

## **Supporting Information: Antagonizing the Androgen Receptor with a Biomimetic Acyltransferase**

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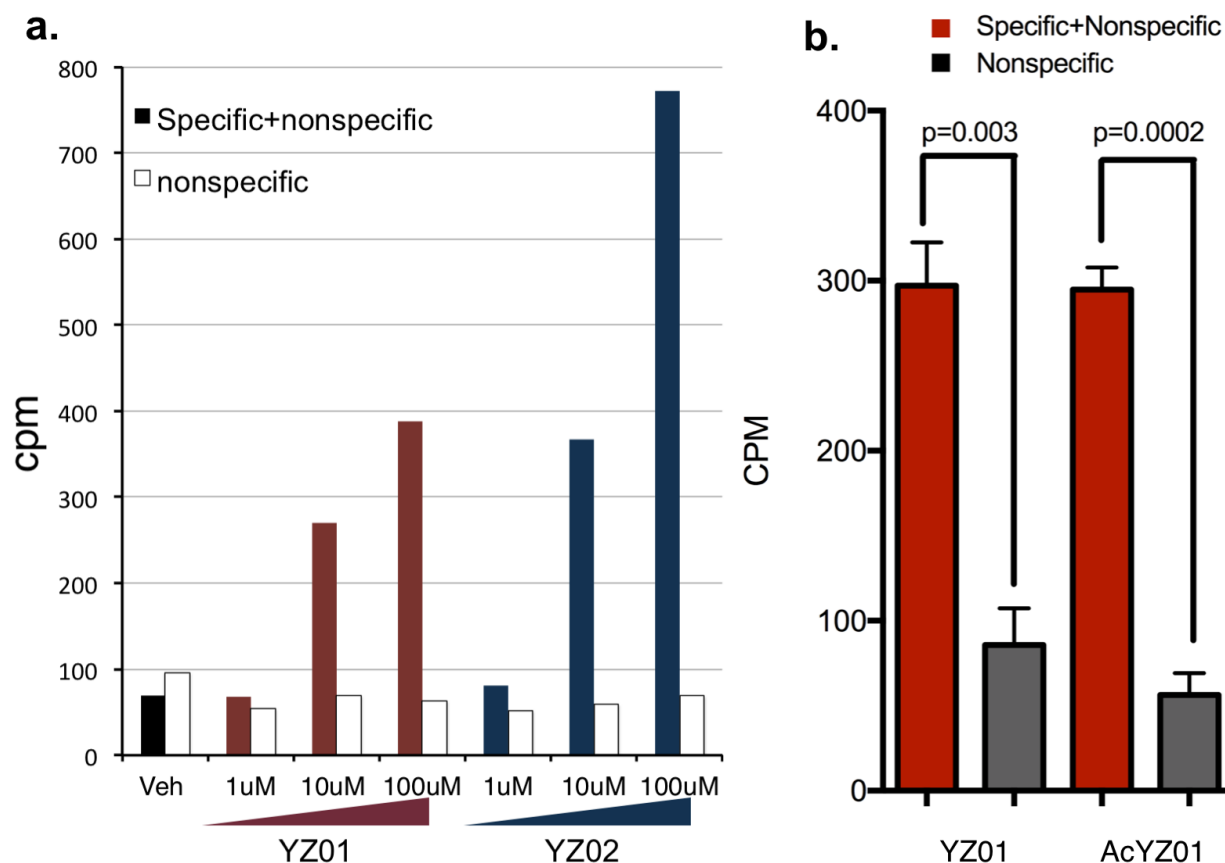
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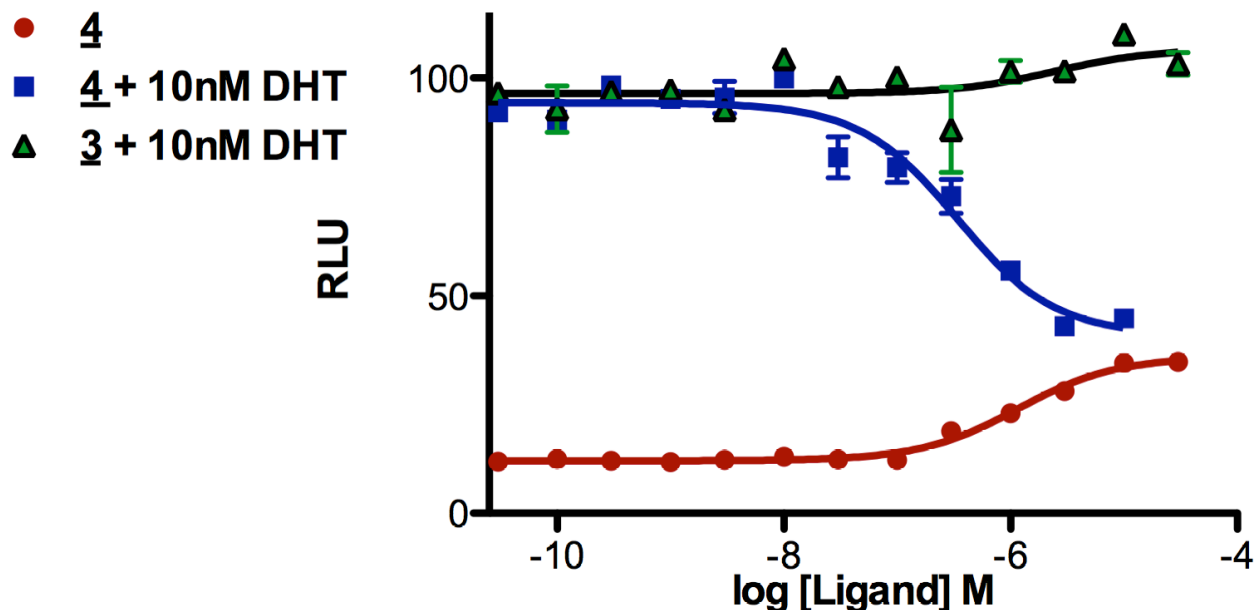


**Figure S1.** a. Total and non-specific radio labeling of YZ01 and YZ02 in  $^{14}\text{C}$ -pyruvate treated HEK293t cells. b. Total and non-specific radio labeling of YZ01 and AcYZ01 in  $^{14}\text{C}$ -acetate treated HEK293t cells.

**Methods for Cellular radio-labeling studies of Biotin Probes YZ01 and YZ02 with  $^{14}\text{C}$ -acetate or Pyruvate :**

HEK293t Cells (300,000 cells/well) were seeded individually into 32 mm wells (6-well plate) in DMEM supplemented with 10% CCS, 4 mM L-Glutamine. After 12-16 hours the media was changed to pyruvate-free DMEM supplemented with 10% dialyzed CCS (1 ml). After 5 h acetylation probe YZ01 or YZ02 was added along with 2 uCi  $^{14}\text{C}$ -pyruvate (or 5 uCi  $^{14}\text{C}$ -acetate) at indicated concentrations. The cells were incubated at 37°C for 16 h before the media was remove and retained. The cells were lysed with 200 uL lysis buffer (5% glycerol, 0.05% tritonx-100 in PBS) followed by washing with 200 uL DPBS. Lysates were then combined with the cell media and centrifuged (12,000 rpm, 20 min). The supernatant was then divided into two 450 uL aliquots. Control aliquots were treated with 50 uL of 200 mM “cold” (unlabeled) biotin (buffered with an equivalent of triethylamine) in DMSO to block specific binding of YZ01 or YZ02. To the remaining aliquot was added 50 uL DMSO. Streptavidin-agarose Beads (50 uL, Thermo Scientific, #20357) were added to both aliquots and rocked on a rocker platform for 3.5 hrs at 4°C. The slurry was added to a spin column and washed with lysis buffer (7 x 500uL). The beads were wetted with 100uL of methanol dried for 12 hrs prior to addition of scintillation cocktail. After mixing with scintillation cocktail the specific activity was measured in a microbeta scintillation counter (Figure 1 and S1a).

**Reacylation of S-acetylthiosalicylamide conjugates:** To demonstrate that thiosalicylamides are capable of repetitive acylation, cellular acetylation reactions were performed using  $^{14}\text{C}$ -acetate and the pre-acetylated form of YZ01, AcYZ01 (Figure S1b).



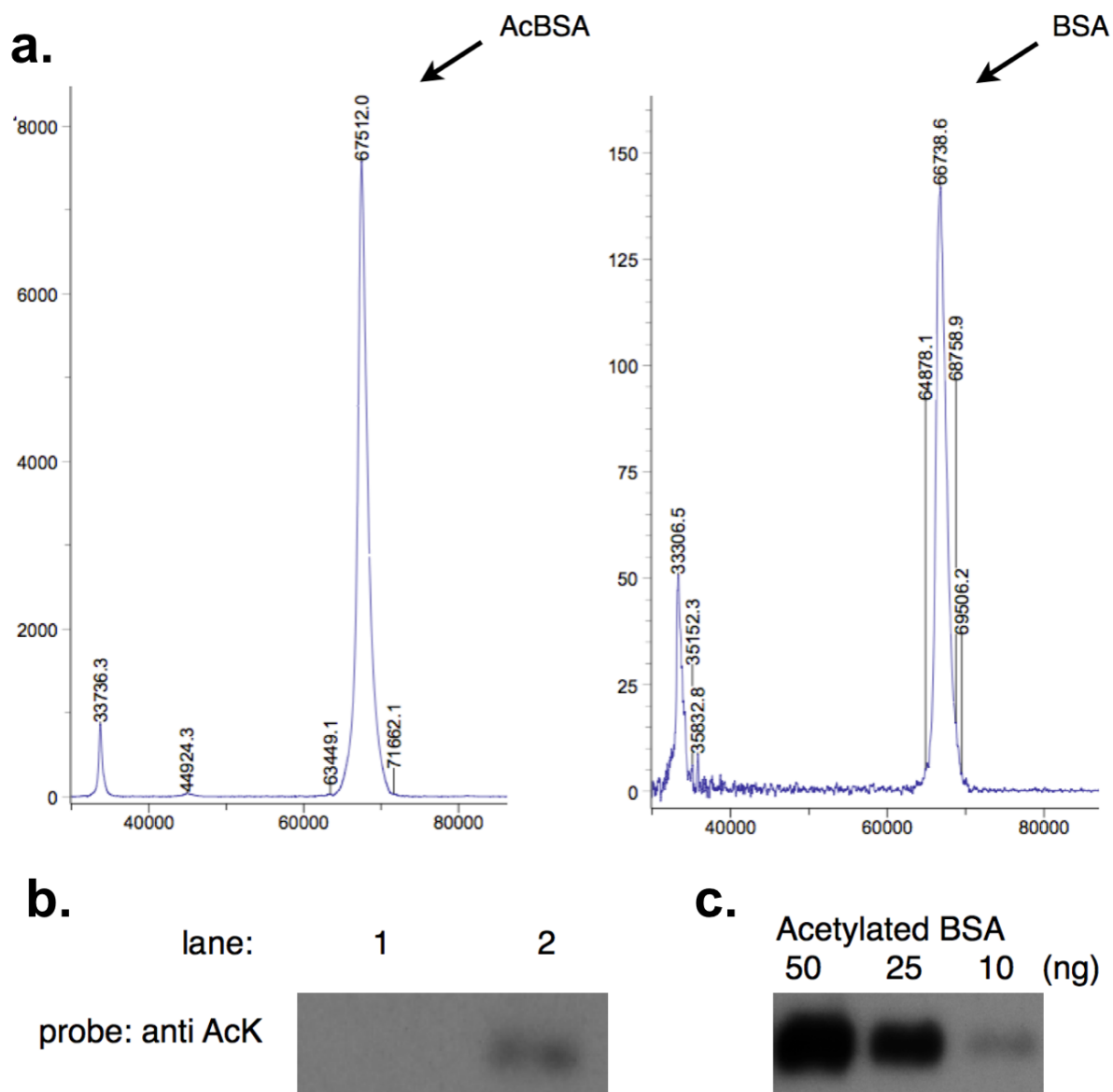
**Figure S2.** Ligand dependent AR transcription response determined by cellular reporter gene assays using AR-responsive luciferase reporter (ARE-Luc): (green) tolfenamic acid (3) + 10 nM DHT; (red) amide (4) ; (blue) amide (4) + 10 nM DHT. (RLU = relative light units)

**Propargyl tolfenamic amide** is a moderately potent BF-3 site binding partial antagonist of Androgen Receptor: Tolfenamic acid binds to a recently discovered allosteric site on the ligand-binding domain of the AR (termed BF-3), which is flanked by several lysine residues on the receptor surface. Tolfenamic acid is a poor AR antagonist ( $IC_{50} > 30 \mu M$ ) in cells, however, we have found the propargyl amide (4) to be a submicromolar potent AR antagonist ( $IC_{50} = 673.33 \pm 101 nM$ ) in cellular reporter gene assays (Figure S2).

#### **Transcription/antagonist response by cellular (luciferase) reporter gene assay:**

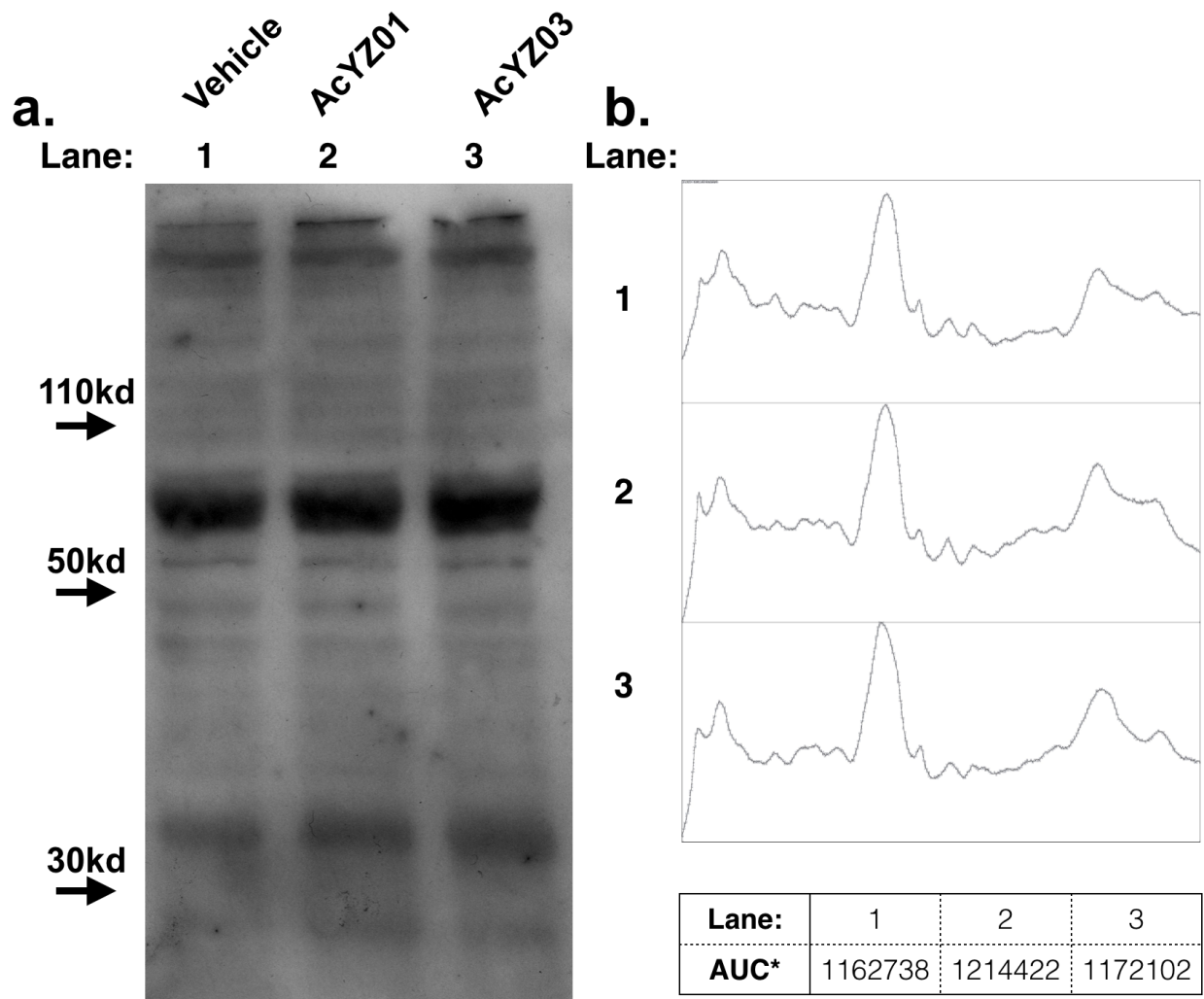
Twenty-four hours prior to transfection, HEK293T cells were seeded at a density of 75,000 cells per well in 24-well cell culture plates and grown in phenol red free Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% cosmic calf serum (CCS) (HyClone, Logan, UT). Transfections were performed using  $Ca_3(PO_4)_2$  following general protocol and using 0.08  $\mu g$  hAR(wt), 0.16  $\mu g$  of ARE-Luciferase reporter, 0.08  $\mu g$  of Renilla-luc as internal standard. Five hours after transfection the wells were washed with DPBS and media was added containing appropriate ligands. The cells were allowed to incubate for 36 h before harvesting by passive lysis buffer. Cell extracts were immediately assayed using the Dual Luciferase Assay (Promega #E1960, Madison, WI) with a Perkin-Elmer Microbeta Luminometer. All experiments were run in triplicates. Activity is reported in relative light unit (RLU), determined as the ratio of inducible firefly luciferase luminescence divided by the luminescence of the renilla luciferase control (Figure S2).





**Figure S3.** Quantification of Acetylation of AR by YZ03 by Western Blot. a. Assessment of number of acetylated lysine on the AcBSA; b. Western Blot of acetylated AR treated by YZ03; c. Western Blot of AcBSA Standard.

**Quantitative analysis of AR acetylation from western blot:** The level of AR acetylation by YZ03 can be approximated by comparing anti-AcK response in western blots with known standards. We used acetylated BSA (Promega) as a standard and determined the level of acetylation by MALDI mass spectrometry. It was determined that commercial AcBSA contained on average 18 acyl groups per molecule based on the average molecular weight difference between acetylated and unacetylated BSA (i.e.  $(67512 - 66738)/42 \approx 18$ ) (Figure 3a). Anti-AcK blots were probed and analyzed by densitometry. The bands for acetylated AR and acetylated BSA were obtained simultaneously from the same blot (Figure S3b and S3c). Densitometry values indicates that the AR band contains 1.9 pmol of AcK. The amount of total AR was determined to be 36 pmol by silver staining gels of same sample aliquots with protein standards. Therefore the percent acetylation was determined as follows: percent acetylation = AcK on AR (25ul loaded) / AR loaded =  $1.90 \text{ pmol} / 36.3 \text{ pmol} = 5.2\%$ .



**Figure S4.** Western blot Analysis of Global Acetylation. a. Crude lysate from treated cells probed with anti-AcK antibody. b. Analysis of lysate acetylation by densitometry. \*AUC=Area Under the Curve

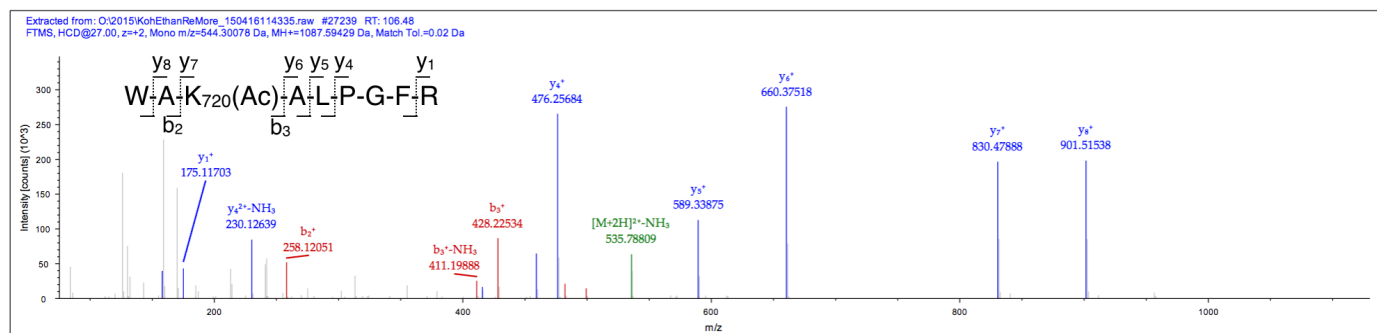
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4	sp	AR(LBD)_Human				36.10	47.20 %	1	11	22	140	250	29.1	8.72		
	A2	Sequence	# PSMs	# Proteins	# Protein Groups	Protein Group Accessions	Modifications	ΔCn	q-Value	PEP	XCorr	Charge	MH+ [Da]	ΔM [ppm]	RT [min]	
1	WAKALPGFR		4	1	1	sp	K3(Acetyl)	0.0000	0	0.0054	2.18	2	1087.59526	-8.76	107.50	
2	RFYQLTK		15	1	1	sp		0.0000	0	0.0134	2.03	2	955.52745	-8.95	56.00	
3	QLVHVVK		9	1	1	sp		0.0000	0	0.00247	2.00	2	822.51183	-9.49	39.03	
4	FYQLTK		5	1	1	sp		0.0000	0	0.00191	1.78	2	799.42742	-9.34	62.79	
5	LLDSVQPIAR		21	1	1	sp		0.0000	0	0.00111	1.70	2	1111.63652	-9.46	81.41	
6	LDSDVQPIAR		1	1	1	sp		0.0000	0	0.00833	1.47	2	998.55419	-8.80	79.77	
7	FFDELRL		56	1	1	sp		0.0000	0	0.000345	1.42	2	826.40166	-9.39	84.75	
8	VKPIYFH		1	1	1	sp		0.0000	0	0.00539	1.05	2	903.50060	-8.99	58.87	
9	SFTNVNSR		6	1	1	sp		0.0000	0	0.0209	0.97	2	924.44426	-9.89	38.23	
10	DSVQPIAR		1	1	1	sp		0.0000	0.023	0.262	1.24	1	885.47003	-10.02	79.86	
11	SFTNVN		1	1	1	sp		0.0000	0.032	0.316	1.32	1	681.31390	-9.32	47.39	
12	QKFFDELRL		1	1	1	sp		0.0000	0.191	0.83	0.19	3	1082.56830	4.92	72.16	
13	VLEAIEPGVVCAGH		4	1	1	sp		0.0000	0.731	1	0.06	3	1393.71769	2.29	64.65	
14	MYSQcVR		1	1	1	sp	C5(Carbamyl)	0.0000	0.757	1	0.13	2	929.39513	-1.82	71.76	
15	PTScSRR		3	1	1	sp	C4(Carbamyl)	0.0000	0.761	1	0.14	2	849.40282	3.79	48.61	
16	DSVQPIAREL		1	1	1	sp		0.0000	0.806	1	0.03	2	1127.59636	-8.17	98.62	
17	PGFRNL		1	1	1	sp		0.0000	0.826	1	0.03	2	703.39141	3.96	13.24	
18	LSQEFGLW		1	1	1	sp		0.0000	0.835	1	0.05	2	979.49645	8.21	46.19	
19	QFTFDLLK		3	1	1	sp		0.0000	0.836	1	0.03	2	1124.62395	-9.89	129.45	
20	WAKALPGFR		1	1	1	sp		0.0000	0.844	1	0.24	2	1045.59099	-3.09	135.25	
21	ELDRIIACK		1	1	1	sp	C8(Carbamyl)	0.1176			0.15	2	1103.58806	0.22	96.81	
22	VkPIYFH		1	1	1	sp	K2(Acetyl)	0.7727			0.05	2	945.51842	-0.92	63.54	
23	IIACKrk		1	1	1	sp	C4(Carbamyl); K5(Acetyl);...	0.2500			0.03	3	958.55692	6.91	34.18	
24	YIKELDR		1	1	1	sp	K3(Acetyl)	0.9787			0.01	2	978.52678	1.30	57.09	

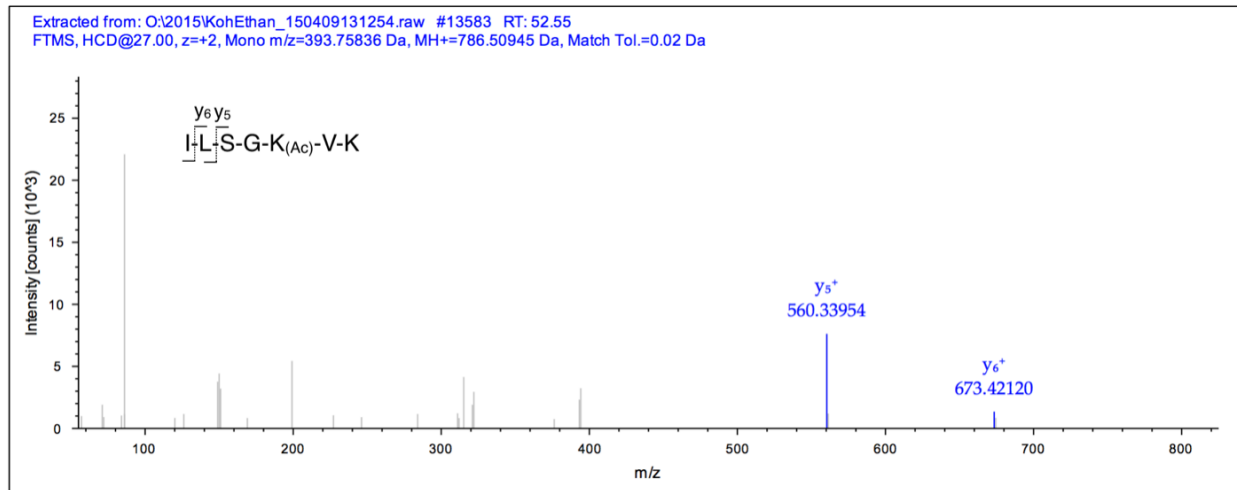
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2	sp	AR(LBD)_Human				65.83	74.40 %	1	10	30	119	250	29.1	8.72		
	A2	Sequence	# PSMs	# Proteins	# Protein Groups	Protein Group Accessions	Modifications	ΔCn	q-Value	PEP	XCorr	Charge	MH+ [Da]	ΔM [ppm]	RT [min]	
1	SHMVSVDFFEMMAEISVQV...		4	1	1	sp		0.0000	0	0.000541	6.58	3	2474.21683	1.57	141.48	
2	LLDSVQPIAR		10	1	1	sp		0.0000	0	0.0485	2.95	2	1111.64849	1.30	81.53	
3	QLVHVVK		8	1	1	sp		0.0000	0	0.00781	2.23	2	822.52117	1.87	40.96	
4	RFYQLTK		17	1	1	sp		0.0000	0	0.0158	2.23	2	955.53752	1.59	56.23	
5	SFTNVNSR		3	1	1	sp		0.0000	0	0.0253	2.06	2	924.45586	2.66	40.45	
6	FFDELRL		30	1	1	sp		0.0000	0	0.0507	1.98	1	826.41034	1.11	86.60	
7	FYQLTK		4	1	1	sp		0.0000	0	0.22	1.67	2	799.43608	1.50	64.51	
8	ALPGFR		6	1	1	sp		0.0000	0	0.114	1.20	2	660.38310	0.44	65.99	
9	VkPIYFH		2	1	1	sp	K2(Acetyl)	0.0000	0	0.324	1.02	2	945.51750	-1.89	85.98	
10	VHVvKwAK		2	1	1	sp	K5(Acetyl)	0.0000	0.02	0.517	0.96	2	1008.59984	0.88	84.28	
11	VKPIYFH		1	1	1	sp		0.0000	0.02	0.508	0.77	3	903.50910	0.42	60.54	
12	ILSGkVK		2	1	1	sp	K5(Acetyl)	0.0000	0.106	0.645	1.12	2	786.50945	1.32	52.55	
13	KNPTScSR		1	1	1	sp	C6(Carbamyl)	0.0200	0.334	0.773	0.49	1	935.43542	-1.02	135.51	
14	WAKALPGFR		3	1	1	sp	K3(Acetyl)	0.0000	0.51	0.843	0.36	2	1087.60393	-0.79	94.40	
15	FSIIPVDGLKN		1	1	1	sp		0.0000	0.51	0.841	0.07	2	1202.68682	7.32	90.41	
16	NVLEAIEPGVVCAGH		1	1	1	sp	C12(Carbamyl)	0.0000	0.573	0.864	0.04	2	1550.77641	8.50	137.91	
17	DSVQPIAR		1	1	1	sp		0.0000	0.67	0.91	0.29	1	885.48132	2.74	81.30	
18	QITPQFLcMKAL		1	1	1	sp	C9(Carbamyl); K11(Acetyl)	0.0000	0.867	1	0.24	2	1606.78679	-6.25	128.92	
19	PTScSRR		1	1	1	sp		0.0000	0.879	1	0.27	1	806.39301	-0.96	105.49	
20	SGKvkPIYFH		1	1	1	sp	K3(Acetyl); K5(Acetyl)	0.0000	0.895	1	0.02	3	1259.67734	-0.78	50.35	
21	IIACKRK		3	1	1	sp	K5(Acetyl); K7(Acetyl)	0.0000	0.919	1	0.04	2	915.54094	-3.87	85.41	
22	PTScSRR		1	1	1	sp	C4(Carbamyl)	0.0000	0.922	1	0.03	2	849.39891	-0.80	59.24	
23	KSRMYSQcVR		1	1	1	sp	C8(Carbamyl)	0.3095			0.29	2	1300.61290	-9.25	85.99	
24	YFAPDLVFNEYR		1	1	1	sp		0.1071			0.25	3	1533.72260	-9.59	105.83	
25	VVKWAK		1	1	1	sp		0.6515			0.23	2	730.45781	-4.45	11.56	
26	SQEFGLWQITPQFLcMK		1	1	1	sp	C16(Carbamyl); K18(Acetyl)	0.6182			0.21	3	2270.08060	8.13	120.33	
27	ELDRIIACK		2	1	1	sp	C8(Carbamyl)	0.6000			0.08	2	1103.58757	-0.22	107.37	
28	ELDRIIACK		2	1	1	sp	C8(Carbamyl); K9(Acetyl)	0.1429			0.06	2	1145.60051	1.86	115.70	
29	QITPQFLcMK		1	1	1	sp	C9(Carbamyl)	0.2500			0.06	2	1380.66411	-0.71	119.84	
30	NQPDSSFAAL		1	1	1	sp		0.5714			0.06	3	962.45176	-6.31	38.58	
31	PTScSRRFYQL		1	1	1	sp	C4(Carbamyl)	0.9000			0.04	2	1400.66875	-3.74	93.82	
32	QFTFDLLK		1	1	1	sp		0.3333			0.02	2	1124.63896	3.46	130.24	
33	VVKwAK		1	1	1	sp	K3(Acetyl); K6(Acetyl)	0.8667			0.02	2	814.48033	-2.28	50.49	
34	VkPIYFHT		1	1	1	sp	K2(Acetyl)	0.9130			0.02	2	1046.57085	3.71	84.31	
35	ILSGKVK		1	1	1	sp	K5(Acetyl); K7(Acetyl)	0.8000			0.01	1	828.51715	-2.20	122.76	
36	WAKALPGFR		1	1	1	sp		0.7500			0.01	2	1045.60161	7.07	135.55	

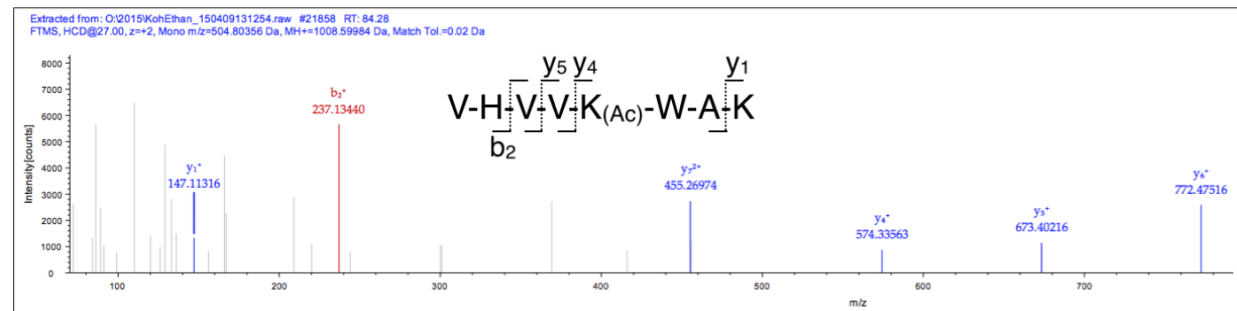
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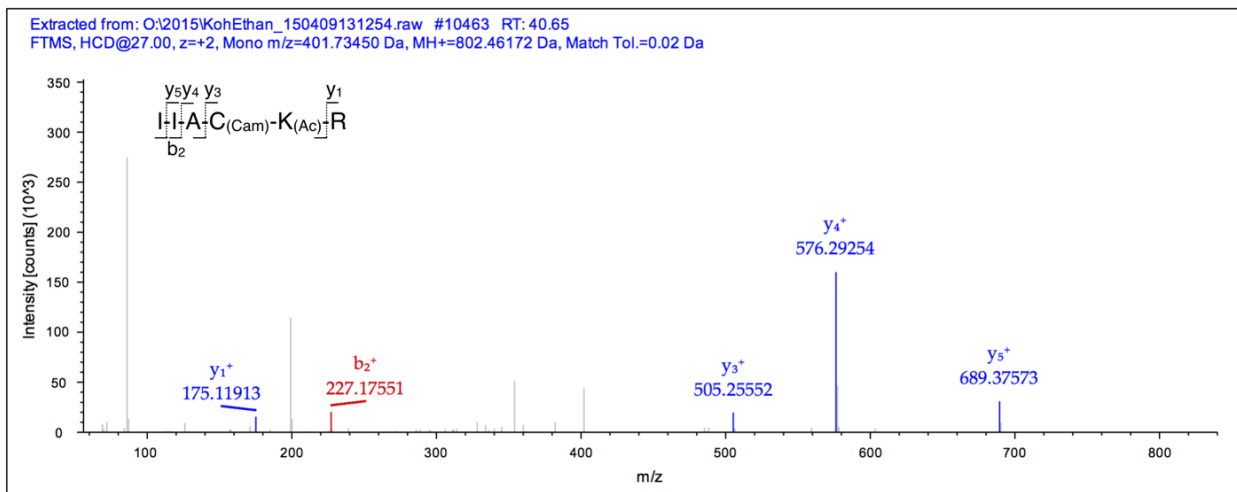
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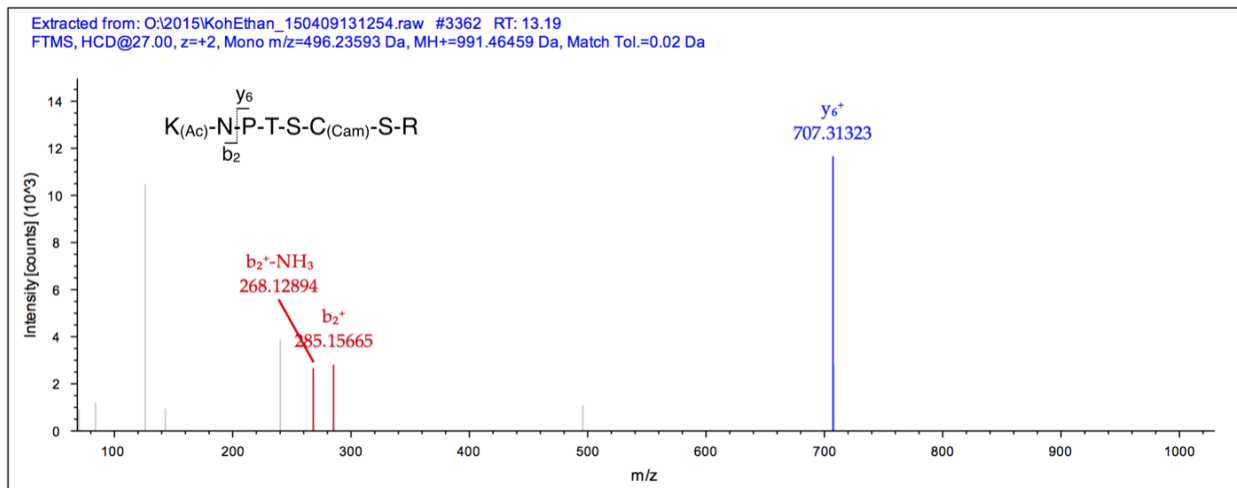
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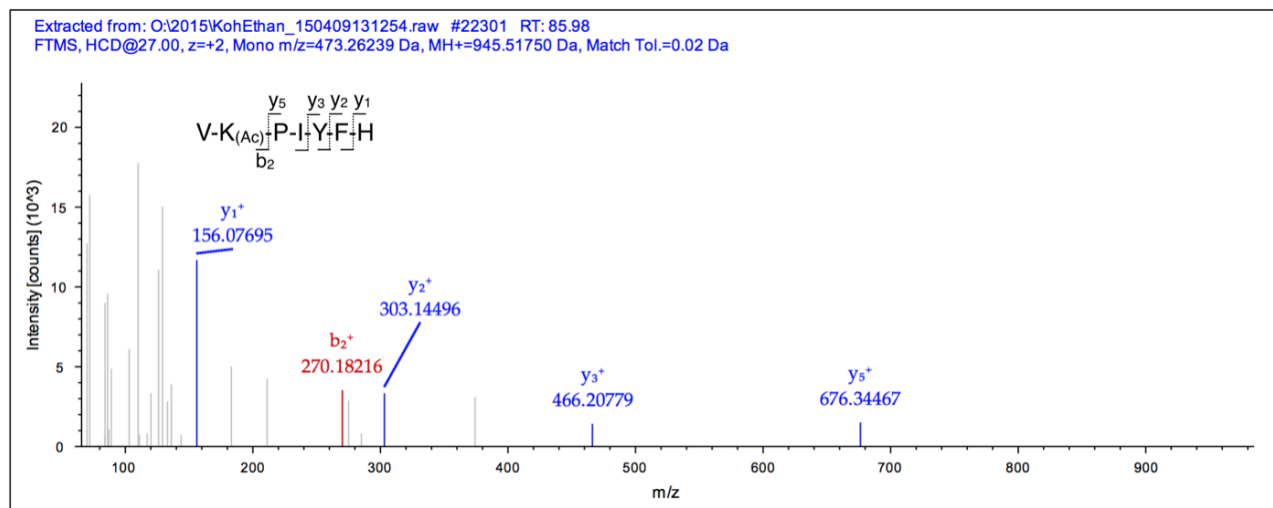
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g.



h.



I.

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670 QPILNVLEAIEPGVVCAGHDNNQPDSFAALLSSLNELGERQLVHVVKWAKALPGFRNLH
730 VDDQMAVIQYSWMGLMVFAMGWSRFTNVNSRMLYFAPDLVFNEYRMHKSRMYSQCVRMRH
790 LSQEFGLWQITPQEFCLMKALLFSIIPVDGLKNQKFFDELRMNYIKELDRIIACKRKNP
850 TSCSRRFYQLTKLLDSVQPIARELHQFTFDLLIKSHMVSVDPEMMAEIIISVQVPKILSG
910 KVKPIYFHT

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**Figure S5.** a. Full peptide analysis of C-18 microcolumn treated AR(LBD) tryptic sample. b. Full peptide analysis of cation exchange microcolumn treated AR(LBD) tryptic sample. c. MS/MS spectrum of Lys(Ac)720 containing peptide d-h. MS/MS spectra of possible Androgen receptor acetylation sites identified By Orbitrap Mass Spectrometry and Proteome Discoverer™ which do not arise to be statistical confidence ( $q > 0.05$ ). I. Protein Sequence of AR(LBD)

**MS/MS analysis of acetylation site of AR(LBD):** The codon-optimized sequence for the Androgen receptor Ligand-binding Domain (AR-LBD; residues 670-919) was subcloned into pET-41a vector with N-terminal GST fusion tag. AR(LBD) was expressed in BL21DE3 cells, supplemented with 10 uM DHT and purified by GST column. Protein quantification was performed by Bradford assay. Approximately 10 uM AR(LBD) was incubated with 50 uM AcYZ03 in PBS buffer (pH 7.4) for one hour at 30°C. Acetylated AR(LBD) was resolved by SDS-PAGE gel, which was stained by coomassie blue. The band corresponding to GST-AR(LBD) (~57 kDa) was excised, dehydrated and digested with trypsin. The digest product was acidified by formic acid and prepped by C18 ZipTip microcolumn with elution by 60% acetonitrile. Alternatively acidified digest was prepped by SCX (strong cation exchange) microcolumn using NH<sub>4</sub>OH elution. Time program on the Orbitrap was from 5% to 95% ACN in 1 h, with C-18 Acclaim PepMap Column and flow rate set to be 300 nL/min. Raw data was obtained, and analyzed by Proteome Discoverer™ Software (Thermo Scientific, V1.4). AR(LBD) sequence along with the cRAP protein (The Common Repository of Adventitious Proteins) sequence data as a source of possible sources of contamination was indexed into the Proteome Discoverer™ and was used to identify the possible peptide hits from the MS/MS data. The tolerance was set to be 10 ppm for the precursor mass and for the fragment mass tolerance was set to be 0.02 Da.





**Figure S6.** Steered Molecular Dynamic Simulation of AcYZ03 to proximal lysine on the surface of AR. a. Linear distance between the amide carbonyl carbon of docked AcYZ03 to proximal surface lysine residues of AR. b. Steered Molecular Dynamic Simulation monitoring the distance between the thioacetate carbonyl carbon of AcYZ03 to each lysine nitrogen over the course of the simulation. c. Bar graph showing time-averaged distance, thioacetate carbonyl carbon to lysine-nitrogen, over the last 100 ps of simulation.

### Molecular Dynamics Simulations:

**Simulation System Setup:** The protein structure was obtained from the crystal structure of flufenamic acid bound AR(LBD) (PDBID: 2PIX). Tolfenamic acid was removed for docking of AcYZ03 by Autodock vina [1] with protonation states assigned using the H++ server [2]. Force field parameters for YZ03 were calculated as follows; RESP charges were calculated with Gaussian09[3] using DFT/6-31G(d) as the basis set. Then, topology parameters were generated with the general Amber force field (GAFF) by ANTECHAMBER [4]. Protein topology parameters were generated using Amber 14SB force field [4]. The protein-ligand system was then solvated with the TIP3PBOX water model in a cubic periodic unit cell. Each side of the unit cell is at least 10 Å from the nearest solute atom. The system was neutralized by adding sodium cations. The Amber14 package was used to perform all MD simulations. The system was first minimized by 50000 steps of steepest descent minimization followed by another 50000 steps conjugated gradient minimization. The system was then heated from 0K to 300K over 500 ps with NVT ensemble, with a 10 Kcal•mol<sup>-1</sup>•Å<sup>-2</sup> restraint on the protein-ligand complex. Then 5ns equilibrium MD was performed with the unconstrained NPT ensemble. SHAKE algorithm was employed to constrain bonds containing hydrogen atoms, 2fs step was used for all MD simulations. The Particle Mesh Ewald method was used to calculate electrostatic interactions with a nonbonded cutoff distance of 10 Å.

**Steered Molecular Dynamics (SMD):** To determine which lysines might be acetylated by YZ03 from its bound state, the closest possible distances between the side-chain nitrogen of each lysine and the carbonyl carbon of the thioester of AcYZ03 were calculated by SMD using a small pulling constant. The equilibrated complex was used as a starting point for each SMD. For each simulation, a 0.5 Kcal•mol<sup>-1</sup>•Å<sup>-2</sup> constant pulling force between the carbonyl carbon of the YZ03 thioester and the epsilon nitrogen of lysine was used to pull the YZ03 side chain thioester to each surface lysine within 36Å (linear distance) from the tolfenamic acid carbonyl. To avoid drifting of the protein or pulling YZ03 from the binding site, a 5 Kcal•mol<sup>-1</sup>•Å<sup>-2</sup> force was used to constraint the lysine backbone and YZ03's tolfenamic acid core.

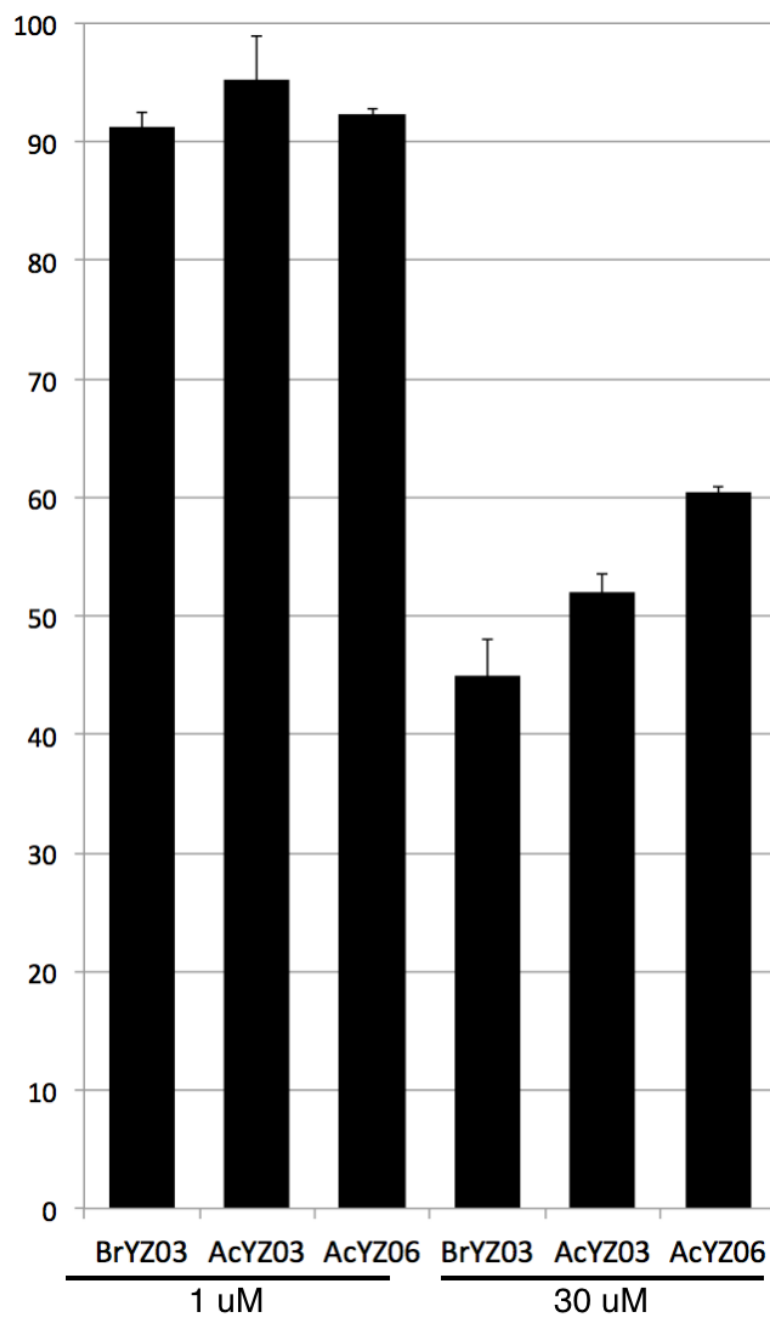
For each lysine, 500ps SMD was performed at 300K. All trajectories were visually inspected to confirm their unimpeded access to the target lysine, or to the closest distance thereof. Trajectories of the last 100 ps were used to calculate average distances for Figure S6c.

### Reference:

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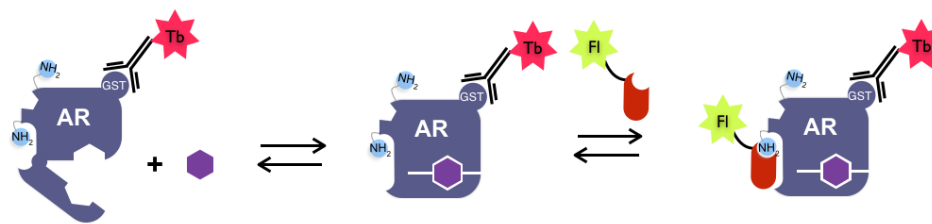
Gomperts, R.; Stratmann, R. E.; Yazyev, O.; Austin, A. J.; Cammi, R.; Pomelli, C.; Ochterski, J. W.; Martin, R. L.; Morokuma, K.; Zakrzewski, V. G.; Voth, G. A.; Salvador, P.; Dannenberg, J. J.; Dapprich, S.; Daniels, A. D.; Farkas, Ö.; Foresman, J. B.; Ortiz, J. V.; Cioslowski, J.; Fox, D. J.; Gaussian, Inc.: Wallingford, CT, USA, 2009.  
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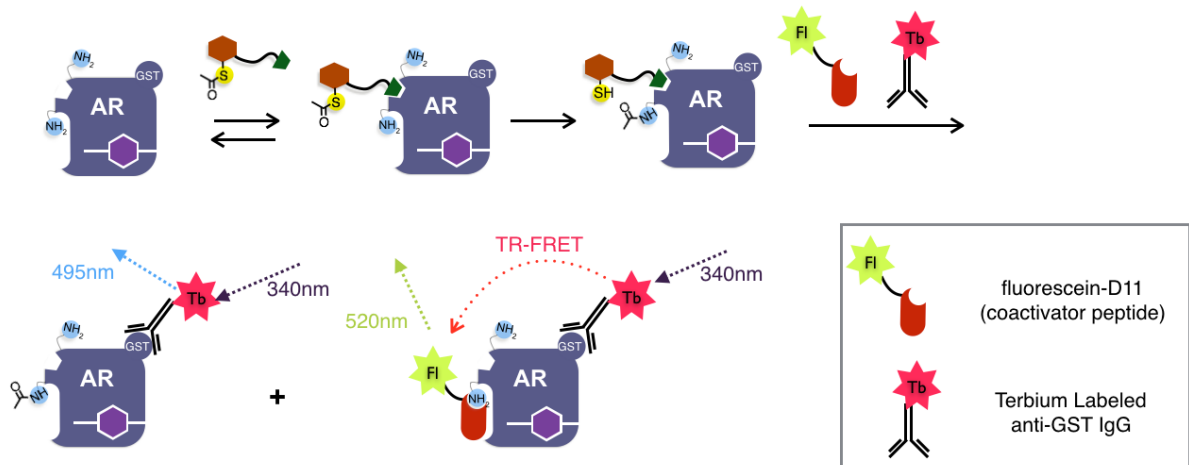


**Figure S7.** Cellular Ligand Dependent Transcriptional Activity of AR-driven luciferase reporter gene (ARE-Luc).

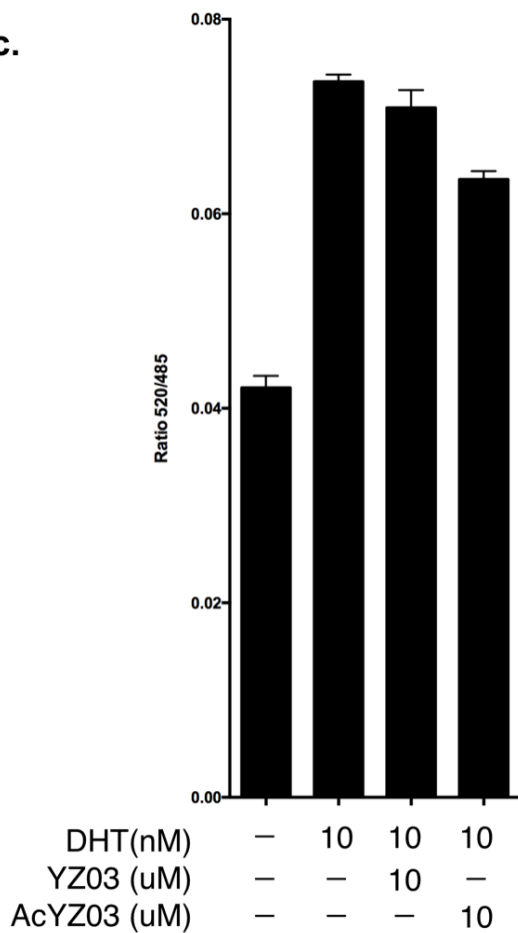
**a. Standard TR-FRET Coactivator Association Assay (Lanthascreen)**



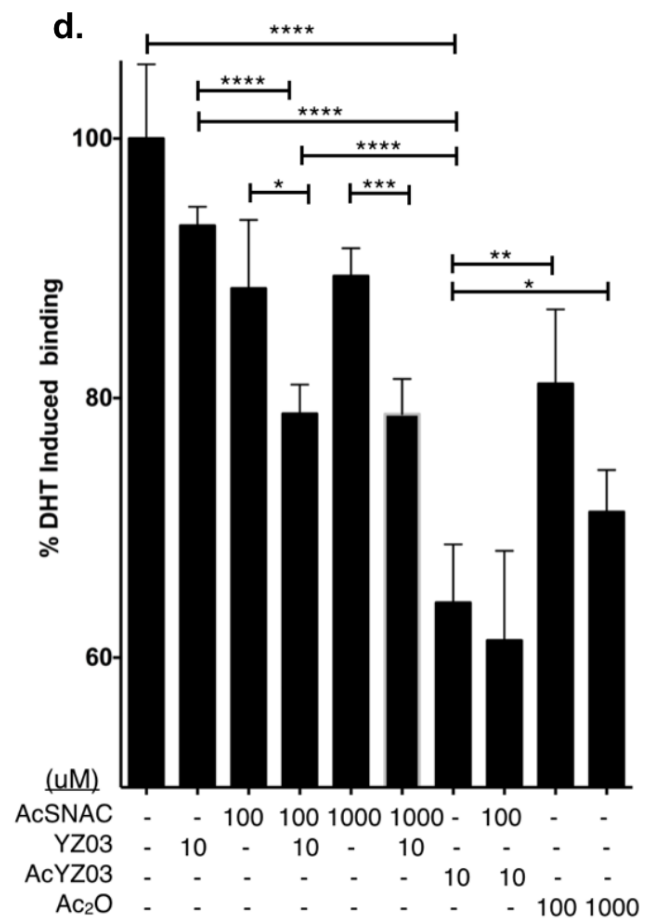
**b. TR-FRET Coactivator Association Assay**



**c.**

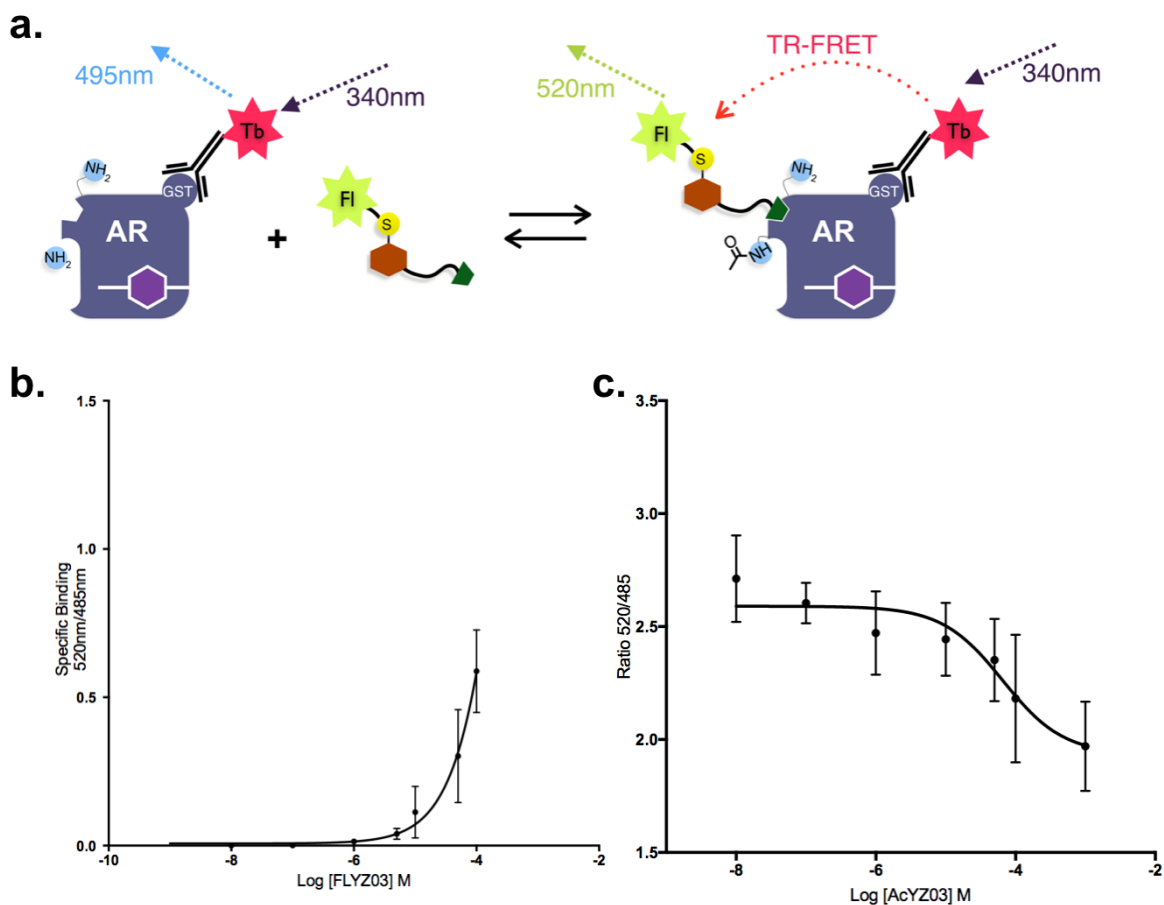


**d.**



**Figure S8.** In vitro Coactivator Recruitment Activity of YZ03 and AcYZ03 by modified TR-FRET Assay. a. Standard TR-FRET Coactivator Association Assay (Lanthascreen, Fisher). b. Modified TR-FRET assay used to test coactivator association in the presence of acyl-transfer agents (this work). c. DHT-induced association was established by modified TR-FRET from measuring background fluorescence ratio in the absence of DHT. d. Complete data set and controls for assessing YZ03 as an antagonist of coactivator peptide association by TR-FRET.

**Inhibition of Coactivator Association by YZ03 by TR-FRET:** TR-FRET Androgen Receptor Coactivator Assay, Lanthascreen (FisherScientific, A15878) was performed following manufacturer's protocol. The AR(LBD) was incubated with 5nM DHT plus indicated amounts of test compounds or controls for 3 h at 30°C. After incubation, fluorescein labeled coactivator peptide was added and the fluorescence signal was measure using 520nm and 485nm filters using 100 microsecond delay, and the signal determined ratiometrically. Evaluation of coactivator association in the absence and presence of 5 nM DHT was used to determine the DHT-inducible response (Figure S8).

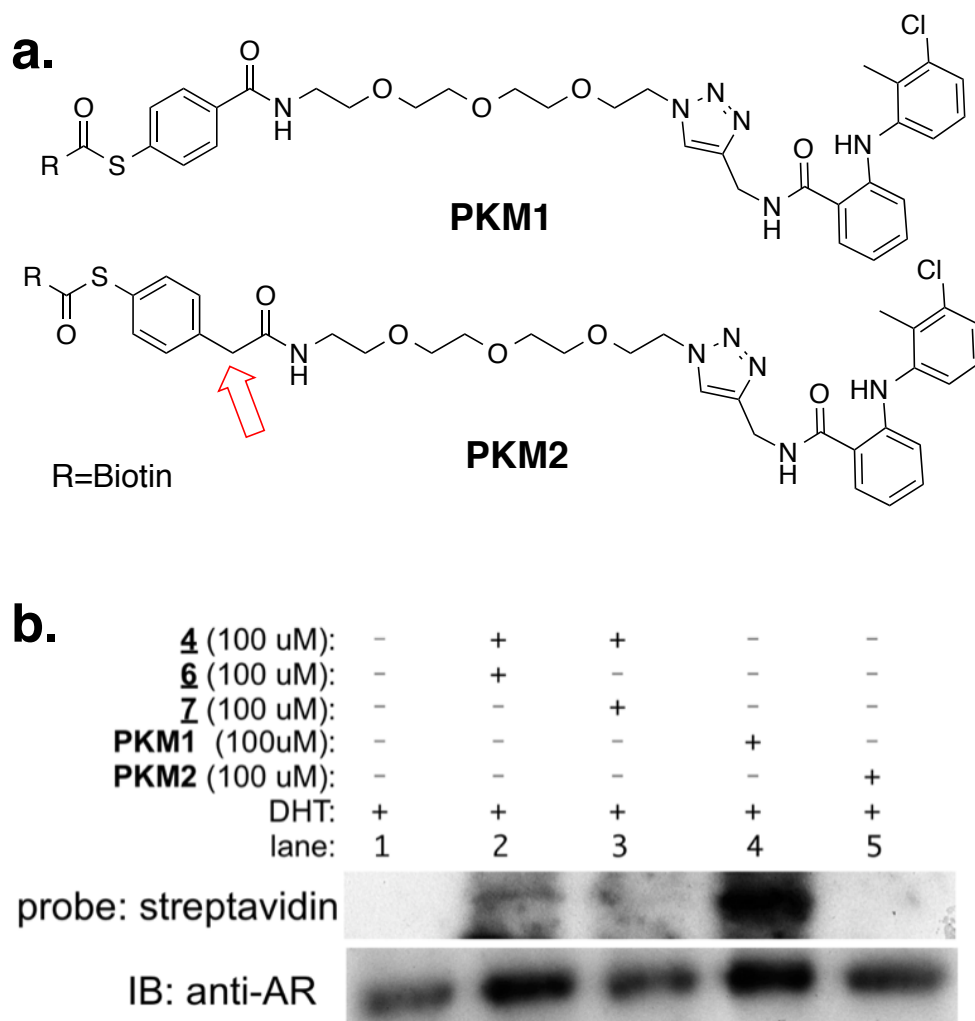


**Figure S9.** Direct and competitive binding of BF-3 site ligands by TR-FRET Assay. **a.** Scheme of modified TR-FRET assay using FLYZ03. **b.** Specific binding of FLYZ03 to AR(LBD) was evaluated by measuring difference between specific binding and nonspecific binding. **c.** Competitive assay of AcYZ03 versus 50  $\mu$ M FLYZ03 for AR(LBD).

#### Assessment of Binding Constant of FLYZ03:

A TR-FRET Androgen Receptor Coactivator Assay based on the commercial Lanthascreen (FisherScientific, A15878) coactivator binding assay was developed to assess the direct binding of the YZ03 derivative FLYZ03. AR(LBD) was incubated with 10nM DHT, anti-GST-terbium antibody and increasing concentrations of FLYZ03. Fluorescence signal was measured using 520nm and 485nm emission filters using a 100 microsecond delay, and the signal determined ratiometrically. The signal from nonspecific controls was determined from incubation of DHT and anti-GST-terbium antibody (i.e. no GST-AR) at the same concentration of FLYZ03. The specific binding signal was obtained by subtracting the signal obtained from nonspecific controls. The signal was plot against FLYZ03 concentration using Prism software, with nonlinear regression fit to a sigmoidal dose-response equation. From the plot which did not fully saturate due to solubility limitations, the  $K_d$  of FLYZ03 was estimated to be to be 59  $\mu$ M.

To confirm the validity of this approximate  $K_d$ , a competitive binding assay was performed with AcYZ03. For the competition assay, FLYZ03 was kept at a constant 50 $\mu$ M concentration and titrated against increasing concentrations of AcYZ03. The signal was measured using the same method described above. Based on the estimated  $K_d$  of FLYZ03 calculated above (59  $\mu$ M), and the observed  $EC_{50}$  of AcYZ03, the calculated  $K_i$  of AcYZ03 was 60  $\mu$ M which is consistent with the  $K_d$  of FLYZ03 as the probe and competitor share the same binding core. (Figure S9)



**Figure S10.** Proximity directed biotinylation by PKM1 and PKM2 in CWR22Rv1 cell lysates. a. PKM Biotin conjugated compounds. b. Cell lysates treated with indicated compounds at 30°C, followed by immunoprecipitation (anti-AR) and by western analysis with HRP-streptavidin. Total AR controls run with identical aliquots on parallel gels.

**Biotinylation of CWR22Rv1 cell lysates by biotin thioesters PKM1 and PKM2:**

Model biotin-thioester acyltransfer probes PKM1 and PKM2 (see synthesis below) were used to access acyl transfer efficiency in cell lysates: CWR22Rv1 cells were seeded in 100 mm dishes with RPMI 1640 media supplemented with 10% FBS. After cells reached 90% confluence, they were washed with cold DPBS and lysed using lysis buffer (DPBS, 5% Glycerol, 0.5% Tritonx-100, 0.1mM PMSF and 2 Roche protease minitab tablets/10ml). Cells were lysed by sonication (Branson Sonifier 200 watt 2 x 5s pulses @40%) and centrifuged at 20,000 rpm for 20 minutes at 4°C. The final concentration of lysate was adjusted to 2mg/ml. To 1 ml of the lysate was teated with 100uM of the reagents and were incubated for 1-6 hrs at 37°C or 30°C. After which 5uL of anti-AR antibody was added and rocked for 1 h and 40 uL Protein A beads (50% solution) was added and rocked overnight. The beads were then washed with cold PBS buffer (3 x 500μl), denatured with Lammeli buffer and the protein was analyzed by 12% SDS-PAGE followed by western blotting onto PVDF membrane which was probed with Streptavidin-HRP (1: 1000, Cell signaling) and imaged on film by chemiluminescence (Figure S10).

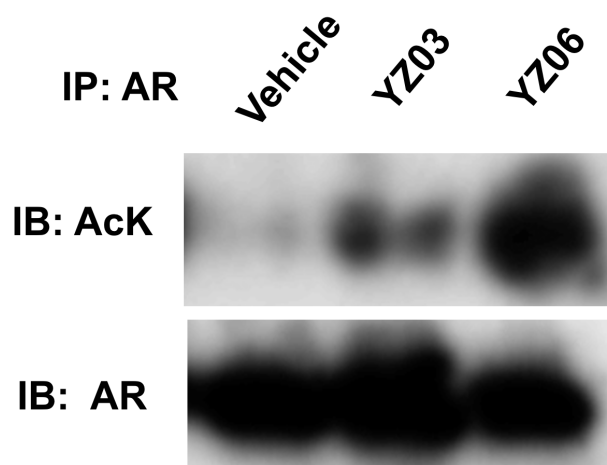


Figure 11. Acetylation of Endogenous AR From CWR22Rv1 Cells Treated With AR-targeting (Free-Thiols) YZ03 and YZ06 for 16 hours. Immunoprecipitated AR was analyzed by immunoblot with anti-AcK or anti-AR antibodies.

### **General Methods and Materials:**

All chemicals were bought from Sigma Aldrich and Acros Organics unless otherwise noted. Tolfenamic acid was purchased from Cayman chemicals and ASDI, Newark, DE.

$^1\text{H}$  and  $^{13}\text{C}$  spectra were recorded on Bruker AV 400, DRX-400 or AV-600 instruments. Chemical shifts are reported in  $\delta$  and  $J$  values are reported in Hz.

RPMI 1640, Dulbecco's modified Eagle Media (DMEM), Sodium pyruvate solution, DPBS were purchased from MediaTech. Phenol red free RPMI 1640 was purchased from Gibco. EDTA free protease cocktail minitab was purchased from Roche Applied science. Anti-acetyllysine, Streptavidin-HRP, Agarose-A beads were purchased from cell signaling. Anti-AR (N-20)antibody and secondary antibodies were purchased from Santa Cruz biotechnologies, CA. High capacity streptavidin beads were purchased from Thermo Scientific. Coactivator association assay kit based on TR-FRET was purchased from Fisher. HEK293t Cells were purchased from ATCC.

### **Androgen Receptor Acetylation in CWR22Rv1 cells by Synthetic Acyltransferase YZ03 (IP:AR followed by IB: Anti-AcK):**

CWR22Rv1 cells were seeded in 100 mm dish with RPMI 1640 media supplemented with 10% FBS. After the cell reached 60% confluence, the media was changed to phenol red-free RPMI 1640 supplemented with 1mM pyruvate and incubated for an additional 12 h. The media was then changed to phenol red-free RPMI containing YZ03, HDAC inhibitor SAHA (50uM) and incubated for 16 hrs. The cells were then washed with ice cold DPBS and lysed with RIPA buffer (pH 7.4 PBS with 0.1% SDS, 1% Sodium Deoxycholate and 1.5M NaCl, 1 ml) followed by sonication (Branson Sonifier 200 watt 2 x 2s pulses @40%) and centrifugation (12,000 rpm, 20 mins). The supernatants were saved and the total protein levels were determined by Bradford assay. To 500 uL of lysate was added 5uL anti-AR (N20, Santa Cruz) and the solution rock at 4°C for 12 hours. Protein A beads (20 uL, Cell Signaling) was added and the solution was rocked for an additional 3.5 h. The beads were washed with PBS buffer (3 x 500 uL), denatured and the protein analyzed by 12% SDS-PAGE followed by western blot onto PVDF membrane which was probed with anti-AcK (1:1000, Cell Signaling, #9441S) and visualized by chemiluminescence using HRP conjugated secondary antibodies and film.

### **Androgen Receptor Acetylation by AcYZ01 (control) vs AcYZ03 in HEK293T cells (IP:AR followed by IB: Anti-AcK):**

HEK293T (1,000,000 cells/well) cells were seeded individually into 100 mm dish. Standard calcium phosphate transfection of 20 ug pSG5-hAR DNA were performed when the cells reached 20% confluent. The cells were grown over night before the media was changed to phenol red-free DMEM containing 5uM SAHA and either 20uM AcYZ01 or AcYZ03 (or vehicle DMSO) for three hours. The cells were then washed with ice cold DPBS and lysed with RIPA buffer (pH 7.4 PBS with 0.1% SDS, 1% sodium deoxycholate and 1.5M NaCl, 1 ml). To 500 uL of lysate was added 5uL anti-AR (N20, Santa Cruz) and the solution rocked at 4°C for 12 hours. Protein A beads (20 uL, GenScript, L00273) was added and the solution was rocked for an additional 3.5 hrs. The beads were washed with PBS buffer (3 x 500 ul), denatured and the protein analyzed by 12% SDS-PAGE followed by western blotting onto PVDF membrane which was probed with anti-AcK (1:1000, Cell Signaling) and visualized by chemiluminescence using HRP conjugated secondary antibodies and film.

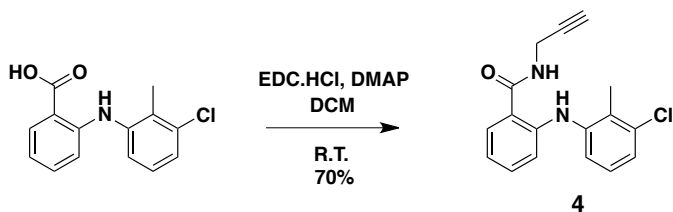
### **Androgen Receptor Acetylation Selectivity Assay; AR vs HSP70 by AcYZ03 and AcYZ06 (IP:anti-AcK followed by IB: AR or HSP70):**

HEK293T (1,000,000 cells/well) cells were seeded individually into 100 mm dish. Standard calcium phosphate transfection of 20 ug hAR DNA were performed HEK293T cells when the cells were 20% confluent. The HEK293T cells were grown over night before the media was changed to phenol red-free DMEM containing 5uM SAHA, and either 20uM AcYZ03 or AcYZ06 (or DMSO as vehicle control) for three hours. The cells were then washed with ice cold DPBS and lysed with RIPA buffer (pH 7.4 PBS with 0.1% SDS, 1% Sodium Deoxycholate and 1.5M

NaCl, 1 ml). To 500 uL of lysate was added 5uL anti-AcK and the solution rocked at 4°C for 12 hours. Protein A beads (20 uL, GenScript, L00273) was added and the mixture was rocked for an additional 3.5 hrs. The beads were washed with PBS buffer (3 x 500 ul), denatured and the protein analyzed by 12% SDS-PAGE followed by western blotting onto PVDF membrane. The blot was cut along the 90kD mark of the protein ladder and the top portion (>90kD) probed with anti-AR and the bottom portion (<90 kD) probed with anti-HSP70. Both were visualized together by chemiluminescence using HRP conjugated secondary antibodies and film.



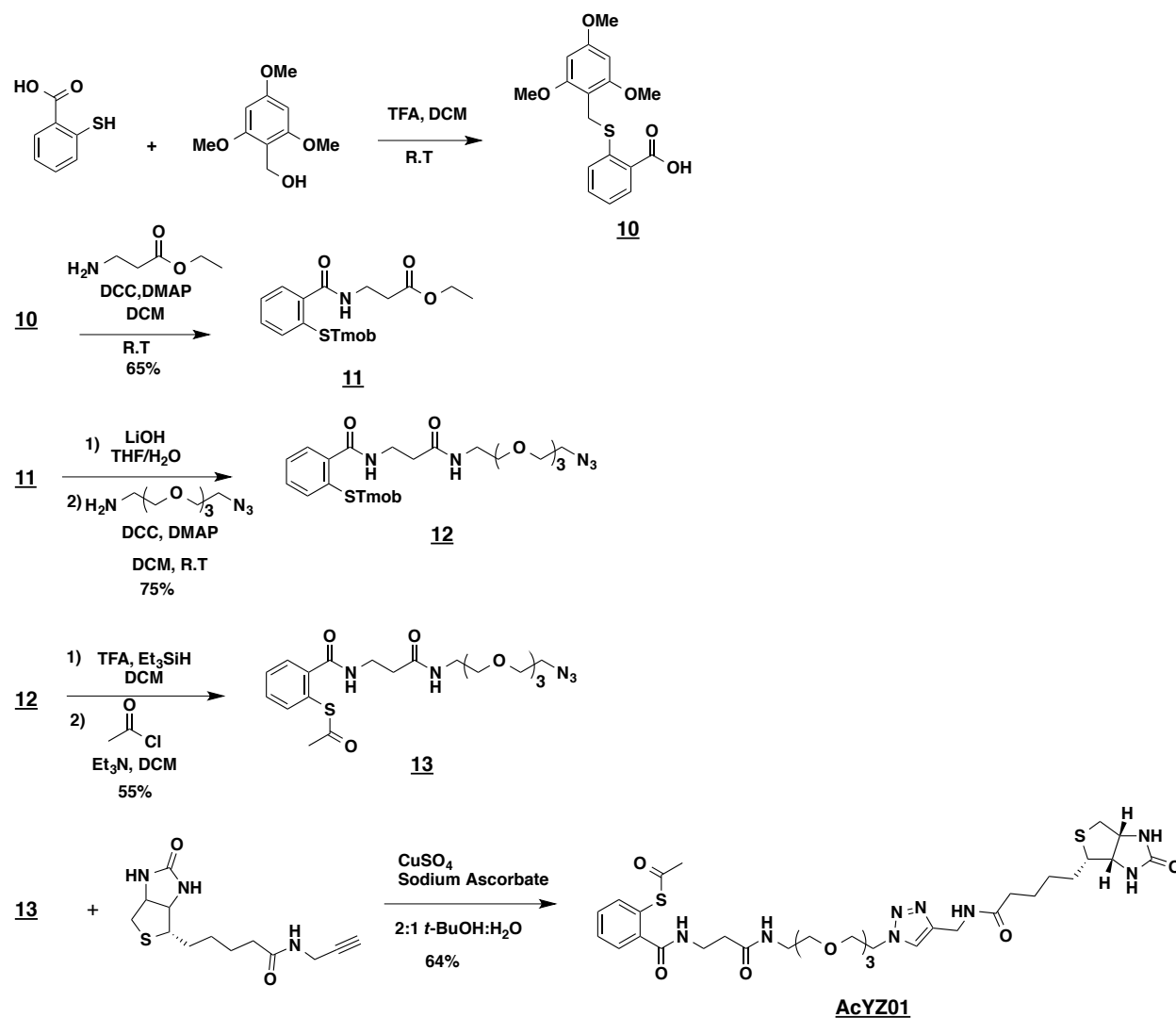
#### Scheme S1. Synthesis of propargyl amide of tolfenamic acid(**4**)



#### Synthesis of 2-((3-chloro-2-methylphenyl)amino)-N-(prop-2-yn-1-yl)benzamide (**4**):

To 1 g (3.9 mmol) of Tolfenamic acid in 40 ml methylene chloride was added 1.12 g (5.85 mmol, 1.5eq.) of EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide). After stirring for 5 min at room temperature 250 mg (4.7 mmol, 1.2 eq) of freshly distilled propargyl amine was added. After an additional 5 min at room temperature 950 mg of DMAP (7.82 mmol, 2.0 eq) was added and the reaction stirred overnight. The reaction mixture was partitioned between H<sub>2</sub>O and methylene chloride. The aqueous layer was extracted 3x with DCM. The combined organic extracts were washed with 3x sat. NaHCO<sub>3</sub> and brine, dried over MgSO<sub>4</sub> and reduced in vacuo. The product was purified by column chromatography (2% MeOH/DCM) to afford **4** as a yellow powder; yield 70% (817mg). <sup>1</sup>H NMR (600 MHz, CDCl<sub>3</sub>) δ 9.27 (s, 1H), 7.44(d, 1H, *J* = 7.8 Hz), 7.21(d, 1H, *J* = 7.8 Hz), 7.13 (d, 1H, *J* = 7.8 Hz), 7.08 (t, 1H, *J* = 8.4 Hz), 6.95 (d, 1H, *J* = 8.4 Hz), 6.76 (t, 1H, *J* = 8.4 Hz), 6.29 (s, 1H), 4.24 (q, 2H, *J* = 2.4 Hz), 2.34 (s, 3 H), 2.29 (s, 1H). <sup>13</sup>C NMR (150 MHz, CDCl<sub>3</sub>) δ 169.1, 146.57, 141.07, 135.53, 132.75, 130.28, 127.46, 126.74, 124.66, 120.98, 117.64, 116.39, 115.27, 79.36, 72.03, 29.63, 14.90. HRMS calcd for [C<sub>17</sub>H<sub>15</sub>ClN<sub>2</sub>O] 298.08730, found 298.08761

## Scheme S2. Synthesis of YZ01



**Ethyl 3-(2-((2,4,6-trimethoxybenzyl)thiol)benzamido)propanoate, **11****: To a solution of 2-mercaptobenzoic acid (1 g 6.49 mmol) and (2,4,6-trimethoxyphenyl)methanol (1.29 g, 6.49 mmol) in distilled DCM (65 ml) was added trifluoroacetic acid (0.626 ml, 8.43 mmol). After 30 min at RT the solution was washed with sat.  $\text{NaHCO}_3$  (3 x 20 ml). The combined aqueous washings were back extracted with DCM (3 x 10 ml). The combined organic extracts were dried over  $\text{Na}_2\text{SO}_4$  followed by concentration in vacuo to afford a light yellow solid. The metastable product **10** was applied to next step without further purification.  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ )  $\delta$  8.27 (dd,  $J$  = 7.9, 1.6 Hz, 1H), 7.59 (dd,  $J$  = 7.8, 1.3 Hz, 1H), 7.48 (td,  $J$  = 7.6, 1.6 Hz, 1H), 7.37 (td,  $J$  = 7.6, 1.3 Hz, 1H), 6.06 (s, 2H), 4.20 (s, 2H), 3.82 (s, 3H), 3.71 (s, 6H). To a solution of **10** (800 mg, 2.39 mmol) in 24 ml distilled DCM was added DCC (822.64 mg, 3.99 mmol). The solution was stirred for 15 min before ethyl-3-aminopropanoate (311.2 mg, 2.66 mmol) and DMAP was added. The reaction was stirred under  $\text{N}_2$  for 16 h. The solution was washed with  $\text{H}_2\text{O}$  (3 x 10 ml), dried over  $\text{Na}_2\text{SO}_4$  then concentration in vacuo. The residue was purified by

flash column chromatography (EtOAc: Hexane = 3:7, R<sub>f</sub> = 0.45) to give **11** as white solid. <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ 7.75 (dd, J = 7.4, 1.7 Hz, 1H), 7.62 (bs, 1H), 7.42 (dd, J = 7.3, 1.7 Hz, 1H), 7.30-7.23 (m, 2H), 6.06 (s, 2H), 4.18-4.11 (m, 4H), 3.81 (s, 3H), 3.73-3.65 (m, 8H), 2.66 (t, J = 6.4 Hz, 2H), 1.25 (t, J = 7.1 Hz, 3H). <sup>13</sup>C NMR (101 MHz, CDCl<sub>3</sub>) δ 172.36, 167.92, 160.73, 158.84, 137.09, 134.35, 133.60, 130.19, 129.44, 126.69, 105.80, 90.28, 60.74, 55.64, 55.07, 35.51, 34.19, 28.77, 14.22. HR-ESI MS m/e calcd for [C<sub>22</sub>H<sub>27</sub>NO<sub>6</sub>S] 434.1637, found 434.1648.

**N-(1-azido-13-oxo-3,6,9-trioxa-12-azapentadecan-15-yl)-2-(2,4,6-trimethoxybenzyl)thio)benzamide, 12**: To a solution of **11** (267.3 mg 0.62 mmol) in 3 ml distilled THF was added 1.23 ml 1M LiOH solution. The reaction was stirred at RT for 90 min before 2 ml of 1M HCl was added followed by 5 ml Sat. NaCl. The mixture was extracted with DCM (5 x 15 ml). The combined organic extracts were dried over Na<sub>2</sub>SO<sub>4</sub> then concentration in vacuo to afford crude acid intermediate, which was applied to next step without further purification.

To a solution of acid intermediate (250 mg, 0.62 mmol) in 6 ml distilled DCM was added DCC (190.8 mg 0.92 mmol). After 15 mins, 11-azido-3,6,9-trioxaundecan-1-amine (116.2 μl, 0.95 mmol) and DMAP were added sequentially. The solution was stirred under N<sub>2</sub> for 16 hrs and then was washed with H<sub>2</sub>O (3 x 5 ml). The aqueous washings were back extracted with DCM (3 x 15 ml). The combined organic extracts were combined and dried over Na<sub>2</sub>SO<sub>4</sub> and concentration in vacuo. The residue was purified by flash column chromatography (MeOH:DCM = 3 : 97 R<sub>f</sub> = 0.3) to give **12** as oil. <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ 7.68 (dd, J = 7.6, 1.6 Hz, 1H), 7.54 (bt, J = 5.9 Hz, 1H), 7.47-7.31 (dd, J = 7.6, 1.6 Hz, 1H), 7.31-7.21 (m, 2H), 6.39 (bs, 1H), 6.07 (s, 2H), 4.13 (s, 2H), 3.81 (s, 3H), 3.74-3.62 (m, 12H), 3.64-3.58 (m, 2H), 3.61-3.54 (m, 2H), 3.51 (m, 2H), 3.41 (m, 4H), 2.53 (t, J = 6.3 Hz, 2H), 1.82 (bs, 2H). <sup>13</sup>C NMR (101 MHz, CDCl<sub>3</sub>) δ 171.38, 168.25, 160.72, 158.98, 137.14, 134.85, 133.16, 130.11, 128.95, 126.61, 105.71, 90.34, 70.68, 70.59, 70.56, 70.22, 70.05, 69.69, 55.66, 55.38, 50.67, 39.19, 36.33, 36.00, 28.53. HR-ESI MS m/e calcd for [C<sub>28</sub>H<sub>39</sub>N<sub>5</sub>O<sub>8</sub>S] 606.2598, found 606.2607.

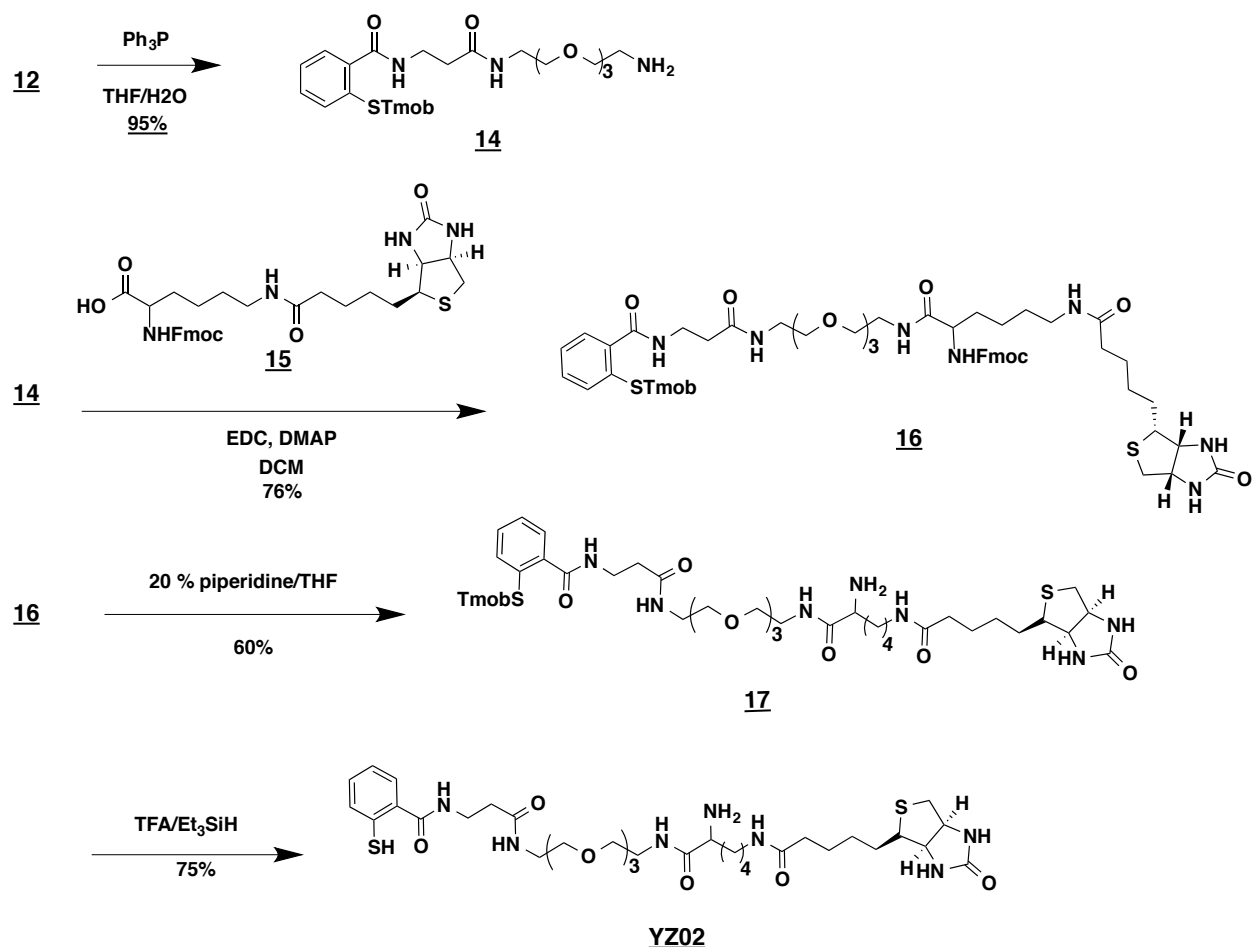
**S-(2-((1-azido-13-oxo-3,6,9-trioxa-12-azapentadecan-15-yl)carbamoyl)phenyl)ethanethioate, 13**: To a solution of **12** (117.4 mg 0.194 mmol) in 2 ml distilled DCM was added 245 μl TFA and 144 μl triethylsilane. After 1 h at RT, 5 ml H<sub>2</sub>O was added to the solution. The mixture partitioned and the aqueous layer extracted with DCM (5 x 20 ml). The combined organic extracts were dried over Na<sub>2</sub>SO<sub>4</sub> and concentration in vacuo to afford crude intermediates. The residue was applied to next step without further purification.

To the residue in 2 ml distilled DCM was added sequentially acetyl chloride (16 μl, 0.233 mmol) and triethylamine (54.5 μl 0.388 mmol). The reaction was stirred at RT under N<sub>2</sub> for 16 h and then was washed with 5 ml H<sub>2</sub>O and dried over Na<sub>2</sub>SO<sub>4</sub> and concentration in vacuo. The residue was purified by flash column chromatography (MeOH : DCM = 1 : 33, R<sub>f</sub> = 0.25) to give **13** as light yellowish oil. <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ 7.60 (dd, J = 5.8, 3.0 Hz, 1H), 7.55-7.41 (m, 3H), 6.88 (bs, 1H), 6.34 (bs, 1H), 3.75-3.60 (m, 12H), 3.60-3.53 (m, 2H), 3.48 (q, J = 5.1 Hz, 2H), 3.43-3.35 (m, 2H), 2.52 (t, J = 7.1 Hz, 2H), 2.46 (s, 3H). <sup>13</sup>C NMR (101 MHz, CDCl<sub>3</sub>) δ 194.65, 171.40, 168.21, 140.84, 136.52, 130.43, 129.96, 128.44, 125.39, 70.69, 70.64, 70.54, 70.21, 70.06, 69.64, 50.67, 39.28, 35.83, 35.39, 30.36. HR-ESI MS m/e calcd for [C<sub>20</sub>H<sub>29</sub>N<sub>5</sub>O<sub>6</sub>S] 468.1917, found 468.1927.

**S-(2-((13-oxo-1-(4-((5-(2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanamido)methyl)-1H-1,2,3-triazol-1-yl)-3,6,9-trioxa-12-azapentadecan-15-yl)carbamoyl)phenyl)ethanethioate, AcYZ01**: To a solution of **13** (63 mg 0.135 mmol) and biotin propargylamide (39.93 mg 0.142 mmol) (*JACS*, Vol. 128, No. 37, 2006, pp. 12174.) in dry DMF was added 14.2 μL 1M CuSO<sub>4</sub>, 14.2 μL 1M ascorbic acid and TBTA (7.53 mg

0.0142mmol). The solution was stirred at RT under N<sub>2</sub> for 16 h, followed by removal of DMF by rotary evaporation. The residue was purified by flash column chromatography on SiO<sub>2</sub> (MeOH : DCM = 1 : 6 R<sub>f</sub> = 0.3) to give **AcYZ01** as a yellowish oil. A solution of **AcYZ01** in degassed MeOH was purged in NH<sub>3</sub> for 20 min to give the free thiol **YZ01** quantitatively. The solution was concentrated in vacuo, and used as a stock solution in DMSO. <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ 7.86 (bs, 2H), 7.62-7.48 (m, 4H), 7.07 (bs, 1H), 7.01 (s, 1H), 6.36 (s, 1H), 4.55 (m, 4H), 4.38 (m, 2H), 3.92 (t, J = 7.0 Hz, 2H), 3.87 (t, J = 4.9 Hz, 2H), 3.64-3.56 (m, 4H), 3.53 (t, J = 5.1 Hz, 2H), 3.47-3.34 (m, 2H), 3.20-3.09 (m, 1H), 2.94 (dd, J = 12.8, 4.9 Hz, 1H), 2.79 (d, J = 12.8 Hz, 1H), 2.54 (t, J = 7.0 Hz, 2H), 2.42 (s, 3H), 2.29 (s, 2H), 2.22 (m, 3H), 1.79-1.61 (m, 8H). <sup>13</sup>C NMR (101 MHz, CDCl<sub>3</sub>) δ 192.87 , 173.34 , 171.86 , 170.61 , 164.44 , 145.15 , 140.69 , 136.83 , 131.20 , 130.02 , 128.23 , 125.26 , 123.77 , 70.37 , 70.06 , 69.92 , 69.19 , 61.55 , 60.27 , 55.56 , 50.12 , 42.53 , 40.75 , 39.48 , 35.65 , 34.82 , 34.38 , 30.38 , 29.66 , 27.83 , 26.39 , 25.28. HR-ESI MS m/e calcd for [C<sub>33</sub>H<sub>48</sub>N<sub>8</sub>O<sub>8</sub>S<sub>2</sub>] 747.2958, found 747.2964

### Scheme S3. Synthesis of YZ02

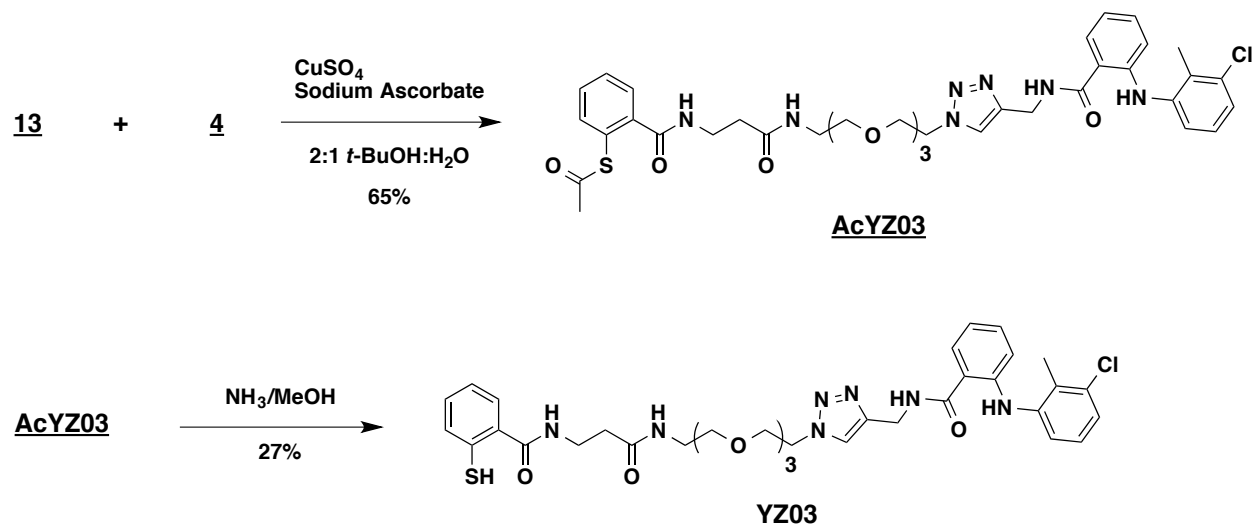


***N*-(1-amino-13-oxo-3,6,9-trioxa-12-azapentadecan-15-yl)-2-((2,4,6-trimethoxybenzyl)thio)benzamide, **14**:** To a solution of **12** (203 mg 0.335 mmol) in 3.4 ml distilled THF is added Ph<sub>3</sub>P (169.2 mg 0.67 mmol) and 0.13 ml H<sub>2</sub>O. The solution was reflux for 2 h then was partitioned between 1M HCl and ethyl ether. The organic layer was extracted with 1M HCl (2 x 10 ml). The combined aqueous extracts were made basic with NaOH to pH > 10, and back extracted with DCM (5 x 15 ml). The organic extracts were combined, dried over Na<sub>2</sub>SO<sub>4</sub>, and concentration in vacuo. The residue was purified by flash column chromatography (NH<sub>3</sub> Sat. MeOH : DCM = 1:12 R<sub>f</sub> = 0.3 ) to give pure **14** as light yellow oil. <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ 7.67 (dd, *J* = 7.6, 1.6 Hz, 1H), 7.56 (bs, 1H), 7.43 (dd, *J* = 7.7, 1.1 Hz, 1H), 7.35 (bs, 1H), 7.31-7.21 (m, 2H), 6.07 (s, 2H), 4.13 (s, 2H), 3.81 (s, 3H), 3.75-3.65 (m, 8H), 3.62-3.53 (m, 8H), 3.52-3.48 (m, 4H), 3.45-3.39 (m, 2H), 2.85 (t, *J* = 5.0 Hz, 2H), 2.54 (t, *J* = 6.4 Hz, 2H), 2.1-1.7 (bs, 2H). <sup>13</sup>C NMR (101 MHz, CDCl<sub>3</sub>) δ 171.56 , 168.28 , 160.73 , 158.98 , 137.26 , 134.97 , 133.07 , 130.08 , 128.89 , 126.55 , 105.74 , 90.34 , 73.19 , 70.51 , 70.50 , 70.18 , 70.06 , 69.94 , 55.66 , 55.37 , 41.56 , 39.17 , 36.39 , 35.79 , 28.40. HR-ESI MS *m/e* calcd for [C<sub>28</sub>H<sub>41</sub>N<sub>3</sub>O<sub>8</sub>S] 580.2693, found 580.2690.

**(9H-fluoren-9-yl)methyl (1,5,19,26-tetraoxo-30-(2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)-1-(2-((2,4,6-trimethoxybenzyl)thio)phenyl)-9,12,15-trioxa-2,6,18,25-tetraazatriacontan-20-yl)carbamate, 16:** To a solution of 14 (80.7 mg 0.1357 mmol) in 1.5 ml distilled DCM was added 1-[3-(Dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (EDC) (39 mg 0.204 mmol). The solution was stirred for 15 min before 15 (70.78 mg 0.1221 mmol) (JACS, 2004, 126 (44), pp 14435–14446) and DMAP (4.98 mg 0.041 mmol) were added sequentially. The reaction was stirred under N<sub>2</sub> for 16 h and then was washed with H<sub>2</sub>O (3 x 5ml). The combined aqueous washings were back extracted with DCM (3 x 15 ml). The combined organic extracts were combined and dried over Na<sub>2</sub>SO<sub>4</sub> and concentration in vacuo. The residue was purified by flash column chromatography (NH<sub>3</sub> Sat. MeOH: DCM = 1:9 R<sub>f</sub> = 0.3) to give pure 16 as yellowish oil. <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ 7.87 (s, 1H), 7.77 (d, *J* = 7.5 Hz, 2H), 7.65-7.53 (m, 4H), 7.45-7.25 (m, 5H), 7.19 (t, *J* = 7.5 Hz, 1H), 6.85 (bs, 1H), 6.16 (bs, 1H), 6.05 (s, 2H), 6.04 (d, *J* = 8.6 Hz, 1H), 5.76 (s, 1H), 4.49-4.41 (bs, 1H), 4.32 (d, *J* = 7.2 Hz, 2H), 4.13 (s, 2H), 3.80 (s, 3H), 3.75-3.39 (m, 24H), 3.16-2.98 (m, 2H), 2.71 (d, *J* = 12.8 Hz, 1H), 2.56 (t, *J* = 6.1 Hz, 2H), 2.09-2.02 (m, 3H), 1.82-1.57 (m, 8H), 1.55-1.35 (m, 4H), 0.98-0.73 (m, 6H). <sup>13</sup>C NMR (101 MHz, CDCl<sub>3</sub>) δ 173.30, 172.46, 171.88, 168.83, 163.91, 160.79, 158.99, 156.34, 143.92, 143.73, 141.25, 141.22, 136.58, 135.69, 131.84, 130.24, 128.48, 127.75, 127.14, 126.12, 125.22, 120.00, 105.39, 90.37, 70.30, 70.27, 69.89, 69.76, 69.69, 67.04, 61.88, 60.21, 55.95, 55.70, 55.39, 54.65, 47.07, 39.23, 39.15, 39.03, 36.67, 35.69, 35.04, 32.88, 31.94, 29.75, 27.94, 27.53, 25.14, 23.07, 22.71. HR-ESI MS *m/e* calcd for [C<sub>59</sub>H<sub>77</sub>N<sub>7</sub>O<sub>13</sub>S<sub>2</sub>] 1156.4735, found 1156.4723.

***N*-(18-amino-3,17,24-trioxa-28-(2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)-7,10,13-trioxa-4,16,23-triazaoctacosyl)-2-mercaptobenzamide, YZ02:** A solution of 16 (30.9 mg 0.027mmol) in 20% piperidine/ THF was stirred for 60 min at RT followed by removal of solvent by rotary evaporation to afford crude 17; HR-ESI MS *m/z* calcd for [C<sub>44</sub>H<sub>67</sub>N<sub>7</sub>O<sub>11</sub>S<sub>2</sub>] 934.4418, found 934.4414, The residue was applied to next step without further purification. To a solution of 17 (15.1 mg 0.0162 mmol) in distilled 0.2 ml DCM was added TFA (20.42 uL 0.275 mmol) and Triethylsilane (12 uL 0.05 mmol). The solution was stirred under N<sub>2</sub> for 60 mins, followed by removal of solvent by rotary evaporation. The residue was purified by flash column chromatography on C-18 reverse phase column (MeOH : H<sub>2</sub>O (w/ 3% TFA) = 3 : 2) to give pure YZ02 as the free thiol. <sup>1</sup>H NMR (400 MHz, MeOD) δ 7.64 (d, *J* = 7.8 Hz, 1H), 7.48 (d, *J* = 7.6 Hz, 1H), 7.41-7.25 (t, *J* = 13.6 Hz, 1H), 7.25-7.11 (t, *J* = 13.6 Hz, 1H), 4.49-4.36 (m, 1H), 4.25-4.13 (m, 1H), 3.75 (t, *J* = 6.5 Hz, 2H), 3.65-3.51 (m, 14H), 3.47-3.18 (m, 5H), 3.16-3.00 (m, 3H), 2.82 (dd, *J* = 12.6, 5.0 Hz, 1H), 2.60 (d, *J* = 12.8 Hz, 1H), 2.48 (t, *J* = 6.3 Hz, 2H), 2.09 (t, *J* = 6.8 Hz, 2H), 1.85-1.10 (m, 13H). <sup>13</sup>C NMR (101 MHz, MeOD) δ 174.81, 172.47, 169.12, 168.85, 164.80, 136.55, 134.16, 130.99, 127.69, 126.53, 126.17, 70.19, 70.12, 69.80, 69.77, 69.11, 68.88, 62.07, 60.26, 55.65, 53.00, 39.69, 39.14, 39.00, 38.46, 36.30, 35.39, 35.25, 30.87, 28.70, 28.39, 28.11, 25.48, 21.80. HR-ESI MS *m/e* calcd for 1156.4735, found 1156.4723. HR-ESI MS *m/e* calcd for [C<sub>34</sub>H<sub>55</sub>N<sub>7</sub>O<sub>8</sub>S<sub>2</sub>] 753.3520, found 753.3535.

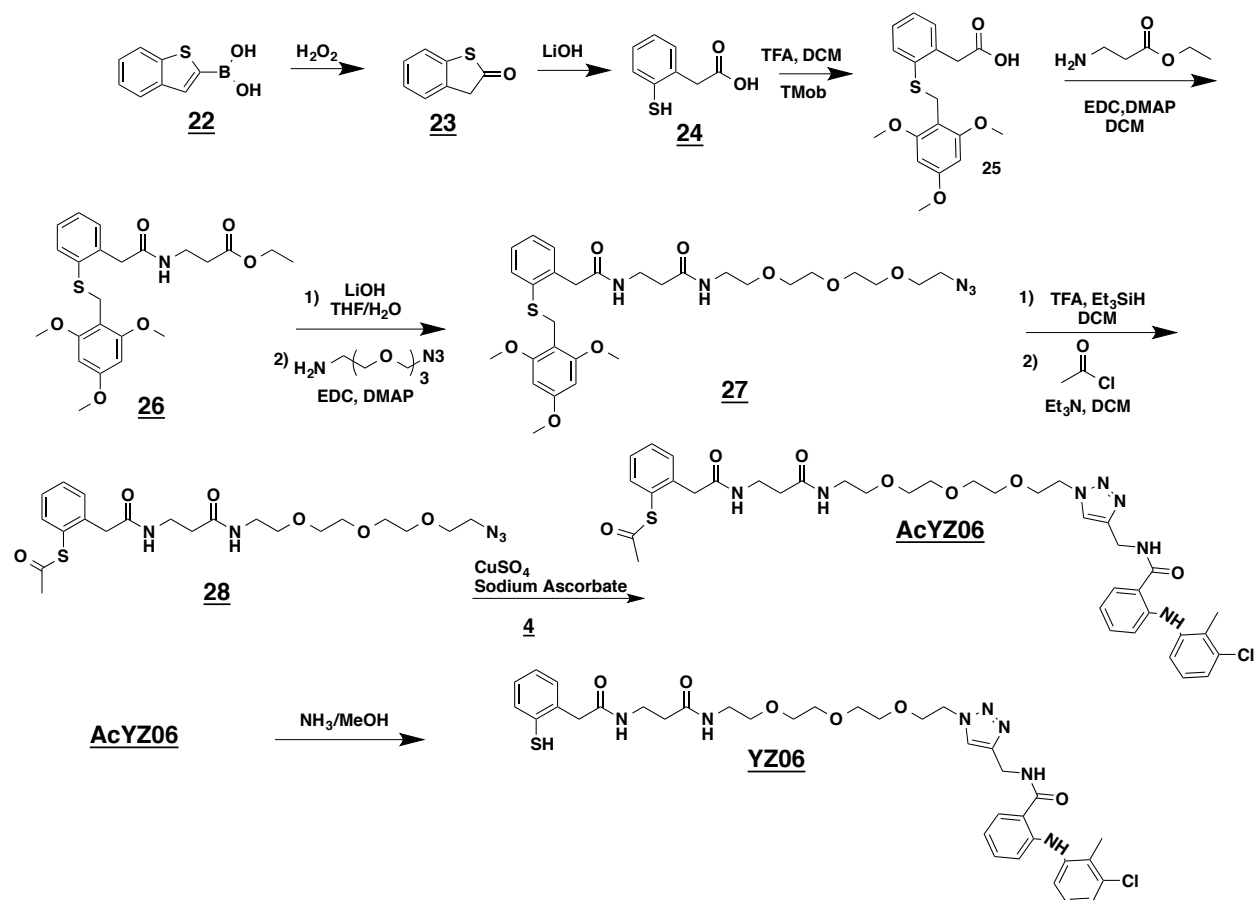
### Scheme S3. Synthesis of YZ03



#### S-(2-((1-(4-((3-((3-chloro-2-methylphenyl)amino)benzamido)methyl)-1H-1,2,3-triazol-1-yl)-13-oxo-3,6,9-trioxo-12-azapentadecan-15-yl)carbamoyl)phenyl) ethanethioate **AcZ03**:

To a solution of **13** (40 mg 0.085 mmol) and **4** (25.53 mg 90  $\mu$ mol) in dry DMF was added 9  $\mu$ l 1M CuSO<sub>4</sub>, 9  $\mu$ l 1M ascorbic acid and TBTA (4.78 mg 9  $\mu$ mol). The solution was stirred at room temperature under N<sub>2</sub> for 16 hr, followed by removal of DMF by rotary evaporation. The residue was purified by flash column chromatography (MeOH : DCM = 1 : 19) to give **AcYZ03** as a yellowish oil. A solution of **AcYZ03** in degassed MeOH was purged in NH<sub>3</sub> for 20 min to give **YZ03** as the free thiol. The solution was concentrated in vacuo, redissolved in DMSO to use without further purification. <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>)  $\delta$  9.40 (s, 1H), 7.79-7.75 (m, 1H), 7.62-7.54 (m, 1H), 7.51 (d, *J* = 7.9 Hz, 1H), 7.48-7.41 (m, 2H), 7.37 (bs, 1H), 7.24 (d, *J* = 7.7 Hz, 2H), 7.17-7.06 (m, 2H), 7.02-6.95 (m, 2H), 6.89 (bs, 1H), 6.77- 6.69 (m, 1H), 4.75-4.69 (m, 1H), 4.66 (d, *J* = 5.6 Hz, 2H), 4.59-4.51 (m, 2H), 3.95-3.85 (m, 2H), 3.71 -3.39 (m, 15H), 2.53 (t, *J* = 12 HZ, 1H), 2.44 (s, 2H), 2.37 (s, 3H), 1.98 (s, 1H). <sup>13</sup>C NMR (101 MHz, CDCl<sub>3</sub>)  $\delta$  194.81, 171.59, 169.55, 168.35, 146.20, 144.48, 141.29, 140.90, 136.54, 135.47, 132.47, 130.43, 129.96, 129.73, 128.44, 127.97, 126.77, 125.32, 124.28, 123.51, 120.37, 117.79, 117.02, 115.17, 70.56, 70.48, 70.44, 70.05, 69.76, 69.40, 50.36, 39.18, 36.31, 35.47, 35.17, 30.37, 14.94. HR-ESI MS *m/e* calcd for [C<sub>37</sub>H<sub>44</sub>ClN<sub>7</sub>O<sub>7</sub>S] 766.2790, found 766.2800. HPLC trace of **AcYZ03** was reported with >95% purity.

## Scheme S4. Synthesis of YZ06



**ethyl 3-(2-(2-((2,4,6-trimethoxybenzyl)thio)phenyl)acetamido)propanoate, **26****: Compound **26** was made from 2-(2-mercaptophenyl)acetic acid, **24** following the reported method of Chen et al. and the crude reaction product was used directly in the next reaction to avoid oxidation (Chen, Shuyi; Zhao, Xianrui; Chen, Jingyi; Chen, Jin; Kuznetsova, Larisa; Wong, Stanislaus S.; Ojima, Iwao *Bioconjugate Chemistry*, 2010, vol. 21, # 5 p. 979 - 987): To a solution of **24** (157 mg, 0.935 mmol) and (2,4,6-trimethoxyphenyl)methanol (185.2 mg, 0.935 mmol) in 10 mL dried DCM was added drop wise TFA (90  $\mu\text{L}$ , 1.22 mmol). The reaction was stirred under  $\text{N}_2$  at room temperature for 30 mins, solvent and TFA was reduced in vacuo. The crude product, **25** was applied to the next reaction without further purification.

To a solution of **25** (180 mg, 1.07 mmol) in 10 mL dry DCM was added 1-[3-(Dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (EDC) (307.7 mg, 1.605 mmol). The reaction was stirred for 15 mins under  $\text{N}_2$  before ethyl 3-aminopropanoate (164.2mg, 1.07mmol) was added followed by the addition of DMAP (261.3mg, 2.14 mmol). The reaction was stirred under  $\text{N}_2$  for 16 h before 1 ml 1N HCl was added and aqueous layer was extracted with DCM (2 X 10 mL). The combined organic extracts were combined, dried over  $\text{Na}_2\text{SO}_4$  and reduced in vacuo. The product was purified by flash chromatography (20% EtOAc/DCM) to give **26** as an oil.  $^1\text{H}$  NMR (400 MHz, Chloroform-d)  $\delta$  7.50 – 7.48 (m, 1H), 7.30 – 7.27 (m, 1H), 7.23 – 7.20 (m, 2H), 6.09 (s, 2H), 4.10 (s, 2H), 4.06 (q,  $J$  = 7.1 Hz, 2H), 3.82 (s, 3H), 3.72 (s, 6H), 3.44 (q,  $J$  = 6.2 Hz, 2H), 2.48 (t,  $J$  = 6.3 Hz, 2H), 1.698 (s, 2H), 1.20 (t,  $J$  = 7.1 Hz, 3H).  $^{13}\text{C}$  NMR (101 MHz, Chloroform-d)  $\delta$  172.13, 171.03, 160.67, 159.02, 136.81, 136.62, 133.17, 130.32,



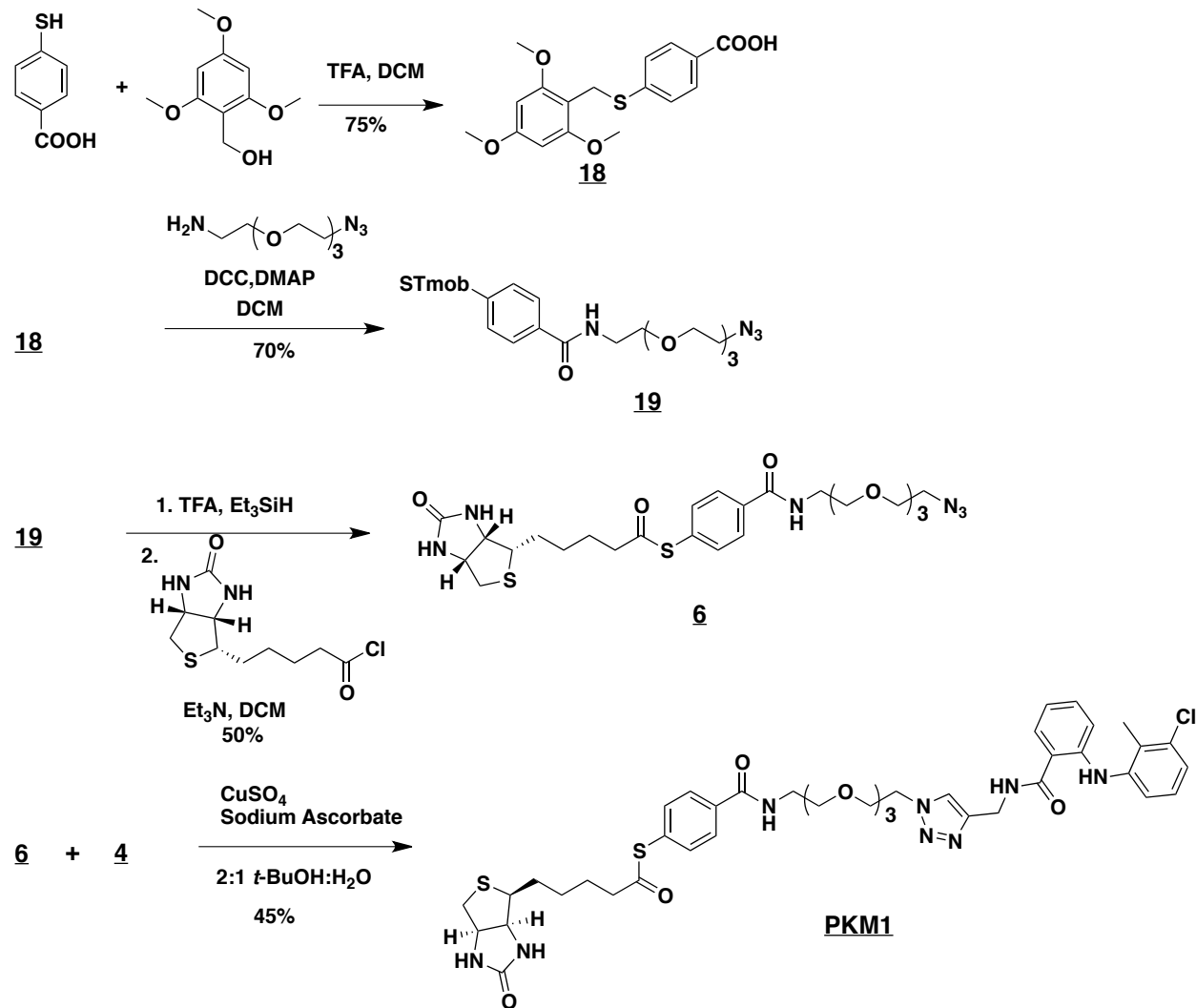
127.68 , 127.26 , 105.96 , 90.39 , 60.59 , 55.67 , 55.36 , 42.04 , 35.07 , 34.08 , 28.22 , 14.14.  
HR-ESI MS m/e calcd for [C<sub>23</sub>H<sub>29</sub>NO<sub>6</sub>S] 447.1716, found 447.1718.

**N-(2-(2-(2-(2-azidoethoxy)ethoxy)ethoxy)ethyl)-3-(2-(2-((2,4,6-trimethoxybenzyl)thio)phenyl)acetamido)propanamide, 27:** To a solution of 26 (54.35 mg, 0.1216 mmol) in 1 mL THF was added 1N LiOH (0.2432 mL). The reaction was stirred for 4 hours before 1 mL 1M HCl was added. The THF was removed in vacuo, and the aqueous layer was extracted exhaustively with DCM. The organic layers were combined, dried over Na<sub>2</sub>SO<sub>4</sub> and reduced in vacuo. To a solution of the crude acid product (51 mg, 0.1216 mmol) in 3 mL dried DCM was added 1-[3-(Dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (EDC) (47 mg, 0.2432 mmol). The reaction was stirred for 15 mins under N<sub>2</sub> before ethyl 11-azido-3,6,9-trioxaundecan-1-amine (48  $\mu$ L, 0.2432 mmol) was added followed by the addition of DMAP (261.3 mg, 2.14 mmol). The reaction was stirred under N<sub>2</sub> for 16 hours before 1 mL 1N HCl was added and aqueous layer was extracted with DCM (2 X 10 mL). The organic layers were combined, dried over Na<sub>2</sub>SO<sub>4</sub> and reduced in vacuo. The product was purified by flash chromatography (5% MeOH/DCM) to give 27 as an oil. <sup>1</sup>H NMR (400 MHz, Chloroform-d)  $\delta$  7.50 – 7.43 (m, 1H), 7.28 – 7.26 (m, 1H), 7.25 – 7.17 (m, 2H), 6.24 – 6.17 (m, 2H), 6.10 (s, 2H), 4.10 (s, 2H), 3.83 (s, 3H), 3.73 (s, 6H), 3.71 (s, 2H), 3.70 – 3.64 (m, 8H), 3.63 – 3.59 (m, 2H), 3.51 (t, J = 5.2 Hz, 2H), 3.47 (q, J = 6.2 Hz, 2H), 3.4 (t, J = 5.0 Hz, 2H), 3.37 (q, J = 5.3 Hz, 2H), 2.36 (t, J = 6.3 Hz, 2H). <sup>13</sup>C NMR (101 MHz, Chloroform-d)  $\delta$  171.47 , 171.38 , 160.73 , 159.05 , 136.93 , 136.35 , 132.89 , 130.34 , 127.70 , 127.71 , 105.98 , 90.50 , 70.70 , 70.64 , 70.59 , 70.05 , 69.54 , 55.71 , 55.37 , 50.69 , 41.91 , 39.29 , 35.97 , 35.90 , 28.14. HR-ESI MS m/e calcd for [C<sub>29</sub>H<sub>41</sub>N<sub>5</sub>O<sub>8</sub>S] 619.2676, found 619.2655.

**S-(2-(1-(4-((2-((3-chloro-2-methylphenyl)amino)benzamido)methyl)-1H-1,2,3-triazol-1-yl)-13,17-dioxo-3,6,9-trioxa-12,16-diazaoctadecan-18-yl)phenyl) ethanethioate, AcZ06** was made from S-(2-(1-azido-13,17-dioxo-3,6,9-trioxa-12,16-diazaoctadecan-18-yl)phenyl) ethanethioate, 28: To a solution of 27 (85.3 mg, 0.138 mmol) in 2 mL DCM was added TFA (174  $\mu$ L, 2.34 mmol) and Et<sub>3</sub>SiH (70  $\mu$ L, 0.434 mmol) at 0°C. The reaction was stirred for 30 mins then the solvent was removed in vacuo, and applied to high vacuum for 3 hours to remove the trace amount of the TFA. To the residue in 2 mL dried DCM was added Acetyl chloride (25.6  $\mu$ L, 29 mmol) and Et<sub>3</sub>N (78  $\mu$ L, 55 mmol) at -78°C. The reaction was slowly warmed up to RT and stirred for 16 hours. The solvent was removed in vacuo and the crude product 28 was applied to next step without purification. HR-ESI MS m/e calcd for [C<sub>21</sub>H<sub>31</sub>N<sub>5</sub>O<sub>6</sub>S+Na] 504.1887, found 504.1879.

To a solution of 28 (17 mg, 0.035 mmol) in 1 mL DMF was added 2-((3-chloro-2-methylphenyl)amino)-N-(prop-2-yn-1-yl)benzamide (12.6 mg, 0.042 mmol), 1N CuSO<sub>4</sub> (4  $\mu$ L), 1N ascorbic acid (4  $\mu$ L) and TBTA (2 mg, 0.0035 mmol). The reaction was stirred for 16 hours before the solvent was removed under vacuum. The product was purified by flash column chromatography (5 % MeOH/DCM) to give AcYZ06 as an oil. HR-ESI MS m/e calcd for C<sub>38</sub>H<sub>46</sub>ClN<sub>7</sub>O<sub>7</sub>S 802.2700, found 802.2693. A solution of AcYZ06 in degassed MeOH was purged in NH<sub>3</sub> for 20 min to give YZ06 with free thiol. The solution was concentrated in vacuo, redissolved in DMSO to use without further purification. <sup>1</sup>H NMR (400 MHz, Chloroform-d)  $\delta$  9.35 (s, 1H), 7.72 (s, 2H), 7.51 – 7.43 (m, 1H), 7.39 – 7.35 (m, 2H), 7.35 – 7.29 (m, 2H), 7.24 (td, J = 7.3, 2.2 Hz, 1H), 7.19 – 7.12 (m, 2H), 7.04 (dd, J = 8.0, 1.4 Hz, 1H), 7.00 (t, J = 7.8 Hz, 1H), 6.93 – 6.85 (m, 1H), 6.70 – 6.60 (m, 2H), 6.21 (d, J = 6.7 Hz, 1H), 4.61 (d, J = 5.3 Hz, 2H), 4.45 (t, J = 5.0 Hz, 2H), 3.81 (t, J = 5.0 Hz, 2H), 3.56 (s, 2H), 3.54 – 3.33 (m, 13H), 3.29 (q, J = 5.3 Hz, 2H), 2.34 (s, 3H), 2.27 (s, 3H). <sup>13</sup>C NMR (101 MHz, CDCl<sub>3</sub>)  $\delta$  194.39, 171.38, 170.42, 169.54, 146.30, 141.35, 138.94, 136.94, 135.49, 133.09, 132.43, 131.31, 131.18, 130.75, 129.85, 128.30, 128.04, 127.94, 126.73, 124.34, 120.55, 117.77, 117.09, 115.17, 70.58, 70.48, 70.47, 70.13, 69.70, 69.37, 50.38, 42.18, 39.15, 36.03, 35.56, 35.19, 30.28, 14.89. HR-ESI MS m/e calcd for [C<sub>38</sub>H<sub>46</sub>ClN<sub>7</sub>O<sub>7</sub>S+Na] 802.2700, found 802.2693. **HPLC** trace of AcYZ06 was reported with >95% purity.

## Scheme S5. Synthesis of PKM1



### N-(2-(2-(2-(2-azidoethoxy)ethoxy)ethoxy)ethyl)-4-((2,4,6-trimethoxybenzyl)thio)benzamide 19:

To a solution of 4-mercaptobenzoic acid (1 g, 6.49 mmol) and (2,4,6-trimethoxyphenyl)methanol (1.29 g, 6.49 mmol) in distilled DCM (65 ml) was added trifluoroacetic acid (0.626 ml, 8.43 mmol) dropwise. Trifluoroacetic acid was removed under reduced pressure to yield a light yellow solid. The product **18** is prone to oxidation and was applied to next step without further purification. <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ 7.98 (d, *J* = 8.4 Hz 2H), 7.44 (d, *J* = 8.4 Hz 2H), 6.15 (s, 2H), 4.30 (s, 2H), 3.83 (d, *J* = 2.5 Hz, 9H). <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>) δ 170.62, 160.92, 159.10, 147.35, 147.35, 130.21, 126.69, 124.99, 104.93, 90.57, 55.83, 55.39, 25.56.

To 250 mg (0.75 mmol) of **18** was added 230 mg of DCC and stirred for 5 mins. After which 155 mg (0.711 mmol, 0.95 eq.) of 2-(2-(2-(2-azidoethoxy)ethoxy)ethoxy)ethanamine was added and stirred for 5 mins at room temperature. After which 27 mg of dimethylaminopyridine was added and stirred overnight at room temperature. The reaction was worked up with 0.1 N HCl and

extracted with 3x 50ml DCM. The organic layers were pooled and washed with 3x 50ml brine. Dried over  $\text{Mg}_2\text{SO}_4$  and concentrated in vacuo. Purified using column chromatography with 2% MeOH/DCM to afford **19** as yellow oil in 70% yield (280mg).  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ )  $\delta$  7.69 (d,  $J$  = 6.73, 2H), 7.42 (dd,  $J$  = 2 Hz, 1H), 7.40 (td,  $J$  = 4.6 Hz, 1H), 6.73 (bs, 1H), 6.13 (s, 2H), 4.25 (s, 2H), 3.82 (s, 3H), 3.80 (s, 6H), 3.71 - 3.62 (m, 14H), 3.36 (t,  $J$  = 5.2 Hz, 2H).  $^{13}\text{C}$  NMR (100 MHz,  $\text{CDCl}_3$ )  $\delta$  167.07, 160.81, 159.05, 143.52, 130.89, 127.71, 127.11, 105.38, 90.54, 70.69, 70.65, 70.62, 70.59, 70.25, 70.08, 69.84, 55.81, 55.37, 50.65, 50.62, 39.70, 26.08. HRMS  $m/z$  calcd for  $[\text{C}_{25}\text{H}_{35}\text{N}_4\text{O}_7\text{S}]$  535.6330 found 535.2362.

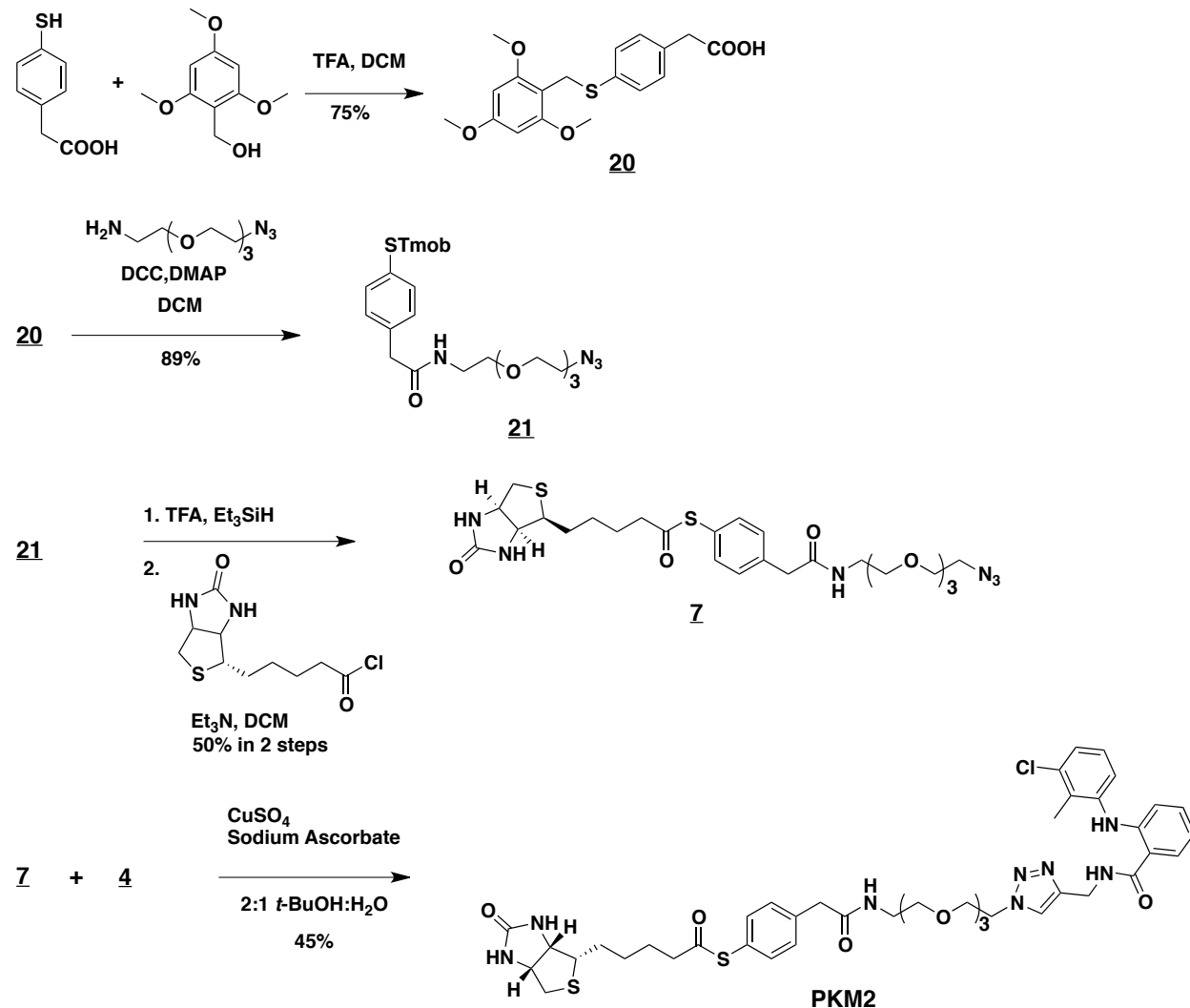
**S-(4-((2-(2-(2-(2-azidoethoxy)ethoxy)ethoxy)ethyl)carbamoyl)phenyl) 5-(2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanethioate **6**:**

To **19** 165 mg (0.3 mmol) in 3ml DCM was added 0.5ml  $\text{Et}_3\text{SiH}$  and 0.6 ml TFA. The mixture was stirred for 1h after which the reaction was extracted with sat.  $\text{NaHCO}_3$  and DCM. The aqueous layer was extracted 3x with DCM. The organic layer was washed with brine, dried over  $\text{MgSO}_4$  and concentrated in vacuo. The residue was used in the next step. To the residue in 5ml DCM 130 mg (0.49 mmol, 1.2 eq.) of biotin chloride (JACS, 2005, vol. 127, # 38 p. 13094 - 13095) was added. 130 mg (0.49 mmol, 1.2 eq.) of biotin chloride was added. To this 115  $\mu\text{l}$  of triethylamine was added dropwise. The reaction was stirred at room temperature overnight. After which it was extracted with 3x DCM and  $\text{H}_2\text{O}$ . The organic layer was washed with 3x brine, dried over  $\text{Mg}_2\text{SO}_4$  and concentrated in vacuo. Purified by column chromatography (MeOH:DCM = 1:9) to give **6** in 31.5% yield (55 mg).  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ )  $\delta$  7.85 (d,  $J$  = 8.4Hz, 2H), 7.45 (d,  $J$  = 8.4Hz, 2H), 6.92 (bs, 1H), 4.55-4.52 (m, 1H), 4.36 - 4.33 (m, 1H), 3.77 - 3.63 (m, 12H), 3.41 (t,  $J$  = 4.8 Hz, 2H), 3.19-3.16 (m, 1H), 2.95 (dd,  $J$  = 12.8, 5.6 Hz, 1H), 2.78 - 2.71 (m, 3H), 1.84 - 1.64 (m, 4H), 1.58 - 1.47 (m, 2H), 1.30-1.25 (m, 2H).  $^{13}\text{C}$  NMR (100 MHz,  $\text{CDCl}_3$ )  $\delta$  196.69, 166.73, 163.06, 135.44, 134.39, 131.44, 127.80, 77.36, 77.05, 76.73, 70.67, 70.64, 70.59, 70.27, 70.06, 69.72, 61.95, 60.11, 55.27, 50.63, 40.56, 39.85, 28.30, 28.19, 25.35, 14.29, HRMS  $m/z$  calcd for  $[\text{C}_{25}\text{H}_{37}\text{N}_6\text{O}_6\text{S}_2]$  581.2216 found 581.2215.

**S-(4-((2-(2-(2-(2-(4-((3-chloro-2-methylphenyl)amino)benzamido)methyl)-1H-1,2,3-triazol-1-yl)ethoxy)ethoxy)ethoxy)ethyl)carbamoyl)phenyl) 5-(2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanethioate, **PKM1**:**

To a solution of 7.6 mg **6** (13.2  $\mu\text{mol}$ ) and **4** (10 mg 14.5  $\mu\text{mol}$ ) in 1ml dry DMF was added 7.5  $\mu\text{l}$  of 2mM  $\text{CuSO}_4$  (0.13  $\mu\text{mol}$ , 0.1 eq), 75 $\mu\text{l}$  of 2mM ascorbic acid (33  $\mu\text{mol}$ , 1eq) and TBTA (1.0 mg 1.9  $\mu\text{mol}$ ). The solution was stirred at room temperature under  $\text{N}_2$  for 16 h, followed by removal of DMF by rotary evaporation. The residue was purified by flash column chromatography (MeOH : DCM = 1 : 9) to give **PKM1** in 45 % yield (5.7 mg).  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ )  $\delta$  9.40 (bs, 1H), 8.04 (bs, 1H), 7.73 (d,  $J$  = 8 Hz, 2H), 7.52 (s, 1H), 7.33 (d,  $J$  = 7.6 Hz, 2H), 7.16- 7.04(m, 2H), 7.04 - 6.89 (m, 4H), 6.77 (t,  $J$  = 6.8 Hz, 1H), 6.17 (bs, 1H), 4.62 (bs, 1H), 4.51 (bs, 1H), 4.35 (bs, 1H), 4.3 (bs, 1H), 3.6 (s, 1H), 3.59 - 3.43 (m, 12H), 3.10 (bs, 1H), 2.86 (bs, 1H), 2.68 (d,  $J$  = 10.8 Hz, 1H), 2.58 (t,  $J$  = 8Hz, 2H), 2.26 (s, 3H), 1.67 - 1.60 (m, 7H), 1.43 - 1.40 (m, 2H), 1.21 - 1.18 (s, 2H).  $^{13}\text{C}$  NMR (100 MHz,  $\text{CDCl}_3$ )  $\delta$  196.97, 166.95, 146.23, 141.39, 135.41, 135.35, 134.40, 131.41, 129.61, 127.90, 126.75, 124.15, 120.31, 117.94, 115.06, 70.33, 70.28, 70.08, 69.62, 68.80, 60.48, 55.64, 53.49, 50.84, 43.40, 40.71, 39.87, 31.00, 29.72, 28.29, 25.43, 24.77, 14.94, 14.28. HRMS calcd for  $[\text{C}_{42}\text{H}_{51}\text{ClN}_8\text{O}_7\text{S}_2]$  879.4867 found 879.3077.

## Scheme S6. Synthesis of PKM2



**N-(2-(2-(2-(2-azidoethoxy)ethoxy)ethoxy)ethyl)-2-(4-((2,4,6-trimethoxybenzyl)thio)phenyl)acetamide **21**:** To a solution of 4-mercaptobenzoic acid (1g 6.49mmol) and (2,4,6-trimethoxyphenyl)methanol (1.29 g, 6.49mmol) in distilled DCM (65 ml) was added trifluoroacetic acid (0.626 ml, 8.43 mmol) dropwise. Trifluoroacetic acid was removed under reduced pressure to afford a light yellow solid. The product **20** was prone to oxidation and applied to next step without further purification.  $^1\text{NMR}$  (400 MHz,  $\text{DMSO-d}_6$ )  $\delta$  7.25- 7.16 ( m, 5H), 6.21 (s, 2H), 4.05 (s, 2H), 3.76 (s, 3H), 3.71 (s, 7H), 3.52 (s 2H).  $^{13}\text{C}$  NMR (100 MHz,  $\text{DMSO-d}_6$ )  $\delta$  172.75, 160.46, 158.59, 136.00, 132.38, 130.19, 129.86, 128.46, 128.37, 104.91, 90.78, 55.74, 55.28, 26.36.

To 300 mg (0.86 mMol) of **20** in 8.6ml was added, 265 mg (1.29 mM) of DCC and stirred at room temperature for 5 min. To this 290 mg (0.95 mmol, 1.1 eq.) of 2-(2-(2-(2-azidoethoxy)ethoxy)ethoxy)ethanamine was added and stirred for 5 min. To this 30mg (0.26 mMol, 0.3 eq.) was added and stirred overnight at room temperature after which it was worked up using 0.1 N HCl and DCM. The organic layer was pooled together and washed with 3x 50ml

brine solution, dried and reduced under vacuo. Purified on column using MeOH:DCM = 2:98 to Yield **21** 89% (470 mg)

$^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ )  $\delta$  7.38 (dd,  $J$  = 2 Hz, 1H), 7.36 (dd,  $J$  = 2 Hz, 1H), 7.18 (td,  $J$  = 2, 1.6 Hz, 1H), 7.16 (dd,  $J$  = 1.6 Hz, 1H), 6.12 (s, 2H), 5.97 (bs, 1H), 4.19 (s, 2H), 3.82 (s, 3H), 3.77 (s, 6H), 3.71 - 3.57 (m, 12H), 3.53 - 3.51 (m, 4H).  $^{13}\text{C}$  NMR (100 MHz,  $\text{CDCl}_3$ )  $\delta$  170.94, 160.63, 159.00, 137.20, 132.12, 129.50, 106.14, 90.51, 70.70, 70.61, 70.60, 70.38, 70.30, 70.07, 69.74, 55.77, 55.36, 50.65, 49.16, 39.38, 33.94, 27.15, 25.61, 24.96.

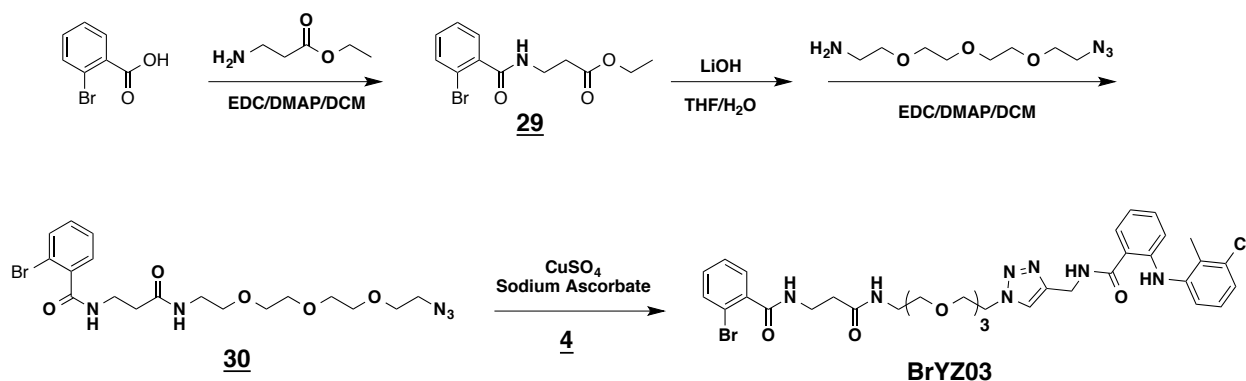
**S-(4-(14-azido-2-oxo-6,9,12-trioxa-3-azatetradecyl)phenyl) 5-(2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanethioate **7**:**

To 150 mg of **21** (0.47 mmol) in 4ml DCM was added  $\text{Et}_3\text{SiH}$  and TFA. The mixture was stirred for 1hr after which the reaction was extracted with sat.  $\text{NaHCO}_3$  and DCM. The aqueous layer was extracted 3x with DCM. The organic layer was washed with brine, dried over  $\text{MgSO}_4$  and reduced under vacuo. The residue was used in the next step. To the residue in 4ml DCM 130 mg (0.49 mmol, 1.2 eq.) of biotin chloride was added. To this 115  $\mu\text{l}$  of triethylamine was added drop wise. The reaction was stirred at room temperature overnight. After which it was extracted with 3x DCM and  $\text{H}_2\text{O}$ . The organic layer was washed with 3x brine, dried over  $\text{Mg}_2\text{SO}_4$  and concentrated in vacuo. Purified by column chromatography (MeOH:DCM = 1:9) to give **7** in 47% yield (130mg).  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ )  $\delta$  7.37 (q,  $J$  = 8.3 Hz, 4H), 6.17 (bs, 1H), 5.67 (bs, 1H), 5.16 (bs, 1H), 4.54 - 4.51 (m, 1H), 4.34 - 4.31 (m, 1H), 3.70 - 3.50 (m, 14H), 3.47 - 3.39 (m, 4H), 3.20 - 3.15 (m, 1H), 2.93 (dd,  $J$  = 12.8, 4.8 Hz, 1H), 2.78 - 2.68 (m, 3H), 1.79 - 1.69 (m, 4H), 1.52 - 1.47 (m, 2H).  $^{13}\text{C}$  NMR (100 MHz,  $\text{CDCl}_3$ )  $\delta$  197.53, 170.33, 163.30, 136.62, 134.85, 130.20, 126.48, 70.70, 70.61, 70.56, 70.27, 70.08, 69.69, 61.94, 60.10, 55.36, 50.66, 43.38, 43.19, 40.58, 39.45, 28.28, 28.21, 25.41. HRMS-ESI calcd for  $[\text{C}_{26}\text{H}_{39}\text{N}_6\text{O}_6\text{S}_2]$  595.24 found 595.2362.

**S-(4-(14-(4-((2-((3-chloro-2-methylphenyl)amino)benzamido)methyl)-1H-1,2,3-triazol-1-yl)-2-oxo-6,9,12-trioxa-3-azatetradecyl)phenyl) 5-((3aS,4S,6aR)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanethioate **PKM2**:**

To a solution of **7** (18.5 mg 32  $\mu\text{mol}$ ) and **4** (10 mg 33  $\mu\text{mol}$ ) in 1 ml of dry DMF was added 3  $\mu\text{l}$  of 1M  $\text{CuSO}_4$ , 33.5  $\mu\text{l}$  of 1M ascorbic acid and TBTA (1.7 mg 3.2  $\mu\text{mol}$ ). The solution was stirred at room temperature under  $\text{N}_2$  for 16 h, followed by removal of DMF by rotary evaporation. The residue was purified by flash column chromatography (MeOH : DCM = 1 : 9) to give yield 45% (12.7 mg) **PKM2** as a yellowish oil.  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ )  $\delta$  9.42 (bs, 1H), 8.01 (bs, 1H), 7.71 (s, 1H), 7.50 (d,  $J$  = 8 Hz, 1H), 7.27 - 7.21 (m, 3H), 7.16 - 7.13 (m, 2H), 7.03 - 6.96 (m, 2H), 6.9 (d,  $J$  = 8.4 Hz, 1H), 6.67 (t,  $J$  = 7.6 Hz, 1H), 6.23 (s, 2H), 4.58 (bs, 2H), 4.42 (bs, 2H), 4.28 - 4.25 (m, 1H), 3.76 (t,  $J$  = 4.8 Hz, 2H), 3.49 - 3.40 (m, 12H), 3.47 - 3.31 (m, 2H), 3.01 (m, 1H), 2.84 (dd,  $J$  = 12.8, 5.6 Hz, 1H), 2.66 (d,  $J$  = 12.8 Hz, 2H), 2.26 (s, 3H), 2.10 (s, 3H), 1.71 - 1.53 (m, 5H), 1.44 (m, 2H).  $^{13}\text{C}$  NMR (100 MHz,  $\text{CDCl}_3$ )  $\delta$  197.45, 170.46, 169.64, 163.46, 146.13, 141.42, 136.60, 135.43, 134.83, 132.32, 130.18, 129.54, 128.30, 126.73, 126.53, 124.09, 120.17, 117.89, 117.15, 115.05, 70.54, 70.43, 70.38, 70.25, 69.66, 69.32, 60.01, 55.60, 50.39, 43.42, 43.20, 40.68, 39.40, 34.69, 31.00, 28.43, 28.36, 25.47, 14.95. HRMS-ESI calcd for  $[\text{C}_{43}\text{H}_{54}\text{ClN}_8\text{O}_7\text{S}_2]$  893.32 found 893.3250.

## Scheme S7. Synthesis of BrYZ03



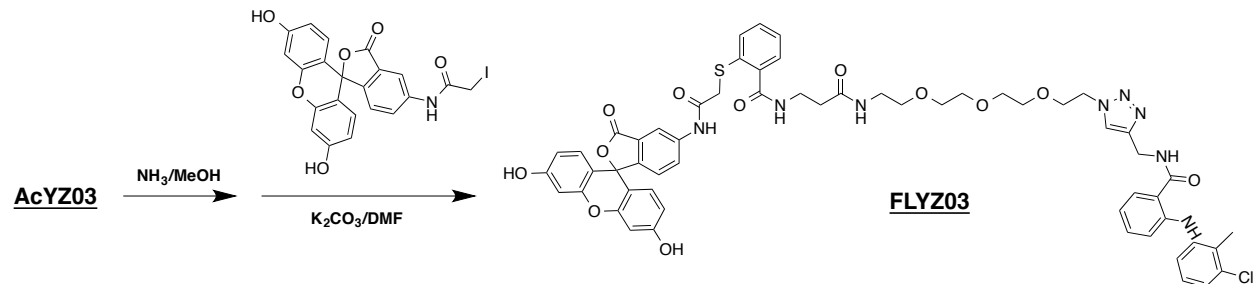
**Ethyl 3-(2-bromobenzamido)propanoate, 29:** To a solution of 2-Bromobenzoic acid (100mg, 0.497mmol) in 5 mL dried DCM was added 1-[3-(Dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (EDC) (143mg, 0.746mmol). The reaction was stirred for 15 mins under N<sub>2</sub> before ethyl 3-aminopropanoate (76.3mg, 0.497mmol) was added followed by the addition of DMAP (91mg, 0.7455 mmol). The reaction was stirred under N<sub>2</sub> for 16 hours before 1ml 1N HCl was added and aqueous layer was extracted with DCM (2 X 10mL). The organic layers were combined, dried over Na<sub>2</sub>SO<sub>4</sub> and reduced in vacuo. The product was purified by flash chromatography (20% EtOAc/DCM) to give **29** as an oil.

<sup>1</sup>H NMR (400 MHz, Chloroform-d) δ 7.56 (d, J = 8.0, 1H), 7.48 (d, J = 7.6, 1H), 7.34 (t, J = 7.5, 1H), 7.30 – 7.22 (m, 1H), 6.66 (bs, 1H), 4.15 (q, J = 7.1 Hz, 2H), 3.72 (q, J = 6.0 Hz, 2H), 2.67 (t, J = 5.9 Hz, 2H), 1.27 (t, J = 7.1 Hz, 3H). <sup>13</sup>C NMR (101 MHz, CDCl<sub>3</sub>) δ 172.60, 167.68, 137.76, 133.29, 131.23, 129.34, 127.53, 119.24, 60.87, 35.34, 33.84, 14.23. HR-ESI MS m/e calcd for [C<sub>12</sub>H<sub>14</sub>BrNO<sub>3</sub>] 299.0153, found 299.0157.

**N-(1-azido-13-oxo-3,6,9-trioxa-12-azapentadecan-15-yl)-2-bromobenzamide, 30:** To a solution of **18** (100mg, 0.334mmol) in 1 mL THF was added 1N LiOH (0.668 mL). The reaction was stirred for 2 hours before 1 mL HCl was added to quench the reaction. The THF was removed in vacuo, and the aqueous layer was extracted copiously with DCM. The organic layers were combined, dried over Na<sub>2</sub>SO<sub>4</sub> and reduced in vacuo. To a solution of the acid product in 3 mL dried DCM was added 1-[3-(Dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (EDC) (96.8mg, 0.501 mmol). The reaction was stirred for 15 mins under N<sub>2</sub> before ethyl 11-azido-3,6,9-trioxaundecan-1-amine (66uL, 0.334mmol) was added followed by the addition of DMAP (12.2mg, 0.1 mmol). The reaction was stirred under N<sub>2</sub> for 16 hours before 1ml 1N HCl was added and aqueous layer was extracted with DCM (2 x 10mL). The organic layers were combined, dried over Na<sub>2</sub>SO<sub>4</sub> and reduced in vacuo. The product was purified by flash chromatography (5% MeOH/DCM) to give **30** as an oil. <sup>1</sup>H NMR (400 MHz, Chloroform-d) δ 7.54 (dd, J = 8.0, 1.3 Hz, 1H), 7.44 (dd, J = 7.6, 1.8 Hz, 1H), 7.35 – 7.26 (m, 1H), 7.23 (td, J = 7.7, 1.8 Hz, 1H), 7.00 (t, J = 5.6 Hz, 1H), 6.59 (t, J = 5.5 Hz, 1H), 3.74 – 3.66 (m, 4H), 3.64 – 3.55 (m, 6H), 3.52 (dd, J = 5.6, 4.5 Hz, 2H), 3.45 – 3.33 (m, 6H), 2.57 – 2.49 (m, 2H). <sup>13</sup>C NMR (101 MHz, CDCl<sub>3</sub>) δ 171.62, 167.90, 138.02, 133.21, 131.07, 129.12, 127.46, 119.33, 72.48, 70.62, 70.49, 70.18, 69.99, 69.60, 61.65, 39.22, 36.03, 35.07. HR-ESI MS m/e calcd for [C<sub>18</sub>H<sub>26</sub>BrN<sub>5</sub>O<sub>5</sub>] 471.1182, found 471.1196.

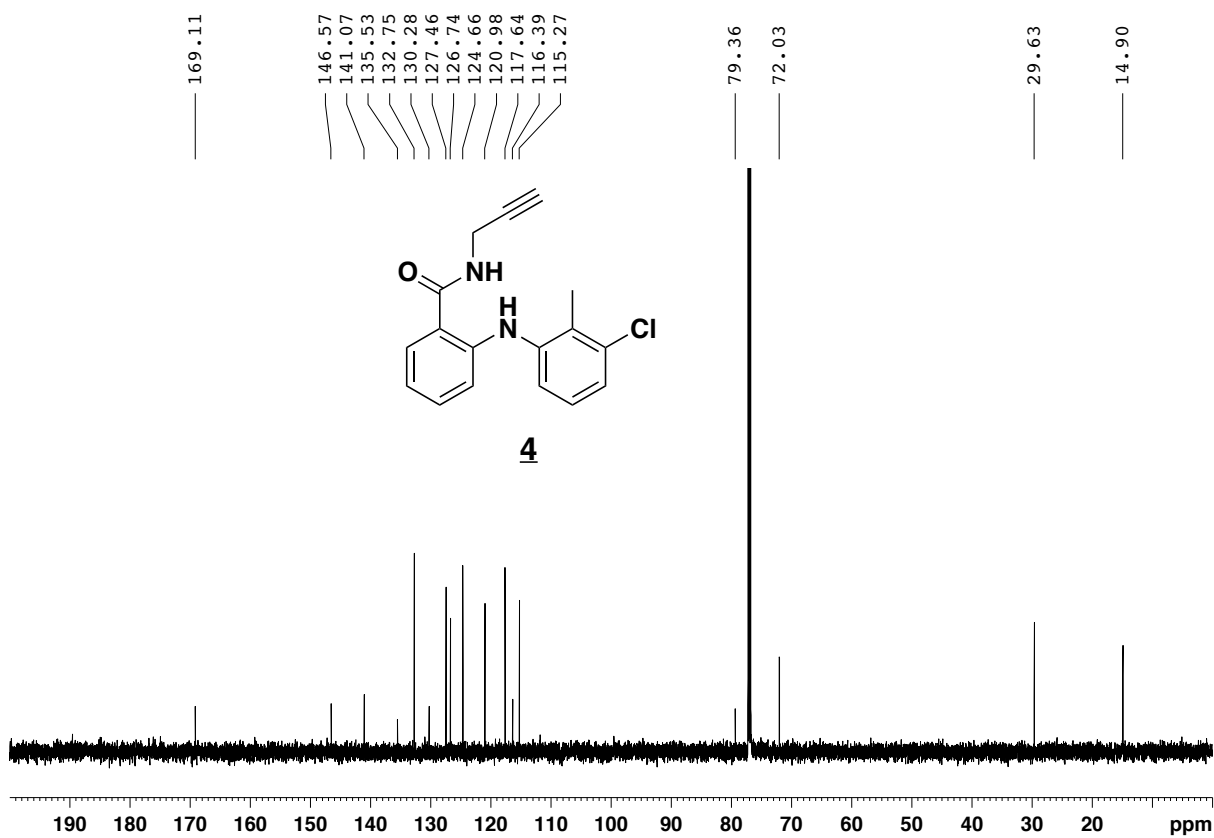
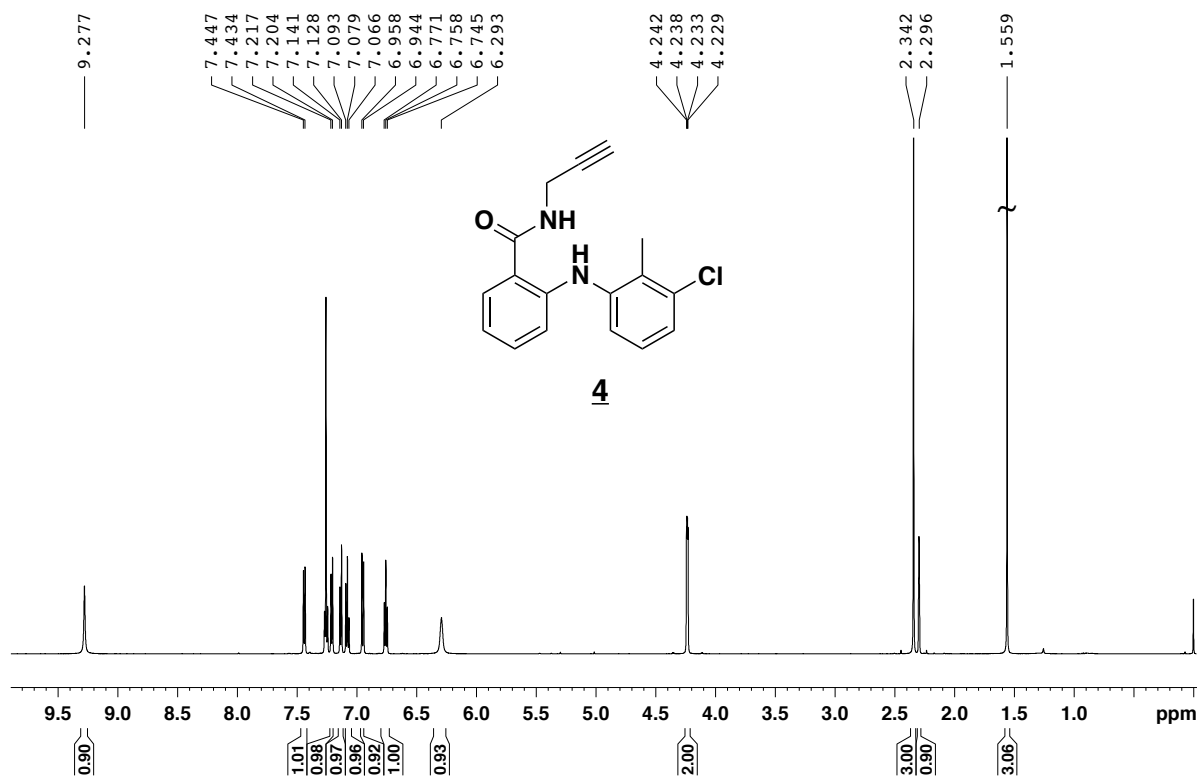
**2-bromo-N-(1-(4-((2-((3-chloro-2-methylphenyl)amino)benzamido)methyl)-1H-1,2,3-triazol-1-yl)-13-oxo-3,6,9-trioxa-12-azapentadecan-15-yl)benzamide, BrYZ03** :To a solution of **19** (50mg, 0.106 mmol) in 5 mL DMF was added 2-((3-chloro-2-methylphenyl)amino)-N-(prop-2-yn-1-yl)benzamide (31.8 mg, 0.106 mmol), 1N CuSO<sub>4</sub> (10 uL), 1N ascorbic acid (10uL) and TBTA (5.8mg, 0.011mmol). The reaction was stirred for 16 hours before the solvent was removed by high vacuum. The product was purified by flash column chromatography (5 % MeOH/DCM) to give **BrYZ03** as an oil. <sup>1</sup>H NMR (400 MHz, Chloroform-d) δ 9.42 (s, 1H), 7.79 (s, 1H), 7.54 (m, 2H), 7.46 (dd, J = 7.6, 1.8 Hz, 1H), 7.33 (d, J = 1.3 Hz, 2H), 7.30 – 7.19 (m, 3H), 7.19 – 7.04 (m, 2H), 6.99 (dd, J = 8.5, 1.1 Hz, 1H), 6.92 (t, J = 6.1 Hz, 1H), 6.82 (t, J = 5.5 Hz, 1H), 6.72 (t, J = 0.9 Hz, 1H), 4.70 (d, J = 5.6 Hz, 2H), 4.54 (t, J = 5.0 Hz, 2H), 3.90 (t, J = 5.5, 2H), 3.72 (q, J = 5.9 Hz, 2H), 3.64 – 3.58 (m, 2H), 3.58 – 3.47 (m, 8H), 3.44 (t, J = 5.1 Hz, 2H), 2.58 (t, J = 6.0 Hz, 2H), 2.36 (s, 3H). <sup>13</sup>C NMR (101 MHz, CDCl<sub>3</sub>) δ 171.66, 169.56, 167.99, 146.16, 144.43, 141.28, 137.96, 135.46, 133.25, 132.46, 131.13, 129.69, 129.17, 127.93, 127.49, 126.76, 124.27, 123.47, 120.36, 119.33, 117.79, 117.03, 115.17, 70.55, 70.46, 70.42, 70.11, 69.71, 69.40, 50.31, 39.20, 36.16, 35.24, 35.13, 14.94. HR-ESI MS m/e calcd for [C<sub>35</sub>H<sub>41</sub>BrClN<sub>7</sub>O<sub>6</sub>] 769.1965, found 769.1990.

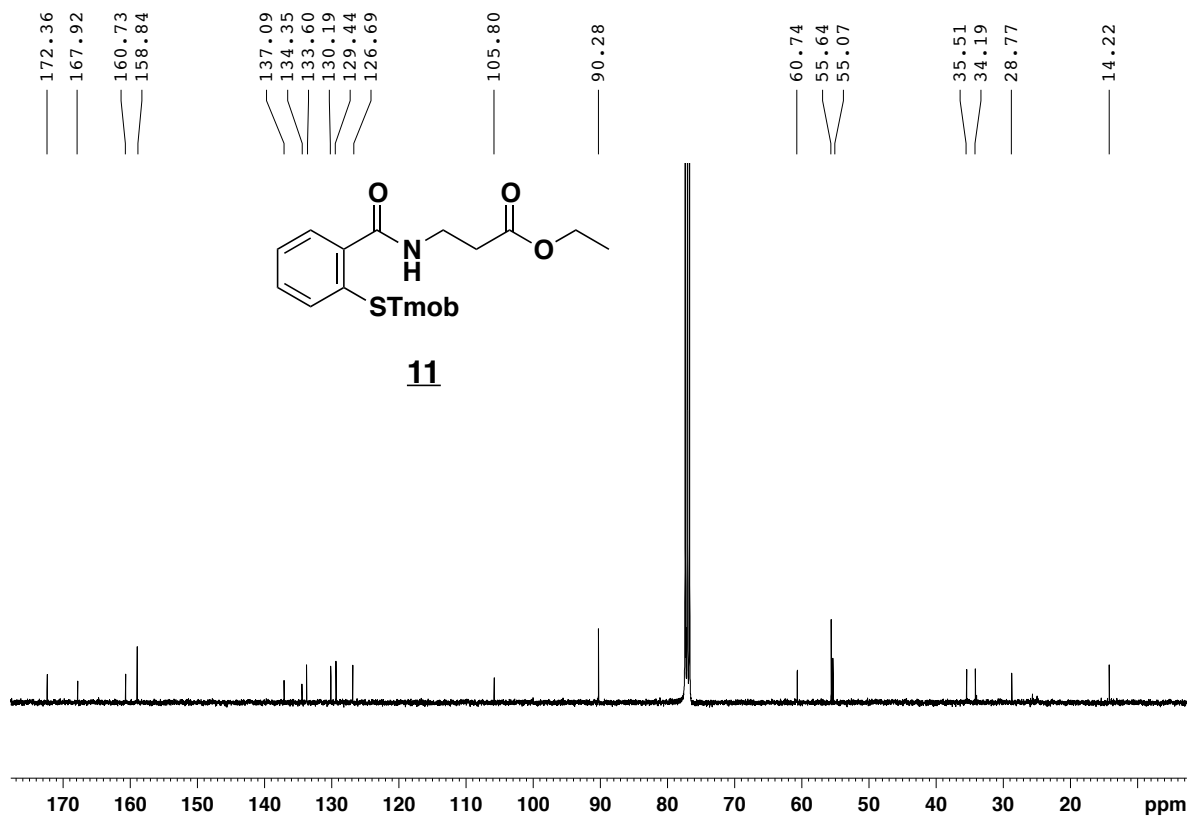
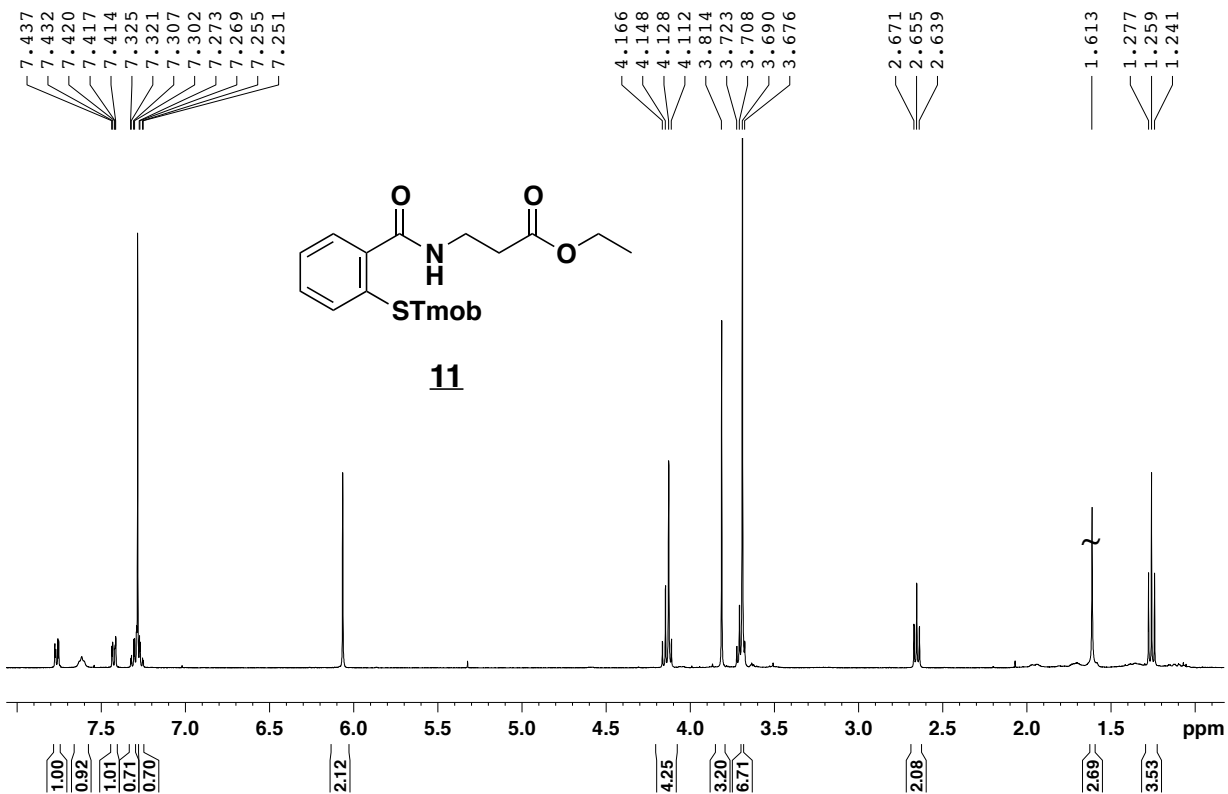
## Scheme S7. Synthesis of FIYZ03

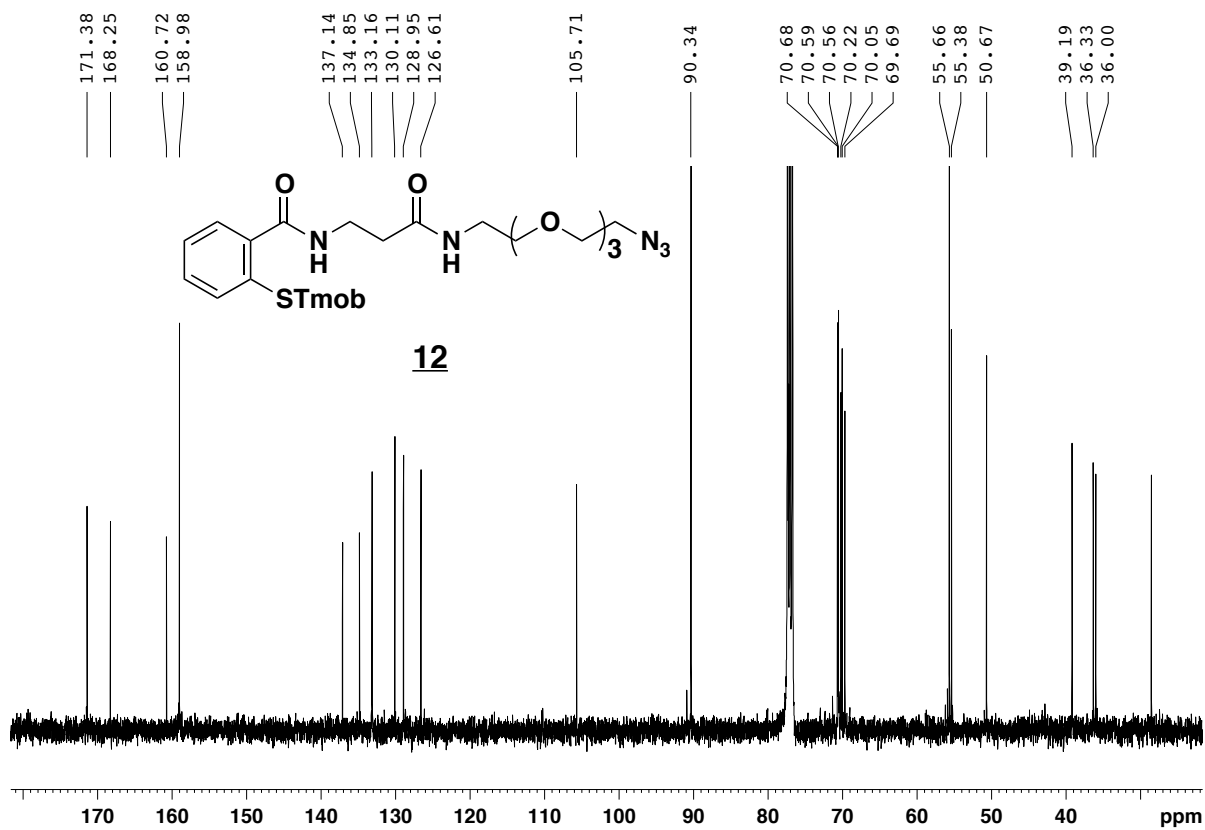
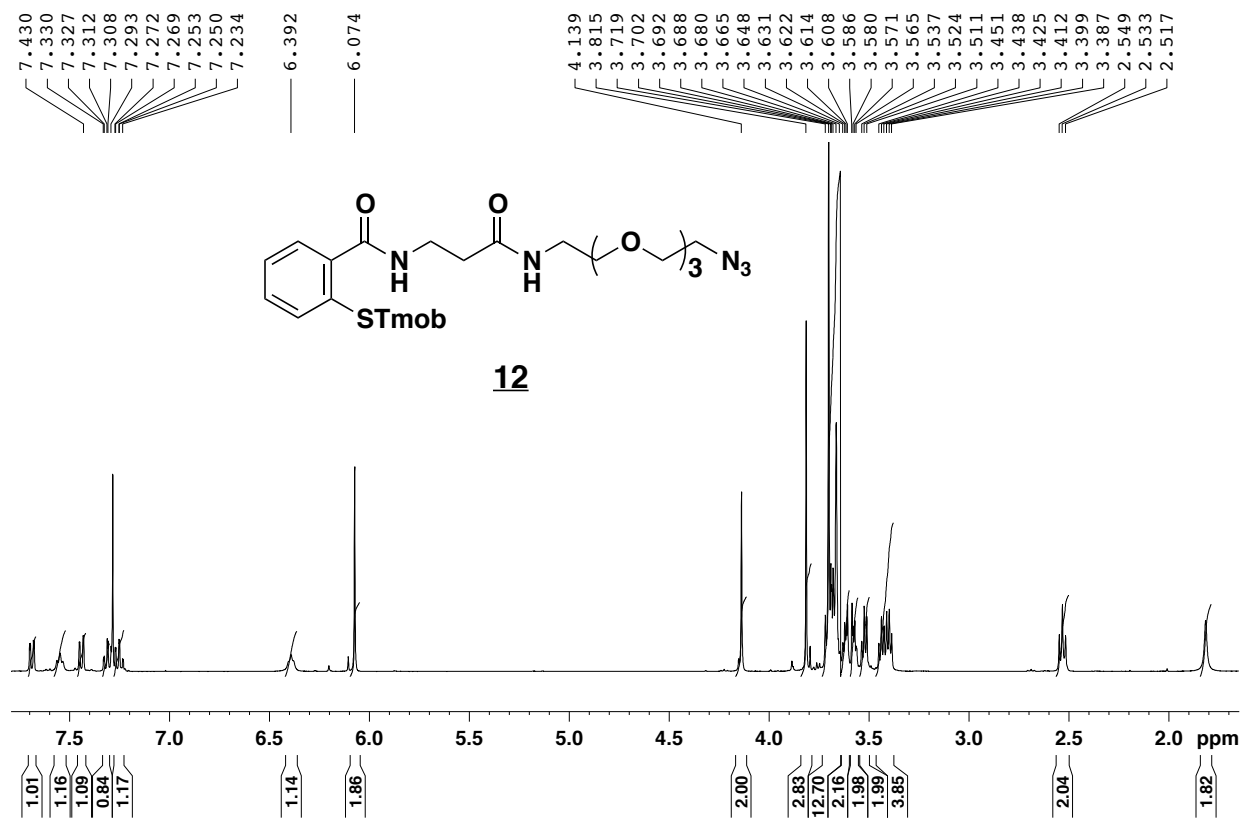


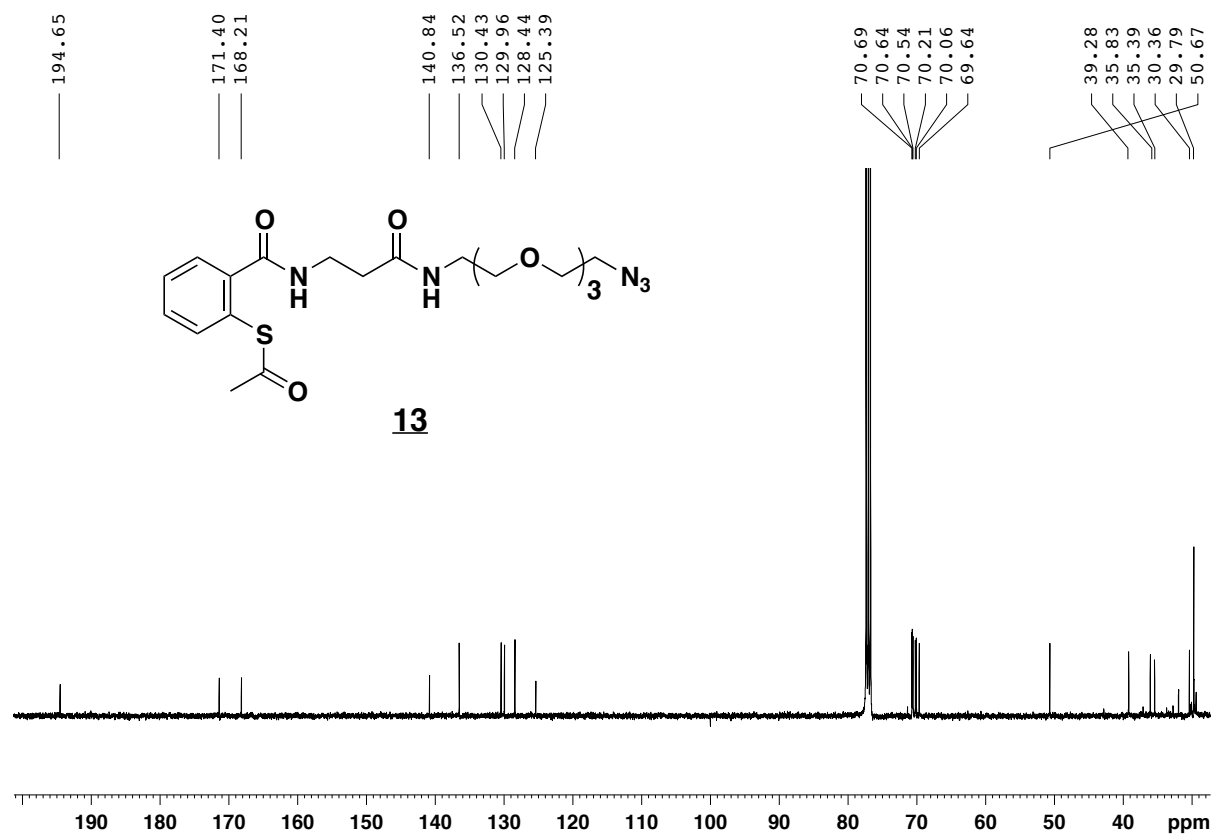
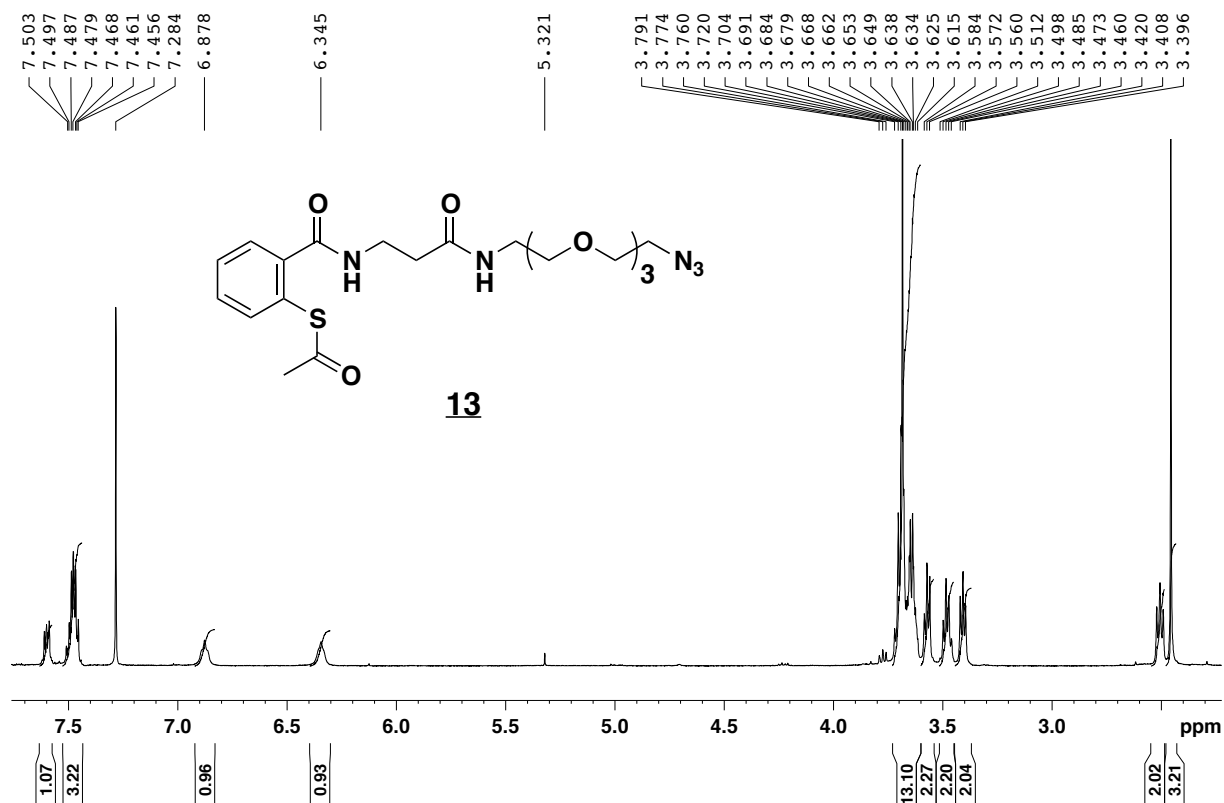
**2-((3-chloro-2-methylphenyl)amino)-N-((1-(1-(2-((2-((3',6'-dihydroxy-3-oxo-3H-spiro[isobenzofuran-1,9'-xanthen]-5-yl)amino)-2-oxoethyl)thio)phenyl)-1,5-dioxo-9,12,15-trioxa-2,6-diazaheptadecan-17-yl)-1H-1,2,3-triazol-4-yl)methyl)benzamide, FLYZ03**: To a solution of **AcYZ03** (30mg, 0.039 mmol) in MeOH was purged with  $\text{NH}_3$  to give the **YZ03** as free thiol. The MeOH and  $\text{NH}_3$  was removed in vacuo after which the residue was dissolved in 1 ml DMF followed by addition of 5-(iodoacetamido)fluorescein (20.2mg, 0.039 mmol) and  $\text{K}_2\text{CO}_3$  (11mg, 0.078 mmol). The reaction was stirred for 16 h before the solvent was removed in vacuo. The residue was purified by C18 flash chromatography to give **FLYZ03** as an orange oil.  $^1\text{H}$  NMR (400 MHz,  $\text{DMSO-d}_6$ )  $\delta$  10.49 (s, 1H), 9.82 (s, 1H), 9.35 (s, 1H), 8.48 (s, 1H), 8.33 (s, 1H), 8.13 (s, 1H), 8.06 (d,  $J = 2.3$  Hz, 1H), 7.97 (s, 1H), 7.77 – 7.70 (m, 2H), 7.59 (d,  $J = 8.0$  Hz, 2H), 7.48 – 7.36 (m, 2H), 7.34 – 7.10 (m, 5H), 6.99 (d,  $J = 8.3$  Hz, 1H), 6.92 (d,  $J = 8.2$  Hz, 1H), 6.80 (t,  $J = 7.5$  Hz, 1H), 6.57 (d,  $J = 9.2$  Hz, 2H), 5.97 (dd,  $J = 9.3, 2.2$  Hz, 2H), 5.89 (d,  $J = 2.1$  Hz, 2H), 4.54 – 4.45 (m, 4H), 3.88 (s, 2H), 3.77 (t,  $J = 5.2$  Hz, 2H), 3.59 – 3.40 (m, 8H), 3.26 – 3.12 (m, 6H), 2.39 (t,  $J = 7.4$  Hz, 2H), 2.26 (s, 3H).  $^{13}\text{C}$  NMR (101 MHz,  $\text{CDCl}_3$ )  $\delta$  170.66, 169.28, 168.99, 167.93, 167.61, 159.93, 152.34, 147.64, 145.42, 145.19, 141.81, 140.89, 139.61, 134.81, 133.07, 132.66, 131.24, 129.66, 129.54, 129.32, 129.21, 128.09, 127.97, 127.89, 127.51, 126.73, 125.10, 123.80, 119.61, 119.35, 118.45, 118.03, 115.15, 113.87, 113.03, 110.04, 102.64, 83.57, 70.11, 70.07, 69.96, 69.95, 69.51, 69.22, 49.74, 38.93, 36.32, 35.49, 35.13, 31.17, 15.01. HR-ESI MS  $m/e$  calcd for  $[\text{C}_{57}\text{H}_{55}\text{ClN}_8\text{O}_{12}\text{S} + \text{Na}]$  1133.3235, found 1133.3246.

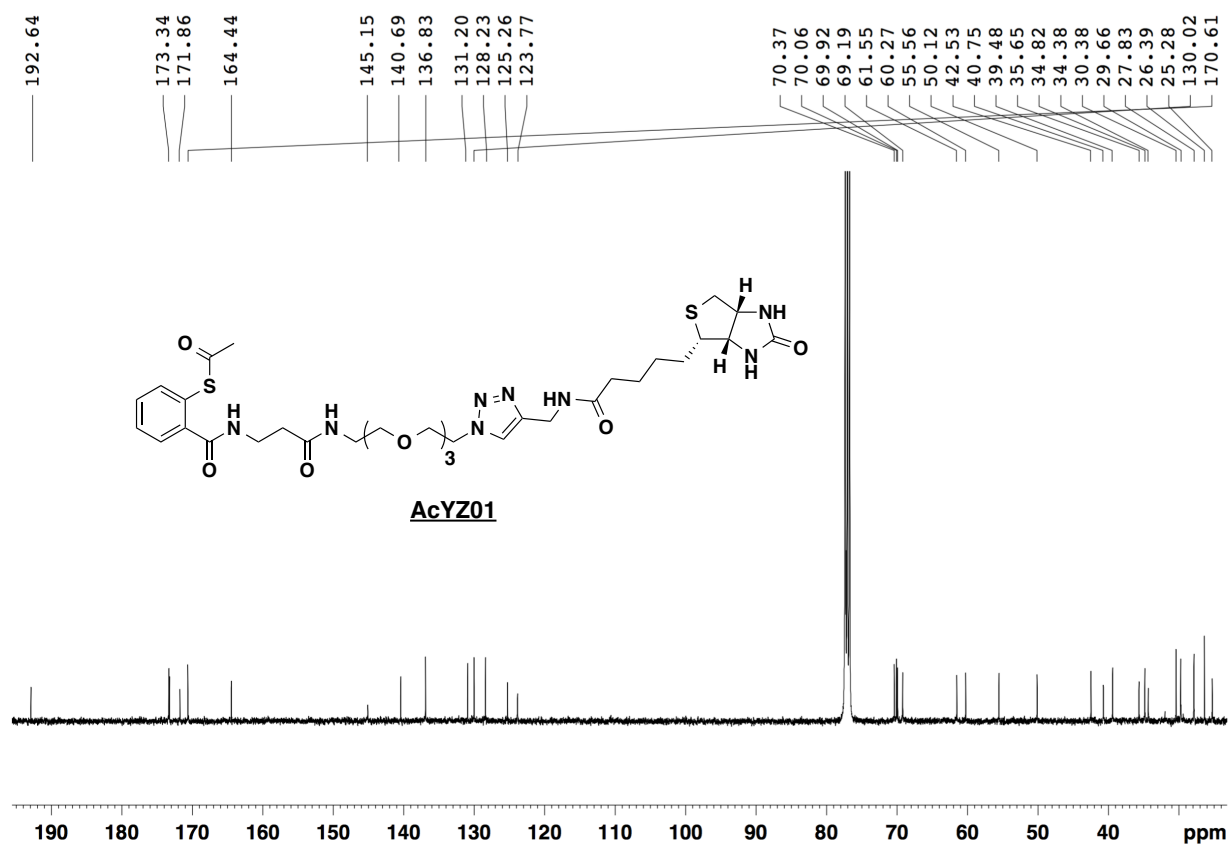
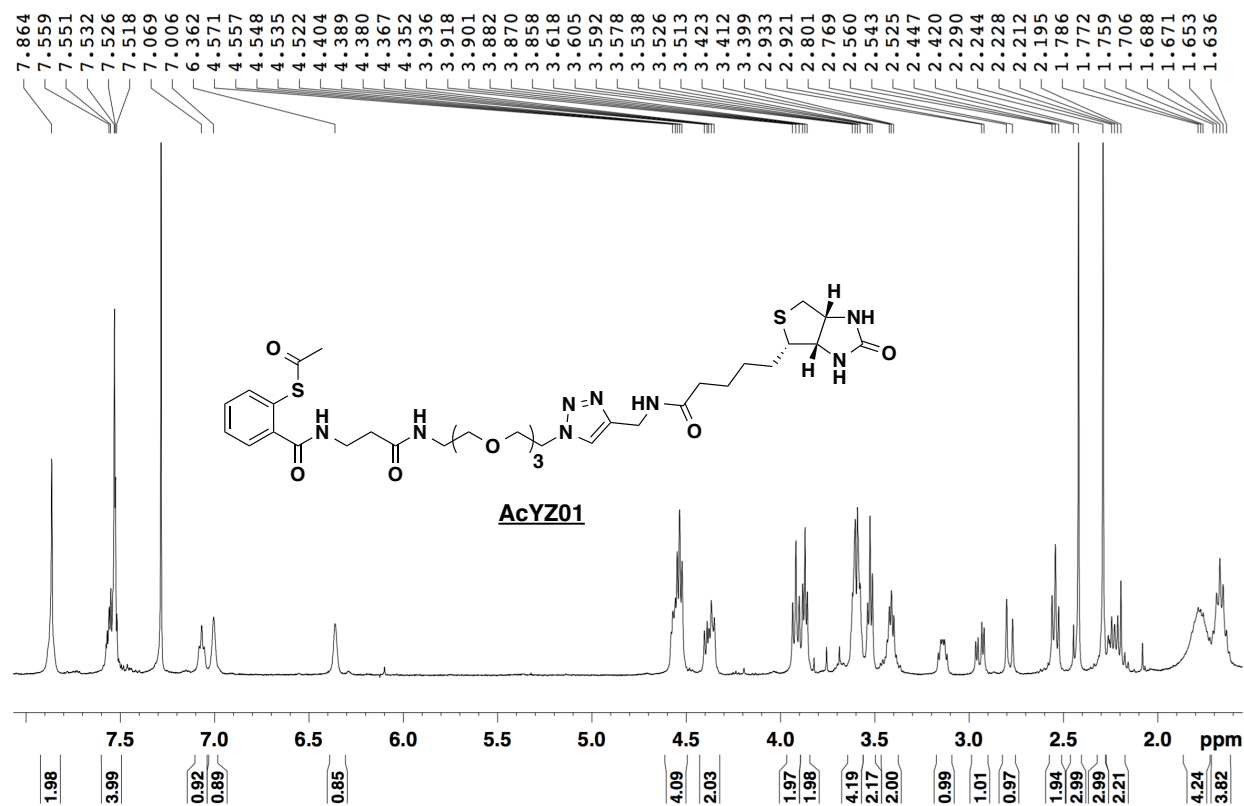


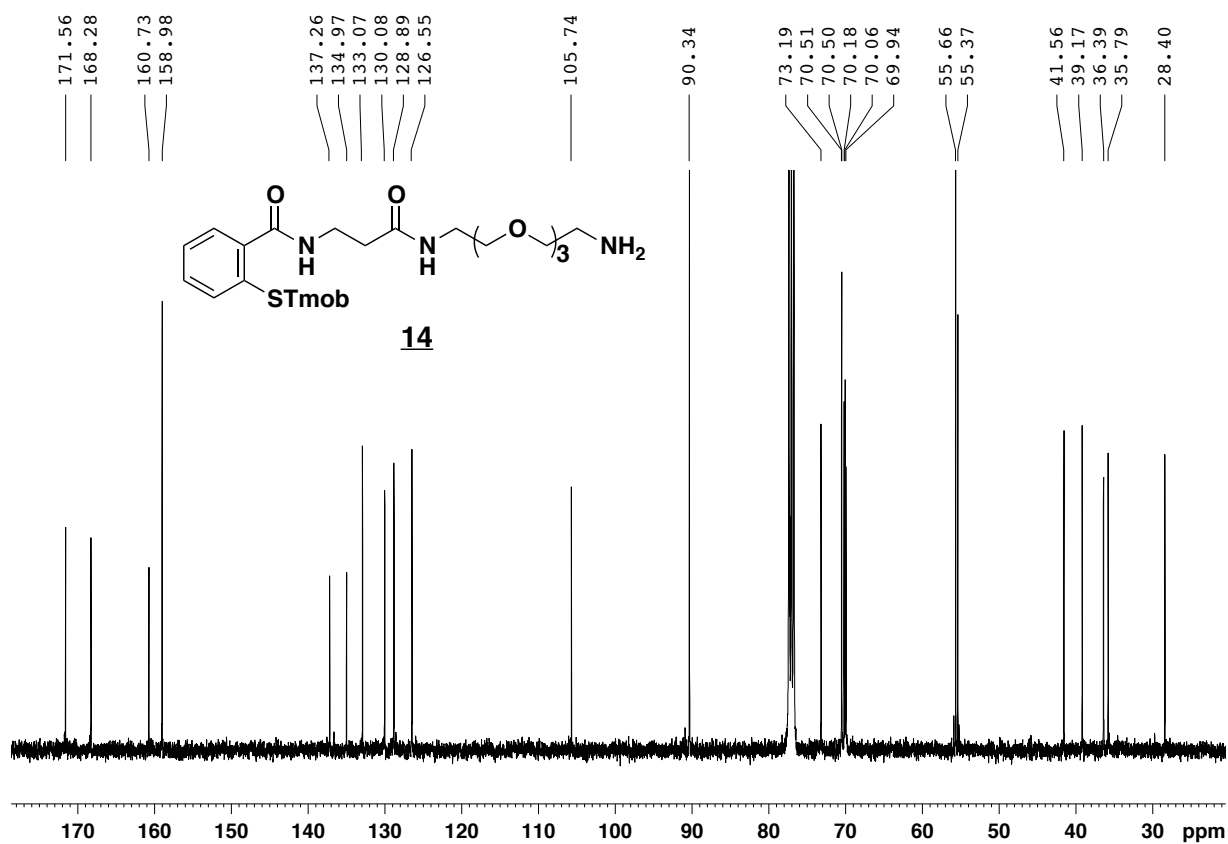
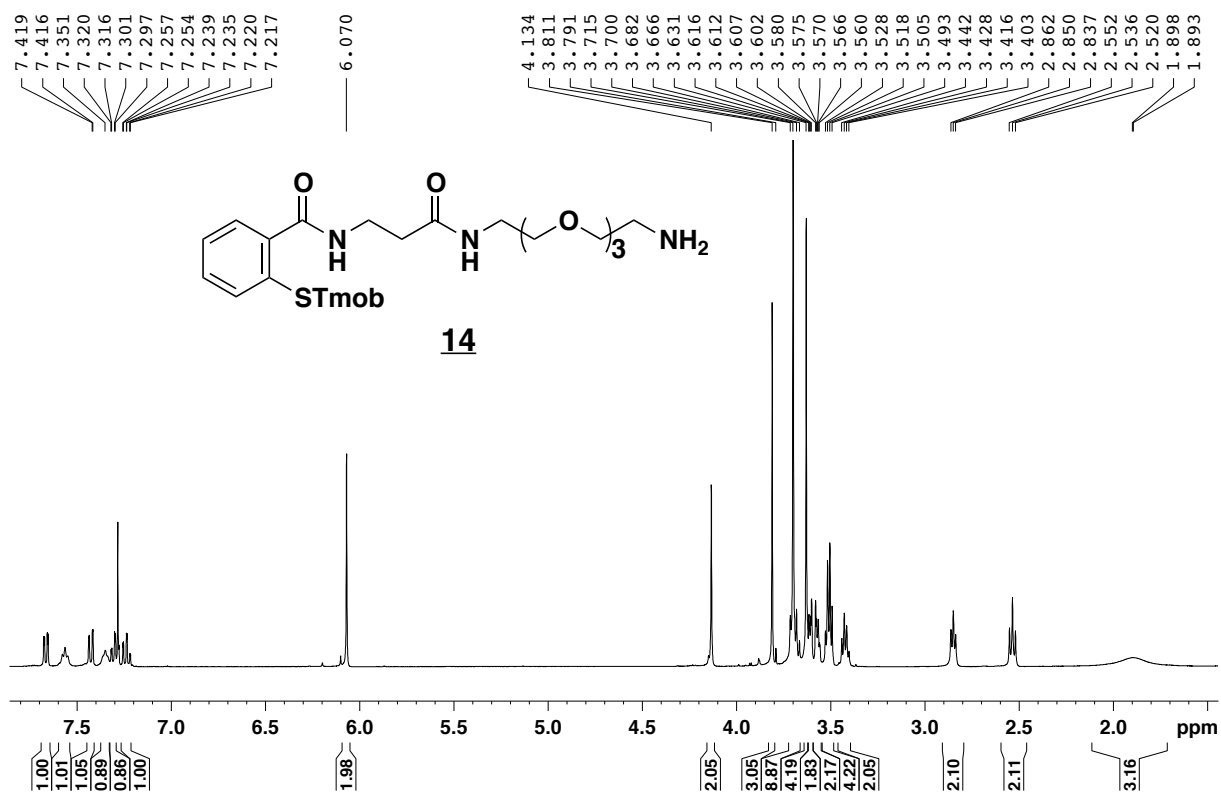


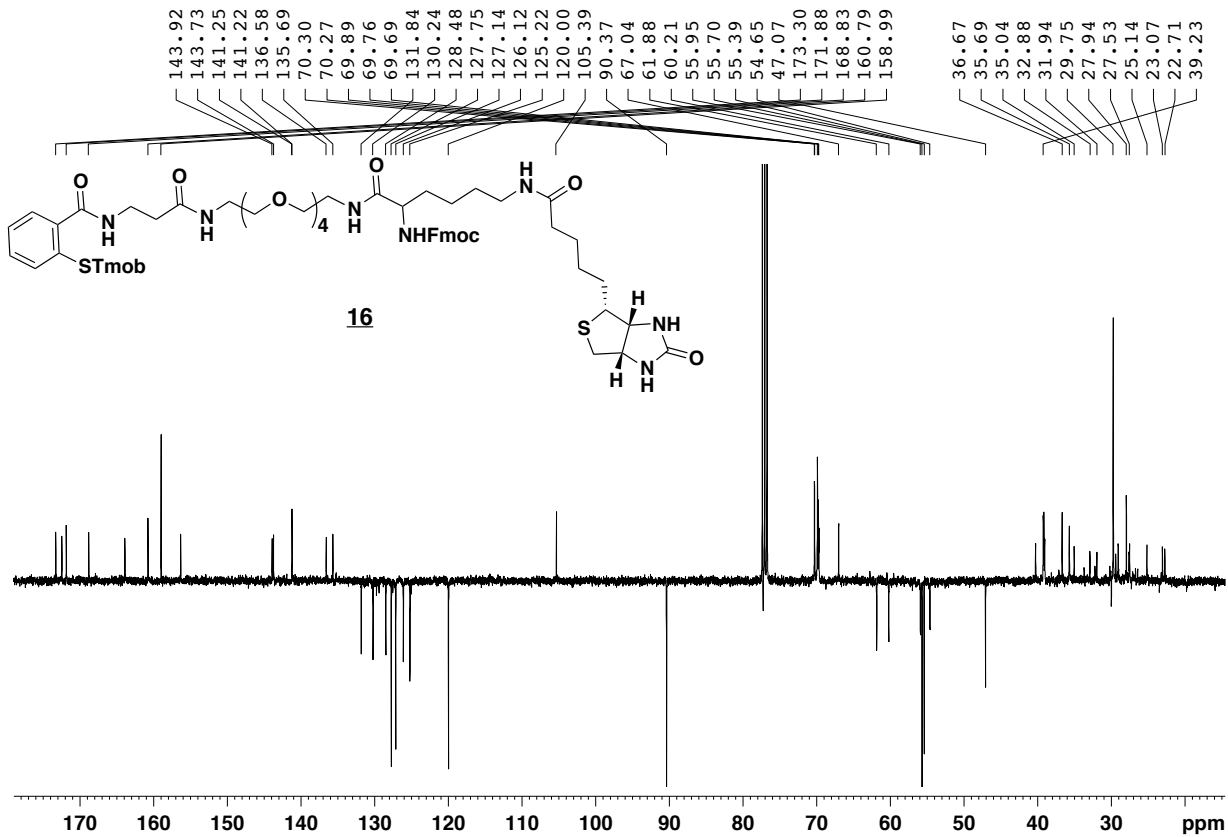
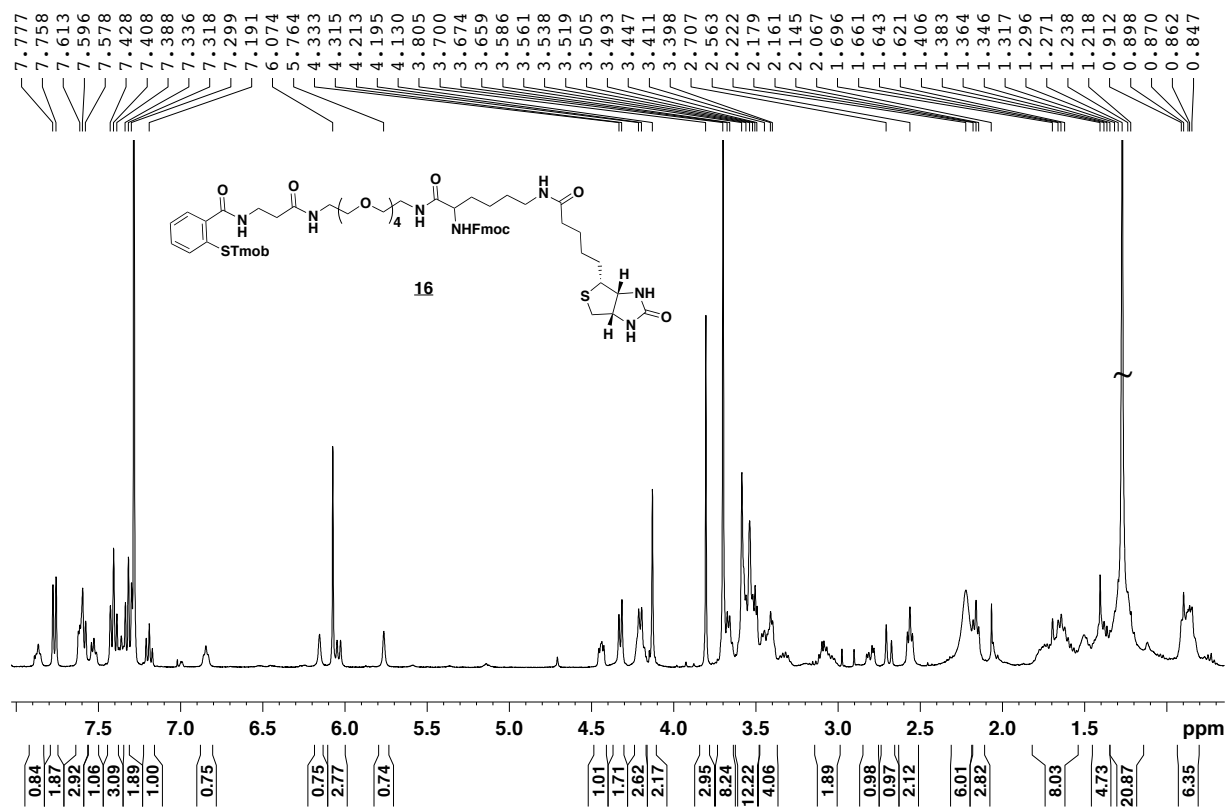


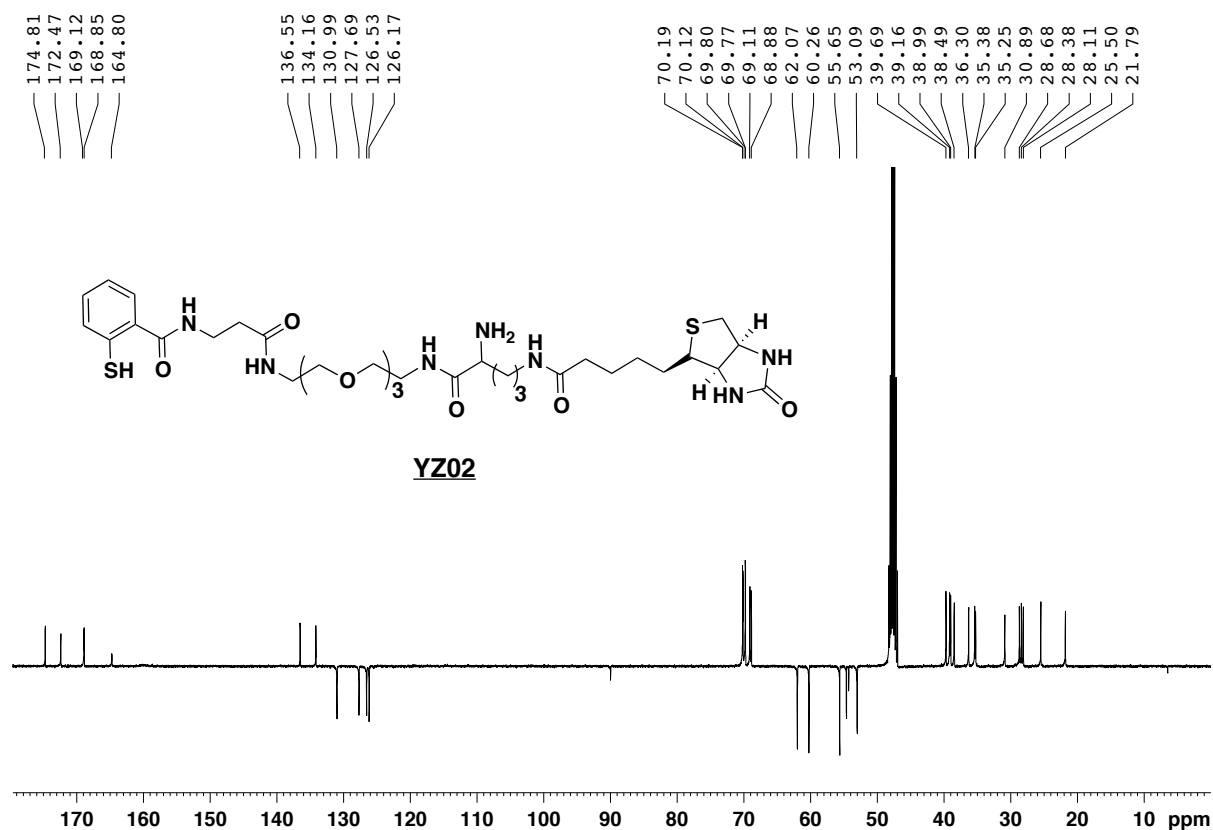
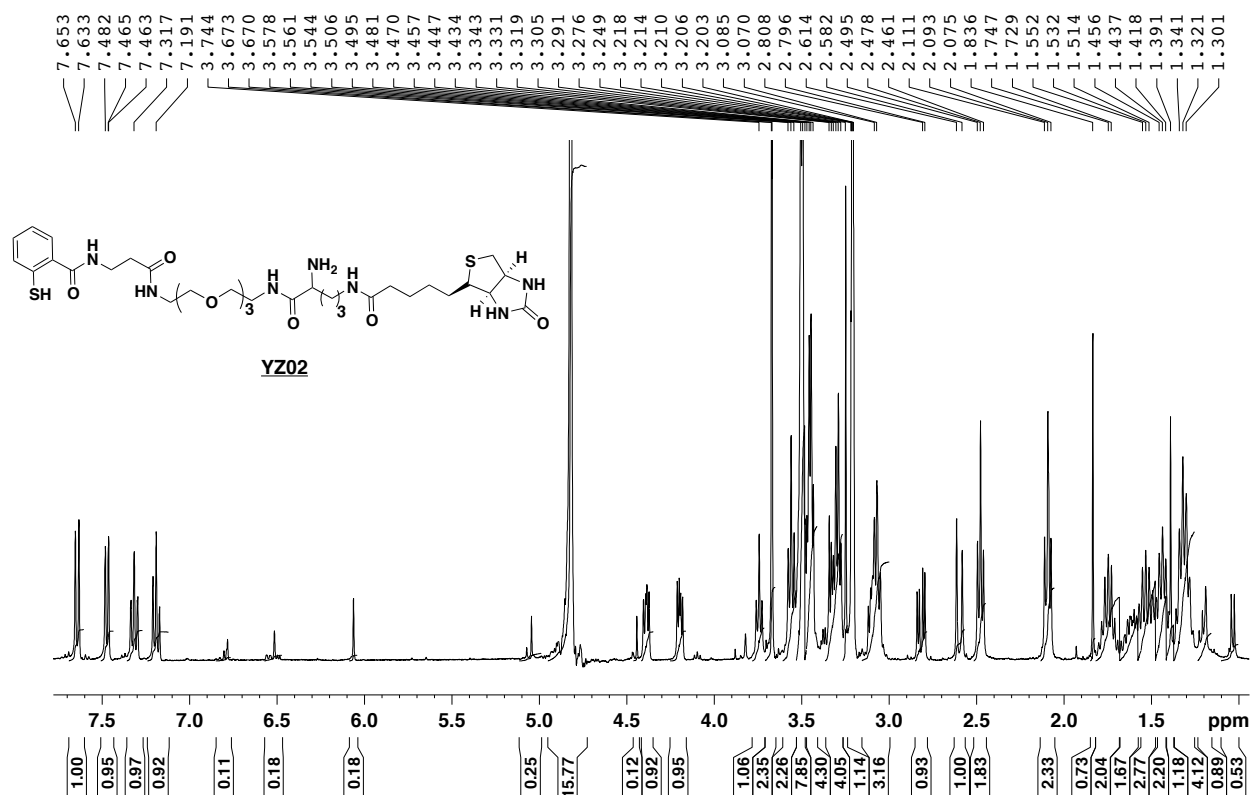




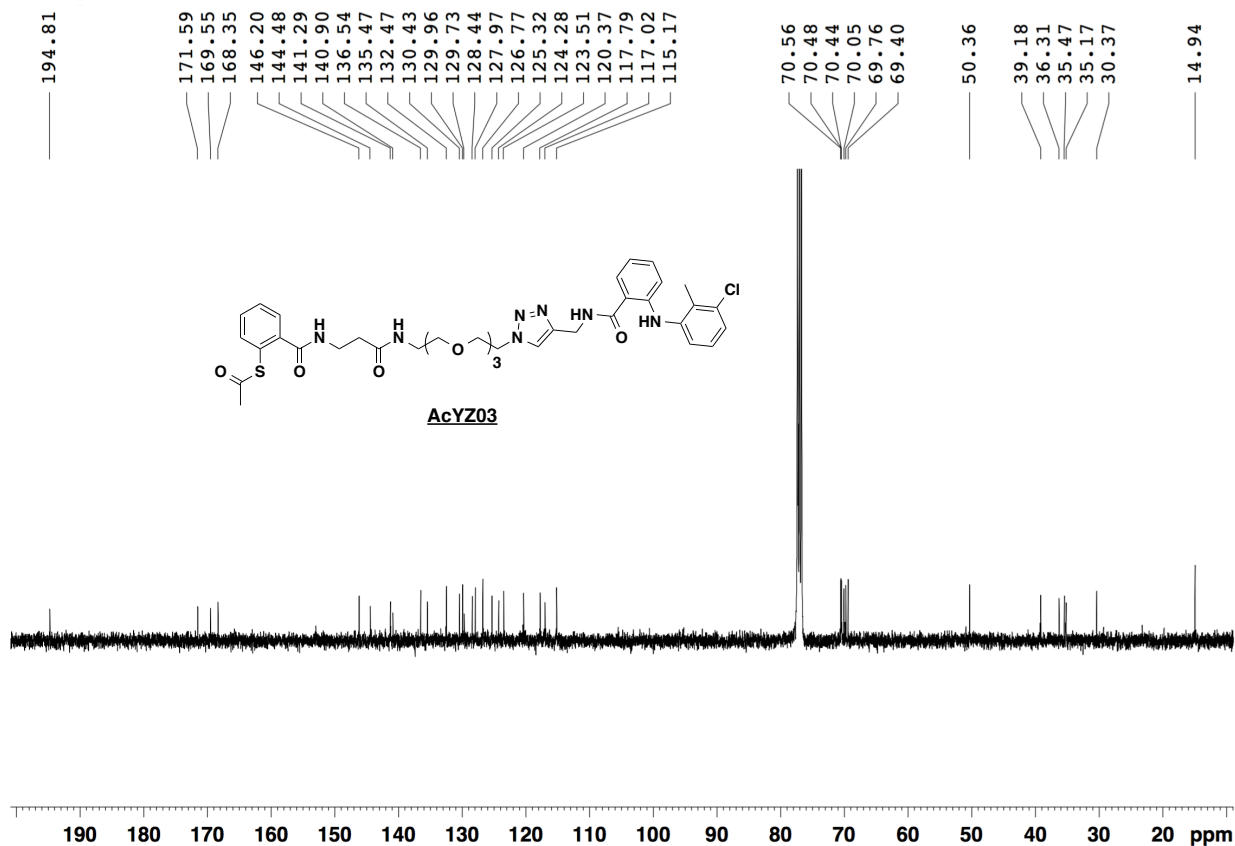
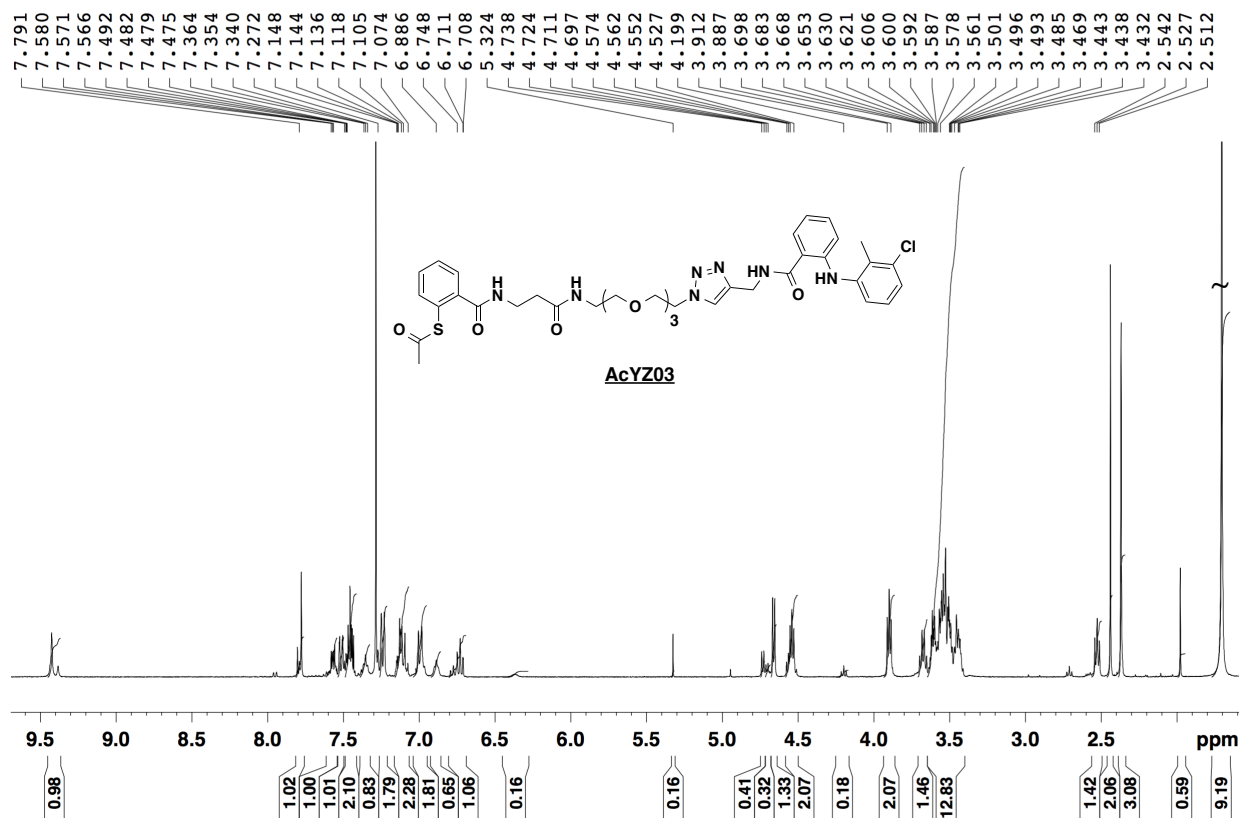


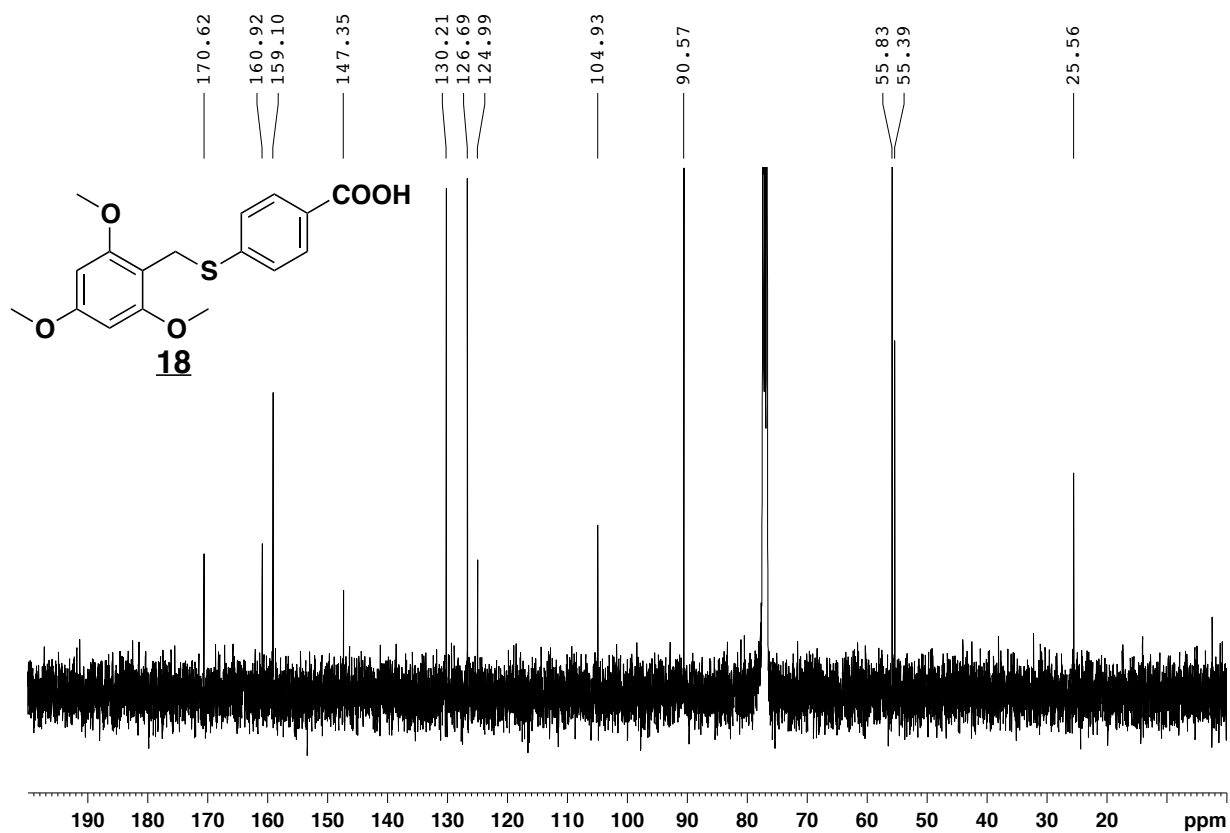
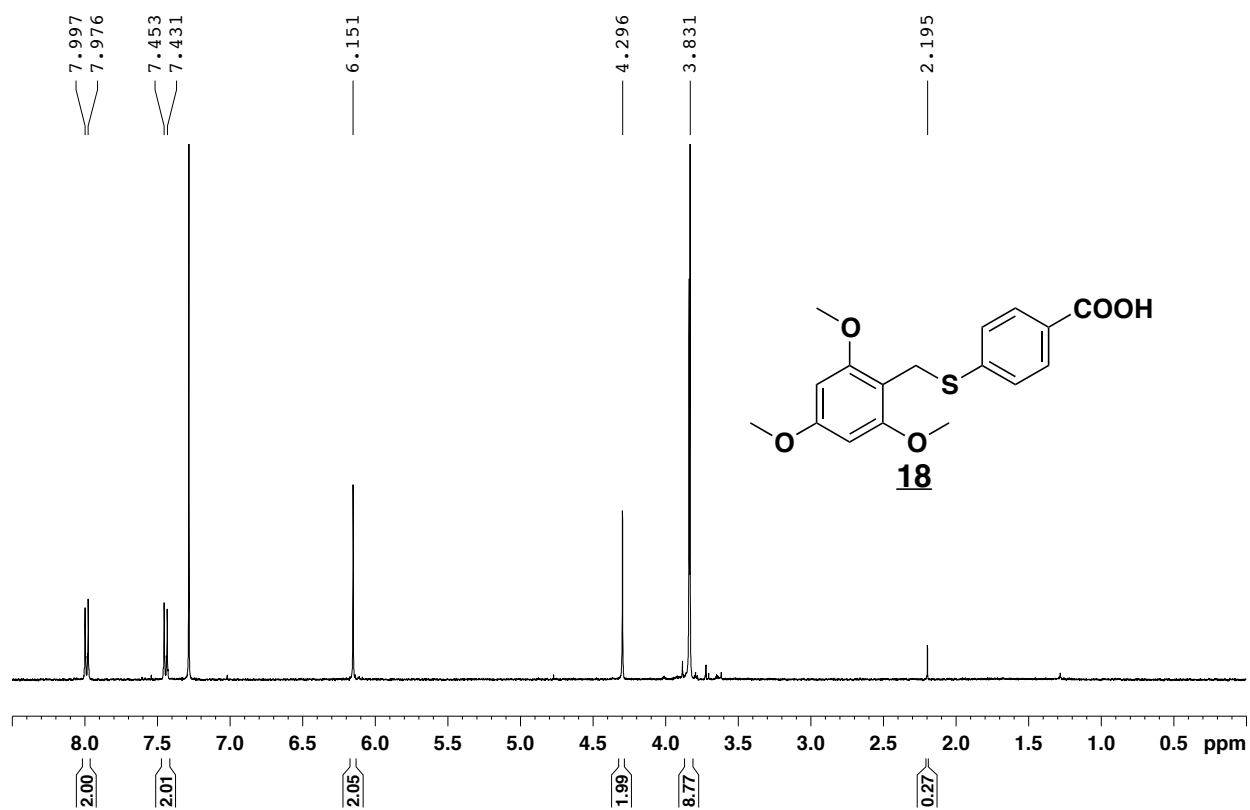


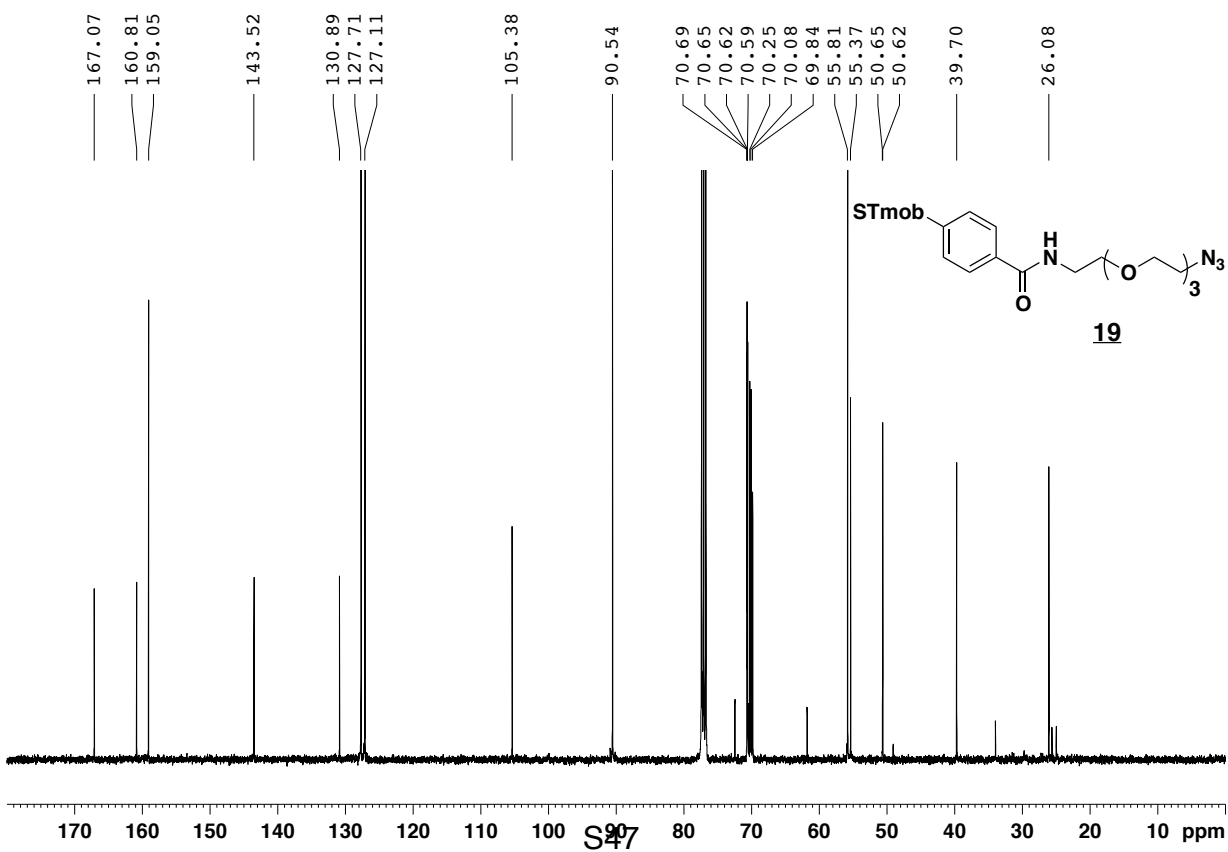
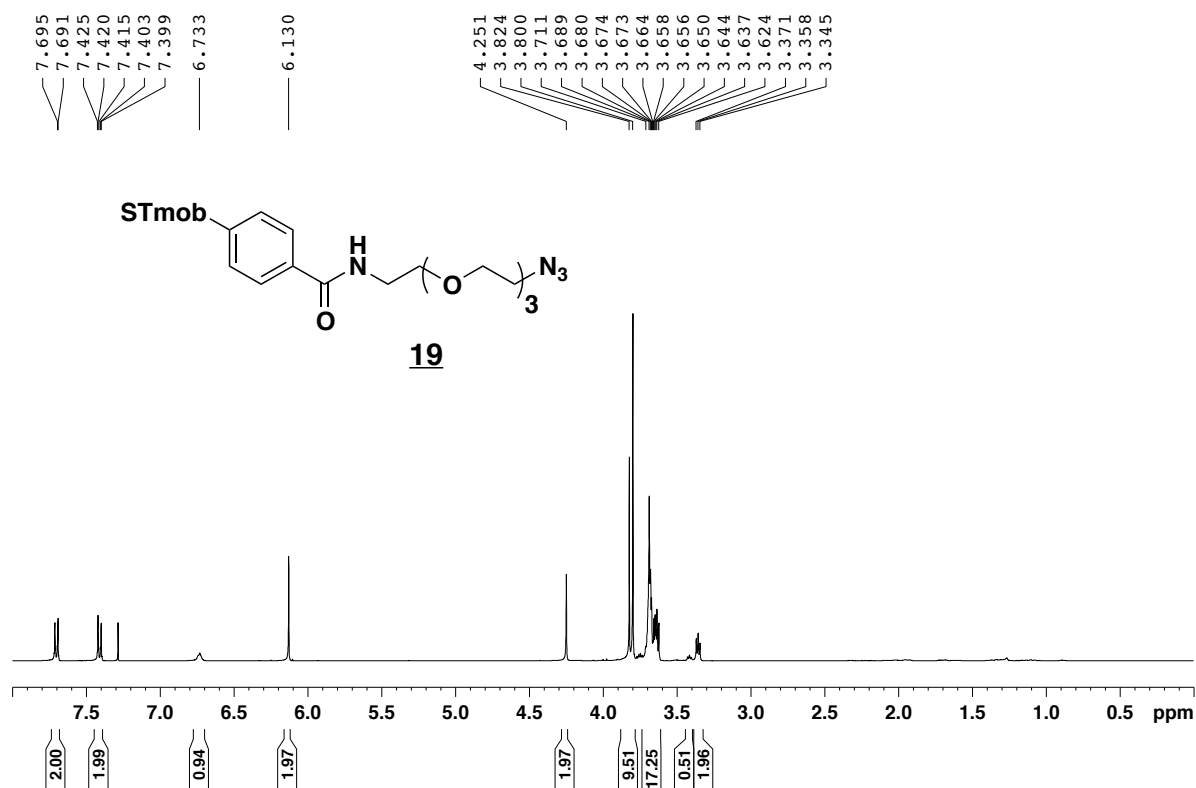


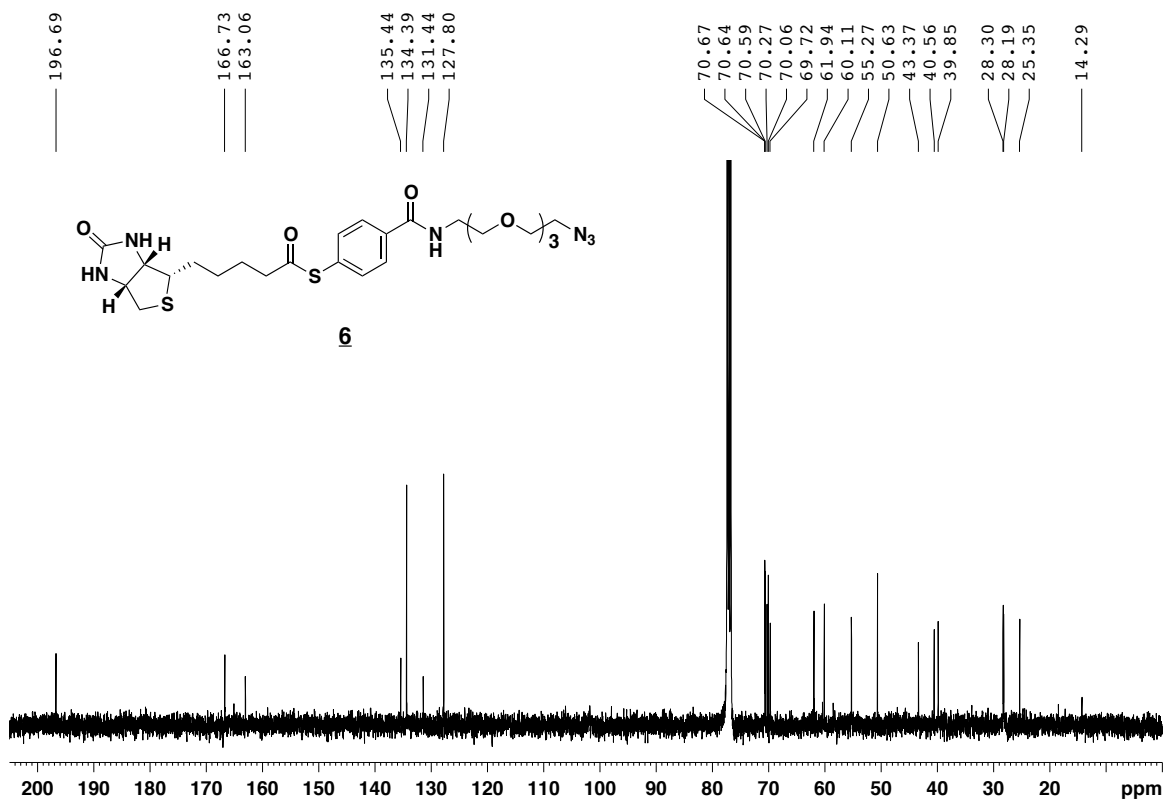
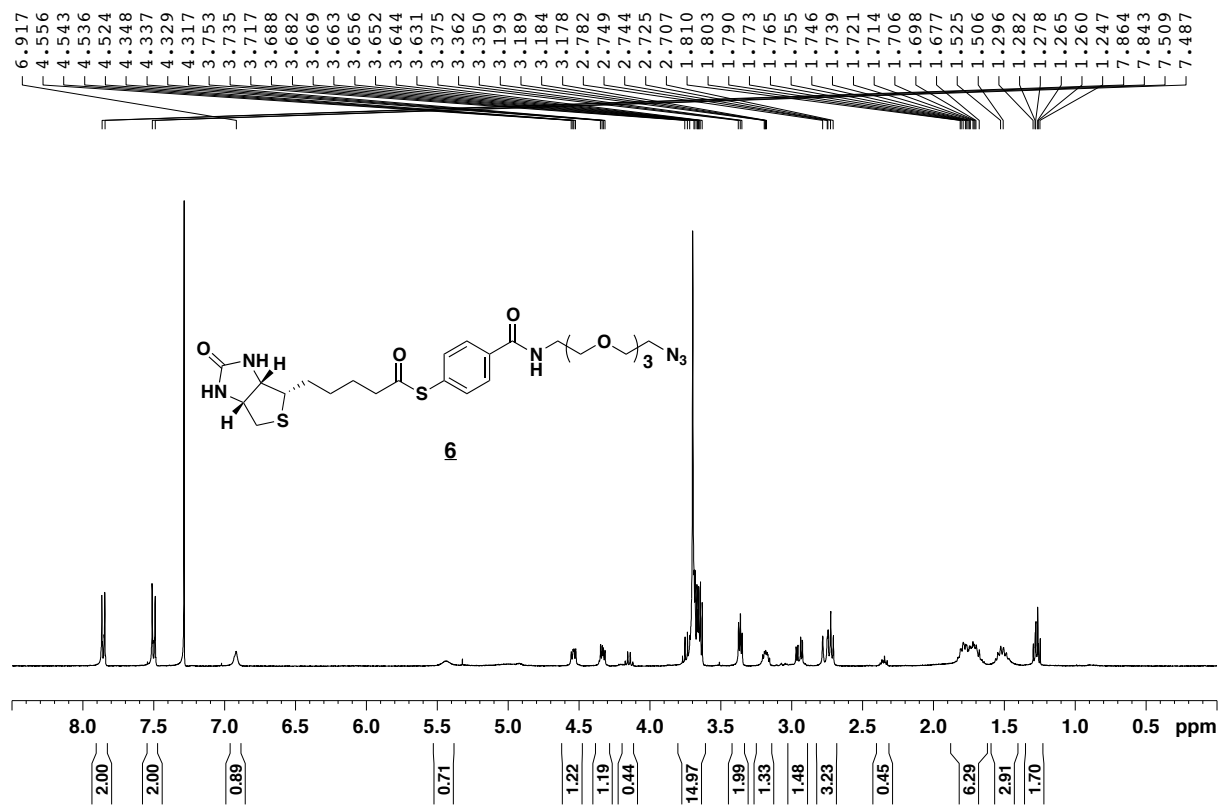


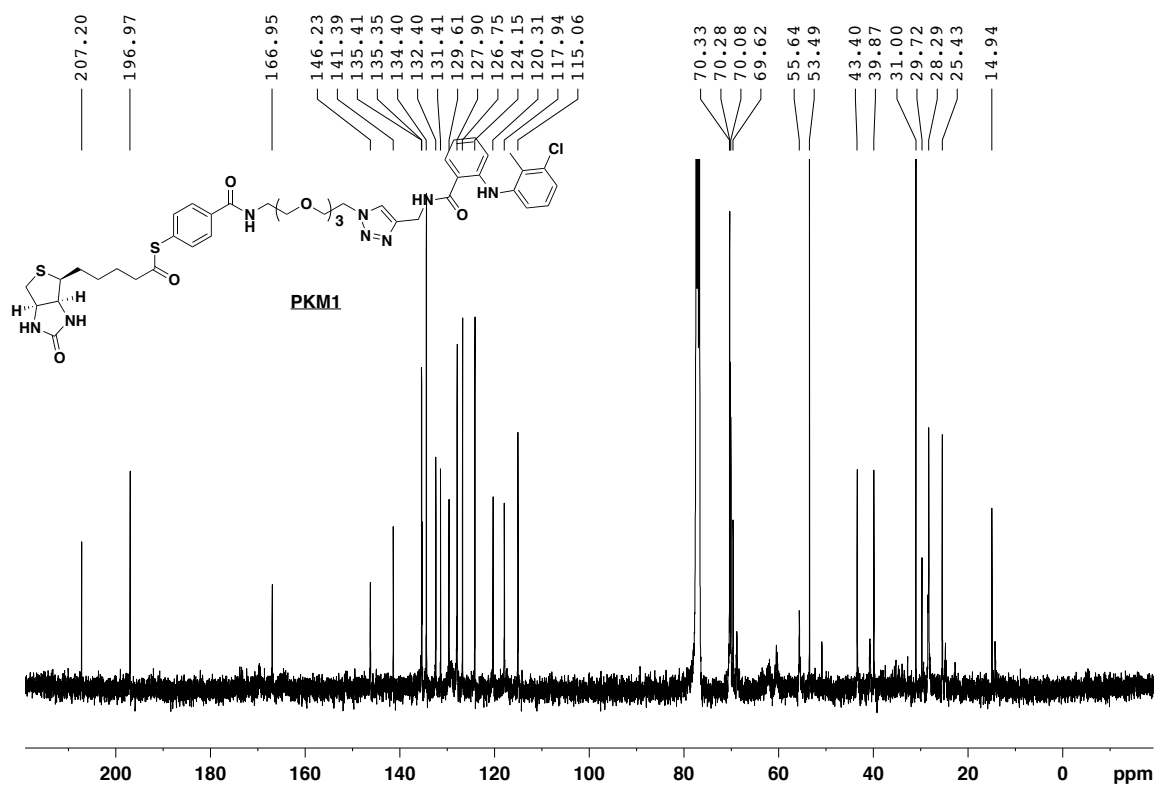
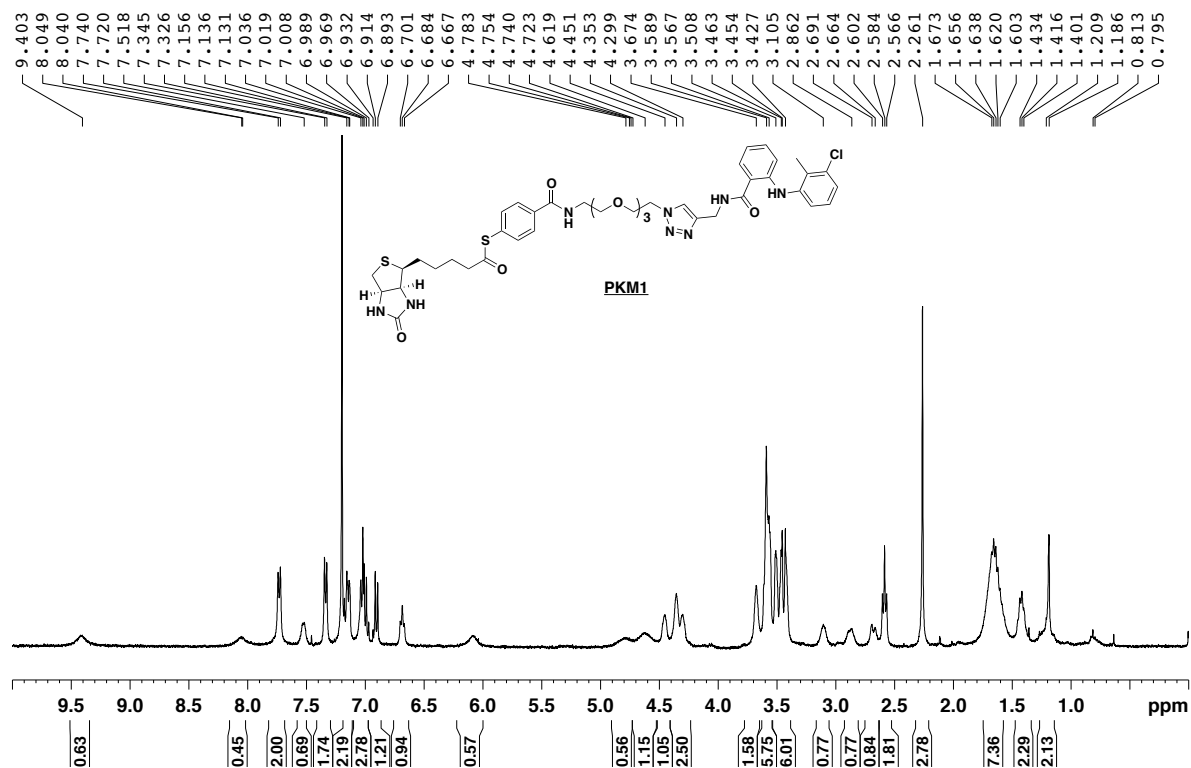


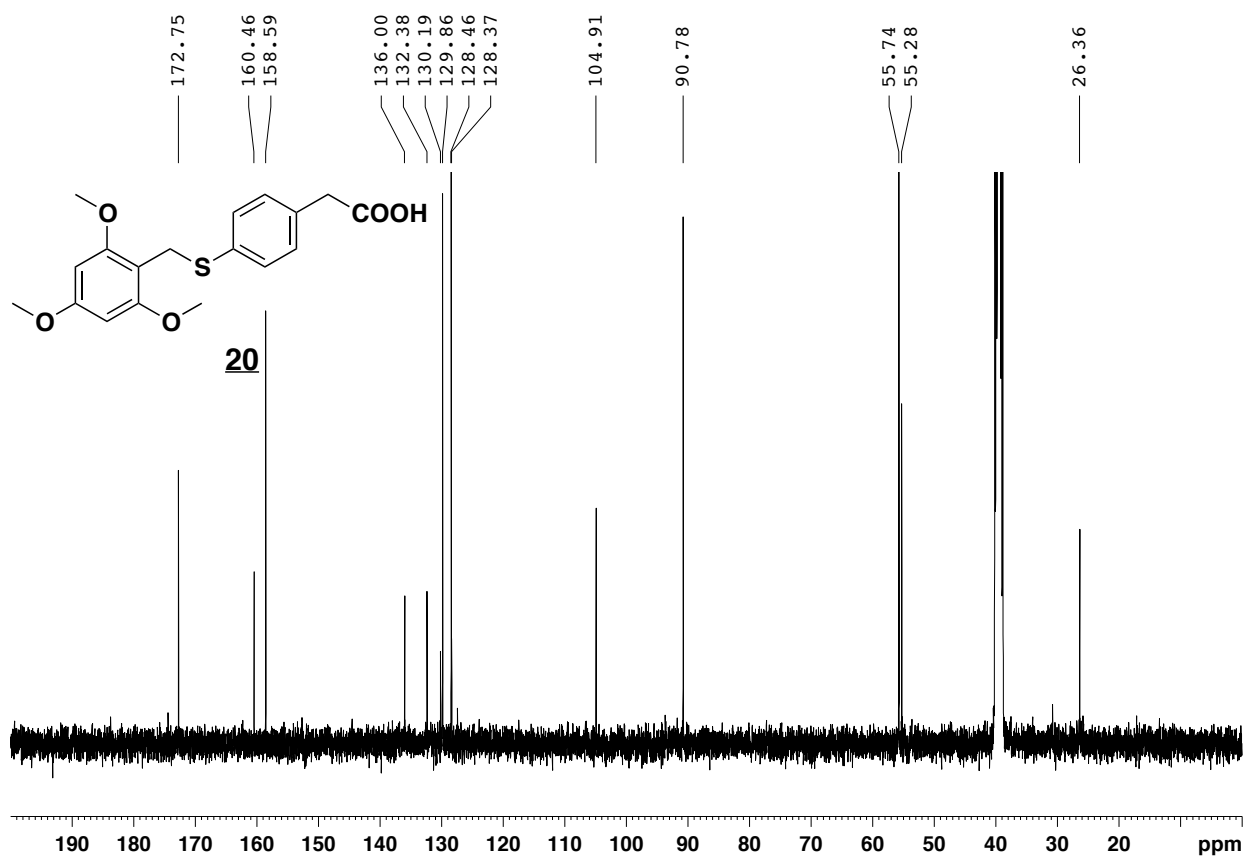
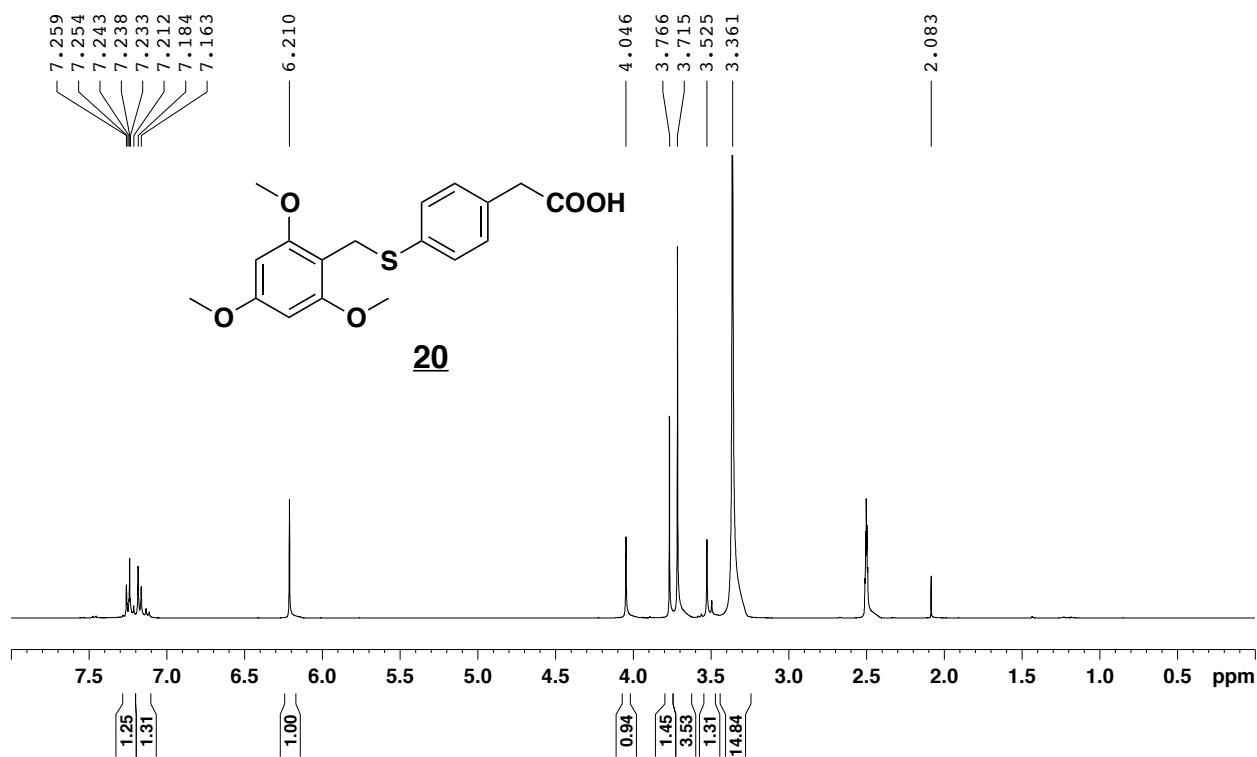


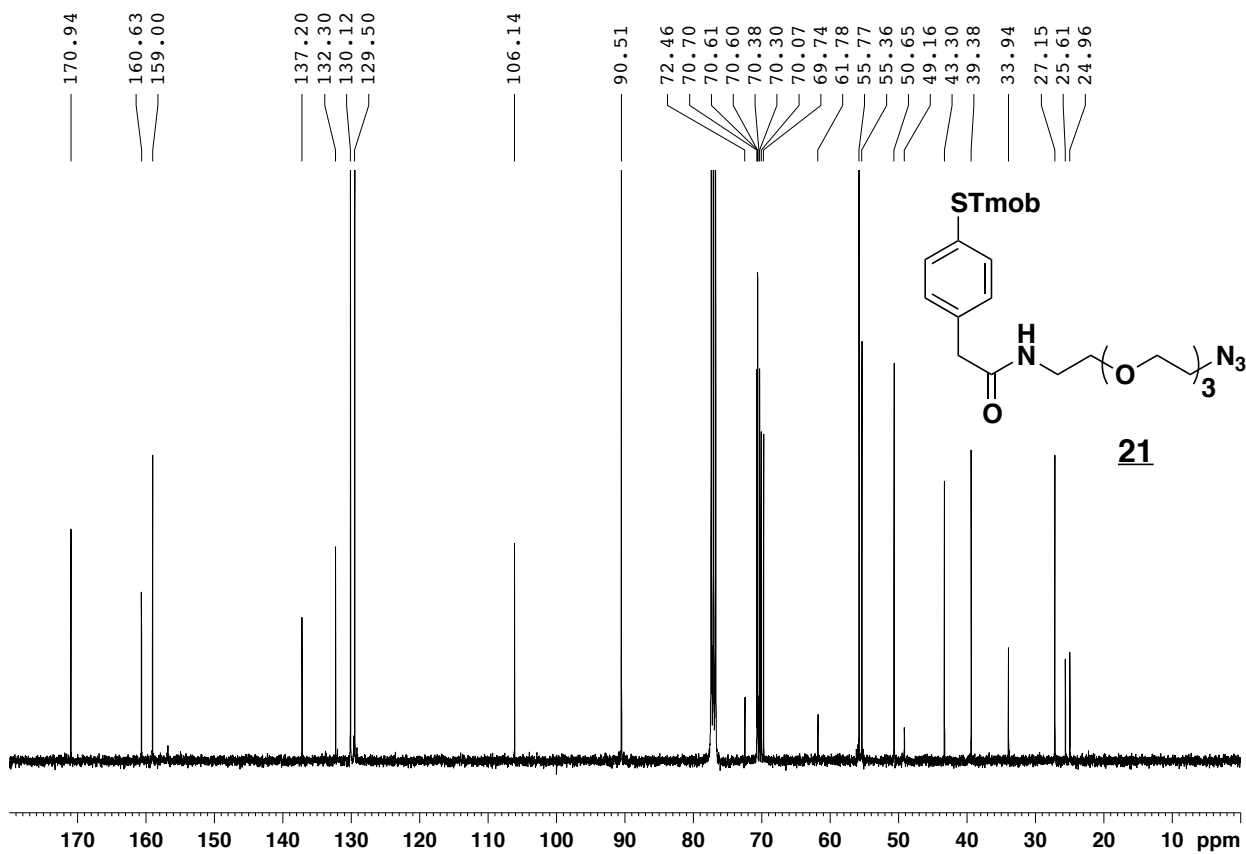
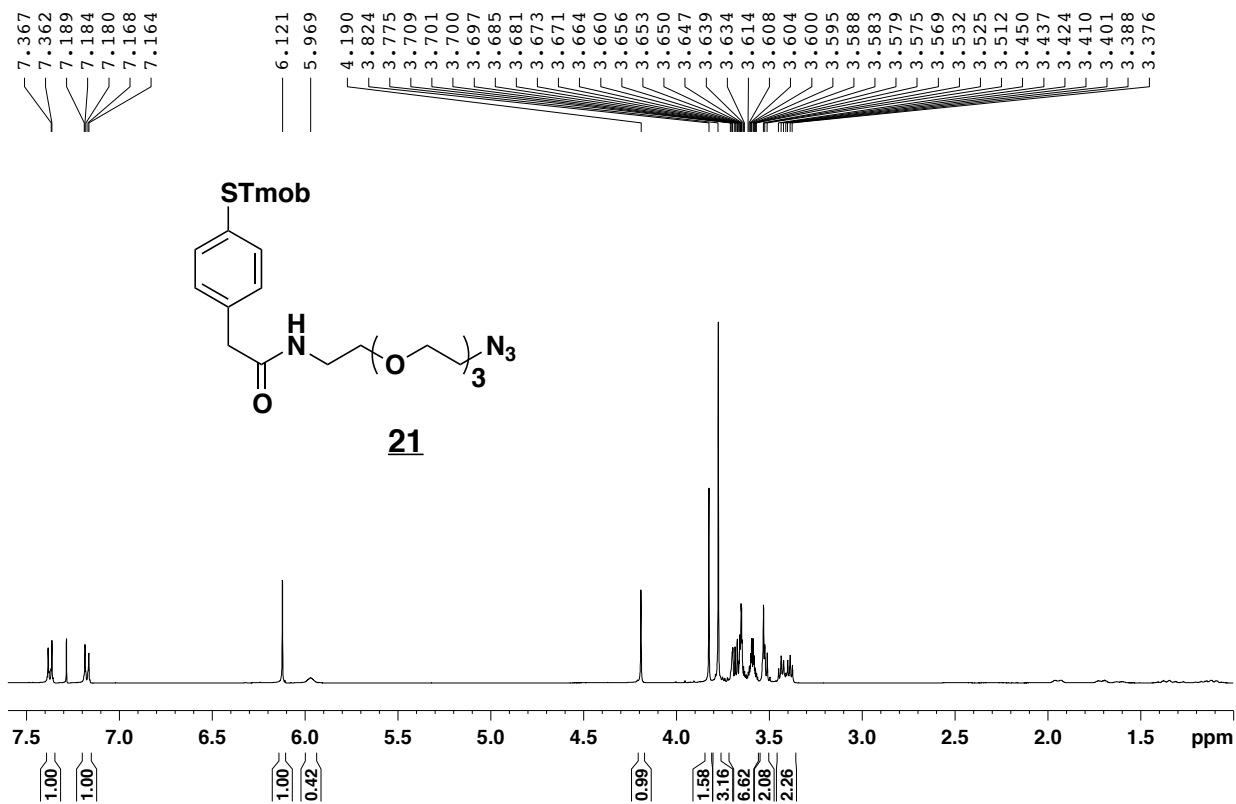


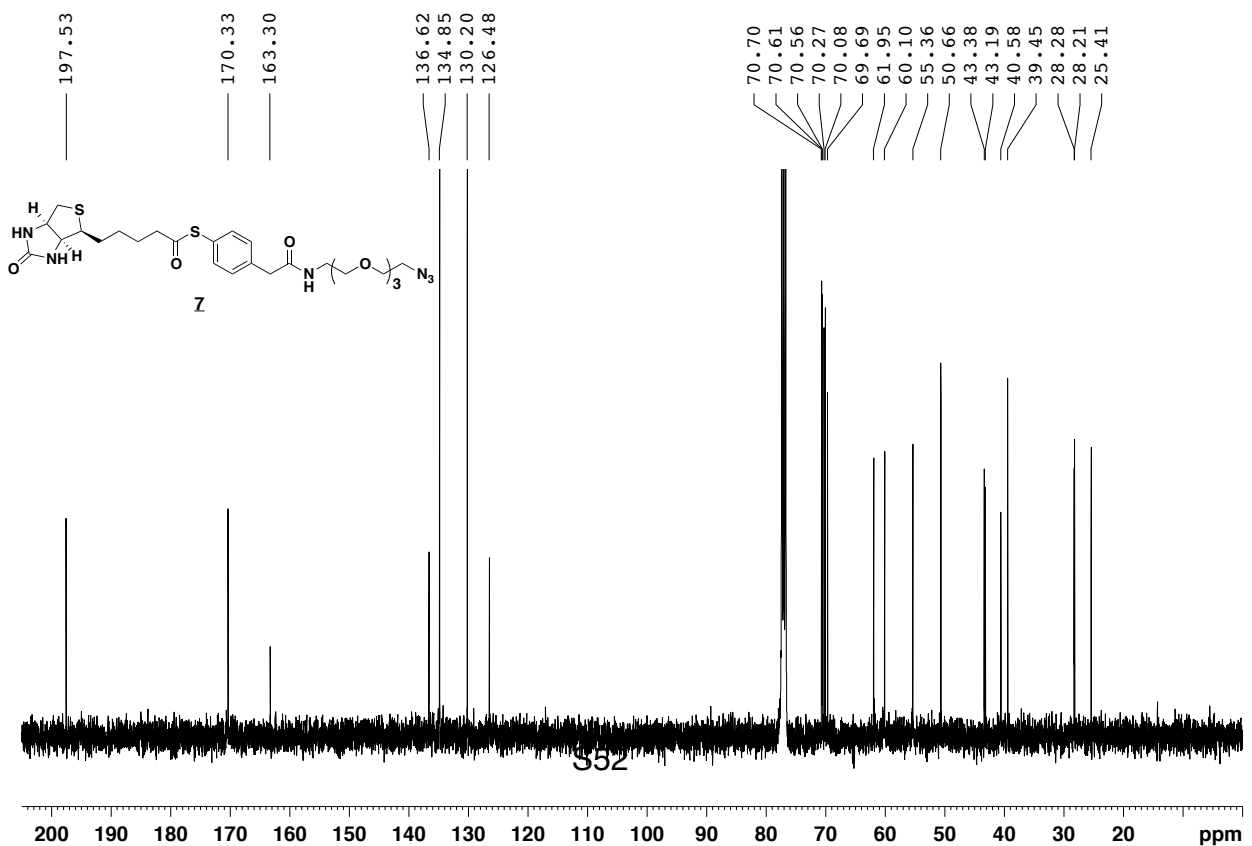
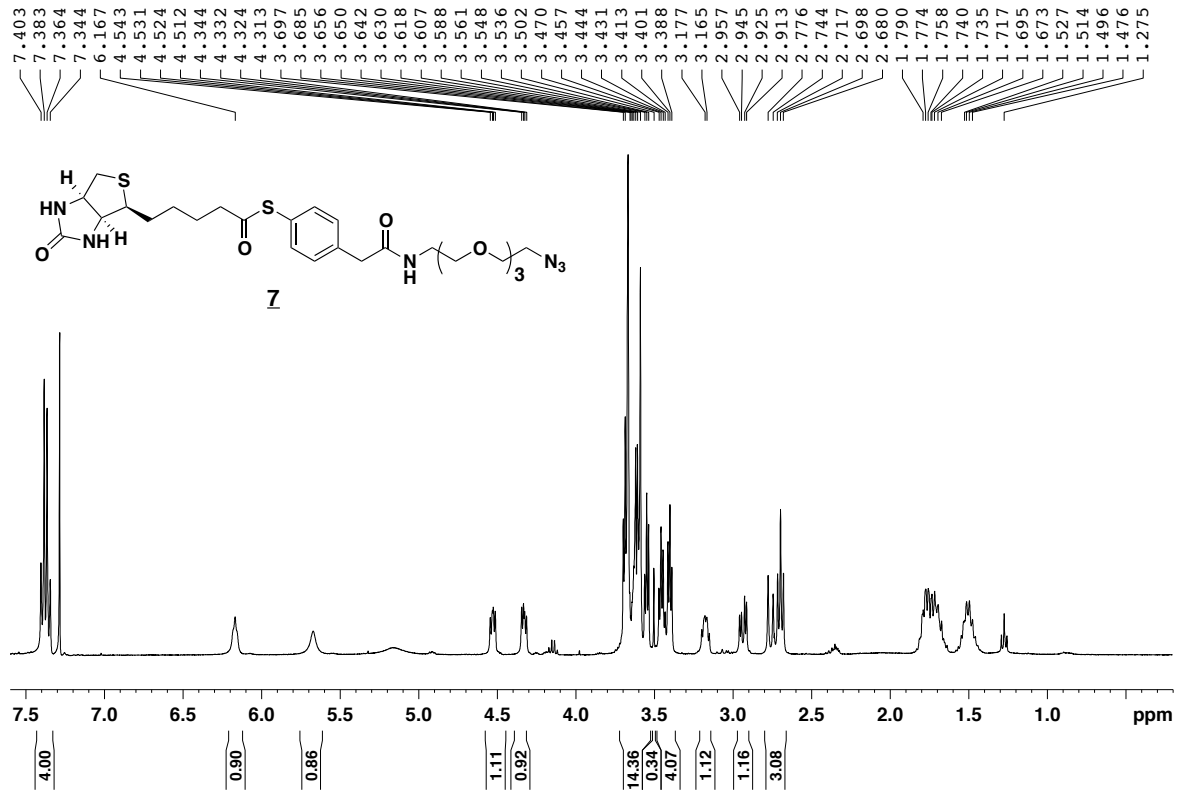




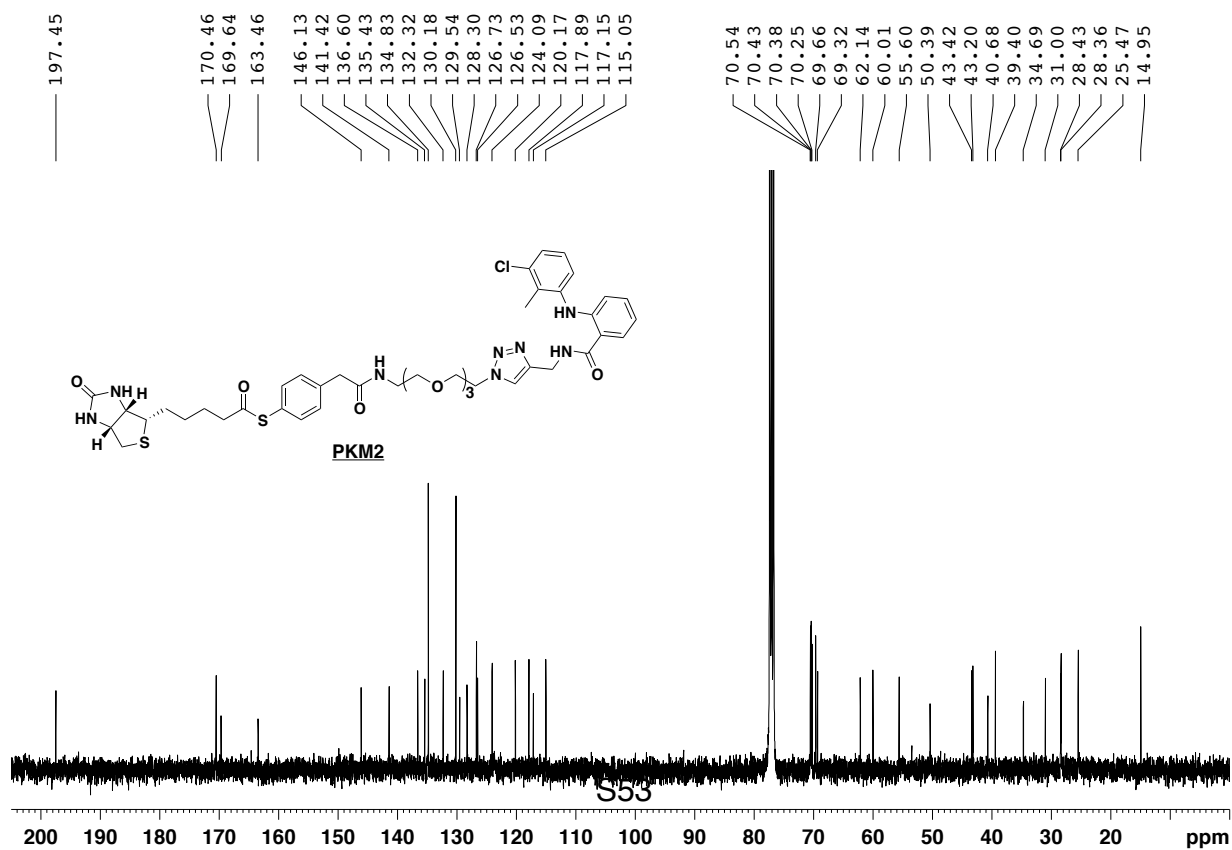
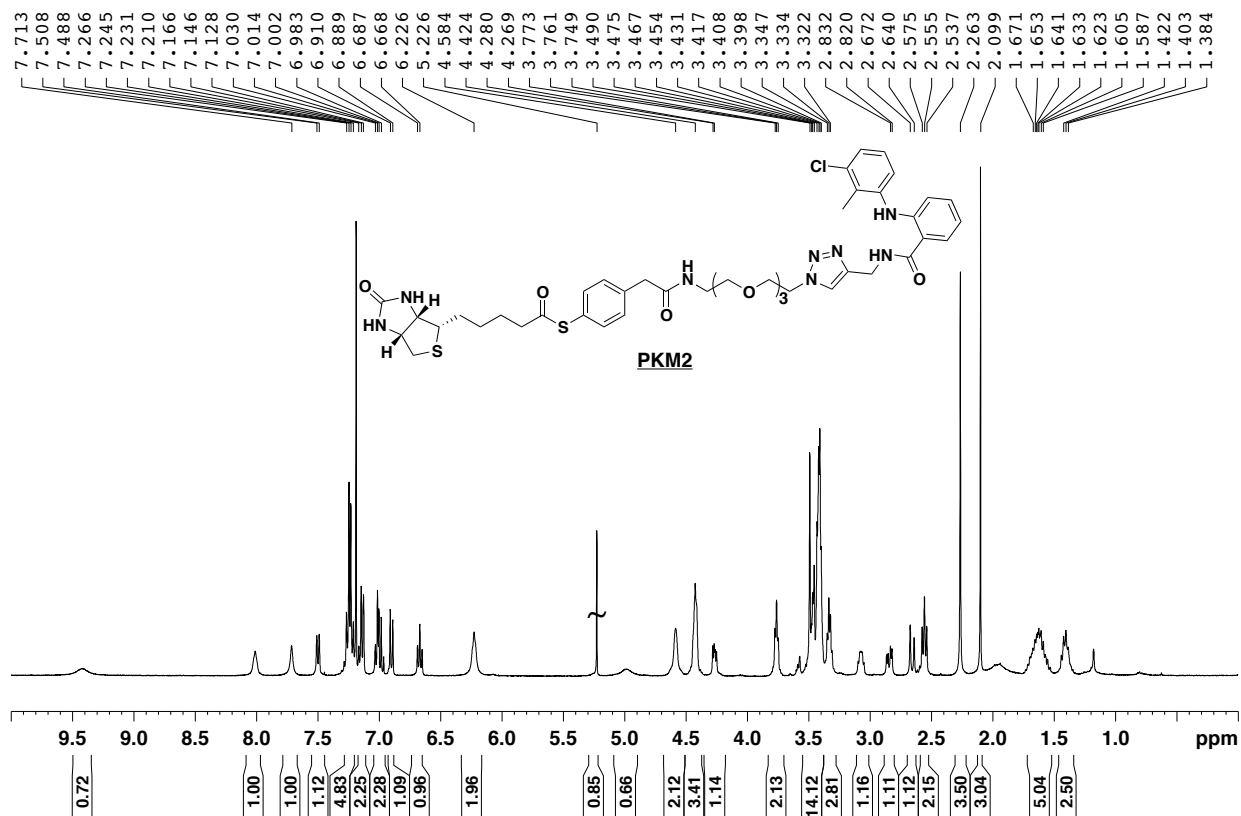


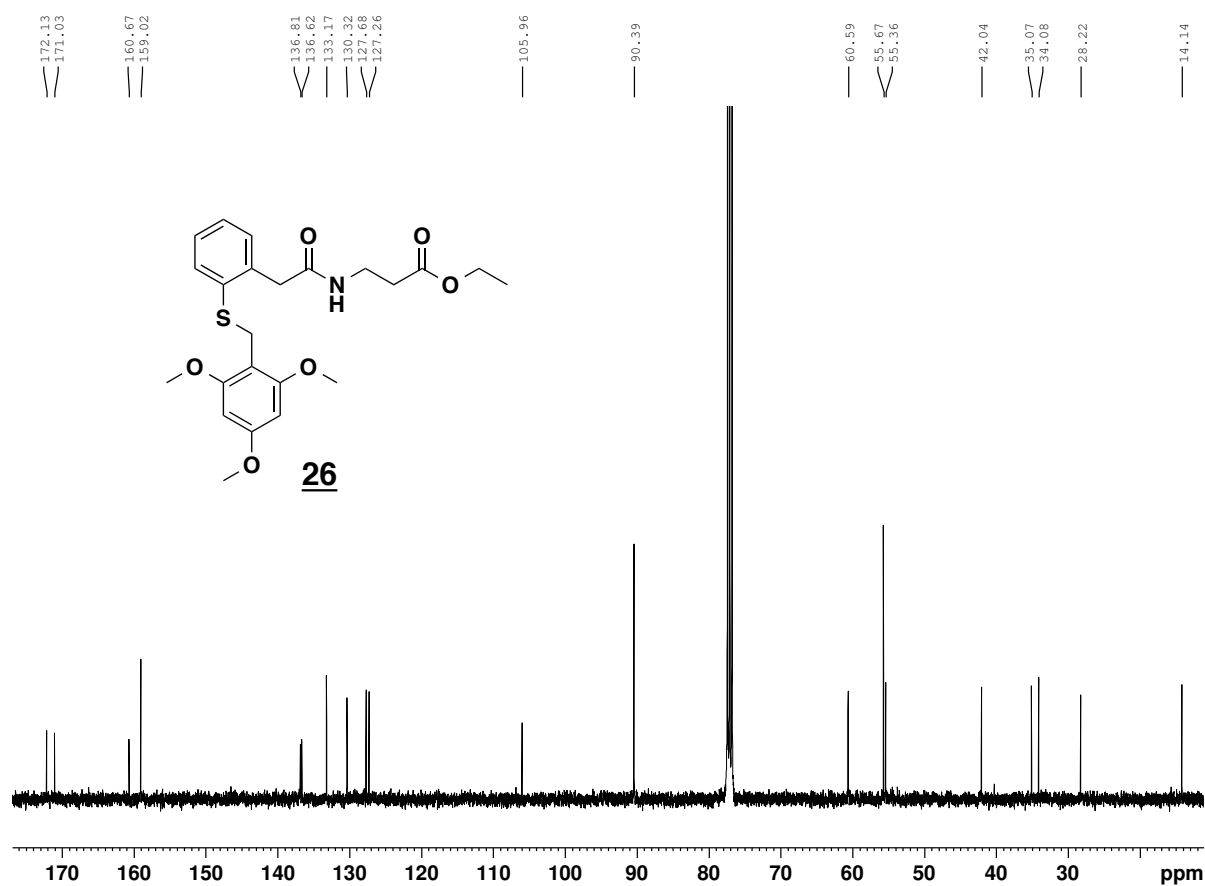
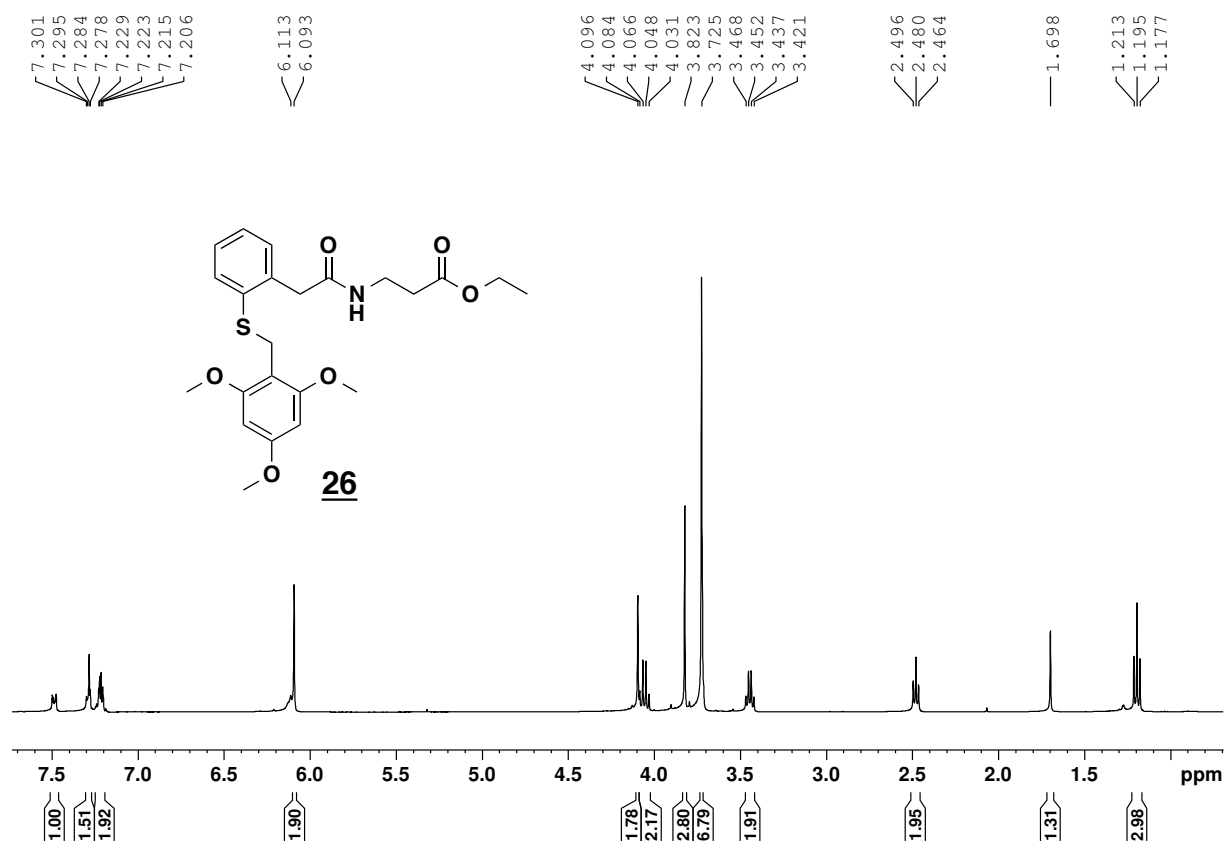


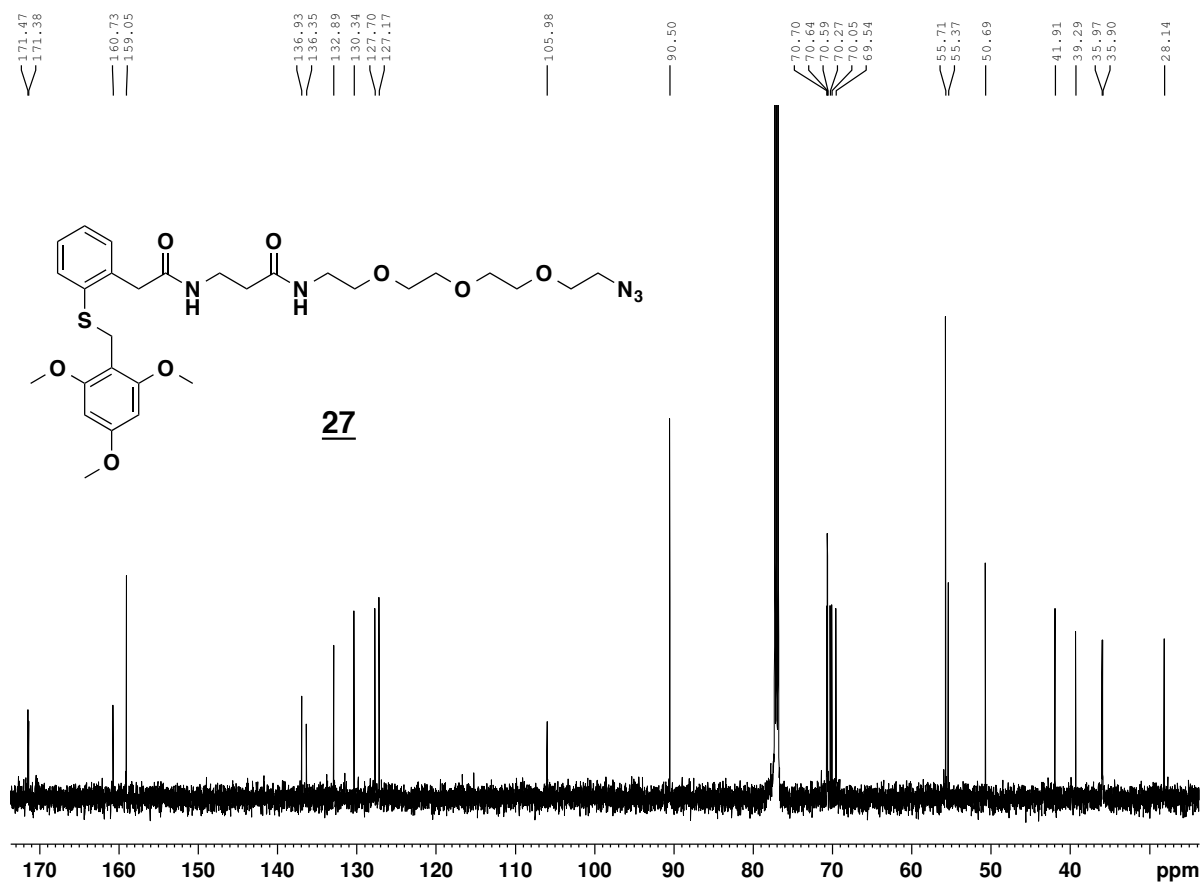
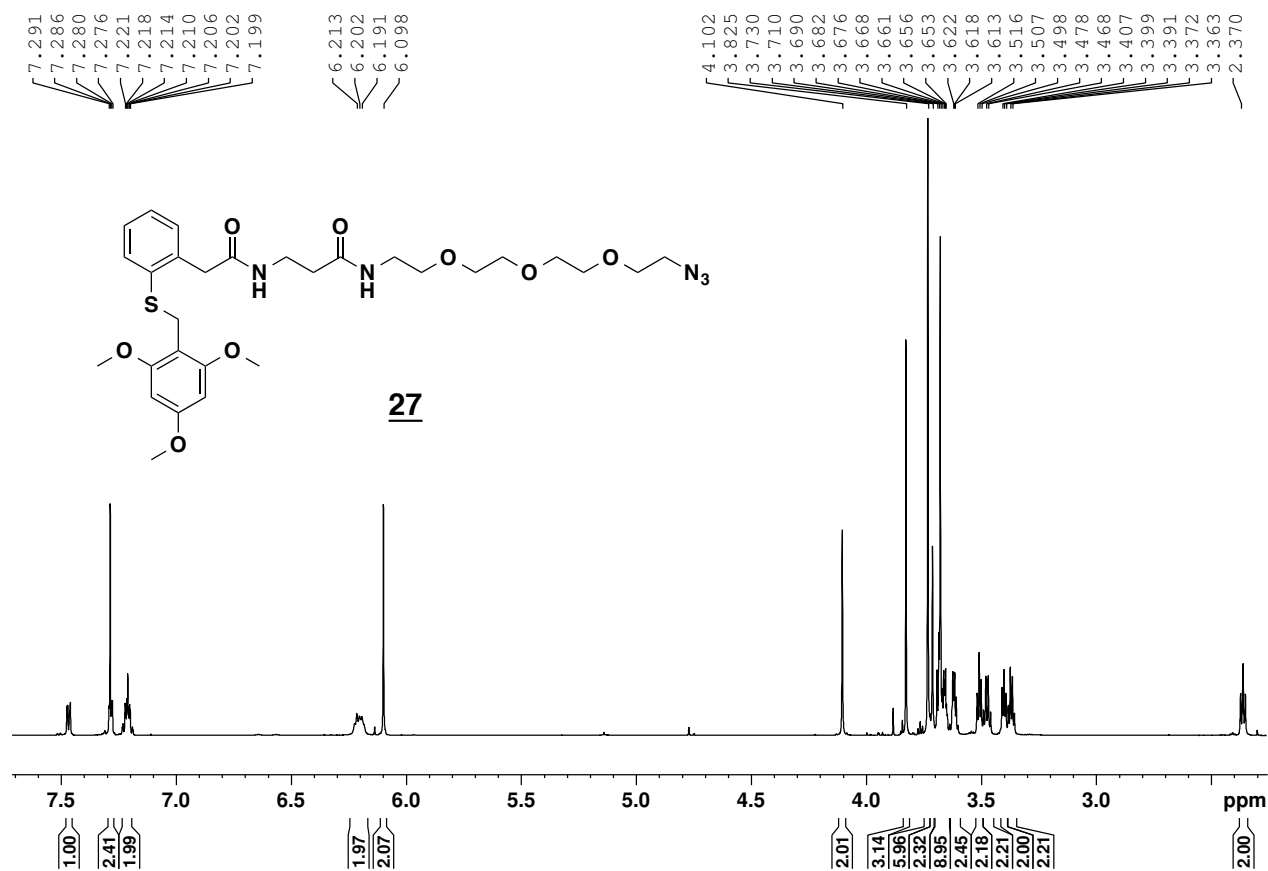


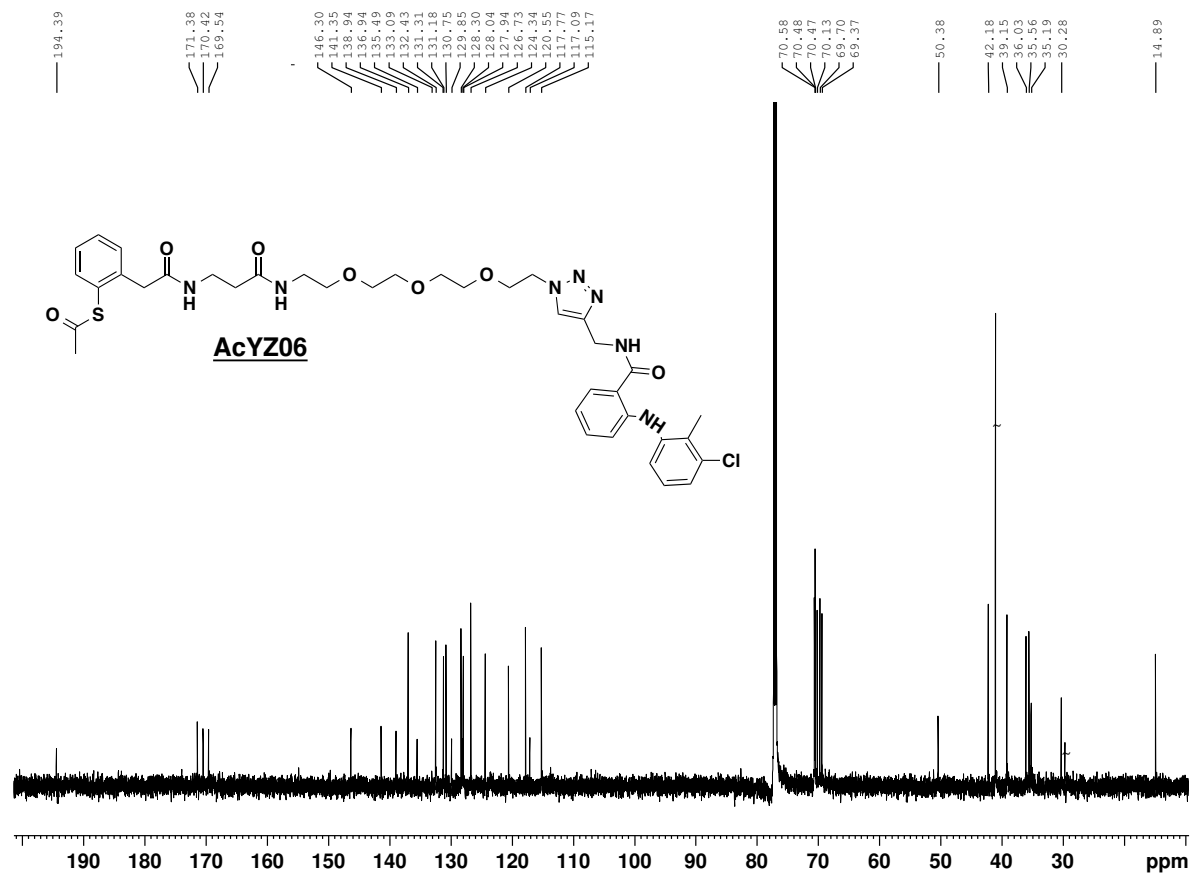
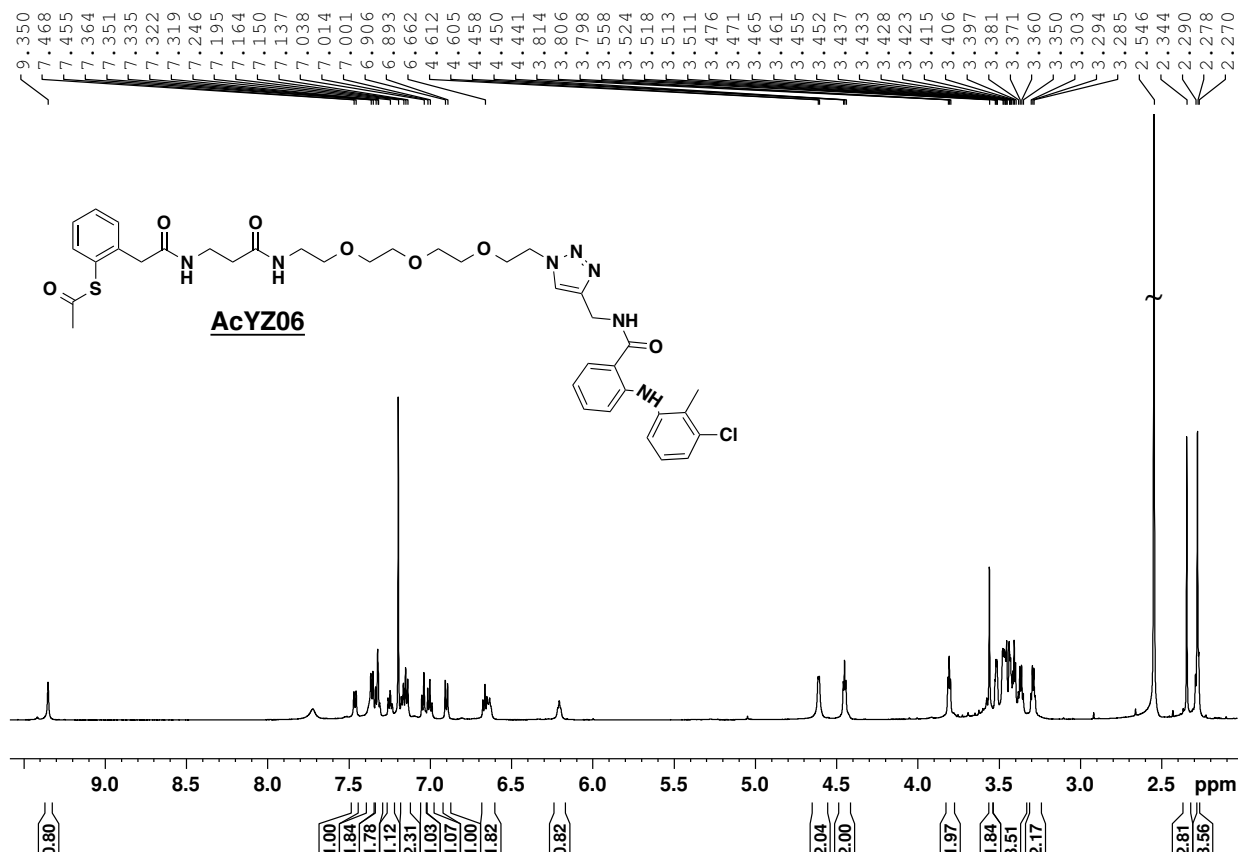


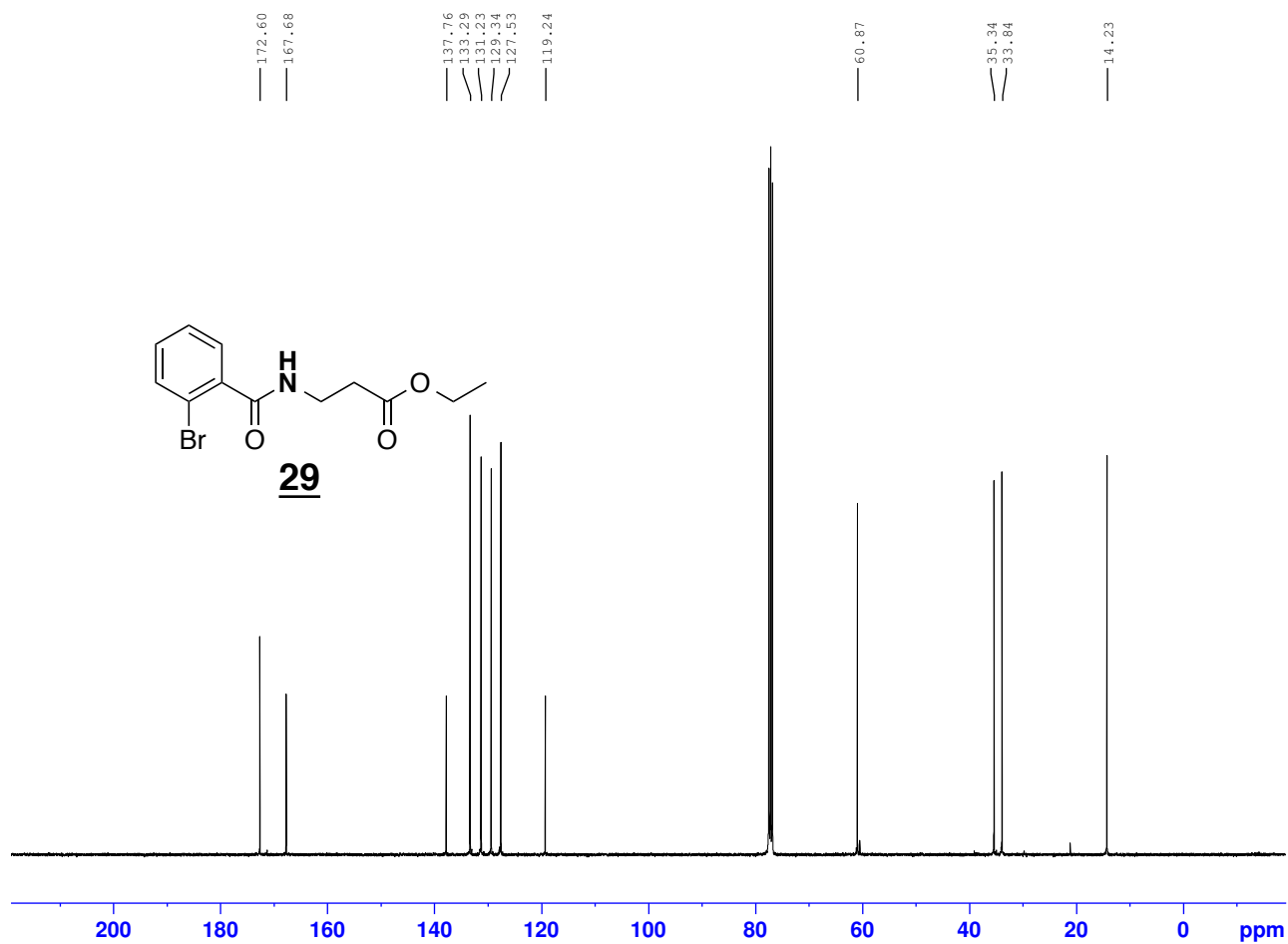
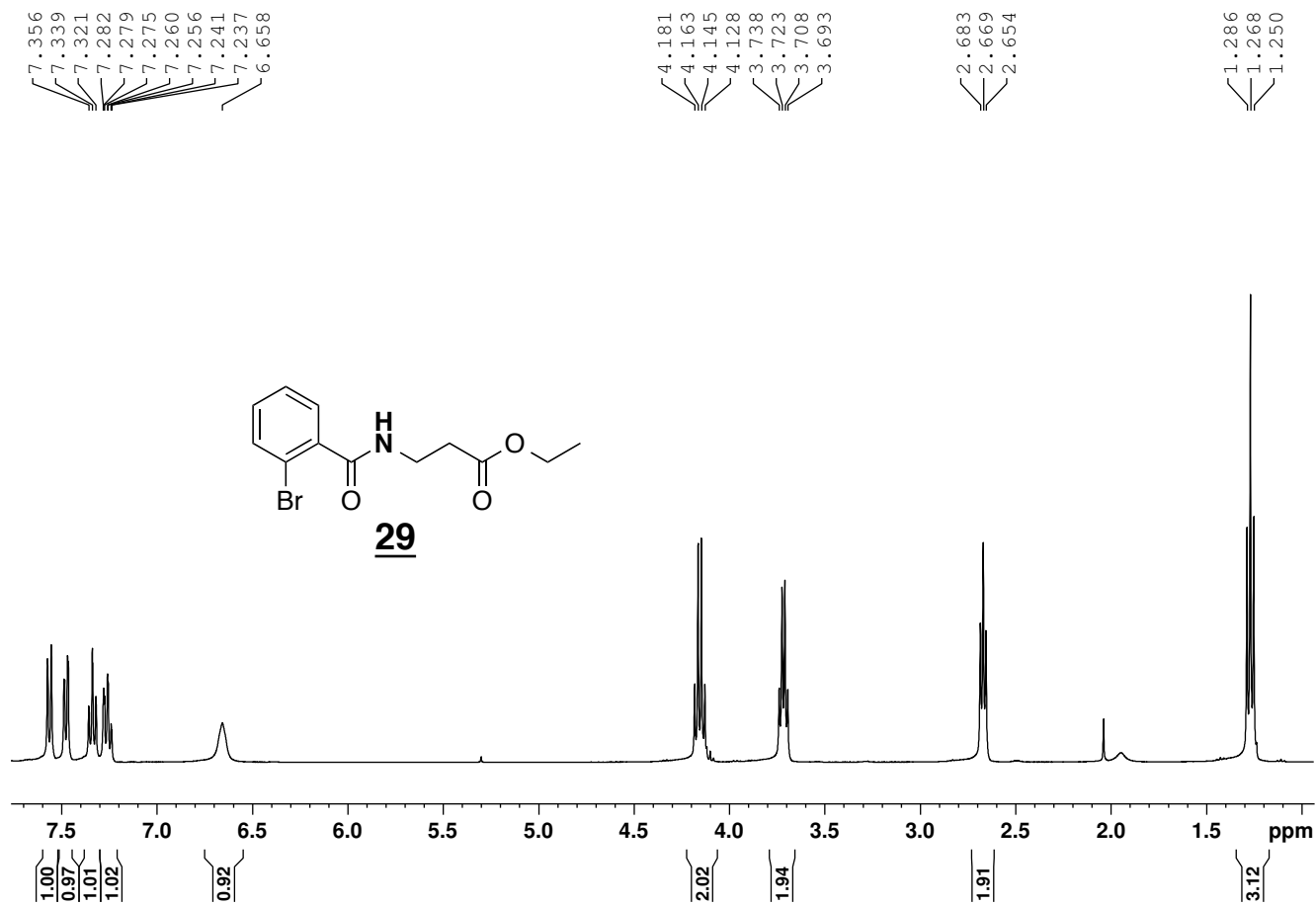


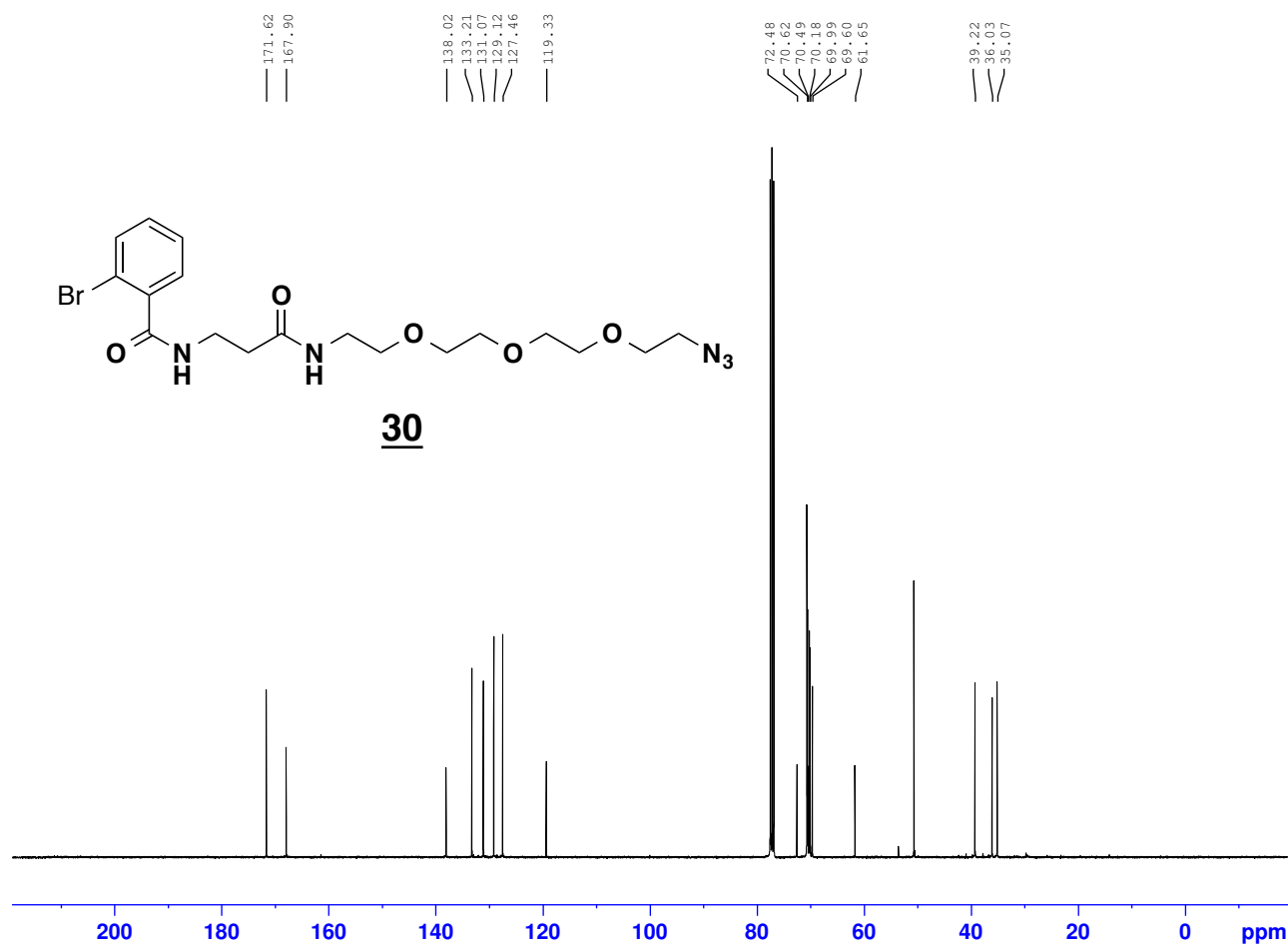
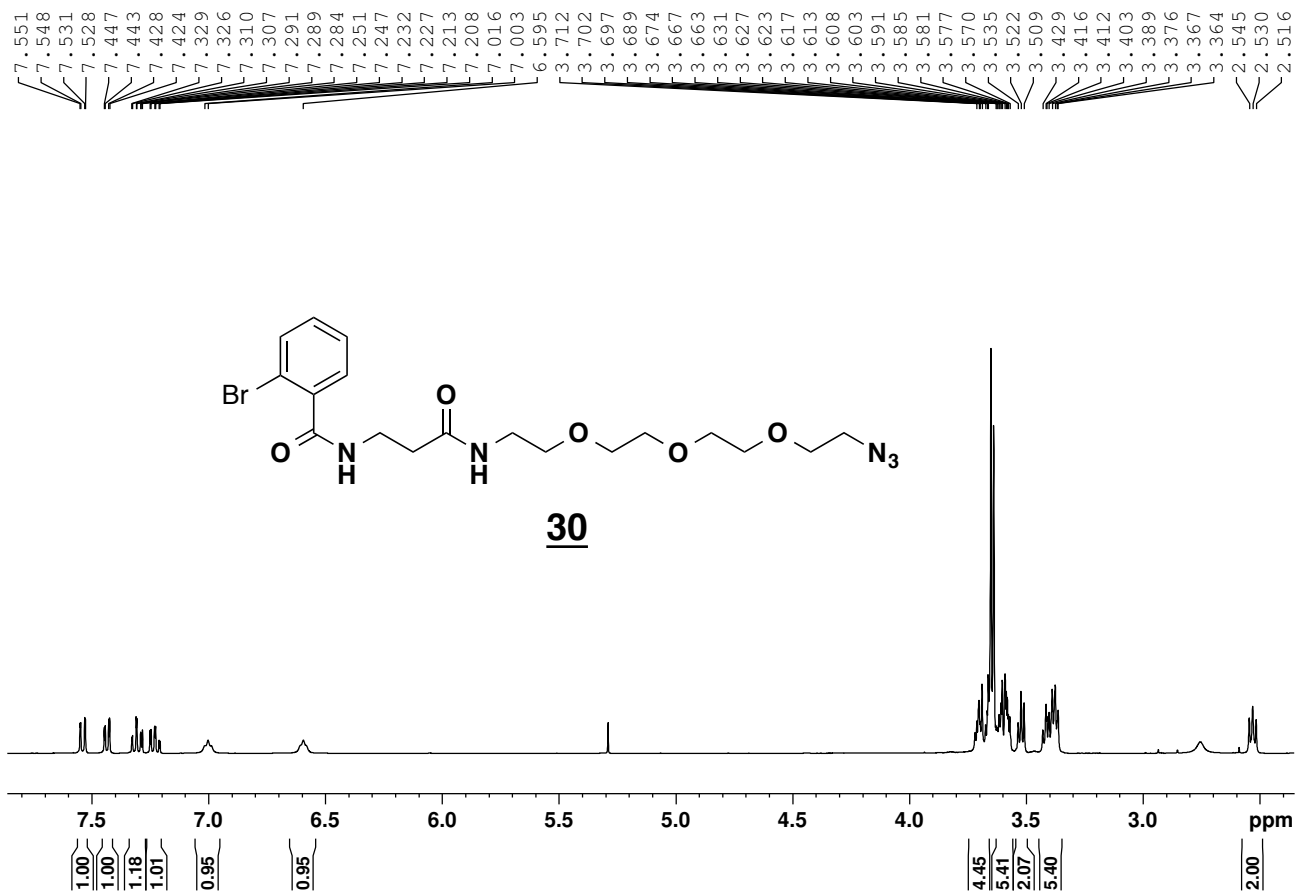


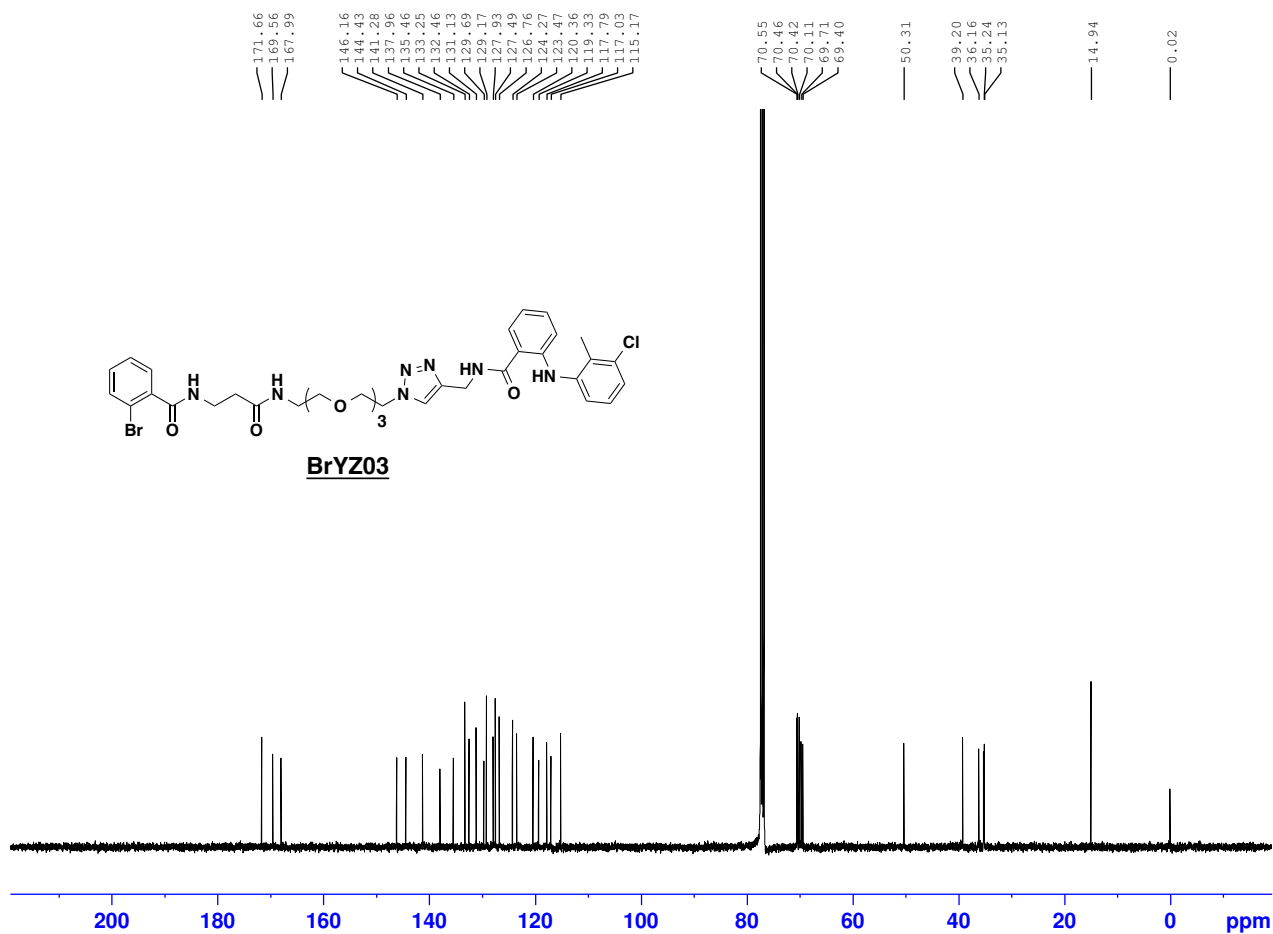
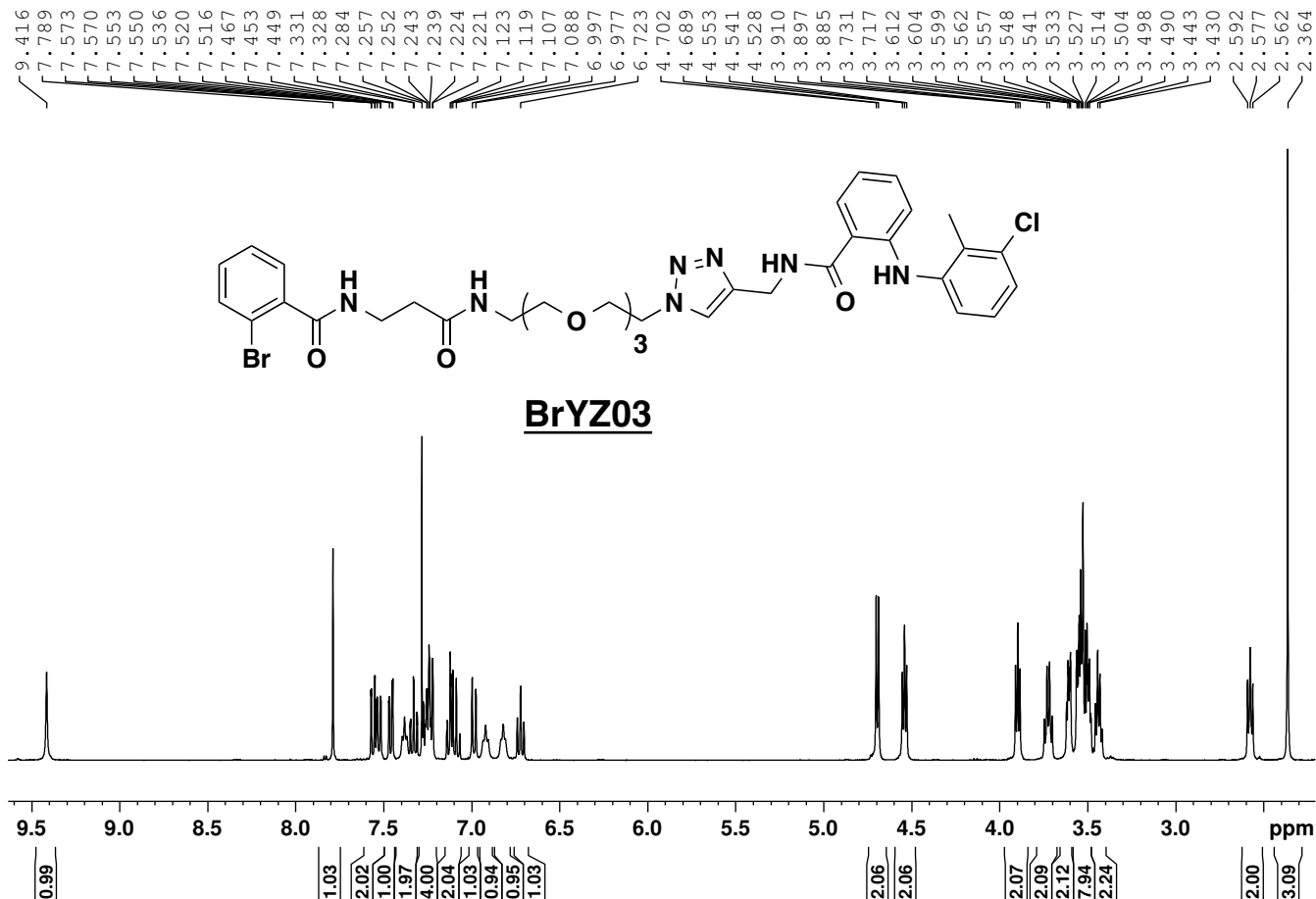


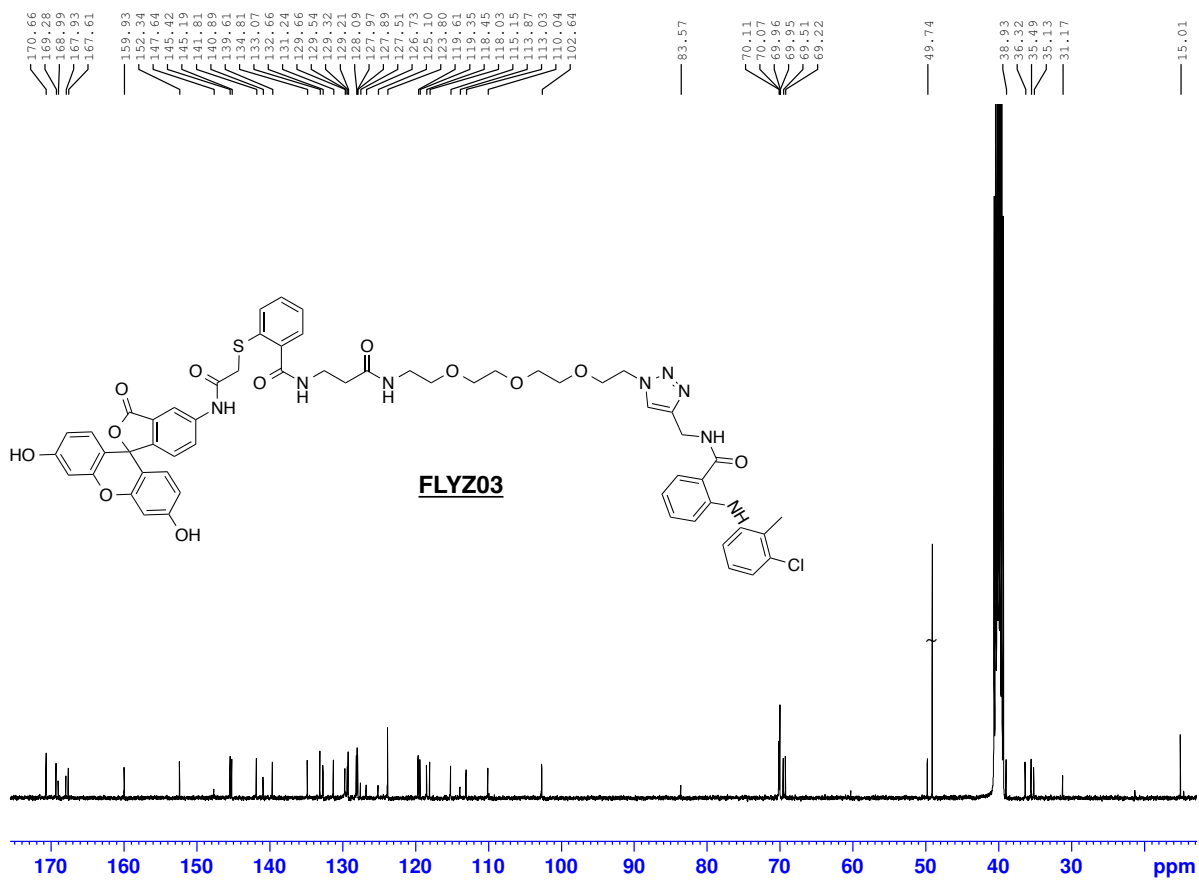
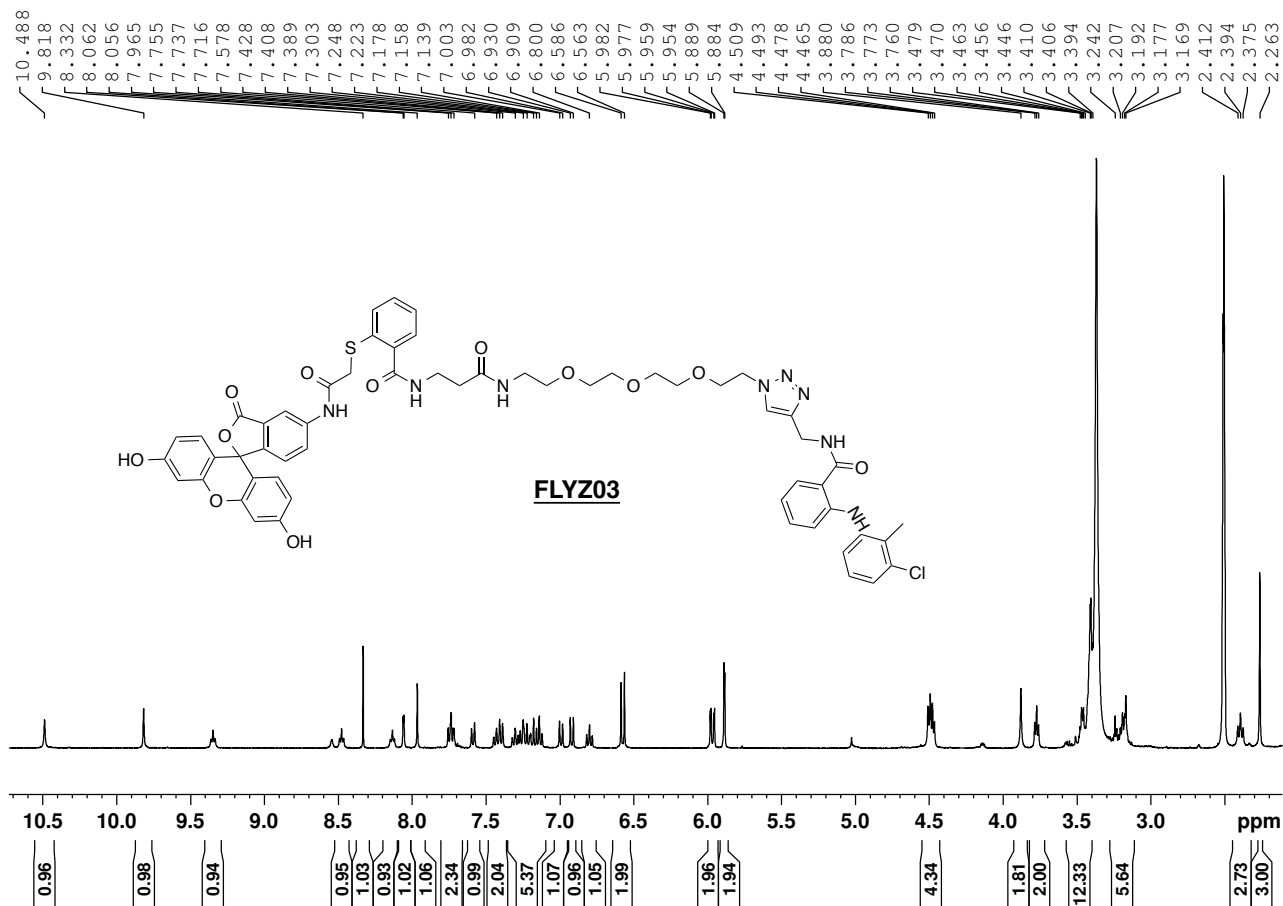








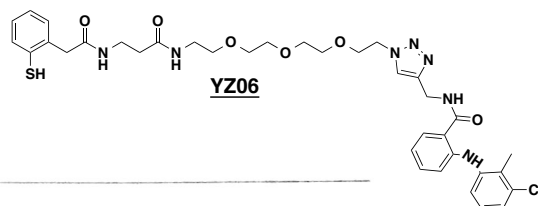




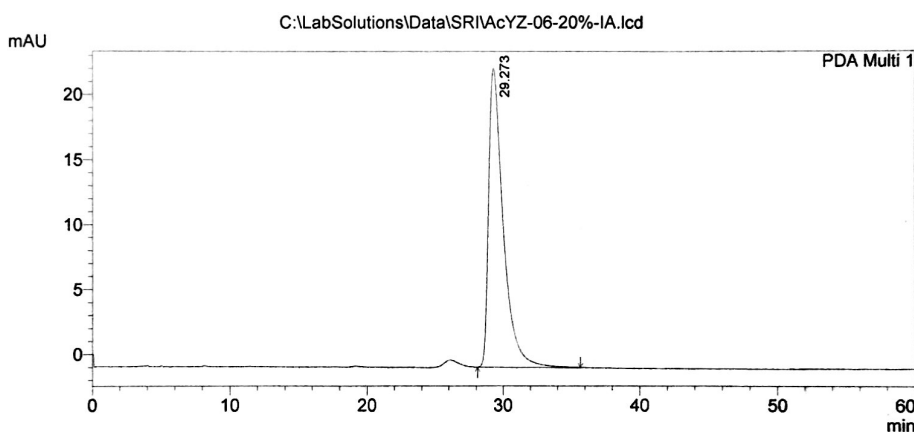


# ==== Shimadzu LCsolution Analysis Report ====

Acquired by : Admin  
 Sample Name :  
 Sample ID :  
 Tray# : 1  
 Vial # : 2  
 Injection Volume : 2 uL  
 Data File Name : AcYZ-06-20%-IA.lcd  
 Method File Name : Col5\_Ethan\_20%B\_1mL\_60min.lcm  
 Batch File Name : Ethan.lcb  
 Report File Name : Default.lcr  
 Data Acquired : 7/3/2016 10:58:47 PM  
 Data Processed : 7/5/2016 12:43:28 PM



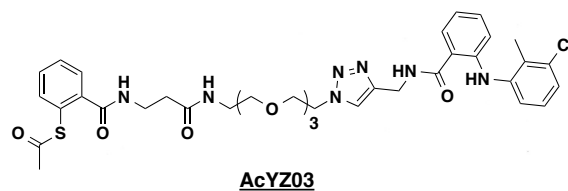
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7/14/2016 14:59:01 1 / 1

# ==== Shimadzu LCsolution Analysis Report ====

Acquired by : Admin  
 Sample Name :  
 Sample ID :  
 Tray# : 1  
 Vial # : 1  
 Injection Volume : 5 uL  
 Data File Name : AcYZ-03-3-40%-IA.lcd  
 Method File Name : Col5\_Ethan\_40%B\_1mL\_60min.lcm  
 Batch File Name : Ethan3.lcb  
 Report File Name : Default.lcr  
 Data Acquired : 7/6/2016 12:23:18 PM  
 Data Processed : 7/6/2016 1:23:22 PM



## <Chromatogram>

