Activating Human 15-Lipoxygenase-1 Beyond Flatland: Discovery of Non-Aromatic Modulators

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Supporting Information

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Biochemistry: Methods/protocols

Enzyme Activity assay: The h-15-LOX-1 was expressed in BL21 (DE3) *E. coli* cells and the cell lysate was used for the activity assay with no further purification. The conversion of linoleic acid to 13S-hydroperoxy-9Z,11E-octadecadienoic acid (13(S)-HpODE) was observed through UV absorbance at 234 nm over time with a ThermoFisher Varioskan Plate Reader and a Greiner Bio-One F- Bottom 96-well plate. The measurement took place for 20 min with an interval time of 20 sec. Only the linear part was used for the determination of the enzymatic activity, typically extending over the first 1–5 min depending on the enzyme concentration. After that, the conversion rate slows down due to the consumption of the substrate. The activity assay was used for the determination of the optimum concentration of the cell lysate (×200 times dilution in assay buffer: 50 mM HEPES, 50 mM NaCl, pH 7,5). Linoleic acid (Sigma Aldrich, L1376) was diluted in ethanol. Each measurement was performed in triplicate and all data were processed with Microsoft Excel Professional Plus 2016 and GraphPad Prism 8 software.

Enzyme inhibition assay: For the evaluation of the inhibitory potency of the compounds the same experimental approach based on the absorption of 13(S)-HpODE at 234 nm was used. All the compounds were dissolved in DMSO at a final concentration of 2 mM. Then, they were diluted with assay buffer and tested at 50 μM. Each compound was mixed with the diluted cell lysate and after a 10-minute incubation at RT, the linoleic acid (or arachidonic) was added at a final concentration of 25 μM. All the values were normalized by setting as 100% the absence of the inhibitor. Compounds in test samples with enzyme activity less than 50% were considered as hits. Each measurement was performed in triplicate and all data were processed with Microsoft Excel Professional Plus 2016 and Graph- Pad Prism 8 software.

Determination of the half maximal potency concentration: The half maximal inhibitor or activator concentration (IC_{50}/EC_{50}) was also determined by a comparable method using the absorption of 13(S)-HpODE at 234 nm. All the compounds were firstly dissolved in DMSO and then diluted with assay buffer at a final concentration of 200 μM. The desired concentrations, ranging from 0.78 to 100 μM, were achieved by serial dilution. Each concentration was mixed with the diluted cell lysate and after a 10-minute incubation at RT, the linoleic acid (or arachidonic acid) was added at the same final concentration as above. The 100% was set by the absence of the compound, while the 0% was set by the absence of the substrate. DMSO, diluted in assay buffer, was added to both the positive control and the blank sample at the same concentration as the test sample. Each measurement was performed in triplicate and all data were processed with Microsoft Excel Professional Plus 2016 and Graph- Pad Prism 8 software.

Michaelis–Menten kinetics: The absorption of 13(S)-HpODE at 234 nm was employed once again for the kinetics study. Four different concentrations of the linoleic acid were tested this time: 5 μM, 10 μM, 20 μM and 40 μM. The enzyme activity was determined in the absence or presence of specified concentrations of the inhibitors based on their IC50 value. As previously, 100 μl of the inhibitor or activator solution were mixed with 40 μl of assay buffer and 50 μl of the diluted cell lysate. In absence of compound, the corresponding volume is replaced with assay buffer. After a 10-minute incubation at RT, 10 μl of linoleic acid ranging from 800 μM to 100 μM. The reaction velocity V was plotted against the substrate concentration [S] in a Michaelis – Menten plot for the determination of the K_m and V_{max} in the presence of the inhibitor. The reciprocal of the substrate concentration 1/[S] was plotted against the reciprocal of the reaction velocity 1/V in a Lineweaver Burk plot. Each measurement was performed in triplicate and all data were processed with Microsoft Excel Professional Plus 2016 and GraphPad Prism 8 software.

Enzyme activation and inhibition studies

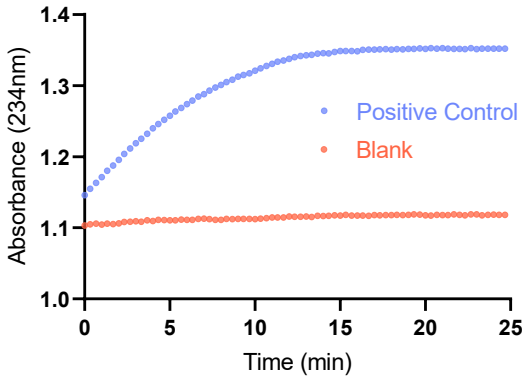

Figure S1. Conversion of substrate (linoleic acid) from enzyme (Positive Control) and absent of linoleic acid (Blank) following with UV-absorbance protocol at 234nm as time progresses.

Chemical library

Table S1. Library comprising all compounds that are screened (80). The name of every compound arises from a number and a letter, correspond with the position in the table.

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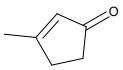
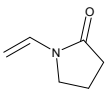
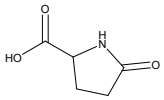
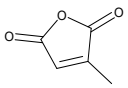
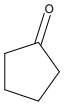
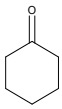
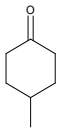
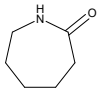
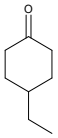
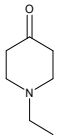
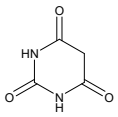
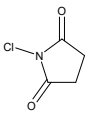
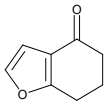
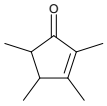
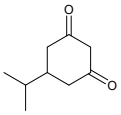
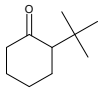
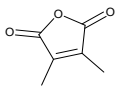
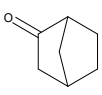
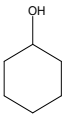
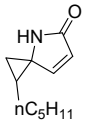
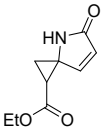
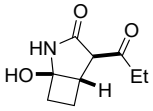
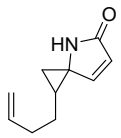
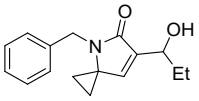
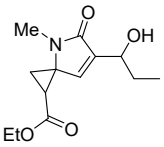
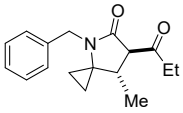
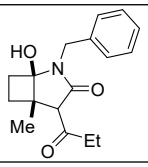
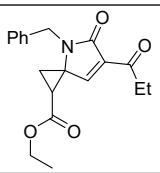
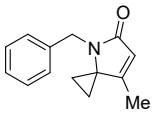
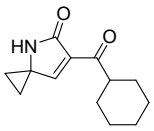
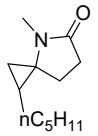
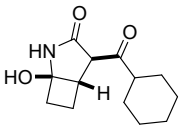
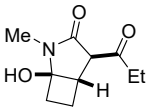
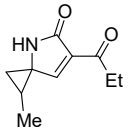
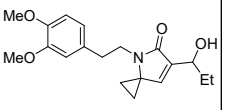
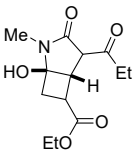
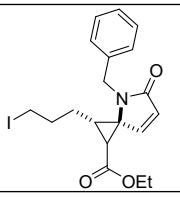
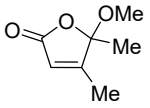
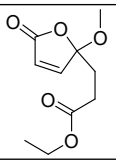
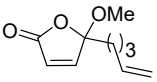
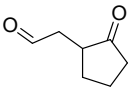
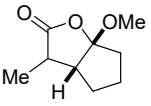
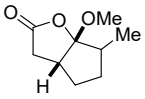
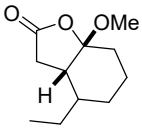
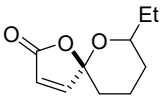
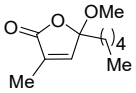
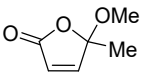
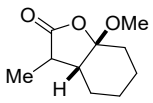
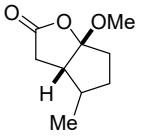
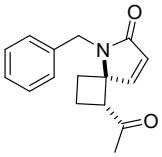
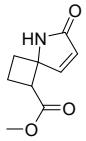
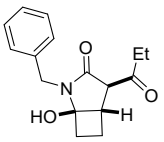
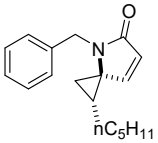
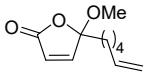
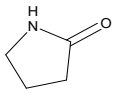
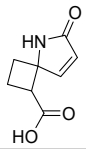
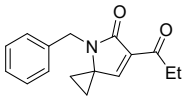
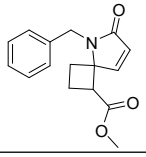
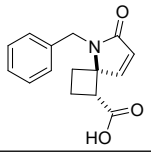
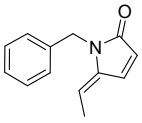
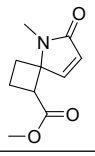
1					
2					
3					
4					
5					

6					
7					
8					
9					
10					
11					
12					
13					
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15					
16					

Screening Results

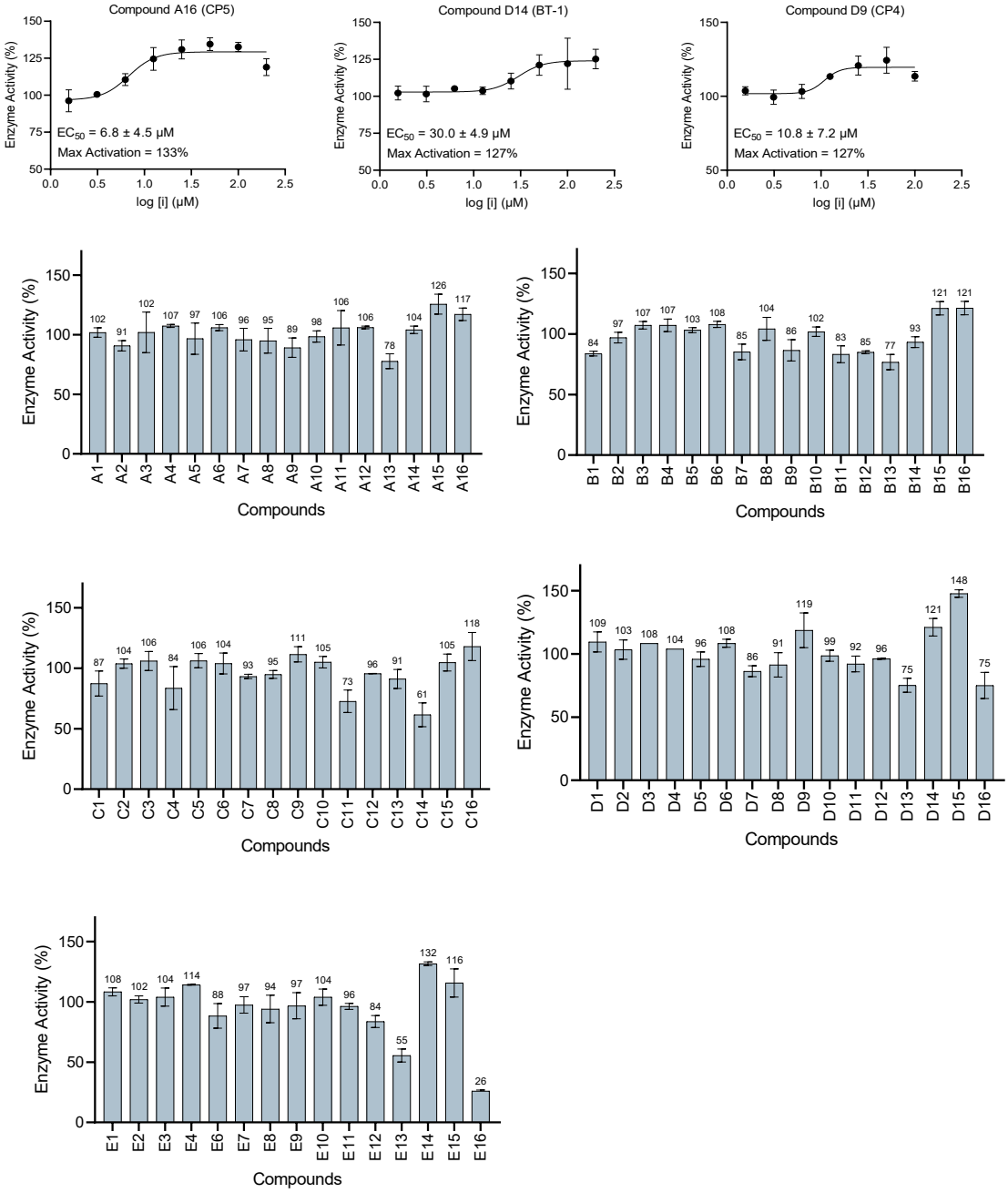


Figure S2. Column graphs with remaining enzyme activity (%) that was detected during the screening of a chemical library for regulation of h15-LOX-1. All compounds were screened at 50 µM.

IC_{50}/EC_{50} graphs

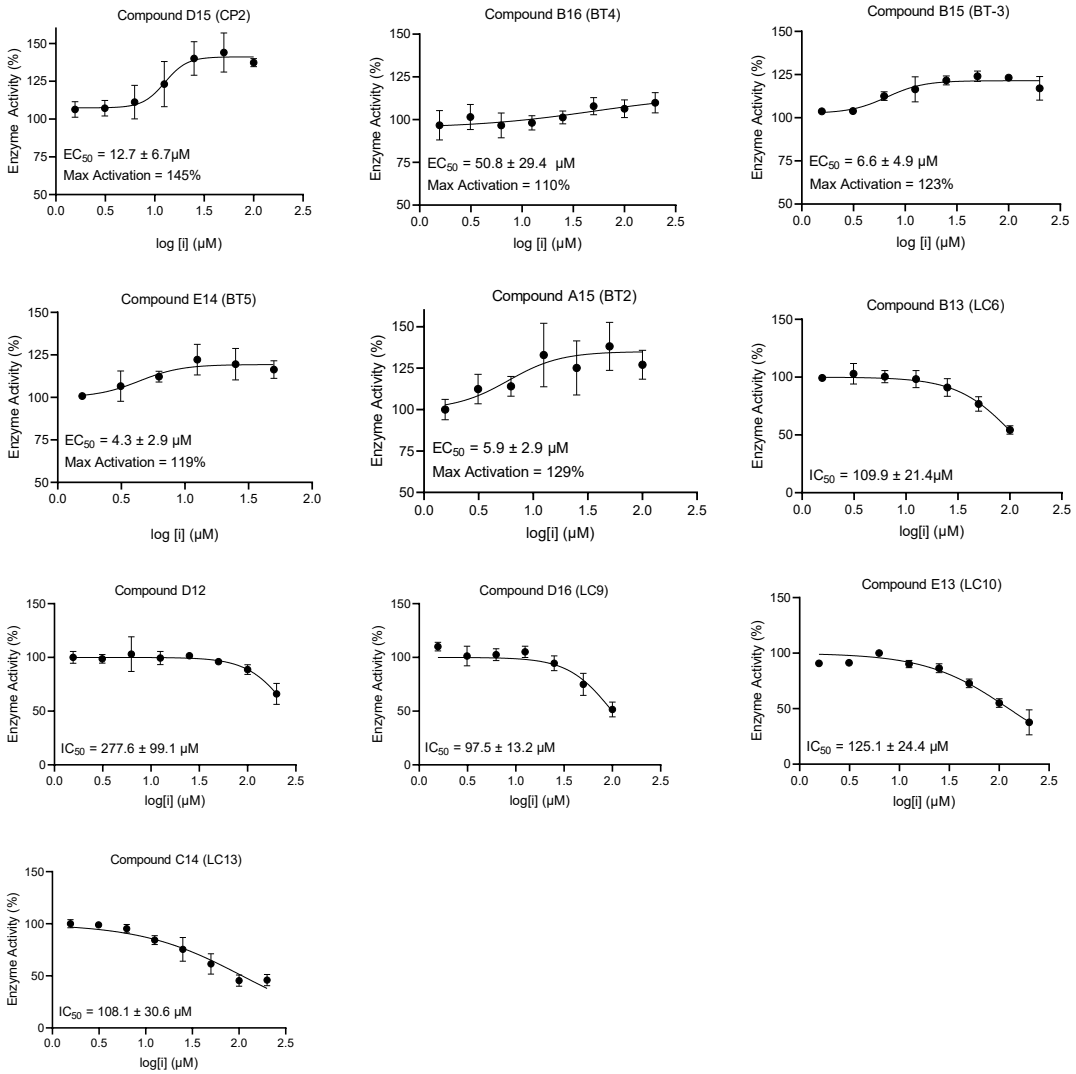


Figure S3. The graphs of IC_{50} (inhibitors: B13 (LC6), D12, D16 (LC9), E13 (LC10), C14 (LC13)) or EC_{50} (activators: A16 (CP5), D14 (BT1), D9 (CP4), D15 (CP2), B16 (BT4), B15 (BT3), E14 (BT5), A15 (BT2)) values of the most promising compounds. Maximum percentage of enzyme activation is also indicated for each compound.

Selectivity Profile

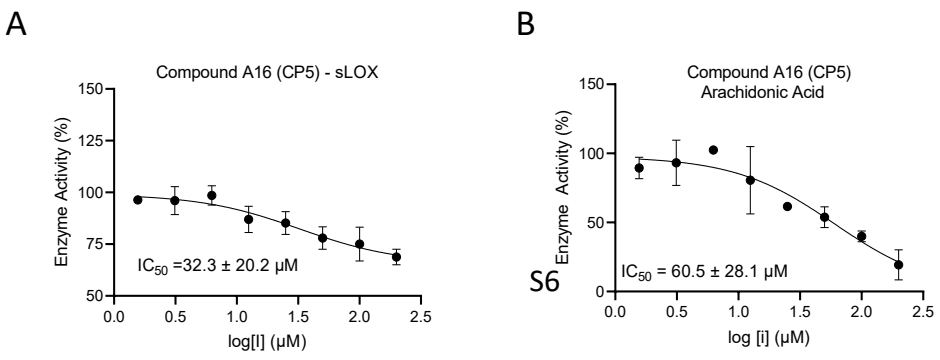


Figure S4. The IC_{50} graphs of CP5 **A)** with soybean LOX (sLOX) and Linoleic acid; **B)** with h15-LOX-1 and Arachidonic acid.

Enzyme Kinetics

Table S2. Enzyme kinetic parameters for inhibition of 15-LOX-1 by compound LC9. These are the best fit values calculated by Prism 8.

LC9 (μM)	K_m^{app} (μM)	V_{max}^{app} (absorbance/s)

0	1.4	3.1×10^4
10	2.9	3.0×10^4
40	6.8	3.0×10^4

Table S3. Enzyme kinetic parameters for inhibition of 15-LOX-1 by compound CP2. These are the best fit values calculated by Prism 8.

CP2 (μM)	**K_m^{app} (μM)**	**V_{max}^{app} (absorbance/s)**
0	4.3	3.2×10^4
10	1.9	3.3×10^4
40	0.19	3.3×10^4

Molecular Modeling

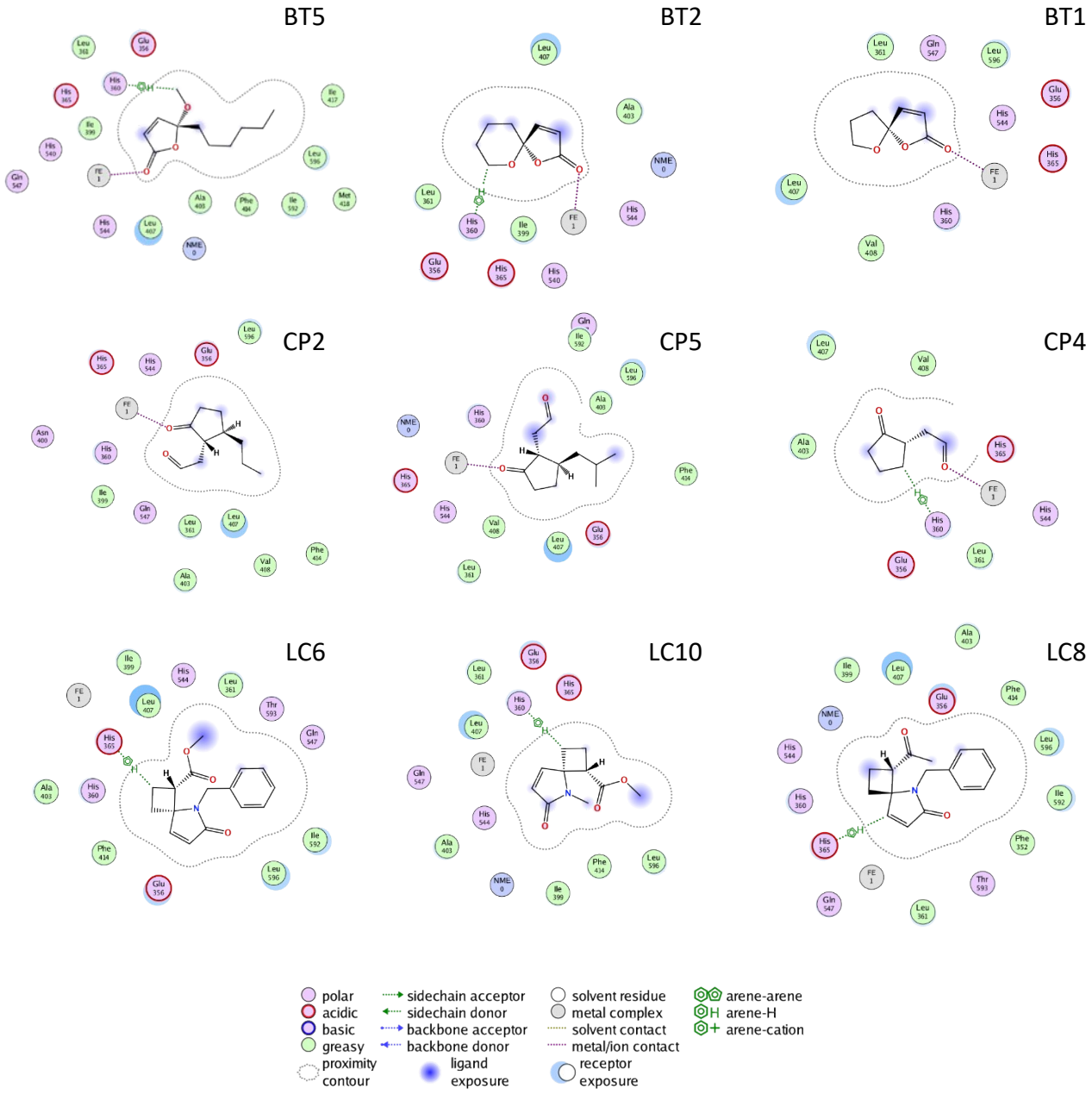


Figure S5. 2D illustration of the interactions between BT, CP, and LC compounds and the h15-LOX-1 active site, as modeled using MOE software.

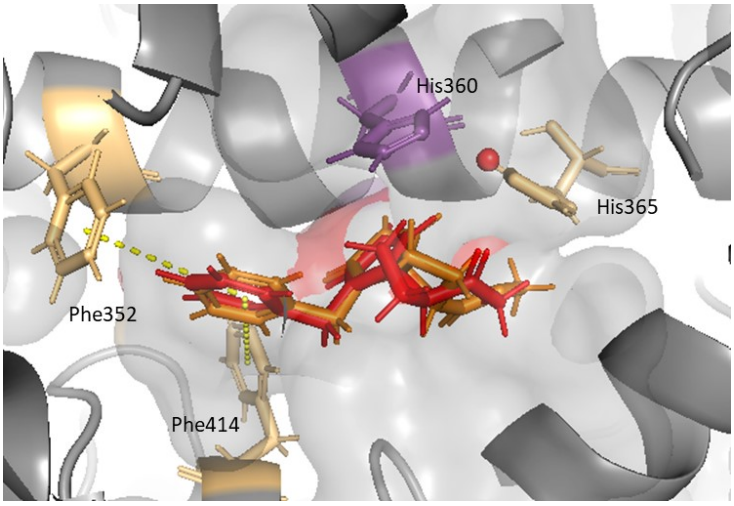


Figure S6: Predicted binding poses of compounds **LC6** (orange) and **LC8** (red) in the h15-LOX-1 active site, modeled using MOE software. Key predicted interactions with residues Phe352 and Phe414 are highlighted.

Organic Synthesis: General methods/experimental procedures

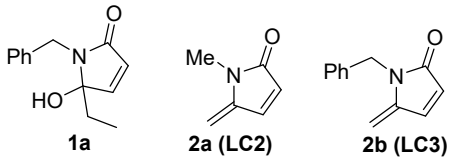
General methods

Reactions were monitored by thin layer chromatography (TLC) carried out on Millipore precoated silica gel plates with a layer thickness of 0.20 mm, using UV light as a visualizing agent. TLC plates were also visualized with a solution of H_2O (94 mL), $Ce(SO_4)_2{\cdot}H_2O$ (1 g), phosphomolybdic acid (1.5 g) and H_2SO_4 (6 mL). NMR data were obtained for ^1H at 500 MHz and for ^{13}C at 125 MHz. ^1H NMR splitting patterns are designated as singlet (s), doublet (d), triplet (t), multiplet (m), doublet of doublets (dd) and quartet of doublets (qd).

HRMS data was recorded on a Q-Exactive Plus Orbitrap MS, using ESI as ionization source.

Many of the compounds included in this study are known compounds from the synthetic studies previously published by the Vassilikogiannakis group where their synthesis and characterization data can be found.[1-3] The synthesis and characterization of all new compounds included in the current study are given below.

Starting substrates



1a **2a (LC2)** **2b (LC3)**

Compounds above were prepared as previously reported: **1a**,[1] **2a**,[2] & **2b**.[2]

Synthesis of compound D13

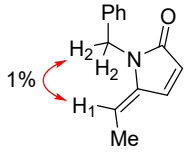
Compound **1a** (0.2 mmol, 43.5 mg) was dissolved in DCM and afterwards p-TSA·H_2O (0.02 mmol, 3.8 mg) was added. Reaction was monitored by tlc. After completion (30 min.), the mixture was quenched with 2 drops of Et_3N and concentrated. Product **D13** was purified by flash column chromatography (silica gel neutralized with Et_3N, petroleum ether: EtOAc = 5:1 → 3:1) to furnish **D13** as a single isomer and as a brown oil (yield = 93%, 37.0 mg).

^{1}H NMR (500 MHz, $CHCl_3$) δ 7.31-7.16 (m, 6H), 6.26 (d, *J*=5.9 Hz, 1H), 5.37 (qd, J_1=7.5 Hz, J_2=1.1 Hz, 1H), 4.82 (s, 2H), 1.88 (d, *J*=7.5 Hz, 3H) ppm.

^{13}C NMR (125 MHz, $CDCl_3$) δ 170.1, 139.9, 137.4, 132.3, 128.6, 127.2, 126.8, 123.9, 110.8, 42.5, 13.1 ppm.

HRMS (Orbitrap ESI): $[M+H]^+$ calcd. for $C_{13}H_{14}NO$, 200.1069; found 200.1069.

Representative NOE for compound **D13**



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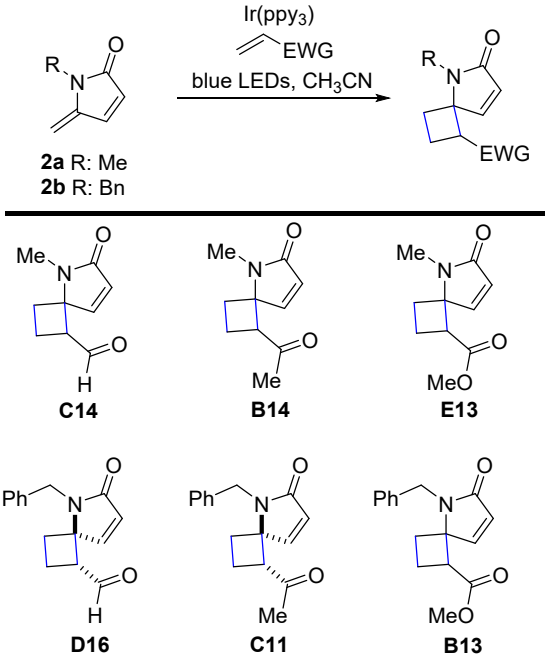
[1] D. Kalaitzakis, A. Bosveli, T. Montagnon and G. Vassilikogiannakis, *Chem. Eur. J.*, 2022, **28**, e202200322.
[2] D. Kalaitzakis, I. Kampouropoulos, M. Sofiadis, T. Montagnon and G. Vassilikogiannakis, *Chem. Commun.*, 2022, **58**, 8085-8088.
[3] L.-P. Apostolina, A. Bosveli, A. Profyllidou, T. Montagnon, V. Tsopanakis, M. Kaloumenou, D. Kalaitzakis and G. Vassilikogiannakis, *Org. Lett.*, 2022, **24**, 8786–8790.

General procedure for the photocatalytic transformation of compounds of type 2, via intermolecular [2+2] cycloaddition.[2]

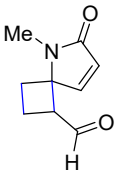


N-Bn bearing compounds **D16**, **C11** & **B13** were already synthesized according to our previous studies, utilizing 10 eq of the corresponding α, β-unsaturated compound.[2]

For the N-methyl bearing compounds **C14**, **B14** & **E13**, 20 eq of the α, β-unsaturated compounds were used, due to dimerization of **2a**. The experimental procedure is described below.

To a solution of compounds of type **2** (0.1 mmol, 10.9 mg for **2a**, 18.5 mg for **2b**) in dry CH_3CN (1 mL, 0.1 M) at rt, the photocatalyst $Ir(ppy)_3$ (0.5%, 0.3 mg, 0.0005 mmol) was added and argon (balloon) was gently bubbled through the solution for 10 min. Afterwards, freshly distilled amount of the corresponding α, β-unsaturated compound (2 mmol, 133.5 µL for acrolein, 165.0 µL for methyl vinyl ketone, 180.0 µL for methyl acrylate) was added and the solution was irradiated using blue LED light strips (60 LEDs/m, 10.8 w/m, 1000 lm/m, λ_{max} = 420 nm) at room temperature. After completion of the reaction, as indicated by TLC analysis (usually 20 h), the solution was concentrated *in vacuo* and the desired products were purified by flash column chromatography.

5-methyl-6-oxo-5-azaspiro[3.4]oct-7-ene-1-carbaldehyde (C14)



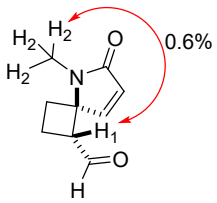
Product **C14** was synthesized according to the experimental procedure described above. The crude product was purified by flash column chromatography (silica gel, petroleum ether: EtOAc = 6:1 → 1:1) to furnish **C14** as an inseparable mixture of two isomers 1:0.16 (see ^{1}H NMR integrations at 9.71 and 9.45 ppm) and as yellow solid (yield = 50%, 8.3 mg).

^{1}H NMR (500 MHz, $CHCl_3$) δ 9.71 (s, 1H for minor isomer), 9.45 (s, 1H for major isomer), 7.27 (d, *J*=5.8 Hz, 1H for minor isomer), 7.24 (d, *J*=6.0 Hz, 1H for major isomer), 6.16 (d, *J*=6.0 Hz, 1H for major isomer), 6.15 (d, *J*=5.8 Hz, 1H for minor isomer), 3.67 (t, *J*=9.2 Hz, 1H for major isomer), 3.60 (dd, J_1=7.6 Hz, J_2=6.7 Hz, 1H for minor isomer), 3.04 (s, 3H for major isomer), 2.89 (s, 3H for minor isomer), 2.52 (m, 1H for both isomers), 2.36 (m, 1H for both isomers), 2.00 (m, 2H for both isomers) ppm.

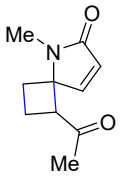
^{13}C NMR (125 MHz, $CDCl_3$) δ 199.1 (minor), 198.5 (major), 170.0 (both), 150.3 (minor), 146.6 (major), 126.3 (major), 125.3 (minor), 69.9 (both), 52.7 (minor), 51.3 (major), 26.7 (minor), 26.4 (minor), 25.7 (major), 24.4 (major), 14.7 (minor), 13.7 (major) ppm.

HRMS (Orbitrap ESI): $[M+H]^+$ calcd. for $C_9H_{12}NO_2$, 166.0862; found 166.0860.

Representative NOE for the major diastereomer of compound **C14**



1-acetyl-5-methyl-5-azaspiro[3.4]oct-7-en-6-one (B14)



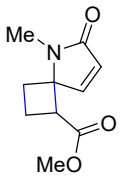
Product **B14** was synthesized according to the experimental procedure described above. The crude product was purified by flash column chromatography (silica gel, petroleum ether: EtOAc = 6:1 → 1:1) to furnish **B14** as an inseparable mixture of two isomers 1:0.6 (see ^{1}H NMR integrations at 7.19 and 7.14 ppm) and as yellow solid (yield = 63%, 11.3 mg).

^{1}H NMR (500 MHz, $CHCl_3$) δ 7.19 (d, *J*=5.8 Hz, 1H for minor isomer), 7.14 (d, *J*=6.0 Hz, 1H for major isomer), 6.17 (d, *J*=5.8 Hz, 1H for minor isomer), 6.15 (d, *J*=6.0 Hz, 1H for major isomer), 3.65 (m, 1H for both isomers), 3.06 (s, 3H for major isomer), 2.88 (s, 3H for minor isomer), 2.64 (m, 1H for minor isomer), 2.51-2.47 (m, 1H for minor isomer), 2.44-2.40 (m, 1H for major isomer), 2.38-2.32 (m, 1H for both isomers), 1.95 (m, 1H for both isomers), 1.89 (s, 3H for minor isomer), 1.85 (m, 1H for major isomer), 1.77 (s, 3H for major isomer) ppm.

^{13}C NMR (125 MHz, $CDCl_3$) δ 204.9 (minor), 204.1 (major), 170.5 (minor), 170.1 (major), 150.6 (minor) 147.2 (major), 126.3 (major), 125.7 (minor), 70.5 (minor), 70.4 (major), 52.4 (minor), 51.9 (major), 29.0 (minor), 28.7 (major), 25.8 (minor), 25.7 (major), 24.9 (major), 24.4 (minor), 16.2 (minor), 14.0 (major) ppm.

HRMS (Orbitrap ESI): $[M+H]^+$ calcd. for $C_{10}H_{14}NO_2$, 180.1019; found 180.1018.

methyl 5-methyl-6-oxo-5-azaspiro[3.4]oct-7-ene-1-carboxylate (E13)



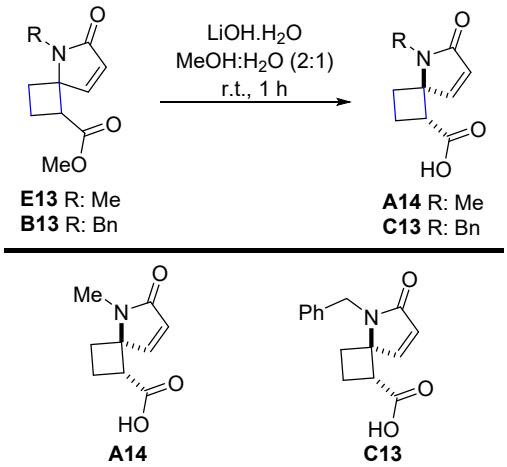
Product **E13** was synthesized according to the experimental procedure described above. The crude product was purified by flash column chromatography (silica gel, petroleum ether: EtOAc = 6:1 → 1:1) to furnish **E13** as an inseparable mixture of two isomers 1:0.9 (see ^{1}H NMR integrations at 7.29 and 7.12 ppm) and as yellow solid (yield = 71%, 13.9 mg). The reaction was scaled up to 1 mmol scale and the results were very similar (yield = 69%, 134.7 mg).

^{1}H NMR (500 MHz, $CHCl_3$) δ 7.29 (d, *J*=6.0 Hz, 1H for major isomer), 7.12 (d, *J*=5.8 Hz, 1H for minor isomer), 6.10 (d, *J*=5.8 Hz, 1H for minor isomer), 6.09 (d, *J*=6.0 Hz, 1H for major isomer), 3.65 (s, 3H for minor isomer), 3.55 (s, 3H for major isomer), 3.52-3.47 (m, 1H for both isomers), 2.97 (s, 3H for major isomer), 2.93 (s, 3H for minor isomer), 2.53 (m, 1H for both isomers), 2.44-2.39 (m, 1H for both isomers), 2.34 (m, 1H for major isomer), 2.09 (m, 1H for minor isomer), 2.03-1.98 (m, 1H for both isomers) ppm.

^{13}C NMR (125 MHz, $CDCl_3$) δ 171.5 (minor), 170.8 (major), 170.7 (minor), 170.2 (major), 150.1 (minor), 147.1 (major), 125.9 (major), 125.2 (minor), 69.8 (major), 69.6 (minor), 52.2 (major), 51.8 (minor), 45.2 (minor), 43.9 (major), 26.4 (major), 25.6 (major), 25.5 (minor), 24.3 (minor), 17.4 (major), 15.8 (minor) ppm.

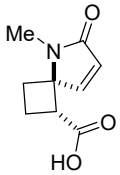
HRMS (Orbitrap ESI): $[M+H]^+$ calcd. for $C_{10}H_{14}NO_3$, 196.0968; found 196.0965.

Synthesis of compounds A14 & C13



Compounds **E13** and **B13** (0.12 mmol, 23.4 mg for **E13**, 32.5 mg for **B13**) were dissolved in MeOH: H_2O (0.1 M, 0.8 mL MeOH and 0.4 mL H_2O) and afterwards LiOH·H_2O (0.14 mmol, 5.9 mg) was added. Reactions were monitored by tlc. After completion (1 h), the solutions were acidified with 1 M HCl (pH = 2-3) and the mixtures were extracted with EtOAc (3× 2 mL). The combined organic layers were dried over $MgSO_4$, filtered and concentrated under reduced pressure to give the desired acids **A14** and **C13** without the need of further purification.

5-methyl-6-oxo-5-azaspiro[3.4]oct-7-ene-1-carboxylic acid (A14)



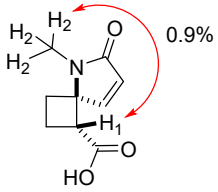
Product **A14** was synthesized and isolated according to the experimental procedure described above as a single diastereomer and as a yellow solid (yield = 50%, 10.9 mg).

^{1}H NMR (500 MHz, CD_3OD) δ 7.52 (d, *J*=6.0 Hz, 1H), 6.09 (d, *J*=6.0 Hz, 1H), 3.70 (t, *J*=9.6 Hz, 1H), 3.00 (s, 3H), 2.53 (m, 1H), 2.30 (m, 1H), 2.06-2.02 (m, 1H), 2.00-1.96 (m, 1H) ppm.

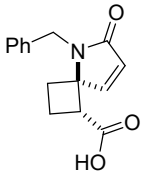
^{13}C NMR (125 MHz, CD_3OD) δ 173.8, 172.7, 150.6, 125.7, 71.9, 44.9, 25.9, 24.7, 16.9 ppm.

HRMS (Orbitrap ESI): $[M+H]^+$ calcd. for $C_9H_{12}NO_3$, 182.0811; found 182.0811.

Representative NOE for compound **A14**



5-benzyl-6-oxo-5-azaspiro[3.4]oct-7-ene-1-carboxylic acid (C13)



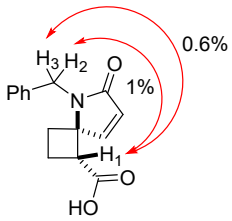
Product **C13** was synthesized and isolated according to the experimental procedure described above as a single diastereomer and as a white solid (yield = 80%, 24.7 mg).

^{1}H NMR (500 MHz, $CHCl_3$) δ 7.36 (d, *J*=6.0 Hz, 1H), 7.30 (t, *J*=7.4 Hz, 2H), 7.25 (m, 3H), 6.22 (d, *J*=6.0 Hz, 1H), 4.76 (d, *J*=16.2 Hz, 1H), 4.70 (d, *J*=16.2 Hz, 1H), 3.44 (t, *J*=9.5 Hz, 1H), 2.30 (m, 1H), 2.22 (m, 1H), 1.91 (m, 2H) ppm.

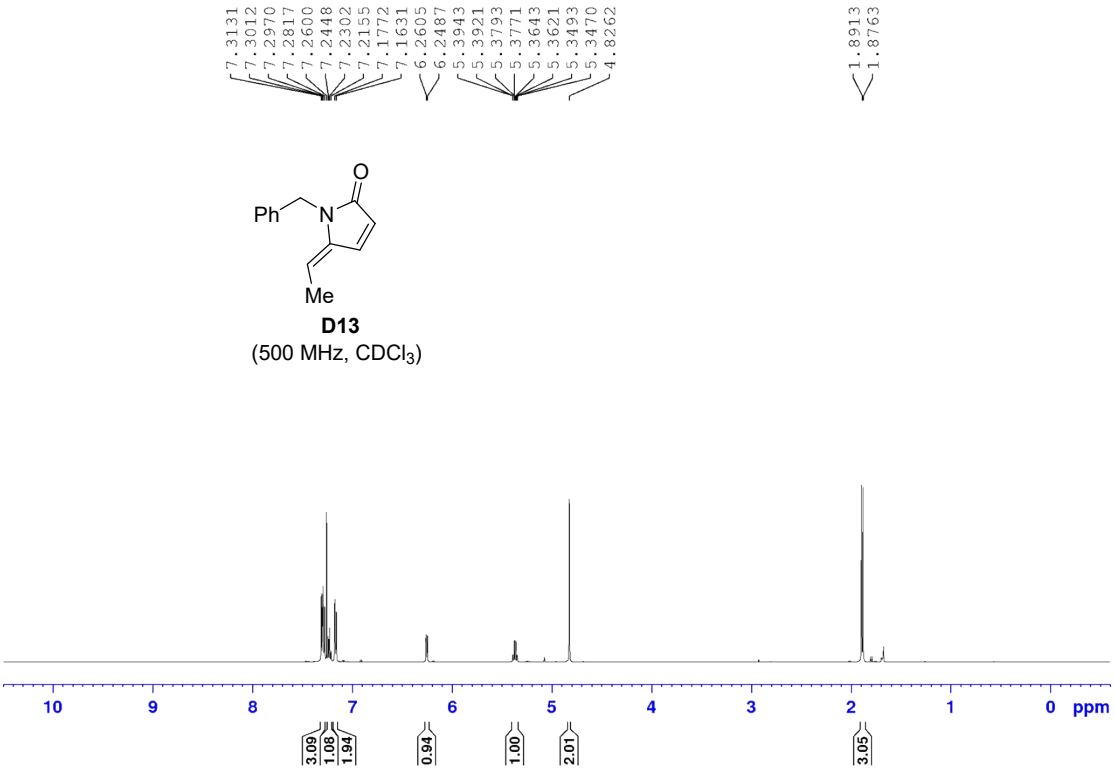
^{13}C NMR (125 MHz, $CDCl_3$) δ 174.0, 171.3, 148.2, 137.2, 128.7, 127.4, 127.1, 125.3, 70.2, 44.5, 42.6, 26.3, 16.0 ppm.

HRMS (Orbitrap ESI): $[M+H]^+$ calcd. for $C_{15}H_{16}NO_3$, 258.1124; found 258.1123.

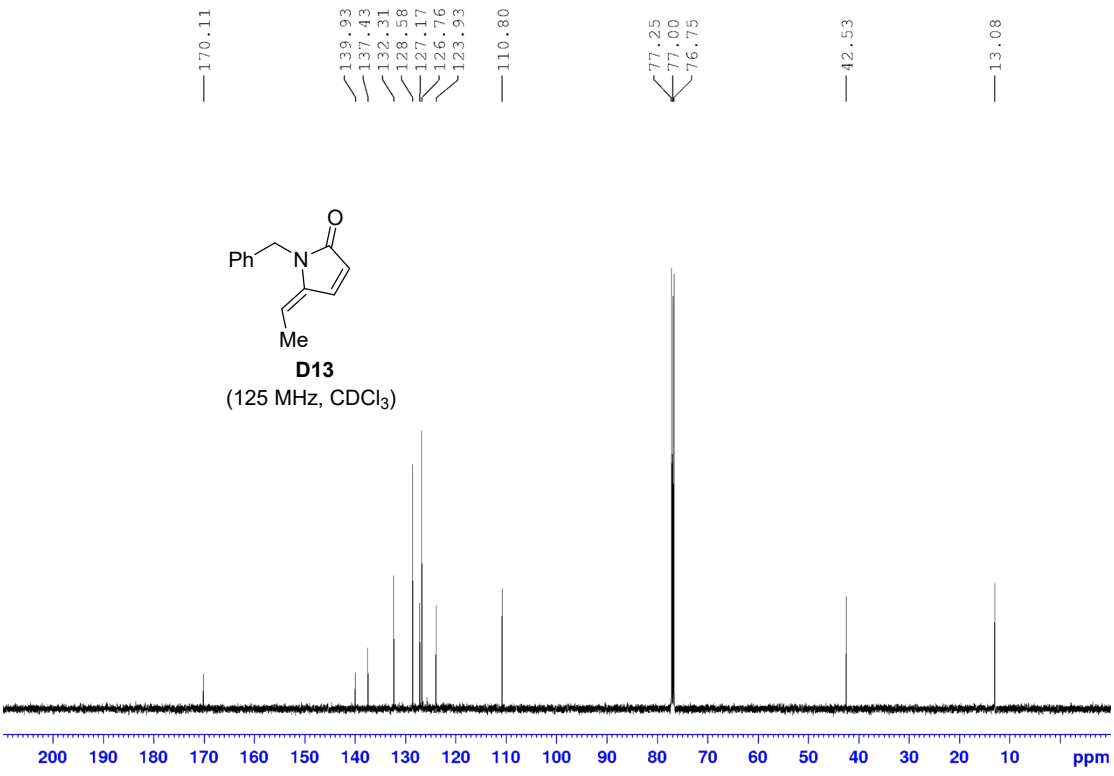
Representative NOE for compound **C13**


0.6%
1%
H3 H2
O
Ph
N
H1
O
HO

Copies of ^{1}H-NMR, ^{13}C-NMR and NOE spectra

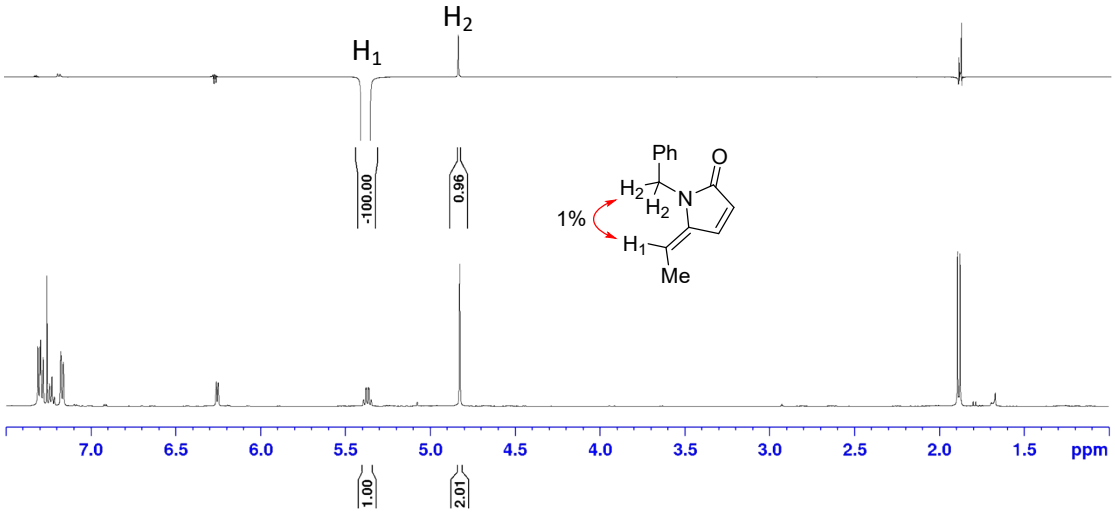


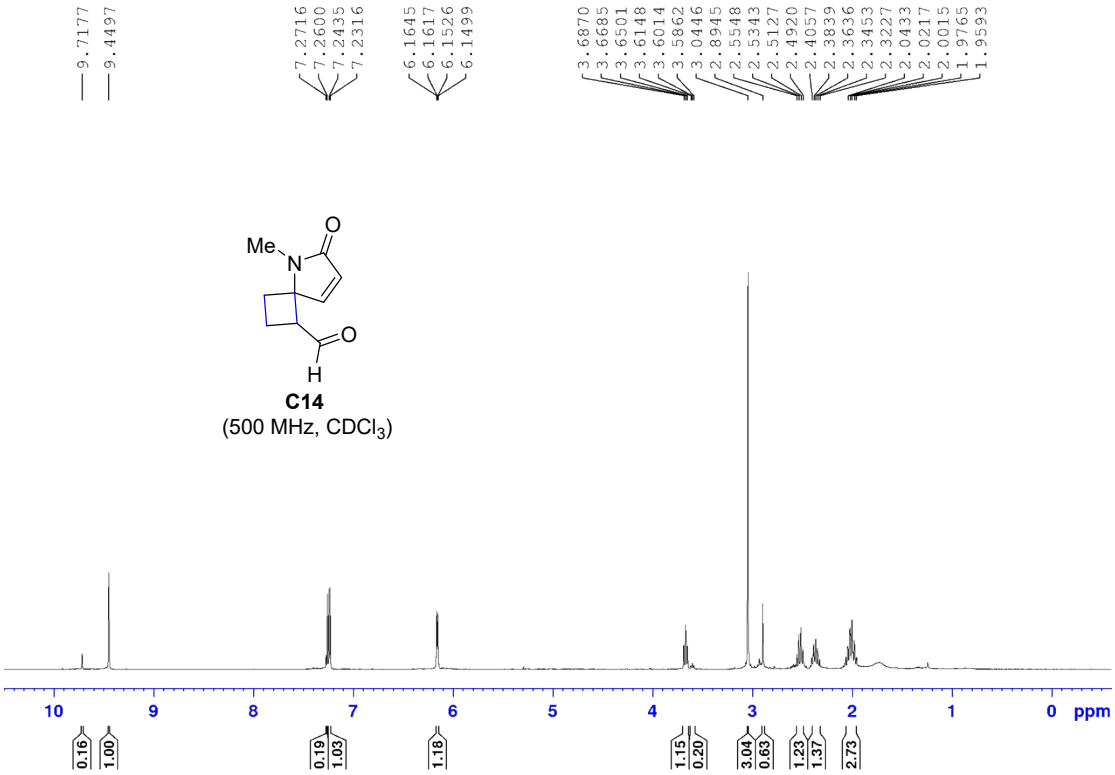
D13
(500 MHz, $CDCl_3$)



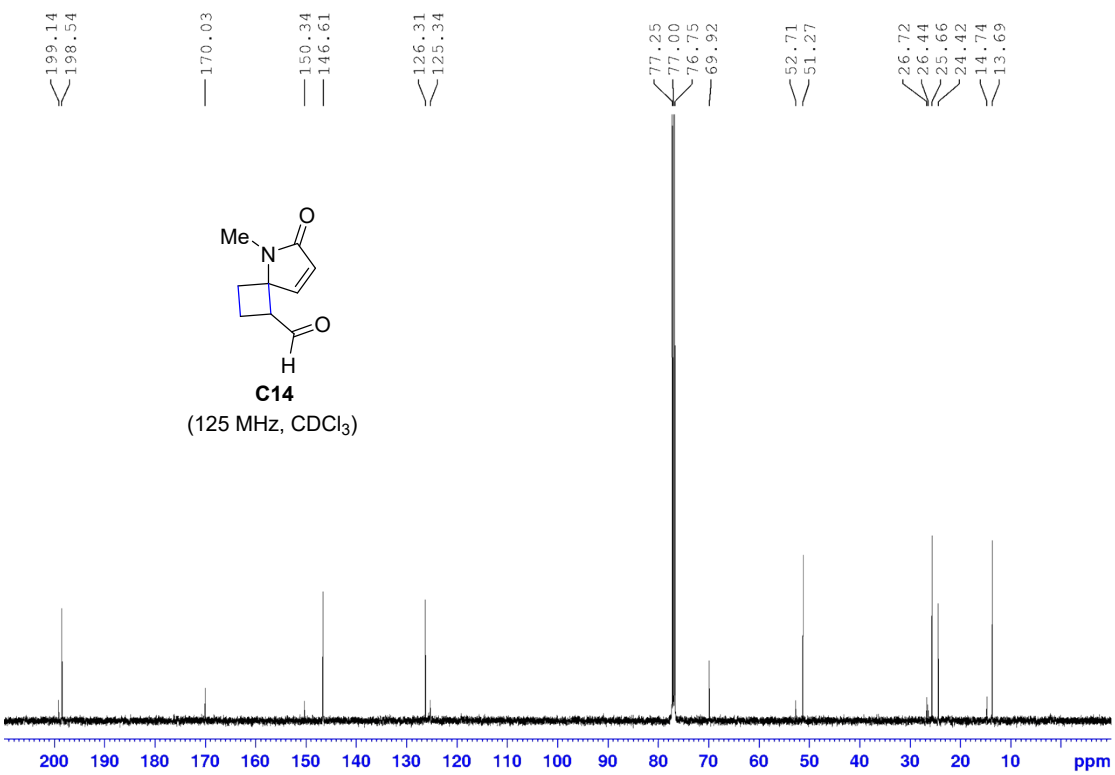
D13
(125 MHz, $CDCl_3$)

Representative NOE of compound **D13**



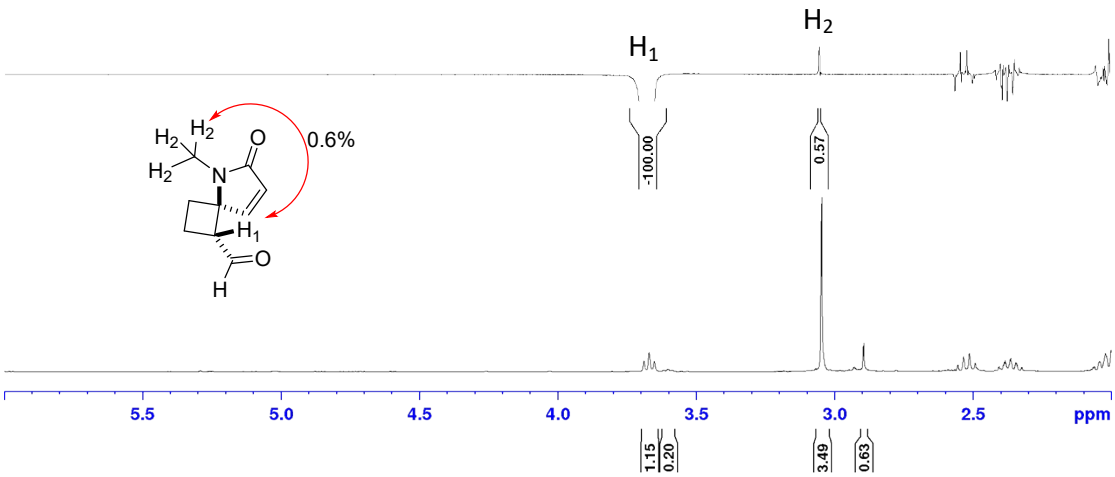


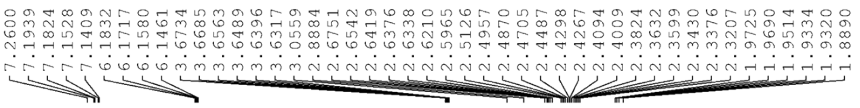
C14
(500 MHz, $CDCl_3$)

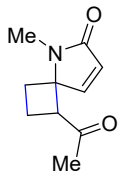


C14
(125 MHz, $CDCl_3$)

Representative NOE of the major diastereomer of compound **C14**

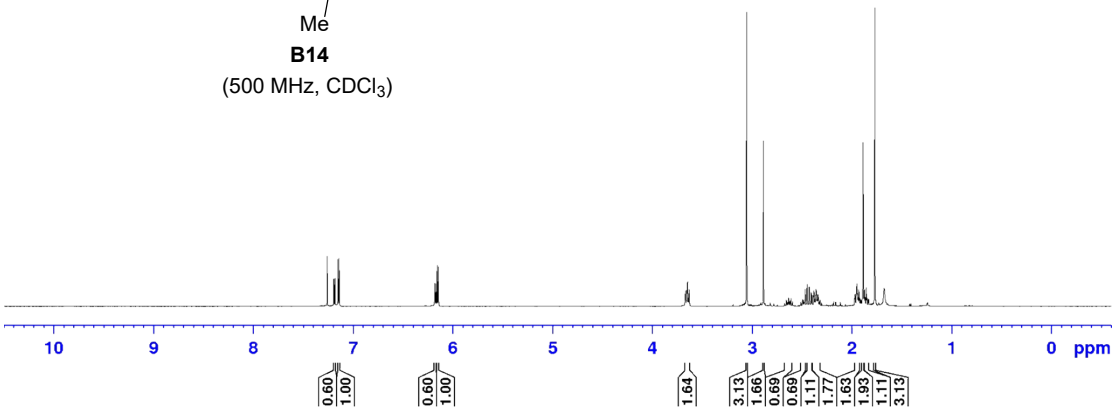


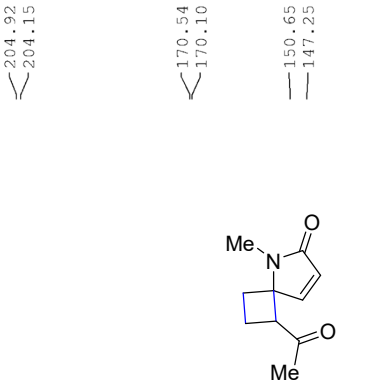




B14

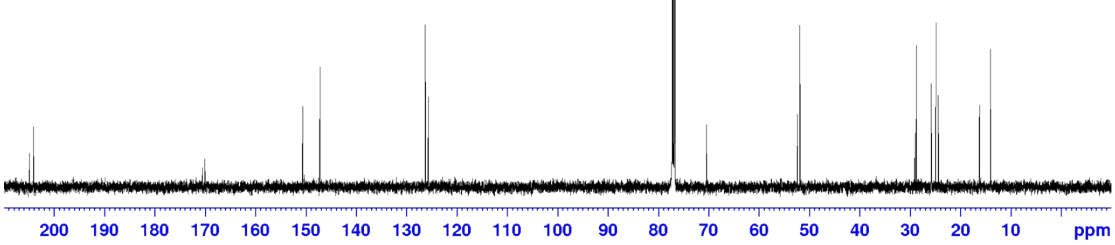
(500 MHz, $CDCl_3$)

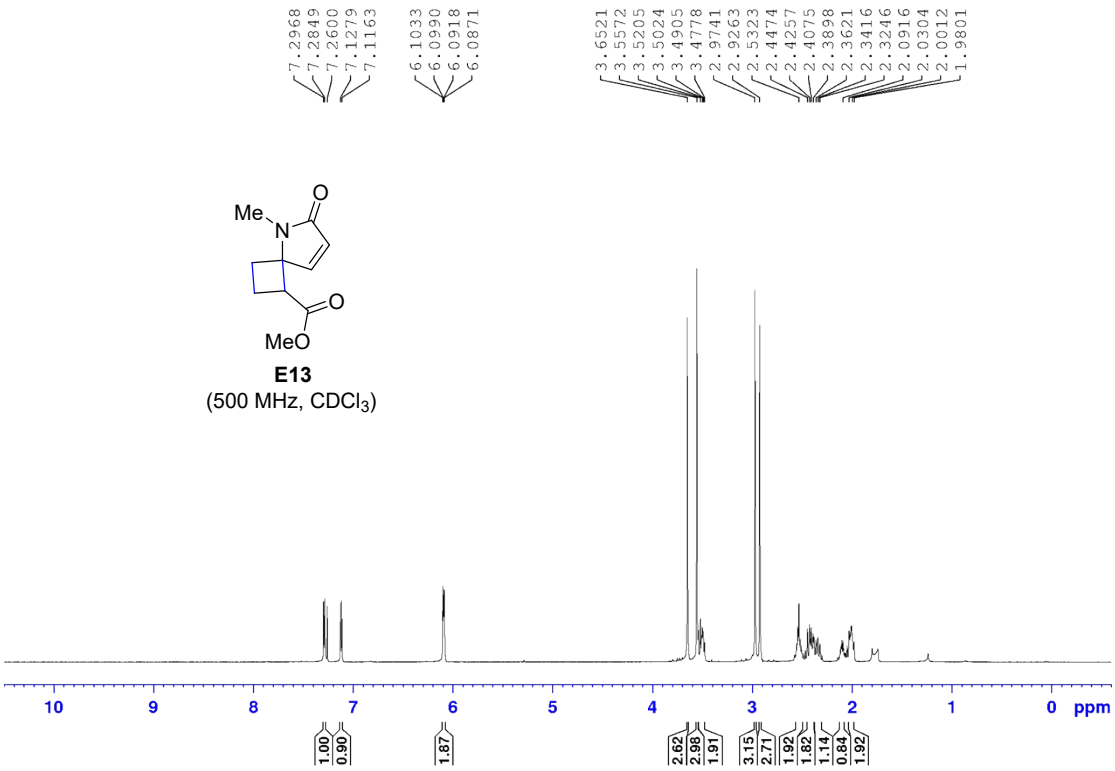




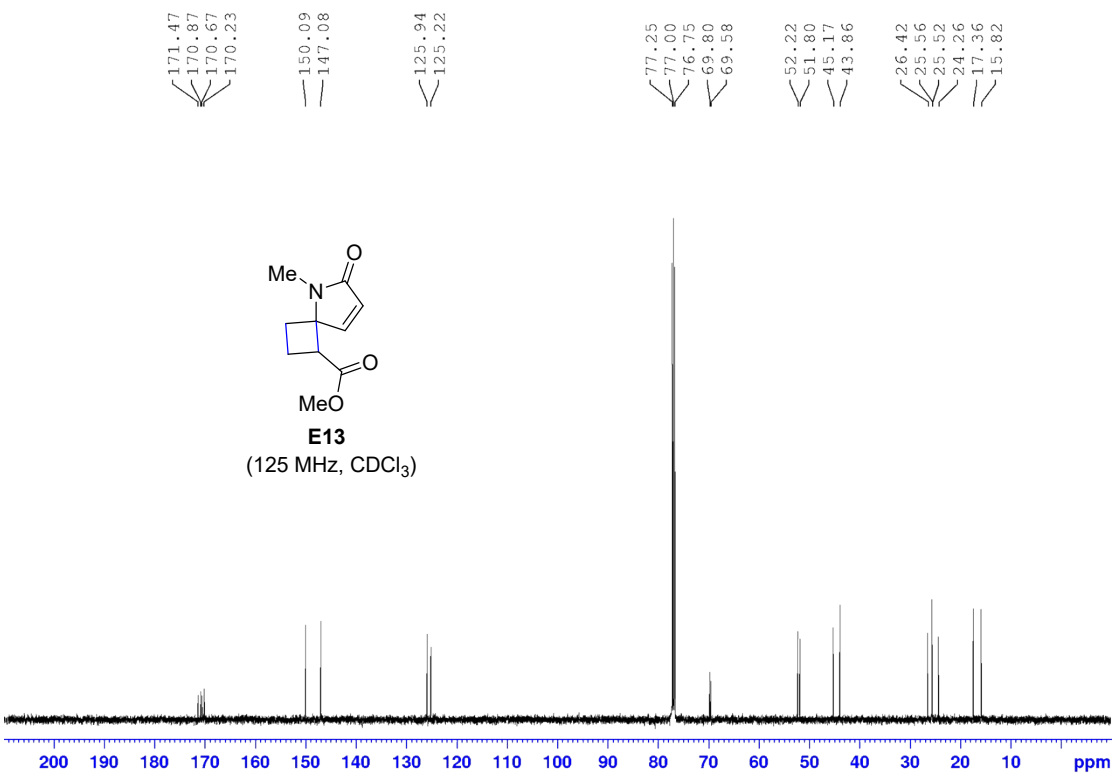
B14

(125 MHz, $CDCl_3$)

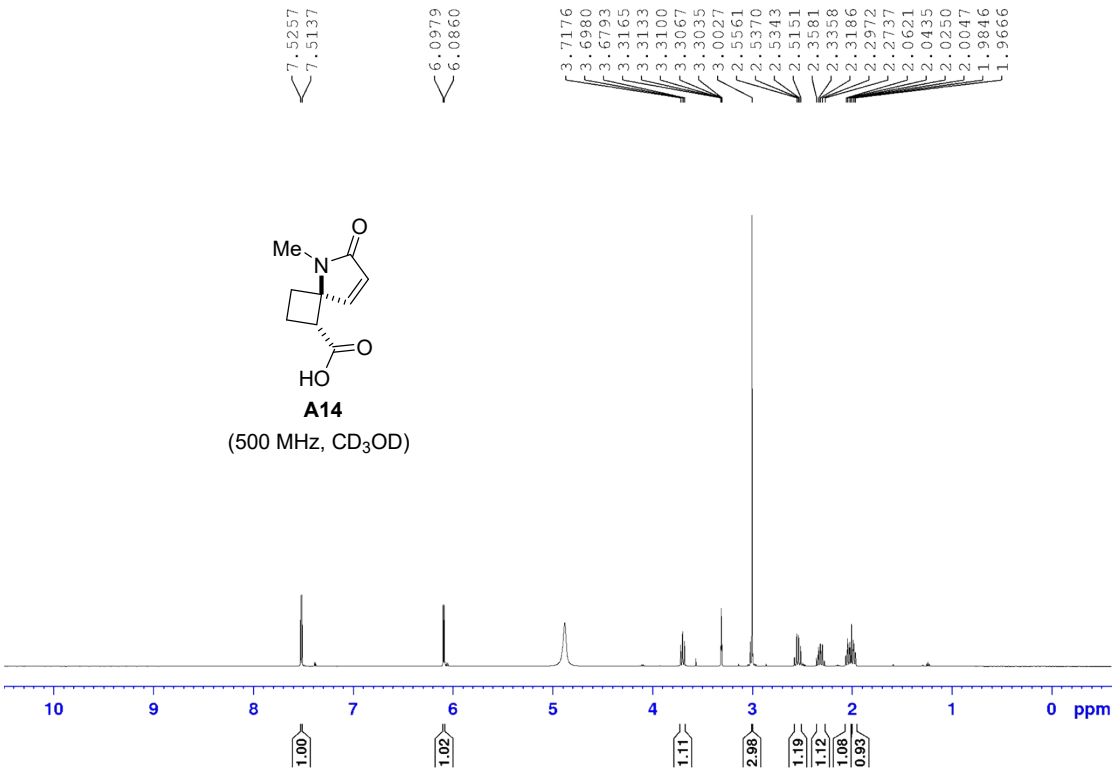




E13
(500 MHz, $CDCl_3$)

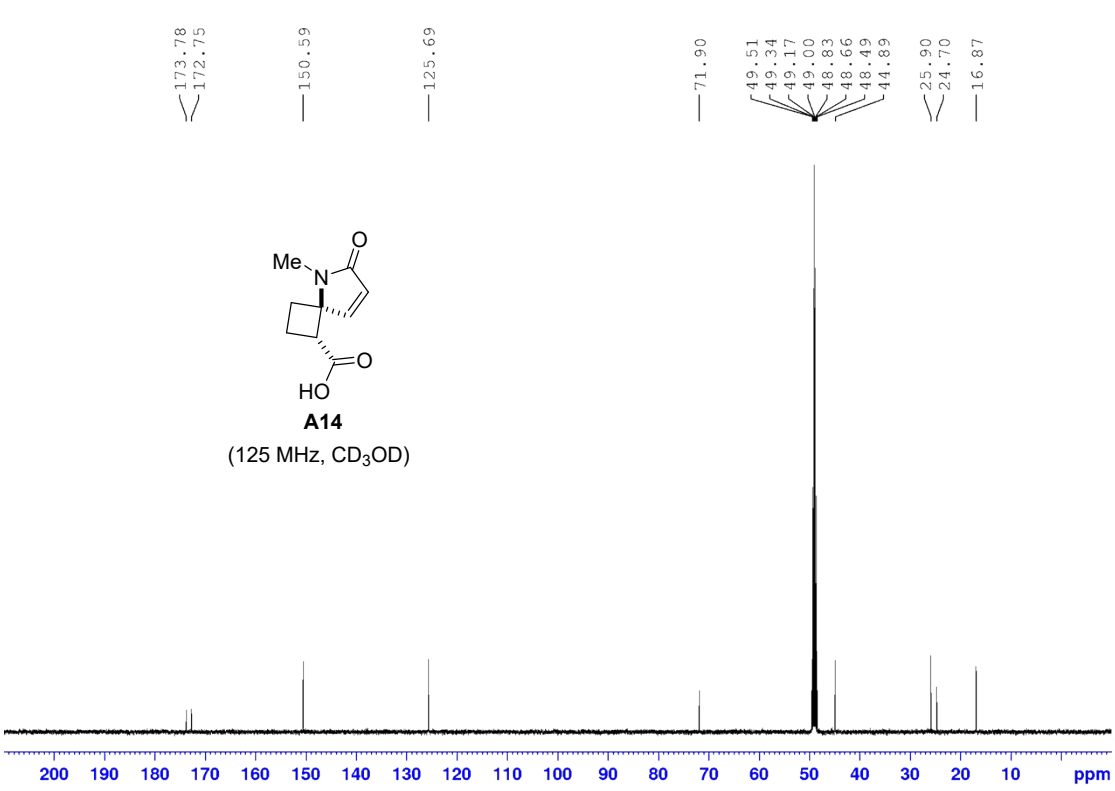


E13
(125 MHz, $CDCl_3$)



A14

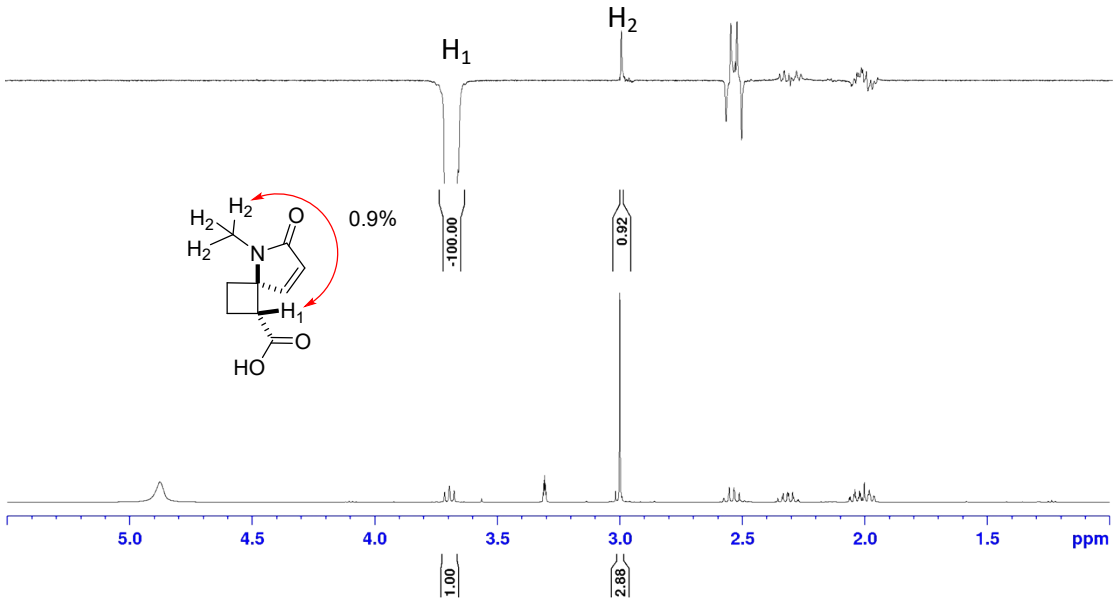
(500 MHz, CD_3OD)

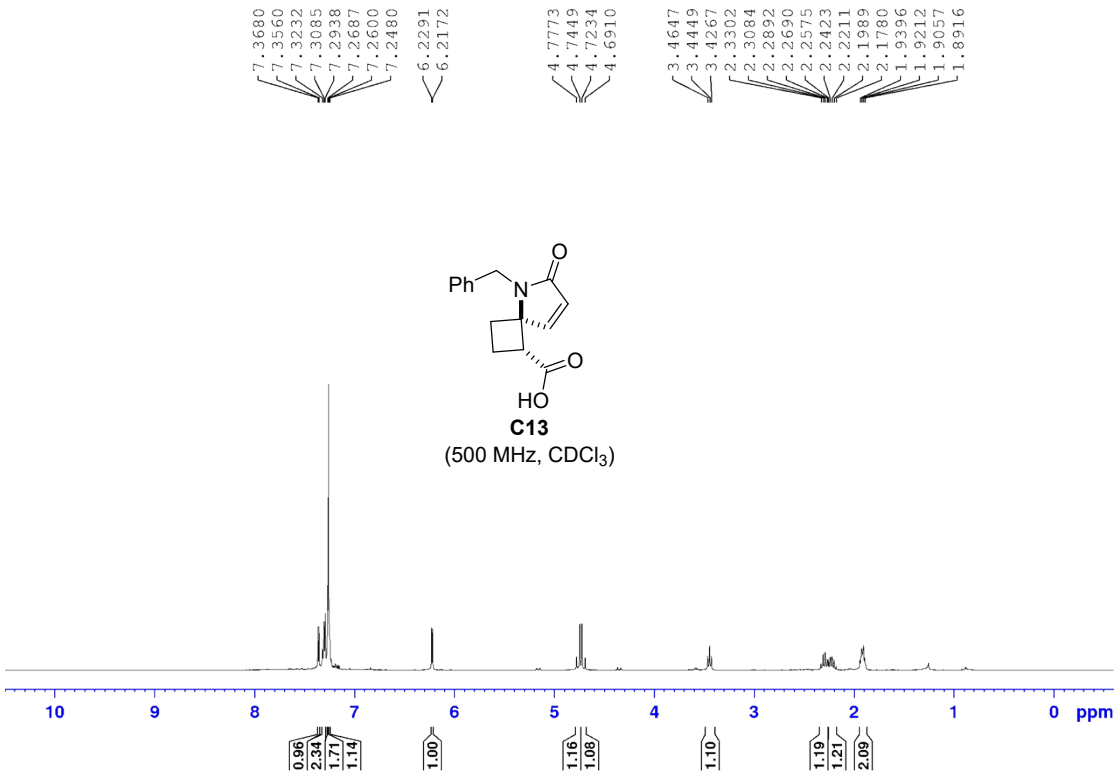


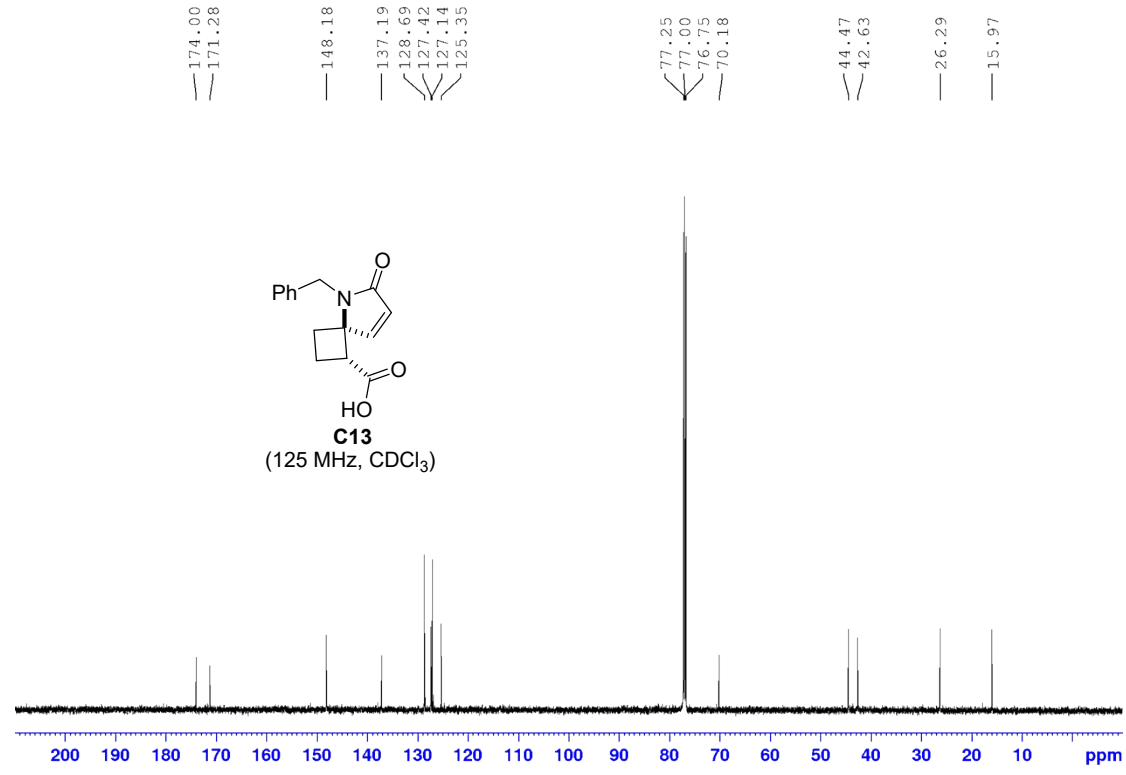
A14

(125 MHz, CD_3OD)

Representative NOE of compound **A14**




7.3680
7.3560
7.3232
7.3085
7.2938
7.2687
7.2600
7.2480
6.2291
6.2172
4.7773
4.7449
4.7234
4.6910
3.4647
3.4449
3.4267
2.3302
2.3084
2.2892
2.2690
2.2575
2.2423
2.2211
2.1989
2.1780
1.9396
1.9212
1.9057
1.8916
Ph
N
O
O
HO
C13
(500 MHz, CDCl3)
10
9
8
7
6
5
4
3
2
1
0
ppm
0.96
2.34
1.71
1.14
1.00
1.16
1.08
1.10
1.19
1.21
2.09


174.00
171.28
148.18
137.19
128.69
127.42
127.14
125.35
77.25
77.00
76.75
70.18
44.47
42.63
26.29
15.97
Ph
N
O
O
HO
C13
(125 MHz, CDCl3)
200
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
ppm

Representative NOE of compound **C13**