Supporting Information

Novel Benzohydroxamate-Based Potent and Selective Histone Deacetylase 6 (HDAC6) Inhibitors Bearing a Pentaheterocyclic Scaffold: Design, Synthesis and Biological Evaluation

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In vitro determination of specific HDAC6 inhibitory activity (data related to Figure 8)

Table S1 - Tubulin and H3 acetylation in human 697 B-precursor acute lymphoblastic leukemia

(B-Pre-ALL) cell line.

	α-tubulin[a]				H3[a]		
	Conc (nM)				Conc (nM)		
compd	**1000**	**333**	**111**	**37**	**1000**	**333**	**111**
13	16±2	13±1	7±1	3±1	2±1	1±1	1±1
29	7±1	6±1	4±1	2±1	8±3	n.a.	n.a.
34	6±1	4±1	3±1	2±1	1±1	1±1	1±1
35	11±1	8±1	3±1	2±1	3±1	n.a.	n.a.
39	22±1	20±1	19±1	10±1	2±1	3±1	1±1
42	14±3	9±2	3±1	2±1	3±1	2±1	1±1
43	12±2	9±1	4±1	3±1	1±1	1±1	1±1
44	10±2	9±2	8±1	3±1	2±1	2±1	1±1
45	17±1	21±1	15±1	7±1	1±1	1±1	1±1
47	14±1	10±1	11±1	6±1	1±1	n.a.	n.a.
48	7 ±1	5±1	2±1	2±1	2±1	1±1	1±1
49	19±1	15±1	7±1	2±1	1±1	n.a.	n.a.
Givinostat	13±1	9±1	3±1	2±1	23±7	17±5	8±3

a. *fold increase* of the ratio of acetylated tubulin and total tubulin and of acetylated H3 and total H3 ratio towards control. The test has been carried out in dose/response (4-points for tubulin and 3-points for histone H3) starting from 1 μM with 3-fold serial dilution (3 technical replicates).

n.a. not available.

In vivo determination of specific HDAC6 inhibitory activity (data related to Figure 9)

Table S2 - Levels of tubulin and histone H3 acetylation and concentrations of compound **42** in the spleen and in plasma of mice following oral administration of the inhibitor (data presented in Figure 9A).

Time (hrs)	Ac-tubulin	Ac-H3	Compound 42 concentration	Compound 42 plasma concentration
	Fold increase (average ± sem)		(ng/g spleen)	(ng/ml)
1	10.54 ± 0.97	2.03 ± 0.38	1357 ± 492	958 ± 362
4	1.68 ± 0.21	0.83 ± 0.07	106.1 ± 26.6	17.8 ± 3.3
24	0.84 ± 0.02	0.72 ± 0.05	17.1 ± 2.6	0.7 ± 0.4
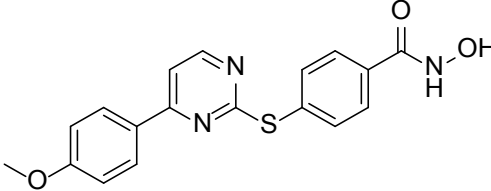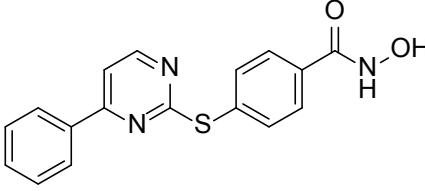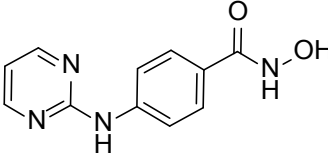
Table S3 - Levels of tubulin and histone H3 acetylation and concentrations of compound 13 in the spleen and in plasma of mice following oral administration of the inhibitor (data presented in Figure 9B).

Time (hrs)	Ac-tubulin	Ac-H3	Compound 13 concentration	Compound 13 plasma concentration
	Fold increase (average ± sem)		(ng/g spleen)	(ng/ml)
1	11.04 ± 1.25	3.12 ± 0.59	6140 ± 1583	1989 ± 638
4	1.94 ± 0.21	1.14 ± 0.04	109.2 ± 24.1	16.0 ± 4.8
24	0.80 ± 0.04	0.85 ± 0.05	17.5 ± 3.3	1.3 ± 0.1

Six-membered heteroaromatic central scaffold compounds

Some compounds bearing a six-membered heteroaromatic ring as a central scaffold were synthesized and tested. These compounds show quite low potency in HDAC6 inhibitory activity compared to compounds with five-membered heteroaromatic ring core, even if they retain a certain selectivity toward HDAC3.

Table S4 – Enzymatic activity of some Six-membered heteroaromatic central scaffold compounds

Compd code	Structure	HDAC6 IC$_{50}$ (nM)[a]	HDAC3 IC$_{50}$ (nM)[a]	Selectivity (HDAC3 IC$_{50}$/HDAC6 IC$_{50}$)
ITF3858		621±16	5414±95	8.7
ITF3857		701±8	6691±154	9.5
ITF3743		129±2	1558±31	12.1

a - Enzymatic data (IC$_{50}$) in nM unit were obtained from curve-fitting of a 5-point enzymatic assay starting from 100, 30 or 10 μM with 10-fold serial dilution. Experiments were done in triplicate (single experiment). SDs were calculated on technical replicates.

Repeats of enzyme assay IC$_{50}$ determination (for key compounds) and standard deviations.

Table S5 – IC$_{50}$ calculation - Single experiments results

Enzyme	Compd	IC$_{50}$ (nM)				Mean	Std Dev	repeats
HDAC6	13	9	8	8		8	1	3
	29	8	8	5		7	1	3
	33	8	7	7		7	1	3
	34	6	5	5		5	1	3
	35	3	5	2		3	2	3
	39	6	8	4		6	2	3
	42	27	15	15	9	17	8	4
	43	9	8	9		9	1	3
	44	4	4	1		3	2	3
	45	4	2	6		4	2	3
	46	5	4	6		5	1	3
	47	5	6	6		6	1	3
	48	6	9	15		10	4	3
	49	11	13	16		13	3	3
HDAC3	13	487	532	493		504	24	3
	29	974	1026	681	804	871	158	4
	33	6510	7831	6680	6357	6845	671	4
	34	5988	5243	4306		5179	843	3
	35	641	750	617		669	71	3
	39	2470	2803	2450		2574	198	3
	42	979	794	712	975	865	134	4

	43	2827	2074	1871	1606	2095	524	4
	44	2635	2273	2348		2419	191	3
	45	1776	1870	1885	1584	1779	138	4
	46	2608	2729	1887		2408	455	3
	47	3005	2661	2669		2778	196	3
	48	2936	3635	3741	3851	3541	413	4
	49	13717	10953	10382	8614	10917	2116	4
HDAC1	13	1015	1004			1010	8	2
	29	812	1047			929	166	2
	33	7512	6768			7140	526	2
	34	4447	3472			3960	689	2
	35	1425	700			1063	513	2
	39	2898	2436			2667	327	2
	42	1094	953	829	820	924	129	4
	43	3991	4288			4140	210	2
	44	2517	2107			2312	290	2
	45	2878	2983			2931	74	2
	46	3659	4677			4168	720	2
	47	3182	3452			3317	191	2
	48	5162	5269			5216	76	2
	49	7377	8838			8107	1033	2

Repeats of cytotoxicity on PBMC and on 697 B-precursor acute lymphoblastic leukemia (B-Pre-ALL) cell line experiments (on key compounds) and standard deviations

Table S6 – Cytotoxicity on PBMC

Compd	PBMC cytotoxicity (nM)	Standard deviation	repeats
13	5857	4230	6
29	4274	3507	6
33	>10000	-	2
34	7387	7608	4
35	5524	6334	2
39	>10000	-	2
42	5387	5128	5
43	>10000	-	3
44	>10000	-	3
45	>10000	-	1
46	>10000	-	3
47	5987	3892	3
48	7445	6210	3
49	3963	4108	5
Givinostat	313		

Table S7 – Cytotxicity on 697 B-precursor acute lymphoblastic leukemia (B-Pre-ALL) cell line

Compd	697 cell line cytotoxicity (nM)	Standard deviations	repeats
13	>10000	-	3
29	3634	2271	6
33	>10000	-	5
34	>10000	-	7
35	6489	2603	6
39	>10000	-	3
42	8934	5531	6
43	5506	3031	4
44	2749	1865	7
45	>10000	-	6
46	5983	2844	5
47	>10000	-	5
48	>10000	-	4
49	>10000	-	3
Givinostat	100		

Both cytotoxicity test on 697 (B-Pre-AL) and hPBMC were performed in a concentration range between 10 and 10000 nM (Higher concentrations lead to phenomena which could compromise the assay, such as inhibitor precipitation). The IC_{50} of the majority of tested compounds could not be determined, being higher than 10000 nM. The high standard deviations of the other compounds

are due to the closeness of the IC_{50} value to the experiment highest dose. These data state that all the tested compounds are not cytotoxic.

Cell cytotoxicity assay. Cytotoxic activities of the compounds were evaluated in the human 697 promyelocytic B leukemia cell line (ACC 42, DSMZ- Deutsche Sammlung von Mikroorganismen und Zellkulturen) and on human peripheral blood mononuclear cells (PBMCs) using a commercial viability assay which measures the mitochondrial activity. 697 cells were plated at $2x10^4$ cells per well, and the compounds were added in concentrations ranging from 10 to 10000 nM. After 48 h of incubation, cell viability was evaluated with CellTiter 96® Aqueous One Solution Cell Proliferation Assay (Promega) according to the manufacturer's instructions.

hPBMC from healthy donors were separated by density gradient on Ficoll Hypaque.

PBMCs were plated at $5x10^5$ cell per well and compounds were added in a concentration range from 10 to 10000 nM. After 72 h of incubation, cell viability was evaluated as described before.

Homology model protocol

The homology model h-HDAC6-CD2.pdb was prepared using "Build Homology Model" protocols available in Discovery Studio, and based on MODELER (Sali, A.; Blundell, T.L., *J. Mol. Biol*. **1993**, *234*(3), 779-815.). Parameters are set to the default values suggested by supplier.

Sequences of templates (h-HDAC8: PDB code 1T64, h-HDAC2: PDB code 4LXZ and h-HDAC3: PDB code 4A69) and the portion of h-HDAC6 target of homology modeling (h-HDAC6-CD2, residues from 485 to 835, GenBank: AAH69243.1 - Ref. Strausberg et al., *Proc. Natl. Acad. Sci*. **2002**, *99*, 16899-16903). Water molecules available in templates were removed, whereas all ligands coprecipitated with template were conserved and transferred to the model. Optimization level was set to the highest level.

Docking protocol

Docking calculation were performed using libdoc protocol of Discovery Studio (DS) Suite, focusing the conformational search of candidates in a sphere centered in the center of mass of aligned inhibitors and having radius equal to 8.0 A. The volume includes both L1 and L2 loops, the catalytic core, the zinc cation and its residue directly bound to the metal ion.

The best score function for poses ranking was detected in the preliminary calculation, where a training set of HDAC6 selective and pan inhibitors are mixed with compounds exhibiting low isoform 6 potency (at least 10-fold).

Final Receiving Operator Curves (ROC) were based on the Dreiding scores, which exhibited the highest number of true positive hits, on the basis of the top scoring function value.

Comparison between the best docking poses of compound 35 (related to Figure 7), Bavarostat and ACY-1083 into HDAC6 active site.
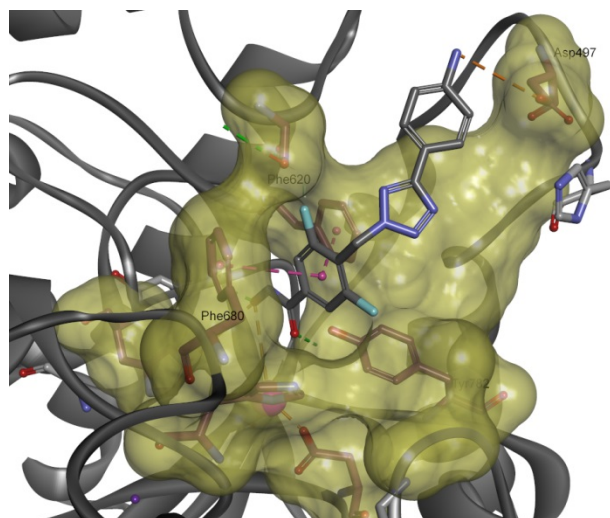
Figure S1.1 (related to Figure 7). Compound 35 h-CD-HDAC6 complex: conformer detected in the docking experiment. Protein PDB code: 5EDU
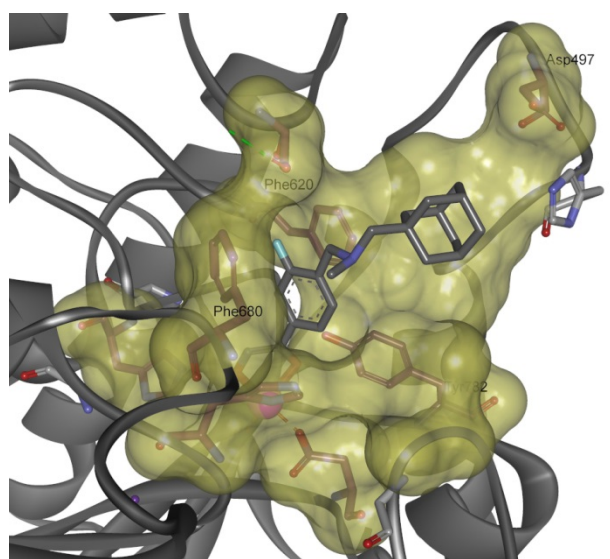
Figure S1.2. Bavarostat h-CD-HDAC6 virtual complex: the "binding" conformer was extracted from Bavarostat-z-CD2-HDAC6 complex (PDB code 6DVO), preliminary superimposed to h-CD2-HDAC6 (PDB code 5EDU) by using sequences alignment.

Figure S1.3. ACY-1083 h-CD-HDAC6 virtual complex: the "binding" conformer was extracted from ACY-1083-z-CD2-HDAC6 complex (PDB code 5WGM), preliminary superimposed to h-CD2-HDAC6 (PDB code 5EDU) by using sequences alignment.

HPLC chromatograms of key compounds

Compound **13**
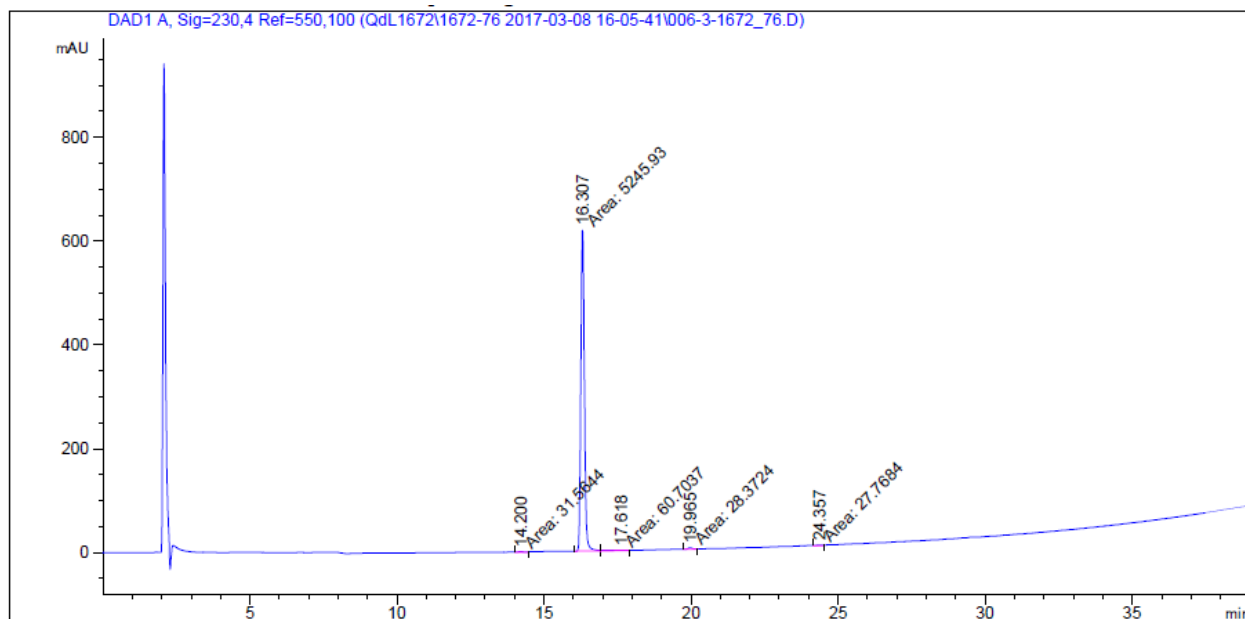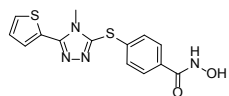
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  3  17.618 FM   0.8307   60.70369    1.21788    1.1253
  4  19.965 MM   0.1715   28.37240    2.75685    0.5260
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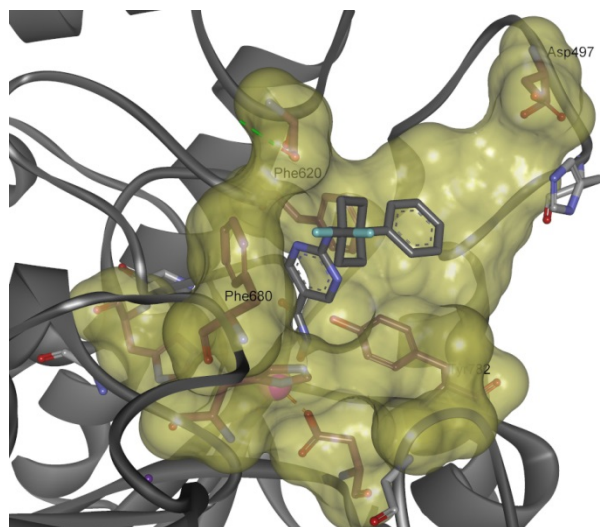
Compound **29**


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   2  13.439 FM    0.3375  136.16881    6.72462    1.2704
   3  14.663 MM    0.1033   21.78426    3.51447    0.2032
   4  17.013 MM    0.1082  253.14133   38.99165    2.3617

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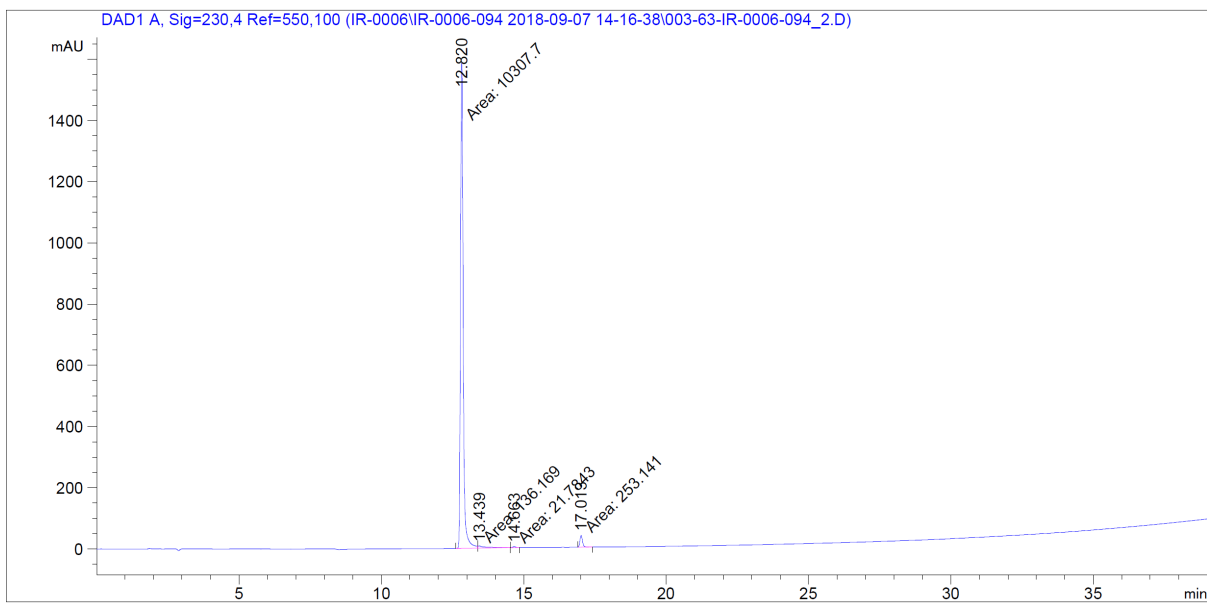
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   4   14.786   VV    147.0223    0.123     16.631    0.628
   5   15.727   BB     43.1524    0.094      6.988    0.184
   6   18.576   BB    100.9549    0.108     14.001    0.431
   7   23.995   BB     88.4048    0.134      9.752    0.378
   8   24.460   BB     84.4804    0.115     11.134    0.361

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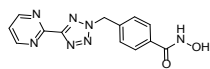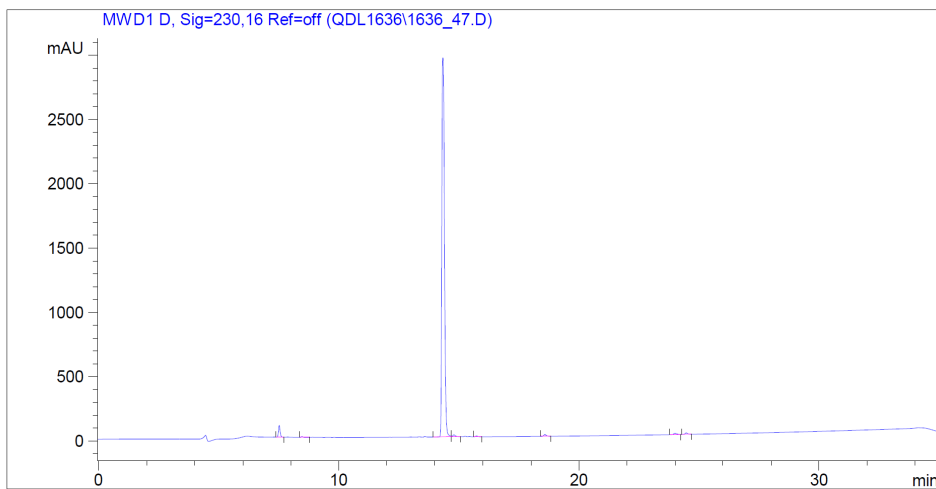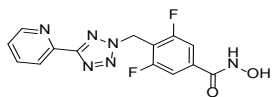
Compound **34**

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   5  13.721 BB   0.1305   35.58794   4.30420    0.2646
   6  14.141 MM   0.2270    9.66364 7.09389e-1   0.0719
   7  15.857 MM   0.2234   12.64052 9.43196e-1   0.0940
   8  16.344 BB   0.1218   77.54416   9.64533    0.5767
   9  20.235 MM   0.2125    8.81314 6.91092e-1   0.0655
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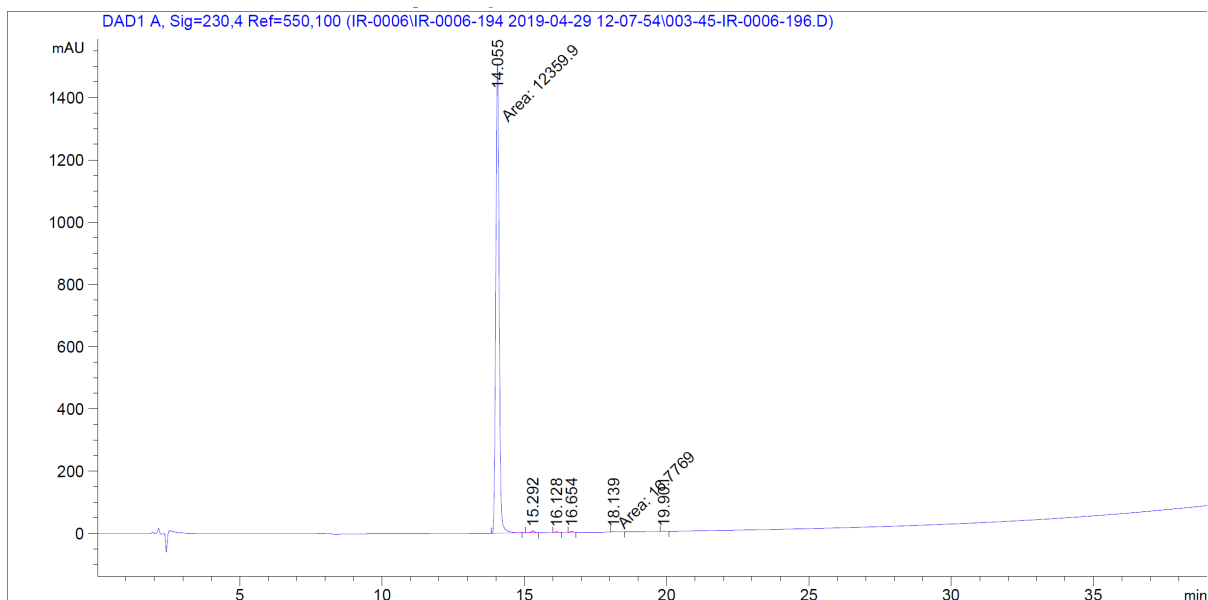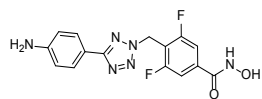

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Use Multiplier & Dilution Factor with ISTDs


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   4  16.654 BB   0.0960   25.01641    4.05269   0.2004
   5  18.139 MM   0.1979   16.77694    1.41315   0.1344
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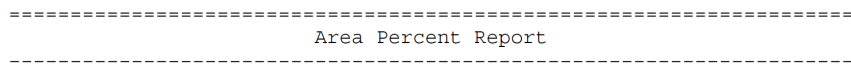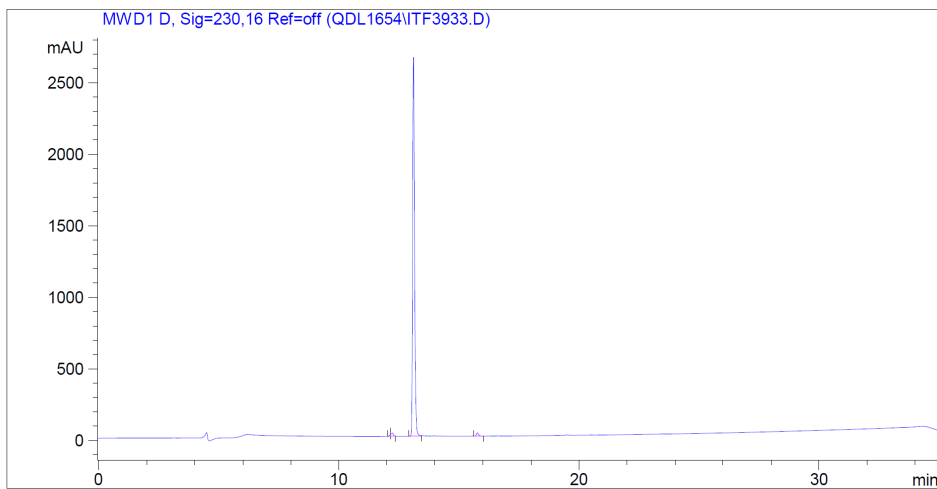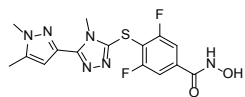
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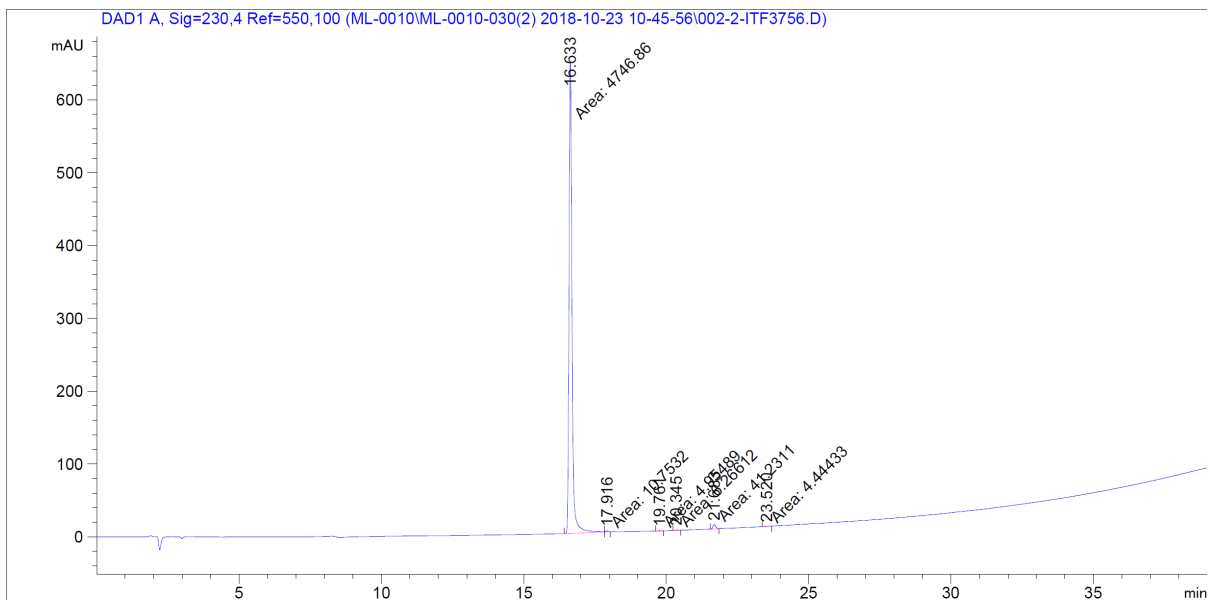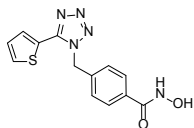Use Multiplier & Dilution Factor with ISTDs

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4	15.761	VB	147.1682	0.096	23.239	0.978

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Compound **42**


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Use Multiplier & Dilution Factor with ISTDs


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   2  17.916 FM    0.1213   10.75318    1.47803   0.2233
   3  19.761 MM    0.1569    4.95489 5.26211e-1   0.1029
   4  20.345 MM    0.1245    6.26612 8.39117e-1   0.1302
   5  21.683 MM    0.1185   41.23109    5.79919   0.8564
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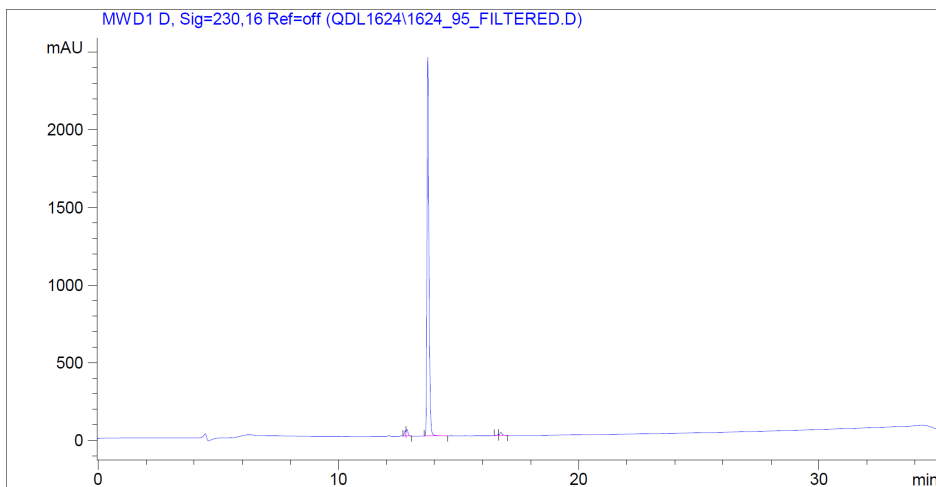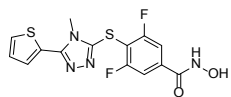
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Signal 1       :          MWD1 D, Sig=230,16 Ref=off

Peak  RetTime  Type     Area      Width     Height    Area %
 #     [min]          [mAU*s]     [s]       [mAU]
|---|---------|-----|---------|---------|---------|--------|
   1   12.765  VV    142.2237    0.062    34.635     0.996
   2   12.847  VB    243.4736    0.080    44.129     1.705
   3   13.713  VV    1.3720e4    0.086  2442.290    96.064
   4   16.632  BV     27.0258    0.071     5.910     0.189
   5   16.749  VB    149.4247    0.104    20.842     1.046

---- --------- ----- --------- --------- --------- ---------

 Totals :         14282.465            2547.806
=======================================================================
                    *** End of Report ***
```

Compound **44**


```
                          AREA PERCENT REPORT

Sorted By         :         Retention Time
Multiplier        :         1.000000
Dilution          :         1.000000
Use Multiplier & Dilution Factor with ISTDs

Signal 1          :         MWD1 A, Sig=220,8 Ref=off

Peak  RetTime  Type    Area      Width     Height    Area %
  #    [min]          [mAU*s]     [s]       [mAU]
|---|--------|-----|---------|---------|---------|--------|
   1   15.439  BV    2.0405e4    0.112   2847.222    98.966
   2   17.019  VB     77.4276    0.119      9.334     0.376
   3   19.905  BB    135.7432    0.112     18.491     0.658
---- --------- ----- --------- --------- --------- ---------

 Totals  :          20617.825             2875.048
==============================================================================
                     *** End of Report ***
```
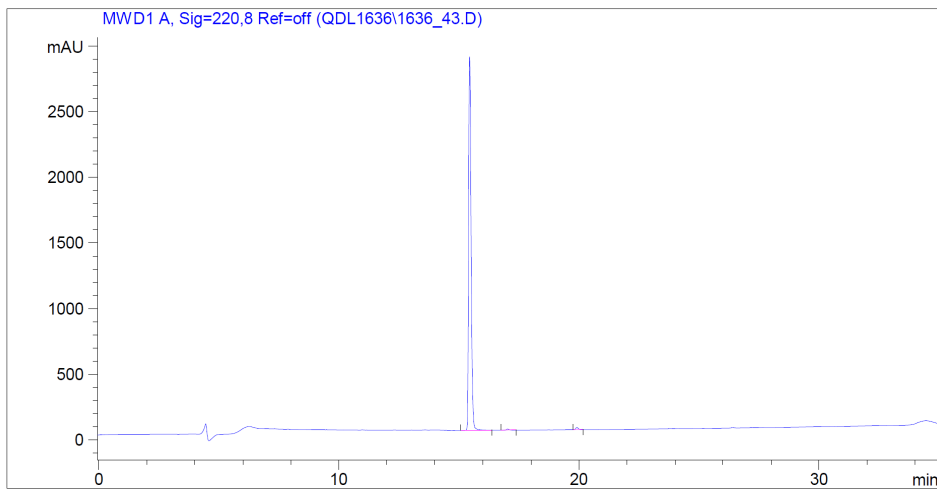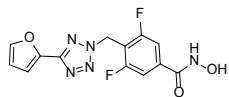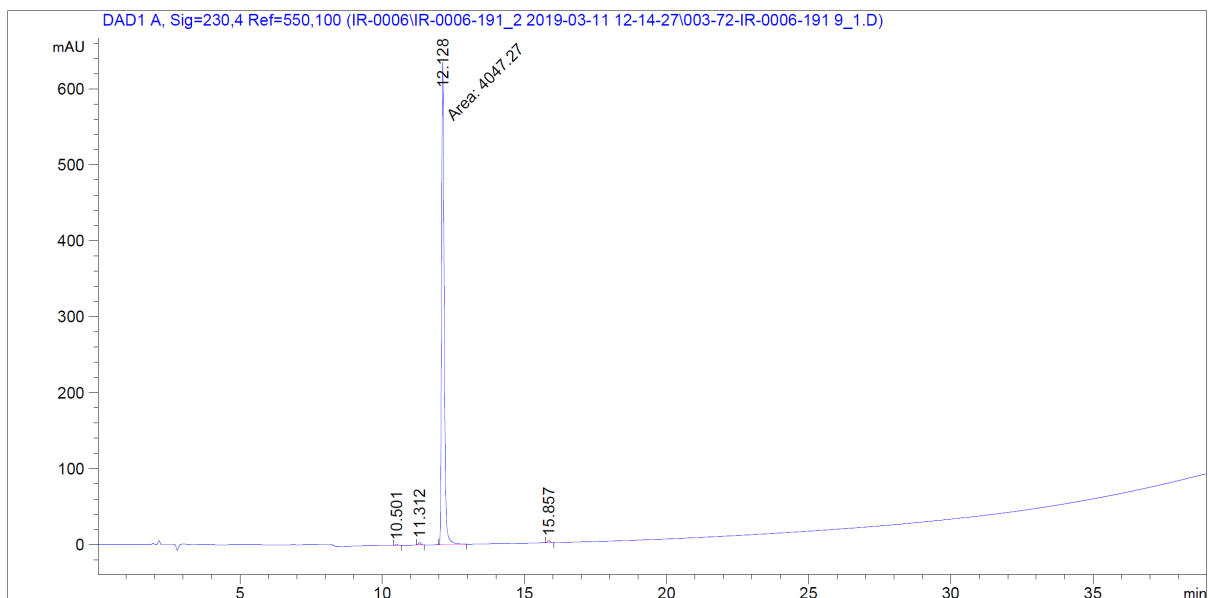
Compound **45**
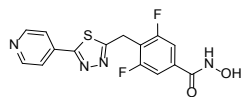

```
=====================================================================
                        Area Percent Report
=====================================================================

Sorted By            :        Signal
Multiplier           :        1.0000
Dilution             :        1.0000
Use Multiplier & Dilution Factor with ISTDs


Signal 1: DAD1 A, Sig=230,4 Ref=550,100

Peak RetTime Type  Width     Area        Height      Area
  #    [min]       [min]   [mAU*s]       [mAU]        %
----|-------|----|-------|----------|----------|--------|
   1  10.501 BB    0.0938   6.83267     1.11008   0.1669
   2  11.312 BB    0.0938  21.14806     3.43508   0.5167
   3  12.128 MM    0.1059 4047.27197  636.98608  98.8851
   4  15.857 BB    0.0956  17.65212     2.87751   0.4313

Totals :                  4092.90483  644.40875


=====================================================================
                        *** End of Report ***
```

Compound 46
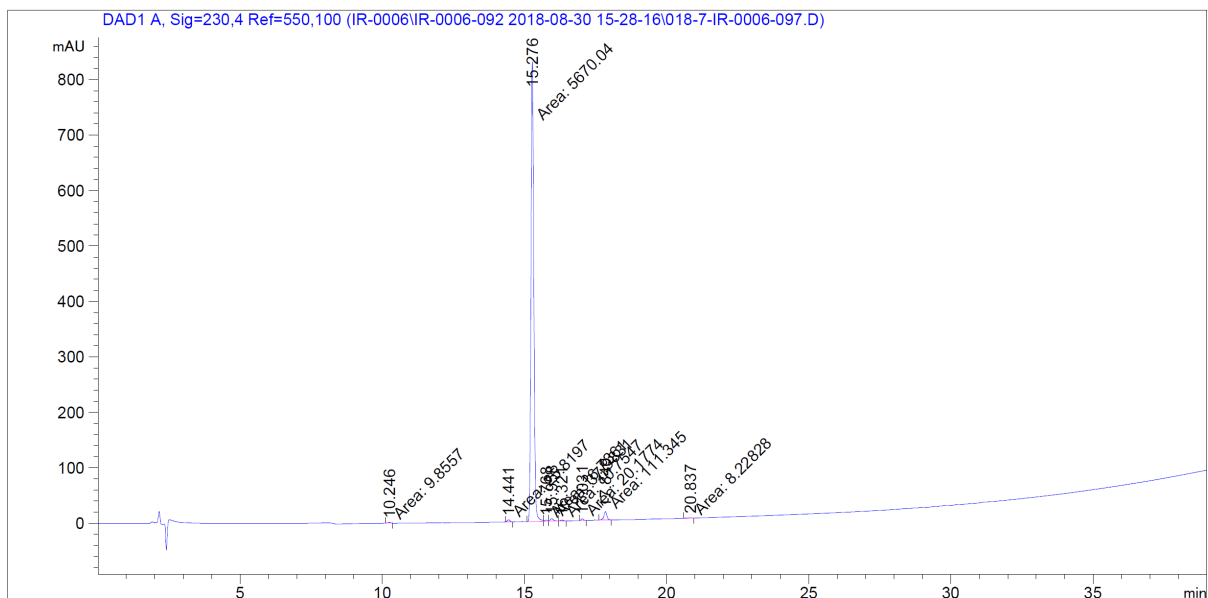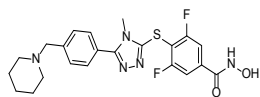
```
=====================================================================
                          Area Percent Report
=====================================================================

Sorted By          :      Signal
Multiplier         :      1.0000
Dilution           :      1.0000
Use Multiplier & Dilution Factor with ISTDs


Signal 1: DAD1 A, Sig=230,4 Ref=550,100

Peak RetTime Type  Width     Area       Height      Area
  #   [min]        [min]   [mAU*s]      [mAU]        %
----|-------|----|-------|----------|----------|--------|
   1  10.246 MM    0.1214    9.85570    1.35345    0.1667
   2  14.441 MM    0.1169   25.81969    3.68123    0.4368
   3  15.276 MF    0.1139 5670.03955  829.62567   95.9245
   4  15.768 FM    0.1392   16.73606    2.00368    0.2831
   5  15.958 FM    0.1398   37.98315    4.52803    0.6426
   6  16.321 FM    0.1110   10.75467    1.61549    0.1819
   7  17.031 MM    0.1052   20.17744    3.19765    0.3414
   8  17.849 MM    0.1235  111.34515   15.02161    1.8837
   9  20.837 MM    0.2144    8.22828 4.97986e-1    0.1392

Totals :                  5910.93969  861.52480
```
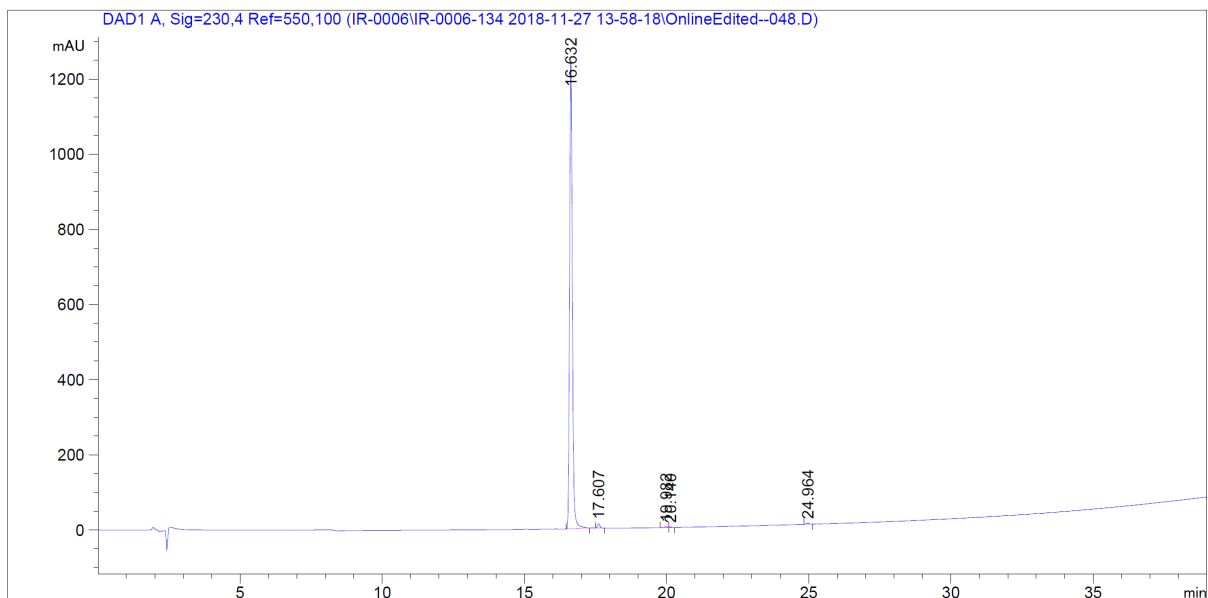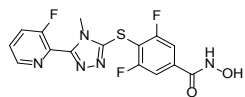
Compound **47**


```
===================================================================
                        Area Percent Report
===================================================================

Sorted By          :        Signal
Multiplier         :        1.0000
Dilution           :        1.0000
Use Multiplier & Dilution Factor with ISTDs


Signal 1: DAD1 A, Sig=230,4 Ref=550,100

Peak RetTime Type  Width     Area       Height     Area
  #   [min]        [min]   [mAU*s]      [mAU]        %
----|-------|----|-------|----------|----------|--------|
  1  16.632 BB    0.1059 8536.11914 1246.60913  98.5507
  2  17.607 BB    0.0962   73.10281   11.81493   0.8440
  3  19.982 BV    0.1227   19.25979    2.32263   0.2224
  4  20.140 VB    0.0995   10.28613    1.54806   0.1188
  5  24.964 BB    0.1070   22.88335    3.29540   0.2642

Totals :                 8661.65123 1265.59016
```
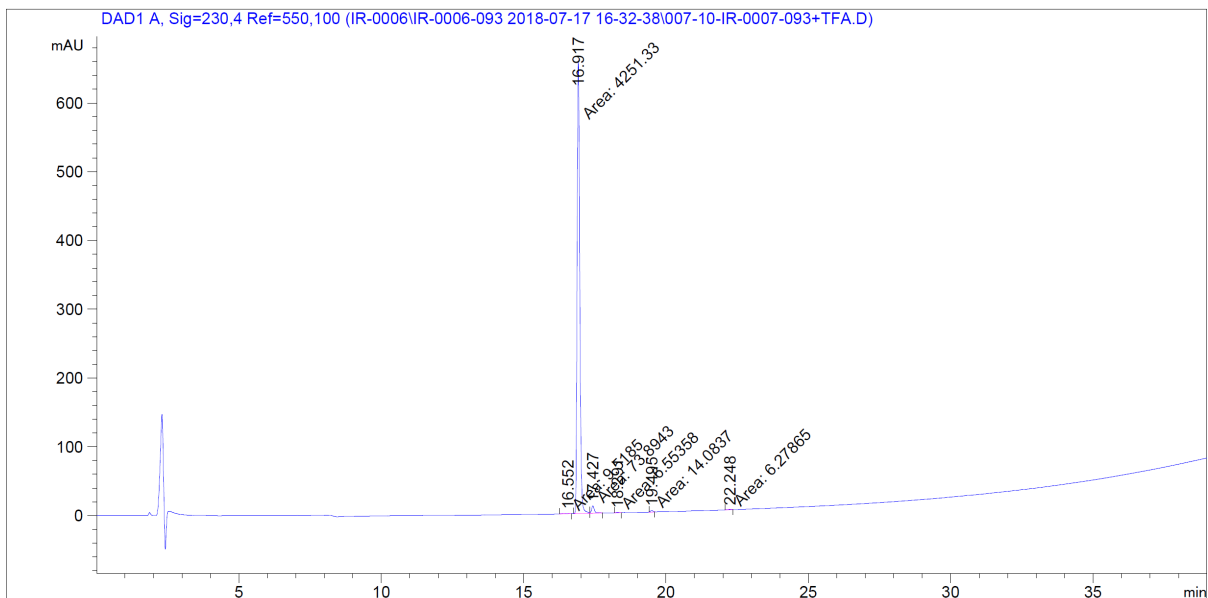
Compound **48**

```
=====================================================================
                         Area Percent Report
=====================================================================


Sorted By            :        Signal
Multiplier           :        1.0000
Dilution             :        1.0000
Use Multiplier & Dilution Factor with ISTDs


Signal 1: DAD1 A, Sig=230,4 Ref=550,100

Peak RetTime Type  Width     Area       Height      Area
  #   [min]        [min]   [mAU*s]       [mAU]        %
----|-------|----|-------|----------|----------|--------|
  1  16.552 MM    0.2451    9.51850 6.47343e-1   0.2182
  2  16.917 MF    0.1075 4251.33350  658.83563  97.4705
  3  17.427 FM    0.1160   73.89431   10.61377   1.6942
  4  18.291 MM    0.1224    6.55358 8.92673e-1   0.1503
  5  19.495 MM    0.1005   14.08372    2.33581   0.3229
  6  22.248 MM    0.1673    6.27865 6.25553e-1   0.1440

Totals :                 4361.66227  673.95077


=====================================================================
```
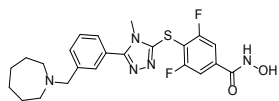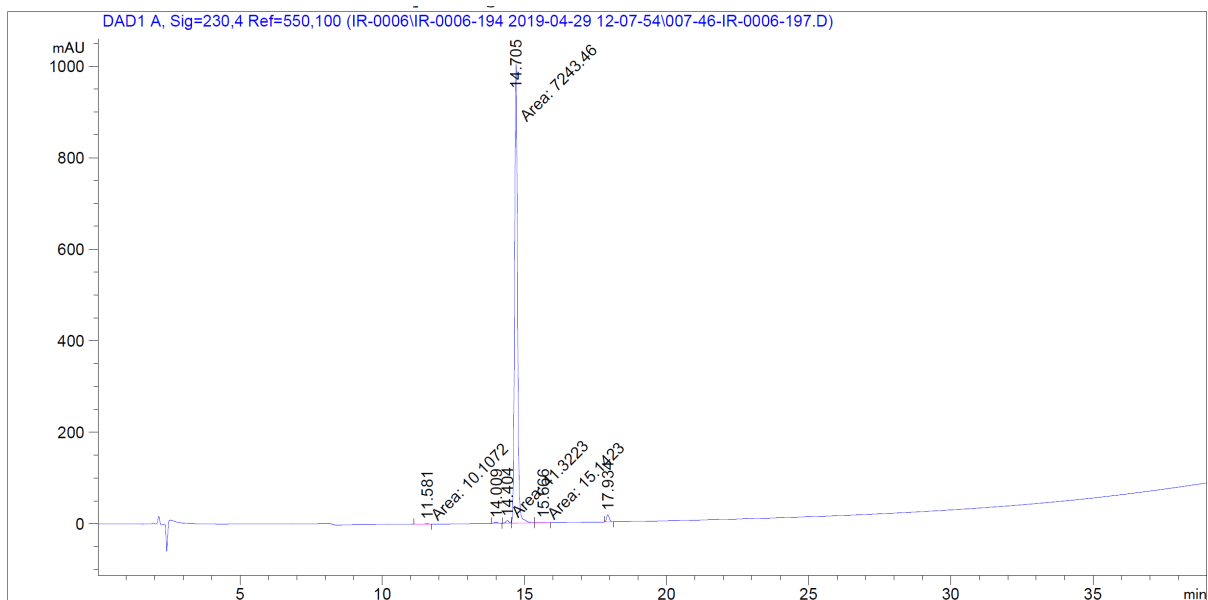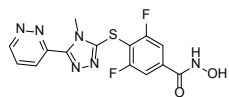
Compound **49**

DAD1 A, Sig=230,4 Ref=550,100 (IR-0006\IR-0006-194 2019-04-29 12-07-54\007-46-IR-0006-197.D)

```
=======================================================================
                         Area Percent Report
=======================================================================

Sorted By              :       Signal
Multiplier             :       1.0000
Dilution               :       1.0000
Use Multiplier & Dilution Factor with ISTDs


Signal 1: DAD1 A, Sig=230,4 Ref=550,100

Peak RetTime Type  Width     Area       Height     Area
  #   [min]        [min]    [mAU*s]      [mAU]       %
----|-------|----|-------|----------|----------|--------|
   1  11.581 MM   0.1920    10.10718 8.77479e-1   0.1360
   2  14.009 BV   0.1380    23.20093    2.76172   0.3121
   3  14.404 MF   0.1244    41.32228    5.53506   0.5558
   4  14.705 FM   0.1201 7243.45898 1005.46704  97.4330
   5  15.666 FM   0.3124    15.14232 8.07779e-1   0.2037
   6  17.934 BB   0.0991   101.06865   15.70725   1.3595

Totals :                  7434.30035 1031.15633


=======================================================================
```