SUPPORTING INFORMATION

Title: Click Chemistry-Mediated Synthesis of Selective Melanocortin Receptor-4 Agonists

Authors: Daniel Palmer[1*], Juliana P L Gonçalves[1], Louise Valentin Hansen[1], Boqian Wu,[2], Helle Hald[1], Sanne Schoffelen[1], Frederik Diness[1], Sebastian T Le Quement[3], Thomas E Nielsen[4,5] and Morten Meldal[1]*

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Receptor family

- Angiotensin receptors
- Apelin receptors
- Relaxin family peptide receptors
- Somatostatin receptors
- Tachykinin receptors
- Thyrotropin-releasing hormone receptors
- Urotensin receptors
- Vasopressin and oxytocin receptors
- Bombesin receptors
- Bradykinin receptors
- Cholecystokinin receptors
- Complement peptide receptors
- Endothelin receptors
- Formylpeptide receptors
- Galanin receptors
- Ghrelin receptors
- Gonadotrophin-releasing hormone receptors
- Kisspeptin receptors
- Melanin-concentrating hormone
- Melanocortin receptors
- Motilin receptors
- Neuromedin U receptors
- Neuropeptide FF/neuropeptide AF receptors
- Neuropeptide S receptors
- Neuropeptide W/neuropeptide B receptors
- Neuropeptide Y receptors
- Neurotensin receptors
- Opioid receptors
- Orexin receptors
- Peptide P518 receptors
- Prolactin-releasing peptide receptors
- Proteinase-activated receptors

Sequence Identity

	Human MC1R	Human MC2R	Human MC3R	Human MC4R	Human MC5R
Human MC1R	-	33	38	39	35
Human MC2R	46	-	35	33	35
Human MC3R	52	52	-	51	56
Human MC4R	52	46	66	-	55
Human MC5R	51	51	72	69	-

Sequence Similarity

Figure S1. Phylogenetic analysis of melanocortin receptor family using www.GPCRdb.org. The dendrogram displays the evolutionary relationship between human MCRs as part of the entire peptide receptor complement within the rhodopsin subset of GPCRs. Comparative primary sequence similarity and identity is displayed as percentages in the lower- right table. Identity provides an index as to how many identical amino acid residues are conserved at the comparable loci whereas sequence similarity refers to loci where amino acids with common functional groups (e.g., acidic, basic, polar, lipophilic, etc.) are shared.

Table S1. The alignment of the transmembrane domaíns of MC4R and β2-Adrenergic receptor.

Domain	Receptor	Alignment of domain[a]
TM1:	MC4R	LFVSPEVFVTLGVISLLENILVIVAI
	β2-Adrenergic	VVGMGIVMSLIVLAIVFGNVLVITAI
TM2:	MC4R	FICSLAVADMLVSVSNGSETIVITL
	β2-Adrenergic	FITSLACADLVMGLAVVPFGAAHIL
TM3:	MC4R	VIDSVICSSLLASICSLLSIAV
	β2-Adrenergic	FWTSIDVLCVTASIETLCVIAV
TM4:	MC4R	GIIISCIWAACTVSGILFII
	β2-Adrenergic	RVIILMVWIVSGLTSFLPIQ
TM5:	MC4R	AVIICLITMFFTMLALMASLYV
	β2-Adrenergic	YAIASSIVSFYVPLVIMVFVYS
TM6:	MC4R	LTILIGVFVVCWAPFFLHLIFYIS
	β2-Adrenergic	LGIIMGTFTLCWLPFFIVNIVHVI
TM7:	MC4R	SHFNLYLILIMCNSIIDPLIYAL
	β2-Adrenergic	EVYILLNWIGYVNSGFNPLIYCR

[a]The alignment provides the best helical register fit considering identical residues, conservative substitutions, structural residues small amino acids and hydrophobicity.

a

b

D122
N123
R
D126
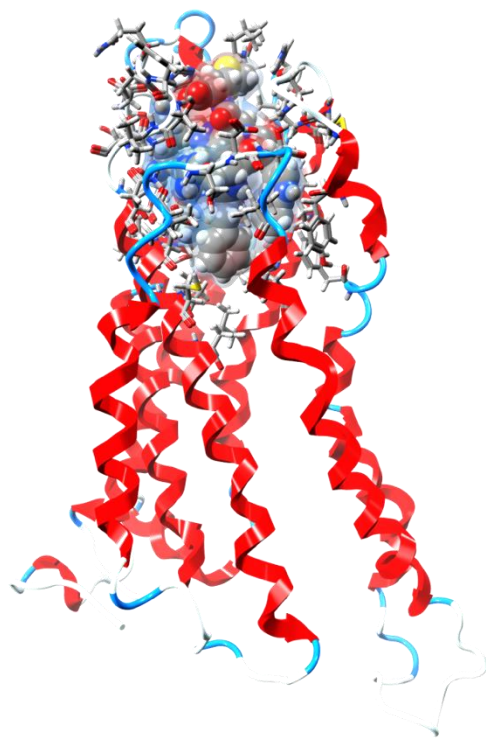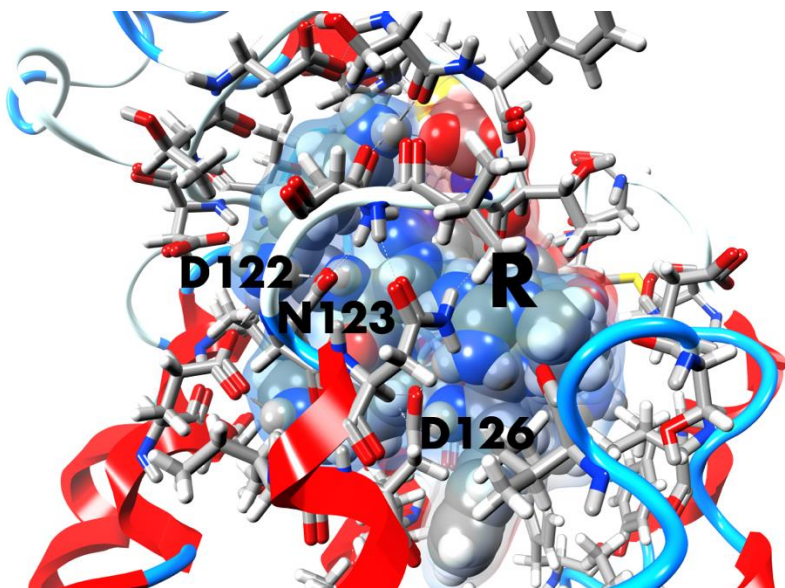
c
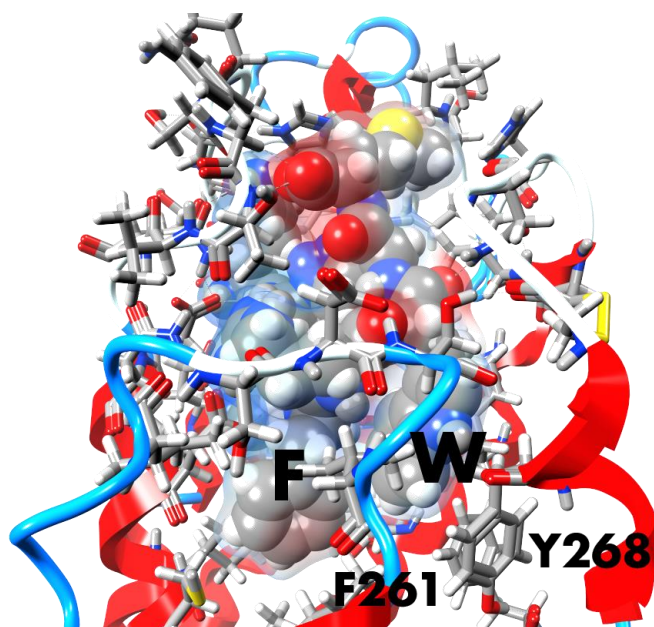
d
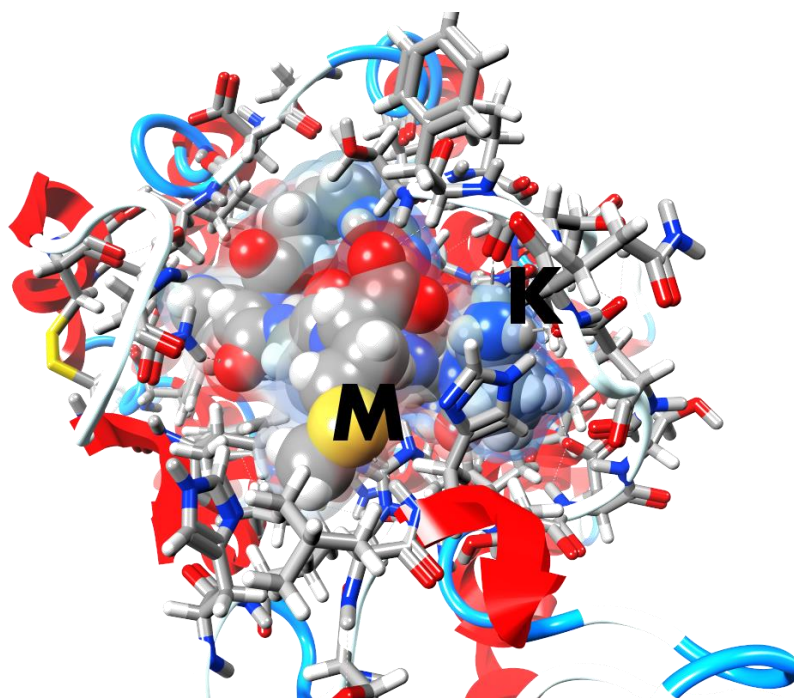
e
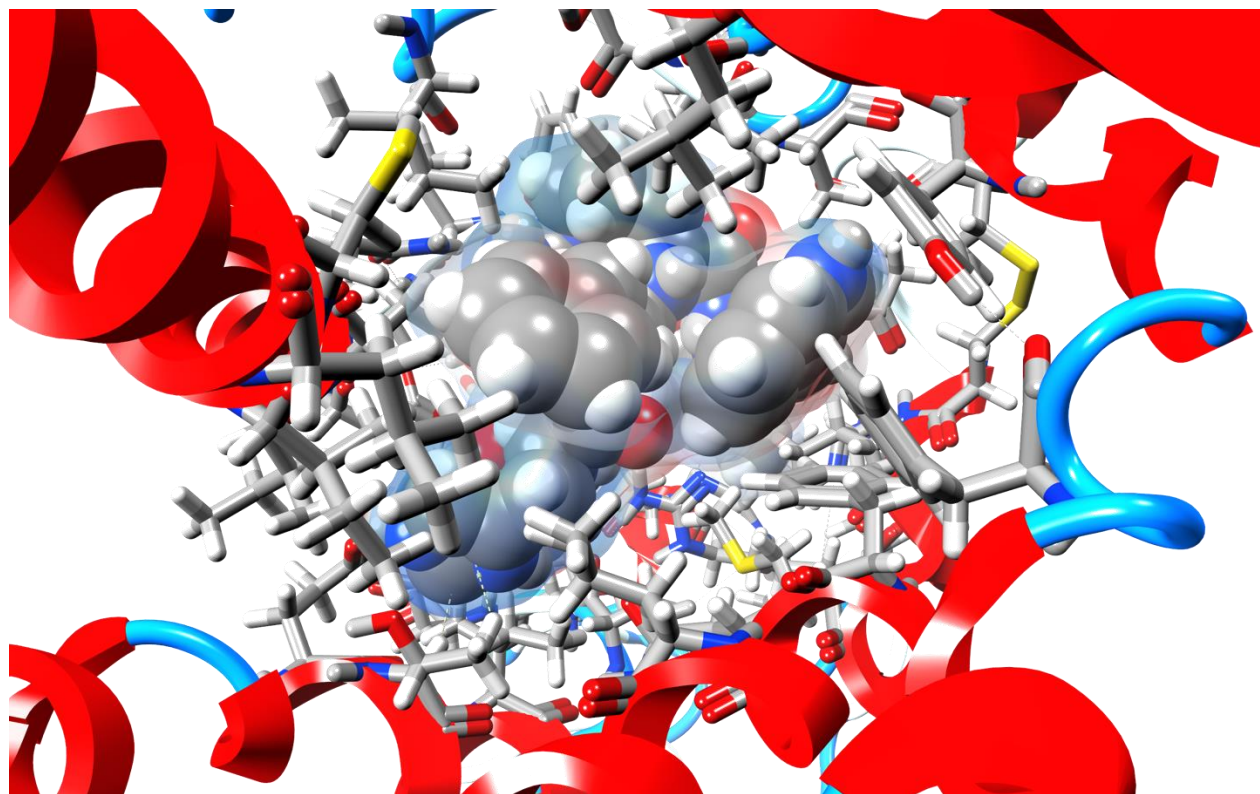
Figure S2. Molecular model of NOE-restricted compound **1** in complex with human MC4R. **A.** Ligand-receptor complex derived by aligning MC4R with the model from Rasmussen *et al.*[63] Compound **1** is displayed using the space-filling view while the receptor is shown with both secondary structures and wire frame formats. **B.** Model centered on R residue in **1** with key contact points in MC4R denoted. **C.** Model centered on the hydrophobic residues F and W of **1** with key contact points denoted. **D.** Modelled complex displayed from a vantage point along the axis of the transmembrane helical bundle towards the bound ligand from above. The extra-cyclic M and K residues are denoted on the agonist for the complexed structure. **E.** Modelled complex displayed from a vantage point along the axis of the transmembrane helical bundle towards the bound ligand from below. The pdb file used for the generation of these images is included in the supplementary information (JMC-NOE-1-MC4R.pdb).

Table S2. Key MC4R Residues in Contact with Compound **1** in Modelled Molecular Structure.

Pharmacophore Residue of Compound **1**	H	f	R	W
Contacting Residues in MC4R	E100^{2x60} T101^{2x61} I104^{2x64} I125^{3x28} N285^{7x35}	I129^{3x32} I185^{4x61} L197^{5x43} L290^{7x40}	D122^{3x25} N123^{3x26} D126^{3x29}	F261^{6x51} Y268^{6x58} F284^{7x34} L290^{7x40}

Figure S3. The general Fmoc-based solid phase peptide synthesis protocol used to synthesize cyclic peptides **1 – 17** in Figure S4.
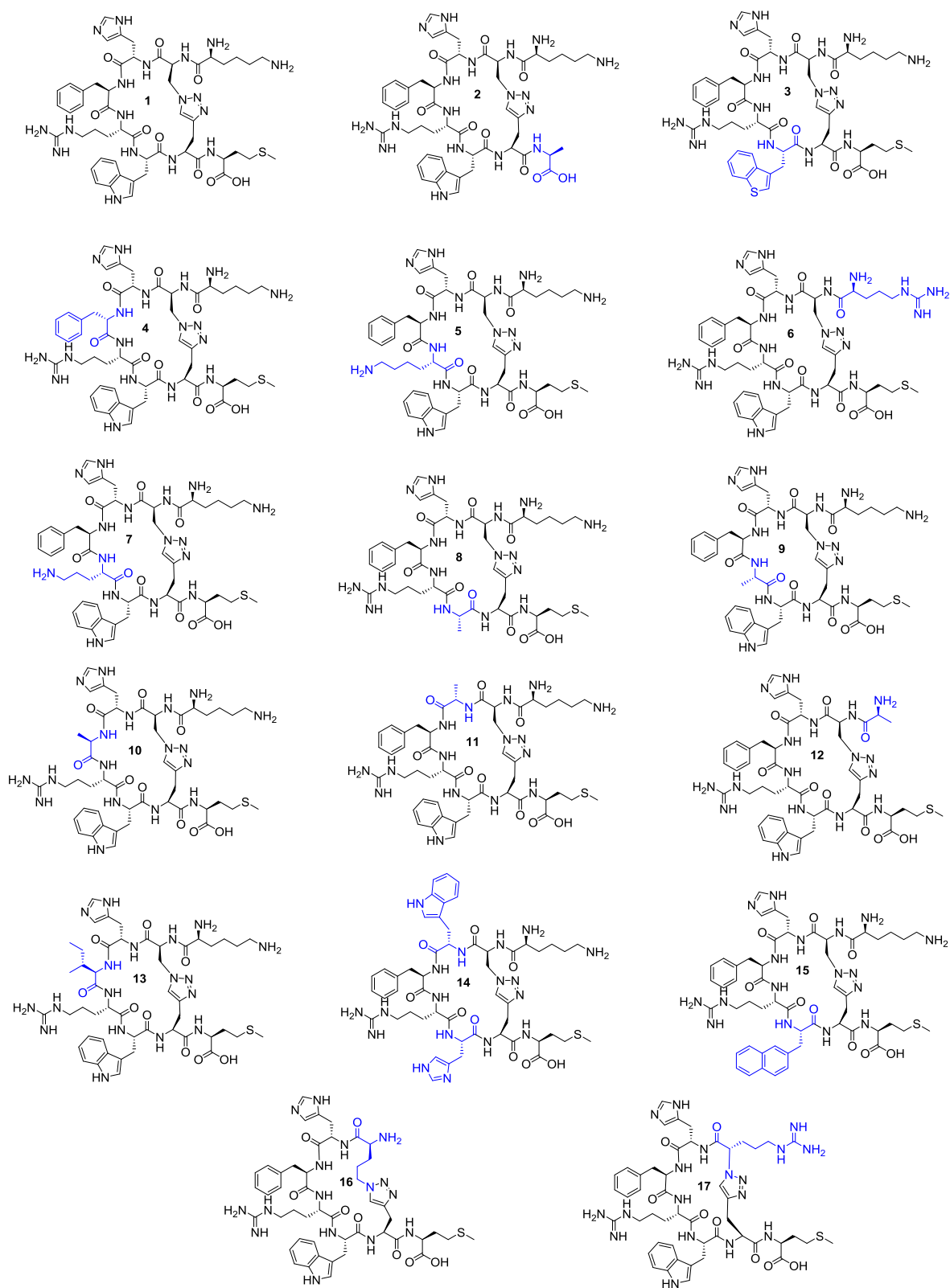
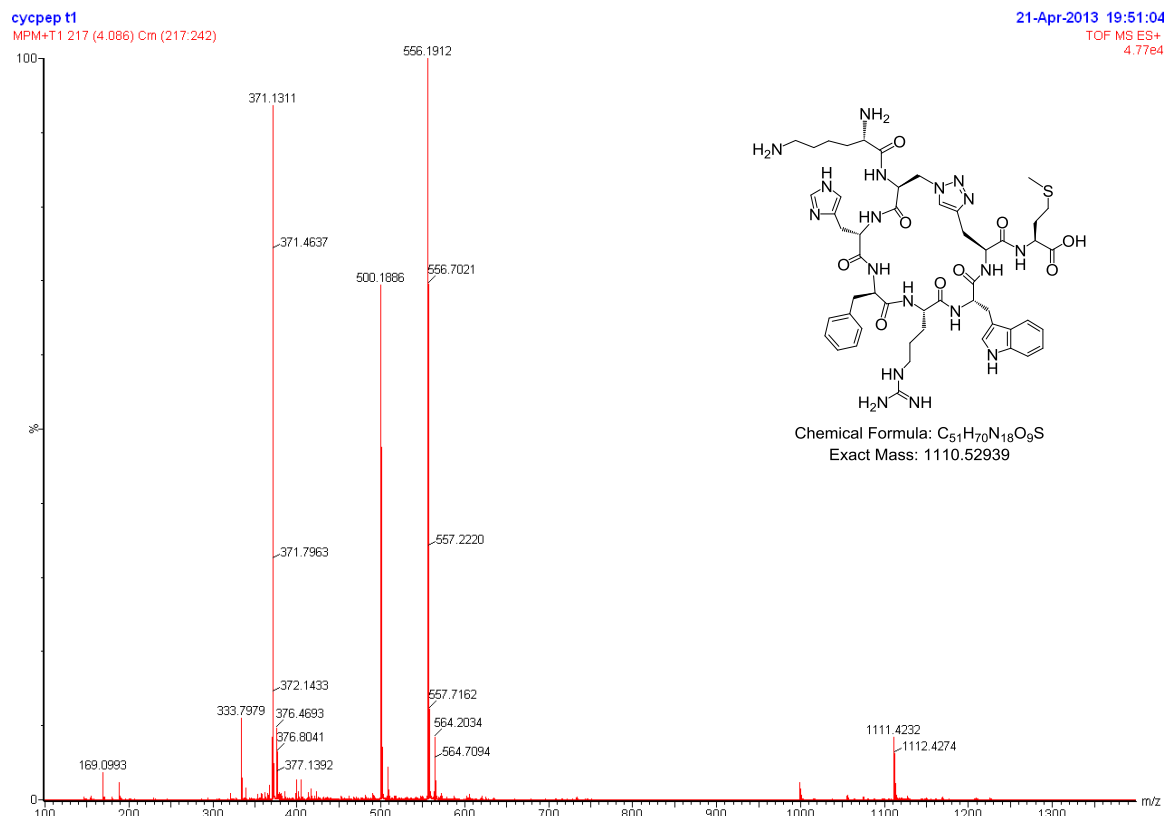**Figure S4**. CuAAC cyclized MC3R, MC4R and MC5R ligands **1**-**17** used in the present study**.**

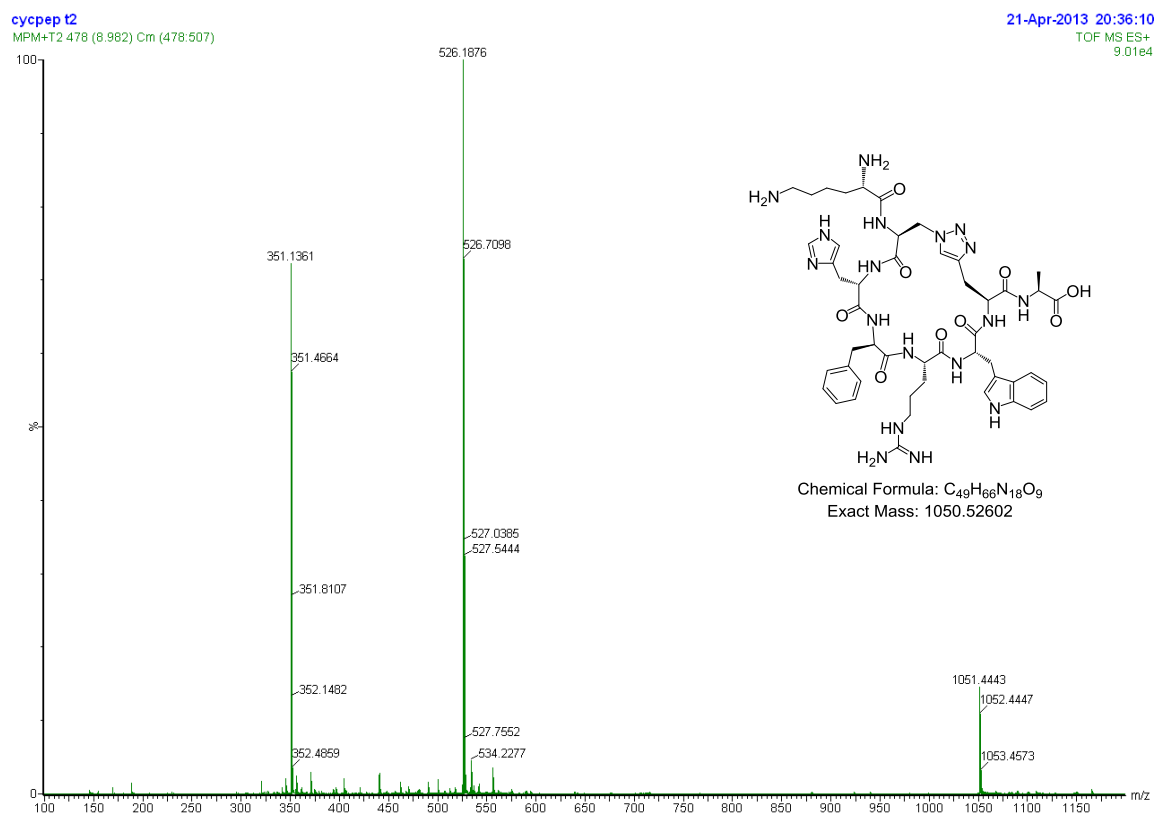**Figure. S5.** High resolution mass spectrum of compound **1**

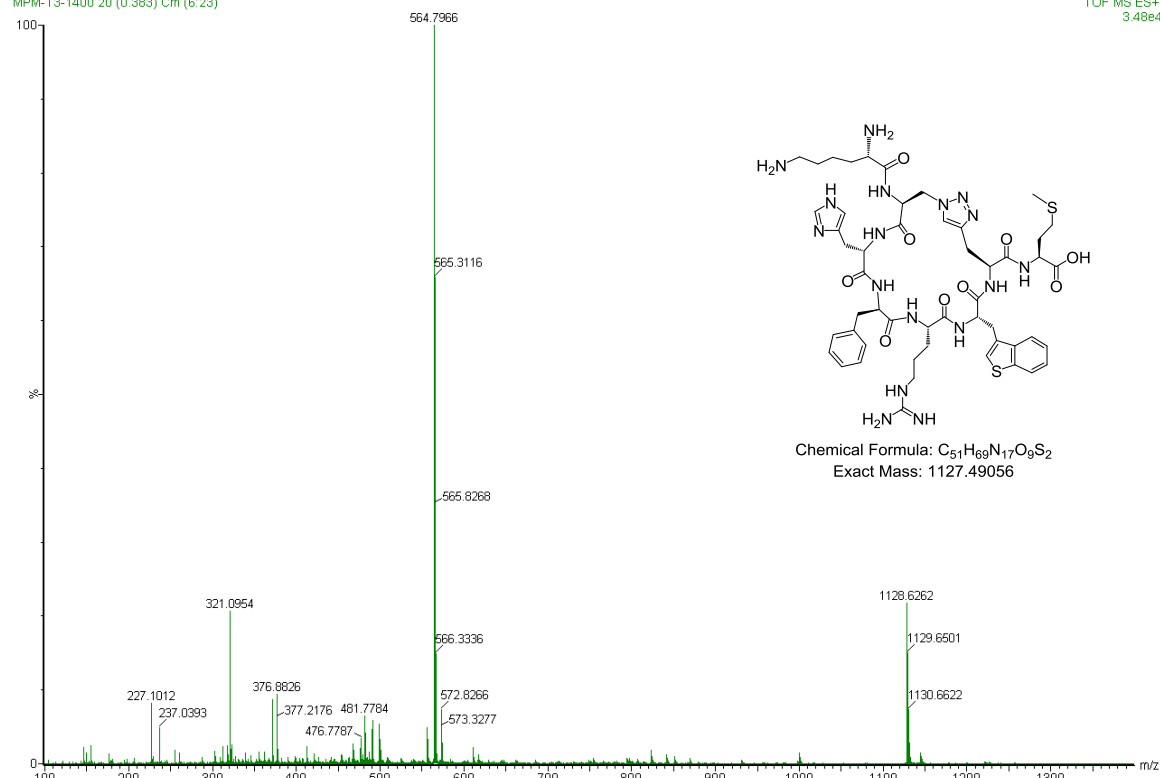**Figure. S6.** High resolution mass spectrum of compound **2**

Figure. S7.   High resolution mass spectrum of compound **3**
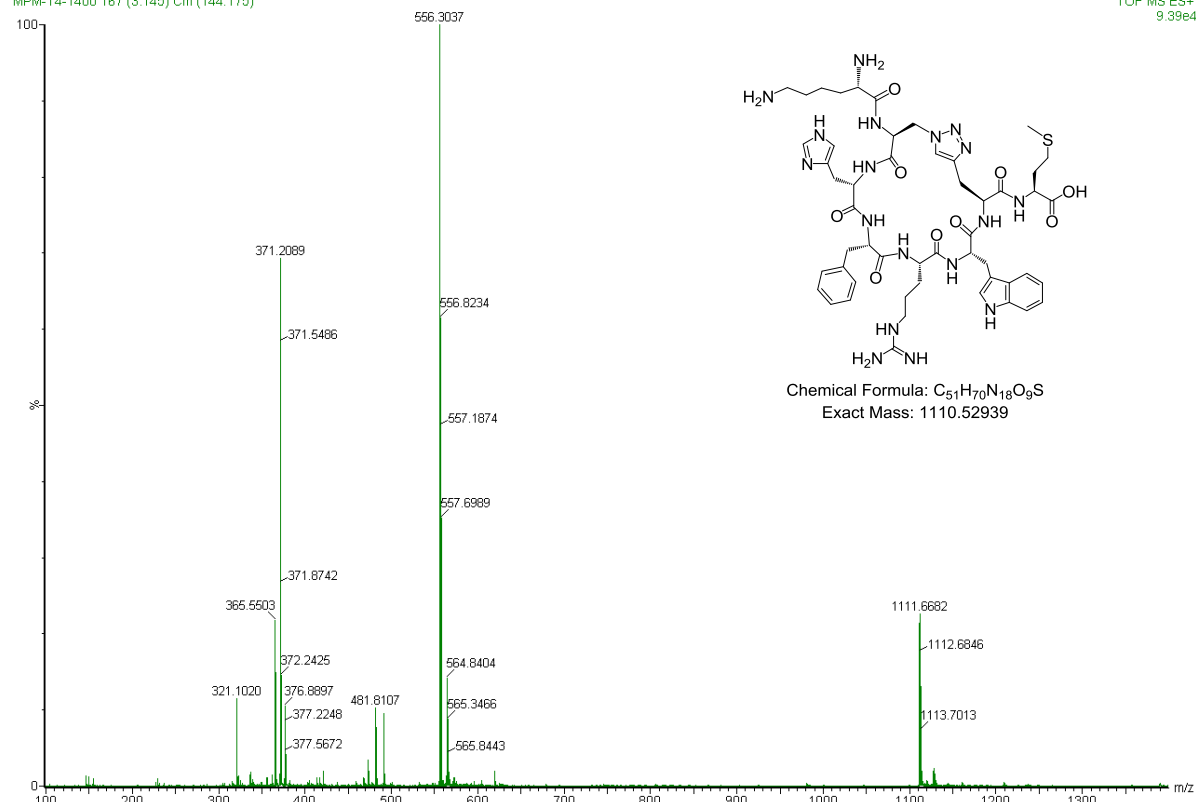

Figure. S8.   High resolution ESI mass spectrum of compound **4**
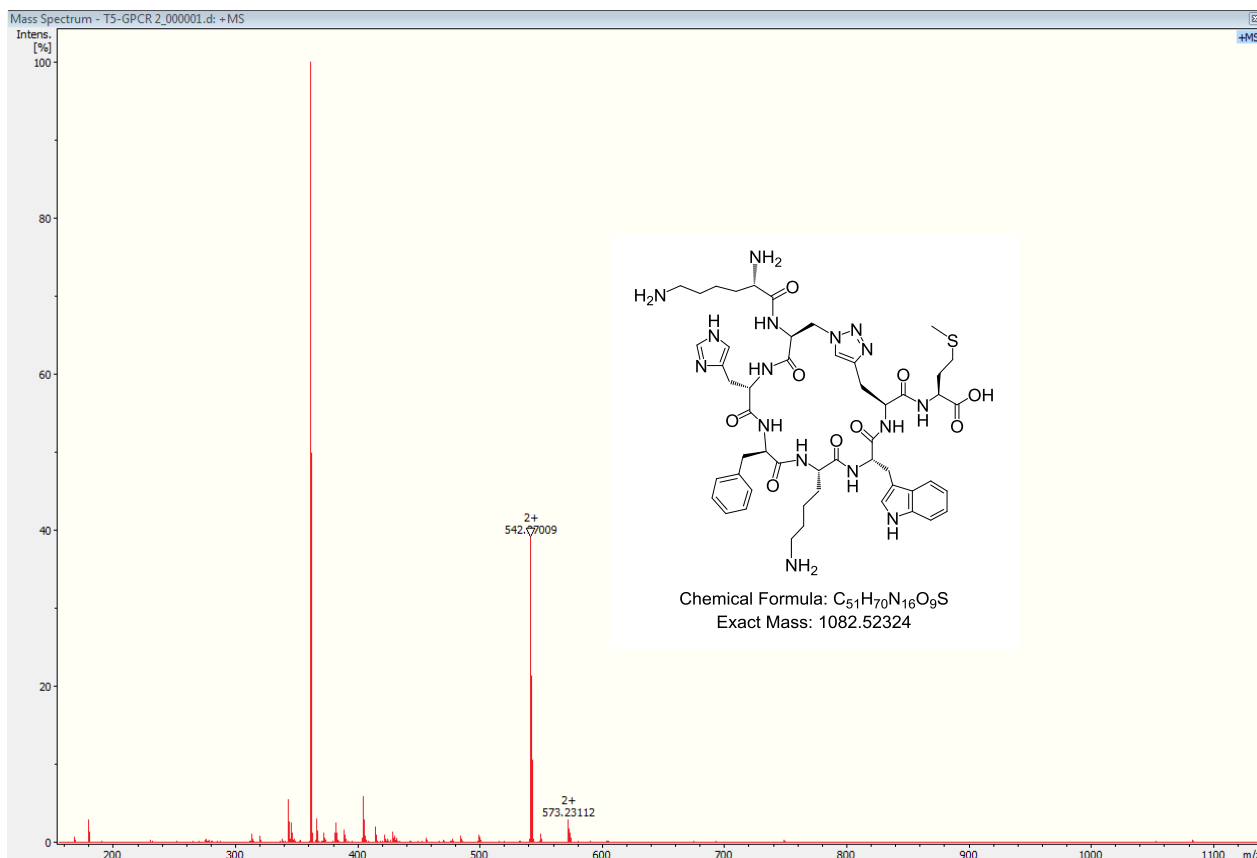
S11

Figure. S9. High resolution ICR mass spectrum of compound **5,** only the double and triple charged ions were observed.
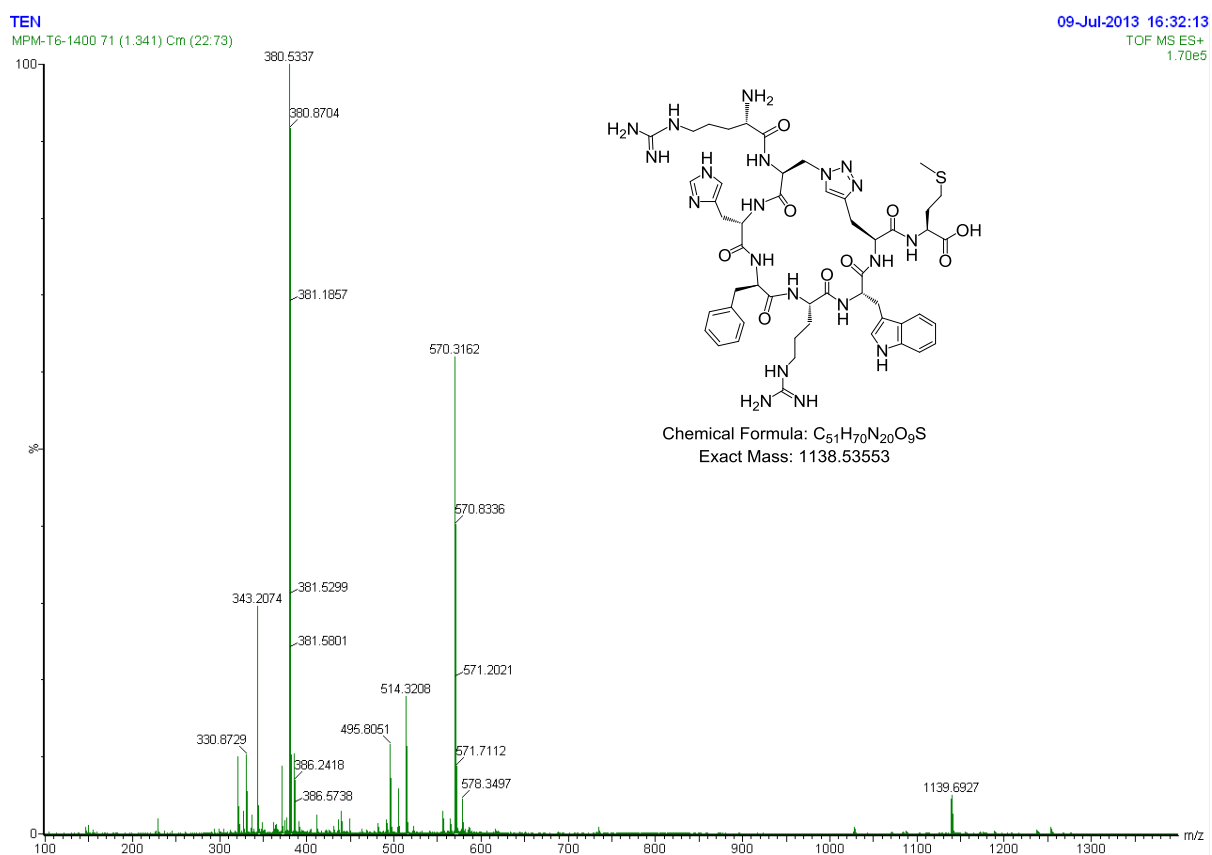

Figure. S10. High resolution mass spectrum of compound **6**
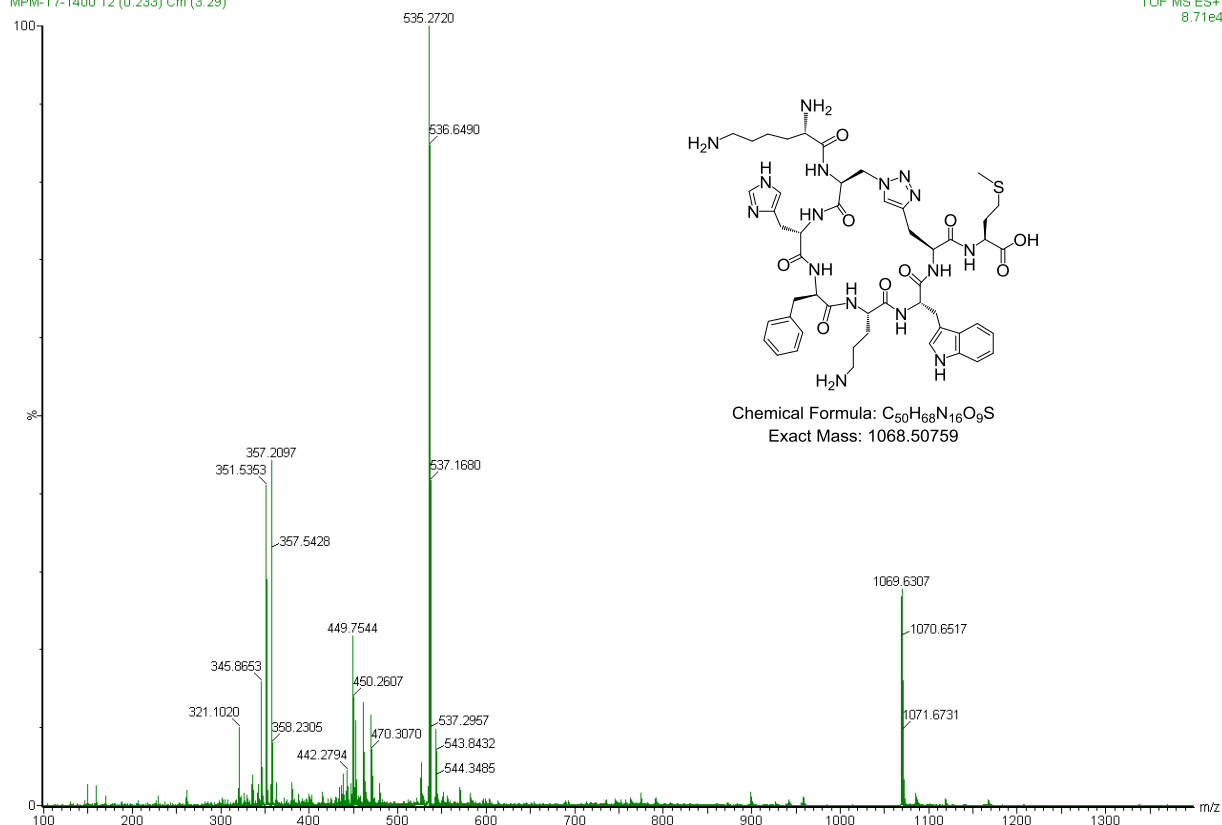
Figure. S11. High resolution mass spectrum of compound **7**

Figure. S12. High resolution mass spectrum of compound **8**

100

513.7713

514.2958

515.1287

%

521.8171
522.3204
522.8240

321.1086

1026.6029
1027.6267
1028.6392

Chemical Formula: C₄₈H₆₃N₁₅O₉S

Chemical Formula: $C_{48}H_{63}N_{15}O_9S$
Exact Mass: 1025.46539

0

100 200 300 400 500 600 700 800 900 1000 1100 1200 1300   m/z

Figure. S13. High resolution mass spectrum of compound **9**

100

518.3170

518.8354

%

345.8926

321.1086

346.2273

519.3373

346.5621

519.8479
526.3392
526.8362

155.0987

351.2255

435.2883

Chemical Formula: $C_{45}H_{66}N_{18}O_9S$
Exact Mass: 1034.49809

1035.6576
1036.6741
1037.6791

0

100 200 300 400 500 600 700 800 900 1000 1100 1200 1300   m/z
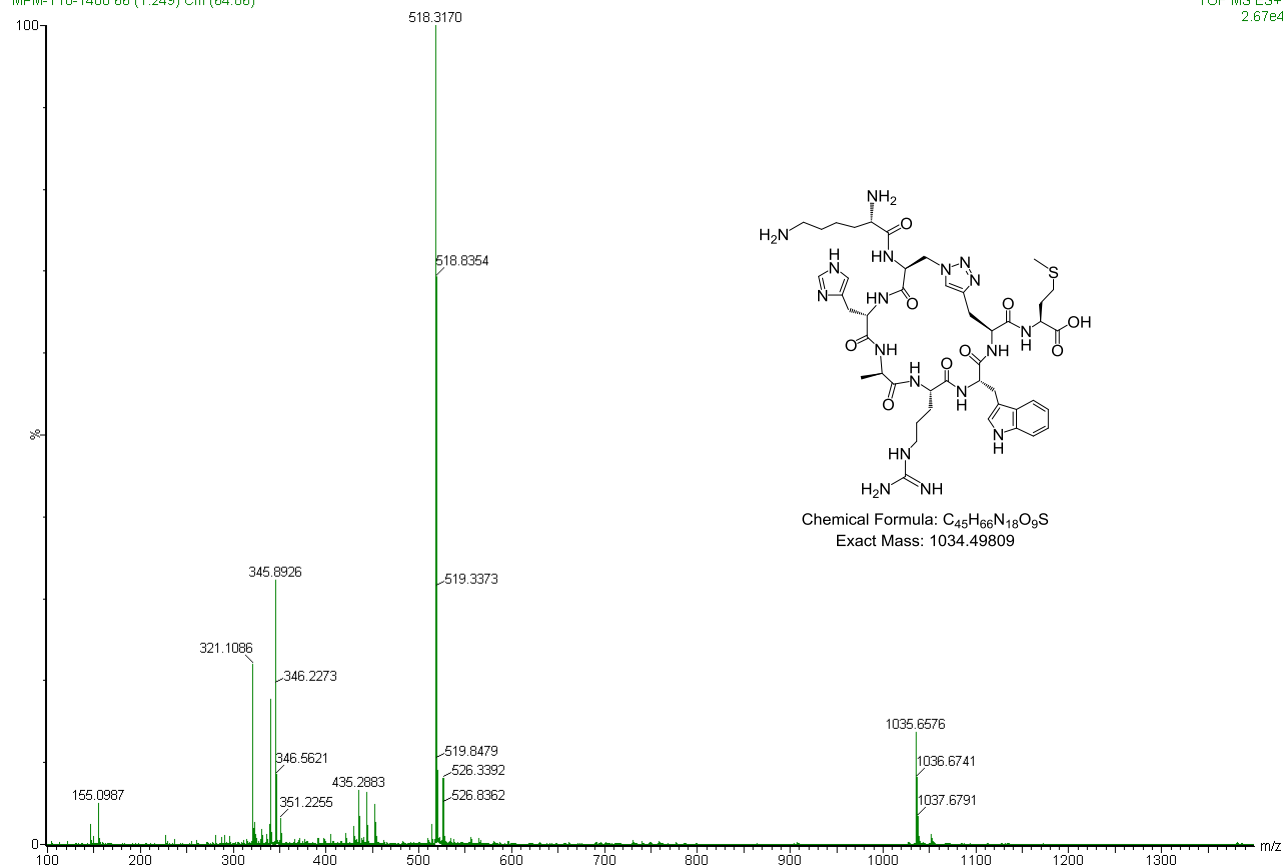
Figure. S14. High resolution mass spectrum of compound **10**
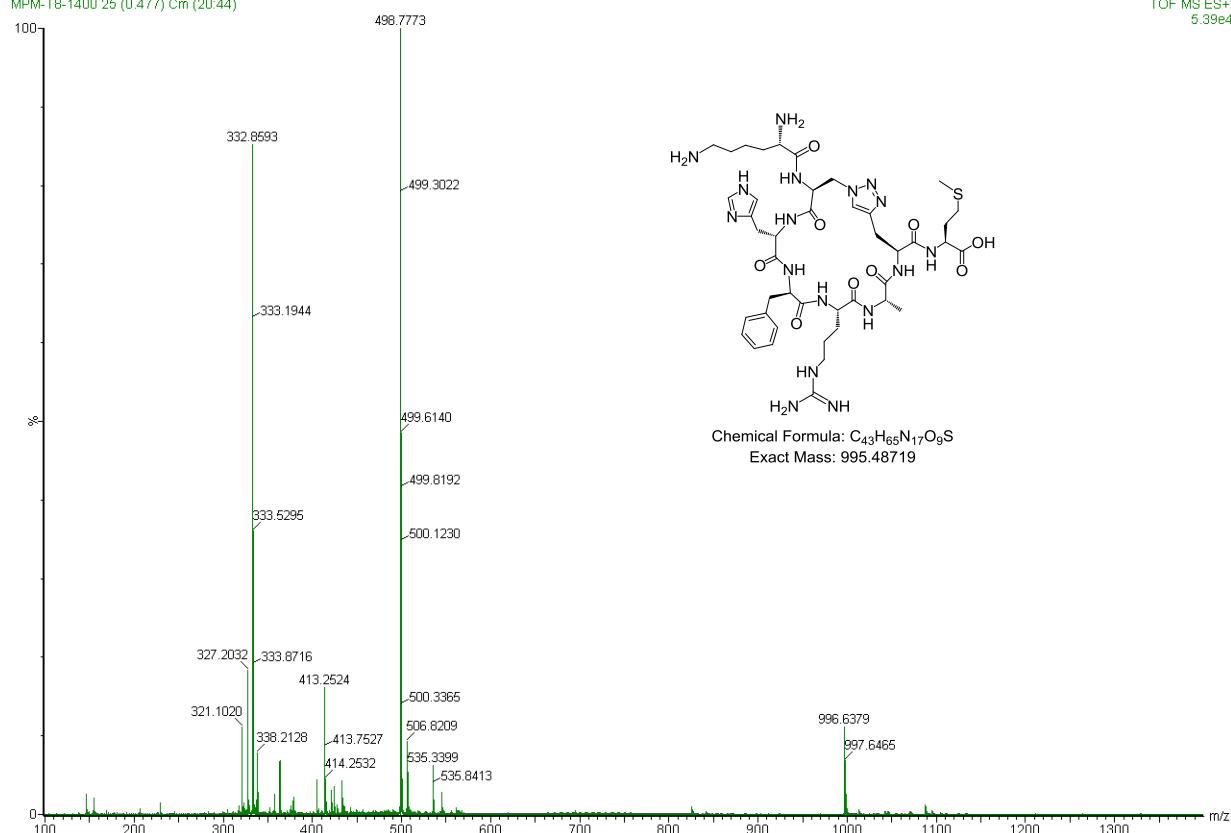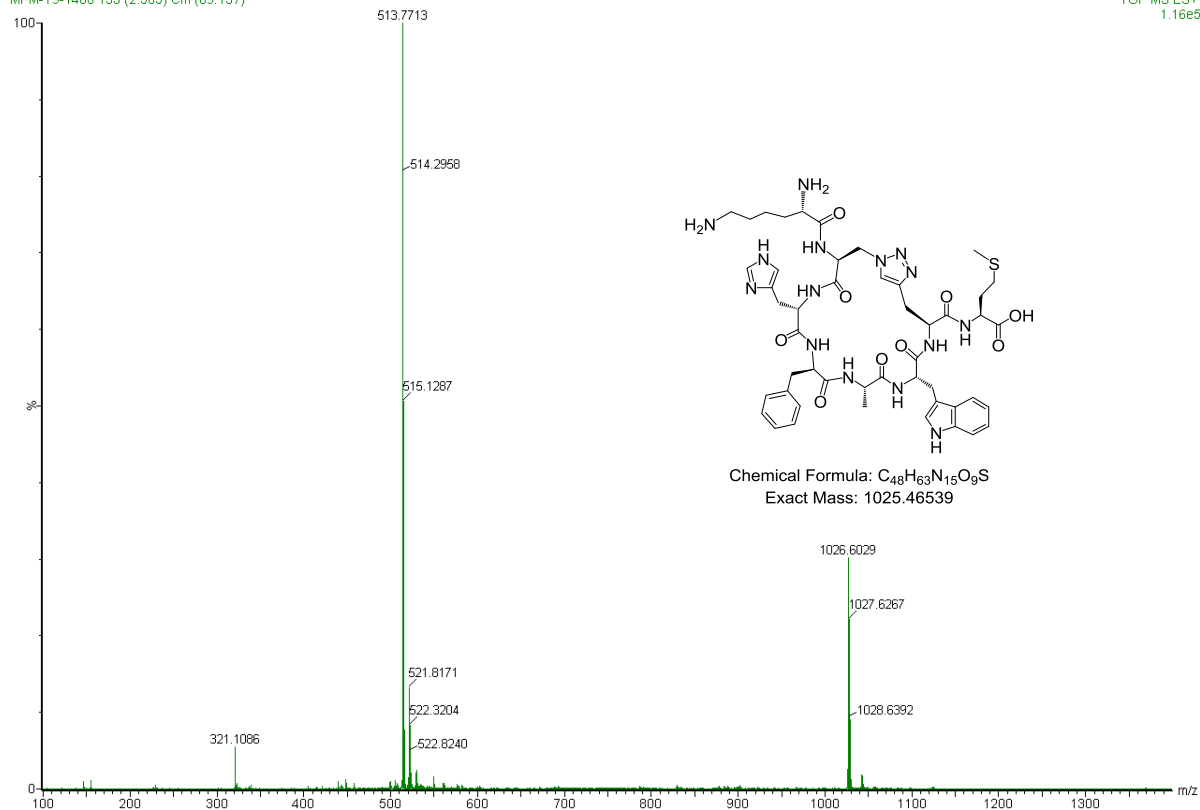
S14
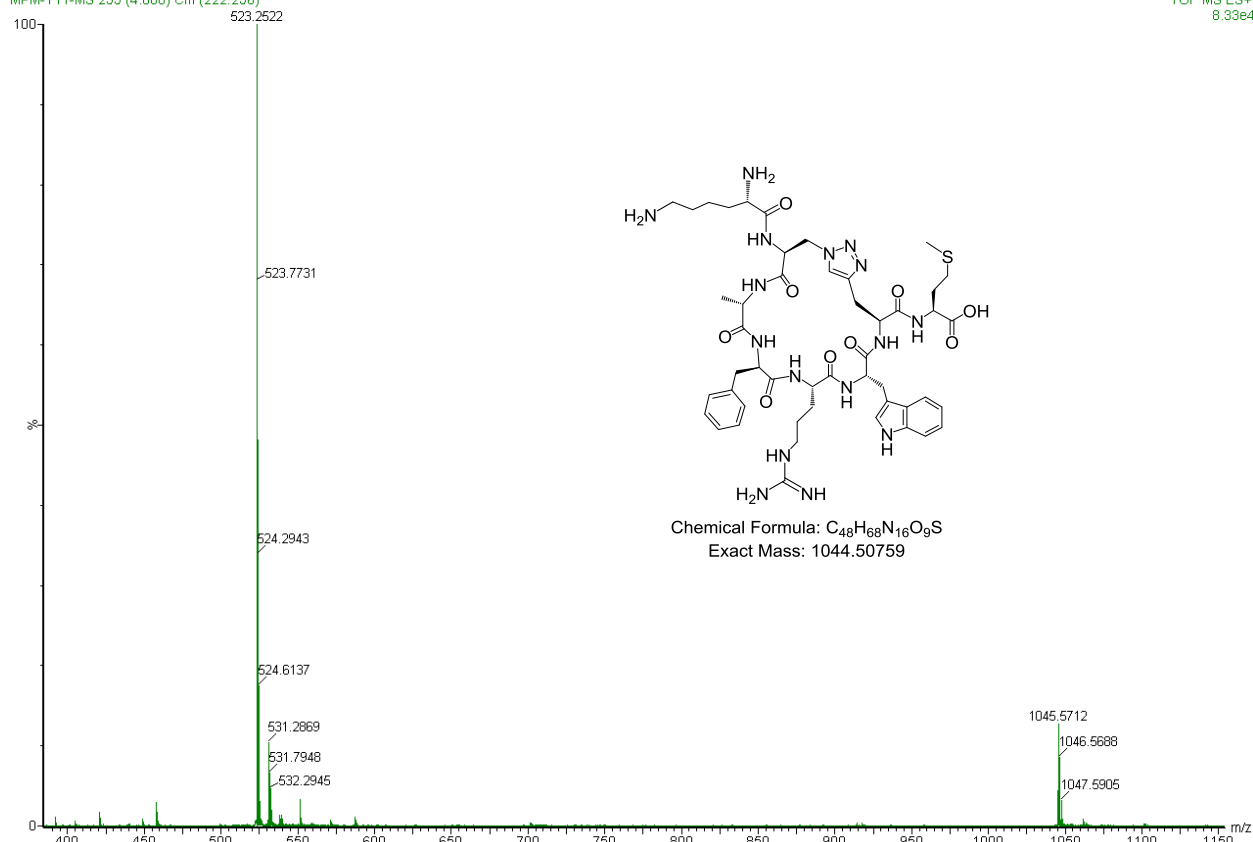
Figure. S15. High resolution mass spectrum of compound **11**
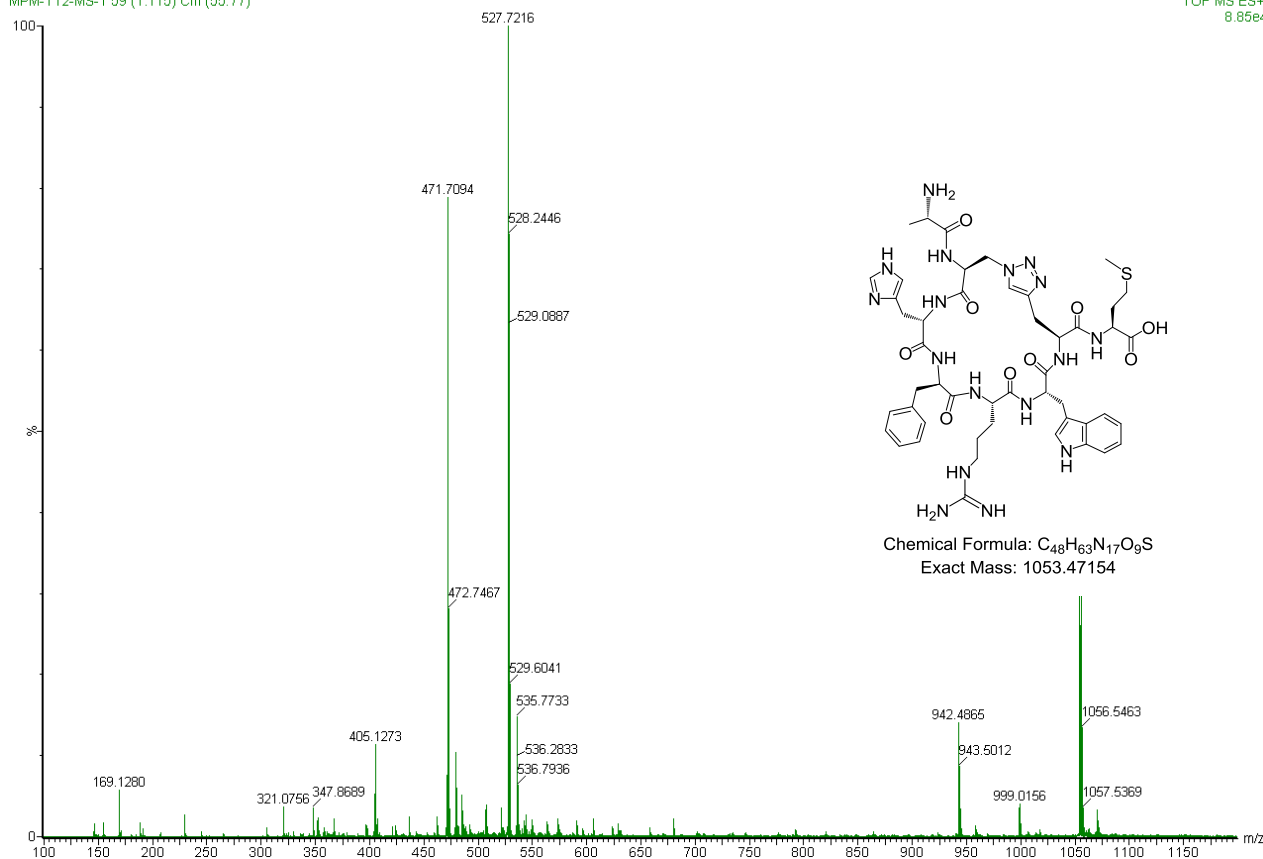
Figure. S16. High resolution mass spectrum of compound **12**
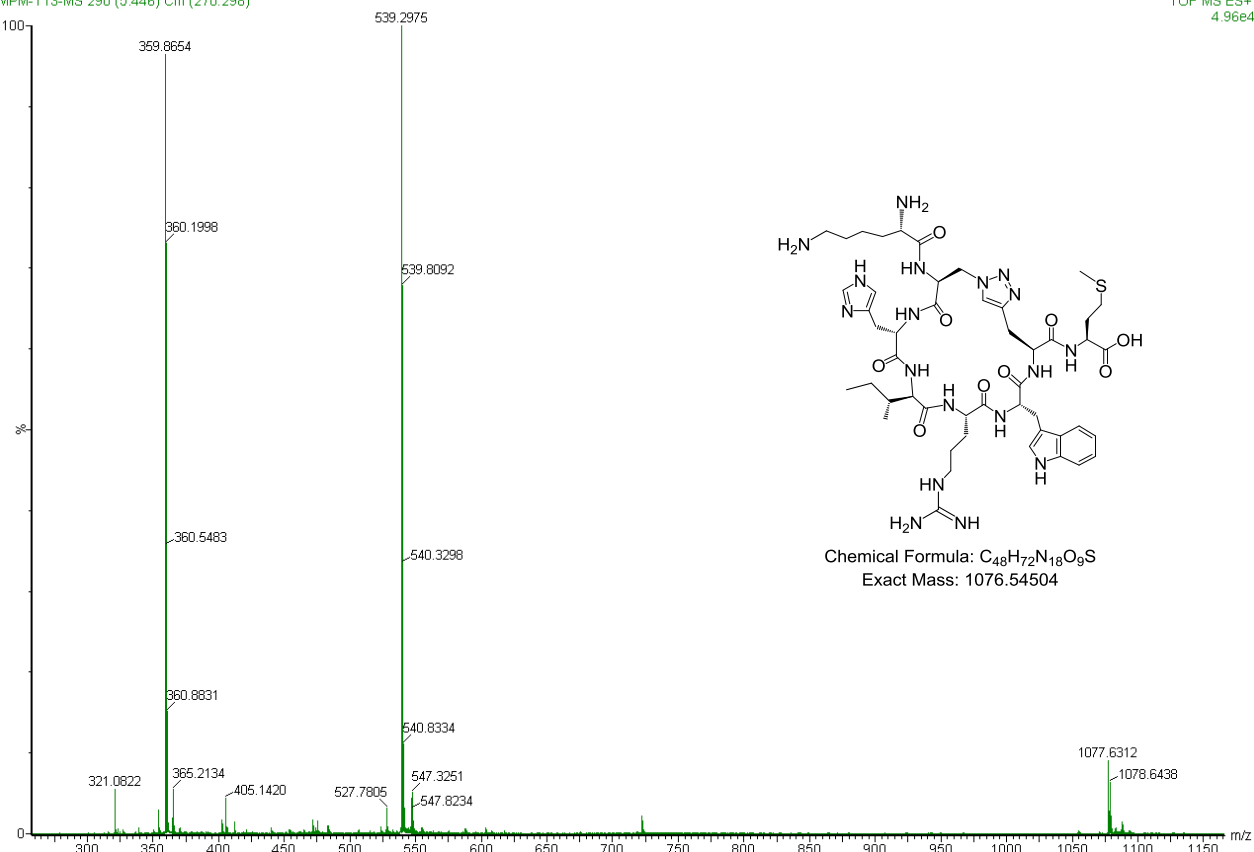
Figure. S17. High resolution mass spectrum of compound **13**
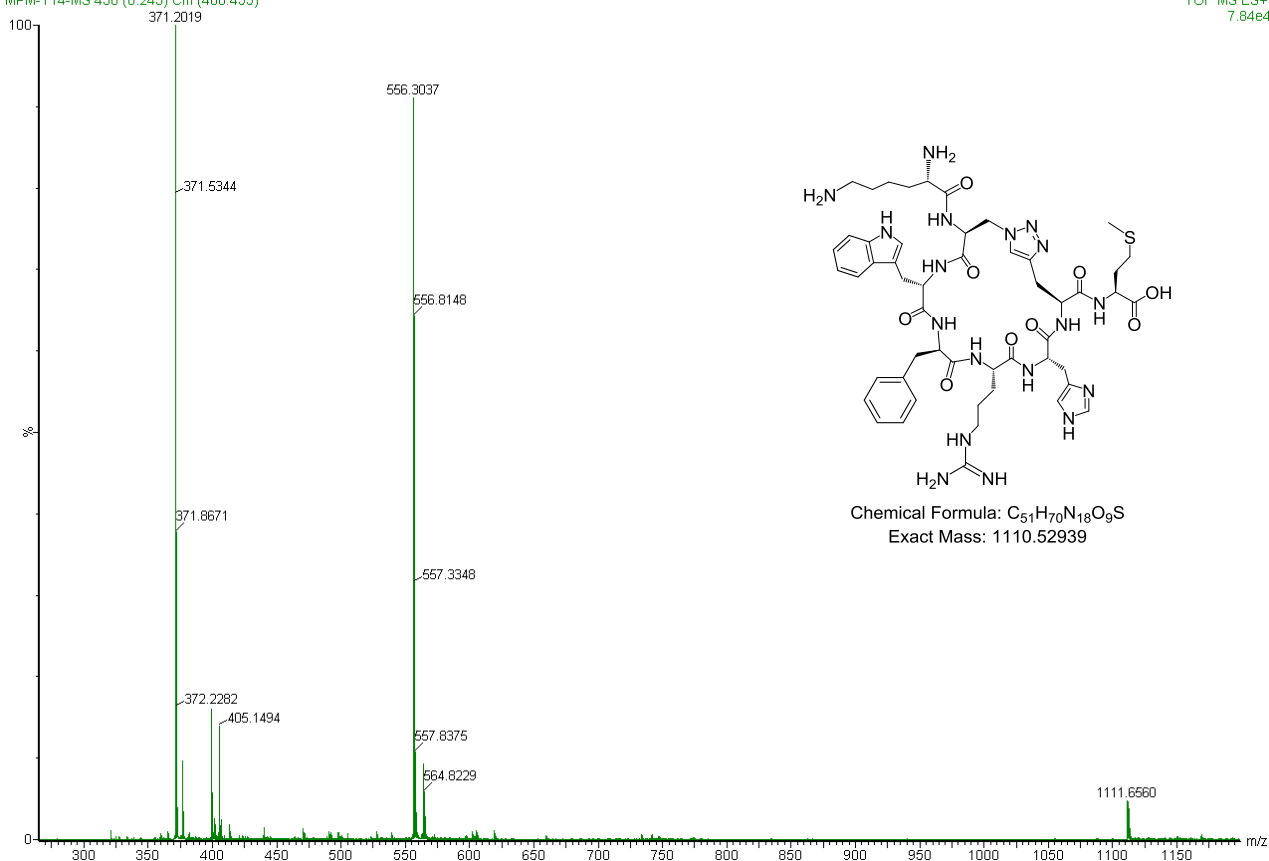
Figure. S18. High resolution mass spectrum of compound 1**4**

S16

Chemical Formula: $C_{53}H_{71}N_{17}O_9S$
Exact Mass: 1121.53414
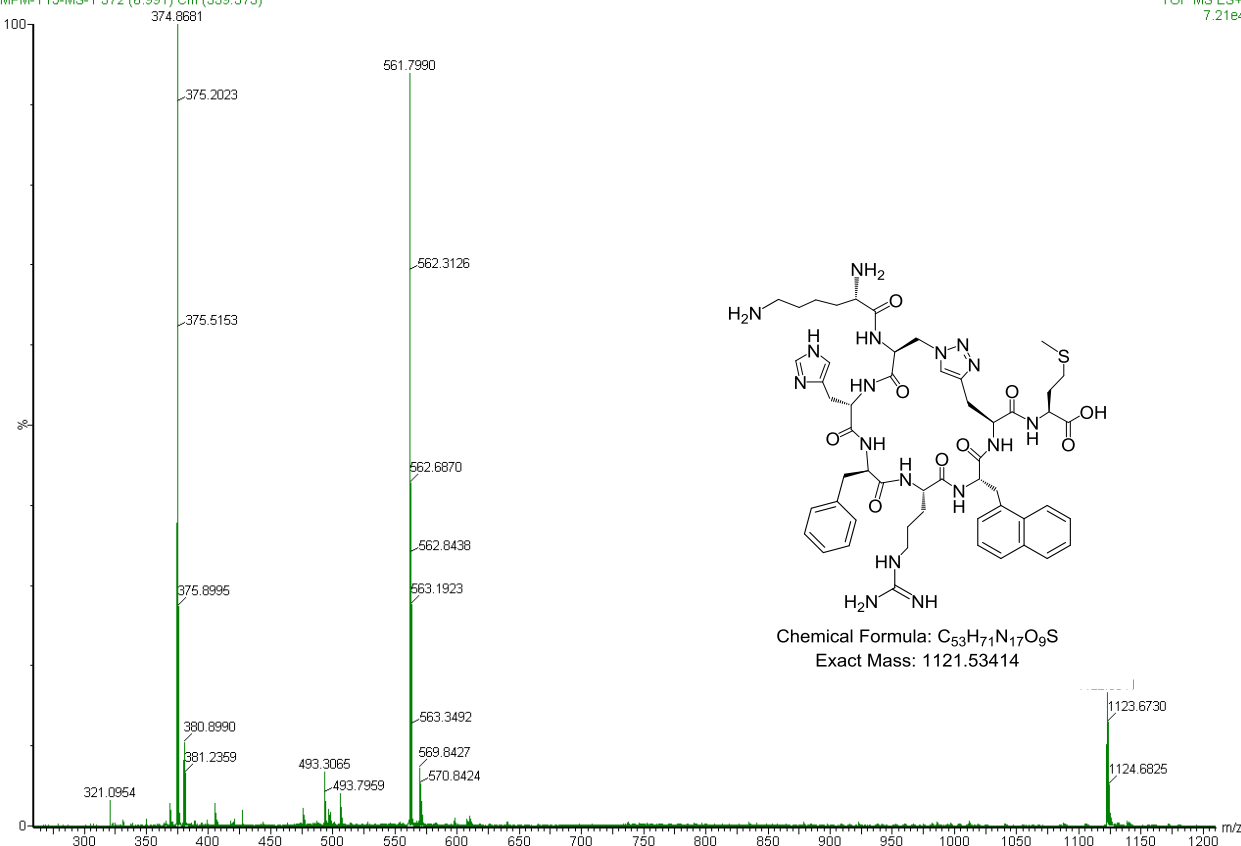
Figure. S19. High resolution mass spectrum of compound **15**

Chemical Formula: $C_{47}H_{62}N_{16}O_8S$
Exact Mass: 1010.46572
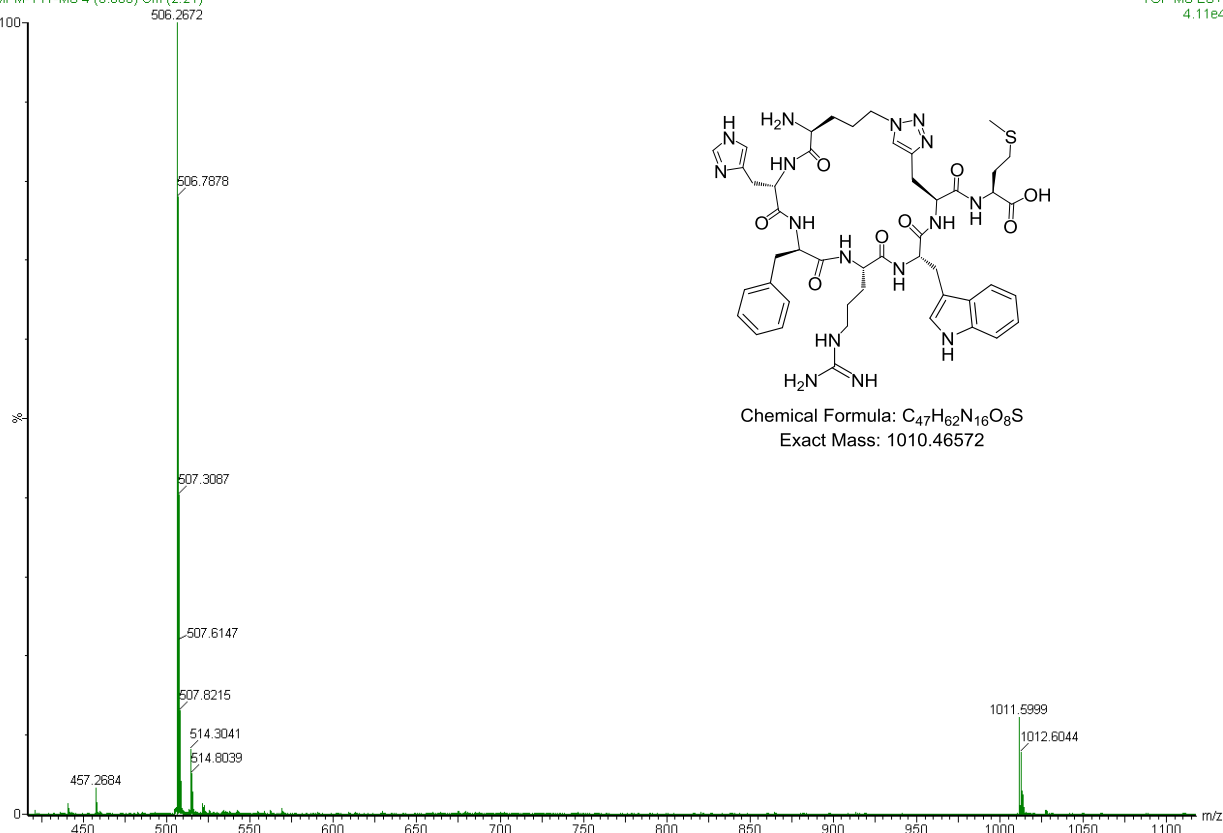
Figure S20. High resolution mass spectrum of compound **16**

S17

Chemical Formula: $C_{48}H_{64}N_{18}O_8S$
Exact Mass: 1052.49

Figure. S21. High resolution mass spectrum of compound **17**

Table S3. Assignment of the ^{1}H-NMR spectrum of compound **1** (in H_2O/D_2O: 9/1 containing 2 % deuterated-acetic acid) and the relative NOEs (NOESY, 800 mHz, 1024 x 512, 50 ms and 300 ms mixing time) obtained by peak volume integration using Topspin (Bruker). For assigning the COSY experiment and NOSEY/ROESY, experiments with various mixing times (50, 100, 200, 300, 400 and 600 ms) were recorded. Correlations with known fixed distances are marked in grey. The CH_2 NOE-correlations were generally too close to the diagonal to be used as reference for the distance calculation except that of Met-H^β. However, the best reference was that of the Trp indole NH - H^2 providing a balanced calculation of the 73 NOE-distances used in the MD-simulations.

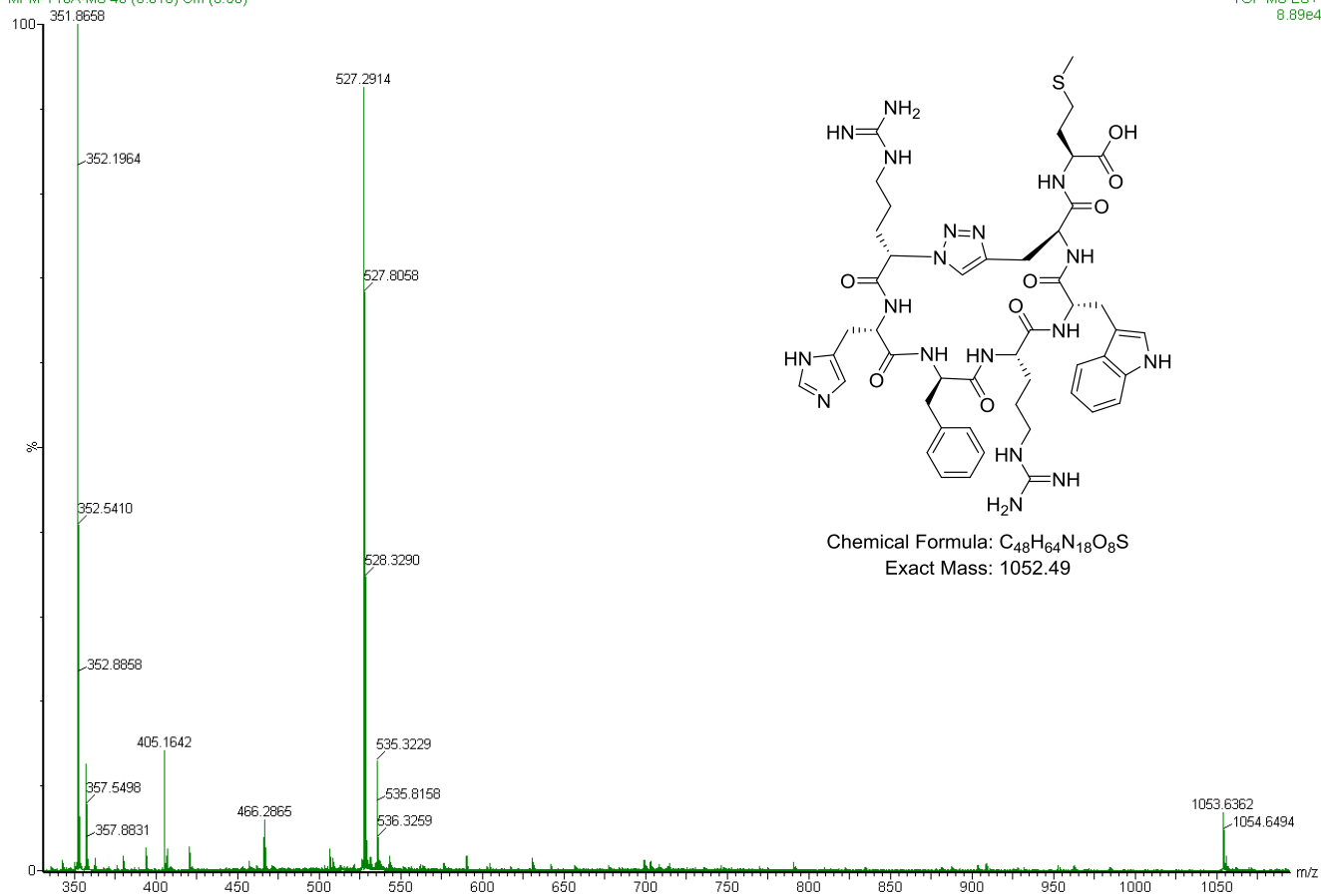| Aa	^{1}H	δ/ppm	Number (multiplicity)	NOE	^{1}H	dist	NOE	^{1}H	dist	NOE	^{1}H	dist	NOE	^{1}H	dist	^{13}C -δ/ppm
K	K^a	3.958	1 (t)	0.0072	K^{b1}	3.13	0.0122	K^{b2}	2.87							52.65
	K^{b1}	1.729	1 (m)													30.22
	K^{b2}	1.765	1 (m)	0.01	Dap^N	2.90										
	K^g	1.319	2 (t)	0.1281	K^{b1}	1.94	0.1006	K^{b2}	2.02							21.12
	K^d	1,38	2 (t)	0.0286	K^g	2.49										26.27
	K^e	2.869	2 (t)													38.89
	K^N	4.735	2 (bs)	0.0270	Dap^N	2.51										
Dap	Dap^a	4.668	-	0.0186	H^N	2.67										53.41
	Dap^{b1}	2,823	1 (d)	0.0239	R^{Gua}	2.56	0.0083	Dap^N	3.06							38.89
	Dap^{b2}	2,907	1 (d)	0.0410	R^{Gua}	2.34	0.0146	Dap^N	2.78							
	Dap^N	8.675	1 (d)	0.0173	H^b	2.71	0.0341	K^a	2.42	0.0200	Dap^a	2.64				
H	H^a	4.508	-	0.0367	Pra^{b2}	2.39	0.0438	H^{b1}	2.32	0.0382	H^{b2}	2.37					55.35
	H^{b1}	2.856	1 (dd)	1	H^{b2}	1.38										26.87
	H^{b2}	2.941	1 (dd)	0.0168	H^N	2.72										
	H^2	8.411	1 (s)													133.29
	H^4	6.928	1 (s)	0.0087	R^{d2}	3.03										117.18
	H^N	8.639	0.3 (m)	0.0256	H^a	2.53	0.0075	$Pra^{5\text{-}Tria}$	3.10							
	H^{ImNH}	9.874	1 (m)	0.0035	H^{b1}	3.53	0.0024	H^{b2}	3.76							
f	f^a	4.557	-	0.0479	f^{b2}	2.28	0.0179	f^{b1}	2.69	0.0201	$f^{2.6}$	2.64					55.01
	f^{b1}	2.808	1 (dd)	0.4179	f^{b2}	1.59	0.0416	f^N	2.34							37.43
	f^{b2}	2.941	1 (dd)	0.0162	R^{g2}	2.74	0.0559	f^N	2.23							
	$f^{2,6}$	7.107	1 (m)	0.0488	f^{b1}	2.28	0.0421	f^{b2}	2.33	0.0088	W^N	3.03	0.0049	f^N	3.34	129.00
	$f^{3,5}$	7.230	1 (m)	0.3620	$f^{2.6}$	1.63	0.0117	f^{b1}	2.89	0.0158	f^{b2}	2.75					128.73
	f^4	7.185	1 (d)	0.6539	$f^{3.5}$	1.48										127.28
	f^N	8.585	1 (d)	0.0405	H^{1b}	2.35	0.1030	H^a	2.01	0.0304	f^a	2.46	0.0058	R^N	3.25	
R	R^a	3.868	1 (m)	0.0380	R^b	2.37	0.0430	R^a	2.33							54.07
	R^b	1.217	1 (m)	0.0250	R^N	2.55	0.02	W^N	2.64	0.0101	R^{d2}	2.96					27.81
	R^{g1}	0.779	1 (m)	0.0475	R^b	2.29	0.0097	R^N	2.98	0.0022	W^N	3.82					23.61
	R^{g2}	0.89	1 (m)	0.4330	R^{g1}	1.58	0.0112	R^N	2.91	0.0058	W^N	3.25	0.0634	R^b	2.18	
	R^{d1}	2.718	1 (m)	0.0085	R^{g2}	3.05	0.0244	R^{g1}	2.55							40.27
	R^{d2}	2.728	1 (m)	0.0168	R^{g1}	2.72										
	R^N	8.103	1 (m)	0.0190	R^a	2.66	0.0821	f^a	2.09	0.0060	f^{b1}	3.23	0.0089	f^{b2}	3.03	

	Label	δ	Mult	J	P1	v	J	P2	v	J	P3	v	J	P4	v	
	R^{Gua1}	6.898	1 (m)													
	R^{Gua2}	7.247	1 (m)													
W	W^a	4.413	-	0.0337	W^N	2.42	0.0057	W^2	3.25							51.57
	W^{b1}	3.054	1(dd)	0.6736	W^{b2}	1.47	0.0323	W^a	2.44	0.0186	W^N	2.67				25.58
	W^{b2}	3.145	1(dd)	0.0396	W^a	2.36	0.0210	W^N	2.62							
	W^2	7.100	1 (s)	0.0155	W^{b1}	2.76	0.0085	W^{b2}	3.05	0.0021	R^N	3.85				121.93
	W^4	7.406	1 (d)	0.0093	W^a	3.00	0.0113	W^{b2}	2.91	0.0083	W^{b1}	3.06				118.01
	W^5	7.008	1 (t)	0.0715	W^4	2.14										119.22
	W^6	7.111	1 (t)	0.1856	W^5	1.82										127.17
	W^7	7.366	1 (d)	0.0215	W^6	2.61										111.77
	W^N	8.074	1 (d)	0.0642	R^b	2.18	0.0292	W^{b1}	2.48	0.0042	W^2	3.43	0.0016	W^4	4.02	
	W^{InNH}	9.974	1 (s)	0.0245	W^2	2.55	0.0038	W^7	3.49	0.0277	$Pra^{5\text{-}Tria}$	2.50				
Pra	Pra^a	4.499	-	0.0150	Pra^b	2.77	0.0332	M^N	2.43							52.42
	Pra^{b1}	2.966	1 (m)	0.0265	Pra^N	2.52	0.0091	$Pra^{5\text{-}Tria}$	3.01							26.97
	Pra^{b2}	3.198	1 (m)	0.2736	Pra^{b1}	1.71	0.0147	$Pra^{5\text{-}Tria}$	2.78							
	Pra^N	7.741	1 (d)	0.0142	Pra^{b2}	2.80	0.0302	W^a	2.47	0.0190	Pra^a	2.67				
	$Pra^{5\text{-}Tria}$	7.486	1 (s)	0.0107	H^a	2.93	0.0087	Dap^N	3.04	0.0100	Pra^N	2.97	0.0022	f^N	3.82	125.24
M	M^a	4.332		0.0083	M^{b2}	3.06	0.0163	M^{b1}	2.73							51.57
	M^{b1}	1.882	1 (m)	0.2078	M^{b2}	1.79	0.0076	M^N	3.11							29.71
	M^{b2}	2.064	1 (m)	0.0145	M^{g1}	2.79	0.0072	M^N	3.13							
	M^{g1}	2.402	1 (m)	0.4800	M^{g2}	1.56										29.29
	M^{g2}	2.462	1 (m)	0.0258	M^{b2}	2.53										
	M^{Me}	2.004	3 (s)													14.08
	M^N	7.948	1 (d)	0.0095	M^a	2.99	0.0190	Pra^N	2.66	0.0139	M^{b1}	2.81				

Table S4. The distances from a NOESY experiment of compound **1** applied in the restrained molecular dynamic calculations using Molecular Operation Environment (MOE, CCG). Weights for short range NOE's smaller than 3 Å were set at 1, while weights for short range distances larger than 3 Å were set at 0.5 in all restrained MD calculations. The final simulated distances correlated quite accurately with those listed in table S3.

```
 Type      Weight     [L,U]        Atoms
> distance  5.000e-001 [3.13,3.14]  7:LYS4.(HB2 HA)
> distance  1.000e+000 [2.87,2.88]  7:LYS4.(HB3 HA)
> distance  1.000e+000 [1.94,2.1]   7:LYS4.(HG2 HB3)
> distance  1.000e+000 [2.02,2.03]  7:LYS4.(HG3 HB2)
> distance  1.000e+000 [2.49,2.7]   7:LYS4.(HD2 HG2)
> distance  1.000e+000 [2.49,2.7]   7:LYS4.(HD3 HG3)
> distance  1.000e+000 [2.51,2.52]  7:ALA5.H 7:LYS4.H2
> distance  1.000e+000 [2.42,2.43]  7:ALA5.H 7:LYS4.HA
> distance  1.000e+000 [2.9,2.92]   7:ALA5.H 7:LYS4.HB3
> distance  1.000e+000 [2.67,2.68]  7:ALA5.(H HA)
> distance  5.000e-001 [3.06,3.07]  7:ALA5.(H HB2)
> distance  1.000e+000 [2.78,2.79]  7:ALA5.(H HB3)
> distance  1.000e+000 [2.67,2.68]  7:HIS6.H 7:ALA5.HA
> distance  1.000e+000 [3.1,3.2]    7:HIS6.H 7:ALA5.H
> distance  1.000e+000 [2.53,2.54]  7:HIS6.(HA H)
> distance  1.000e+000 [2.32,2.33]  7:HIS6.(HB2 HA)
> distance  1.000e+000 [2.7,2.75]   7:HIS6.(HB3 H)
> distance  1.000e+000 [2.37,2.38]  7:HIS6.(HB3 HA)
> distance  5.000e-001 [3.53,3.54]  7:HIS6.(HD2 HB2)
> distance  5.000e-001 [3.76,3.77]  7:HIS6.(HD2 HB3)
> distance  2.000e+000 [3.82,3.83]  7:DPN7.H 7:ALA5.H
> distance  1.000e+000 [2.01,2.02]  7:DPN7.H 7:HIS6.HA
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> distance  1.000e+000 [2.28,2.29]  7:DPN7.(HD2 HB2)
> distance  1.000e+000 [2.33,2.34]  7:DPN7.(HD2 HB3)
> distance  1.000e+000 [3.25,3.26]  7:ARG8.H 7:DPN7.H
> distance  1.000e+000 [2.28,2.29]  7:ARG8.H 7:DPN7.HA
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> distance  5.000e-001 [3.23,3.24]  7:ARG8.H 7:DPN7.HB3
> distance  1.000e+000 [2.66,2.67]  7:ARG8.(HA H)
> distance  1.000e+000 [2.55,2.56]  7:ARG8.(HB2 H)
> distance  1.000e+000 [2.37,2.38]  7:ARG8.(HB3 HA)
> distance  5.000e-001 [2.98,2.99]  7:ARG8.(HG2 H)
> distance  1.000e+000 [2.29,2.3]   7:ARG8.(HG2 HB2)
> distance  1.000e+000 [2.74,2.75]  7:ARG8.HG3 7:DPN7.HB3
> distance  5.000e-001 [2.91,2.92]  7:ARG8.(HG3 H)
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> distance  5.000e-001 [3.05,3.06]  7:ARG8.(HD2 HG2)
> distance  1.000e+000 [2.79,2.8]   7:ARG8.(HD2 HG3)
> distance  1.000e+000 [2.72,2.73]  7:ARG8.(HD3 HG3)
> distance  1.000e+000 [2.34,2.35]  7:ARG8.HH22 7:ALA5.HB2
> distance  1.000e+000 [2.56,2.57]  7:ARG8.HH22 7:ALA5.HB3
> distance  3.000e+000 [2.18,2.19]  7:TRP9.H 7:ARG8.HB3
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> distance  1.000e+000 [2.62,2.63]  7:TRP9.(HB2 H)
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> distance  1.000e+000 [2.67,2.68]  7:TRP9.(HB3 H)
> distance  1.000e+000 [2.36,2.37]  7:TRP9.(HB3 HA)
> distance  1.000e+000 ]3.85,3.84[  7:TRP9.HD1 7:ARG8.H
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> distance  1.000e+000 [2.97,2.98]  7:ALA10.H 7:ALA5.H
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> distance  1.000e+000 [2.67,2.68]  7:ALA10.(H HA)
> distance  1.000e+000 [2.67,2.68]  7:ALA10.(H HB2)
> distance  1.000e+000 [2.8,2.81]   7:ALA10.(H HB3)
> distance  1.000e+000 [2.53,2.54]  7:MET11.(HG3 HB2)
> distance  1.000e+000 [2.43,2.44]  7:MET11.H 7:ALA10.HA
> distance  1.000e+000 [2.66,2.67]  7:MET11.H 7:ALA10.H
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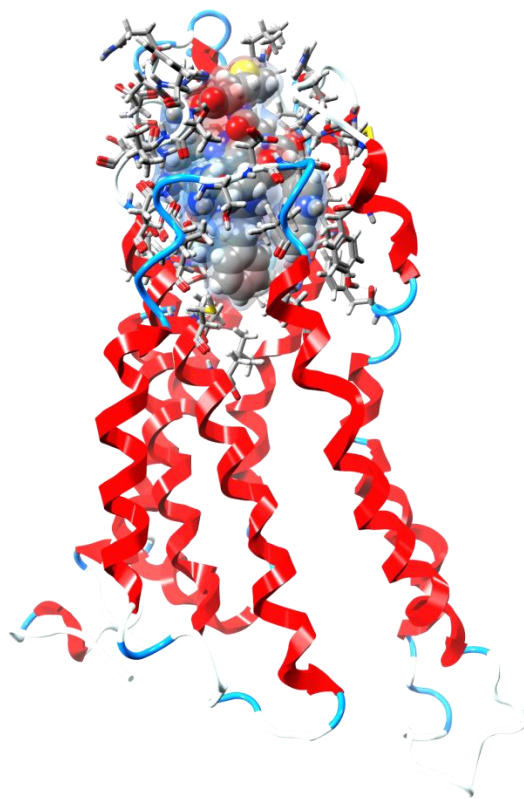
Figure S22. The structure of compound **1** obtained by implementing the 73 NOEs observed in the NOESY spectrum of the compound as indicated in table S4. The compound was subjected to 3 rounds of simulated annealing from 930, 730, and 530 to 230 K respectively. The resulting structure was subjected to 5 ns of MD-calculation prior the energy minimization. The restrained structure was inserted into the fixed binding site of the receptor, without any serious collisions with receptor residues. It was subjected to 200 ps MD-simulation, then receptor contact residues were released and simulation was continued for 500 ps. Finally, the NOE-restraints were removed and the simulation was allowed to continue for 1 ns without any structural changes.

Figure S23. The NOE restraints imposed upon compound **1** were removed and the free ligand was energy minimized to show that the used restraints did not induce significant strain to the cyclic structure, which was largely conserved.

Figure S24. Compound **1** in its NOE restrained conformation bound to MC4R in its active conformation. The ligand is shown with its solvent accessible surface. The extracellular face of the receptor-ligand complex is at the top of the figure with the intracellular face at the bottom.
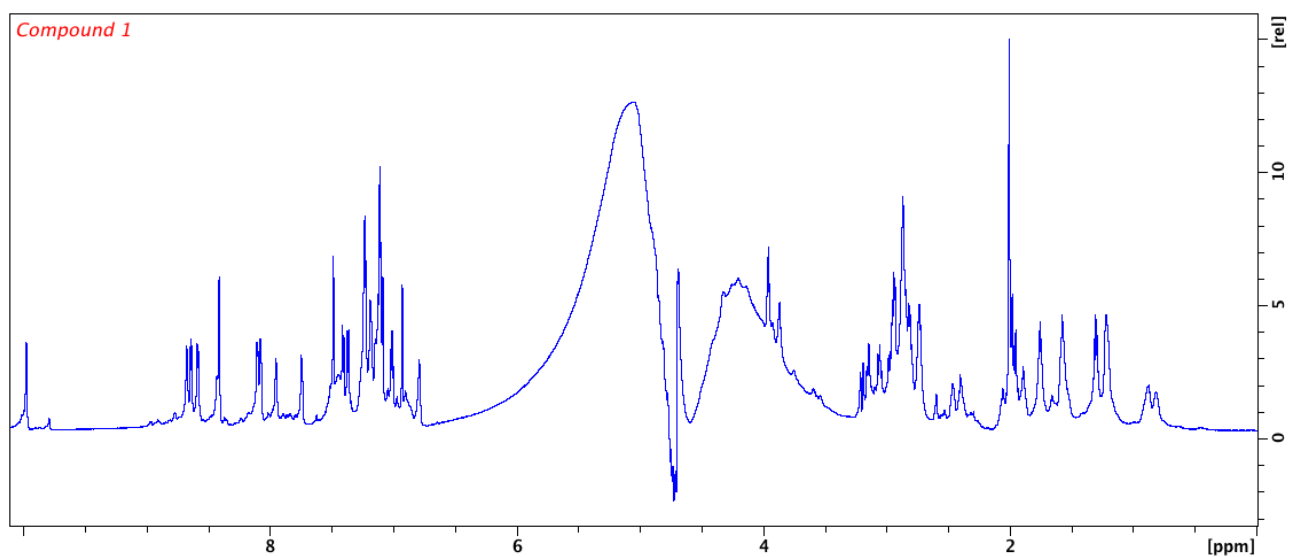
Figure S25: 1D ^{1}H-NMR spectrum of compound **1** (800 MHz in H_2O / D_2O / $HOAc_{d3}$ - 88:10:2 recorded with water suppression.
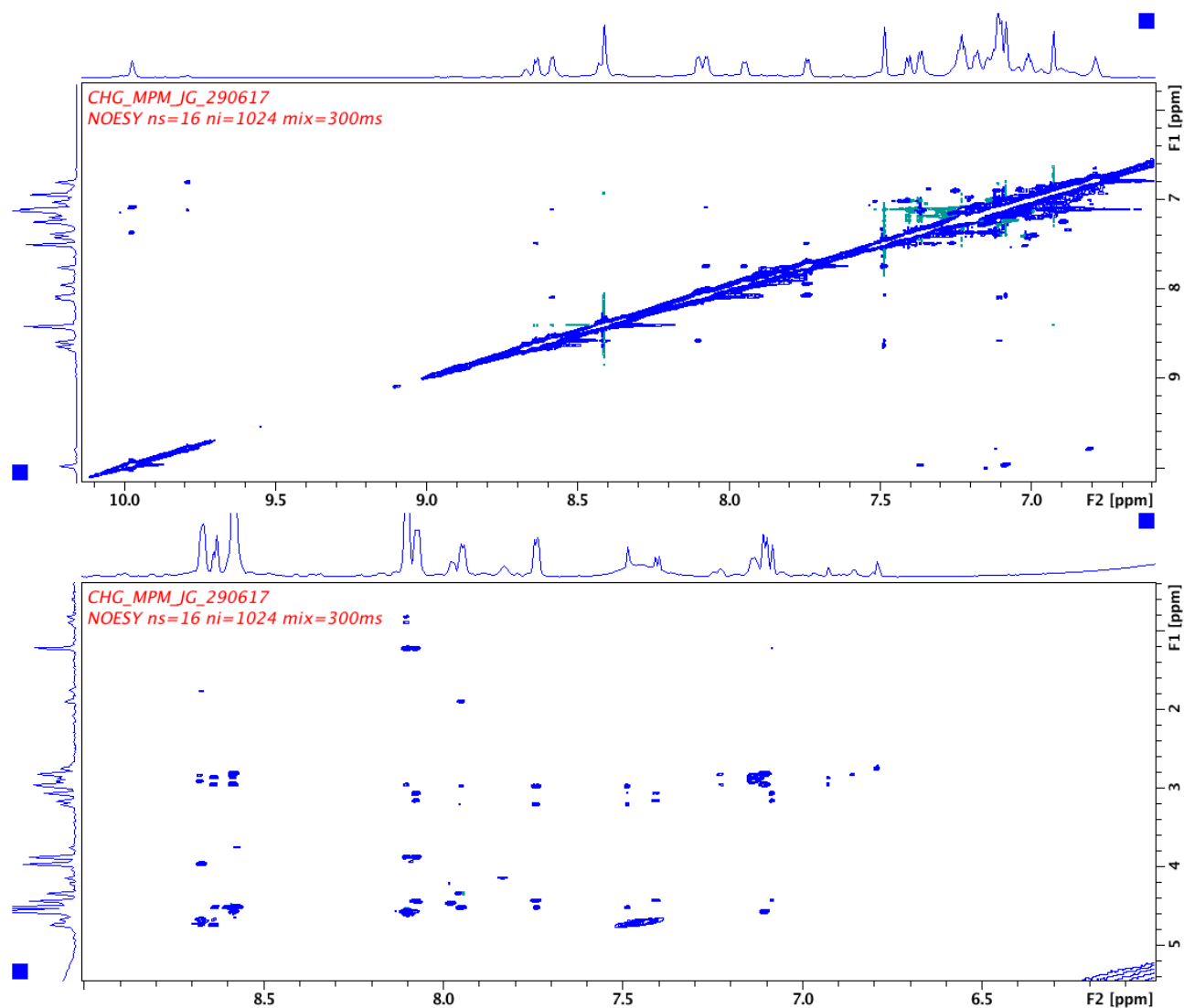
Figure S26. The NH-NH, NH-H$^{a/b}$, and the aliphatic region of the recorded NOESY spectrum (800 MHz, 300 ms mixing time) used in the structural analysis of compound **1**.
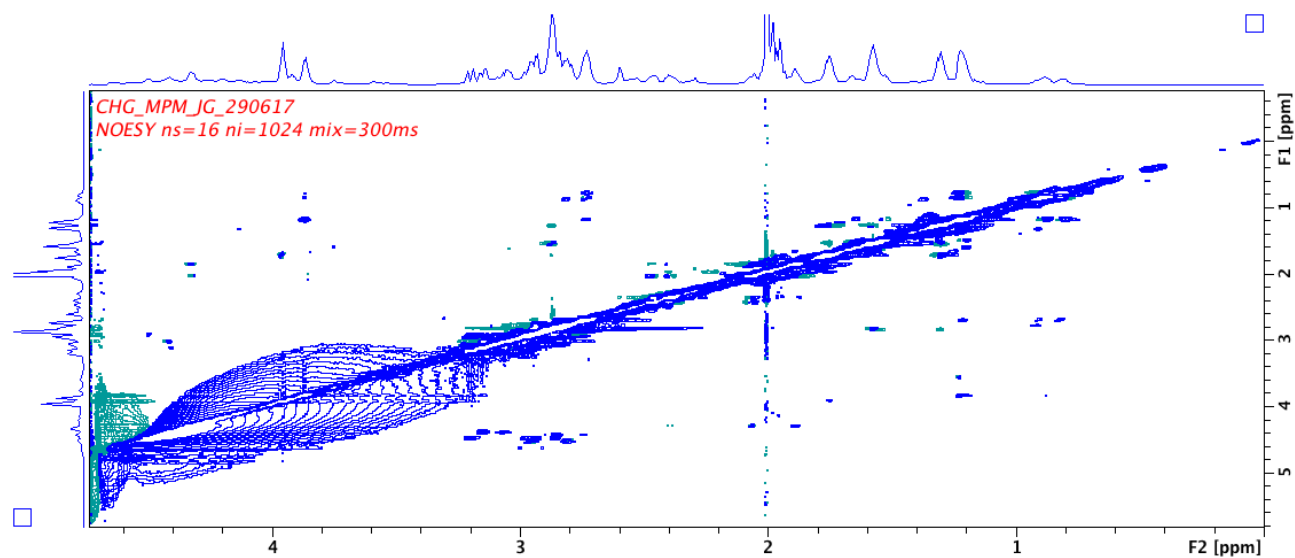
Figure S27. The H^a-H^b and the aliphatic region of the recorded NOESY spectrum (800 MHz, 300 ms mixing time) used in the structural analysis of compound **1**.

a
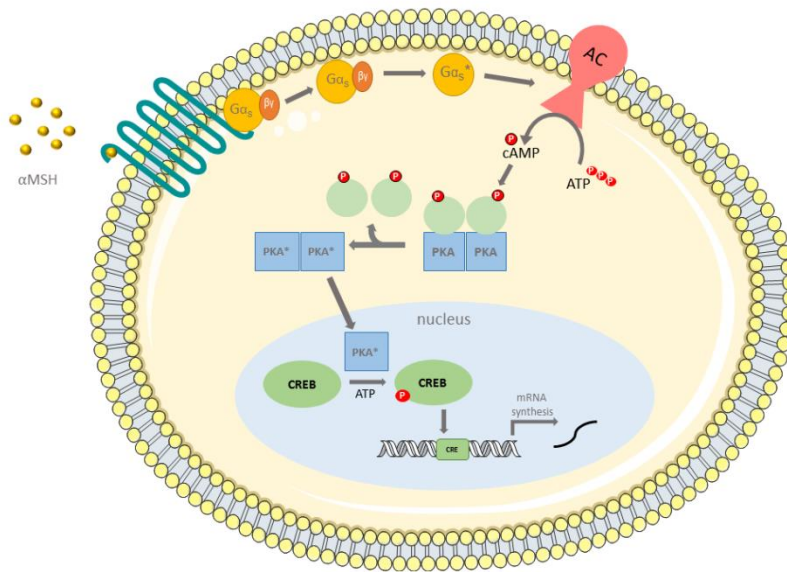
b
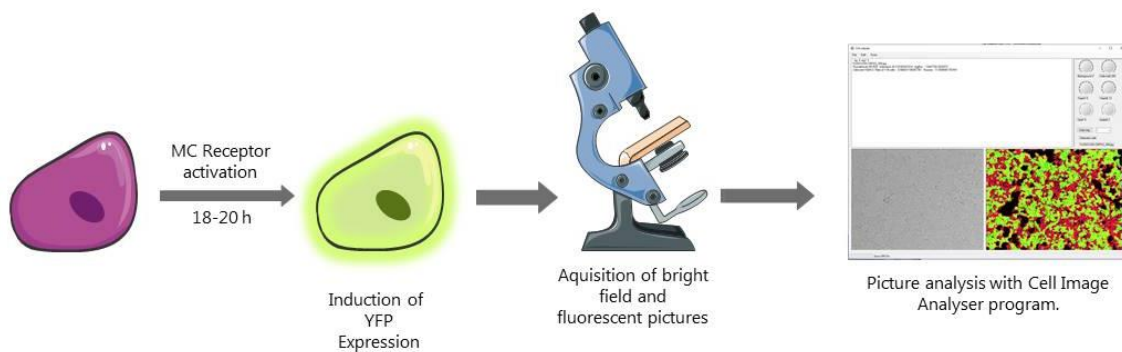
Figure S28. Overview of the cAMP-mediated transcriptional reporter assay system. a. Schematic for canonical cAMP signaling cascade as a mechanism for Melanocortin-mediated regulation of gene expression. α-MSH stimulation of melanocortin receptors (MCRs) leads to activation of the α-subunit of the stimulatory heterotrimeric G-protein, $G_{\alpha s}$, and the subsequent activation of adenylyl cyclase (AC) enzymes, which convert ATP in cAMP. cAMP-mediated activation of protein kinase A (PKA) leads to the dissociation of the catalytic subunit of the PKA complex. Upon translocation of the active PKA catalytic subunit into the nucleus, PKA can phosphorylate the transcription factor CREB (cAMP-Response Element Binding Protein; CREB-P denoting the phosphorylated variant). CREB-P, when bound to DNA with defined consensus sequences called cAMP Response Elements (CREs) upstream of genes can enhance the transcription of these genes via facilitation of the function of the core transcriptional machinery. b. Schematic representation of CRE reporter assay. Stimulation of cells expressing MCRs over 18-20 hours results in an increase in EYFP expression. This is visualized microscopically and aanalyzed with custom-designed data acquisition software.
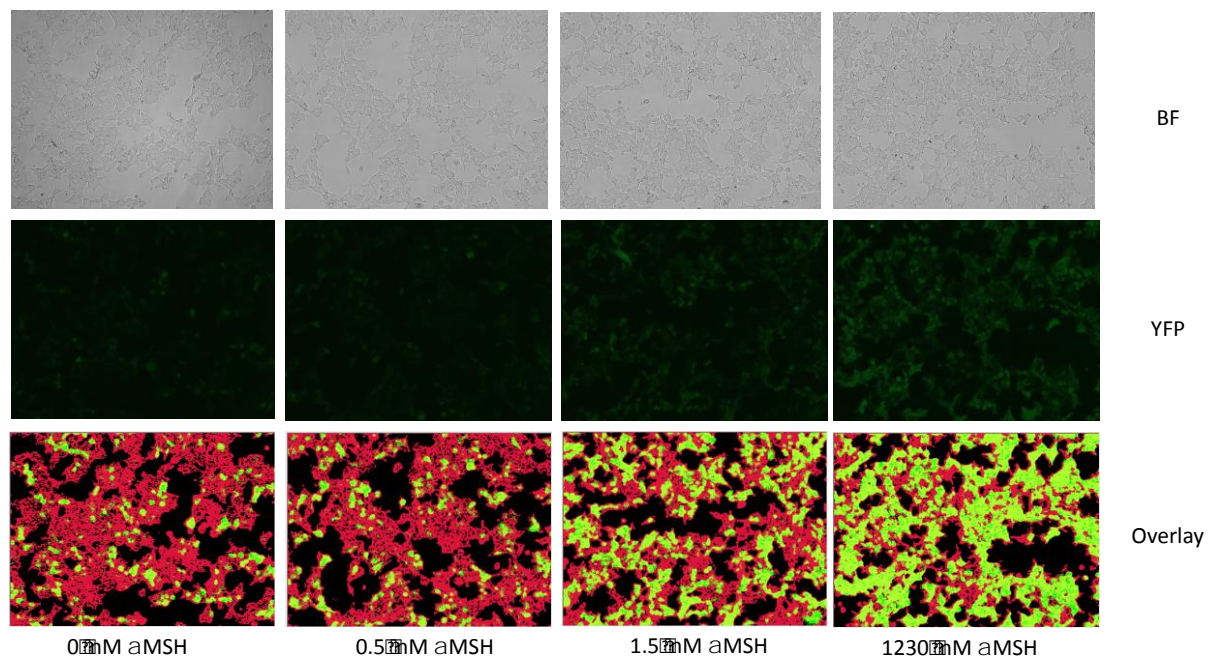
Figure S29. Comparison of bright field, fluorescence images and overlaid analysis results from the CIA program, for a CRE transcriptional reporter gene assay for MC4R expressing cells stimulated with α−MSH. Bright field (BF), and fluorescence images (YFP; filter cube: ex: 500/20 nm, em: 530/30 nm)) are shown with the image analysis result (Overlay) for a range encompassing 0-1200 nM concentrations of α-MSH.
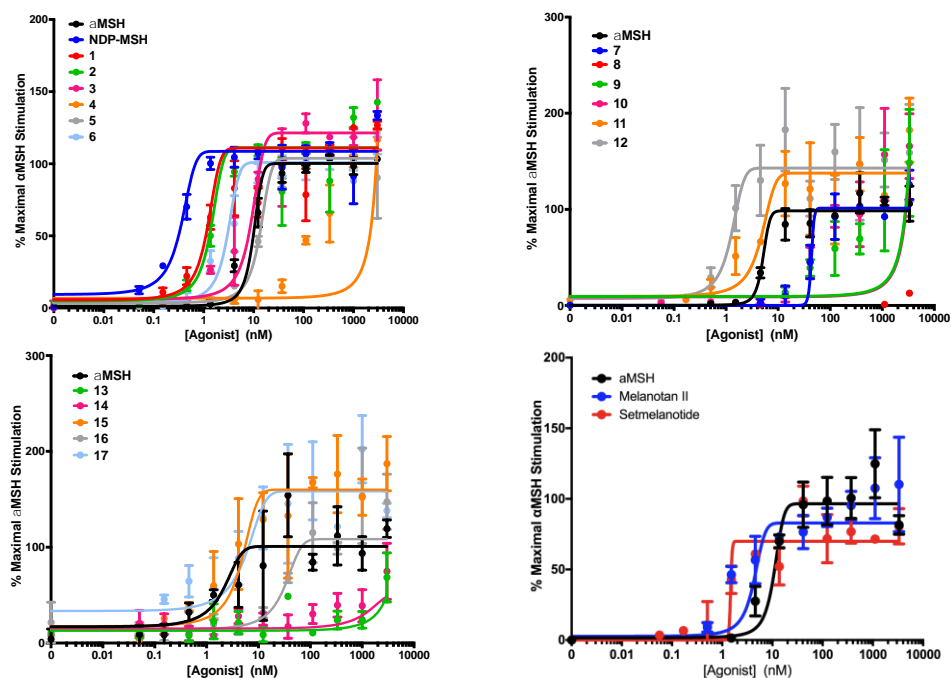
Figure S30. Concentration response curves for single vector reporter gene assay. Depicted are representative tracings for the indicated compounds from individual experiments using MC4R expressing cells. Data are displayed as the average of normalized, background-corrected responses ± standard error of the mean. Curves have been generated and fitted using GraphPad Prism 7.0 analytical software.
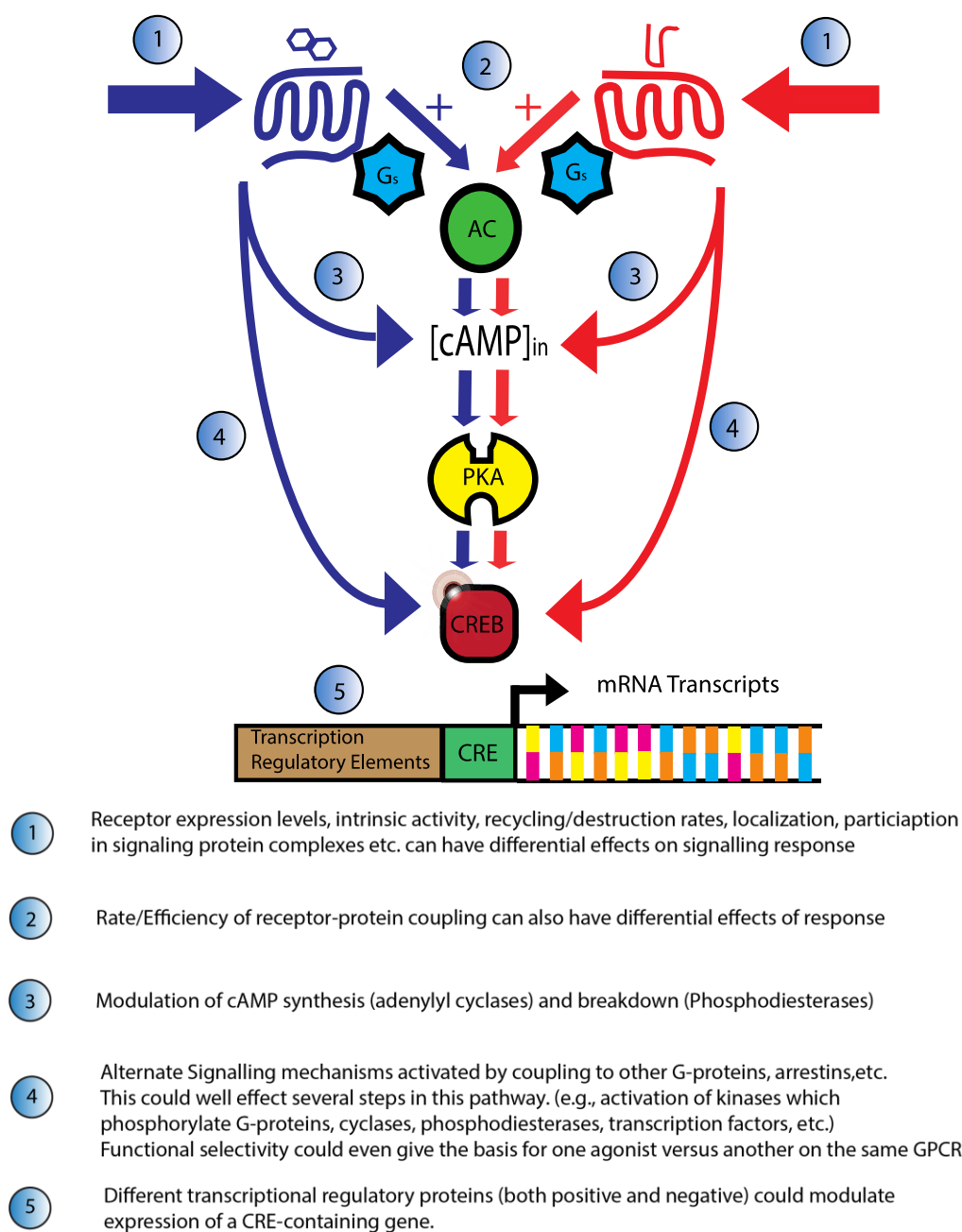
Figure S31. Overview of variations and redundancies in Gs-mediated modulation of CRE-regulated gene expression. Displayed, and expanded upon in the legend, are points of convergence, as well as places where divergence can occur, between two different GPCRs (one displayed as blue with a small molecule ligand, and a red receptor with a hypothetical peptide ligand) that utilize cAMP signaling. The net effect on a given gene's expression following the activation of different G_s-coupled receptors is the integration of the extent of specific signalling pathways activated as well as the timing of their activation.