Electronic Supplementary Material (ESI) for ChemComm.

Supporting Information

Protein-protein inhibitor designed *de-novo* to target the MEEVD region on the C-terminus of Hsp90 and block co-chaperone activity

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Abbreviations

Boc	di-*tert*-butyl dicarbonate
cLogP	calculated partition coefficient
D2O	deuterium oxide
d6-DMSO	deuterated DMSO
DIC	*N,N'*-diisopropylcarbodiimide
DIPEA	*N,N*-Diisopropylethylamine
DMF	dimethylformamide
DMSO	dimethylsulfoxide
DMTMM	4-(4,6-Dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride
EDTA	ethylenediaminetetraacetic acid
ESI	electrospray ionisation
Fmoc	fluorenomethoxycarbonyl
HATU	1-[Bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-*b*]pyridinium 3-oxid hexafluorophosphate
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HOAt	1-hydroxy-7-azabenzotriazole
HOBt	hydroxybenzotriazole
HPLC	high performance liquid chromatography
iPrOH	isopropanol, propan-2-ol
LC/MS	liquid chromatography/mass spectrometry
MeOH	methanol
MeOD	deuterated methanol
NMR	nuclear magnetic resonance
PBS	phosphate buffered saline
PSA	polar surface area
TBTU	O-(Benzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium tetrafluoroborate
tBu	*tert*-butyl
TFA	trifluoroacetic acid
TFE	trifluoroethanol
Trt	trityl

Experimental Procedures: Biological Methods

Materials and Methods

Protein Expression and Purification

Bacterial expression constructs for Hsp90 domains were cloned as N-terminal 6xHis tag fusion proteins in the pET-15 vector (Novagen). Proteins were transformed into *Escherichia coli* strain BL21(DE3) (Stratagene) for expression. Bacterial cell pellets containing His-tagged Hsp90 domains were suspended in 50 mM Tris-HCl pH 8.0, 500 mM NaCl, 10 mM imidazole, and lysed using an ultrasonic homogenizer sonicator. Crude lysate was clarified by centrifugation at 10,000 x g for 30 minutes. The supernatant was collected and filtered through 0.22µm syringe filters (Millipore). Filtered supernatant was loaded onto a 5mL Ni-NTA Superflow cartridge (Qiagen). Cell lysate was loaded onto column over 90 minutes. Protein was then eluted via a linear gradient up to 300mM imidazole.

Eluted protein was dialysed against ddH_2O overnight, followed by buffer exchange with 25mM Tris-HCl, 50mM NaCl buffer. Protein was purified again using anion exchange chromatography on a 5 mL HiTrapQ column (GE Healthcare) via a linear NaCl gradient, up to 1M NaCl. Eluted protein was combined, concentrated and dialysed against ddH_2O to yield final protein.

Cell Lysate Pull Down Assay

Human Colon Cancer HCT116 cells were grown to confluency, harvested and lysed in lysis buffer. Protein concentration was determined *via* BCA assay. All test compounds were dissolved in 100% DMSO and were diluted to a final concentration of 1% DMSO in each reaction. Tagged compounds were incubated with known concentrations of cell lysate in binding buffer (20mM Tris-HCl, 150mM NaCl, 1% Triton-X-100, pH 7.4) for 16 hours. . Protein pull-down was achieved by incubating each reaction with NeutrAvidin™ Agarose Resins (Thermo Scientific, 29201 or 29204), for an hour. Following the incubation with NeutrAvidin®, the supernatant was removed from each reaction, and the resin was washed six times with wash buffer (20mM Tris-HCl, 300mM NaCl, 1% Triton-X-100, pH 7.4). To elute proteins, beads were boiled with Laemmli sample buffer (10% SDS, 30% glycerol, 0.5 M DTT, 0.05% bromophenol blue, 250 mM Tris HCl, pH 6.8). The supernatant of each sample was loaded onto an 8% Tris-Glycine gel. The gel was made using the SureCast Gel Handcast system (Invitrogen, HC1000S) according to the manufacturer's protocol. The proteins were separated using SDS-PAGE gel electrophoresis.

Proteins were transferred onto a PVDF membrane which was blocked with 5% non-fat milk in TBS-T (Tris-buffered saline containing 0.1% Tween-20) for 1 hr at 4◦C. The membrane was then incubated with Hsp90 primary antibody (Anti-Hsp90 antibody, ab13495, 1:8000 dilution) in 2.5% non-fat milk (in TBS-T) at 4 °C overnight. After wash with cold TBS-T, the membrane was incubated with the respective HRP-conjugated secondary antibody (goat anti-Rabbit IgG, ab6721, 1:2000 dilution) at 4 °C for 30 min, followed by six washes with cold TBS-T. Immunoblotting was performed using West Pico chemiluminescent substrate (Thermo scientific, 34580) and the images were captured by ImageQuant LAS 4010 digital imaging system (GE Healthcare). The relative amount of Hsp90 was analysed via Image J and transformed to a fold of Hsp90 pulled down relative to 100µM LB76-tag control, which was set at 100%. Each experiment was completed at least n=3.

Cell lysate pull-down data

a) **b)**

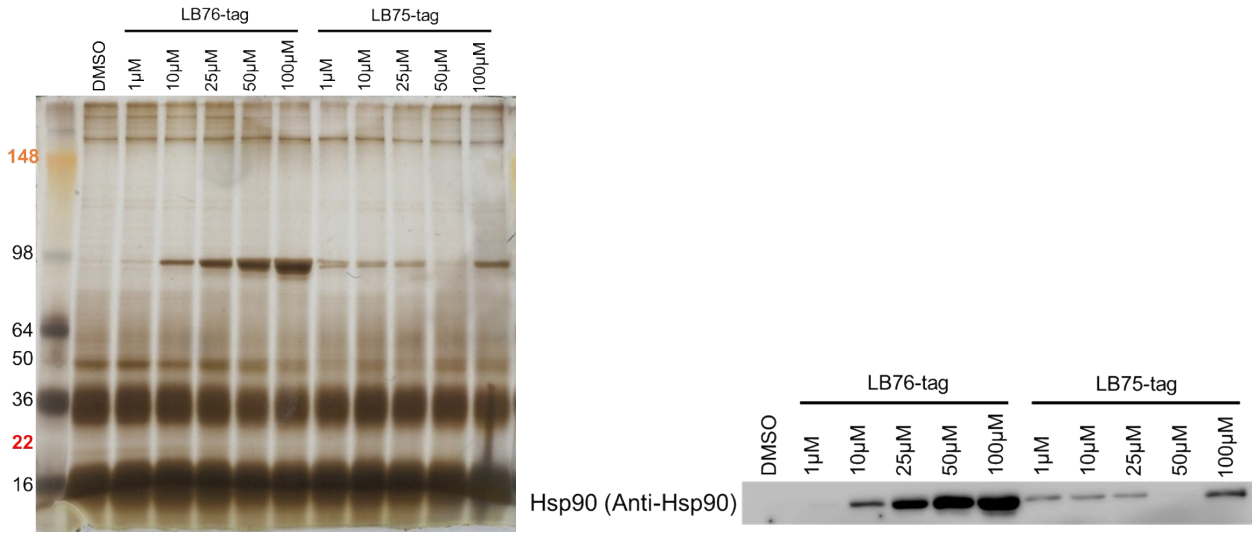


Figure S1: a) Cell lysate pulldown utilising 1-100μM concentrations of tagged molecules LB76-tag and LB75-tag. 1% DMSO was used as a control and gel stained (N=1), b) Cell lysate pulldown utilising tagged molecules, gel was transferred onto membrane and protein target confirmed as Hsp90 using anti-Hsp90. (N=1)

a) **b)**

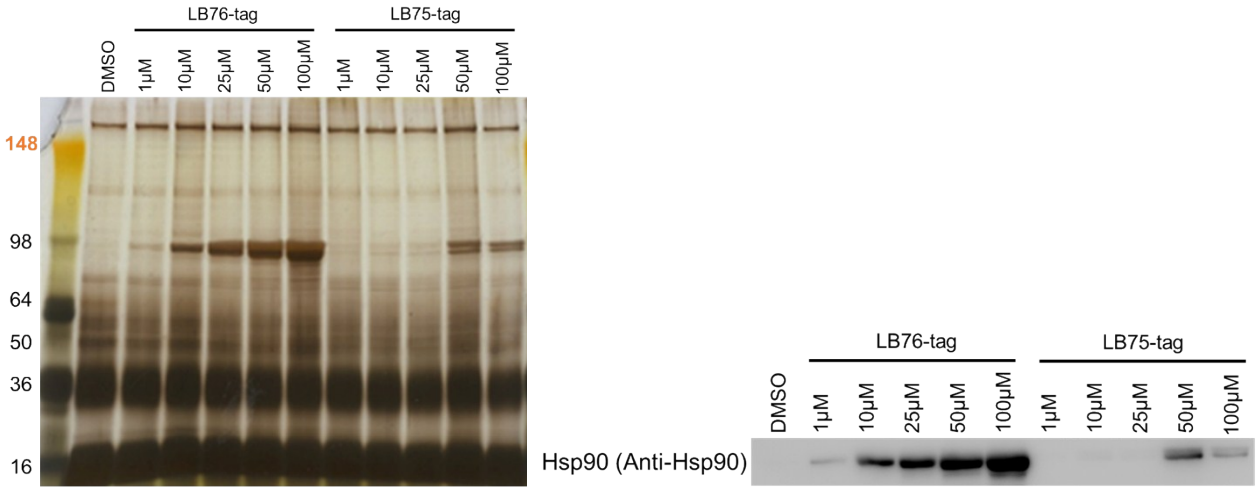


Figure S2: a) Cell lysate pulldown utilising 1-100μM concentrations of tagged molecules LB76-tag and LB75-tag. 1% DMSO was used as a control and gel stained (N=2), b) Cell lysate pulldown utilising tagged molecules, gel was transferred onto membrane and protein target confirmed as Hsp90 using anti-Hsp90. (N=2)

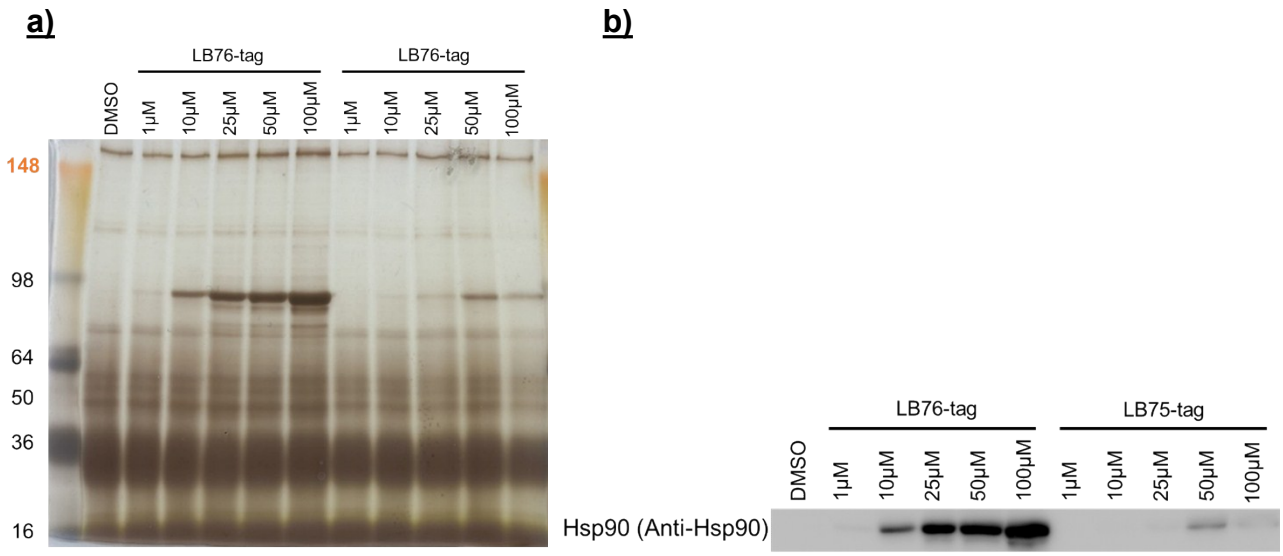


Figure S3: a) Cell lysate pulldown utilising 1-100µM concentrations of tagged molecules LB76-tag and LB75-tag. 1% DMSO was used as a control and gel stained (N=3), b) Cell lysate pulldown utilising tagged molecules, gel was transferred onto membrane and protein target confirmed as Hsp90 using anti-Hsp90. (N=3)

Protein Mass Spectrometry

Gels that contained protein bands of interest for mass spectrometric analysis were firstly washed with deionized water for 5 min. The bands of interest were excised using a fresh scalpel blade with excision being made as close as possible to the boundary of the band. Gel pieces were then placed into a 1.5 ml screwcap tube with a small amount of liquid left to keep the gel slice hydrated. Coomassie stain was removed by incubating gel bands in 200 µl NH_4HCO_3 (25 mM) in ACN until clear. Silver stained bands were digested by incubation of the gel bands with 40 µl DTT (10 mM) in NH_4HCO_3 (50 mM) for 30 min at 37°C. After removal of the solvent, the bands were incubated with 40 µl iodoacetamide (25 mM) in NH_4HCO_3 (50 mM) for 30 min at 37°C. The bands were then washed with 50 µl ACN twice, 10 min each and 40 µl trypsin (~100 ng) in NH_4HCO_3 (20 mM) was added then the solution incubated at 37°C for 14h. The bands were then washed with 50 µl deionized water with 1% v/v formic acid and 100 µl of ACN for 15 min. The extracted peptides were then dried and dissolved in 10 µl deionized water with 0.05% v/v heptafluorobutyric acid and 0.1% v/v formic acid.

Digest peptides were separated by nanoLC using an Ultimate nanoRSLC UPLC and autosampler system (Dionex, Amsterdam, Netherlands). Samples (2.5 µl) were concentrated and desalted onto a micro C18 precolumn (300 µm x 5 mm, Dionex) with H2O:CH_3CN (98:2, 0.1 % TFA) at 15 µl/min. After a 4 min wash the pre-column was switched (Valco 10 port UPLC valve, Valco, Houston, TX) into line with a fritless nano column (75µ x ~15cm) containing C18AQ media (1.9µ, 120 Å Dr Maisch, Ammerbuch-Entringen Germany). Peptides were eluted using a linear gradient of H2O:CH_3CN (98:2, 0.1 % formic acid) to H_2O:CH_3CN (64:36, 0.1 % formic acid) at 200 nl/min over 30 min. High voltage 2000 V was applied to low volume Titanium union (Valco) and the tip positioned ~ 0.5 cm from the heated capillary (T=275°C) of a Orbitrap Fusion Lumos (Thermo Electron, Bremen, Germany) mass spectrometer. Positive ions were generated by electrospray and the Fusion Lumos operated in data dependent acquisition mode (DDA).

A survey scan m/z 350-1750 was acquired in the orbitrap (resolution = 120,000 at m/z 200, with an accumulation target value of 400,000 ions) and lockmass enabled (m/z 445.12003). Data-dependent tandem MS analysis was performed using a top-speed approach (cycle time of 2s). MS2 spectra were fragmented by HCD (NCE=30) activation mode and the ion-trap was selected as the mass analyzer. The intensity threshold for fragmentation was set to 25,000. A dynamic exclusion of 20 s was applied with a mass tolerance of 10ppm.

Peak lists were generated using Mascot Daemon/Mascot Distiller (Matrix Science, London, England) using default parameters, and submitted to the database search program Mascot (version 2.5.1, Matrix Science). Search parameters were: Precursor tolerance 4 ppm and product ion tolerances ± 0.4 Da; Met (O) carboxyamidomethyl-Cys specified as variable modification, enzyme specificity was trypsin, 1 missed cleavage was possible and the non-redundant protein database from Unirpot (2018) searched.

Peak-list results

a)

Protein hits : HS90A_HUMAN Heat shock protein HSP 90-alpha OS=Homo sapiens GN=HSP90AA1 PE=1 SV=5
HS90B_HUMAN Heat shock protein HSP 90-beta OS=Homo sapiens GN=HSP90AB1 PE=1 SV=4
K2C1_HUMAN Keratin, type II cytoskeletal 1 OS=Homo sapiens GN=KRT1 PE=1 SV=6
PLEC_HUMAN Plectin OS=Homo sapiens GN=PLEC PE=1 SV=3

1. HS90A_HUMAN **Mass:** 84607 **Score:** 4881 **Matches:** 142(142) **Sequences:** 47(47) **emPAI:** 16.25
Heat shock protein HSP 90-alpha OS=Homo sapiens GN=HSP90AA1 PE=1 SV=5

Query	Observed	Mr(expt)	Mr(calc)	ppm	Miss	Score	Expect	Rank	Unique	Peptide
369	405.6944	809.3743	809.3742	0.13	0	35	0.00075	1		K.FENLCK.I
449	408.2606	814.5067	814.5065	0.21	0	37	0.00022	1	U	R.ALLFVPR.R 445 446 447 448
802	429.7739	857.5333	857.5334	-0.15	1	36	0.0011	1	U	K.KLSELLR.Y
817	432.2182	862.4219	862.4218	0.05	0	38	0.0012	1		R.EMLQQSK.I
1537	473.7786	945.5426	945.5429	-0.31	1	43	0.00013	1		R.IMKAQALR.D
1545	474.7265	947.4385	947.4389	-0.41	0	36	0.00098	1	U	K.FYEQFSK.N 1546 1547
1696	482.2137	962.4129	962.4127	0.16	0	35	0.0012	1	U	R.TDTGEPMGR.G
1777	486.3113	970.6080	970.6076	0.37	1	15	0.03	1	U	R.ALLFVPRR.A
1968	495.2885	988.5623	988.5627	-0.32	1	51	1.4e-005	1	U	K.IMKDILEK.K 1969
2088	503.2860	1004.5574	1004.5576	-0.14	1	(41)	0.00022	1	U	K.IMKDILEK.K
2345	515.3065	1028.5984	1028.5978	0.51	1	36	0.00076	1	U	K.VEKVVVSNR.L
2411	520.2656	1038.5167	1038.5168	-0.07	1	60	5.9e-006	1	U	K.TKFENLCK.I
3078	554.7746	1107.5347	1107.5349	-0.19	0	44	0.00017	1	U	R.APFDLFENR.K
3481	576.2823	1150.5500	1150.5506	-0.46	0	67	8.7e-007	1		K.YIDQEELNK.T 3483
3597	584.7889	1167.5632	1167.5632	0.01	0	60	5.3e-006	1	U	K.LGIHEDSQNR.K 3596
3715	594.7546	1187.4947	1187.4951	-0.27	0	54	3.9e-006	1	U	R.DNSTMGYMAAK.K
3842	602.7520	1203.4894	1203.4900	-0.52	0	(28)	0.0017	1	U	R.DNSTMGYMAAK.K
3843	602.7520	1203.4895	1203.4900	-0.43	0	(47)	2e-005	1	U	R.DNSTMGYMAAK.K

b)

Protein hits : AVID_CHICK Avidin OS=Gallus gallus GN=AVD PE=1 SV=3
K2C1_HUMAN Keratin, type II cytoskeletal 1 OS=Homo sapiens GN=KRT1 PE=1 SV=6
K1C10_HUMAN Keratin, type I cytoskeletal 10 OS=Homo sapiens GN=KRT10 PE=1 SV=6
K22E_HUMAN Keratin, type II cytoskeletal 2 epidermal OS=Homo sapiens GN=KRT2 PE=1 SV=2

1. AVID_CHICK **Mass:** 16758 **Score:** 1593 **Matches:** 56(56) **Sequences:** 12(12) **emPAI:** 18.51
Avidin OS=Gallus gallus GN=AVD PE=1 SV=3

Query	Observed	Mr(expt)	Mr(calc)	ppm	Miss	Score	Expect	Rank	Unique	Peptide
396	418.2282	834.4418	834.4422	-0.43	0	38	0.0047	1	U	K.TMWLLR.S 397 398 399
969	460.2716	918.5286	918.5287	-0.10	0	66	2.3e-005	1	U	R.VGINIFTR.L 957 958 960 961 962 963 964
2416	618.2800	1234.5454	1234.5466	-1.00	0	66	7.8e-006	1	U	R.SSVNDIGDDWK.A 2417
2611	435.5847	1303.7321	1303.7322	-0.07	1	32	0.022	1	U	K.EVLKTMWLLR.S
2942	713.3610	1424.7074	1424.7089	-1.02	0	74	2.2e-006	1	U	R.TQPTFGFTVNWK.F 2943 2945 2947
3414	782.3729	1562.7313	1562.7325	-0.77	1	100	5e-009	1	U	R.SSVNDIGDDWKATR.V 3411 3412 3413 3415
3417	521.9180	1562.7323	1562.7325	-0.15	1	(83)	2.1e-007	1	U	R.SSVNDIGDDWKATR.V 3419 3421
3572	527.9440	1580.8102	1580.8100	0.12	1	49	0.0008	1	U	K.RTQPTFGFTVNWK.F
3689	532.2815	1593.8227	1593.8223	0.24	1	46	0.0015	1	U	K.ESPLHGTQNTINKR.T
5244	632.2924	1893.8553	1893.8567	-0.74	0	84	1.2e-007	1	U	K.FSESTTVFTGQCFIDR.N
5245	947.9356	1893.8567	1893.8567	-0.03	0	(51)	0.00024	1	U	K.FSESTTVFTGQCFIDR.N
5672	668.3368	2001.9885	2001.9895	-0.52	0	(52)	0.00041	1	U	R.GEFTGTYITAVTATSNEIK.E
5673	1002.0020	2001.9894	2001.9895	-0.03	0	114	2.5e-010	1	U	R.GEFTGTYITAVTATSNEIK.E 5671
6314	732.0119	2193.0139	2193.0161	-1.00	1	74	1.3e-006	1	U	K.FSESTTVFTGQCFIDRNGK.E
10470	856.4316	3421.6973	3421.7001	-0.82	1	40	0.0037	1	U	R.GEFTGTYITAVTATSNEIKESPLHGTQNTINK.R

Figure S4: MASCOT results identifying proteins on lane LB76-tag 100µM. a) Proteins sequenced from 50-148kDa b) Proteins sequenced from 6-49kDa.

a)

Protein hits	:	
	K2C1_HUMAN	Keratin, type II cytoskeletal 1 OS=Homo sapiens GN=KRT1 PE=1 SV=6
	K1C9_HUMAN	Keratin, type I cytoskeletal 9 OS=Homo sapiens GN=KRT9 PE=1 SV=3
	K22E_HUMAN	Keratin, type II cytoskeletal 2 epidermal OS=Homo sapiens GN=KRT2 PE=1 SV=2
	K1C10_HUMAN	Keratin, type I cytoskeletal 10 OS=Homo sapiens GN=KRT10 PE=1 SV=6
	PLEC_HUMAN	Plectin OS=Homo sapiens GN=PLEC PE=1 SV=3
	HS90B_HUMAN	Heat shock protein HSP 90-beta OS=Homo sapiens GN=HSP90AB1 PE=1 SV=4
	HS90A_HUMAN	Heat shock protein HSP 90-alpha OS=Homo sapiens GN=HSP90AA1 PE=1 SV=5

6. HS90B_HUMAN **Mass:** 83212 **Score:** 783 **Matches:** 27(27) **Sequences:** 20(20) **emPAI:** 2.05
Heat shock protein HSP 90-beta OS=Homo sapiens GN=HSP90AB1 PE=1 SV=4

Query	Observed	Mr(expt)	Mr(calc)	ppm	Miss	Score	Expect	Rank	Unique	Peptide
593	415.2681	828.5217	828.5221	-0.47	0	49	1.1e-005	1	U	R.ALLFIPR.R
1449	473.7786	945.5425	945.5429	-0.41	1	17	0.043	1		R.IMKAQALR.D
1478	476.2356	950.4567	950.4570	-0.31	0	72	2e-007	1	U	R.ADHGEPIGR.G
2067	510.3034	1018.5922	1018.5923	-0.16	1	31	0.0016	1		K.NNIKLYVR.R
3102	571.2832	1140.5519	1140.5523	-0.34	0	43	0.0002	1	U	K.LGIHEDSTNR.R
3173	576.2826	1150.5506	1150.5506	0.02	0	27	0.011	1		K.YIDQEELNK.T
3388	597.8280	1193.6415	1193.6404	0.90	0	30	0.0023	1	U	K.IDIIPNPQER.T
3714	619.3178	1236.6210	1236.6211	-0.04	1	(27)	0.01	1	U	R.ADHGEPIGRGTK.V
3715	413.2143	1236.6211	1236.6211	-0.02	1	36	0.0014	1	U	R.ADHGEPIGRGTK.V
3743	621.8554	1241.6962	1241.6979	-1.36	0	73	1.7e-007	1		K.ADLINNLGTIAK.S
4194	656.2885	1310.5625	1310.5626	-0.08	0	42	9.3e-005	1		K.EDQTEYLEER.R
4396	675.3695	1348.7245	1348.7272	-2.02	0	73	1.3e-007	1	U	R.TLTLVDTGIGMTK.A
4527	683.3688	1364.7230	1364.7221	0.65	0	(54)	1.1e-005	1	U	R.TLTLVDTGIGMTK.A 4526
4798	704.8512	1407.6879	1407.6881	-0.15	1	58	7.7e-006	1		K.EKYIDQEELNK.T
4799	470.2368	1407.6887	1407.6881	0.38	1	(33)	0.0028	1		K.EKYIDQEELNK.T
4854	708.8225	1415.6304	1415.6303	0.03	0	34	0.00078	1		K.EGLELPEDEEEK.K
5344	499.5988	1495.7744	1495.7743	0.10	1	24	0.012	1	U	K.NLKLGIHEDSTNR.R
5412	757.3962	1512.7779	1512.7784	-0.33	0	83	2.2e-008	1		R.GVVDSEDLPLNISR.E 5413 5414
5675	782.9387	1563.8628	1563.8620	0.49	1	14	0.036	1	U	K.ELKIDIIPNPQER.T
7086	924.4024	1846.7902	1846.7897	0.24	0	99	1.6e-010	1	U	R.NPDDITQEEYGEFYK.S
8087	726.3199	2175.9380	2175.9379	0.06	0	70	1.2e-007	1	U	R.YHTSQSGDEMTSLSEYVSR.M
8687	816.7047	2447.0923	2447.0920	0.14	0	20	0.022	1	U	R.LVSSPCCIVTSTYGWTANMER.I
10233	1379.2929	4134.8569	4134.8586	-0.43	1	39	0.00012	1	U	K.LGLGIDEDEVAAEEPNAAVPDEIPPLEGDEDASRMEEVD.-
10246	1384.6251	4150.8535	4150.8536	-0.02	1	(39)	0.00013	1	U	K.LGLGIDEDEVAAEEPNAAVPDEIPPLEGDEDASRMEEVD.-

b)

Protein hits	:	
	AVID_CHICK	Avidin OS=Gallus gallus GN=AVD PE=1 SV=3
	K2C1_HUMAN	Keratin, type II cytoskeletal 1 OS=Homo sapiens GN=KRT1 PE=1 SV=6
	K1C10_HUMAN	Keratin, type I cytoskeletal 10 OS=Homo sapiens GN=KRT10 PE=1 SV=6
	K1C9_HUMAN	Keratin, type I cytoskeletal 9 OS=Homo sapiens GN=KRT9 PE=1 SV=3
	K22E_HUMAN	Keratin, type II cytoskeletal 2 epidermal OS=Homo sapiens GN=KRT2 PE=1 SV=2

1. AVID_CHICK **Mass:** 16758 **Score:** 424 **Matches:** 8(8) **Sequences:** 6(6) **emPAI:** 3.41
Avidin OS=Gallus gallus GN=AVD PE=1 SV=3

Query	Observed	Mr(expt)	Mr(calc)	ppm	Miss	Score	Expect	Rank	Unique	Peptide
772	460.2721	918.5296	918.5287	0.99	0	57	0.00018	1	U	R.VGINIFTR.L
1917	618.2802	1234.5459	1234.5466	-0.54	0	80	3.4e-007	1	U	R.SSVNDIGDDWK.A
2337	713.3613	1424.7080	1424.7089	-0.64	0	54	0.00026	1	U	R.TQPTFGFTVNWK.F
2660	782.3730	1562.7315	1562.7325	-0.65	1	92	3.1e-008	1	U	R.SSVNDIGDDWKATR.V 2661
3627	947.9370	1893.8594	1893.8567	1.41	0	63	1.5e-005	1	U	K.FSESTTVFTGQCFIDR.N
3818	1002.0018	2001.9890	2001.9895	-0.23	0	88	9.3e-008	1	U	R.GEFTGTYITAVTATSNEIK.E 3819

Figure S5: MASCOT results identifying proteins on lane LB75-tag 100μM. a) Proteins sequenced from 50-148kDa b) Proteins sequenced from 6-49kDa.

Domain Pull Down Assay

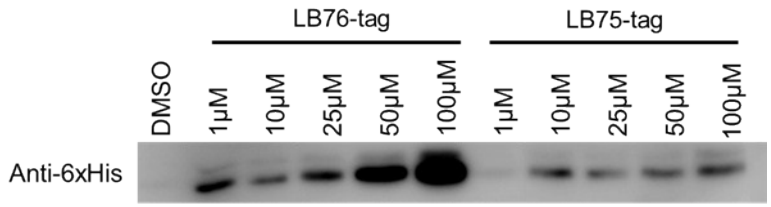
Pure recombinant Hsp90 CTD encompassing residues 553-734 was utilised in a pull-down assay with 1-100μM of tagged molecules and 25μM of purified Hsp90 domain. Tagged molecules were incubated with protein for 1 hours at room temperature (RT) while rocking. NeutrAvidin™ agarose resin was prepared according to manufacturer's protocol (Thermo Scientific, Cat#29202), added to each reaction, and incubated for an additional 1 hour at room temperature. Following the incubation with NeutrAvidin®, the supernatant was removed from each reaction, and the resin was washed six times with wash buffer (20mM Tris-HCl, 300mM NaCl, 1% Triton-X-100, pH 7.4). To elute proteins, beads were boiled with Laemmli sample buffer (10% SDS, 30% glycerol, 0.5 M DTT, 0.05% bromophenol blue, 250 mM Tris HCl, pH 6.8). The supernatant of each sample was loaded onto an 12% Tris-Glycine gel. The gel was made using the SureCast Gel Handcast system (Invitrogen, HC1000S) according to the manufacturer's protocol. The proteins were separated using SDS-PAGE gel electrophoresis.

Proteins were transferred onto a PVDF membrane which was blocked with 5% non-fat milk in TBS-T (Tris-buffered saline containing 0.1% Tween-20) for 1 hr at 4°C. The membrane was then incubated with 6xHis antibody (Anti-6X His tag, ab18184, 1:1000 dilution) in 2.5% non-fat milk (in

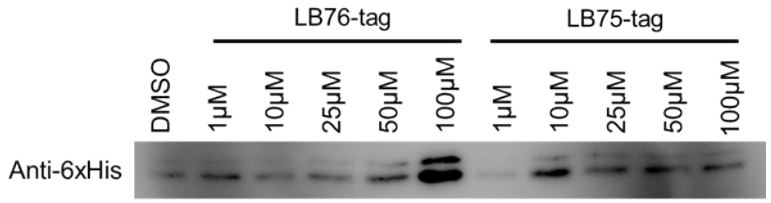
TBS-T) at 4 °C overnight. After washing with cold TBS-T, the membrane was incubated with the respective HRP-conjugated secondary antibody (rabbit anti-mouse, ab6728, 1:2000 diltuion) at 4 °C for 30 min, followed by six washes with cold TBS-T. Immunoblotting was performed using West Pico chemiluminescent substrate (Thermo scientific, 34580) and the images were captured by ImageQuant LAS 4010 digital imaging system (GE Healthcare). The relative amount of Hsp90 was analysed via Image J and transformed to a fold of Hsp90 pulled down relative to 100μM LB76-tag control, which was set at 100%. Each experiment was completed at least n=3.

Domain pull-down data

a)



b)



c)

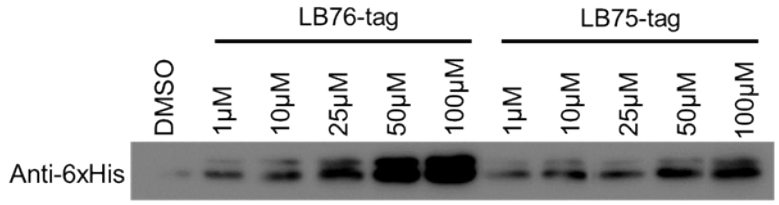


Figure S6: a) Pull-down of Hsp90α CTD using 1-100μM concentrations of compounds LB76-tag and LB75-tag (n=1), b) pull-down of Hsp90α CTD using 1-100μM concentrations of compounds LB76-tag and LB75-tag (n=2), c) Pull-down of Hsp90α CTD using 1-100μM concentrations of compounds LB76-tag and LB75-tag (n=3).

Binding Assay

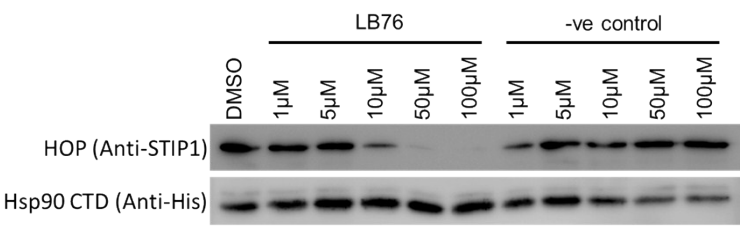
The binding affinity between Hsp90 domains and its co-chaperones (i.e., HOP, FKBP8) were completed using 100nM (final concentration) of recombinant 6xHis tagged Hsp90 CTD protein and 200nM of GST or FLAG tagged recombinant co-chaperone. Experiments were carried out with concentrations ranging from 0-100μM of tagged molecules. Hsp90 CTD was incubated with compounds in binding buffer (20mM Tris-HCl, 150mM NaCl, 1% Triton-X-100, pH 7.4) at various concentrations for 1 hour at room temperature (RT) with rocking. Co-chaperone was then added, and the reaction mixture was incubated for another 1 hour at RT with rocking. The Hsp90 CTD was fished out with Talon-Metal Affinity Resin. The resin underwent six washes in wash buffer (20mM Tris-HCl, 300mM NaCl, 1% Triton-X-100, pH 7.4). To elute protein complexes, samples were boiled in 5 x Laemmli sample buffer (10% SDS, 30% glycerol, 0.5 M DTT, 0.05% bromophenol blue, 250 mM Tris HCl, pH 6.8). The supernatant of each sample was loaded onto an 8% Tris-Glycine gel. The gel was made using the SureCast Gel Handcast system (Invitrogen, HC1000S) according to the manufacturer's protocol. The proteins were separated using SDS-PAGE gel electrophoresis.

Proteins were transferred onto a PVDF membrane which was blocked with 5% non-fat milk in TBS-T (Tris-buffered saline containing 0.1% Tween-20) for 1 hr at 4 0C. The membrane was then cut appropriate lengths and incubated with 6xHis antibody (Anti-6X His tag, ab18184, 1:1000 dilution) to detect Hsp90 CTD and either HOP primary antibody (abcam, ab126724,1:15000 dilution) or GST antibody to detect FKBP38 (anti-GST antibody, ab19256, 1:1000 dilution). Membranes were incubated in 2.5% non-fat milk (in TBS-T) at 4 °C overnight. The previous steps were repeated to detect HOP on the PVDF membrane. The respective ratio of Hsp90 CTD to either HOP or FKBP8 was analysed via Image J and transformed to a fold co-chaperone bound to CTD. Each experiment was completed at least n=3.

Binding Data

HOP Binding Assay

a)



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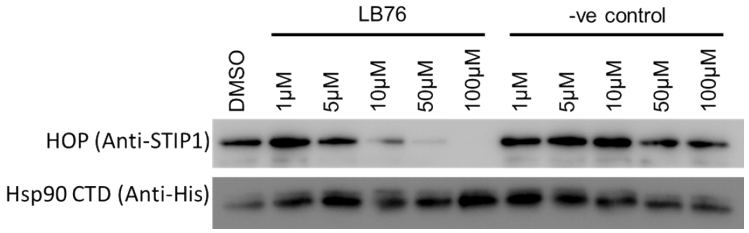


c)

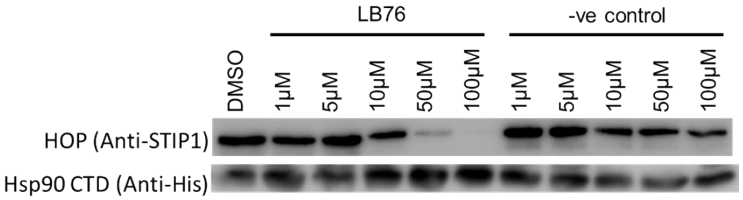
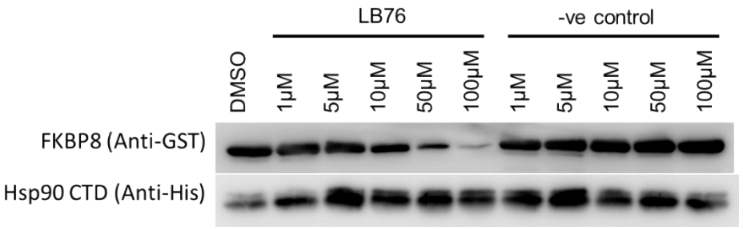


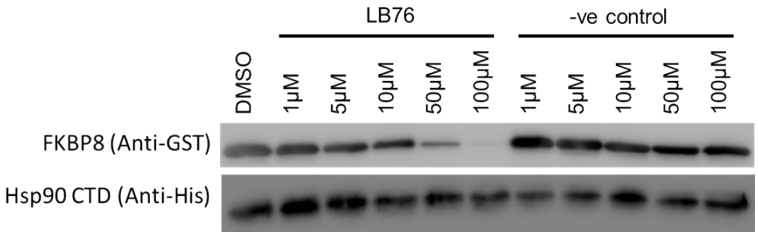
Figure S7: Binding assay between Flag-HOP and Hsp90α CTD using LB76 and -ve control molecule to determine inhibitory effect of compounds a) n=1, b) n=2, and c) n=3

FKBP38 Binding Assay

a)



b)



c)

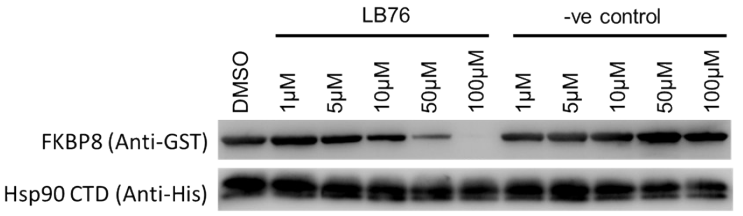


Figure S8: Binding assay between GST-FKBP8 and Hsp90α CTD using LB76 and -ve control molecule to determine inhibitory effect of compounds a) n=1, b) n=2, and c) n=3

Cyp40 Binding Assay

a) **b)**

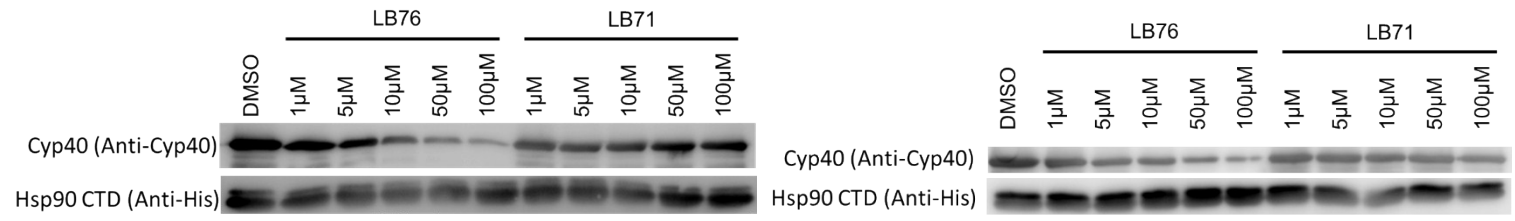


Figure S9: Binding assay between GST-Cyp40 and Hsp90α CTD using LB76 and -ve control molecule to determine inhibitory effect of compounds a) n=1, and b) n=2.

FKBP51 Binding Assay

a) **b)**

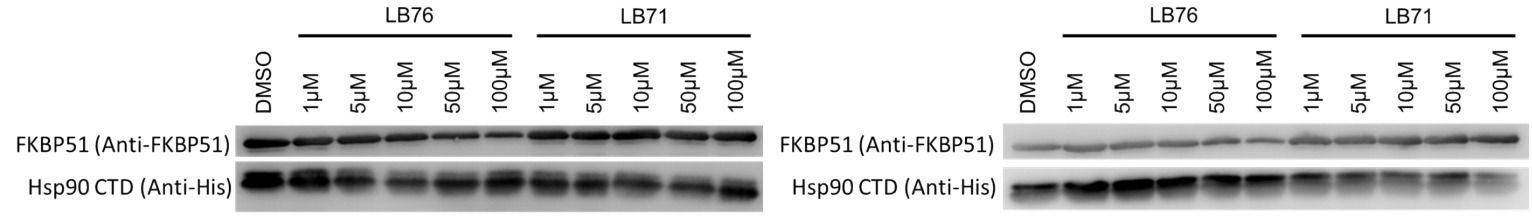


Figure S10: Binding assay between GST-Cyp40 and Hsp90α CTD using LB76 and -ve control molecule to determine inhibitory effect of compounds a) n=1, and b) n=2.

Luciferase Assay

The luciferase refolding assay was adapted from literature methods[1]. Luciferase (QuantiLum recombinant luciferase, Promega, E1701) was denatured in 2x denaturing buffer (25 mM HEPES, 50 mM potassium acetate, 5 mM DTT and 6 M Guanidine HCl, pH 7.2) at a concentration of 0.5 mg/mL for 60 mins at room temperature. Native luciferase was used as a control and was incubated in 1 x diluting buffer (25 mM HEPES (pH 7.6), 50 mM potassium acetate, 5 mM DTT) at a concentration of 0.5 mg/mL for 60 mins at room temperature. Both the native and denatured luciferase were then diluted 1 in 40 using 1 x diluting buffer on ice for 20 minutes.

Compounds to be tested were dissolved in 100% DMSO and diluted to final concentration of 50μM in reaction vessel by diluting in 1 x refolding buffer (28 mM HEPES (pH 7.6) 120 mM potassium acetate, 12 mM magnesium acetate, 2.2 mM DTT, 0.25 mM ATP, 8.8 mM creatine phosphate, 35 U/mL creatine kinase). Reactions were made up to a final volume of 50μL by adding the following proteins to final concentrations: 500nM Hsp72 (ENZO, ADI-NSP-555), 100 nM DnaJ (abcam, ab91598), 50 nM GrpE (abcam, ab63839), 50 nM Hsp90 (ENZO ADI-SPP-770-F), 50 nM HOP (StressMarq Biosciences, SPR-302-C) and finally 2 μL of denatured luciferase. Two control reactions were set up and consisted of 2 μL of either native or denatured luciferase, 1% DMSO and refolding buffer to a final volume of 50 μL.The reactions were incubated at room temperature. 2 μL aliquots of each reaction were taken at various time points and were combined with 48 μL of Bright-Glo reagent (Promega, E2610) in a white 384-well polystyrene microplate (PerkinElmer, 6007290). The luminescence of the respective wells was measured using a Tecan F200 Pro multimode plate reader. The final data was plotted as the % of luciferase refolded over time relative to the maximum luminescence signal from DMSO at 5 hours.

To determine whether the compounds interacted with native or denatured luciferase the following reactions were set-up: 2 µL of native or denatured luciferase, compound at 50 µM concentrations and 1 x refolding buffer to final volume of 50 µL. The reactions were incubated for 20 minutes on ice. 2 µL aliquots of each reaction was combined with 23 µL of Bright-Glo reagent (Promega, E2610) in a white 384-well polystyrene microplate. The luminescence of these wells was measured, and the raw luminescence values were plotted.

Luciferase Data: Raw Data

Table S1: Raw luminescence values of luciferase assay (n=1).

	Raw Luminescence				
Time/ mins	**Denatured luciferase**	**Native luciferase**	**1% DMSO**	**LB76 50µM**	**LB75 50 µM**
0	124	31139	1088	488	1520
60	78	32237	1672	1132	1558
120	48	25462	2993	1760	3581
180	42	24166	5000	2285	5222
240	34	14124	6302	2182	6621
300	34	15290	8040	2530	8070

Table S2: Protein refolding relative to 1% DMSO control (n=1).

	Relative refolding (%)				
Time/ mins	**Denatured luciferase**	**Native luciferase**	**1% DMSO**	**LB76 50µM**	**LB75 50 µM**
0	1.54	387.30	13.53	6.07	18.91
60	0.97	400.96	20.80	14.08	19.38
120	0.60	316.69	37.23	21.89	44.54
180	0.52	300.57	62.19	28.42	64.95
240	0.42	175.67	78.38	27.14	82.35
300	0.42	190.17	100.00	31.47	100.37

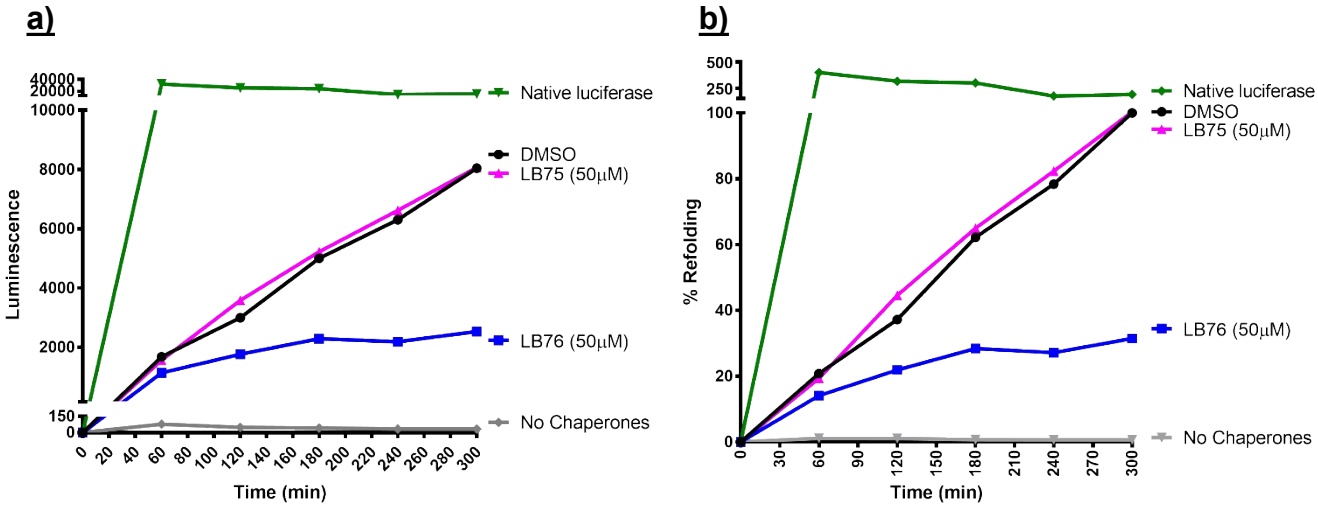


Figure S11: a) Raw luminescence data of luciferase assay (n=1) and b) Protein refolding relative to 1% DMSO control (n=1).

Table S3: Raw luminescence values of luciferase assay (n=2).

	Raw Luminescence				
Time/ mins	**Denatured luciferase**	**Native luciferase**	**1% DMSO**	**LB76 50µM**	**LB75 50 µM**
30	236	433495	856	138	692
60	268	420229	1498	812	1490

120	184	422882	2138	1252	2610
180	176	351435	3408	1352	3816
240	128	271228	4619	1880	4379
300	122	243756	6388	2109	6139

Table S4: Protein refolding relative to 1% DMSO control (n=2).

	Relative refolding (%)				
Time/ mins	**Denatured luciferase**	**Native luciferase**	**1% DMSO**	**LB76 50µM**	**LB75 50 µM**
30	3.69	6786.08	13.40	2.16	10.83
60	4.20	6578.41	23.45	12.71	23.32
120	2.88	6619.94	33.47	19.60	40.86
180	2.76	5501.49	53.35	21.16	59.74
240	2.00	4245.90	72.31	29.43	68.55
300	1.91	3815.84	100.00	33.02	96.10

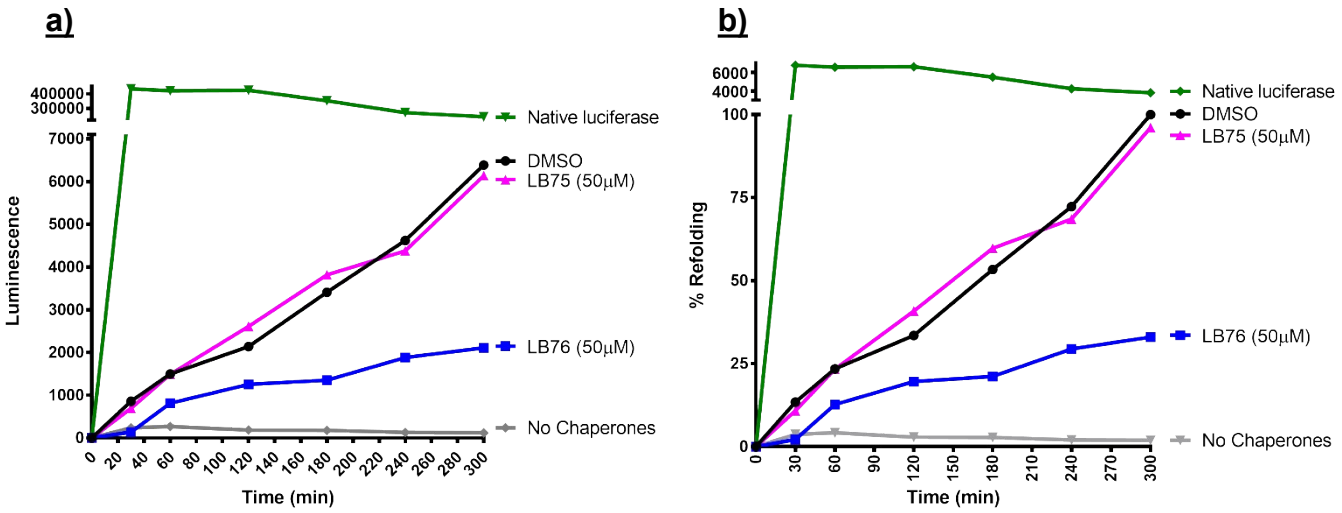


Figure S12: a) Raw luminescence data of luciferase assay (n=2) and b) Protein refolding relative to 1% DMSO control (n=2).

Table S5: Raw luminescence values of luciferase assay (n=3).

	Raw Luminescence				
Time/ mins	**Denatured luciferase**	**Native luciferase**	**1% DMSO**	**LB76 50µM**	**LB75 50 µM**
30	462	147018	1228	388	1462
60	436	95819	3486	1824	3102
120	438	161850	6426	3140	6978
180	582	177743	10430	3622	11487
240	424	139290	13816	4458	13820
300	304	132291	18579	5935	17140

Table S6: Protein refolding relative to 1% DMSO control (n=3).

	Relative refolding (%)				
Time/ mins	**Denatured luciferase**	**Native luciferase**	**1% DMSO**	**LB76 50µM**	**LB75 50 µM**
30	2.49	791.31	6.61	2.09	7.87
60	2.35	515.74	18.76	9.82	16.70
120	2.36	871.14	34.59	16.90	37.56
180	3.13	956.69	56.14	19.50	61.83
240	2.28	749.72	74.36	23.99	74.39

300	1.64	712.05	100.00	31.94	92.25

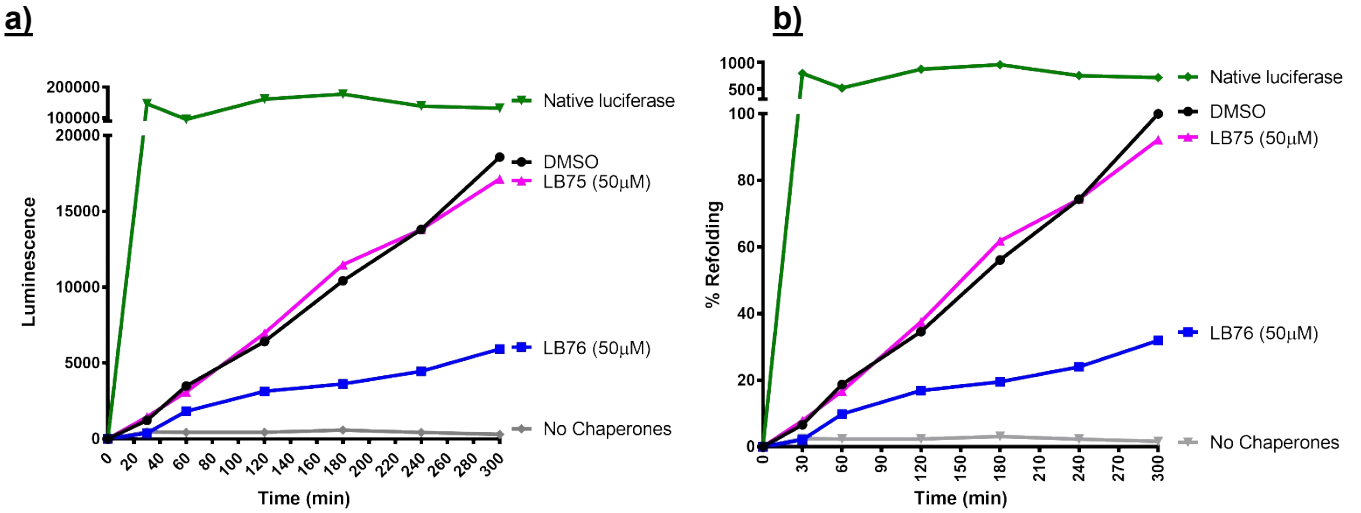


Figure S13: a) Raw luminescence data of luciferase assay (n=3) and b) Protein refolding relative to 1% DMSO control (n=3).

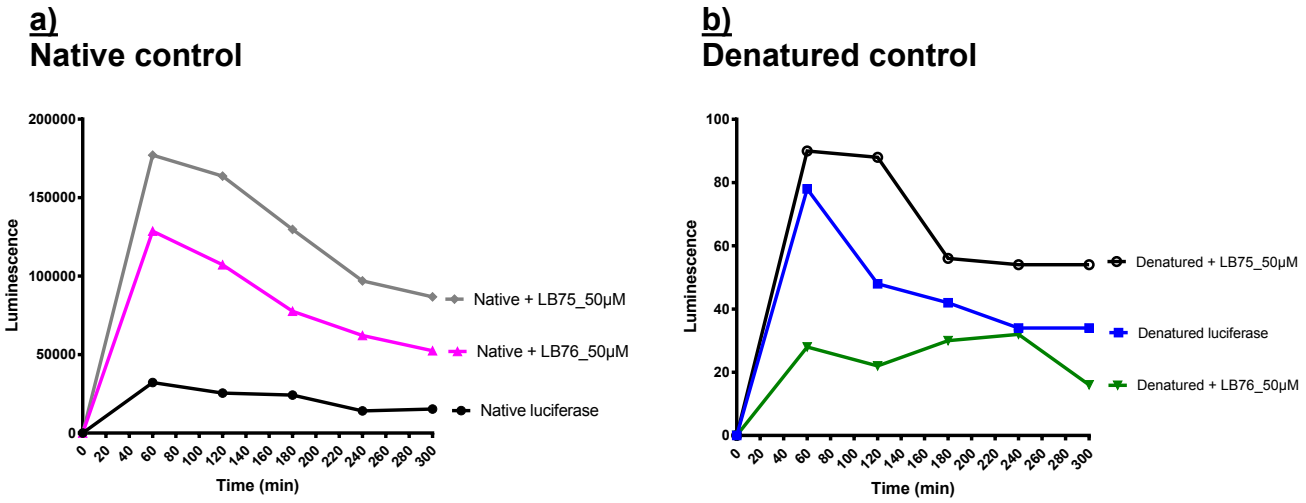


Figure S14: a) Luciferase control assay of native protein interacting with compounds. Compounds do not have inherent refolding or denaturing characteristics, b) Luciferase control assay of denatured protein interacting with compounds. Compounds do not have inherent refolding or denaturing characteristics,

Statistical Analysis

All Mean±SEM was calculated using GraphPad Prism 7 Software and Microsoft Excel. All western blot analysis was completed with Image J software.

General Synthetic Remarks

All chemicals were purchased from commercial suppliers (Chem-Impex International and Sigma Aldrich) and used without further purification. All moisture sensitive reactions were performed using anhydrous solvents under nitrogen gas. Removal of solvent was carried out under reduced pressure using a Buchi R-210 rotary evaporator.

Thin Layer Chromatography (TLC) was performed on aluminium silica gel sheets (Merck TLC silica gel 60 F254). Spots were visualised under ultraviolet light (λ = 254 nm) and developed by heating with ninhydrin solution.LC/MS analyses were performed using a Phenomenex Aeris XB-C18 column (3.6 µm, 2.1 x 100 mm) on either a Shimadzu LCMS 2020, Shimadzu LCMS 8030 or LCQ Deca XP Plus (Thermo Finnigan). The mobile phase consisted of milli-Q water with 0.1% (v/v) formic acid (Mobile Phase A), and HPLC grade acetonitrile with 0.1% (v/v) formic acid (Mobile Phase B) at a flow rate of 0.2 mL/min, starting at 95% Mobile Phase A and 5% Mobile Phase B.

Semi-preparative HPLC for purification was performed using a GRACE VisionHT C18 column (5 µm, 22 x 150 mm) or a Phenomenex Aeris XB-C18 column (5 µm, 21.2 x 150 mm) on aShimadzu Prominence system. The mobile phase consisted of milli-Q water with 0.1% (v/v) formic acid (Mobile Phase A), and HPLC grade acetonitrile with 0.1% (v/v) formic acid (Mobile Phase B) at a flow rate of 5 mL/min, starting at 95% Mobile Phase A and 5% Mobile Phase B.

^{1}H NMR spectras were obtained on Bruker Avance III 600 MHz. Multiplicity of NMR signals were represented by the following abbreviations: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, br = broad, dd = doublet of doublet. Assignment of resonances for each residue was accomplished using ^{1}H, HSQC, HMBC and COSY spectra.

General Synthetic Procedures

Solid Phase Peptide Synthesis

Stepwise SPPS was performed in a polypropylene solid-phase extraction cartridge fitted with a 20 µm polyethylene frit purchased from Applied Separations (Allentown, PA).

Resin Loading

The resin was weighed, transferred to the cartridge and swelled in CH_2Cl_2 for 1 hour prior to the resin loading reaction. The appropriate Fmoc-protected amino acid was dissolved in the minimum amount of 0.4M DIPEA in CH_2Cl_2. The swelled resin was then drained, and the dissolved amino acid was added. The suspension was agitated for 4 hours. The resin was then washed 3 times with CH_2Cl_2, 3 times with DMF and 3 times with CH_2Cl_2. The resin was then dried in vacuo overnight. A 5 mg sample of resin was used to determine the amino acid loading. 20% piperidine in DMF was added to the sample to cleave the Fmoc protecting group. The resin was filtered away and the remaining solution was diluted 1 in 20 and the UV absorbance measured at 301 nm using a Cary 50 Bio UV-Vis instrument. DMF was used as a blank and samples were measured in a 1 mL quartz cuvette.

Coupling Reaction

Couplings were performed in DMF at a concentration of 0.3 M. Fmoc-protected amino acid (2 equiv.) and HOBt (2 equiv.) were mixed with the resin. DIC (4 equiv.) was then added to activate the reaction. Coupling reaction was run for a minimum of 2 hours while shaking (Labquake tube shaker, Thermo Fisher Scientific) at room temperature. A negative ninhydrin test was used to confirm reaction completion. Once completed, the reaction mixture was drained and the resin was

subjected to *Fmoc Removal*. (Note: For particularly hindered coupling reactions, HOBt was replaced with HOAt.

Fmoc Removal

The Fmoc protecting group was removed using the following washes: DMF (3 x 1 min), 20% piperidine in DMF (1 x 5 min), 20% piperidine in DMF (1 x 10 min), DMF (2 x 1 min), *i*-PrOH (1 x 1 min), DMF (1 x 1 min), *i*-PrOH (1 x 1 min) and DMF (3 x 1 min). The resin was then ready for the next coupling reaction.

Resin Cleavage of Linear Peptide

Once the desired peptide was generated, the final Fmoc protecting group was removed following Fmoc Removal procedure with the following additional washes: DMF (3 x 1 min), *i*-PrOH (3 x 1 min) and MeOH (3 x 1 min). The resin-bound peptide was then dried *in vacuo* overnight. The resin was then cleaved from the linear peptide using TFE and CH_2Cl_2 (1:1 v/v) at a concentration of 10 mL/g resin. The reaction mixture was stirred at room temperature for 24 hours before filtering the resin. The filtrate was concentrated and washed at least 10 times with CH_2Cl_2 to remove residual entrapped TFE. The product was then dried *in vacuo* overnight to produce the linear peptide.

Macrocyclisation

Macrocyclisation of the linear peptide was achieved using a cocktail of 3 coupling reagents: HATU (1 eq.), TBTU (0.5 equiv.) and DMTMM (0.5 equiv.). The reaction was performed in dilute conditions using anhydrous solvents at concentration of 0.001 M. The linear peptide and coupling reagents were dissolved separately in CH_2Cl_2, where 20% of the final volume was used to dissolve the linear peptide and the other 80% dissolved the coupling reagents. DIPEA (4 equiv.) was added to each solution. The linear peptide solution was then added drop-wise to the coupling reagents solution via a syringe pump over approximately 2 hours. The reaction was stirred overnight and monitored via LC/MS. (Note: if the reaction failed to reach completion after stirring overnight, additional HATU (1 equiv.) was added and the reaction was monitored using LC/MS.) Upon completion, the reaction mixture was evaporated, and the dry solid was redissolved in CH_2Cl_2 and extracted 3 times with milli-Q water. The aqueous layers were combined and extracted 3 times with fresh CH_2Cl_2. All organic layers were combined and dried over Na_2SO_4, filtered and evaporated under reduced pressure before the compound was dried in vacuo overnight.

Side Chain Deprotection

Amino acid side chain protecting groups were removed using TFA in CH_2Cl_2 (9:1 v/v) with anisole (2 equiv. per protecting group). Anisole was added to the peptide, whilst stirring, followed by the TFA solution at a concentration of 4 mL/g compound. The reaction was left stirring at room temperature for 4 hours. The reaction was monitored using LC/MS and once complete the reaction solution was dried under a stream of nitrogen before redissolving in CH_2Cl_2 and evaporating multiple times to remove residual entrapped TFA. The product was triturated in diethyl ether, collected via centrifugation and lyophilised to produce the crude cyclic peptide.

Biotin tagging of Lysine Side Chain

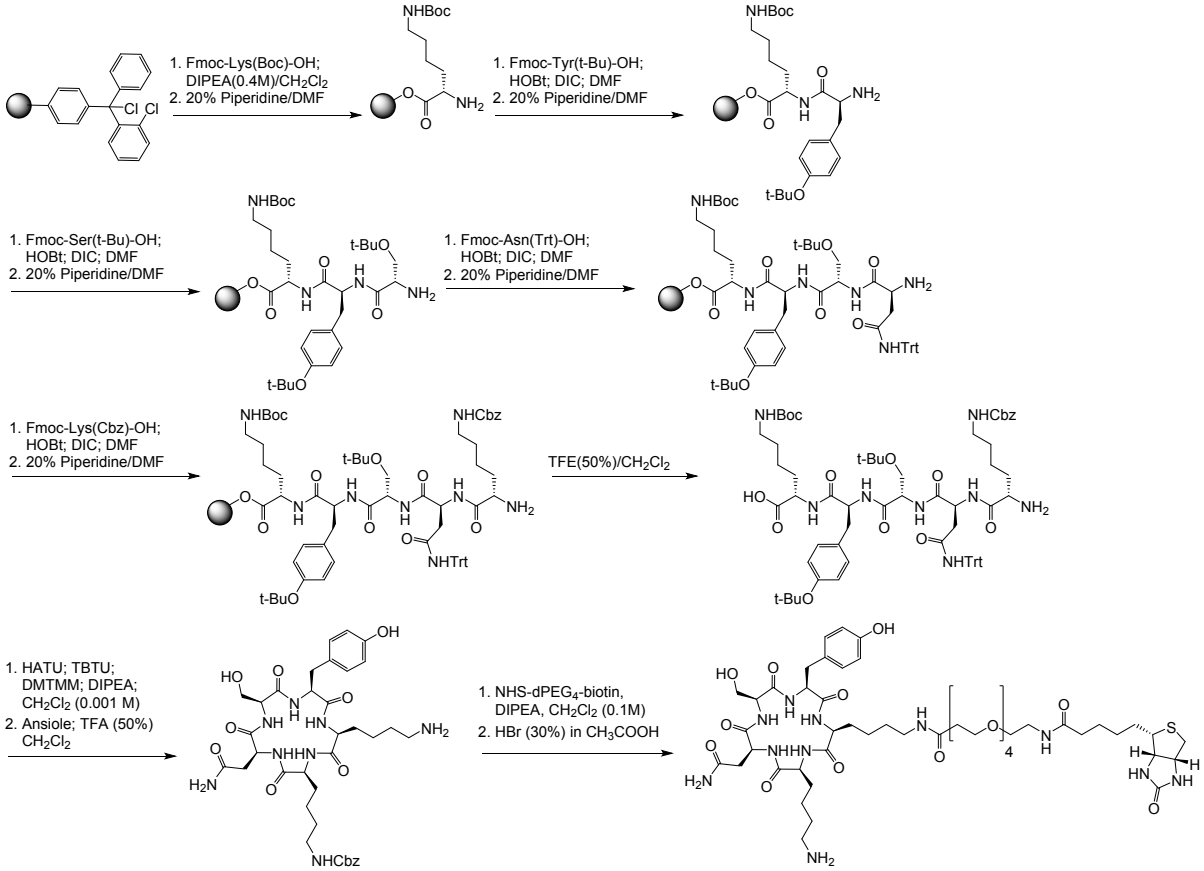
Peg4 biotin tag was coupled onto the free lysine residue using N-hydroxysuccinimydyl-d-biotin-15-amino-4,7,10,13-tetraoxapentadecylate (NHS-Peg-4-biotin) (2 equiv.) with DIPEA (8 equiv.). Partially protected peptide and NHS Peg4 Biotin were mixed in solution. While stirring, DIPEA was added dropwise to start reaction. The reaction was left stirring at room temperature for 4 hours. The reaction was monitored using LC/MS and once complete the reaction solution was dried under a stream of nitrogen before redissolving in CH_2Cl_2 and evaporating multiple times to remove DIPEA.

Cbz removal

Final cbz protecting group was removed from tagged peptide using hydrogen bromide solution in acetic acid. Tagged molecule was dissolved in CH_2Cl_2 (0.1M) and allowed to stir. To this was added anisole. Following stirring, HBr in acetic acid was added dropwise to the stirring mixture. The reaction was left stirring at room temperature for 4 hours. The reaction was monitored using LC/MS and once complete the reaction solution was dried under a stream of nitrogen before redissolving in CH_2Cl_2 and evaporating multiple times to remove residual acid. The product was triturated in diethyl ether, collected via centrifugation and lyophilised to produce the crude tagged cyclic peptide.

Experimental Procedure for LB76-tag

Supplementary Scheme 1



Resin-O-Lys(Boc)-NH_2

The resin bound amino acid Resin-O-Lys(Boc)-NH_2 was synthesized following the *Resin Loading* procedure. A sample of 2-chlorotrityl chloride resin (1g, 1.1 mmol/g loading, 1 equiv.) was placed in a solid phase tube, swelled with CH_2Cl_2 for 1 hour and then drained. Fmoc-Lys(Boc)-OH (0.56g,1.1 mmol, 2 equiv.) was pre-dissolved in the minimum volume of DIPEA in CH_2Cl_2 (0.4 M) and was added to the swelled resin. The reaction was agitated at room temperature for 4 hours. The reaction mixture was drained, and the resin was washed according to the *Resin Loading* protocol to produce Resin-O-Lys(Boc)-NHFmoc. A sample of resin was removed, and the resin loading was determined to be 0.72 mmol/g. The Fmoc group was then removed using the *Fmoc Removal* procedure to produce Resin-O-Lys(Boc)-NH_2.

Resin-O-Lys(Boc)-Tyr(t-Bu)-NH_2

Resin-O-Lys(Boc)-Tyr(t-Bu)-NH_2 was synthesized according to the *Coupling Reaction* procedure, using Resin-O-Lys(Boc)-NH_2 (0.72 mmol, 1 equiv.), 0.66 g Fmoc-Tyr(tBu)-OH (1.4 mmol, 2 equiv.), 3.6 mL HOAt (2.1 mmol, 3 equiv.), 0.67 mL DIC (4.3 mmol, 6 equiv.) and 0.7 mL of DMF to generate a concentration of 0.1 M. The coupling reaction was run for 2 hours and a negative ninhydrin test was used to confirm reaction completion. The reaction mixture was drained to afford Resin-O-Lys(Boc)-Tyr(t-Bu)-NHFmoc. The Fmoc group was then removed following the *Fmoc Removal* procedure to afford Resin-O-Lys(Boc)-Tyr(t-Bu)-NH_2.

Resin-O-Lys(Boc)-Tyr(t-Bu)-Ser(t-Bu)-NH_2

Resin-O-Lys(Boc)-Tyr(t-Bu)-Ser(t-Bu)-NH_2 was synthesized according to the *Coupling Reaction* procedure, using Resin-O-Lys(Boc)-Tyr(t-Bu)-NH_2 (0.72 mmol, 1 equiv.), 0.55 g Fmoc-Ser(tBu)-OH (1.4 mmol, 2 equiv.), 3.6 mL HOAt (2.1 mmol, 3 equiv.), 0.67 mL DIC (4.3 mmol, 6 equiv.)

and 0.7 mL of DMF to generate a concentration of 0.1 M. The coupling reaction was run for 2 hours and a negative ninhydrin test was used to confirm reaction completion. The reaction mixture was drained to afford Resin-O-Lys(Boc)-Tyr(t-Bu)-Ser(t-Bu)-NHFmoc. The Fmoc group was then removed following the *Fmoc Removal* procedure to afford Resin-O-Lys(Boc)-Tyr(t-Bu)-Ser(t-Bu)-NH_2.

Resin-O-Lys(Boc)-Tyr(t-Bu)-Ser(t-Bu)-Asn(Trt)-NH_2

Resin-O-Lys(Boc)-Tyr(t-Bu)-Ser(t-Bu)-Asn(Trt)-NH_2 was synthesized according to the *Coupling Reaction* procedure, using Resin-O-Lys(Boc)-Tyr(t-Bu)-Ser(t-Bu)-NH_2 (0.72 mmol, 1 equiv.), 0.86 g Fmoc-Asn(Trt)-OH (1.4 mmol, 2 equiv.), 3.6 mL HOAt (2.1 mmol, 3 equiv.), 0.67 mL DIC (4.3 mmol, 6 equiv.) and 0.7 mL of DMF to generate a concentration of 0.1 M. The coupling reaction was run for 2 hours and a negative ninhydrin test was used to confirm reaction completion. The reaction mixture was drained to afford Resin-O-Lys(Boc)-Tyr(t-Bu)-Ser(t-Bu)-Asn(Trt)-NHFmoc. The Fmoc group was then removed following the *Fmoc Removal* procedure to afford Resin-O-Lys(Boc)-Tyr(t-Bu)-Ser(t-Bu)-Asn(Trt)-NH_2.

Resin-O-Lys(Boc)-Tyr(t-Bu)-Ser(t-Bu)-Asn(Trt)-Lys(Cbz)-NH_2

Resin-O-Lys(Boc)-Tyr(t-Bu)-Ser(t-Bu)-Asn(Trt)-Lys(Cbz)-NH_2 was synthesized according to the *Coupling Reaction* procedure, using Resin-O-Lys(Boc)-Tyr(t-Bu)-Ser(t-Bu)-Asn(Trt)-NH_2 (0.72 mmol, 1 equiv.), 0.71 g Fmoc-Lys(Cbz)-OH (1.4 mmol, 2 equiv.), 3.6 mL HOAt (2.1 mmol, 3 equiv.), 0.67 mL DIC (4.3 mmol, 6 equiv.) and 0.7 mL of DMF to generate a concentration of 0.1 M. The coupling reaction was run for 2 hours and a negative ninhydrin test was used to confirm reaction completion. The reaction mixture was drained to afford Resin-O-Lys(Boc)-Tyr(t-Bu)-Ser(t-Bu)-Asn(Trt)-Lys(Cbz)-NHFmoc. The Fmoc group was then removed following the *Fmoc Removal* procedure to afford Resin-O-Lys(Boc)-Tyr(t-Bu)-Ser(t-Bu)-Asn(Trt)-Lys(Cbz)-NH_2.

HO-Lys(Boc)-Tyr(t-Bu)-Ser(t-Bu)-Asn(Trt)-Lys(Cbz)-NH_2

The protected linear peptide HO-Lys(Boc)-Tyr(t-Bu)-Ser(t-Bu)-Asn(Trt)-Lys(Cbz)-NH_2 was generated following the *Resin Cleavage* procedure. The linear peptide was cleaved from the resin using a solution of 5 mL of trifluoroethanol and 5 mL of CH_2Cl_2. The resin containing solution was filtered and dried in vacuo to yield the protected linear pentapeptide as a pale yellow solid (0.69 g, 72% overall).

cyclo-Lys(Boc)-Tyr(t-Bu)-Ser(t-Bu)-Asn(Trt)-Lys(Cbz)

The protected cyclic peptide *cyclo*-Lys(Boc)-Tyr(t-Bu)-Ser(t-Bu)-Asn(Trt)-Lys(Cbz) was synthesised following the *Macrocyclisation* procedure using 0.12 g HO-Lys(Boc)-Tyr(t-Bu)-Ser(t-Bu)-Asn(Trt)-Lys(Cbz)-NH_2 (0.1 mmol, 1 equiv.), 0.06 g HATU (0.15 mmol, 1.5 equiv.), 0.02 g TBTU (0.05 mmol, 0.5 equiv.), 0.01 g DMTMM (0.05 mmol, 0.5 equiv.), 0.14 mL DIPEA (0.8 mmol, 8 equiv.) in anhydrous CH_2Cl_2 (101 mL, 0.001 M), The reaction was then stirred for 2 hours and monitored via LC/MS. Upon completion, the reaction mixture was worked up as described in the *Macrocyclisation* procedure and dried in vacuo to produce the crude, protected, cyclic peptide *cyclo*-Lys(Boc)-Tyr(t-Bu)-Ser(t-Bu)-Asn(Trt)-Lys(Cbz).

cyclo-Lys-Tyr-Ser-Asn-Lys(Cbz)

The partially deprotected cyclic peptide *cyclo*-Lys-Tyr-Ser-Asn-Lys(Cbz) was synthesised following the *Side Chain Deprotection* procedure using 0.1 g *cyclo*-Lys(Boc)-Tyr(t-Bu)-Ser(t-Bu)-Asn(Trt)-Lys(Cbz) (0.08 mmol, 1 equiv.), 1.4 mL of a mixed solution of TFA and CH_2Cl_2 (9:1 v/v) and 0.24 mL anisole (2.2 mmol, 15 equiv.) to remove the side chain protecting groups. The reaction mixture was stirred for 4 hours and monitored via LC/MS. Upon completion, the reaction

mixture was worked up as described in the *Global Deprotection* procedure to afford the crude cyclic peptide *cyclo*-Lys-Tyr-Ser-Asn-Lys(Cbz).

cyclo-Lys-Tyr-Ser-Asn-Lys(Cbz) tagged Peg4 Biotin

The partially protected cyclic tagged peptide *cyclo*-Lys-Tyr-Ser-Asn-Lys(Cbz) tagged Peg4 was synthesised following the *Biotinylation* procedure using 0.09 g *cyclo*-Lys-Tyr-Ser-Asn-Lys(Cbz) (0.1 mmol, 1 equiv.), 0.14 g EZ-Link NHS- PEG_4-Biotin (0.23 mmol, 2 equiv.), 0.33 mL DIPEA (1.9 mmol, 16 equiv.) in anhydrous CH_2Cl_2 (0.68 mL, 0.1 M). The reaction was then stirred for 4 hours and monitored via LC/MS. Upon completion, the reaction mixture was drained, and the crude material washed multiple times with CH_2Cl_2 before being carried to final Cbz removal.

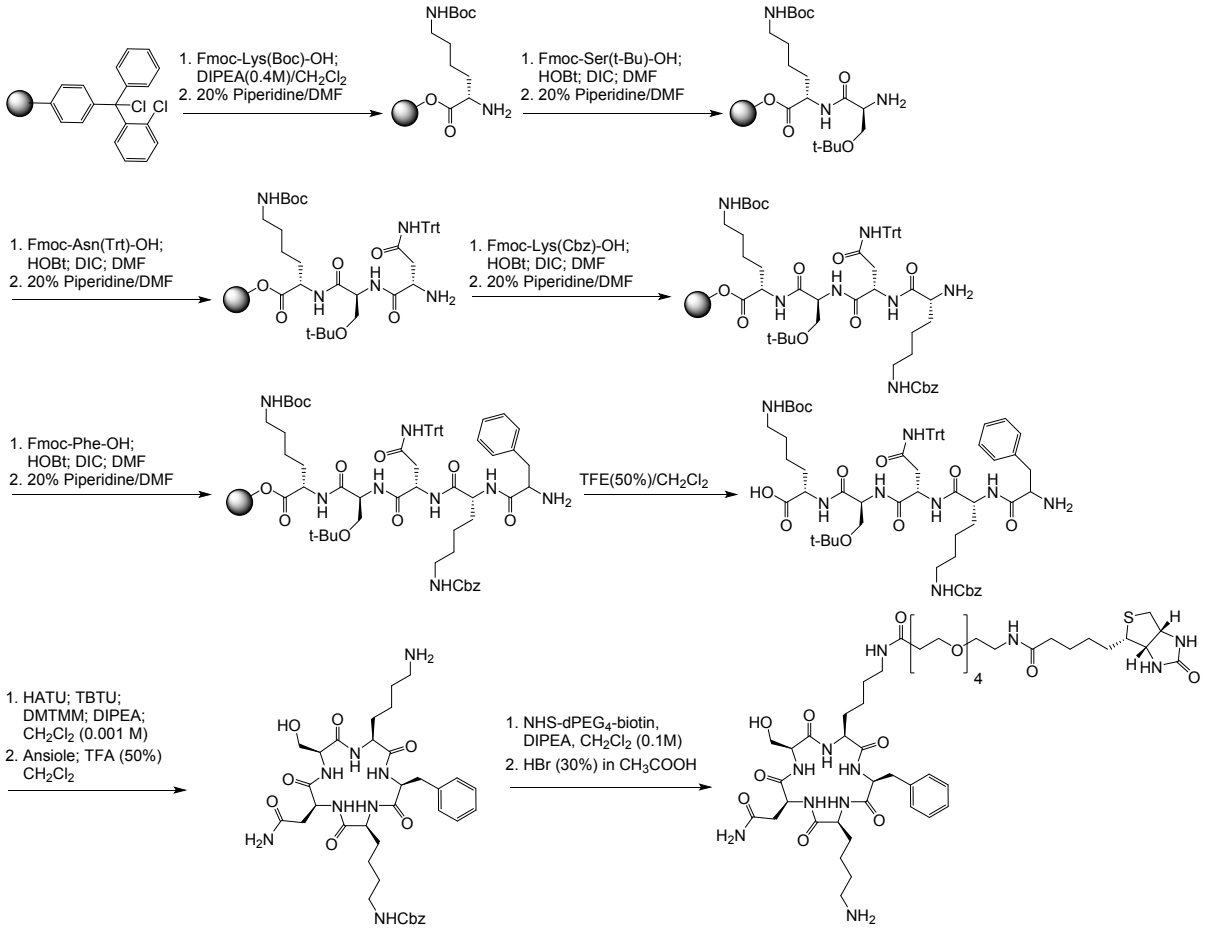
cyclo-Lys-Tyr-Ser-Asn-Lys tagged Peg4 Biotin

The final cyclic tagged peptide *cyclo*-Lys-Tyr-Ser-Asn-Lys tagged Peg4 Biotin was synthesised following *Cbz removal* procedure using 30mg *cyclo*-Lys-Tyr-Ser-Asn-Lys(Cbz) tagged Peg4 Biotin (0.02 mmol, 1 equiv.), 0.25 mL hydrogen bromide solution (33%) in acetic acid (0.1 M) and 0.02 mL anisole (0.18 mmol, 8 equiv.). The reaction was stirred for 4 hours and monitored via LC/MS. Upon completion, the reaction mixture was washed multiple times with CH_2Cl_2. The crude product was purified using HPLC, then lyophilised to generate pure compound as a white solid (9 mg, 15% overall).

LC/MS (ESI) *m/z;* $[M+H]^+$ calculated for $C_{49}H_{79}N_{11}O_{15}S^+$ 1093.55, found 1094.77

Experimental Procedure for LB75-tag

Supplementary Scheme 2



Resin-O-Lys(Boc)-NH_2

The resin bound amino acid Resin-O-Lys(Boc)-NH_2 was synthesized following the *Resin Loading* procedure. A sample of 2-chlorotrityl chloride resin (1g, 1.1 mmol/g loading, 1 equiv.) was placed in a solid phase tube, swelled with CH_2Cl_2 for 1 hour and then drained. Fmoc-Lys(Boc)-OH (0.75g,1.1 mmol, 2.5 equiv.) was pre-dissolved in the minimum volume of DIPEA in CH_2Cl_2 (0.4 M) and was added to the swelled resin. The reaction was agitated at room temperature for 4 hours. The reaction mixture was drained, and the resin was washed according to the *Resin Loading* protocol to produce Resin-O-Lys(Boc)-NHFmoc. A sample of resin was removed, and the resin loading was determined to be 0.72 mmol/g. The Fmoc group was then removed using the *Fmoc Removal* procedure to produce Resin-O-Lys(Boc)-NH_2.

Resin-O-Lys(Boc)-Ser(t-Bu)-NH_2

Resin-O-Lys(Boc)-Ser(t-Bu)-NH_2 was synthesized according to the *Coupling Reaction* procedure, using Resin-O-Lys(Boc)-NH_2 (0.72 mmol, 1 equiv.), 0.55 g Fmoc-Ser(tBu)-OH (1.4 mmol, 2 equiv.), 3.6 mL HOAt (2.1 mmol, 3 equiv.), 0.67 mL DIC (4.3 mmol, 6 equiv.) and 0.7 mL of DMF to generate a concentration of 0.1 M. The coupling reaction was run for 2 hours and a negative ninhydrin test was used to confirm reaction completion. The reaction mixture was drained to afford Resin-O-Lys(Boc)-Ser(t-Bu)-NHFmoc. The Fmoc group was then removed following the *Fmoc Removal* procedure to afford Resin-O-Lys(Boc)-Ser(t-Bu)-NH_2.

Resin-O-Lys(Boc)-Ser(t-Bu)-Asn(Trt)-NH_2

Resin-O-Lys(Boc)-Ser(t-Bu)-Asn(Trt)-NH_2 was synthesized according to the *Coupling Reaction* procedure, using Resin-O-Lys(Boc)-Ser(t-Bu)-NH_2 (0.72 mmol, 1 equiv.), 0.85 g Fmoc-Asn(Trt)-OH (1.4 mmol, 2 equiv.), 3.6 mL HOAt (2.1 mmol, 3 equiv.), 0.67 mL DIC (4.3 mmol, 6 equiv.) and 0.7 mL of DMF to generate a concentration of 0.1 M. The coupling reaction was run for 2 hours and a negative ninhydrin test was used to confirm reaction completion. The reaction mixture was drained to afford Resin-O-Lys(Boc)-Ser(t-Bu)-Asn(Trt)-NHFmoc. The Fmoc group was then removed following the *Fmoc Removal* procedure to afford Resin-O-Lys(Boc)-Ser(t-Bu)-Asn(Trt)-NH_2.

Resin-O-Lys(Boc)-Ser(t-Bu)-Asn(Trt)-Lys(Cbz)-NH_2

Resin-O-Lys(Boc)-Ser(t-Bu)-Asn(Trt)-Lys(Cbz)-NH_2 was synthesized according to the *Coupling Reaction* procedure, using Resin-O-Lys(Boc)-Ser(t-Bu)-Asn(Trt)-NH_2 (0.72 mmol, 1 equiv.), 0.67 g Fmoc-Lys(Cbz)-OH (1.4 mmol, 2 equiv.), 3.6 mL HOAt (2.1 mmol, 3 equiv.), 0.67 mL DIC (4.3 mmol, 6 equiv.) and 0.7 mL of DMF to generate a concentration of 0.1 M. The coupling reaction was run for 2 hours and a negative ninhydrin test was used to confirm reaction completion. The reaction mixture was drained to afford Resin-O-Lys(Boc)-Ser(t-Bu)-Asn(Trt)-Lys(Cbz-)NHFmoc. The Fmoc group was then removed following the *Fmoc Removal* procedure to afford Resin-O-Lys(Boc)-Ser(t-Bu)-Asn(Trt)-Lys(Cbz)-NH_2.

Resin-O-Lys(Boc)-Ser(t-Bu)-Asn(Trt)-Lys(Cbz)-Phe-NH_2

Resin-O-Lys(Boc)-Ser(t-Bu)-Asn(Trt)-Lys(Cbz)-Phe-NH_2 was synthesized according to the *Coupling Reaction* procedure, using Resin-O-Lys(Boc)-Ser(t-Bu)-Asn(Trt)-Lys(Cbz)-NH_2 (0.72 mmol, 1 equiv.), 0.71 g Fmoc-Phe-OH (0.61 mmol, 2 equiv.), 3.6 mL HOAt (2.1 mmol, 3 equiv.), 0.67 mL DIC (4.3 mmol, 6 equiv.) and 0.7 mL of DMF to generate a concentration of 0.1 M. The coupling reaction was run for 2 hours and a negative ninhydrin test was used to confirm reaction completion. The reaction mixture was drained to afford Resin-O-Lys(Boc)-Ser(t-Bu)-Asn(Trt)-Lys(Cbz)-Phe-NHFmoc. The Fmoc group was then removed following the *Fmoc Removal* procedure to afford Resin-O-Lys(Boc)-Ser(t-Bu)-Asn(Trt)-Lys(Cbz)-Phe-NH_2.

HO-Lys(Boc)-Ser(t-Bu)-Asn(Trt)-Lys(Cbz)-Phe-NH_2

The protected linear peptide HO-Lys(Boc)-Tyr(t-Bu)-Ser(t-Bu)-Asn(Trt)-Lys(Cbz)-NH_2 was generated following the *Resin Cleavage* procedure. The linear peptide was cleaved from the resin using a solution of 5 mL of trifluoroethanol and 5 mL of CH_2Cl_2. The resin containing solution was filtered and dried in vacuo to yield the protected linear pentapeptide as a pale yellow solid (0.59 g, 63% overall).

cyclo-Lys(Boc)-Ser(t-Bu)-Asn(Trt)-Lys(Cbz)-Phe

The protected cyclic peptide *cyclo*-Lys(Boc)-Ser(t-Bu)-Asn(Trt)-Lys(Cbz)-Phe was synthesised following the *Macrocyclisation* procedure using 0.25 g HO-Lys(Boc)-Ser(t-Bu)-Asn(Trt)-Lys(Cbz)-Phe-NH_2 (0.21 mmol, 1 equiv.), 0.08 g HATU (0.21 mmol, 1 equiv.), 0.03 g TBTU (0.1 mmol, 0.5 equiv.), 0.03 g DMTMM (0.1 mmol, 0.5 equiv.), 0.3 mL DIPEA (1.0 mmol, 8 equiv.) in anhydrous CH_2Cl_2 (216 mL, 0.001 M), The reaction was then stirred for 2 hours and monitored via LC/MS. Upon completion, the reaction mixture was worked up as described in the *Macrocyclisation* procedure and dried in vacuo to produce the crude, protected, cyclic peptide *cyclo*-Lys(Boc)-Ser(t-Bu)-Asn(Trt)-Lys(Cbz)-Phe.

cyclo-Lys-Ser-Asn-Lys(Cbz)-Phe

The partially deprotected cyclic peptide *cyclo*-Lys-Ser-Asn-Lys(Cbz)-Phe was synthesised following the *Side Chain Deprotection* procedure using 0.2 g *cyclo*-Lys(Boc)-Ser(t-Bu)-Asn(Trt)-Lys(Cbz)-Phe (0.17 mmol, 1 equiv.), 3 mL of a mixed solution of TFA and CH_2Cl_2 (9:1 v/v) and

0.3 mL anisole (1.4 mmol, 8 equiv.) to remove the side chain protecting groups. The reaction mixture was stirred for 4 hours and monitored via LC/MS. Upon completion, the reaction mixture was worked up as described in the *Global Deprotection* procedure to afford the crude cyclic peptide *cyclo*-Lys-Ser-Asn-Lys(Cbz)-Phe.

cyclo-Lys-Ser-Asn-Lys(Cbz)-Phe tagged Peg4 Biotin

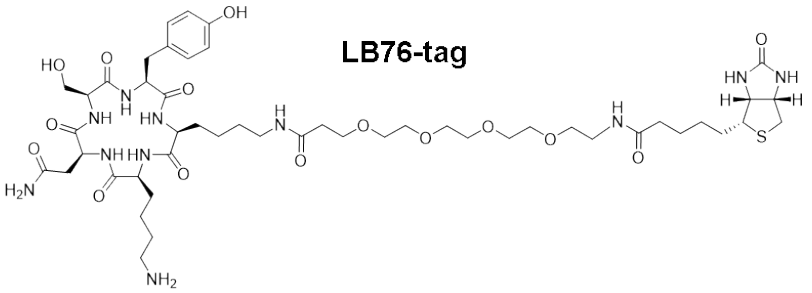
The partially protected cyclic tagged peptide *cyclo*- Lys-Ser-Asn-Lys(Cbz)-Phe tagged Peg4 Biotin was synthesised following the *Biotinylation* procedure using 0.1 g *cyclo*- Lys-Ser-Asn-Lys(Cbz)-Phe (0.1 mmol, 1 equiv.), 0.12 g EZ-Link NHS- PEG_4-Biotin (0.2 mmol, 1.5 equiv.), 0.38 mL DIPEA (2.1 mmol, 16 equiv.) in anhydrous CH_2Cl_2 (1.35 mL, 0.1 M). The reaction was then stirred for 4 hours and monitored via LC/MS. Upon completion, the reaction mixture was drained, and the crude material washed multiple times with CH_2Cl_2 before being carried to final Cbz removal.

cyclo-Lys-Ser-Asn-Lys-Phe tagged Peg4 Biotin

The final cyclic tagged peptide *cyclo*-Lys-Ser-Asn-Lys-Phe tagged Peg4 Biotin was synthesised following *Cbz removal* procedure using 23mg *cyclo*-Lys-Ser-Asn-Lys-Phe tagged Peg4 Biotin (0.02 mmol, 1 equiv.), 1.96 mL hydrogen bromide solution (33%) in acetic acid (0.1 M) and 0.17 mL anisole (1.5 mmol, 8 equiv.). The reaction was stirred for 4 hours and monitored via LC/MS. Upon completion, the reaction mixture was washed multiple times with CH_2Cl_2. The crude product was purified using HPLC, then lyophilised to generate pure compound as a white solid (17 mg, 15% overall).

LC/MS (ESI) *m/z;* $[M+H]^+$ calculated for $C_{49}H_{79}N_{11}O_{14}S^+$ 1077.55, found 1078.65

LC-MS of LB76-tag



Chemical Formula: $C_{49}H_{79}N_{11}O_{15}S$
Exact Mass: 1093.55

SHIMADZU LabSolutions **Analysis Report**

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Batch Filename	: 17-04-11.2.lcb		
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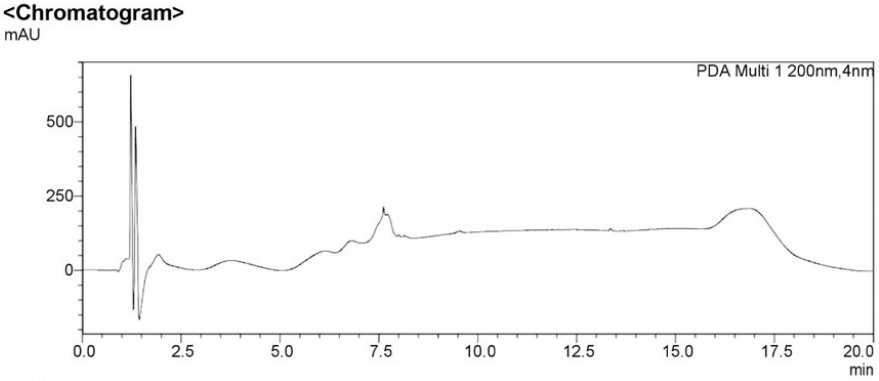

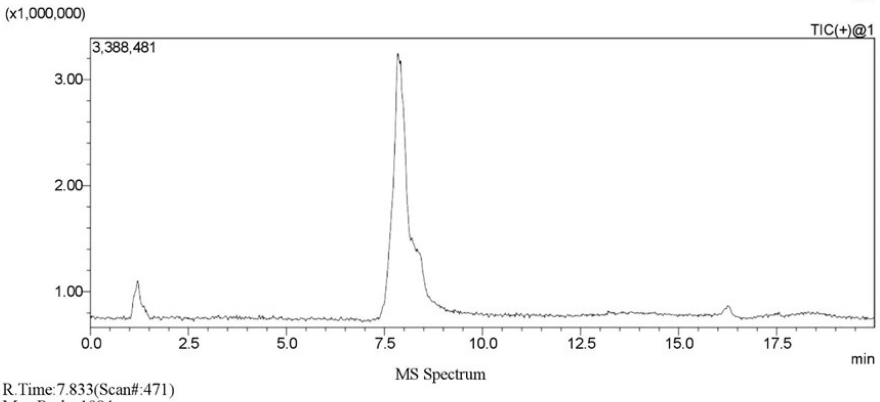

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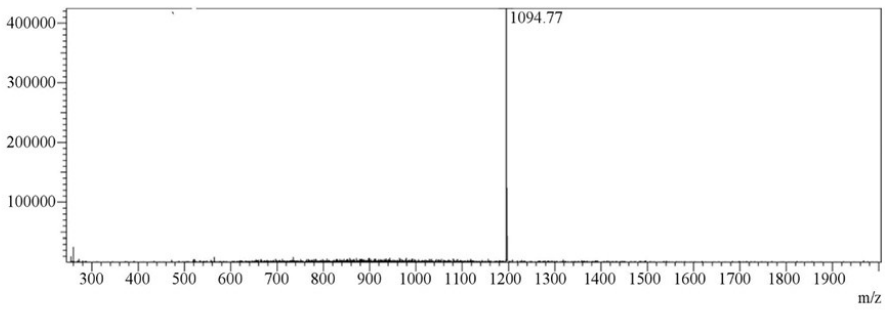

^{1}H NMR of


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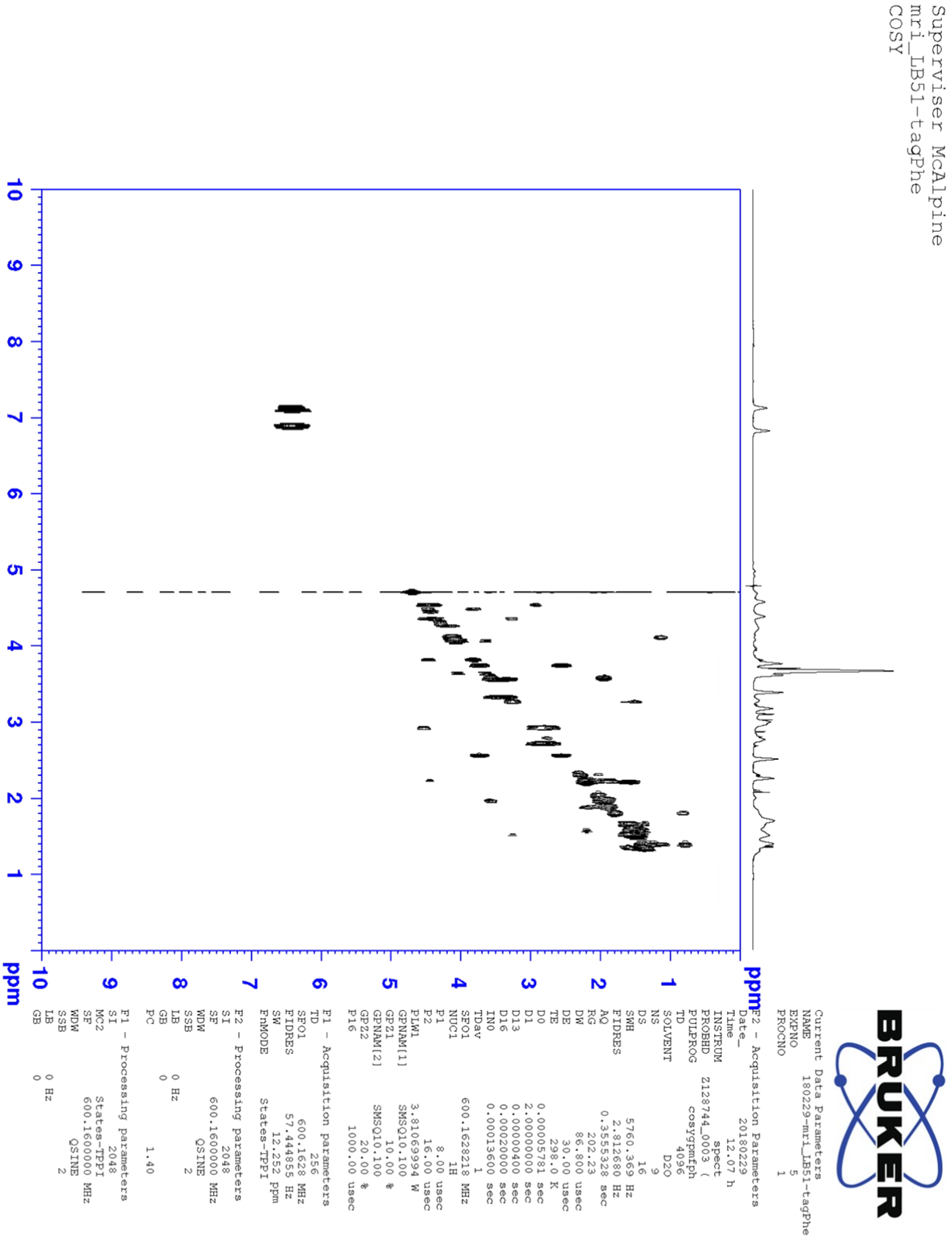

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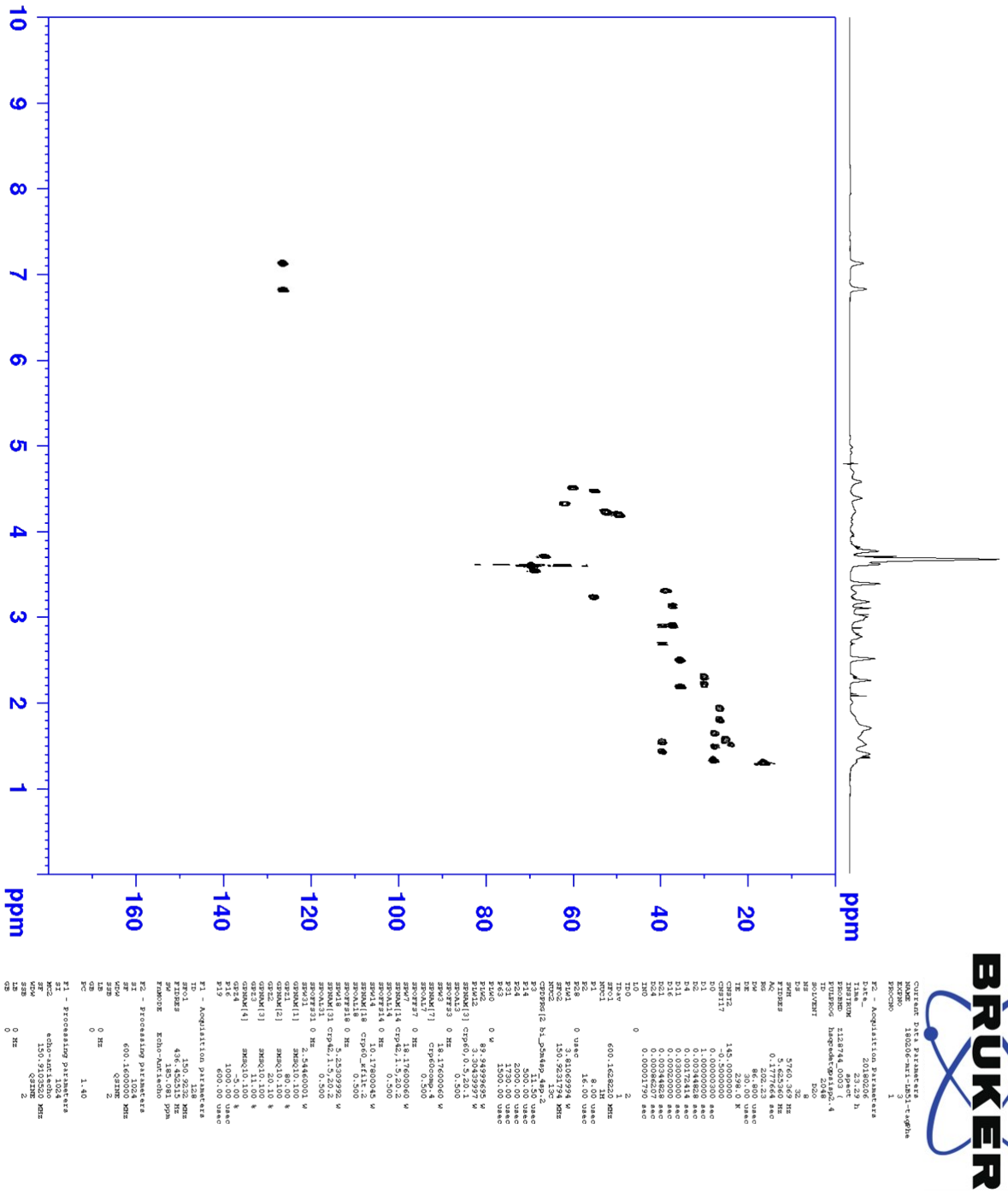

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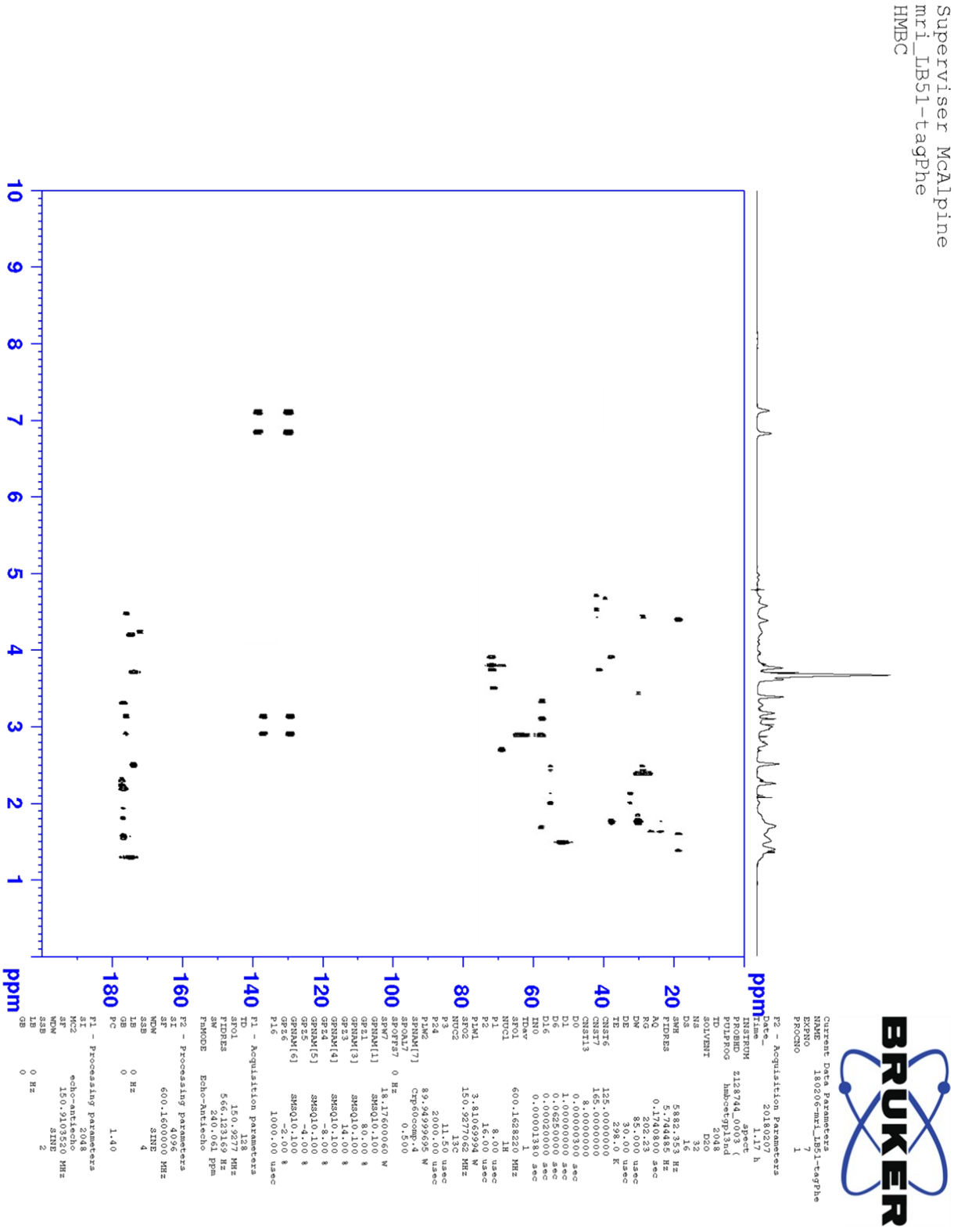
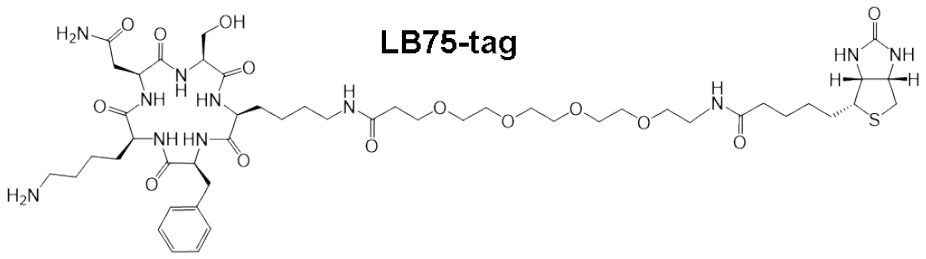


LC-MS of LB75-tag



Chemical Formula: $C_{49}H_{79}N_{11}O_{14}S$
Exact Mass: 1077.55

SHIMADZU LabSolutions **Analysis Report**

Sample Name	: LB51-tagTyr_1.6		
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Batch Filename	: 17-04-15.lcb		
Vial #	: 1-7	Sample Type	: Unknown
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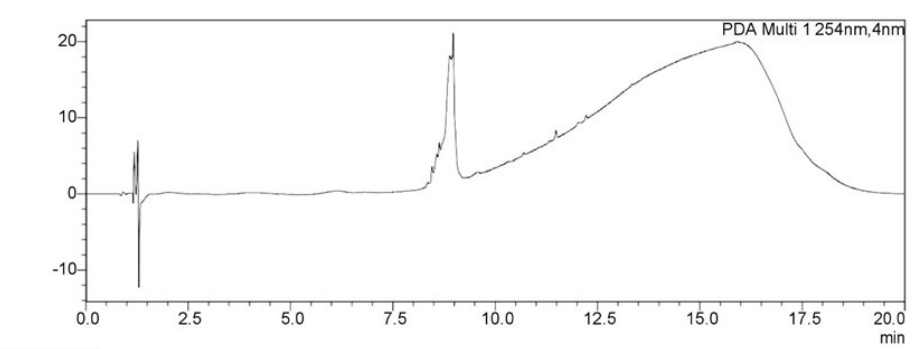

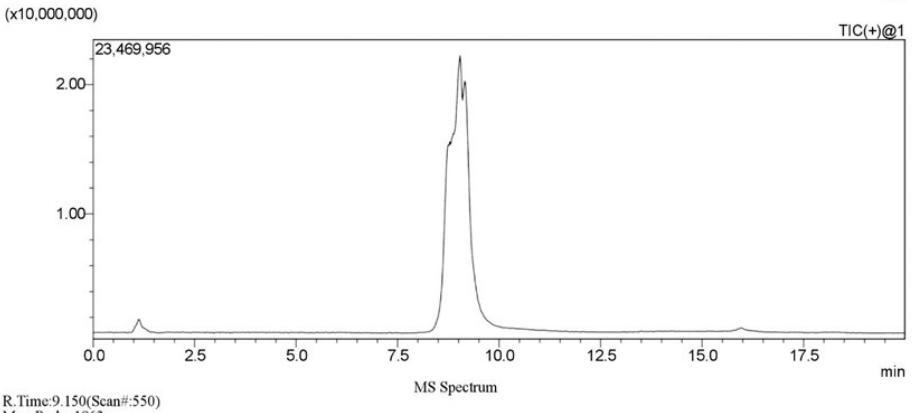

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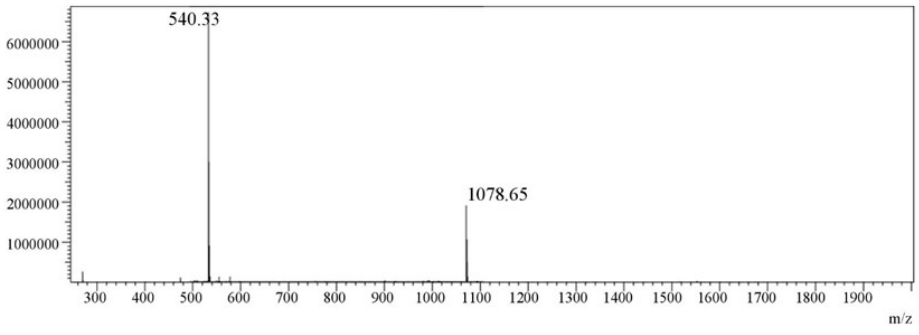

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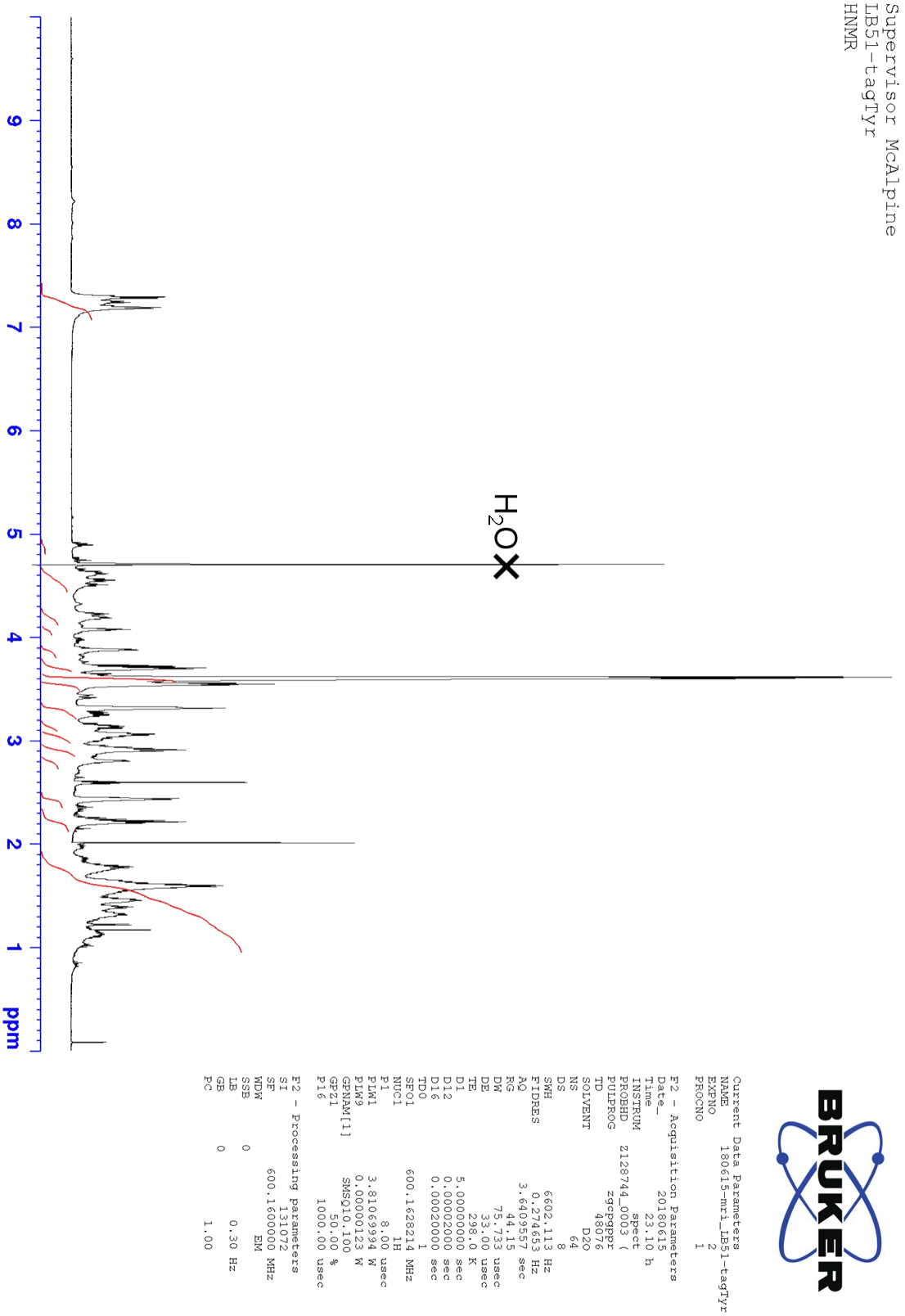

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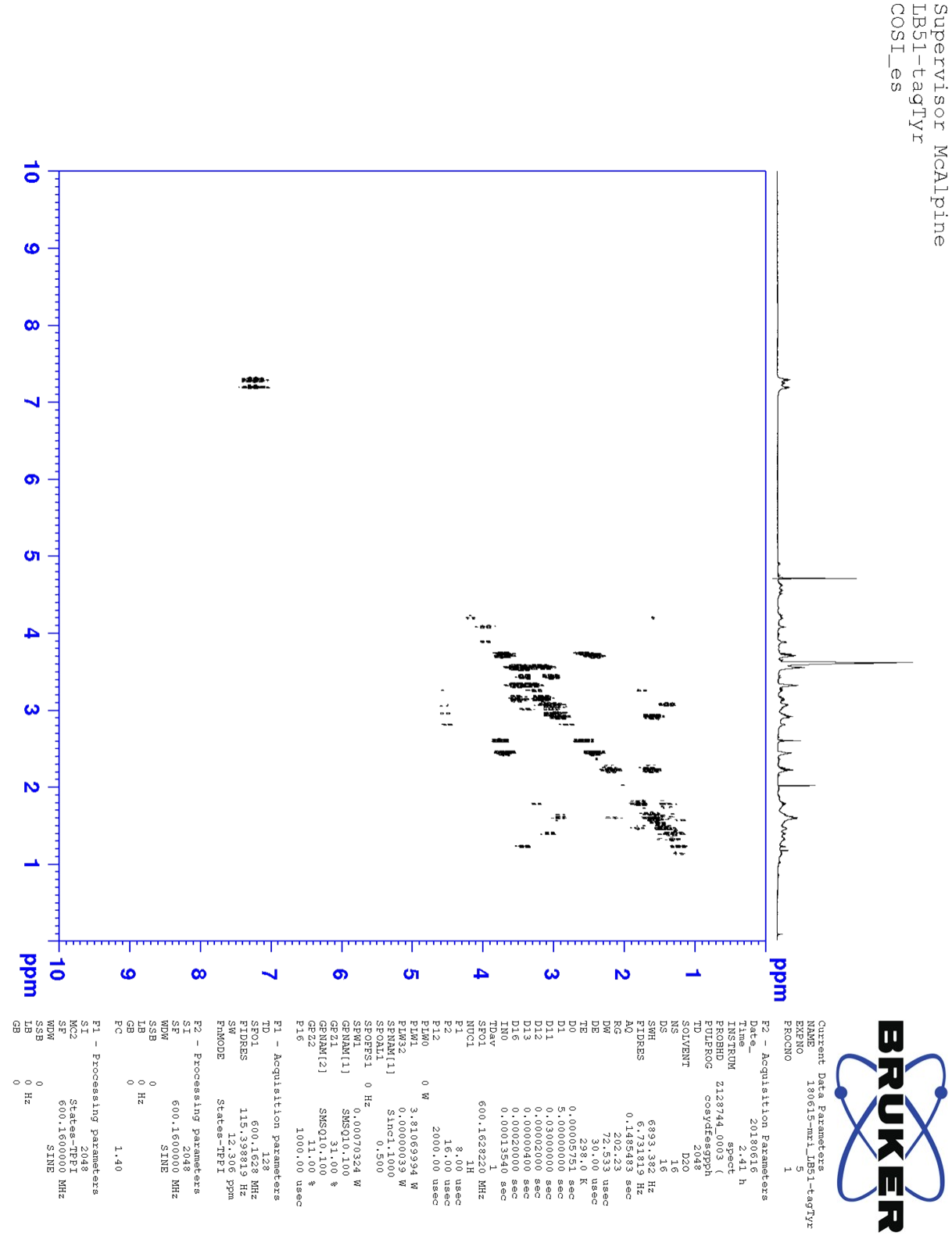

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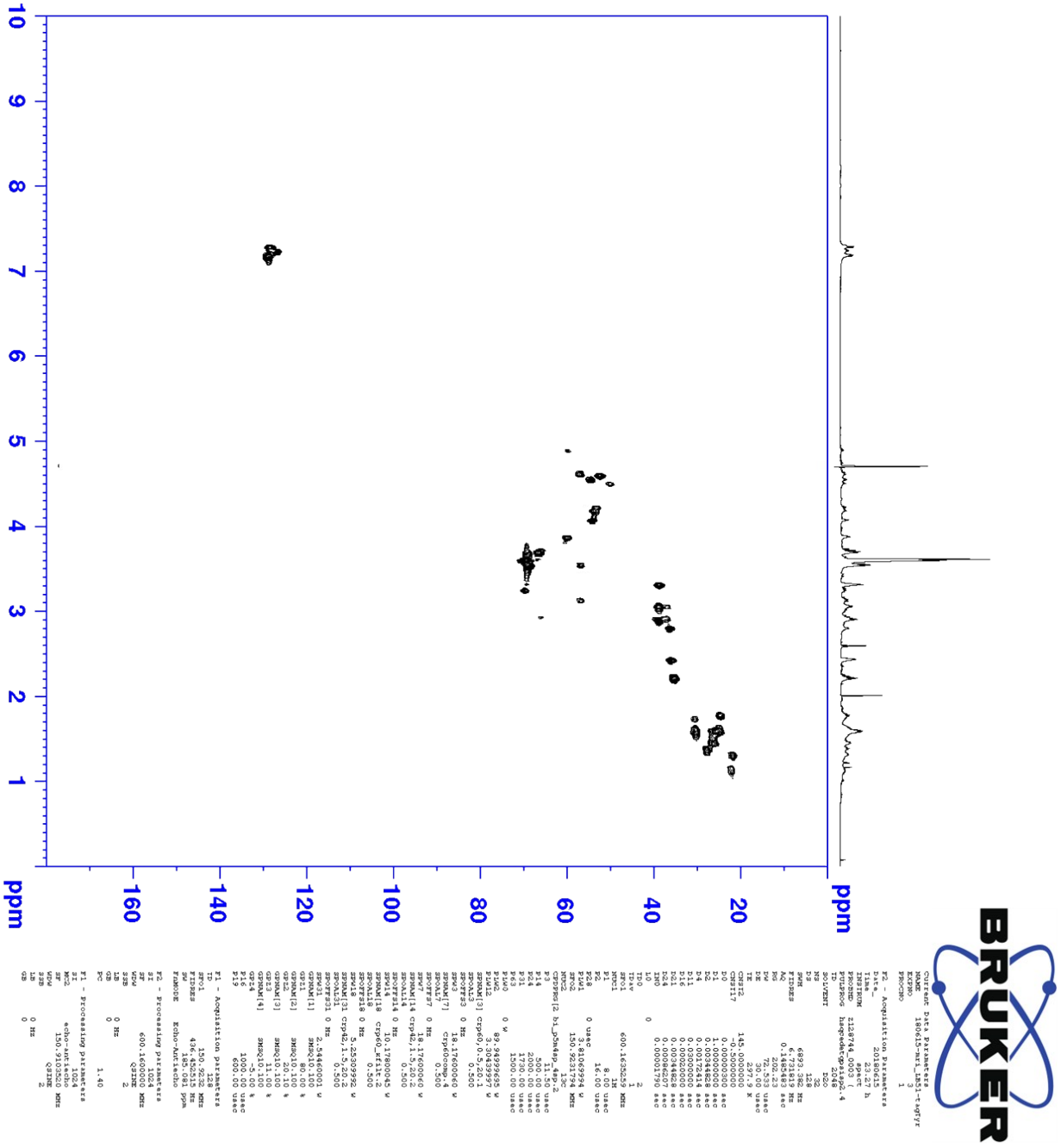

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