Electronic Supplementary Material (ESI) for Organic & Biomolecular Chemistry.

SUPPORTING INFORMATION

for

Accessing diverse bicyclic peptide conformations using 1,2,3-TBMB as a linker

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1 Experimental methods

1.1 General protocol for peptide synthesis

Manual peptide synthesis was performed on Merck Rink Amide MBHA resin (0.52-0.65 mmol/g loading). Couplings were carried out by adding HATU (4 eq) and *N*,*N*-diisopropylethylamine (4 eq) to a solution of the Fmoc-protected amino acid (4 eq) in DMF. This pre-activated mixture was then added to the resin in DMF and shaken for 1 h. The side chain protecting groups used were: *t*-Bu for Asp, Glu, Ser, Thr, Tyr; Boc for Lys, Trp; Pbf for Arg; Trt for Asn, Gln, His. *N*-terminal acetyl capping was carried out by adding Ac_2O (4 eq) and DIPEA (4 eq) in DMF to resin and shaking for 45 min.

Completion of peptide couplings was determined by a chloranil test, in which acetaldehyde (200 µL) and a saturated solution of chloranil in toluene (50 µL) were added to a small amount of resin swelled in CH_2Cl_2. After five minutes shaking at rt, no change in colour indicated complete coupling, whilst green colouration of the resin indicated incomplete coupling. Any incomplete couplings were submitted to a second round of coupling.

Fmoc deprotection was carried out with 20% piperidine in DMF (2 × 1 min, 1 × 10 min). Cleavage from the resin was achieved with TFA containing 2.5% triisopropylsilane and 2.5% H_2O for 2 h. After cleavage, the mixture was concentrated under a stream of nitrogen. The crude residue was triturated with diethyl ether (15-20 mL) before purification by reverse-phase chromatography.

Automated flash reverse-phase column chromatography was carried out on a Biotage Selekt using pre-packed Biotage Sfär C18 column (30 g or 60 g cartridges) for initial purification of peptides.

Semi-preparative reverse-phase HPLC was performed on a Waters 2545 quaternary pump with a Waters 2707 autosampler using a Waters Sunfire C18 OBD Prep Column (10 mm × 250 mm, 5 µm), or on a Waters 1525 binary pump with a Waters 2707 autosampler using a XBridge Peptide BEH C18 OBD Prep Column (10 mm × 250 mm, 5 µm). Peptide samples were eluted with a linear gradient system running with 0.1% (v/v) TFA in MilliQ water (solvent A) and 0.1% (v/v) TFA in MeCN (solvent B) over 30 min at a flow rate of 4 mL/min. Fractions were collected with Waters Fraction Collector lll. Peptides were monitored by UV absorbance at 220 nm on a Waters 2998 PDA Detector or Waters 2489 UV Detector with semi-prep cell.

LCMS chromatograms were obtained using a Shimadzu Nexera-I LC-2040C Plus coupled to a Shimadzu LCMS-2020 mass spectrometer ESI single quadrupole mass detector. Reverse phase separation was performed on a Shimadzu Shim-Pack Sceptor C18-120 (2.1 mm × 100 mm, 3 µm) run at 0.4 mL/min or Shimadzu ShimPack Velox SP-C18 (2.1 mm × 50 mm, 2.7 µm) run at 0.5 mL/min. The mobile phases were water (solvent A) and MeCN (solvent B), both containing 0.1% (v/v) formic acid. The gradient used was 5-95% B over 12 or 6.4 min respectively for each column. UV absorbance was monitored at 220 and 254 nm. Mass spectra were obtained by electrospray ionisation in both positive and negative modes, scanning between *m/z* 200 and 2000.

1.2 Linker synthesis

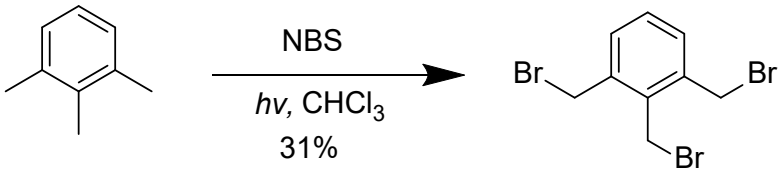
1.2.1 Synthetic methods

N-Bromosuccinimide was recrystallised from water prior to use. UV illumination was carried out using LED light (Detroit, 10W Heavy Duty clamp LED work light).

Flash chromatography was carried out on a Biotage Selekt using Silica Gel 60 LR 0.04-0.06 mm (230-400 mesh ASTM, ChemSupply).

NMR spectra were collected on a Bruker Avance III 300 MHz spectrometer. Nuclear magnetic resonance (NMR) were collected on a Bruker Avance III 300 MHz Spectrometer. Where $CDCl_3$ was used as the solvent and internal lock, spectra were referenced to residual solvent for $CHCl_3$ (δ_H 7.26 ppm) for ^1H NMR and (δ_C 77.0 ppm) for ^{13}C NMR. Chemical shift values are reported in parts per million, ^1H-^1H coupling constants are reported in hertz and H multiplicity is abbreviated as; s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, br = broad signal.

1.2.2 Synthesis of 1,2,3-TBMB



Scheme S1. Synthesis of 1,2,3-tris(bromomethyl)benzene **1,2,3-TBMB** using a modified method based on a literature protocol. [1]

A mixture of 1,2,3-trimethylbenzene (500 mg, 4.16 mmol, 1 eq) and *N*-bromosuccinimide (2.37 g, 13.3 mmol, 3.2 eq) in chloroform (20 mL) was heated at reflux overnight under UV illumination. The reaction mixture was cooled to room temperature then washed with aqueous $NaHCO_3$ (2 × 10 mL) and saturated brine (2 × 10 mL) and concentrated *in vacuo* before purification by flash chromatography with hexane to give **1,2,3-TBMB** as a white solid (454 mg, 31%).

^{1}H NMR (300 MHz, $CDCl_3$): δ_H 7.28–7.37 (3H, m, aromatic CH), 4.83 (2H, s, CH_2), 4.62 (4H, s, CH_2). **^{13}C NMR** (75 MHz, $CDCl_3$): δ_C 138.1 (2 × aromatic C_q), 135.8 (aromatic C_q), 131.9 (2 × aromatic CH), 129.8 (aromatic CH), 29.8 (2 × CH_2), 24.7 (CH_2).

Characterisation data matches that reported in the literature. [1]

1.2.3 NMR spectra of 1,2,3-TBMB

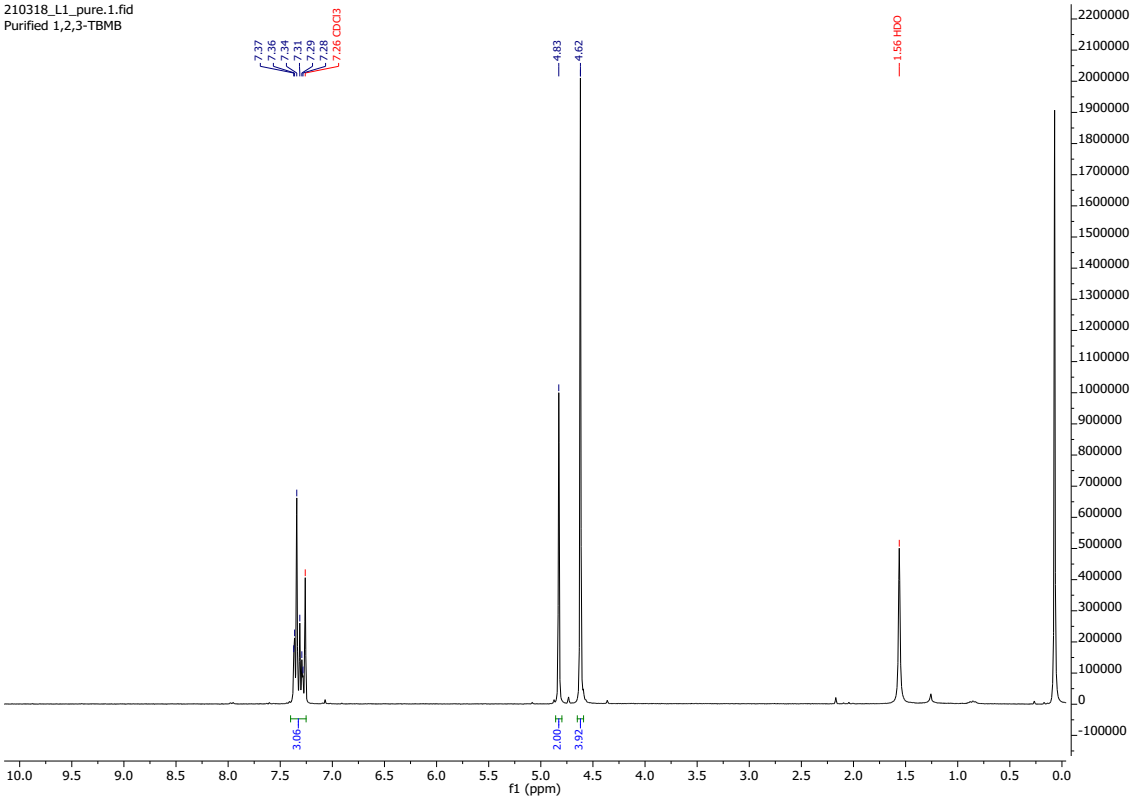


Figure S1. ^{1}H NMR spectrum of 1,2,3-tris(bromomethyl)benzene.

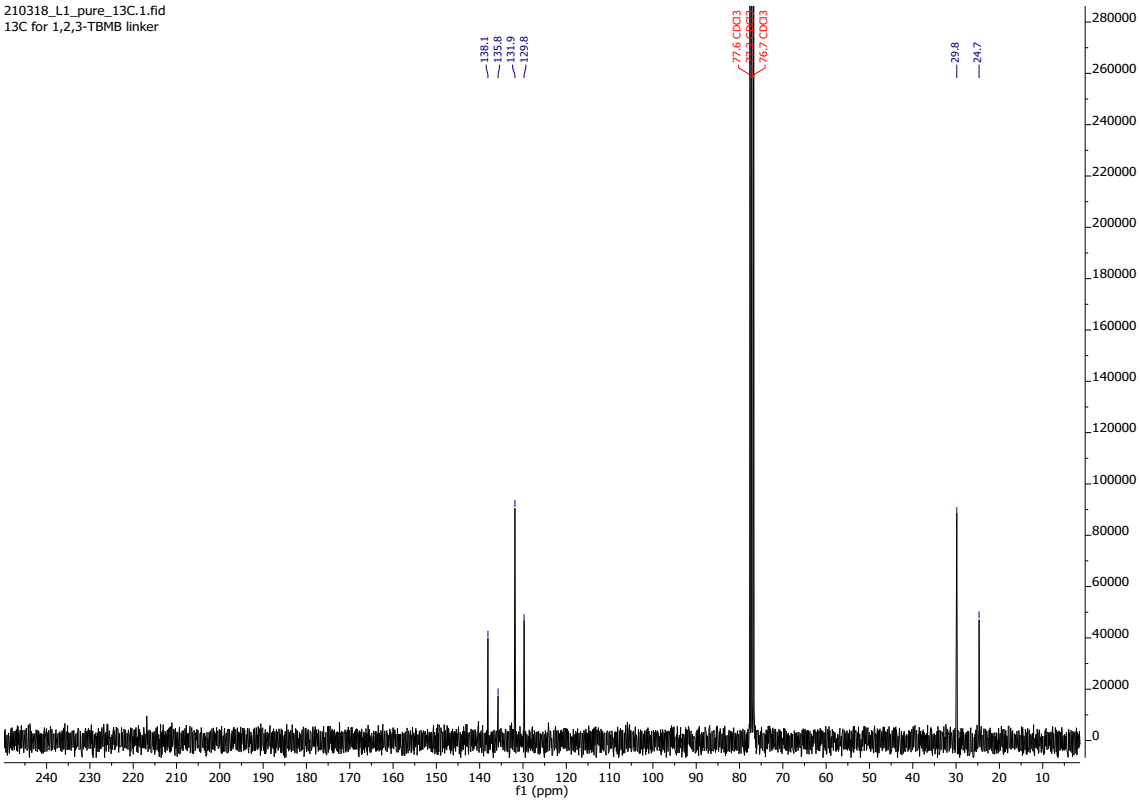


Figure S2. ^{13}C NMR spectrum of 1,2,3-tris(bromomethyl)benzene.

1.3 Bicyclisation protocols

Bicyclisation reactions were carried out on an Eppendorf ThermoMixer C, shaken at 1000 rpm at the temperatures specified.

Percent conversion was calculated based on a ratio of the area underneath the extracted ion chromatogram peaks corresponding to the expected masses of the linear and bicyclised peptides. In the few cases where full clean conversion was not achieved, the percentage conversion represents an upper bound and may be lower due to the formation of byproducts.

1.3.1 Method A bicyclisation

From a 20 mM stock solution of HPLC-purified linear peptide in DMSO, the peptide was diluted to 520 µM using 50% MeCN in 100 mM $NaHCO_3$ buffer at pH 8 (final volume 500 µL). TCEP was added (1 mM, 2 eq) and the mixture was shaken at 30 °C for 30 min. **1,2,3-TBMB** (750 µM, 1.5 eq) in MeCN (15 µL of a 25 mM stock) was added and the reaction mixture was shaken at 30 °C for 1-2 h. Where required, the bicyclic peptides were purified by semi-preparative HPLC.

1.3.2 Method B bicyclisation

This method was used for hydrophobic peptide sequences **P16-18**.

HPLC-purified linear peptide was dissolved to 1 mM in 1:1 MeCN/H_2O (1 mL). TCEP (1 mM, 1 eq) was added, followed by DIPEA (0.1% v/v 1 µL). **1,2,3-TBMB** (1.5 mM, 1.5 eq) in MeCN (13 µL of a 25 mM stock) was then added and the reaction mixture was shaken at 30 °C for 1-2 h. Where required, the crude bicyclic peptides were then purified by semi-preparative HPLC.

1.3.3 Bicyclisation of peptides from 96-well plates

Peptides were obtained from Genscript in crude purity in 96-well plate format.

Crude peptides were dissolved in DMSO (20 mM), then diluted to 520 µM using 50% MeCN in 100 mM $NaHCO_3$ buffer at pH 8 (final volume 280 µL). TCEP was added (1 mM, 2 eq) and the mixtures were shaken at 30 °C for 30 min. **1,2,3-TBMB** (750 µM, 1.5 eq) in MeCN (6.5 µL of a 25 mM stock) was added and the reactions were shaken at 30 °C for 1 h then analysed by LCMS.

2 Pilot study of bicyclisation with 1,2,3-TBMB using Method A

2.1 Summary of peptides used in pilot study

Table S1. List of peptide sequences tested in bicyclisation reactions with **1,2,3-TBMB** using Method A, showing the calculated *m/z*, observed *m/z*, and estimated percentage conversion by LCMS. hC = homocysteine. Further LCMS data for the bicyclisation reactions are shown in ESI Section 9.1.

Name	Sequence	Calc. *m/z*	Obs. *m/z*	%
P1	Ac-GTEP**C**L**C**S**C**HN-NH_2	$[M+H]^+$ =1318.50	1318.55	>95
P2	Ac-YIE**C**QP**C**D**C**W-NH_2	$[M+H]^+$ =1414.53	1414.32	>95
P3	Ac-A**C**QY**C**DG**C**ER-NH_2	$[M+H]^+$ =1302.47	1302.50	>95
P4	Ac-**C**FV**C**G**C**MSENQ-NH_2	$[M+H]^+$ =1375.49	1375.60	>95
P5	Ac-VG**C**K**C**D**C**WYQ-NH_2	$[M+H]^+$ =1359.53	1359.60	>95
P6	Ac-M**C**T**C**S**C**NER-NH_2	$[M+H]^+$ =1201.43	1201.40	>95
P6-hCys	Ac-M**hC**T**hC**S**hC**NER-NH_2	$[M+H^+$ = 1243.47	1243.45	>95
P7	Ac-AL**C**G**C**NR**C**WE-NH_2	$[M+H]^+$ =1309.53	1309.55	>95
P8	Ac-AW**C**NI**C**F**C**MR-NH_2	$[M+H]^+$ =1401.57	1401.60	>95
P9	Ac-DNGFH**C**WI**C**R**C**-NH_2	$[M+H]^+$ =1508.59	1508.60	>95
P10	Ac-N**C**W**C**H**C**LT-NH_2	$[M+H]^+$ =1134.43	1134.40	>95
P11	Ac-A**C**KRT**C**LNP**C**-NH_2	$[M+H]^+$ =1263.56	1263.60	>95
P12	Ac-A**C**NEDG**C**RKTH**C**-NH_2	$[M+H]^+$ =1491.68	1492.95	>95
P13	Ac-**C**FQYEW**C**LAEGI**C**-NH_2	$[M+H]^+$ =1719.70	1719.20	>95
P14	Ac-**C**RPQKWY**C**NMSTEA**C**-NH_2	$[M+2H]^+$ =988.41	998.50	91

2.2 Reactivity of selected peptides with 1,2,3-TBMB

The following LC UV chromatograms show conversion from linear to bicyclic peptides for selected examples with different cysteine spacings and sequences. The LCMS full data set is available in ESI Section 9.1.

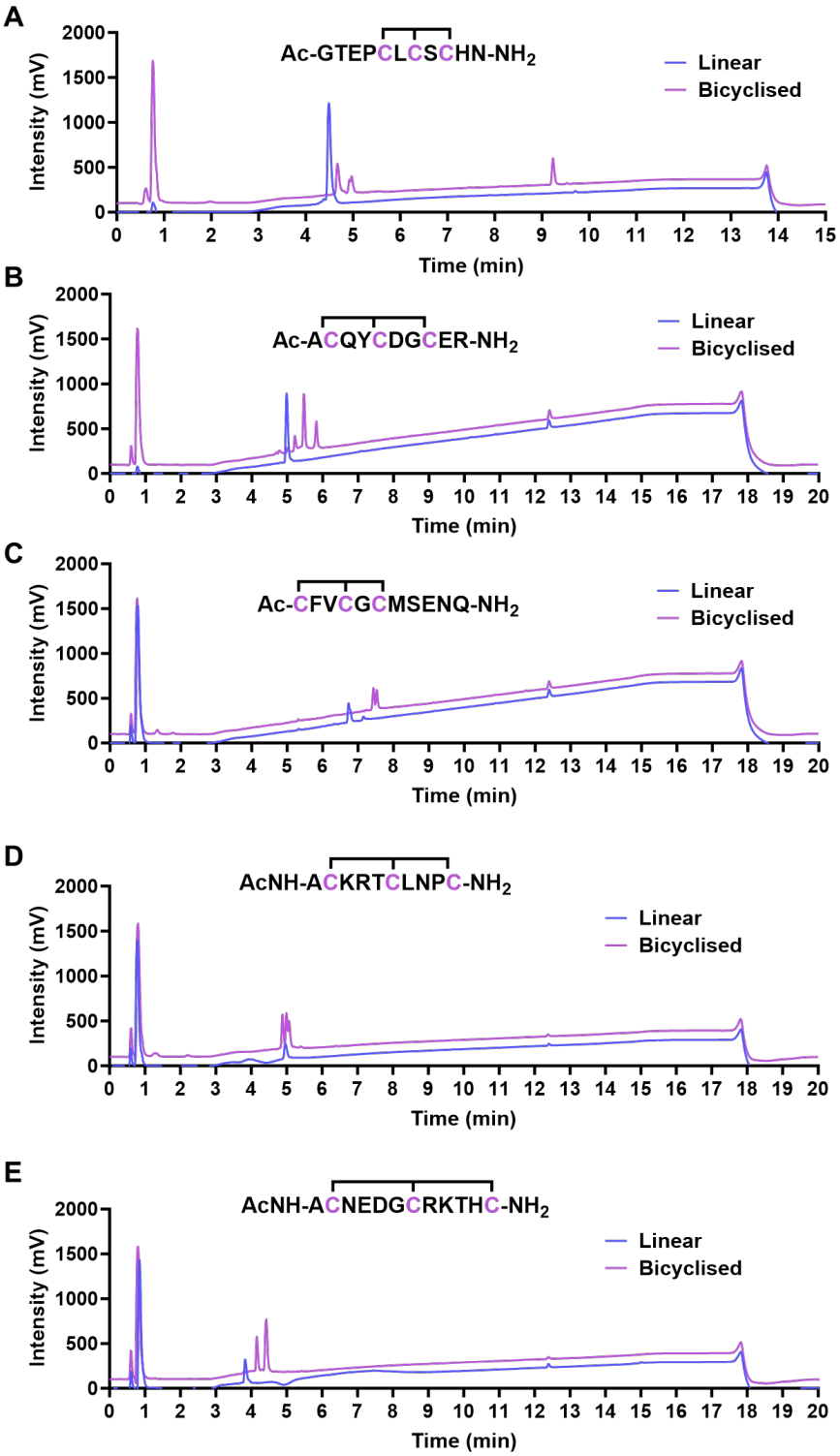


Figure S3. LC UV chromatograms monitored at 220 nm, showing overlay of linear peptide (blue) and crude bicyclisation reaction (purple) for peptides **(A) P1**, **(B) P3**, **(C) P4, (D) P11, (E) P12**. Multiple peaks correspond to different isomers of bicyclic peptides.

3 Detection of free thiols by Ellman's assay

3.1 Method for Ellman's assay

Reaction buffer consisting of 0.1 M sodium phosphate at pH 8.0 with 1 mM EDTA was prepared. To construct the standard curve (Figure S4a), cysteine hydrochloride (Cys-HCl) was dissolved in the reaction buffer to a concentration of 2 mM, and two-fold serial dilutions performed. Ellman's reagent (4 mg) was dissolved in the prepared buffer (1 mL). In a 96-well plate, Ellman's reagent (5 µL), reaction buffer (250 µL), and the appropriate concentration of Cys-HCl solution (25 µL) were added to each well. The plate was incubated at room temperature for 15 min, after which absorbance was measured at 412 nm using an Enspire Multimodal Plate Reader (Perkin-Elmer).

Peptide samples were dissolved in 50% MeCN in water to a concentration of 1 mM and used in the assay in place of Cys-HCl. The concentration of free thiols in the peptide samples was calculated using the standard curve.

3.2 Results of Ellman's assay

The ratio of observed thiol concentration to prepared peptide concentration (1 mM) was calculated, where the theoretical ratio for linear peptides is 3, while bicyclised peptides is 0.

Experimentally, ratios of less than 3 are expected due to the formation of peptide disulfide bonds during the assay, as Ellman's reagent is incompatible with reducing agents that lead to false positive signals.

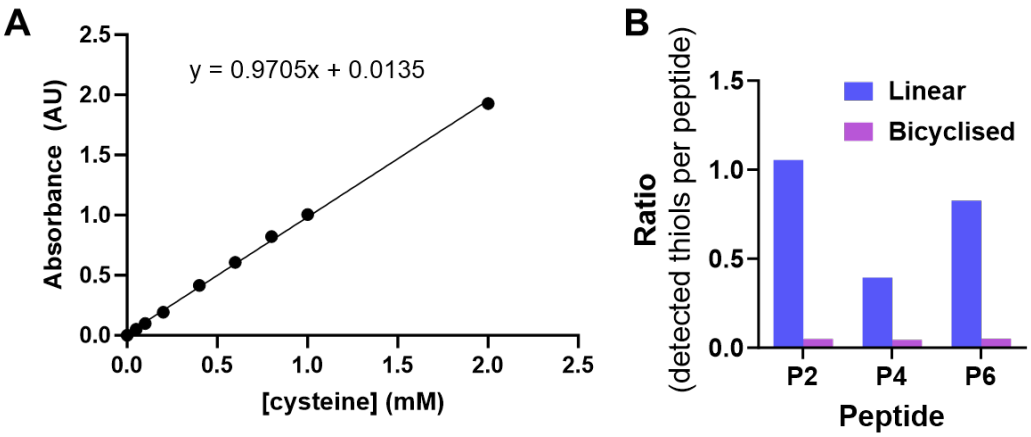


Figure S4. (A) Standard curve of cysteine thiols determined using Ellman's assay. The equation was then used to determine the number of free cysteines in the linear and cyclic peptides based on a 1:1 reactivity of Ellman's reagent with cysteine. **(B)** Ellman's assay results showing the ratio of detected thiols to peptide concentration for linear and cyclic peptides (**P2**, **P4, P6**). A ratio of 1 indicates one free thiol detected per peptide.

4 Comparison with peptides bicyclised with 1,3,5-TBMB

4.1 Summary of peptides bicyclised with 1,3,5-TBMB

Table S2. List of peptides with different cysteine spacings used in bicyclisation reactions with **1,3,5-TBMB**. The calculated *m/z*, measured *m/z*, and estimated percentage conversion by LCMS is reported. LCMS data for the bicyclisation reactions are shown in ESI Section 9.4.

Name	Sequence	Calc. *m/z*	Obs. *m/z*	1,3,5-TBMB (%)
P3	Ac-A**C**QY**C**DG**C**ER-NH_2	$[M+H]^+$ = 1302.47	1302.50	>95
P7	Ac-AL**C**G**C**NR**C**WE-NH_2	$[M+H]^+$ = 1309.53	1309.50	>95

4.2 Characterisation of P3 with different linkers

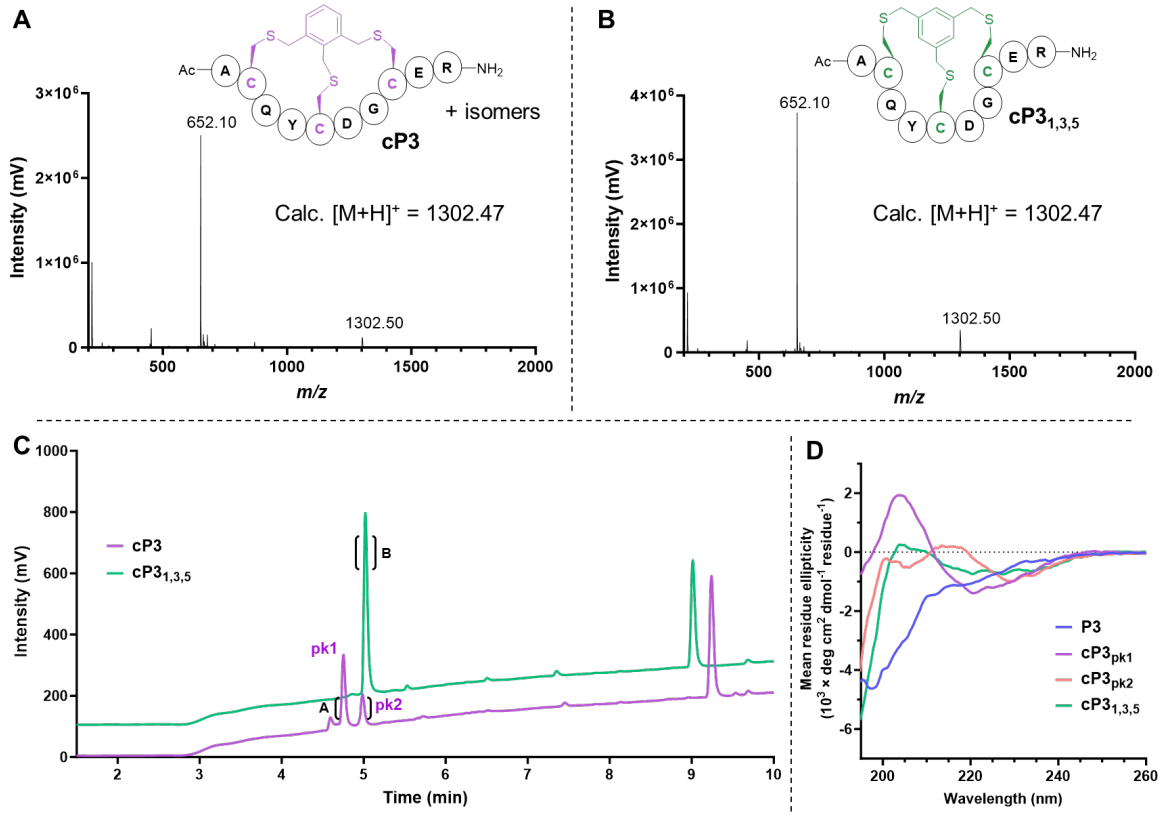


Figure S5. Comparison of **1,2,3-TBMB** and **1,3,5-TBMB** linker reactivity and resulting conformational changes with peptide **P3**. **(A)** Structure and mass spectrum of **cP3**, with one possible isomer shown. **(B)** Structure and mass spectrum of **cP3$_{1,3,5}$**. **(C)** LCMS UV chromatograms monitored at 220 nm for crude bicyclisation reaction mixtures of **P3** using Method A with either **1,2,3-TBMB** (purple) or **1,3,5-TBMB** (green). The **1,2,3-TBMB** reaction shows two distinct peaks with identical *m/z*, while the **1,3,5-TBMB** reaction has a single peak. No linear product is observed in either trace after bicyclisation. Brackets indicate peaks that correspond to the mass spectra displayed in panels A and B. **(D)** CD spectra of linear **P3** (blue), bicyclised **cP3$_{pk1}$** (purple), **cP3$_{pk2}$** (pink), and **cP3$_{1,3,5}$** (green).

5 2D NMR analysis of cP15

5.1 Characterisation of cP15

Table S3. LCMS characterisation of **P15** bicyclisation with **1,3,5-TBMB**. The calculated *m/z*, measured *m/z*, and estimated percentage conversion is reported. Further LCMS data for the **cP15** bicyclisation reaction are shown in ESI Section 9.2. LCMS data for purified **cP15$_{pk1}$** and **cP15$_{pk2}$** are shown in ESI Section 10.3.

Name	Sequence	Calc. *m/z*	Obs. *m/z*	%
P15	Ac-R**C**T**C**YA**C**G-NH_2	$[M+H]^+$ = 1031.39	1031.35	>95

5.2 2D NMR methods

Peptides were dissolved in DMSO-d_6 as 2 mM stocks. All spectra were acquired at 298 K using a Bruker Avance III 600 MHz NMR spectrometer fitted with TCI probe heads and spectra were processed using TOPSPIN3 (Bruker, Karlsruhe, Germany). All 2D spectra were analysed using CCPN software. [2]

5.3 2D NMR spectra of cP15 isomers

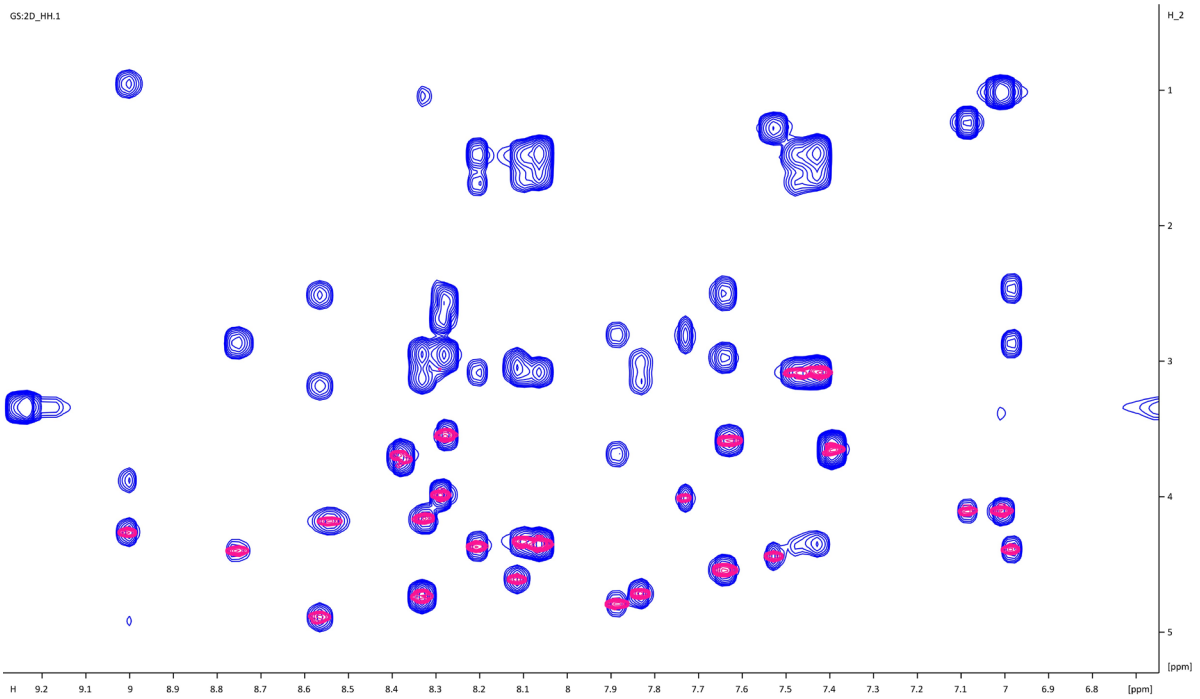


Figure S6. 2D ^{1}H-^{1}H TOCSY (blue) and 2D ^{1}H-^{1}H COSY (pink) of **cP15$_{pk1}$** showing the fingerprint region between 6.5-9 ppm on the horizontal axis. The TOCSY and COSY overlapping signals in this spectrum are the alpha amide (α-NH) of which there are >9, indicating more than one species is present.

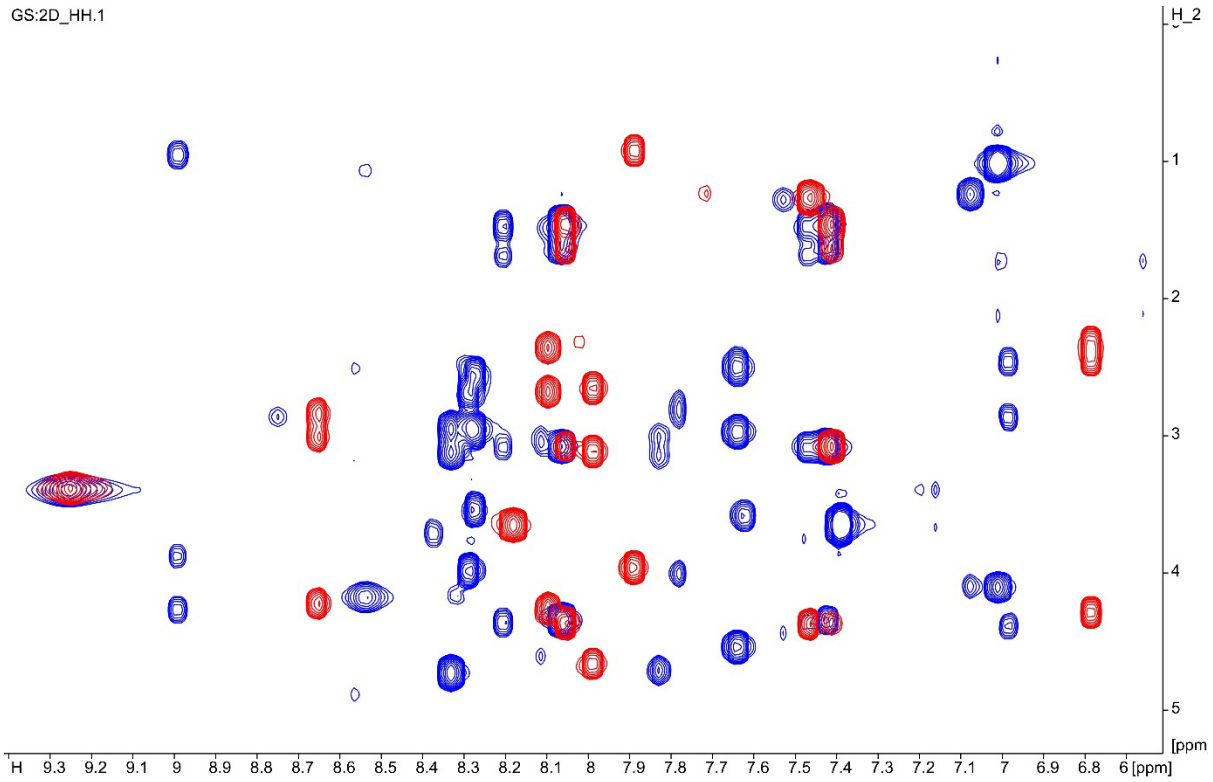


Figure S7. 2D ^{1}H-^{1}H TOCSY overlay of **cP15$_{pk1}$** (blue) and **cP15$_{pk2}$** (red) showing the fingerprint region between 6.5-9 ppm on the horizontal axis. There are no significant overlaps between the spectra showing that the two samples have different peptide backbone signals and hence, different conformations.

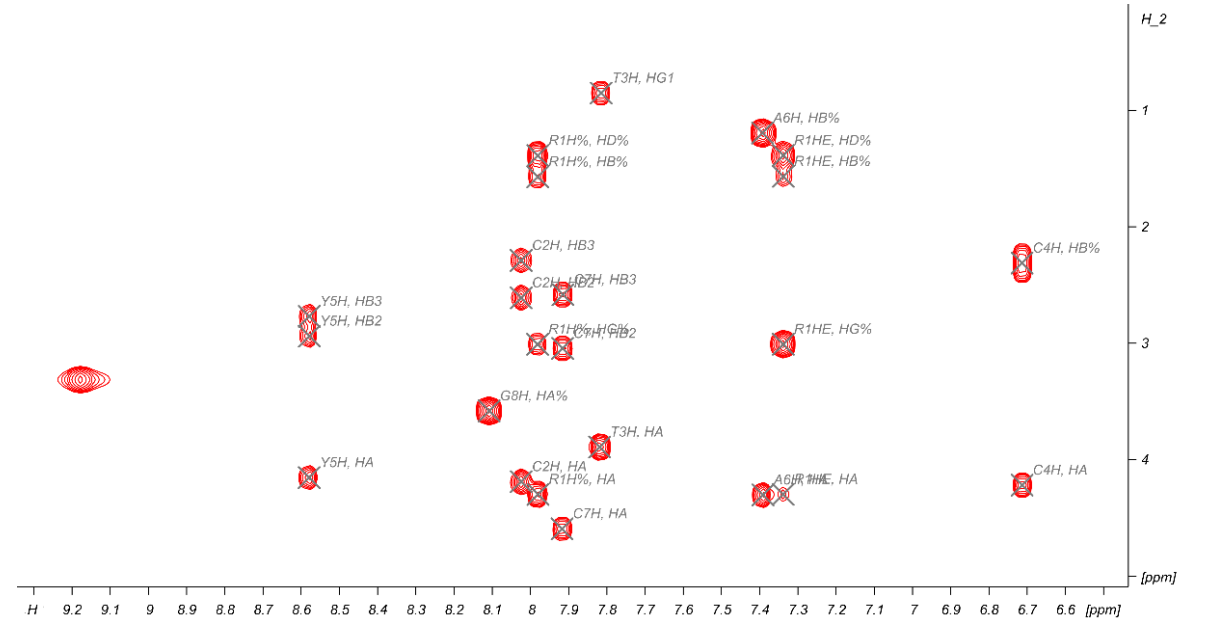


Figure S8. Labelled 2D ^{1}H-^{1}H TOCSY of **cP15$_{pk2}$** showing the fingerprint region between 6.5-9 ppm on the horizontal axis. There are 9 alpha amide (α-NH) signals, indicating one species is present.

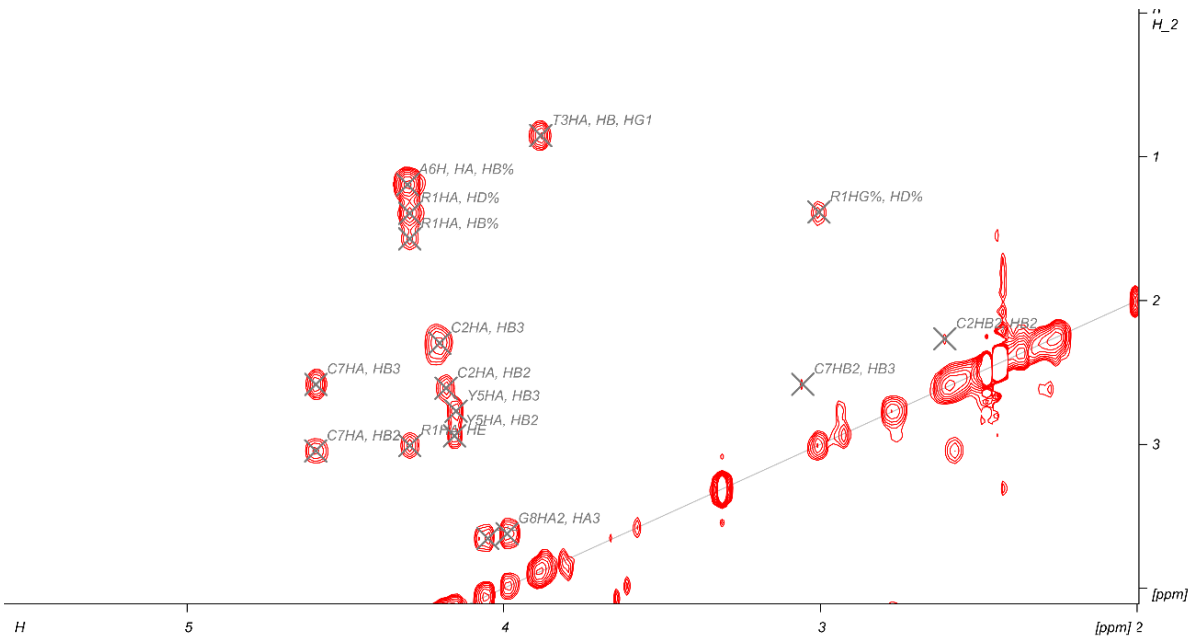


Figure S9. Labelled 2D ^{1}H-^{1}H TOCSY of **cP15$_{pk2}$** showing the region between 2-5 ppm on the horizontal axis.

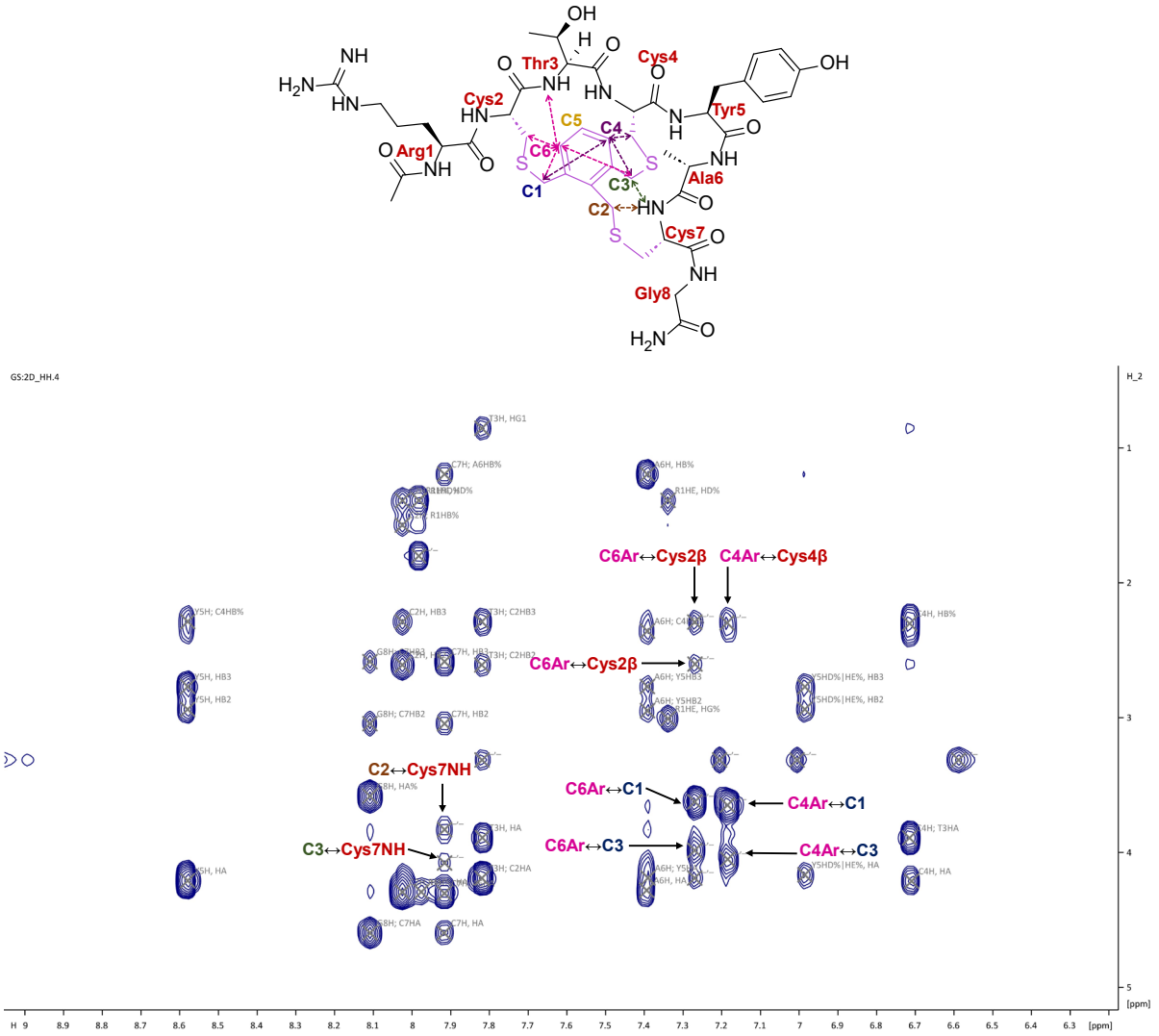


Figure S10. 2D ^{1}H-^{1}H NOESY of **cP15$_{pk2}$** showing the fingerprint region between 6.5-9 ppm on the horizontal axis. Full structure of the corresponding isomer **cP15$_{b}$** is shown, with dashed arrows indicating additional NOE cross peaks between peptide backbone amide protons and benzylic CH_2 protons at C2 (brown), and C3 (green). NOE cross peaks involving linker aromatic CH protons at C4Ar (purple) and C6Ar (pink) are also shown.

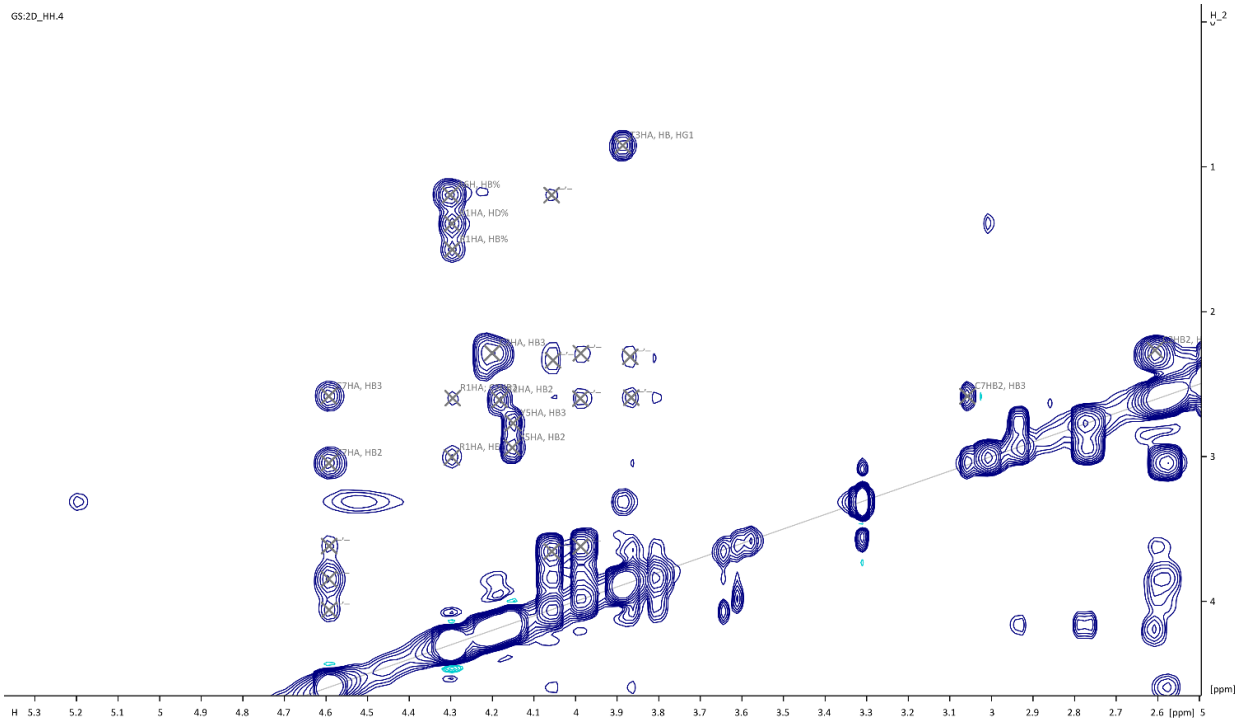


Figure S11. 2D ^{1}H-^{1}H NOESY of **cP15$_{pk2}$** showing the region between 2-5 ppm on the horizontal axis.

6 Study of bicyclisation reactions using Method B

6.1 Summary of hydrophobic peptides

Table S4. List of hydrophobic peptide sequences tested in bicyclisation reactions with **1,2,3-TBMB** using Method B, showing the calculated *m/z*, the observed *m/z*, and estimated percentage conversion. hC = homocysteine. Full LCMS data is available in ESI Section 9.3.

Name	Sequence	Calc. *m/z*	Obs. *m/z*	%
P16	Ac-DRR**C**V**C**F**C**LGF-NH_2	$[M+H]^+$ =1474.66[a]	1474.80	>95
P17	Ac-DRR**hC**V**hC**F**hC**LGF-NH_2	$[M+H]^+$ =1515.71	1515.90	53
P18	Ac-DRR**hC**V**C**F**hC**LGF-NH_2	$[M+H]^+$ =1502.69[a]	1502.90	>95

[a] Calculated masses correspond to the most abundant monoisotopic mass observed by LCMS.

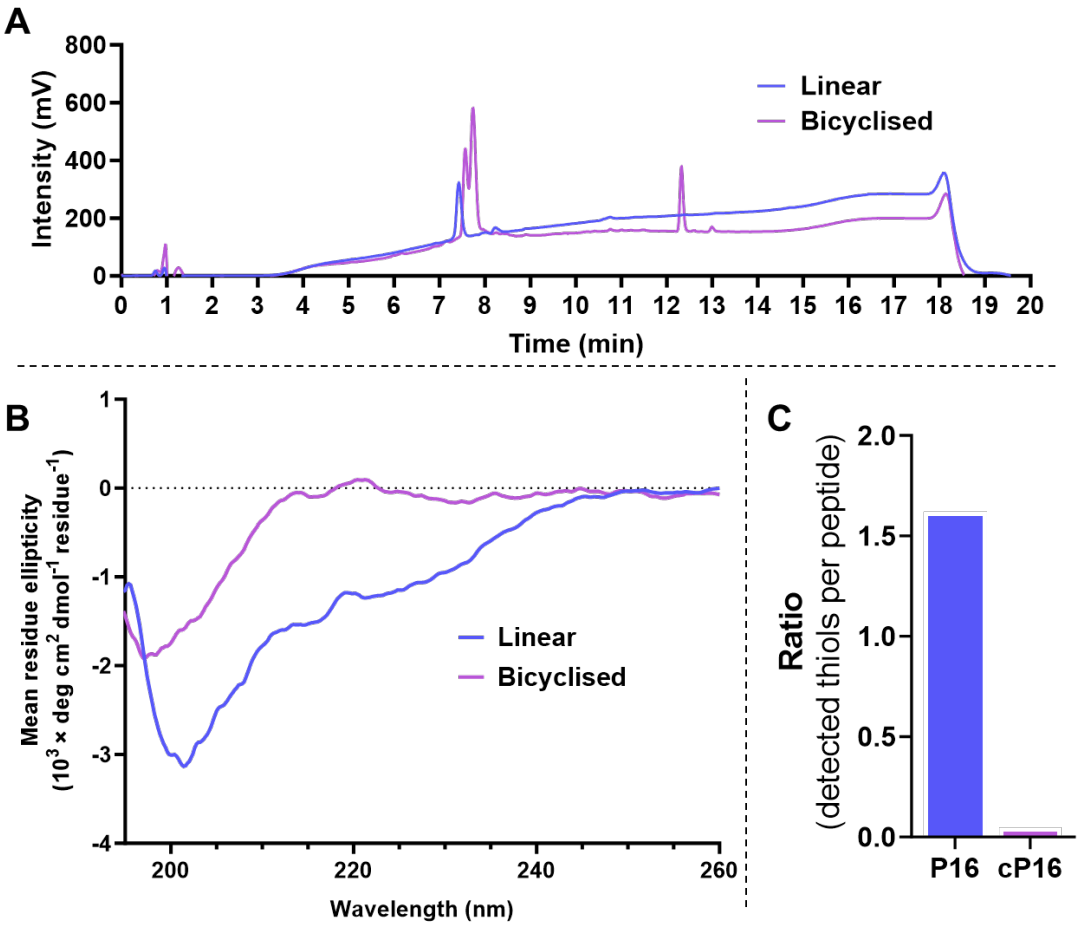


Figure S12. (A) LC UV chromatograms monitored at 220 nm showing overlay of linear peptide (blue) and crude bicyclisation reaction using Method B (purple) for peptide **P16**. **(B)** Circular dichroism spectra of linear **P16** and bicyclic **cP16** as a mixture of isomers. **(C)** Ellman's assay for linear **P16** and bicyclic **cP16**.

6.2 Peptide bicyclisation at homocysteine using Method B

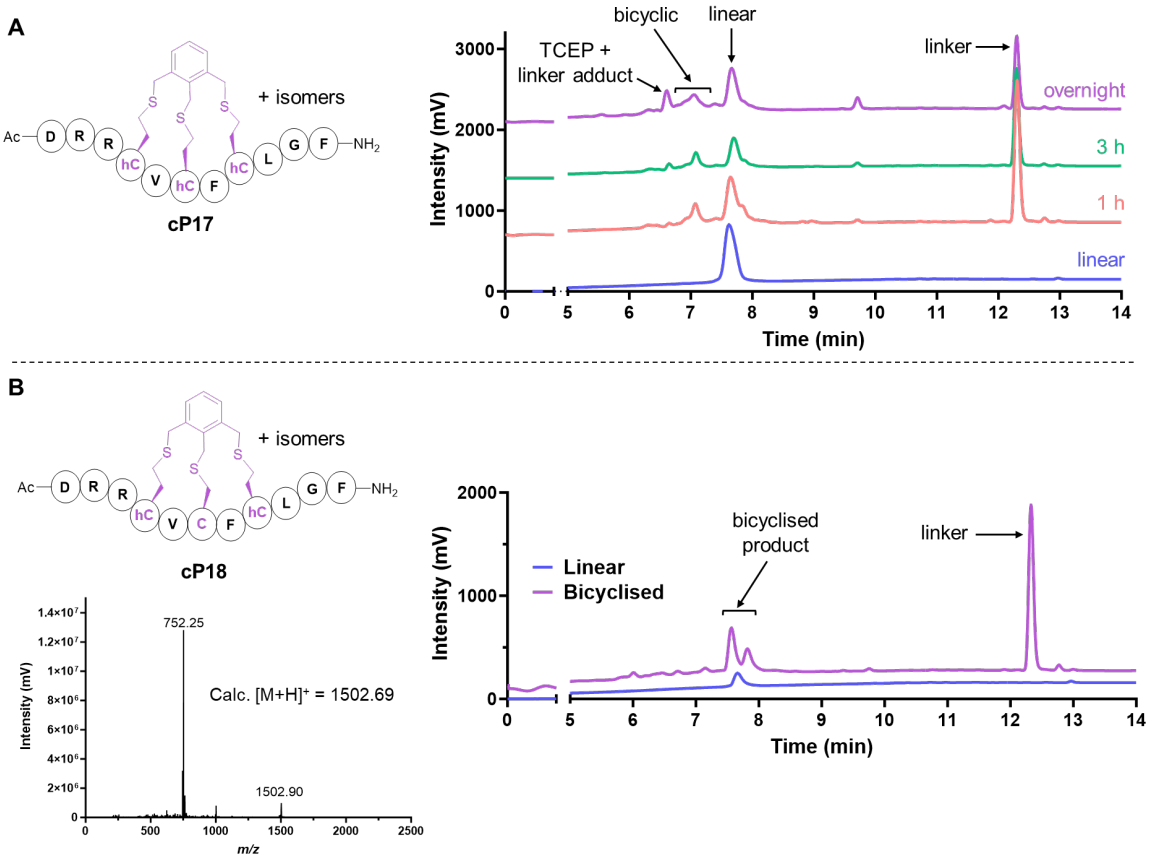


Figure S13. Bicyclisation under Method B for peptides containing homocysteine. **(A)** LCMS UV chromatograms monitoring at 220 nm for the attempted bicyclisation reaction on **P17**, showing the linear peptide (blue) and crude reaction mixture at 1 h (orange), 3 h (green) and overnight (purple). Linear product can still be observed after overnight reaction with only ~53% conversion to the expected bicyclised product. Adduct formation between TCEP and linker increases over time. **(B)** LCMS UV chromatograms of **P18** showing linear (blue) and crude bicyclised reaction (purple). The mass spectrum of the product peak is shown, where the two distinct LCMS peaks have the identical *m/z*. No mass corresponding to the linear product was observed.

6.3 Reactivity of TCEP with various crosslinkers

Similar reactivity of TCEP with maleimide and α-haloacyl groups has been observed in the literature. [3] The phosphine of TCEP can likely act as a nucleophile in a substitution reaction with crosslinkers containing alkyl bromides. The observed *m/z* match the possible structures postulated below, although no further characterisation was performed to confirm these proposed structures.

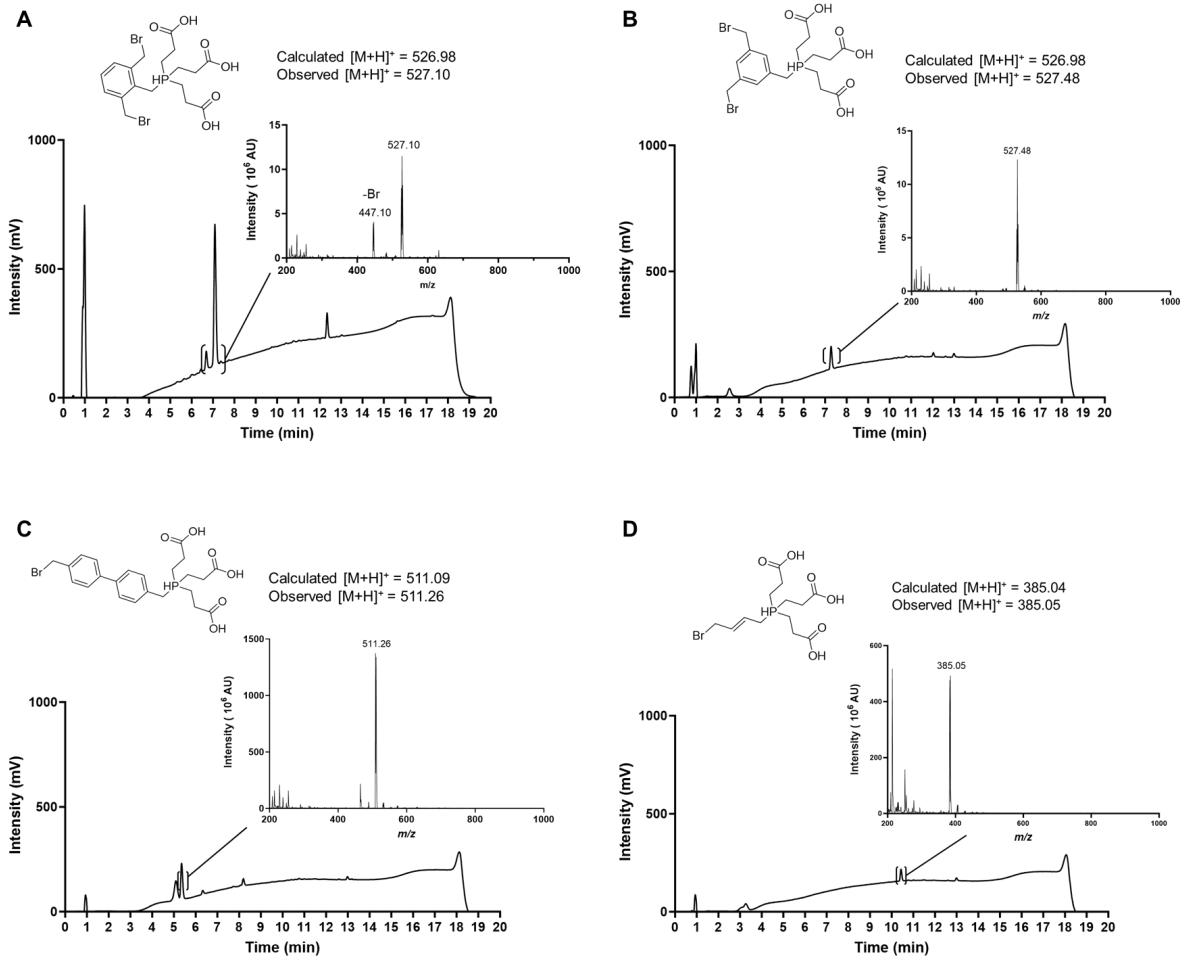


Figure S14. Reactivity of a range of commonly used crosslinkers with TCEP using Method B conditions. **(A)** Mass spectrum and possible structure of adduct with **1,2,3-TBMB** linker. **(B)** Mass spectrum and possible structure of adduct with **1,3,5-TBMB** linker. **(C)** Mass spectrum and possible structure of adduct with 4,4-bis(bromomethyl)biphenyl linker. **(D)** Mass spectrum and possible structure of adduct with *trans*-1,4-dibromo-2-butene linker.

7 CD spectroscopy methods

CD spectra were obtained on a JASCO J-815 Circular Dichroism Spectrometer at 20 °C with a 0.1 cm path-length quartz cuvette, scanning from 260 to 190 nm at 20 nm/min, bandwidth 1.0 nm and response time 2 s. Each spectrum is an average of three measurements and smoothed with Savitzky-Golay at a convolution width of 21 on a JASCO spectra analysis 2.0 software.

HPLC purified peptides were dissolved up to 1 mg/mL using 20% MeCN in water, then diluted to 0.3 mg/mL using 20% MeCN in 10 mM sodium phosphate buffer at pH 7.4 with 1 mM TCEP. Peptide quantities were determined by weighing on a five-digit analytical balance. The molar residue ellipticity (MRE) was calculated using:

$$MRE = \frac{\theta}{l \times r \times c}$$

Where θ is the CD signal (mdeg), l is path length (mm), r is number of residues in peptide and c is total molar concentration of peptide (M).

8 Bicyclisation of crude peptides from a 96-well plate

The peptides were tested as crude purity directly obtained from commercial vendors in a 96-well plate. LCMS was conducted on the crude linear starting materials and the reaction mixtures after bicyclisation to calculate percentage conversion by integration of *m/z* peaks, assuming the linear and cyclised peptides have similar ionisation efficiency in MS. This method for determining %conversion by MS has previously been reported for linear and cyclic peptides. [4]

Table S5. Data for 96-well plate format peptide bicyclisation showing the expected *m/z* for linear and bicyclic peptides, *m/z* peak integration for the linear and bicyclic peptides, and estimated percentage conversion based on these integrals. All sequences are *N*-terminal acetylated and *C*-terminal amidated. LCMS data for the selected **$_{96w}$P15** and **$_{96w}$P51** bicyclisation reaction are shown in ESI Section 9.5.

		Expected *m/z* $[M+H]^+$		Peak integration by m/z		
ID	**Sequence**	**Linear**	**Cyclic**	**Linear**	**Cyclic**	**%**
$_{96w}$P1	YIECQPCDCW	1300.49	1414.49	2191053	85209597	>95
$_{96w}$P2	KLWCLCTCFAA	1299.63	1413.63	948630	171826480	>95
$_{96w}$P3	EDACRCWCMAW	1414.66	1528.66	112058479	107766020	49.0
$_{96w}$P4	YTDCHCDCLTK	1342.53	1456.53	2071338	245903282	>95
$_{96w}$P5	GFICICECAPH	1233.49	1347.49	88977676	167900465	65.4
$_{96w}$P6	QYECMCKCIEH	1427.70	1541.70	7725488	299435943	>95
$_{96w}$P7	AYLCHCGCDHL	1275.49	1389.49	215375190	498219711	69.8
$_{96w}$P8	VVQCGCRCEHI	1287.54	1401.54	3925119	215944314	>95
$_{96w}$P9	AQMCQCNCVNP	1251.49	1365.49	2935303	100985624	>95
$_{96w}$P10	MNDCNCMCPRA	1298.57	1412.57	12304616	136602534	91.7
$_{96w}$P11	GLMCQCGCNHE	1235.44	1349.44	86159254	184648794	68.2
$_{96w}$P12	LSICTCDCHSP	1219.42	1333.42	49524224	147579186	74.9
$_{96w}$P13	GQDCDCRCSPM	1255.43	1369.43	22511345	105211871	82.4
$_{96w}$P14	ENMCLCYCFPI	1376.69	1490.69	65829834	108730670	62.3
$_{96w}$P15	TYMCECRCPWD	1447.69	1561.69	74727478	109074026	59.3
$_{96w}$P16	WTRCHCPCMMD	1423.74	1537.74	137494541	212859399	60.8
$_{96w}$P17	MYGCDCMCLQF	1354.67	1468.67	19317868	88409793	82.1
$_{96w}$P18	SRMCKCMCENF	1392.72	1506.72	46303602	321029225	87.4
$_{96w}$P19	SMICQCPCKWM	1370.76	1484.76	4466679	159403626	>95
$_{96w}$P20	TQSCFCACHYS	1290.45	1404.45	13099804	90273050	87.3
$_{96w}$P21	NSGCHCSCHSY	1238.34	1352.34	73164995	160205859	68.6
$_{96w}$P22	IETCHCRCADL	1304.53	1418.53	126296266	272241784	55.9
$_{96w}$P23	GHDCWCMCWMH	1300.49	1414.49	957419	81033099	>95
$_{96w}$P24	FMPCSCFCWYE	1195.40	1309.40	3443725	141216980	>95
$_{96w}$P25	AACTLCKCLLQ	1207.54	1321.54	565043	15343512	>95
$_{96w}$P26	QYCWICTCDMQ	1434.69	1548.69	2268095	63077787	>95
$_{96w}$P27	PGCDLCNCNLL	1205.43	1319.43	9049658	149633237	94.3
$_{96w}$P28	TRCHFCLCIYH	1436.73	1550.73	1421342	117978659	>95
$_{96w}$P29	IRCVRCNCILM	1364.78	1478.78	16820021	270970670	94.2
$_{96w}$P30	QPCDVCYCGRV	1283.51	1397.51	1323112	128701836	>95
$_{96w}$P31	VFCMVCLCNSR	1315.66	1429.66	10123771	234505160	>95
$_{96w}$P32	LFCKPCFCYMH	1432.81	1546.81	4012069	265839914	>95
$_{96w}$P33	GPCWLCVCMVG	1208.55	1322.55	874316	79135984	>95
$_{96w}$P34	FFCVRCHCVPH	1388.69	1502.69	1254746	110071089	>95

$_{96w}$**P35**	PRCLHCRCYKI	1432.79	1546.79	1105210	55488938	>95
$_{96w}$**P36**	EQCHNCECKLN	1361.53	1475.53	4549086	232504050	>95
$_{96w}$**P37**	PHCFGCRCKFE	1367.63	1481.63	1425754	250054605	>95
$_{96w}$**P38**	RGCFICDCKQV	1312.59	1426.59	7084532	318658650	>95
$_{96w}$**P39**	YGCQDCSCRAS	1233.36	1347.36	1464141	75825525	>95
$_{96w}$**P40**	RLCPSCMCGHS	1234.50	1348.50	1778687	284381177	>95
$_{96w}$**P41**	PQCNGCACVWY	1284.49	1398.49	916290	57577900	>95
$_{96w}$**P42**	YLCNQCMCVVM	1347.72	1461.72	1918006	28983221	93.8
$_{96w}$**P43**	KPCKECRCIWP	1403.75	1517.75	4446998	71677043	94.2
$_{96w}$**P44**	PLCIACGCTDW	1222.46	1336.46	166210608	153795219	48.1
$_{96w}$**P45**	WGCVYCSCWLI	1373.67	1487.67	747521	50531763	>95
$_{96w}$**P46**	RECTPCSCIML	1296.61	1410.61	132631740	107504818	44.8
$_{96w}$**P47**	STCDTCKCKQQ	1285.48	1399.48	873258	164215226	>95
$_{96w}$**P48**	ANCKICWCARK	1195.40	1309.40	2634421	128481971	>95
$_{96w}$**P49**	SRDCFCRECKM	1318.58	1432.58	326935	85497145	>95
$_{96w}$**P50**	VSECGCQLCLK	1223.49	1337.49	2662979	242387399	>95
$_{96w}$**P51**	YGACFCKWCDR	1392.64	1506.64	749244	280793932	>95
$_{96w}$**P52**	RRACFCKKCYL	1431.80	1545.80	228460	81323358	>95
$_{96w}$**P53**	EINCGCDHCDL	1262.40	1376.40	4745814	159162495	>95
$_{96w}$**P54**	DRPCDCVECLK	1321.55	1435.55	1967223	495735654	>95
$_{96w}$**P55**	IKECMCPDCNK	1324.62	1438.62	1669761	307987297	>95
$_{96w}$**P56**	GQHCICNACLT	1203.42	1317.42	2995434	182224046	>95
$_{96w}$**P57**	GKACWCIQCMF	1330.67	1444.67	1330901	197060397	>95
$_{96w}$**P58**	VSMCACKVCVS	1170.50	1284.50	1264312	143284913	>95
$_{96w}$**P59**	VYNCKCKHCHH	1412.67	1526.67	1351068	162776218	>95
$_{96w}$**P60**	SYGCNCAVCHW	1283.47	1397.47	3852799	132480262	>95
$_{96w}$**P61**	FIPCDCTQCGP	1224.44	1338.44	866129	173717015	>95
$_{96w}$**P62**	KLKCTCEYCEP	1357.63	1471.63	26415423	338958669	92.8
$_{96w}$**P63**	HMFCGCWVCKD	1369.67	1483.67	794293	305541141	>95
$_{96w}$**P64**	LMGCYCNYCWF	1443.74	1557.74	645994	109499687	>95
$_{96w}$**P65**	IRICFCSQCKM	1372.75	1486.75	3297581	320933363	>95
$_{96w}$**P66**	THACGCKDCNM	1223.43	1337.43	1648556	275291527	>95
$_{96w}$**P67**	MFFCTCVTCPT	1293.61	1407.61	357618	116876923	>95
$_{96w}$**P68**	NMNCYCNPCEY	1394.58	1508.58	1510116	38261208	>95
$_{96w}$**P69**	SYSCTCYGCSV	1213.37	1327.37	271193	37652452	>95
$_{96w}$**P70**	LPVCSCTNCLF	1240.52	1354.52	3946181	174051371	>95
$_{96w}$**P71**	RWPCWCNECIV	1449.73	1563.73	9146828	130419372	93.4
$_{96w}$**P72**	DKFCYCNWCHE	1318.58	1432.58	596884	47324406	>95
$_{96w}$**P73**	PLCDVCLLCLK	1188.32	1302.32	2105701	154839684	>95
$_{96w}$**P74**	MNCQVCGSCAP	1153.38	1267.38	6078698	86141111	93.4
$_{96w}$**P75**	MPCLVCGECMM	1257.66	1371.66	2652658	179206156	>95
$_{96w}$**P76**	EFCLHCSFCFR	1432.70	1546.70	7648851	336224424	>95
$_{96w}$**P77**	SYCRHCLRCLG	1351.63	1465.63	2422847	204328726	>95
$_{96w}$**P78**	AACKKCPHCTY	1265.53	1379.53	2411424	261691936	>95
$_{96w}$**P79**	NKCWDCQTCNF	1402.59	1516.59	7048278	133671661	>95
$_{96w}$**P80**	LGCMICKECGA	1168.48	1282.48	48278870	148986838	75.5
$_{96w}$**P81**	NFCNACKACTY	1278.49	1392.49	20052949	45261583	69.3
$_{96w}$**P82**	EACAFCTVCRN	1257.47	1371.47	987921	246638821	>95
$_{96w}$**P83**	YNCQECMGCYR	1410.63	1524.63	874645	53939920	>95
$_{96w}$**P84**	RTCYICMGCHS	1314.59	1428.59	4466990	206225896	>95
$_{96w}$**P85**	SLCKNCKPCRF	1339.66	1453.66	2728795	184264370	>95
$_{96w}$**P86**	YTCIVCMSCFH	1347.66	1461.66	1186016	88531685	>95
$_{96w}$**P87**	TECWNCPWCAD	1368.52	1482.52	3104203	69917034	>95

$_{96w}$**P88**	GYCEWCKICNF	1406.66	1520.66	7441305	173062618	>95
$_{96w}$**P89**	SKCNACPYCSE	1245.41	1359.41	762289	107820646	>95
$_{96w}$**P90**	LTCIYCVRCDN	1343.60	1457.60	7660020	225950172	>95
$_{96w}$**P91**	DQCANCNPCMP	1236.43	1350.43	3178027	102484977	>95
$_{96w}$**P92**	ASCAVCWYCKP	1271.54	1385.54	9367165	215362599	>95
$_{96w}$**P93**	EECPICTQCPW	1349.56	1463.56	543933	172032330	>95
$_{96w}$**P94**	DQCIICGDCLE	1252.44	1366.44	6717334	146929430	>95
$_{96w}$**P95**	FTCGTCHDCKQ	1283.46	1397.46	1120848	202834702	>95
$_{96w}$**P96**	GHLHICMLCHY	1188.32	1302.32	2277512	147922641	>95

9 LCMS data for crude peptide bicyclisation reactions

Note: Raw file names do not necessarily match final compound numbers in the manuscript.

9.1 Pilot study peptides

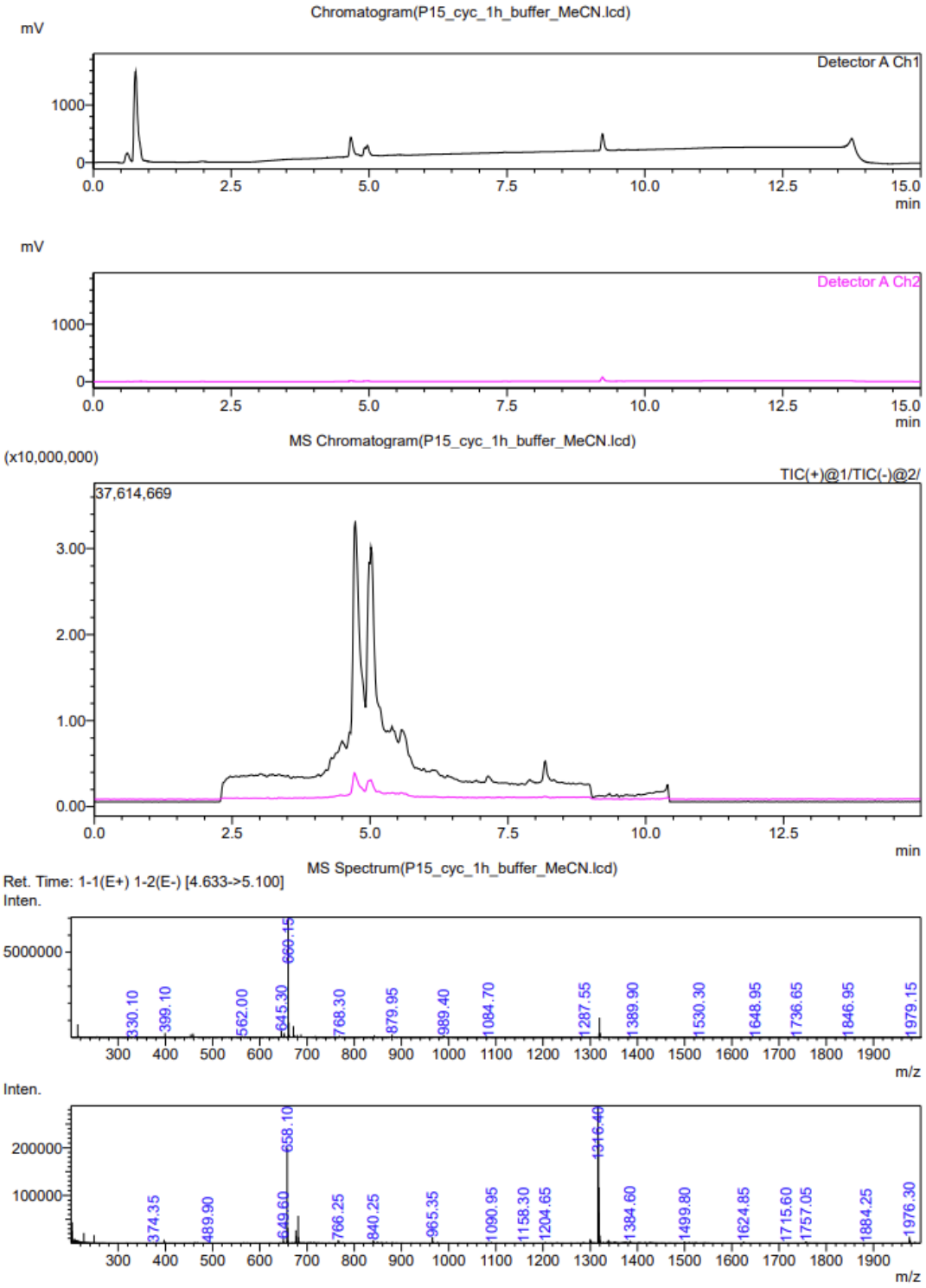


Figure S15. LCMS of crude reaction using Method A for **cP1** showing LC UV trace at 220 and 254 nm (top), TIC (middle), and MS (+ve and -ve) at selected product peak (bottom).

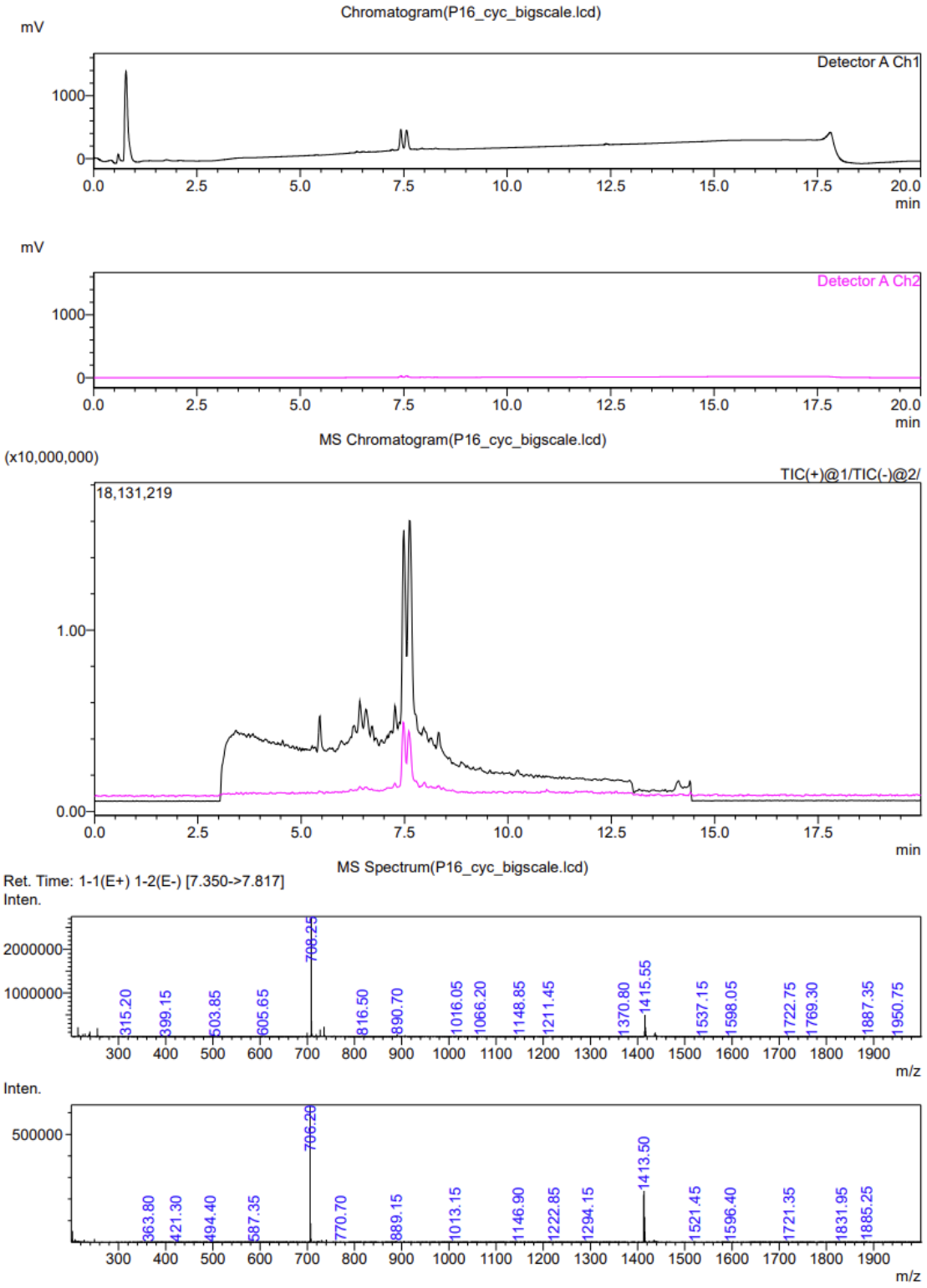


Figure S16. LCMS of crude reaction using Method A for **cP2** showing LC UV trace at 220 and 254 nm (top), TIC (middle), and MS (+ve and -ve) at selected product peak (bottom).

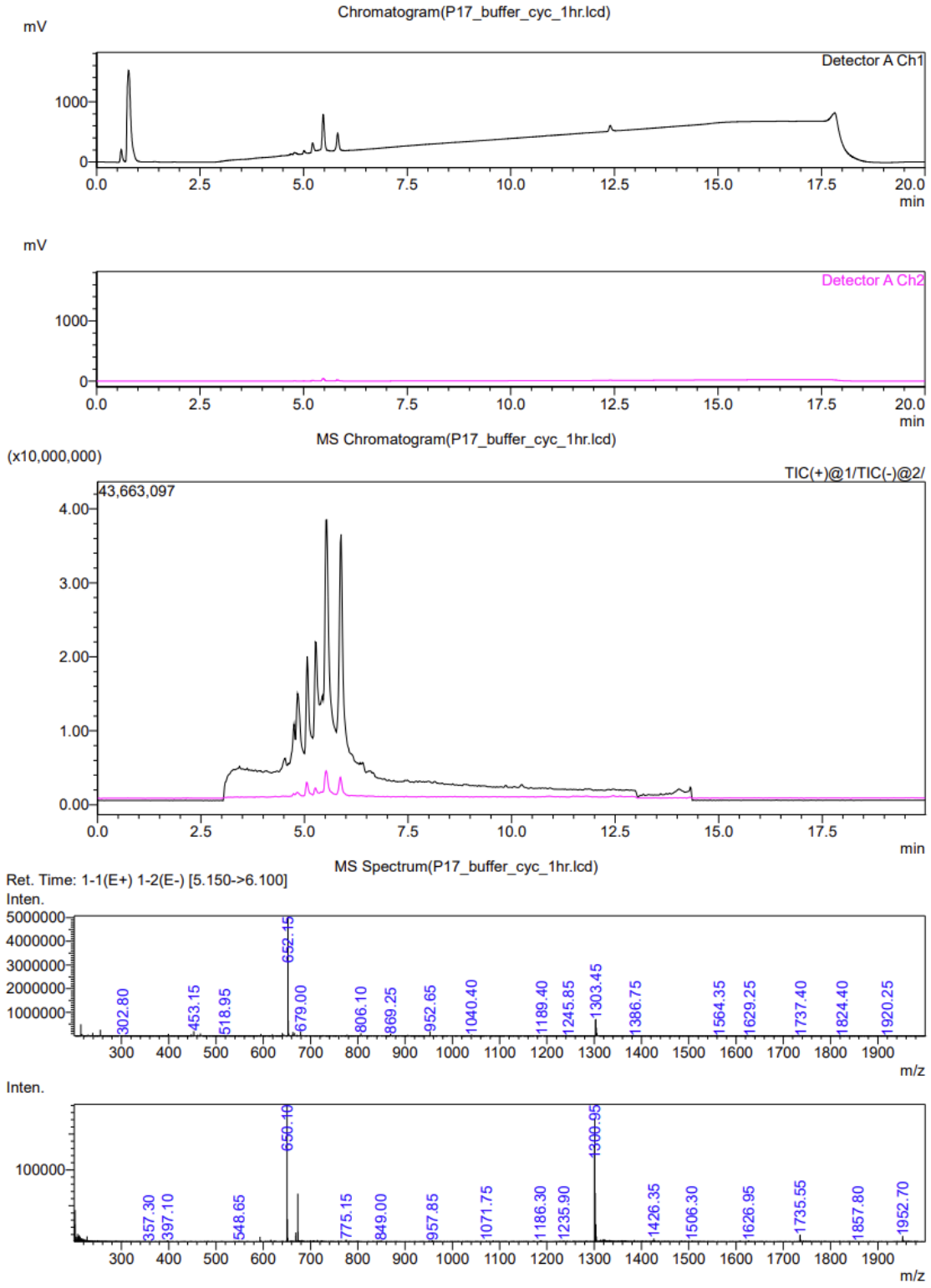


Figure S17. LCMS of crude reaction using Method A for **cP3** showing LC UV trace at 220 and 254 nm (top), TIC (middle), and MS (+ve and -ve) at selected product peak (bottom).

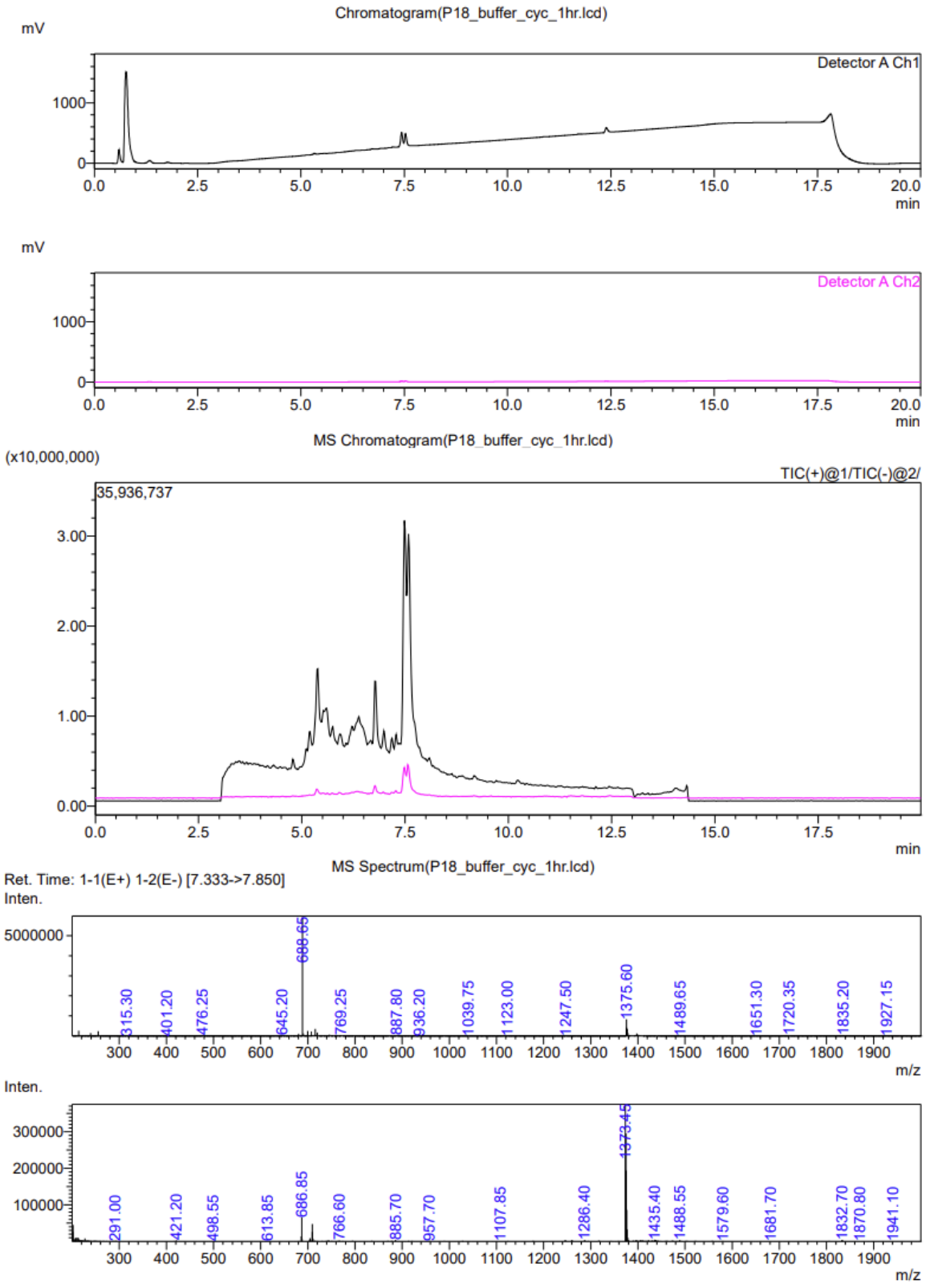


Figure S18. LCMS of crude reaction using Method A for **cP4** showing LC UV trace at 220 and 254 nm (top), TIC (middle), and MS (+ve and -ve) at selected product peak (bottom).

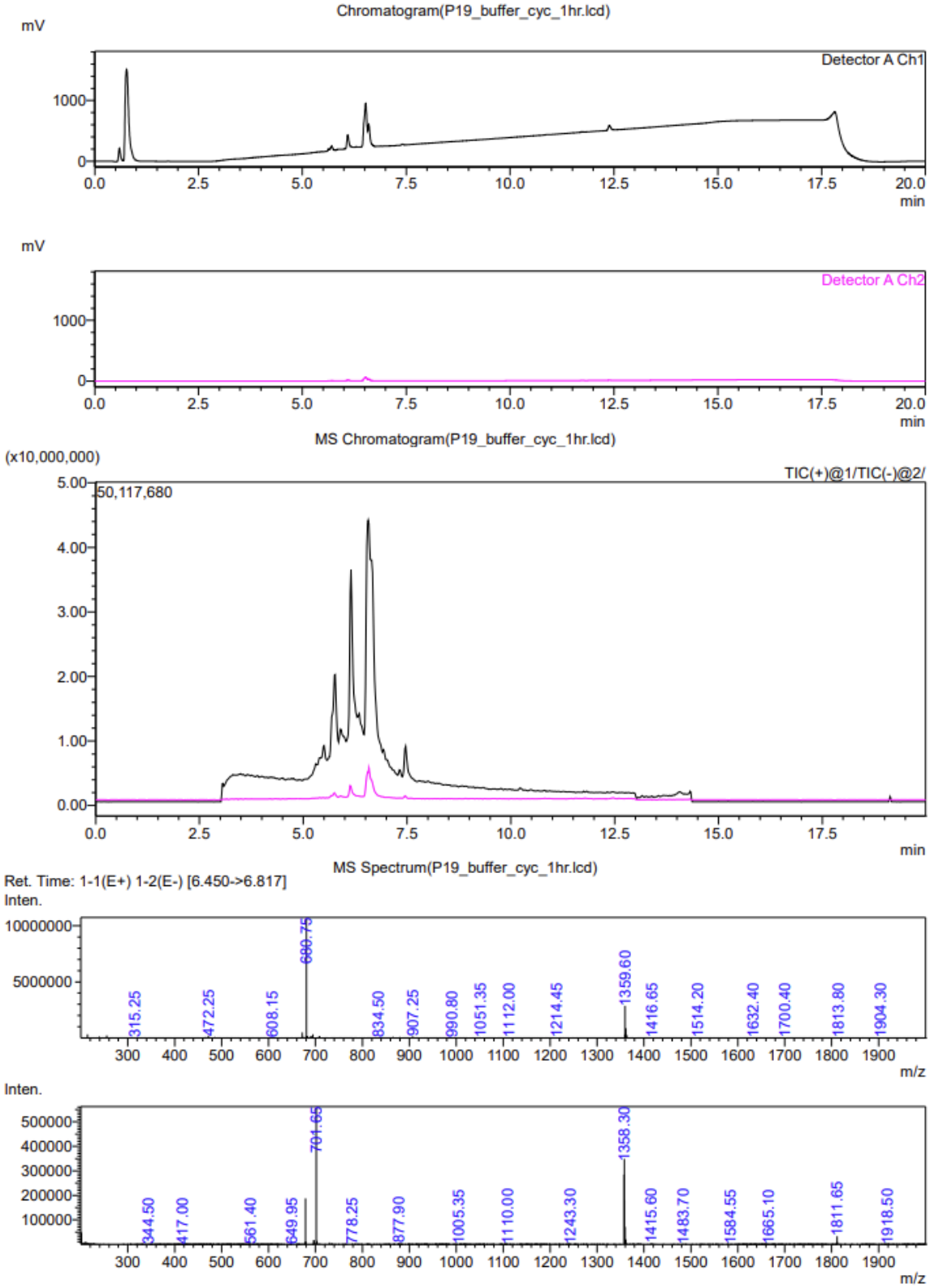


Figure S19. LCMS of crude reaction using Method A for **cP5** showing LC UV trace at 220 and 254 nm (top), TIC (middle), and MS (+ve and -ve) at selected product peak (bottom).

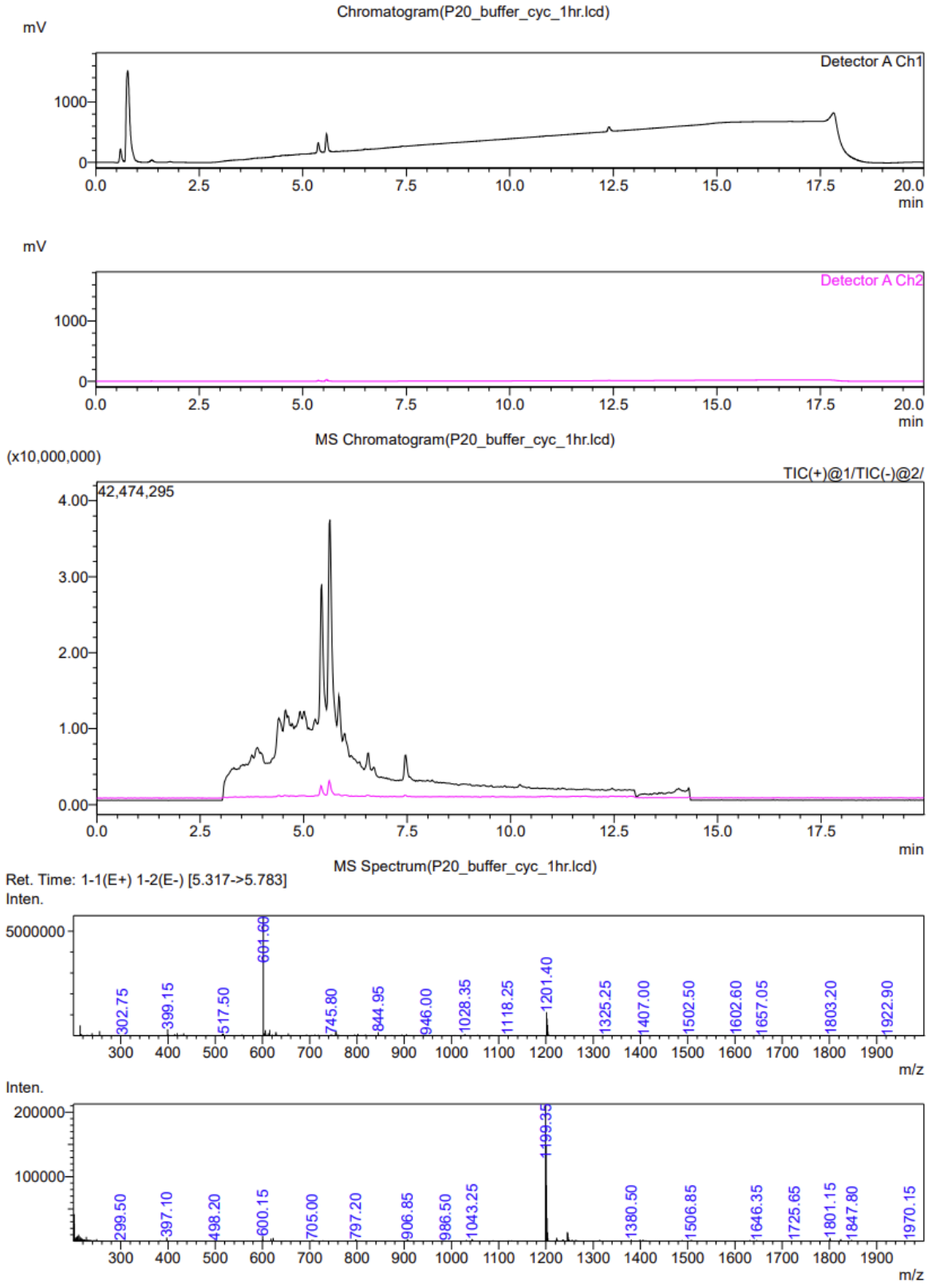


Figure S20. LCMS of crude reaction using Method A for **cP6** showing LC UV trace at 220 and 254 nm (top), TIC (middle), and MS (+ve and -ve) at selected product peak (bottom).

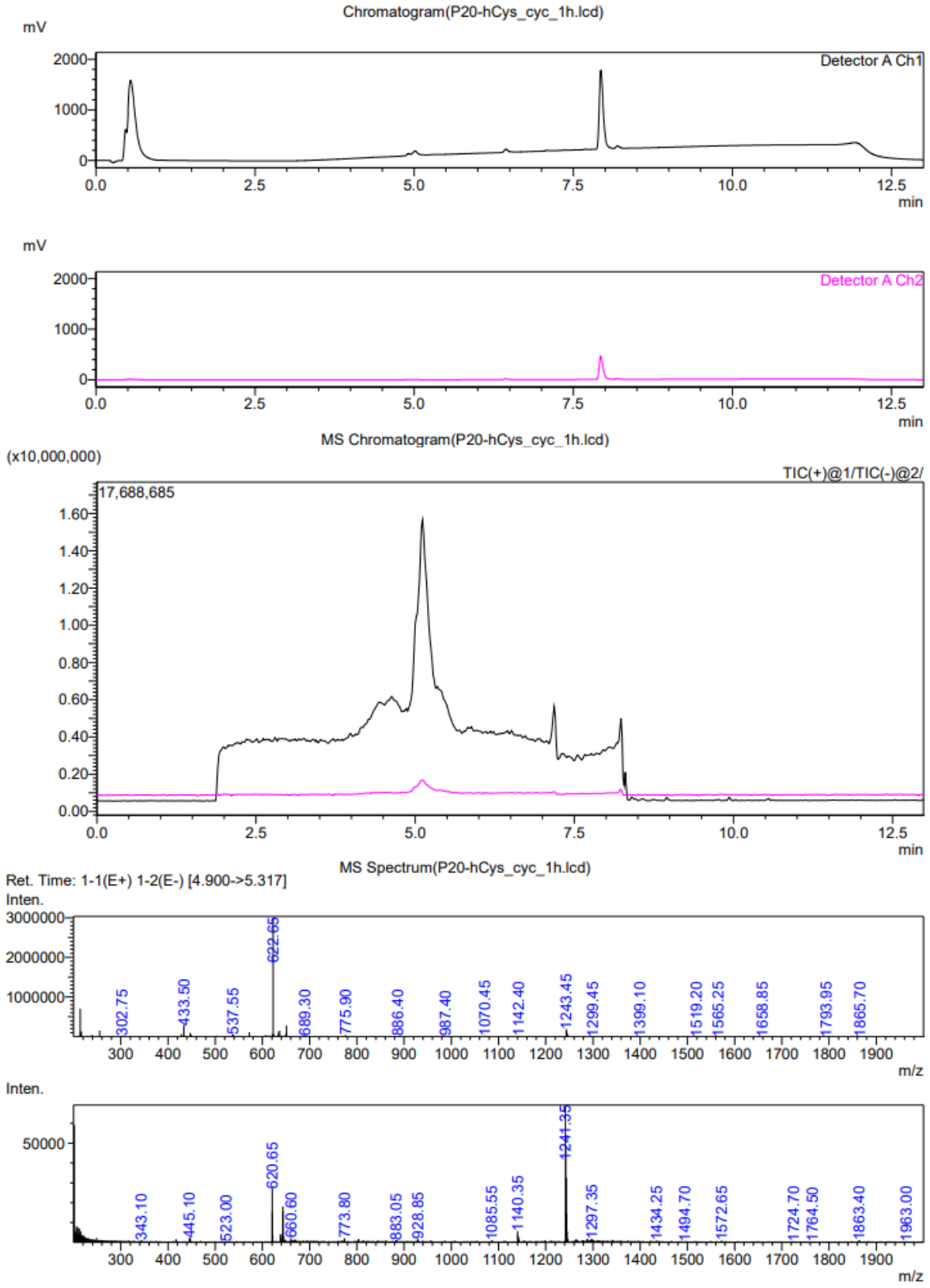


Figure S21. LCMS of crude reaction using Method A for **cP6-hCys** showing LC UV trace at 220 and 254 nm (top), TIC (middle), and MS (+ve and -ve) at selected product peak (bottom).

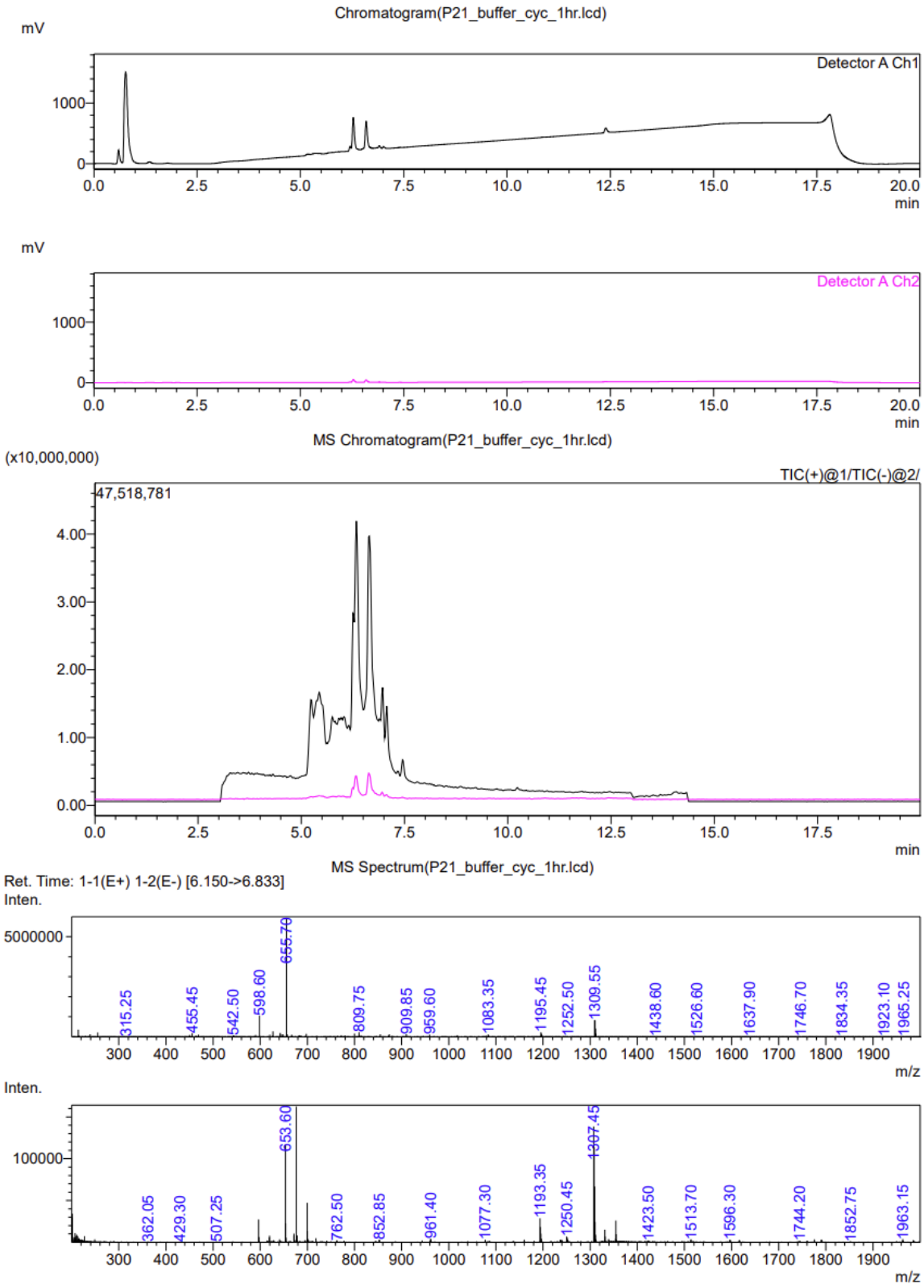


Figure S22. LCMS of crude reaction using Method A for **cP7** showing LC UV trace at 220 and 254 nm (top), TIC (middle), and MS (+ve and -ve) at selected product peak (bottom).

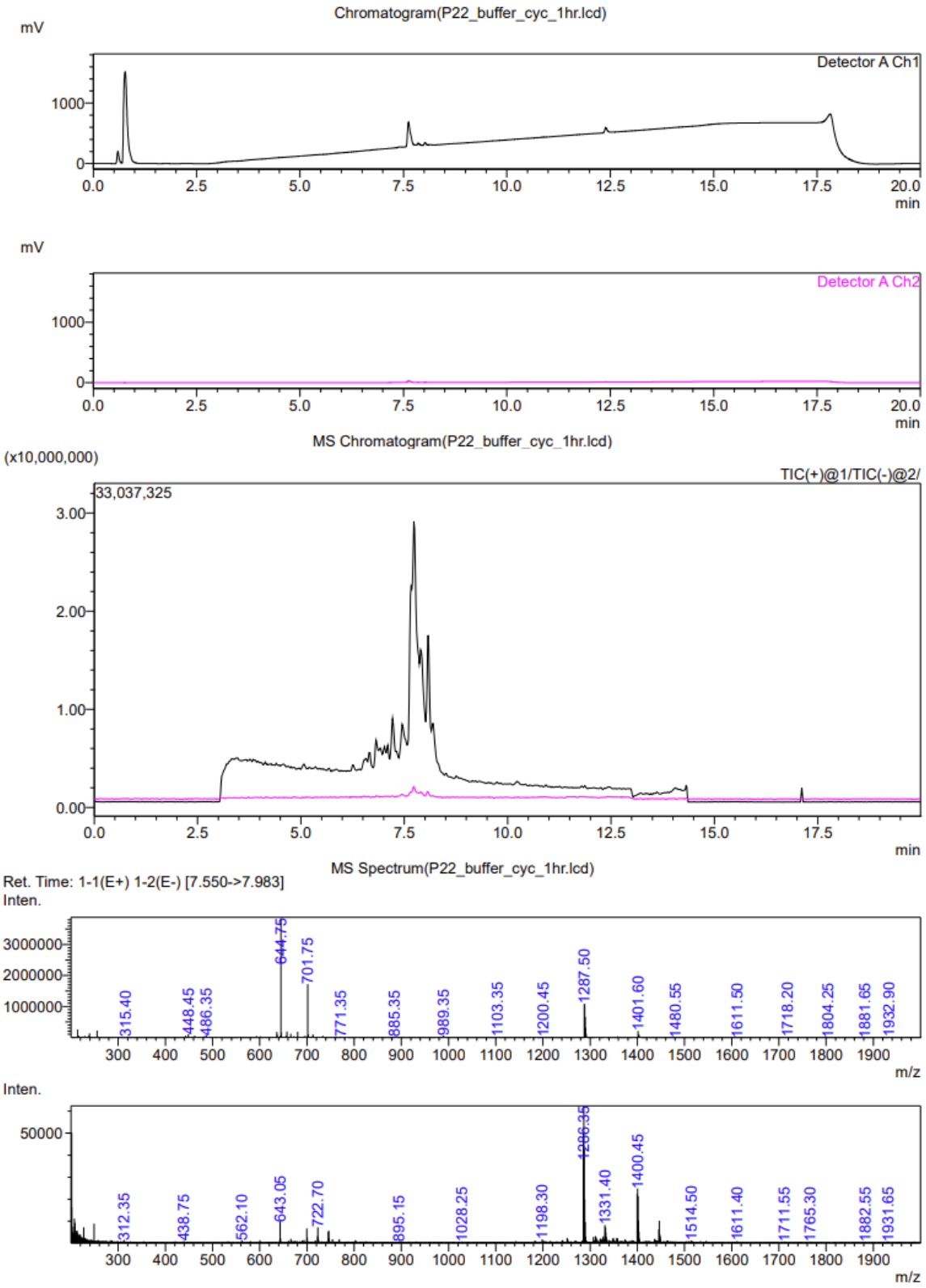


Figure S23. LCMS of crude reaction using Method A for **cP8** showing LC UV trace at 220 and 254 nm (top), TIC (middle), and MS (+ve and -ve) at selected product peak (bottom).

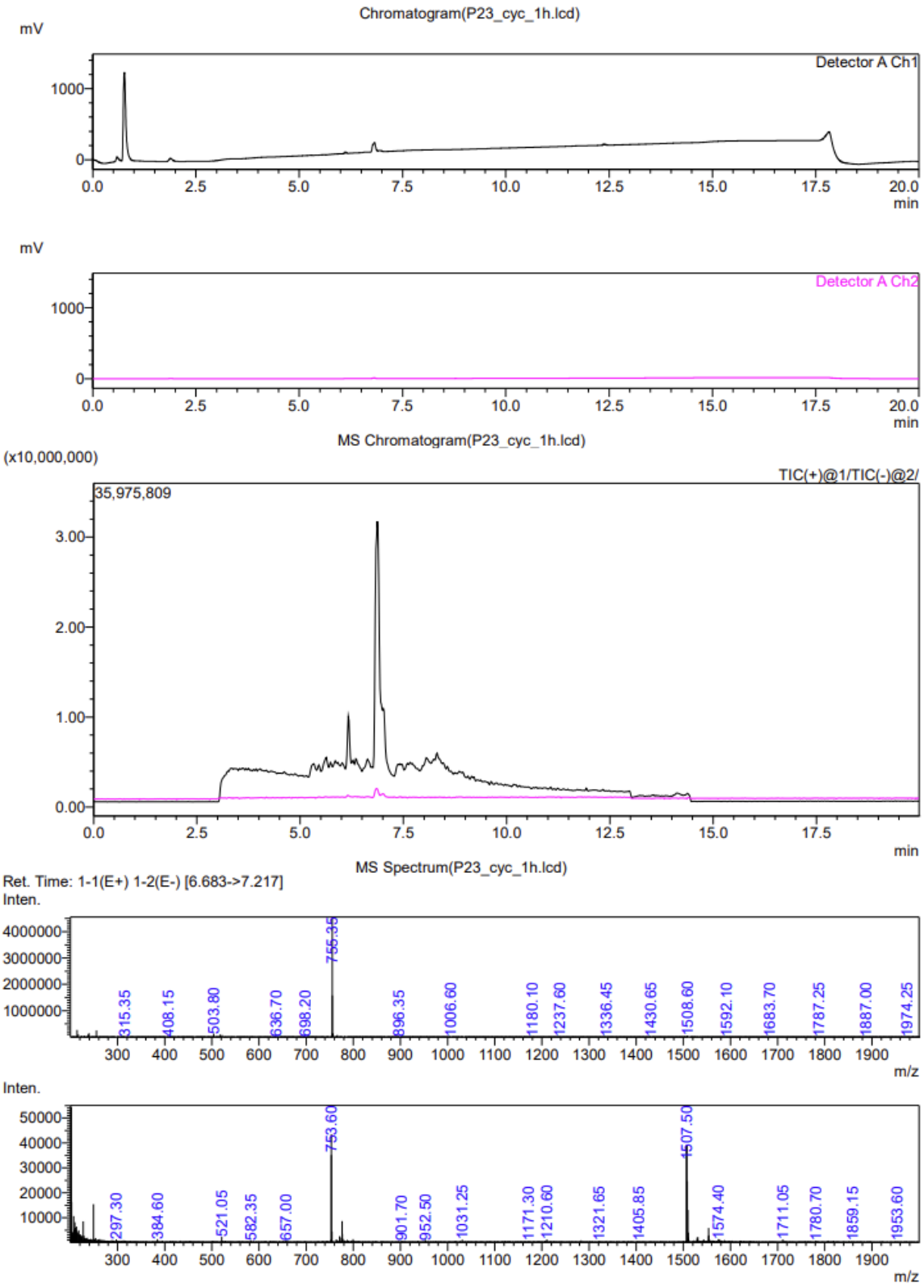


Figure S24. LCMS of crude reaction using Method A for **cP9** showing LC UV trace at 220 and 254 nm (top), TIC (middle), and MS (+ve and -ve) at selected product peak (bottom).

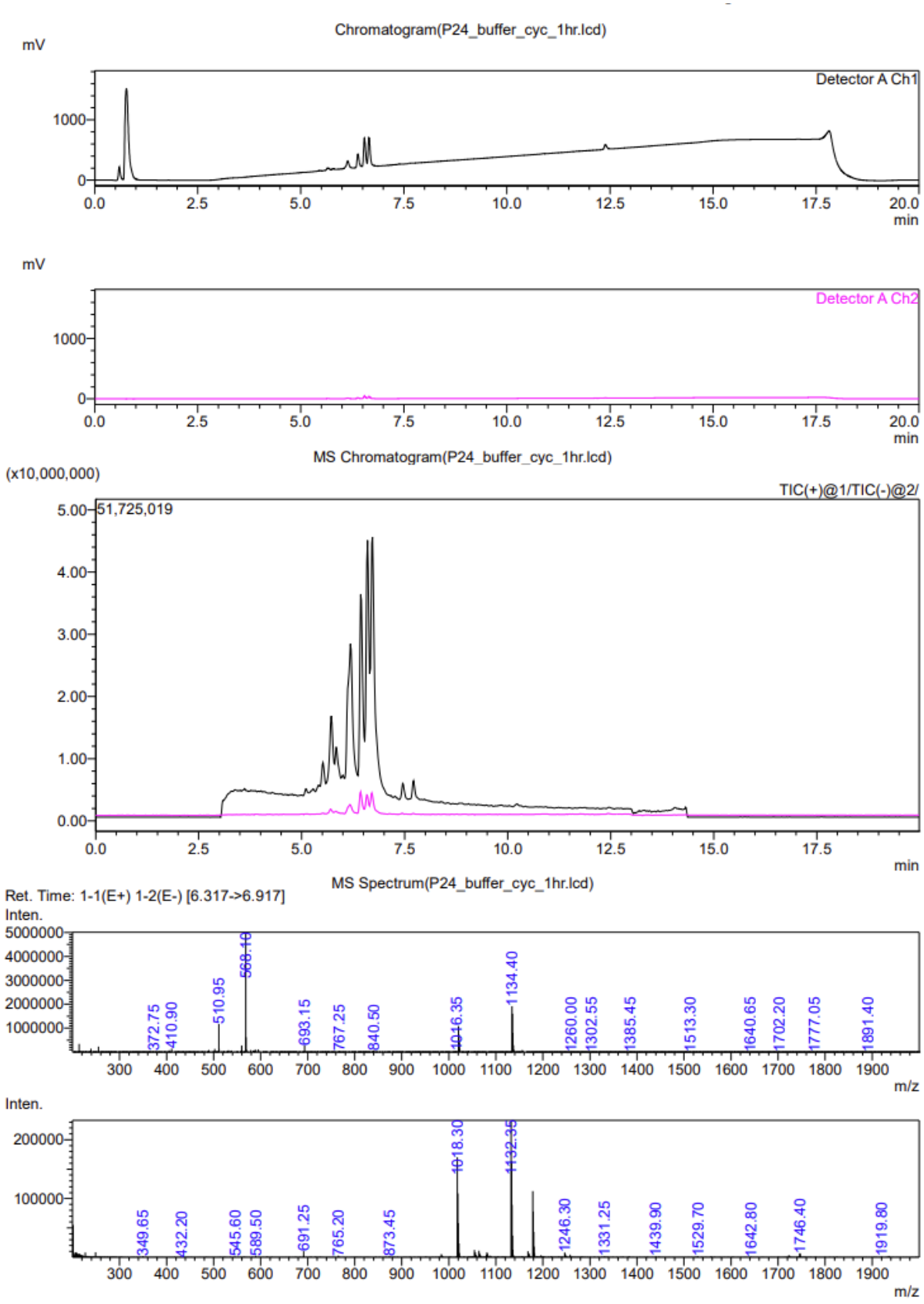


Figure S25. LCMS of crude reaction using Method A for **cP10** showing LC UV trace at 220 and 254 nm (top), TIC (middle), and MS (+ve and -ve) at selected product peak (bottom).

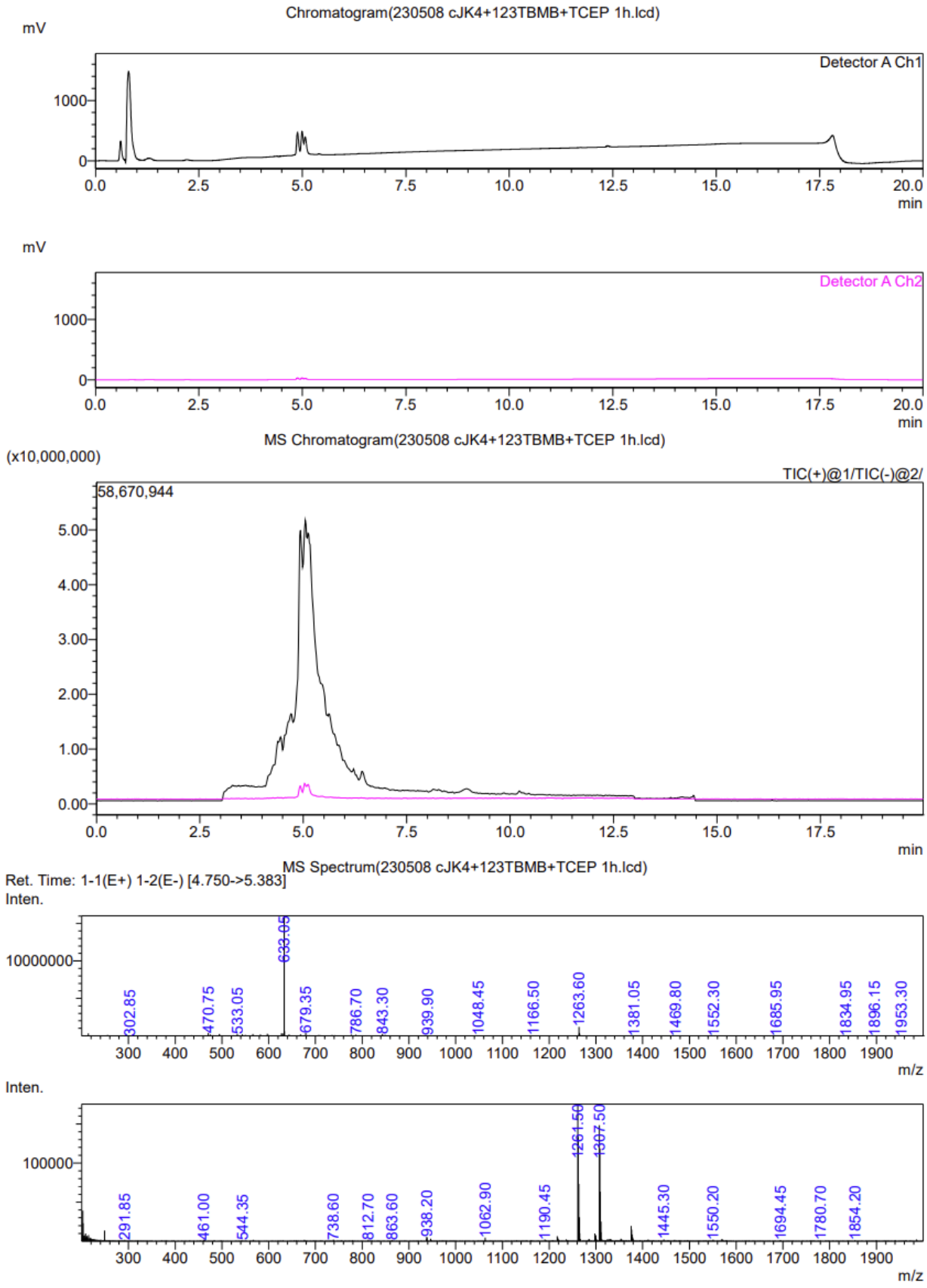


Figure S26. LCMS of crude reaction using Method A for **cP11** showing LC UV trace at 220 and 254 nm (top), TIC (middle), and MS (+ve and -ve) at selected product peak (bottom).

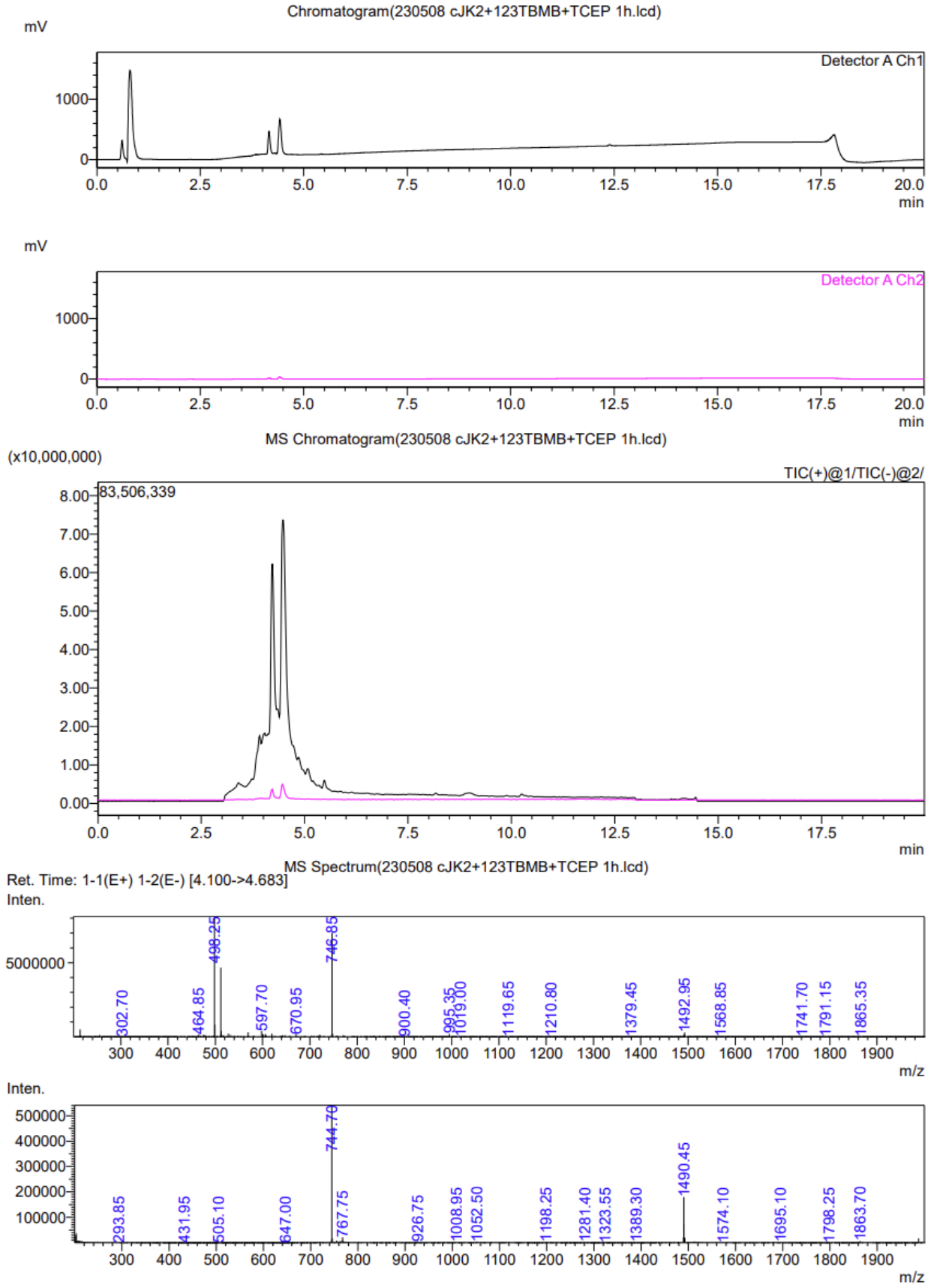


Figure S27. LCMS of crude reaction using Method A for **cP12** showing LC UV trace at 220 and 254 nm (top), TIC (middle), and MS (+ve and -ve) at selected product peak (bottom).

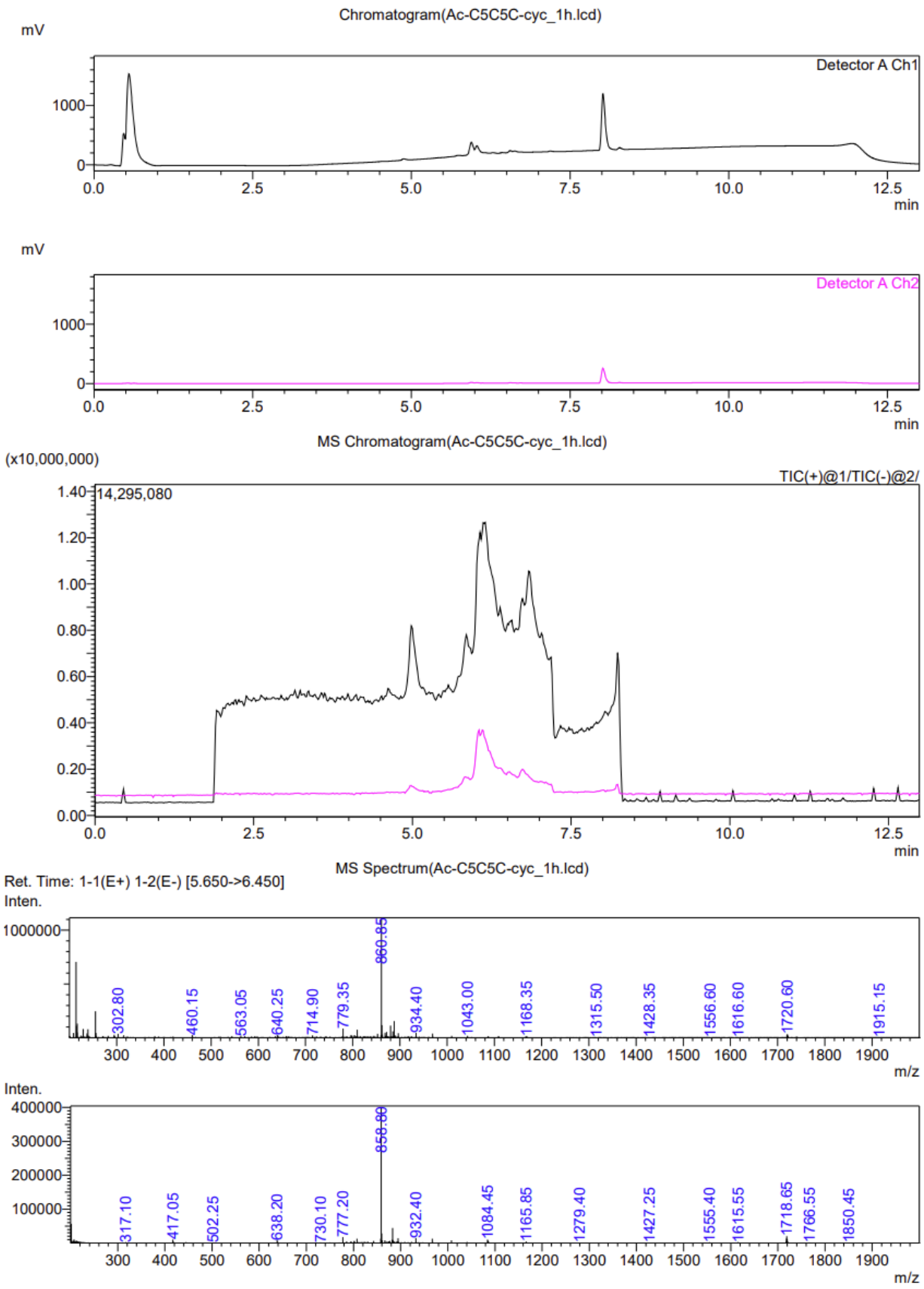


Figure S28. LCMS of crude reaction using Method A for **cP13** showing LC UV trace at 220 and 254 nm (top), TIC (middle), and MS (+ve and -ve) at selected product peak (bottom).

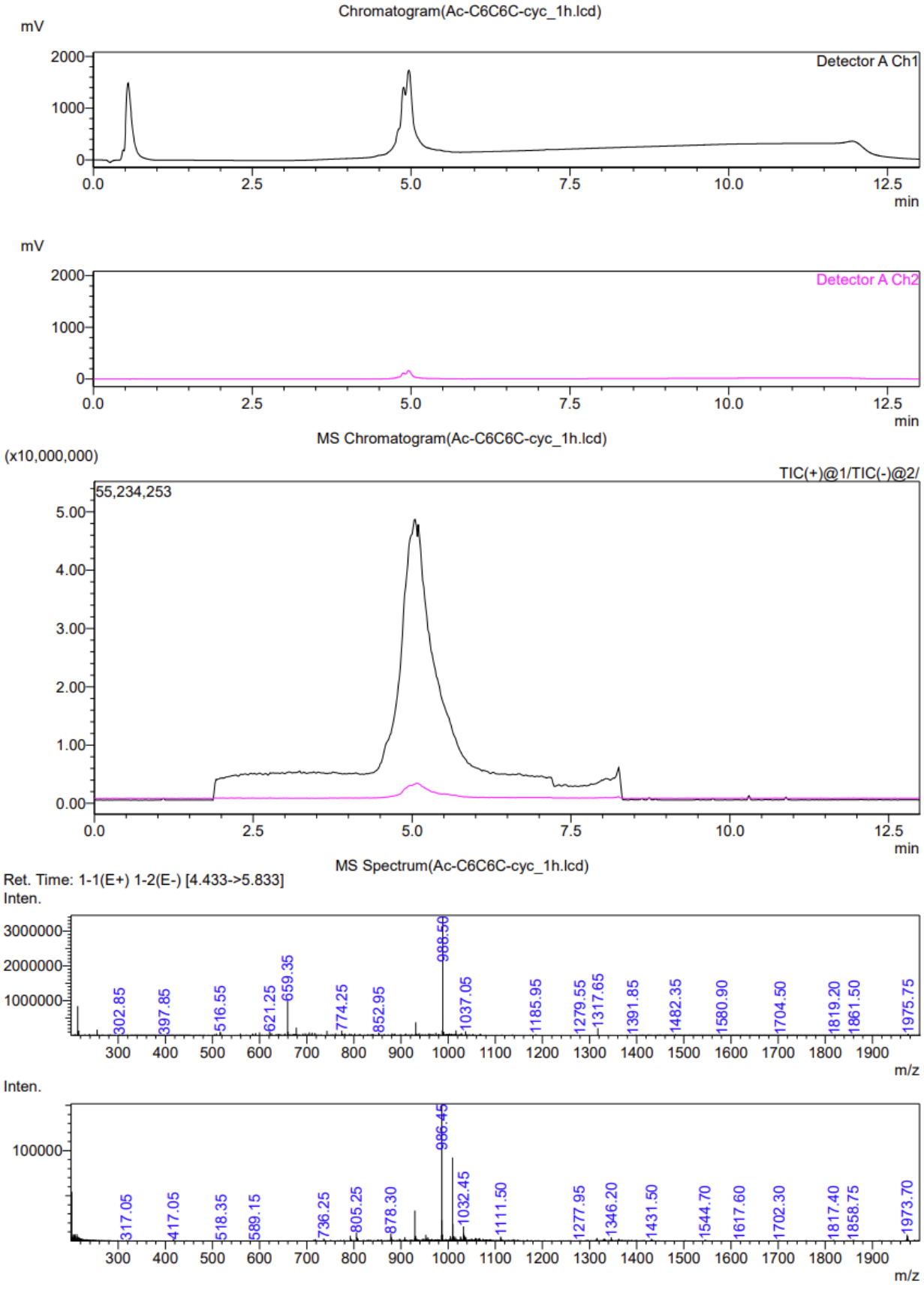


Figure S29. LCMS of crude reaction using Method A for **cP14** showing LC UV trace at 220 and 254 nm (top), TIC (middle), and MS (+ve and -ve) at selected product peak (bottom).

9.2 Peptides for 2D NMR

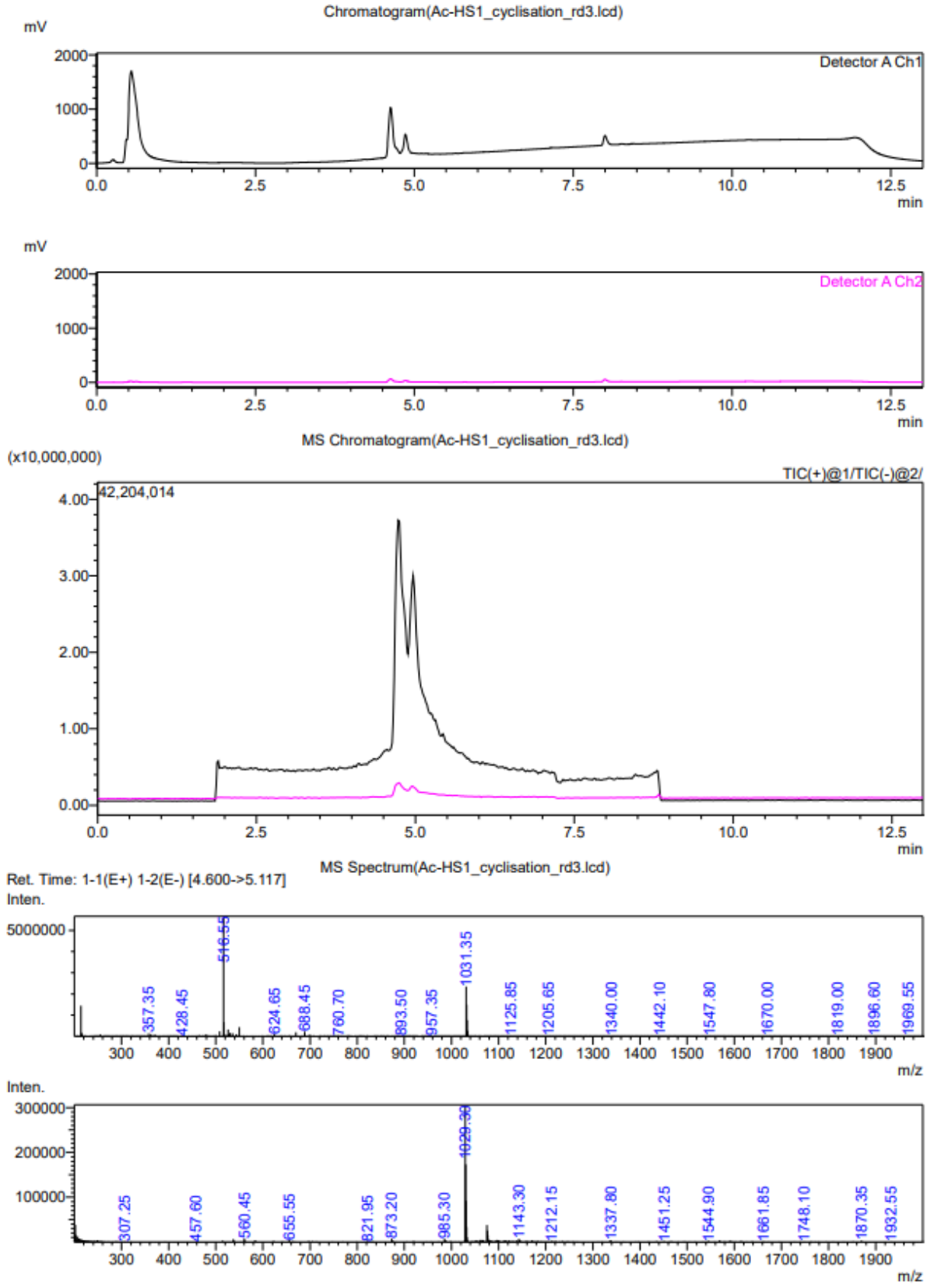


Figure S30. LCMS of crude reaction using Method A for **cP15** showing LC UV trace at 220 and 254 nm (top), TIC (middle), and MS (+ve and -ve) at selected product peak (bottom).

9.3 Hydrophobic peptides by Method B

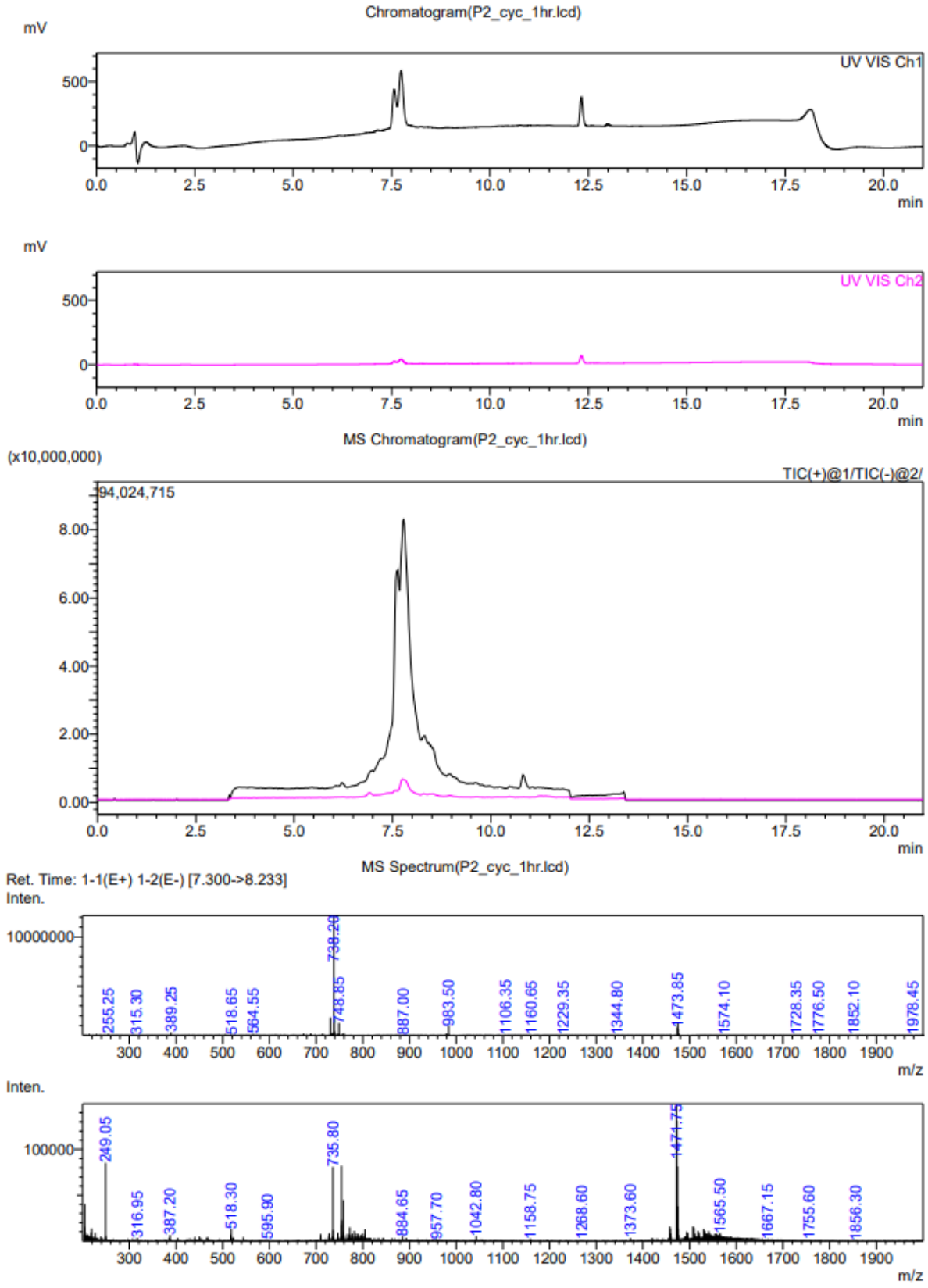


Figure S31. LCMS of crude reaction using Method B for **cP16** showing LC UV trace at 220 and 254 nm (top), TIC (middle), and MS (+ve and -ve) at selected product peak (bottom).

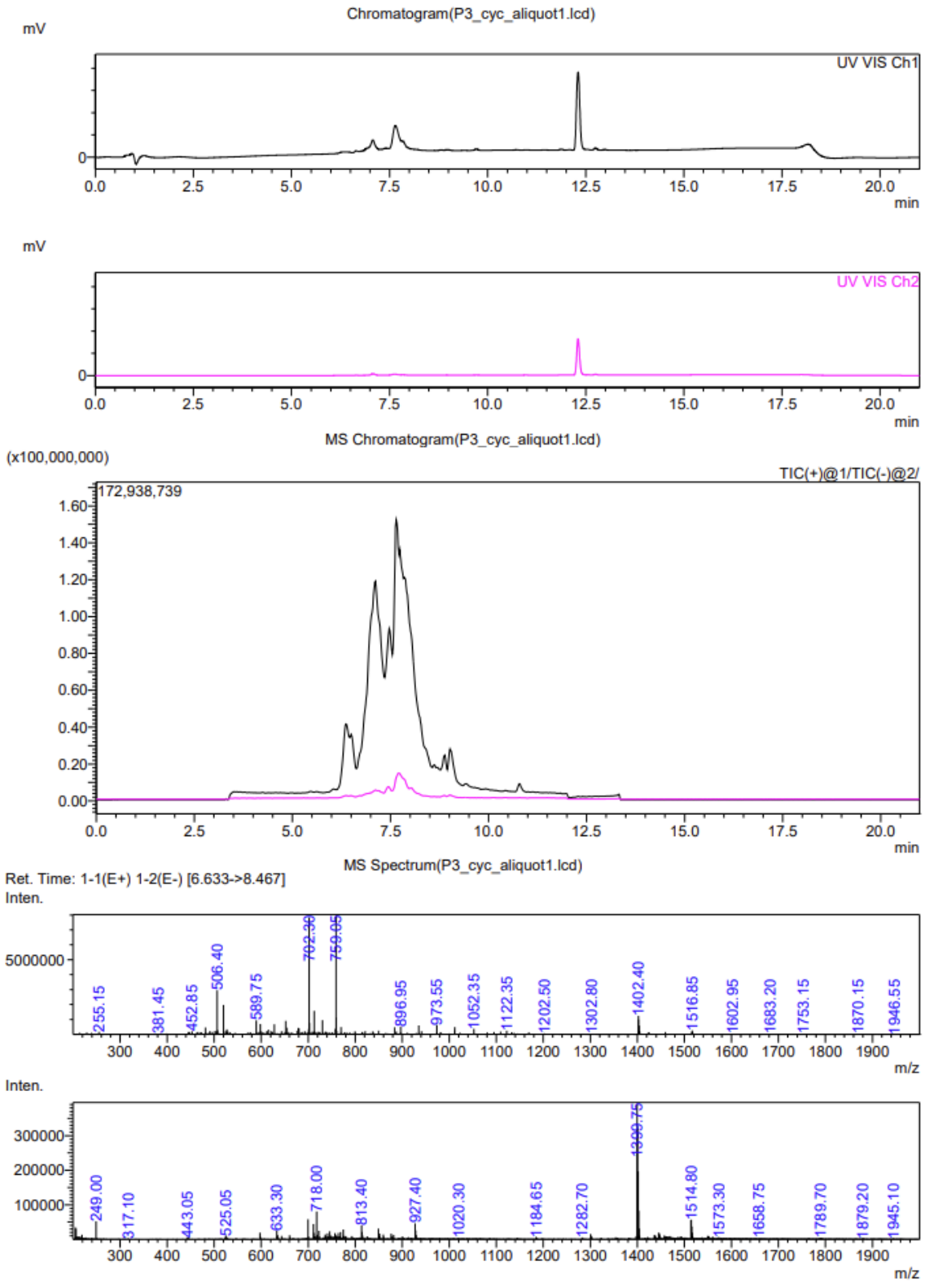


Figure S32. LCMS of crude reaction at 1 h using Method B for **cP17** showing LC UV trace at 220 and 254 nm (top), TIC (middle), and MS (+ve and -ve) at selected product peak (bottom).

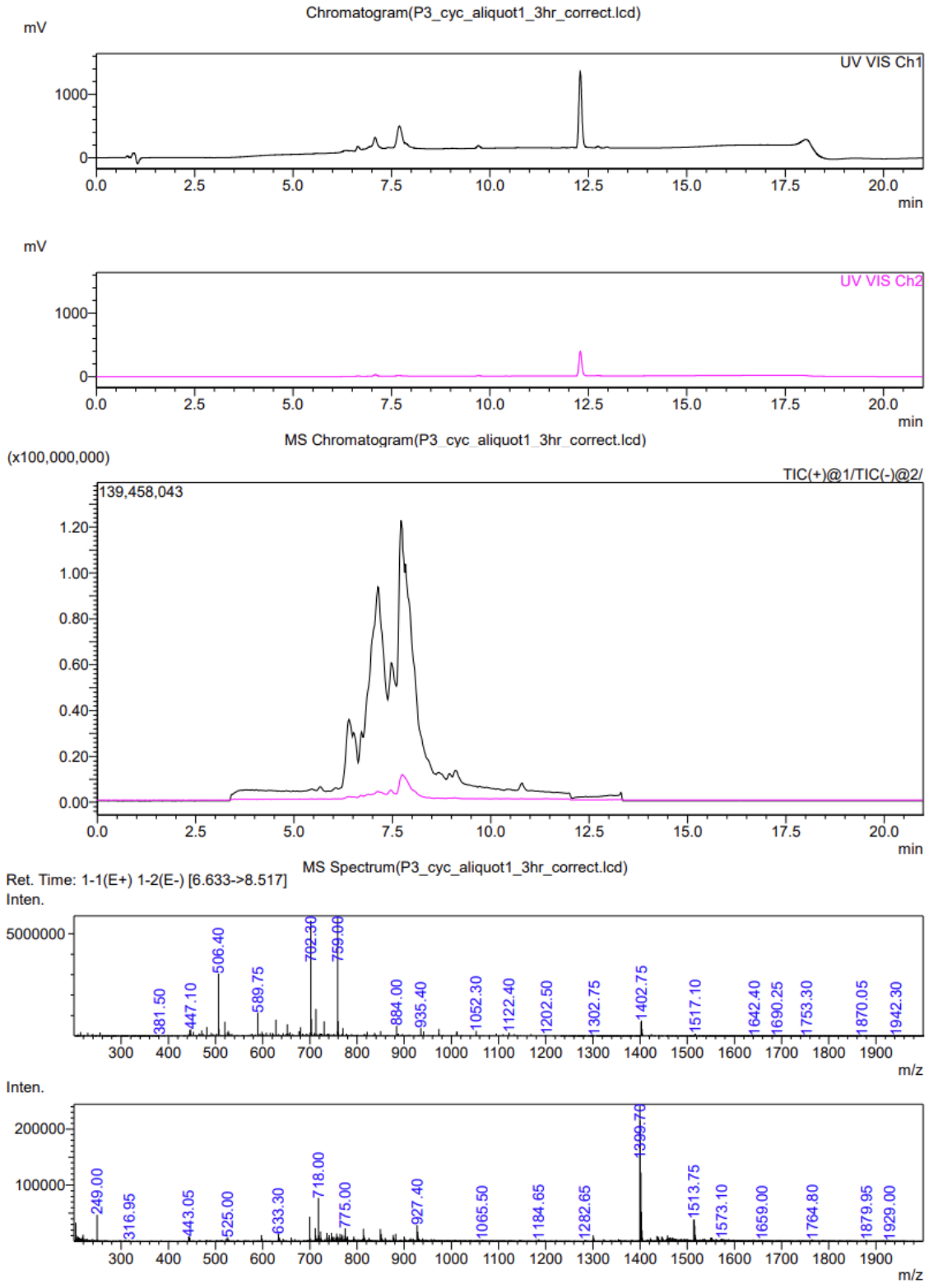


Figure S33. LCMS of crude reaction at 3 h using Method B for **cP17** showing LC UV trace at 220 and 254 nm (top), TIC (middle), and MS (+ve and -ve) at selected product peak (bottom).

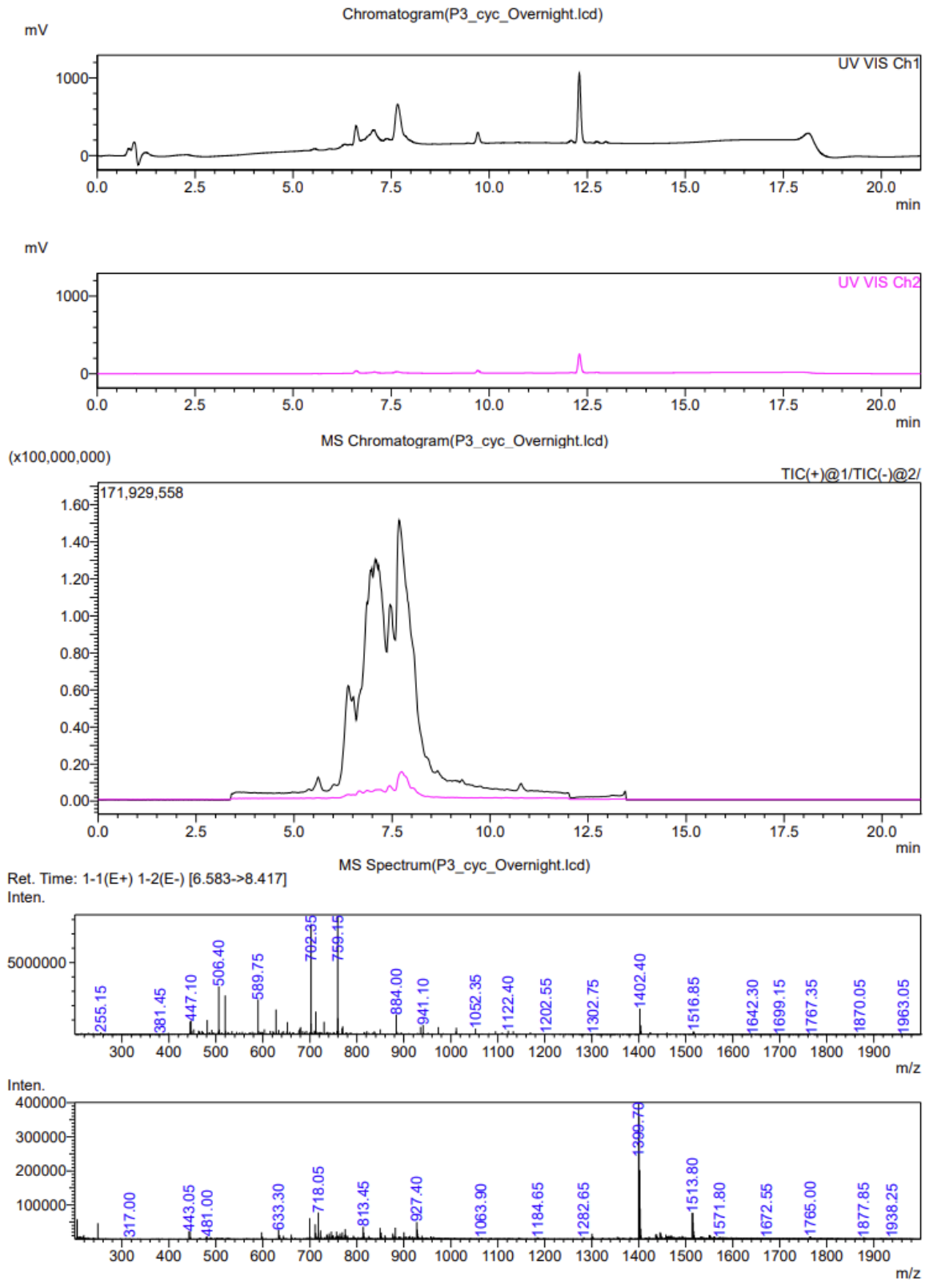


Figure S34. LCMS of crude reaction left overnight using Method B for **cP17** showing LC UV trace at 220 and 254 nm (top), TIC (middle), and MS (+ve and -ve) at selected product peak (bottom).

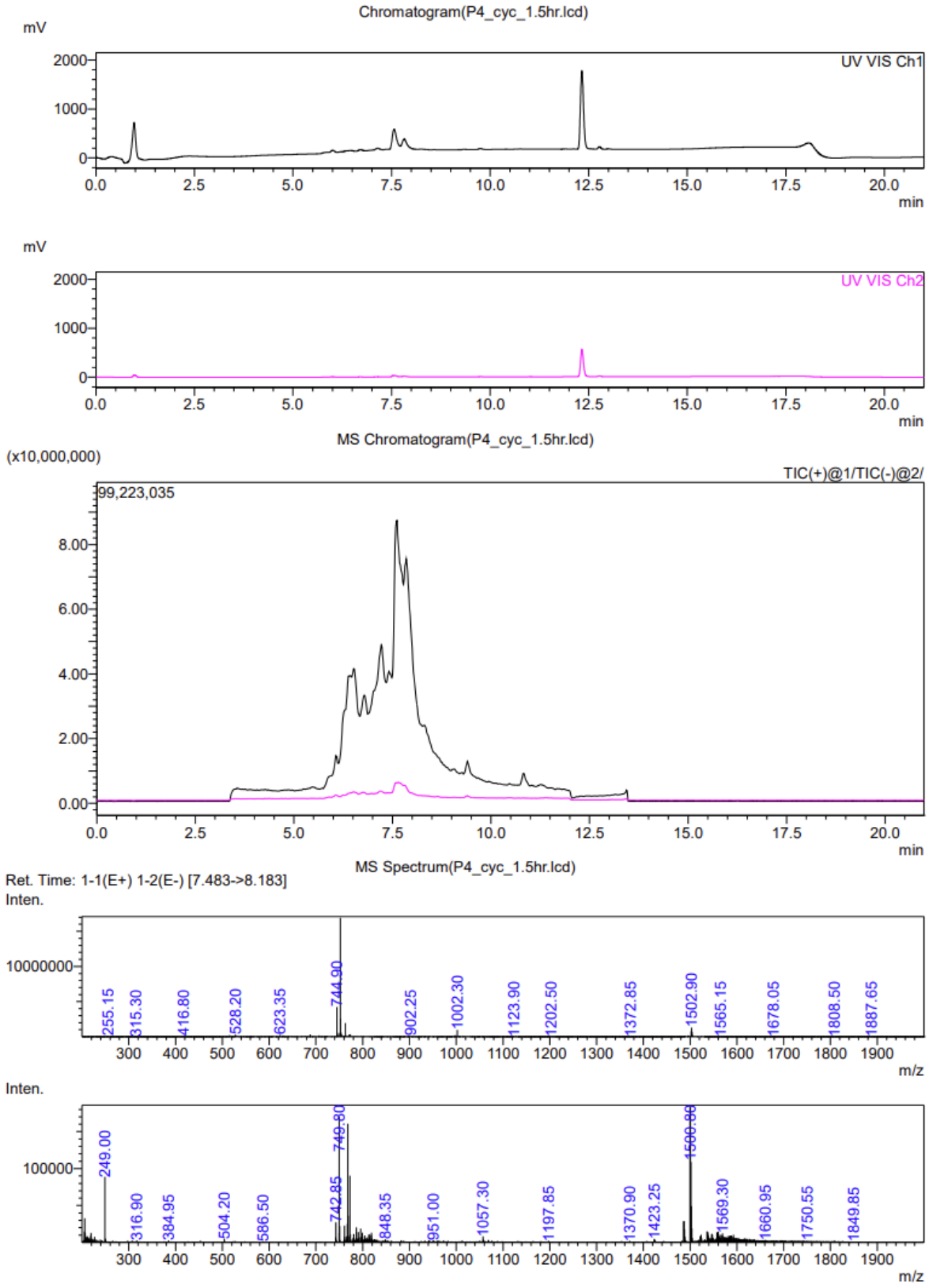


Figure S35. LCMS of crude reaction using Method B for **cP18** showing LC UV trace at 220 and 254 nm (top), TIC (middle), and MS (+ve and -ve) at selected product peak (bottom).

9.4 Bicyclisation reactions with 1,3,5-TBMB

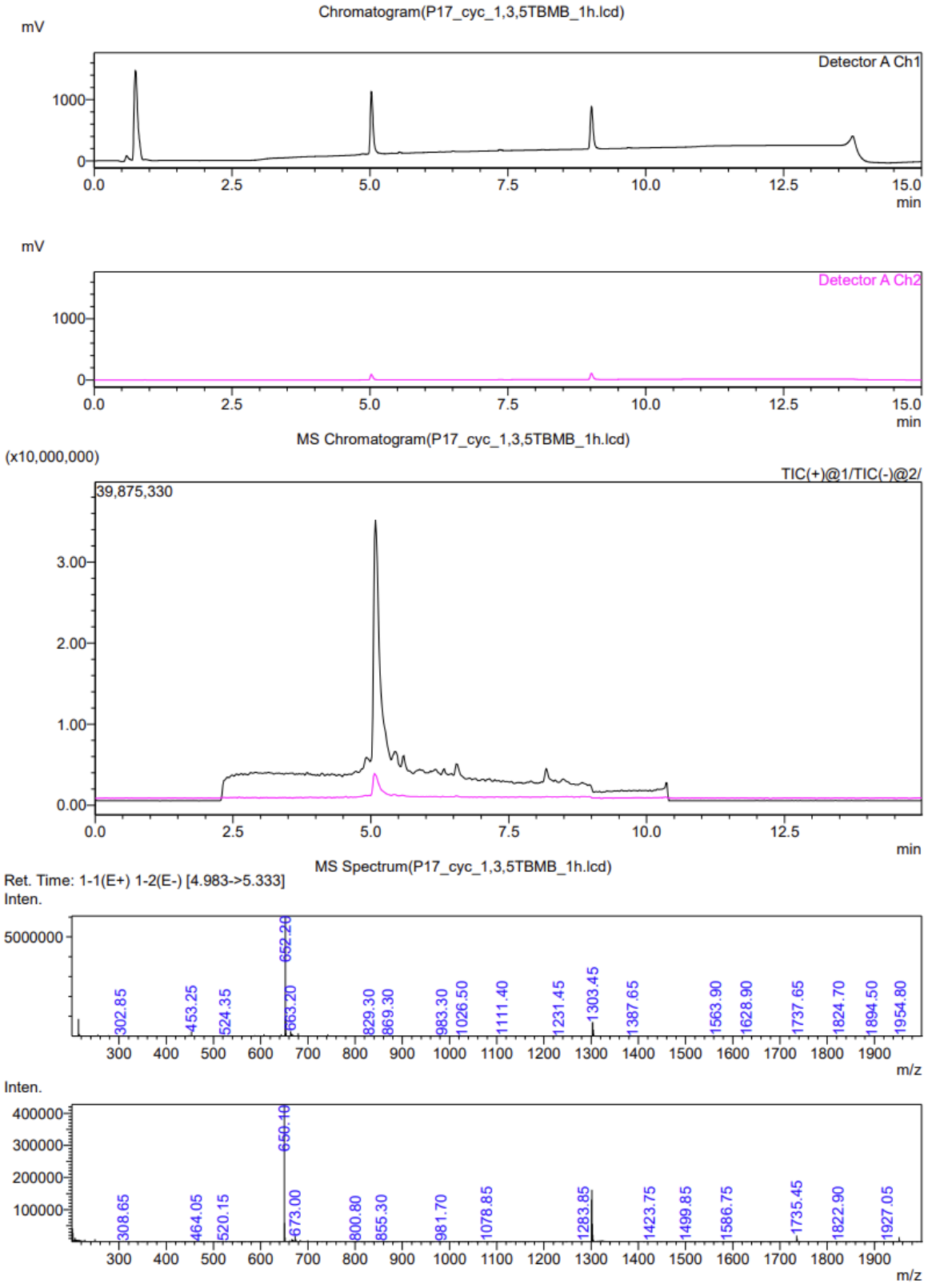


Figure S36. LCMS of crude reaction using Method A for **cP3$_{1,3,5}$** showing LC UV trace at 220 and 254 nm (top), TIC (middle), and MS (+ve and -ve) at selected product peak (bottom).

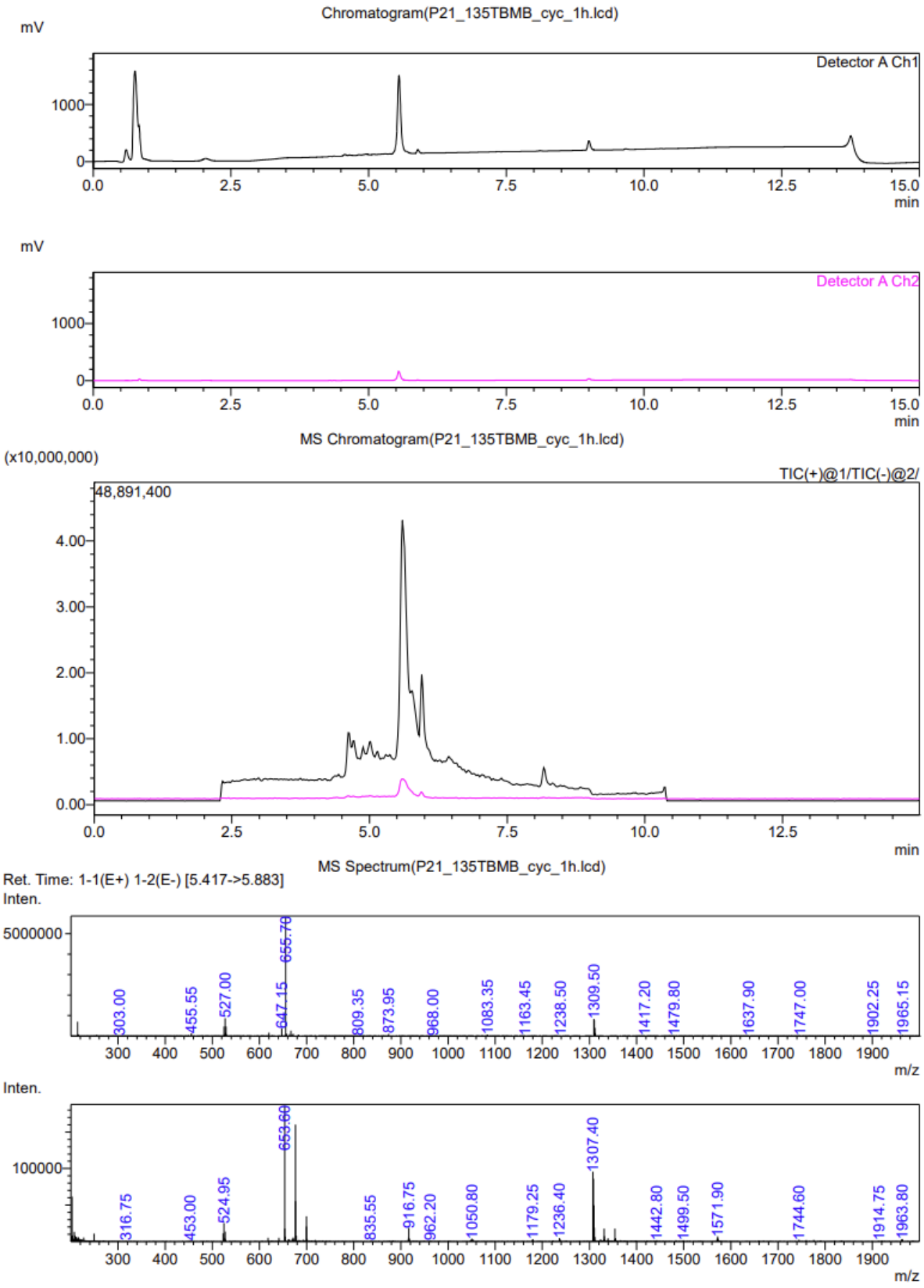


Figure S37. LCMS of crude reaction using Method A for **cP7$_{1,3,5}$** showing LC UV trace at 220 and 254 nm (top), TIC (middle), and MS (+ve and -ve) at selected product peak (bottom).

9.5 Selected bicyclisation reactions in 96-well plates

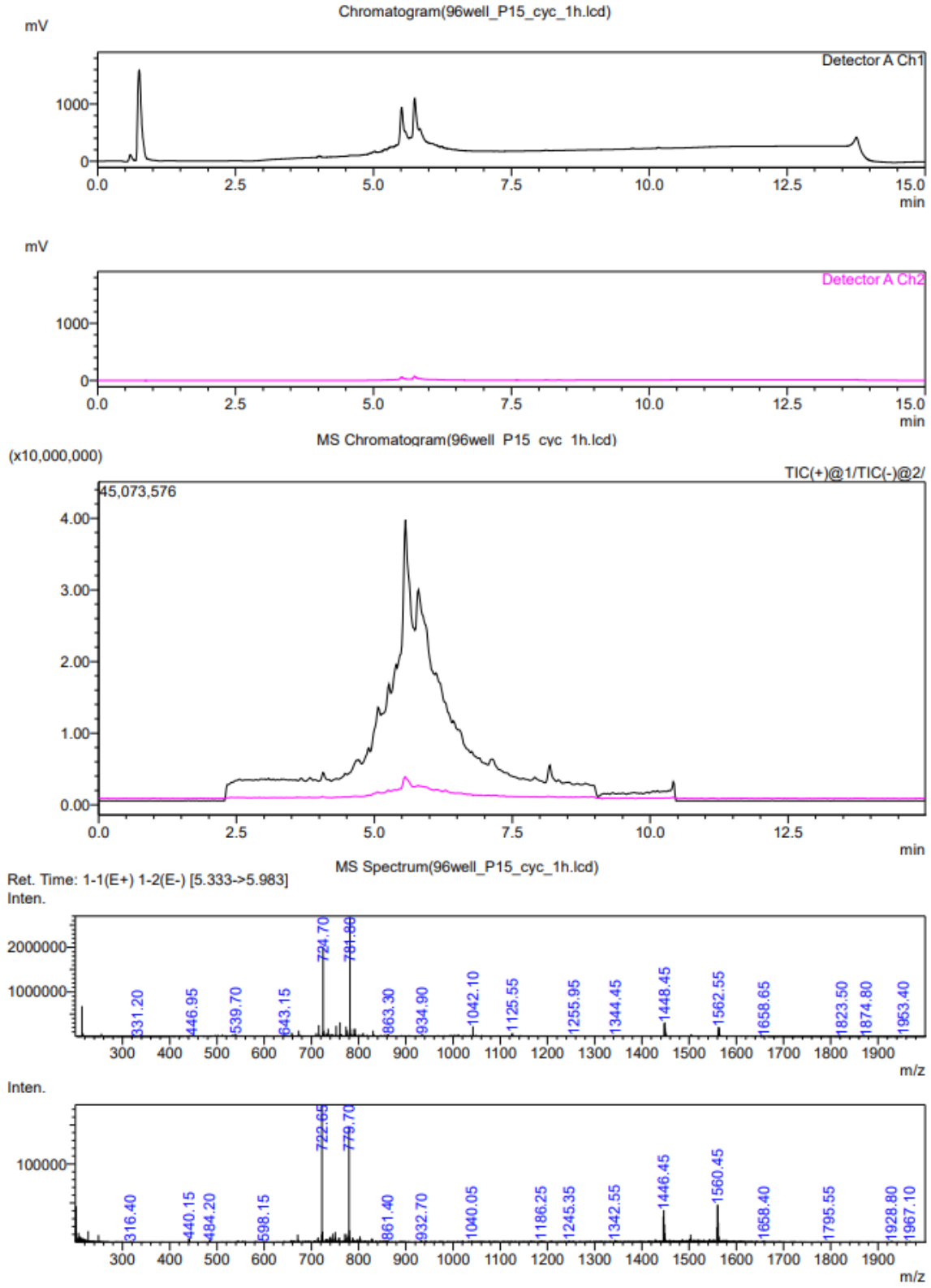


Figure S38. LCMS of crude reaction using Method A for **$_{96w}$cP15** showing LC UV trace at 220 and 254 nm (top), TIC (middle), and MS (+ve and -ve) at selected product peak (bottom).

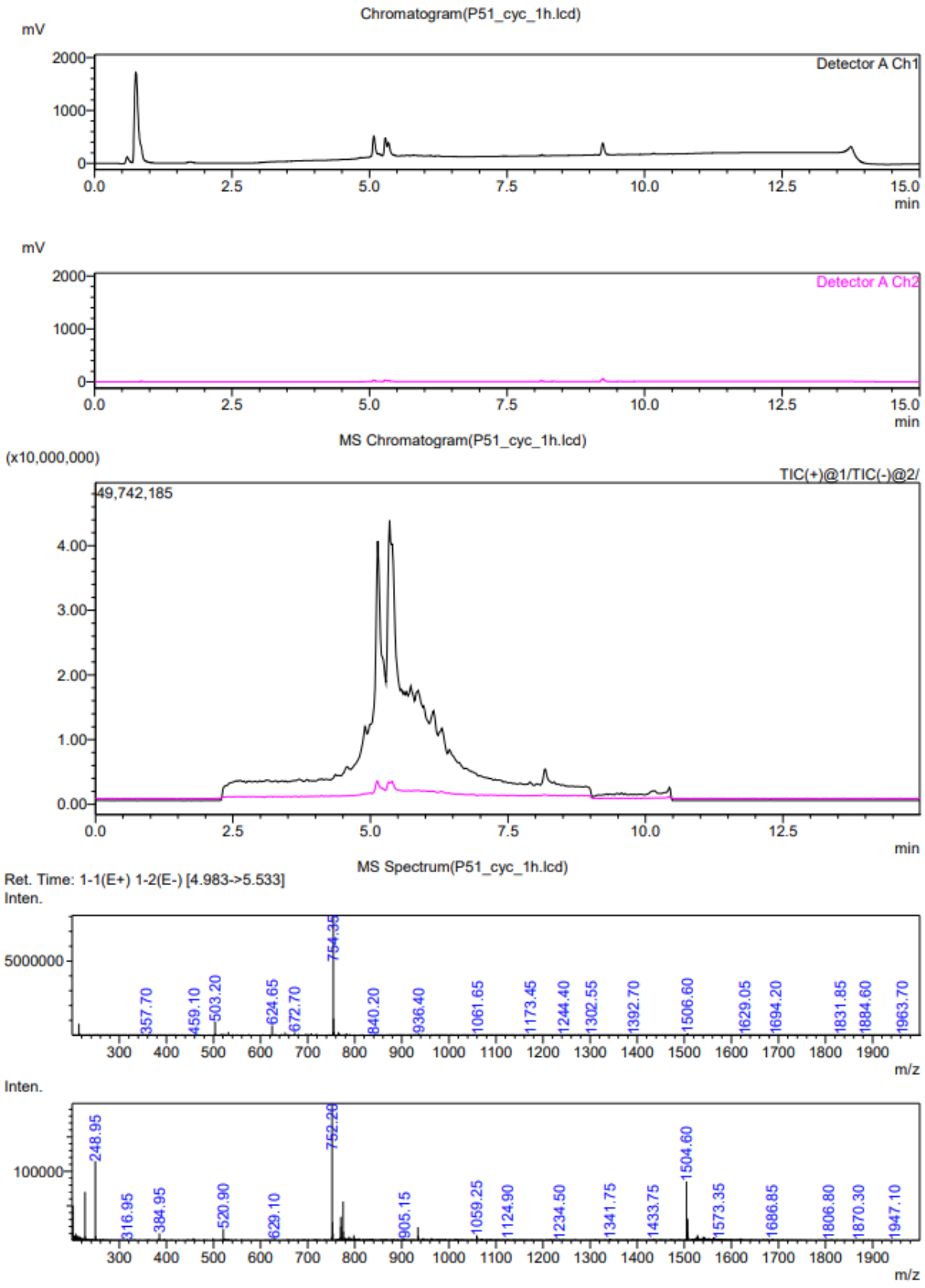


Figure S39. LCMS of crude reaction using Method A for **$_{96w}$cP51** showing LC UV trace at 220 and 254 nm (top), TIC (middle), and MS (+ve and -ve) at selected product peak (bottom).

10 LCMS characterisation of HPLC-purified peptides

10.1 Pilot peptides for Ellman's assay

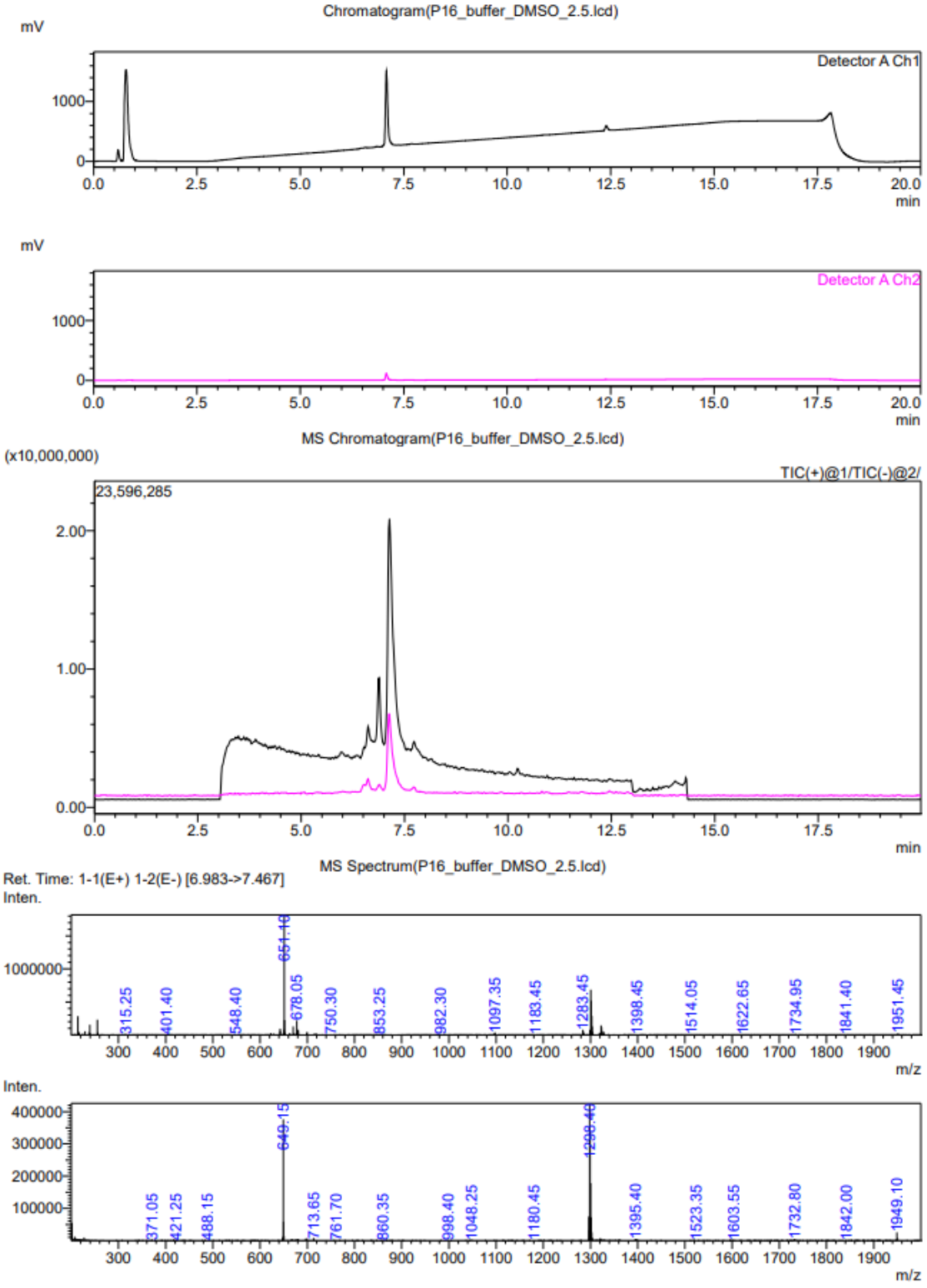


Figure S40. LCMS for purified peptide **P2** showing LC UV trace at 220 and 254 nm, (top), TIC (middle), and MS (+ve and -ve) at selected product peak (bottom).

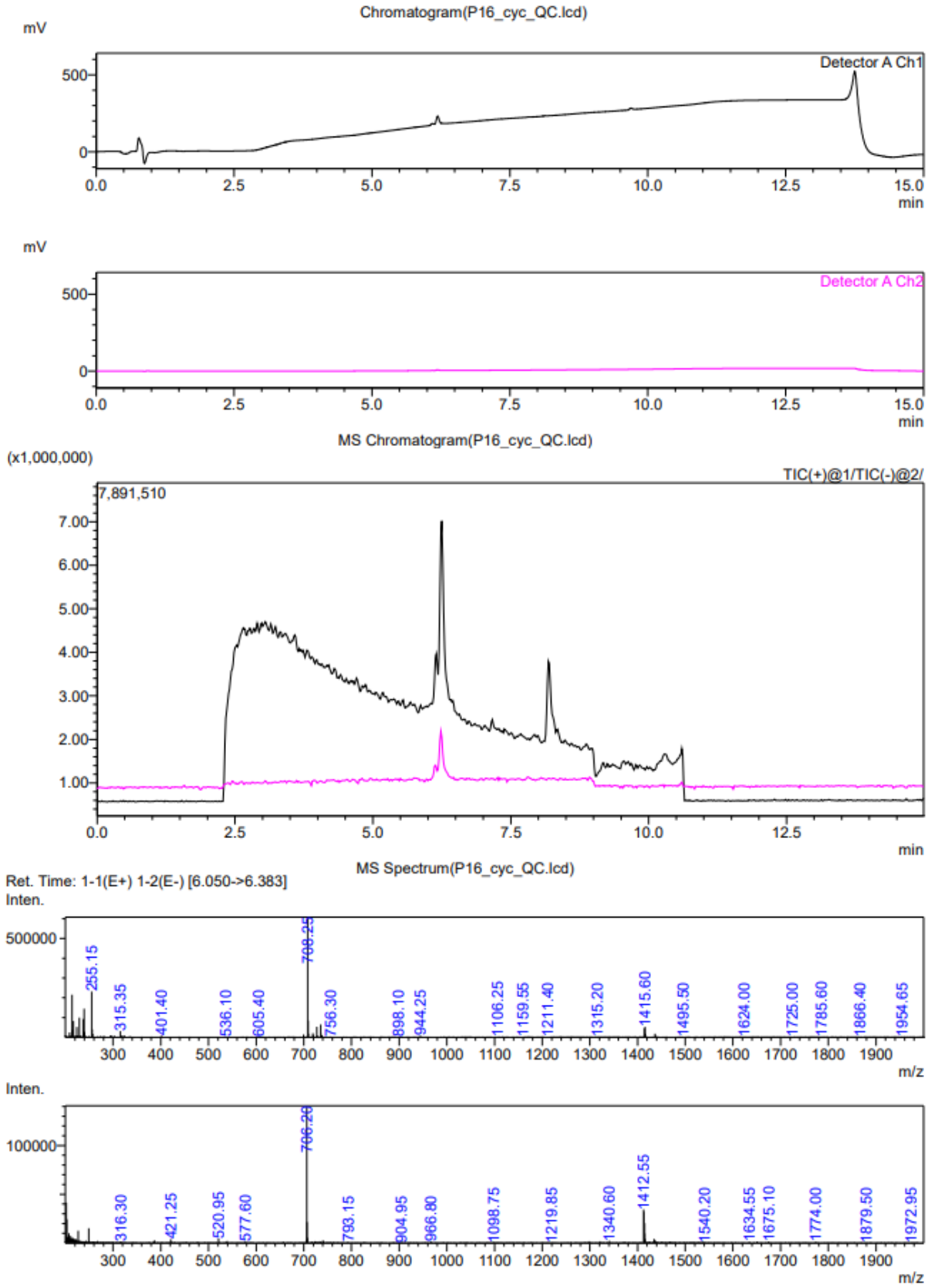


Figure S41. LCMS for purified peptide **cP2** showing LC UV trace at 220 and 254 nm, (top), TIC (middle), and MS (+ve and -ve) at selected product peak (bottom).

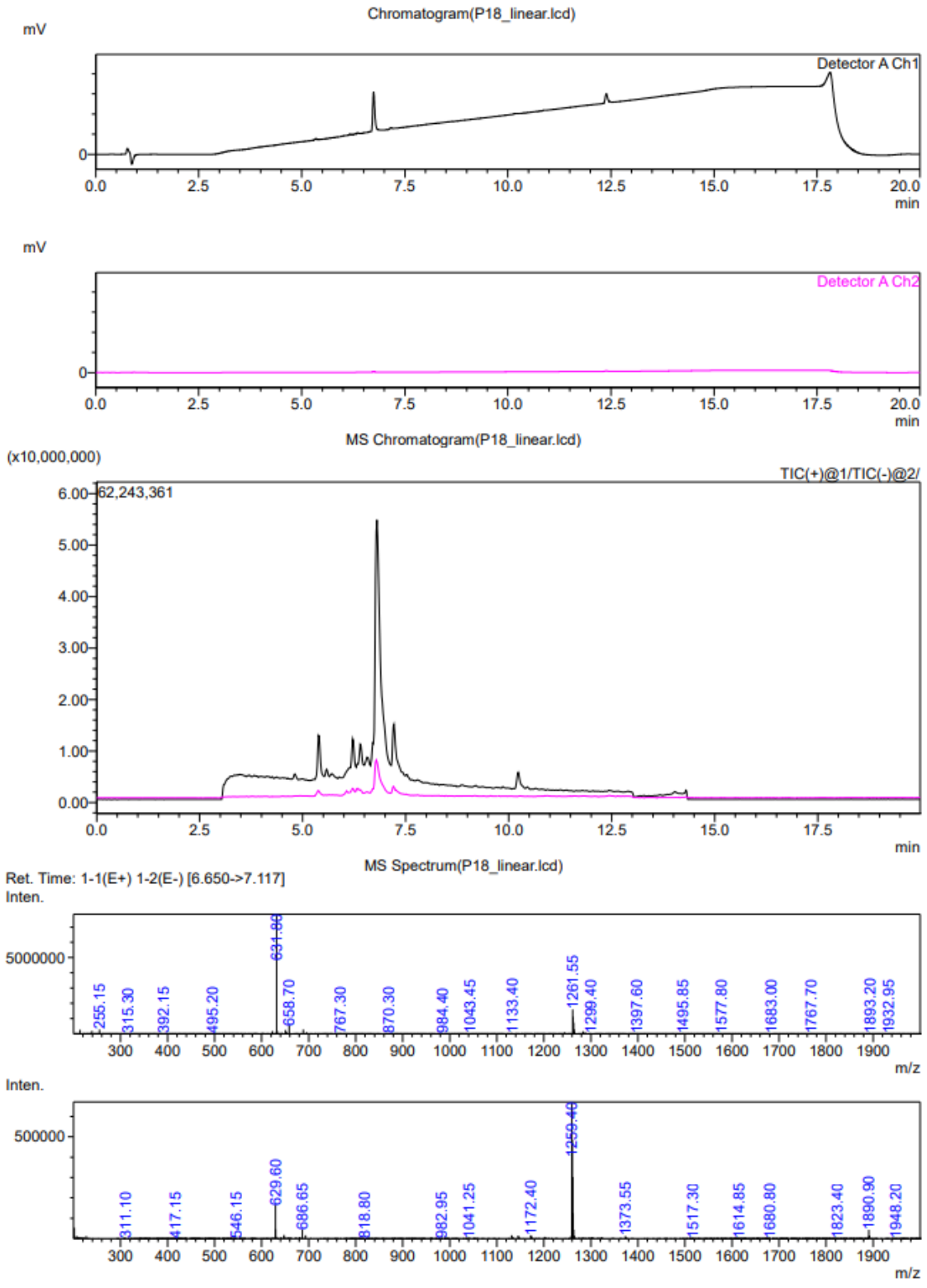


Figure S42. LCMS for purified peptide **P4** showing LC UV trace at 220 and 254 nm, (top), TIC (middle), and MS (+ve and -ve) at selected product peak (bottom).

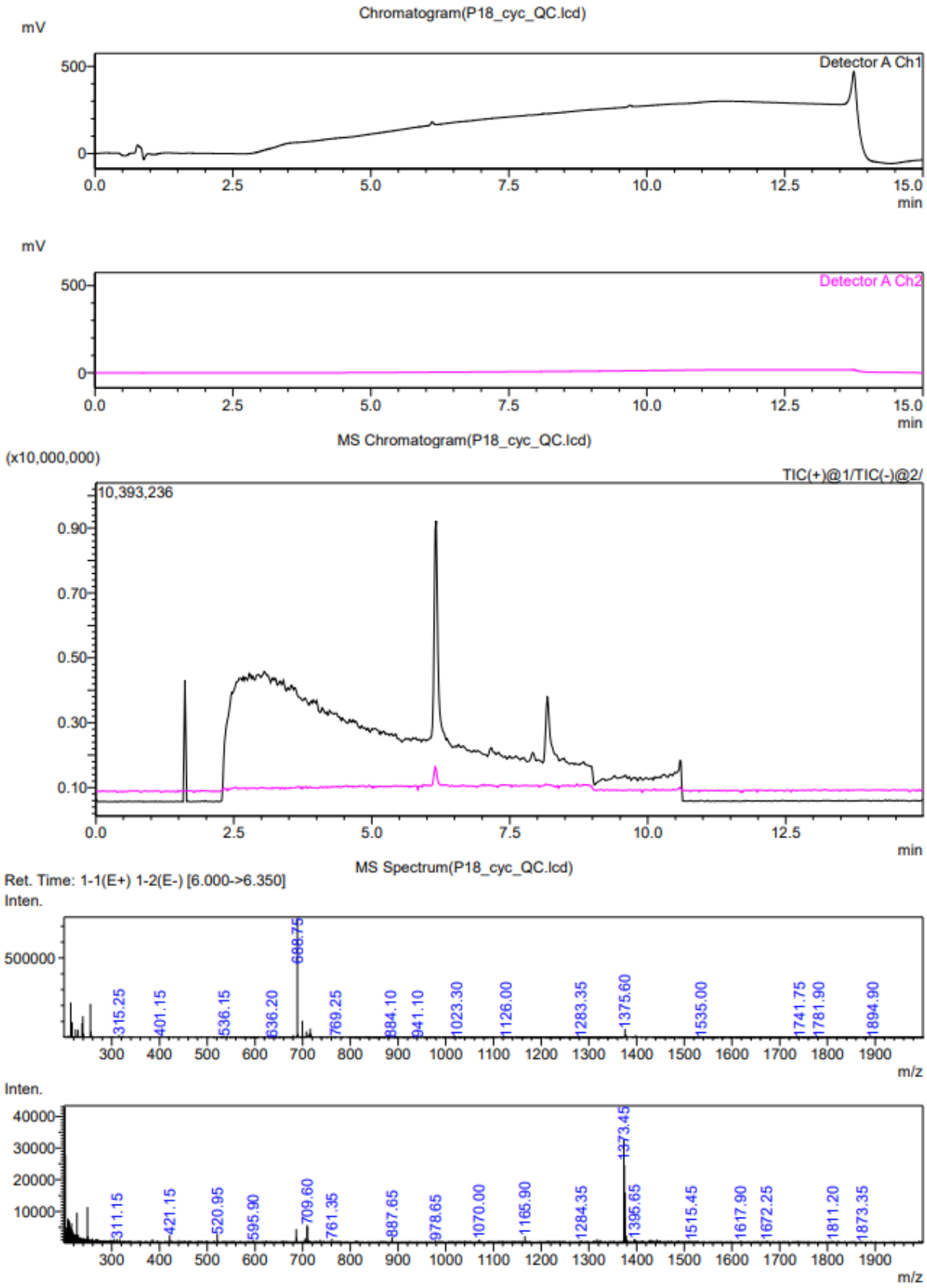


Figure S43. LCMS for purified peptide **cP4** showing LC UV trace at 220 and 254 nm, (top), TIC (middle), and MS (+ve and -ve) at selected product peak (bottom).

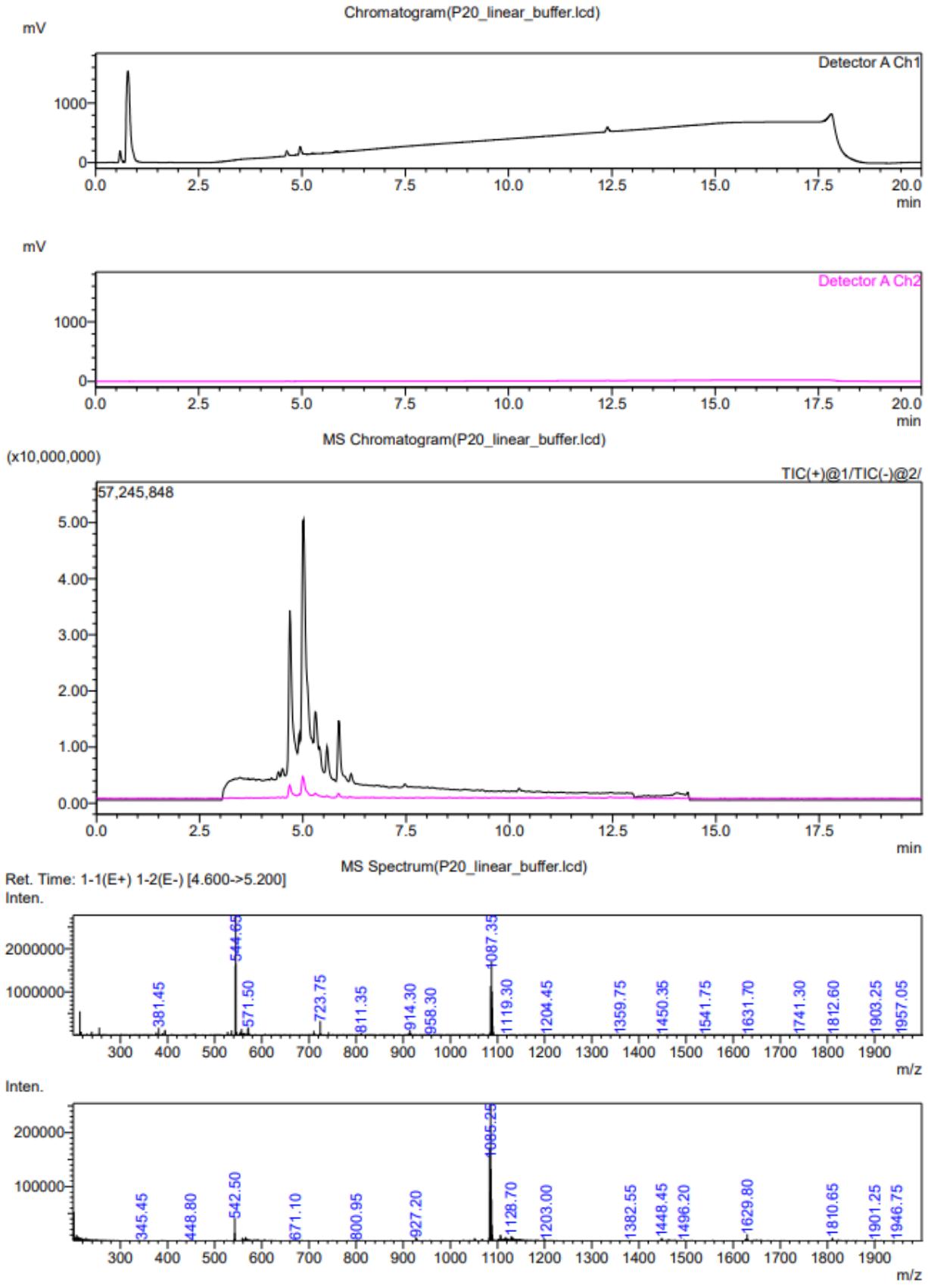


Figure S44. LCMS for purified peptide **P6** showing LC UV trace at 220 and 254 nm, (top), TIC (middle), and MS (+ve and -ve) at selected product peak (bottom).

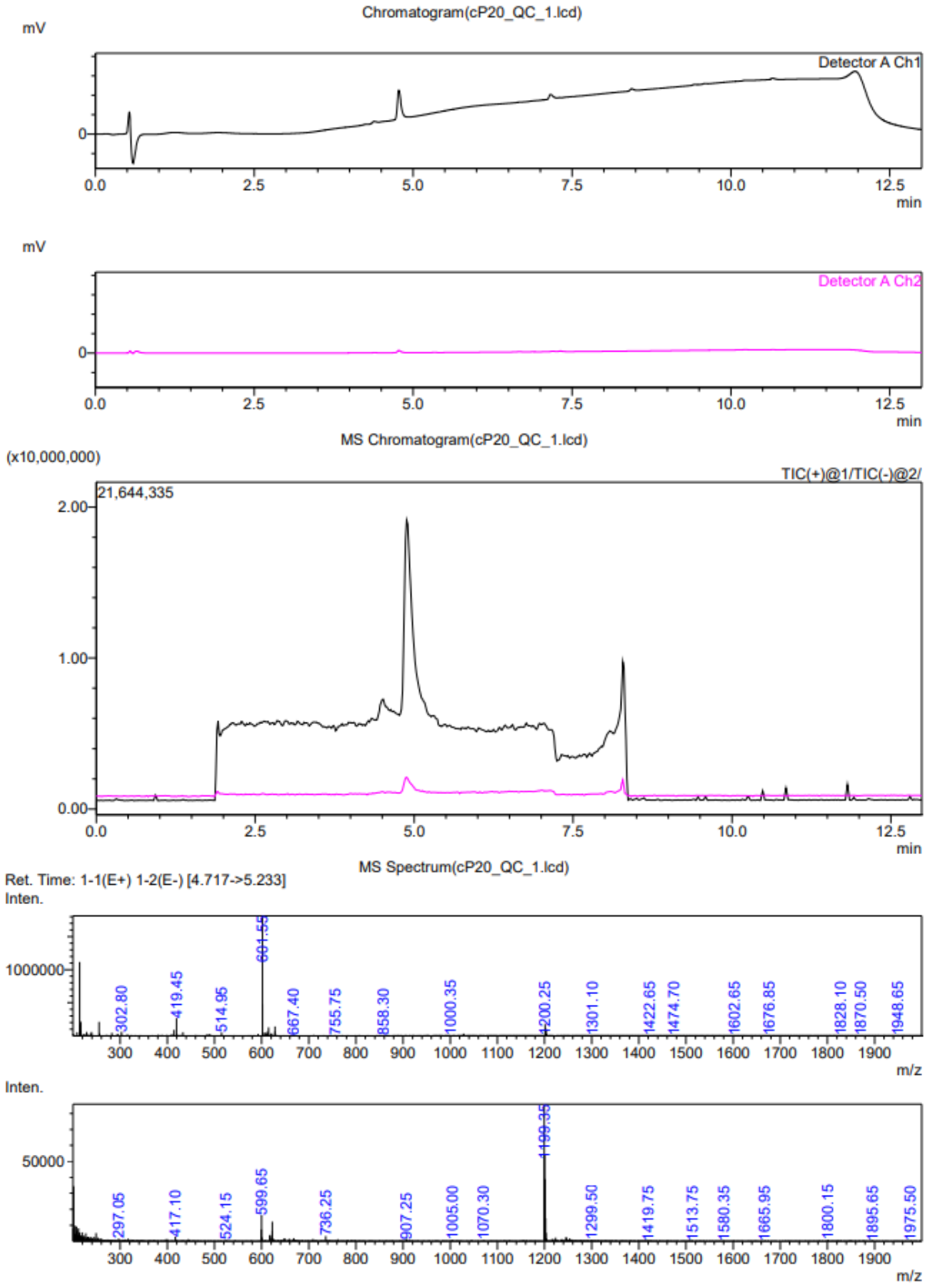


Figure S45. LCMS for purified peptide **cP6** showing LC UV trace at 220 and 254 nm, (top), TIC (middle), and MS (+ve and -ve) at selected product peak (bottom).

10.2 Peptides for bicyclisation with 1,3,5-TBMB

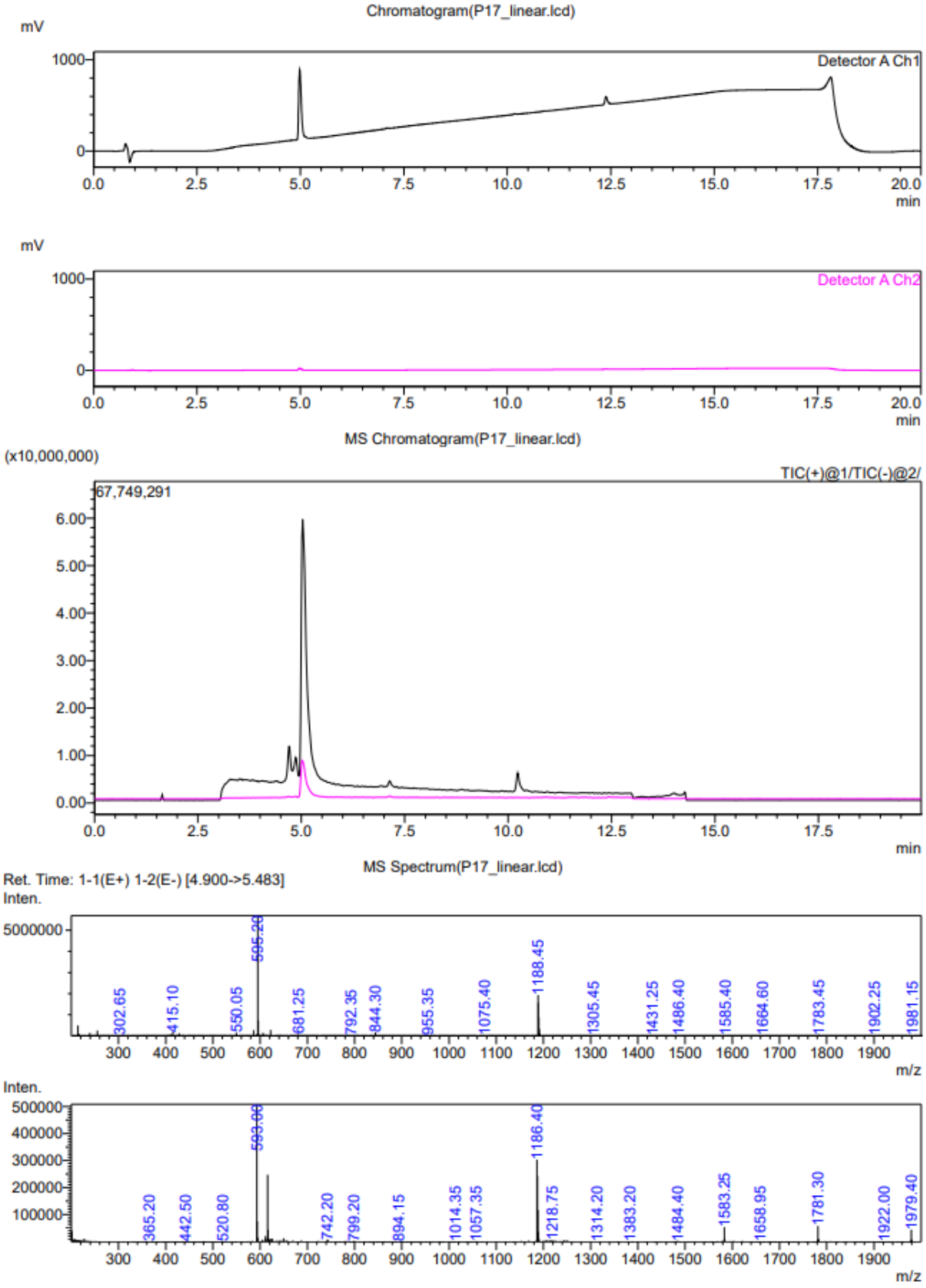


Figure S46. LCMS for purified peptide **P3** showing LC UV trace at 220 and 254 nm, (top), TIC (middle), and MS (+ve and -ve) at selected product peak (bottom).

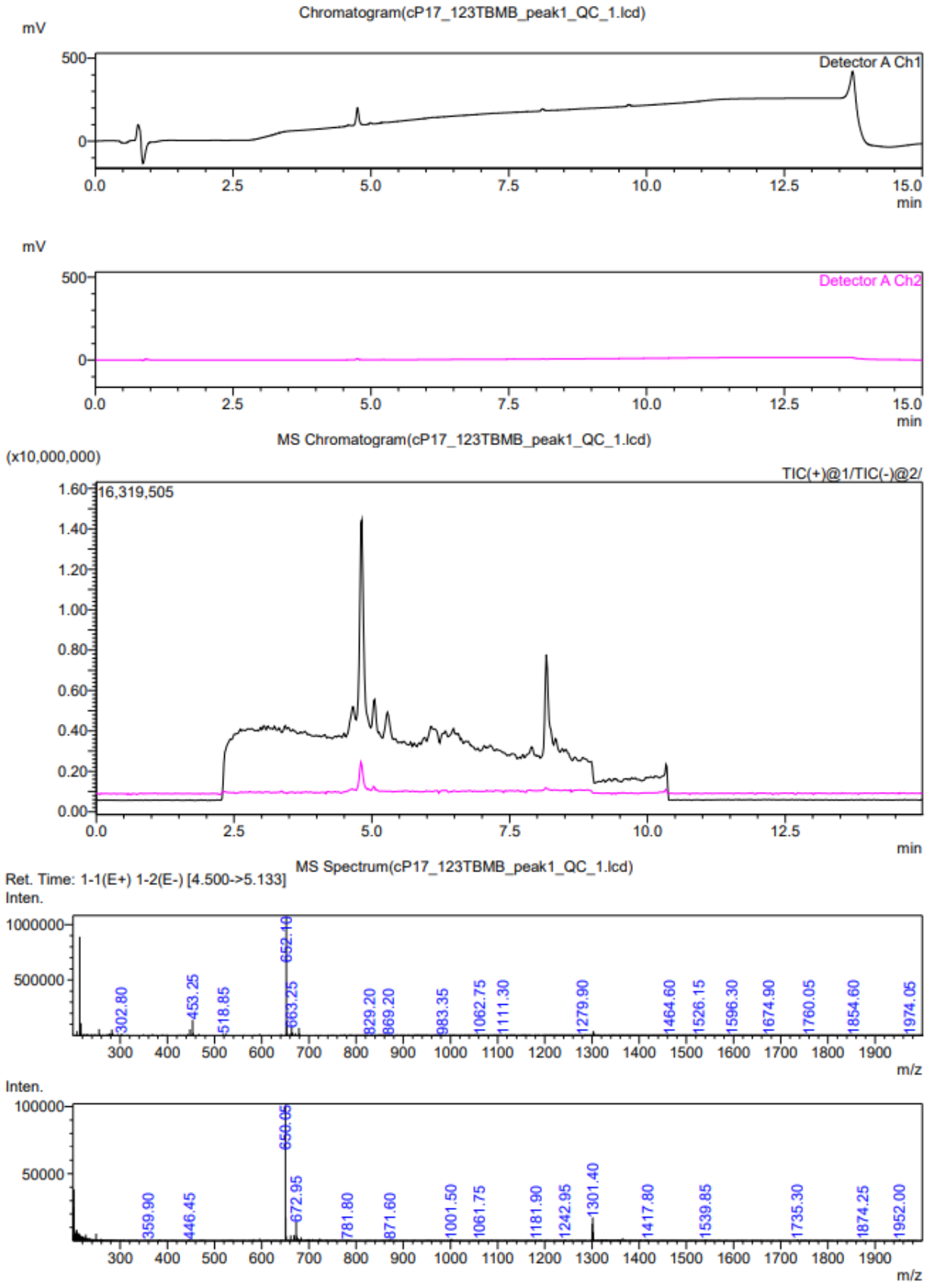


Figure S47. LCMS for purified peptide **cP3$_{pk1}$** showing LC UV trace at 220 and 254 nm, (top), TIC (middle), and MS (+ve and -ve) at selected product peak (bottom).

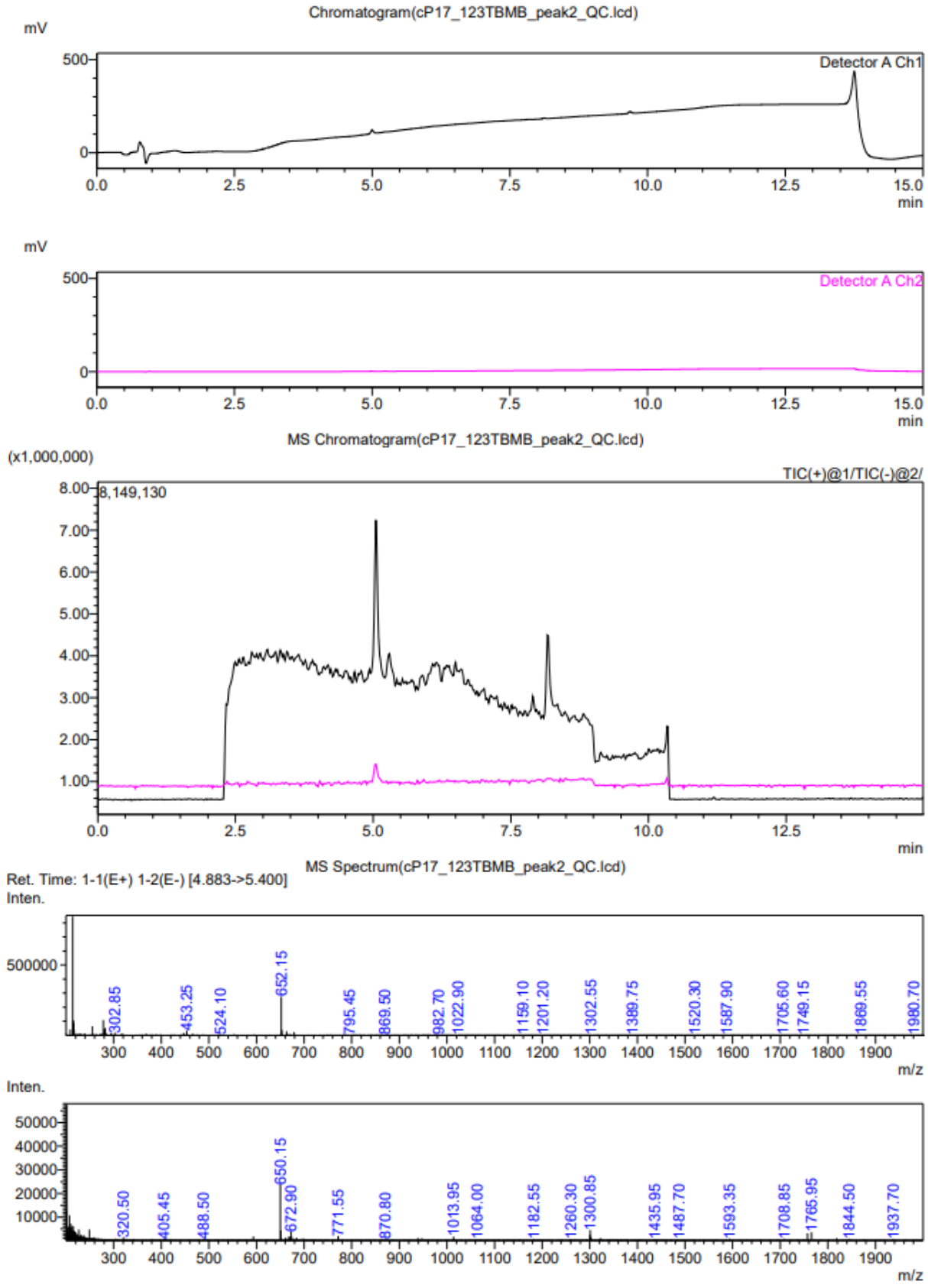


Figure S48. LCMS for purified peptide **cP3$_{pk2}$** showing LC UV trace at 220 and 254 nm, (top), TIC (middle), and MS (+ve and -ve) at selected product peak (bottom).

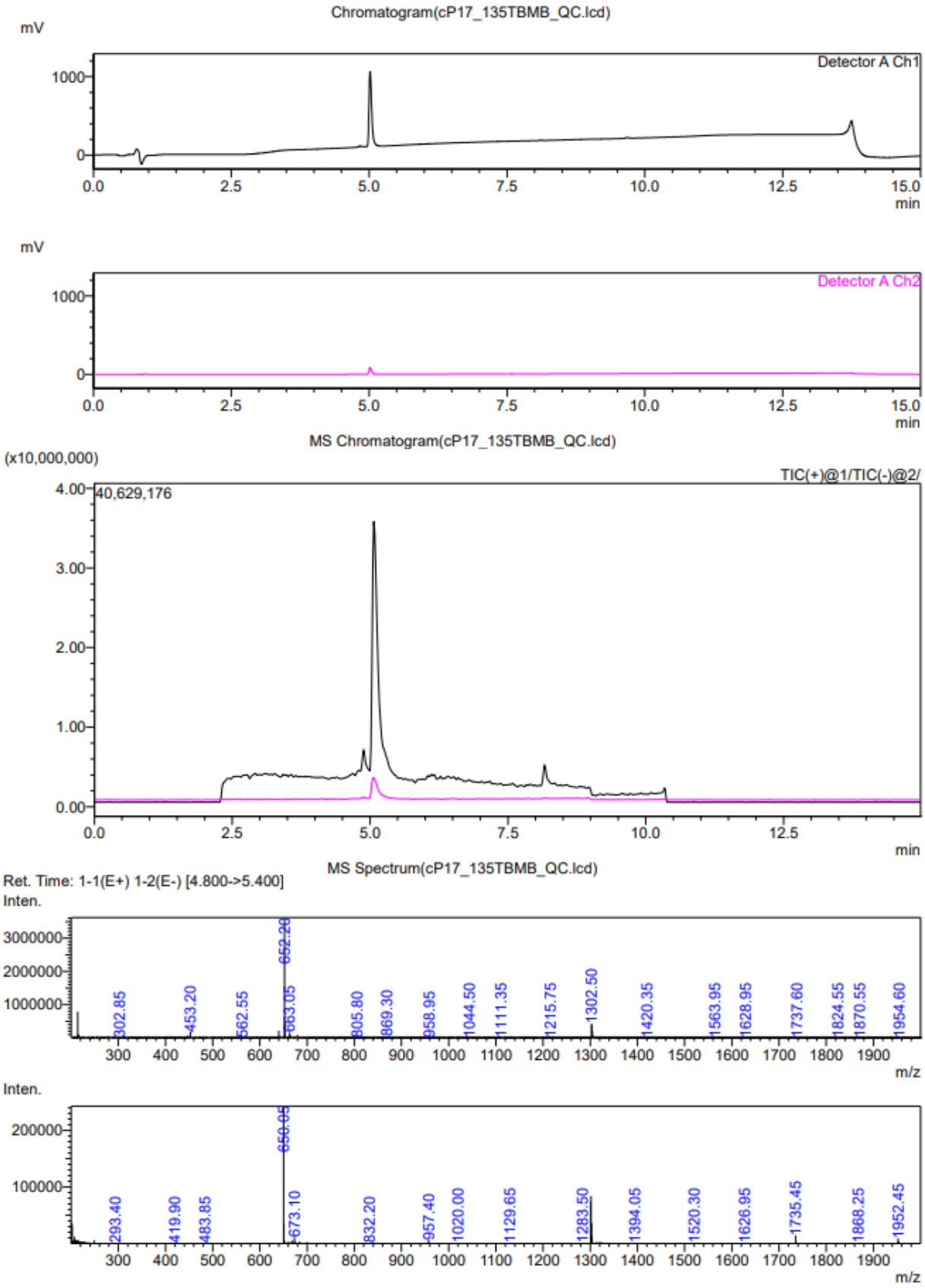


Figure S49. LCMS for purified peptide **cP3$_{1,3,5}$** showing LC UV trace at 220 and 254 nm, (top), TIC (middle), and MS (+ve and -ve) at selected product peak (bottom).

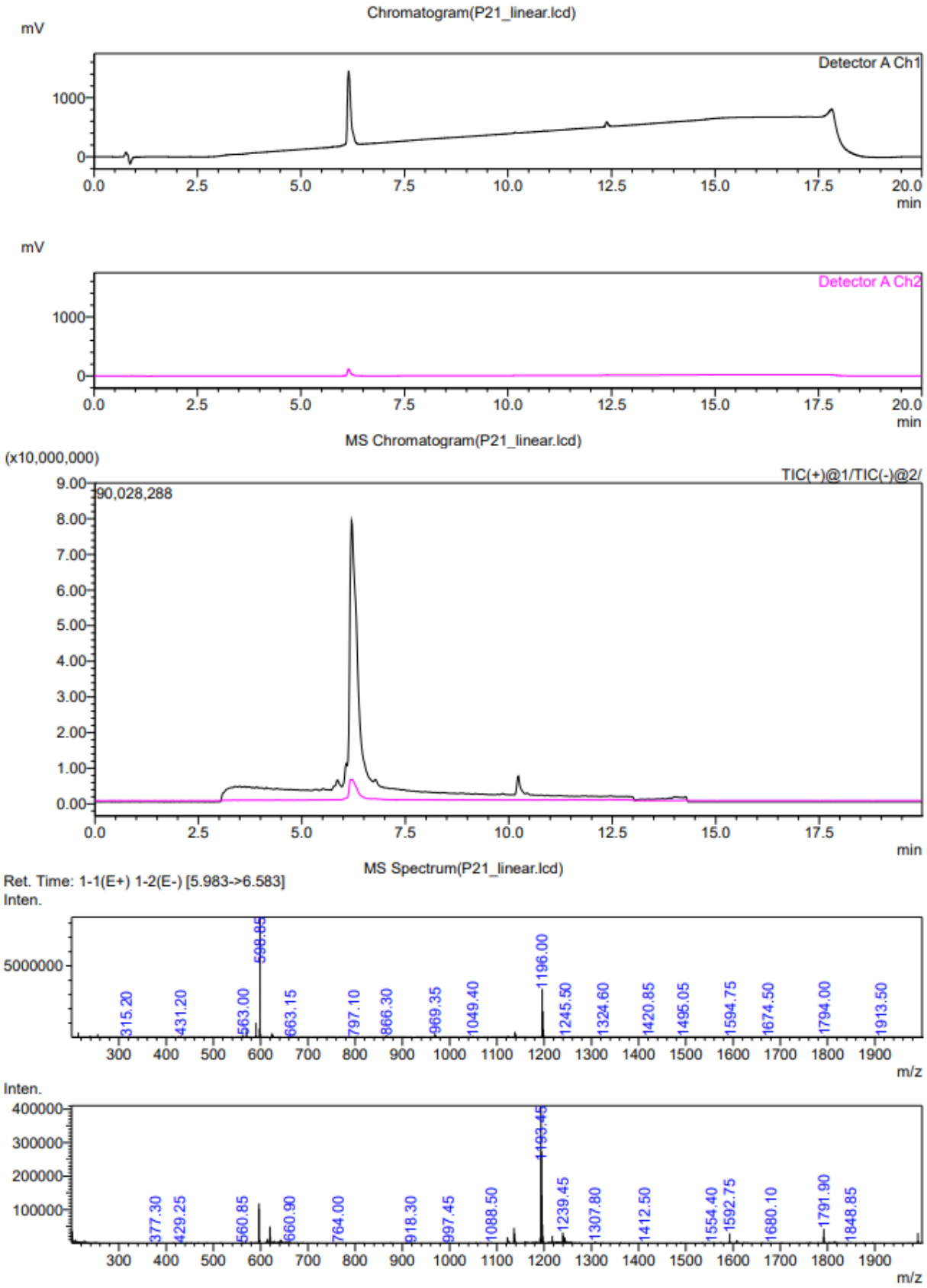


Figure S50. LCMS for purified peptide **P7** showing LC UV trace at 220 and 254 nm, (top), TIC (middle), and MS (+ve and -ve) at selected product peak (bottom).

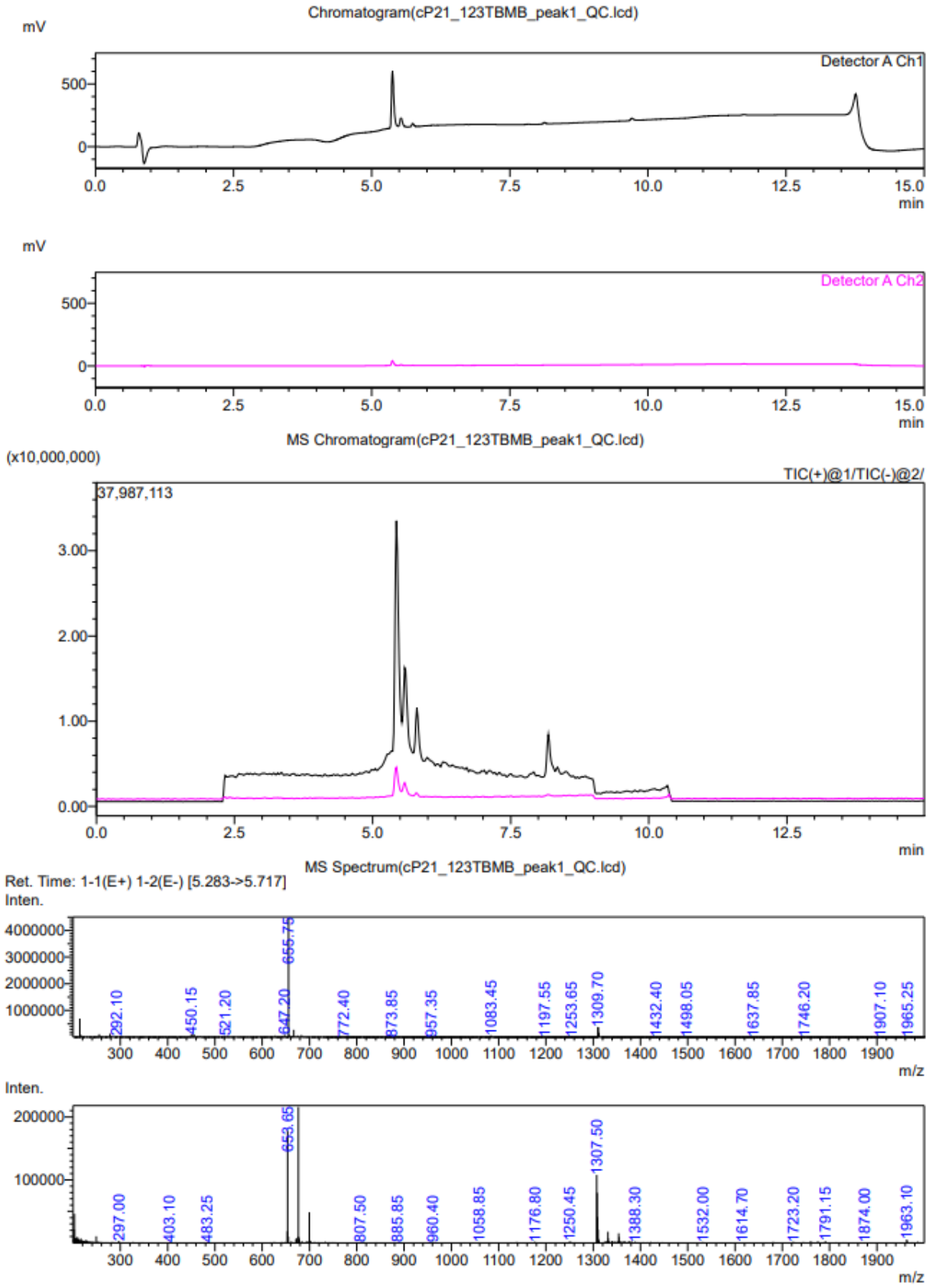


Figure S51. LCMS for purified peptide **cP7$_{pk1}$** showing LC UV trace at 220 and 254 nm, (top), TIC (middle), and MS (+ve and -ve) at selected product peak (bottom).

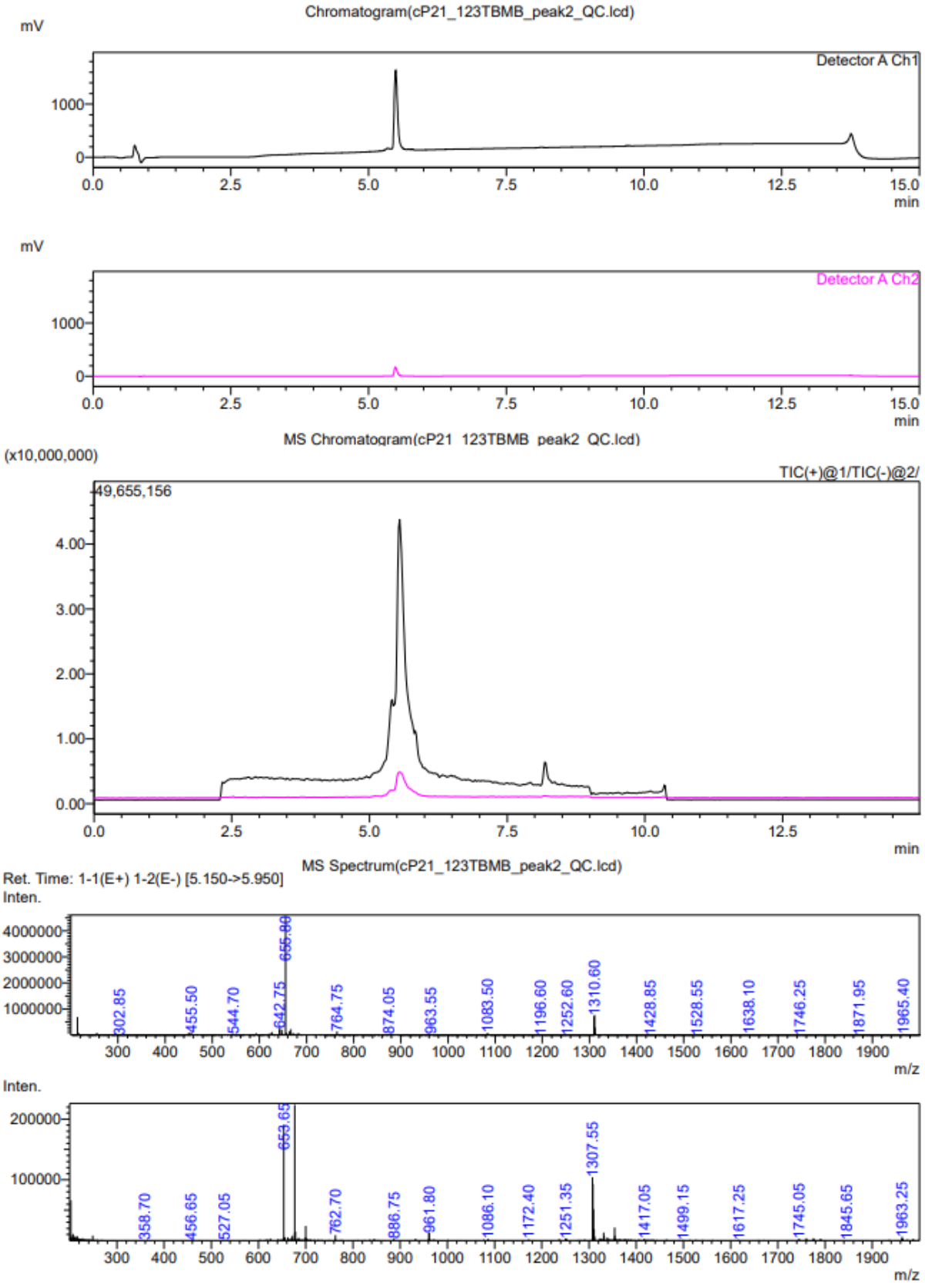


Figure S52. LCMS for purified peptide **cP7$_{pk2}$** showing LC UV trace at 220 and 254 nm, (top), TIC (middle), and MS (+ve and -ve) at selected product peak (bottom).

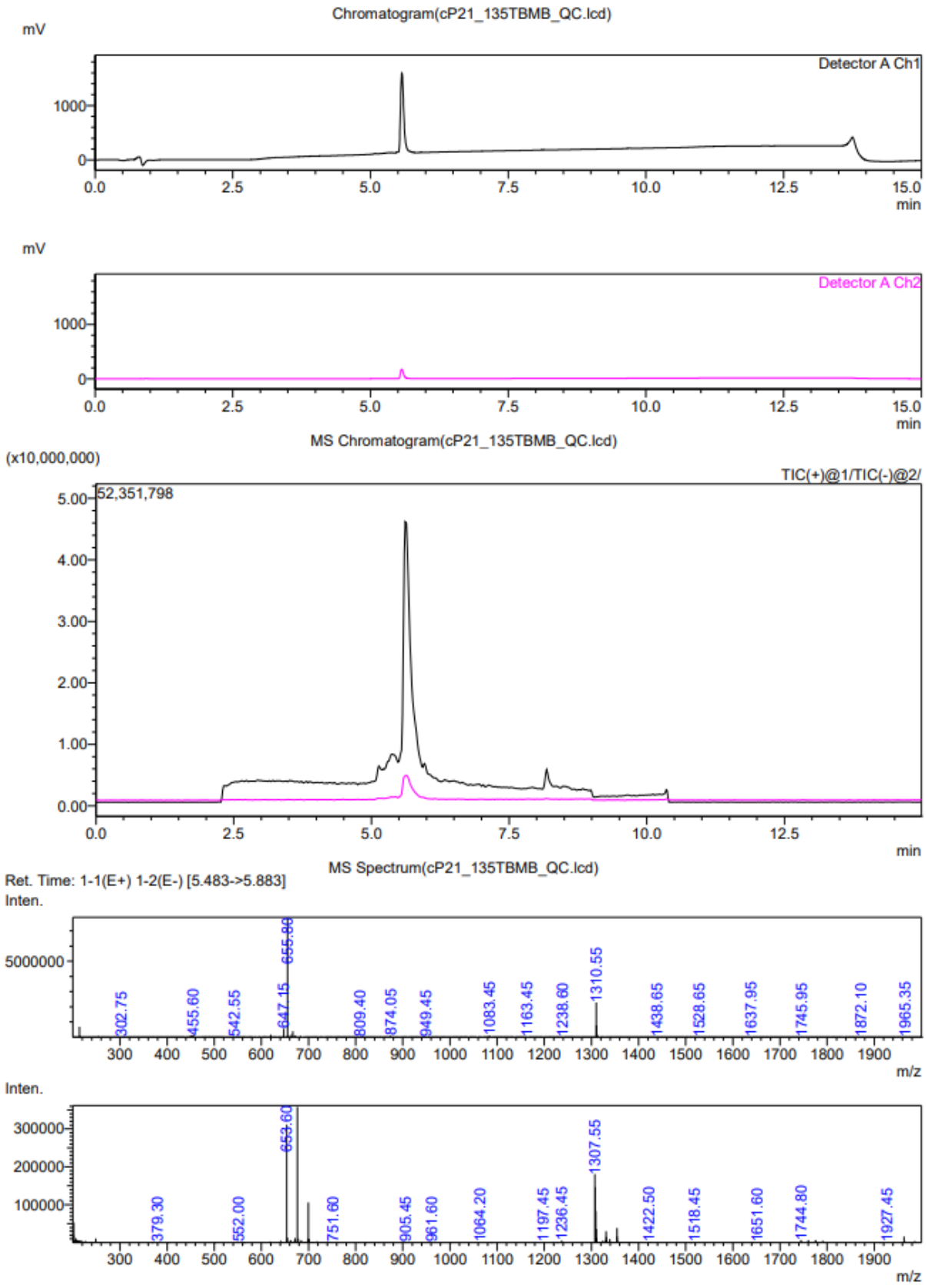


Figure S53. LCMS for purified peptide **cP7$_{1,3,5}$** showing LC UV trace at 220 and 254 nm, (top), TIC (middle), and MS (+ve and -ve) at selected product peak (bottom).

10.3 Peptides for 2D NMR

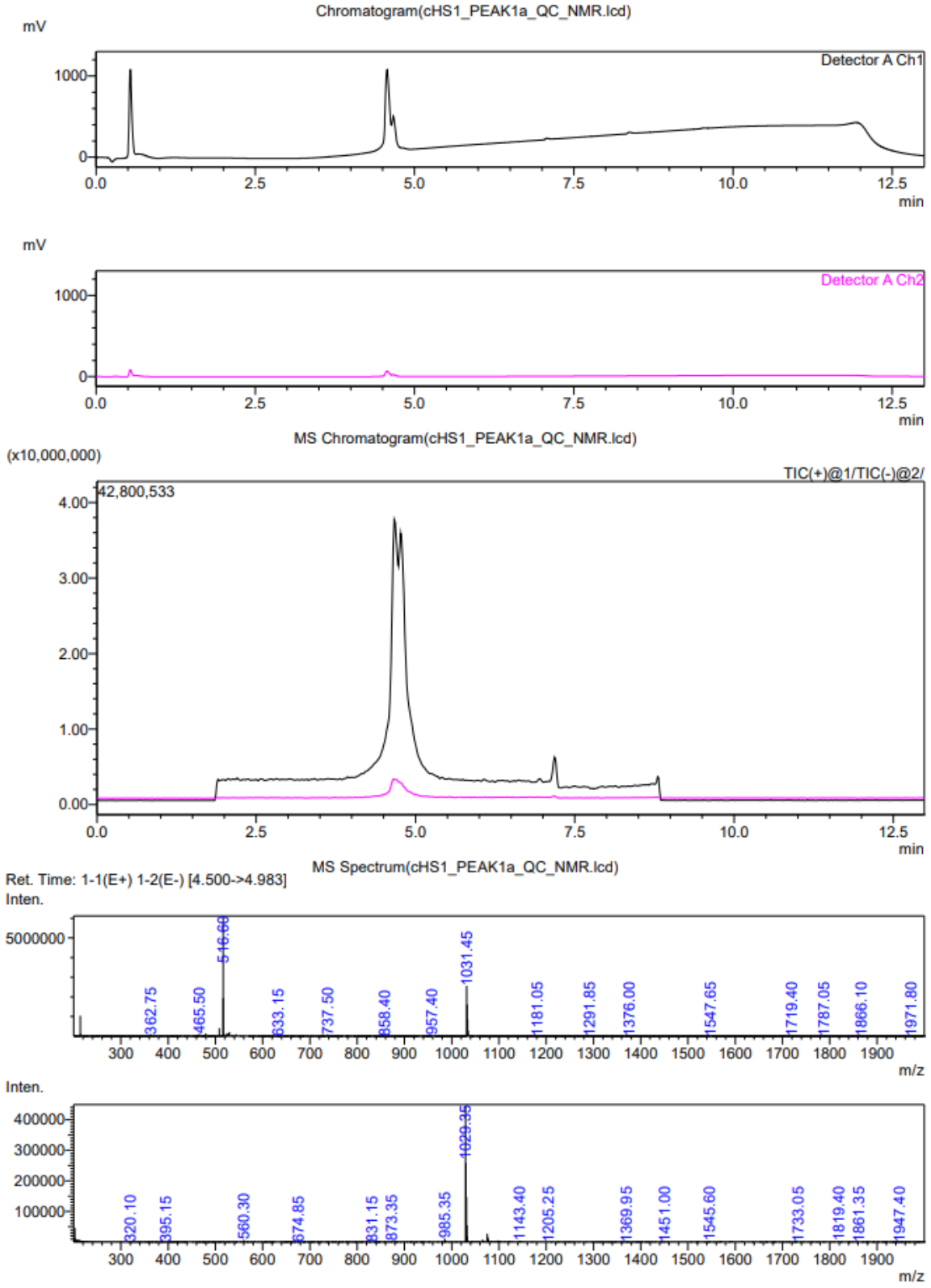


Figure S54. LCMS for purified peptide **cP15$_{pk1}$** for NMR showing LC UV trace at 220 and 254 nm (top), TIC (middle), and MS (+ve and -ve) at selected product peak (bottom).

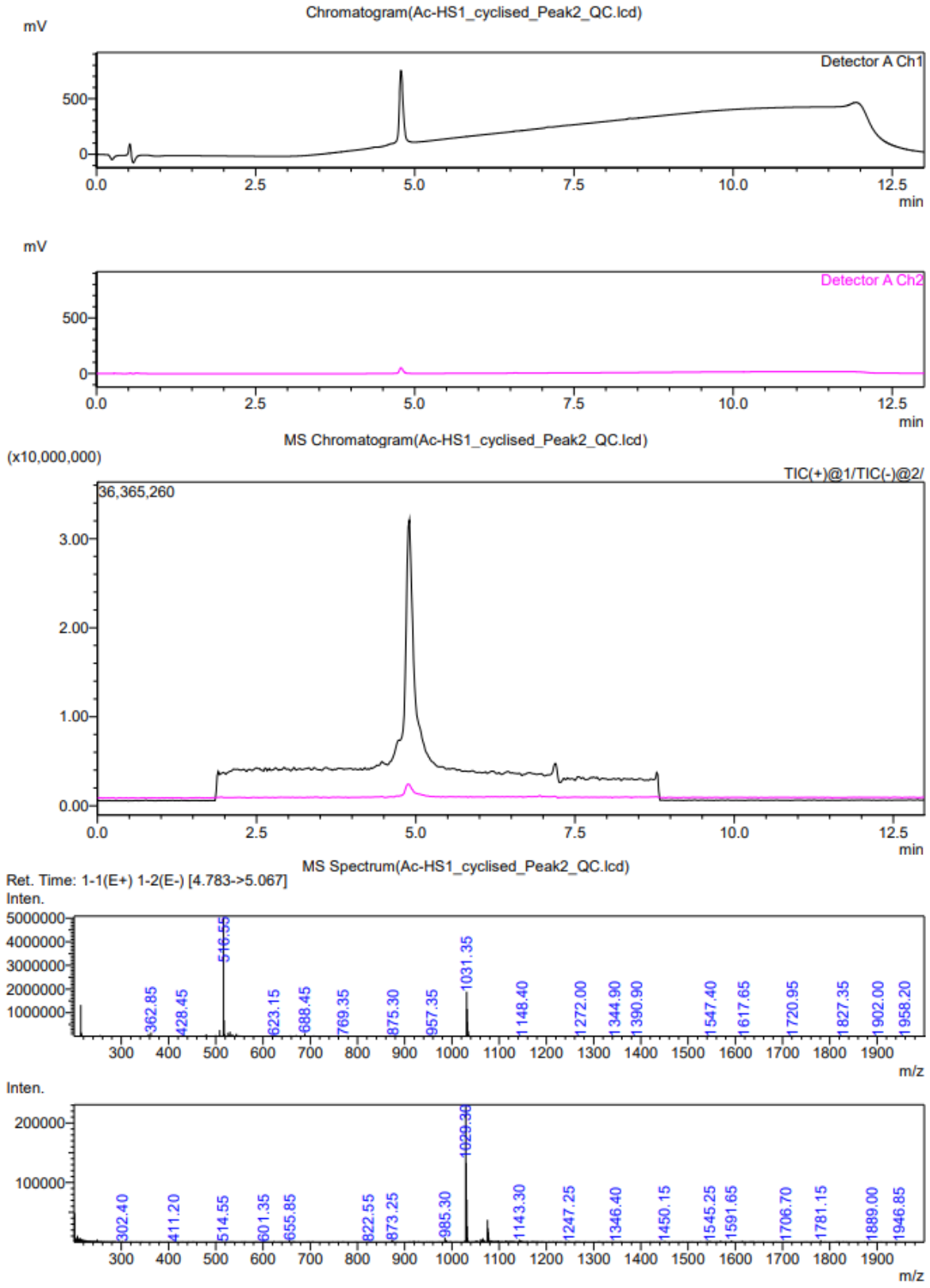


Figure S55. LCMS for purified peptide **cP15$_{pk2}$** for NMR showing LC UV trace at 220 and 254 nm (top), TIC (middle), and MS (+ve and -ve) at selected product peak (bottom).

11 References

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3. D. E. Shafer, J. K. Inman and A. Lees, *Anal. Biochem.*, 2000, **282**, 161-164.
4. Q. Xiao and D. Pei, *J. Med. Chem.*, 2007, **50**, 3132-3137.