Supplementary Materials for

Di-isatropolone C, a spontaneous isatropolone C dimer derivative with autophagy activity

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Jie Fu and Xiaoyan Liu contributed equally to the paper.

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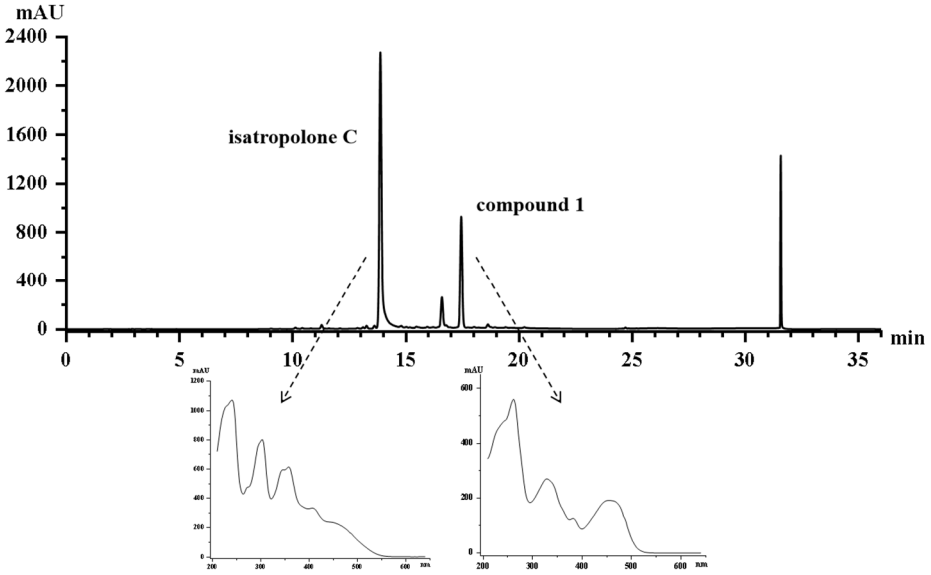


Figure S1. HPLC of isatropolone C and di-isatropolone C (**1**) with their UV-visible spectra

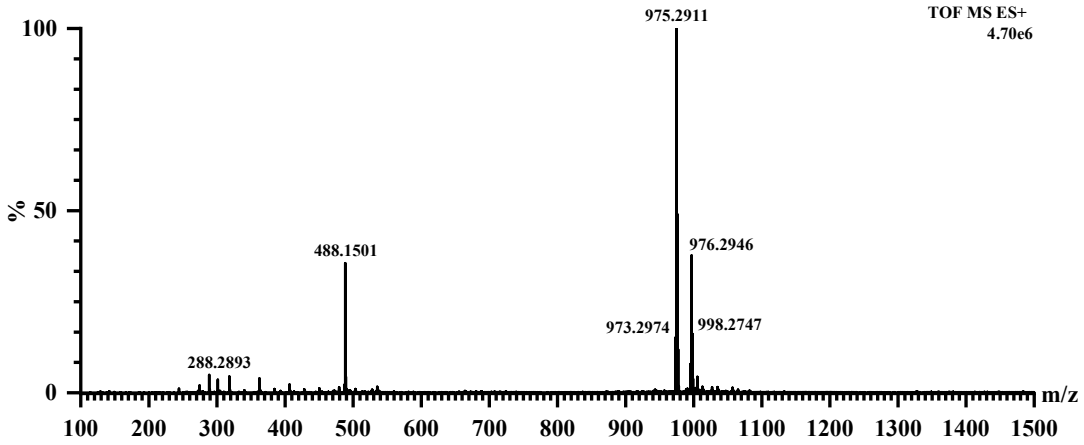
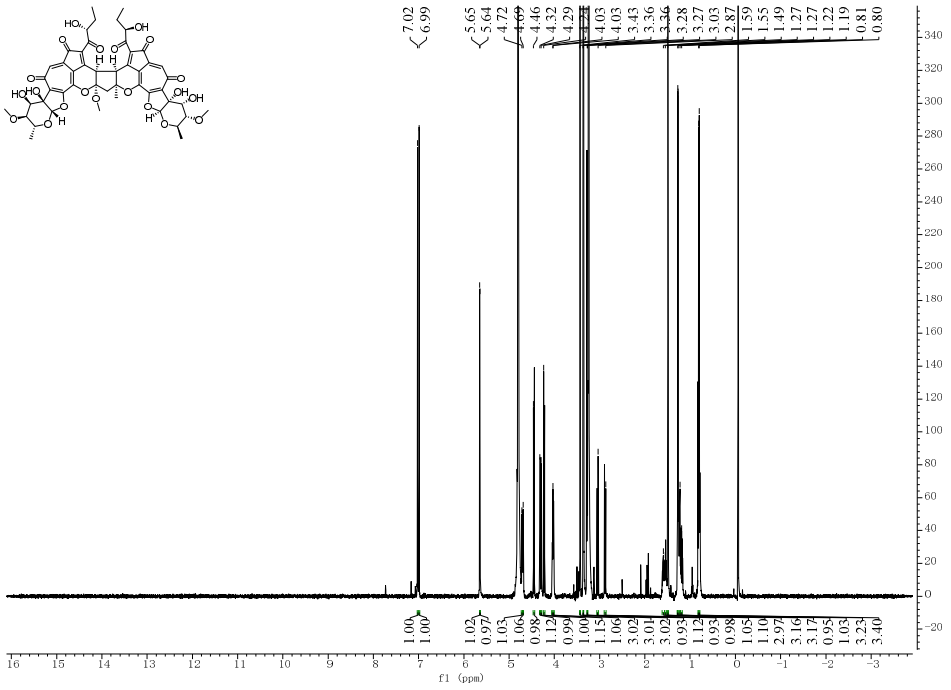


Figure S2. HRESIMS of di-isatropolone C (**1**)



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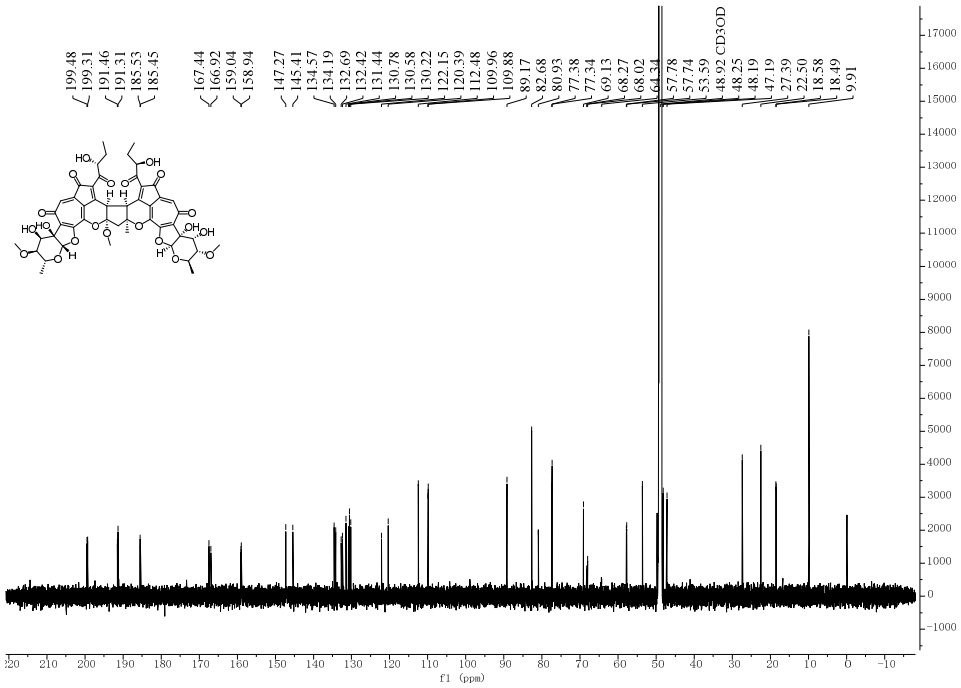


Figure S4. ^{13}C NMR spectrum of di-isatropolone C (**1**)

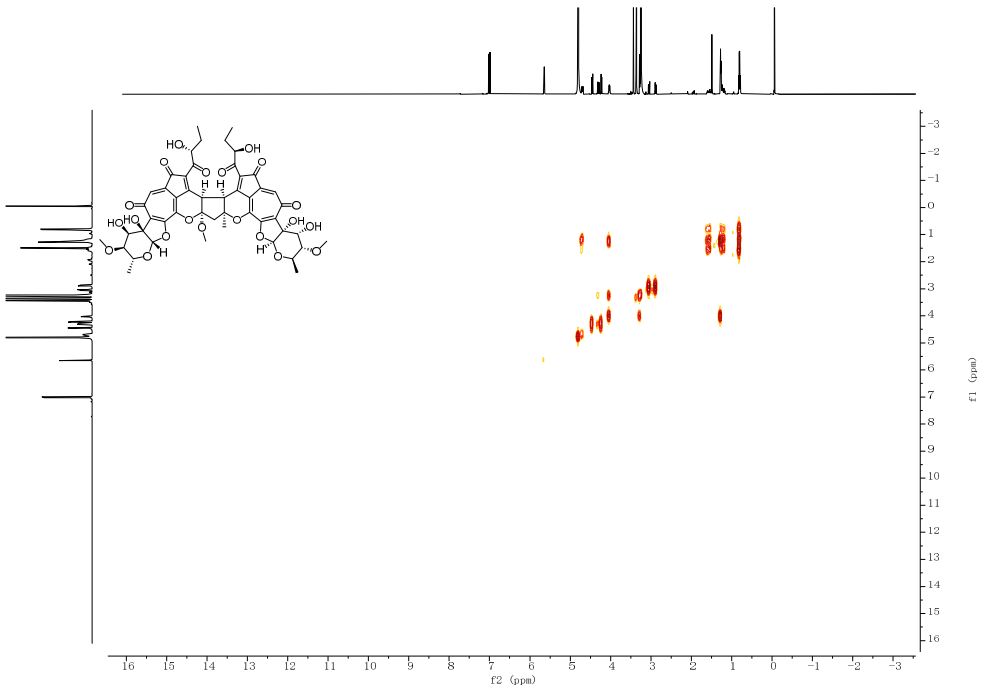
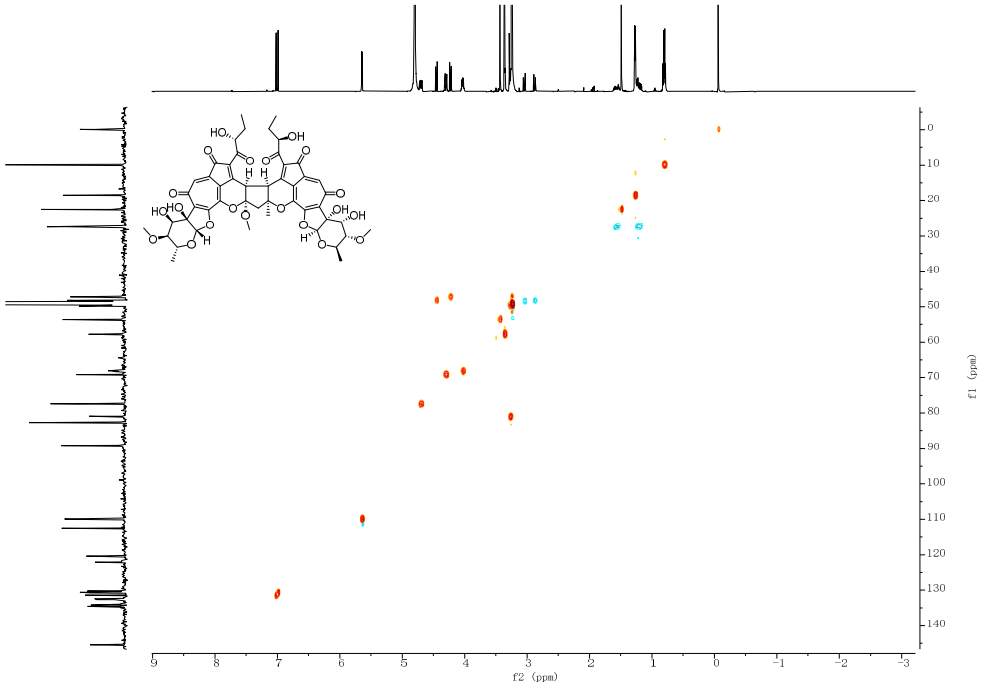


Figure S5. ^{1}H-^{1}H COSY spectrum of di-isatropolone C (**1**)



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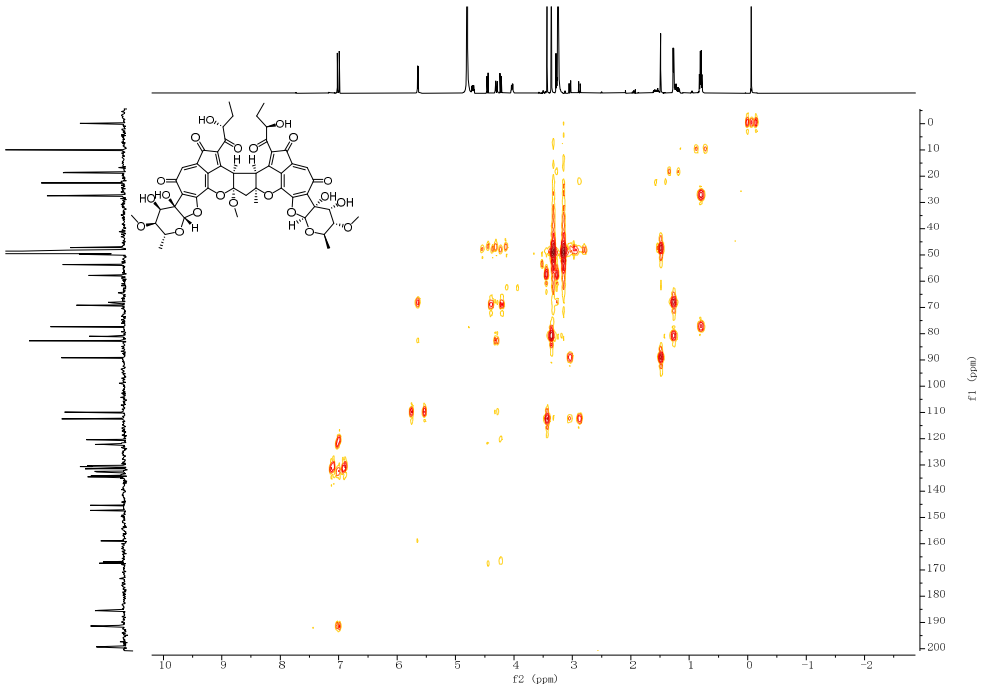


Figure S7. HMBC spectrum of di-isatropolone C (**1**)

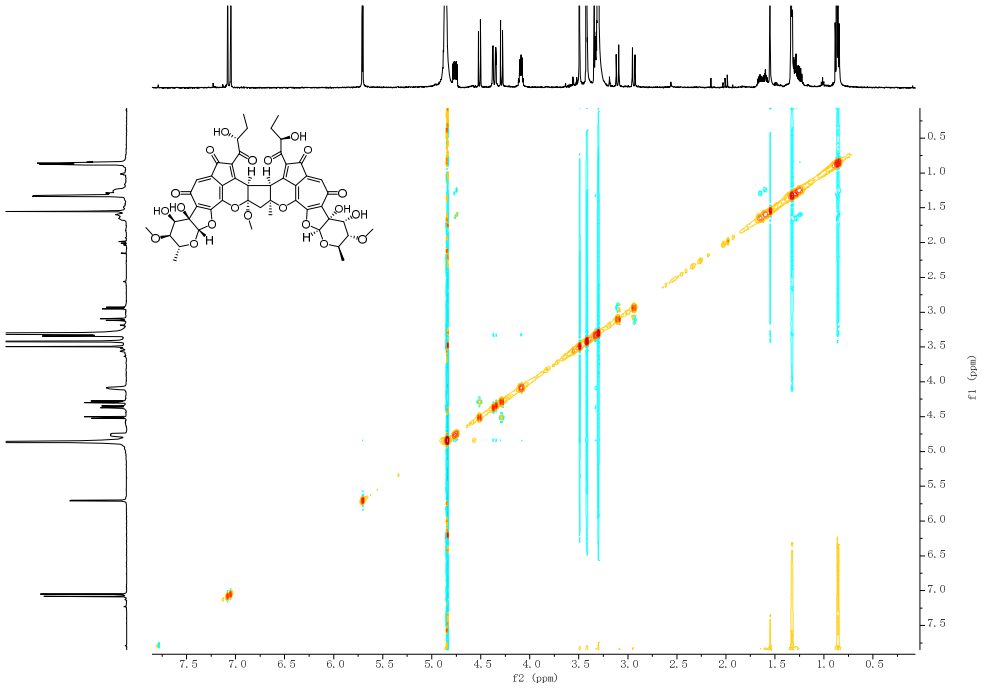
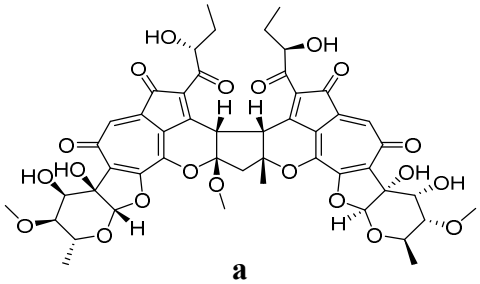


Figure S8. NOESY spectrum of di-isatropolone C (**1**)



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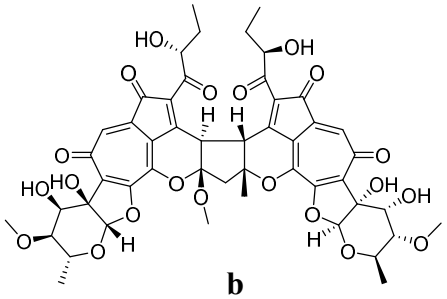

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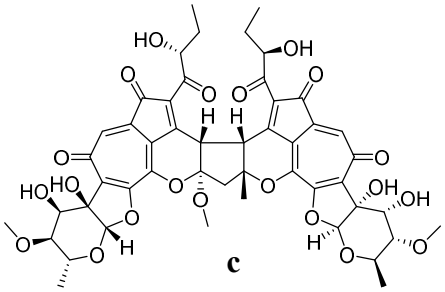

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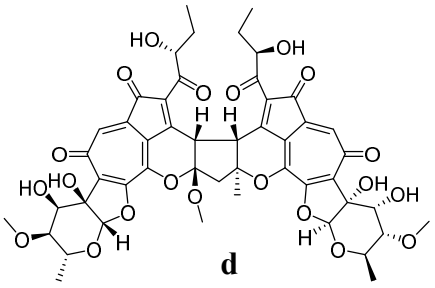

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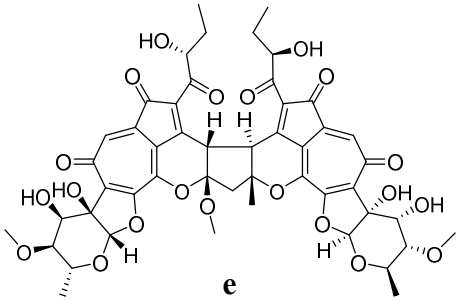

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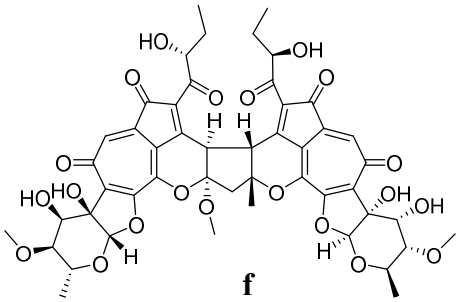

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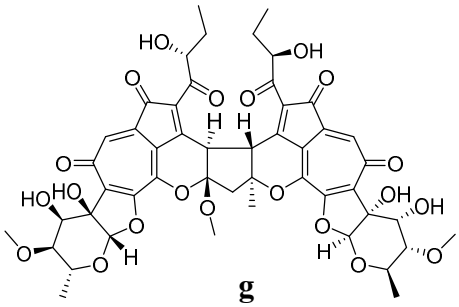

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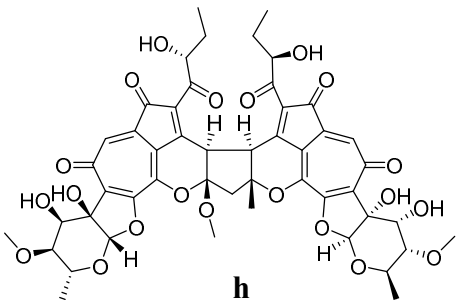

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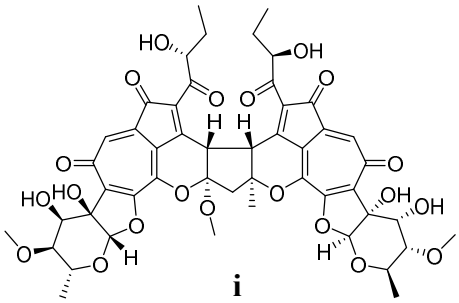

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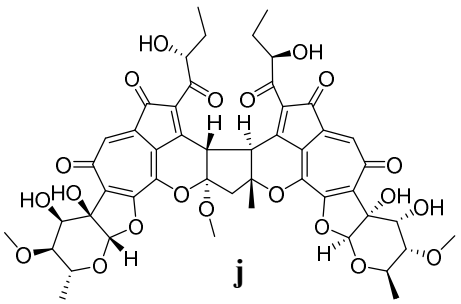

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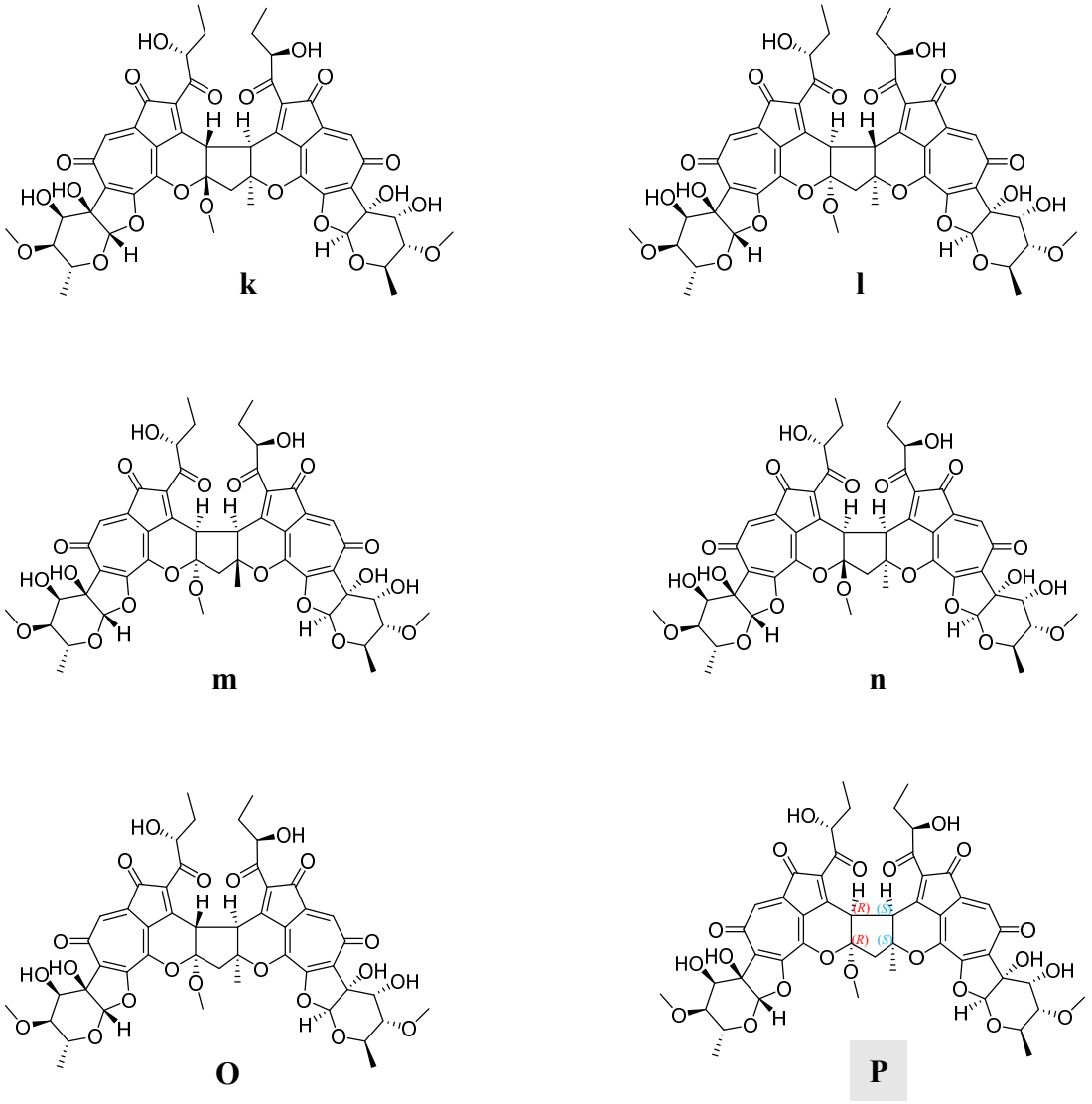


Figure S9. Chemical structures of all 16 probable diastereomers of di-isatropolone C

Table S1. Energies and populations of conformers of diastereomer **a**

Conformer	Population (%)	E (Hartree)
1	16.93	-3475.590599
2	11.95	-3475.590271
3	19.43	-3475.590729
4	8.41	-3475.589939
7	9.14	-3475.590018
16	1.01	-3475.587939
23	8.43	-3475.589941
33	13.01	-3475.590351
40	1.03	-3475.58796
58	10.66	-3475.590163

Table S2. Energies and populations of conformers of diastereomer **b**

Conformer	Population (%)	E (Hartree)
1	10.77	-3475.553422
2	4.14	-3475.552521
4	2.09	-3475.551875
5	1.12	-3475.551287
10	17.11	-3475.553858
14	1.06	-3475.551236
19	4.75	-3475.552649
26	58.96	-3475.555025

Table S3. Energies and populations of conformers of diastereomer **c**

Conformer	Population (%)	E (Hartree)
11	100	-3476.846558

Table S4. Energies and populations of conformers of diastereomer **d**

Conformer	Population (%)	E (Hartree)
1	7.55	-3475.549551
2	1.17	-3475.547793
3	2.14	-3475.54836
11	2.12	-3475.548352
14	26.56	-3475.550737
17	26.49	-3475.550734
20	4.4	-3475.54904
21	9.5	-3475.549767
23	4.38	-3475.549036
24	9.45	-3475.549762
27	6.23	-3475.549368

Table S5. Energies and populations of conformers of diastereomer **e**

Conformer	Population (%)	E (Hartree)
1	15.52	-3475.555587
2	12.34	-3475.555371
3	13.42	-3475.55545
4	4.42	-3475.554402
5	4.99	-3475.554516

6	3.9	-3475.554283
7	1.42	-3475.55333
8	3.86	-3475.554273
11	6.63	-3475.554785
12	4.4	-3475.554397
15	4.49	-3475.554418
16	4.31	-3475.554378
19	8.6	-3475.55503
21	2.92	-3475.554012
22	1.93	-3475.55362
24	5.57	-3475.55462
25	1.29	-3475.553242

Table S6. Energies and populations of conformers of diastereomer **f**

Conformer	Population (%)	E (Hartree)
1	1.01	-3475.563137
11	1.56	-3475.563545
12	65.56	-3475.567071
14	1.94	-3475.563753
16	20.67	-3475.565983
19	1.63	-3475.563587
22	7.62	-3475.565041

Table S7. Energies and populations of conformers of diastereomer **g**

Conformer	Population (%)	E (Hartree)
1	17.77	-3475.554308

2	4.34	-3475.552979
3	6.76	-3475.553396
4	17.83	-3475.554311
5	11.24	-3475.553876
7	6.32	-3475.553333
8	12	-3475.553938
9	7.49	-3475.553493
10	3.94	-3475.552888
16	4.22	-3475.552952
17	2.6	-3475.552493
19	2.31	-3475.552385
20	1.62	-3475.552051
24	1.54	-3475.551999

Table S8. Energies and populations of conformers of diastereomer **h**

Conformer	Population (%)	E (Hartree)
1	14.81	-3475.533528
2	17.09	-3475.533662
3	10.25	-3475.53318
4	9.77	-3475.533135
5	5.87	-3475.532655
6	5.75	-3475.532634
7	4.49	-3475.532401
8	6.69	-3475.532778
9	3.94	-3475.532279
11	3.82	-3475.532249
12	3.01	-3475.532024
13	3.22	-3475.532087

16	1.27	-3475.531208
20	1.46	-3475.531339
22	2.15	-3475.531706
37	3.7	-3475.532219
57	1.46	-3475.531339
60	1.27	-3475.531208

Table S9. Energies and populations of conformers of diastereomer **i**

Conformer	Population (%)	E (Hartree)
1	10.98	-3475.527922
2	10.12	-3475.527845
3	13.99	-3475.52815
4	15.98	-3475.528276
5	6.87	-3475.527479
10	4.49	-3475.527079
13	6.25	-3475.52739
18	1.38	-3475.525968
23	1.3	-3475.525912
24	9.65	-3475.5278
28	2.15	-3475.526384
35	6.22	-3475.527387
36	2.79	-3475.526631
49	1.79	-3475.52621
58	6.04	-3475.527358

Table S10. Energies and populations of conformers of diastereomer **j**

Conformer	Population (%)	E (Hartree)
1	30.26	-3475.533573
2	17.97	-3475.533081
3	20.26	-3475.533194
5	11.53	-3475.532662
6	5.48	-3475.531961
7	6.07	-3475.532056
8	3.51	-3475.53154
12	1.14	-3475.530479
13	1.45	-3475.530706
14	1.17	-3475.530501
17	1.16	-3475.530499

Table S11. Energies and populations of conformers of diastereomer **k**

Conformer	Population (%)	E (Hartree)
2	11.55	-3475.580585
5	4.42	-3475.579679
6	3.76	-3475.579527
8	3.03	-3475.579325
10	1.83	-3475.578847
13	1.83	-3475.578849
14	1.42	-3475.578607
21	4.69	-3475.579735
24	11.02	-3475.580541
26	1.28	-3475.578509
29	1.42	-3475.578606

30	11.22	-3475.580558
31	3.8	-3475.579537
32	2	-3475.578929
36	10.94	-3475.580534
42	3.08	-3475.579338
45	3.02	-3475.579318
47	1.21	-3475.578456
48	3.86	-3475.579552
49	1.32	-3475.578539
50	2.19	-3475.579018
55	1.23	-3475.578472
56	3.78	-3475.579531
60	2.36	-3475.579087
61	1.32	-3475.578541
66	1.3	-3475.578527
75	1.12	-3475.578388

Table S12. Energies and populations of conformers of diastereomer **l**

Conformer	Population (%)	E (Hartree)
1	24.32	-3475.534006
2	1.6	-3475.531439
3	9.39	-3475.533108
4	1.17	-3475.531142
8	36.79	-3475.534396
10	13.33	-3475.533439
11	1.32	-3475.531258
17	9.72	-3475.533141
22	1.24	-3475.531197

45	1.12	-3475.531099

Table S13. Energies and populations of conformers of diastereomer **m**

Conformer	Population (%)	E (Hartree)
1	3.49	-3475.543786
2	2.92	-3475.543618
3	3.02	-3475.54365
4	30.07	-3475.545817
5	1.18	-3475.542761
7	4.09	-3475.543934
9	29.5	-3475.545798
12	1.26	-3475.542827
17	4.14	-3475.543946
18	2.96	-3475.543629
19	3.08	-3475.543666
22	1.21	-3475.542786
31	1.33	-3475.542878
32	3.68	-3475.543835
36	4.19	-3475.543957
37	1.26	-3475.542825
49	1.29	-3475.542847
54	1.31	-3475.542862

Table S14. Energies and populations of conformers of diastereomer **n**

Conformer	Population (%)	E (Hartree)
8	3	-3475.547133

9	1.09	-3475.546175
13	4.59	-3475.547532
15	1.17	-3475.546244
16	1.29	-3475.546335
17	3.11	-3475.547165
18	51.36	-3475.54981
20	2.01	-3475.546752
21	5.62	-3475.547723
36	17.02	-3475.548769
38	2.14	-3475.546811
41	1.86	-3475.546682
68	1.85	-3475.546674
72	1.86	-3475.546682
80	2.02	-3475.54676

Table S15. Energies and populations of conformers of diastereomer **o**

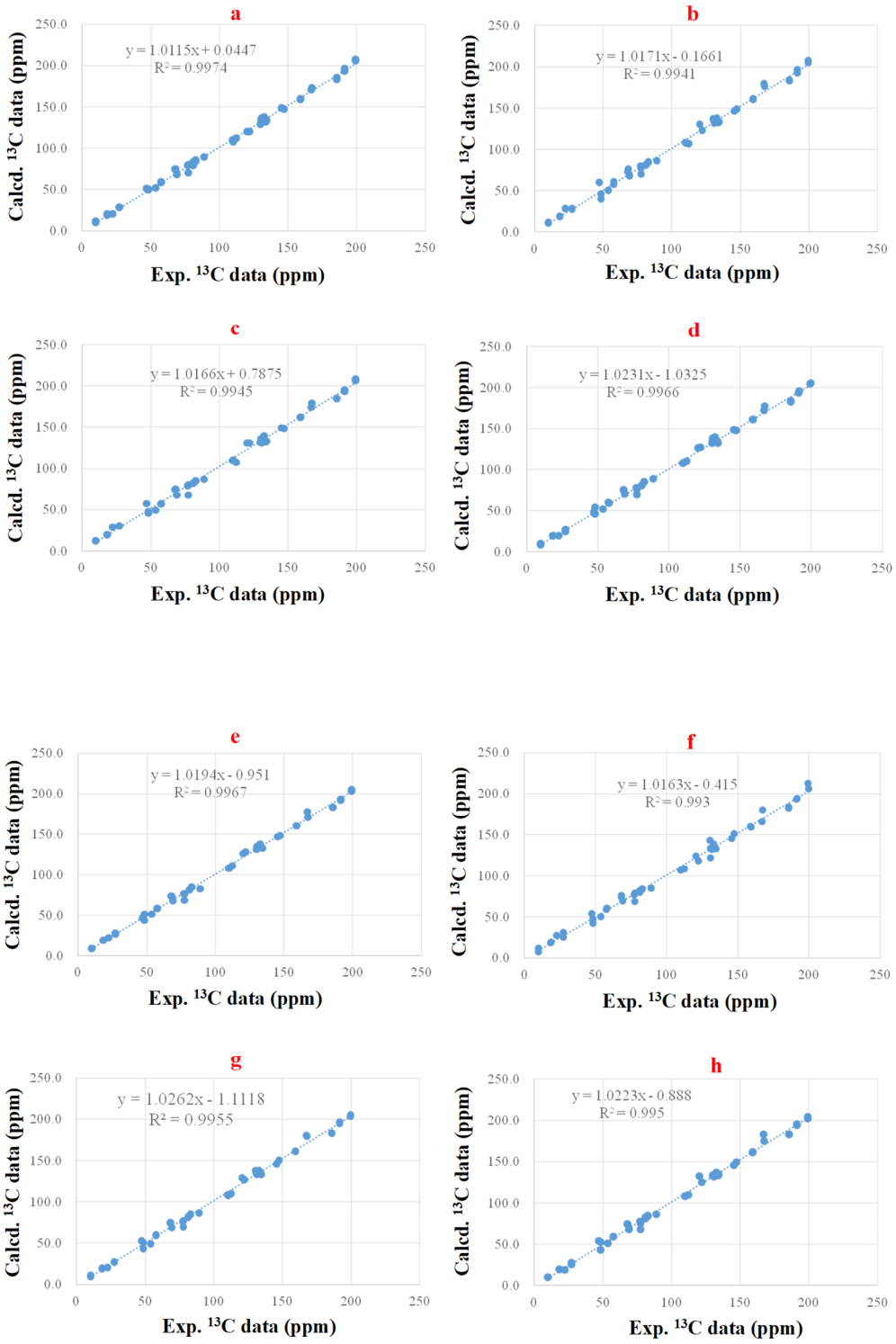
Conformer	Population (%)	E (Hartree)
1	4.78	-3475.561645
2	4.86	-3475.56166
3	3.18	-3475.56126
4	3.13	-3475.561245
5	3.28	-3475.56129
7	3.23	-3475.561274
8	4.77	-3475.561642
9	7.47	-3475.562065
10	4.34	-3475.561553
11	7.33	-3475.562048
12	2.08	-3475.56086

14	2.18	-3475.560903
17	1.71	-3475.560676
20	1.91	-3475.560781
21	1.42	-3475.560499
22	4.84	-3475.561655
23	1.24	-3475.560374
30	2.86	-3475.56116
32	1.25	-3475.560378
34	2.55	-3475.561052
35	2.21	-3475.560916
36	1.52	-3475.560563
38	4.46	-3475.561578
42	1.54	-3475.560573
48	3.61	-3475.561379
49	1.66	-3475.560647
50	1.46	-3475.560524
51	1.45	-3475.560518
53	1.91	-3475.560778
54	1.47	-3475.560533
55	1.46	-3475.560522
58	1.46	-3475.560527
70	2.71	-3475.561109
73	3.43	-3475.561331
75	1.25	-3475.560377

Table S16. Energies and populations of conformers of diastereomer **p**

Conformer	Population (%)	E (Hartree)
1	3.85	-3475.588999

2	2.88	-3475.588726
3	8.2	-3475.589713
4	1.9	-3475.588331
5	1.45	-3475.588082
6	3.84	-3475.588999
7	4.44	-3475.589134
8	4.86	-3475.58922
9	2.88	-3475.588726
10	3.25	-3475.588841
13	8.14	-3475.589706
16	4.2	-3475.589083
17	1.46	-3475.588087
18	1.57	-3475.588157
20	4.88	-3475.589224
25	4.39	-3475.589124
26	3.03	-3475.588775
28	3.27	-3475.588846
31	2.3	-3475.588515
35	8.41	-3475.589737
36	2.12	-3475.588439
40	2.32	-3475.58852
42	1.69	-3475.588224
45	1.36	-3475.588018
49	1.59	-3475.588167
54	4.61	-3475.58917
56	1.02	-3475.587748
61	3.03	-3475.588773
83	1.36	-3475.588016
92	1.69	-3475.588221


a
y = 1.0115x + 0.0447
R² = 0.9974
b
y = 1.0171x - 0.1661
R² = 0.9941
c
y = 1.0166x + 0.7875
R² = 0.9945
d
y = 1.0231x - 1.0325
R² = 0.9966
e
y = 1.0194x - 0.951
R² = 0.9967
f
y = 1.0163x - 0.415
R² = 0.993
g
y = 1.0262x - 1.1118
R² = 0.9955
h
y = 1.0223x - 0.888
R² = 0.995
Calcd. ¹³C data (ppm)
Exp. ¹³C data (ppm)
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50.0
100.0
150.0
200.0
250.0
0
50
100
150
200
250

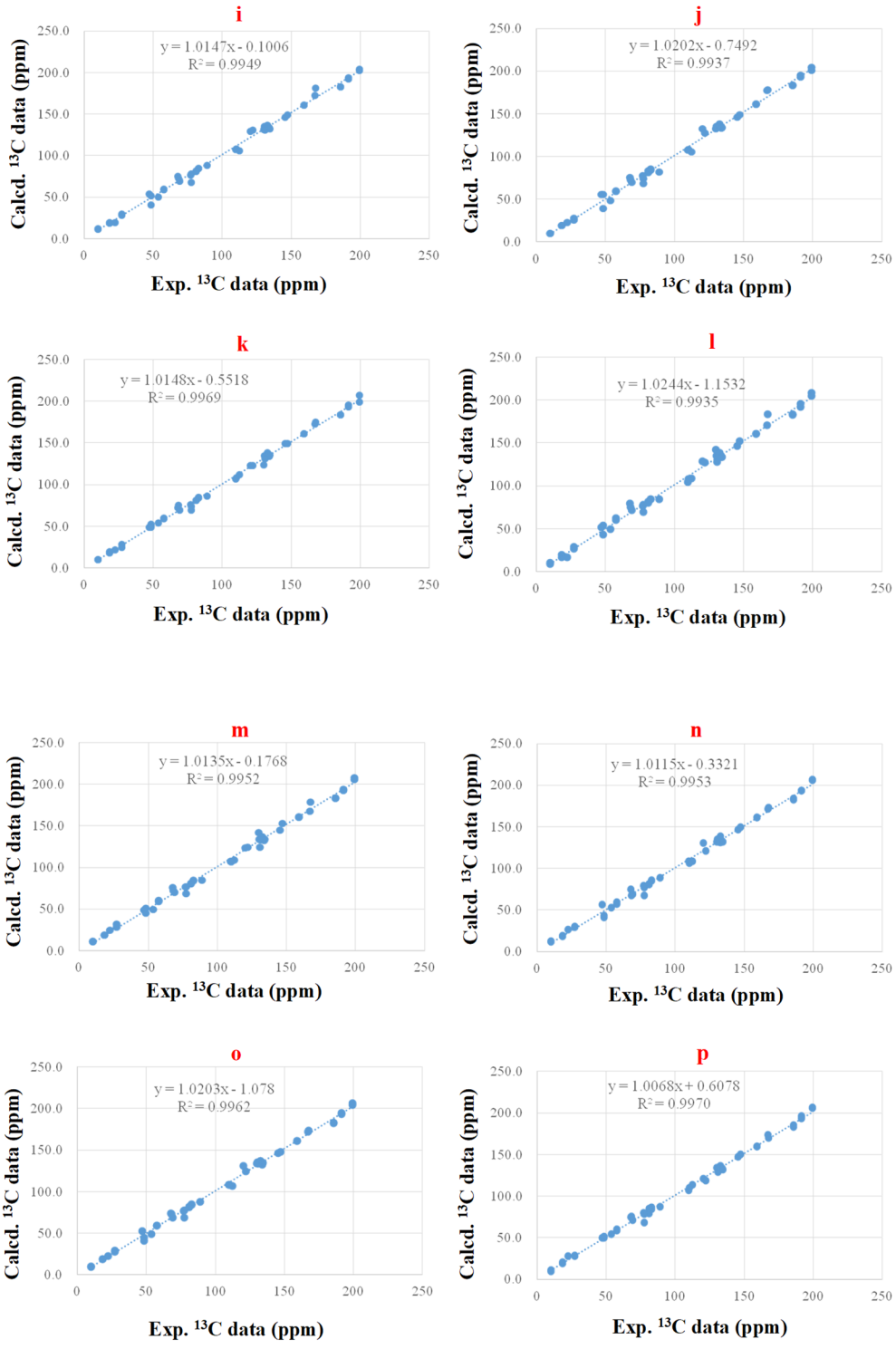

Figure S10. Linear regression fitting of computed ^{13}C NMR chemical shifts of 16 diastereomers of di-isatropolone C (**1**) with experimental data

Table S17. The results of experimental and computed ^{13}C NMR chemical shifts comparison and DP4+ probability analysis of di-isatropolone C (**1**)

Diastereomer	R^2	RMSE	DP4+ (%)
a	0.9974	3.16	0.14
b	0.9941	4.65	0
c	0.9945	4.90	0
d	0.9966	3.75	0
e	0.9967	3.54	0
f	0.9930	4.93	0
g	0.9955	4.33	0
h	0.9950	4.37	0
i	0.9949	4.31	0
j	0.9937	4.76	0
k	0.9969	3.33	0
l	0.9935	4.92	0
m	0.9952	4.09	0
n	0.9953	3.96	0
o	0.9962	3.75	0
p	0.9970	3.33	99.86

Table S18. Conformers of diastereomer **p**

	Conformer
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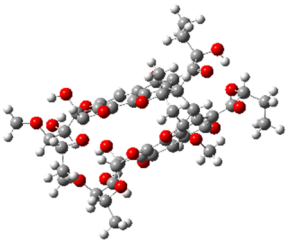
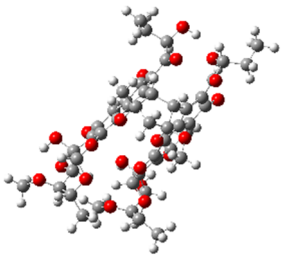
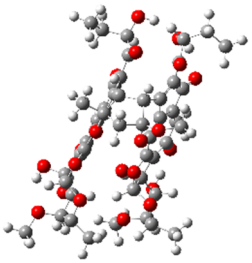
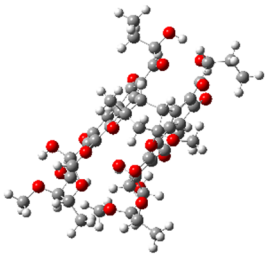
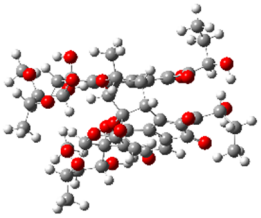
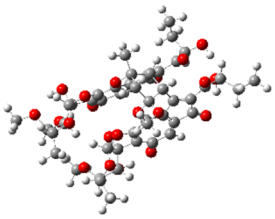
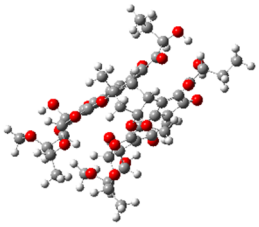
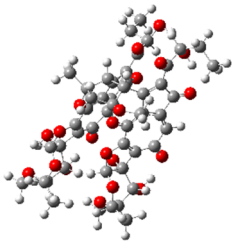
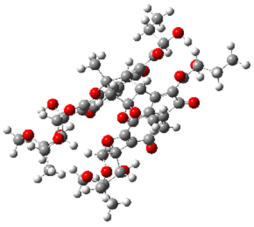
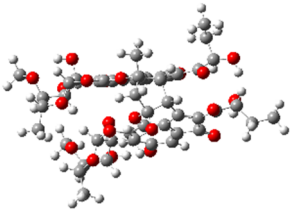
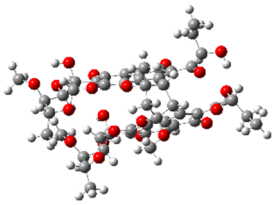
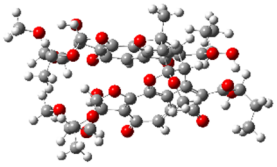
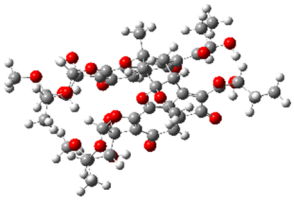
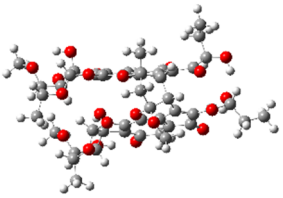
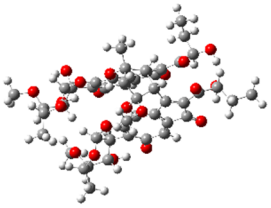
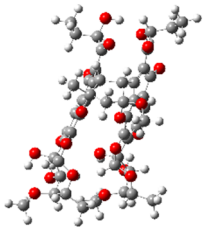
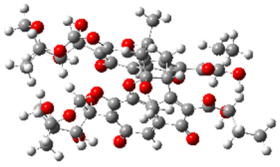
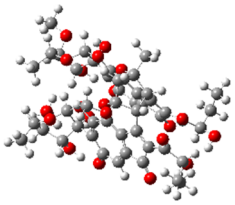
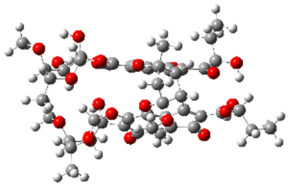
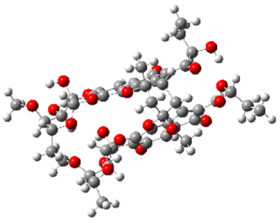
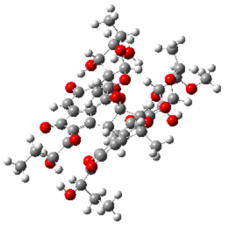
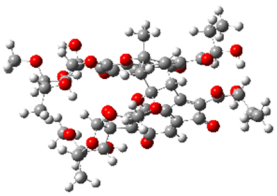
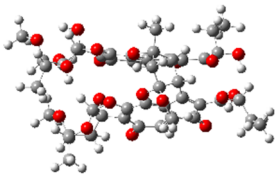
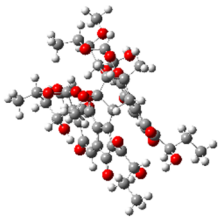
2	
3	
4	
5	

6	
7	
8	
9	
10	
13	

16	
17	
18	
20	
25	
26	

28	
31	
35	
36	
40	
42	
45	

49	
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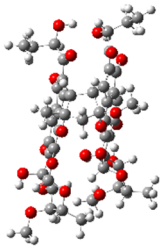
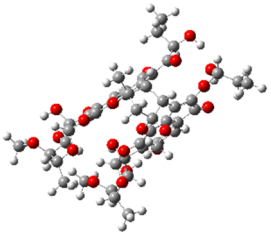
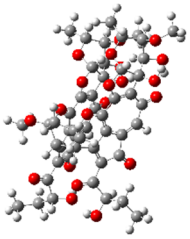
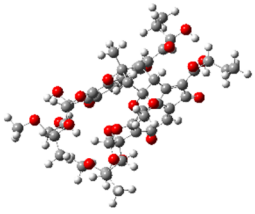
56	
61	
83	
92	

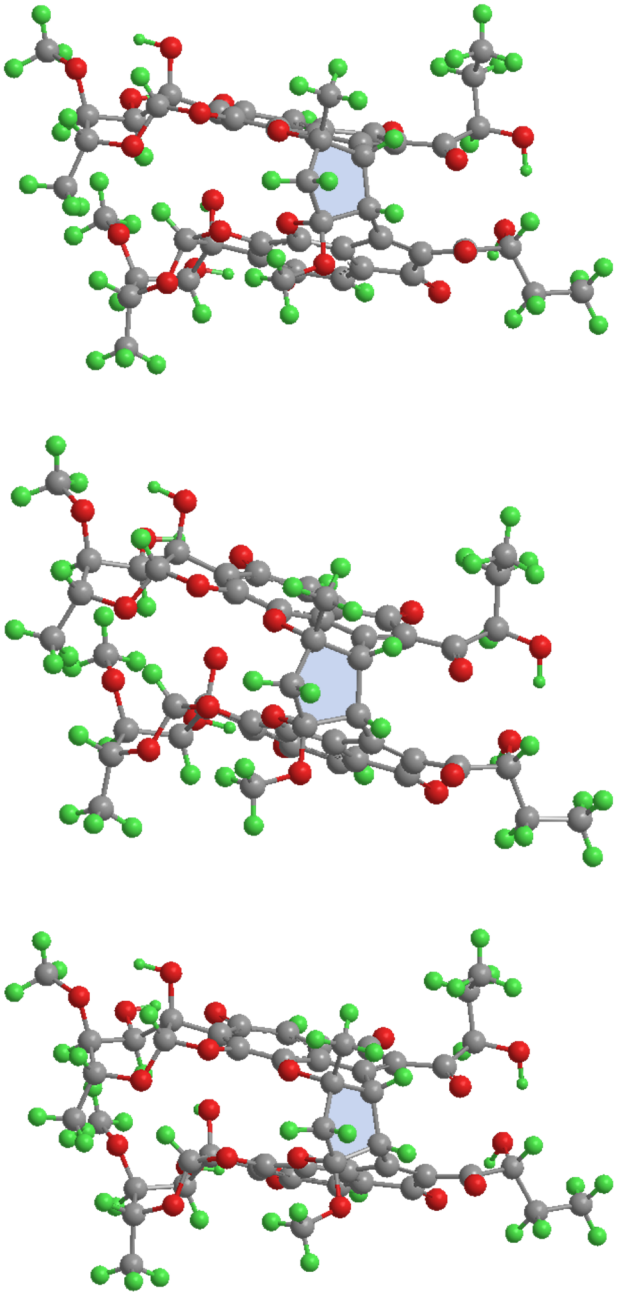


Figure S11. The three most populated conformers of diastereomer **p** (from Table S17 and S18)

The cyclopentane ring is blue-lighted to show better that the hydrogen atom attached to C15, methoxy group attached to C16, hydrogen atom attached to C-15" and methyl group attached to C-16" are all out-positioned in the conformers of **p** diastereomer, which may favor close-stacking of the two cyclopentadienone-tropolone planar moieties in di-isatropolone C.

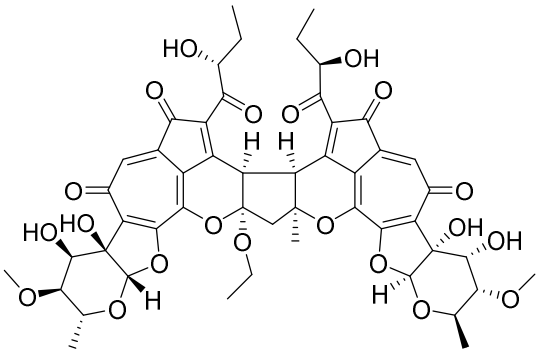


Figure S12. The structure of 16-ethoxy di-isatropolone C

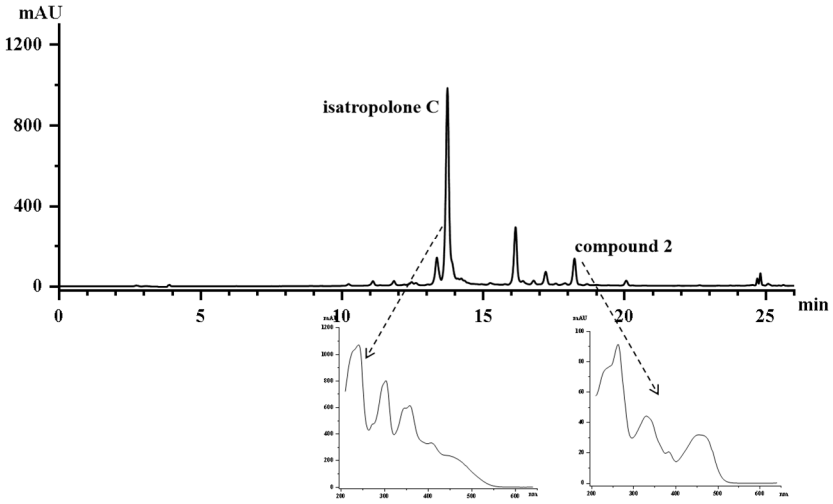


Figure S13. HPLC of isatropolone C and 16-ethoxy di-isatropolone C with their UV-visible spectra

Analytical HPLC of isatropolone C after incubation in ethanol for ten days or more revealed a new peak (compound **2**) at 18.2 min (13.8 min for isatropolone C).

Production and purification of 16-ethoxy di-isatropolone C

Isatropolone C was dissolved in ethanol and incubated at room temperature (20-25 °C) for 20 days. Analytical HPLC of the isatropolone C solution revealed a new peak at 18.2 min. Compound in the new peak was purified by semi-preparative HPLC. A pure preparation of 2.5 mg compound (16-ethoxy di-isatropolone C) was

obtained from 100 mg isatropolone C dissolved in 8.0 ml ethanol. The preparation was used for NMR assays.

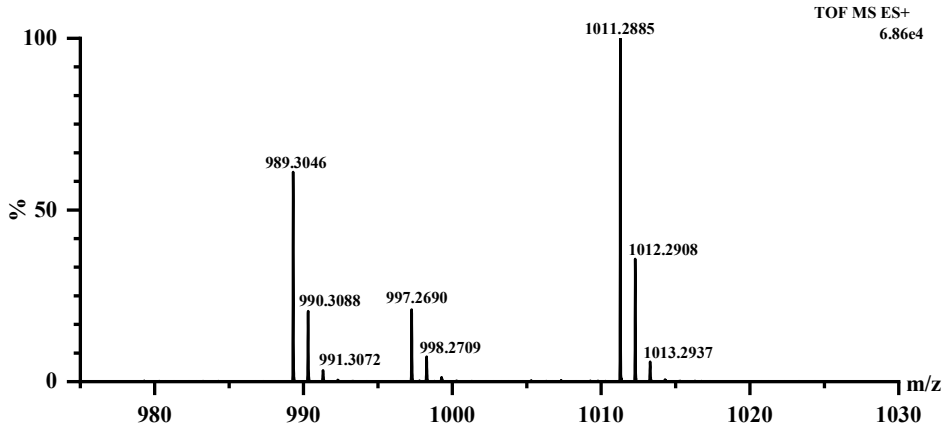


Figure S14. HRESIMS of 16-ethoxy di-isatropolone C

The molecular formula of di-isatropolone C was determined as $C_{50}H_{52}O_{21}$ (*m/z* 989.3046 $[M+H]^+$, calcd for 989.3074)

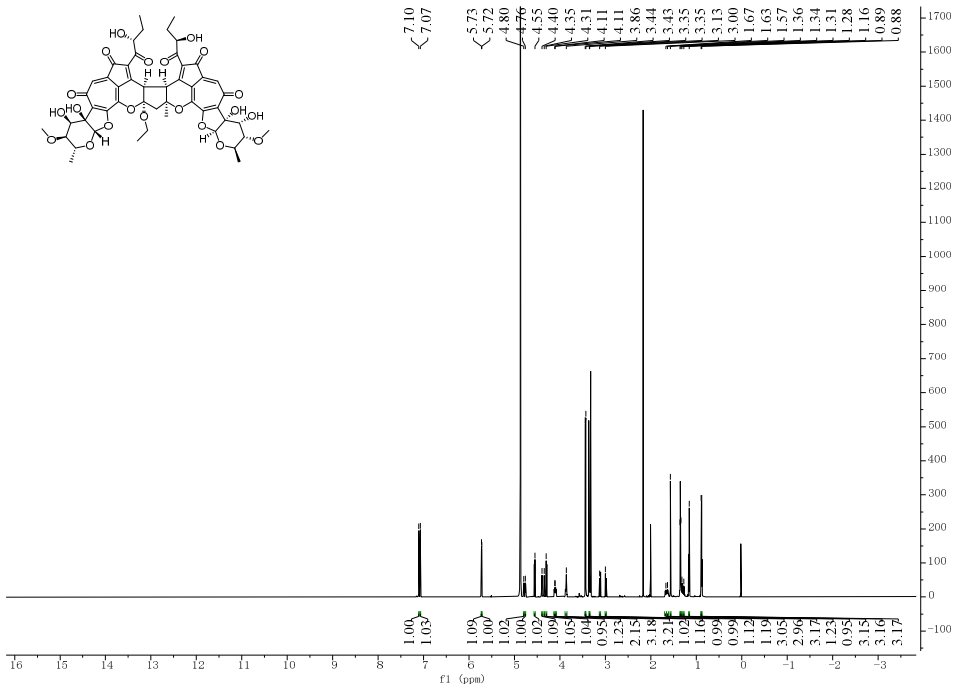


Figure S15. ^{1}H NMR spectrum of 16-ethoxy di-isatropolone C

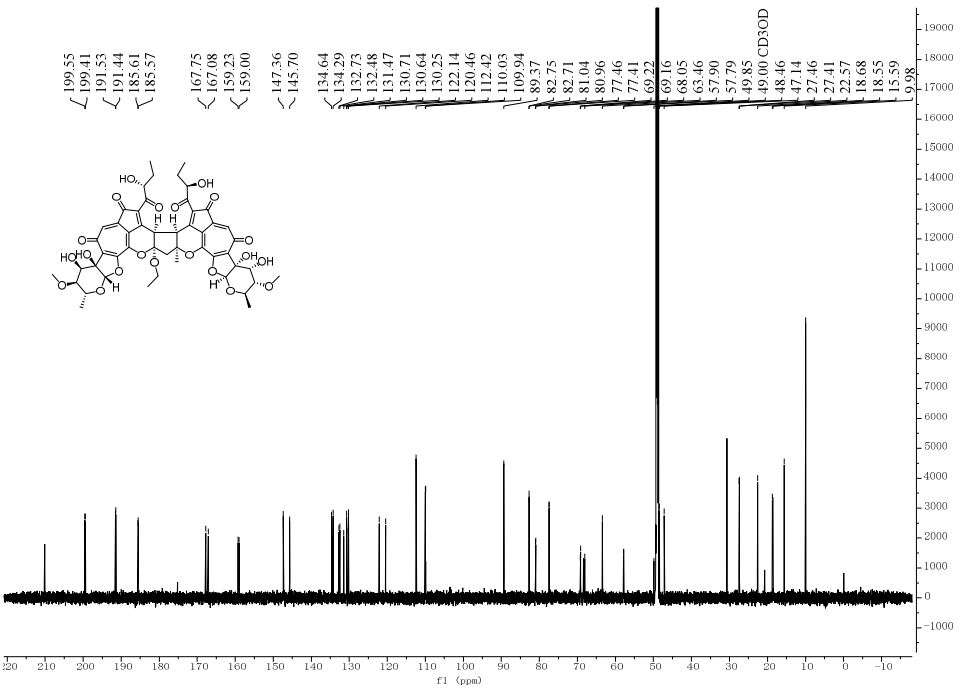


Figure S16. ^{13}C NMR spectrum of 16-ethoxy di-isatropolone C

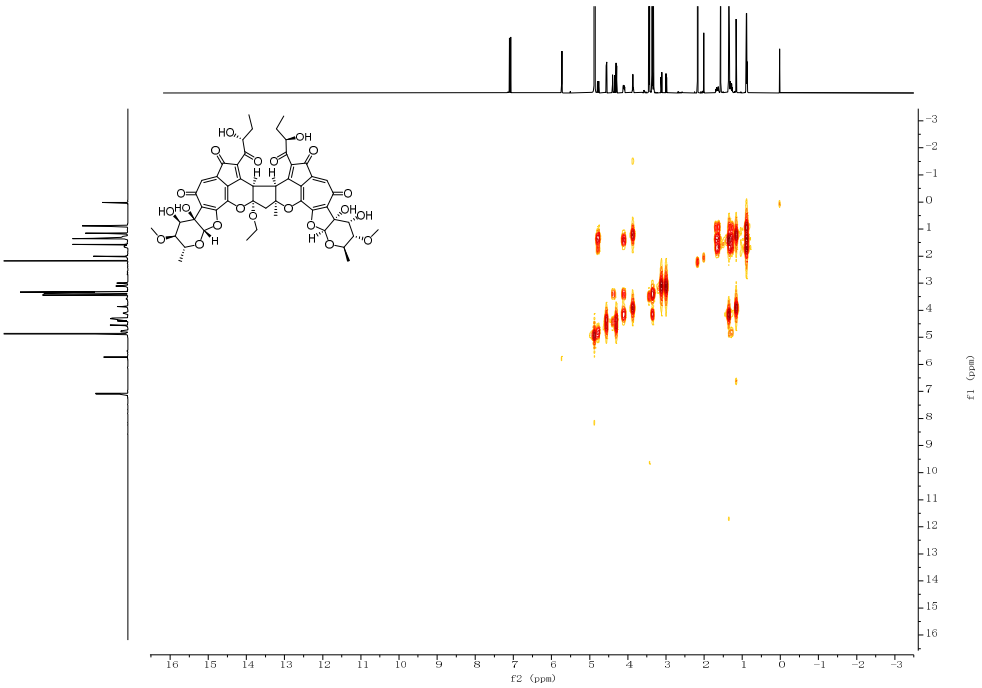


Figure S17. ^{1}H-^{1}H COSY spectrum of 16-ethoxy di-isatropolone C

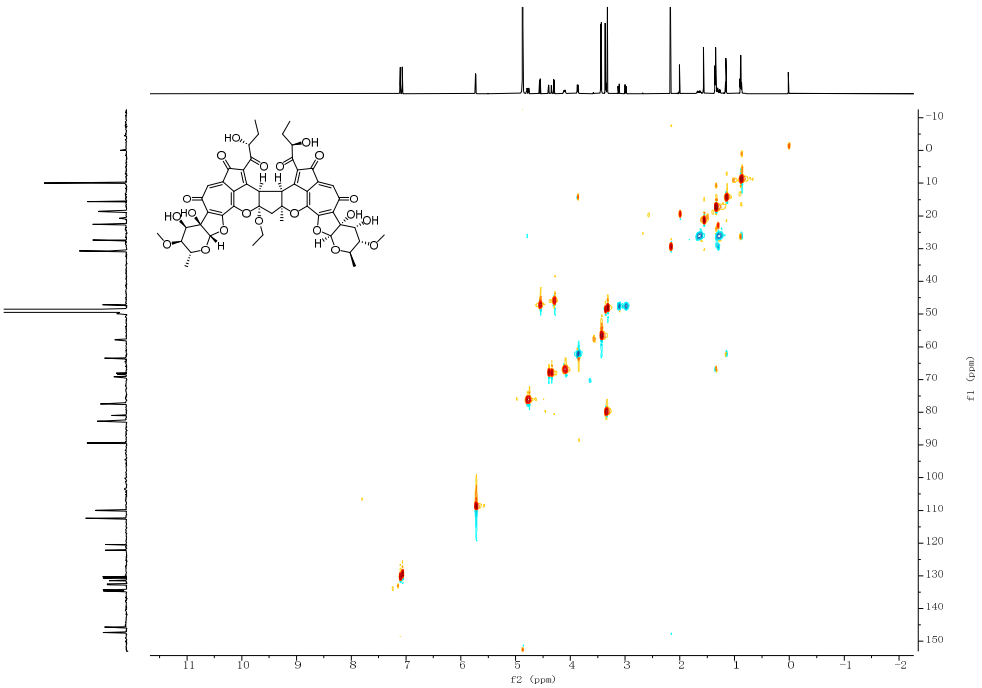


Figure S18. HSQC spectrum of 16-ethoxy di-isatropolone C

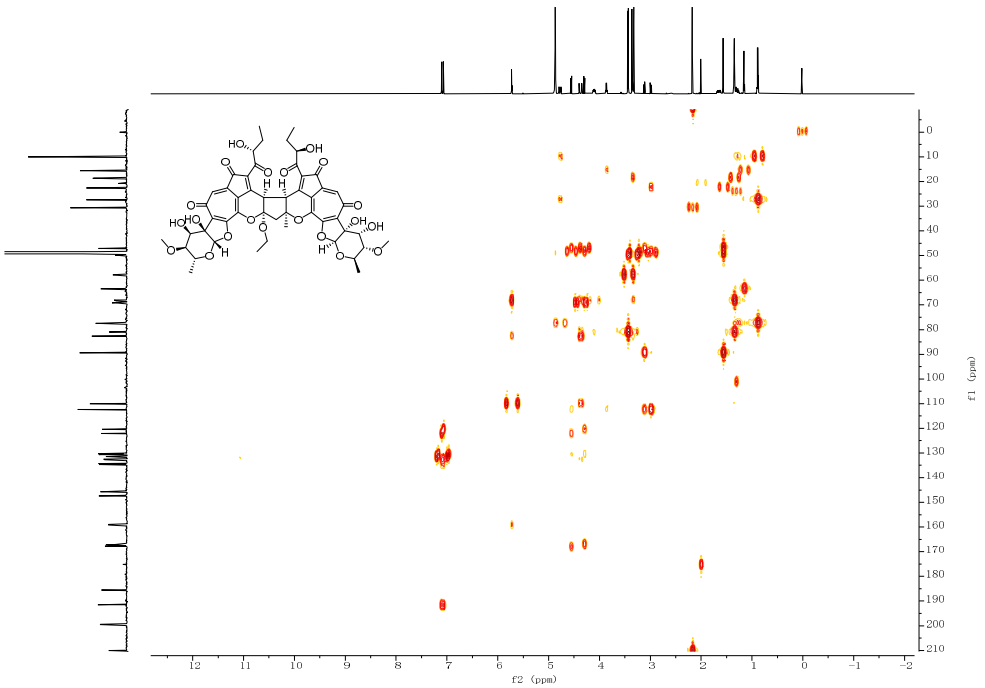


Figure S19. HMBC spectrum of 16-ethoxy di-isatropolone C

Table S19. NMR data of 16-ethoxy di-isatropolone C in methanol-d_4

Position	δ_C, type	δ_H, mult, (J in Hz)	Position	δ_C, type	δ_H, mult, (J in Hz)
1	10.0, CH_3	0.88 t (7.2)	1"	10.0, CH_3	0.89 t (7.2)
2	27.4, CH_2	1.27 m, 1.65 m	2"	27.5, CH_2	1.31 m, 1.67 m
3	77.4, CH	4.76 dd (8.0, 4.0)	3"	77.5, CH	4.79 dd (8.0, 4.0)
4	199.4, C		4"	199.6, C	
5	130.2, C		5"	130.7, C	
6	191.4, C		6"	191.5, C	
7	130.6, CH	7.07 s	7"	131.5, CH	7.15 s
8	185.6, C		8"	185.6, C	
9	132.5, C		9"	132.7, C	
10	159.0, C		10"	159.2, C	
11	145.7, C		11"	147.4, C	

12	134.3, C		12"	134.6, C	
13	120.5, C		13"	122.1,C	
14	167.1, C		14"	167.8, C	
15	47.1, CH	4.30 d (12.8)	15"	48.5, CH	4.55 d (12.8)
16	112.4, C		16"	89.4, C	
17	48.8, CH_2	2.99 d (16.0) 3.12 d (16.0)	17"	22.6, CH_3	1.57 s
18	63.4, CH_2	3.87 m			
19	15.6, CH_3	1.16 t (7.2)			
1'	109.9, CH	5.72 d (5.6)	1'"	110.0, CH	5.72 d (5.6)
2'	82.7, C		2'"	82.8, C	
3'	69.2, CH	4.35 d (3.2)	3'"	69.2, CH	4.40 d (2.4)
4'	81.0, CH	3.35 m overlap	4'"	81.0, CH	3.37 m overlap
5'	68.0, CH	4.10 m	5'"	68.4, CH	4.12 m
6'	18.6, CH_3	1.35 d (6.4)	6'"	18.7, CH_3	1.36 d (6.4)
7'	57.9, CH_3	3.43 s	7'"	58.0, CH_3	3.45 s

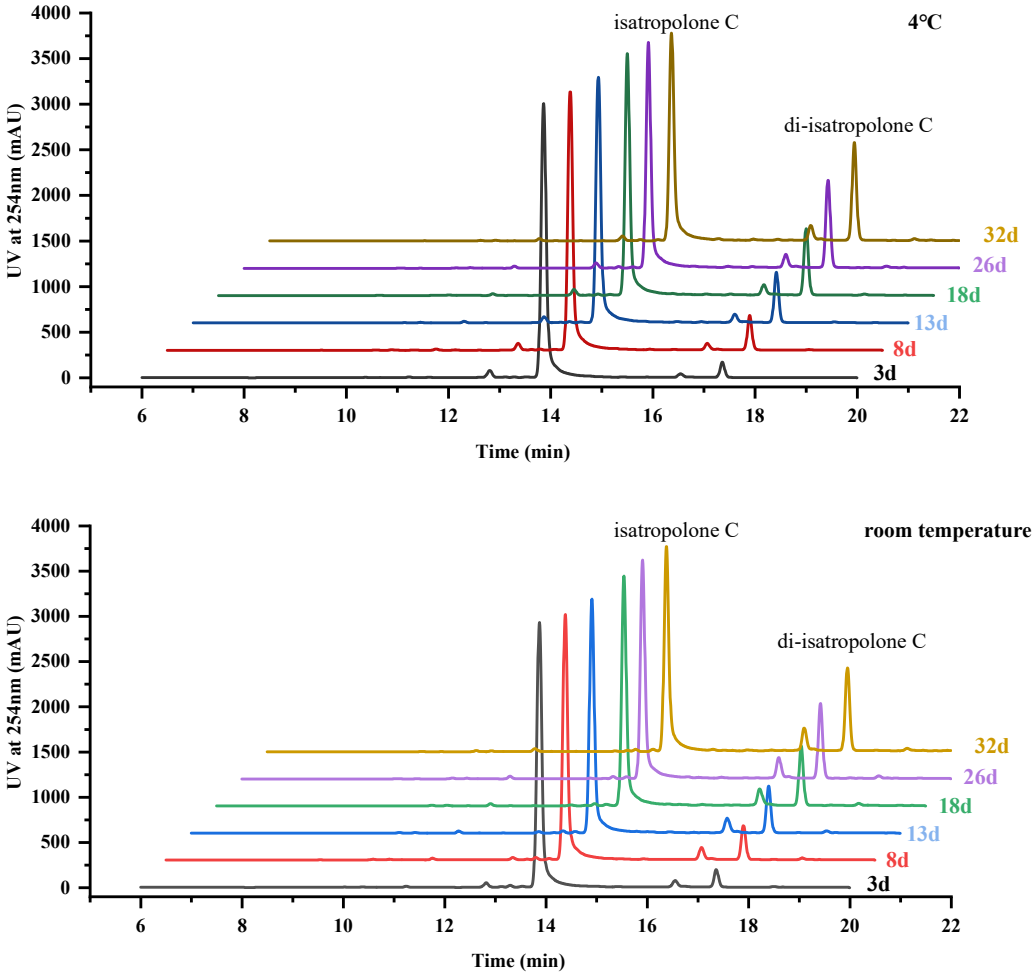


Figure S20. A time-course monitoring of di-isatropolone C production from isatropolone C in methanol incubated at 4 °C or room temperature

Isatropolone C was dissolved in 2.0 mL methanol at a concentration of 2.0 mg/mL, then divided into two equal parts. One part was incubated at 4 °C for a period of over one month, while the other part was incubated at room temperature (20-25 °C) for the same time. Each part was periodically sampled for di-isatropolone C production by analytical HPLC (each with an injection volume of 10 μL).

Di-isatropolone C productions increased with incubation time but displayed no significant differences at 4 °C and room temperature.

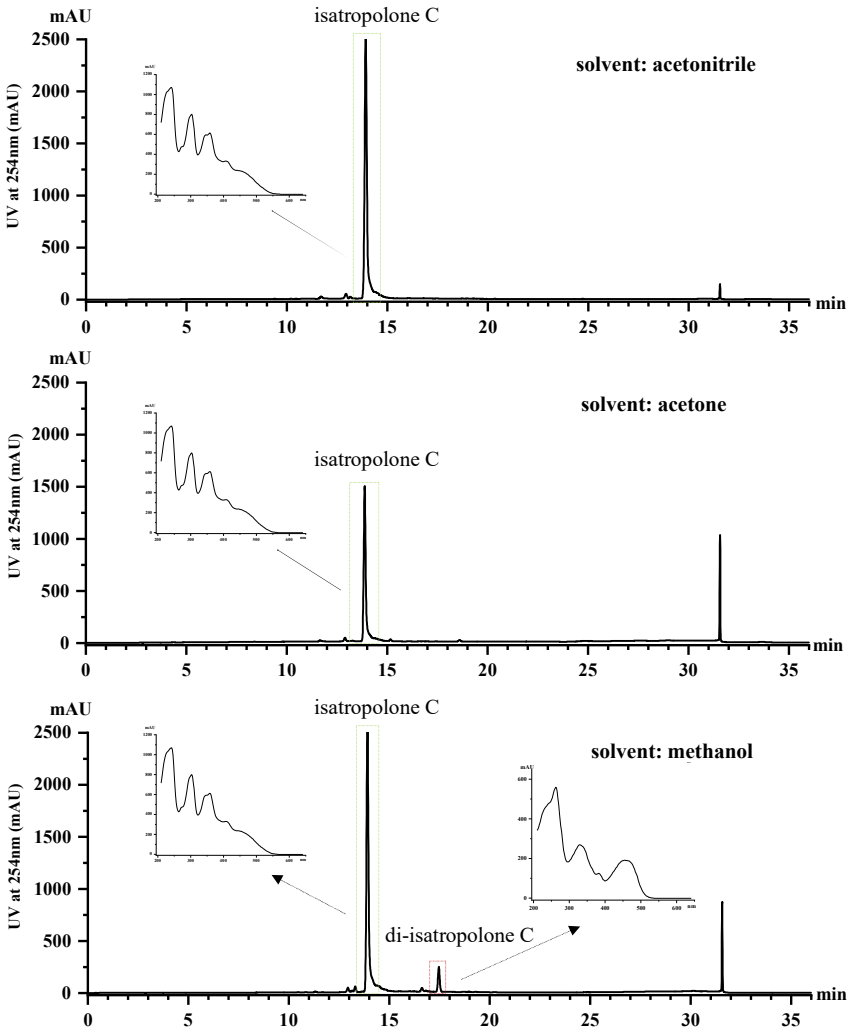


Figure S21. Production of di-isatropolone C from isatropolone C in methanol, acetonitrile, or acetone

Isatropolone C was dissolved in 400 μL methanol, acetonitrile or acetone at a concentration of 0.8 0.8 and 0.4 mg/mL, respectively. The solutions were incubated at 4 °C for 30 days and then analyzed by HPLC for di-isatropolone C production (each with an injection volume of 30 μL).

Di-isatropolone C was not observed in isatropolone C dissolved in acetonitrile or acetone.

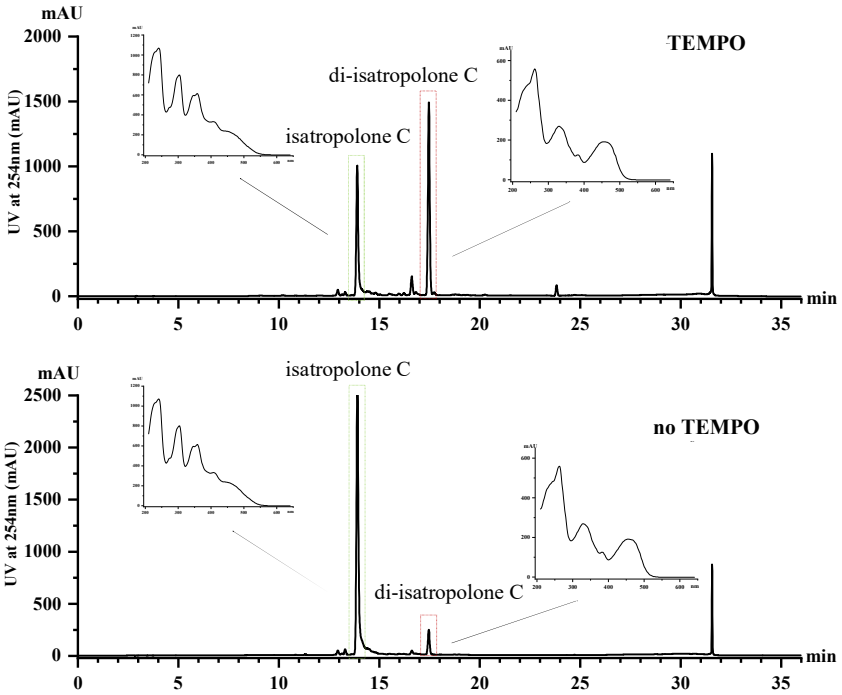


Figure S22. Production of di-isatropolone C from isatropolone C in methanol with TEMPO

Isatropolone C was dissolved in methanol at a concentration of 0.8 mg/mL, and TEMPO was dissolved in H_2O at a concentration of 10.0 mg/mL. The isatropolone C solution (200 μL) was added with 1.0 μL TEMPO solution. The mixed solution was placed at 4 °C for 30 days and then analyzed by HPLC for di-isatropolone C production (with an injection volume of 20 μL). Meanwhile, an identical volume of isatropolone C solution without TEMPO was also placed at 4 °C for 30 days and then analyzed by HPLC for di-isatropolone C production as control.

TEMPO increased significantly (about six folds) the production of di-isatropolone C.

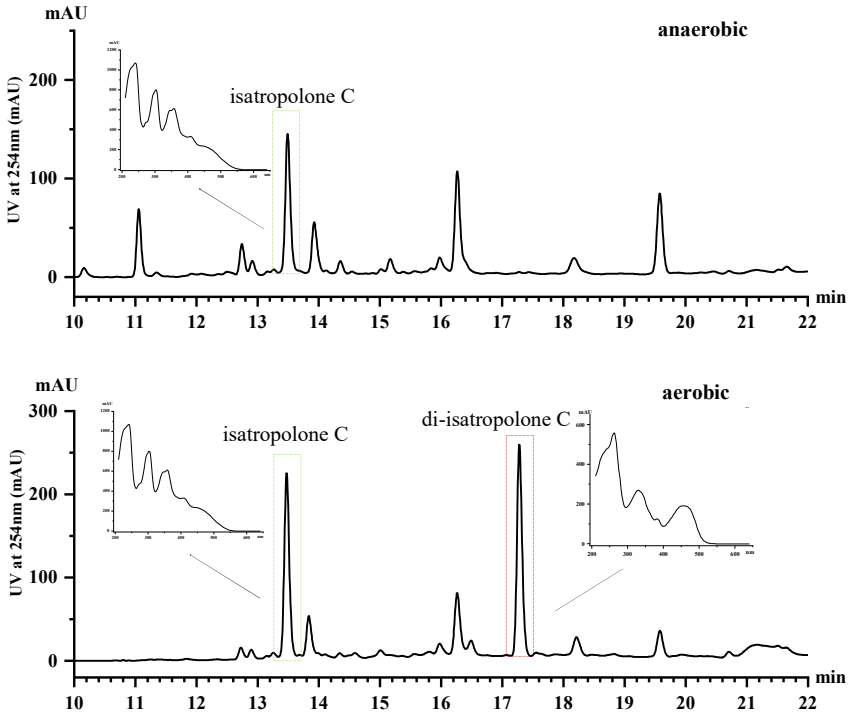


Figure S23. Production of di-isatropolone C from isatropolone C in methanol under air (aerobic) or oxygen-free gas mixture (anaerobic)

Isatropolone C was dissolved in degassing methanol at a concentration of 0.8 mg/mL, and TEMPO was dissolved in degassing H_2O at a concentration of 10.0 mg/mL. The isatropolone C solution (400 μL) was added with 5.0 μL TEMPO solution. The mixed solution was incubated anaerobically (under a gas mixture of nitrogen 90%, carbon dioxide 5% and hydrogen 5%) at 37 °C for 30 days and then analyzed by HPLC for di-isatropolone C production (with an injection volume of 1.0 μL). Meanwhile, an identical volume of the mixed solution was incubated aerobically (under air) at 37 °C for 30 days as control.

Di-isatropolone C was not observed in isatropolone C solution incubated anaerobically.