Supporting Information

Discovery of highly potent benzimidazole derivatives as indoleamine 2,3-dioxygenase-1 (IDO1) inhibitors: from structure-based virtual screening to *in vivo* pharmacodynamic activity

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Molecular modelling

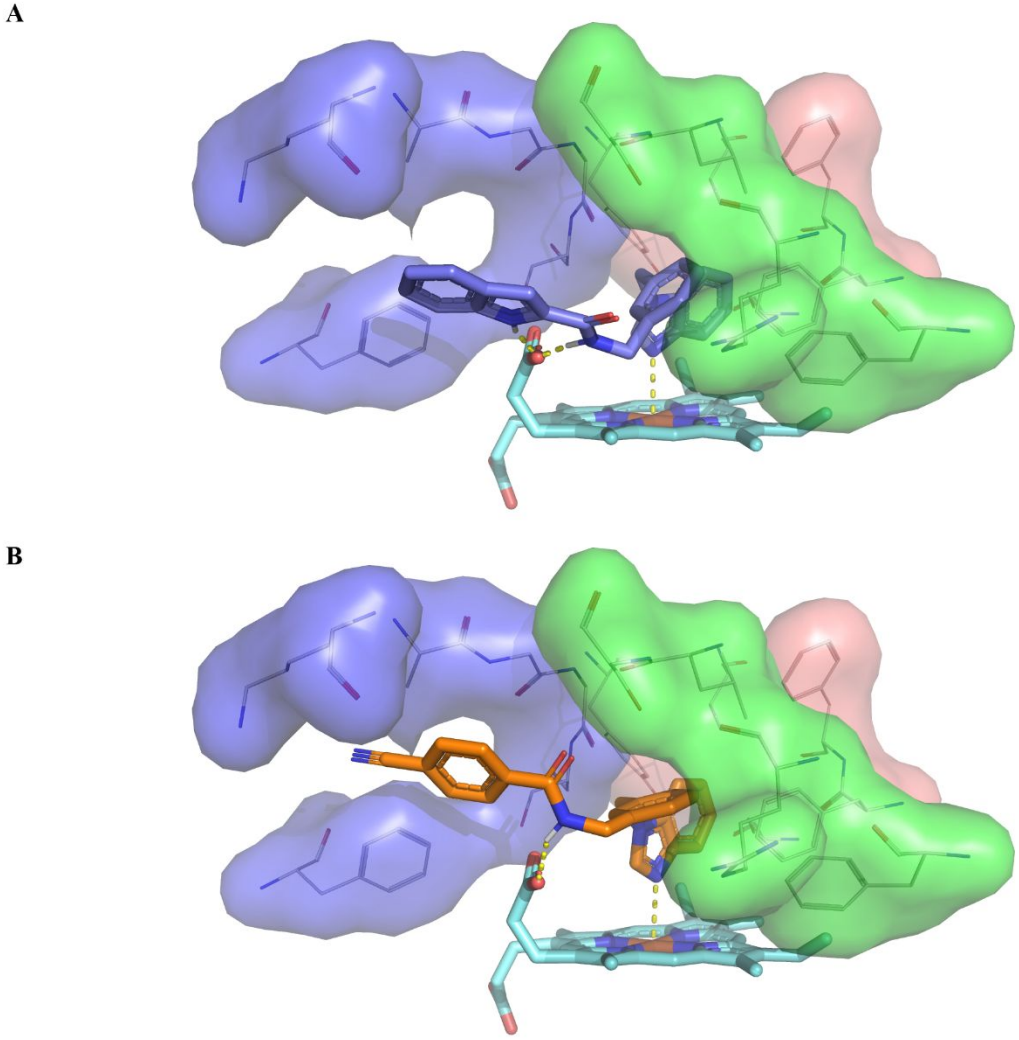


Figure S1. Docking pose of compounds **23** (**A**) and **35** (**B**) on IDO1 crystal structure (PDB id: 2D0T). Amino acids of pocket A, pocket B and pocket C are depicted in red, green and blue, respectively. Heme group, compounds **23** and **35** are depicted as cyan, blue and orange sticks, respectively.

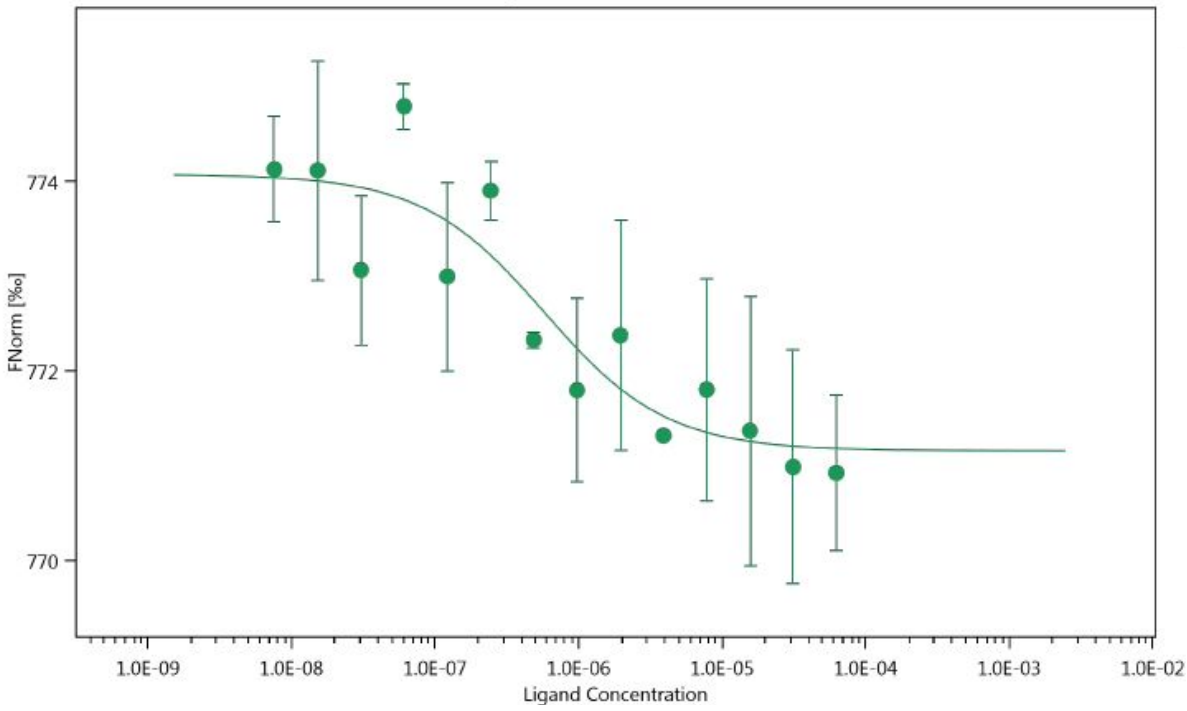


Figure S2. MST binding curve of compound **10** to recombinant human IDO1.

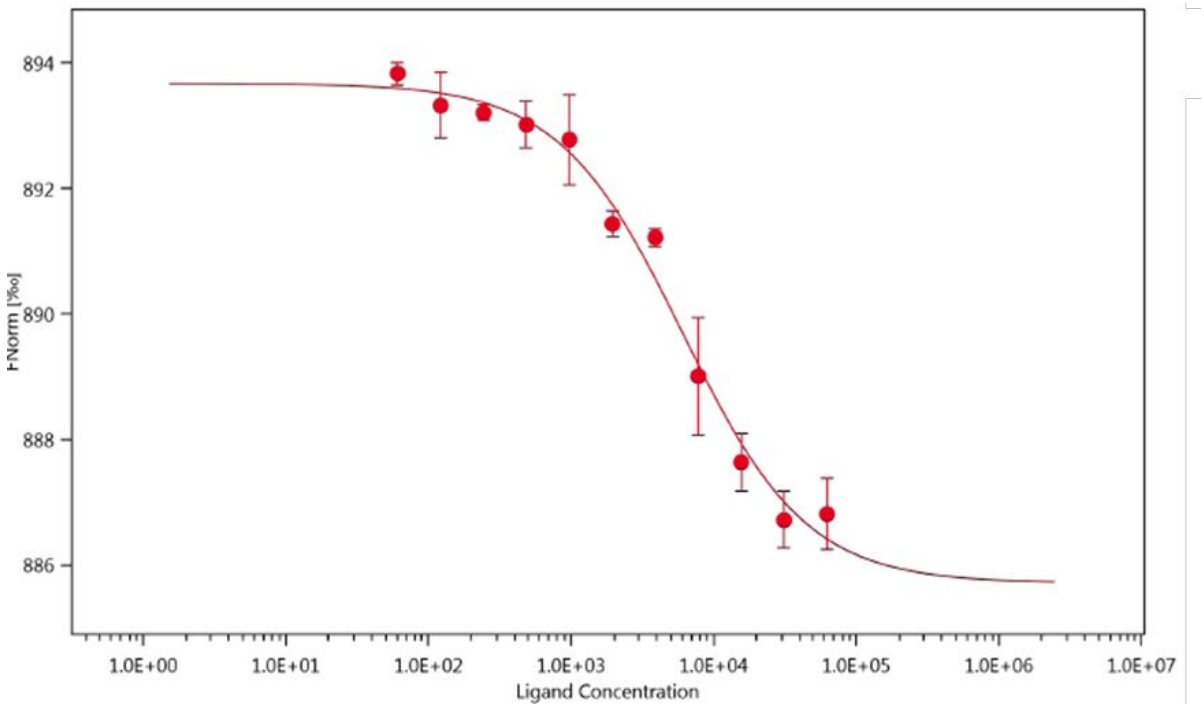


Figure S3. MST binding curve of epacadostat (**1**) to recombinant human IDO1.

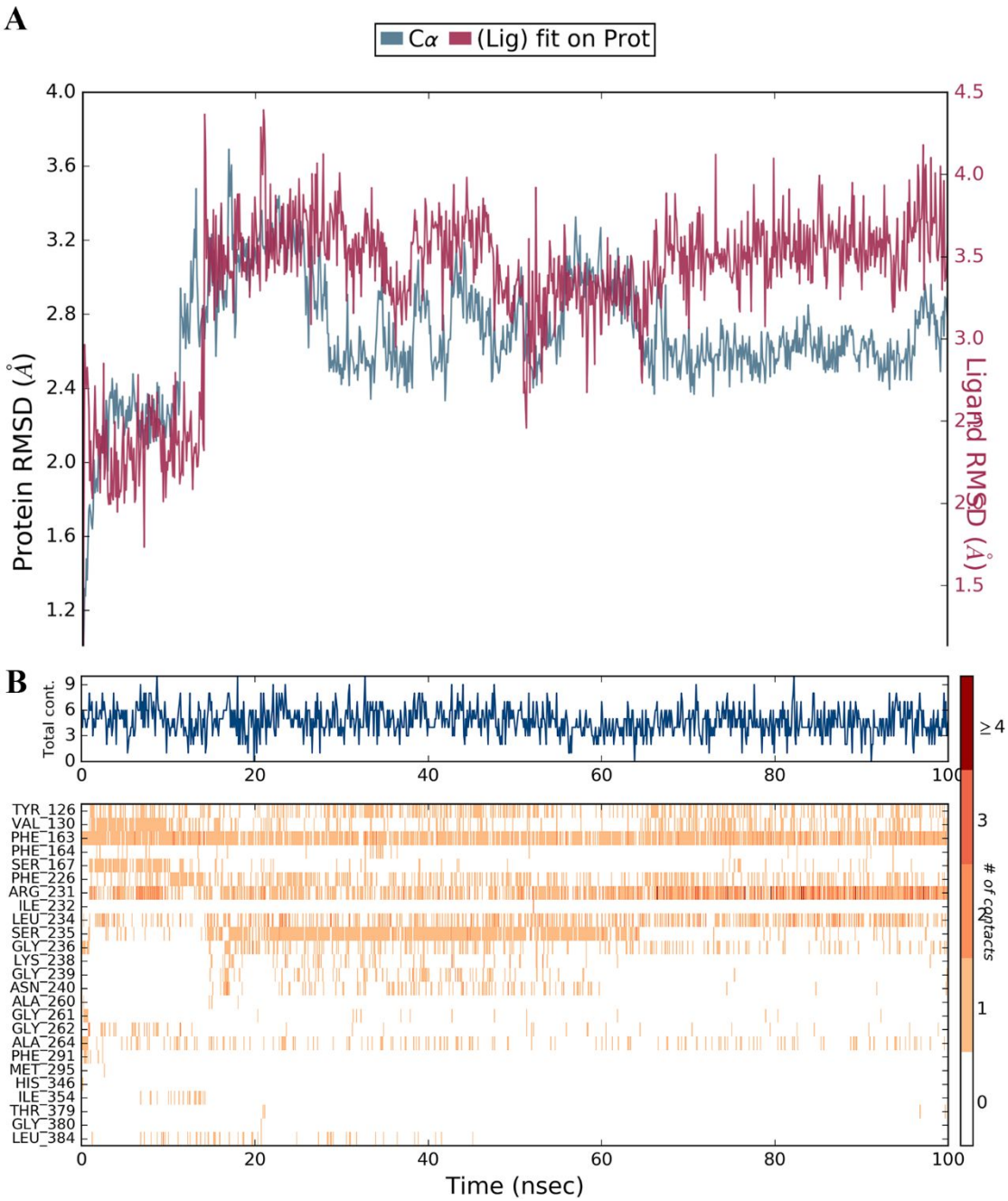


Figure S4. (**A**) RMSD of the atomic positions for compound **10** (in red, Lig fit Prot) and the protein IDO-1 (Cα positions in blue) of the 100 ns molecular dynamics simulations using Desmond package. (**B**) A timeline representation of the interactions and contacts (H-bonds, Hydrophobic, Ionic, Water bridges).

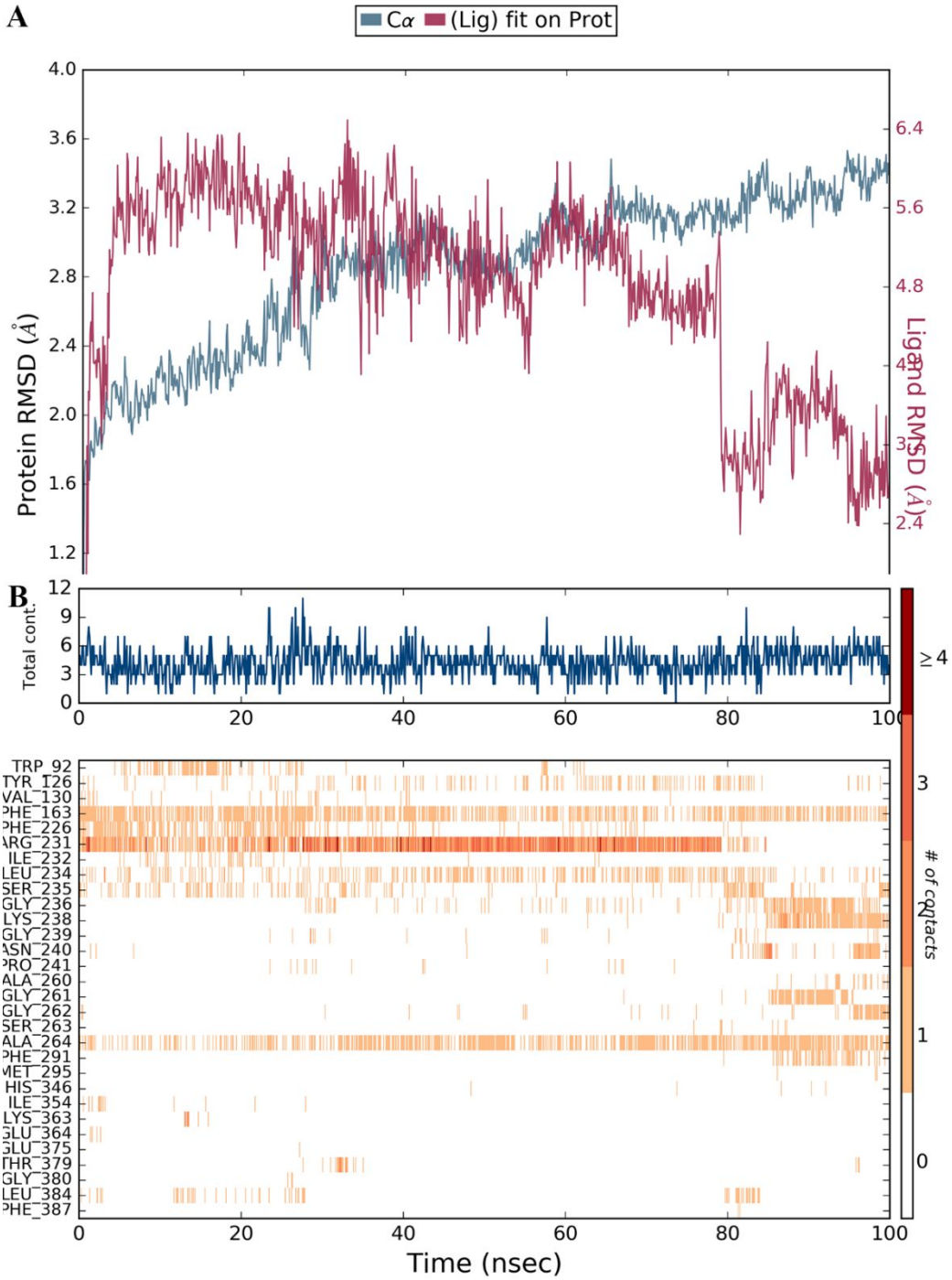


Figure S5. (**A**) RMSD of the atomic positions for compound **23** (in red, Lig fit Prot) and the protein IDO-1 (Cα positions in blue) of the 100 ns molecular dynamics simulations using

Desmond package. (**B**) A timeline representation of the interactions and contacts (H-bonds, Hydrophobic, Ionic, Water bridges).

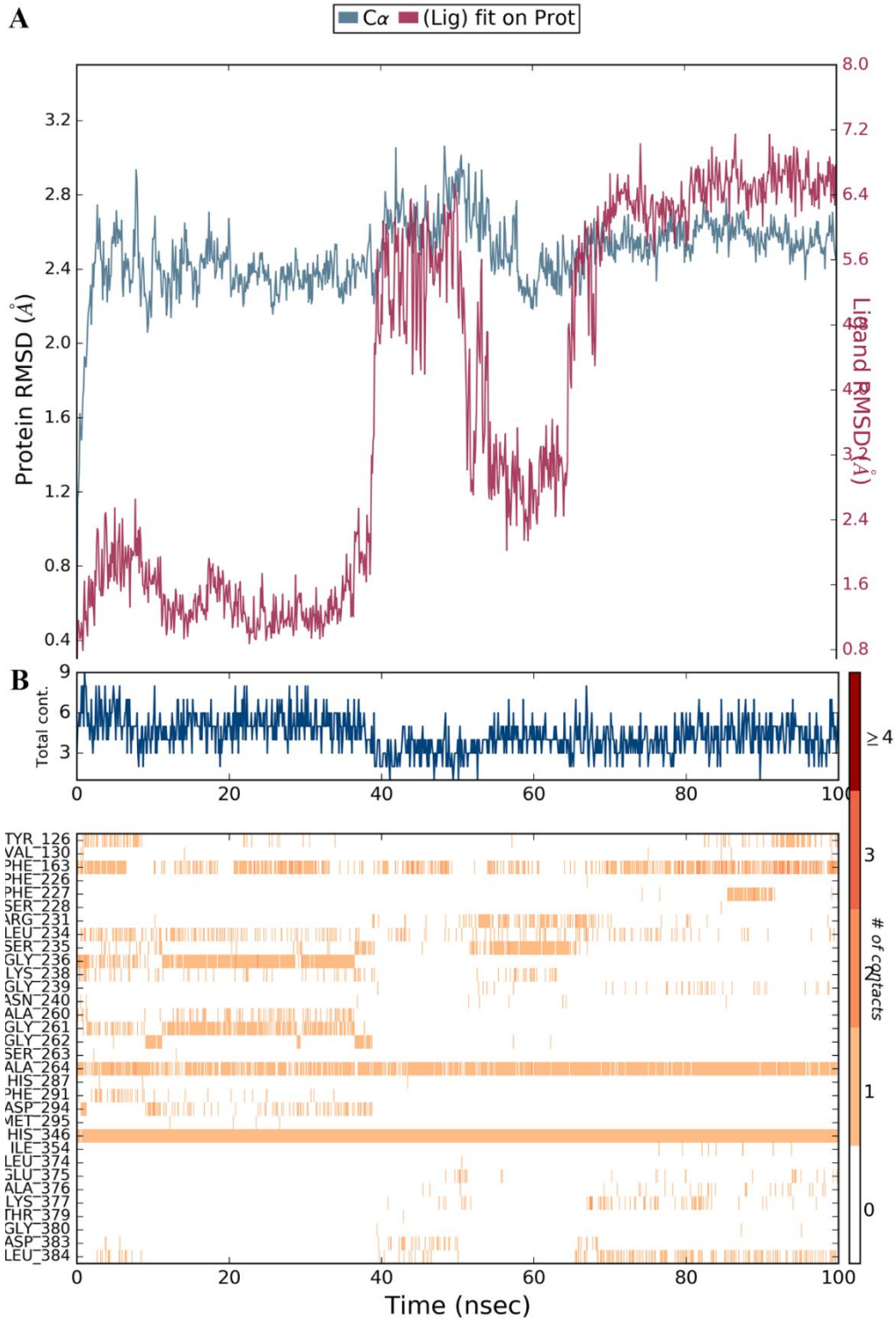
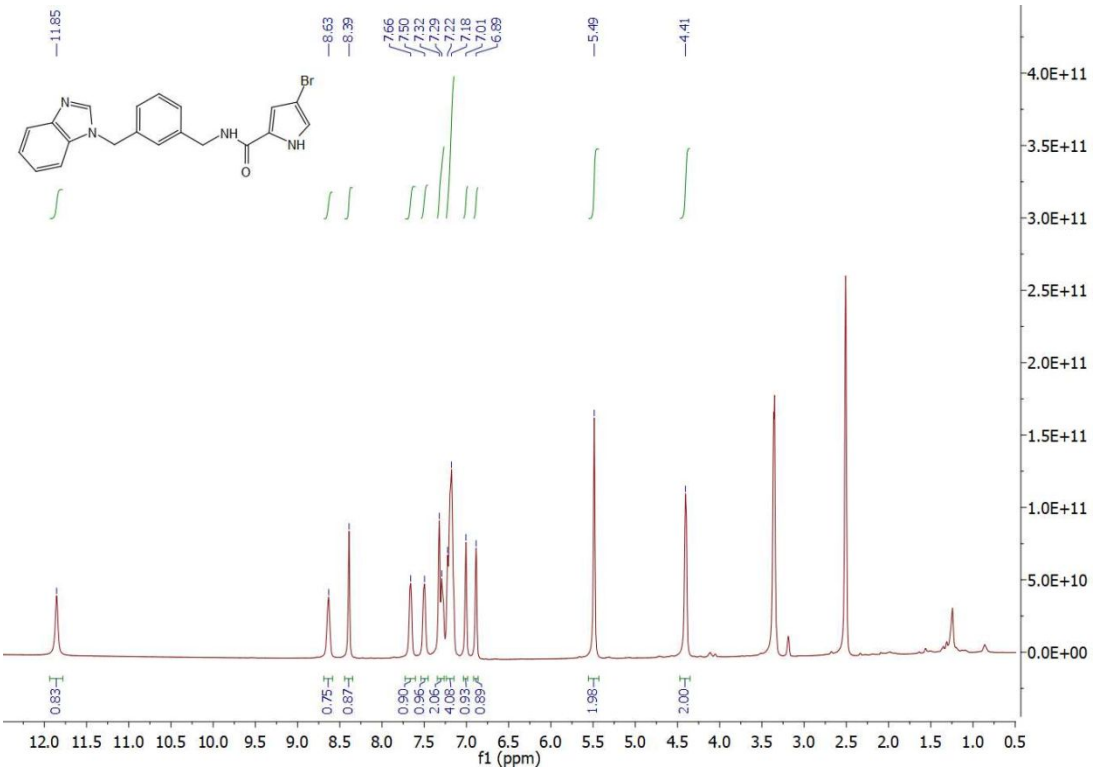
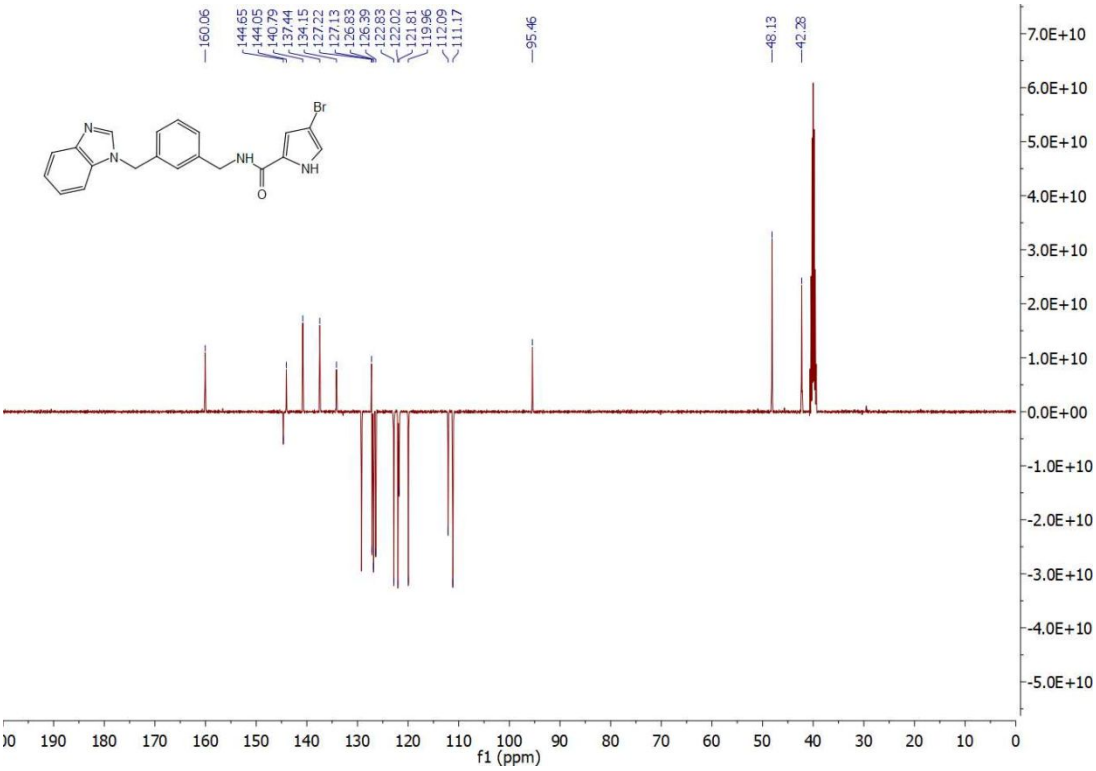


Figure S6. (**A**) RMSD of the atomic positions for compound **35** (in red, Lig fit Prot) and the protein IDO-1 (Cα positions in blue) of the 100 ns molecular dynamics simulations using Desmond package. (**B**) A timeline representation of the interactions and contacts (H-bonds, Hydrophobic, Ionic, Water bridges).

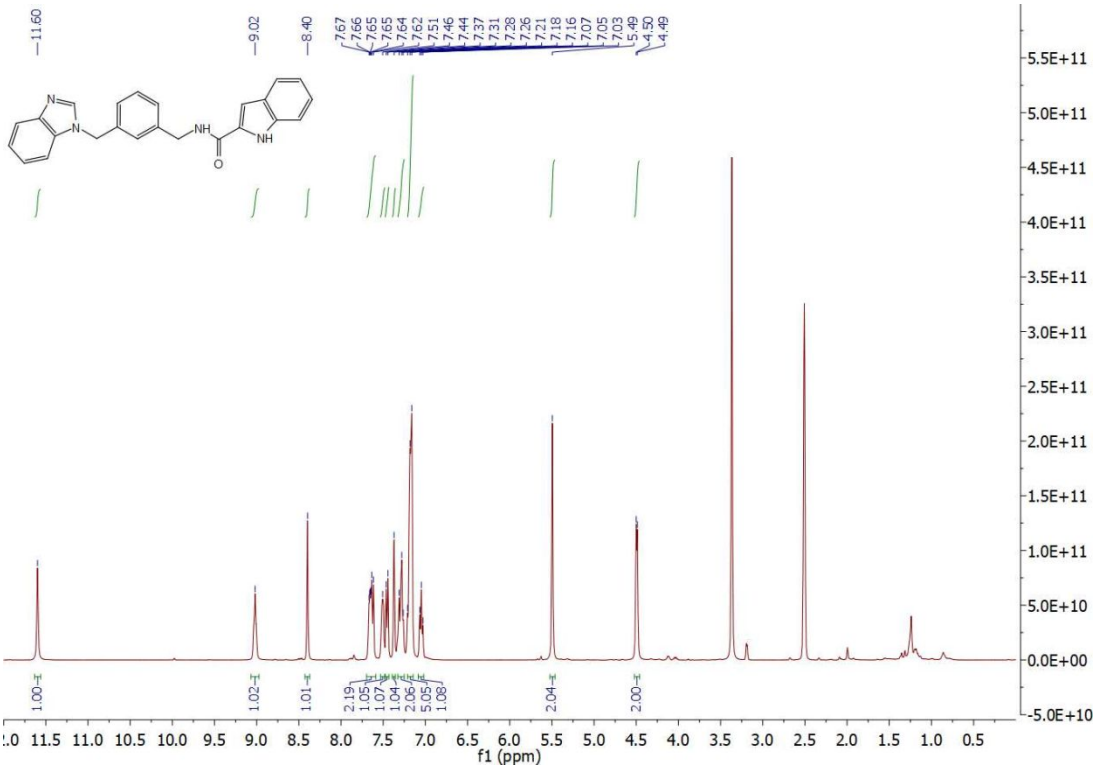
NMR Spectra of compounds

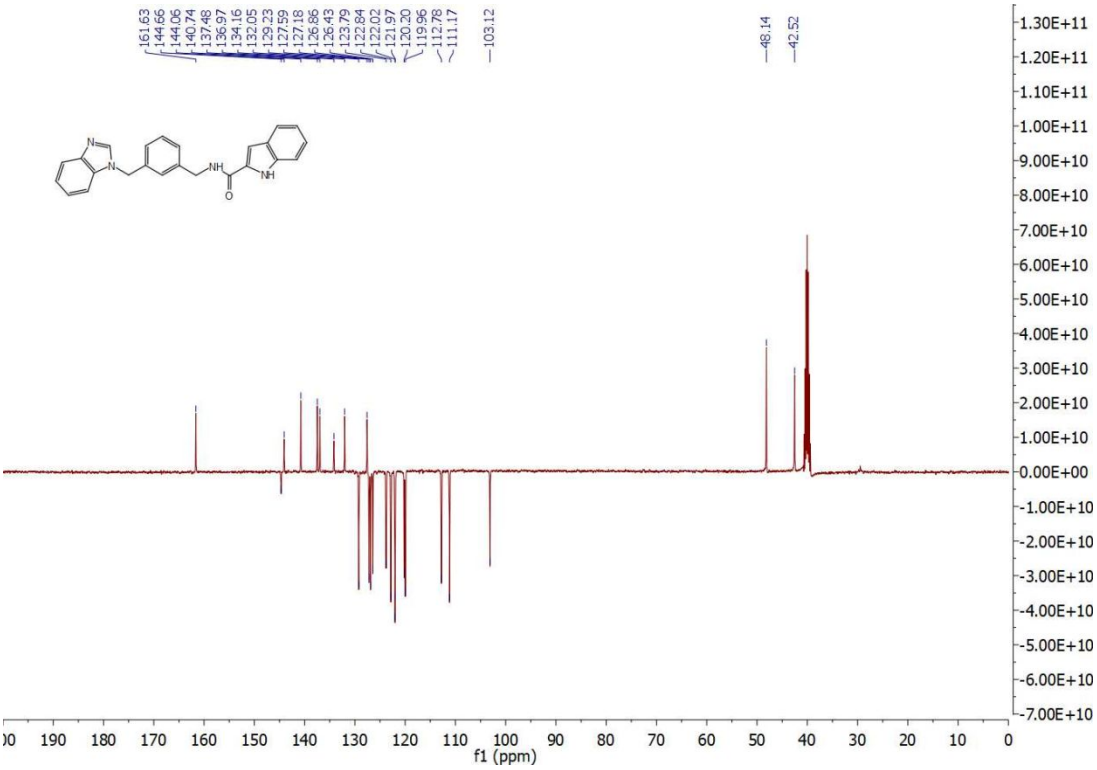
10: ^{1}H (DMSO-d_6), ^{13}C (DMSO-d_6)



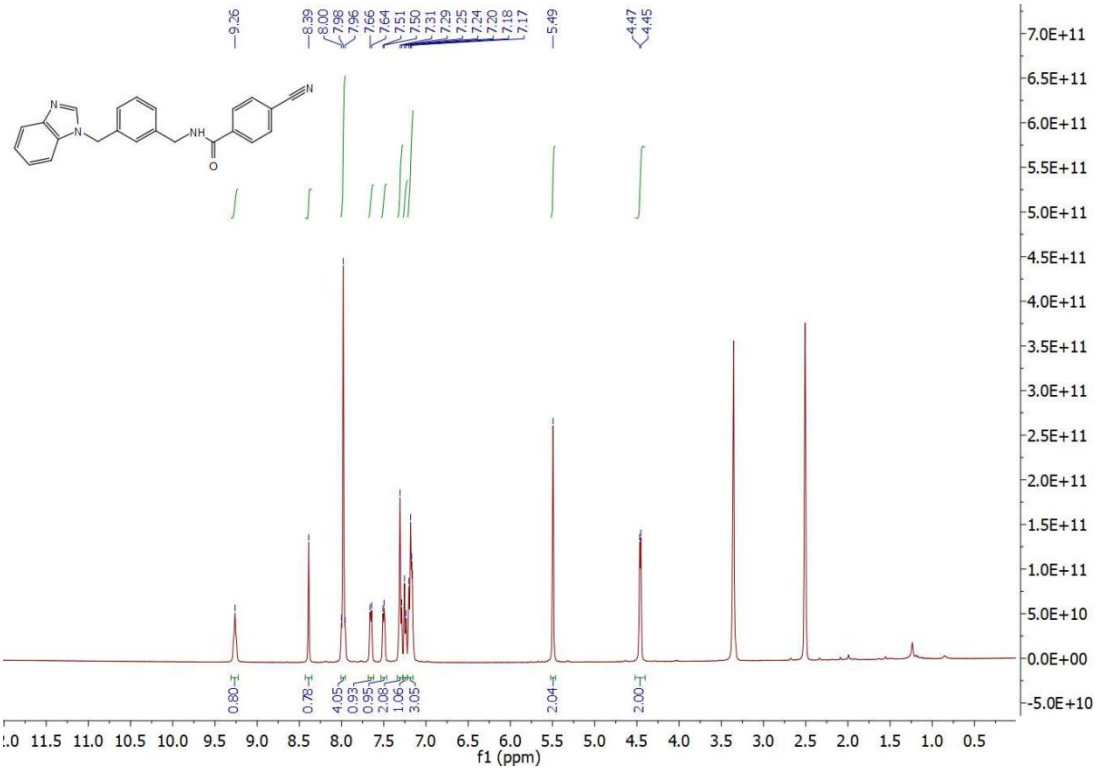


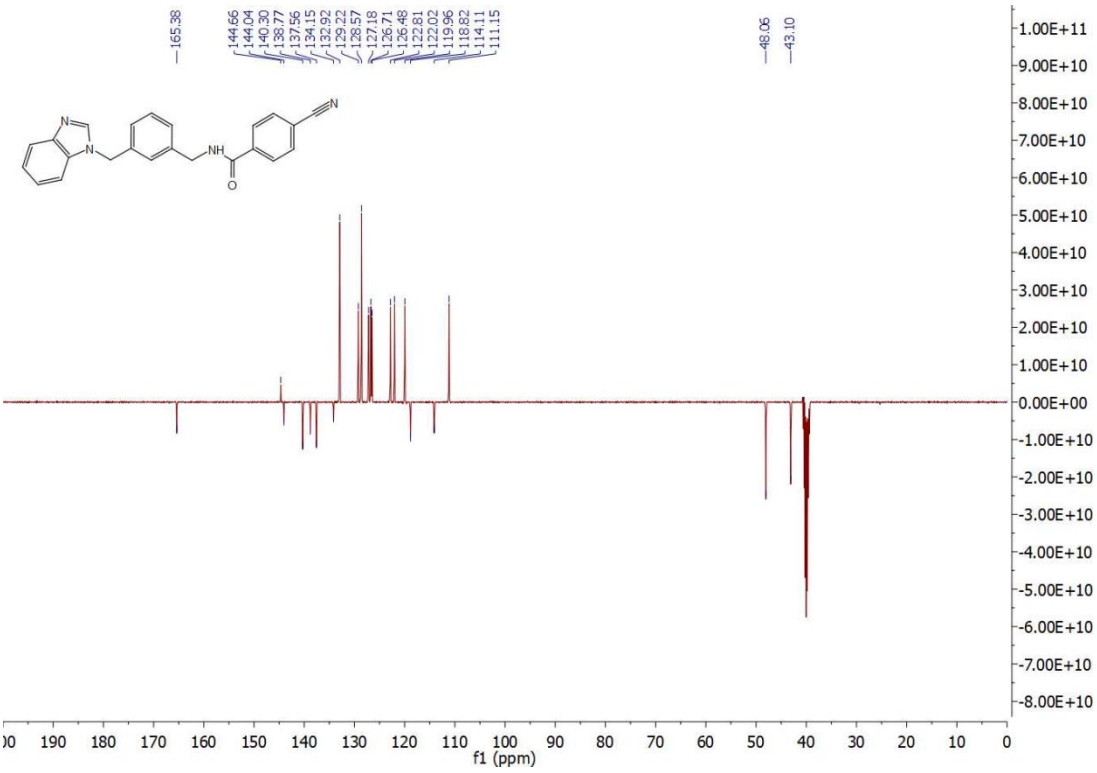
23: ^{1}H (DMSO-d_6), ^{13}C (DMSO-d_6)





35: ^{1}H (DMSO-d_6), ^{13}C (DMSO-d_6)





Biology

Table S1. Biological profiles of the 10 active compounds from virtual screening.

Compound	Cell viability (%) @ 10 μM ± SD	IDO1 cellular assay inhibition (%) @ 10 μM	IC_{50} (μM)
VS1	95 ± 4.5	76 ± 4.9	4.4
VS2	87 ± 6.1	68 ± 6	0.9
VS3	98 ± 3.9	28 ± 4	n.d.
VS4	97 ± 2.7	78 ± 2	0.6
VS5	92 ± 4.2	76 ± 3.5	3.7
VS6	98 ± 2.6	70 ± 5.6	2.6
VS7	98 ± 1.9	32 ± 6	n.d.
VS8	96 ± 3.5	86 ± 9	0.8
VS9	94 ± 3.3	56 ± 7	n.d.
***VS10* (10)**	100 ± 5.5	99 ± 0.7	0.016

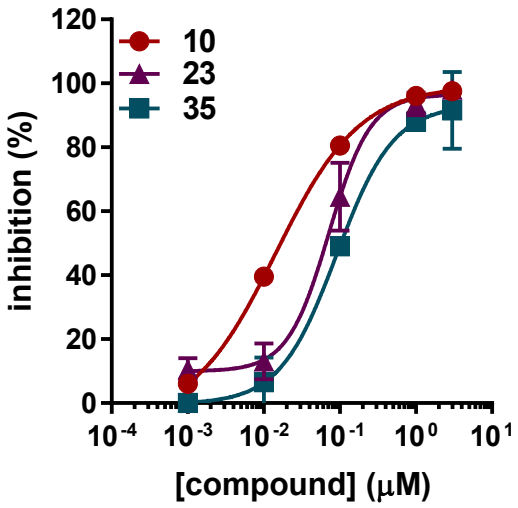


Fig. S7. Concentration-response curves for selected compounds (**10**, **23** and **35**) in IDO1 inhibition cell-based assay. A375 cells were treated (48 h) with IFN-γ (1000 U/mL) and increasing concentrations (0.001-3 μM) of each compound. The L-KYN levels in cell culture supernatants were measured by HPLC method. The data represent mean ± SEM of at least three independent assays run

in triplicate. Percent inhibition against inhibitor concentration was plotted and IC_{50} value was determined. The data were evaluated by GraphPad 7.0.

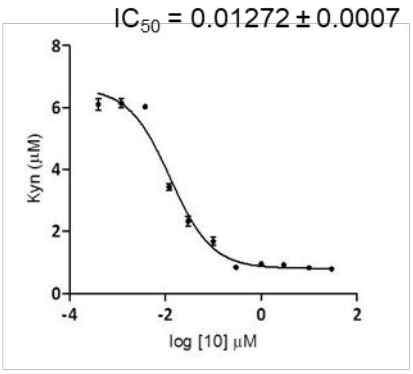
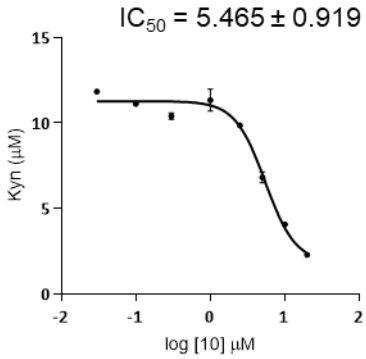
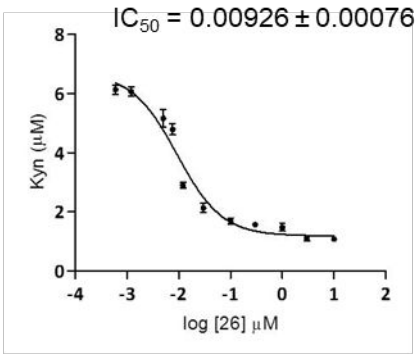
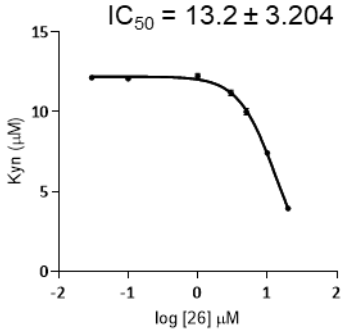
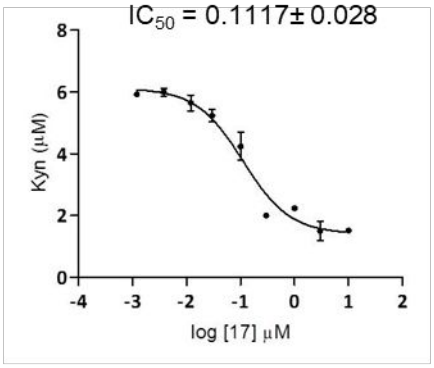
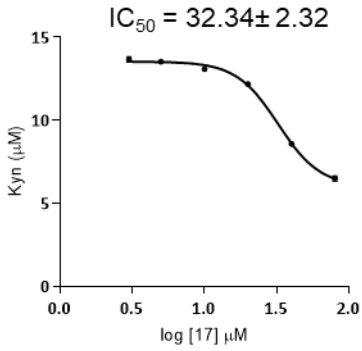
	Cell type	
Cpd	P1.IDO1	P1.TDO
10		
23		
35		

Fig. S8. Concentration-response curves (μM) for selected compounds (**10**, **23** and **35**) in stable transfectant P1.IDO1 and P1.TDO cell lines.

Metabolic stability data

Chromatographic methods

1) LC-UV methods for purity and metabolic stability determination.

A Shimadzu HPLC system (Shimadzu, Kyoto, Japan), consisting of two LC-10AD Vp module pumps, an SLC-10A Vp system controller, an SIL-10AD Vp autosampler, and a DGU-14-A on-line degasser were used for the analysis. The SPD-M10Avp photodiode array detector was used to detect the analytes. LC-Solution 1.24 software was used to process the chromatograms.

- Column: *Phenomenex Synegi Polar, 150 × 4.6 mm (4 µm d.p.)* protected with a SecurityGuard® (Torrance, CA, USA).
- Eluent:

  A: 0.2% formic acid in water.

  B: 0.2% formic acid in acetonitrile.
- Flow rate: 1 mL/min.
- Injection volume: 20 µL.
- Wavelength: 294 nm (**23**), 268 nm (**10**), 254 nm (**35**),
- Gradient program: 0.00 min [B%=20%], 14.50 min [B%=80%], 15.00 min [B%=20%], 20 min [B%=20%].

2) LC-HRMS methods for metabolites characterization.

A Thermo Scientific Q-Exactive Plus system equipped with a Thermo Scientific Vanquish UHPLC system with a binary pump VF-P10, a split sampler VF-A10, and a column compartment VH-C10. Data were acquired and processed using Xcalibur® software.

- Column: *Phenomenex Synergi Polar 150 × 2 mm (4µm d.p.)* protected with a SecurityGuard® kept at 35 °C (Torrance, CA, USA).
- Eluent:

  A: 0.1% formic acid in water.

  B: acetonitrile.
- Flow rate: 0.250 mL/min.
- Injection volume: 5 µL.
- Gradient program: 0.00 min [B%=20%], 14.50 min [B%=80%], 15.00 min [B%=20%], 20.00 min [B%=20%].

The operating conditions of the mass spectrometer were as follows: positive mode; sheath gas flow rate, 45 Auxiliary Units (A.U.), auxiliary gas flow rate, 5 A.U.; sweep gas flow rate, 0 A.U.; spray voltage, 3.2 kV; capillary temperature, 300 °C; auxiliary gas heater temperature, 280 °C.

Data were acquired in full-scan/data dependent MS^2 mode using a mass scan range *m/z* 110-800. The stepped collision energy was set at: 20, 40, 80% levels.

3) LC-HRMS methods for pharmacokinetic analysis and evaluation of L-Kyn trend levels.

A Thermo Scientific Q-Exactive Plus system equipped with a Thermo Scientific Vanquish UHPLC system with a binary pump VF-P10, a split sampler VF-A10, and a column compartment VH-C10. Data were acquired and processed using Xcalibur® software.

- Column: *Phenomenex Synergi Polar 150 × 2 mm (4 µm d.p.)* protected with a SecurityGuard® kept at 35 °C (Torrance, CA, USA).
- Eluent:

  A: 0.1% formic acid in water.

  B: acetonitrile.
- Flow rate: 0.250 mL/min.
- Injection volume: 5 µL.
- Gradient program: 0.00 min [B%=20%], 1.00 min [B%=20%], 7.50 min [B%=95%], 10.00 min [B%=95%], 10.50 min [B%=20%], 16.00 min [B%=20%].

The operating conditions of the mass spectrometer were as follows: positive mode; sheath gas flow rate, 45 Auxiliary Units (A.U.), auxiliary gas flow rate, 5 A.U.; sweep gas flow rate, 0 A.U.; spray voltage, 3.2 kV; capillary temperature, 300 °C; auxiliary gas heater temperature, 280 °C.

Data were acquired in full-scan and parallel reaction monitoring (PRM) using a mass scan range *m/z* 110-800 with the inclusion list and the collision energy parameters reported in Table S2.

Compound	**Mass [m/z]**	**Formula [M]**	**(N)CE**
10	409.06585	$C_{20}H_{17}BrN_4O$	35
23	381.17099	$C_{24}H_{20}N_4O$	35
L-Kyn	209.09207	$C_{10}H_{12}N_2O_3$	10

Table S2

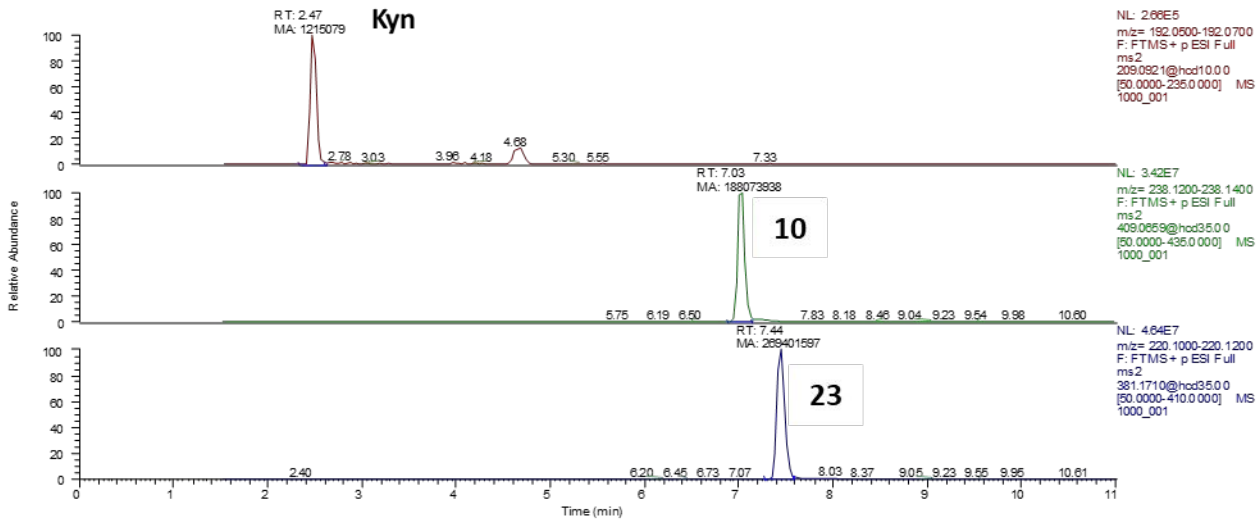
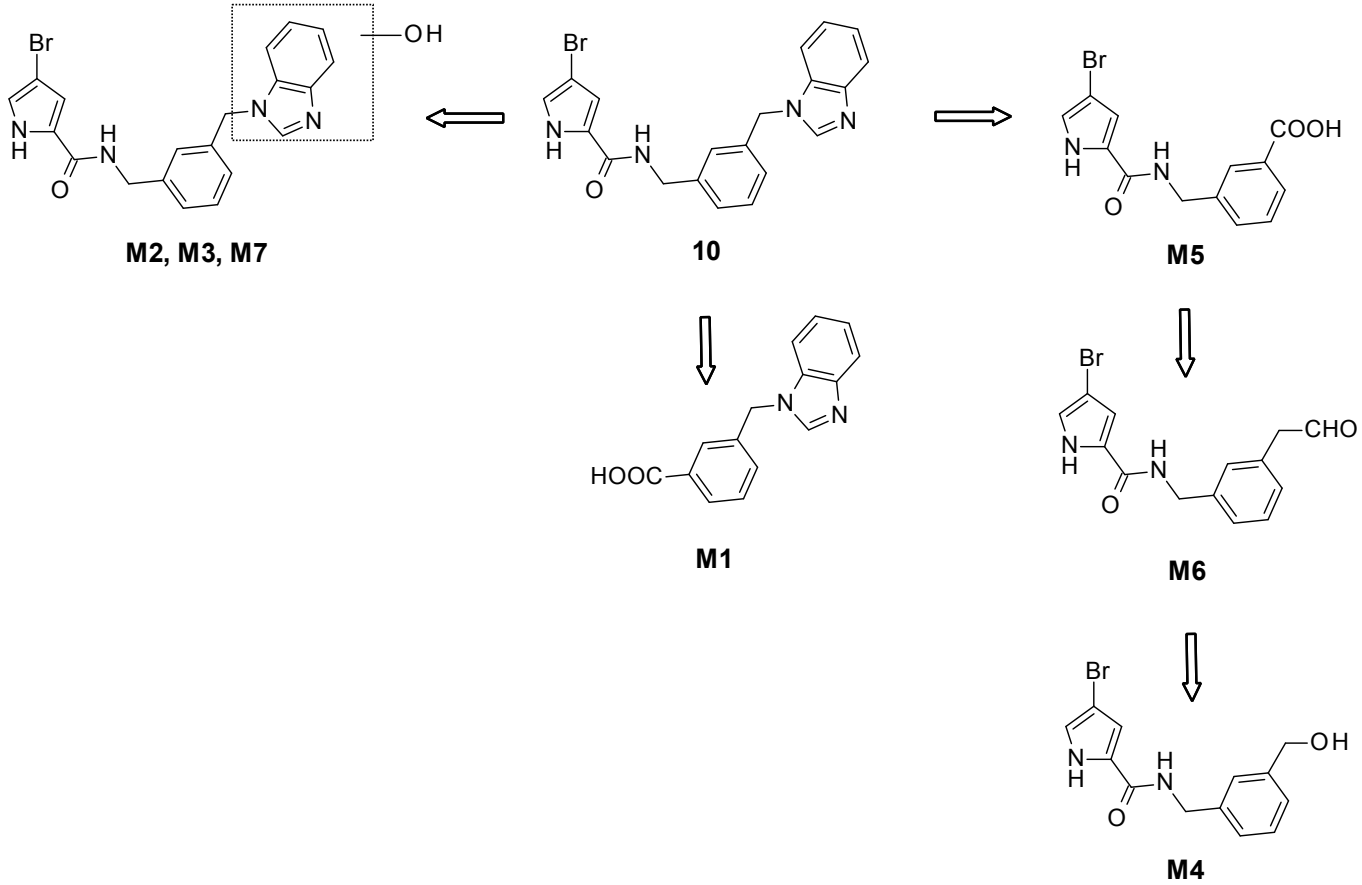


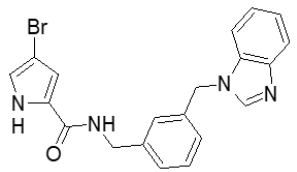
Figure S9. Representative PRM chromatogram of all the analytes.

Structural characterization of metabolites by HRMS

Scheme S1. Metabolic pathways of compound **10**.



	RT (min)	Molecular Formula	Teoretical $[M+H]^+$	Measured $[M+H]^+$	Mass shift (ppm)	Metabolic pathway
10	8.02	$C_{20}H_{17}BrN_4O$	409.06584	409.06607	0.55	
M1	4.40	$C_{15}H_{12}N_2O_2$	253.09715	253.09723	0.30	oxidative *N*-dealkylation
M2	6.92	$C_{20}H_{17}BrN_4O_2$	425.06076	425.06091	0.36	+ O
M3	7.28	$C_{20}H_{17}BrN_4O_2$	425.06076	425.06090	0.34	+ O
M4	8.14	$C_{13}H_{13}BrN_2O_2$	309.02331	309.02347	0.52	oxidative *N*-dealkylation + reduction
M5	8.61	$C_{13}H_{11}BrN_2O_3$	323.00257	323.00266	0.27	oxidative *N*-dealkylation
M6	9.85	$C_{13}H_{11}BrN_2O_2$	307.00760	307.00778	0.59	oxidative *N*-dealkylation + reduction
M7	10.49	$C_{20}H_{17}BrN_4O_2$	425.06076	425.06088	0.29	+ O



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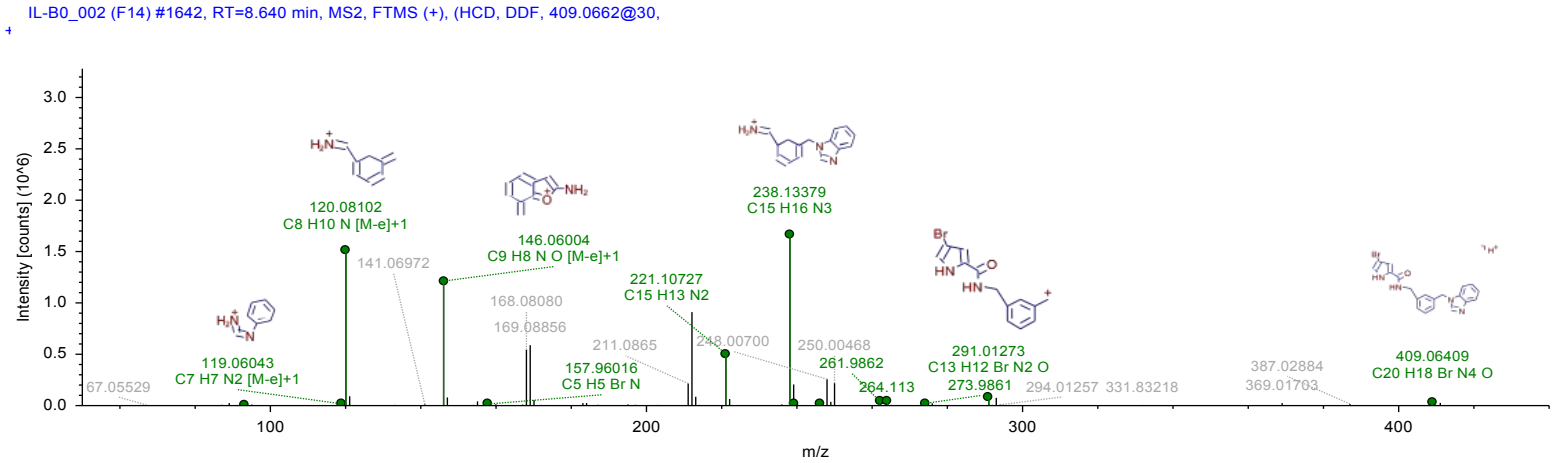

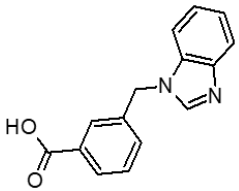

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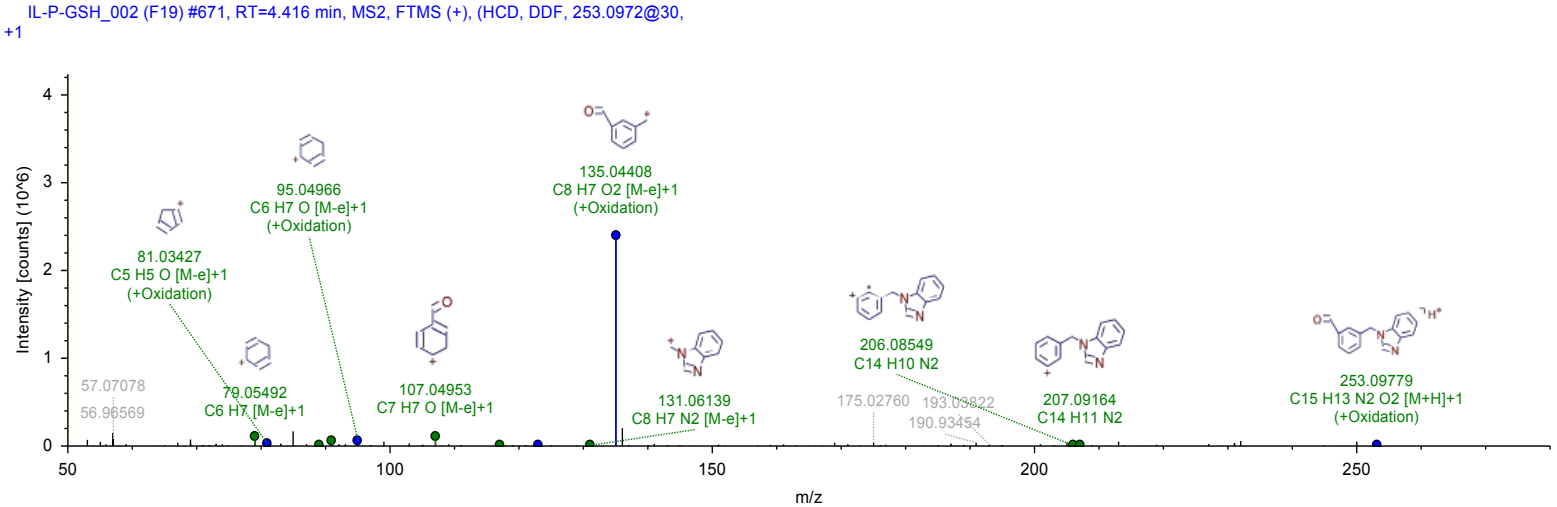
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57.07078
56.96569
81.03427
C5 H5 O [M-e]+1
(+Oxidation)
79.05492
C6 H7 [M-e]+1
95.04966
C6 H7 O [M-e]+1
(+Oxidation)
107.04953
C7 H7 O [M-e]+1
135.04408
C8 H7 O2 [M-e]+1
(+Oxidation)
131.06139
C8 H7 N2 [M-e]+1
175.02760
193.03822
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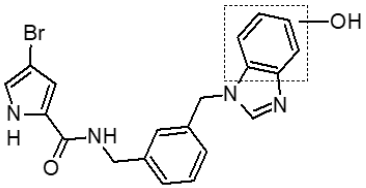
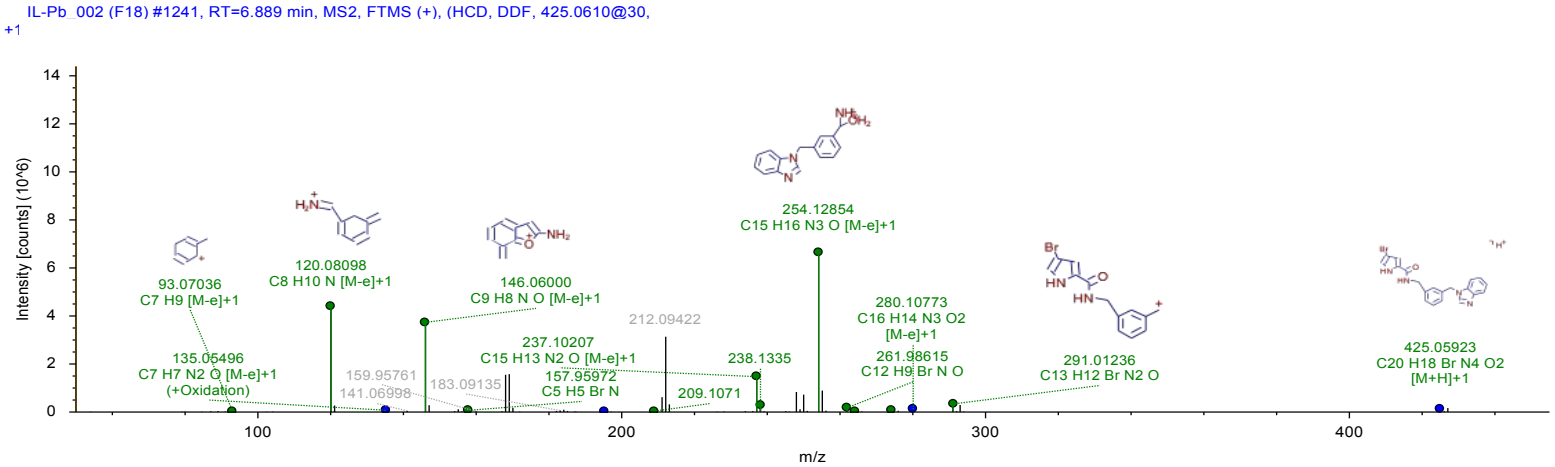
(+Oxidation)



M2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C7 H9 [M-e]+1
120.08098
C8 H10 N [M-e]+1
146.06000
C9 H8 N O [M-e]+1
135.05496
C7 H7 N2 O [M-e]+1
(+Oxidation)
159.95761
141.06998
183.09135
237.10207
C15 H13 N2 O [M-e]+1
157.95972
C5 H5 Br N
212.09422
209.1071
238.1335
254.12854
C15 H16 N3 O [M-e]+1
280.10773
C16 H14 N3 O2
[M-e]+1
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C12 H9 Br N O
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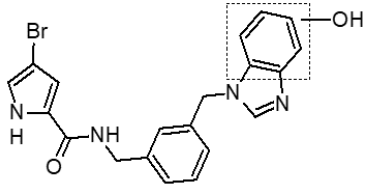
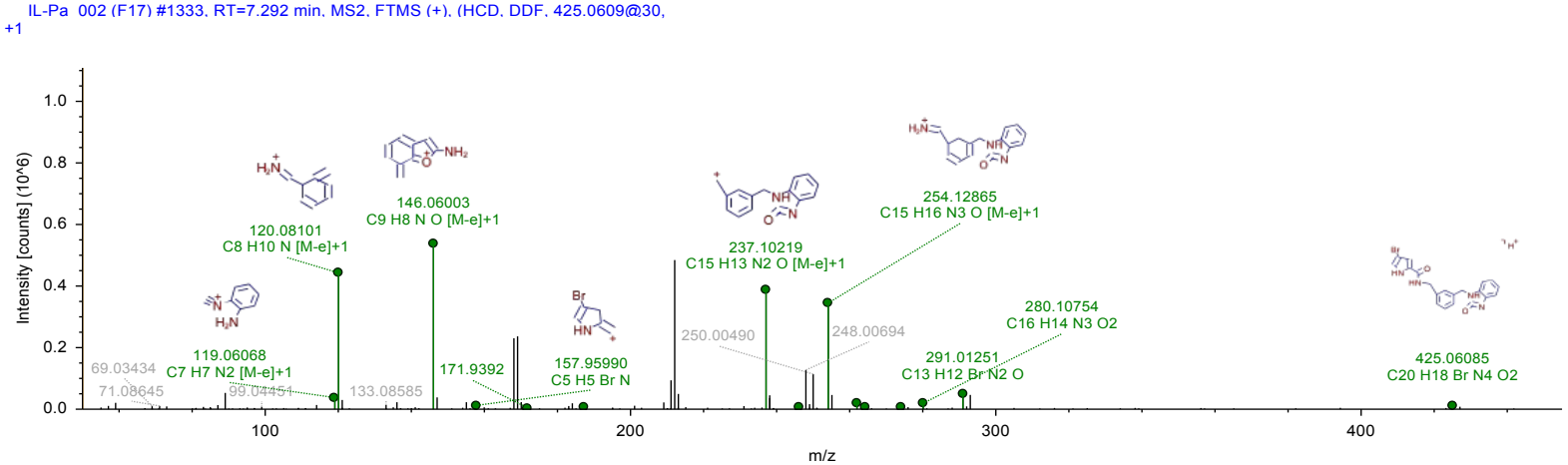
C13 H12 Br N2 O
425.05923
C20 H18 Br N4 O2
[M+H]+1


Br
OH
N
H
NH
O
N
N

M3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+1
Intensity [counts] (10^6)
m/z
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C9 H8 N O [M-e]+1
120.08101
C8 H10 N [M-e]+1
237.10219
C15 H13 N2 O [M-e]+1
254.12865
C15 H16 N3 O [M-e]+1
280.10754
C16 H14 N3 O2
119.06068
C7 H7 N2 [M-e]+1
157.95990
C5 H5 Br N
291.01251
C13 H12 Br N2 O
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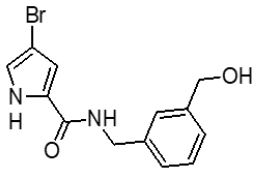
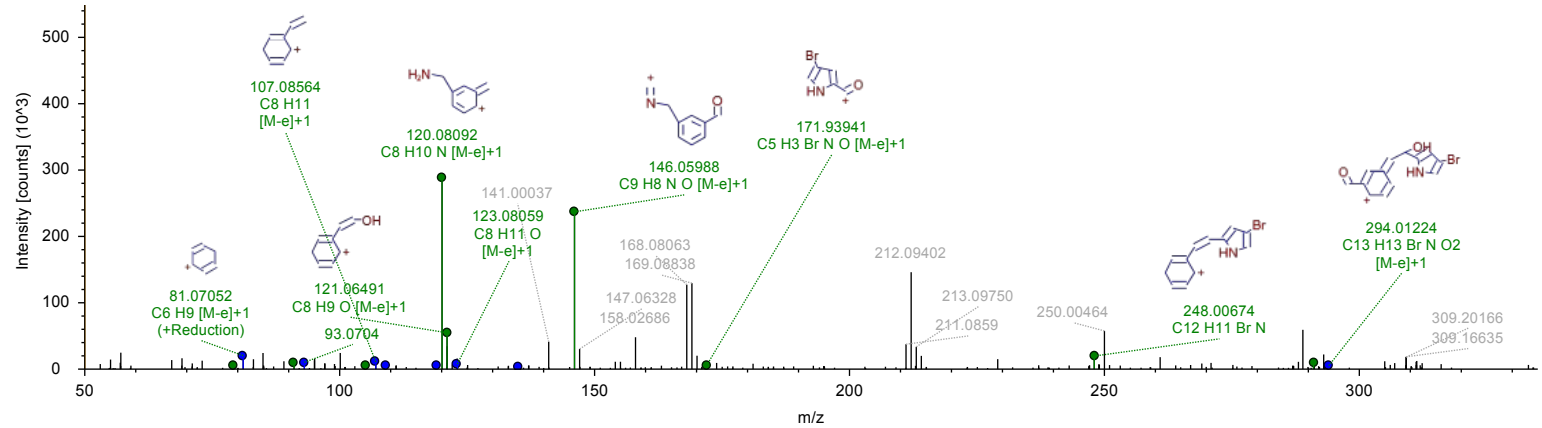
248.00694


Br
OH
N
H
NH
O

M4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+1
Intensity [counts] (10^3)
m/z
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C8 H11
[M-e]+1
120.08092
C8 H10 N [M-e]+1
146.05988
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171.93941
C5 H3 Br N O [M-e]+1
294.01224
C13 H13 Br N O2
[M-e]+1
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C6 H9 [M-e]+1
(+Reduction)
121.06491
C8 H9 O [M-e]+1
123.08059
C8 H11 O
[M-e]+1
248.00674
C12 H11 Br N
141.00037
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169.08838
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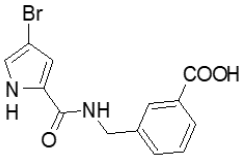
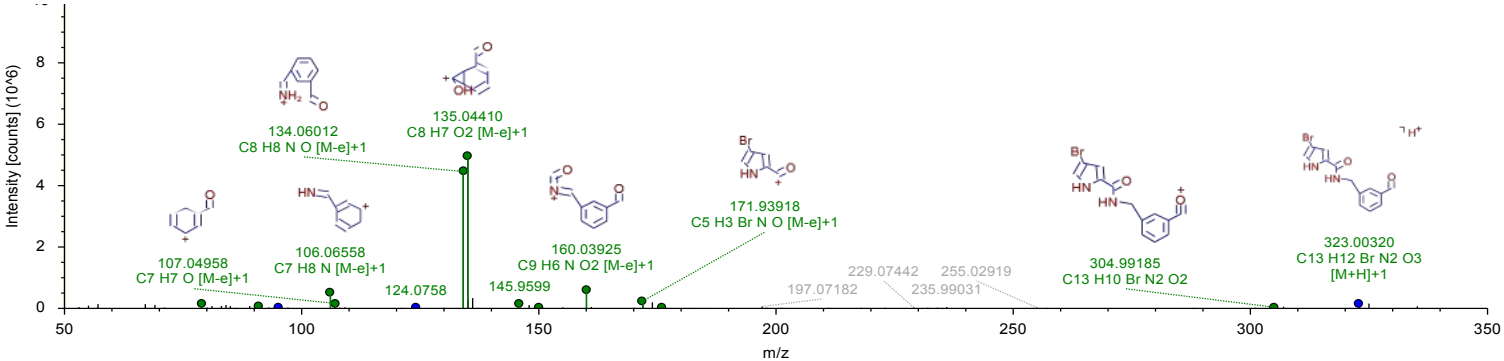
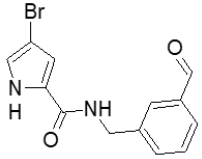
309.20166
309.16635



M5

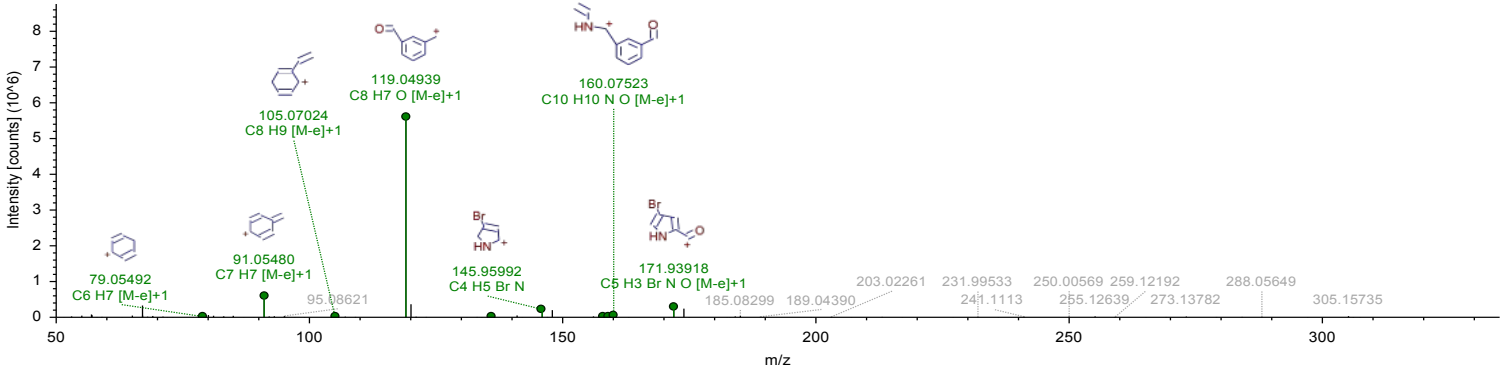
IL-P-GSH_002 (F19) #1648, RT=8.605 min, MS2, FTMS (+), (HCD, DDF, 323.0027@30, +1)

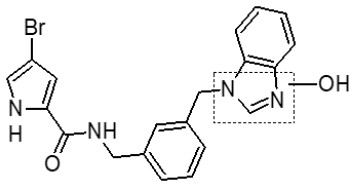




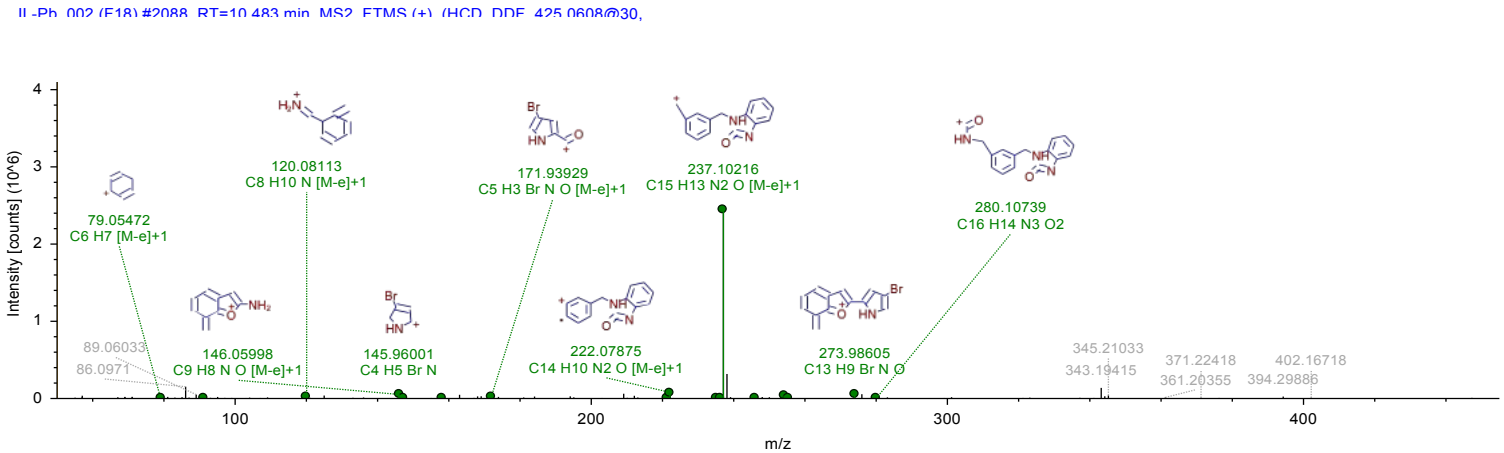
M6

IL-Pb_002 (F18) #1935, RT=9.823 min, MS2, FTMS (+), (HCD, DDF, 307.0078@30,

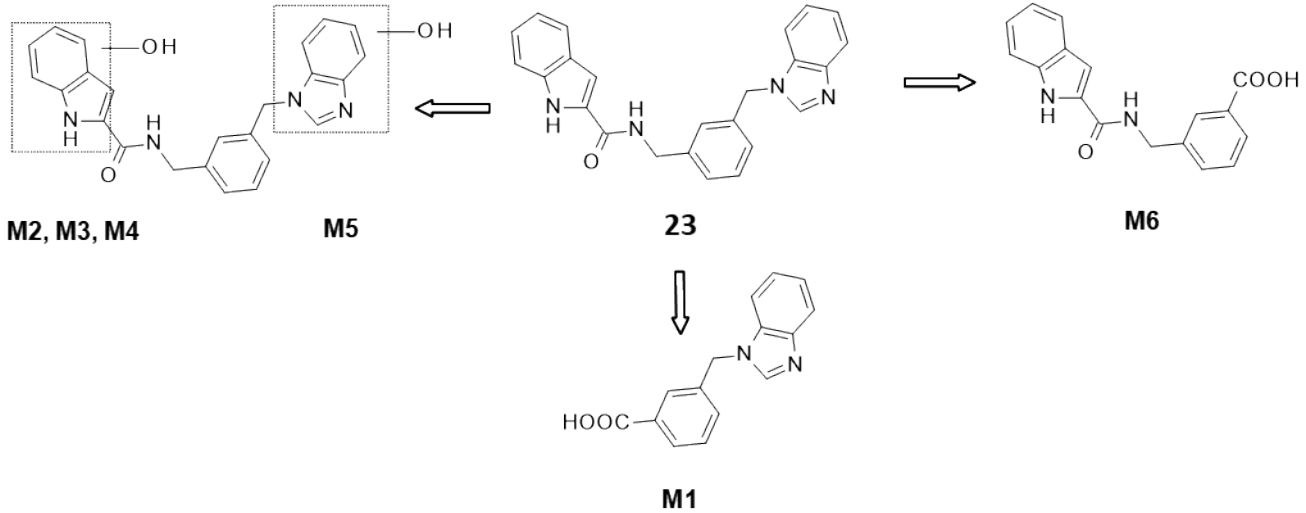



Br
N
H
O
NH
N
N
OH

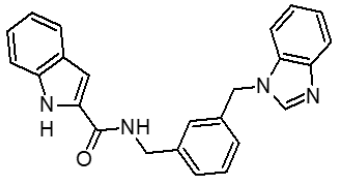
M7


Intensity [counts] (10^6)
m/z
79.05472
C6 H7 [M-e]+1
120.08113
C8 H10 N [M-e]+1
171.93929
C5 H3 Br N O [M-e]+1
237.10216
C15 H13 N2 O [M-e]+1
280.10739
C16 H14 N3 O2
146.05998
C9 H8 N O [M-e]+1
145.96001
C4 H5 Br N
222.07875
C14 H10 N2 O [M-e]+1
273.98605
C13 H9 Br N O
89.06033
86.0971
345.21033
343.19415
371.22418
361.20355
402.16718
394.29886

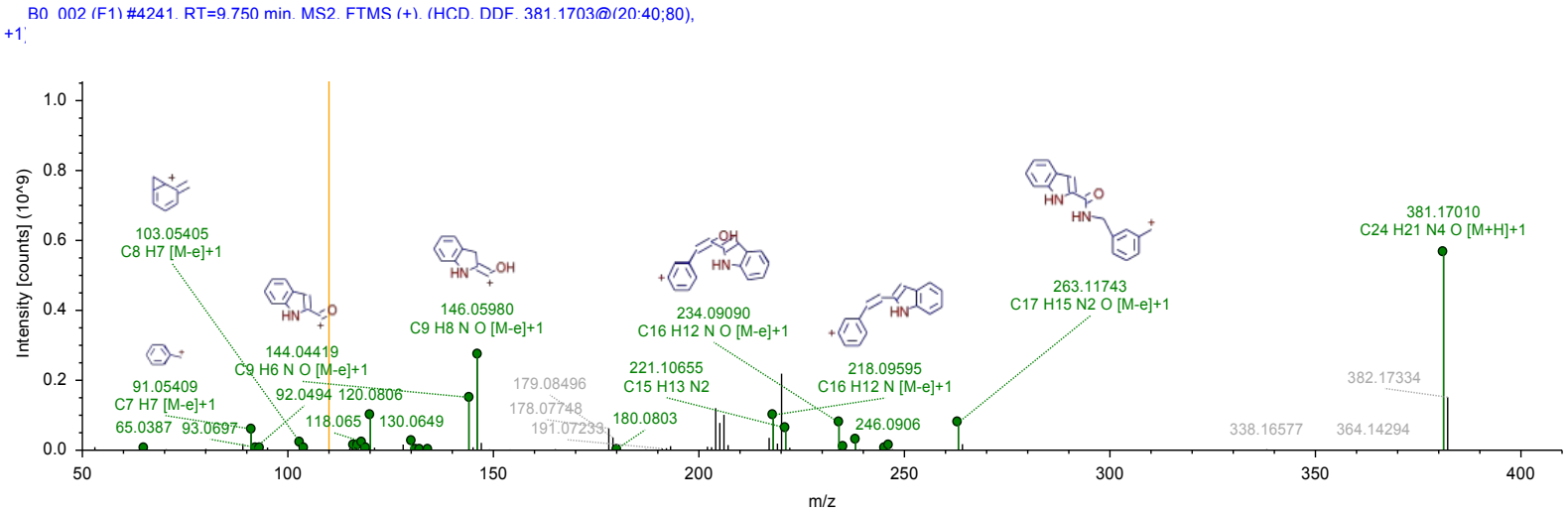
Scheme S2. Metabolic pathways of compound **23.**

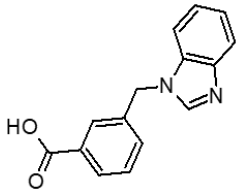


	RT (min)	Molecular Formula	Teoretical $[M+H]^+$	Measured $[M+H]^+$	Mass shift (ppm)	Metabolic pathway
23	9.77	$C_{24}H_{20}N_4O$	381.17099	381.17020	2.07	
M1	4.78	$C_{15}H_{12}N_2O_2$	253.09715	253.09671	1.75	oxidative *N*-dealkylation
M2	7.03	$C_{24}H_{20}N_4O_2$	397.16590	397.16568	0.56	+ O
M3	7.41	$C_{24}H_{20}N_4O_2$	397.16590	397.16565	0.63	+ O
M4	8.24	$C_{24}H_{20}N_4O_2$	397.16590	397.16562	0.71	+ O
M5	8.79	$C_{24}H_{20}N_4O_2$	397.16590	397.16547	1.09	+ O
M6	9.28	$C_{17}H_{14}N_2O_3$	295.10772	295.10742	1.01	oxidative *N*-dealkylation

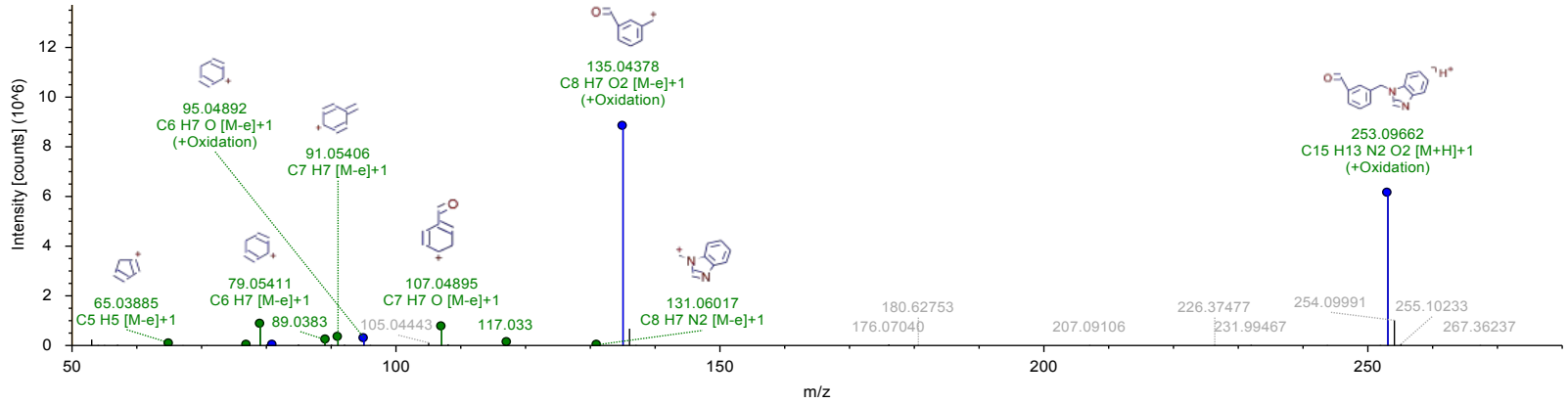


23

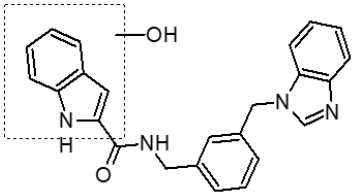

B0_002 (F1) #4241, RT=9.750 min, MS2, FTMS (+), (HCD, DDF, 381.1703@(20;40;80),
+1
Intensity [counts] (10^9)
m/z
103.05405
C8 H7 [M-e]+1
91.05409
C7 H7 [M-e]+1
65.0387
93.0697
144.04419
C9 H6 N O [M-e]+1
92.0494
120.0806
118.065
130.0649
146.05980
C9 H8 N O [M-e]+1
179.08496
178.07748
191.07233
180.0803
221.10655
C15 H13 N2
234.09090
C16 H12 N O [M-e]+1
218.09595
C16 H12 N [M-e]+1
246.0906
263.11743
C17 H15 N2 O [M-e]+1
338.16577
364.14294
382.17334
381.17010
C24 H21 N4 O [M+H]+1



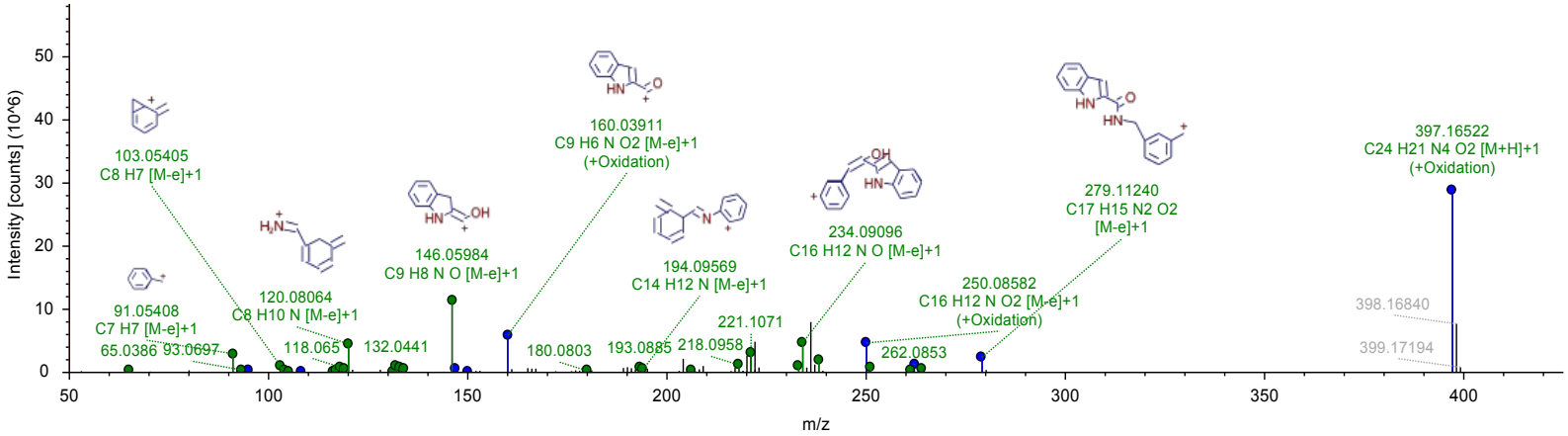
M1


P-GSH_002 (F6) #1709, RT=4.758 min, MS2, FTMS (+), (HCD, DDF, 253.0969@(20;40;80),
Intensity [counts] (10^6)
m/z
65.03885
C5 H5 [M-e]+1
79.05411
C6 H7 [M-e]+1
89.0383
95.04892
C6 H7 O [M-e]+1
(+Oxidation)
91.05406
C7 H7 [M-e]+1
105.04443
107.04895
C7 H7 O [M-e]+1
117.033
135.04378
C8 H7 O2 [M-e]+1
(+Oxidation)
131.06017
C8 H7 N2 [M-e]+1
176.07040
180.62753
207.09106
226.37477
231.99467
254.09991
253.09662
C15 H13 N2 O2 [M+H]+1
(+Oxidation)
255.10233
267.36237

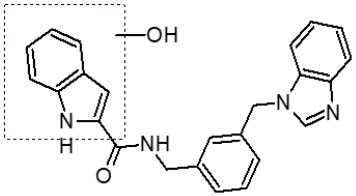
Pa_002 (F4) #2872, RT=7.017 min, MS2, FTMS (+), (HCD, DDF, 397.1656@(20;40;80),



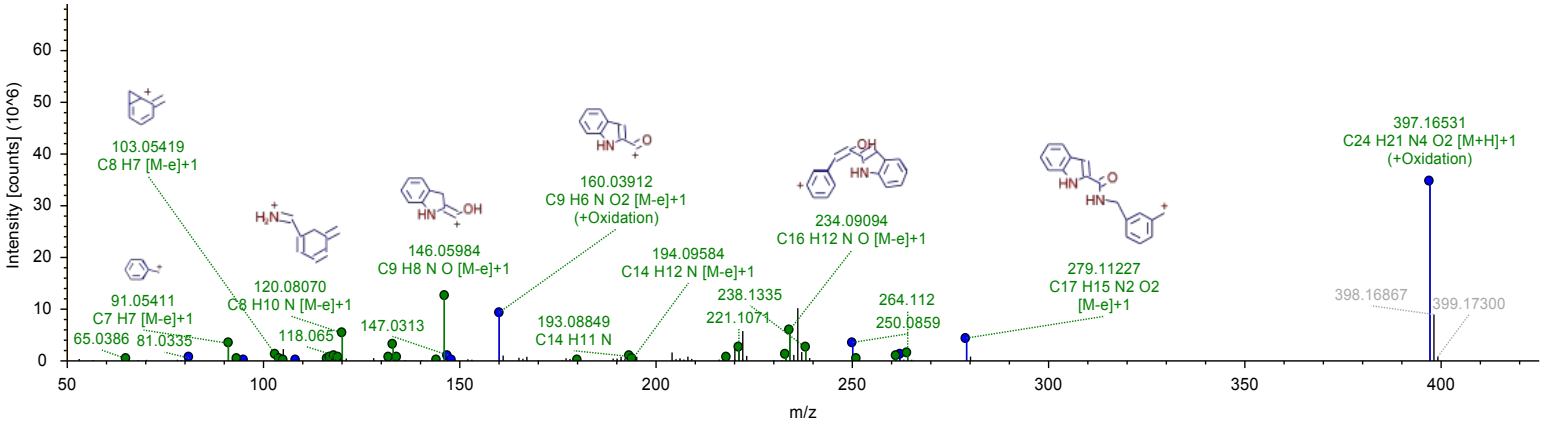
M2

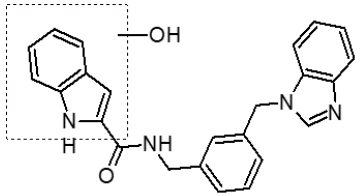


Pa_002 (F4) #3071, RT=7.415 min, MS2, FTMS (+), (HCD, DDF, 397.1656@(20;40;80),
+1)



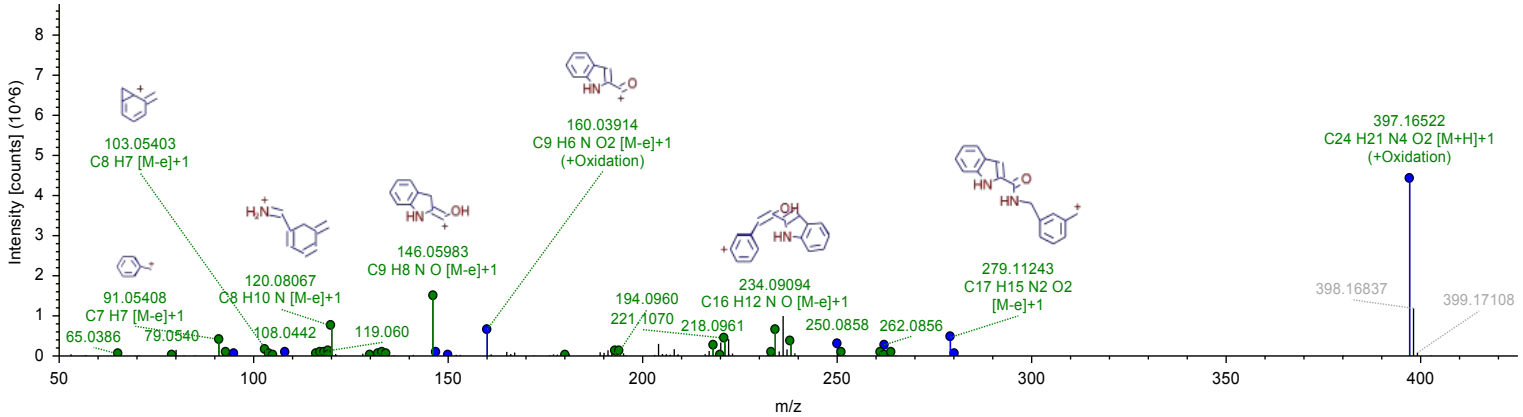
M3

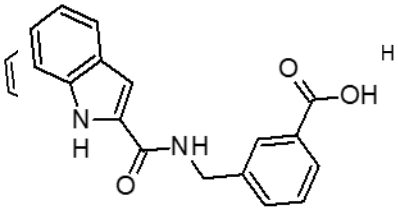




M4

Pa 002 (F4) #3481, RT=8.232 min, MS2, FTMS (+), (HCD, DDF, 397.1657@(20;40;80), +1

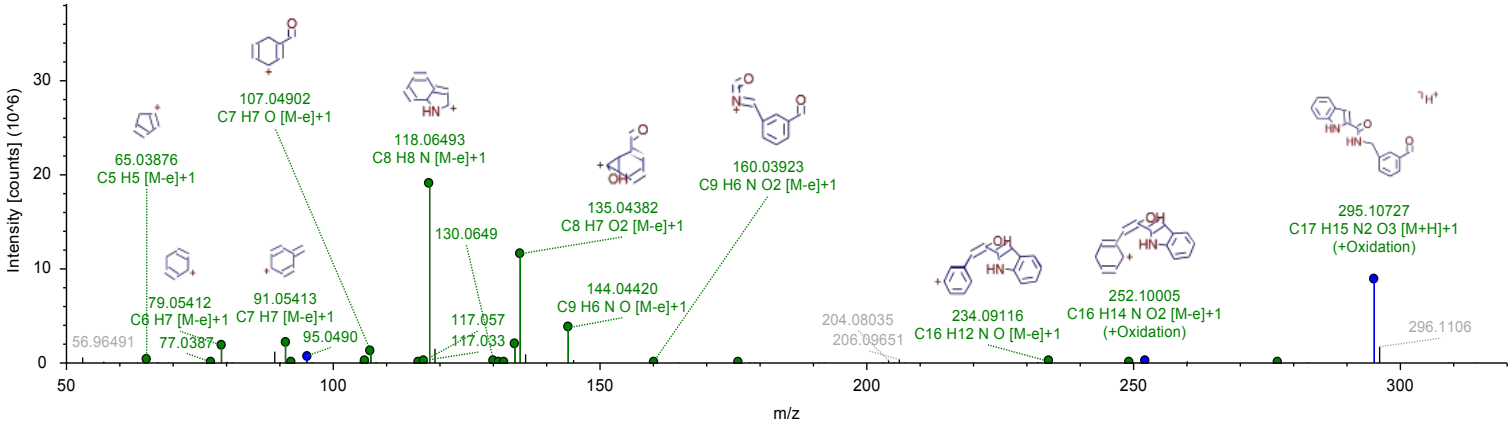




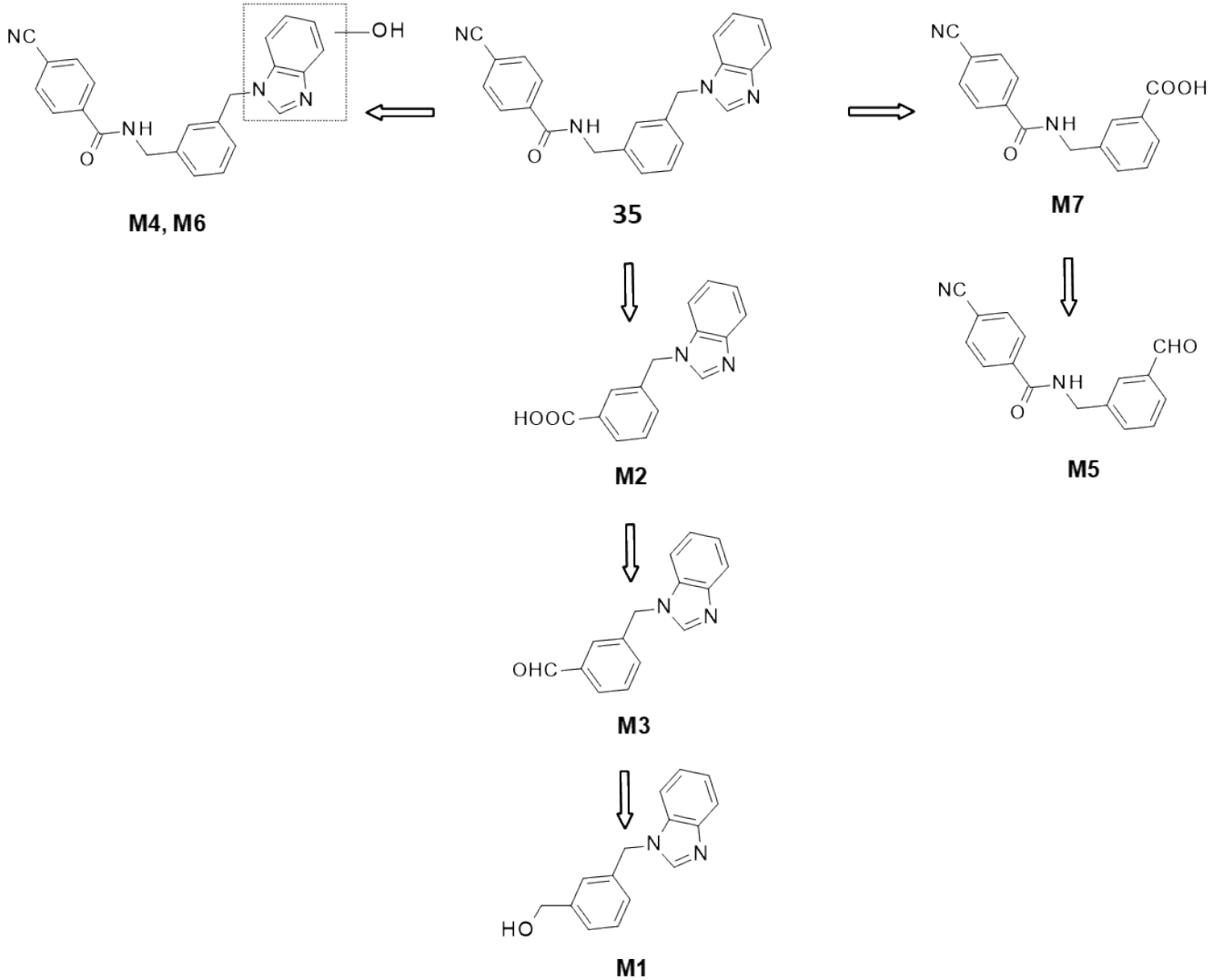
M6

P-GSH 002 (F6) #3751, RT=8.772 min, MS2, FTMS (+), (HCD, DDF, 397.1654@(20;40;80), +1

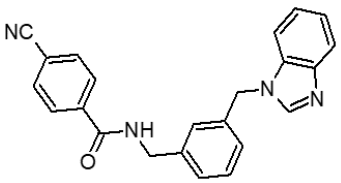
P-GSH 002 (F6) #4007, RT=9.271 min, MS2, FTMS (+), (HCD, DDF, 295.1074@(20;40;80), +1



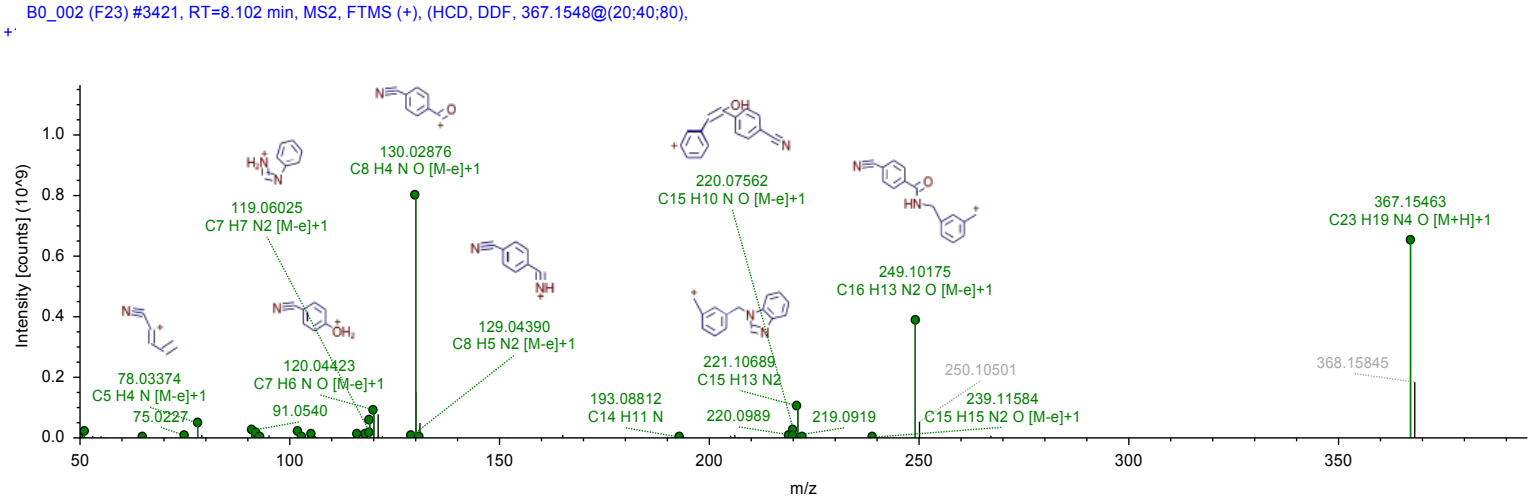
Scheme S3. Metabolic pathways of compound **35.**

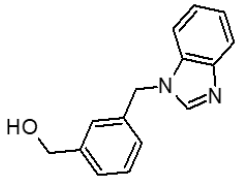

NC
OH
N
N
NH
O
M4, M6
NC
N
N
NH
O
35
NC
COOH
NH
O
M7
HOOC
N
N
M2
NC
CHO
NH
O
M5
OHC
N
N
M3
HO
N
N
M1

	RT (min)	Molecular Formula	Teoretical $[M+H]^+$	Measured $[M+H]^+$	Mass shift (ppm)	Metabolic pathway
35	8.10	$C_{23}H_{18}N_4O$	367.15534	367.15463	1.93	
M1	4.18	$C_{15}H_{14}N_2O$	239.11789	239.11746	1.80	oxidative *N*-dealkylation + reduction
M2	4.78	$C_{15}H_{12}N_2O_2$	253.09715	253.09677	1.52	oxidative *N*-dealkylation
M3	5.81	$C_{15}H_{12}N_2O$	237.10224	237.10193	1.30	oxidative *N*-dealkylation + reduction
M4	6.86	$C_{23}H_{18}N_4O_2$	383.15025	383.14981	1.15	+ O
M5	7.21	$C_{16}H_{14}N_2O_2$	267.11284	267.11243	1.53	oxidative *N*-dealkylation + reduction
M6	7.30	$C_{23}H_{18}N_4O_2$	383.15025	383.14966	1.55	+ O
M7	7.84	$C_{16}H_{12}N_2O_3$	281.09207	281.09180	0.96	oxidative *N*-dealkylation

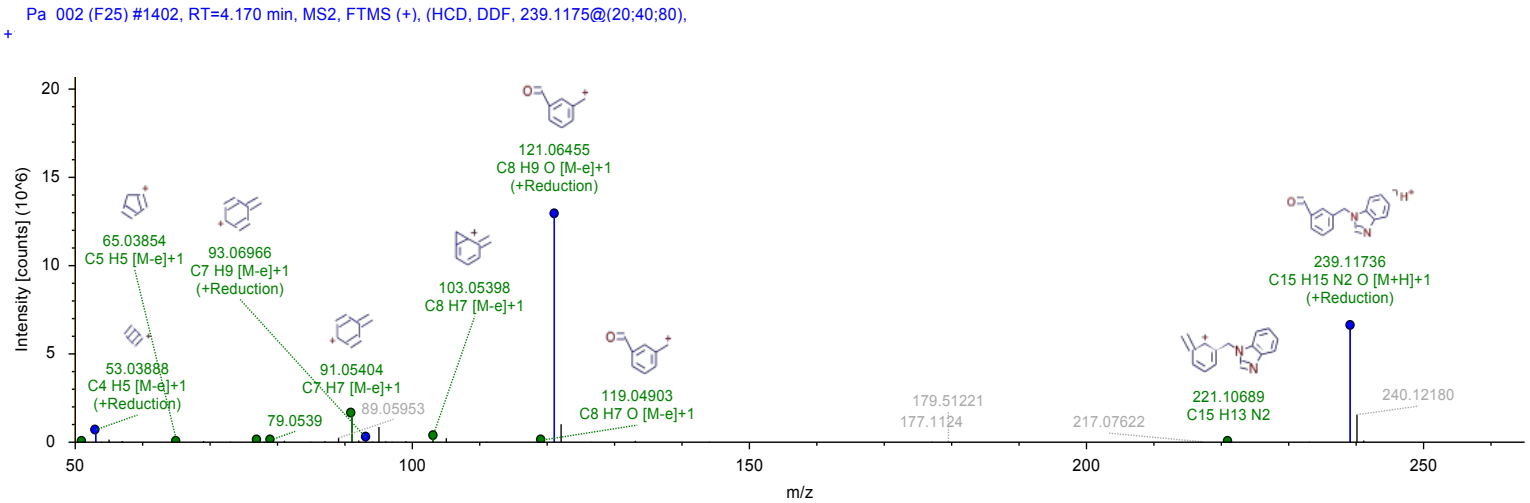


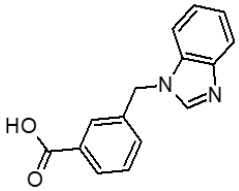
35



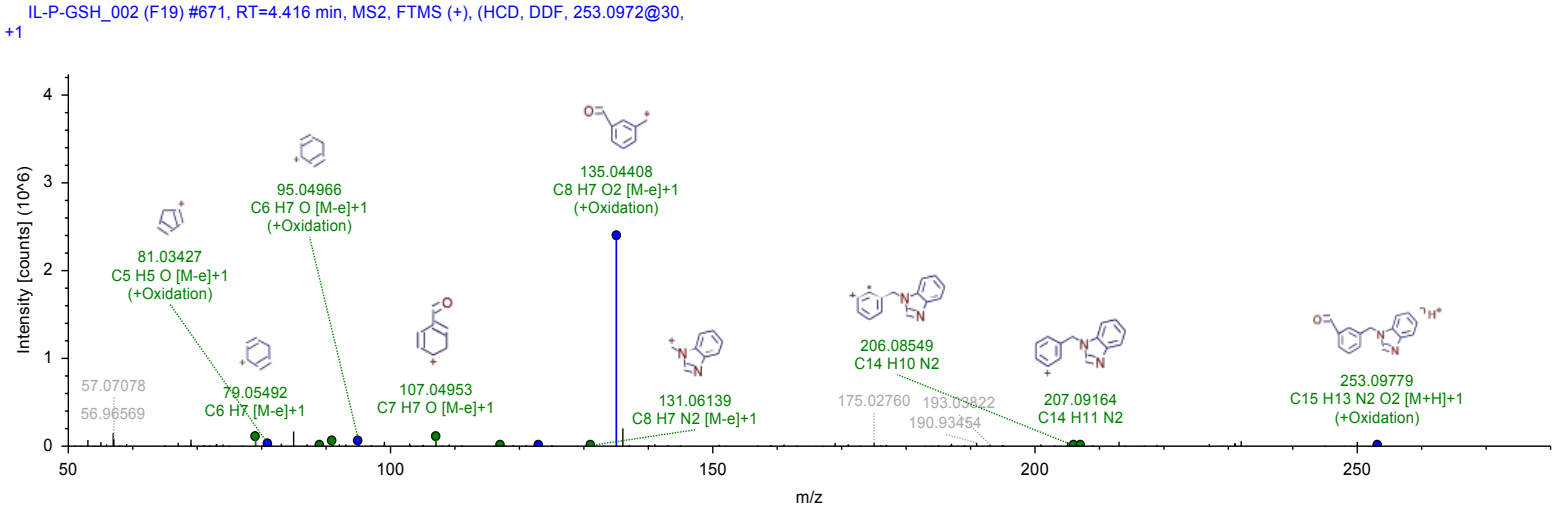


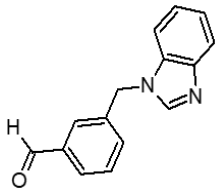
M1


Pa 002 (F25) #1402, RT=4.170 min, MS2, FTMS (+), (HCD, DDF, 239.1175@(20;40;80),
+
Intensity [counts] (10^6)
m/z
121.06455
C8 H9 O [M-e]+1
(+Reduction)
65.03854
C5 H5 [M-e]+1
93.06966
C7 H9 [M-e]+1
(+Reduction)
103.05398
C8 H7 [M-e]+1
239.11736
C15 H15 N2 O [M+H]+1
(+Reduction)
53.03888
C4 H5 [M-e]+1
(+Reduction)
91.05404
C7 H7 [M-e]+1
119.04903
C8 H7 O [M-e]+1
221.10689
C15 H13 N2
79.0539
89.05953
179.51221
177.1124
217.07622
240.12180

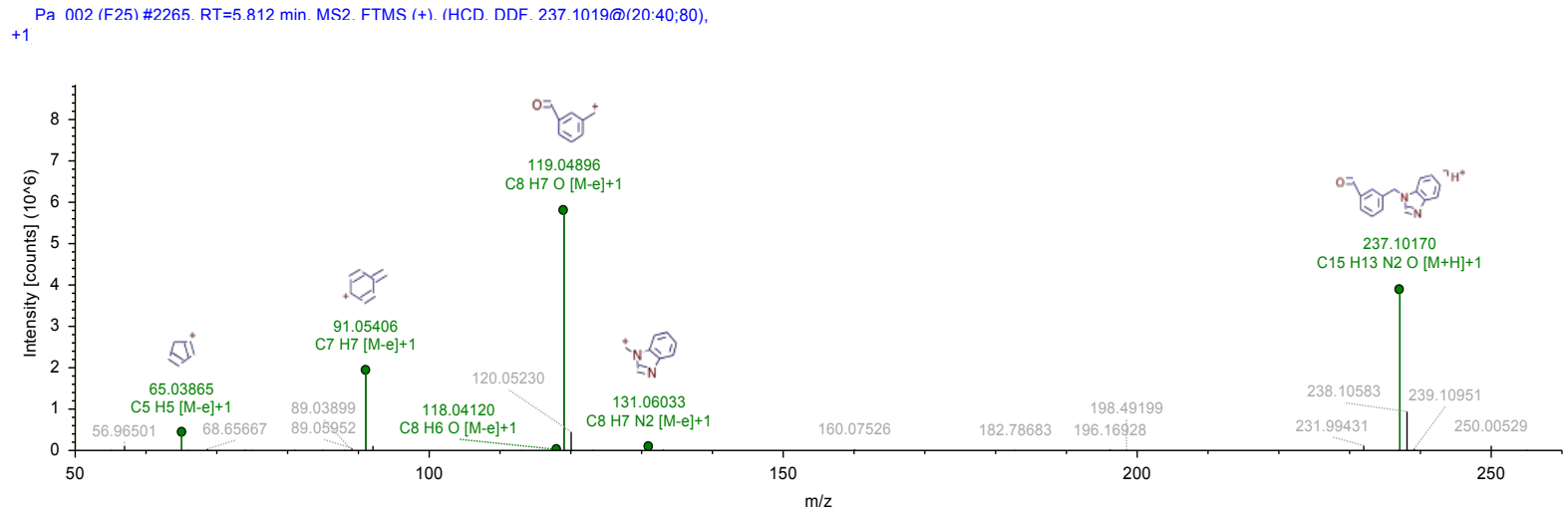


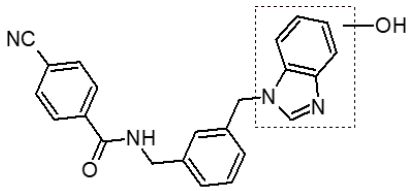
M2


IL-P-GSH_002 (F19) #671, RT=4.416 min, MS2, FTMS (+), (HCD, DDF, 253.0972@30,
+1
Intensity [counts] (10^6)
m/z
135.04408
C8 H7 O2 [M-e]+1
(+Oxidation)
95.04966
C6 H7 O [M-e]+1
(+Oxidation)
81.03427
C5 H5 O [M-e]+1
(+Oxidation)
206.08549
C14 H10 N2
253.09779
C15 H13 N2 O2 [M+H]+1
(+Oxidation)
107.04953
C7 H7 O [M-e]+1
131.06139
C8 H7 N2 [M-e]+1
207.09164
C14 H11 N2
79.05492
C6 H7 [M-e]+1
57.07078
56.96569
175.02760
193.03822
190.93454

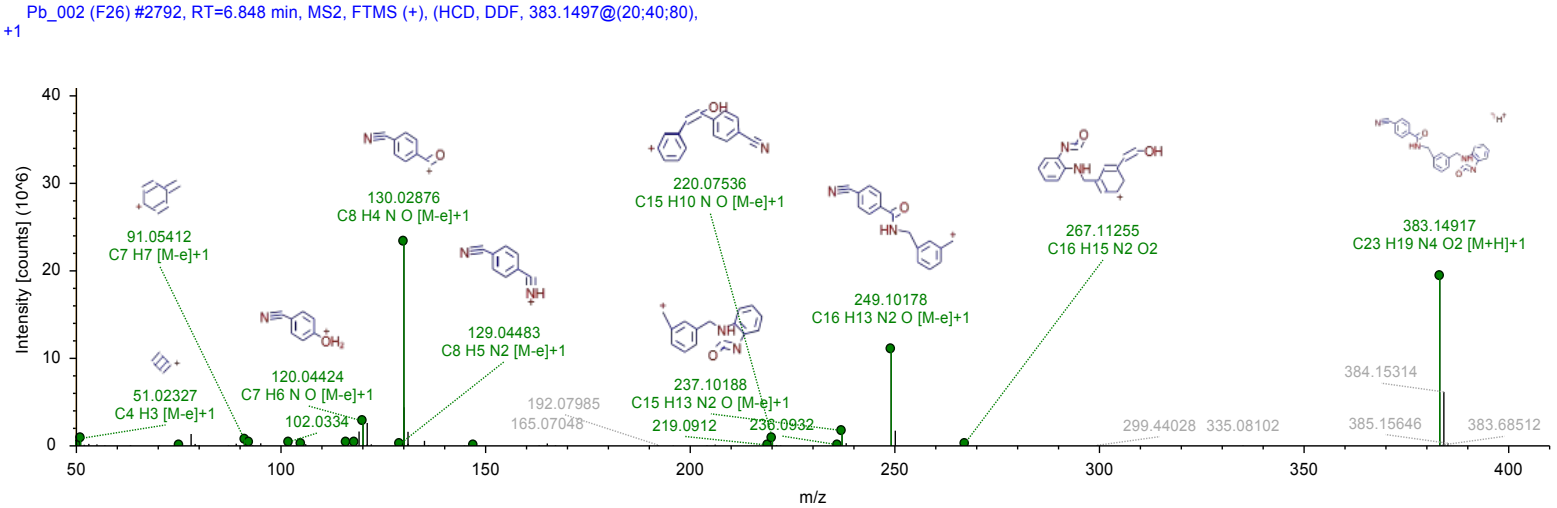


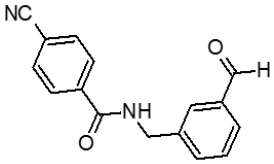
M3


Pa_002 (F25) #2265, RT=5.812 min, MS2, FTMS (+), (HCD, DDF, 237.1019@(20;40;80),
+1
Intensity [counts] (10^6)
m/z
119.04896
C8 H7 O [M-e]+1
237.10170
C15 H13 N2 O [M+H]+1
91.05406
C7 H7 [M-e]+1
65.03865
C5 H5 [M-e]+1
120.05230
118.04120
C8 H6 O [M-e]+1
131.06033
C8 H7 N2 [M-e]+1
56.96501
68.65667
89.03899
89.05952
160.07526
182.78683
198.49199
196.16928
238.10583
239.10951
231.99431
250.00529

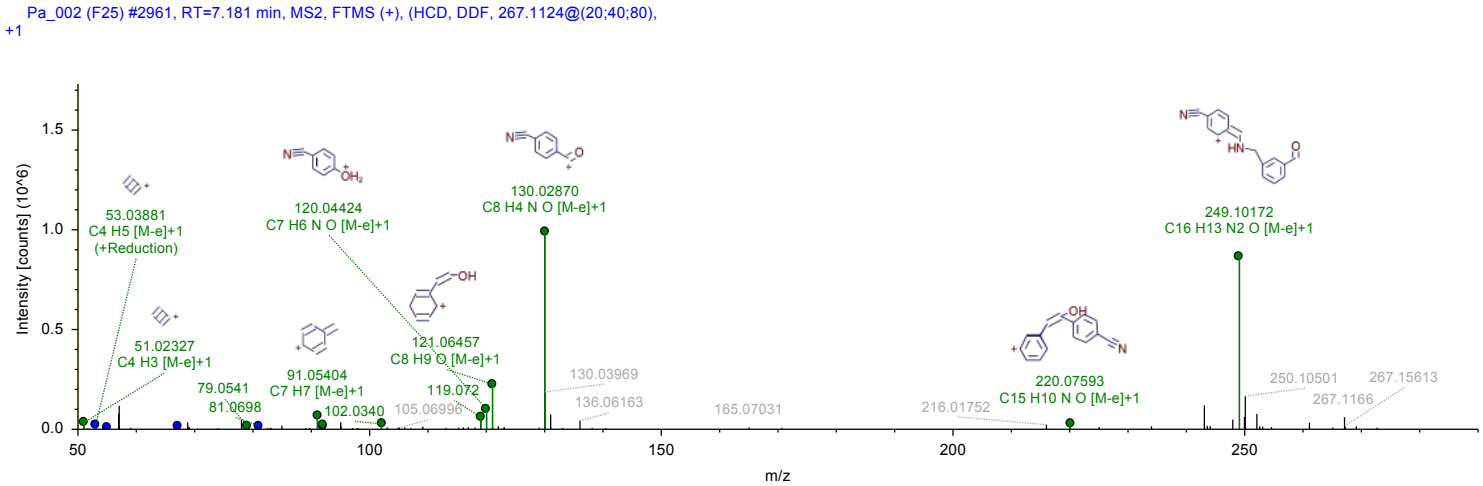


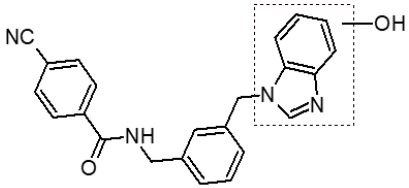
M4


Pb_002 (F26) #2792, RT=6.848 min, MS2, FTMS (+), (HCD, DDF, 383.1497@(20;40;80),
+1
Intensity [counts] (10^6)
m/z
91.05412
C7 H7 [M-e]+1
130.02876
C8 H4 N O [M-e]+1
220.07536
C15 H10 N O [M-e]+1
267.11255
C16 H15 N2 O2
383.14917
C23 H19 N4 O2 [M+H]+1
249.10178
C16 H13 N2 O [M-e]+1
129.04483
C8 H5 N2 [M-e]+1
120.04424
C7 H6 N O [M-e]+1
51.02327
C4 H3 [M-e]+1
237.10188
C15 H13 N2 O [M-e]+1
192.07985
165.07048
102.0334
219.0912
236.0932
299.44028
335.08102
384.15314
385.15646
383.68512

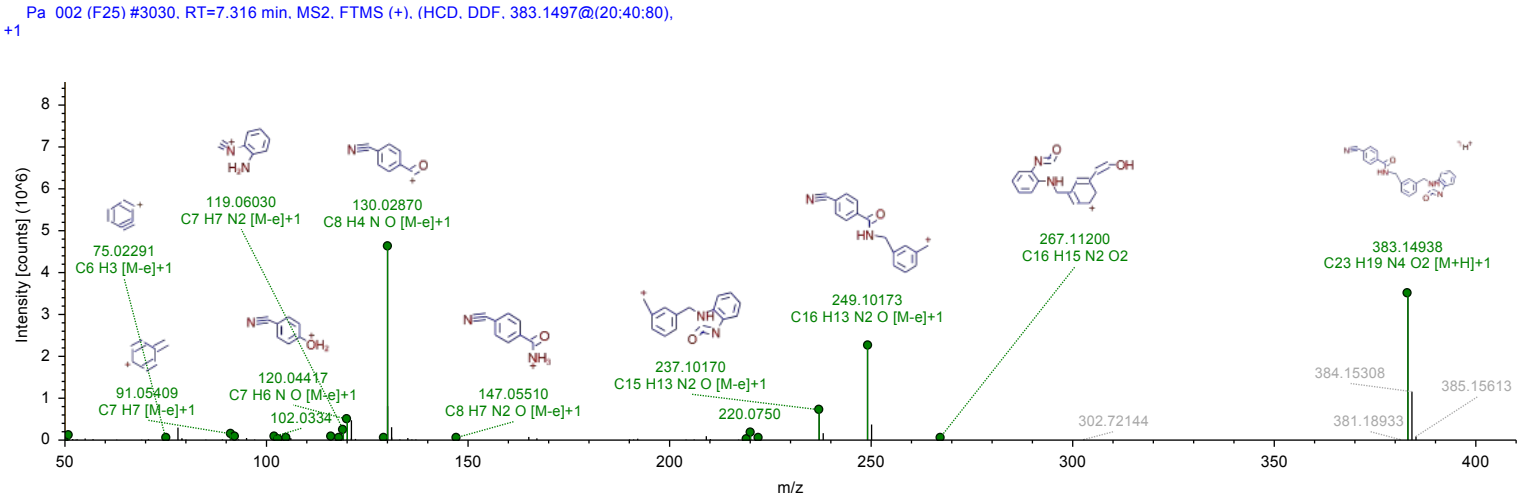


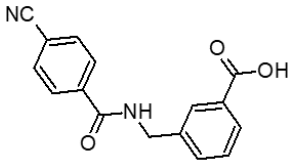
M5


Pa_002 (F25) #2961, RT=7.181 min, MS2, FTMS (+), (HCD, DDF, 267.1124@(20;40;80), +1
Intensity [counts] (10^6)
m/z
53.03881
C4 H5 [M-e]+1
(+Reduction)
51.02327
C4 H3 [M-e]+1
79.0541
81.0698
91.05404
C7 H7 [M-e]+1
102.0340
120.04424
C7 H6 N O [M-e]+1
105.06996
119.072
121.06457
C8 H9 O [M-e]+1
130.02870
C8 H4 N O [M-e]+1
130.03969
136.06163
165.07031
216.01752
220.07593
C15 H10 N O [M-e]+1
249.10172
C16 H13 N2 O [M-e]+1
250.10501
267.15613
267.1166



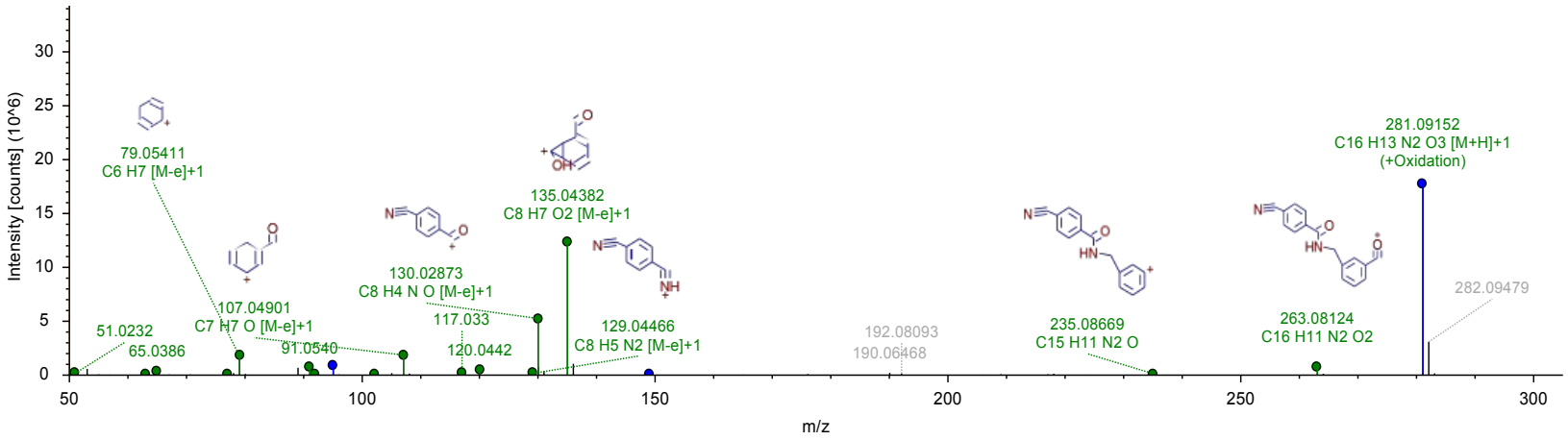
M6


Pa_002 (F25) #3030, RT=7.316 min, MS2, FTMS (+), (HCD, DDF, 383.1497@(20;40;80), +1
Intensity [counts] (10^6)
m/z
75.02291
C6 H3 [M-e]+1
91.05409
C7 H7 [M-e]+1
119.06030
C7 H7 N2 [M-e]+1
120.04417
C7 H6 N O [M-e]+1
102.0334
130.02870
C8 H4 N O [M-e]+1
147.05510
C8 H7 N2 O [M-e]+1
220.0750
237.10170
C15 H13 N2 O [M-e]+1
249.10173
C16 H13 N2 O [M-e]+1
267.11200
C16 H15 N2 O2
302.72144
383.14938
C23 H19 N4 O2 [M+H]+1
384.15308
385.15613
381.18933



M7

P-GSH_002 (F27) #3289, RT=7.833 min, MS2, FTMS (+), (HCD, DDF, 281.0919@(20;40;80), +1)



***In vivo* PK evaluation of compound 23.**

Table S3. PK parameters of single administration of compound **23** (10 mg/kg).

Parameter	*Unit*	*Value*
$t_{1/2}$	h	**0.605**
T_{max}	h	**0.033**
C_{max}	µg/L	**2684.268**
V_d	L/Kg	**6.545**
Cl	L/h/Kg	**7.493**

***In vivo* PD evaluation of compound 23.**

Fig. S10. Residual KYN levels after endovenous administration of **23**.