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Supporting information

Microbial transformation of methyl cyperenoate by *Cunninghamella elegans* AS 3.2028 and the antithrombotic activities of its metabolites

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[d.] *Biology Institute of Shandong Academy of Sciences, Jinan, China*

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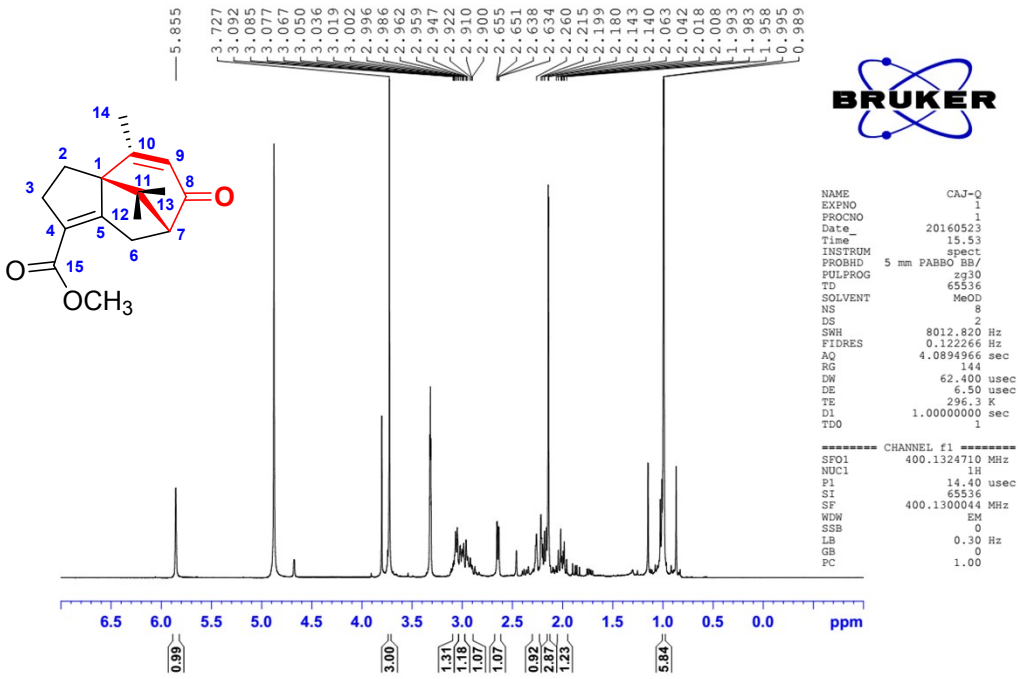

Figure S1. ^{1}H NMR spectrum (400 MHz, CD_3OD-d_4) of compound **1**

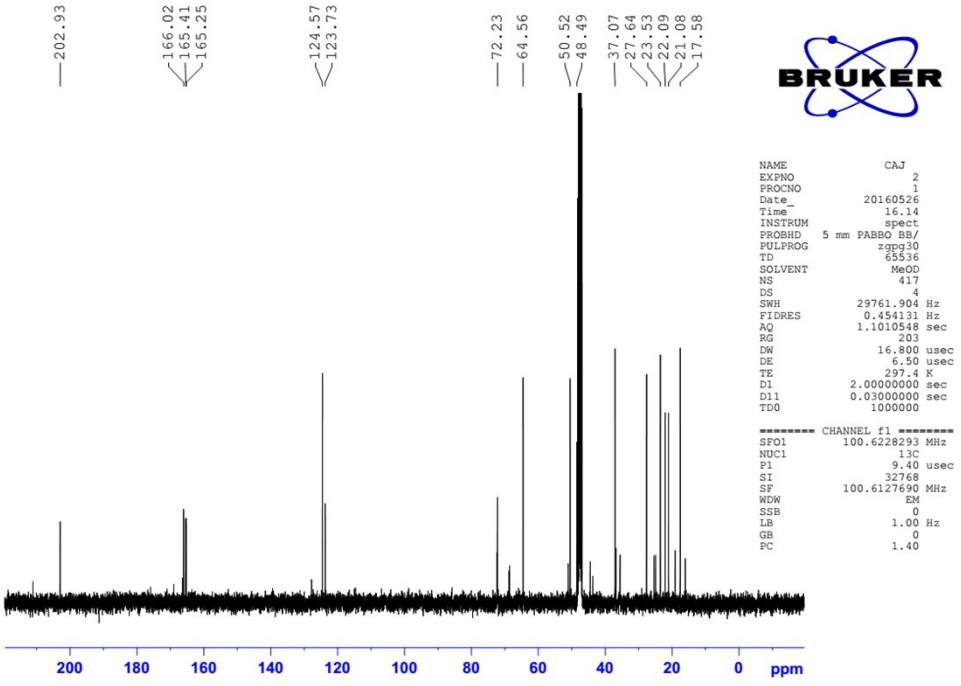

Figure S2. ^{13}C NMR spectrum (100 MHz, CD_3OD-d_4) of compound **1**

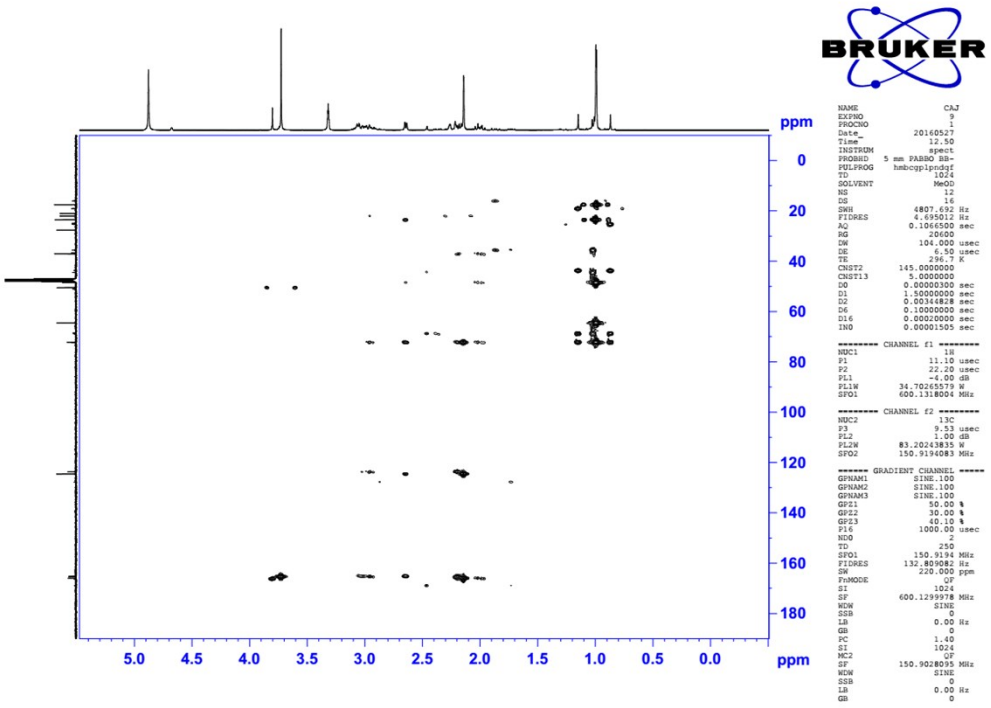

Figure S3. HMBC spectrum (600 MHz, CD_3OD-d_4) of compound **1**

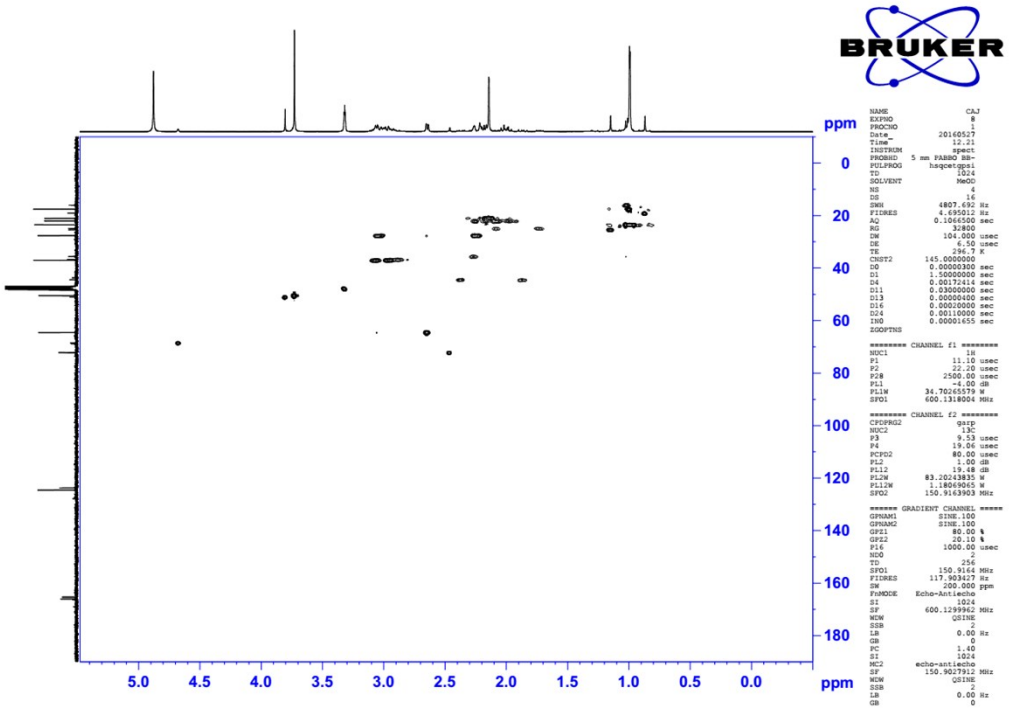

Figure S4. HSQC spectrum (600 MHz, CD_3OD-d_4) of compound **1**

Mass Spectrum Molecular Formula Report

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Comment			

Acquisition Parameter

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Scan End	1000 m/z	Set Collision Cell RF	400.0 Vpp	Set Divert Valve	Source

Generate Molecular Formula Parameter

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Nirogen Rule	Electron Configuration	
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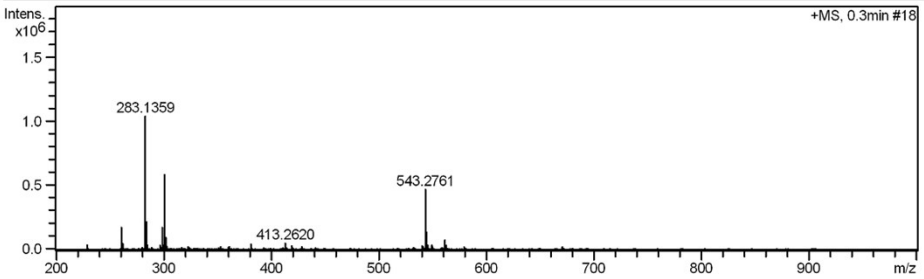

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7	297.1066	29682
8	299.1276	170452
9	300.1244	34374
10	301.1482	581229
11	302.1434	90722
12	303.0751	23132
13	303.1460	22624
14	323.1196	20062
15	353.2595	21438
16	361.1328	20390
17	381.1247	39312
18	413.2620	47246
19	419.1016	27183
20	419.1897	26200
21	428.1990	19384
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Figure S5. HRESIMS spectrum of compound **1**

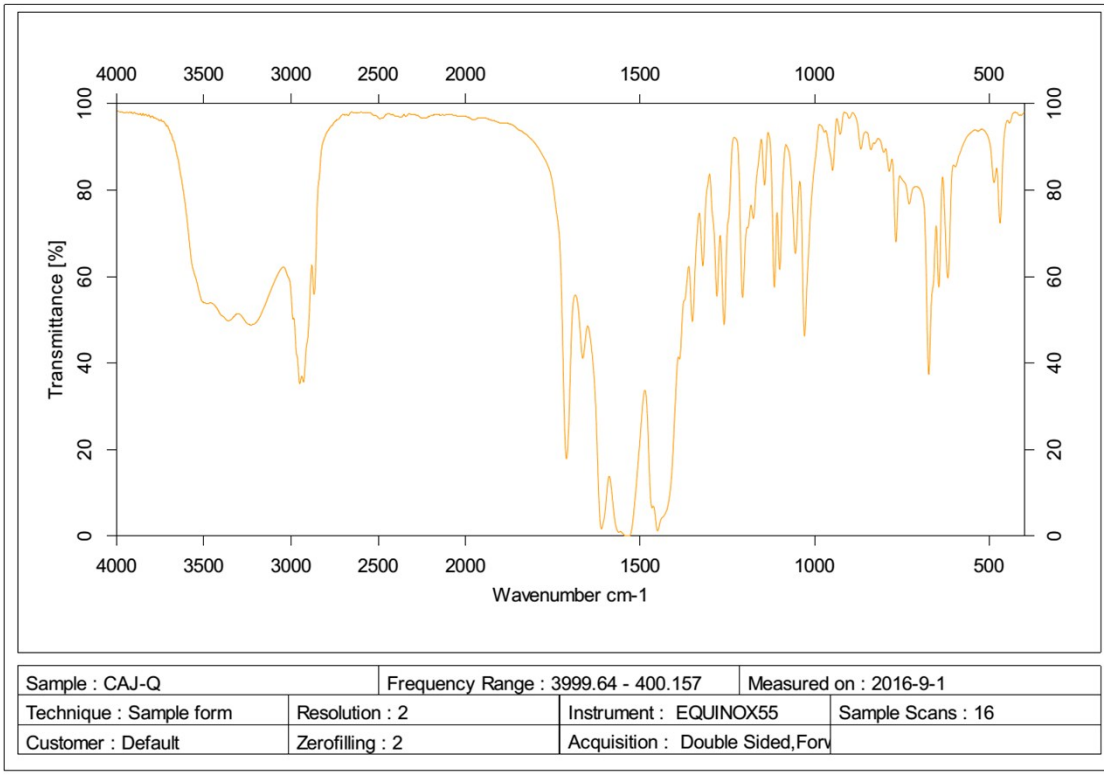

Figure S6. IR spectrum of compound **1**

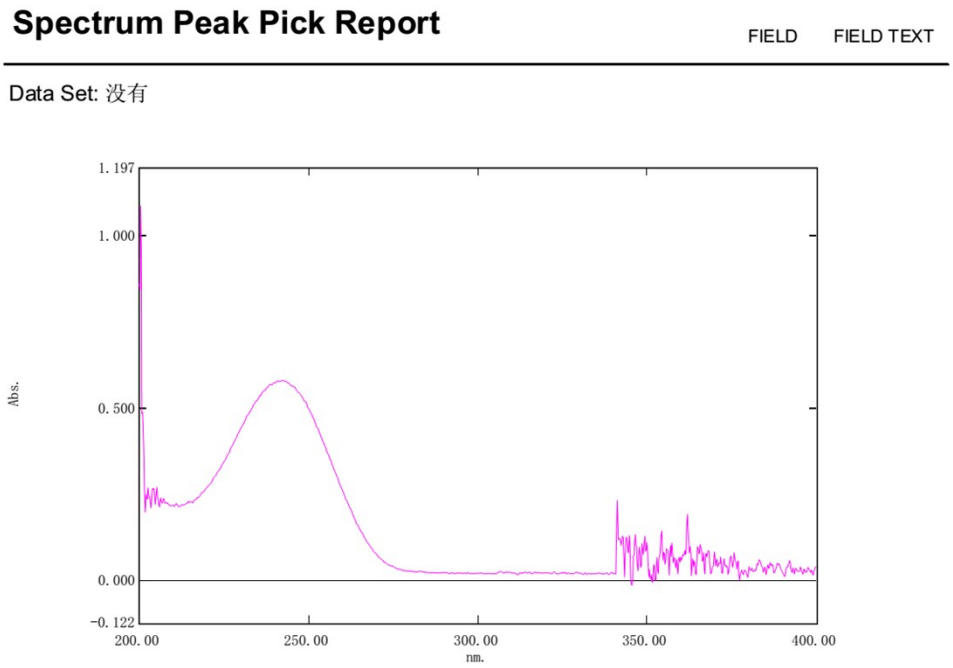

Figure S7. UV spectrum of compound **1**

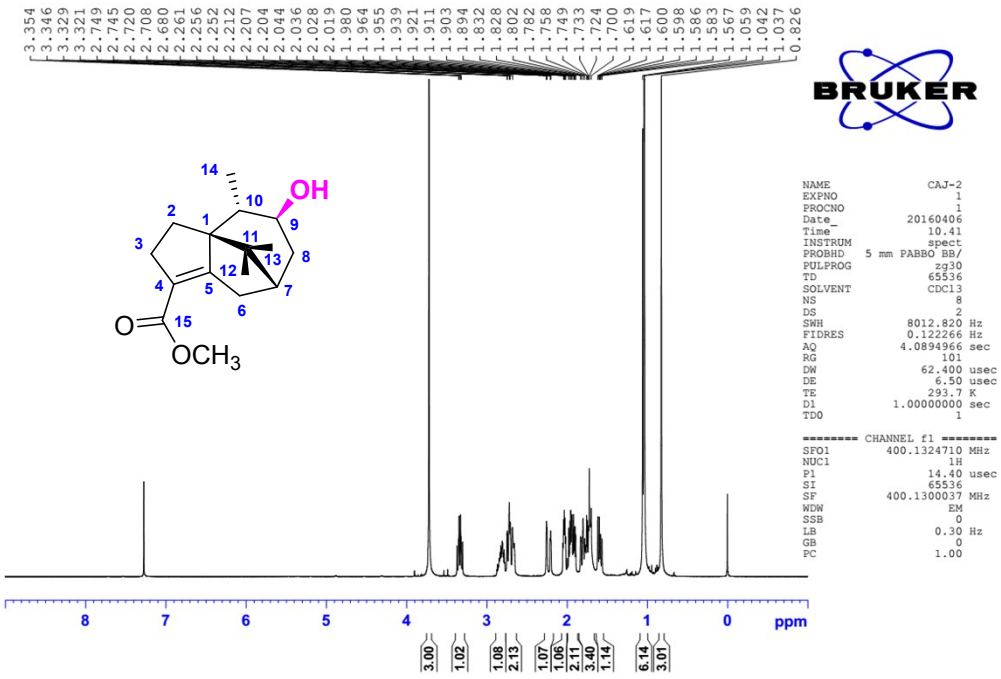

Figure S8. ^{1}H NMR spectrum (400 MHz, $CDCl_3$-*d*) of compound **2**

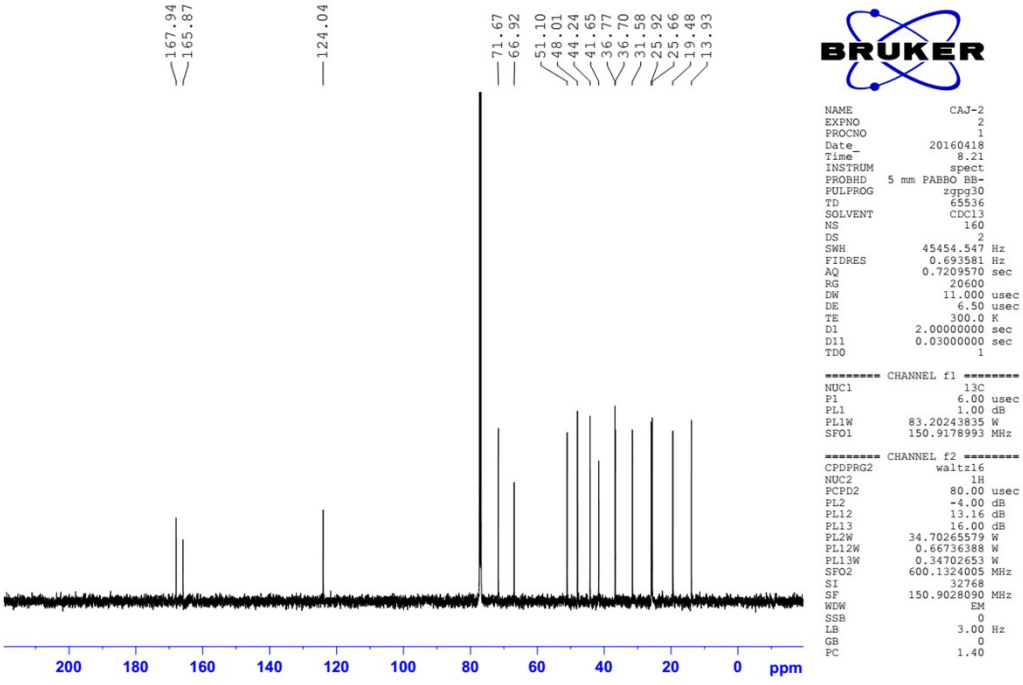

Figure S9. ^{13}C NMR spectrum (100 MHz, $CDCl_3$-*d*) of compound **2**

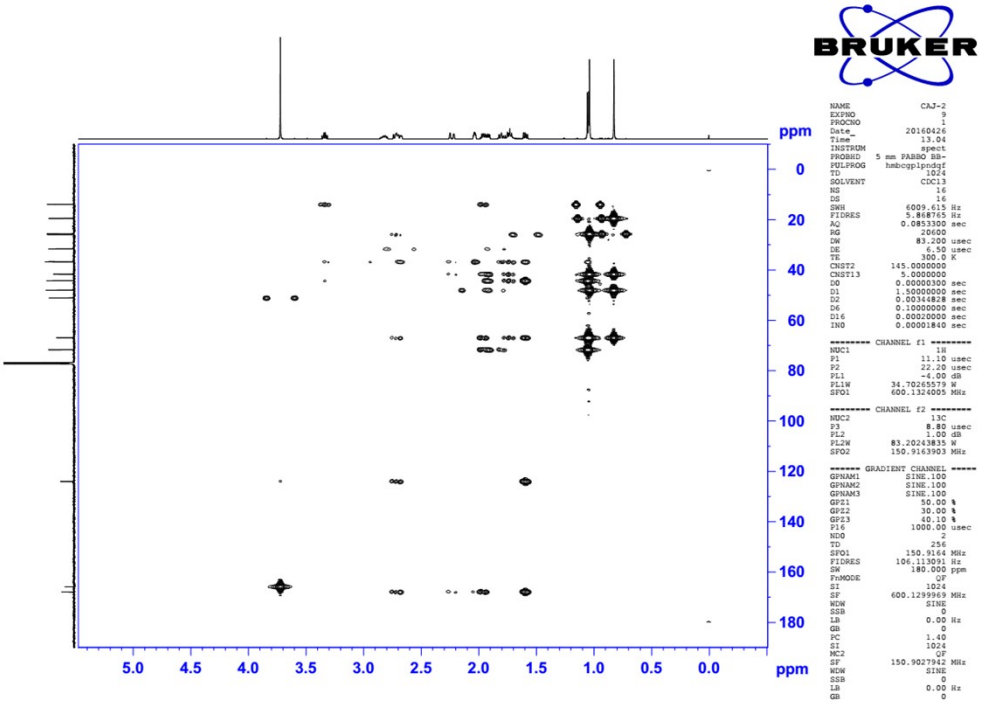

Figure S10. HMBC spectrum (600 MHz, $CDCl_3$-*d*) of compound **2**

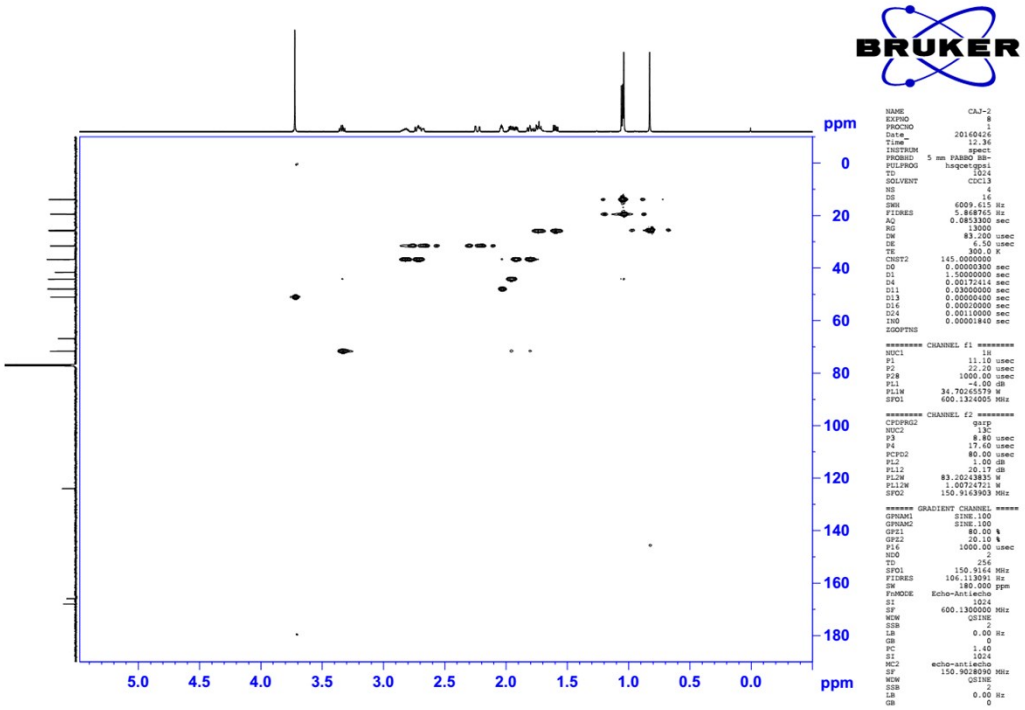

Figure S11. HSQC spectrum (600 MHz, $CDCl_3$-*d*) of compound **2**

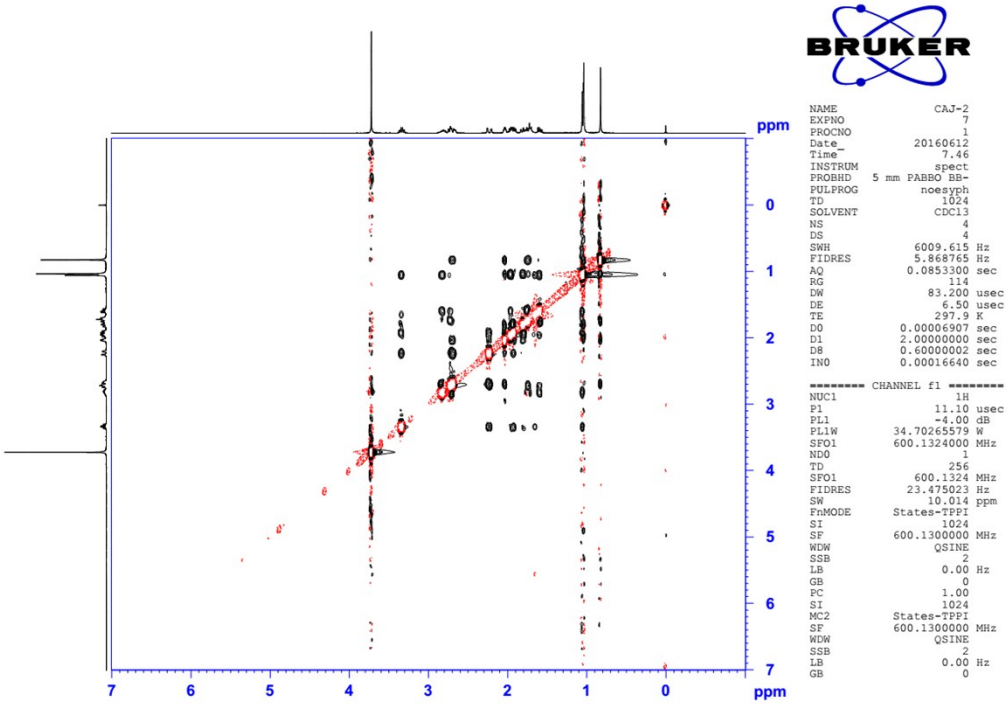

Figure S12. NOESY spectrum (600 MHz, $CDCl_3$-*d*) of compound **2**

Mass Spectrum Molecular Formula Report

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Method	20131026_ceyang.m	Operator	Bruker Customer
Sample Name	CAJ-2	Instrument / Ser#	micrOTOF-Q 125
Comment			

Acquisition Parameter

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Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	8.0 l/min
Scan End	3000 m/z	Set Collision Cell RF	400.0 Vpp	Set Divert Valve	Source

Generate Molecular Formula Parameter

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Formula, max.					
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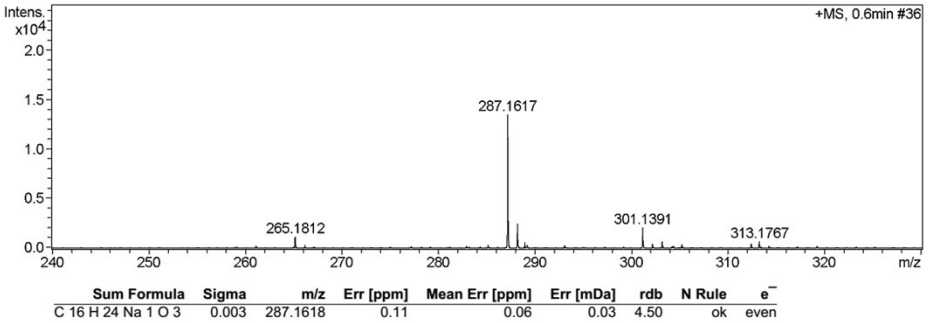

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Figure S13. HRESIMS spectrum of compound **2**

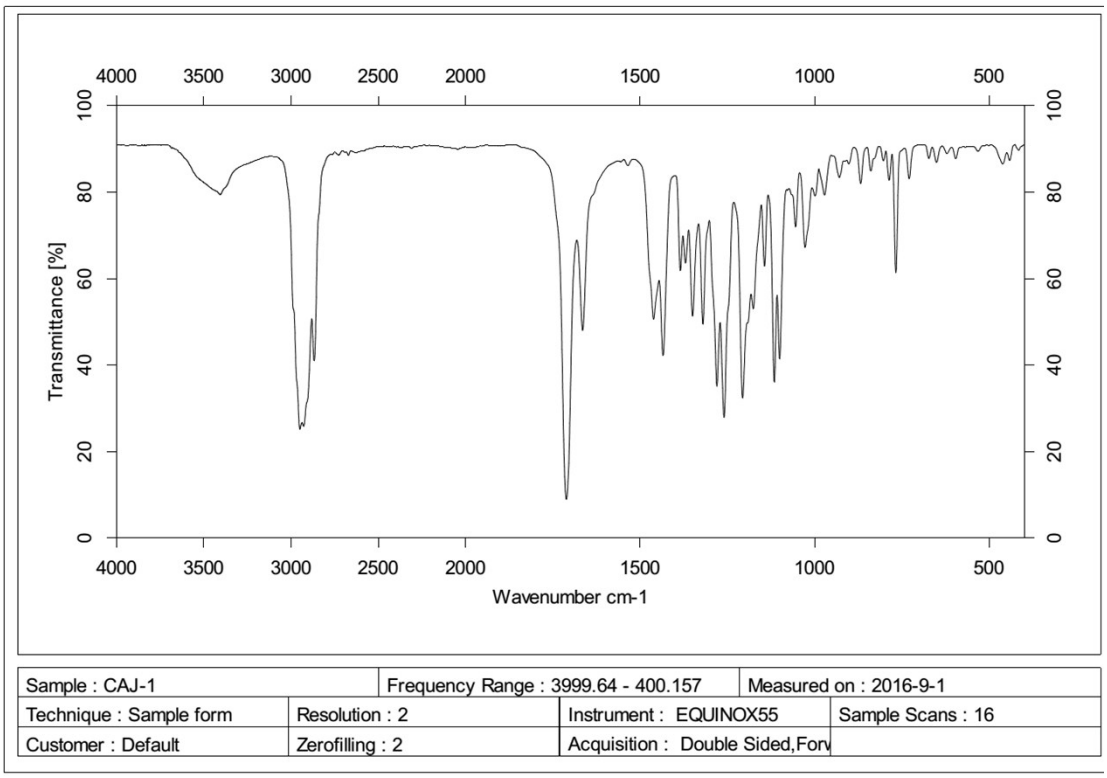


Figure S14. IR spectrum of compound **2**

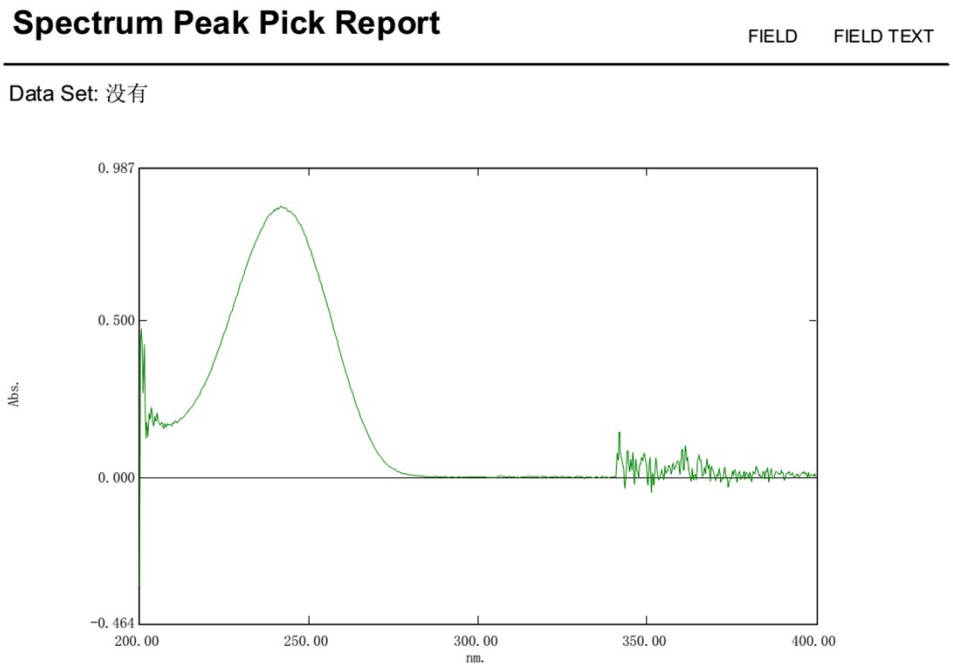


Figure S15. UV spectrum of compound **2**

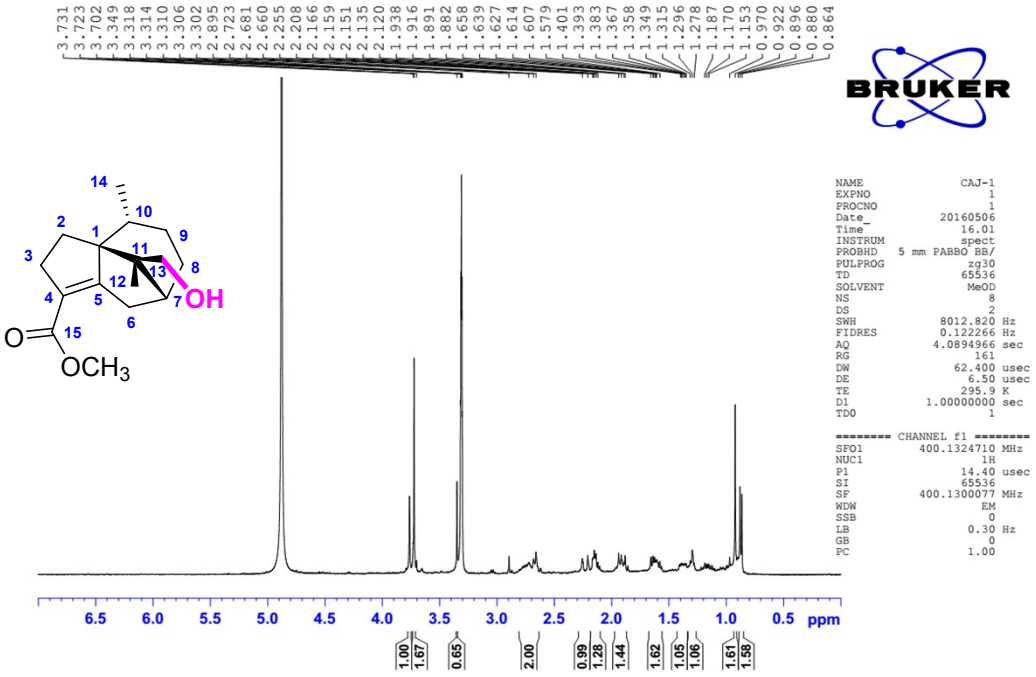


Figure S16. ^{1}H NMR spectrum (400 MHz, CD_3OD-d_4) of compound **3**

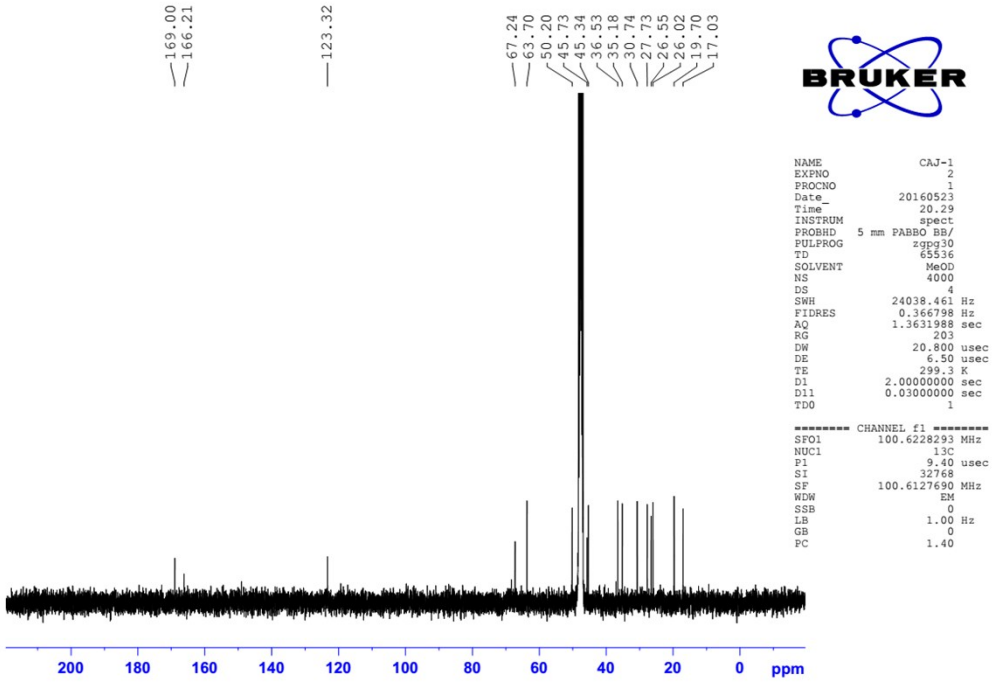


Figure S17. ^{13}C NMR spectrum (100 MHz, CD_3OD-d_4) of compound **3**

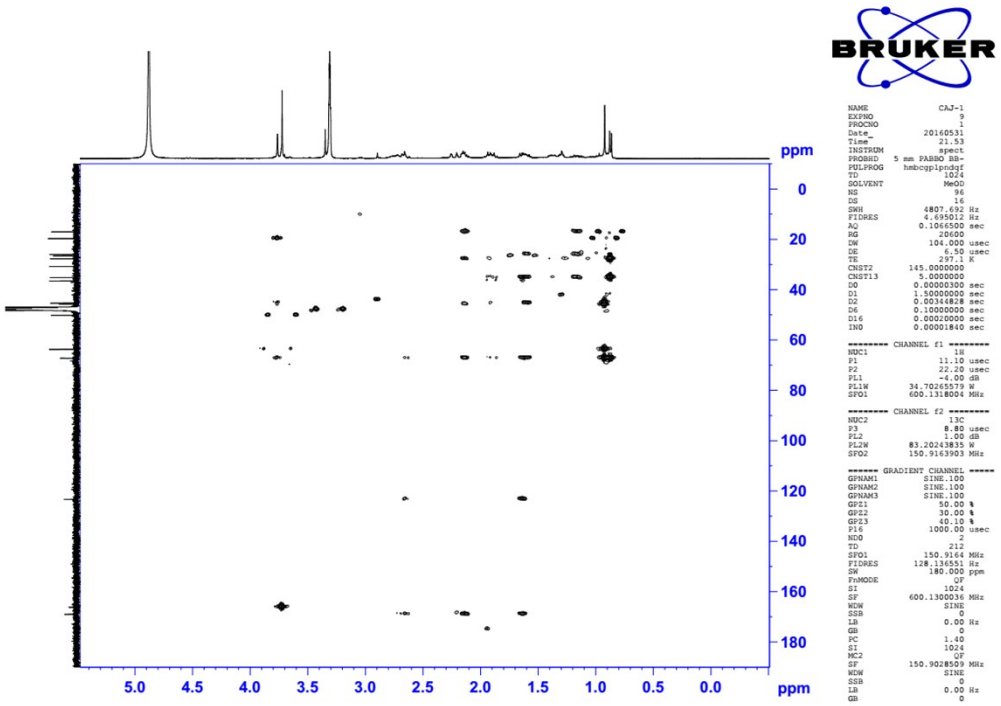


Figure S18. HMBC spectrum (600 MHz, CD_3OD-d_4) of compound **3**

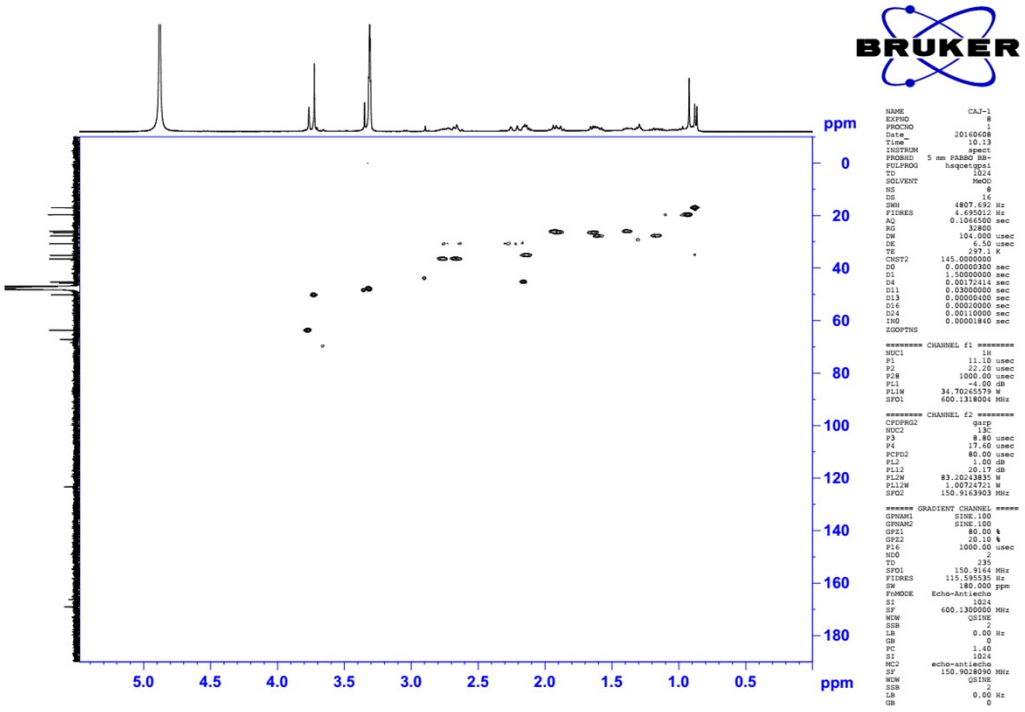


Figure S19. HSQC spectrum (600 MHz, CD_3OD-d_4) of compound **3**

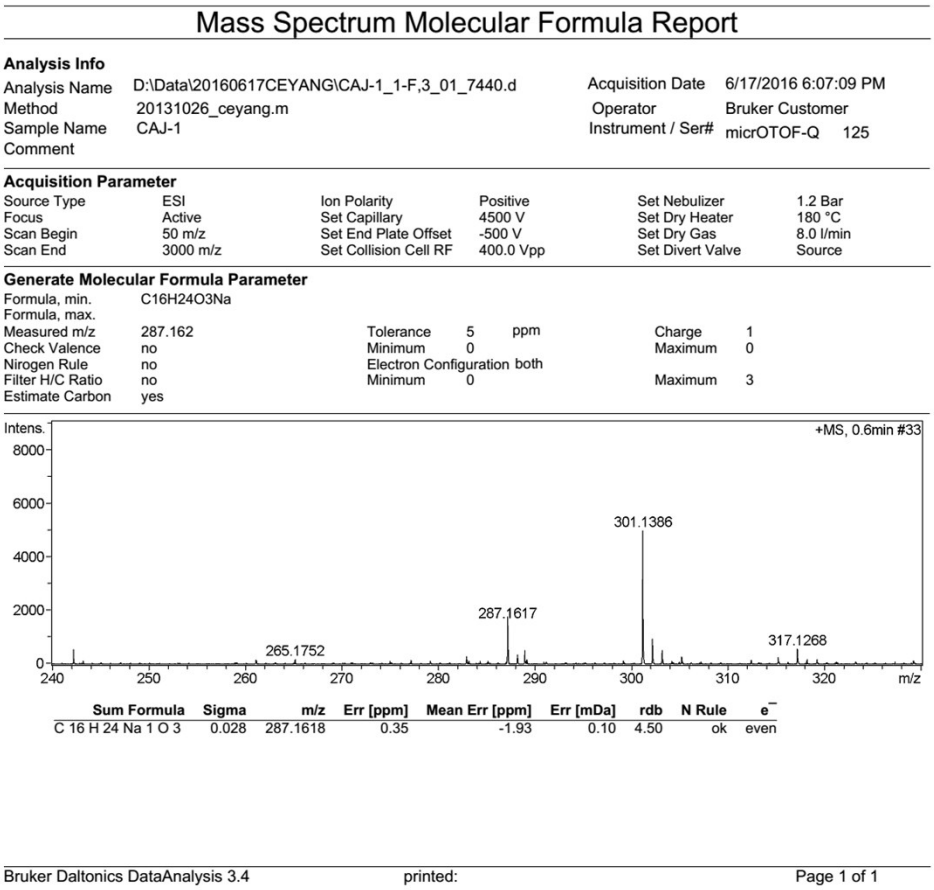


Figure S20. HRESIMS spectrum of compound **3**

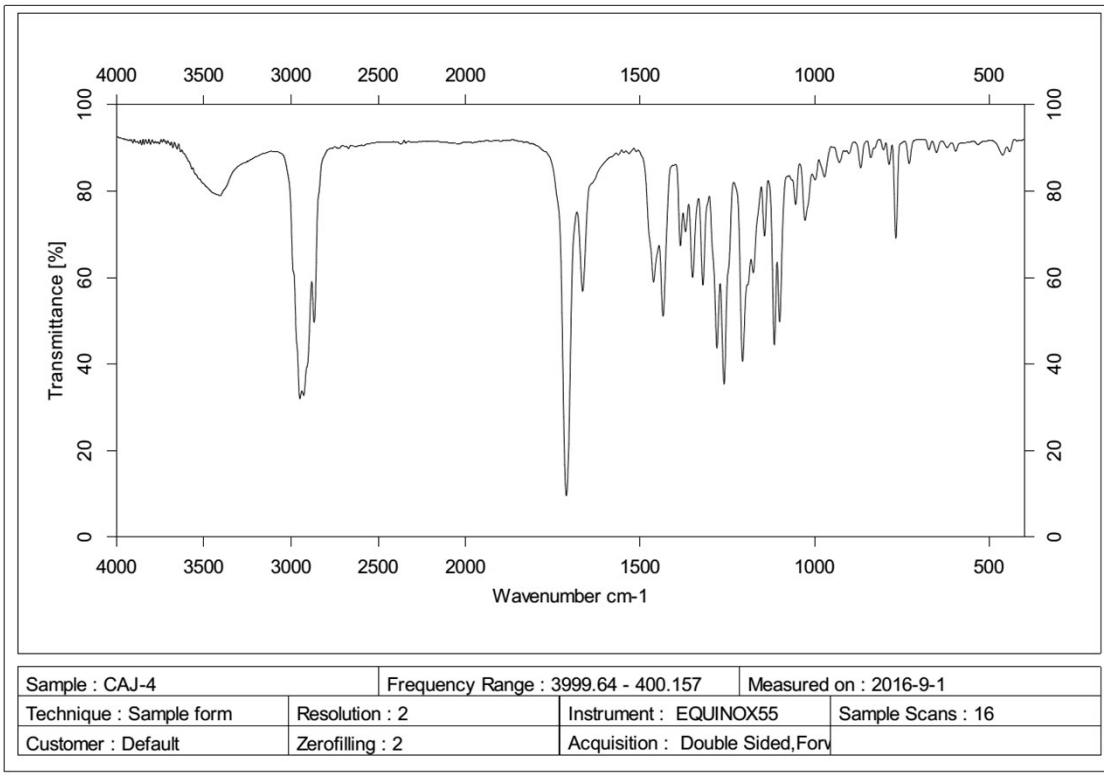


Figure S21. IR spectrum of compound **3**

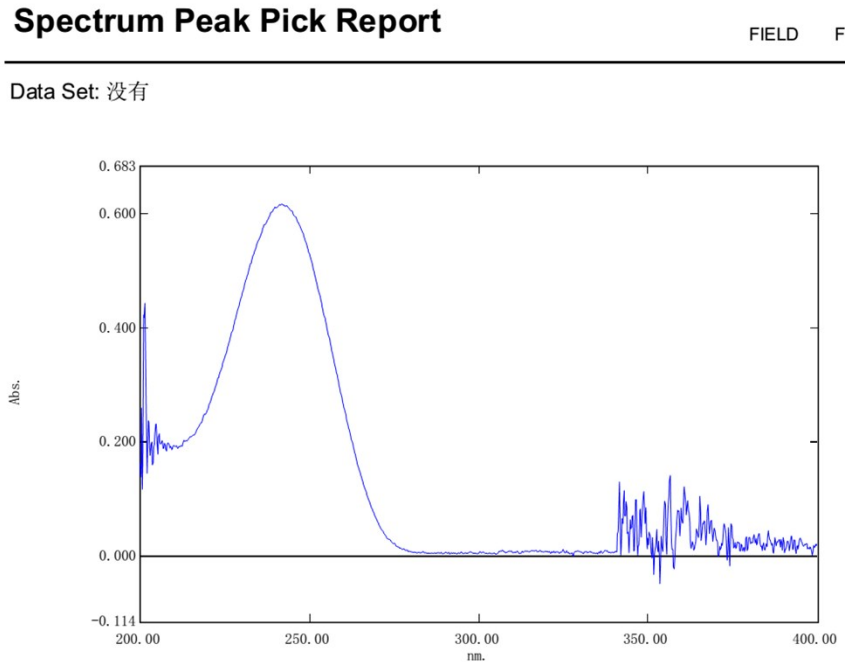


Figure S22. UV spectrum of compound **3**

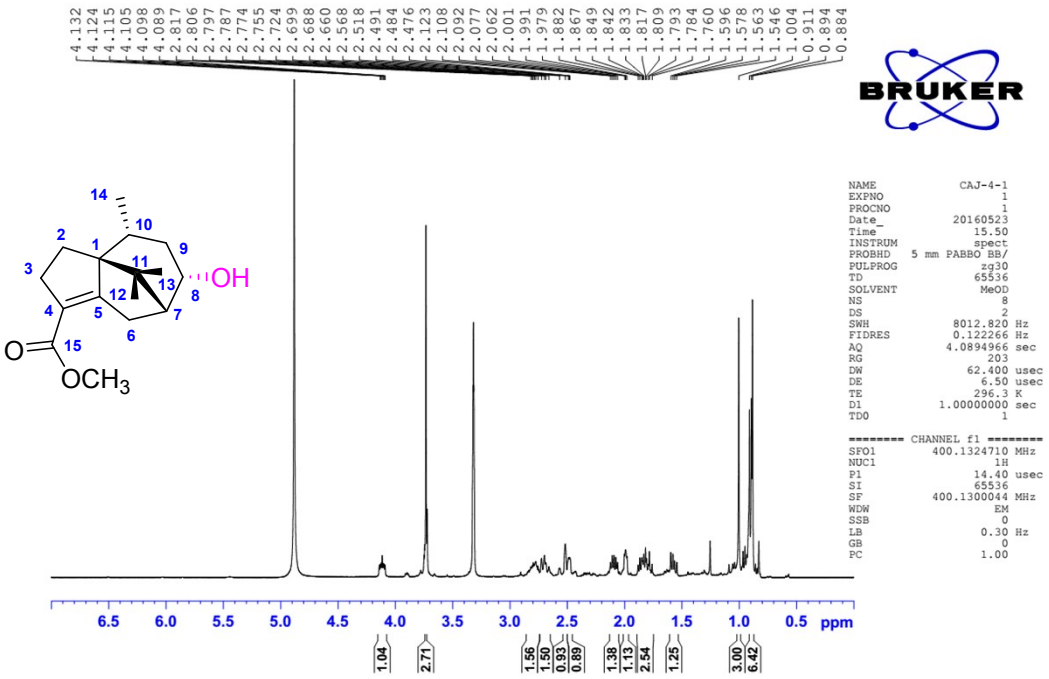


Figure S23. ^{1}H NMR spectrum (400 MHz, CD_3OD-d_4) of compound **4**

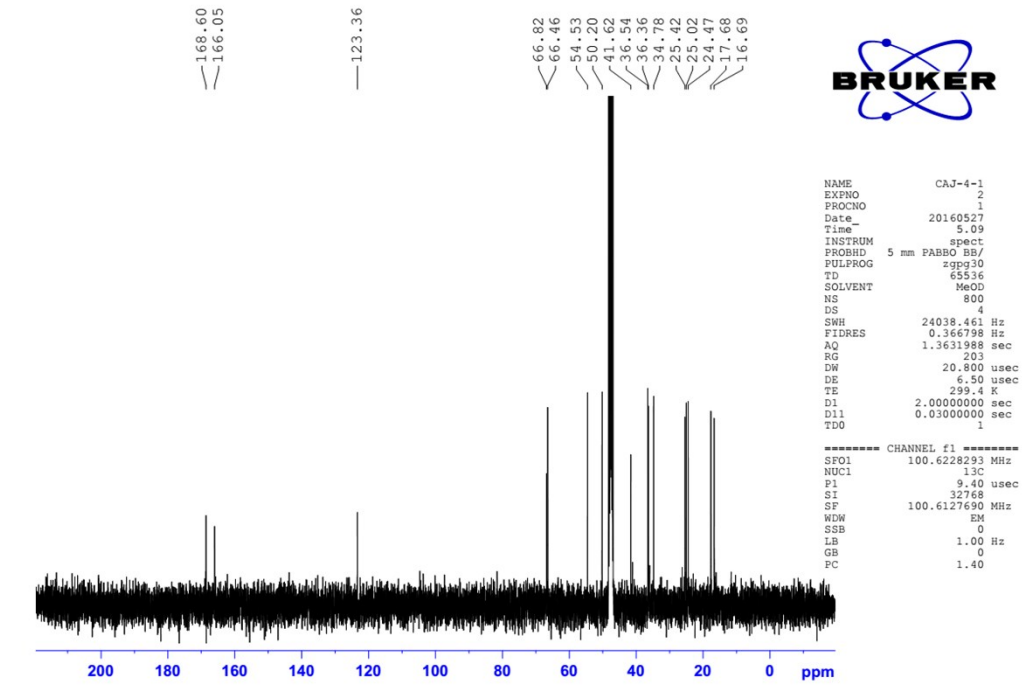


Figure S24. ^{13}C NMR spectrum (100 MHz, CD_3OD-d_4) of compound **4**

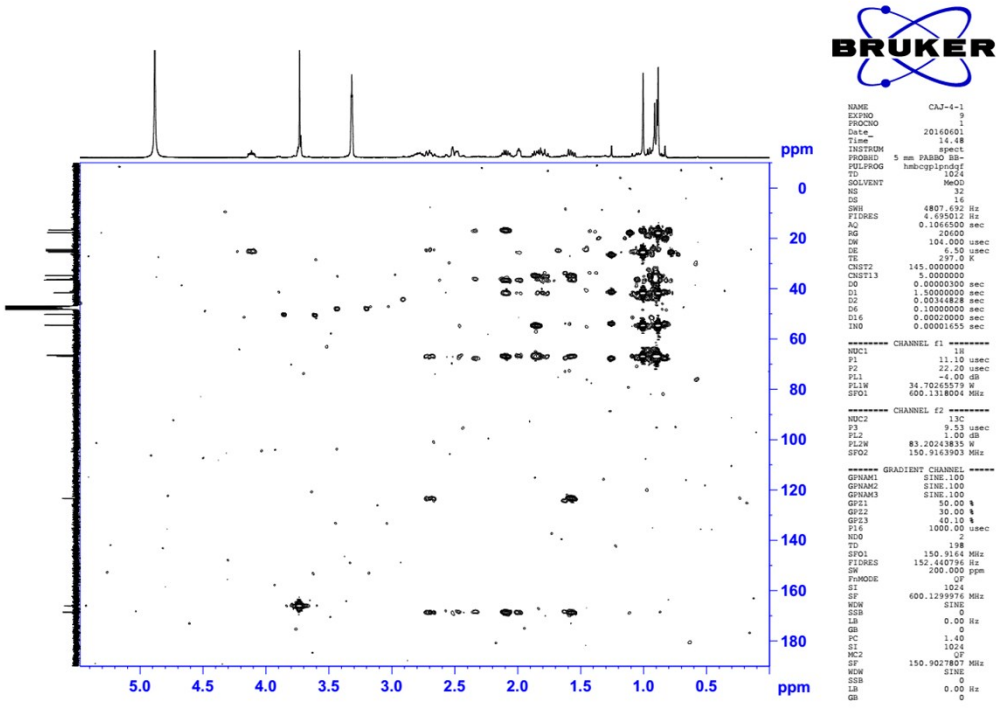


Figure S25. HMBC spectrum (600 MHz, CD_3OD-d_4) of compound **4**

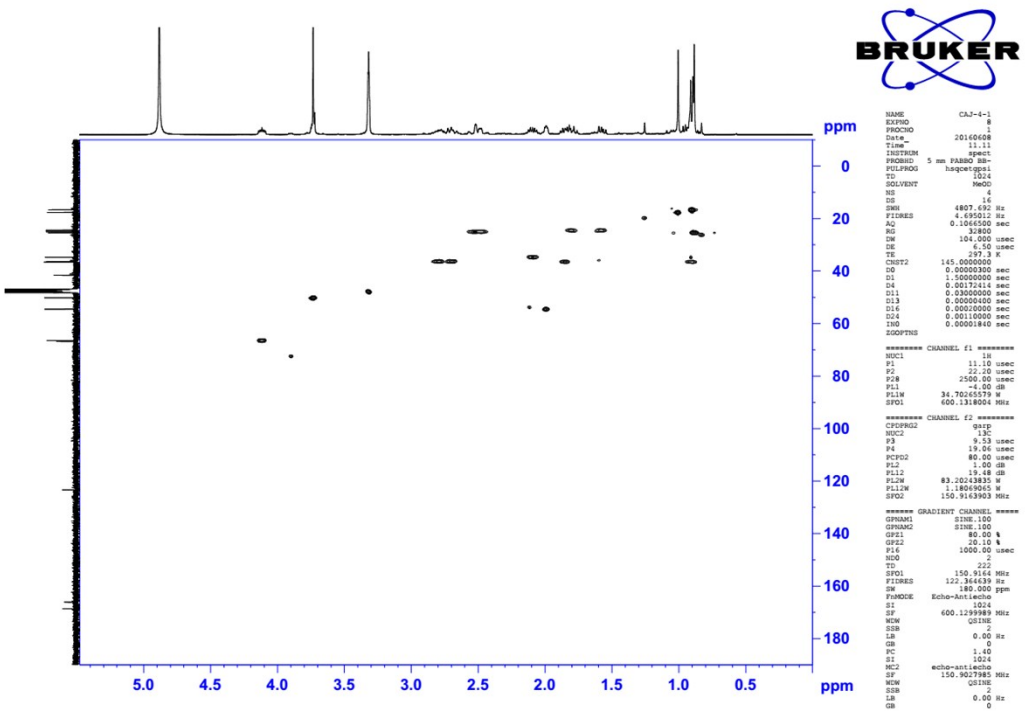


Figure S26. HSQC spectrum (600 MHz, CD_3OD-d_4) of compound **4**

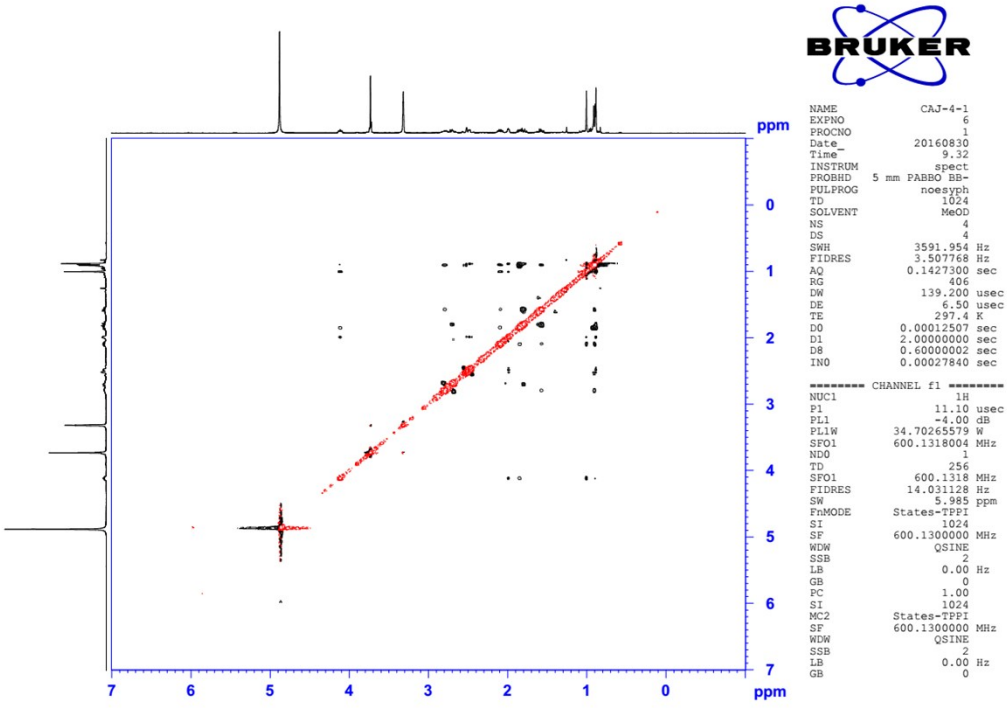
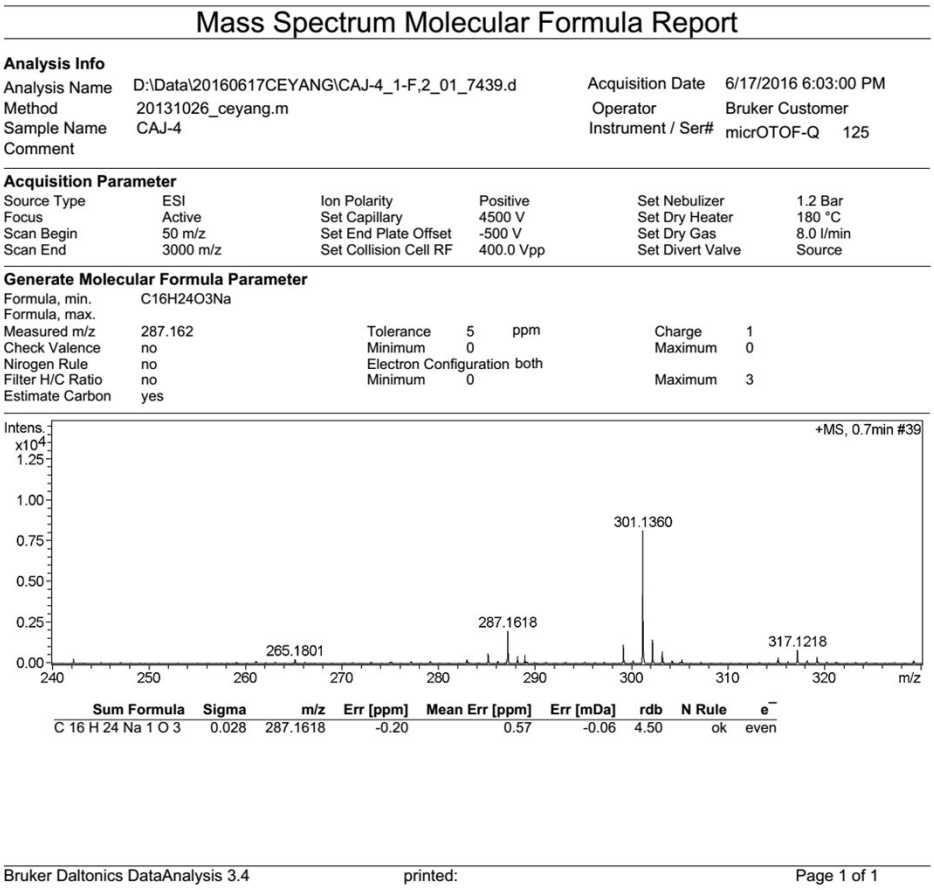


Figure S27. NOESY spectrum (600 MHz, CD_3OD-d_4) of compound **4**



Mass Spectrum Molecular Formula Report

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Sample Name	CAJ-4	Instrument / Ser#	micrOTOF-Q 125
Comment			

Acquisition Parameter

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Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	8.0 l/min
Scan End	3000 m/z	Set Collision Cell RF	400.0 Vpp	Set Divert Valve	Source

Generate Molecular Formula Parameter

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Formula, max.							
Measured m/z	287.162	Tolerance	5	ppm	Charge	1	
Check Valence	no	Minimum	0		Maximum	0	
Nirogen Rule	no	Electron Configuration	both				
Filter H/C Ratio	no	Minimum	0		Maximum	3	
Estimate Carbon	yes						

Sum Formula	Sigma	m/z	Err [ppm]	Mean Err [ppm]	Err [mDa]	rdb	N Rule	e⁻
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Figure S28. HRESIMS spectrum of compound **4**

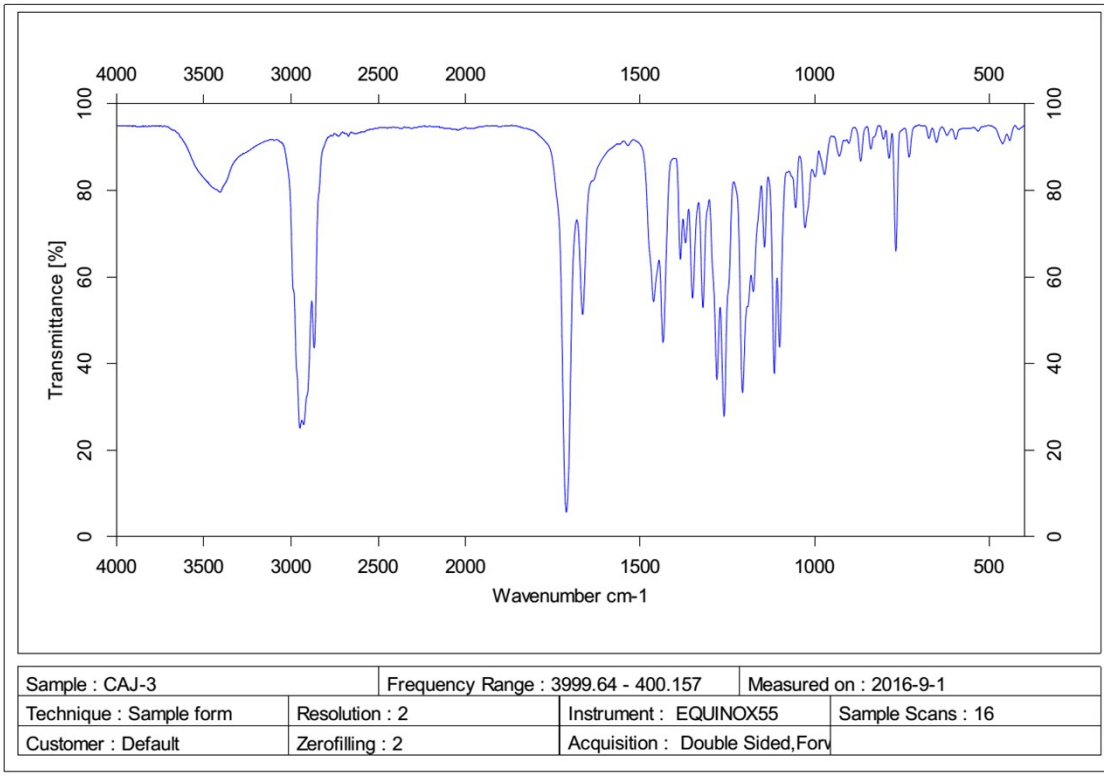


Figure S29. IR spectrum of compound **4**

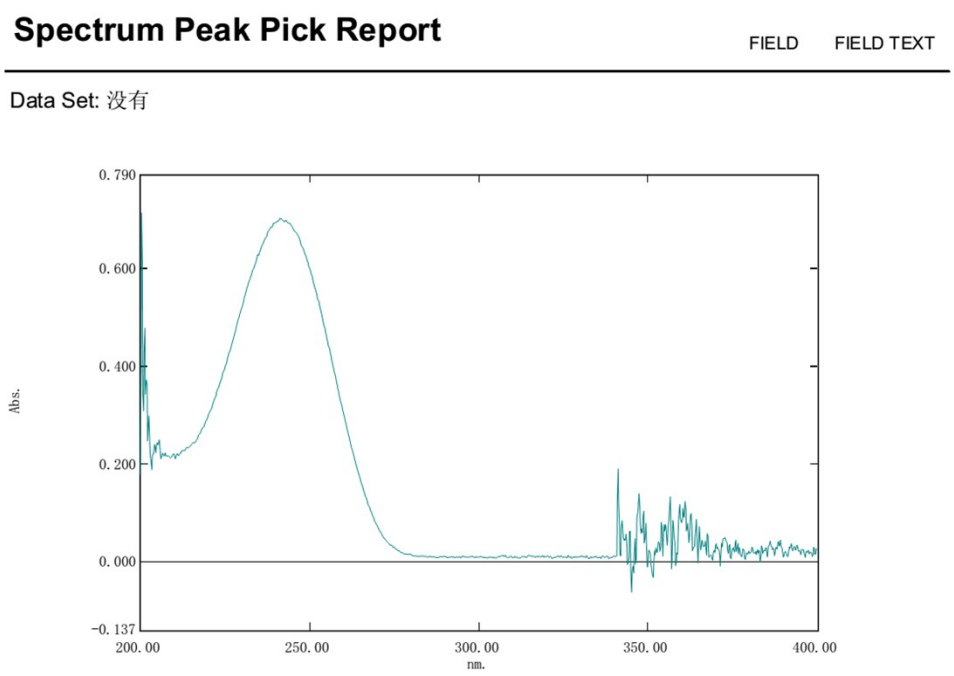


Figure S30. UV spectrum of compound **4**

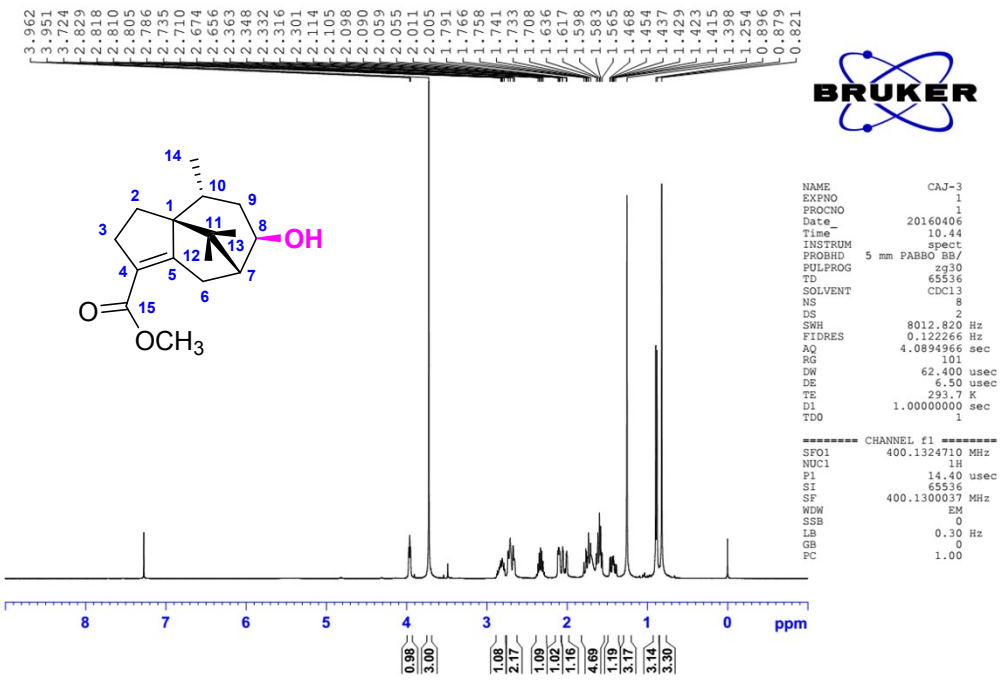


Figure S31. ^{1}H NMR spectrum (400 MHz, $CDCl_3$-*d*) of compound **5**

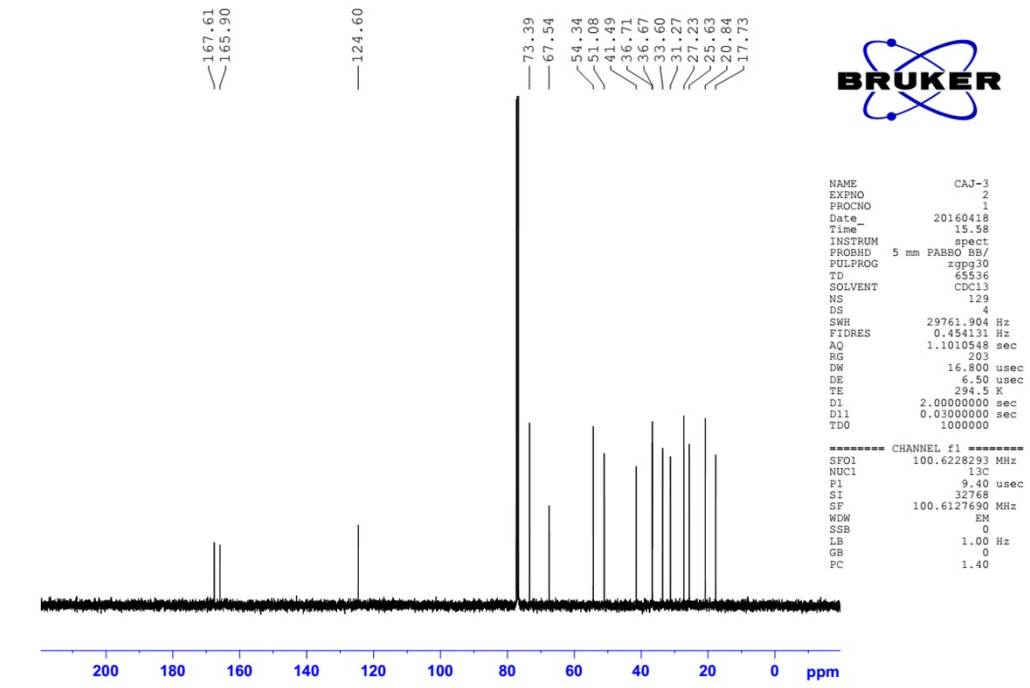


Figure S32. ^{13}C NMR spectrum (100 MHz, $CDCl_3$-*d*) of compound **5**

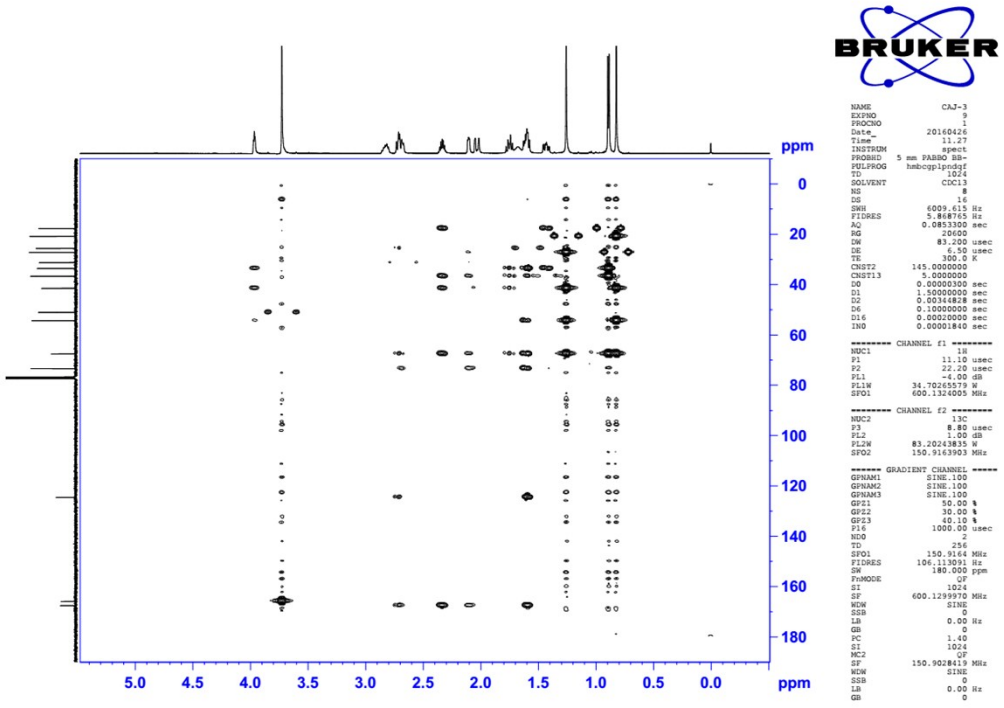


Figure S33. HMBC spectrum (600 MHz, $CDCl_3$-*d*) of compound **5**

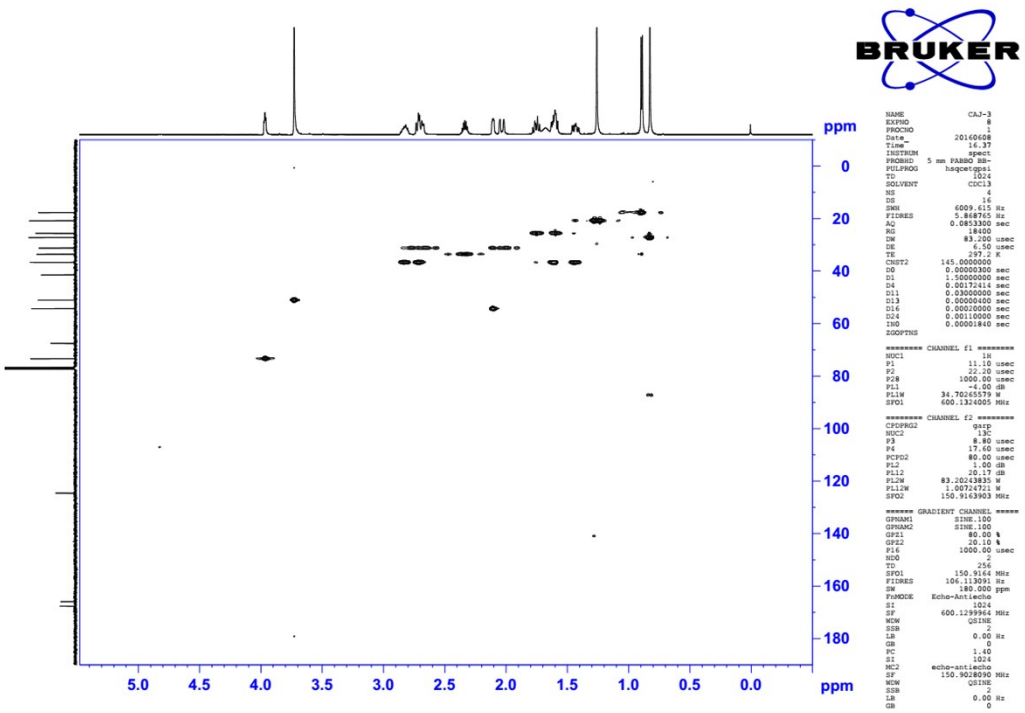


Figure S34. HSQC spectrum (600 MHz, $CDCl_3$-*d*) of compound **5**

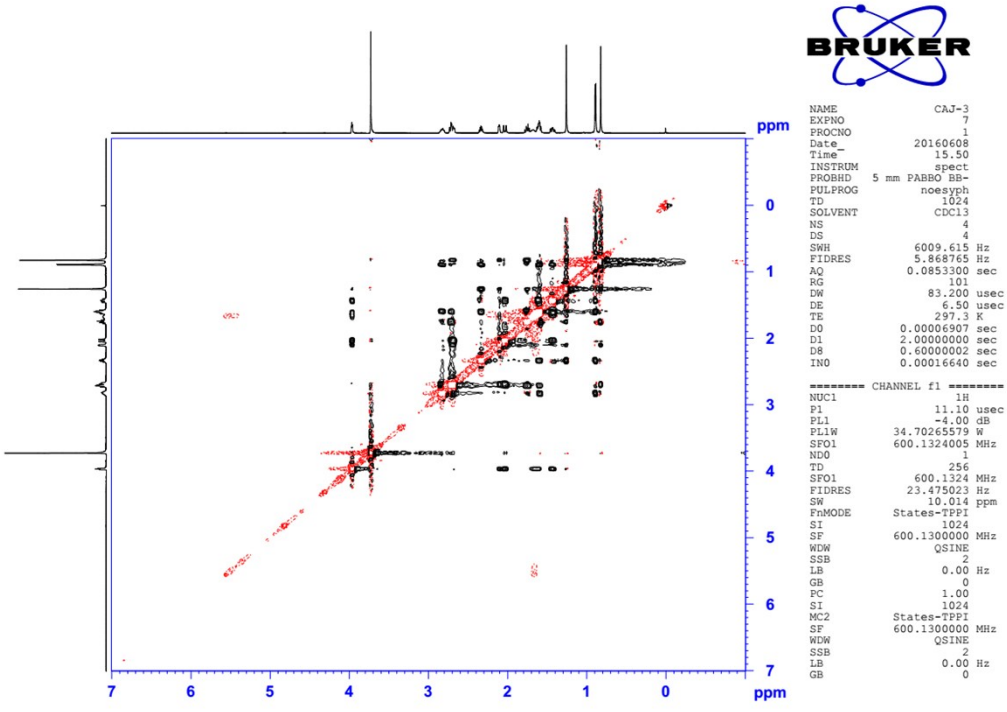


Figure S35. NOESY spectrum (600 MHz, $CDCl_3$-*d*) of compound **5**

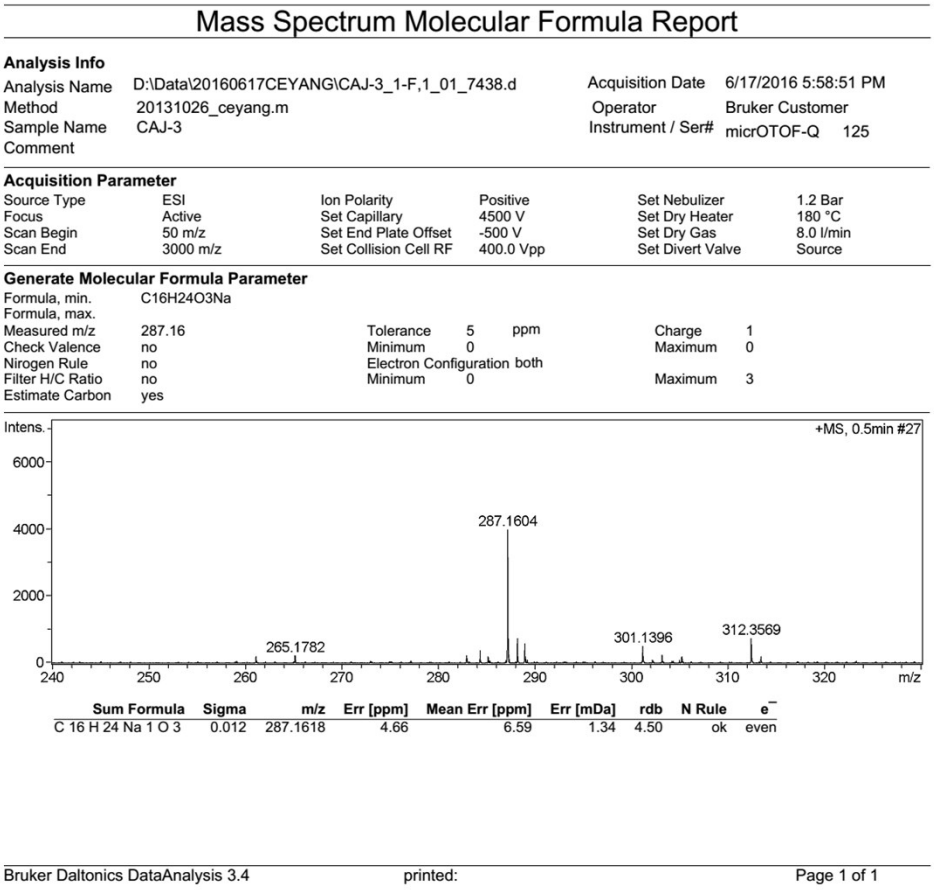


Figure S36. HRESIMS spectrum of compound **5**

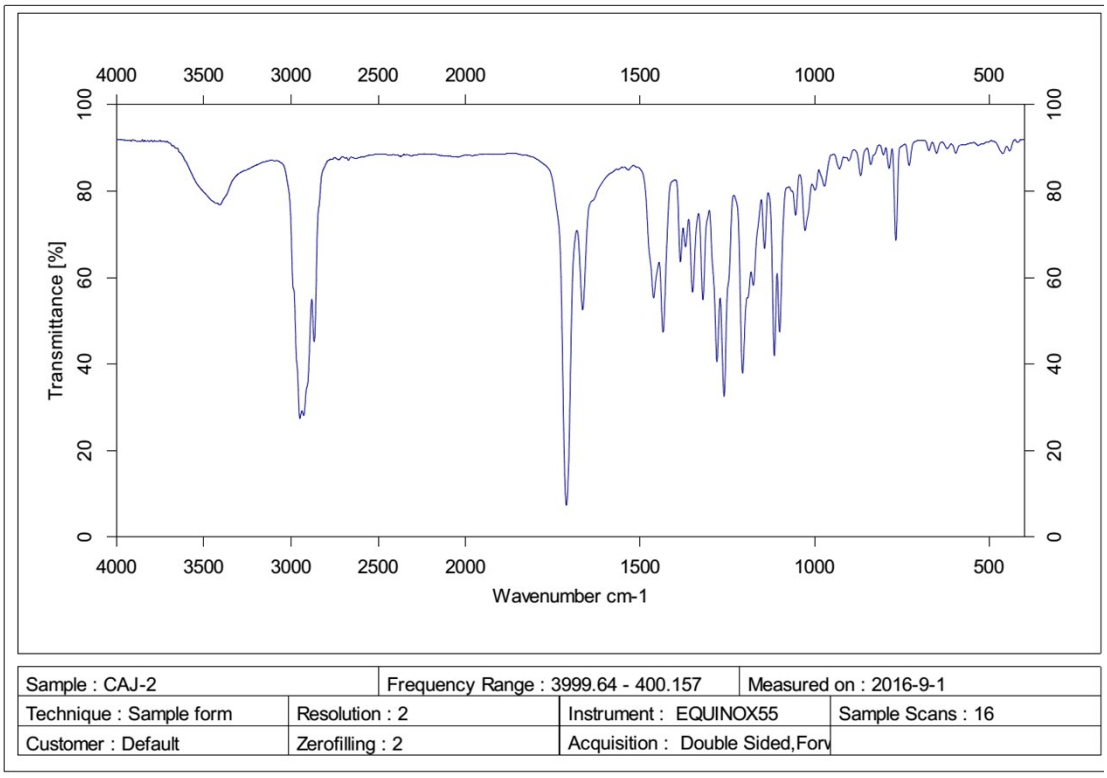


Figure S37. IR spectrum of compound **5**

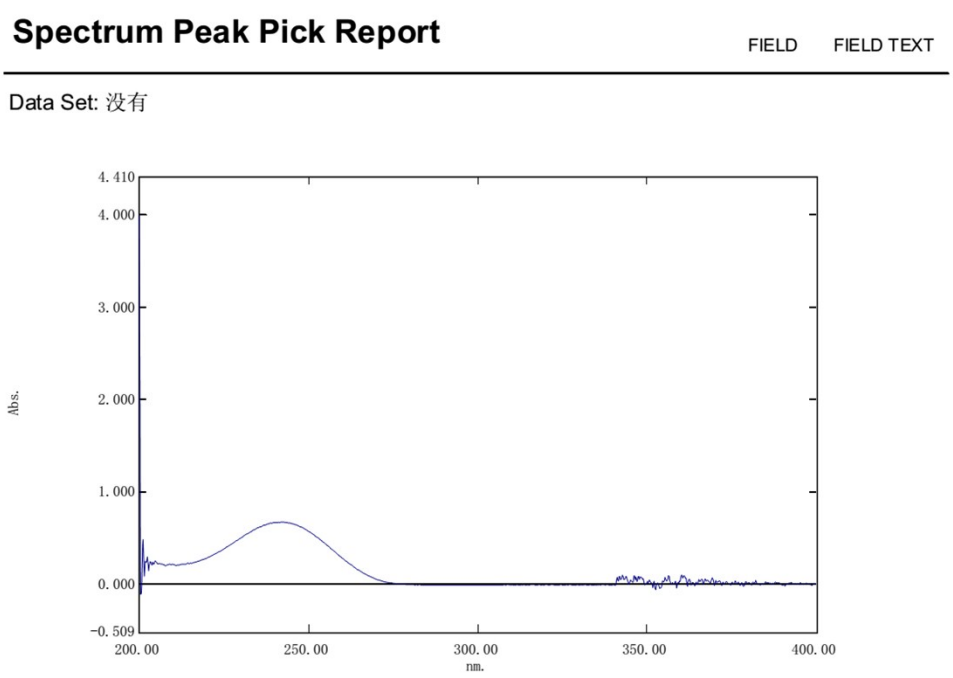


Figure S38. UV spectrum of compound **5**

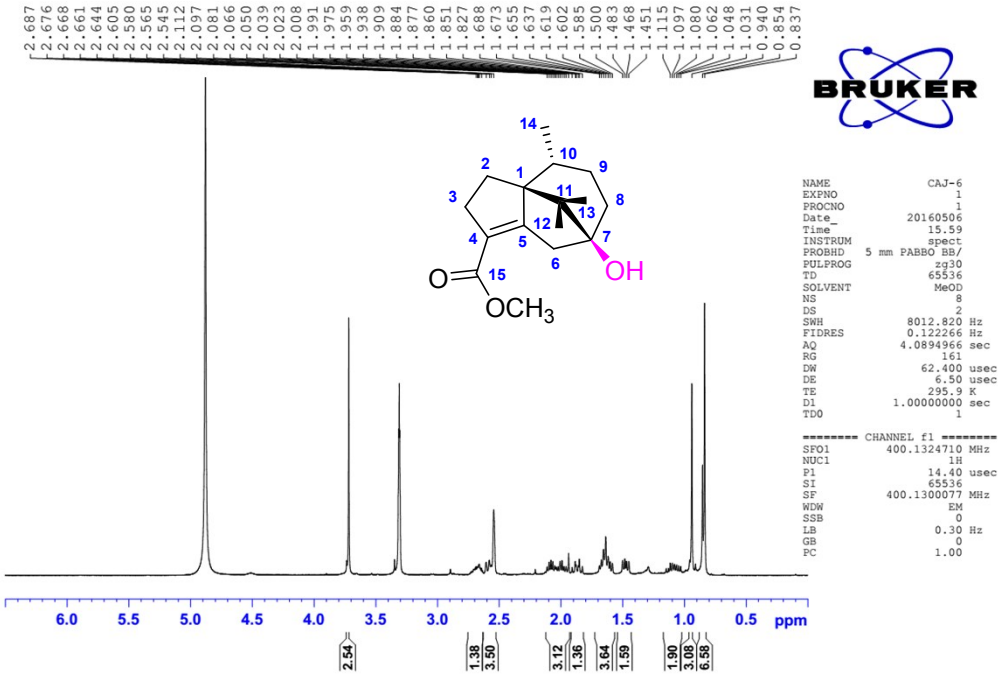


Figure S39. ^{1}H NMR spectrum (400 MHz, CD_3OD-d_4) of compound **6**

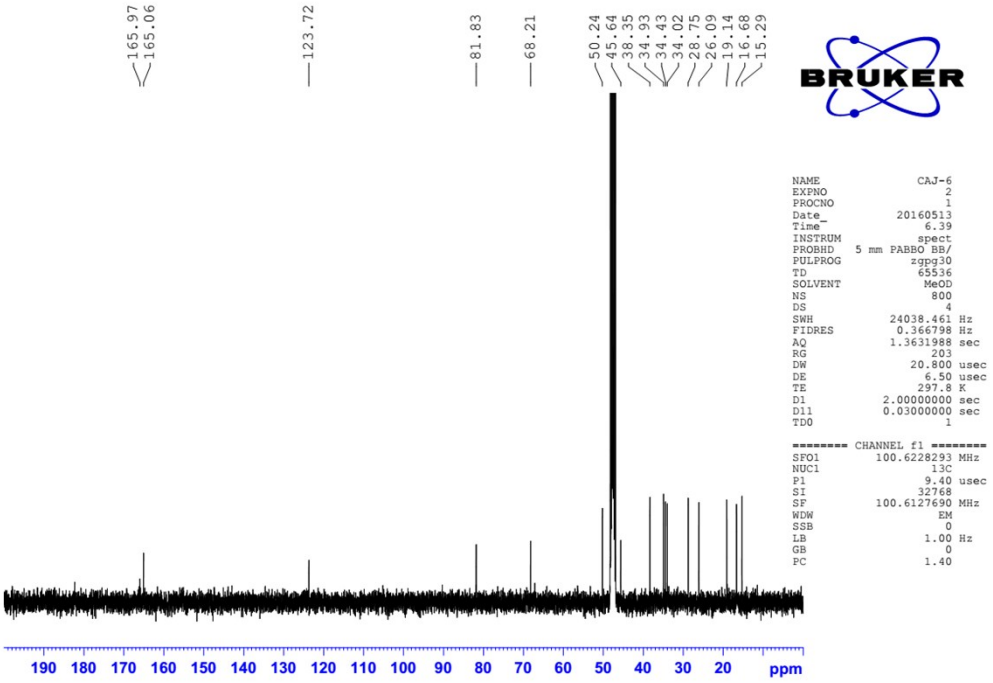


Figure S40. ^{13}C NMR spectrum (100 MHz, CD_3OD-d_4) of compound **6**

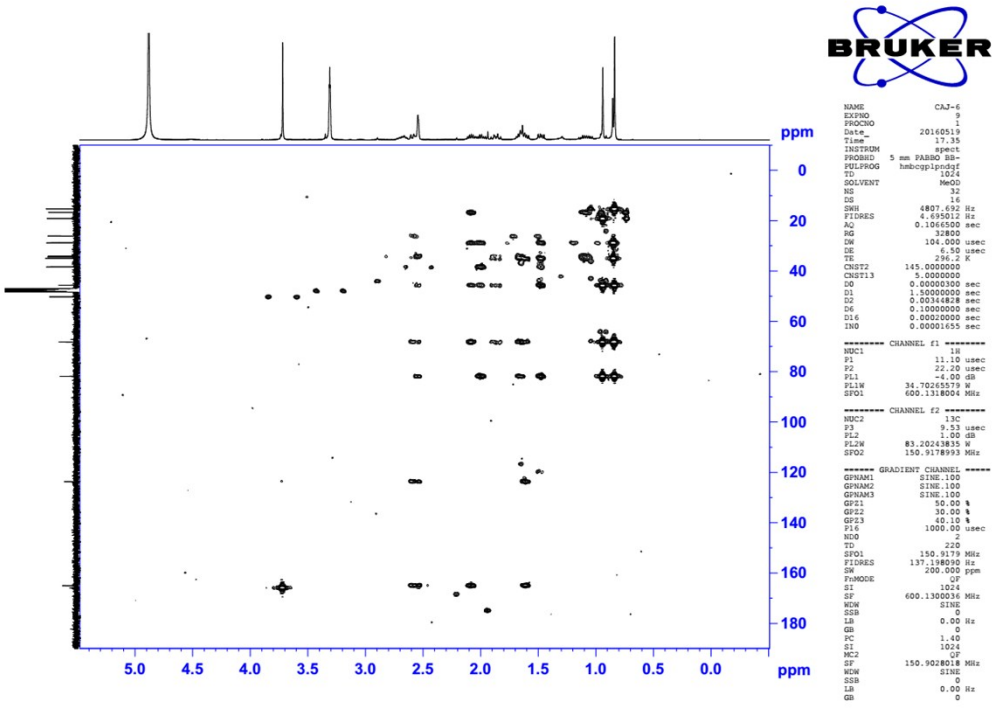


Figure S41. HMBC spectrum (600 MHz, CD_3OD-d_4) of compound **6**

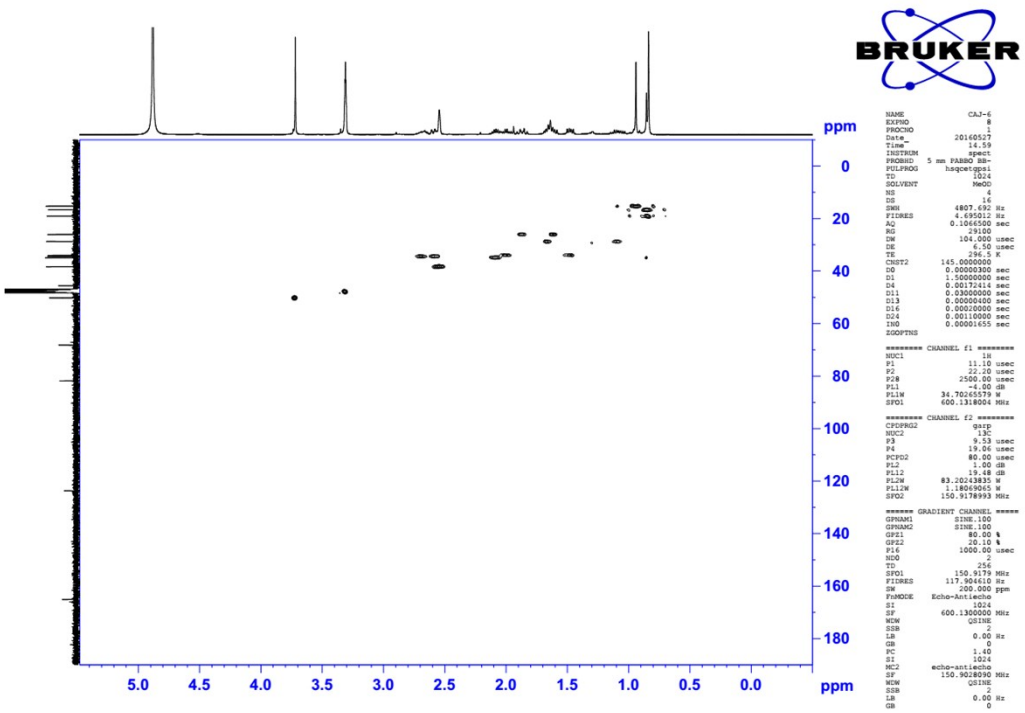
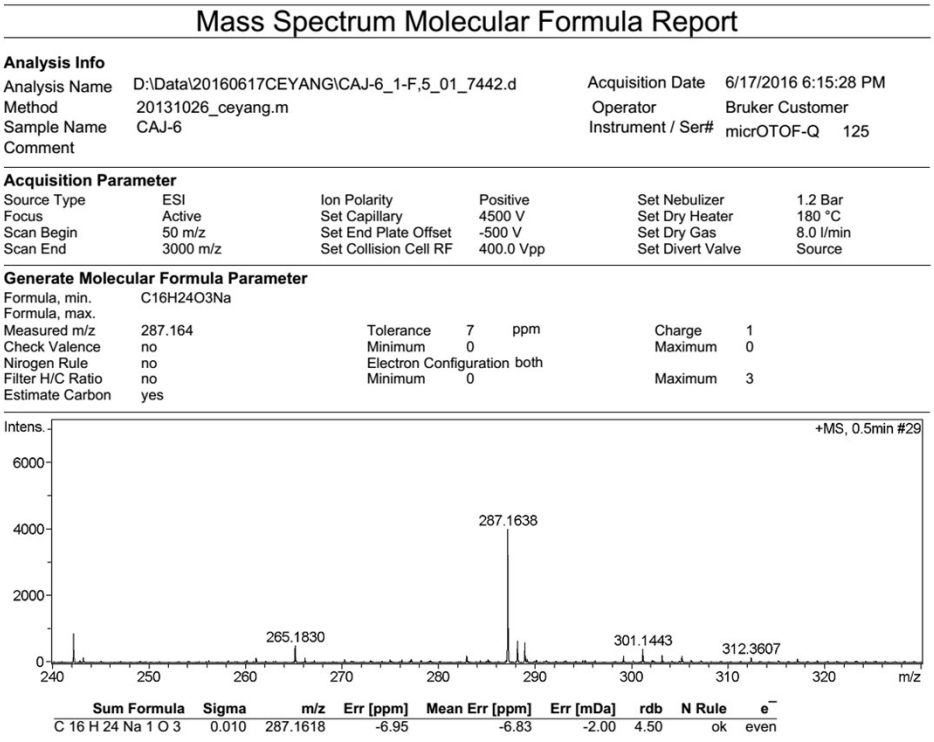


Figure S42. HSQC spectrum (600 MHz, CD_3OD-d_4) of compound **6**



Mass Spectrum Molecular Formula Report

Analysis Info

Analysis Name	D:\Data\20160617CEYANG\CAJ-6_1-F,5_01_7442.d	Acquisition Date	6/17/2016 6:15:28 PM
Method	20131026_ceyang.m	Operator	Bruker Customer
Sample Name	CAJ-6	Instrument / Ser#	micrOTOF-Q 125
Comment			

Acquisition Parameter

Source Type	ESI	Ion Polarity	Positive	Set Nebulizer	1.2 Bar
Focus	Active	Set Capillary	4500 V	Set Dry Heater	180 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	8.0 l/min
Scan End	3000 m/z	Set Collision Cell RF	400.0 Vpp	Set Divert Valve	Source

Generate Molecular Formula Parameter

Formula, min.	C16H24O3Na						
Formula, max.							
Measured m/z	287.164	Tolerance	7	ppm	Charge	1	
Check Valence	no	Minimum	0		Maximum	0	
Nirogen Rule	no	Electron Configuration	both				
Filter H/C Ratio	no	Minimum	0		Maximum	3	
Estimate Carbon	yes						

Sum Formula	Sigma	m/z	Err [ppm]	Mean Err [ppm]	Err [mDa]	rdb	N Rule	e⁻
C 16 H 24 Na 1 O 3	0.010	287.1618	-6.95	-6.83	-2.00	4.50	ok	even

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Figure S43. HRESIMS spectrum of compound **6**

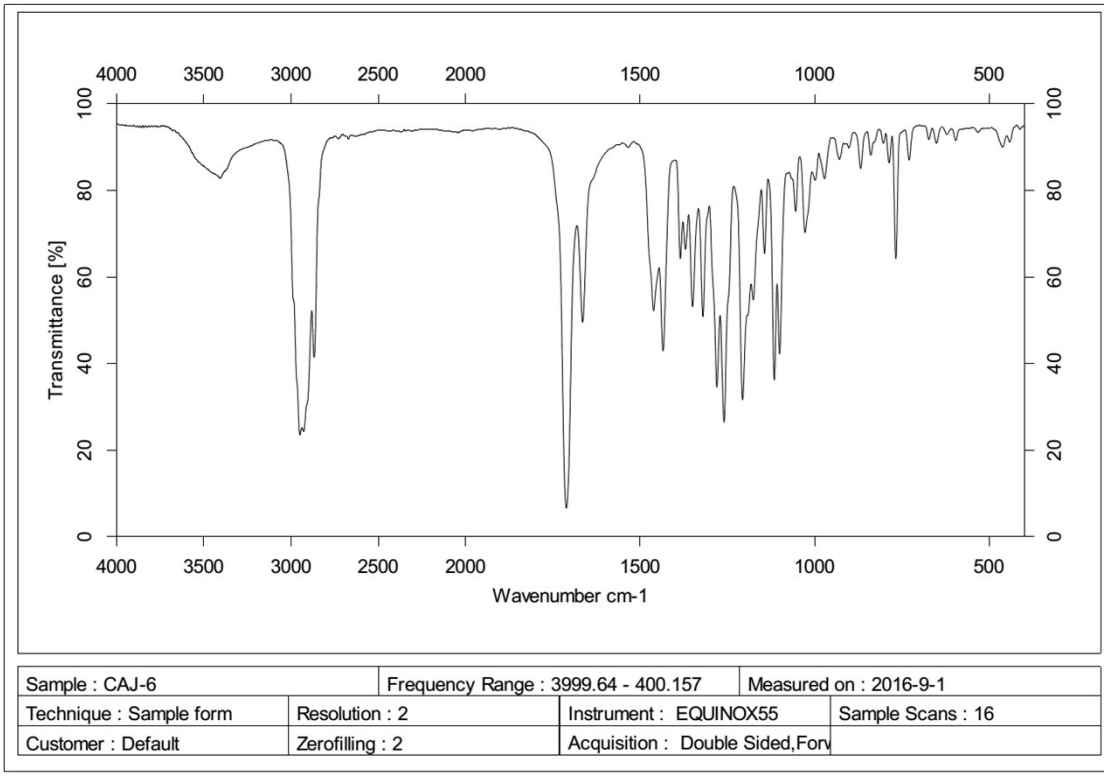


Figure S44. IR spectrum of compound **6**

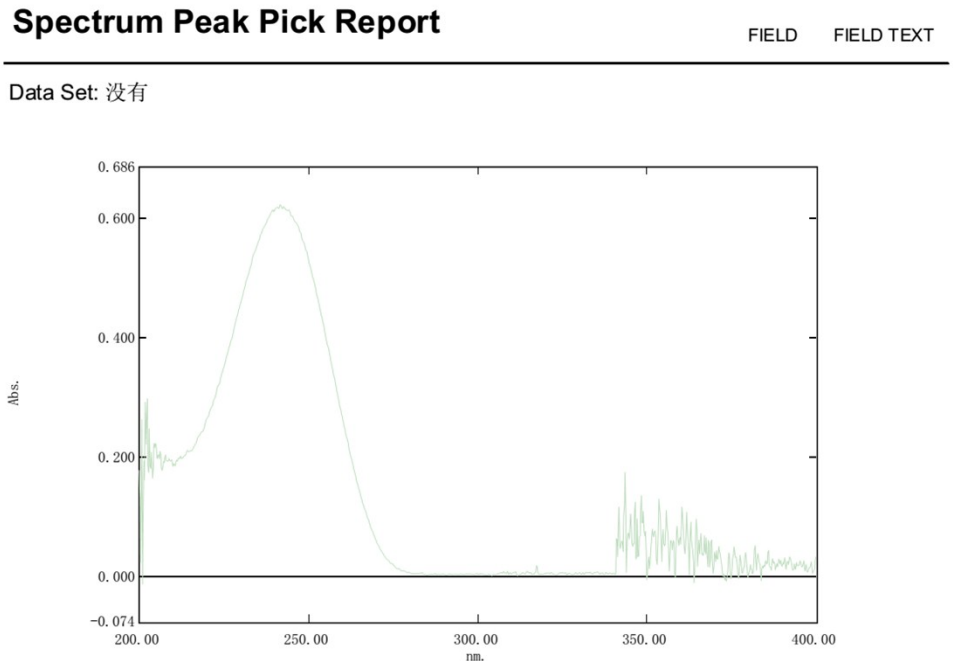


Figure S45. UV spectrum of compound **6**

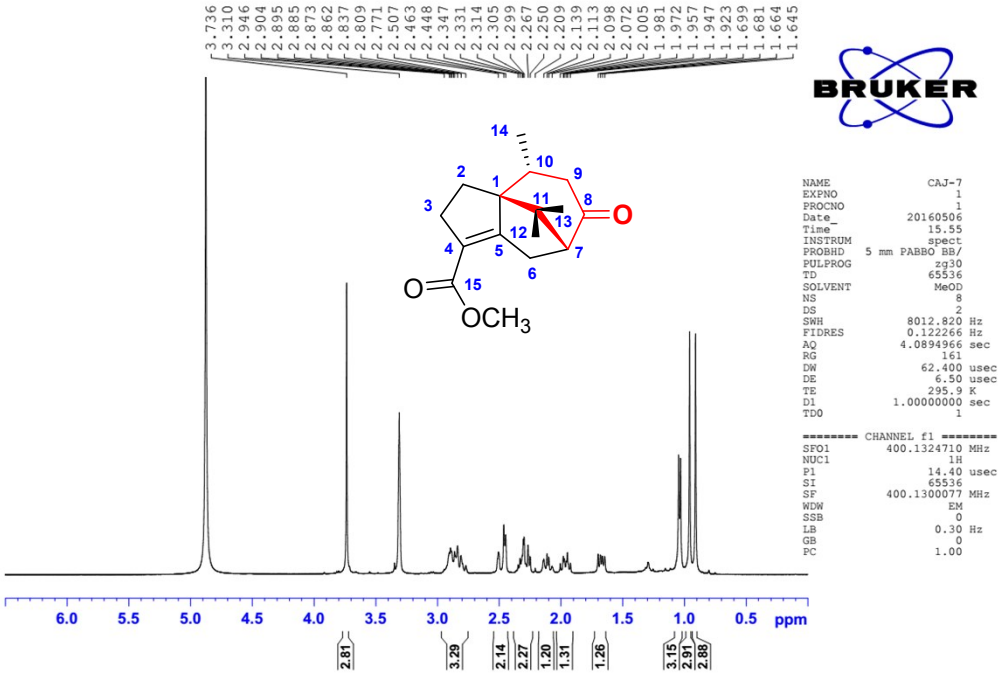


Figure S46. ^{1}H NMR spectrum (400 MHz, CD_3OD-d_4) of compound **7**

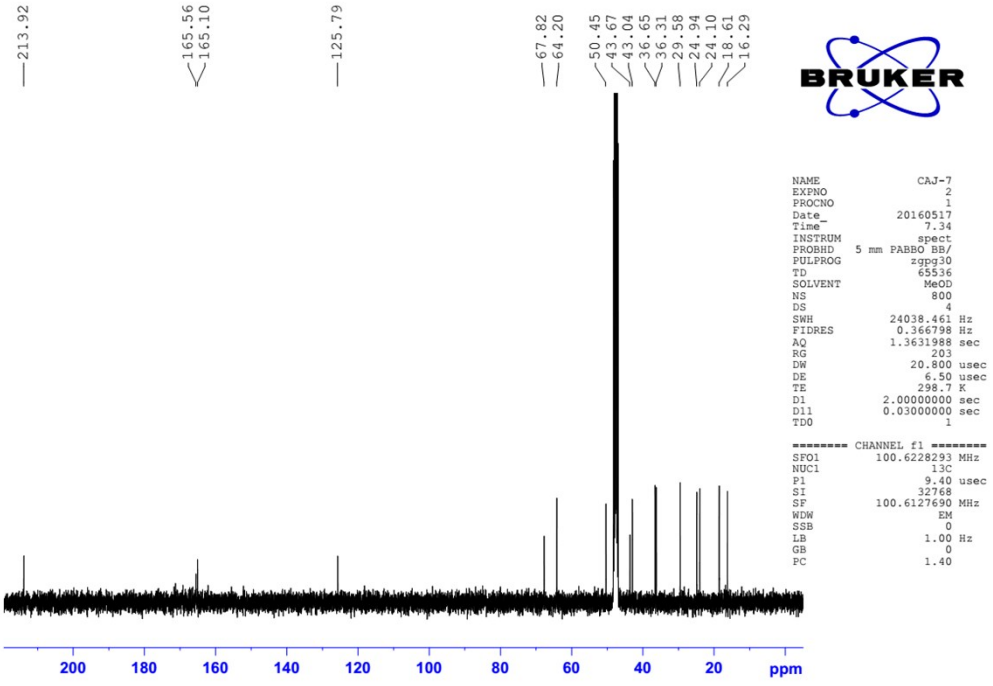


Figure S47. ^{13}C NMR spectrum (100 MHz, CD_3OD-d_4) of compound **7**

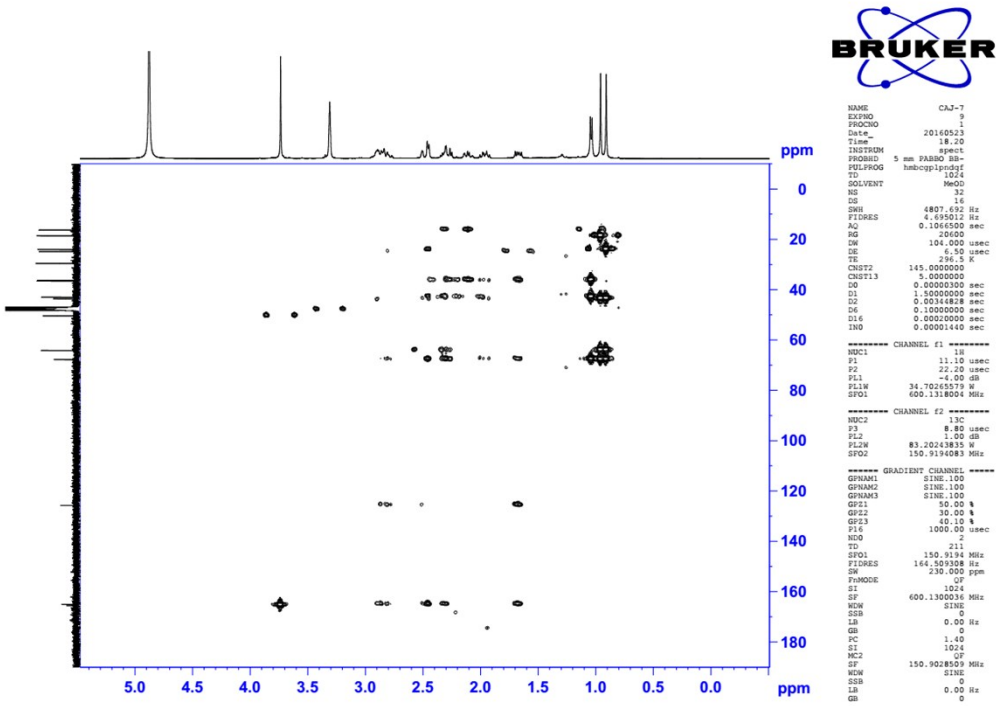


Figure S48. HMBC spectrum (600 MHz, CD_3OD-d_4) of compound **7**

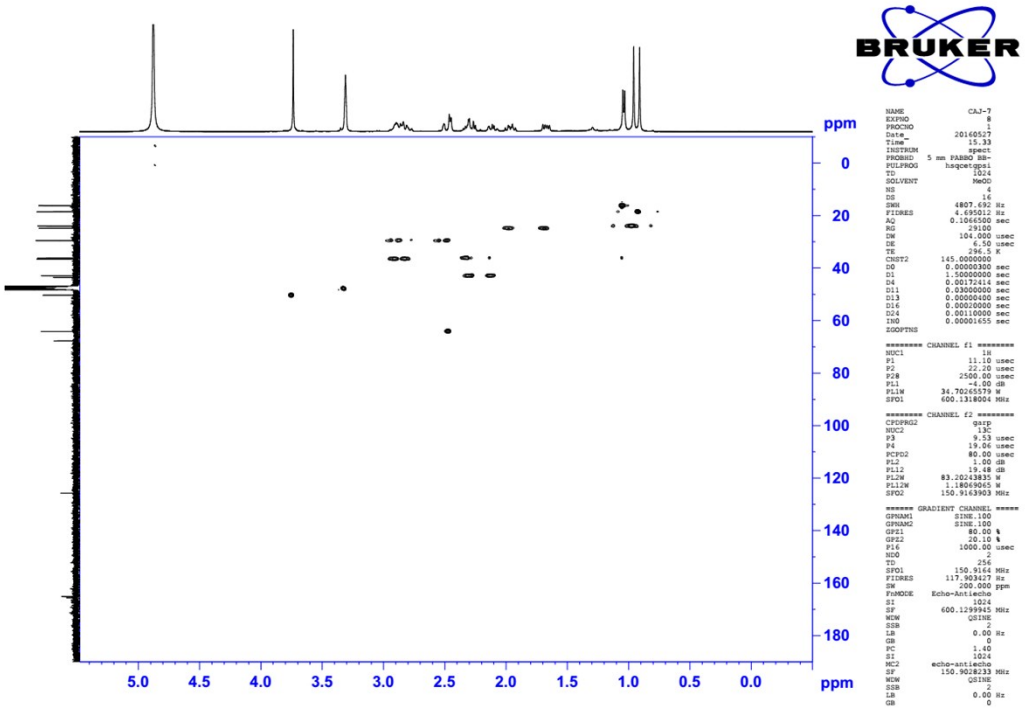


Figure S49. HSQC spectrum (600 MHz, CD_3OD-d_4) of compound **7**

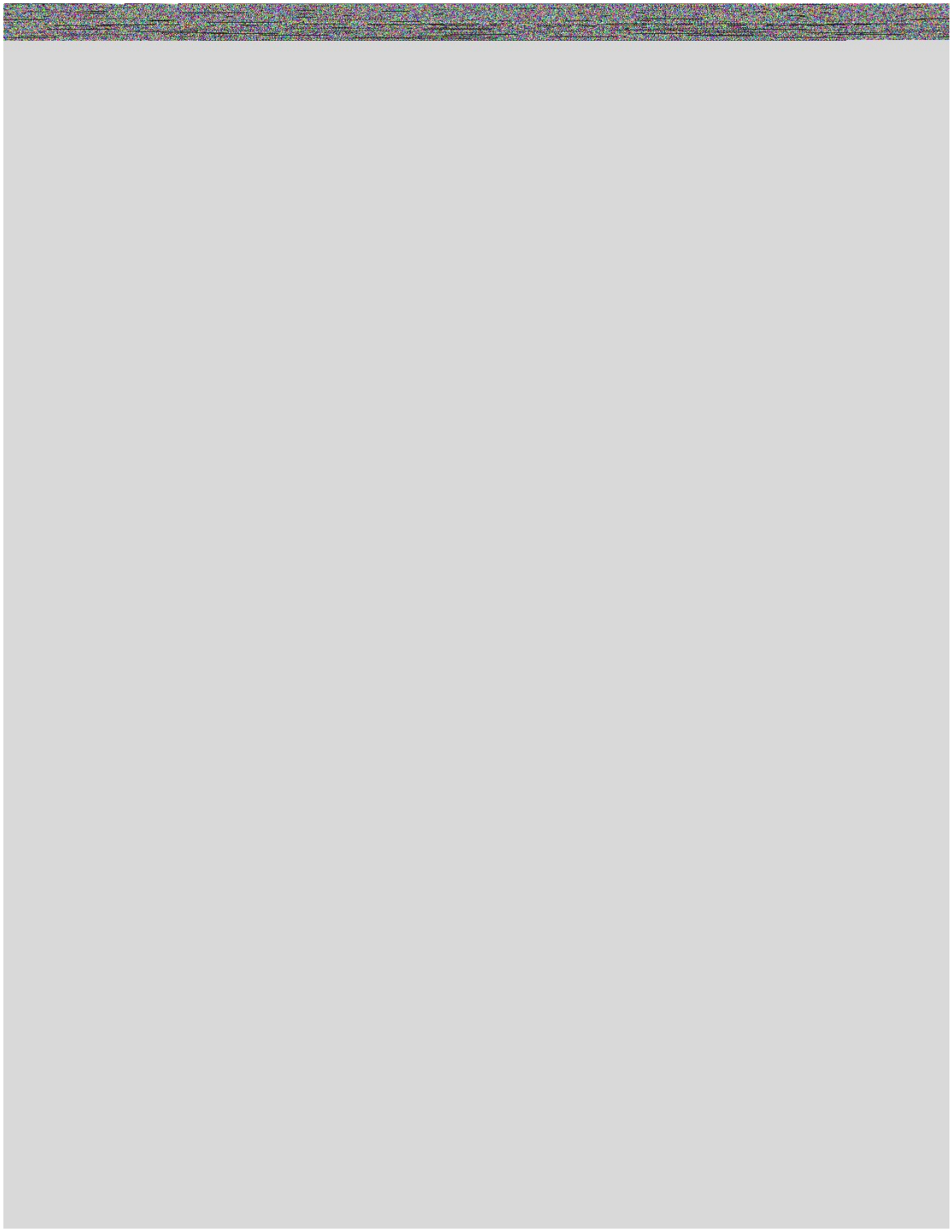


Figure S50. HRESIMS spectrum of compound **7**

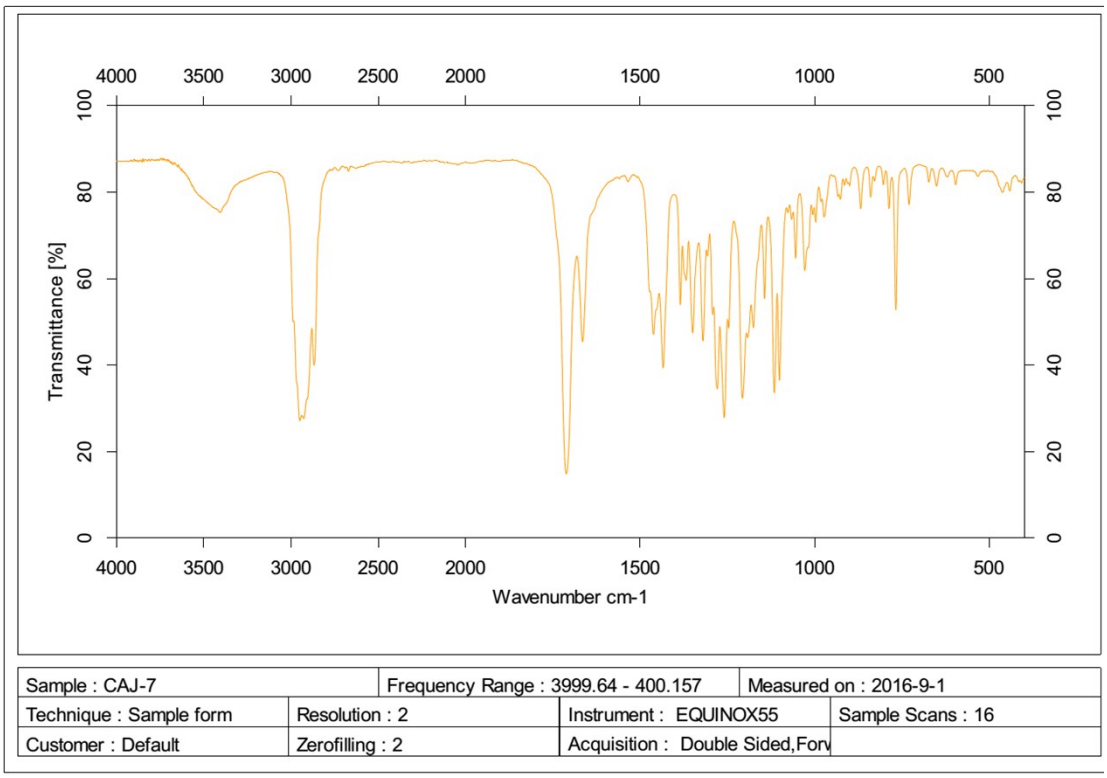


Figure S51. IR spectrum of compound **7**

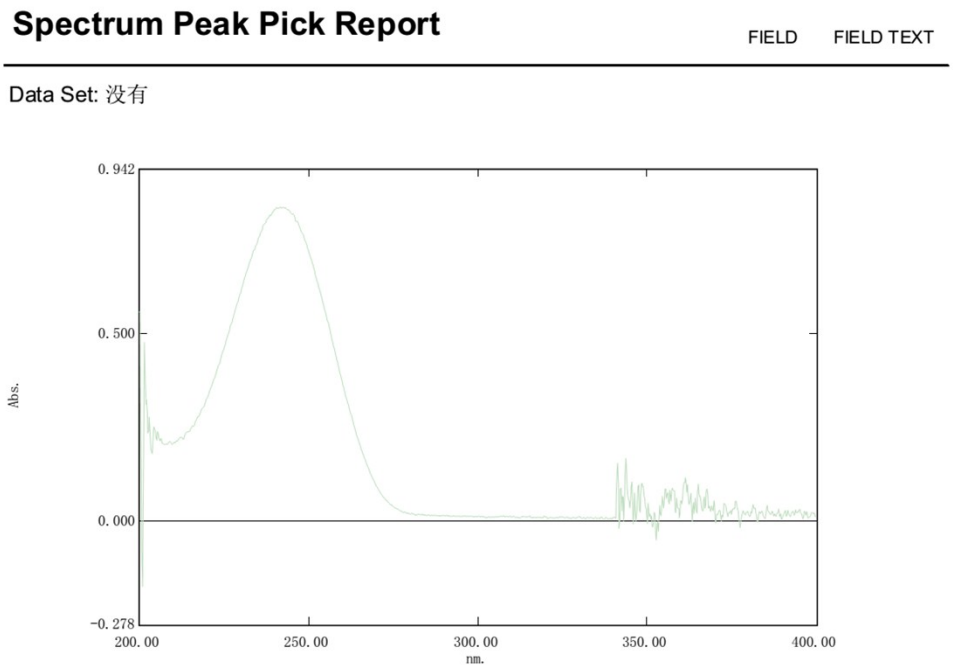


Figure S52. UV spectrum of compound **7**

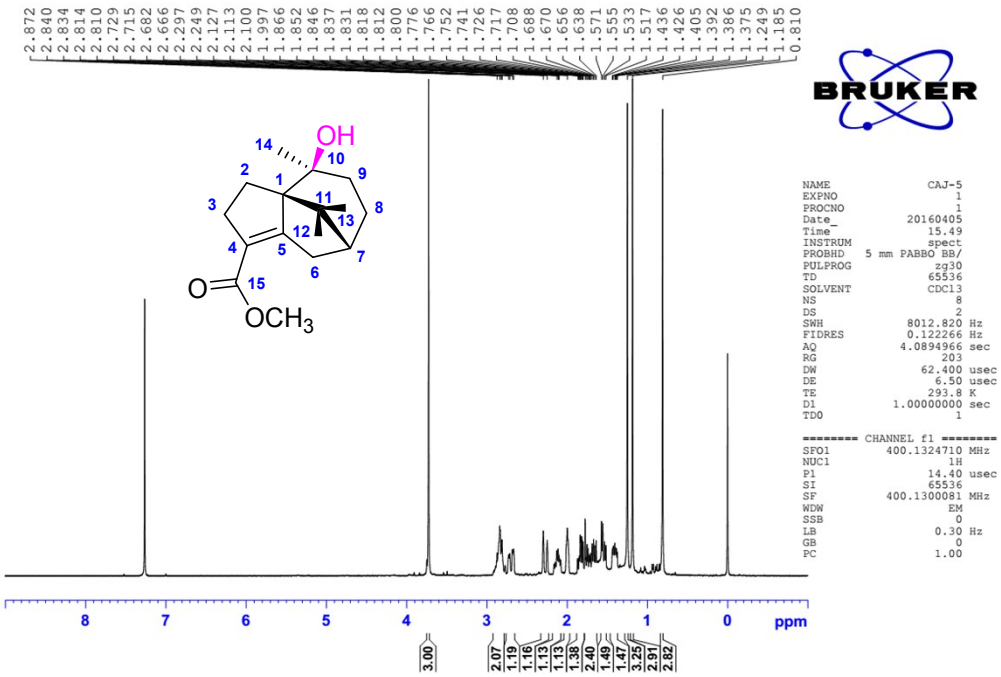


Figure S53. ^{1}H NMR spectrum (400 MHz, $CDCl_3$-*d*) of compound **8**

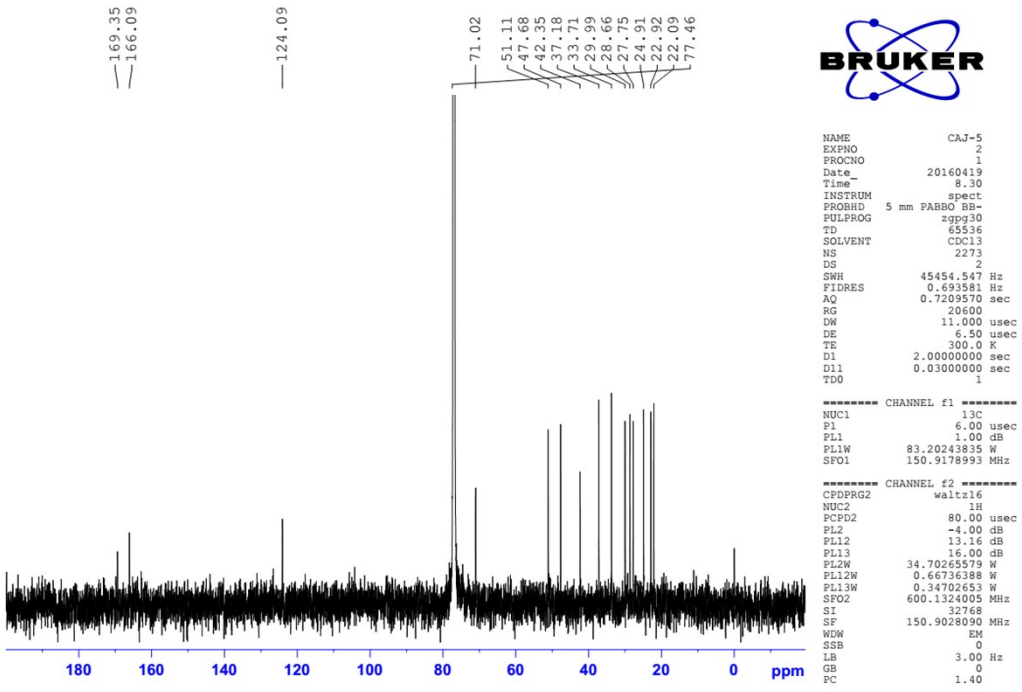


Figure S54. ^{13}C NMR spectrum (100 MHz, $CDCl_3$-*d*) of compound **8**

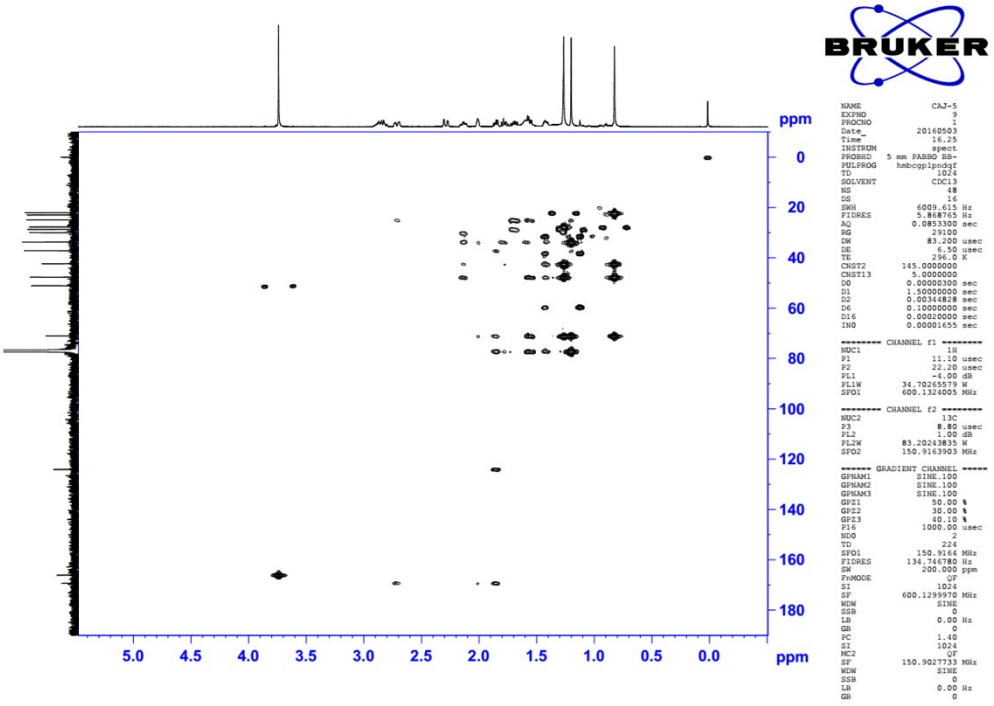


Figure S55. HMBC spectrum (600 MHz, $CDCl_3$-*d*) of compound **8**

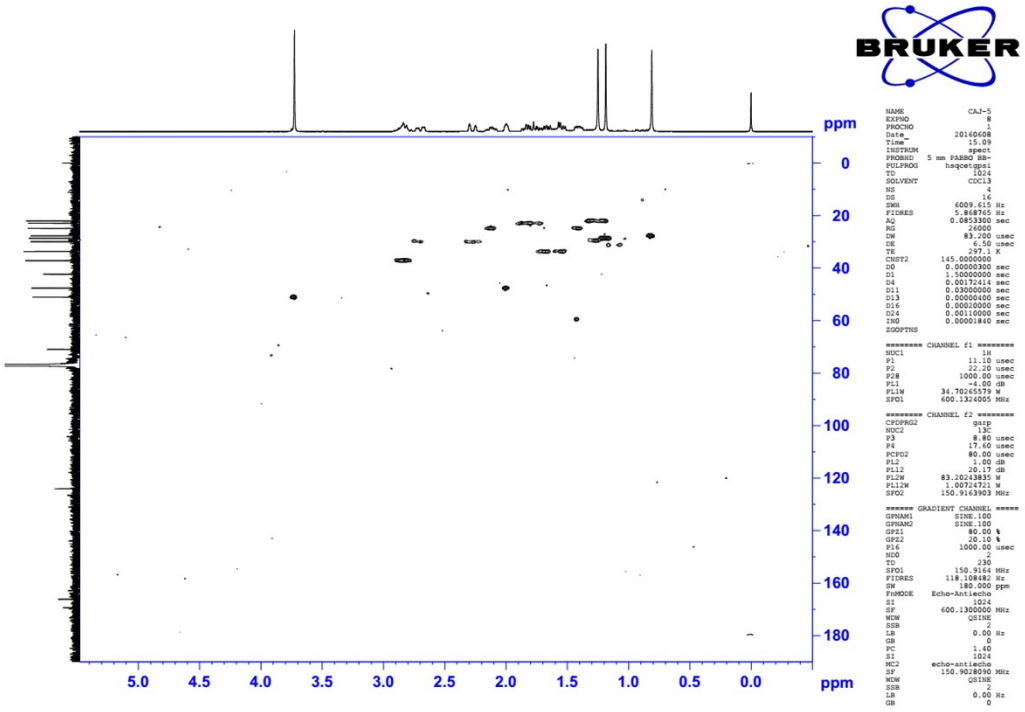


Figure S56. HSQC spectrum (600 MHz, $CDCl_3$-*d*) of compound **8**

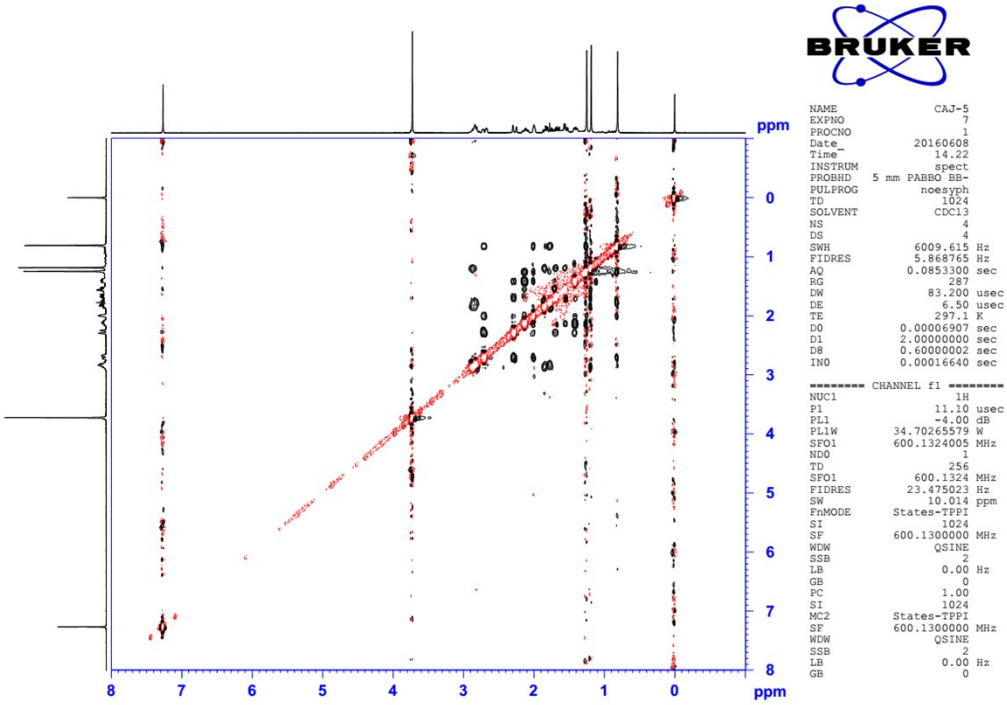


Figure S57. NOESY spectrum (600 MHz, $CDCl_3$-*d*) of compound **8**

Mass Spectrum Molecular Formula Report

Analysis Info

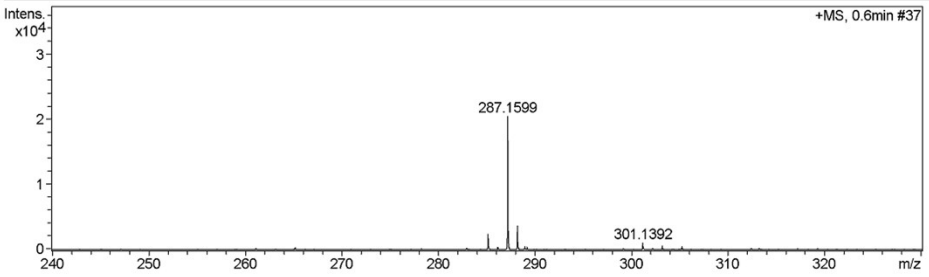
Analysis Name	D:\Data\20160617CEYANG\CAJ-5_1-F,4_01_7441.d	Acquisition Date	6/17/2016 6:11:18 PM
Method	20131026_ceyang.m	Operator	Bruker Customer
Sample Name	CAJ-5	Instrument / Ser#	micrOTOF-Q 125
Comment			

Acquisition Parameter

Source Type	ESI	Ion Polarity	Positive	Set Nebulizer	1.2 Bar
Focus	Active	Set Capillary	4500 V	Set Dry Heater	180 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	8.0 l/min
Scan End	3000 m/z	Set Collision Cell RF	400.0 Vpp	Set Divert Valve	Source

Generate Molecular Formula Parameter

Formula, min.	C16H24O3Na				
Formula, max.					
Measured m/z	287.16	Tolerance	7 ppm	Charge	1
Check Valence	no	Minimum	0	Maximum	0
Nirogen Rule	no	Electron Configuration	both		
Filter H/C Ratio	no	Minimum	0	Maximum	3
Estimate Carbon	yes				



Sum Formula	Sigma	m/z	Err [ppm]	Mean Err [ppm]	Err [mDa]	rdb	N Rule	e⁻
C 16 H 24 Na 1 O 3	0.001	287.1618	6.53	6.94	1.87	4.50	ok	even

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Figure S58. HRESIMS spectrum of compound **8**

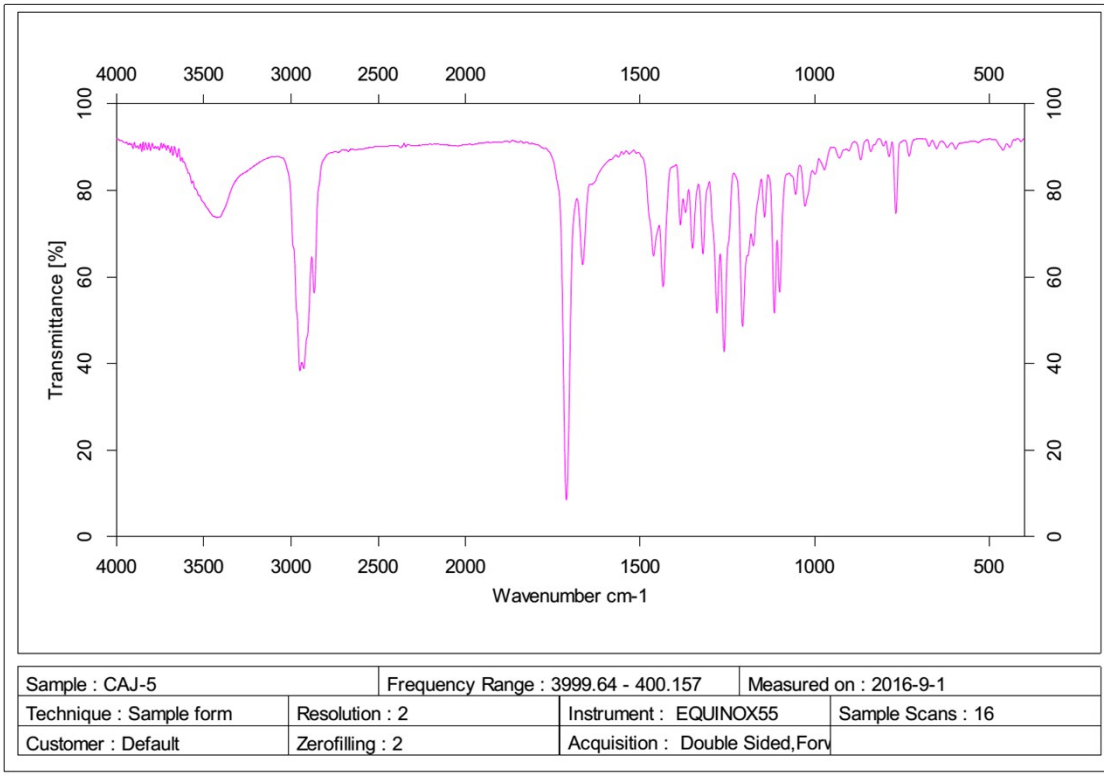


Figure S59. IR spectrum of compound **8**

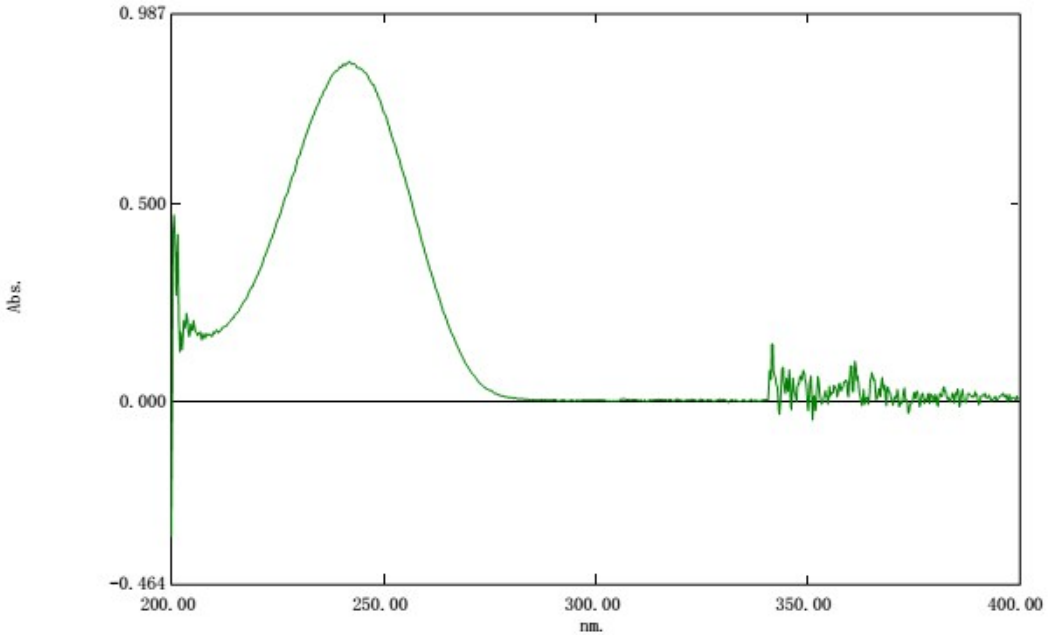


Figure S60. UV spectrum of compound **8**

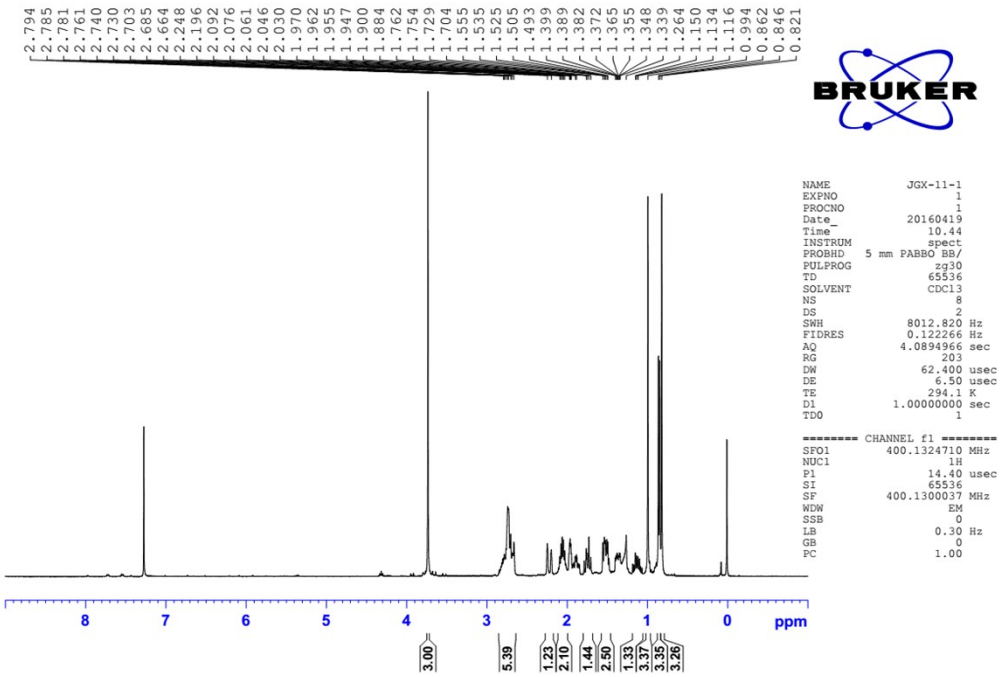


Figure S61. ^{1}H NMR spectrum (400 MHz, $CDCl_3$-*d*) of **MC**

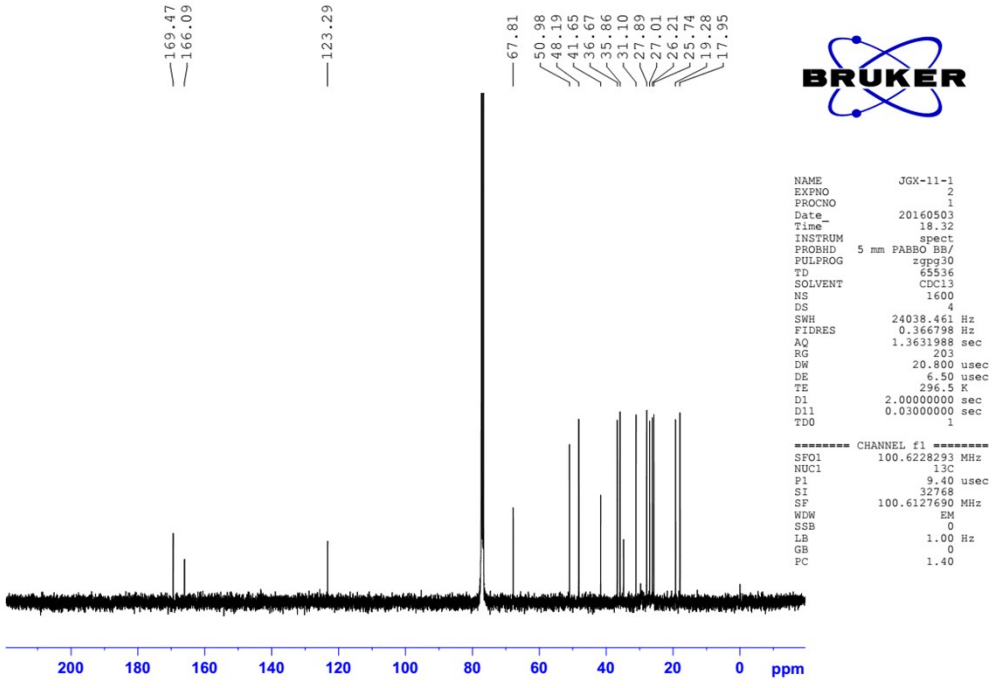


Figure S62. ^{13}C NMR spectrum (100 MHz, $CDCl_3$-*d*) of **MC**

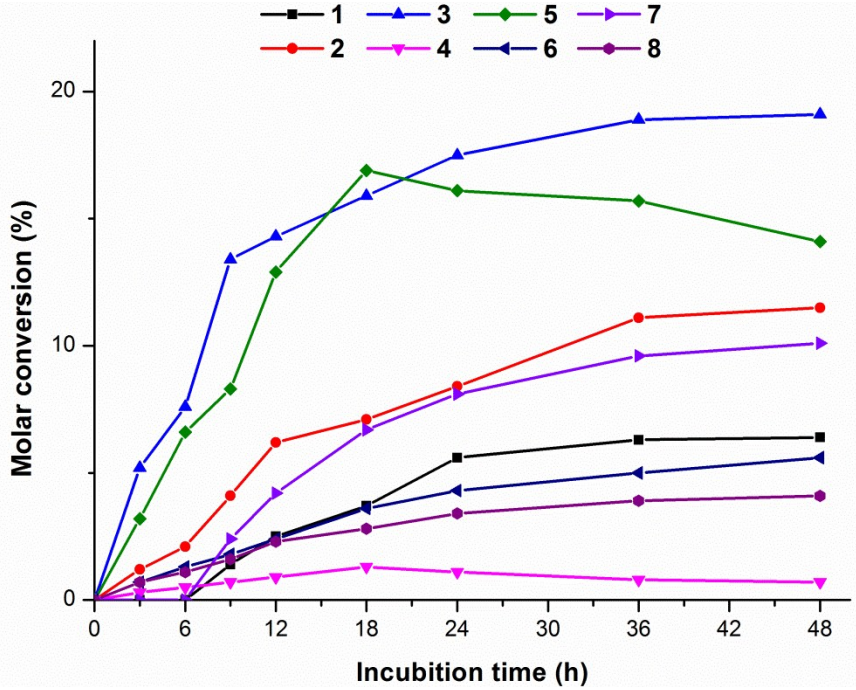


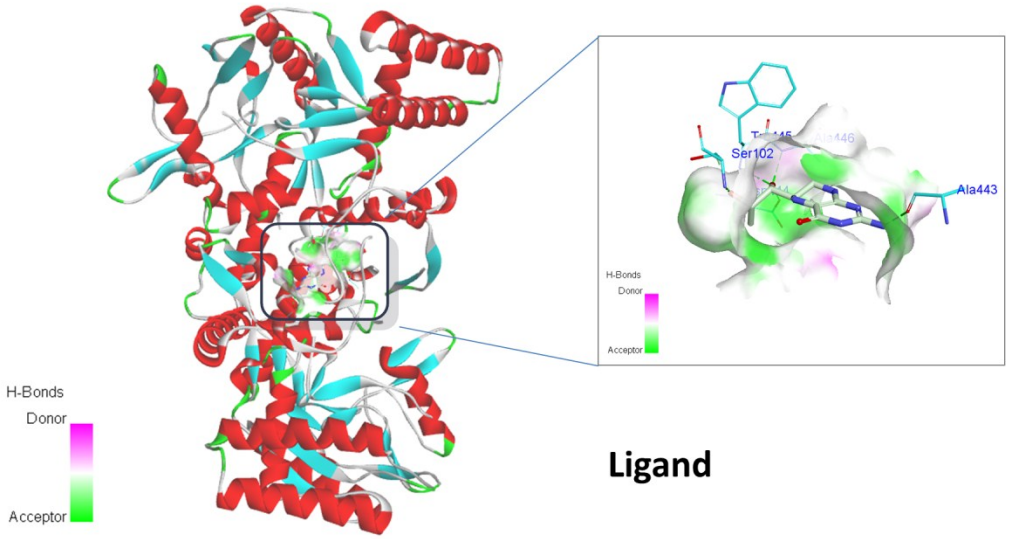
Figure S63. Enlarge figure of time courses of biotransformation.

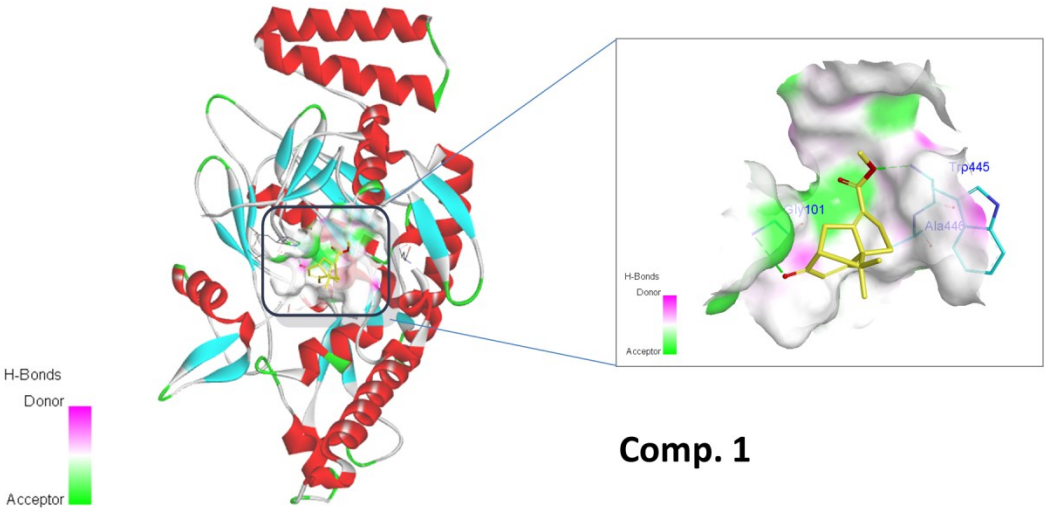
The possible action mechanism of the active compounds was explored by using molecular docking studies. Through the virtual screening of a large number of anti-thrombotic targets, we found that compound **1** had better ligand-receptor interaction with eNOS.

Endothelial nitric oxide synthase (eNOS) generates NO, which plays a crucial role in maintaining vascular function and exerting an antithrombotic action [1]. Nitric oxide (NO) is a chief signaling molecule in cardiovascular regulation. In addition to relaxing vascular tone, NO regulates cardiac contractility, platelet aggregation, angiogenesis, and vascular smooth muscle proliferation. Thus, understanding the mechanisms of eNOS activation and regulation has been the focus of cardiovascular NO research [2-4]. And the literature indicates that eNOS and PT, TT, APTT are related [5].

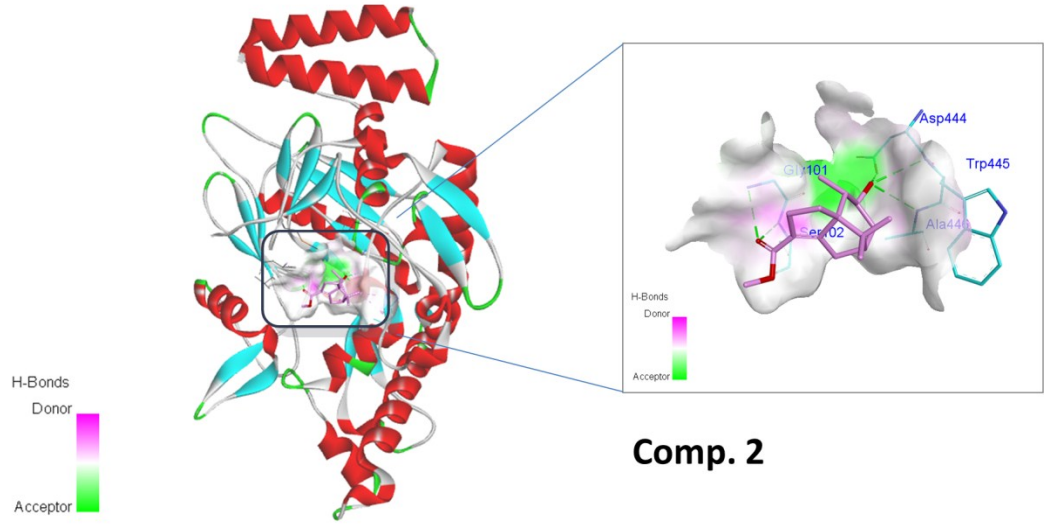
The crystal structure of endothelial nitric oxide synthase (eNOS) was obtained from RCSB Protein Data Bank with PDB Code: 4D1P. The protein was also optimized using the Discovery Studio 3.0 program (Accelrys Inc., San Diego, USA) to remove water molecules and add all hydrogen atoms, and the structures were saved in PDB format for further docking studies. The Molegro Virtual Docker 4.0 (Molegro ApS, Aarhus, Denmark) program was carried out for the docking calculations.

In order to clarify the mode of action of the compounds **1-3** on eNOS, the molecular docking studies were carried out to measure the relative binding energies and localize binding sites. As shown in **Table S2**, compounds **1-3** (Moldock Score: -103.066, -105.809 -102.958 and -103.483) had similar bond in eNOS with the Ligand (Moldock Score: -103.483). Moreover, the hydrogen bonds with amino acids residues could also be observed in **Figure S64**. Compounds **1-3** showed the same hydrogen bonds with ligand at residues. Based on the above conclusions, we can speculate that the compound **1** may be an activator of eNOS.

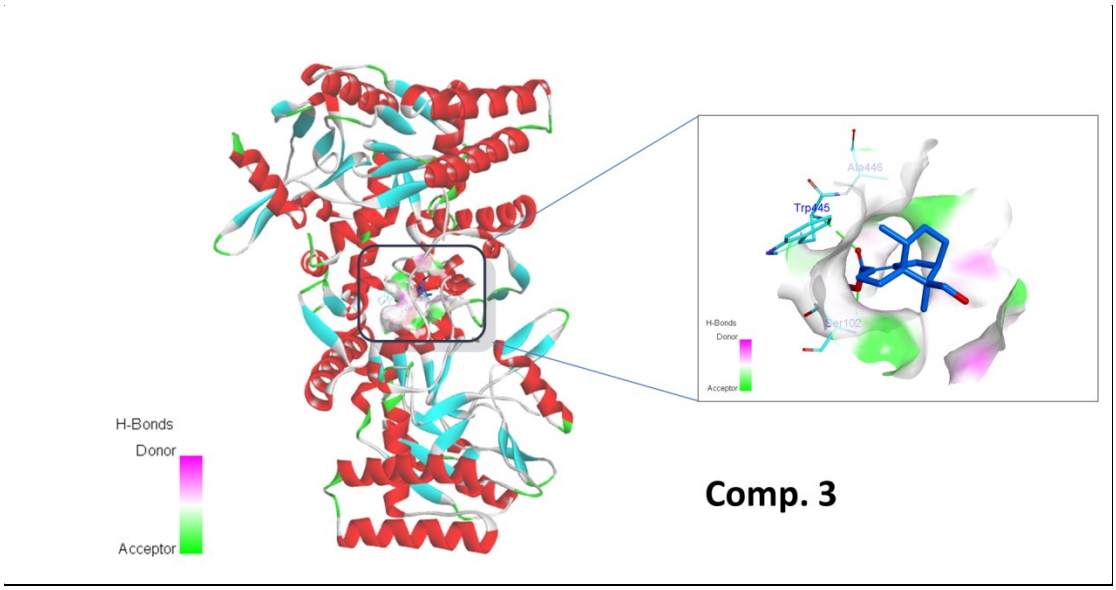



H-Bonds
Donor
Acceptor
Trp445
Gly101
Ala446
H-Bonds
Donor
Acceptor

Comp. 1


H-Bonds
Donor
Acceptor
Asp444
Trp445
Gly101
Ala446
H-Bonds
Donor
Acceptor

Comp. 2



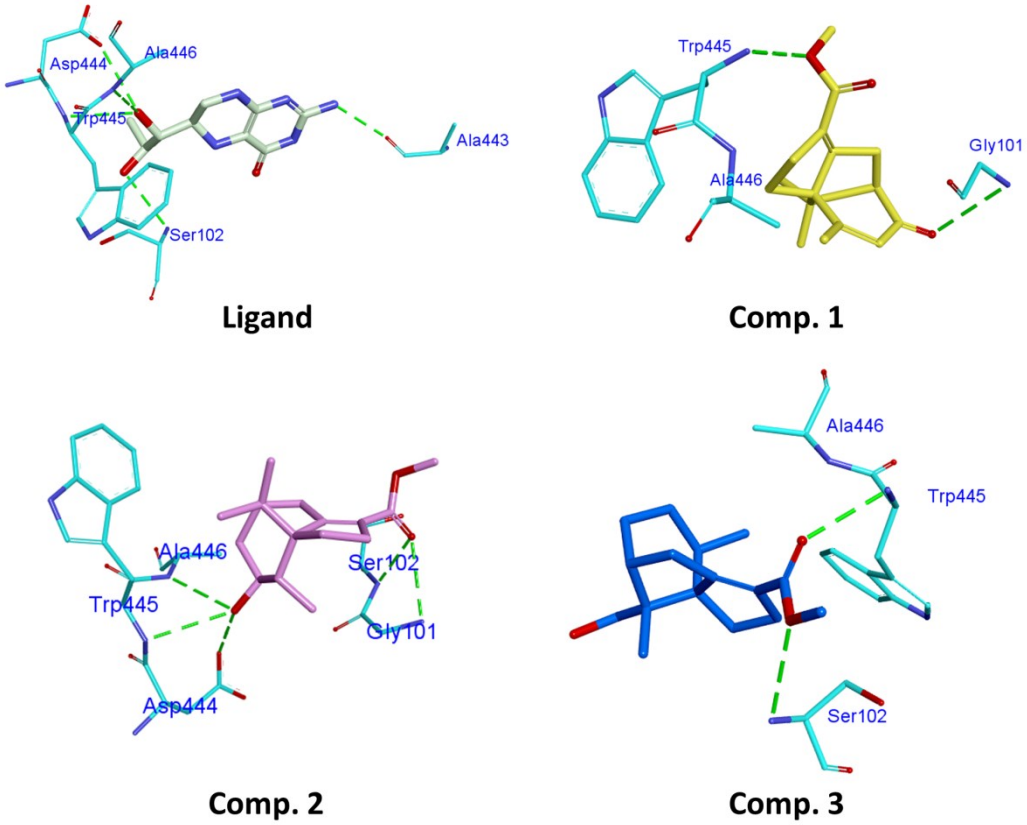


Figure S64. Comp. **1-3** and ligand bound to endothelial nitric oxide synthase (eNOS) (PDB code: 4D1P).

Table S1 Antiplatelet aggregation activity of **MC** and biotransformation derivatives **1**–**8** (x ± s, n = 3).

Samples	Dosage (g mL^{-1})	Inhibition rate (%)
MC	400	43.01% ± 1.98
1	400	93.01% ± 2.38
2	400	96.37% ± 3.76
3	400	82.68% ± 2.21
4	400	35.75% ± 0.65
5	400	36.59% ± 0.75
6	400	25.42% ± 0.66
7	400	74.87% ± 1.32
8	400	65.37% ± 1.13
Aspirin	400	86.32% ± 1.96

Table S2 Moldock Scores of the active compounds **1-3** and the ligand of eNOS.

Name	**Score**
Ligand	-103.483
Comp. **1**	-103.066
Comp. **2**	-105.809
Comp. **3**	-102.956

Notes and references

1. D. M. Dudzinski, T. Michel, Life history of eNOS: Partners and Pathways. *Cardiovasc. Res*. 2007, 75, 247-260.
2. X. L. Tan, Y. Q. Xue, T. Ma, X. F. Wang, J. J. Li, L.B. Lan, K. U. Malik, M. P. McDonald, A. M. Dopico and F. F. Liao, *Mol. Neurodegener.* 2015, **10**, 24-36.
3. H. G. Li and U. Forstermann, *Curr. Pharm. Design,* 2016, **22**, 3595–3606.
4. A. V. Lipeeva, M. V. Khvostov, D. S. Baev, M. M. Shakirov, T. G. Tolstikova and E. E. Shults, Med. Chem., 2016, 12, 674 – 683.
5. C. Wang, F. Yang, Z. H. Xu, D. Q. Shi, D. Y. Chen, J. Dai, N. Gu and Q. Jiang, *Thromb. Res.* 2013, **131**, 31–38.