Trigonal Scaffolds for Multivalent Targeting of Melanocortin Receptors

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Electronic Supplementary Information

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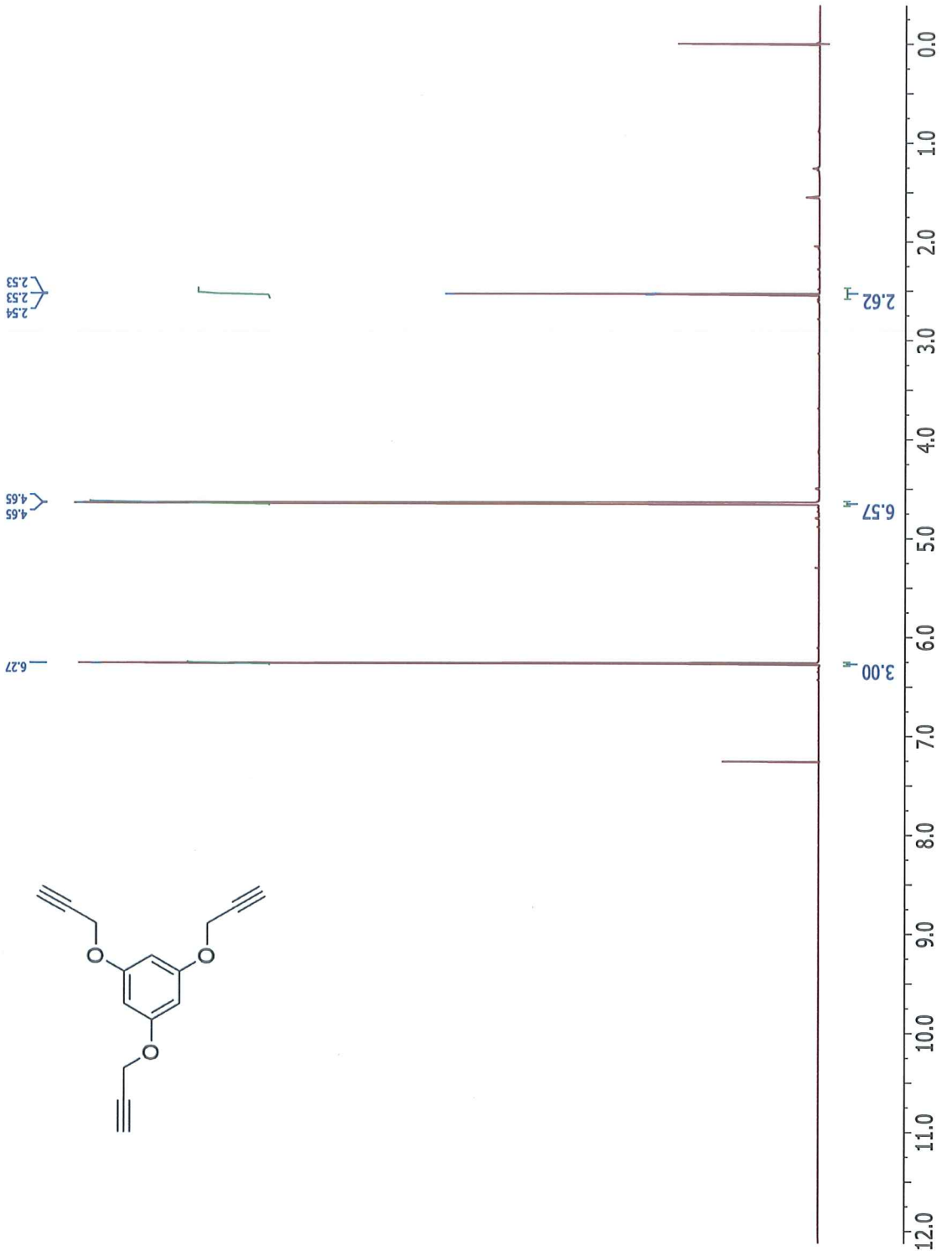
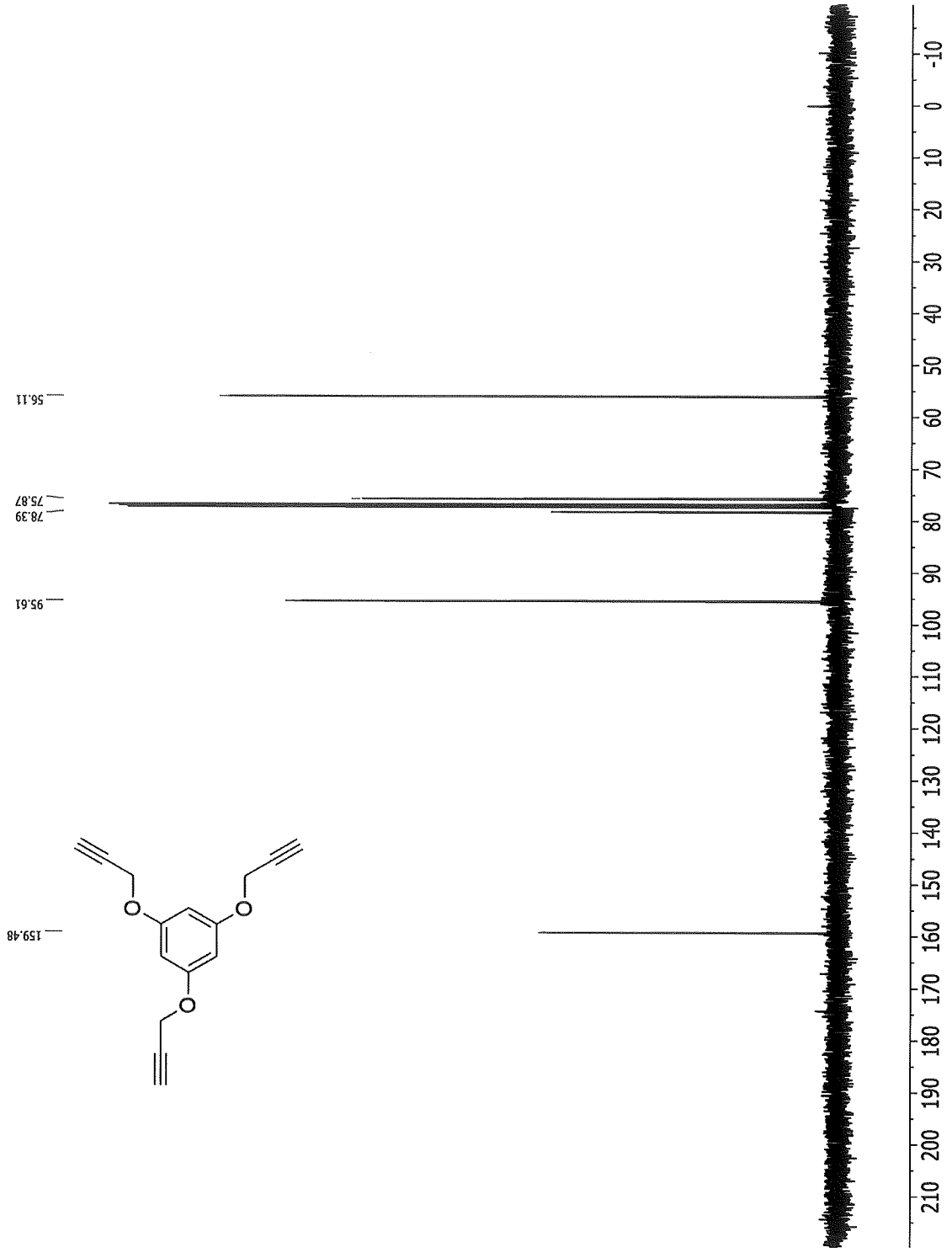
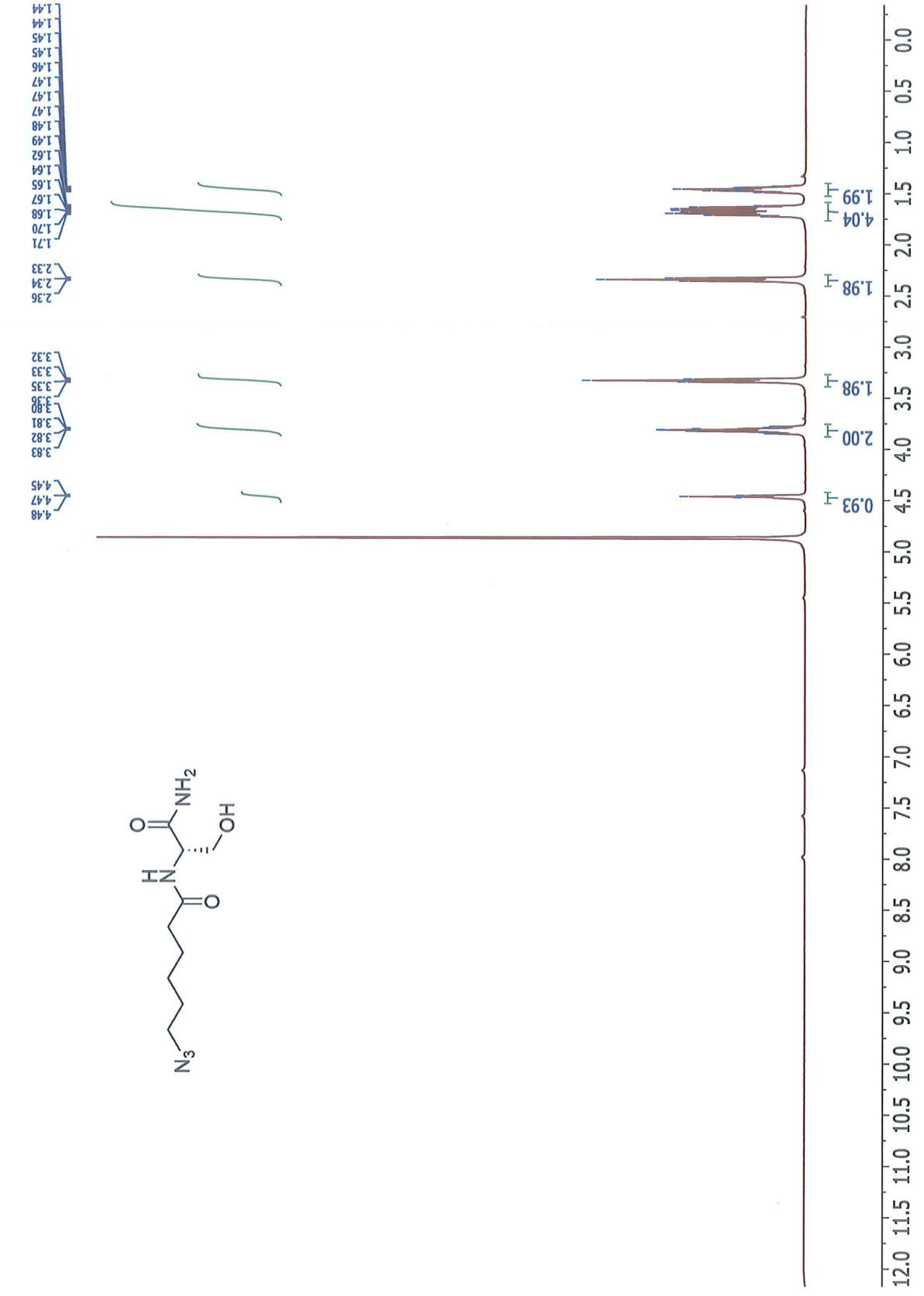
[1]H-NMR Spectrum of **10**



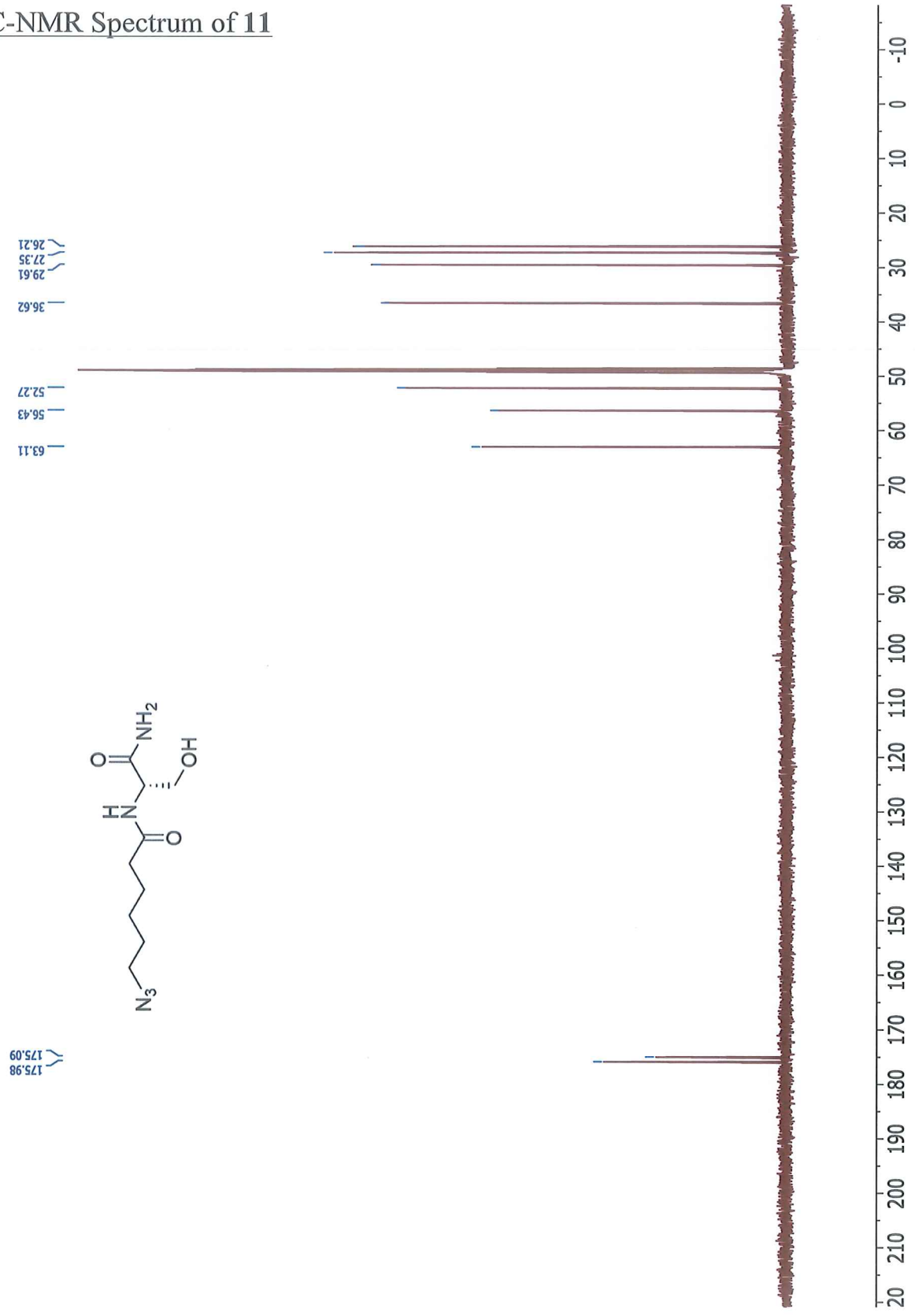
^{13}C-NMR Spectrum of **10**



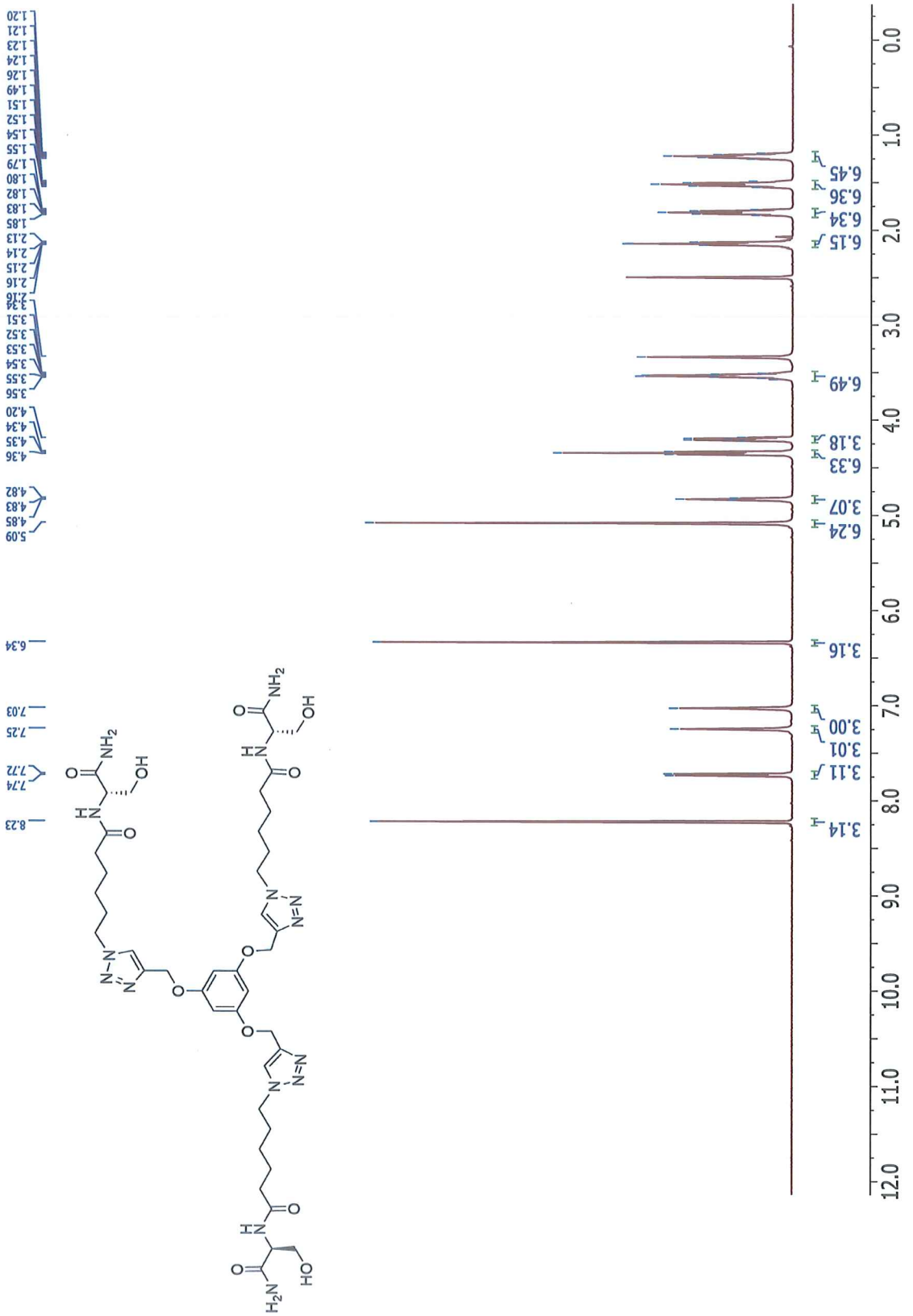
^{1}H-NMR Spectrum of 11



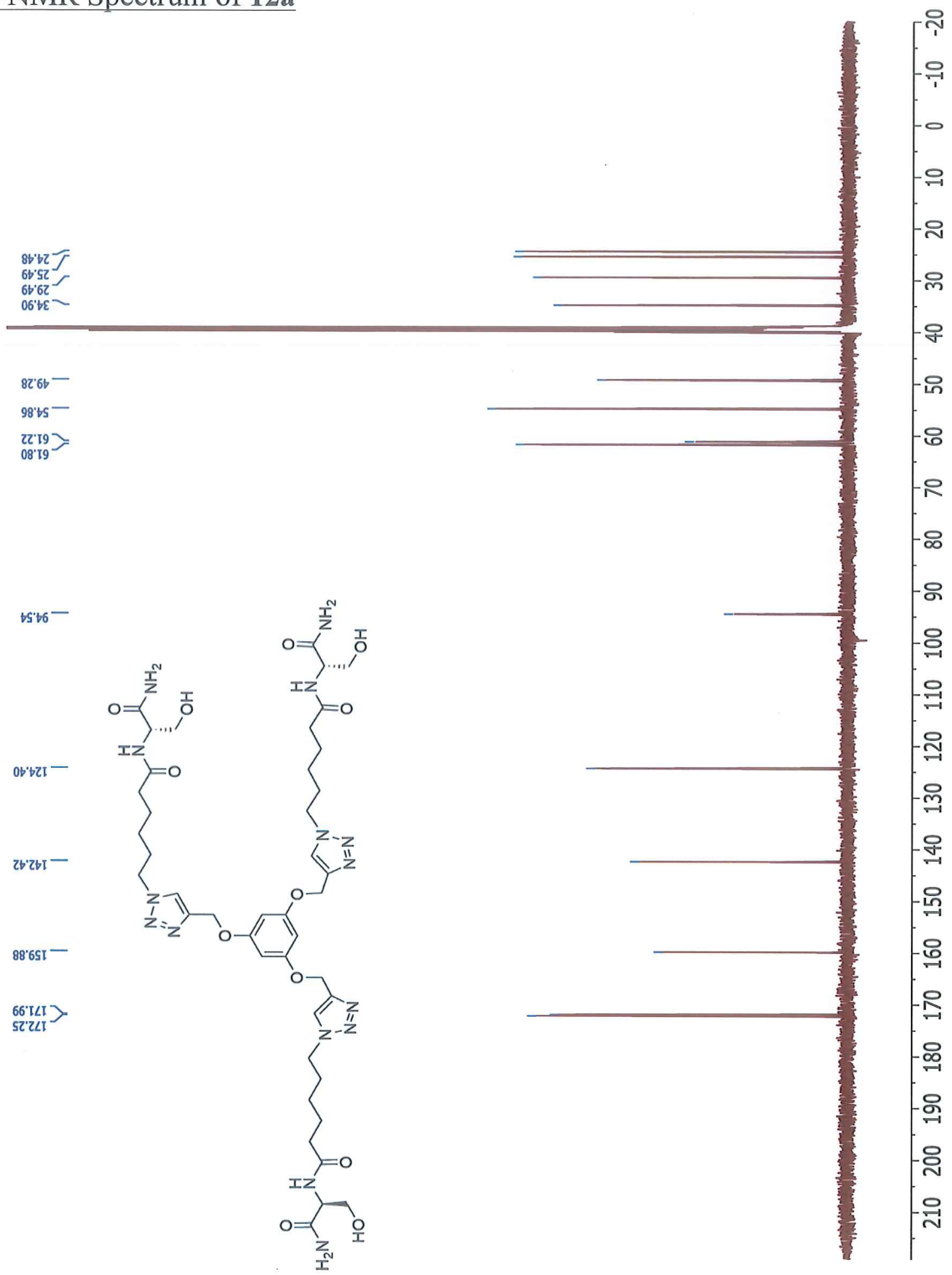
^{13}C-NMR Spectrum of **11**



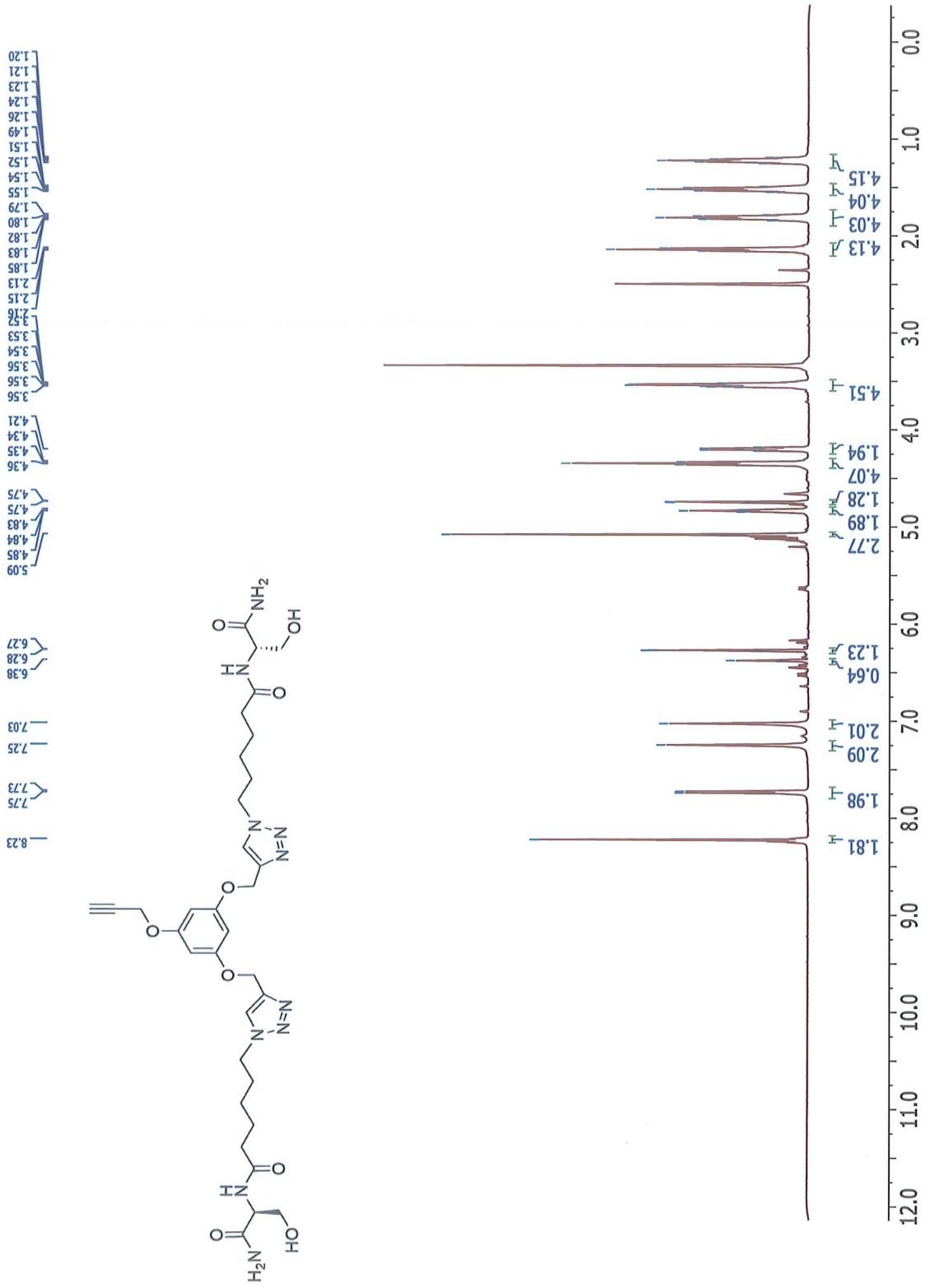
^{1}H-NMR Spectrum of **12a**



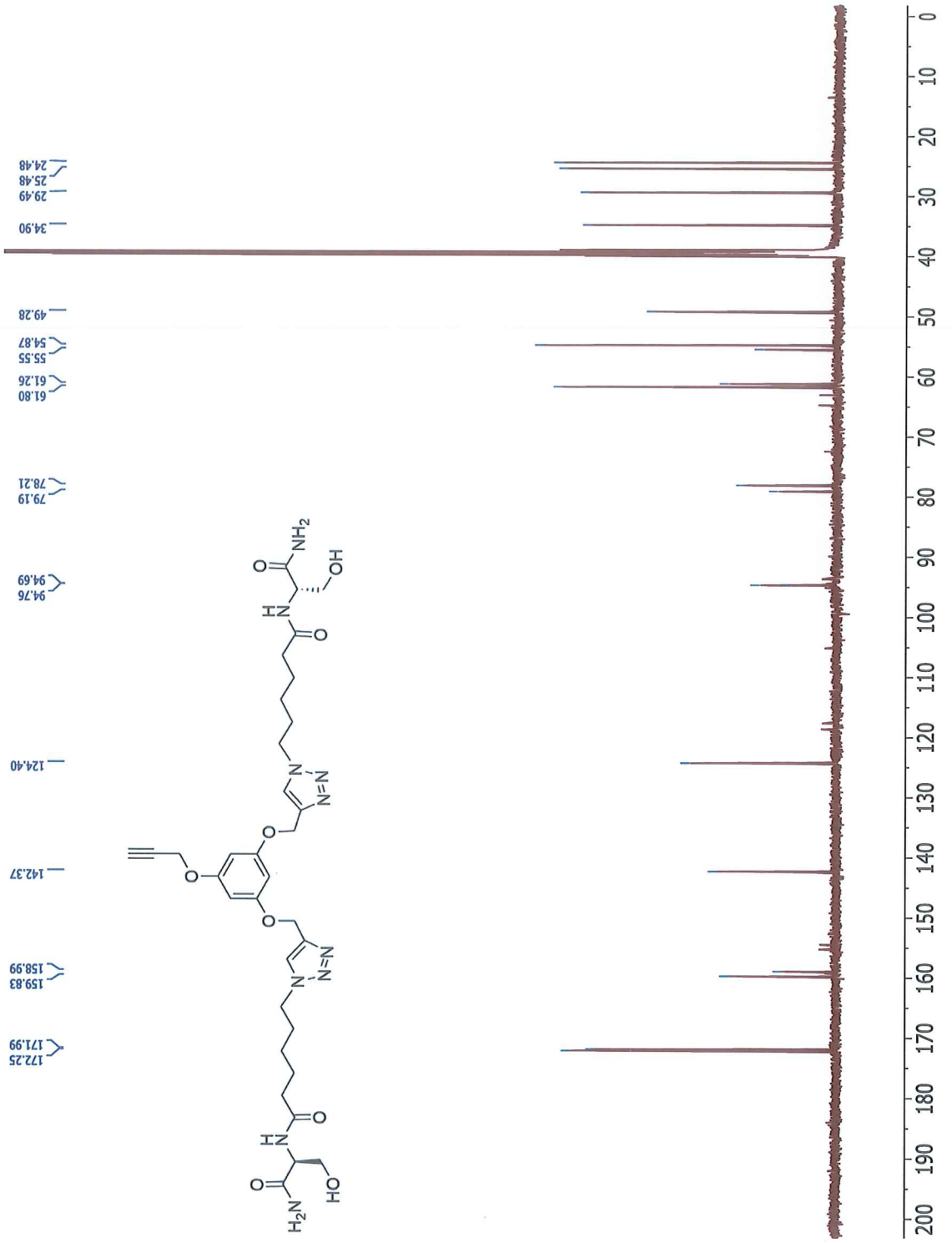
^{13}C-NMR Spectrum of **12a**



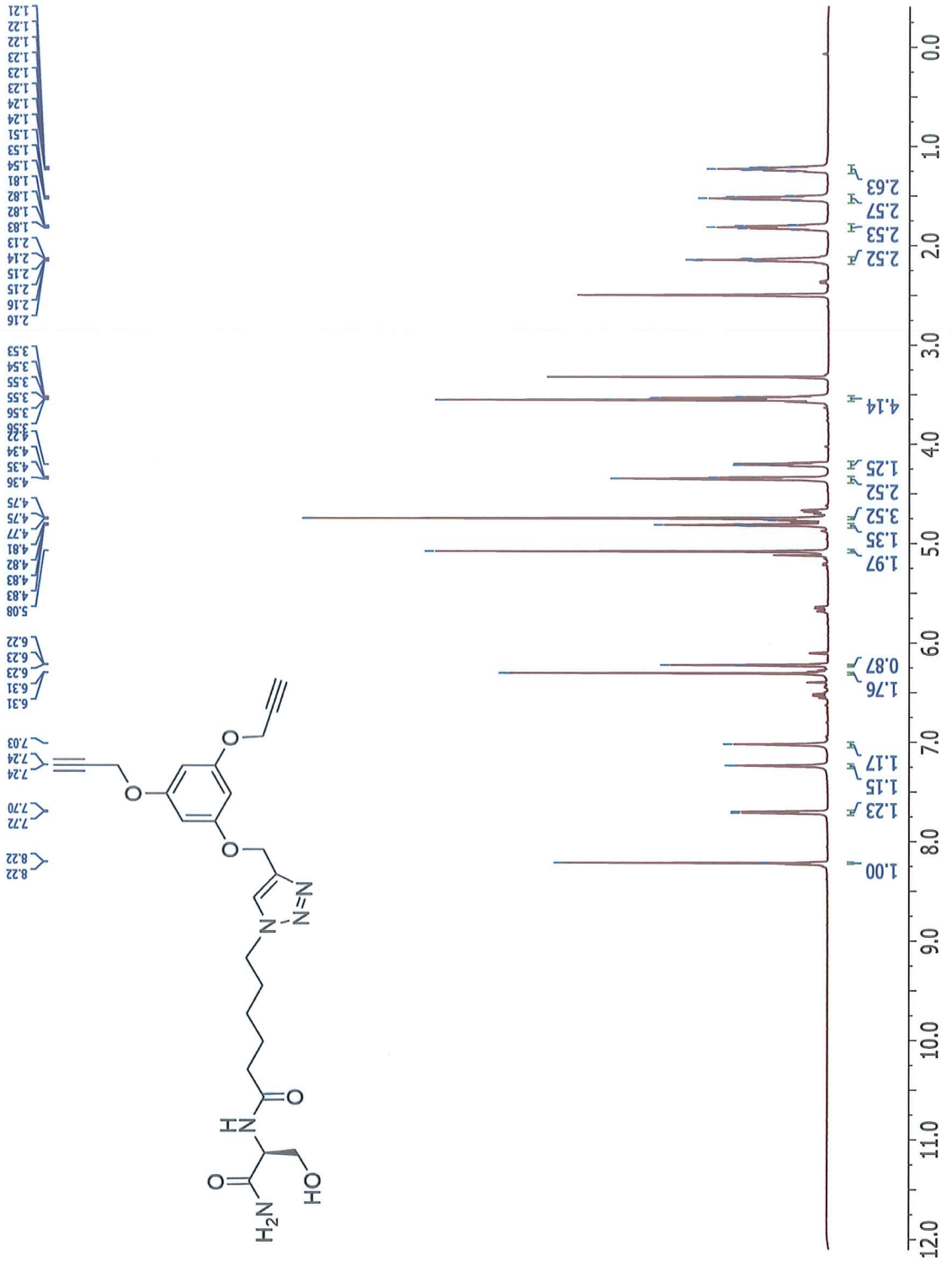
^{1}H-NMR Spectrum of **12b**



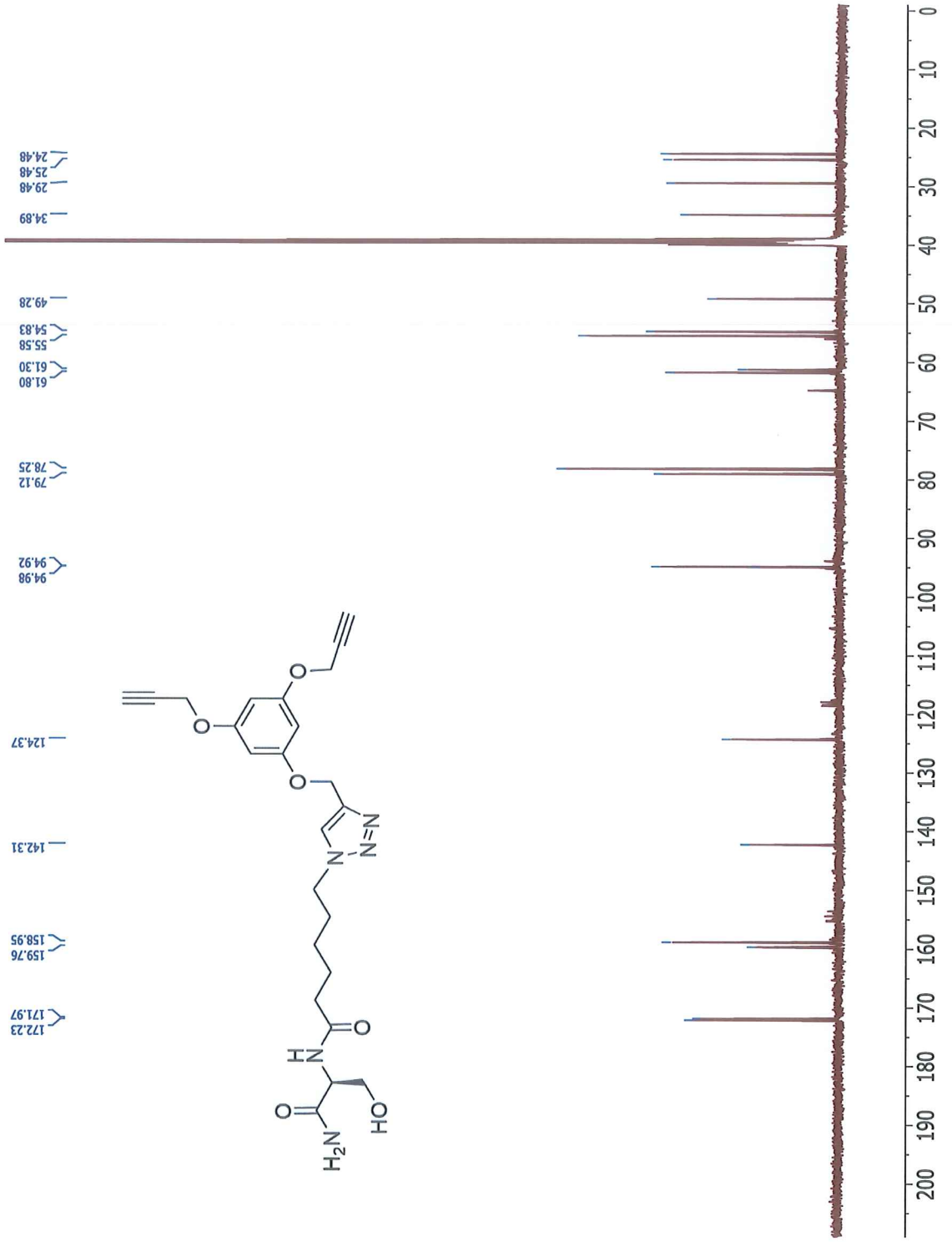
^{13}C-NMR Spectrum of **12b**



^{1}H-NMR Spectrum of **12c**



^{13}C-NMR Spectrum of **12c**



HPLC Chromatogram of **14a**

Empower SOFTWARE

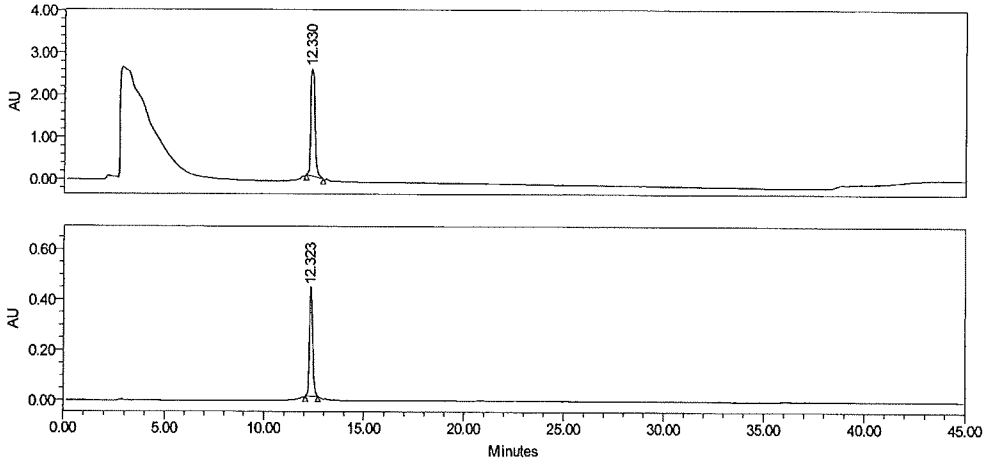
Dual Channel Summary

Reported by User: System

Project Name: BIO5_HPLC1

SAMPLE INFORMATION

Sample Name:	esh B2P148	Acquired By:	System
Sample Type:	Unknown	Date Acquired:	1/7/2014 10:36:53 PM MST
Vial:	106	Acq. Method Set:	10_90B_in
Injection #:	1	Date Processed:	1/8/2014 2:22:26 PM MST,
Injection Volume:	10.00 ul	Processing Method	Peptide_general
Run Time:	45.0 Minutes	Channel Name:	2487Channel 1, 2487Channel 2
Sample Set Name	Prime_Run_07_19_07	Proc. Chnl. Descr.:	220nm, 280nm



Channel 220 nm
Channel: 2487Channel 1

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Channel 280 nm
Channel: 2487Channel 2

	RT	Area (µV*sec)	% Area	Channel
1	12.323	4.90e+006	100.00	2487Channel 2

NOTES:

Report Method: Dual Channel Summary Printed 2:23:13 PM 1/8/2014 Page: 1 of 1

HPLC Chromatogram of **14b**

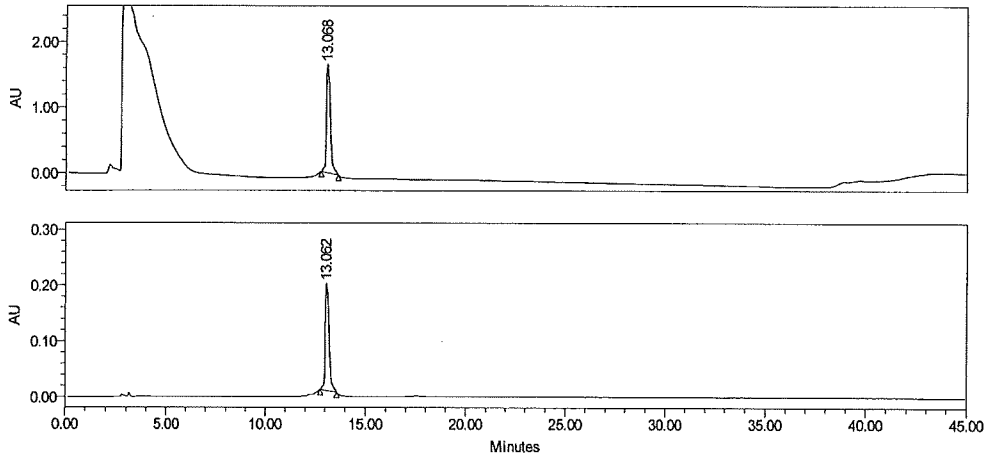
Empower software

Dual Channel Summary

Reported by User: System Project Name: BIO5_HPLC1

SAMPLE INFORMATION

Sample Name:	esh B2p146	Acquired By:	System
Sample Type:	Unknown	Date Acquired:	1/9/2014 7:15:16 PM MST
Vial:	77	Acq. Method Set:	10_90B_in
Injection #:	1	Date Processed:	1/10/2014 1:35:09 PM MST,
Injection Volume:	10.00 ul	Processing Method	Peptide_general
Run Time:	45.0 Minutes	Channel Name:	2487Channel 1, 2487Channel 2
Sample Set Name	Prime_Run_07_19_07	Proc. Chnl. Descr.:	220nm, 280nm



Channel 220 nm
Channel: 2487Channel 1

	RT	Area (µV*sec)	% Area	Channel
1	13.068	2.27e+007	100.00	2487Channel 1

Channel 280 nm
Channel: 2487Channel 2

	RT	Area (µV*sec)	% Area	Channel
1	13.062	2.60e+006	100.00	2487Channel 2

NOTES:

HPLC Chromatogram of **14c**

Empower software

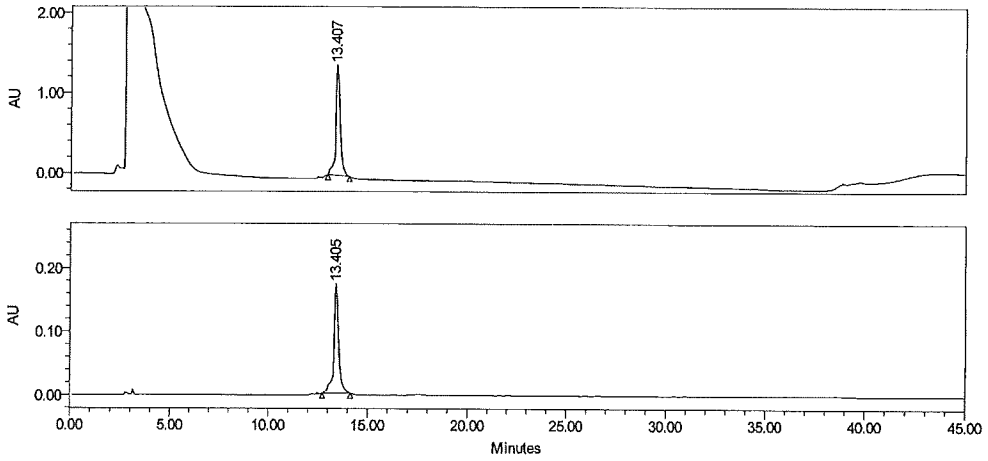
Dual Channel Summary

Reported by User: System

Project Name: BIO5_HPLC1

SAMPLE INFORMATION

Sample Name:	esh B2p82	Acquired By:	System
Sample Type:	Unknown	Date Acquired:	1/9/2014 6:29:07 PM MST
Vial:	76	Acq. Method Set:	10_90B_in
Injection #:	1	Date Processed:	1/10/2014 1:34:17 PM MST,
Injection Volume:	10.00 ul	Processing Method	Peptide_general
Run Time:	45.0 Minutes	Channel Name:	2487Channel 1, 2487Channel 2
Sample Set Name	Prime_Run_07_19_07	Proc. Chnl. Descr.:	220nm, 280nm



Channel 220 nm
Channel: 2487Channel 1

	RT	Area (µV*sec)	% Area	Channel
1	13.407	2.14e+007	100.00	2487Channel 1

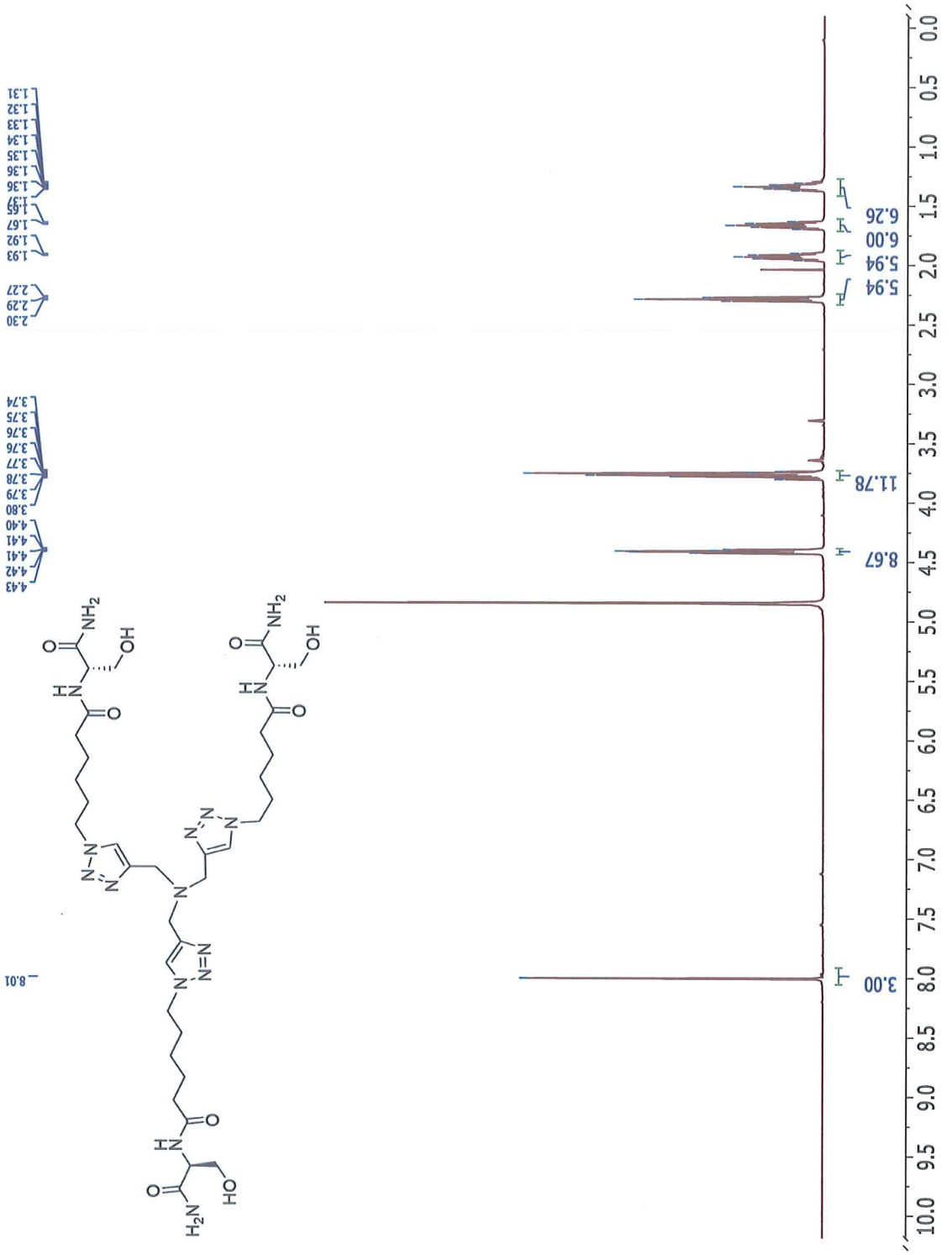
Channel 280 nm
Channel: 2487Channel 2

	RT	Area (µV*sec)	% Area	Channel
1	13.405	2.89e+006	100.00	2487Channel 2

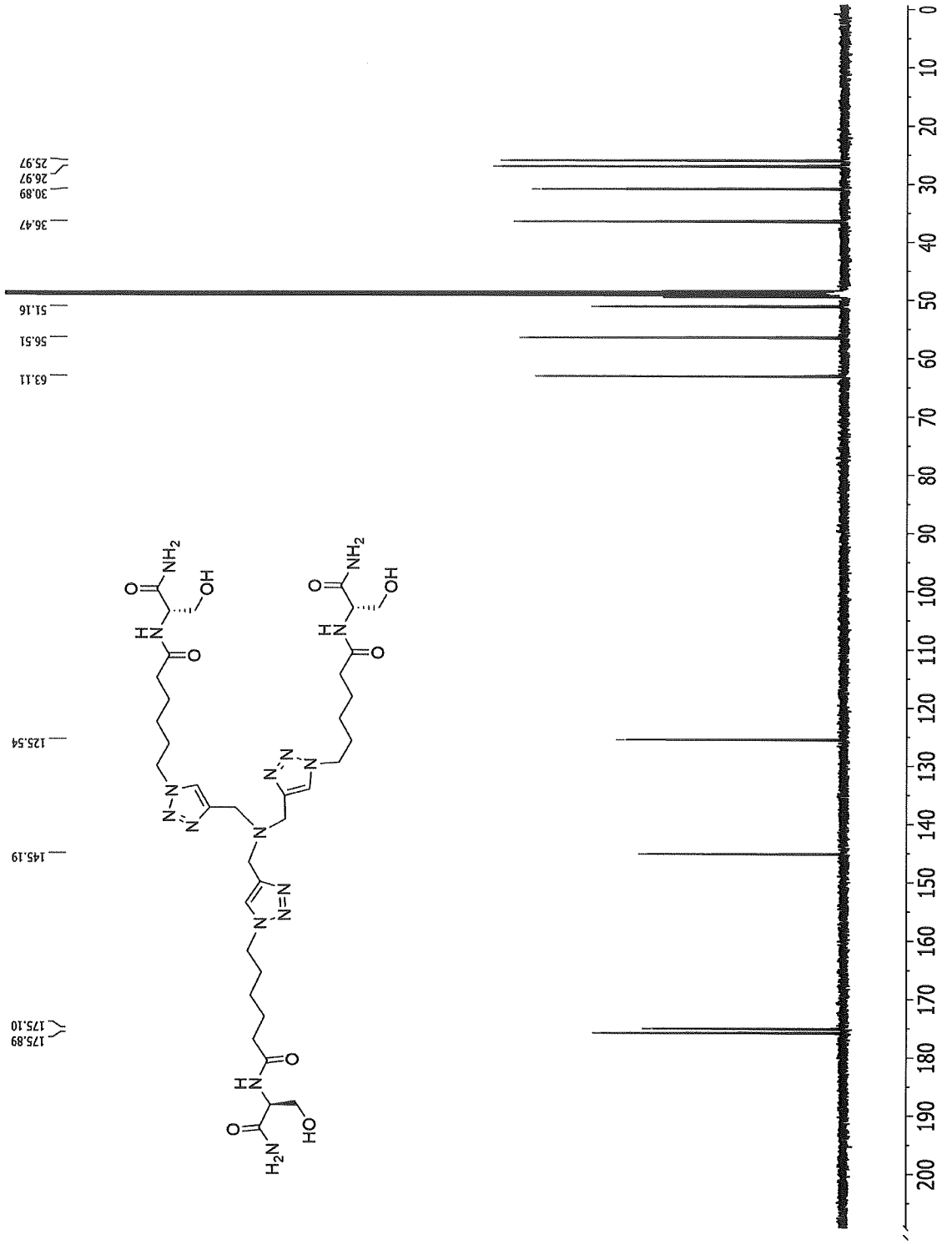
NOTES:

Report Method: Dual Channel Summary Printed 1:36:16 PM 1/10/2014 Page: 1 of 1

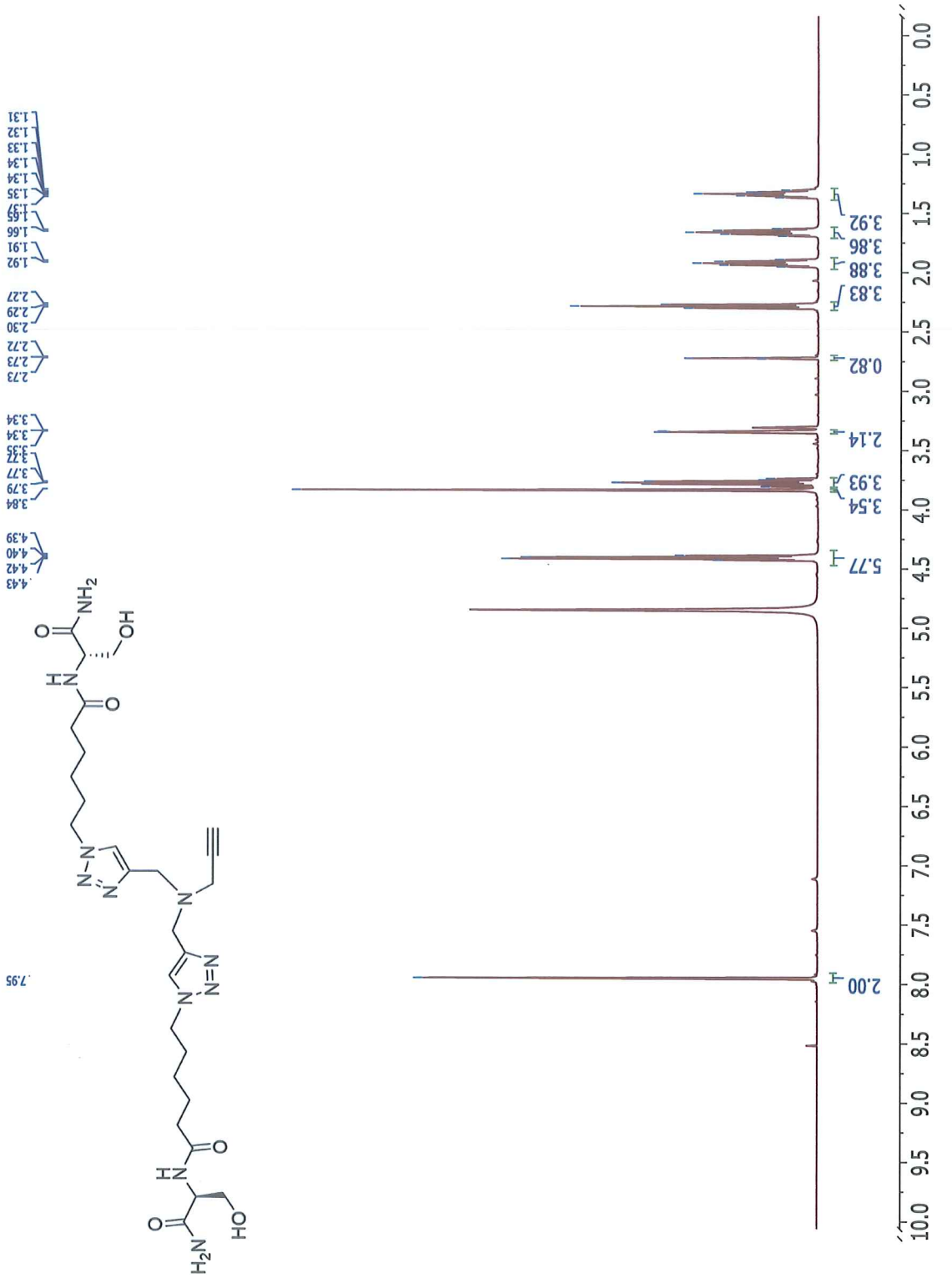
^{1}H-NMR Spectrum of **15a**



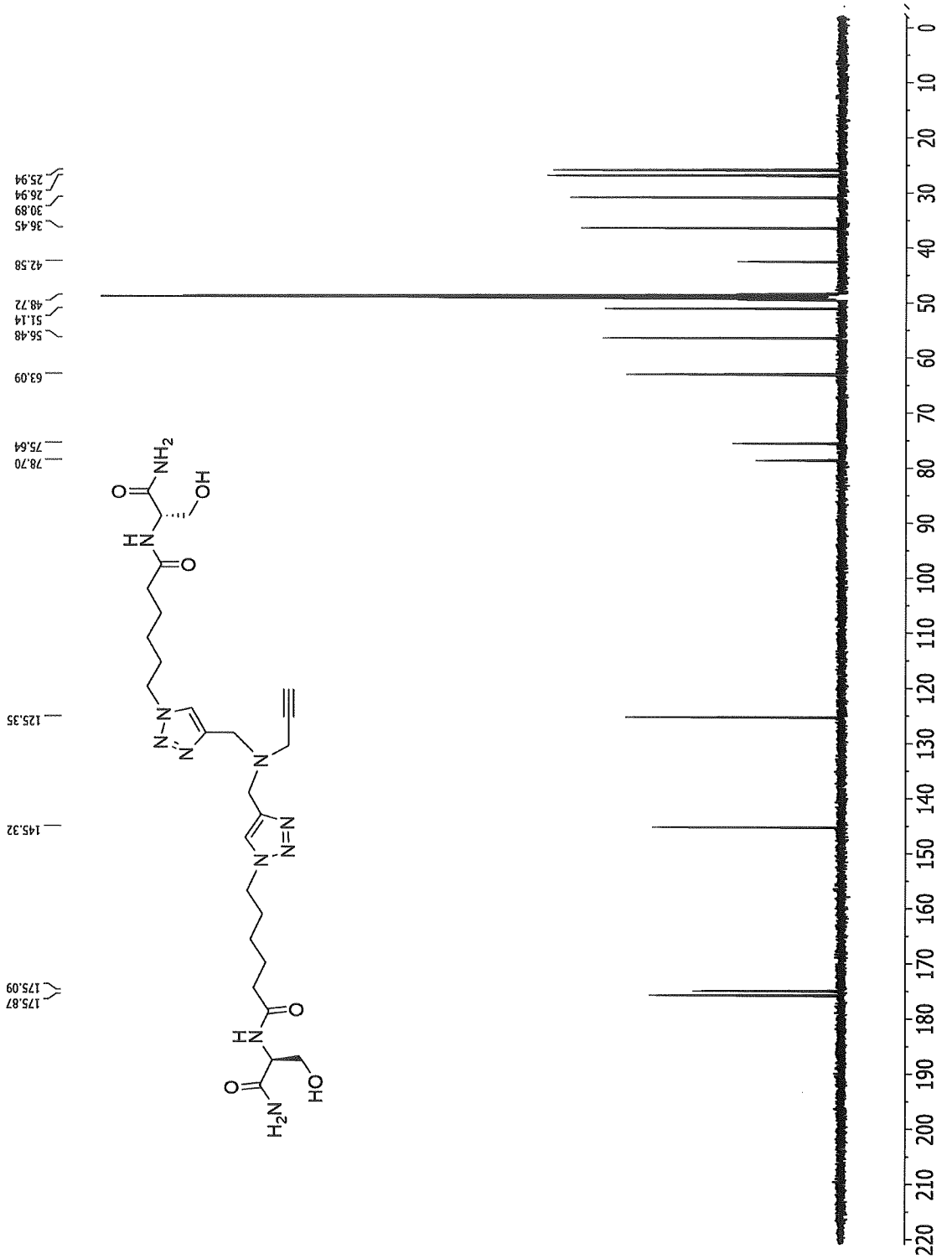
^{13}C-NMR Spectrum of **15a**



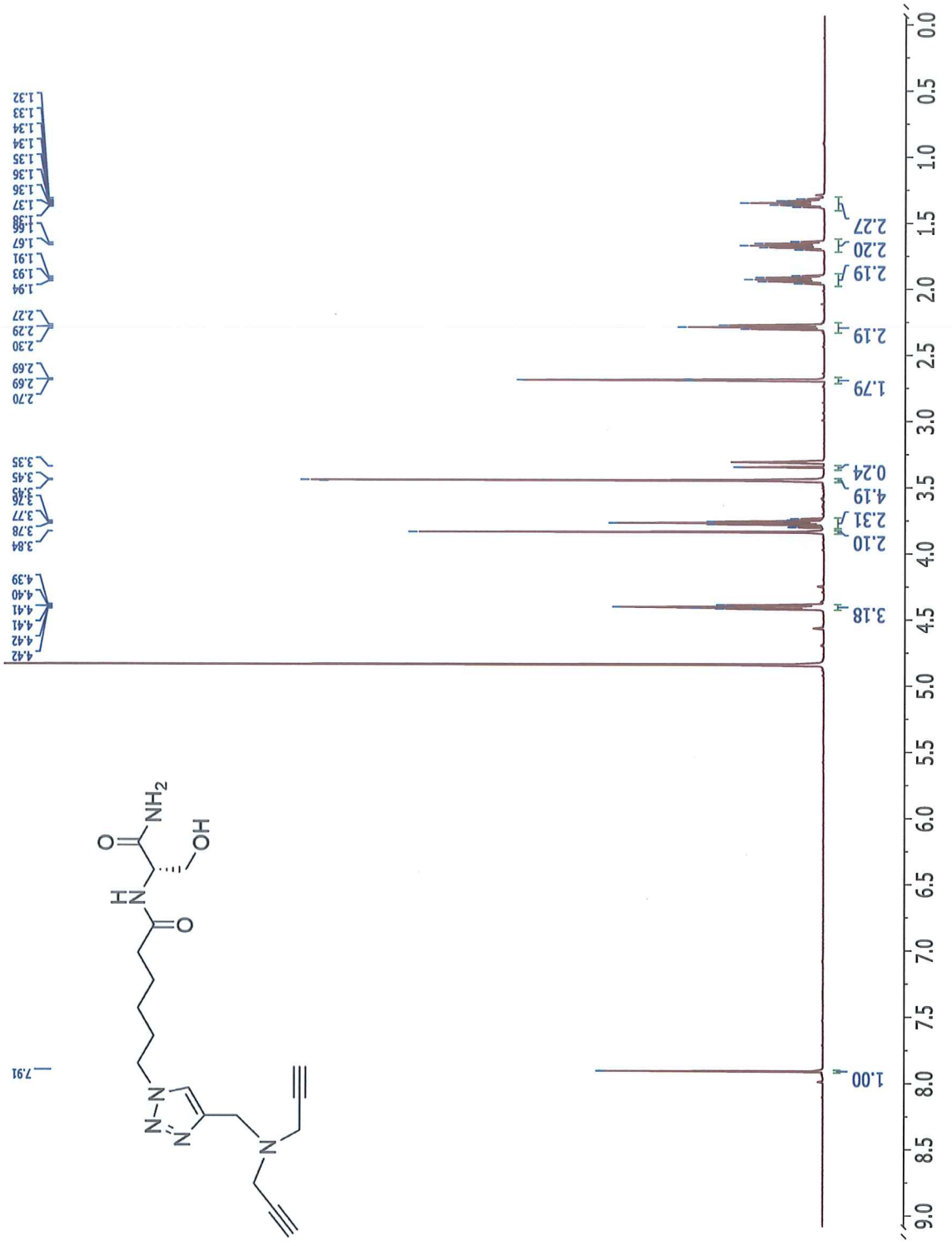
^{1}H-NMR Spectrum of **15b**



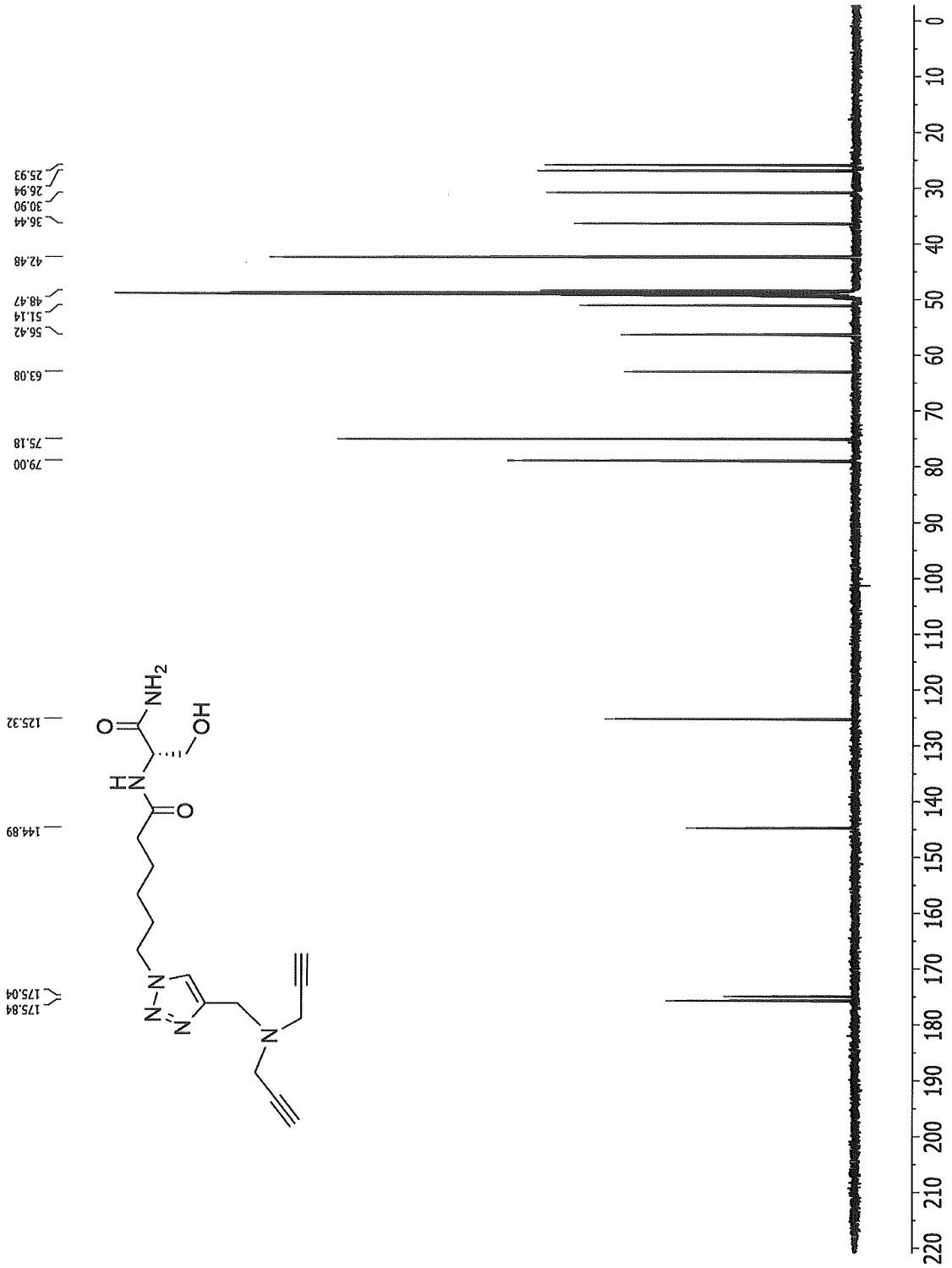
^{13}C-NMR Spectrum of **15b**



^{1}H-NMR Spectrum of **15c**



^{13}C-NMR Spectrum of **15c**



HPLC Chromatogram of **16a**

Empower software

Dual Channel Summary

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Sample Type:	Unknown	Date Acquired:	9/26/2013 5:02:59 PM MDT
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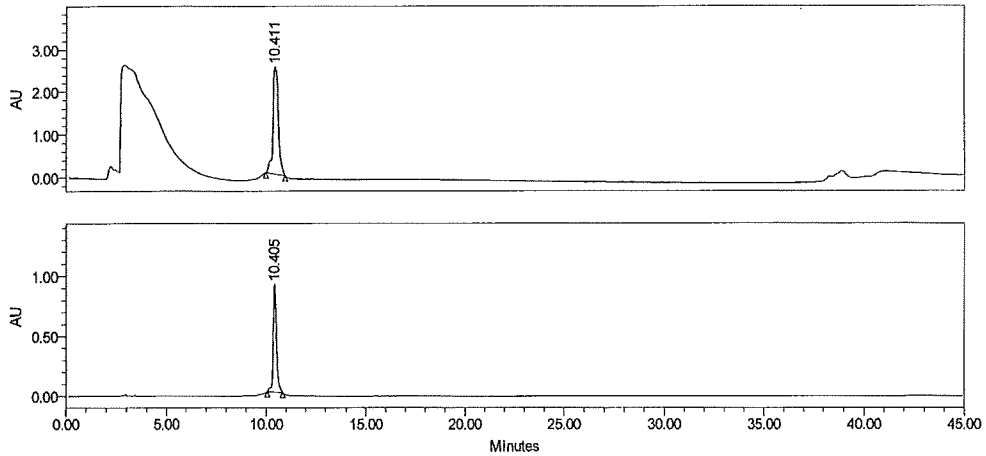
Injection #:	1	Date Processed:	9/27/2013 2:39:35 PM MDT,
Injection Volume:	20.00 ul	Processing Method	Peptide_general
Run Time:	45.0 Minutes	Channel Name:	2487Channel 1, 2487Channel 2
Sample Set Name	Prime_Run_07_19_07	Proc. Chnl. Descr.:	220nm, 280nm



Channel 220 nm
Channel: 2487Channel 1

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1	10.411	4.82e+007	100.00	2487Channel 1

Channel 280 nm
Channel: 2487Channel 2

	RT	Area (µV*sec)	% Area	Channel
1	10.405	1.06e+007	100.00	2487Channel 2

NOTES:

Report Method: Dual Channel Summary Printed 2:41:33 PM 9/27/2013 Page: 1 of 1

HPLC Chromatogram of **16b**

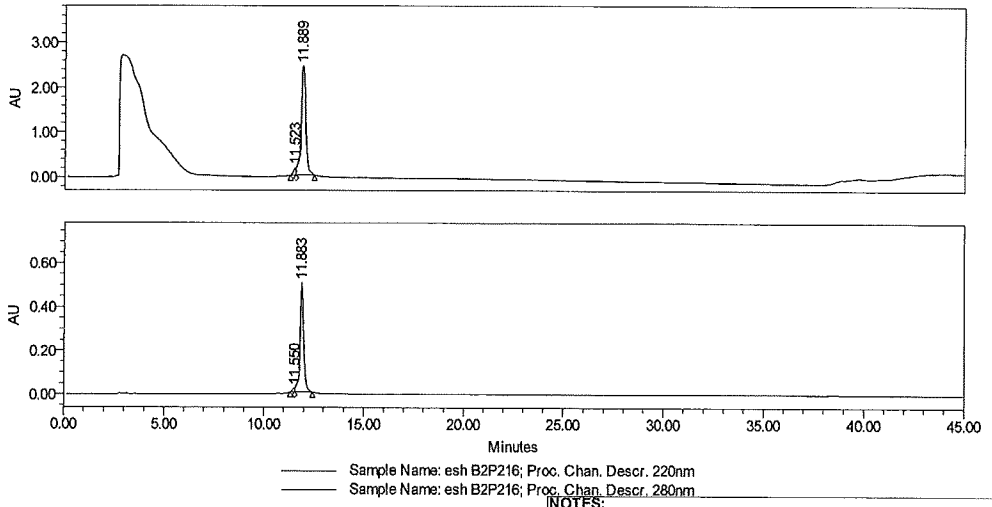
Empower

Dual Channel Summary

Reported by User: System Project Name: BIO5_HPLC1

SAMPLE INFORMATION

Sample Name:	esh B2P216	Acquired By:	System
Sample Type:	Unknown	Date Acquired:	1/7/2014 4:27:57 PM MST
Vial:	98	Acq. Method Set:	10_90B_in
Injection #:	1	Date Processed:	1/8/2014 2:11:48 PM MST,
Injection Volume:	10.00 ul	Processing Method	Peptide_general
Run Time:	45.0 Minutes	Channel Name:	2487Channel 1, 2487Channel 2
Sample Set Name	Prime_Run_07_19_07	Proc. Chnl. Descr.:	220nm, 280nm



Channel 220 nm
Channel: 2487Channel 1

	RT	Area (µV*sec)	% Area	Channel
1	11.889	3.94e+007	96.71	2487Channel 1

Channel 280 nm
Channel: 2487Channel 2

	RT	Area (µV*sec)	% Area	Channel
1	11.883	6.42e+006	98.04	2487Channel 2

NOTES:

Report Method: Dual Channel Summary Printed 2:13:00 PM 1/8/2014 Page: 1 of 1

HPLC Chromatogram of **16c**

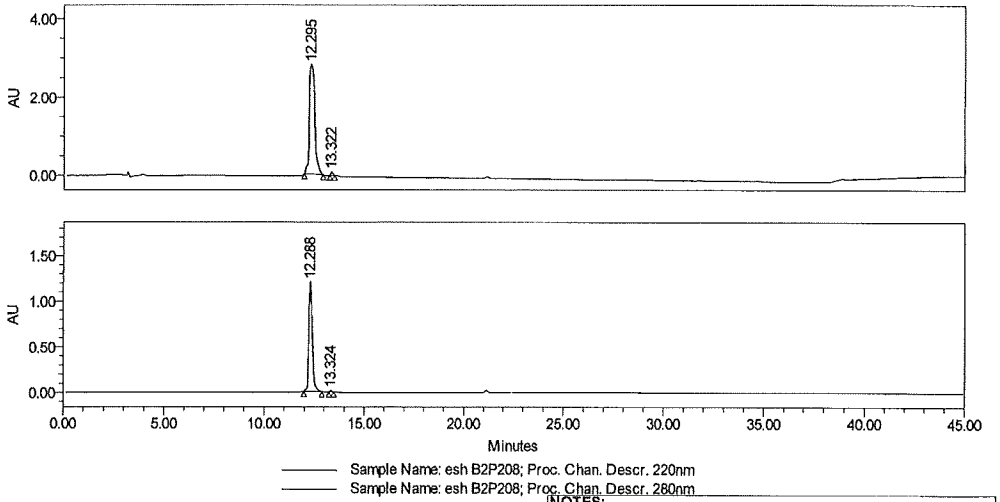
Empower software

Dual Channel Summary

Reported by User: System Project Name: BIO5_HPLC1

SAMPLE INFORMATION

Sample Name:	esh B2P208	Acquired By:	System
Sample Type:	Unknown	Date Acquired:	1/9/2014 4:10:54 PM MST
Vial:	73	Acq. Method Set:	10_90B_in
Injection #:	1	Date Processed:	1/10/2014 1:31:17 PM MST,
Injection Volume:	10.00 ul	Processing Method	Peptide_general
Run Time:	45.0 Minutes	Channel Name:	2487Channel 1, 2487Channel 2
Sample Set Name	Prime_Run_07_19_07	Proc. Chnl. Descr.:	220nm, 280nm



Channel 220 nm
Channel: 2487Channel 1

	RT	Area (µV*sec)	% Area	Channel
1	12.295	5.27e+007	98.95	2487Channel 1

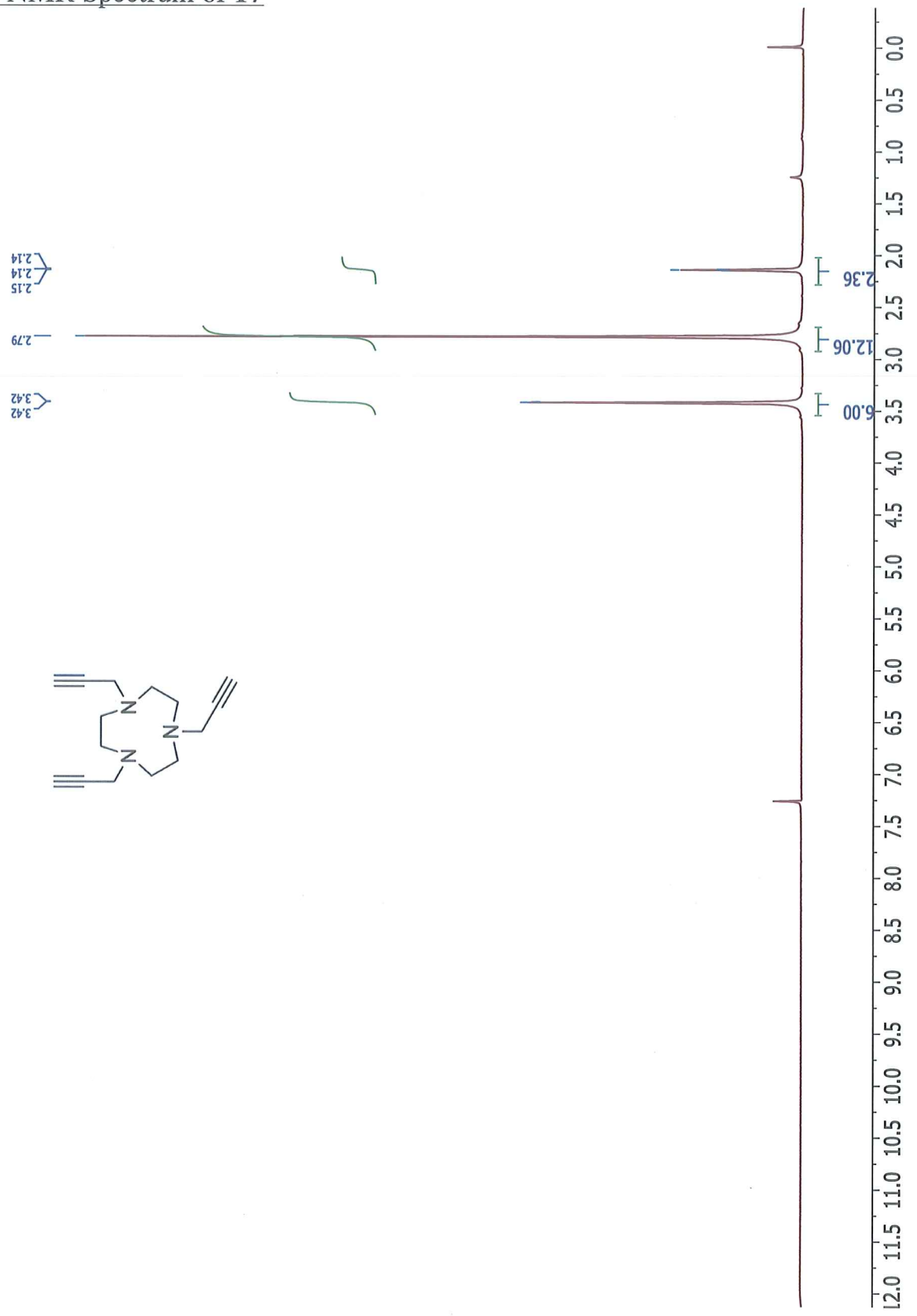
Channel 280 nm
Channel: 2487Channel 2

	RT	Area (µV*sec)	% Area	Channel
1	12.288	1.45e+007	99.34	2487Channel 2

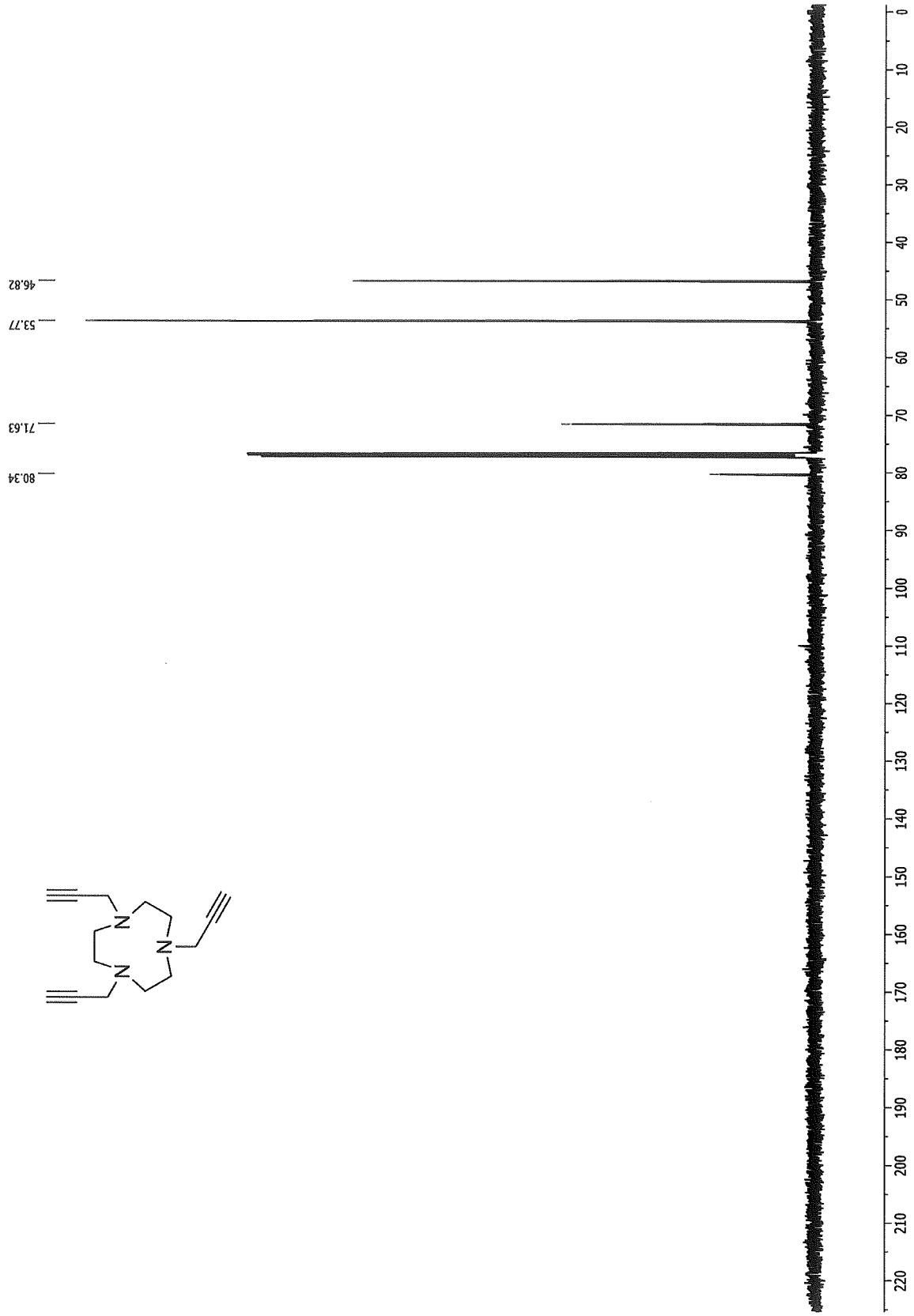
NOTES:

Report Method: Dual Channel Summary Printed 1:36:44 PM 1/10/2014 Page: 1 of 1

^{1}H-NMR Spectrum of **17**



^{13}C-NMR Spectrum of **17**



HPLC Chromatogram of **18**

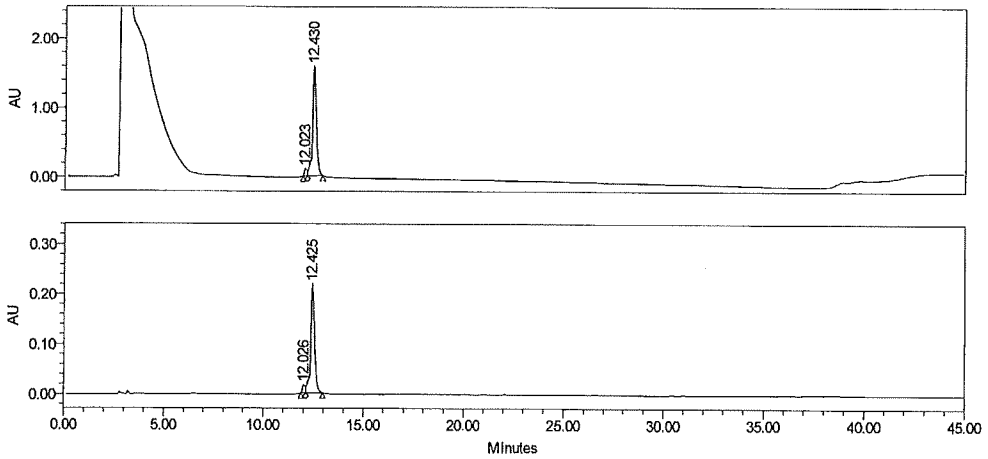
Empower software

Dual Channel Summary

Reported by User: System Project Name: BIO5_HPLC1

SAMPLE INFORMATION

Sample Name:	esh B3P50	Acquired By:	System
Sample Type:	Unknown	Date Acquired:	1/9/2014 5:43:06 PM MST
Vial:	75	Acq. Method Set:	10_90B_in
Injection #:	1	Date Processed:	1/10/2014 1:33:22 PM MST,
Injection Volume:	10.00 ul	Processing Method	Peptide_general
Run Time:	45.0 Minutes	Channel Name:	2487Channel 1, 2487Channel 2
Sample Set Name	Prime_Run_07_19_07	Proc. Chnl. Descr.:	220nm, 280nm



Channel 220 nm
Channel: 2487Channel 1

	RT	Area (µV*sec)	% Area	Channel
1	12.430	2.14e+007	95.88	2487Channel 1

Channel 280 nm
Channel: 2487Channel 2

	RT	Area (µV*sec)	% Area	Channel
1	12.425	2.94e+006	95.14	2487Channel 2

NOTES:

Report Method: Dual Channel Summary Printed 1:36:30 PM 1/10/2014 Page: 1 of 1

Molecular Dynamics Studies

To model the molecular orientations of the MSH(4) ligands of compounds **6**, **14c**, **16c**, and **18** at or near physiologic conditions and temperatures, molecular dynamics simulations were carried out using MOE2013.08 (Chemical Computing Group, Inc., Montreal) and programs therein. The AMBER 99 force field was used. Before starting the dynamics simulation, each molecule was first built and energy minimized, solvated in a water shell (between 4800 and 5900 waters per molecule), then re-minimized to relax unfavorable contacts. In each case, the system was then heated from 300 to 310 K over 100 ps. Following equilibration for 100 ps, the production stage continued for 2 ns, with trajectories recorded every 0.5 ps starting with the heating stage. The lowest energy conformation of each molecule observed during its MD run is depicted in Figure 3 of the article.

Protein Assay for Fluorescence Normalization

The dissolved protein content in each of the micro-centrifuge tubes from the bioassays was quantified using the Bio-Rad protein assay.[1] The manufacturer-supplied lyophilized BSA standard was reconstituted in DI water to provide a standard solution with a concentration of 1.4 mg/mL. By serial dilution, five solutions (0.50, 0.25, 0.12, 0.080, and 0.050 mg/mL) of BSA were prepared. Dye reagent was prepared by dilution of 25 mL of the dye concentrate (Bio-Rad) into 100 mL of DI water, followed by filtration through a Whatman #1 filter. To quantify protein content in each assay sample, 3 × 10 μL of each supernatant solution from the micro-centrifuge tubes was transferred to a 96 well clear bottomed plate (Corning Inc., 353072, Falcon multiwell flat-bottom plates with lids, sterile). Control solutions were also placed in the same plate (3 × 10 μL of each BSA concentration) along with a blank (DI water, 3 × 10 μL). The reagent dye solution was then added to all wells using a multi-channel pipette at 200 μL per well. The sample and the reagent in the well were mixed by repeated (5×) depressing of the pipette plunger. After mixing, the plate was left at rt for 10-15 min, and the absorbance measured at 595 nm. Absorbance values from the standard solutions were used to construct a calibration curve (Figure S1).

[1] http://www.bio-rad.com/webroot/web/pdf/lsr/literature/LIT33.pdf

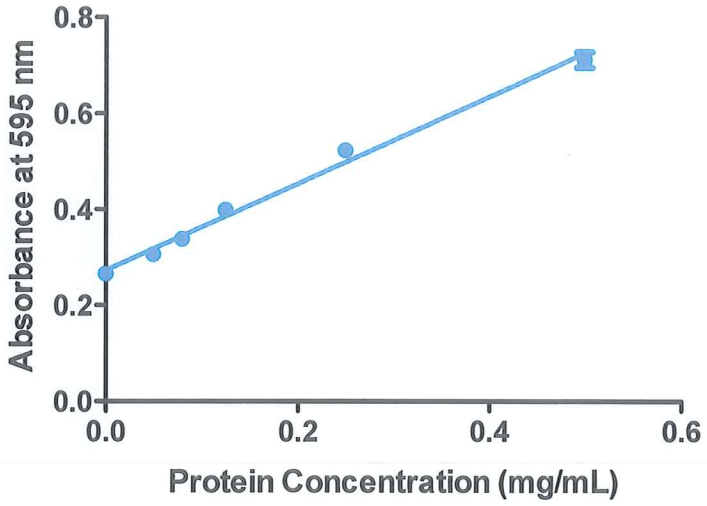


Figure S1. Representative protein assay calibration curve; $y = 0.9038\ x + 0.2725$ ($R^2 = 0.99$).

Using the protein content calibration curves, the dissolved protein content of each of the samples was determined. The observed fluorescence value of each sample (i.e. data point) was normalized to the dissolved protein content according to the following equation. Then, competition binding curves (Figure S2) were constructed using the normalized data values.

$$\text{F/mg protein} = \frac{\text{F}}{100\ \mu\text{L}} \times \frac{1000\ \mu\text{L}}{1\ \text{mL}} \times \frac{1}{[\text{protein}]\ \text{mg/mL}}$$

where F = measured fluorescence

[protein] = concentration of protein

Fluorescence Normalized Competition Binding Curves

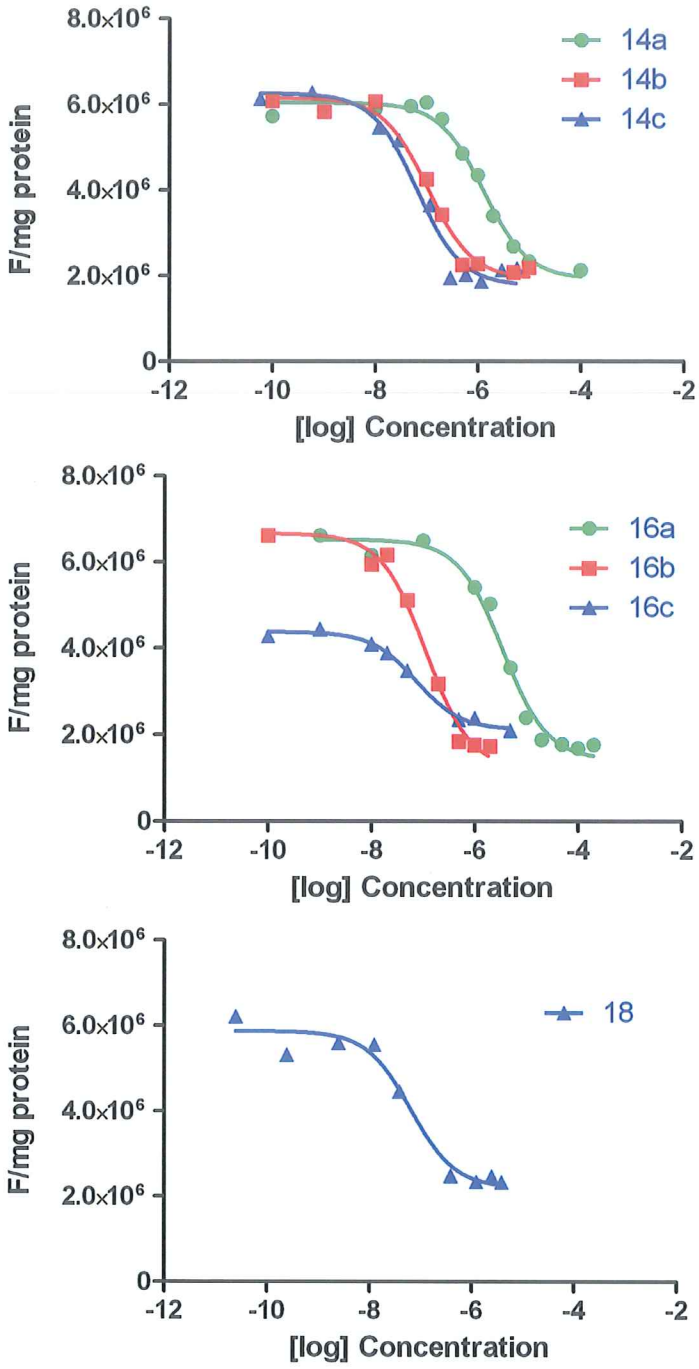


Figure S2. Competition binding curves (normalized to the protein content) for the compounds **14a-c** (*top*), **16a-c** (*middle*), and **18** (*bottom*) against probe **19** (20 nM) generated using the six-well plate assay method. Control compounds **12a** and **15a** were not competitive inhibitors of **19** over the concentration ranges tested.

K_i Values Based on Fluorescence Normalized Competition Binding Curves

Table S1. Results of competitive binding assays based on the fluorescence normalized data.[a]

Compound	$K_i \pm$ SEM (nM)[b]	Relative Potency[c]
12a	nb[d]	Not applicable
14a	600 ± 50	1
14b	64 ± 11	9
14c	45 ± 9	13
15a	nb[d]	Not applicable
16a	1730 ± 200	1
16b	72 ± 6	24
16c	40 ± 8	43
18	29 ± 1	Not applicable

[a]Competition experiments were carried out against probe **19** (K_d = 21 nM, [**19**] = 20 nM) using HEK293 cells overexpressing hMC4R and CCK2R.
[b]SEM = standard error of the mean; n = 5 independent determinations.
[c]Relative potency compared to the monovalent MSH(4) construct with the same scaffold core.
[d]nb = no competitive binding observed.

Data Analysis for the Binding Assays

Biological data analysis was performed using GraphPad Prism software (version 5.04) using the following specific analysis parameters.

Saturation Binding Data

The top graphs in Figure 2 were generated from the binding assay data using nonlinear regression analysis and fitted to the "One-site Total and Nonspecific Binding" equation.

$$\text{Specific} = B_{max}*X / (X + K_d) \qquad \text{(Equation S1)}$$

$$\text{Nonspecific} = NS*X + \text{Background} \qquad \text{(Equation S2)}$$

For Total Binding: Y = specific + nonspecific

For Nonspecific binding: Y = nonspecific

Where

- B_{max} is the maximum specific binding in the same units as Y.
- K_d is the equilibrium binding constant, in the same units as X. It is the labeled ligand concentration needed to achieve a half-maximum binding at equilibrium.
- NS is the slope of nonspecific binding in Y units divided by X units.
- Background is the amount of nonspecific binding with no added labeled ligand. This represents counter background. If the counter automatically subtracts off the background signal, Background can be constrained to a constant value of zero.

The bottom graph in Figure 2 was generated using nonlinear regression analysis and fitted to the "One site - Specific Binding with Hill Slope" equation.

$$Y = B_{max}*X\text{^}h/(K_d\text{^}h + X\text{^}h) \qquad \text{(Equation S3)}$$

Where;

- h = Hill slope.

Competitive Binding Data

Competitive binding data were analyzed using nonlinear regression analysis and fitted to the "One site - Fit K_i" equation.

$$\log EC_{50} = \log (10\text{^}\log K_i*(1 + [L]/[\text{Hot } K_d])) \qquad \text{(Equation S4)}$$

$$Y = \text{Bottom} + (\text{Top-Bottom}) / (1+10\text{^}(X - \text{Log } EC_{50})) \qquad \text{(Equation S5)}$$

Where;

- Top and Bottom are plateaus in the units of Y axis.
- K_i is the molar equilibrium dissociation constant of the unlabeled ligand.
- [L] is the concentration of labeled ligand in nM. Here [L] = 20 nM
- [Hot K_d] is the equilibrium dissociation constant of the labeled ligand in nM. Here [Hot K_d] = 21 nM.