Supporting information

α-Conotoxin GI Triazole-Peptidomimetics: Potent and Stable Blockers of a Human Acetylcholine Receptor

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General Information

Standard Fmoc-protected amino acids were purchased from CEM Corporation or Pepceuticals and HCTU and peptide grade DMF were purchased from Pepceuticals. Fmoc-L-Pra-OH was purchased from TCI Chemicals and Fmoc-L-Aha-OH was purchased from Chiralix. All other reagents were purchased from Sigma Aldrich. Native GI (control native compound) was purchased from Smartox Biotechnology, France.

Peptides were synthesised on a Biotage Initiator+ Alstra microwave assisted peptide synthesiser. Ruthenium catalysis was performed on a CEM Discover SP microwave.

Peptides were purified on a reverse-phase Dionex HPLC system equipped with Dionex P680 pumps and a Dionex UVD170U UV-vis detector (monitoring at 214 nm and 280 nm), using a Phenomenex, Gemini, C18, 5 um, 250 x 21.2 mm column. Gradients were run using solvents consisting of A (H_2O + 0.1% TFA) and B (MeCN + 0.1% TFA) and fractions were lyophilised on a Christ Alpha 2-4 LO plus freeze dryer.

Pure peptides were analysed on a Shimadzu reverse-phase HPLC (RP-HPLC) system equipped with Shimadzu LC-20AT pumps, a SIL-20A autosampler and a SPD-20A UV-vis detector (monitoring at 214 nm and 280 nm) using a Phenomenex, Aeris, 5 µm, peptide XB-C18, 150 x 4.6 mm column at a flow rate of 1 mL/min. RP-HPLC gradients were run using a solvent system consisting of solution A (5% MeCN in H_2O + 0.1% TFA) and B (5% H_2O in MeCN + 0.1% TFA). Two gradients were used to characterise each peptide; a gradient from 0% to 100% solution B over 20 min and a 50 min gradient from 0%-100% solution B. Analytical RP-HPLC data is reported as column retention time (t_R) in minutes (min). Low-resolution mass spectrometry (LRMS) was performed on a Thermo Scientific LCQ Fleet quadrupole mass spectrometer using electrospray ionisation in positive mode (ESI^+). High-resolution mass spectrometry (HRMS) was performed on a Bruker microTOF-Q II (ESI^+).

Peptide content was analysed on a Nanodrop 2000c using UV absorption of peptides at 280 nm.

NMR spectroscopy was performed using a Bruker AVANCE IIIHD 600 MHz instrument. Samples were prepared by dissolving 2.6 mg of peptide into 500 µL 95%H_2O/5%D_2O with 2 µL 3-(trimethylsilyl)propionic acid as an internal reference. pH was 3.1 for all samples.

Peptide synthesis

General information

Peptides were synthesised on 0.1 mmol scale using Tentagel S RAM (Rink amide) resin (Rapp Polymer).

Couplings were performed using 4 equivalents Fmoc-protected amino acid, 4 equivalents HCTU and 8 equivalents DIPEA. Coupling of standard Fmoc-protected amino acids was carried out for 10 min at 75 °C followed by 4 × 45 s washes. Arginine was double coupled; 60 min at room temperature followed by 5 min at 75 °C and repeated with fresh reagents followed by washing. Histidine and cysteine were coupled at room temperature for 5 min followed by 50 °C for 5 min and washes. For coupling of unnatural amino acids only 2 equivalents of amino acid and coupling reagents were used.

Deprotection was carried out in 20% piperidine in DMF + 5% formic acid for 30 s and then 3 min at 75 °C followed by washing.

Test cleavages were performed using 1 mL of cleavage cocktail containing 94% TFA, 2.5% Ethane dithiol (EDT), 2.5% H_2O and 1% Triisopropylsilane (TIS) for 30 min at room temperature. Final cleavage was performed using a cleavage cocktail (10 mL) consisting of 94% TFA, 2.5% EDT, 2.5% H_2O and 1% TIS. The resin was stirred for 3 hours at room temperature and the cleavage cocktail evaporated using a stream of nitrogen. The peptide was precipitated from solution with ice cold Et_2O, centrifuged at 3200 ×g for 5 min and the precipitate washed with ice cold Et_2O. The peptides were dissolved in H_2O/MeCN and lyophilised.

Synthesis of globular GI using orthogonal protection (**1**)

The native linear GI was synthesised using an orthogonal protection strategy for pairs of cysteine residues i.e. Cys2 and Cys7 Fmoc-Cys(Trt)-OH, Cys3 and Cys13 Fmoc-Cys(Acm)-OH.

Following microwave assisted SPPS synthesis the peptide was cleaved from the resin and acid labile protecting groups removed as described above. The crude peptide was oxidised overnight in degassed 0.1 M NH_4HCO_3, pH 7.5, purified and lyophilised as described above. The Acm-protected peptide was then dissolved in degassed 50/50 MeOH/H_2O at a peptide concentration of 0.5 mg/mL and oxidised using 5 eq. of I_2 (dissolved in a small amount of MeOH). The reaction was monitored using LC-MS and quenched by addition of 1M Sodium ascorbate (NaAsc) until colourless. The peptide was lyophilised and purified as described in the general information section above.

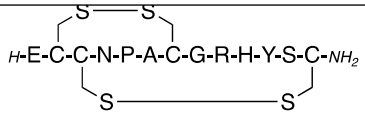
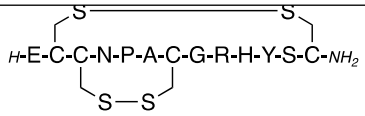
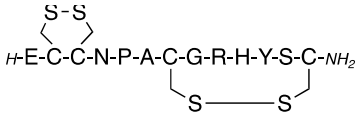
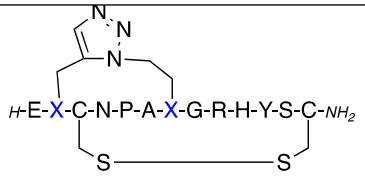
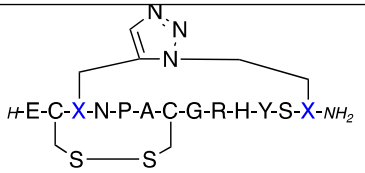
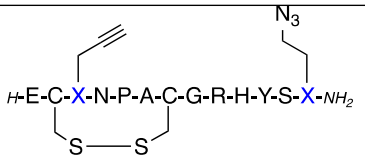
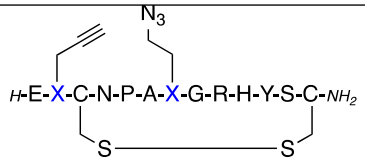
Synthesis of 1,5-triazole peptidomimetics (**10** and **11**)

Linear peptides were prepared using microwave assisted SPPS synthesis using Fmoc-Cys(Trt)-OH for the two cysteines, on a 0.1 mmol scale as specified above, leaving the peptide *N*-terminal Fmoc-protected. The resin was then washed thoroughly with DCM and dried in a desiccator for 24 h. The resin was swollen in 4 mL dry, degassed DMF for 20 min, 20mol% Cp*RuCl(COD) was added and the mixture degassed for 10 min before being reacted in a CEM microwave at 70 ^oC for 1 h. Reaction progress was monitored by IR (disappearance of azide absorbance at 2100 cm^{-1}) and confirmed by test cleavages and analysis by HPLC. If needed the reaction was repeated. When full conversion was observed the *N*-terminal Fmoc-protection was removed in 20% piperidine in DMF 2 x 30 min. The peptide was then cleaved from resin and acid labile protecting groups removed and purified as specified above. The peptide was dissolved in 50/50 degassed MeOH/H_2O at a concentration of 0.5 mg/mL and oxidised by dropwise adding a solution of 0.06 M I_2 in MeOH until a yellow colour persisted. The reaction was monitored by LCMS and quenched by the addition of 1 M NaAsc solution until the solution was colourless. The reaction mixture was lyophilised and the peptide purified as specified in the general information section above.

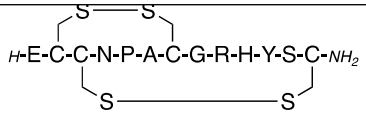
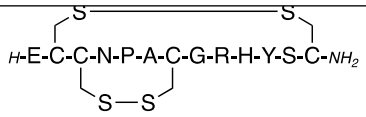
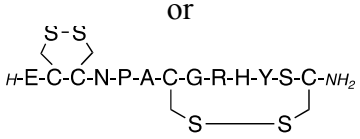
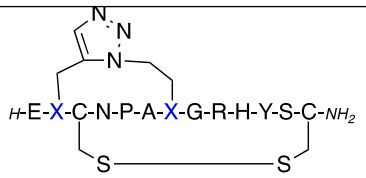
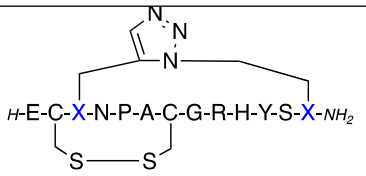
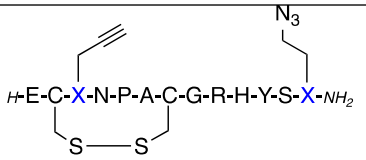
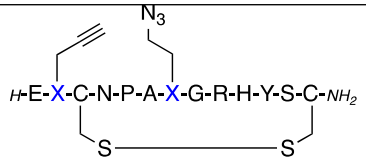
Synthesis of control peptides (**12** and **13**) & oxidative folding of linear native GI (**1**, **2** and **3**)

Linear peptides were synthesised using microwave assisted SPPS using Fmoc-Cys(Trt)-OH for the cysteine residues and cleaved from resin/globally deprotected as described above. The peptide was dissolved in 50/50 degassed MeOH/H_2O at a concentration of 0.5 mg/mL and oxidised by dropwise addition of a solution of 0.06 M I_2 in MeOH until a yellow colour persisted. The reaction was then quenched by the addition of a 1 M NaAsc solution until the solution was colourless. The reaction was lyophilised and purified as specified in the general information section above.

Peptide characterisation

Compound	Structure	t_R (min)[a]	Purity (214 nm/280 nm)[b]	Yield
1		10.16/16.93	>97%/>99%	23%
2		9.48/15.39	>98%/>99%	10%
3	or 	9.91/16.35	>96%/>97%	5%
10		12.00/17.33[c]	>98%/>98%	21%
11		9.57/15.59	>97%/97%	8%
12		10.44/17.35	>98%/>98%	12%
13		10.45/17.27	>99%/>97%	6%

[a] Retention time (t_R) is reported using a gradient from 0-100% solution B over 20 min and over 50 min, respectively.
[b] Lowest purity of the two gradients used.
[c] Analysis was performed at 45 °C

Compound	Structure	Calculated MW [M+2H]	Observed MW [M+2H]	Δ MW (ppm)
1		719.2494	719.2499	-0.7
2		719.2494	719.2477	2.4
3	or 	719.2494	719.2488	0.8
10		727.7937	727.7937	0.0
11		727.7937	727.7922	2.1
12		727.7937	727.7921	2.2
13		727.7937	727.7926	1.4

Calcium Response Assays

Cell culture

CN21 cells, a gift from Dr David Beeson (Institute of Molecular Medicine, John Radcliffe Hospital, Oxford, UK), were grown using standard cell culture techniques in Dulbecco's Modified Eagle Medium (DMEM) (Sigma-Aldrich, UK), supplemented with 10% Foetal Bovine Serum (Sigma-Aldrich, UK), 50 units/ml penicillin, 50 µg/ml streptomycin (ThermoFischer Scientific, UK), 2 mM GlutaMax (ThermoFischer Scientific) and 0.5 mg/mL geneticin (Sigma-Aldrich) and kept in a 37 °C humidified incubator with 5% CO_2. After washing with Ca^{2+}/Mg^{2+}-free phosphate buffered saline, cells were harvested using TrypLE Express (ThermoFischer Scientific) and plated into flasks at ratios of 1:5-1:10 for proliferation.

Calcium response assay

CN21 cells were harvested as described above and plated out onto clear-bottomed, black-walled 96 well tissue culture plates, at densities of 40000 or 20000 cells per well, with assays performed after 24 h or 48 h respectively. The growth medium was removed and 50 µL of dye from a FLIPR calcium 5 kit, made according to manufacturer's instructions (VWR International, UK), was added and diluted 50:50 with DMEM:Hank's balanced salt solution and 20 mM HEPES, pH 7.4 (1:1) (all Sigma-Aldrich). The dye solution contains 20 µM atropine (Sigma-Aldrich) to inhibit muscarinic receptor mediated responses. The cells were then incubated for 30 minutes in the dark, with conotoxins or mimetics (made up as described below) added to the cells 5 minutes prior to analysis.

All test compounds were stored at -20 °C, except during manipulations. Prior to analysis, test compounds were made up to 400 µM stock solutions with phosphate buffered saline and stored at -20 °C in individual aliquots. Aliquots were thawed on the day of the experiment, with further dilutions made in Hank's balanced salt solution with 20 mM HEPES at pH 7.4, to achieve the desired concentrations. Each plate included 11 different concentrations of the compound, with at least 3 technical replicate wells and 'blank' wells where no ACh was added. Each compound was tested in 4-6 independent experiments.

The responses of the cells to ACh were analysed using a FlexStation II fluorescence plate reader (Molecular Devices, UK), with fluorescence measured at approximately 1.5 s intervals for 60 s. Excitation and emission wavelengths were set to 485 and 525 nm respectively, with a cut-off at 515 nm and all measurements were made at room temperature. Addition of ACh to a final concentration of 150 µM was performed automatically by the FlexStation approximately 15 seconds after analysis was started. The response of CN21 cells to ACh in this assay has already been characterised; 150 µM ACh induced a maximal response in CN21 cells, without the inhibitory effects seen at higher doses.[1] Before this series of experiments, the response of CN21 cells to ACh was briefly assessed and it was confirmed the response was consistent with the previously published data (results not shown).

Responses were quantified as the maximum response, minus the baseline (an average of the first 15 seconds prior to the ACh addition) using the SoftMax Pro software (Molecular Devices). All further analysis was conducted using GraphPad Prism version 6.0 for Windows (GraphPad Software, La Jolla California, USA). A sigmoidal dose-response curve with a variable slope was fitted to the raw

fluorescence values to determine the IC_{50}, and data were normalised to the predicted top and bottom best-fit values for presentation. Where complete antagonism of the ACh signal was not achieved, data were normalised to the fitted maximum and agonist-free blank values for presentation. The maximal inhibition at 10 μM has also been reported, to allow comparison of these compounds.

Compound	IC_{50} (nM) (95% CI)	pIC_{50} ± SE	Maximum inhibition (%) at 10 μM ± SEM
1, (Globular GI)	9.8 (7.4 to 12.8)	8.01 ± 0.08	100
Commercial GI	8.8 (6.6 to 11.6)	8.06 ± 0.06	100
2 or **3** (Ribbon/Bead)	857 (548 to 1344)	6.07 ± 0.12	100
2 or **3** (Ribbon/Bead)	969 (713 to 1317)	6.01 ± 0.09	100
10	N.D.	N.D.	1.5 ± 9.7
11	8.2 (6.4 to 10.5)	8.09 ± 0.07	100
12	140 (33.4 to 587)	6.85 ± 0.31	56.2 ± 7.9
13	203 (84.4 to 487)	6.69 ± 0.20	43.9 ± 5.6

Table S1: Full testing data from the CN21 cell inhibition studies.
Comparison of the synthesised GIs and mimetics compared to a commercially obtained GI peptide. Data is represented both as IC50s with 95% confidence intervals (95% CI) and pIC50s with standard error (pIC50 ± SE).

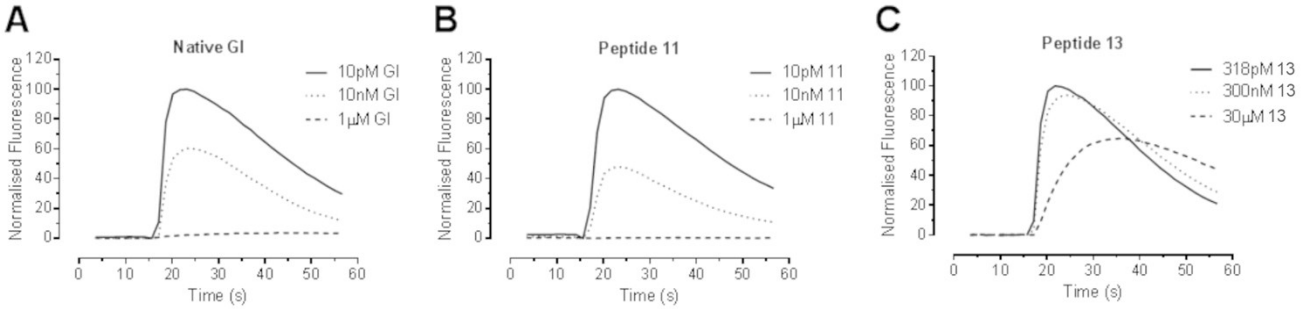


Figure S1: Examples of Calcium Responses.
This figure shows example responses from CN21 cells exposed to different concentrations of conotoxin or mimetics, with each line representing the mean of 4 technical replicates across an individual plate. Responses shown on the same graph were generated on the same plate. Responses were normalised to both baseline values prior to the addition of ACh (0%) and the maximal response seen in the lowest concentration of conotoxin/mimetic (100%). (A) shows the responses to addition of native globular GI, (B) shows the response to the addition of **11**, a potent mimetic and (C) shows the response to addition of **13**, one of the less potent mimetics.

Stability assay

Solutions of peptides were prepared by dissolving in PBS buffer (pH 7.4) to a concentration of 1 mg/mL. To 250 μL of peptide solution was added 250 μL of rat plasma (Sigma-Aldrich). The solutions were incubated at 37 °C and 50 μL aliquots taken at t = 0, 30 min, 1 h, 2 h, 4 h, 24 h and

48 h. Samples were quenched with 50 µL MeCN and 300 µL of 2% TFA/water and were then centrifuged at 13,800 ×g for 5 min. The supernatant was analysed by analytical HPLC as described previously. Controls containing only plasma in buffer and peptide alone in buffer (negative control) were prepared simultaneously. Samples were run with a linear gradient of 0-100% buffer B over 20 mins (buffer A: 95:5 v/v H_2O/MeCN + 0.1% TFA and buffer B: 95:5 v/v MeCN/H_2O + 0.1% TFA).

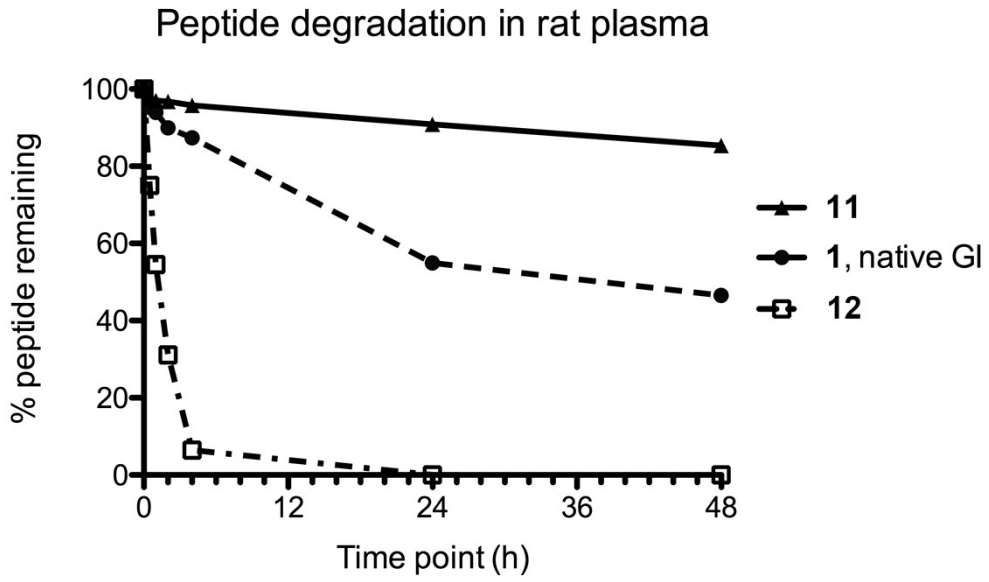


Figure S2: Degradation of peptides and peptidomimetics. **1** (native GI), mimetic **11** (1,5-disubstituted mimetic) and control mimetic **12**. The assay was run in PBS buffer at pH 7.4 using rat plasma (n=3).

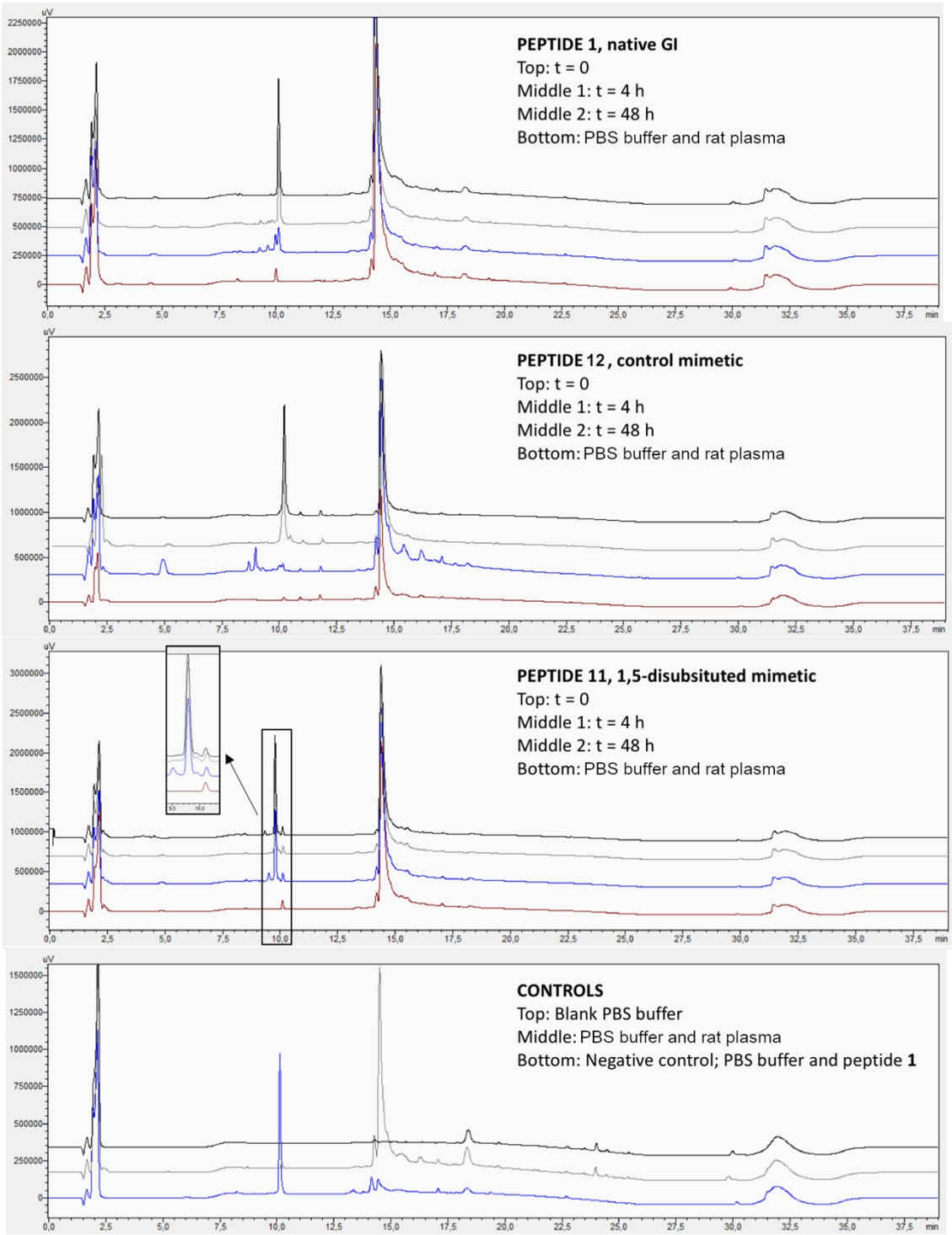


Figure S3: HPLC data degradation at selected time points.
Top chromatogram shows degradation of the native GI **1**; Top trace (black) shows t = 0, Middle 1 (grey) t = 4 h, Middle 2 (blue) t = 24 h and Bottom trace (red) shows the plasma control (PBS buffer and rat plasma). Second and third chromatograms show the control mimetic **12** and the best mimetic **11**, respectively, in a similar manner to the top trace. Bottom panel shows control chromatograms; Top trace is blank PBS buffer, middle trace is the plasma control (PBS buffer and rat plasma) whereas the bottom trace shows the negative control (PBS buffer and peptide).

NMR determination of triazole substitution pattern

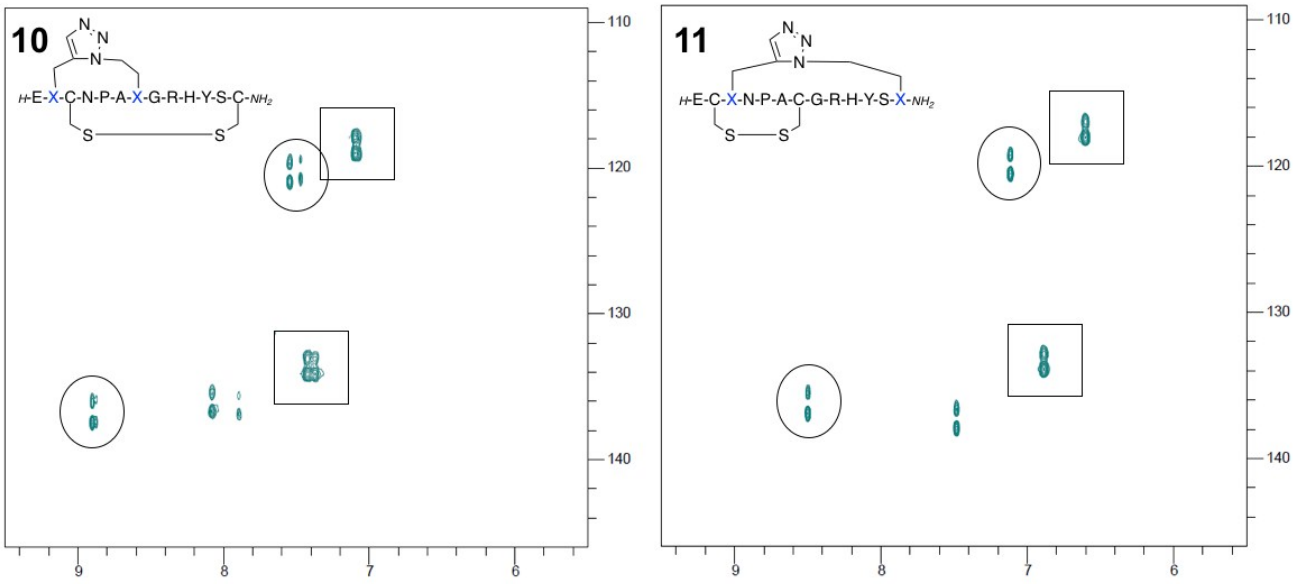


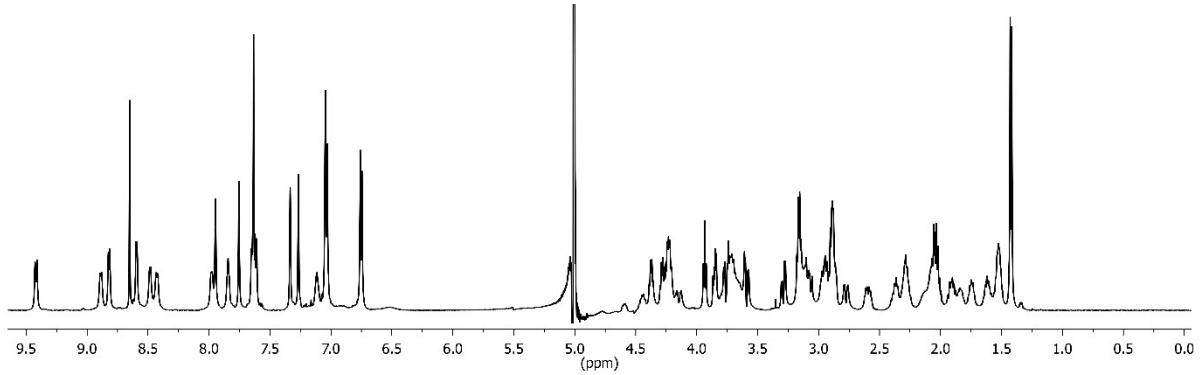
Figure S4: Determination of the 1,2,3-triazole substitution pattern of **10** and **11**.
The aromatic region of non-decoupled HSQC spectra recorded in 95% H_2O/5% D_2O with water suppression at 5 °C. The peaks from the histidine aromatic protons are circled, whereas the tyrosine aromatic peaks are boxed. The remaining peaks originate from the non-substituted carbon from each triazole, where the 1,5-disubstituted 1,2,3-triazole shifts are both above 135 ppm. The triazole multiplets display coupling constants of between 180-190 Hz, as expected. The additional peak for each aromatic group show that mimetic **10** has two different conformations in contrast to the single conformation seen for **11**.

NMR Solution Structure Experiments

1,5-triazole GI mimetic **11** was dissolved in H_2O:D_2O (90:10, 1.00 mM). Spectra were acquired on a Bruker® Avance III HD 600 MHz spectrometer equipped with a TCI cryo-probe at 298 K. Proton chemical shifts were referenced to the H_2O offset frequency and carbon chemical shifts calculated from the proton reference. Frequency-based methods were employed to measure $^3J_{HN\alpha}$ couplings from in-phase/antiphase ^1H-^1H COSY spectra.[1] Signal assignment was performed on CcpNmr Analysis software using TOCSY/COSY/^1H-^{13}C HSQC spectra.[2] Distance restraints for structure calculations were derived from ^1H-^1H NOESY spectra recorded at 80, 120, 200, 300 ms mixing times.

NMR Assignment

1H



aa	Position	δ (ppm)
1 Glu	α-C*H*	3.93
	α-*C*	55.23
	β-CH_a	2.37
	β-CH_b	2.28
	γ-CH_2	2.03
2 Cys	N*H*	8.81
	α-C*H*	4.59
	β-CH_a	3.11
	β-CH_b	2.97
3 Pra	N*H*	9.41
	α-C*H*	4.98
	β-CH_a	3.59
	β-CH_b	3.07
4 Asn	N*H*	7.61
	α-C*H*	5.12
	β-CH_a	2.90
	β-CH_b	2.88
	γ-NH_a	7.94
	γ-NH_b	7.04
5 Pro	α-C*H*	4.29
	α-*C*	65.04
	β-CH_a	2.29
	β-CH_b	1.91
	γ-CH_a	2.11
	γ-CH_b	1.83
	δ-CH_2	3.69
6 Ala	N*H*	8.59
	α-C*H*	4.24
	α-*C*	54.09
	β-CH_2	1.42
7 Cys	N*H*	7.98
	α-C*H*	4.66
	β-CH_a	3.62
	β-CH_b	2.78
8 Gly	N*H*	7.84
	α-CH_a	4.14
	α-CH_b	3.76
	α-*C*	46.02
9 Arg	N*H*	7.11
	α-C*H*	4.28
	α-*C*	43.10
	β-CH_a	1.75
	β-CH_b	1.61
	γ-CH_2	1.52
	δ-CH_2	3.16
10 His	N*H*	8.42
	α-C*H*	4.77
	α-*C*	54.67
	β-CH_a	3.28
	β-CH_b	3.11
	δ-N*H*	8.65
	ε-N*H*	7.26
11 Tyr	N*H*	7.64
	α-C*H*	4.36
	α-*C*	58.56
	β-CH_a	2.94
	β-CH_b	2.87
	δ-C*H**	7.03
	ε-C*H**	6.75
12 Ser	N*H*	8.48
	α-C*H*	4.37
	α-*C*	58.59
	β-CH_a	3.85
	β-CH_b	3.72
13 Tha	N*H*	8.88
	α-C*H*	4.79
	α-*C*	53.78
	β-CH_a	2.59
	β-CH_b	2.13
	γ-CH_a	4.44
	γ-CH_b	4.22
	H4	7.63
14 NH_2	NH_a	7.75
	NH_b	7.33

CCPNmr/ARIA

The non-natural (THA) residue ChemComp was produced using CcpNmr ChemBuild software.[2] Topology and parameter descriptions for THA were derived from crystal structure data (CCDC # 817836) and histidine. The non-natural residue was then included in the TOP/PARALLHDG5.3. force field (Figure S5).[3]

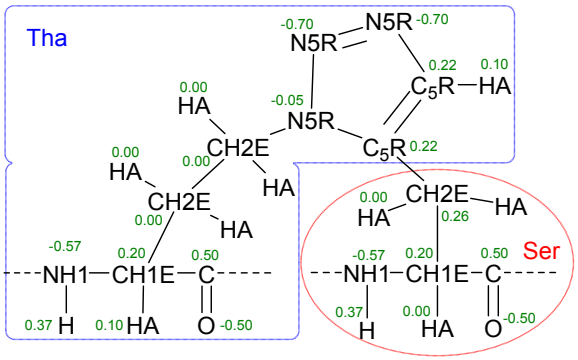


Figure S5: Atom type definitions for the 1,5 triazole linkage used in CNS/ARIA simulations. Atom types and charges used for each atom used in the description of the mimetic link.

CCPN conversion libraries in ARIA (atom/residue descriptors), the ARIA GUI and ARIA patch algorithms were also modified to allow the incorporation of the non-natural residue. Restrained molecular dynamics were performed in ARIA 2.3/CNS 1.2 in Cartesian coordinates and using matrix relaxation refinement. 100 structures were calculated in the final iteration.[4] The disulfide link and the triazole cyclisation were both closed at the beginning of the calculation. The 20 lowest energy structures from the initial iterations were then refined in explicit water. The structural statistics generated are given below (Table S2) and the ensemble superposition shown (Figure S6).

Table S2: Structural statistics for calculated NMR ensemble

NOE distance restraints	
Total NOE	160
Ambiguous	54
Unambiguous	106
Intra-residue	45
Inter-residue	115
Sequential (|i − j| = 1)	47
Short range (1 < |i − j| 4)	31
Long range (< |i − j| 5)	37
Violations > 0.5 Å	0.05
Violations > 0.3 Å	0.30
Distance restraints RMSD (Å)	0.044
Coordinate RMSD (Å)	
Backbone	0.504
All heavy atoms	1.193
Energy values (mean ± S.D.)	
Bond energies (J)	5.176 ± 1.200
Angle energies (J)	50.666 ± 2.161
Impropers (J)	65.930 ± 19.343
Parameter RMSD from idealized geometry (mean ± S.D.)	
Bond lengths (Å)	$0.00520 \pm 5.8 \times 10^{-4}$
Bond angles (◦)	0.997 ± 0.021
Impropers (◦)	1.981 ± 0.286

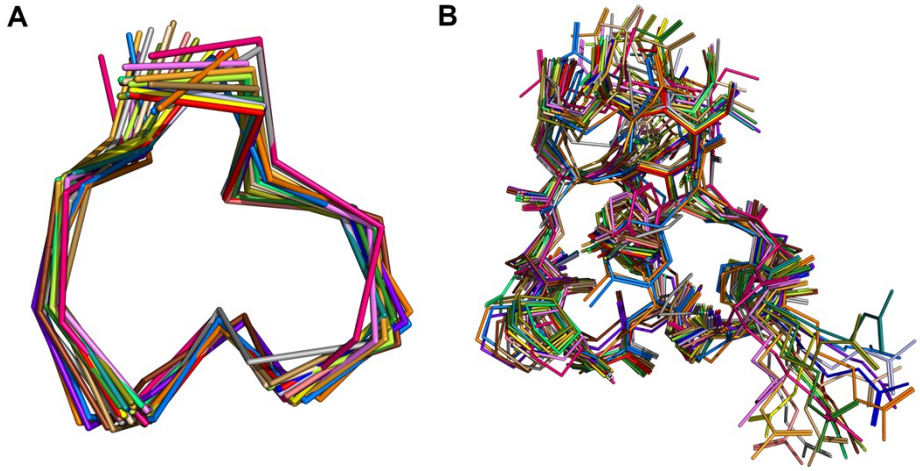
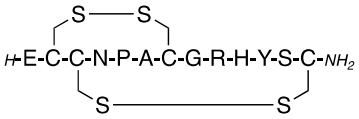


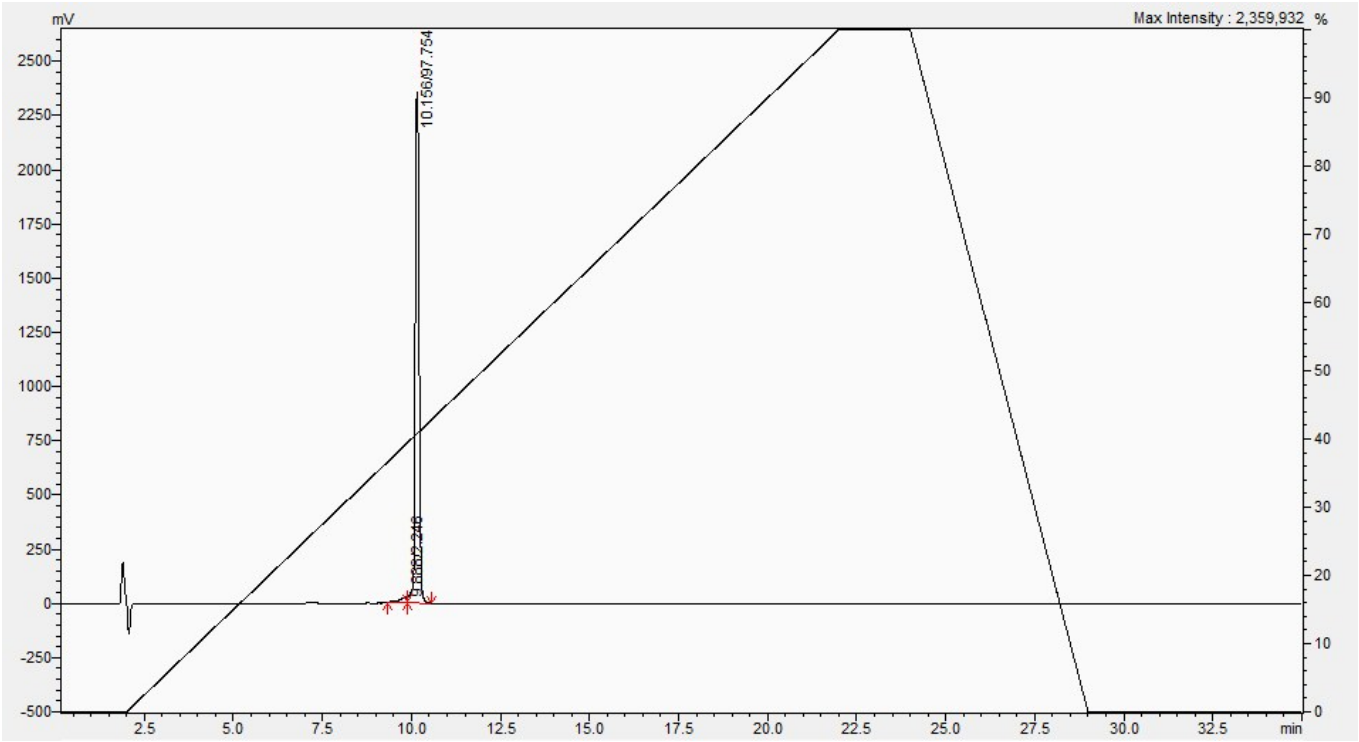
Figure S6: Structure ensemble. **A** The ensemble of 20 structures superimposed on the backbone atoms (RMSD 0.50 Å) with each structure shown as a differently coloured C-alpha trace.**B** The ensemble of 20 structures superimposed on all heavy atoms (RMSD 1.19 Å) shown as sticks with hydrogens omitted.

HPLC peptide chromatograms

Native GI, compound **1**



20 min gradient 214 nm



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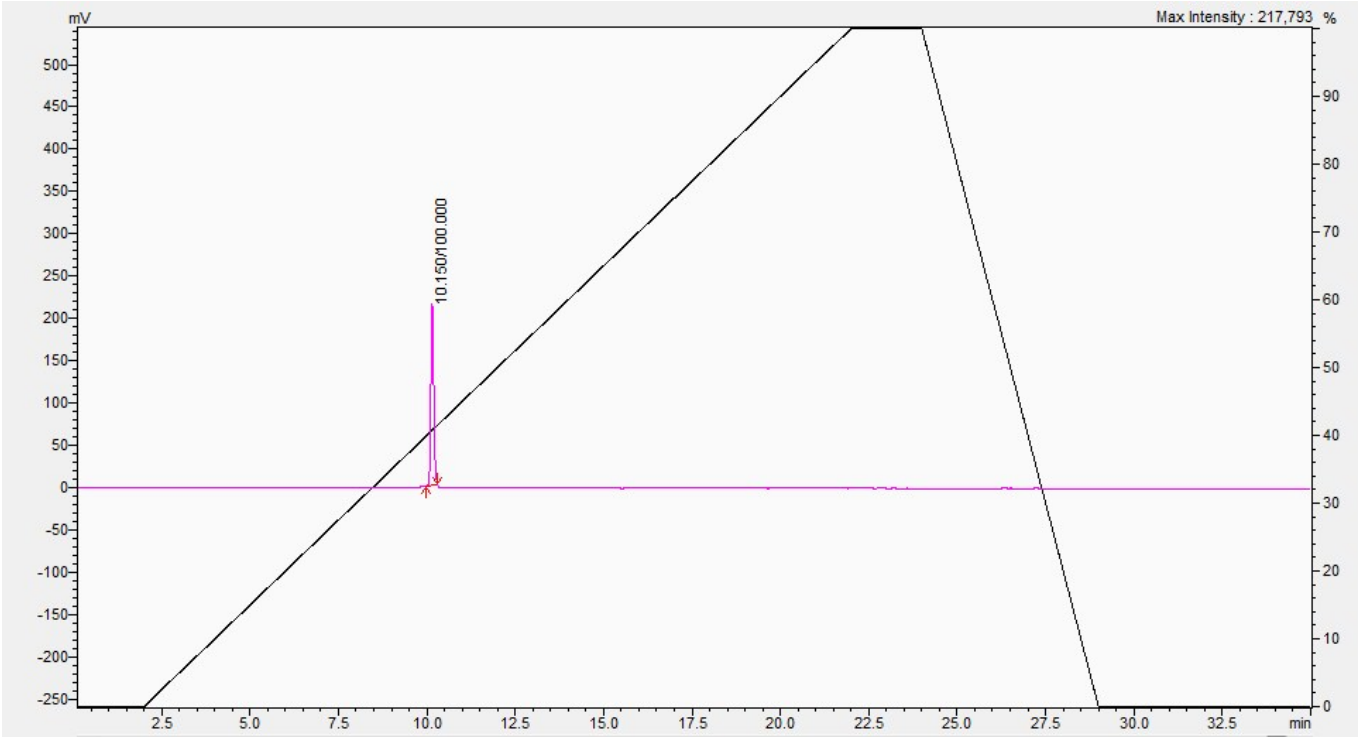

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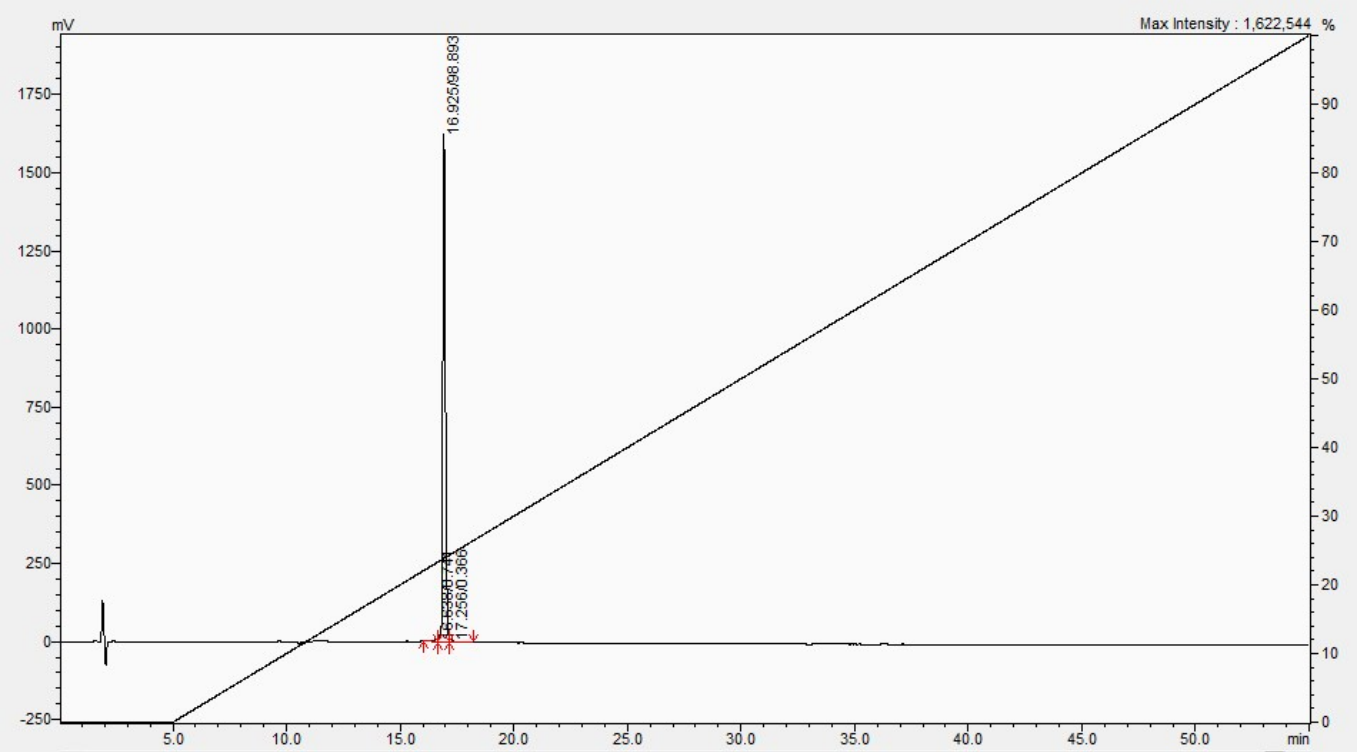

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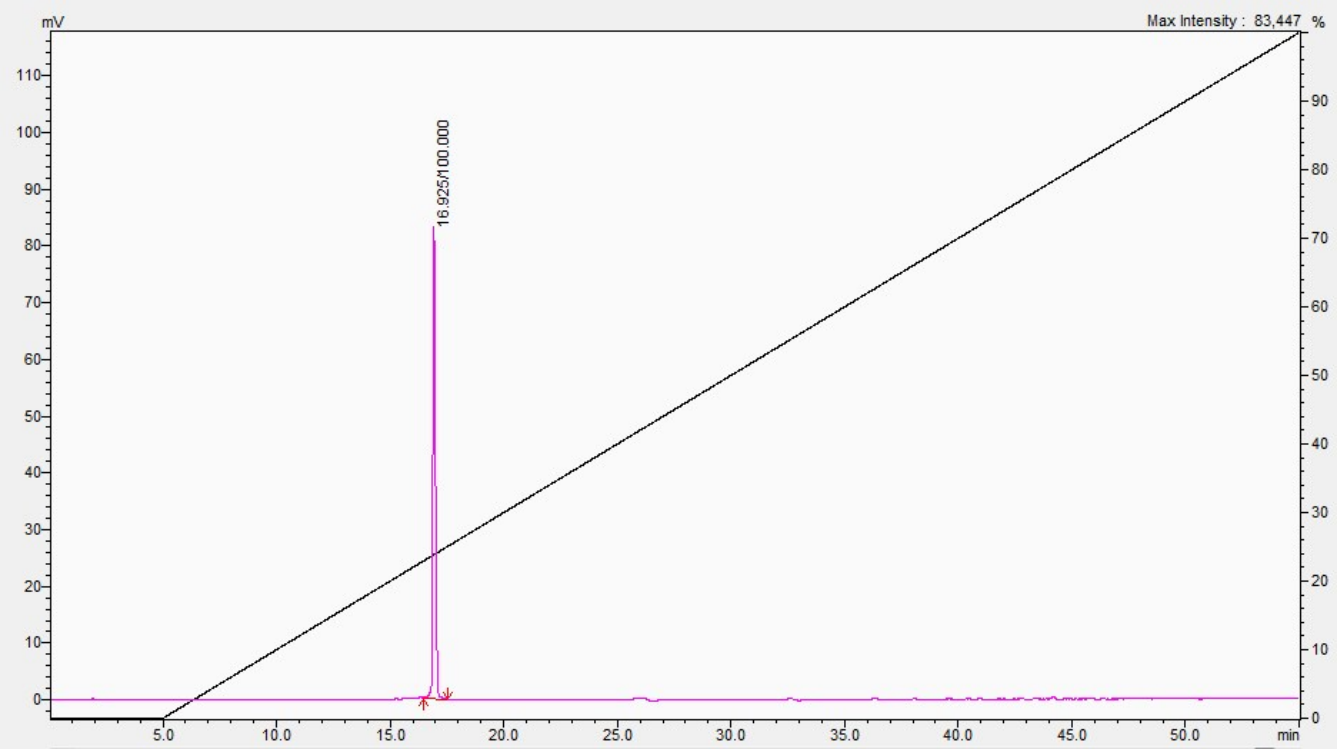
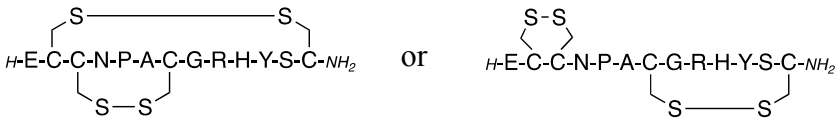


Ribbon or bead, compound **2** or **3**



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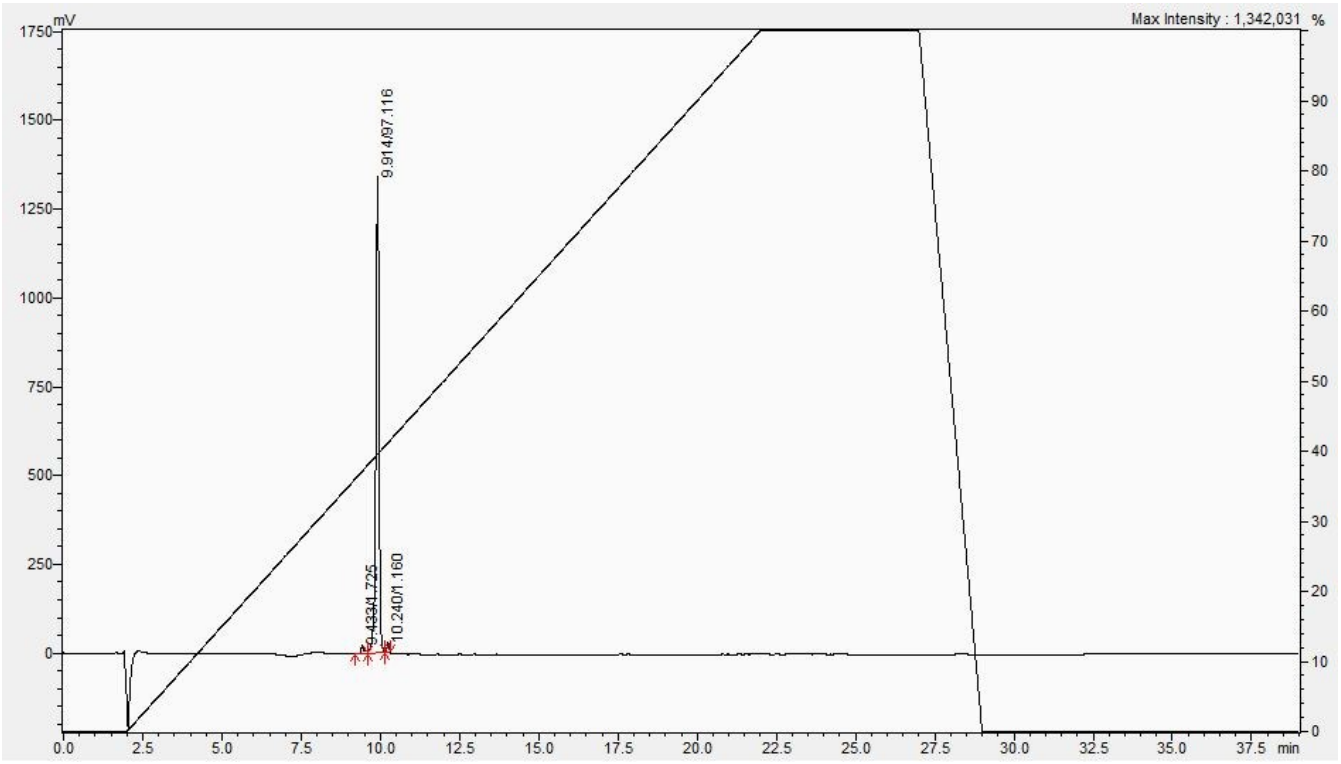

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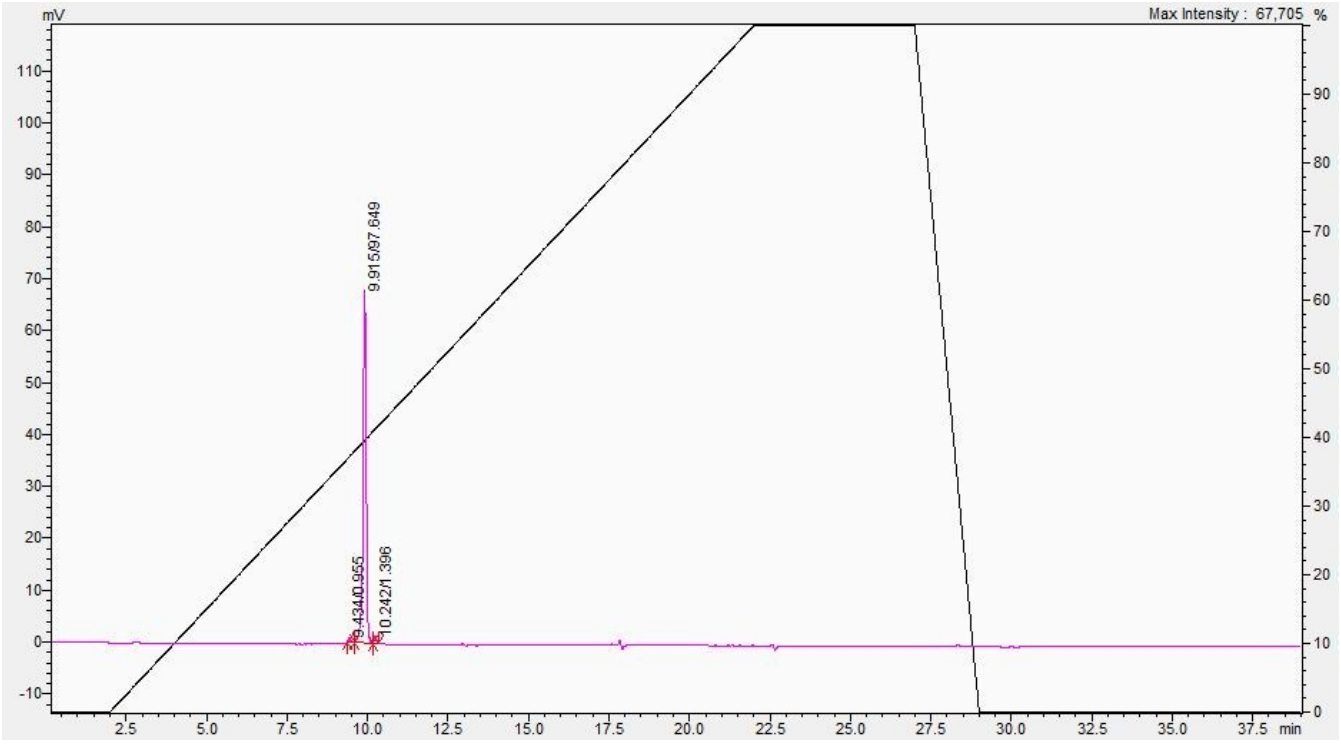

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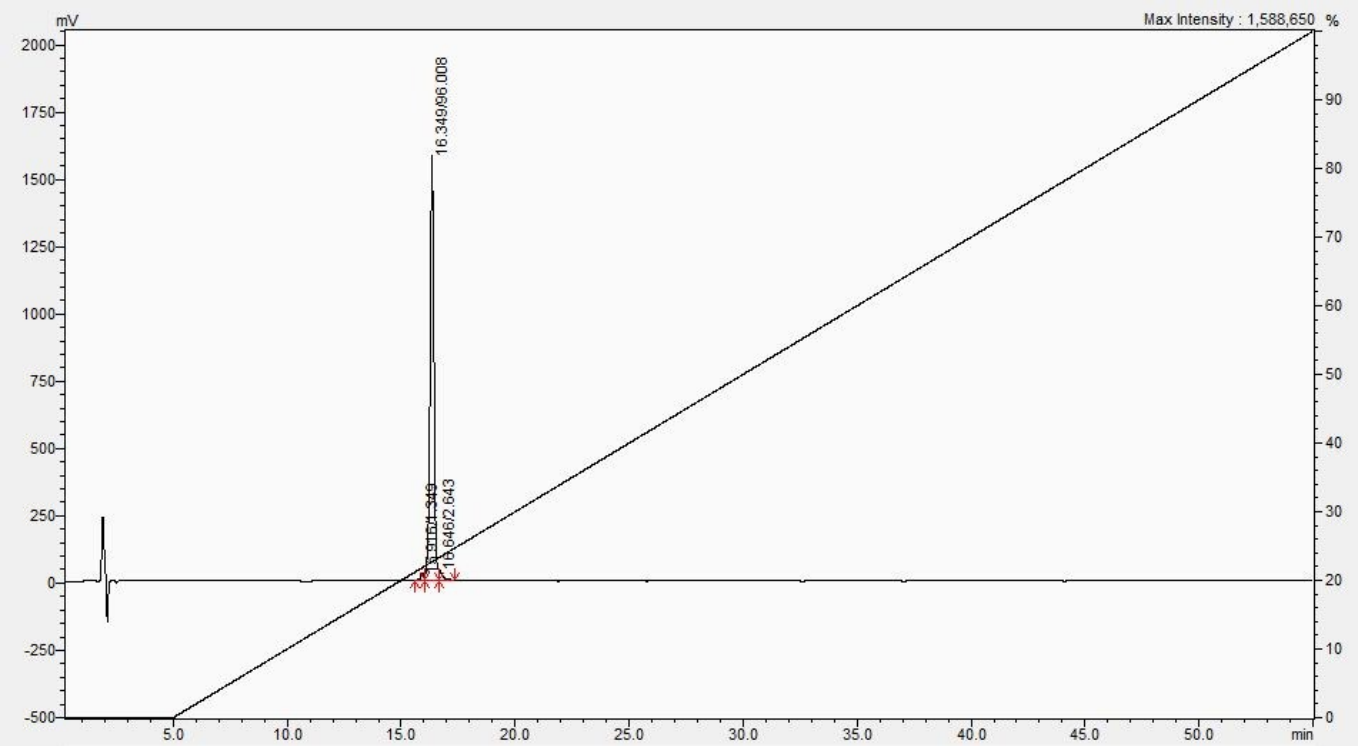

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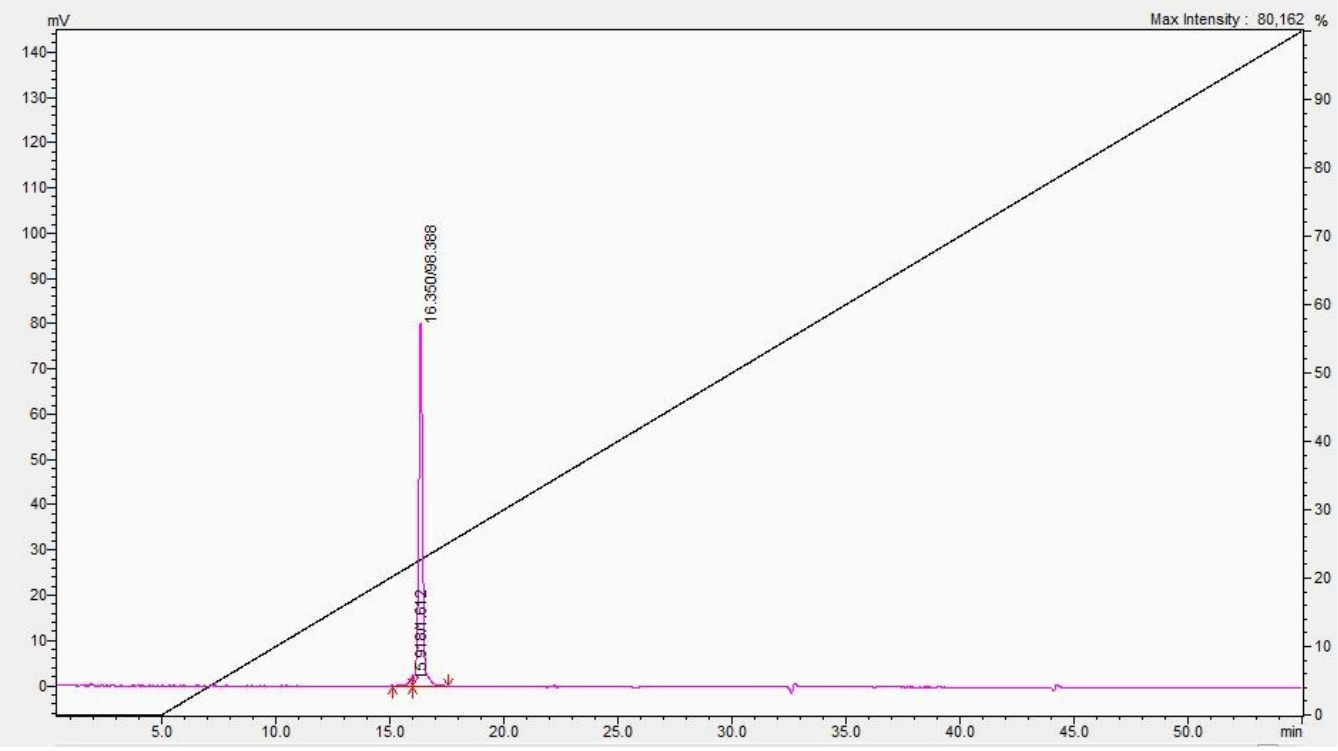
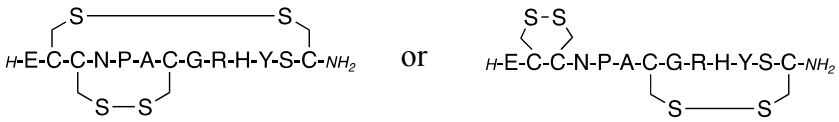


Ribbon or bead, compound **2** or **3**



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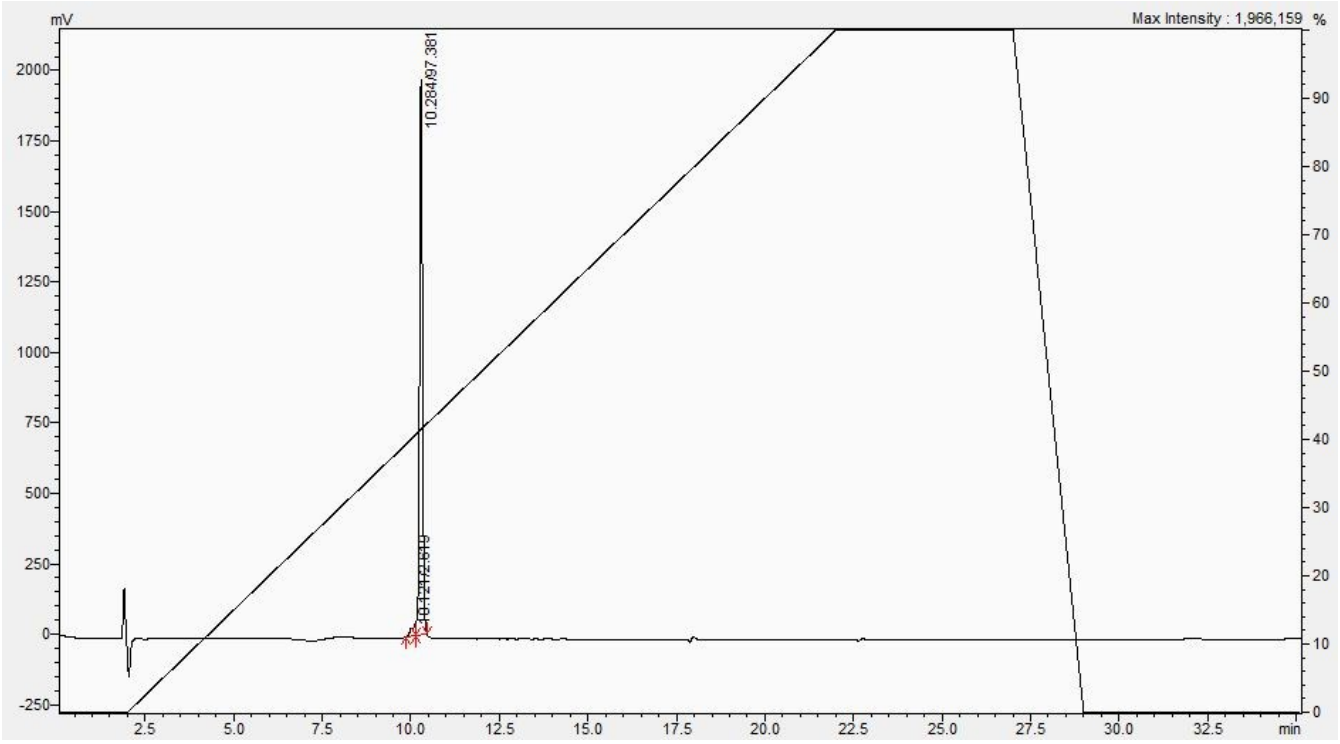

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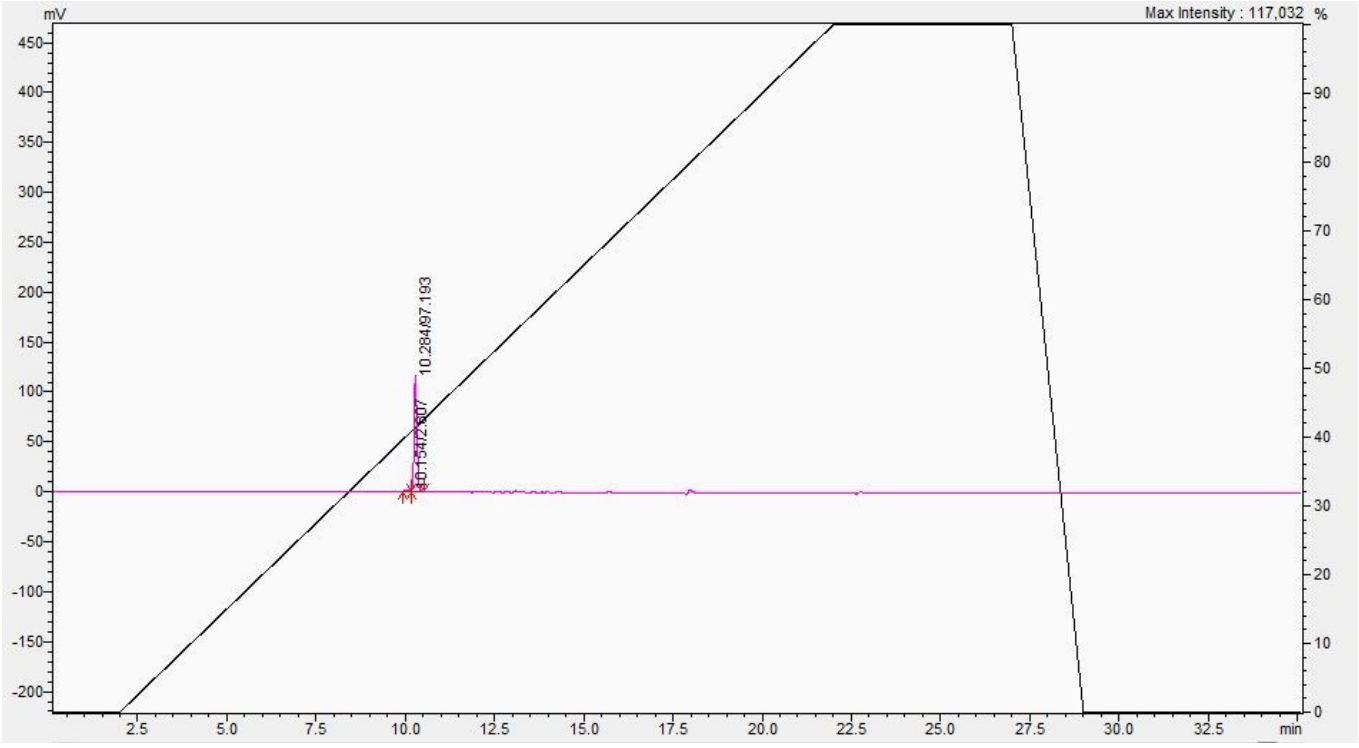

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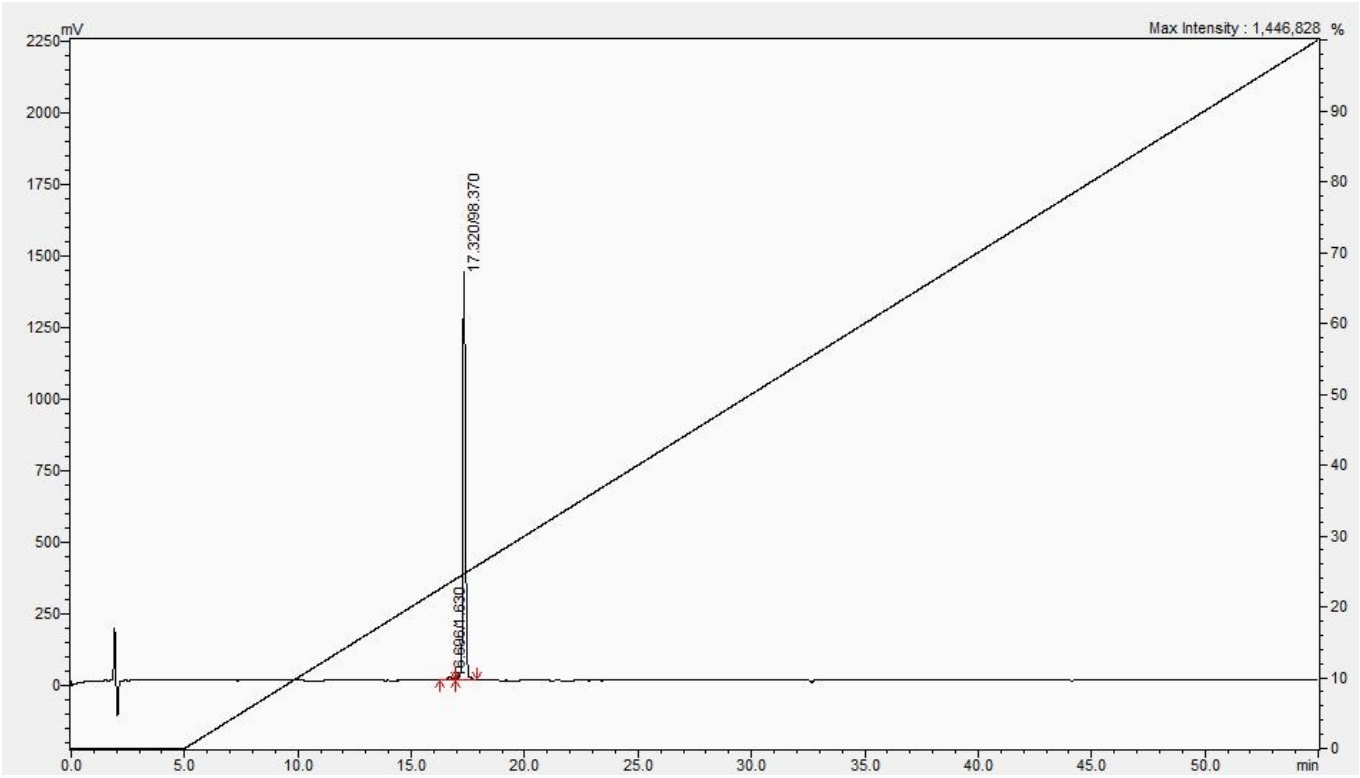

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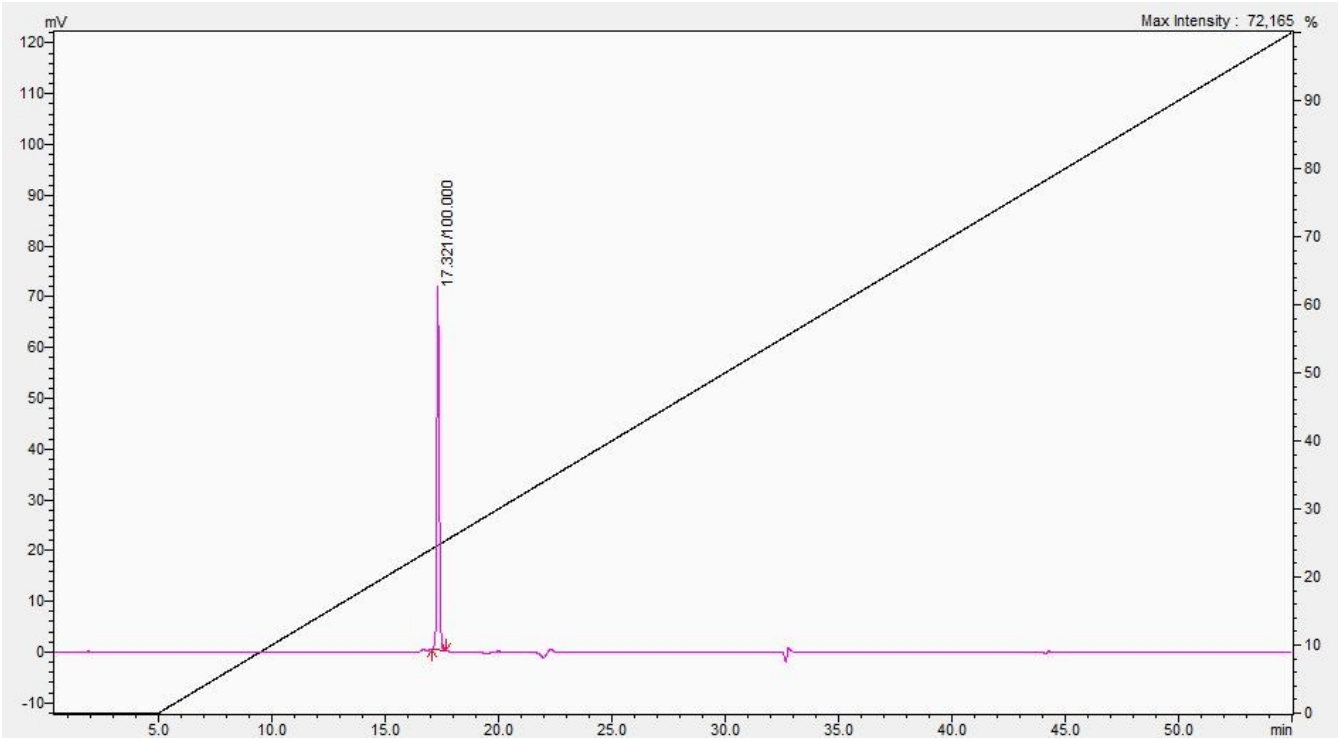
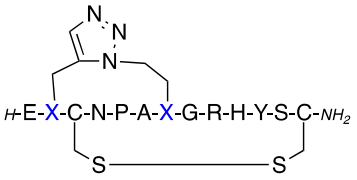
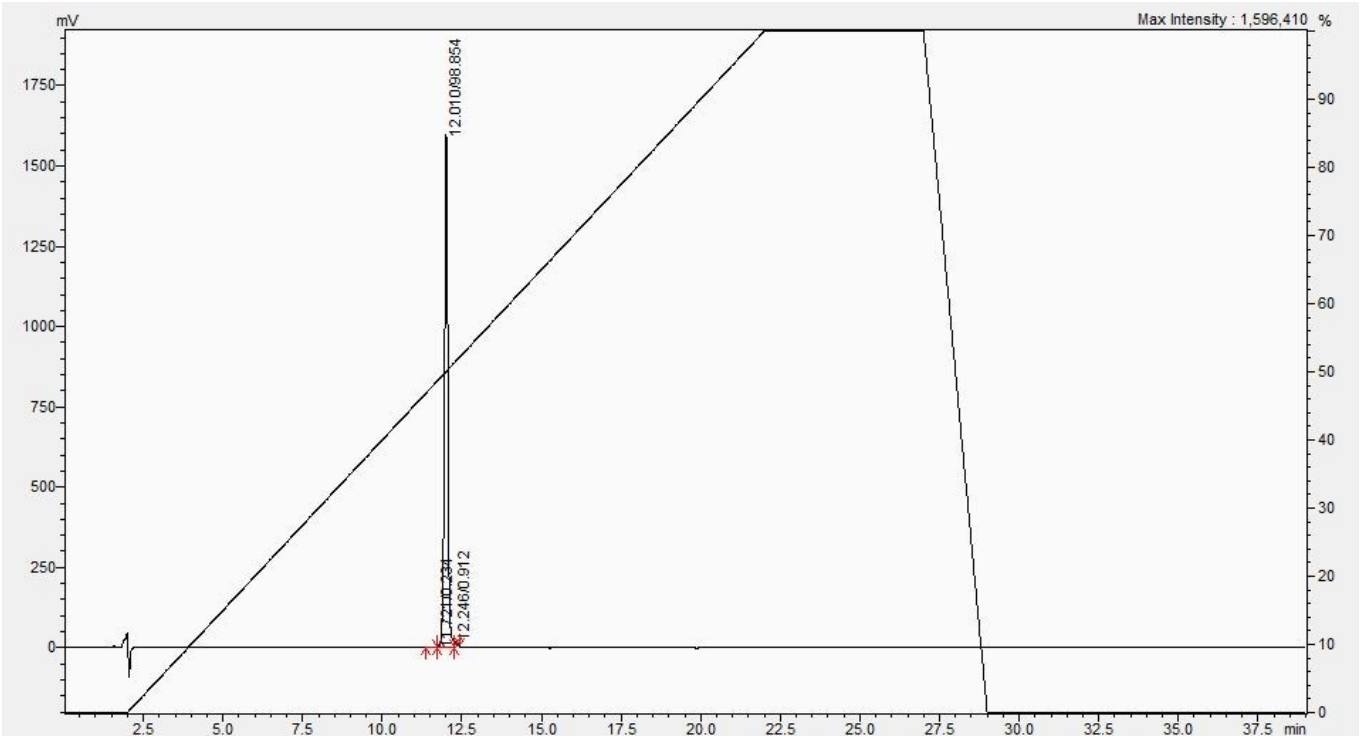


Compound **10**



Note: Analysis column was heated to 45 °C

20 min gradient 214 nm



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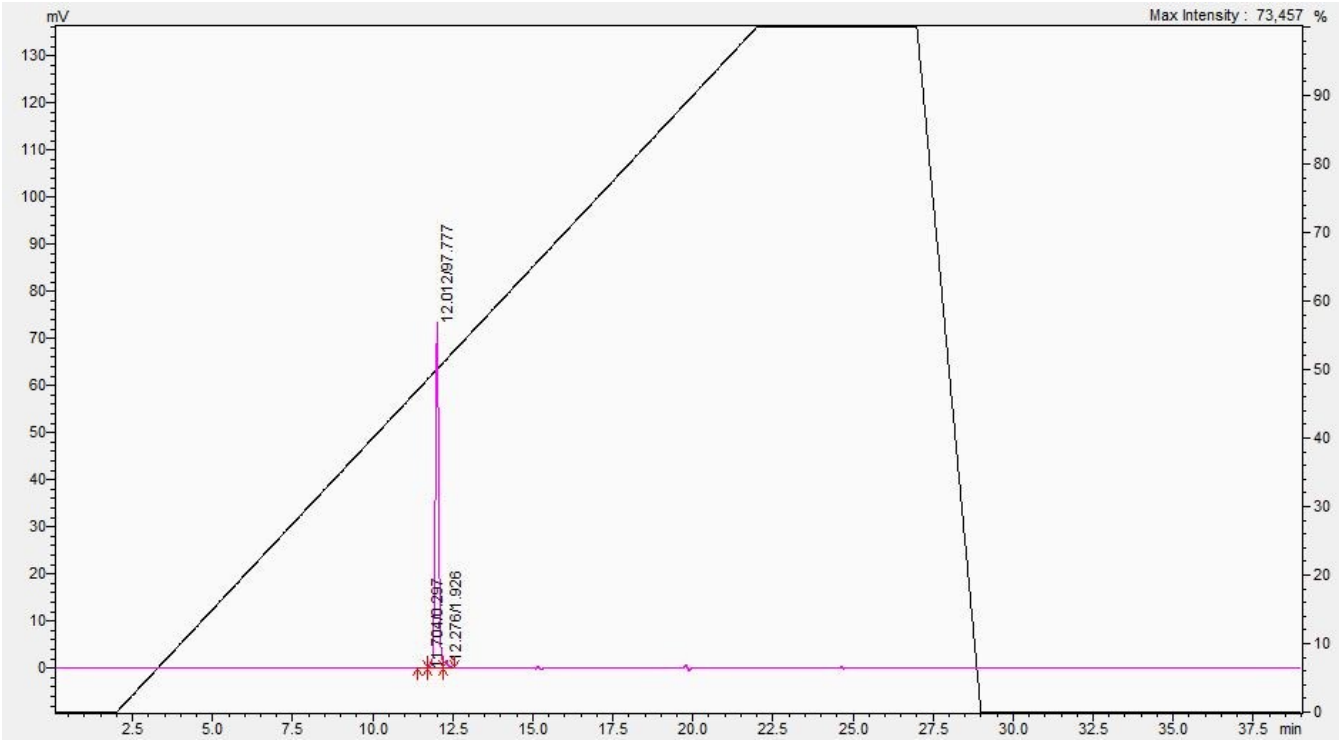

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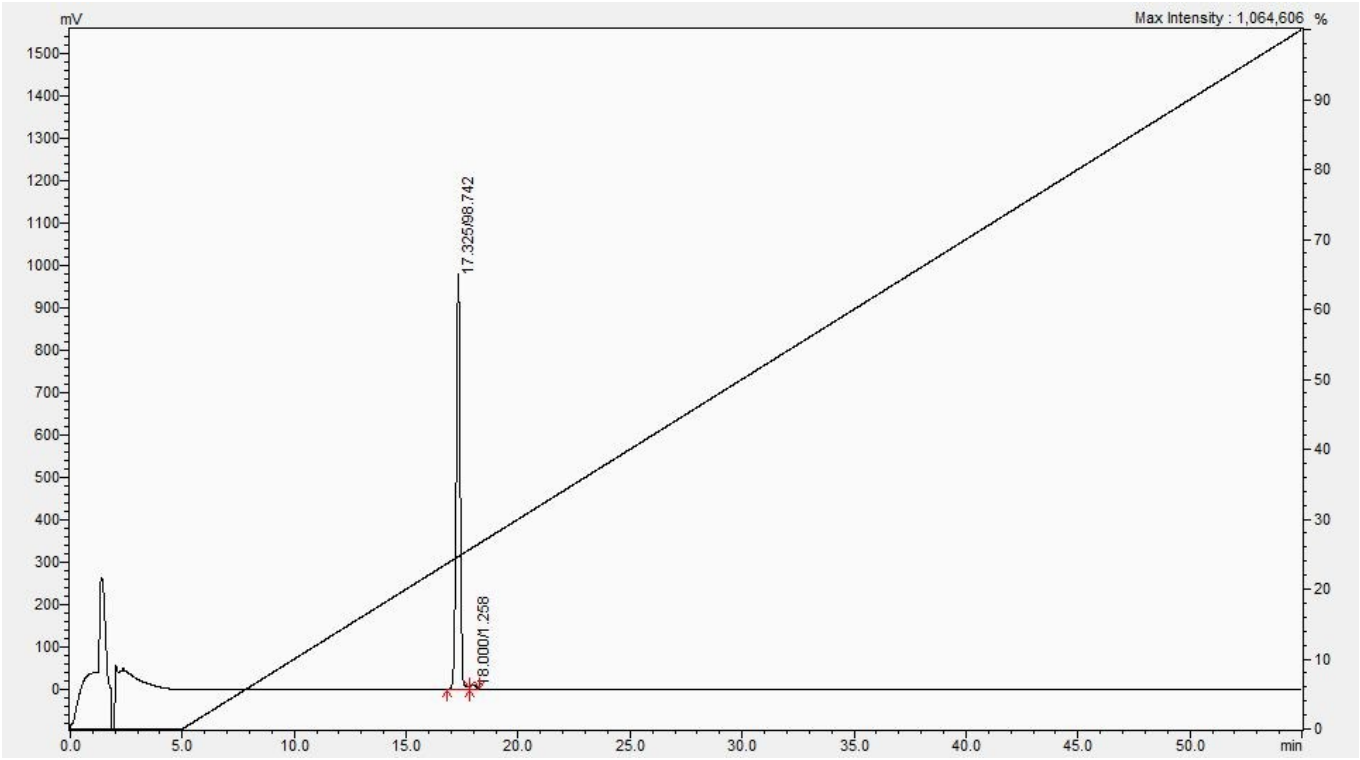

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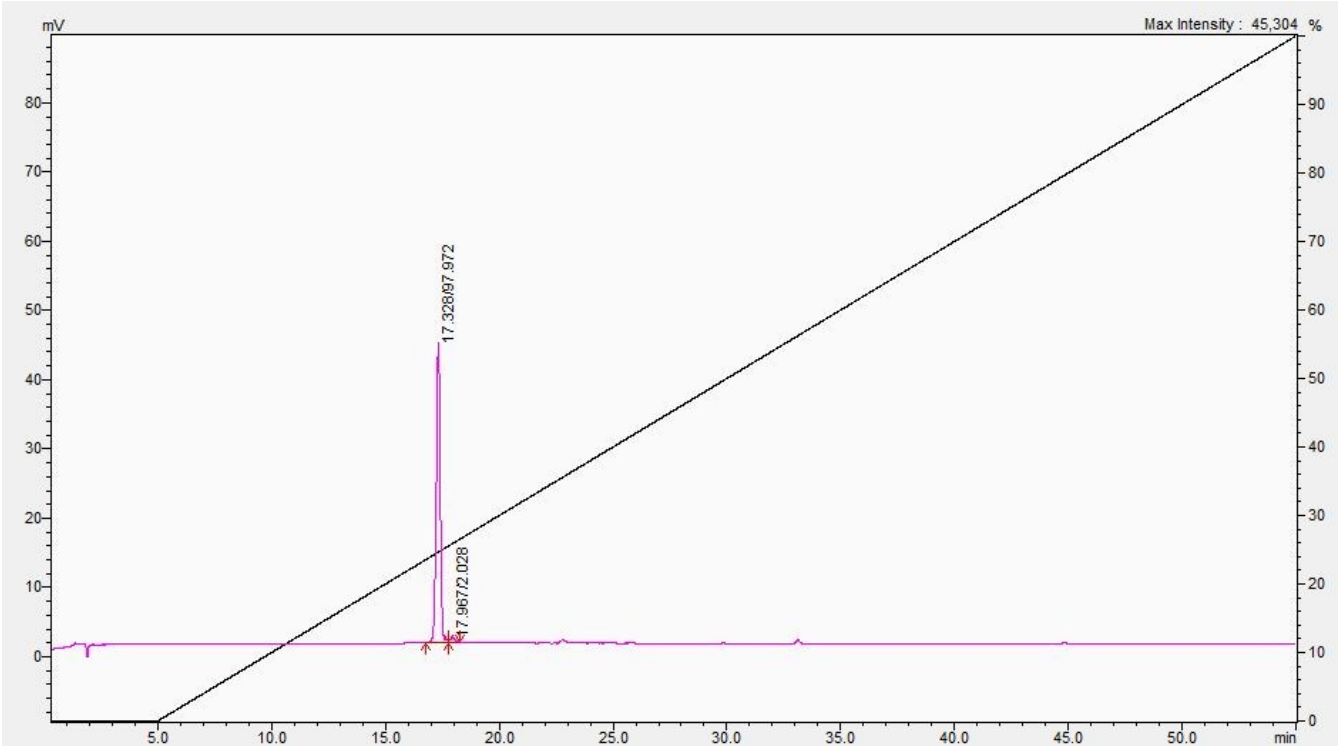
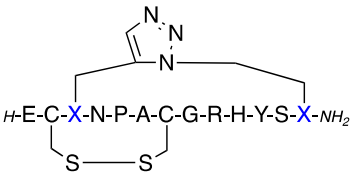


Compound **11**



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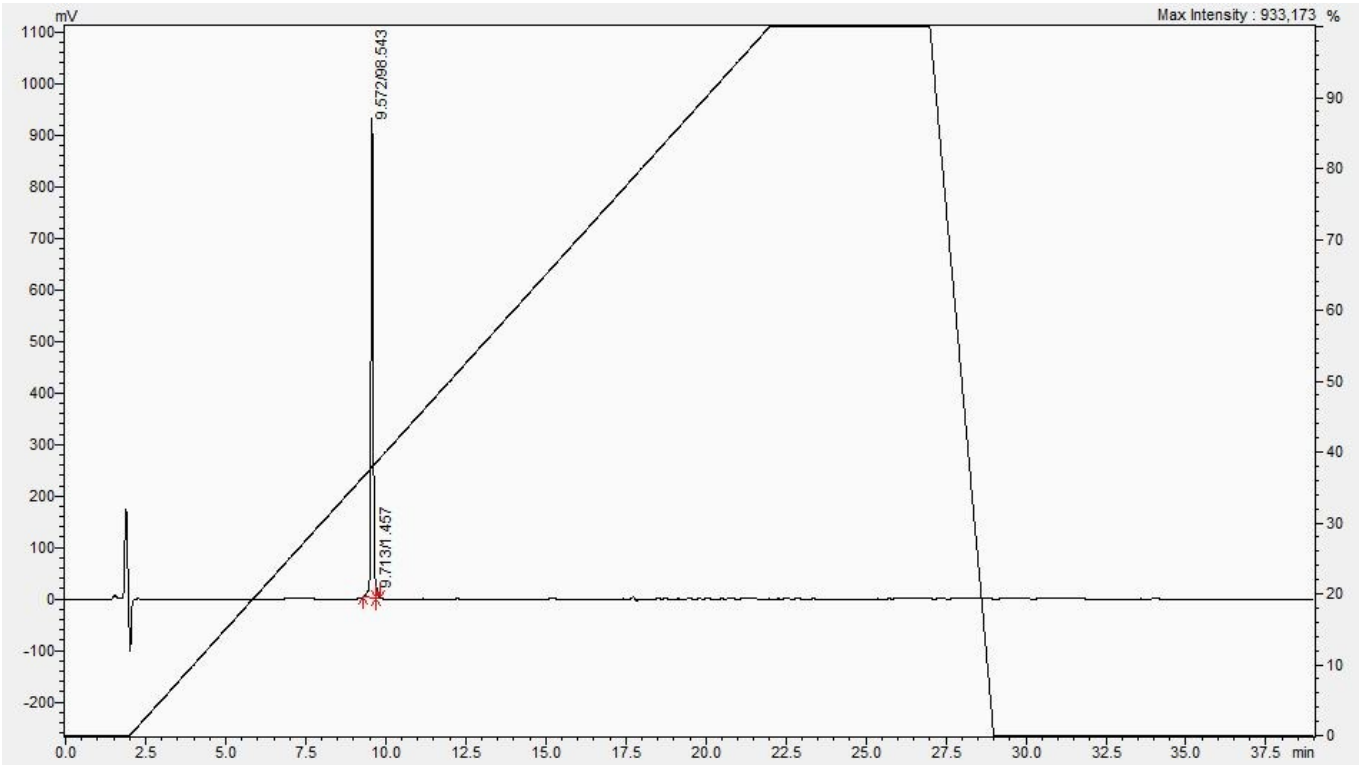

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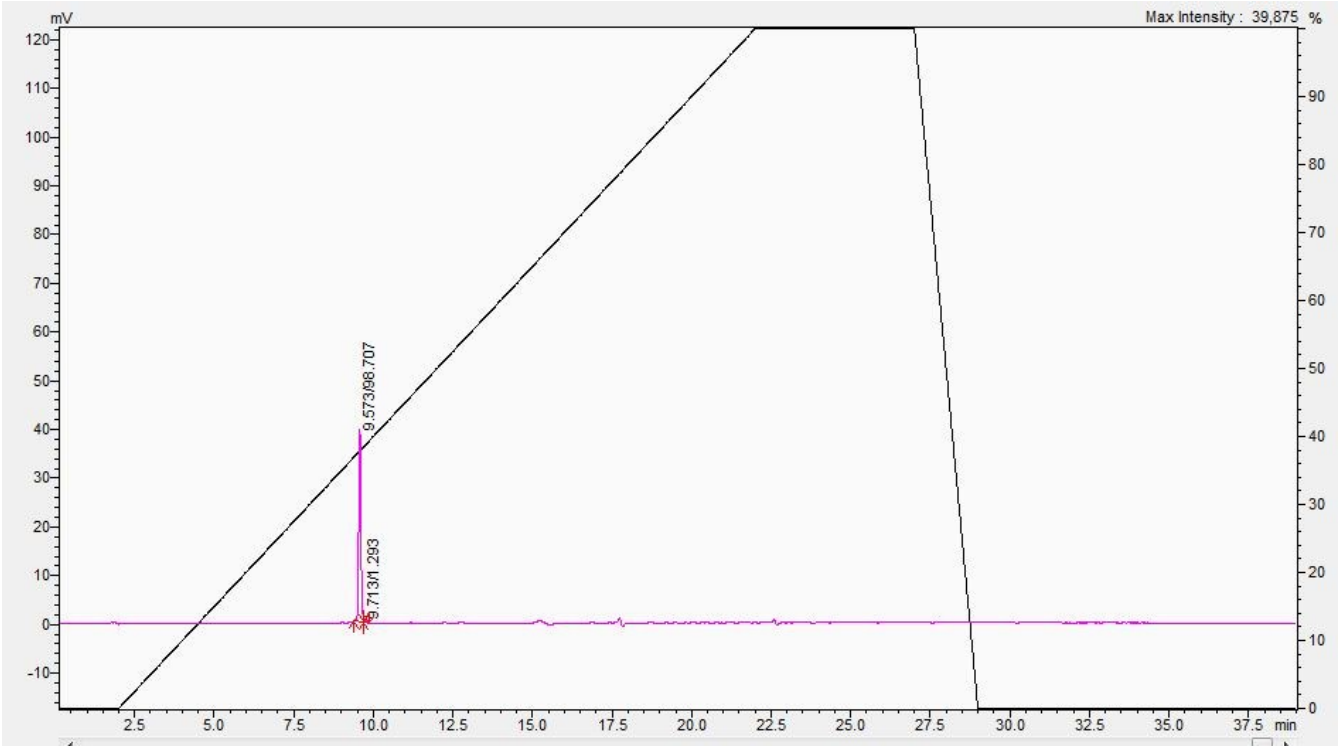

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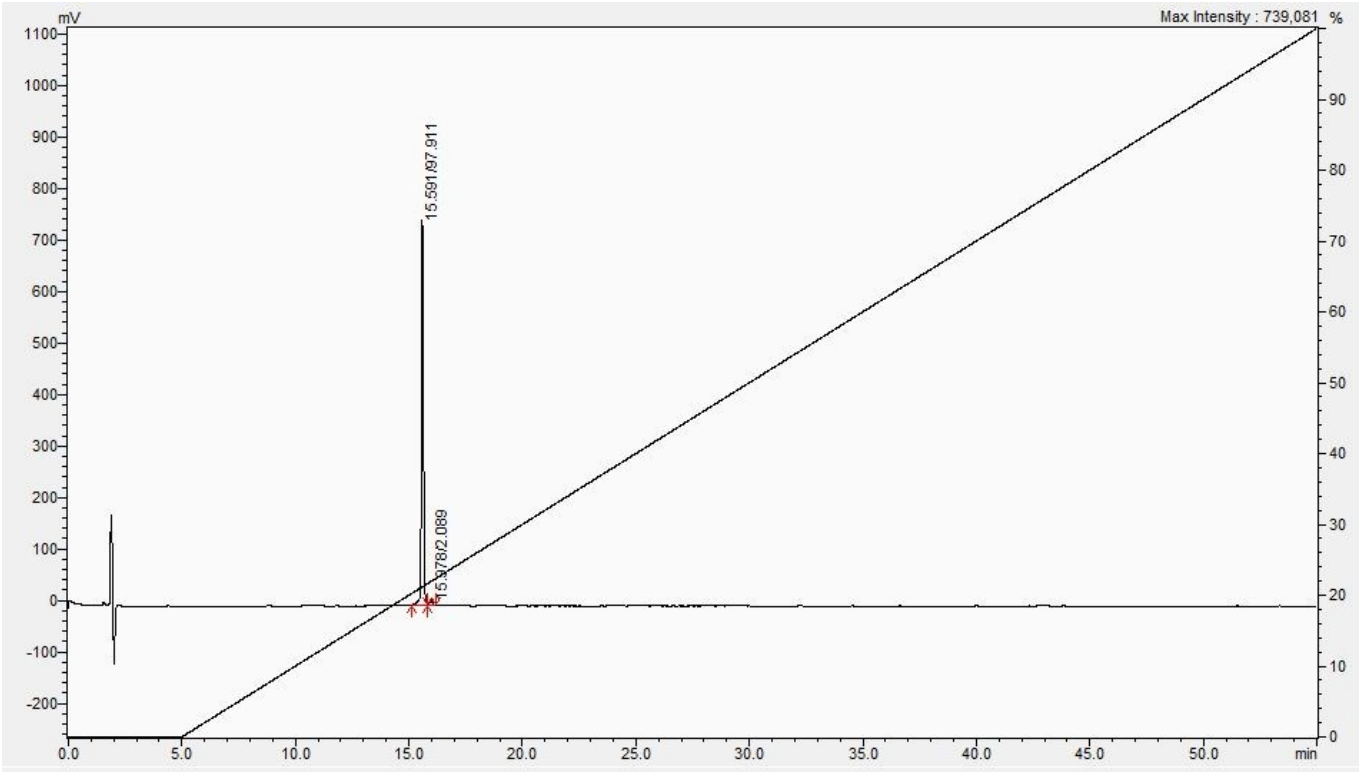

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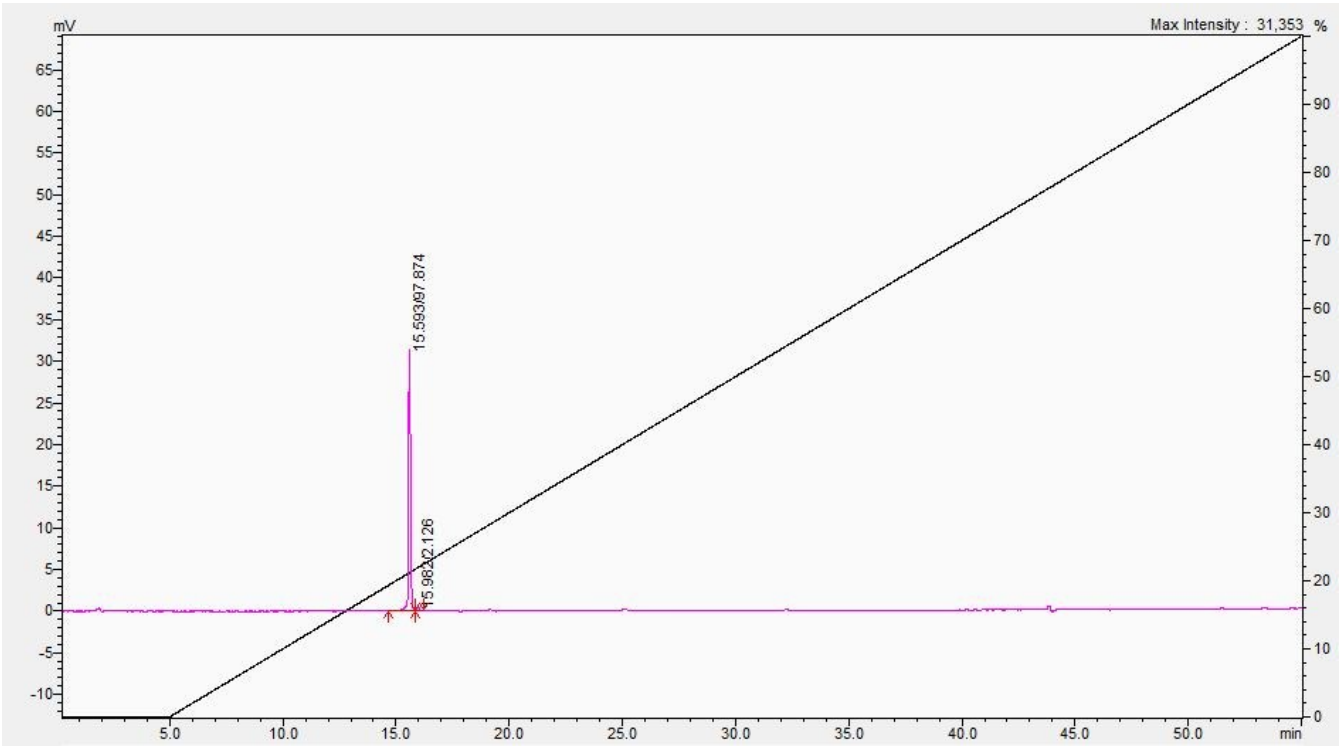
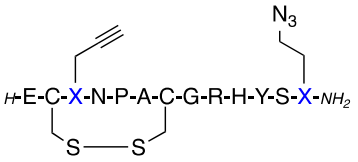


Compound **12**



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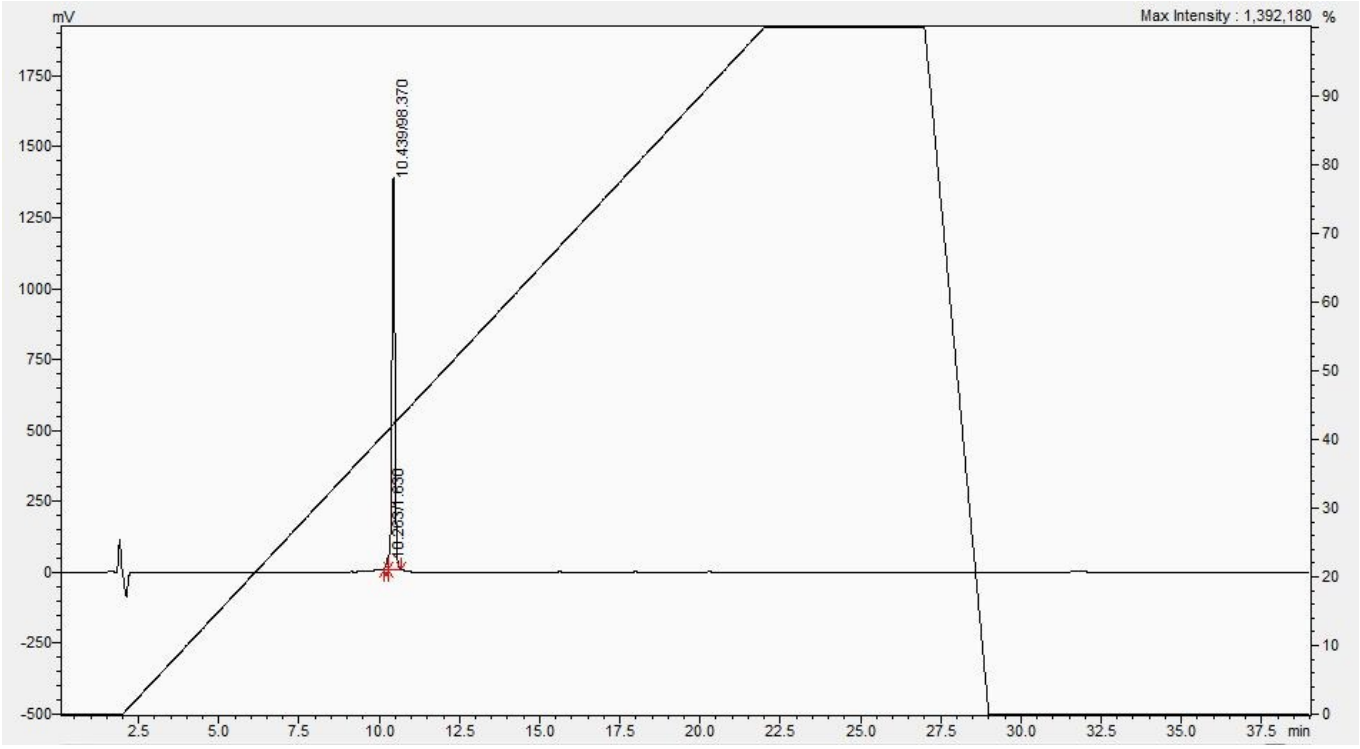

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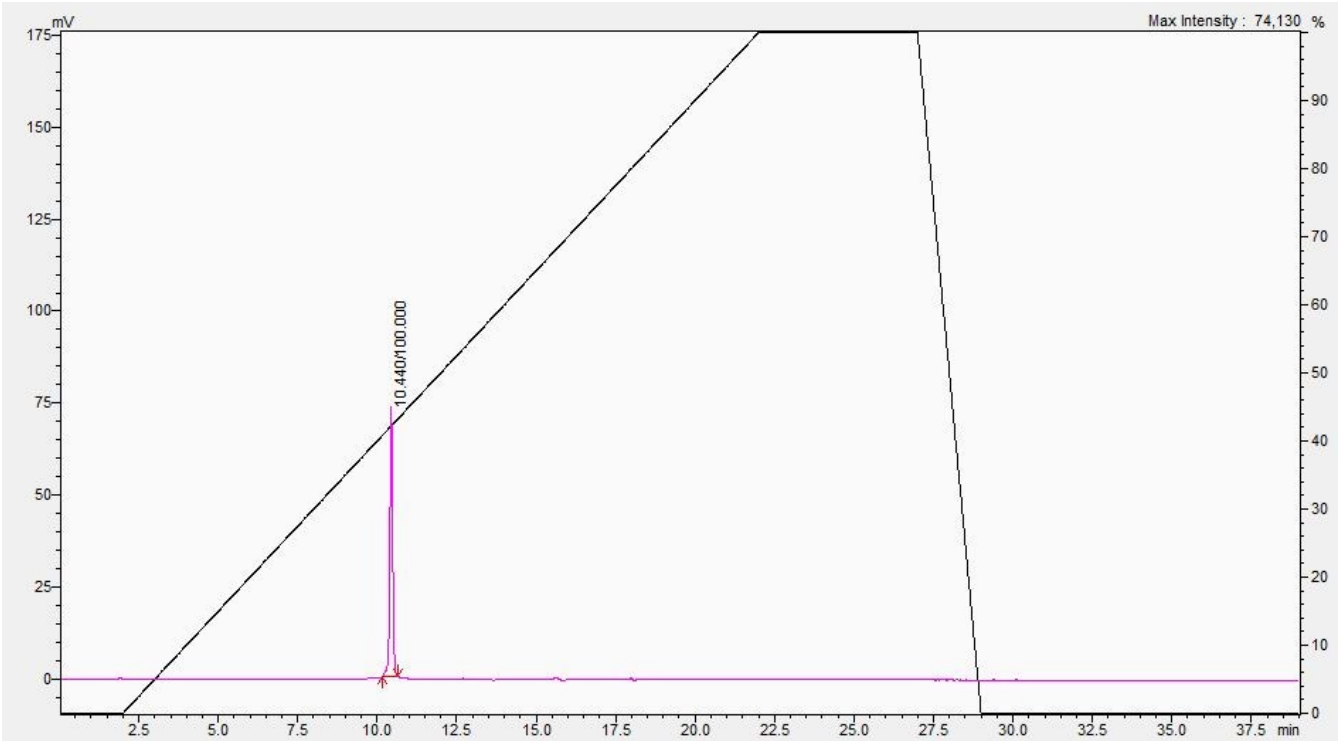

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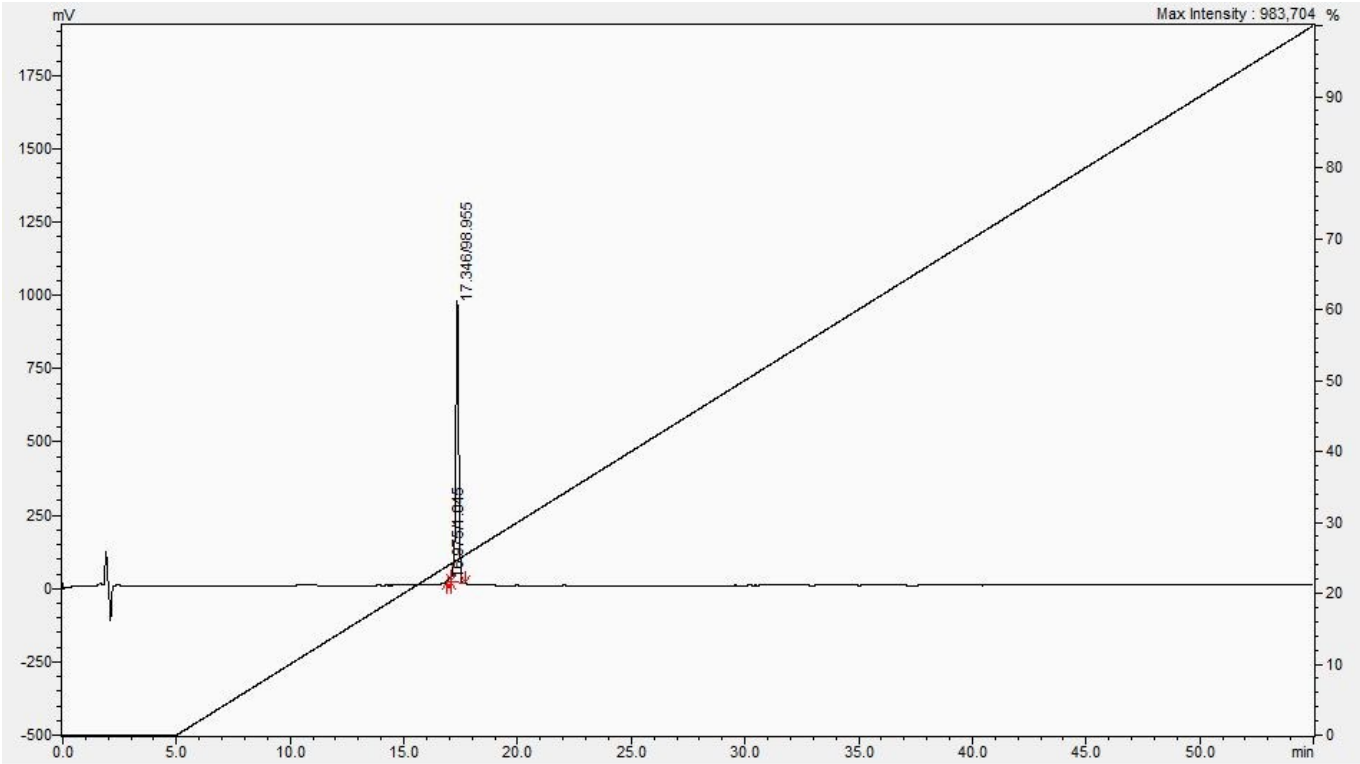

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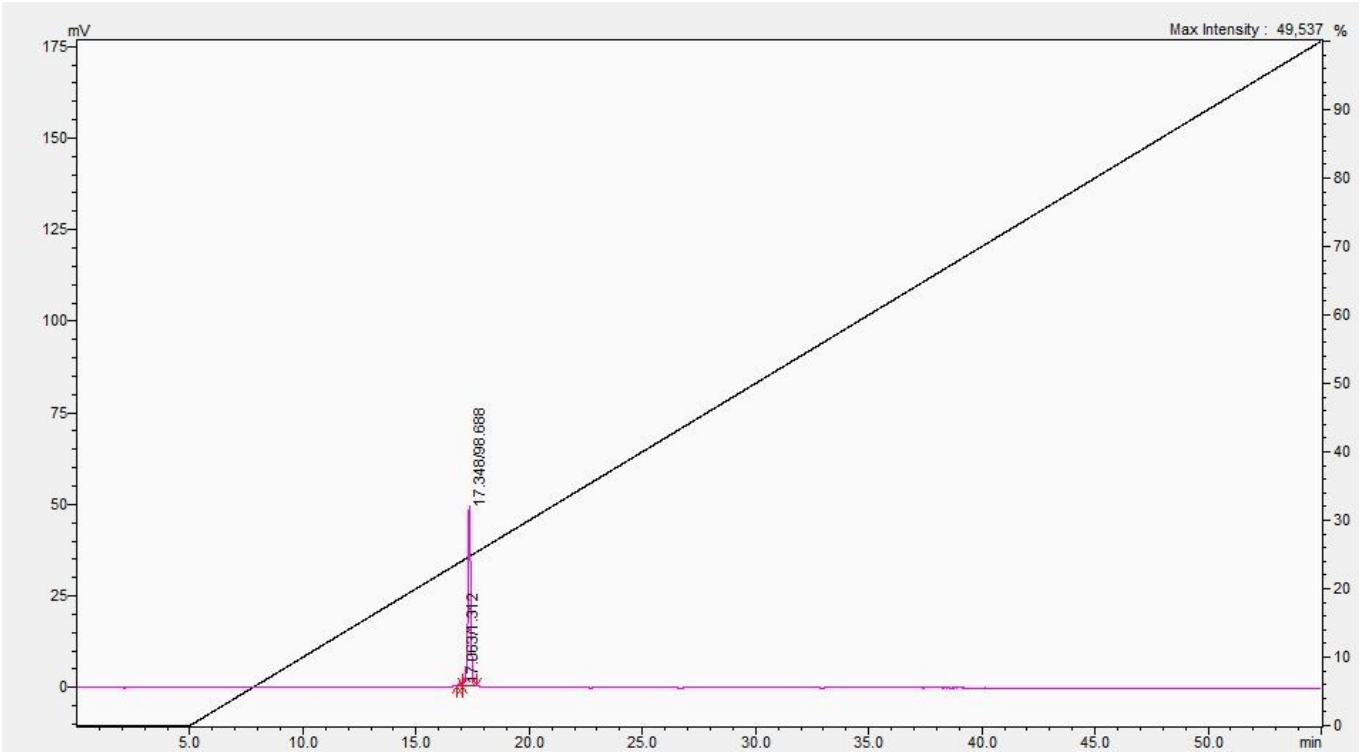
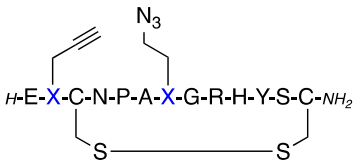


Compound **13**



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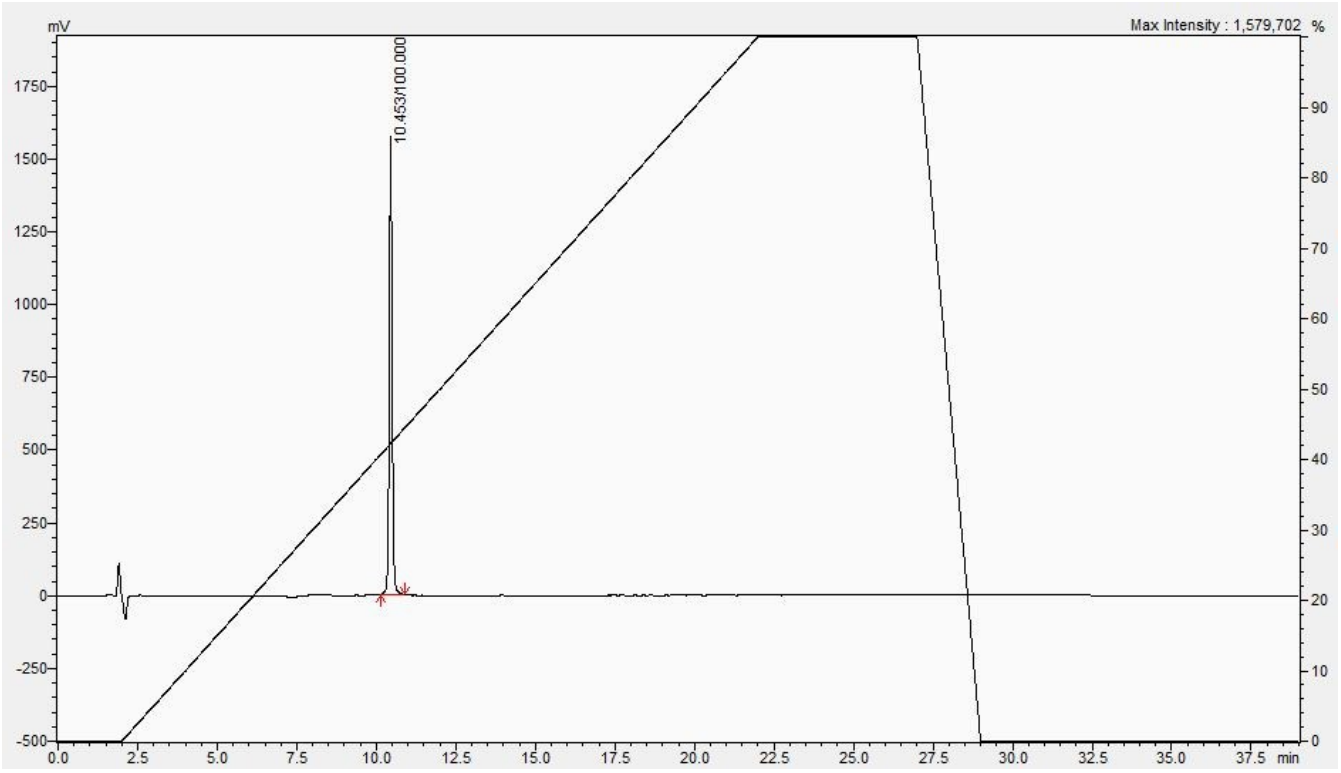

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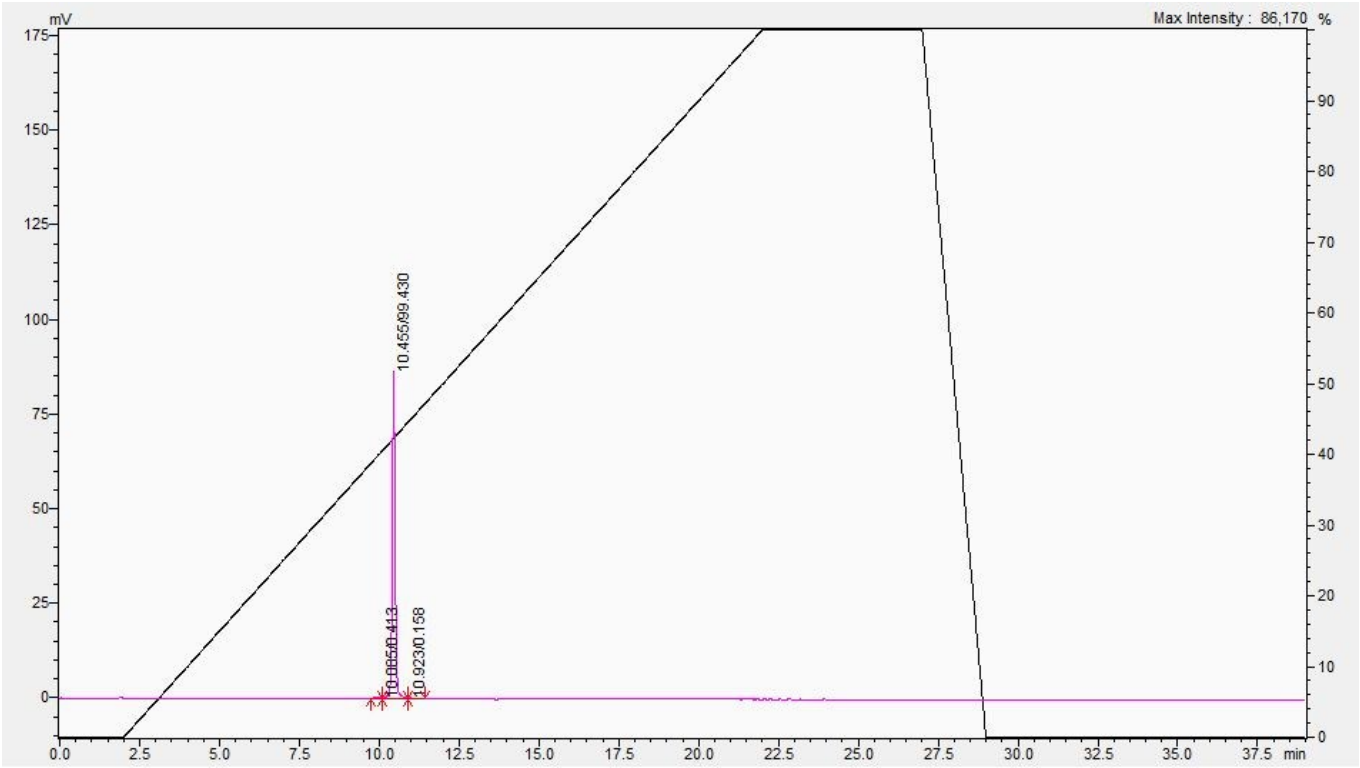

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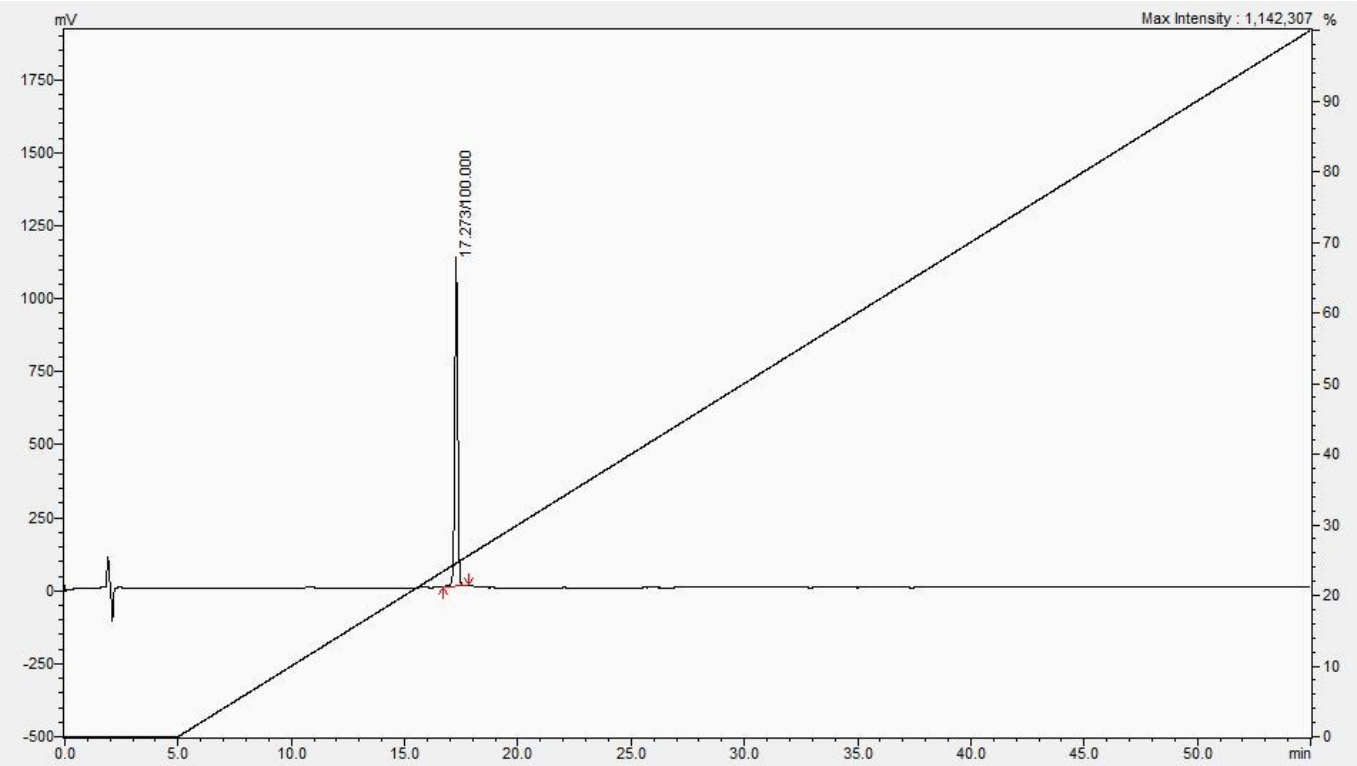

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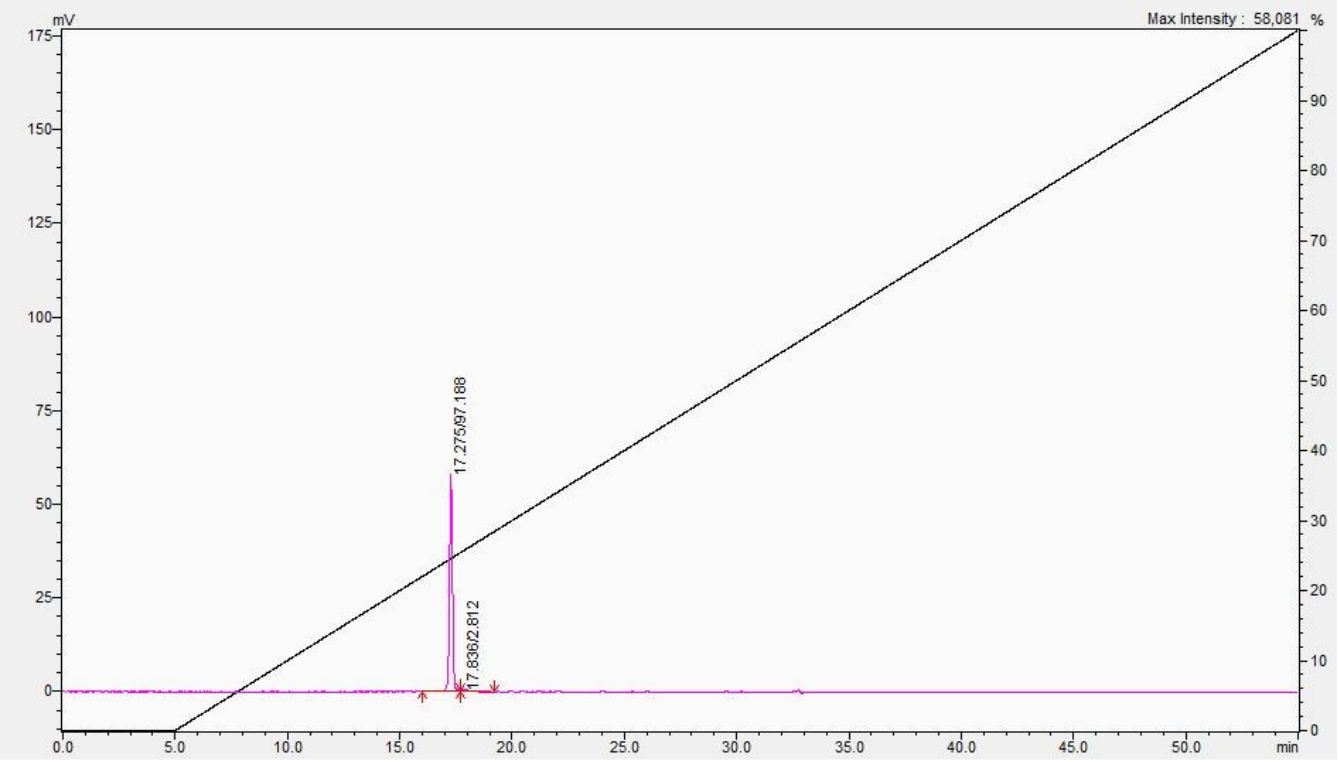

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