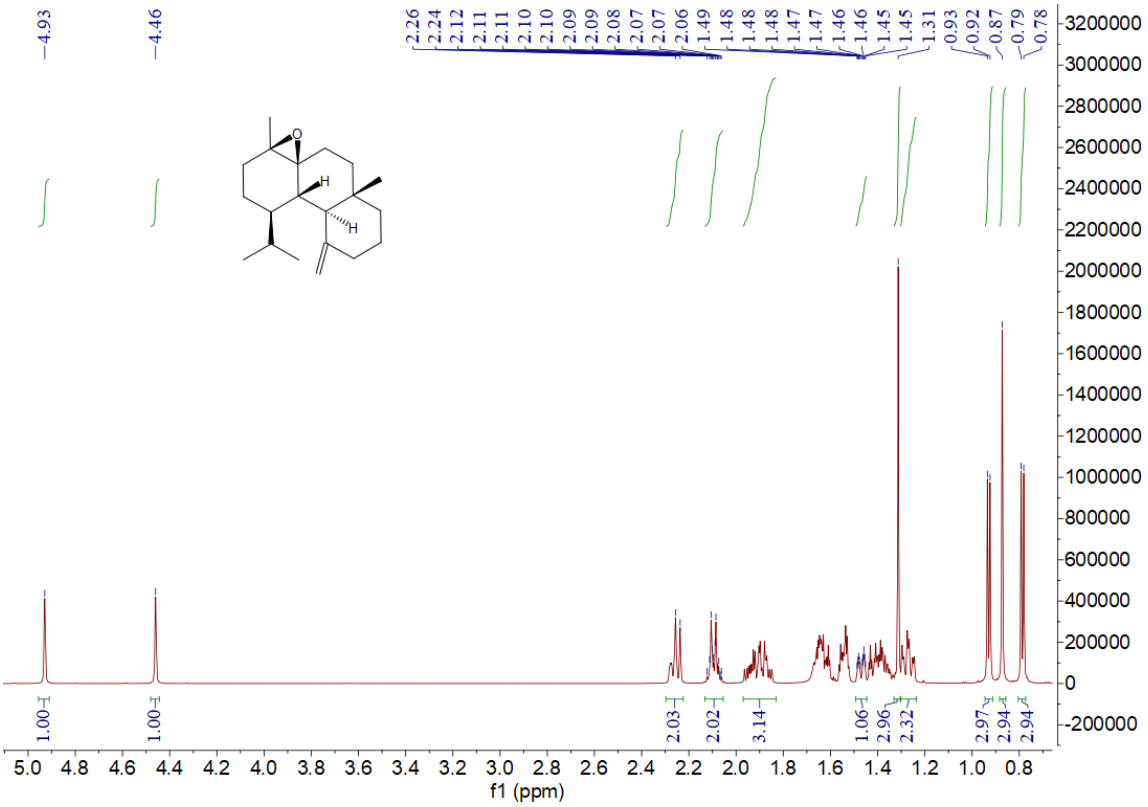


Fig. S22. ^{1}H NMR spectrum (400 MHz) of compound **5a** in $CDCl_3$.

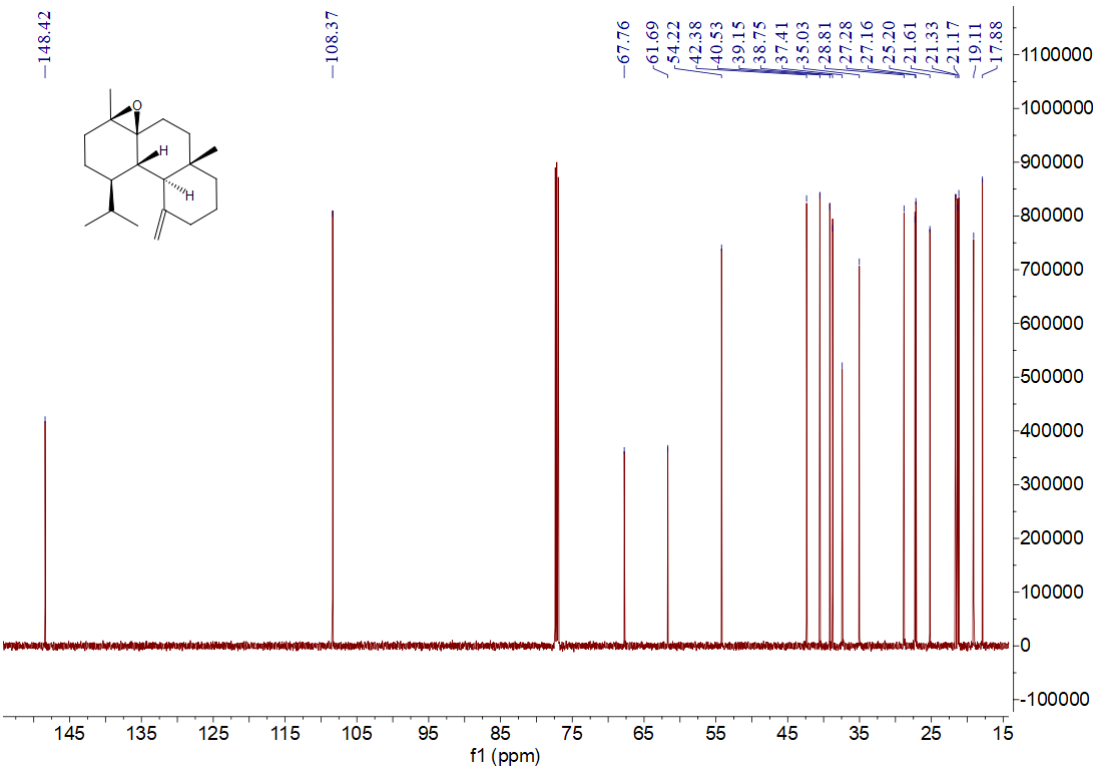


Fig. S23. ^{13}C NMR spectrum (100 MHz) of compound **5a** in $CDCl_3$.

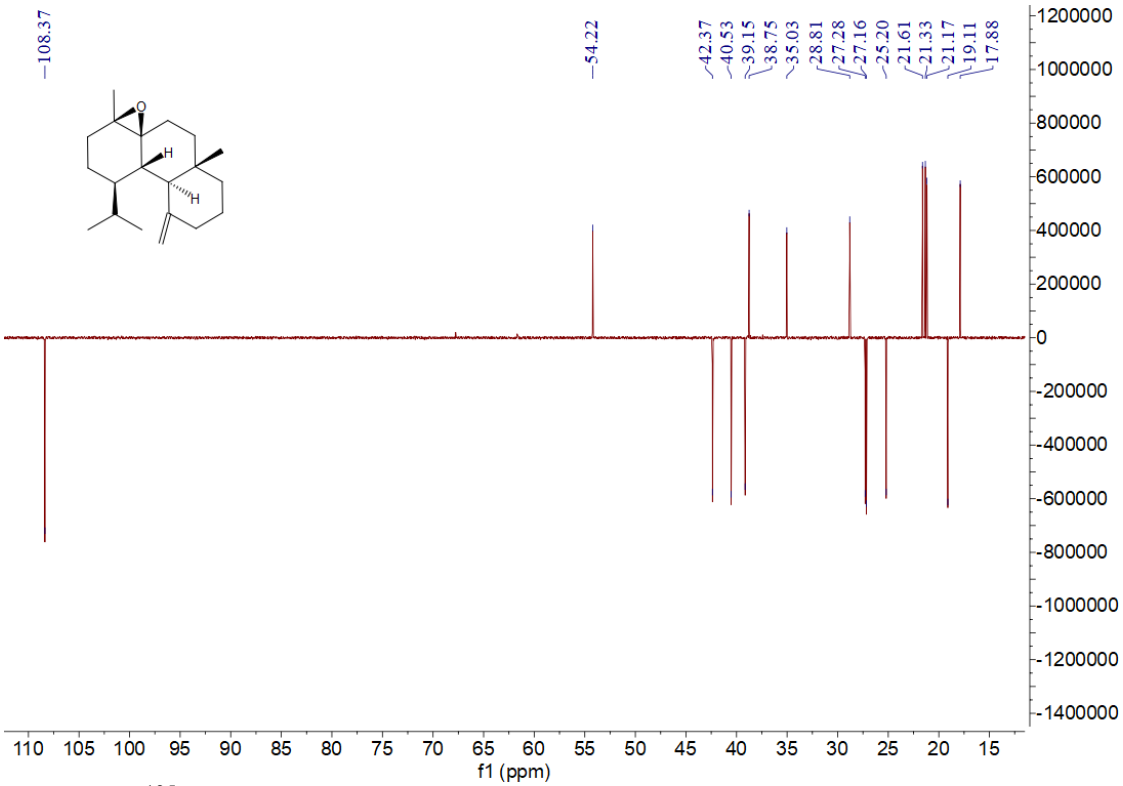


Fig. S24. DEPT^{135} spectrum (100 MHz) of compound **5a** in $CDCl_3$.

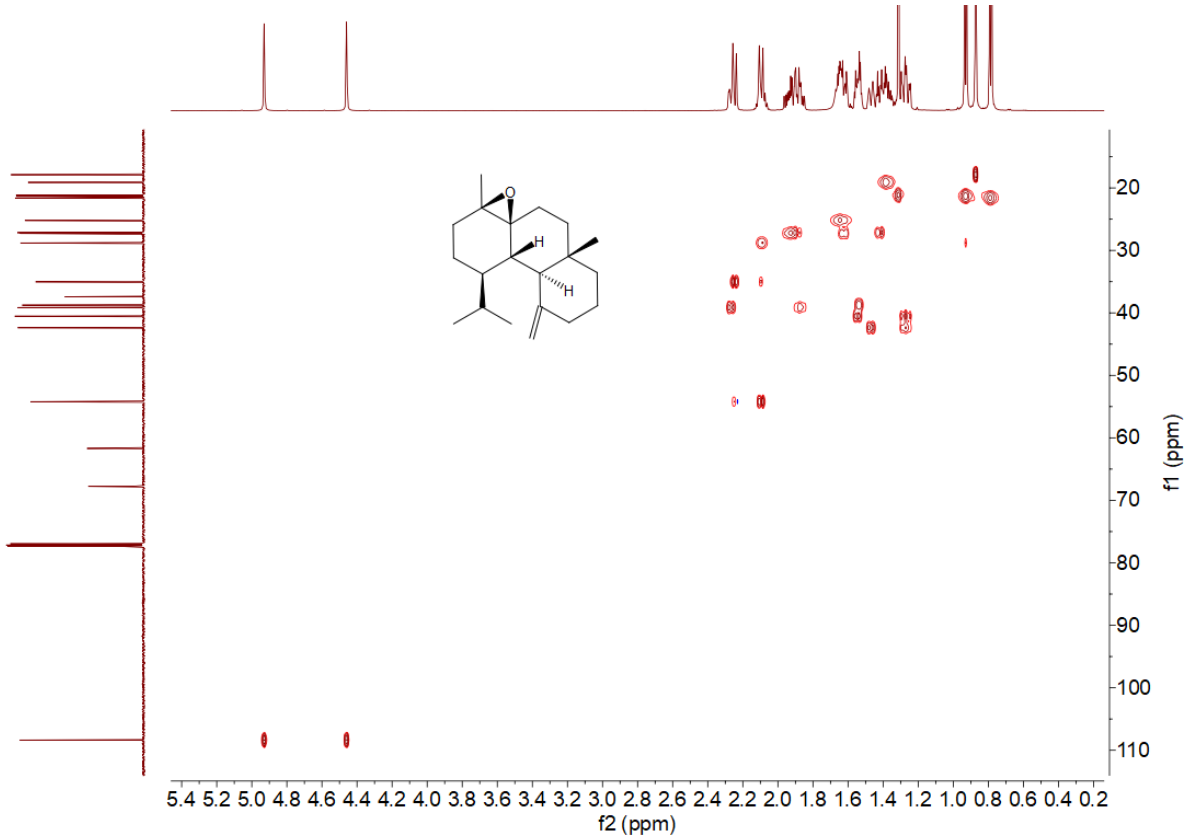


Fig. S25. HSQC NMR spectrum of compound **5a** in $CDCl_3$.

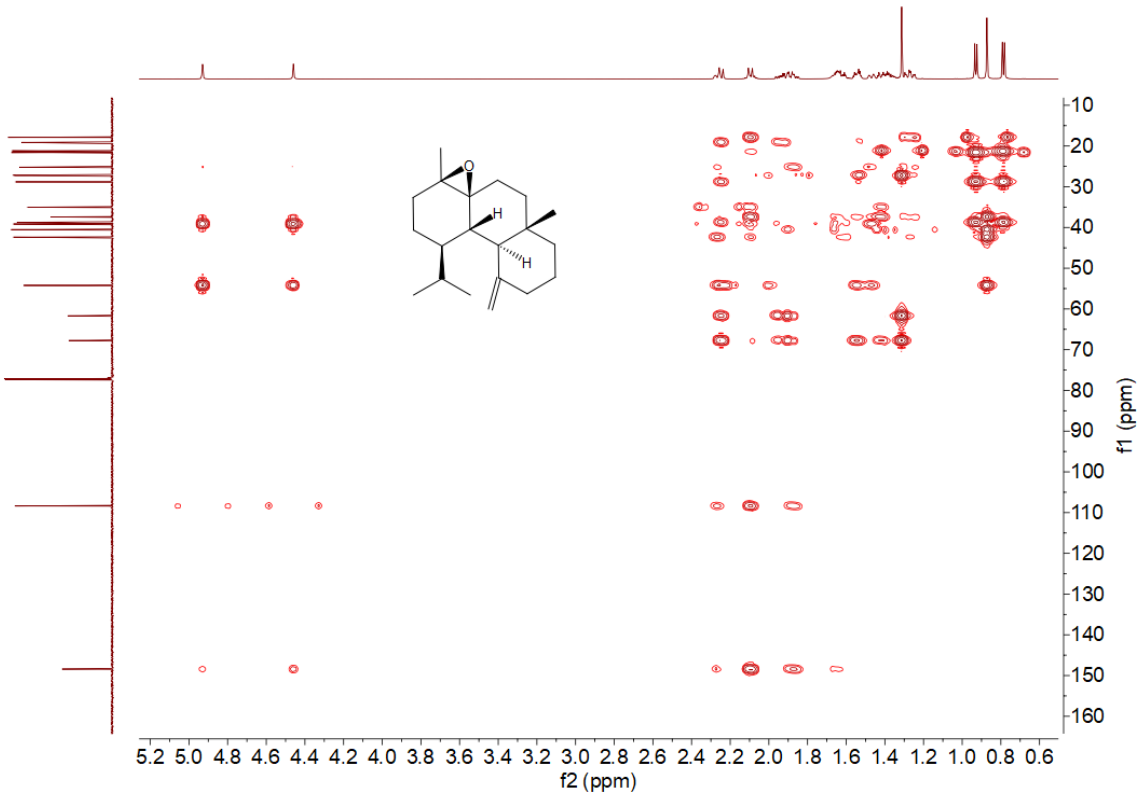


Fig. S26. HMBC NMR spectrum of compound **5a** in $CDCl_3$.

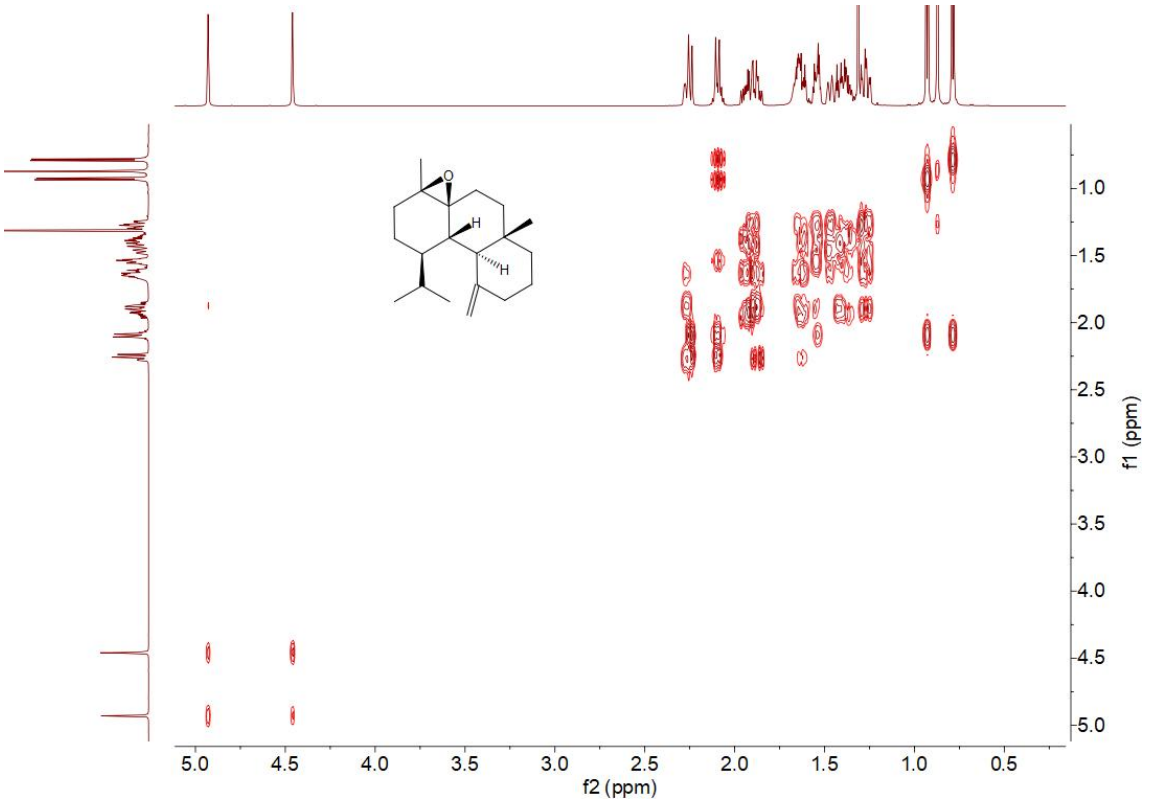


Fig. S27. ^{1}H-^{1}H COSY NMR spectrum of compound **5a** in $CDCl_3$.

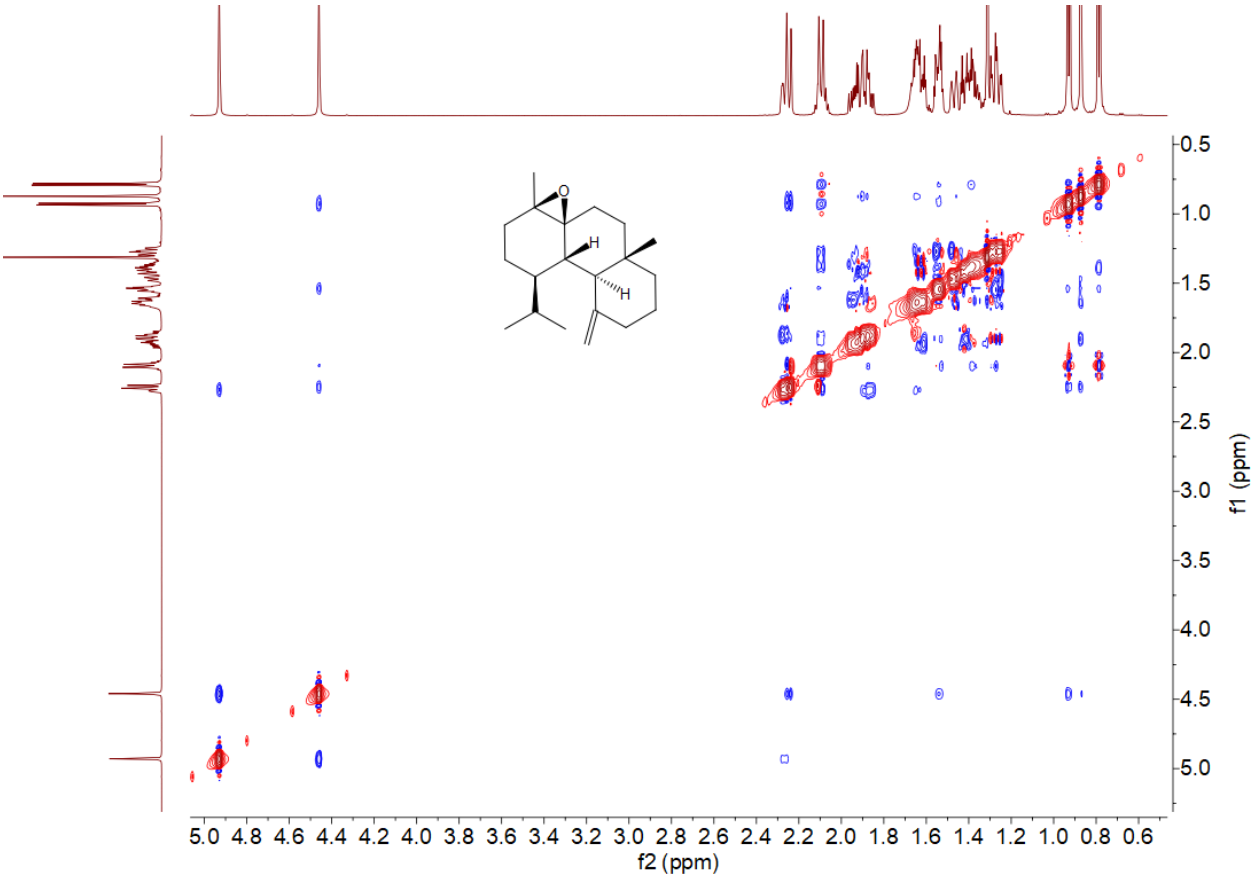


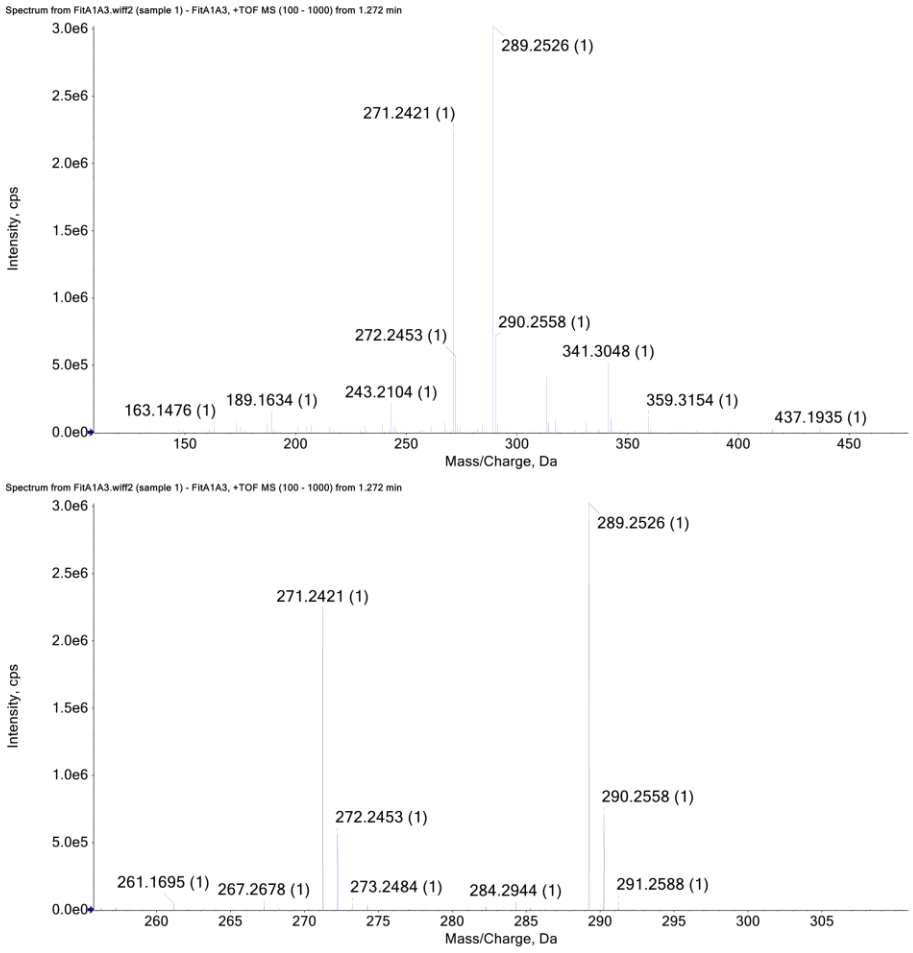
Fig. S28. NOESY NMR spectrum of compound **5a** in $CDCl_3$.

Qualitative Analysis Report

Date: 2025-08-29

File name: FitA1A3
Polarity: +
Instrument: Sciex ZenoTOF™ 7600

MS spectra



Formula Calculator Results

Measured m/z	Cal m/z	Error(mmu)	Error(ppm)	Ion Formula	Ion
289.2526	289.2526	0	0	C20H33O	$[M+H]^+$
271.2421	271.2421	0	0	C20H31	$[M+H-H_2O]^+$

Fig. S29. HRESIMS spectrum of compound **5a**.

Table S1. Strains and plasmids used in this study.

Strain	Description	Source
E. coli Turbo	Host for general cloning	Shanghai Weidi Biotechnology
E. coli BL21 Gold (DE3)	Host for high-level protein production	Shanghai Weidi Biotechnology

Table S2. Primer sequences used in this study.

Primer	Nucleotide Sequence (5′–3′)	Purpose
SsFitTS-F	AGCAAATGGGTCGCGGATCCATGAAGATCCCCCTGCTCGC	SsFitTS mutant amplification for expression in *E. coli*
SsFitTS-R	TCGAGTGCGGCCGCAAGCTTTCAGCGGCCCCCGCCGCGGG	
SsFitTSQ51E-F	ACGACCATACCGGCTTCGGGGAGCTGGCCGCCCGCATCTA	SsFitTS mutagenesis for Q51E
SsFitTSQ51E-R	TAGATGCGGGCGGCCAGCTCCCCGAAGCCGGTATGGTCGT	
SsFitTSI72M-F	TGACCCTGATCTCCCGCTGGATGGCCATCTGGGCCATCTT	SsFitTS mutagenesis for I72M
SsFitTSI72M-R	AAGATGGCCCAGATGGCCATCCAGCGGGAGATCAGGGTCA	
SsFitTSI182M-F	GGTTCGGGGGCATGCGCATGGCGATGGACCTGGCCGAACT	SsFitTS mutagenesis for I182M
SsFitTSI182M-R	AGTTCGGCCAGGTCCATCGCCATGCGCATGCCCCCGAACC	
SsFitTSA185S-F	GGTTCGGGGGCATCCGCATGTCGATGGACCTGGCCGAACT	SsFitTS mutagenesis for A185S
SsFitTSA185S-R	AGTTCGGCCAGGTCCATCGACATGCGGATGCCCCCGAACC	
SsFitTSA218V-F	ACAACCTGGGCGACATCGTCCTGTGGGCCAACGACATCTT	SsFitTS mutagenesis for A218V
SsFitTSA218V-R	AAGATGTCGTTGGCCCACAGGACGATGTCGCCCAGGTTGT	
SsFitTSD230E-F	ATCTTCTCCCTGACCGTGGAACTGGAGGAGGGCAACGTCT	SsFitTS mutagenesis for D230E
SsFitTSD230E-R	AGACGTTGCCCTCCTCCAGTTCCACGGTCAGGGAGAAGAT	
MicA-F	AGCAAATGGGTCGCGGATCCATGACCTTCACCGTCCCGGA	MicA mutant amplification for expression in *E. coli*
MicA-R	TCGAGTGCGGCCGCAAGCTTTCACGGCTGCCCGGCGCGCT	
MicAE53Q-F	CGACCGGACGCGGTTCGGCCAACTCGTGGCCCGGGCCTAT	MicA mutagenesis for E53Q
MicAE53Q-R	ATAGGCCCGGGCCACGAGTTGGCCGAACCGCGTCCGGTCG	
MicAM184I-F	CGGCGCCGCTTTGGCGGCATTCGCCCGTCGATGGACCTGT	MicA mutagenesis for M184I
MicAM184I-R	ACAGGTCCATCGACGGGCGAATGCCGCCAAAGCGGCGCCG	
MicAE232D-F	TTCTCCGTCGAGGCCGACAAGCGCGAGGGCAACGTCAATA	MicA mutagenesis for E232D
MicAE232D-R	TATTGACGTTGCCCTCGCGCTTGTCGGCCTCGACGGAGAA	

Table S3. ^{1}H (600 MHz) and ^{13}C NMR (150 MHz) data of 4 and 5 in $CDCl_3$.

NO.	**4**		**5**	
	δ_H, mult. (*J*, Hz)		δ_H, mult. (*J*, Hz)	δ_C (mult.)
1	2.68 (brd, 11.5)	43.4, CH	2.17 (d, 11.5)	37.5, CH
2	4.65 (d, 10.2)	135.6, CH	2.02 (d, 11.5)	57.1, CH
3	-	129.0, C	-	149.2, C
4a	2.17 (dd, 12.0, 5.0)	39.2, CH_2	2.23 (m)	39.2, CH_2
4b	1.95 (overlapped)		1.87 (overlapped)	
5	2.29 (overlapped)	25.8, CH_2	1.61 (overlapped)	25.2, CH_2
6a	2.04 (m)		1.40 (dtd, 13.1, 3.3, 1.8)	42.6, CH_2
6b	4.72 (brd, 11.1)	126.7, CH	1.24 (m)	
7	-	137.2, C	-	38.4, C
8a	2.28 (overlapped)	41.7, CH_2	1.57 (overlapped)	44.2, CH_2
8b	1.99 (overlapped)		1.18 (m)	
9a	2.64 (t, 8.3)	33.0, CH_2	2.48 (dt, 12.9, 3.5)	27.2, CH_2
9b	1.51 (overlapped)		1.85 (overlapped)	
10	-	134.1, C	-	133.5, C
11	-	128.1, C	-	123.1, C
12a	1.89 (m)	29.0, CH_2	1.96 (m)	27.9, CH_2
12b	1.89 (m)		1.67 (overlapped)	
13a	1.74 (m)	20.6, CH_2	1.56 (overlapped)	21.3, CH_2
13b	1.53 (overlapped)		1.44 (ddd, 13.3, 5.7, 3.4)	
14	1.10 (m)	46.7, CH	2.17 (dt, 10.3, 3.5)	37.5, CH
15	1.53 (overlapped)	27.5, CH	1.57 (overlapped)	27.9, CH
16	0.92 (d, 6.6)	21.9, CH_3	0.90 (d, 6.6)	21.5, CH_3
17	0.87 (d, 6.7)	20.6, CH_3	0.99 (d, 6.6)	21.9, CH_3
18	1.61 (s)	19.6, CH_3	1.62 (d, 1.3)	19.2, CH_3
19	1.53 (s)	16.2, CH_3	0.91 (d, 0.8)	18.4, CH_3
20	1.55 (s)	16.2, CH_3	4.91 (d, 1.8)	108.0, CH_2
			4.48 (d, 1.8)	

Table S4. ^{1}H (400 MHz) and ^{13}C NMR (100 MHz) data of 5a in $CDCl_3$.

NO.	5a	
	δ_H, mult. (*J*, Hz)	δ_C (mult.)
1	2.25 (overlapped)	35.0, CH
2	2.10 (overlapped)	54.2, CH
3		148.4, C
4a	2.26 (overlapped)	39.1, CH_2
4b	1.87 (overlapped)	
5a	1.65 (m)	25.2, CH_2
5b	1.65 (m)	
6a	1.47 (m)	42.4, CH_2
6b	1.27 (overlapped)	
7		37.4, C
8a	1.54 (overlapped)	40.5, CH_2
8b	1.27 (overlapped)	
9a	1.91 (overlapped)	27.2, CH_2
9b	1.42 (m)	
10	-	67.8, C
11	-	61.7, C
12a	1.91 (overlapped)	27.3, CH_2
12b	1.62 (m)	
13a	1.38 (m)	19.1, CH_2
13b	1.38 (m)	
14	1.54 (overlapped)	38.8, CH
15	2.09 (overlapped)	28.8, CH
16	0.79 (d, 6.7)	21.6, CH_3
17	0.93 (d, 6.7)	21.3, CH_3
18	1.38 (s)	19.1, CH_3
19	0.87 (s)	17.9, CH_3
20	4.93 (s)	108.4, CH_2
	4.46 (s)	