Supplementary Information

Hit-to-lead optimization of benzoxazepinoindazoles as human African trypanosomiasis therapeutics

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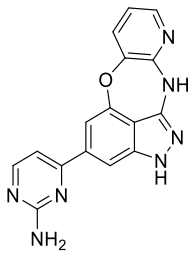
Supplemental Biological and ADME Data

Table S1. Human kinase activity of **NEU-1328**. Compounds with pIC_{50} >7.0 are highlighted in red.



NEU-1328

Kinase	pIC_{50}	Kinase	pIC_{50}
IKK1	7.9	ROCK1	6.9
SYK	7.2	AurB	7.5
p38a	1	JAK3	7.4
JNK1	6.4	JAK2	6.3
ITK	1	EGFR	1
LCK	5.5	LRRK2	7.9
BTK	1	PI3K-α	1
IKK2	6.2		

Table S2. Additional ADME data for compounds **28a**, **31a-b**, **34a-c**, **40**, **49**, **57a, 57c, 57g-j**, **63**, and **68**. *nd* = no data. *Compound too highly cleared for detection.

ID	LogD	Aq. sol. (μM)	PPB (%)	HLM Cl_{int} (μL/min/mg protein)	Rat Hepatocyte Cl_{int} (μL/min/10^6 cells)
28a	1.4	65	*nd*	<3	5.7
31a	0.1	58	*nd*	<3	16
31b	0.8	5	100	<3	46
31c	1.0	<13	100	<3	230
34a	1.4	660	79	19	11
34b	1.8	330	81	28	3.4
34c	1.8	220	83	38	8.5
40	3	0.3	93	63	20
49	2.8	13	88	*nd**	52
57a	1.7	860	89	25	20
57c	2.3	68	76	3	4.9
57g	1.2	750	33	4.5	*nd*
57h	1.3	770	39	3	*nd*
57i	1.4	1000	51	16	*nd*
57j	0.8	380	*nd*	17	9.1
63	3.4	3	100	16	8
68	*nd*	17	96	6.8	20

Table S3. Percent inhibition of individual kinases at 1 μM **18** or **57a**. Kinases that are moderately inhibited (30-50%) are highlighted in yellow; kinases that are potently inhibited (>50%) are highlighted in red.

Kinase	% Inhibition		Kinase	% Inhibition		Kinase	% Inhibition	
	18	**57a**		**18**	**57a**		**18**	**57a**
IRAK4	37	84	HGK (MAP4K4)	93	93	MNK2	11	95
IKKα	13	98	PDK1	22	91	PKCβ2	0.46	37
MAPKAPK2	15	45	FGFR2	7.0	49	EphA3	-20	-4.0
NEK2	8.3	4.1	CDK2 (cycA)	15	84	PAK4	-14	4.0

TAOK2 (TAO1)	-0.32	28	PAK2	7.1	56	Syk	99	27
IRK (InsR)	-26	3.2	SIK	15	97	JNK1	21	17
CDC2/CDK1 (cycB)	16	92	AurA/Aur2	-5.8	42	KDR kinase (VEGFR2)	14	96
CHK1	19	39	FGFR3 kinase	-12	46	ERK2 (P42mapk)	-12	6.3
JAK3	75	100	CHK2	-0.51	91	FGFR1 kinase (h)	-1.0	32
Akt1/PKBα	5.5	45	TRKA	3.3	4.5	Src	1.2	3.7
EphB4	5.7	14	SGK1	-3.5	43	GSK3β	17	59
Pim2	-13	52	EGFR	7.6	51	c-Met	0.42	-2.2
CaMK2α	-7.7	12	Lck	7.1	0.34	p38α	-15	5.6
PKA	39	93	EphA2	9.7	4.8	Abl	3.9	13
RAF-1	5.7	86	PLK1	-15	-2.0	MARK1 (h)	-4.0	86
ROCK	-10	81						

Table S4. *T. cruzi* and *L. donovani* activity of selected analogs. L6 is the host cell line for *T. cruzi* assays; THP-1 is the host cell line for *L. donovani*.

ID	*T. cruzi* pEC_{50}	L6 pTC_{50}	*L. donovani* pEC_{50}	THP-1 pTC_{50}
NEU-1117	7.7	<4.3	<5.7	4.9
9a	<5.0	*nd*	<5.7	4.7
9b	<5.0	*nd*	<5.0	<4.3
9c	5.4	6.0	<5.0	5.3
9d	4.9	4.5	<5.7	4.3
9e	5.7	*nd*	<5.7	4.7
9f	5.0	4.5	<6.3	4.8
9g	<5.0	*nd*	<5.7	4.3
9j	6.0	5.7	<5.3	4.7
9k	5.9	5.5	<6.0	4.9
9l	5.3	<4.3	<5.3	<4.3
9o	<4.7	5.6	5.4	4.6
9p	5.3	4.6	<5.3	<4.3
9q	<4.7	5.0	<5.7	4.8
9r	<4.7	<4.3	<5.3	<4.3
9s	5.1	4.4	*nd*	5.6
15	<4.7	4.6	<5.3	<4.3
18	7.4	*nd*	<5.3	<4.3
19	<4.7	4.3	<5.3	<4.3
20	6.1	5.2	<5.3	5.3
28a	<4.7	*nd*	<5.3	<4.3
28b	<4.7	*nd*	<5.3	<4.3
31a	<4.7	<4.3	<5.3	<4.3
31b	<4.7	<4.3	<5.3	<4.3
31c	<4.7	4.4	<5.3	<4.3
34a	<4.7	4.8	<5.3	4.4
34b	<5.0	*nd*	5.5	<4.3
34c	<4.7	<4.3	<5.3	4.6
40	<4.7	4.3	<5.3	<4.3
49	5.9	5.4	<6.3	<5.3
51	*nd*	4.9	<5.3	4.9

57a	5.4	5.0	<5.3	5.2
57c	*nd*	4.8	<5.3	5.4
57d	*nd*	5.3	<5.3	5.3
57g	<4.7	4.5	<5.3	4.5
57h	<4.7	4.6	<5.3	*nd*
57i	<4.7	4.7	<5.3	*nd*
57j	<4.7	4.6	*nd*	4.8
63	<4.7	*nd*	<5.3	<4.3
68	<4.7	*nd*	<5.3	<4.3

Table S5. Adult *Schistosoma mansoni* activity of selected analogs.

ID	Adult severity score (10 µM)			
	3h	**6h**	**24h**	**48h**
9c	2	2	1	0
9d	2	2	3	3
31c	0	0	0	0
63	2	3	3	3

Table S6. Parasitemia levels of mice treated with vehicle (20% captisol, 5% DMSO) and **18** (30 mg/kg/day, ip). N.D. = Not detected (<5.56E+04 p/ml blood). †Dead.

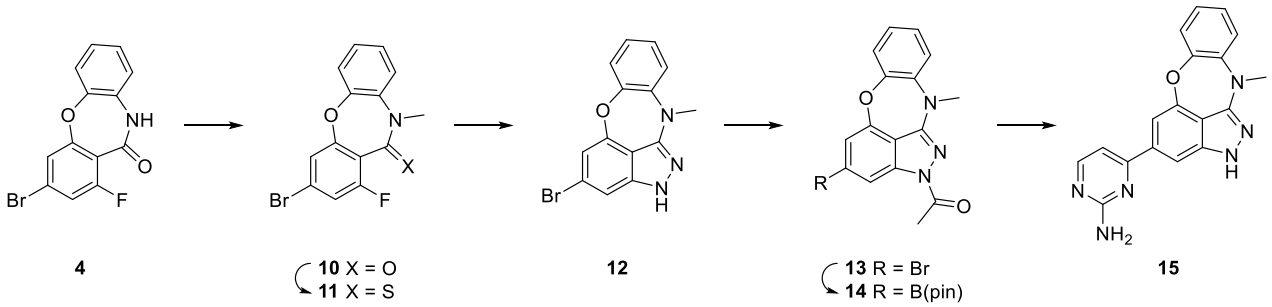
	Infection	Treatment					Post Treatment					
Control	**Day 0**	**Day 3**	**Day 4**	**Day 5**	**Day 6**	**Day 7**	**Day 10-17**	**Day 20**	**Day 24**	**Day 27**	**Day 28**	**Day 31**
1	1.00E+04	7.00E+06	1.35E+08	151E+09	†							
2	1.00E+04	1.05E+07	1.60E+08	1.8E+09	†							
3	1.00E+04	1.05E+07	1.85E+08	1.67E+09	†							
4	1.00E+04	1.10E+07	1.30E+08	8.10E+08	†							
Treated												
1	1.00E+04	1.00E+06	N.D.	N.D.	N.D.	N.D.	N.D.	3.33E+05	1.19E+09	1.13E+09		
2	1.00E+04	8.00E+06	5.56E+04	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
3	1.00E+04	8.00E+06	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
4	1.00E+04	1.05E+07	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
5	1.00E+04	3.00E+06	N.D.	N.D.	N.D.	N.D.	N.D.	3.70E+07	†			

Table S7. Parasitemia levels of control (vehicle -20% captisol, 10% DMSO- and Berenil), Treatment 1, and Treatment 2 mice in CNS efficacy study. †Animal was euthanized.

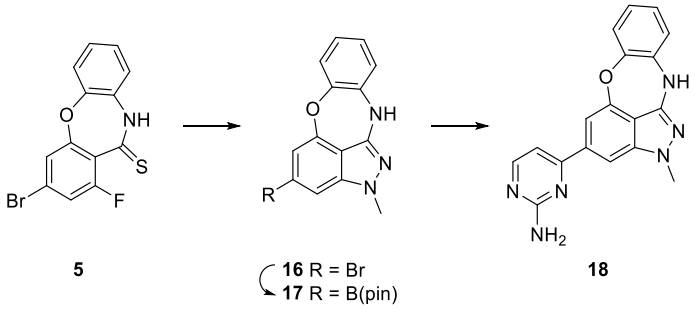
	Infection	Checking	Treatment		Post Treatment					
Control	**Day 0**	**Day 14**	**Day 21**	**Day 24**	**Day 28**	**Day 31**	**Day 35**	**Day 38**	**Day 42**	**Day 45**
1	2.00E+04	1.35E+07			N.D.	N.D.	N.D.	2.50+06 †		
2	2.00E+04	1.22E+08			N.D.	N.D.	2.80E+07 †			
3	2.00E+04	1.20E+07			N.D.	N.D.	N.D.	N.D.	N.D.	7.60E+07 †
4	2.00E+04	1.55E+07			N.D.	N.D.	N.D.	N.D.	6.05E+07 †	
5	2.00E+04	2.20E+07			N.D.	N.D.	N.D.	N.D.	6.60E+07 †	
Treatment 1										
1	2.00E+04	7.60E+07	N.D.		N.D.	N.D.	N.D.	2.00E+06 †		
2	2.00E+04	2.75E+07	N.D.		N.D.	N.D.	N.D.	N.D.	5.56E+04 †	
3	2.00E+04	1.70E+07	N.D.		N.D.	N.D.	N.D.	N.D.	2.80E+07 †	
4	2.00E+04	1.13E+08	N.D.		N.D.	N.D.	N.D.	1.00E+06 †		
Treatment 2										
1	2.00E+04	4.65E+07			N.D.	150E+06 †				
2	2.00E+04	2.55E+07			5.56E+04 †					
3	2.00E+04	8.40E+07			3.50E+06 †					
4	2.00E+04	3.07E+08			1.50E+06 †					

Supplemental Synthetic Schemes

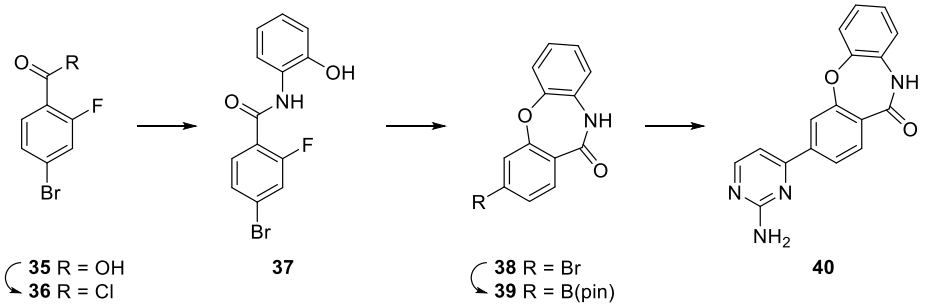
Scheme S1. Synthesis of **15**.



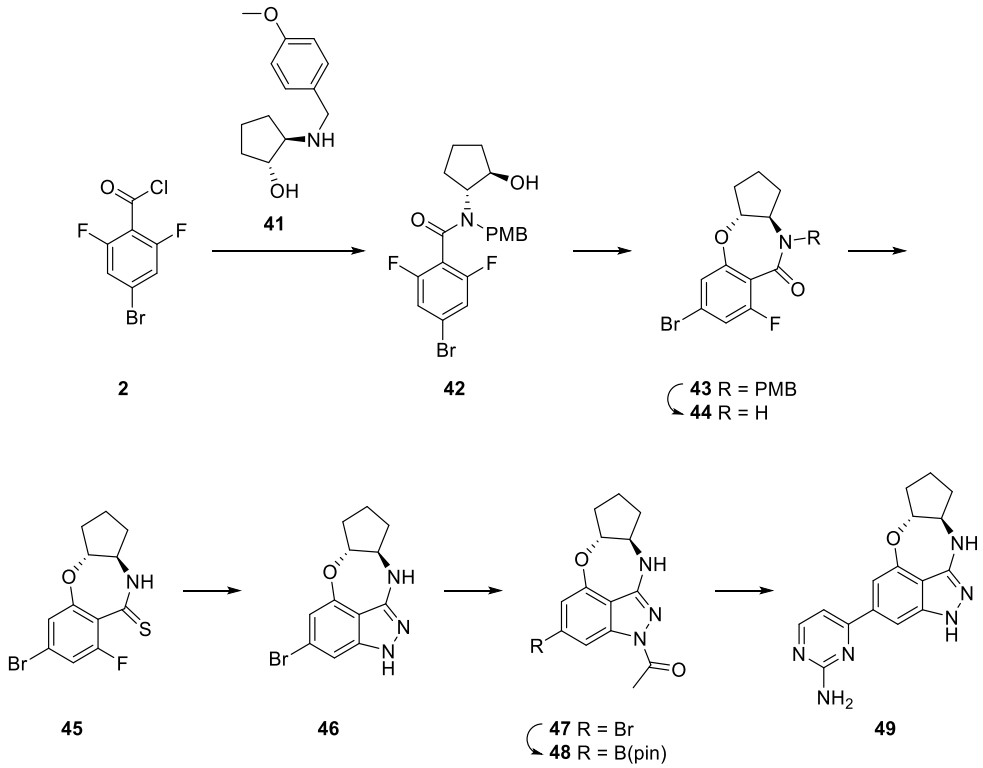
Scheme S2. Synthesis of **18**.



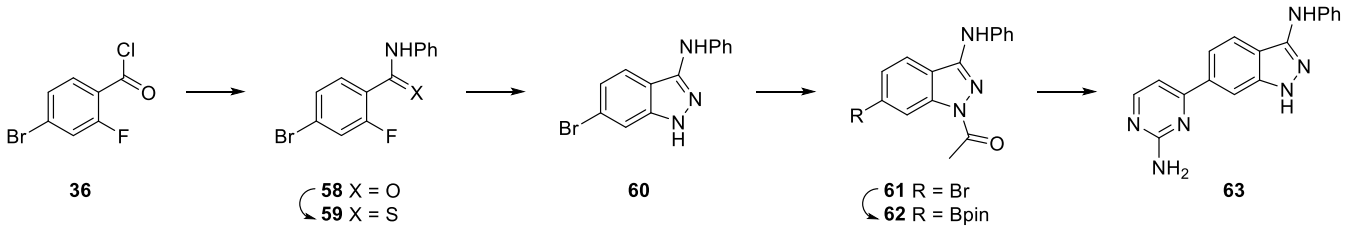
Scheme S3. Synthesis of **40**.



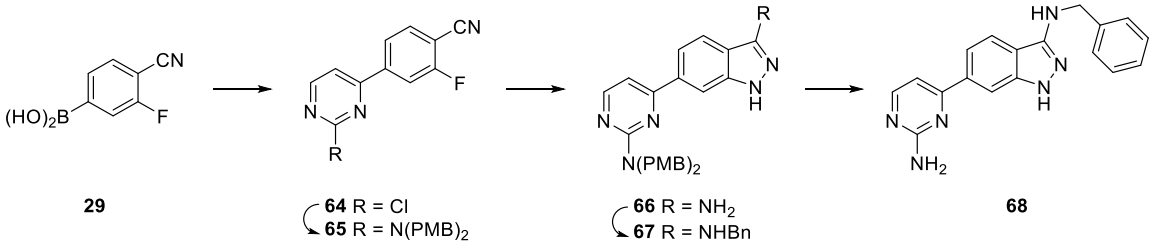
Scheme S4. Synthesis of **49**.



Scheme S5. Synthesis of **63**.



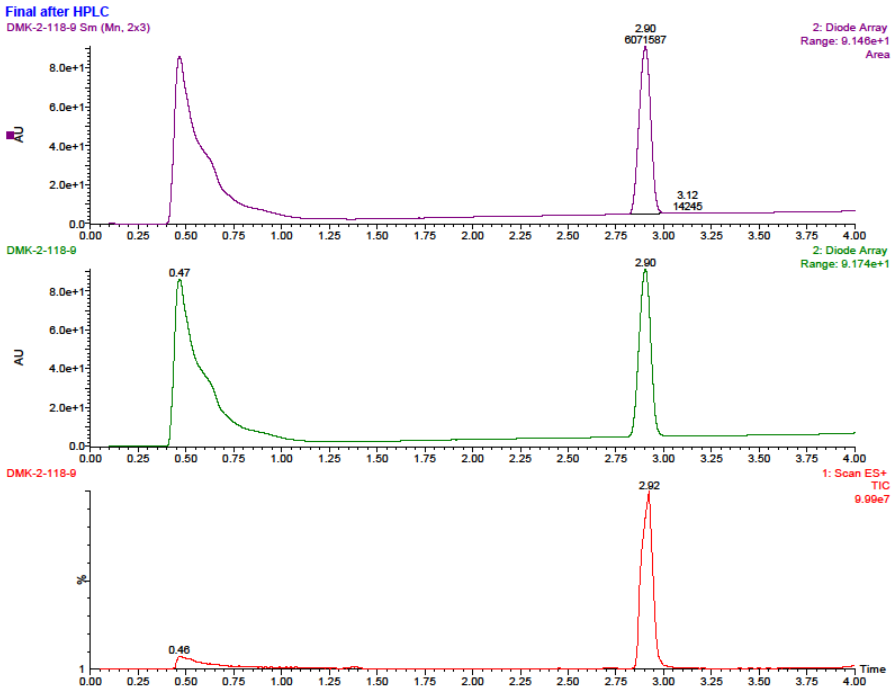
Scheme S6. Synthesis of **68**.



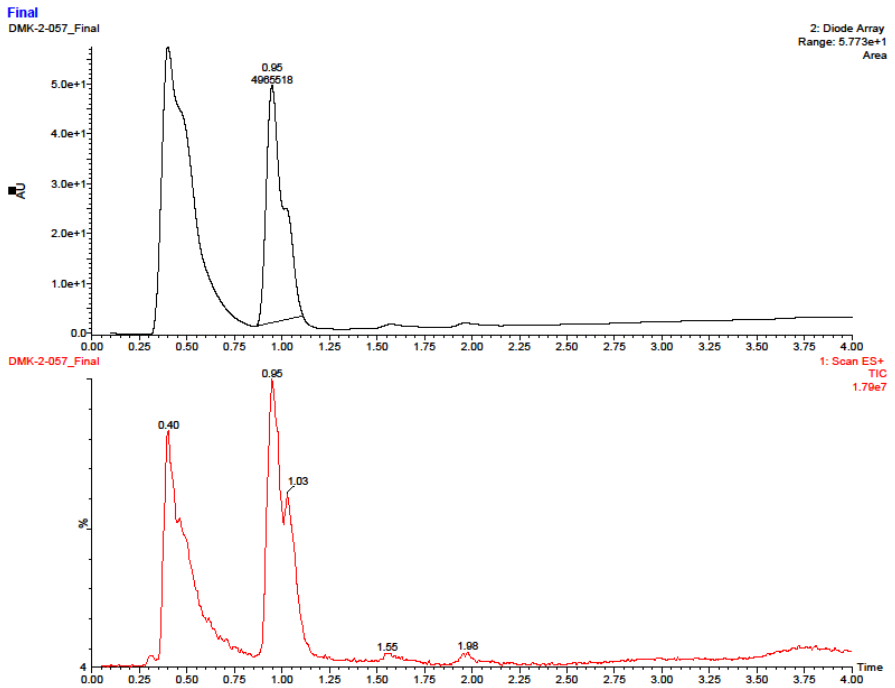
HPLC Chromatograms

LCMS chromatograms from all compounds synthesized by the authors are included. Compounds provided from GSK stock are not characterized. MS traces are included for traces that show peak splitting.

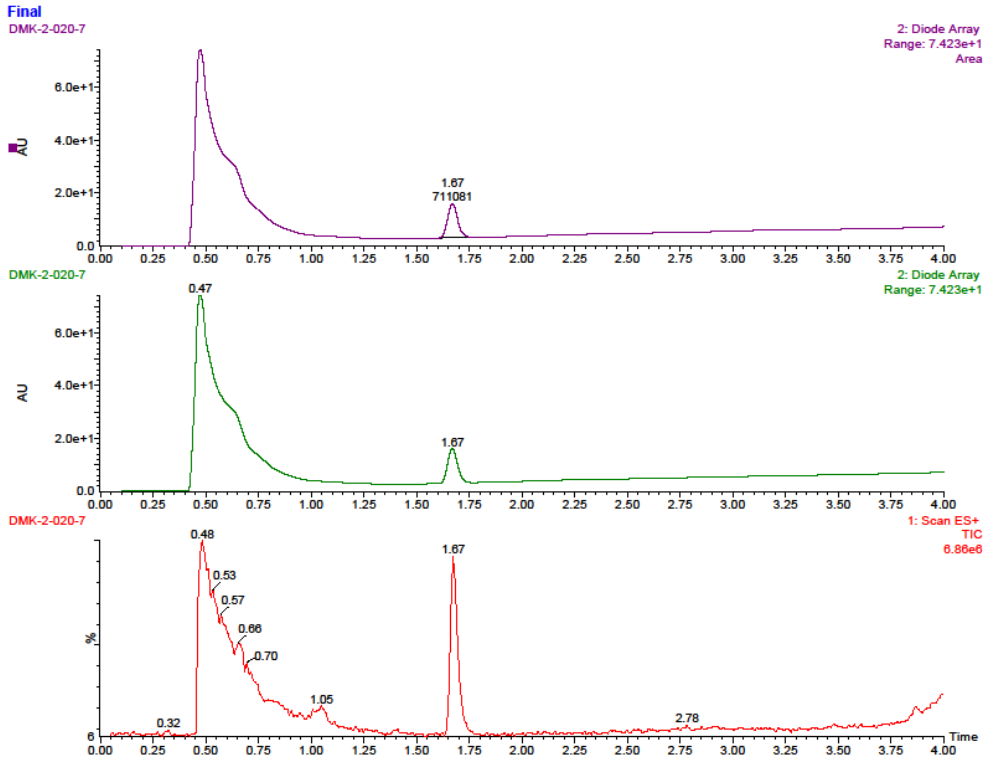
Compound 9a (NEU-1336)



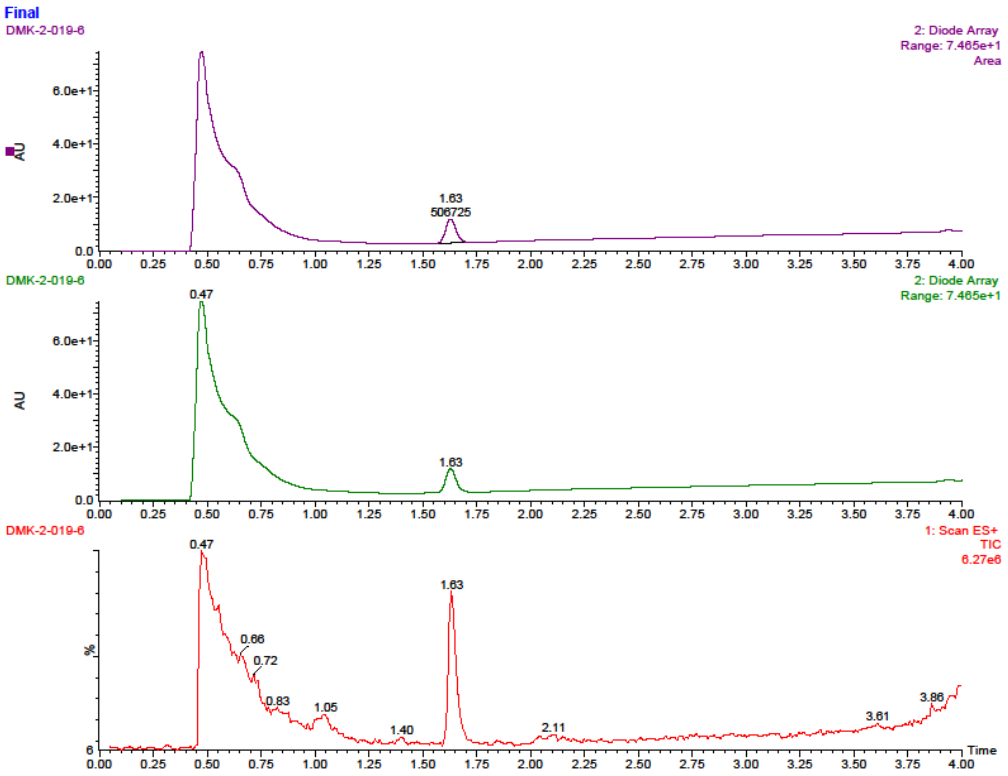
Compound 9b (NEU-2103)



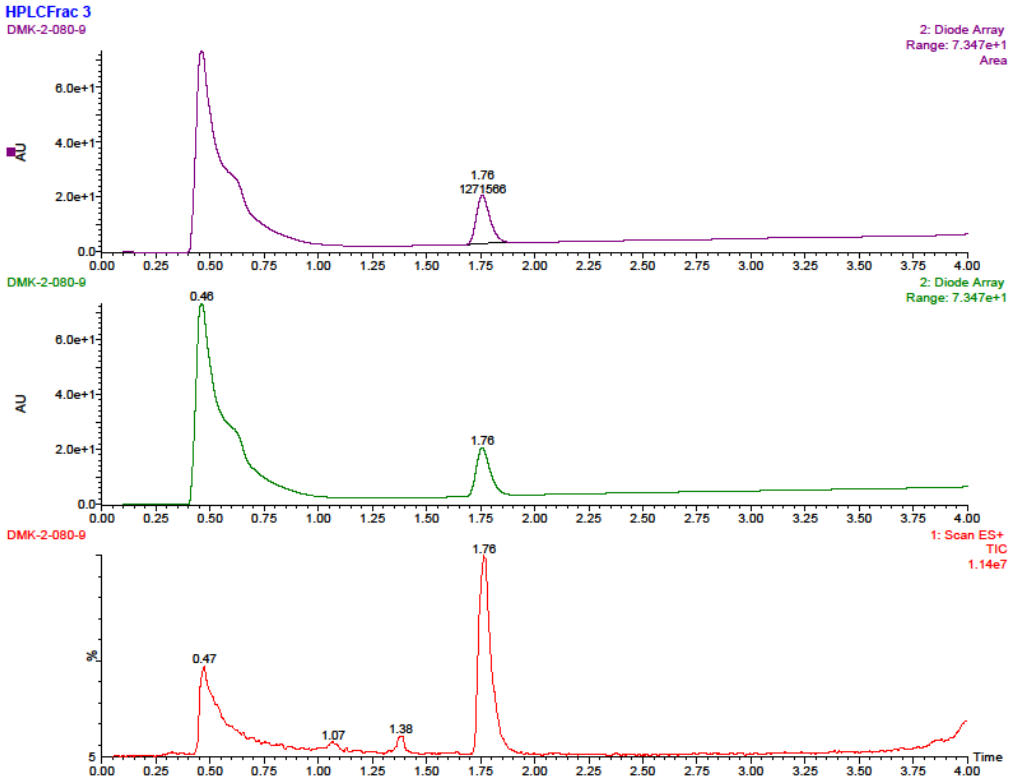
Compound 9c (NEU-1332)



Compound 9d (NEU-2101)



Compound 9e (NEU-2122)



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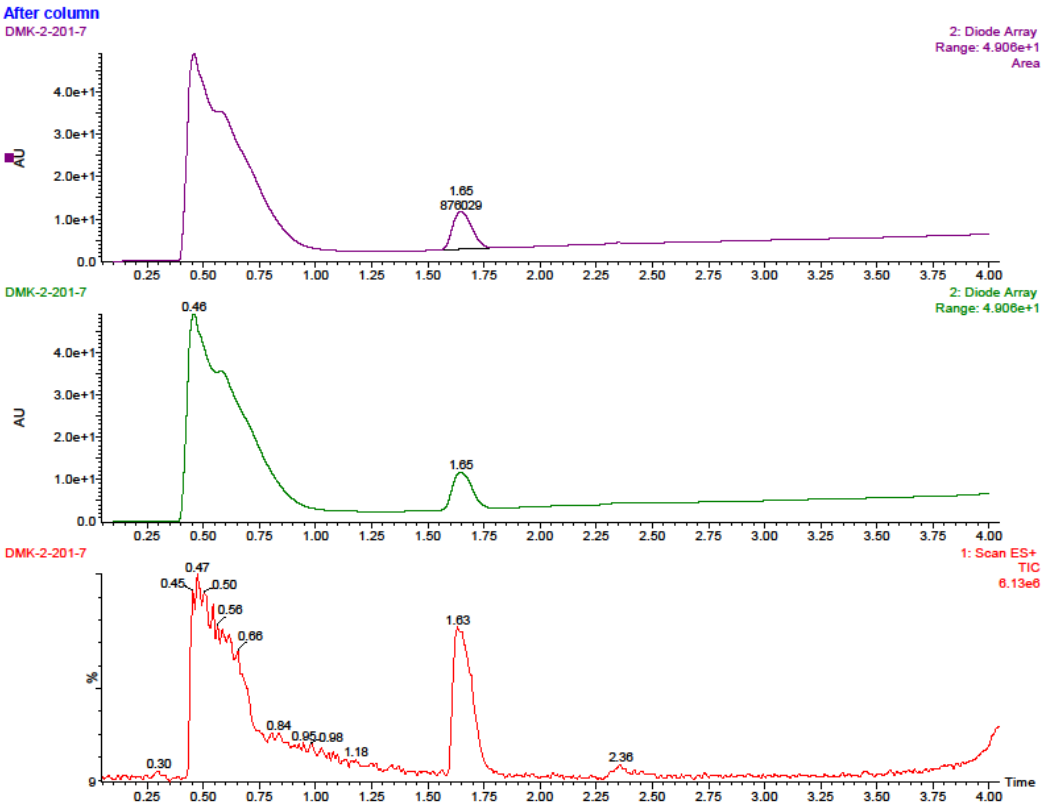
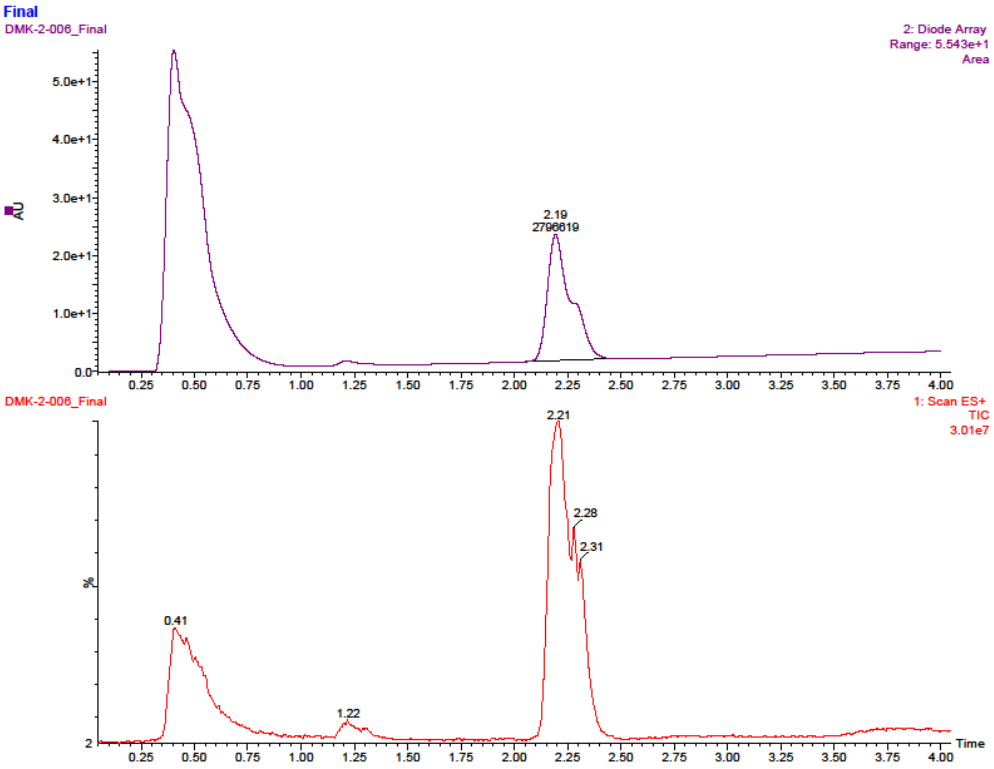
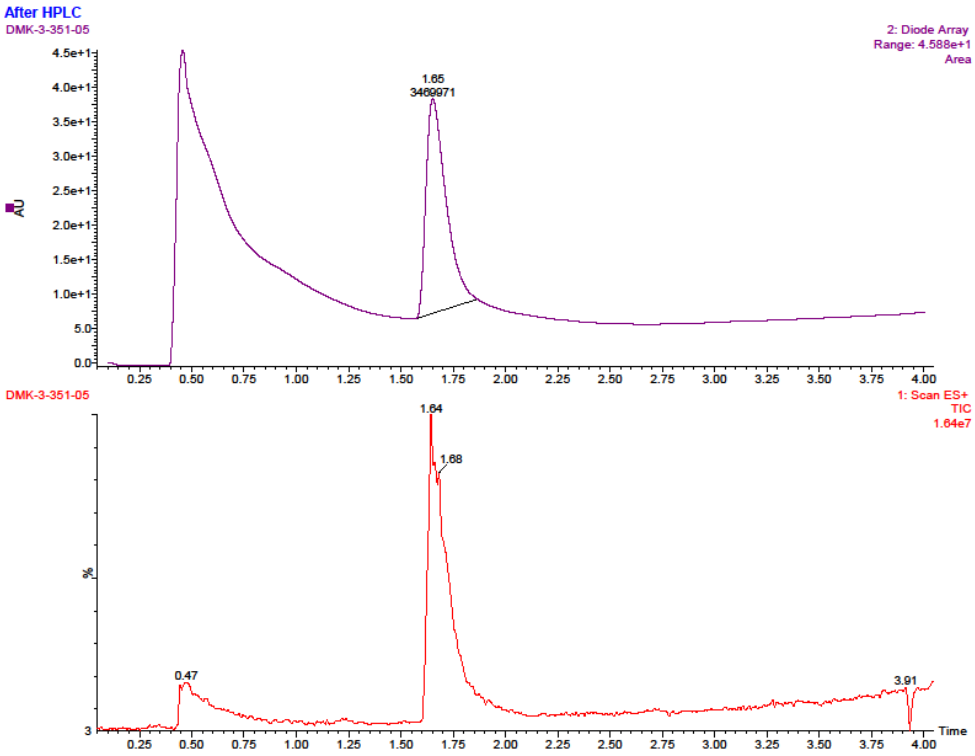


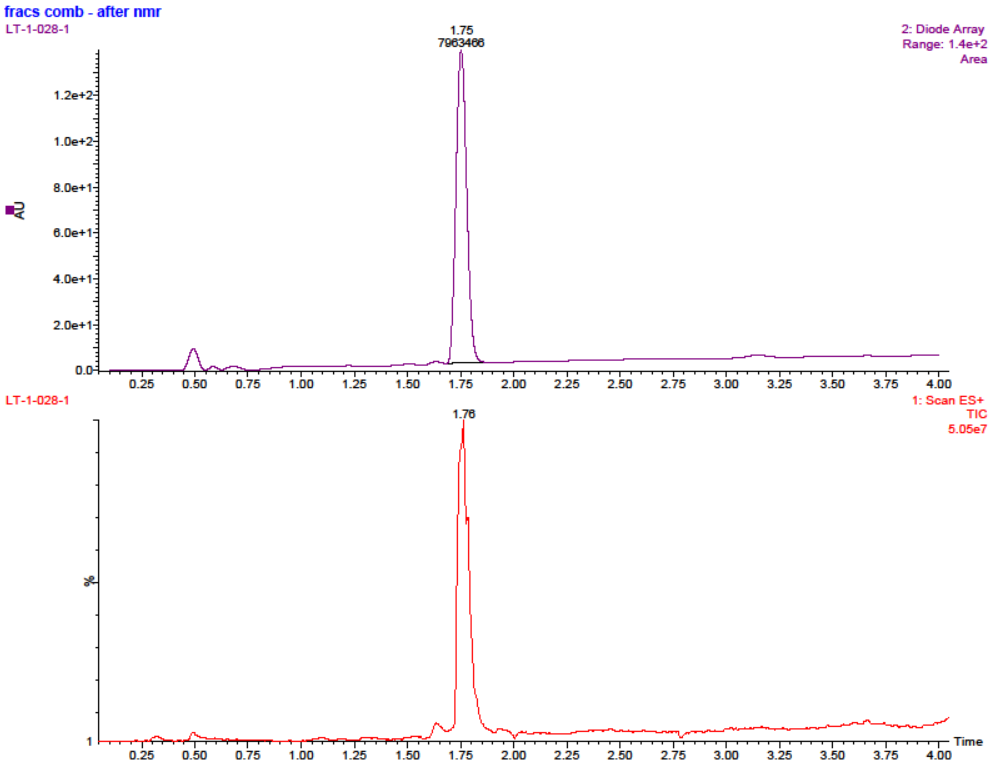
Compound 9g (NEU-2100)



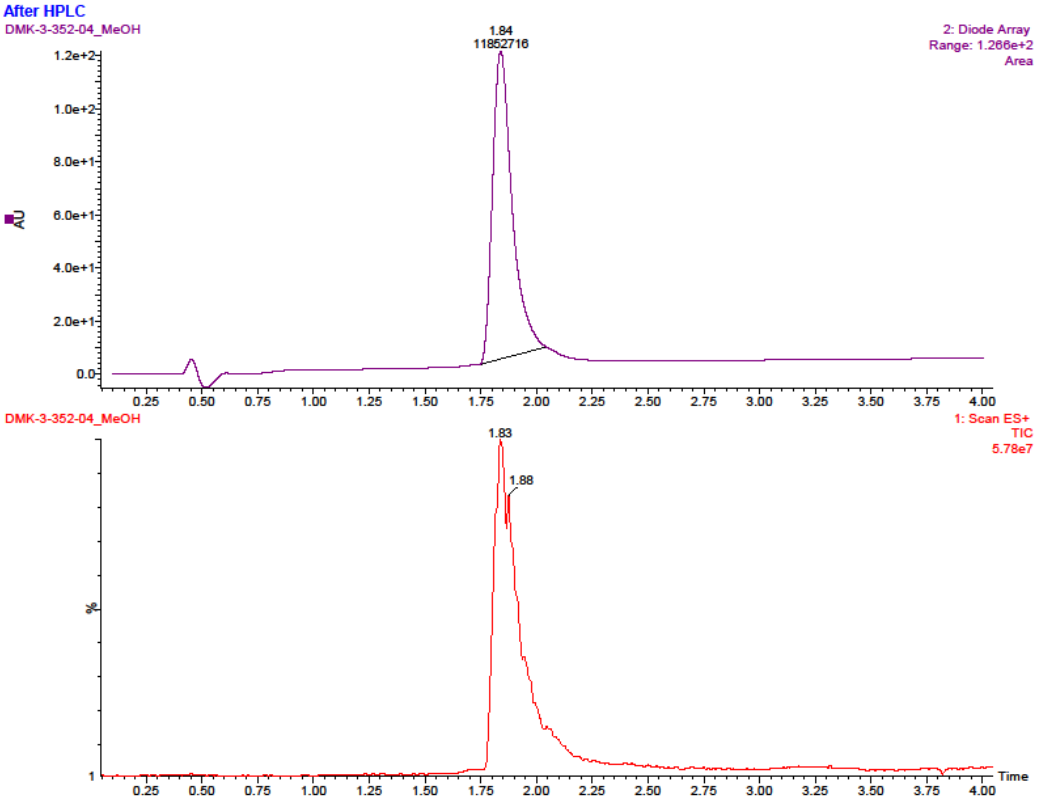
Compound 9j (NEU-1330)



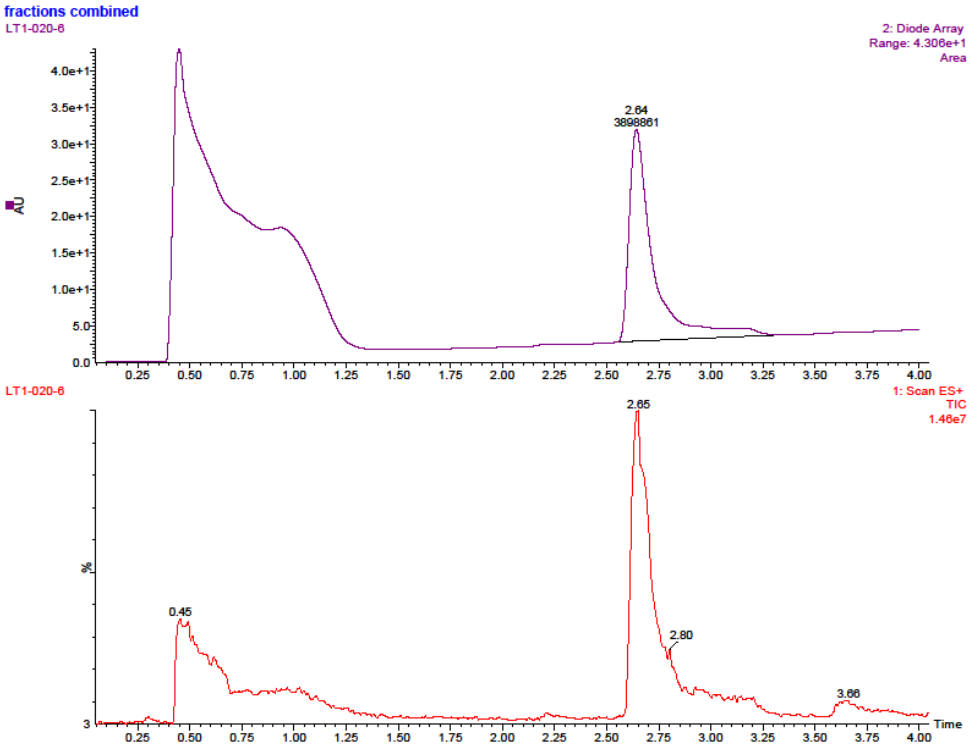
Compound 9k (NEU-4898)



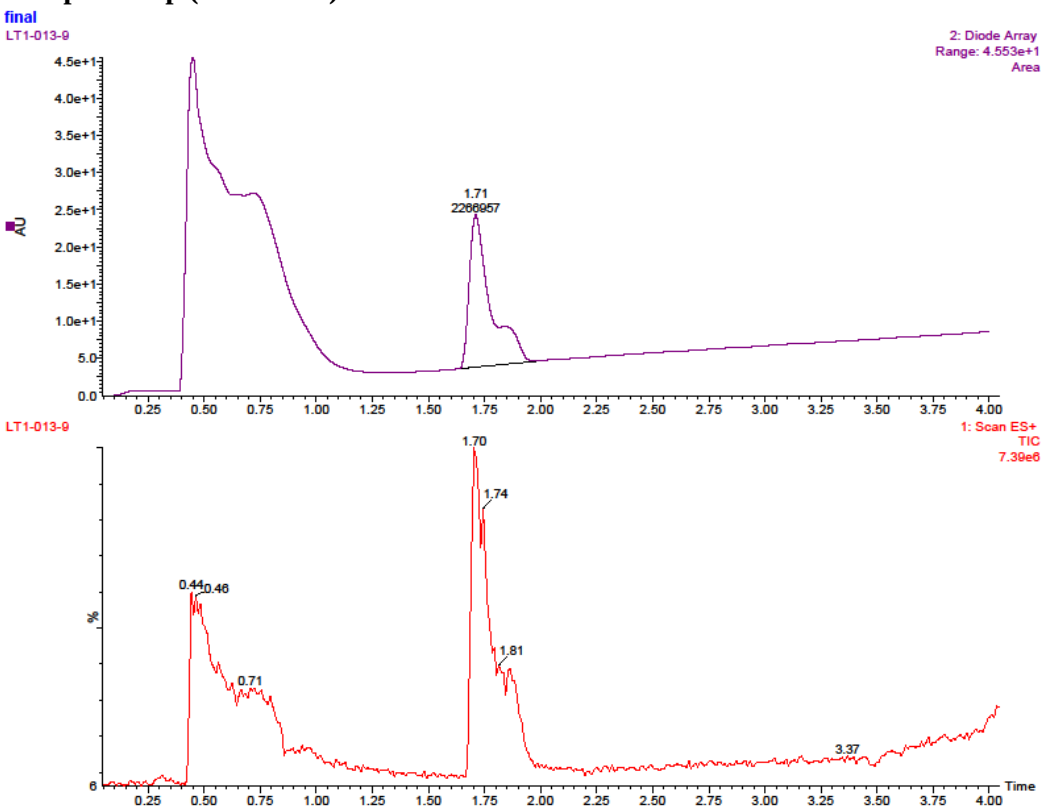
Compound 9l (NEU-4457)

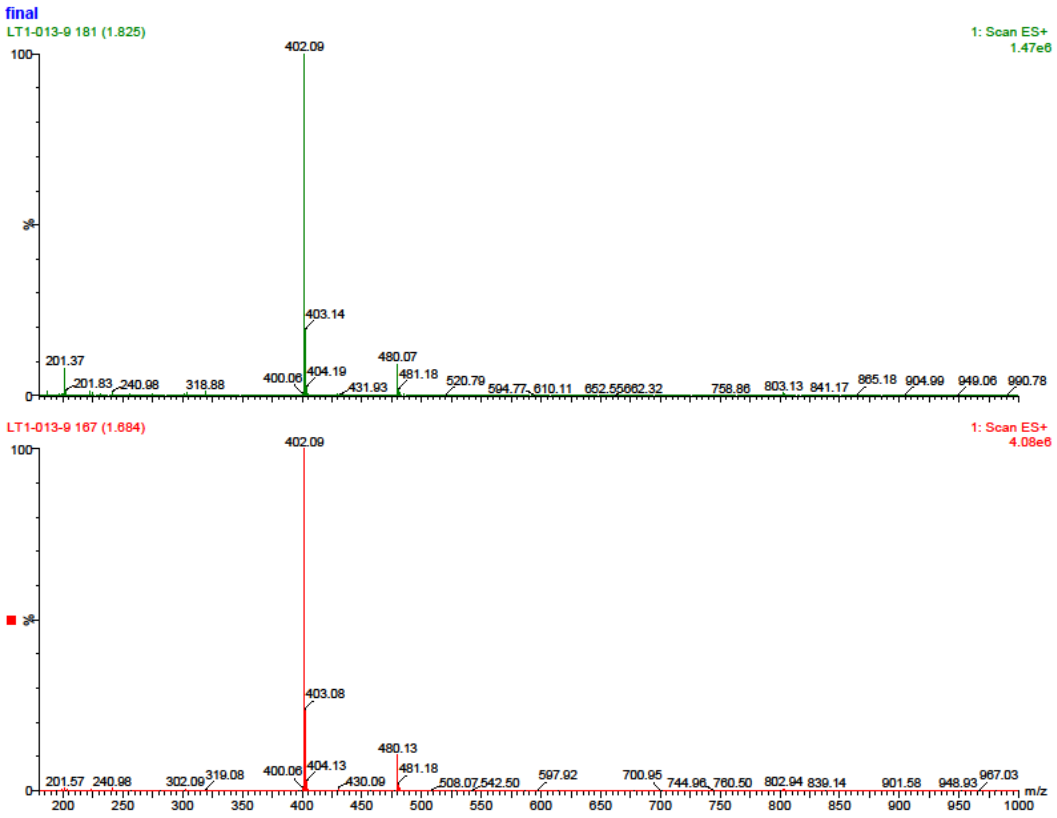


Compound 9o (NEU-4379)

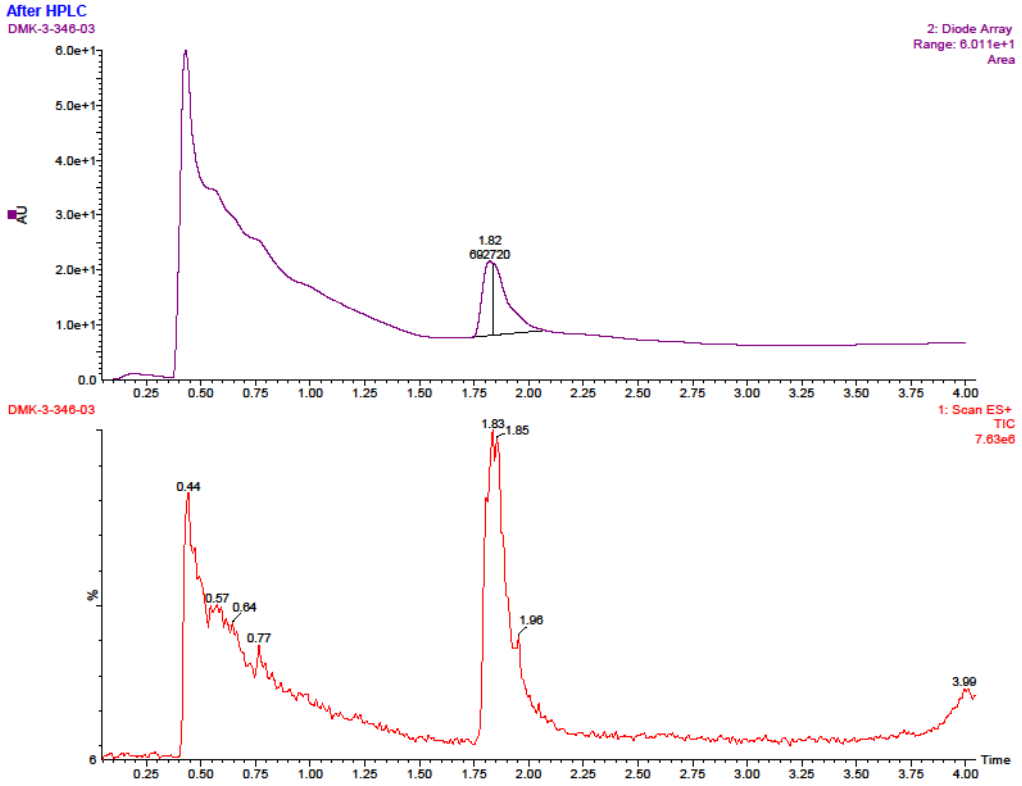


Compound 9p (NEU-4380)

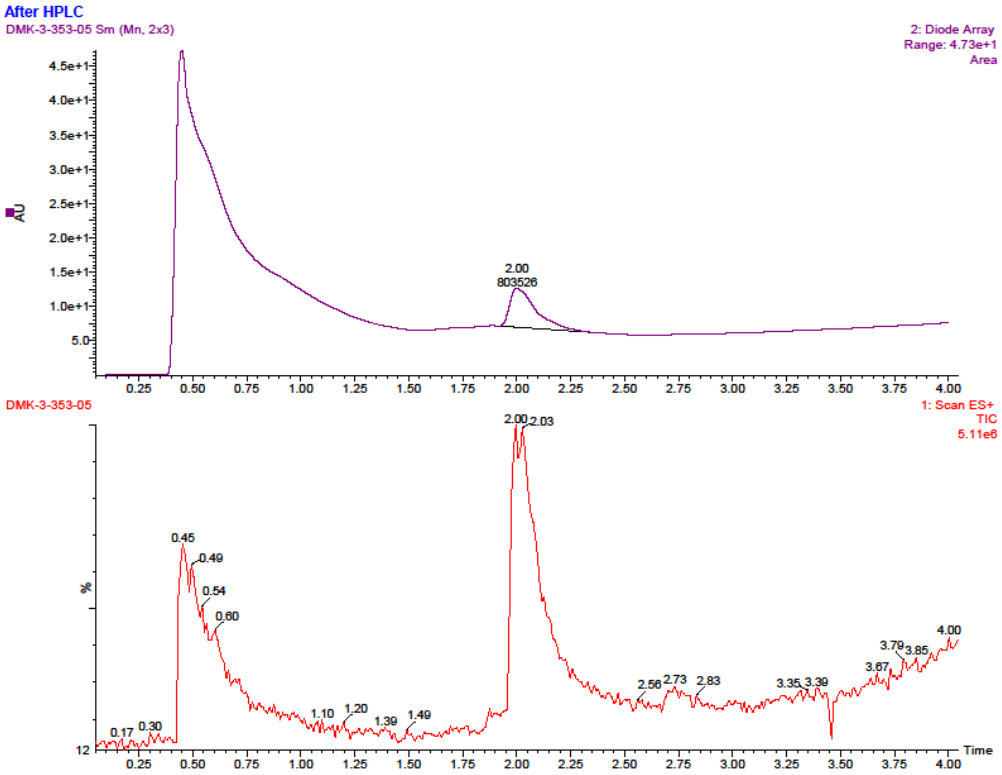




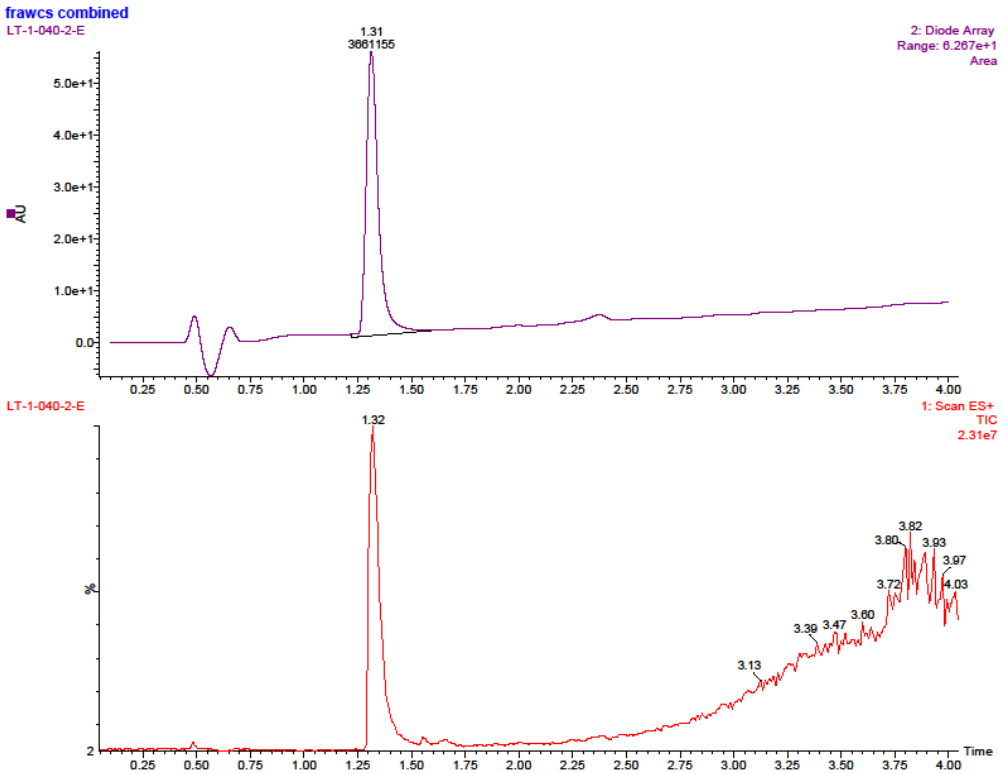
Compound 9q (NEU-4392)



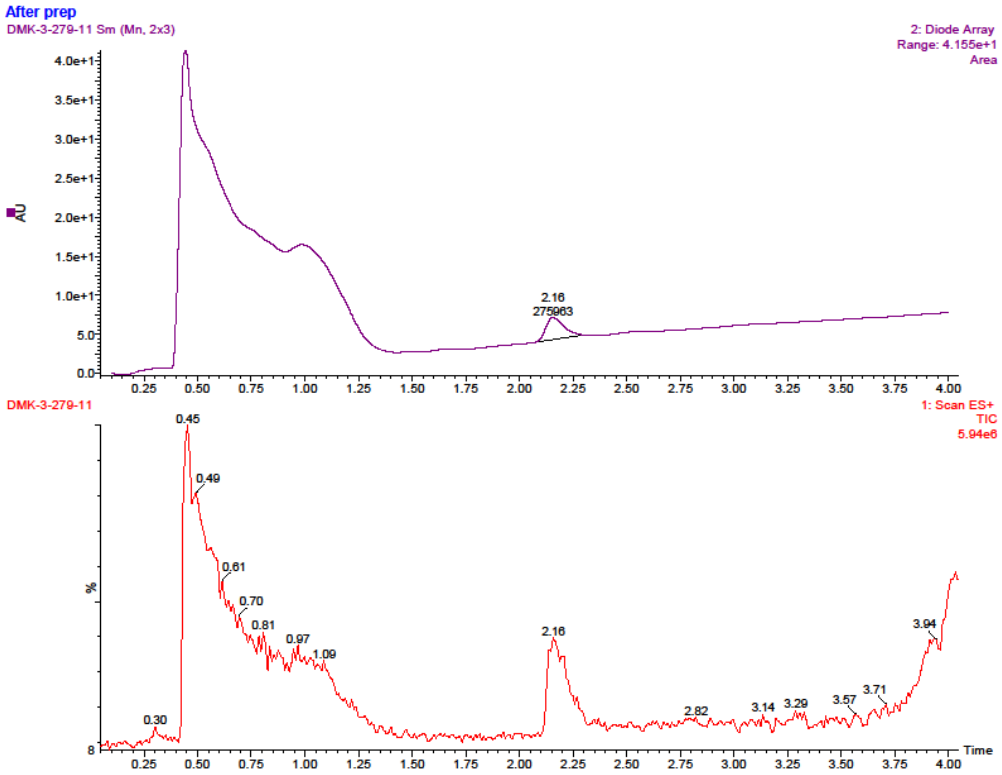
Compound 9r (NEU-4458)



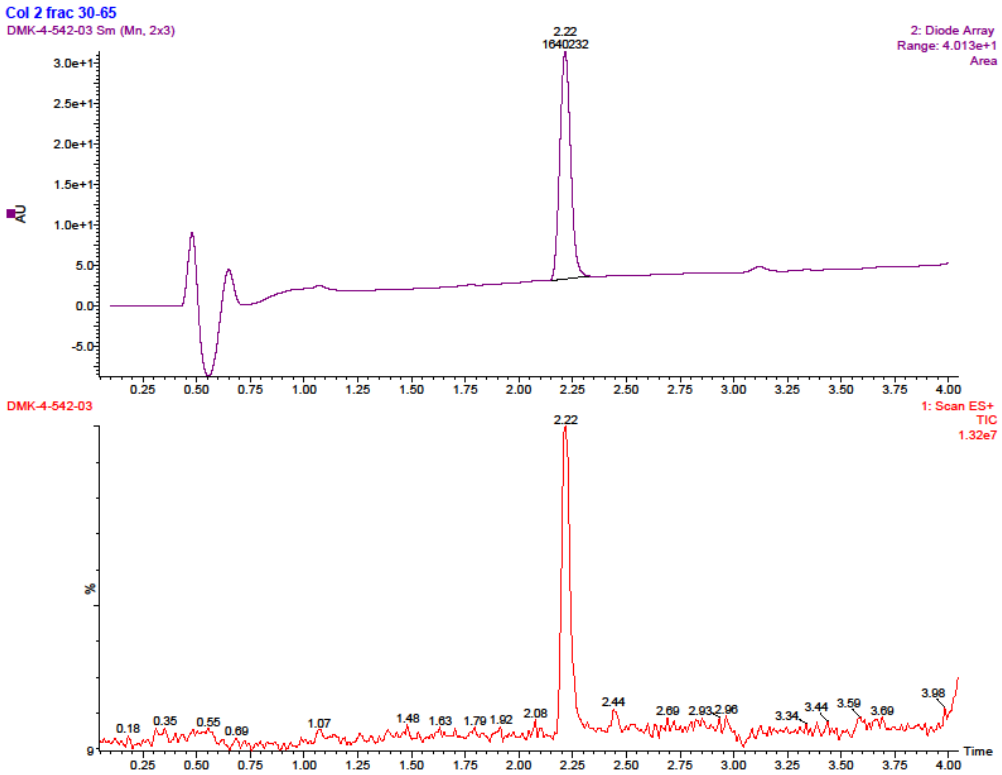
Compound 9s (NEU-4931)



Compound 15 (NEU-4389)



Compound 18 (NEU-4461)



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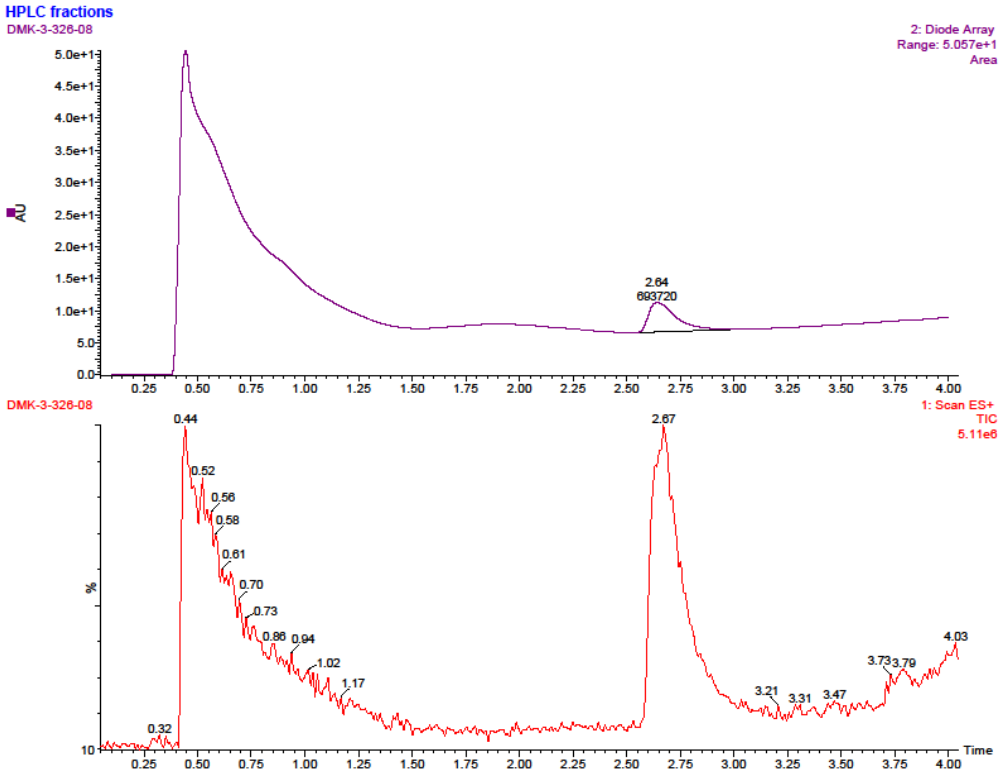

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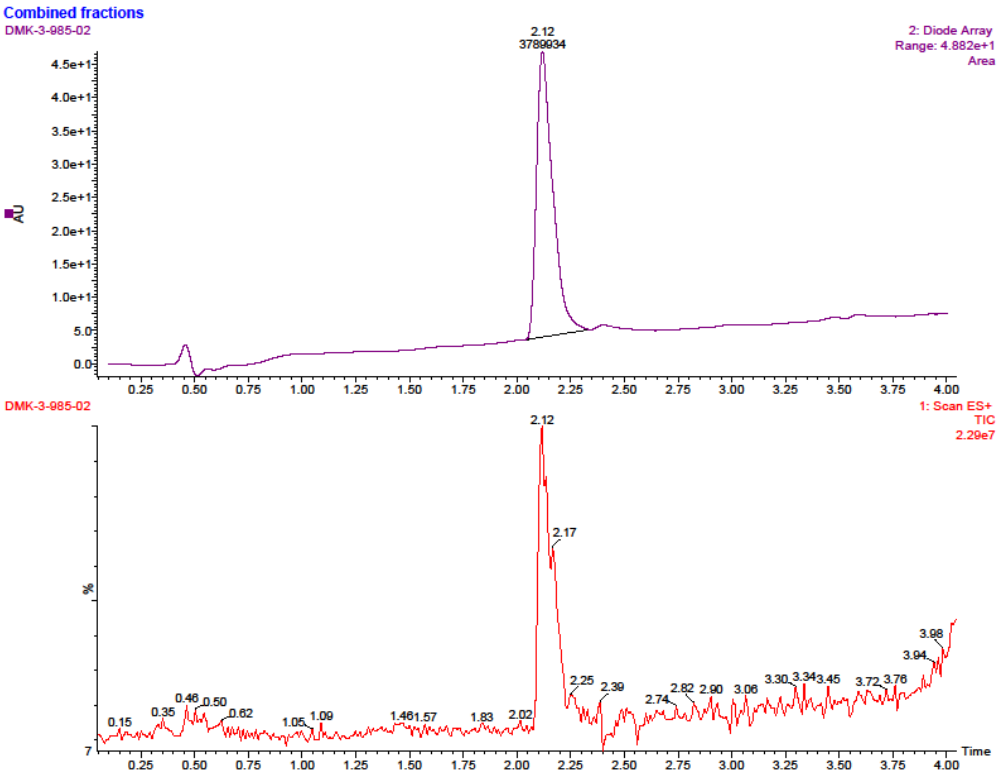
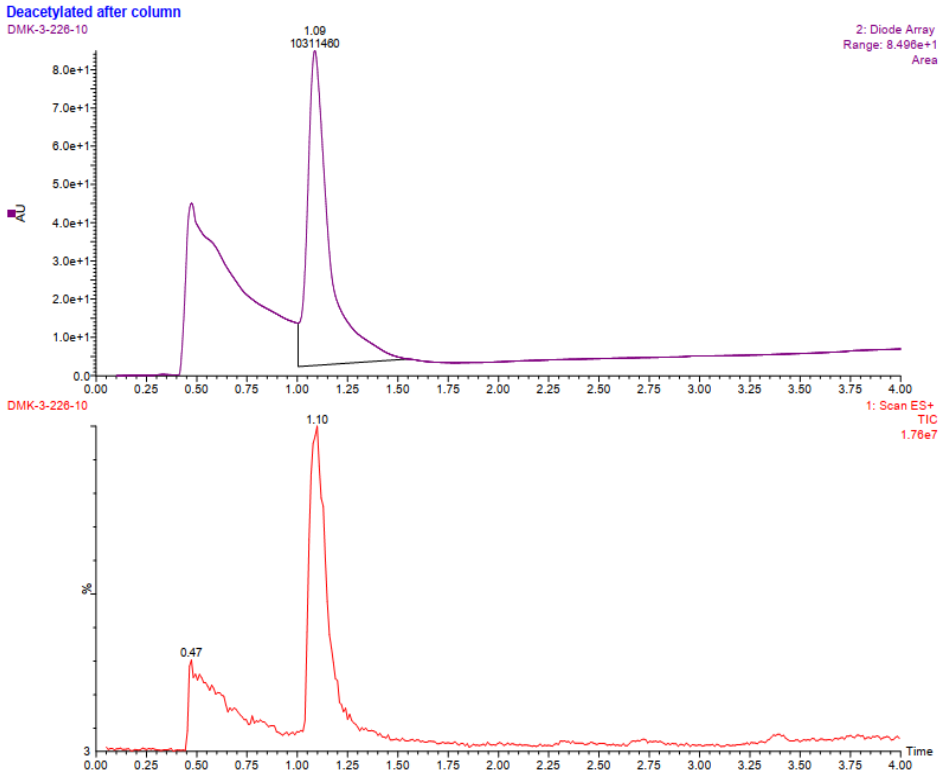
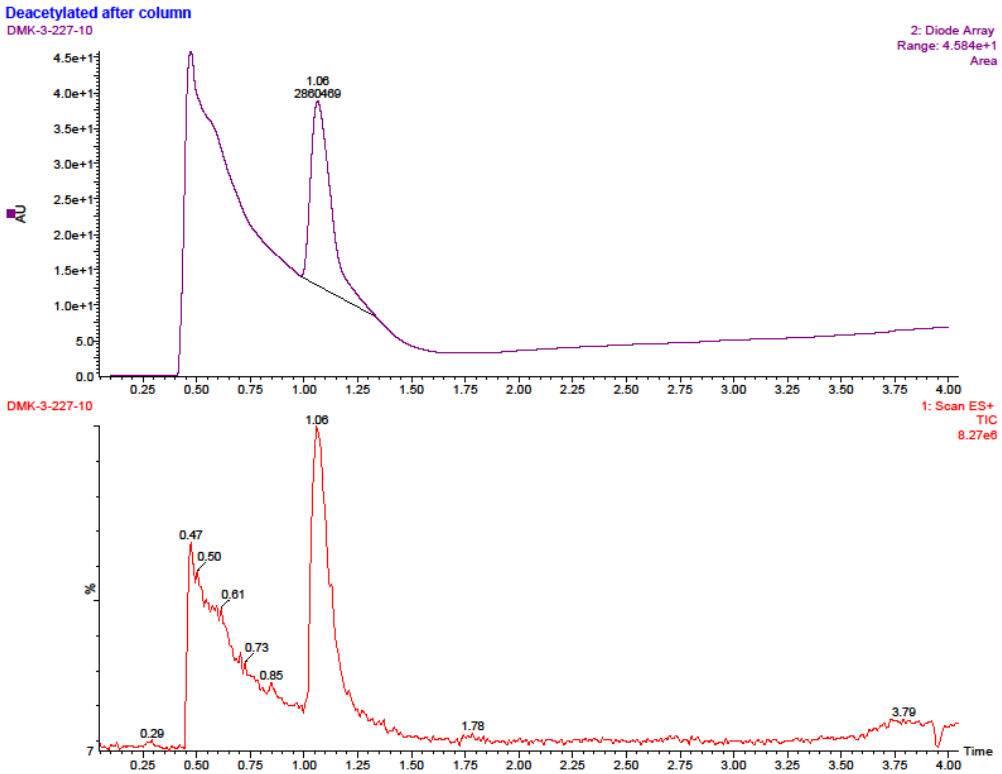


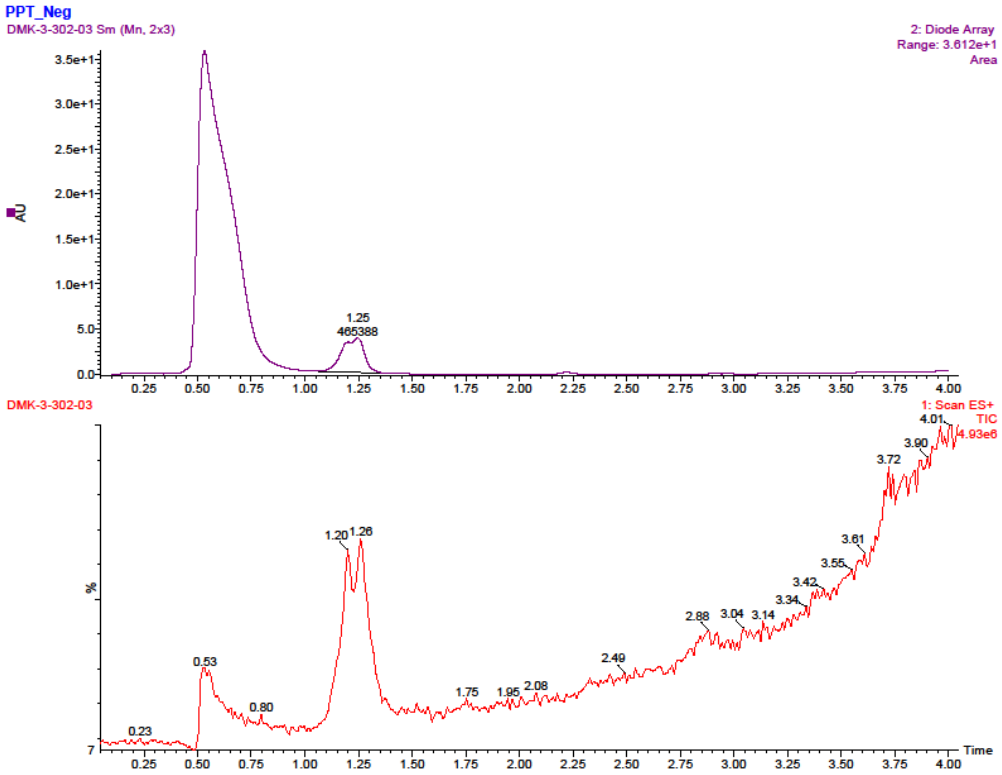
Compound 28a (NEU-2586)



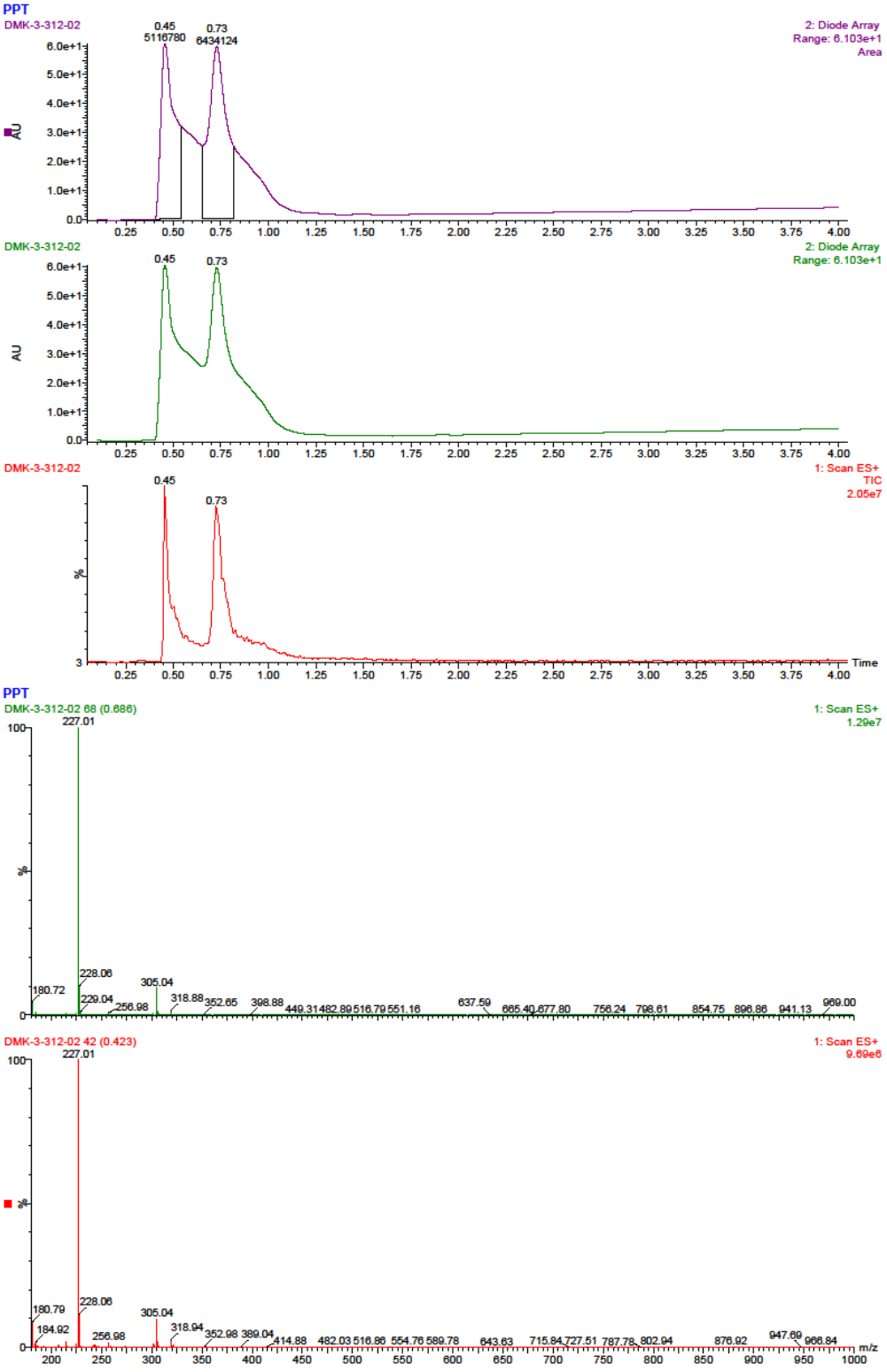
Compound 28b (NEU-2587)



Compound 31a (NEU-4390)

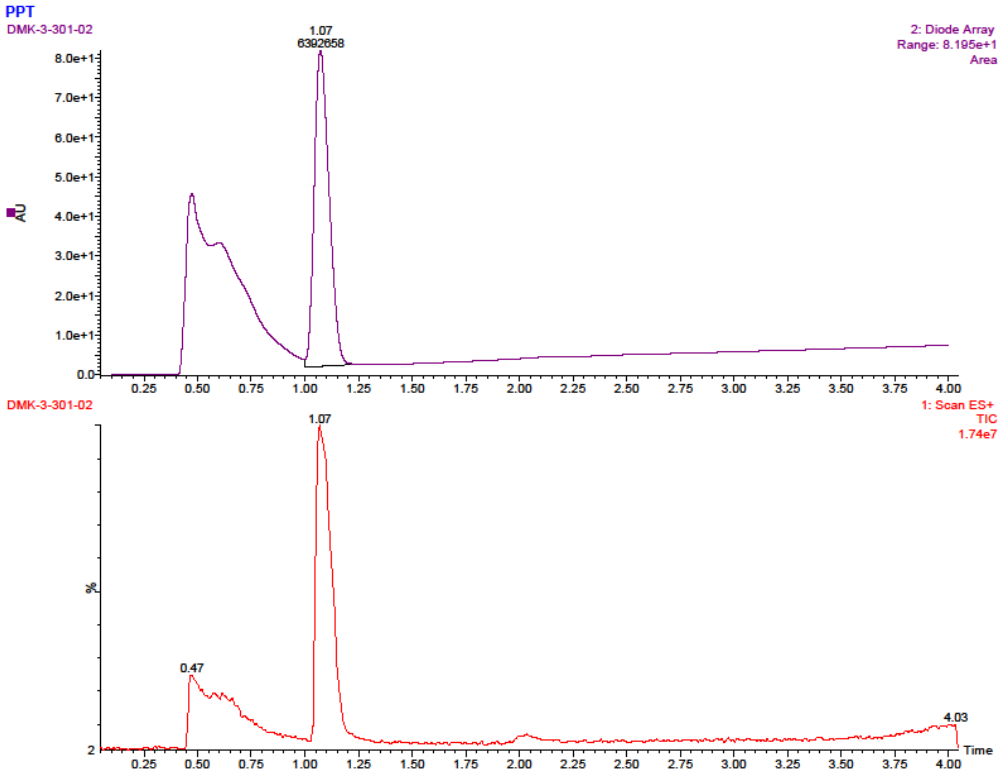


Compound 31b (NEU-4362)

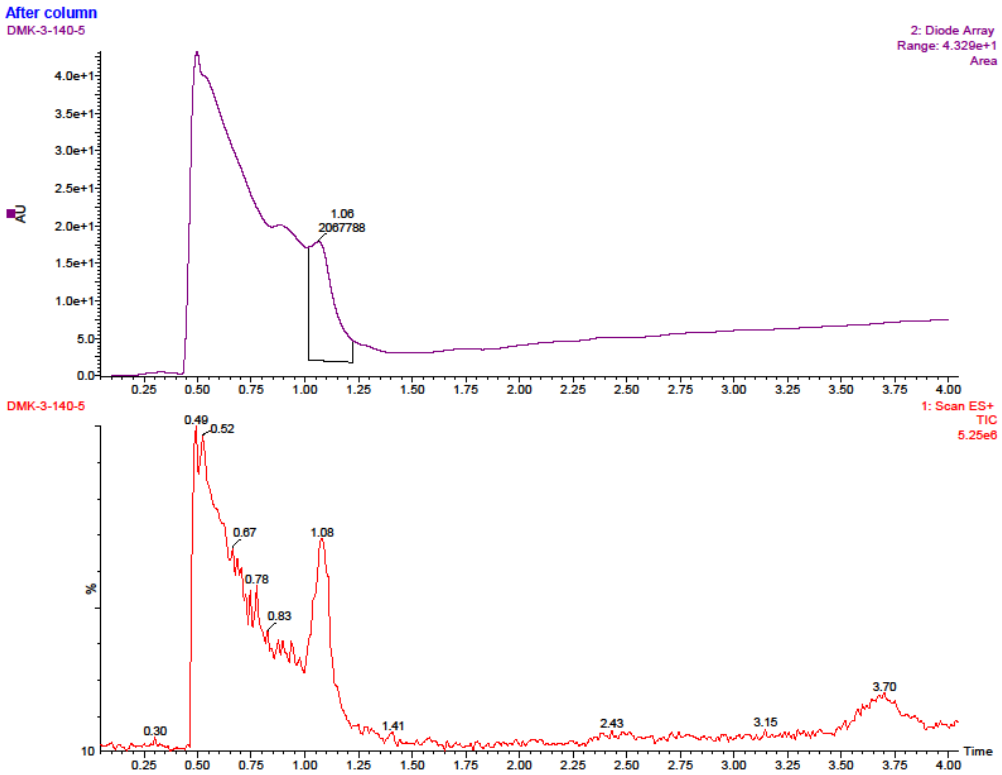


*Note: the compound was very polar and split when eluting off the LCMS despite all our attempts to prevent this.

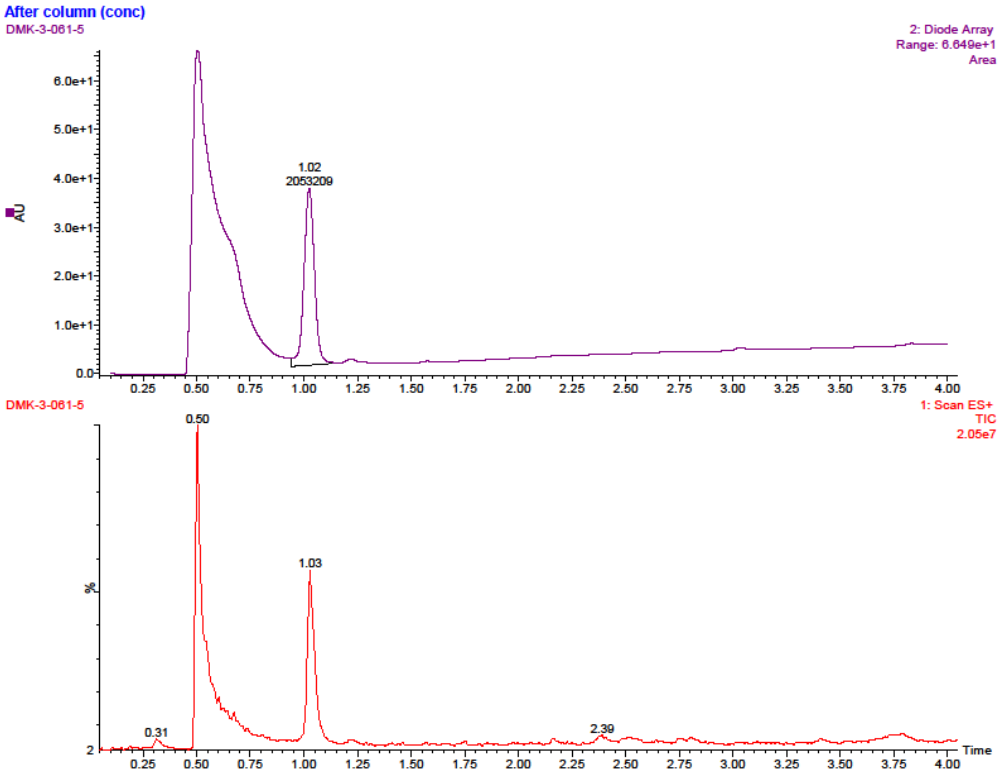
Compound 31c (NEU-4361)



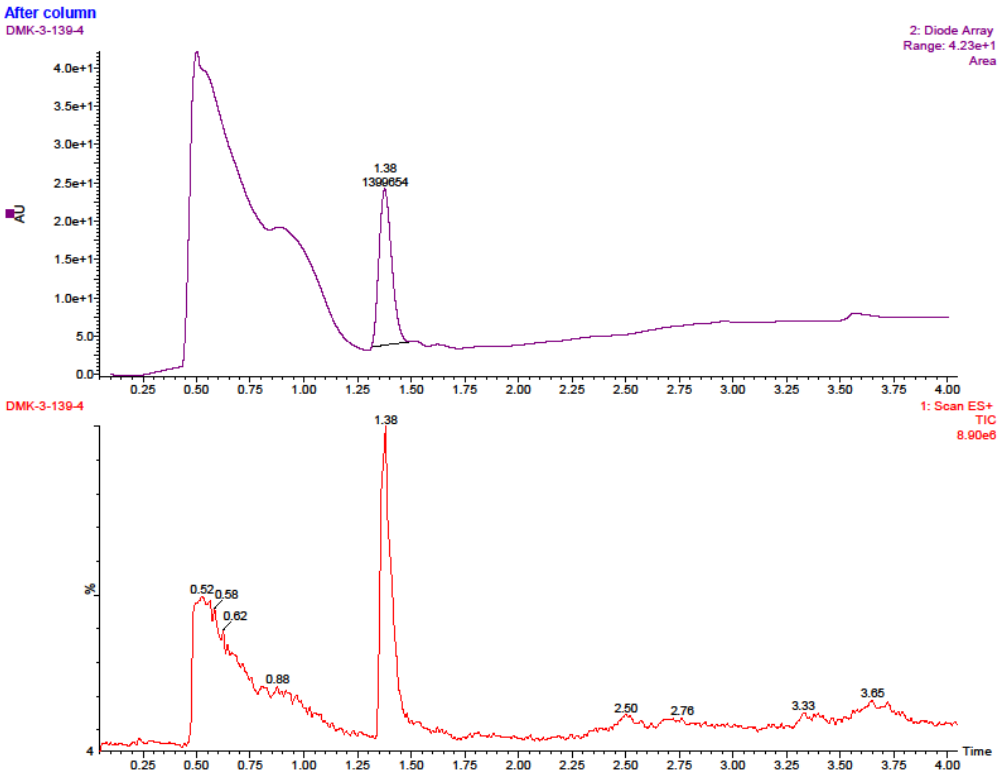
Compound 34a (NEU-2209)



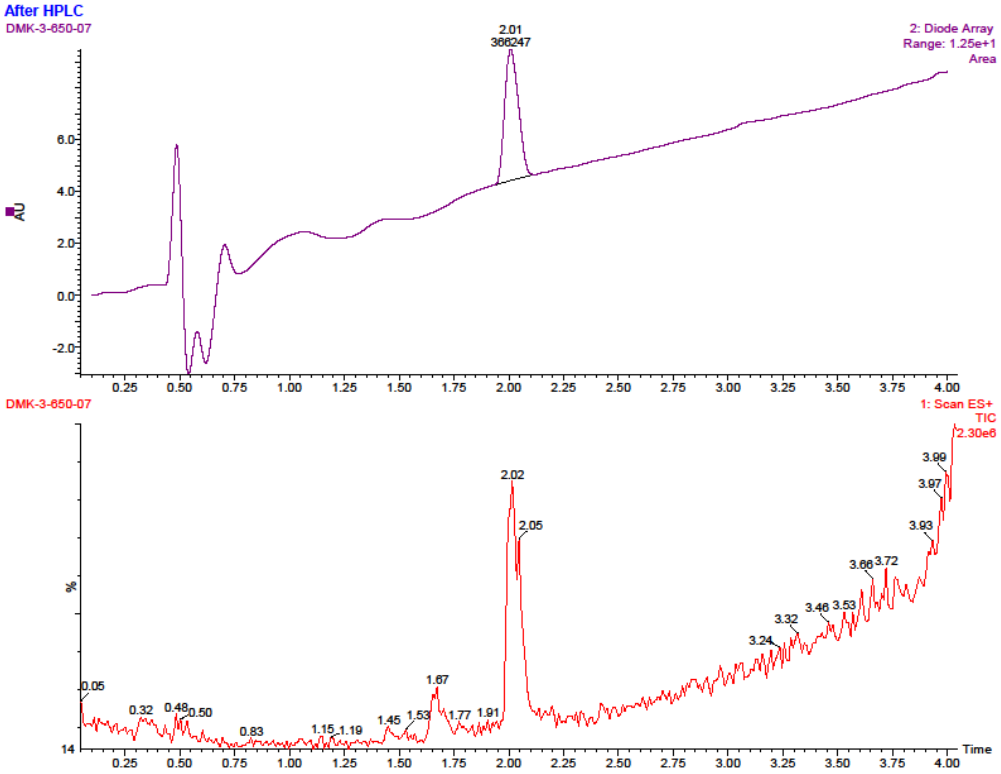
Compound 34b (NEU-2198)



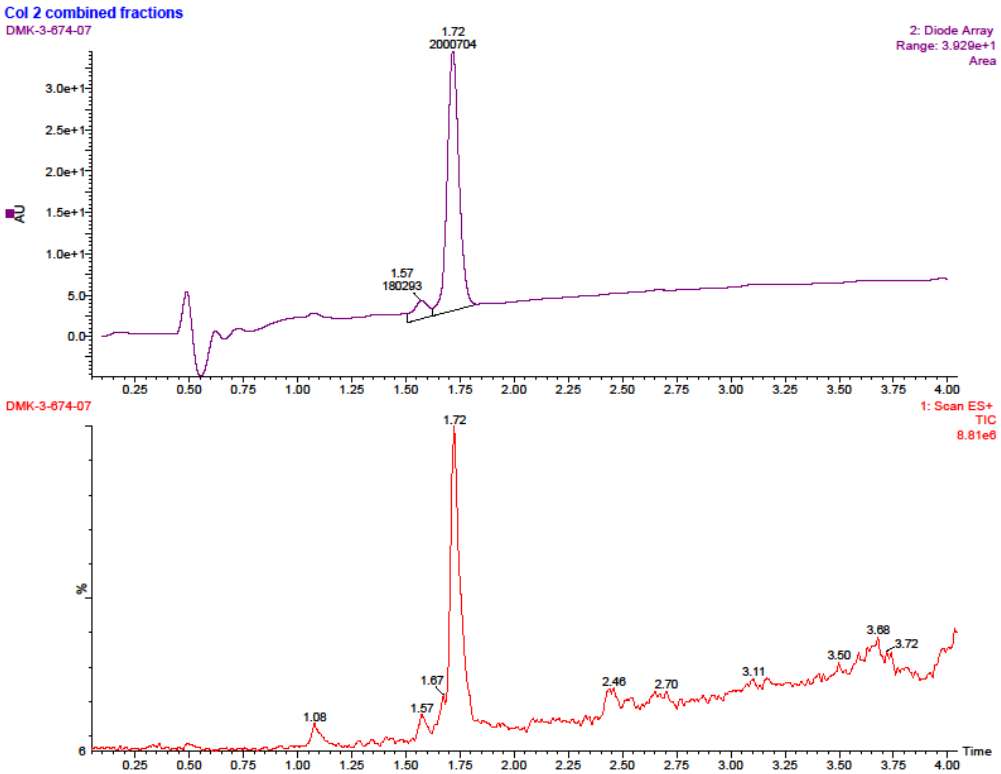
Compound 34c (NEU-2208)



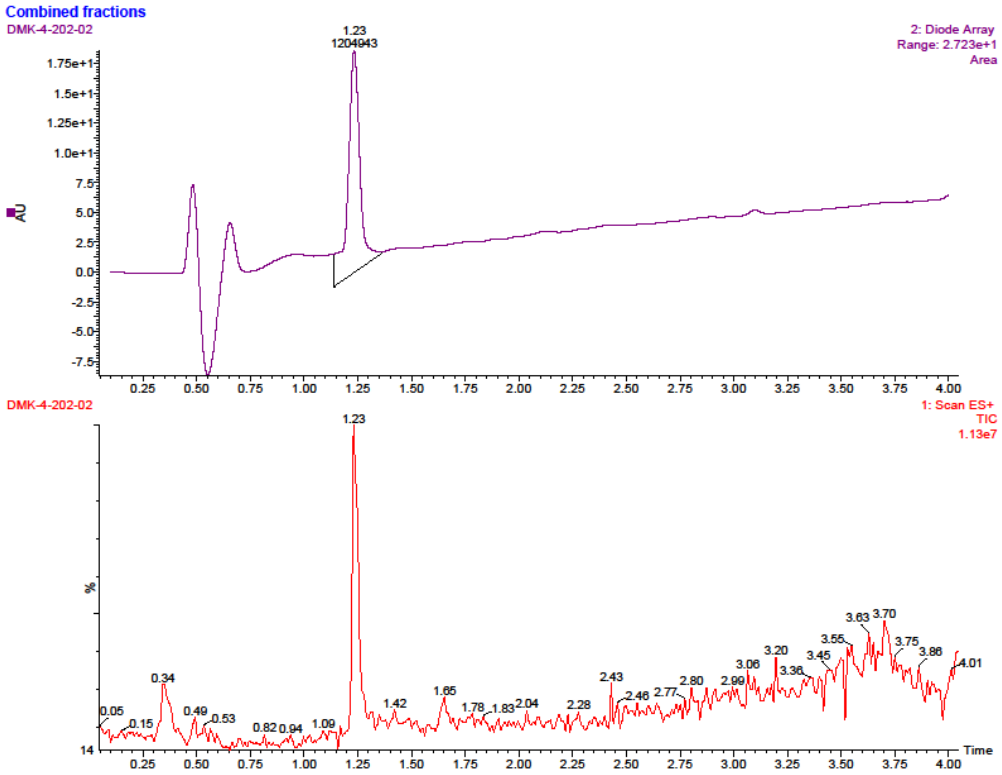
Compound 40 (NEU-4892)



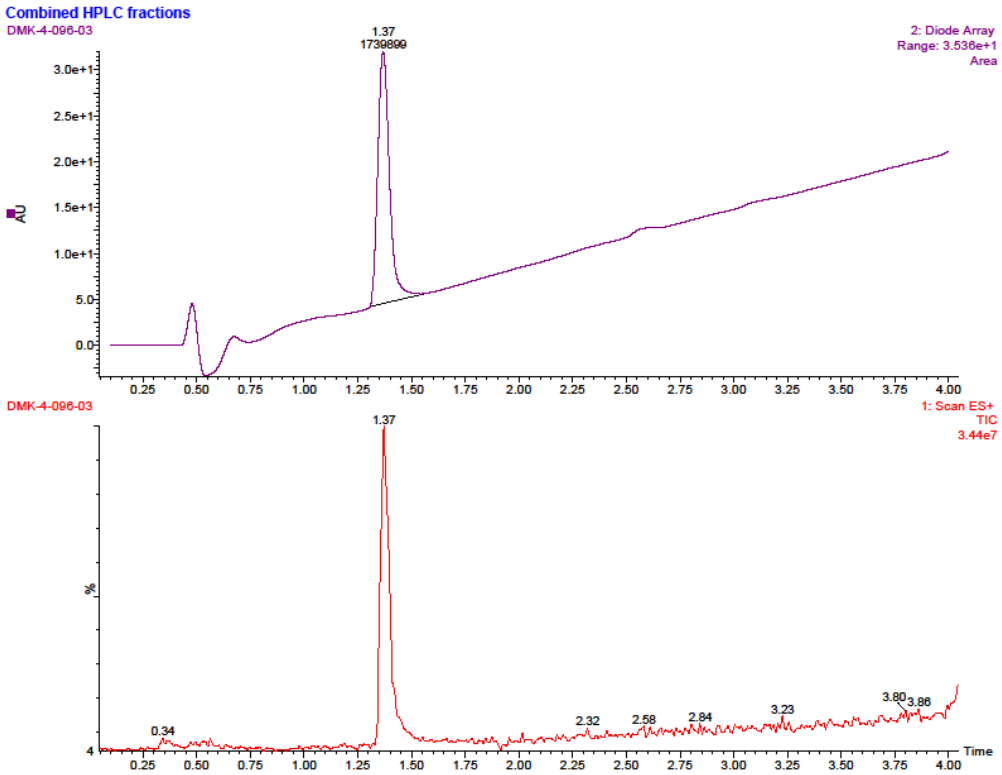
Compound 49 (NEU-4895)



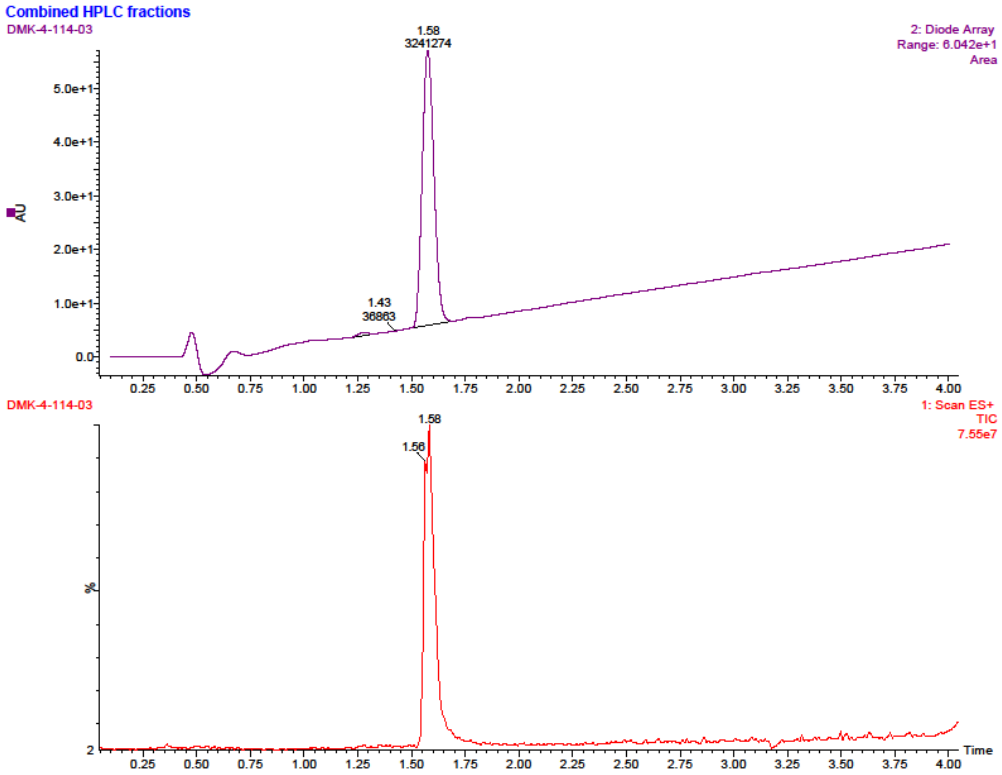
Compound 51 (NEU-1335)



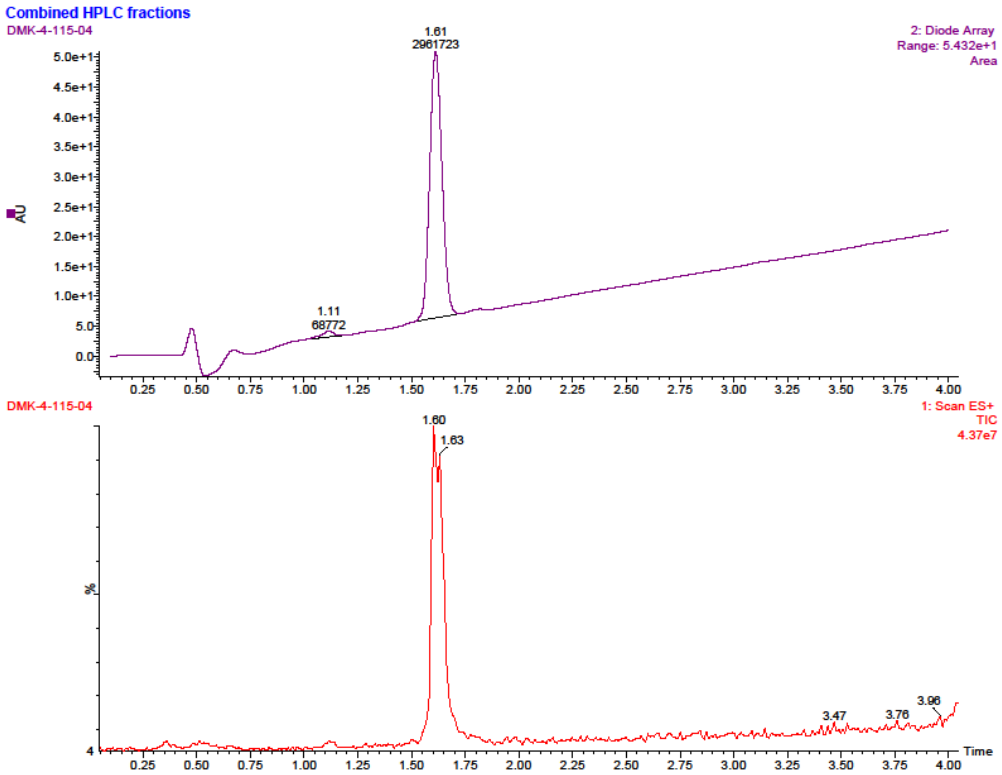
Compound 57a (NEU-5388)



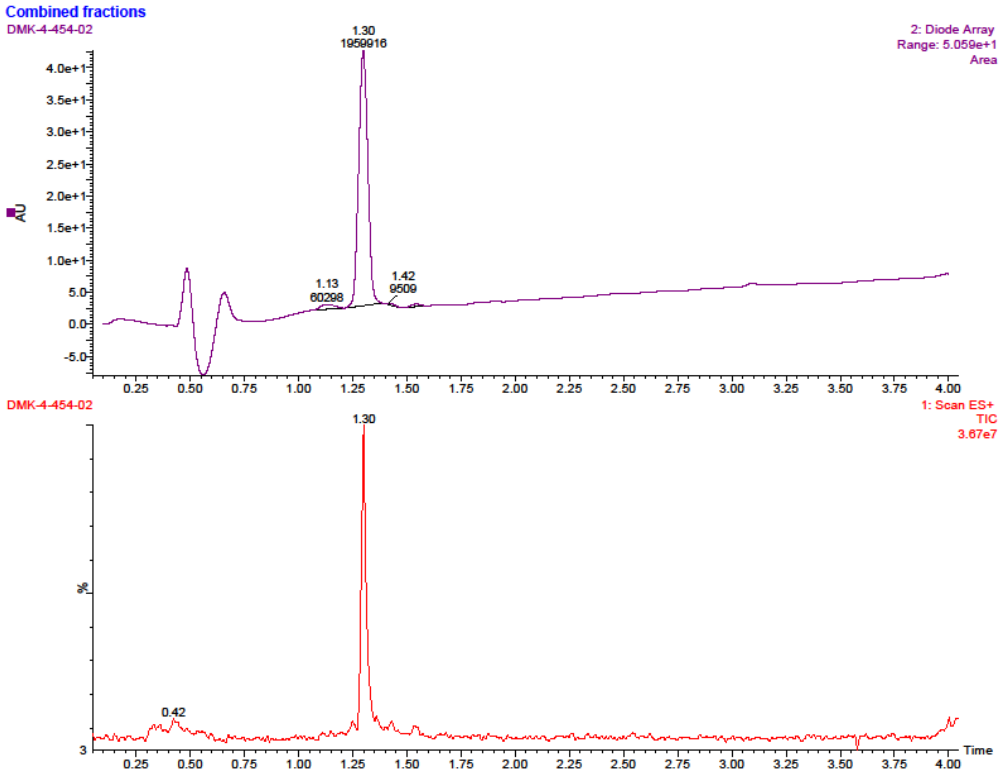
Compound 57c (NEU-5389)



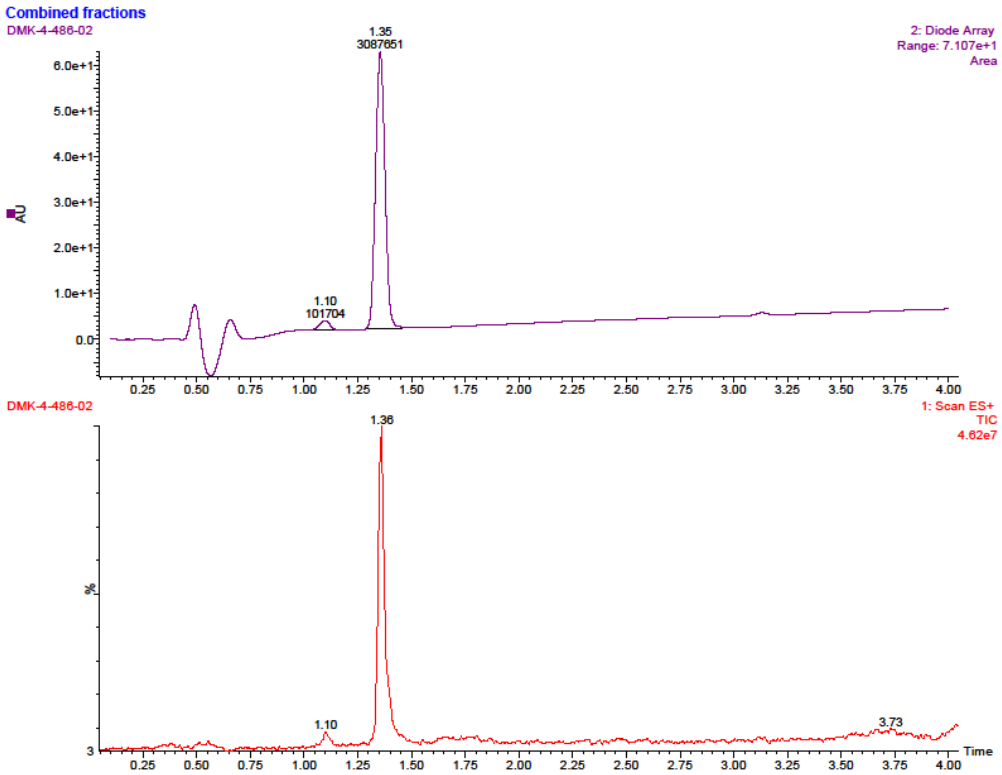
Compound 57d (NEU-5390)



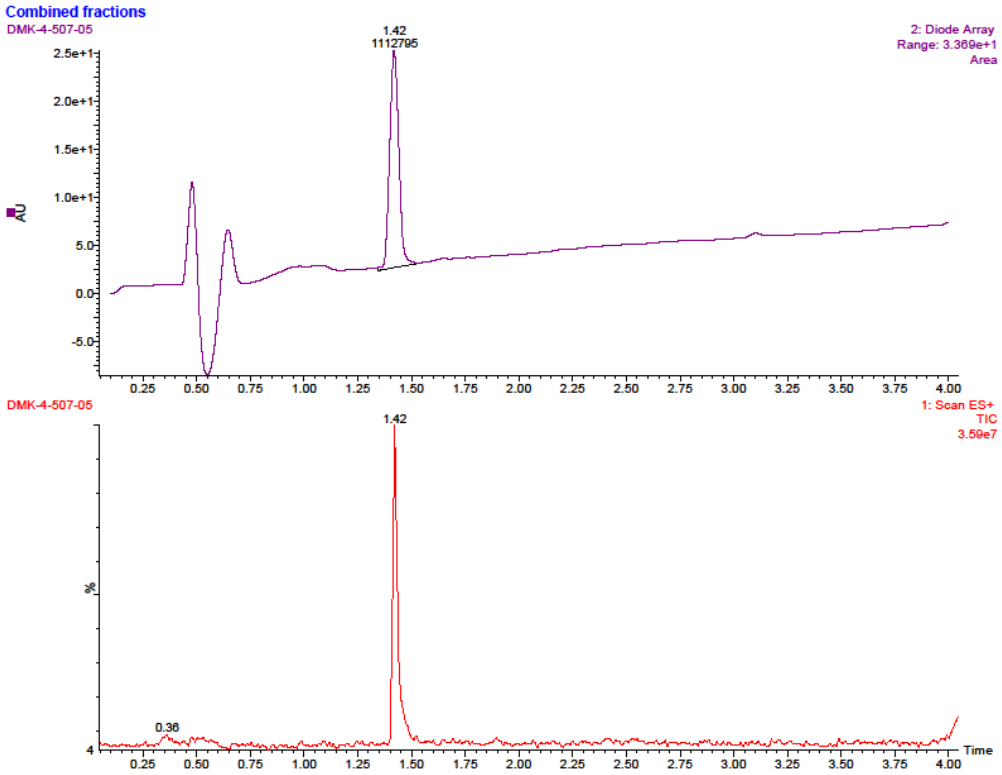
Compound 57g (NEU-5899)



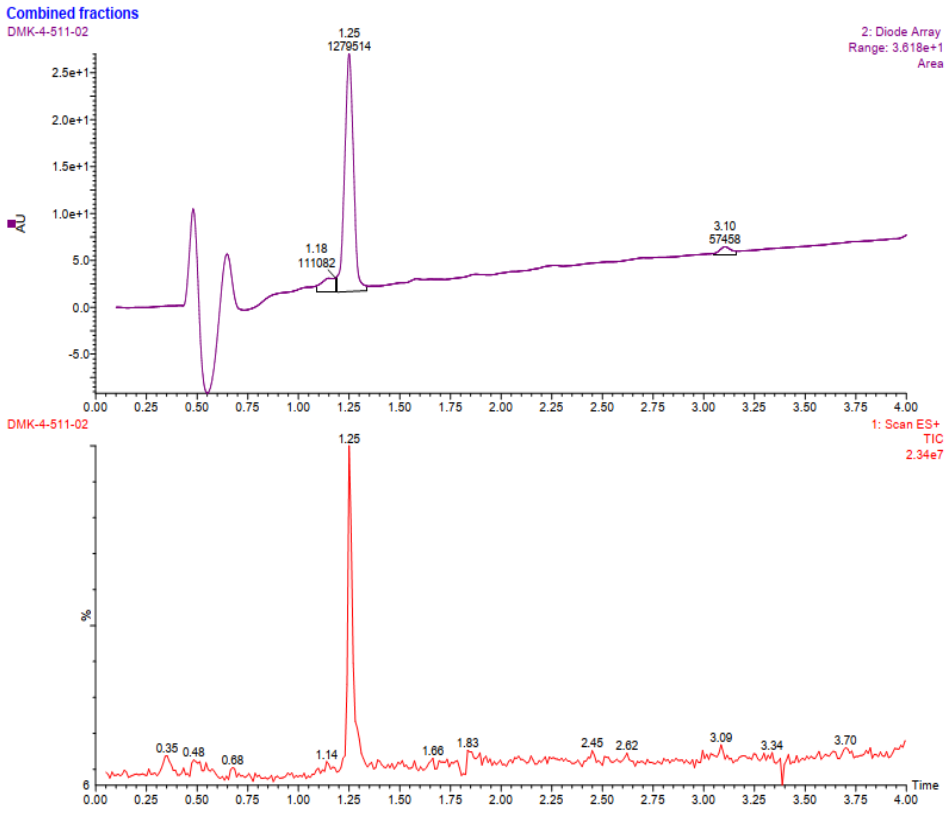
Compound 57h (NEU-5901)



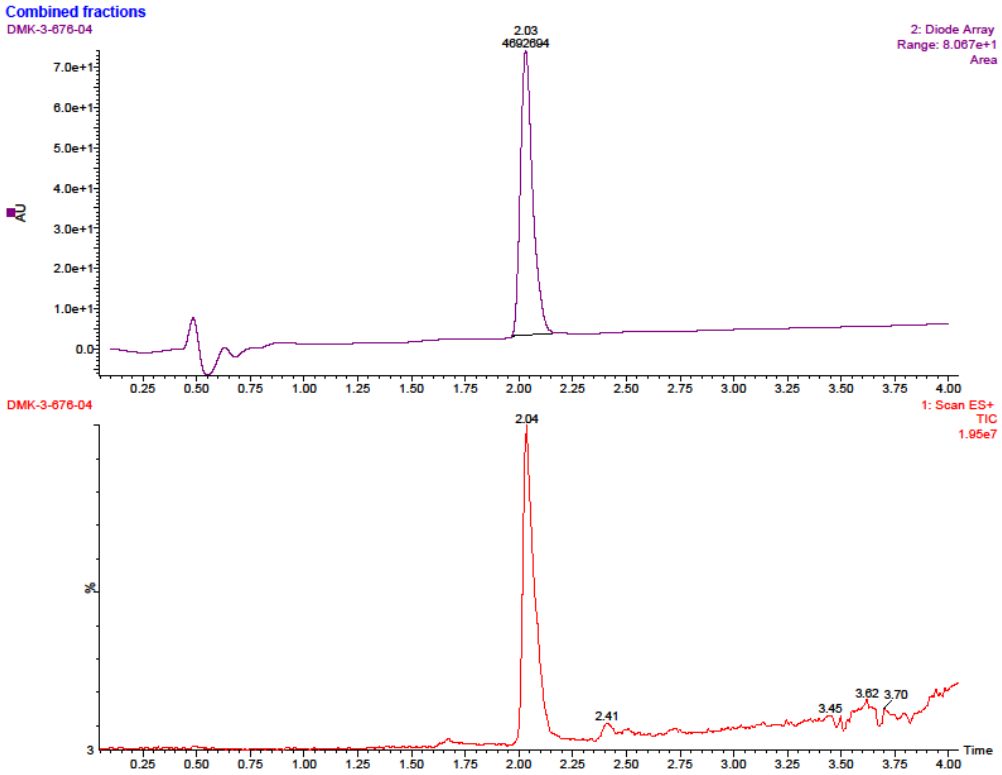
Compound 57i (NEU-5934)



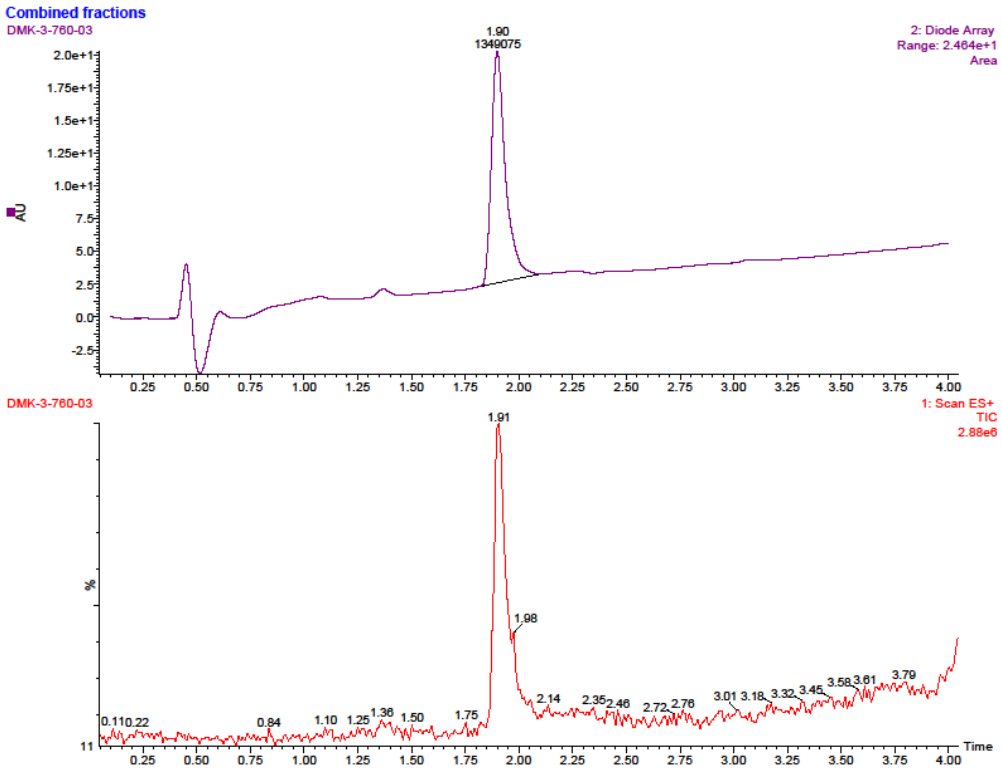
Compound 57j (NEU-5935)



Compound 63 (NEU-4894)



Compound 68 (NEU-4962)



Cell and Whole-Organism Assay Protocols

Strains and media. Bloodstream *Trypanosoma brucei brucei* Lister 427 was cultured in Hirumi's modified Iscove's medium (HMI-9), supplemented with 10% heat-inactivated FBS, at 37 ºC and 5% CO_2 in T-25 vented flask (Corning®). MRC5-SV2 cell line (SV40-transformed human lung fibroblast cell line) was cultured in DMEM medium supplemented with 10% FBS at 37 ºC and 5% CO_2 in T-75 vented flask (Corning®). The *T. cruzi* Tulahuen C4 strain, expressing the β-galactosidase gene (LacZ) and L6 rat skeletal muscle cells, used as host cells, were cultured in RPMI-1640 supplemented with 10% iFBS, 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37 °C and 5% CO_2. *Leishmania donovani* MHOM/ET/67/HU3 cells with the luciferase gene integrated into the parasite genome[1] were grown at 28 °C in RPMI 1640-modified medium (Invitrogen) supplemented with 20% FBS with 100 mg/ml of hygromycin B. Maintenance of the *Schistosoma mansoni* life cycle (NMRI isolate), preparation adult worms (≥42-days-old), and their co-incubation with test compounds were as described.[2-4] Phenotypic responses were visually recorded using a constrained nomenclature and converted to severity scores on a scale from zero (no activity) to 4 (maximal activity).[3-4] Use of hamsters for maintaining the *S. mansoni* life-cycle was approved by the Institutional Animal Care and Use Committee of the University of California San Diego. The Human myelomonocytic cell line THP-1 was grown at 37 °C and 5% CO_2 in RPMI-1640 supplemented with 10% iFBS, 2 mM glutamate, 100 U/mL penicillin and 100 mg/mL streptomycin.

Preparation of compound plates. For dose-response experiments, compound plates were prepared for each analogue by serial 3-fold dilutions in 100% DMSO. Five concentration points (mammalian cytotoxicity) or ten concentration points (parasite growth inhibition), were made in 96-well transparent Nunclon plates. Pentamidine was routinely included in compound plates as internal quality control, and plates were stored sealed at -20 ºC for no more than four weeks.

Rate of Action assays. Mid-log *T. brucei brucei* cultures were diluted to the required cell density, according to the different incubation time points described. Cultures (90 μL per well) were dispensed in final assay Nunclon 96-well flat bottom Solid White plates and 10 μL of intermediate plates were added to each well, as described before. Four sets of assay plates were arranged to assay in order to be sequentially stopped at each indicated time point. Top and bottom rows were dismissed for compound assay, to reduce evaporation effects.

Plates were incubated at 37 ºC and 5% CO_2 for the indicated time points; incubation was stopped by addition of 10 μL of prewarmed Cell Titer Glo reagent (Promega®), and after shaking the plates were

incubated at room temperature for 10 min, to allow the signal to settle. Plate luminescence was read on an Infinite F200 plate reader (Tecan), and raw data were processed and analyzed as previously described.

B-D-Galactosidase Transgenic T. cruzi *Assay.* A Thermo Scientific Multidrop Combi dispenser (MTX Lab Systems, Vienna, VA) was used to dispense 90 μL of *T. cruzi* amastigote–infected L6 cell culture (4×10^3 infected L6 cells per well) into 96-well Corning assay plates (Corning Inc., Corning, NY) already containing 10 μL of the compounds to be screened and controls. The plates were incubated at 37 °C for 96 h. Then, 30 μL of 100 μM CPRG and 0.1% NP40 diluted in PBS were added to each well, and the plates were incubated for 4 h at 37 °C in the dark. Absorbance at 585 nm was measured in a Vmax kinetic microplate reader (Molecular Probes). Compound activities were normalized using the in-plate 100% inhibition (benznidazole at 10 μg/mL) and 0% inhibition (0.2% DMSO) growth controls.

Cytotoxicity assay in MRC5. Intermediate plates were made as described, adding 95 μL of DMEM complete media to 5 μL of compound per well setting a 5% DMSO amount. Log-phase MRC5 cells were removed from a T-75 TC flask using TrypLE® Express (Thermo®) and dispersed by gentle pipetting. Cell density was adjusted to working concentration in prewarmed DMEM medium: 25,000 cells in 90 μL of culture were plated in 96-well transparent Nunclon plates and let to settle for 24 h at 37 ºC and 5% CO_2. After settling incubation, 10 μL of freshly made intermediate plate were added per well: final maximal concentration for compounds was 50 μM in 0.5% DMSO per well. Plates were incubated for 48 h at 37 ºC and 5% CO_2. At 4 h prior to fluorescence measurement, 20 μL of 500 μM resazurin solution was added. Fluorescence was read in an Infinite F200 plate reader (Tecan®) at 550 nm (excitation filter) and 590 nm (emission filter).

A four-parameter equation was used to fit the dose-response curves and determination of EC_{50} by SigmaPlot ® 13.0 software. Assays were performed in duplicate at least twice for positive compounds, to achieve a minimal n=2 per dose response.

Resazurin-Based L6 Assay. One hundred microliters (100 μL) per well of culture medium containing the compounds and controls were added to L6 cells previously cultured (4×10^3 L6 cells per well). After 72 h at 37 °C the medium was exchanged and the viable cell number was determined by resazurin (Sigma–Aldrich) reduction. 20 μl of resazurin (1.1 mg/ml) was added to each well and incubated in the dark for 2 h at 37 ºC. Cell viability was estimated by measuring the final fluorescence at 570-590 nm in an Infinite F200 plate reader (Tecan).

Cytotoxicity assay in THP-1. Cellular toxicity of all compounds was determined using the colorimetric MTT-based assay after incubation at 37 °C for 72 h in the presence of increasing concentrations of compounds (final maximal concentration was 50 μM in 0.5% DMSO per well)[5]. The results are expressed as EC_{50} values, the concentration of compound that reduces cell growth by 50% versus untreated control cells. Assays were performed in duplicate at least twice to achieve a minimal n=3 per dose response.

Determination of EC_{50} in L. donovani. Macrophage-differentiated THP-1 cells were infected at a macrophage/parasite ratio of 1:10 with stationary-phase *L. donovani* promastigotes for 24 h at 35 °C and 5% CO_2, and extracellular parasites were removed by washing three times with PBS. Infected cell cultures were then incubated with different compounds concentrations at 37 °C for 72 h. Luminescence was measured using the Promega kit luciferase assay system (Promega ®, Madison, WI). Assays were performed in duplicate at least twice, to achieve a minimal n=3 per dose response.

ADME Experiment Protocols

Aqueous pH 7.4 Solubility. Compounds were dried down from 10 mM DMSO solutions using centrifugal evaporation technique. Phosphate buffer (0.1 M pH 7.4) was added and StirStix were inserted in the glass vials, with shaking then performed at a constant temperature of 25 °C for 20-24 h. This step was followed by double centrifugation with a tip wash in between, to ensure that no residues of the dried compounds interfere. The solutions were diluted before analysis and quantification using LC/MS/MS was performed.

Log $D_{7.4}$. Shake-flask octanol-water distribution coefficient was determined at pH 7.4 (Log $D_{7.4}$). The aqueous solution used is 10 mM sodium phosphate pH 7.4 buffer. The method has been validated for Log $D_{7.4}$ ranging from -2 to 5.0.

Human Plasma Protein Binding (PPB). PPB was determined using equilibrium dialysis (RED device) to separate free from bound compound. The amount of compound in plasma (10 μM initial concentration) and in dialysis buffer (pH 7.4 phosphate buffer) was measured by LC-MS/MS after equilibration at 37 °C in a dialysis chamber to give the fraction unbound (f_u); percent bound is calculated and reported.

Human Liver Microsomal Cl_{int}. In vitro intrinsic clearance was determined from human liver microsomes using a standard approach.[6] Following incubation and preparation, the samples were analyzed using LC/MS/MS. Refined data were uploaded to IBIS and are displayed as Cl_{int} (intrinsic clearance) in μL/min/mg.

Rat Hepatocyte Cl_{int}. *In vitro* intrinsic clearance was determined from rat hepatocytes using a standard approach.[6] Following incubation and preparation, the samples were analyzed using LC/MS/MS. Refined data are uploaded to IBIS and are displayed as Cl_{int} (intrinsic clearance) μL/min/1 million cells.

Calculated LogP and LogD values. Both LogP and LogD predictions are based on a modified version of the method[7] where the predicted partition coefficients are composed of the molecules' atomic increments.

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