Supporting Information

Novel Allosteric Activators for Ferroptosis Regulator Glutathione Peroxidase 4

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I. Supplementary Figures

a) **b)**

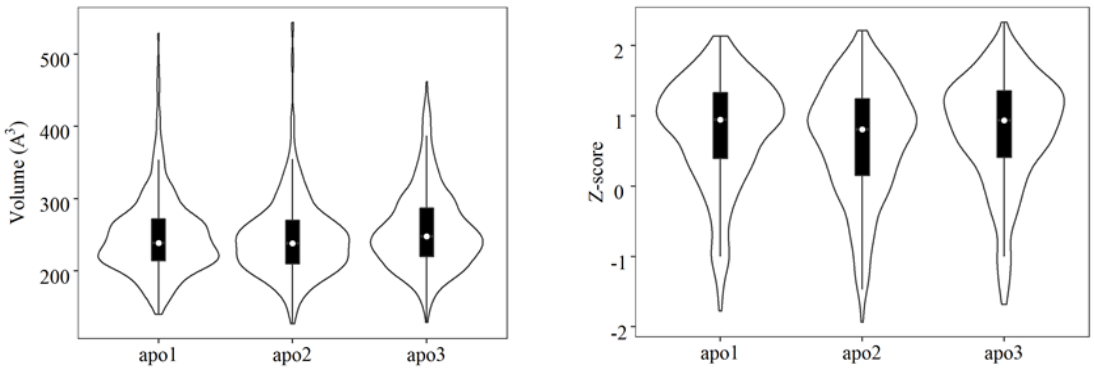


Figure S1. Violin plots for a) the volume distributions and b) Z-score distributions of the binding site in the three trajectories of apo GPX4. Three 500 ns simulations were run for the apo protein. The predicted binding site is persistent with an average volume of 250 Å^3 comparable to that in the crystal structure. The motion correlations between the active site and the predicted allosteric site also remain higher than the cut-off for allosteric site prediction (Z-score > 0.5).

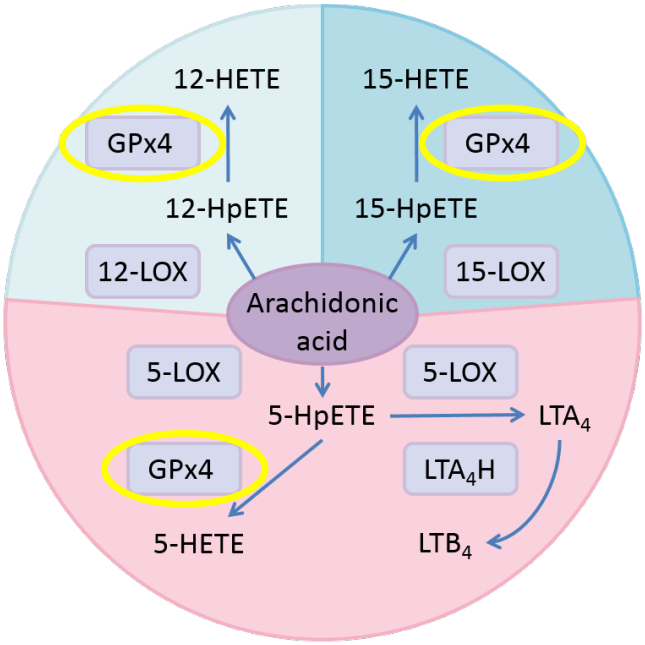


Figure S2. Eicosanoid biosynthesis pathways related to GPX4 in the AA metabolic network. In PMN cells, AA is metabolized into pro-inflammatory leukotrienes through the pathway involving 5-LOX and leukotriene A4 Hydrolase (LTA_4H). Meanwhile, AA is turned into 5-HpETE, 12-HpETE, and 15-HpETE by 5-LOX, 12-lipoxygenase (12-LOX), and 15-lipoxygenase (15-LOX), respectively. Subsequently, GPX4 catalyzes the reduction of HpETEs to generate the corresponding HETEs. Therefore when GPx4 is up-regulated, it is envisioned an increase in 12-HETE and 15-HETE at the downstream of the 12-LOX and 15-LOX pathway, respectively. On the other hand, in the 5-LOX pathway, GPx4 and LTA_4H compete for the same substrate, 5-HpETE. Hence the ratio between the concentration of 5-HETE and leukotriene B_4 (LTB_4) will increase if GPx4 is activated. Pink, 5-LOX pathway; pale blue, 12-LOX pathway; blue, 15-LOX pathway.

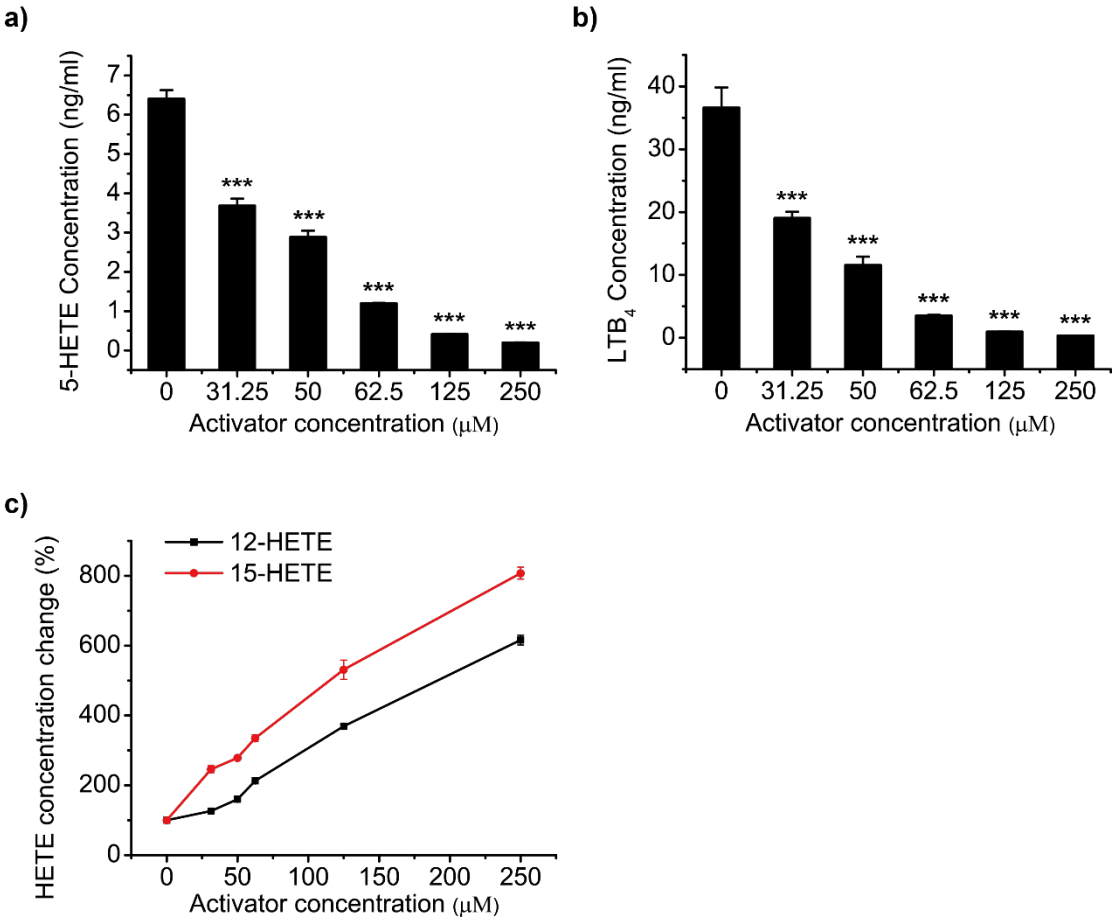


Figure S3. Dose-dependent GPX4 activation in PMN cells. a) The concentration of 5-HETE. b) The concentration of LTB_4. c) The percent changes of 12-HETE and 15-HETE concentration. Data shown represent the mean ± SEM (n = 4). The statistical significance was determined using a two-tailed t-test, ***$p < 0.001$.

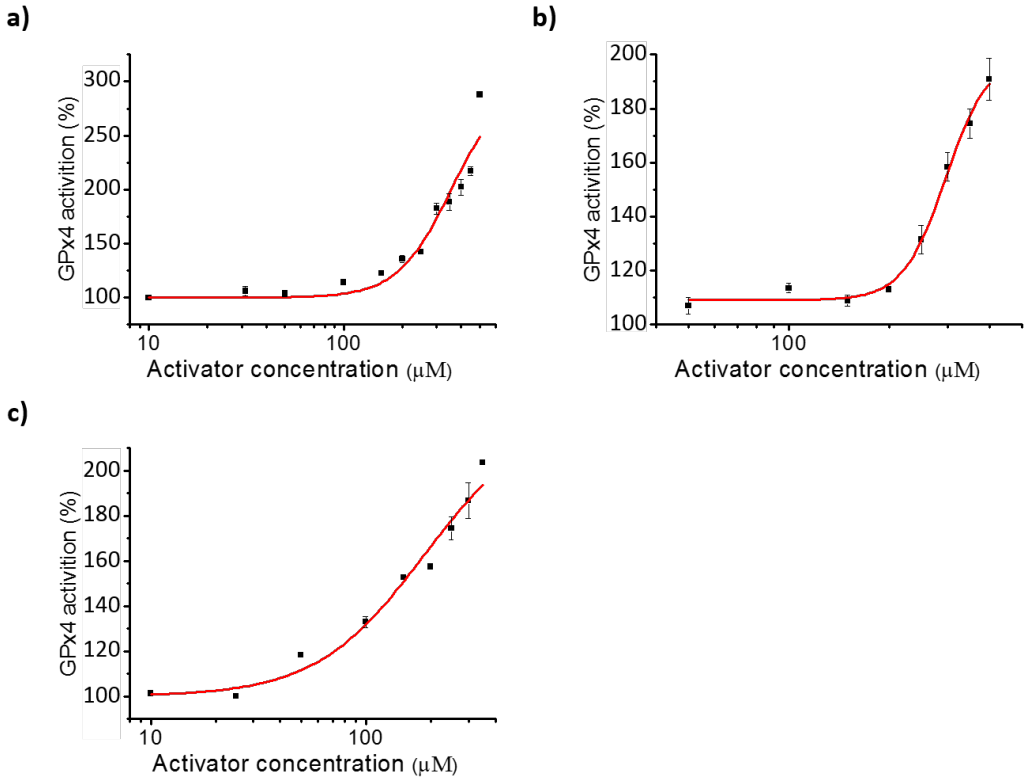


Figure S4. Dose-response curves of **1s1**, **1s2**, and **1s3** in cell-free assays. a) **1s1**. b) **1s2**. c) **1s3**. Data shown represent the mean ± SEM (n = 6).

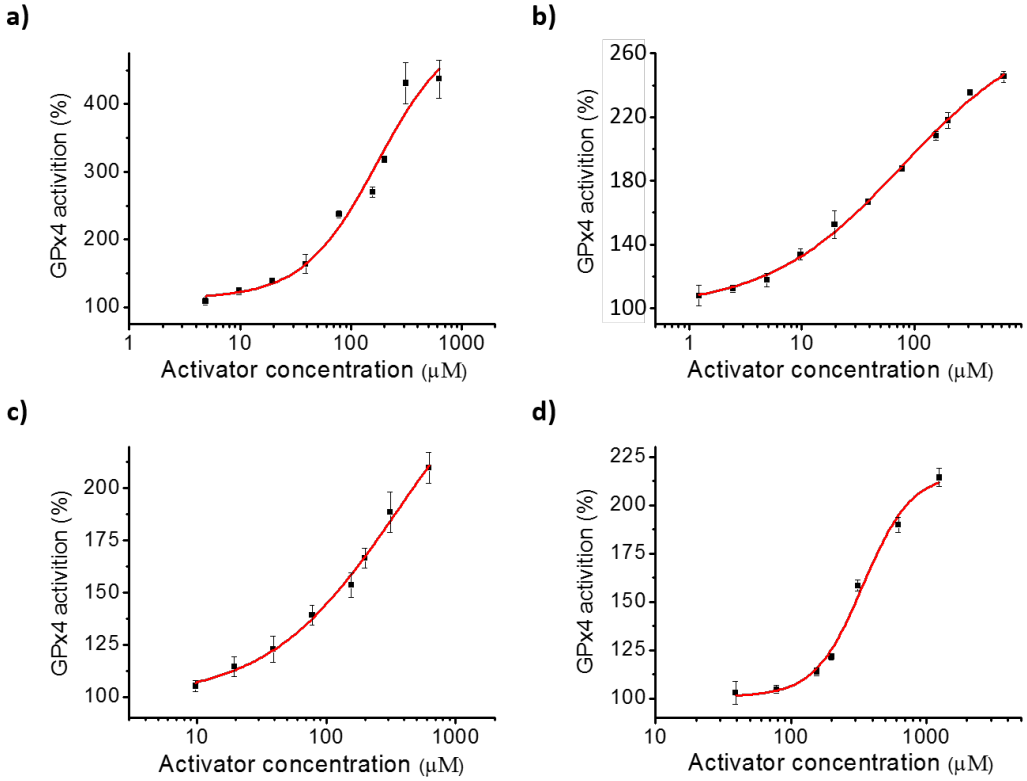


Figure S5. Dose-response curves of **1d3**, **1d4**, **1d10**, and **1d11** in cell-free assays. a) **1d3**. b) **1d4**. c) **1d10**. d) **1d11**. Data shown represent the mean ± SEM (n = 6).

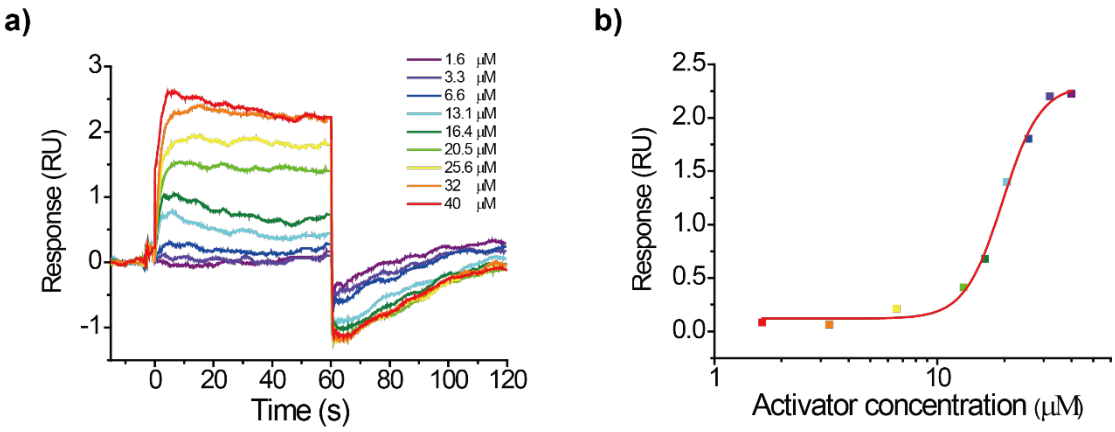


Figure S6. a) SPR dose-response curve of **1d4**. b) Data in Figure S6a at equilibrium were fitted with the Hill model (K_D = 19 ± 1 μM). The color of the dots are consistent with the color of the response curves in Figure S6a.

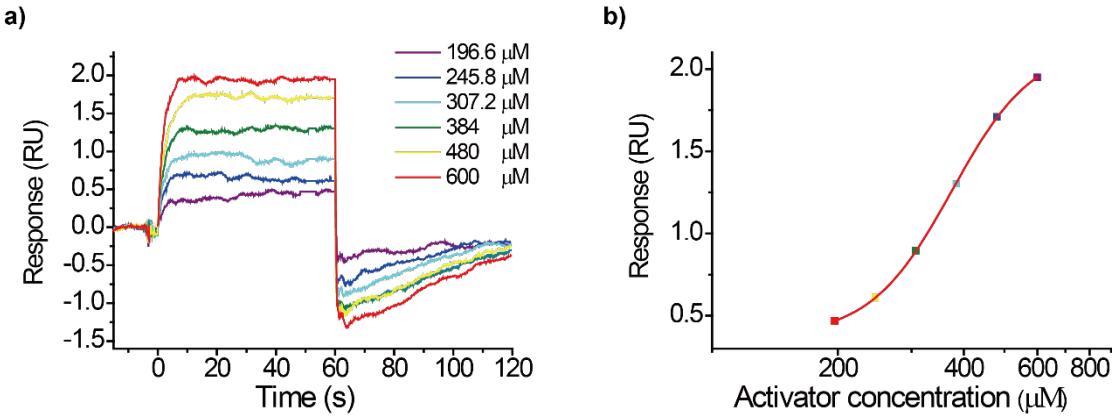


Figure S7. a) SPR dose-response curve of **1d4** with the D21A/D23A mutant. b) Data in Figure S7a at equilibrium were fitted with the Hill model (K_D = 374 ± 8 µM). The color of the dots are consistent with the color of the response curves in Figure S7a.

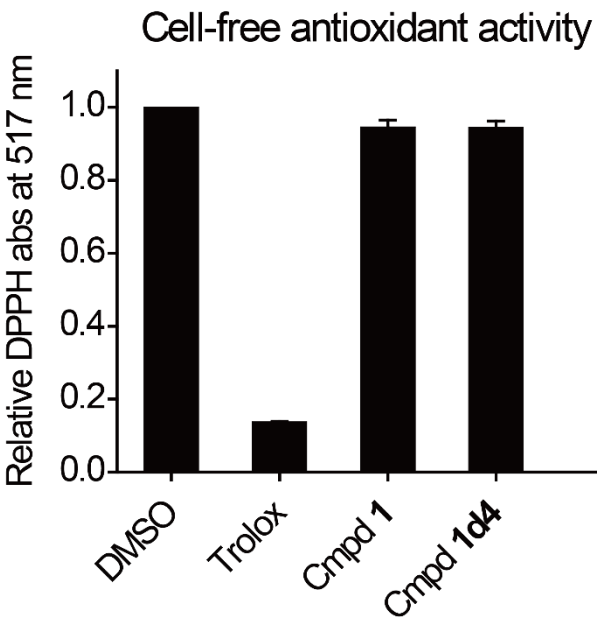


Figure S8. Cell-free antioxidant potential monitored by changes in the absorbance at 517 nm of the stable radical DPPH. The final concentration of each test compound was 500 µM. Data shown represent the mean ± s.d. (n = 3).

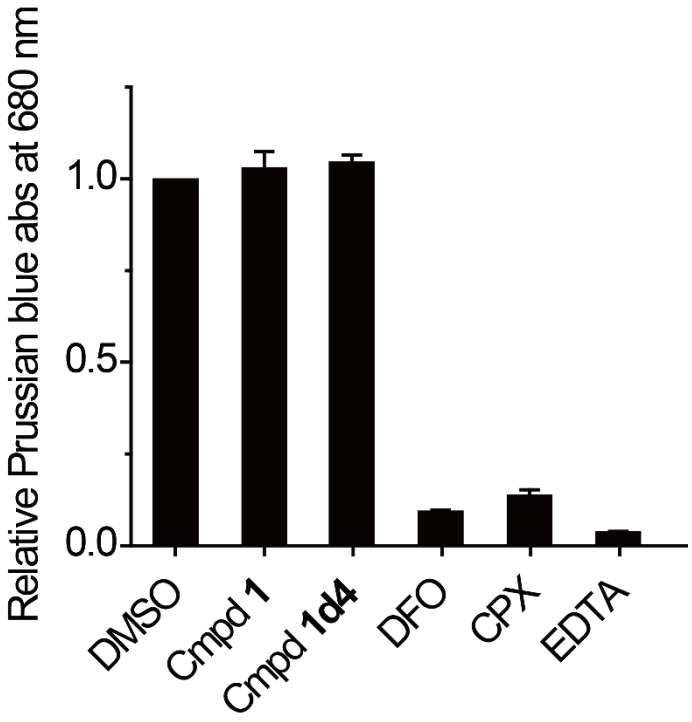


Figure S9. Iron chelating ability monitored by the absorbance at 680 nm of the Prussian blue. The iron chelators deferoxamine (DFO), ciclopirox olamine (CPX), and EDTA were used as the positive control. Data shown represent the mean ± s.d. (n = 3).

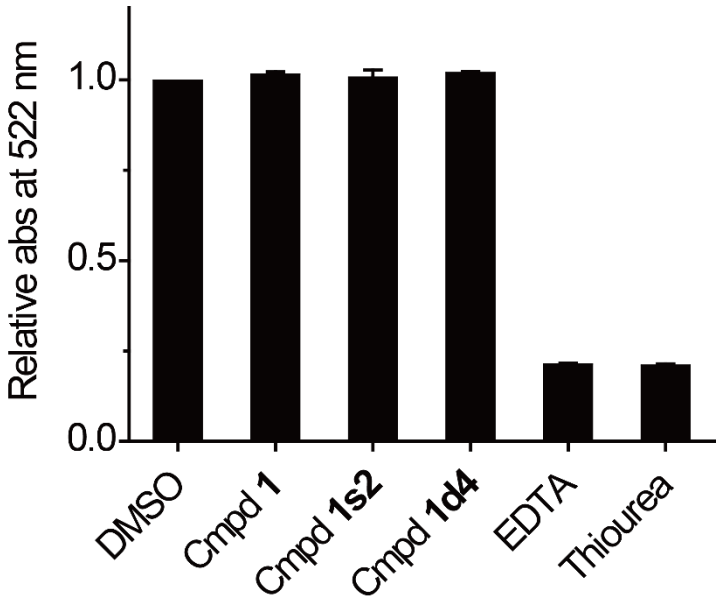


Figure S10. Zinc chelating ability. The zinc chelators EDTA and thiourea were used as the positive control. Data shown represent the mean ± s.d. (n = 3).

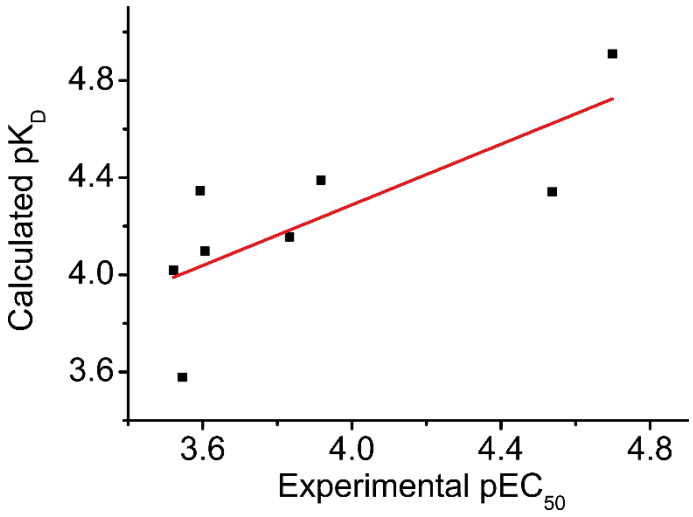


Figure S11. Correlation analysis between experimental pEC_{50} and calculated pK_D. For the 251 compounds tested in the cell-free assay, the EC_{50} values of eight of them were determined (Table 1). The calculated r-square is 0.22.

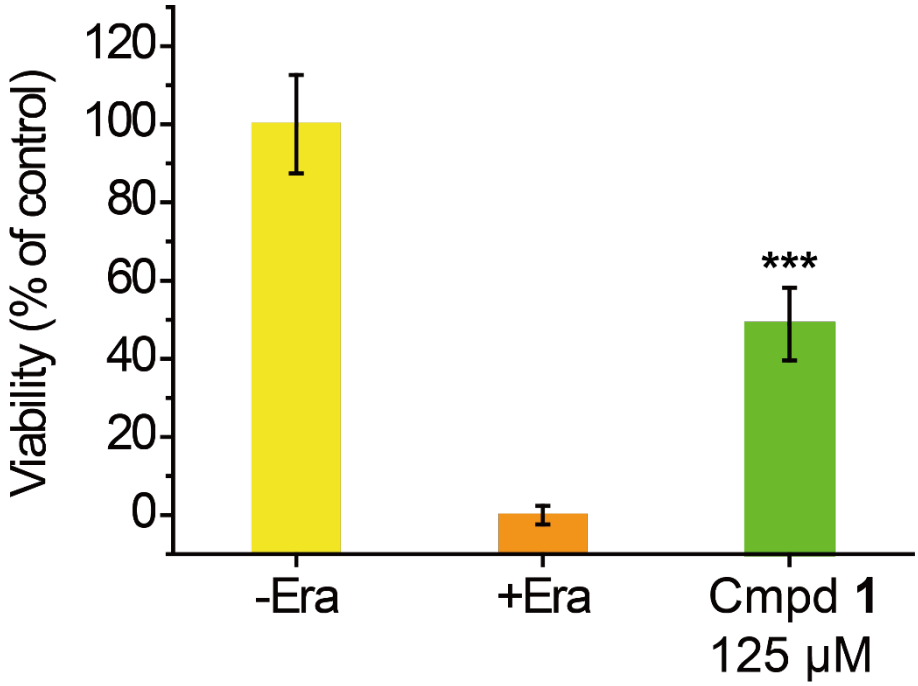


Figure S12. Prevention of erastin-induced ferroptosis by GPX4 activator **1** in HT-1080 cells. Compound **1** prevented cell death with a lethal concentration of erastin (Era, 10 µM). Cell viability was determined by trypan blue exclusion test. Data shown represent the mean ± SEM (n = 5). The statistical significance was determined using a two-tailed t-test, ***$p < 0.001$.

II. Supplementary Table

Table S1. The SPECS ID of the four GPX4 activators.

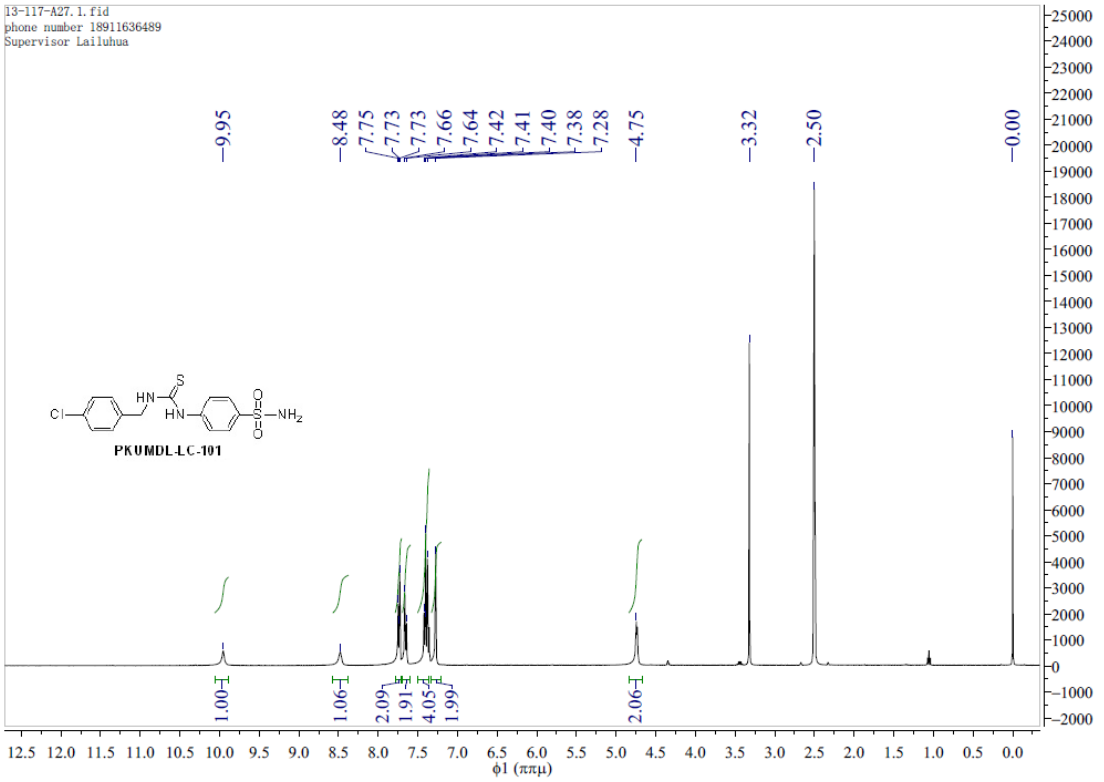
Compound	Name	SPECS ID
1	PKUMDL-LC-101	AJ-292/41694774
1s1	PKUMDL-LC-101-S01	AJ-292/41694767
1s2	PKUMDL-LC-101-S02	AJ-292/41694613
1s3	PKUMDL-LC-101-S03	AJ-292/41694742

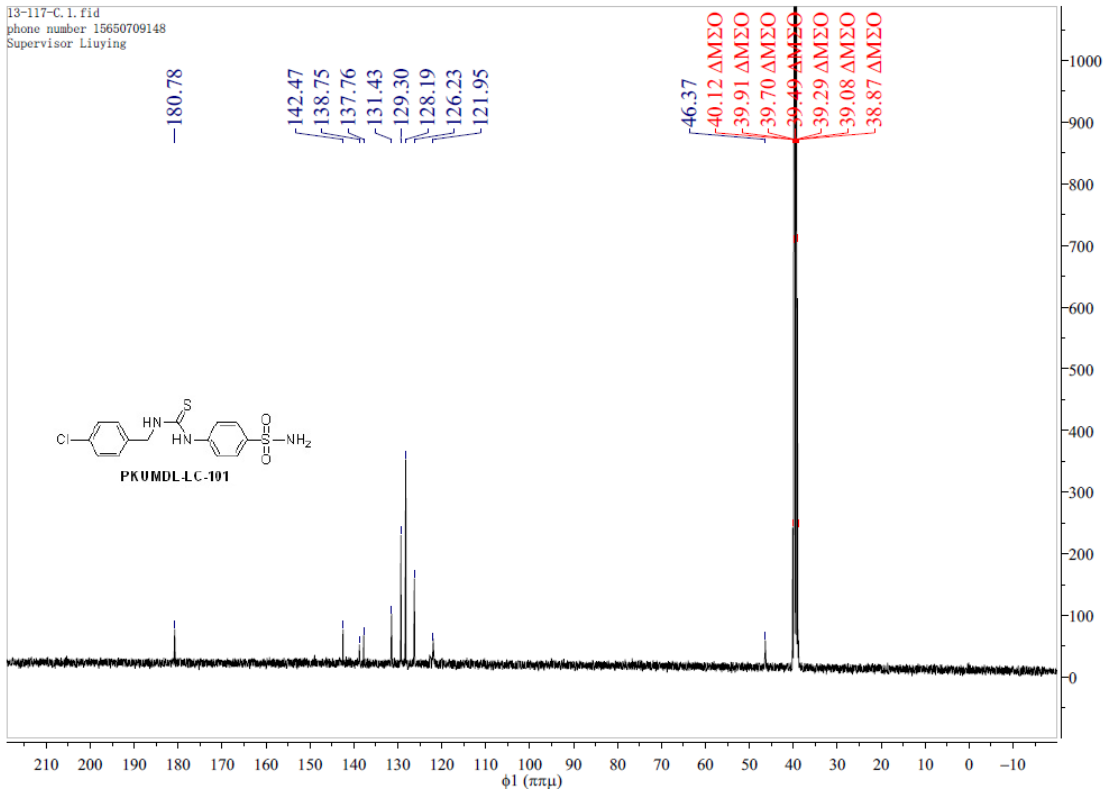
Table S2. The experimental pEC_{50} and calculated pK_D of the eight GPX4 activators.

Compound	Experimental pEC_{50}	Calculated pK_D
1	3.61	4.10
1s1	3.59	4.35
1s2	3.55	3.58
1s3	3.83	4.16
1d3	4.54	4.34
1d4	4.70	4.91
1d10	3.92	4.39
1d11	3.52	4.02

III. Spectra of Compounds

^{1}H-NMR and ^{13}C-NMR of PKUMDL-LC-101 (1)



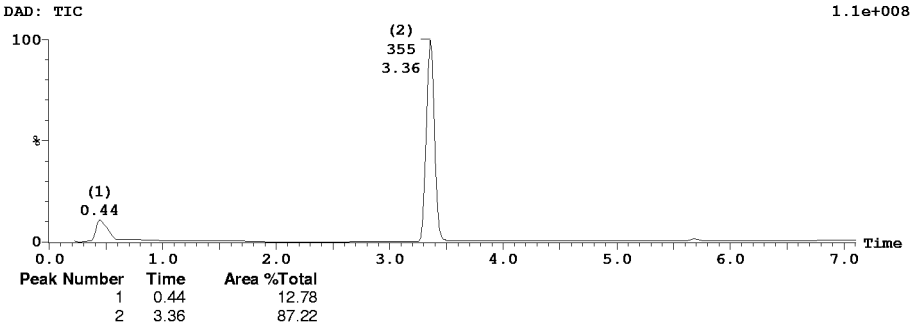


LC-MS spectra of PKUMDL-LC-101 (1)

File:9900141694774 ID:AJ-292/41694774 Description:C14H14ClN3O2S2
Vial:2:48 Date:20-Jul-2002 Time:14:00:14

Printed: Tue Jul 23 13:20:54 2002

Sample Report (continued):



Peak Number	Time	Area %Total
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2	3.36	87.22

Peak ID	Time	Mass Found	BPM
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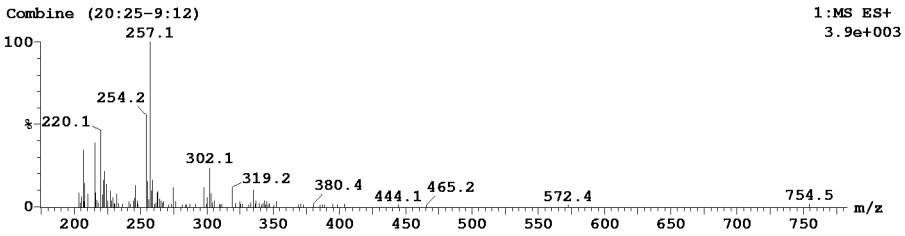
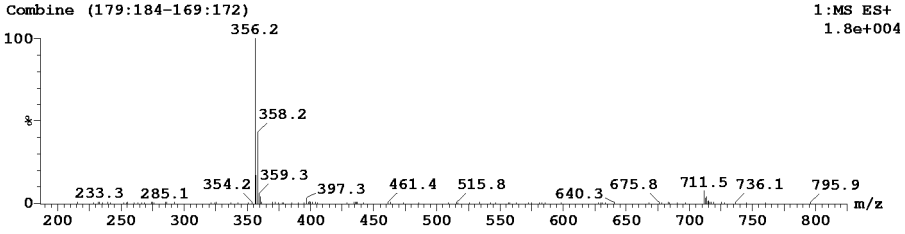


Peak ID	Time	Mass Found	BPM
2	3.36	355.02	356

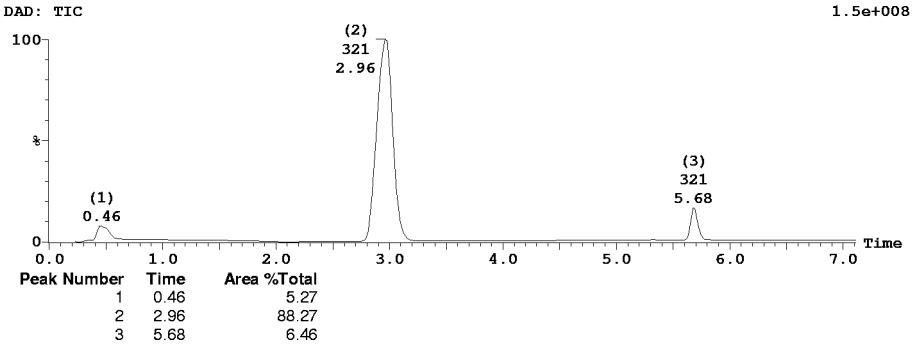


LC-MS spectra of PKUMDL-LC-101-S01 (1s1)

File:9900141694767 ID:AJ-292/41694767 Description:C14H15N3O2S2
Vial:1:132 Date:09-Aug-2002 Time:06:56:06

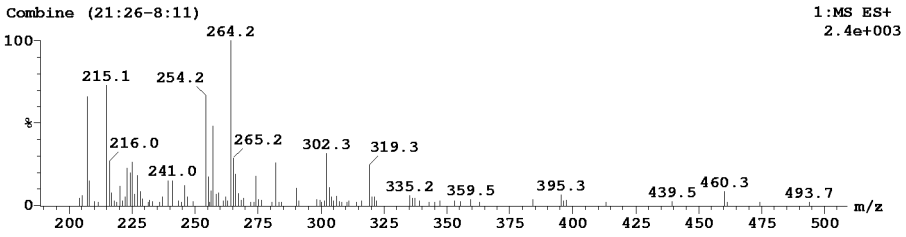
Printed: Tue Aug 13 13:47:57 2002

Sample Report (continued):

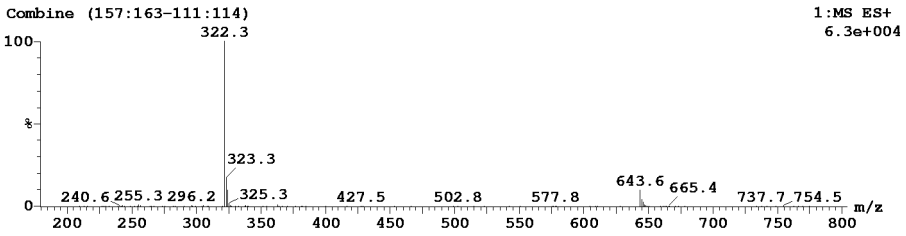


Peak Number	Time	Area %Total
1	0.46	5.27
2	2.96	88.27
3	5.68	6.46

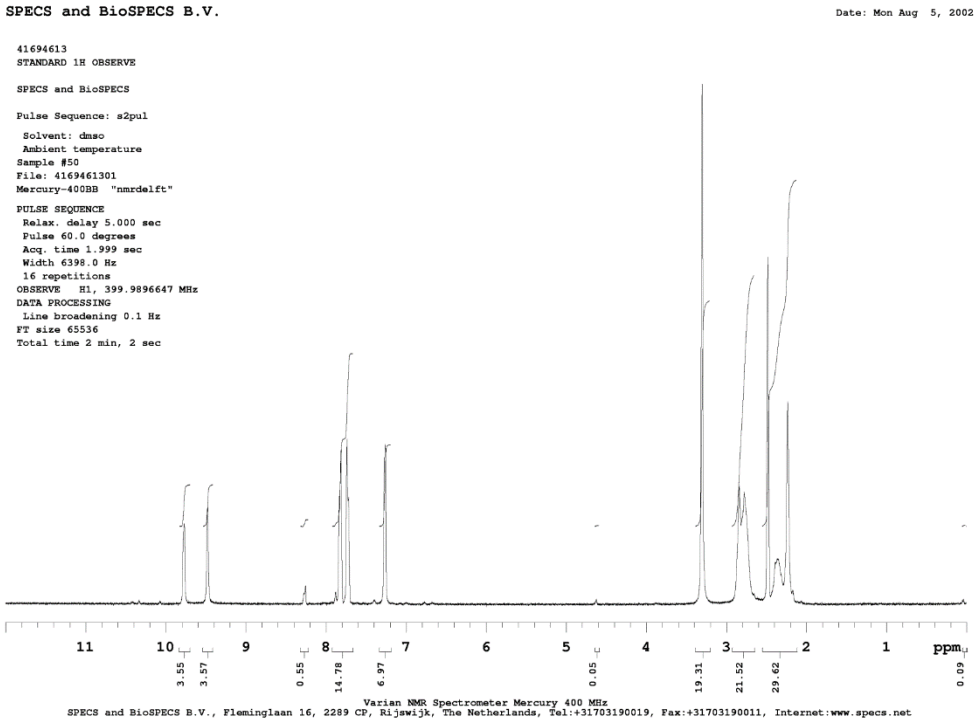
Peak ID	Time	Mass Found	BPM
1	0.46		264



Peak ID	Time	Mass Found	BPM
2	2.96	321.06	322



^{1}H-NMR of PKUMDL-LC-101-S02 (1s2)

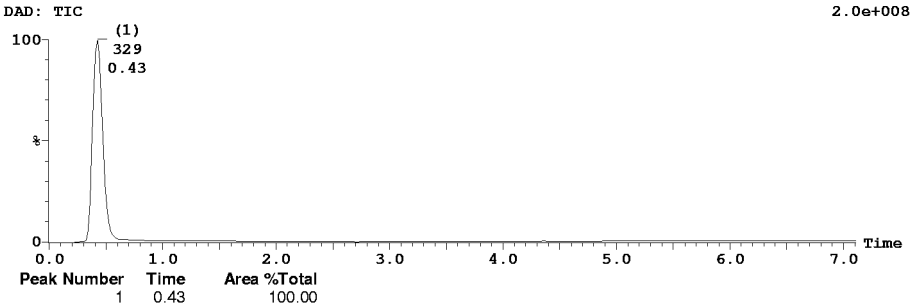


LC-MS spectra of PKUMDL-LC-101-S02 (1s2)

File:9900141694613 ID:AJ-292/41694613 Description:C12H19N5O2S2
Vial:1:29 Date:17-Jul-2002 Time:19:24:44

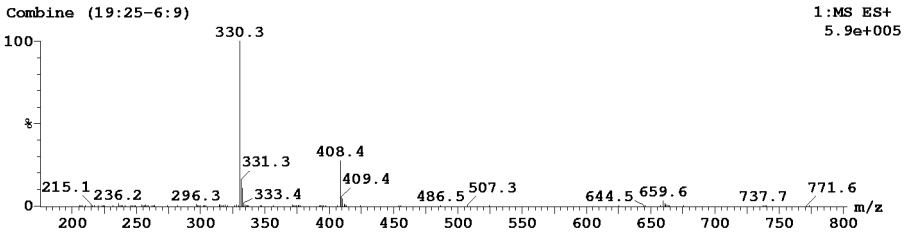
Printed: Fri Jul 19 08:51:59 2002

Sample Report (continued):

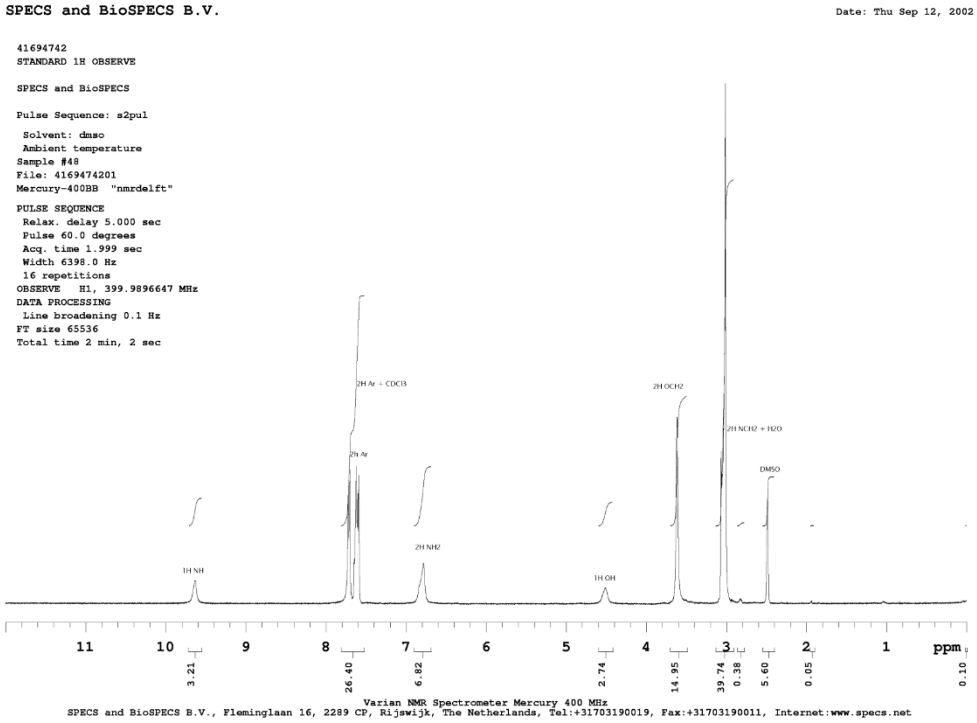


Peak Number	Time	Area %Total
1	0.43	100.00

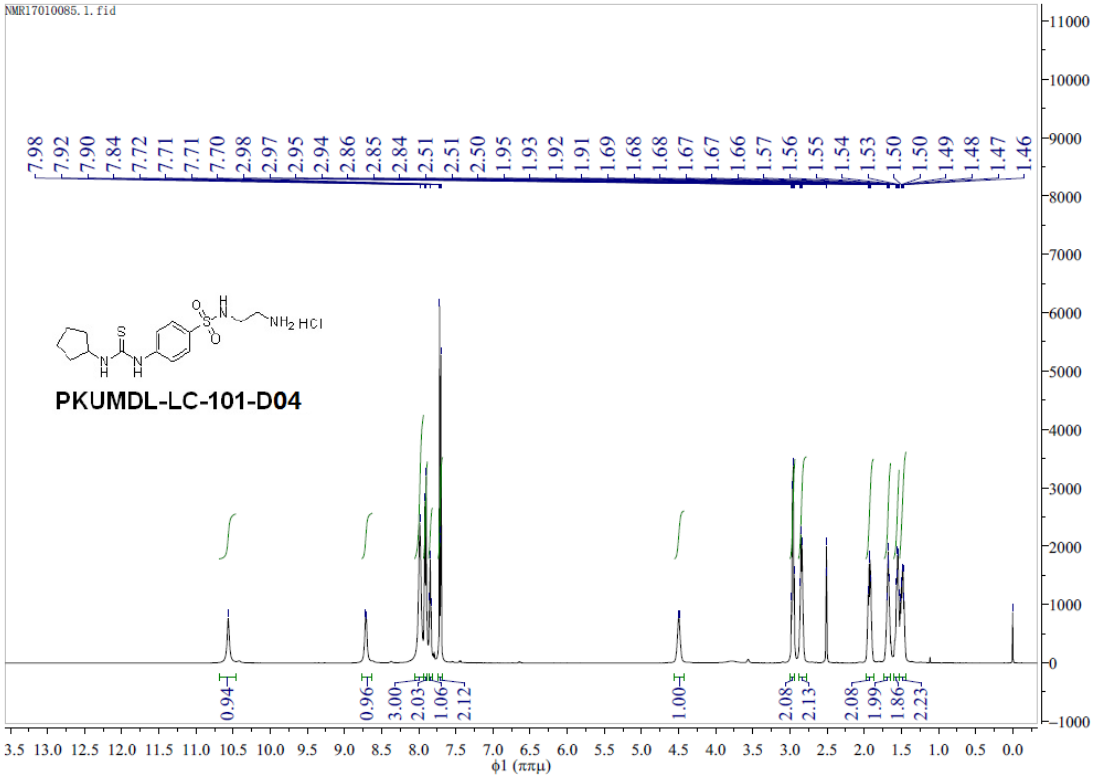
Peak ID	Time	Mass Found	BPM
1	0.43	329.10	330

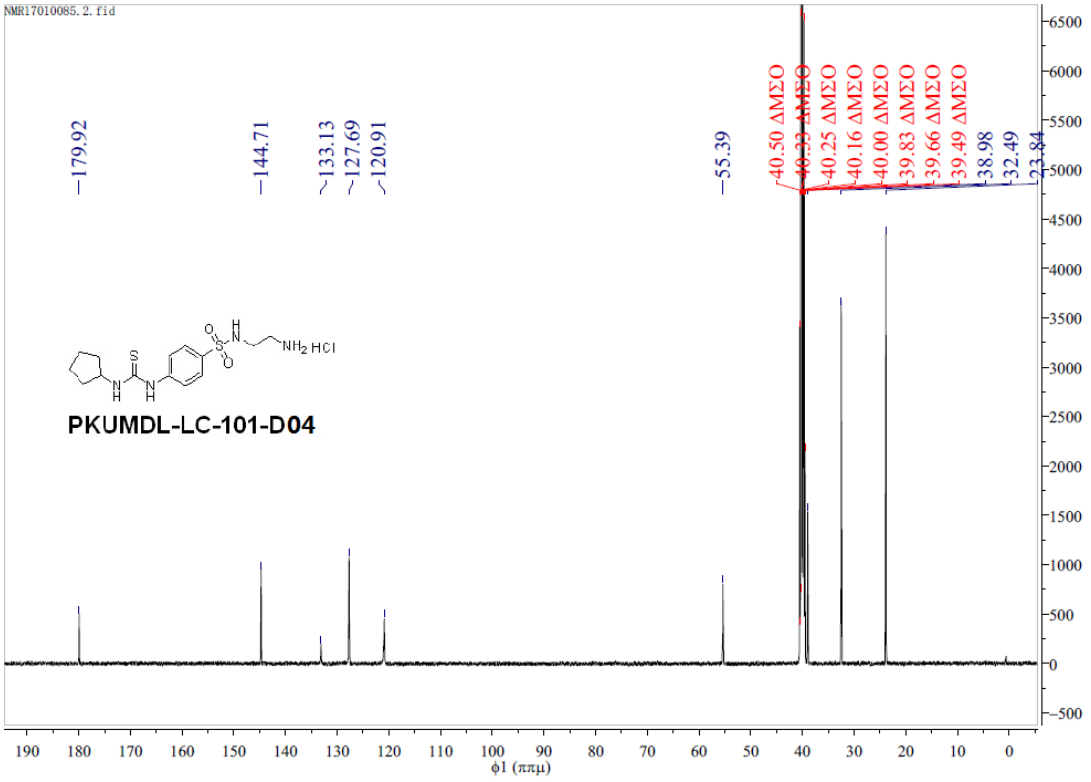


^{1}H-NMR of PKUMDL-LC-101-S03 (1s3)



^{1}H-NMR and ^{13}C-NMR of PKUMDL-LC-101-D04 (1d4)





HRMS of PKUMDL-LC-101-D04 (1d4)

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Peking University HRMS Analysis Report

Analysis Information

Folder:	Company/training/ZJ	Sample Set Created date:	Jul 20, 2016 20:15:49 China Standard Time
Analysis Method Item name:	Small molecule	Sample Set Instrument system name:	ICLASSVion
Modified by:	Zhou, Jiang	Sample Set Instrument system type:	ACQUITY Sample Manager FTN, ACQUITY Binary Solvent Manager, Osprey,

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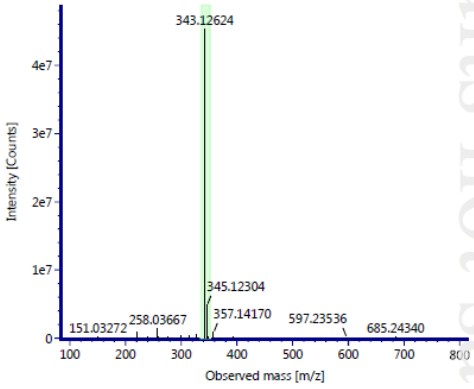

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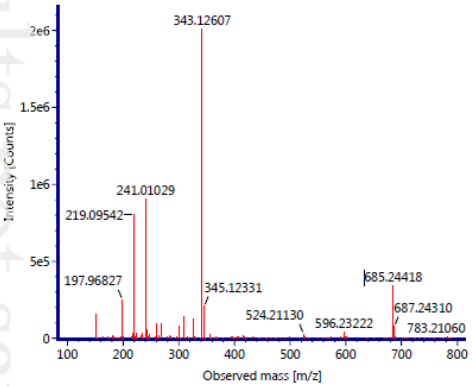

Item name: 13-37-2, Sample position: 2:D,3, Replicate number: 1

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