Macrocycles that inhibit the binding between heat shock protein 90 and TPR-containing proteins

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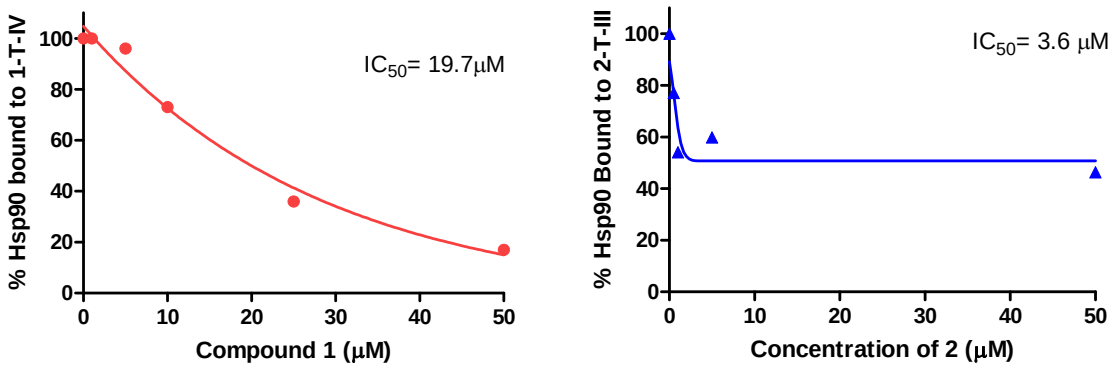
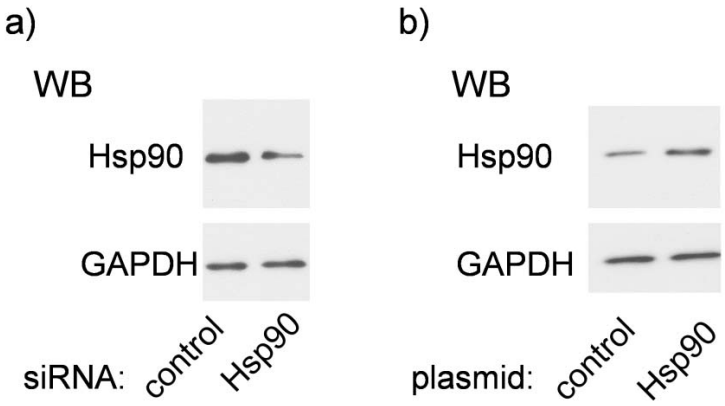
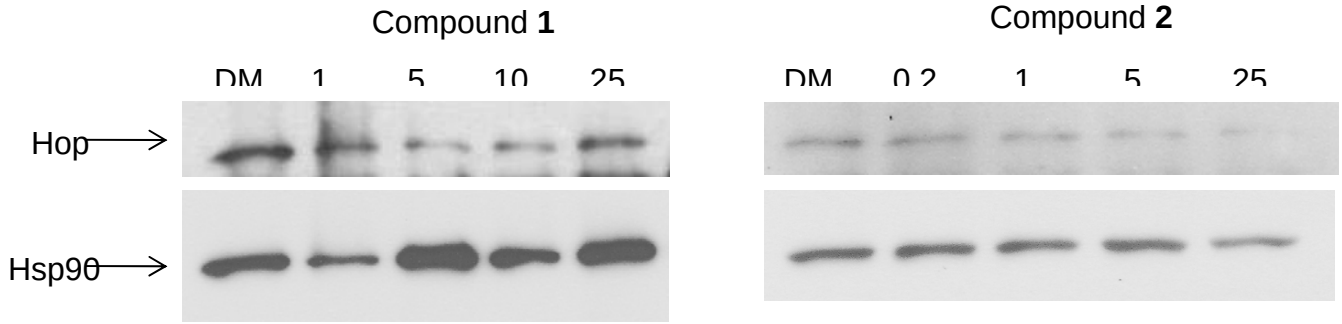
SUPPLEMENTAL FIGURES



SI Figure 1. Competitive binding assays between tagged and untagged compounds. Increasing concentrations of 1 and 2 compete with 1-T-IV and 2-T-III, respectively, for binding to Hsp90.

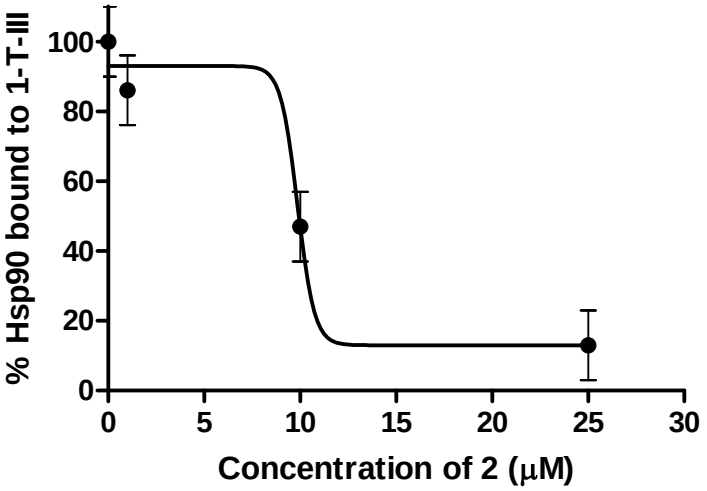


SI Figure 2. Depletion or overexpression of Hsp90. a) HeLa cells were transfected with 1nM of si-control (Qiagen Ctl_AllStars_1) or 0.5nM of si-Hsp90α (AACCCTGACCATTCCATTATT) and 0.5nM of si-Hsp90β (CAAGAATGATAAGGCAGTTAA) for 48 hours. Down-regulation of Hsp90 was confirmed by Western blot. b) HeLa cells were transfected with pCMV (control) or pCMV-Hsp90 plasmid for 24 hours. Overexpression of Hsp90 was confirmed by Western blot. GAPDH was WB for loading control analyses.



SI Figure 3. Western blots from co-immunoprecipitations using compound 1 and 2-treated cell lysates and antibody for Hsp90. Bands were analyzed using Image J densometric software. The amount of Hop in each

lane was normalized to Hsp90. Graphs shown in main manuscript were produced calculating the amount of Hop in compound co-IPs compared to DMSO controls.



SI Figure 4. Competitive binding affinity of compound 1-Tag-III and compound 2 with Hsp90. Purified, native Hsp90 (Stressgen) was incubated in PBS (without calcium or magnesium) with 10μM biotinylated compound 1-Tag-III for 1 h at room temp, and then incubated with 0-25μM of compound 2 for 1 h at room temp. Streptavidin beads were added and incubated for 30 min at room temp followed by removal of the unbound supernatant. The beads were washed four times with PBS and heated for 15 min at 100 ºC in SDS–PAGE sample buffer. Samples were analyzed on SDS page protein gels (Invitrogen), and western blots were done using Hsp90 antibodies. Bands in the western blots were quantified using Image J, and the percentage of Hsp90 still bound to the beads was calculated. This experiment was run two independent times. It was found that compound 2 inhibited binding of compound 1-Tag-III with an IC_{50} of 5.2 μM, thus, confirming our findings that compound 1 and 2 both bind to Hsp90 at the same binding site.

GENERAL SYNTHESIS OF BIOTINYLATED PEPTIDES

General solid phase synthesis remarks

Stepwise solid phase peptide synthesis was performed in a polypropylene solid-phase extraction cartridge fitted with a 20 μM polyethylene frit purchased from Applied Separations (Allentown, PA). 2-Chlorotrityl resins were purchased in pre-loaded form with L-Phe, D-Phe, or L-Leu. Resins were swelled in DMF for 30 min prior to assembly of the linear five-residue peptide sequence. Solid-phase syntheses were performed on a 0.5-0.8 mmol scale based on resin-loading. All operations were performed at room temperature under open atmosphere unless stated otherwise.

General solid phase peptide synthesis

Fmoc-protected amino acids were coupled using 3 equiv of amino acid, 3 equiv of 1-hydroxybenzotriazole, and 6 equiv of diisopropylcarbodiimide. Couplings were performed in DMF at 0.2 M with respect to the incoming Fmoc-protected amino acid. Couplings were allowed to proceed for a minimum of 2 h, and were assayed via ninhydrin test to verify competition. Once complete, the coupling reaction solution was drained, and the resin subjected to Fmoc deprotection. (Note: Fmoc and N-methyl amino acids are coupled according to the cycle above, however for subsequent coupling onto the secondary amino terminus, 1-hydroxybenzotriazole was substituted with 1-hydroxy-7-azabenzotriazole and the coupling was allowed to proceed overnight).

General solid phase amine deprotection
Following coupling completion, the peptide-resin was treated as follows for removal of the Fmoc protecting group: DMF wash (3 x 1 min), 20% Piperdine/DMF (1 x 5 min), 20% Piperdine/DMF (1 x 10 min), DMF wash (2 x 1 min), IPA wash (1 x 1 min), DMF was (1 x 1 min), IPA (1 x 1 min), DMF (3 x 1 min). A ninhydrin test was performed to verify completion.

General N-terminal solid phase amine deprotection
Once the final N-terminal amino acid residue had been coupled, the peptide-resin was treated as follows for removal of the Fmoc protecting group: DMF wash (3 x 1 min), 20% Piperdine/DMF (1 x 5 min), 20% Piperdine/DMF (1 x 10 min), DMF wash (3 x 1 min), IPA wash (3 x 1 min), MeOH (3 x 1 min). The fully-assembled peptide-resin was then drained and dried in vacuo overnight.

Cleavage of linear peptide
The full-length, linear peptide was cleaved from the resin by swelling and shaking the peptide-resin for 24 h in a 1:1 (v/v) 2,2,2-trifluoroethanol/CH_2Cl_2 (10 volumes/gram of dried resin). The cleavage solution was filtered through a Buchner filter, and the drained resin was washed with additional CH_2Cl_2 (5 volumes/ gram of initial dried peptide-resin) to fully extract the cleaved peptide from the resin. Solvents in the combined filtrates were evaporated by rotary evaporation and the solids dried in vacuo overnight. The solids were then reconstituted in CH_2Cl_2, evaporated by rotary evaporation and dried in vacuo overnight again to remove residual entrapped TFE.

Macrocyclization procedure (syringe pump)
Three coupling agents (DEPBT, HATU, and TBTU) were used at 0.5 to 0.75 equiv each. These coupling agents were dissolved in 3/4 of a calculated volume of dry methylene chloride that would give a 0.005–0.007 M overall concentration when included in the volume used for the deprotected peptide. The crude, dry, double deprotected peptide (free acid and free amine) was dissolved in the other 1/4 solvent volume of methylene chloride. DIPEA (8 equiv) was then added to the solution containing coupling reagents dissolved in methylene chloride. The double deprotected peptide was then added to the bulk solution dropwise using a syringe pump at a rate of 30 mL/h. The reaction was monitored via LCMS and generally complete in 1–2 h. Upon completion, the reaction was worked up by washing with aqueous HCl (pH 1) and saturated sodium bicarbonate. After back extraction of aqueous layers with large quantities of CH_2Cl_2, the organic layers were combined, dried, filtered and concentrated. All macrocycles were first purified by flash column chromatography using an ethyl acetate/hexane gradient on silica gel. Finally, when necessary, reversed-phase HPLC was used for additional purification using a gradient of acetonitrile and deionized water with 0.1% TFA.

Benzylation of compounds 2-T-II and 2-T-III
The purified macrocycle was benzylated using 2.0-5.0 equiv of benzyl bromide and 2.0-5.0 equiv of sodium hydride (60% w/w in mineral oil) in 0.1M of dry tetrahydrofuran. Under dry conditions, the macrocycle was dissolved in THF. Sodium hydride was added and allowed to dissolve, followed by the addition of benzyl bromide. The reaction was monitored by LCMS and was complete in 6-12 hours. Upon completion, the reaction was worked up with 2 treatments of deionized water. After back extraction of the aqueous layers with large quantities of CH_2Cl_2, the organic layers were combined, dried, filtered and concentrated *in vacuo*. Benzylated macrocycles were first purified by flash column chromatography using an ethyl acetate/hexane gradient on silica gel. Finally, when necessary, reverse-phase HPLC was used for additional purification using a gradient of acetonitrile and deionized water with 0.1% TFA.

Deprotection of lysine amine (removal of Boc). Boc protected lysine was deprotected using 20% trifluroacetic acid and 80% methylene chloride with 1 eq of anisole at 0.1M overall concentration. The reaction mixture was

stirred under normal atmospheric pressure and room temperature for 45 minutes and was monitored by TLC and LCMS. The final deprotected peptide was taken on to the biotinylation step without further purification.

Coupling of peg-biotin to free lysine-containing macrocycle. All biotin-coupling reactions were carried out using cyclized free-lysine (1.0 equivalent) and biotinylating reagent: NHS-dPEG-Biotin (1.5 equivalents), along with 8 equivalents of DIPEA in 0.1M methylene chloride. The solution was stirred at room temperature and reactions were monitored by TLC and LCMS. The reaction was usually complete in 2-8 hours. Reverse-phase HPLC was used for purification using a gradient of acetonitrile and DI water with 0.1% TFA.

METHODS OF CHROMATOGRAPHIC PURITY

Method A

Instrument: Agilent 1200 Series HPLC
Agilent 62440A LC/MSD Trap
Column: Zorbax SB-C18
2.1x30mm 3.5-Micron

Mobile Phase A: 0.1% (v/v) formic acid, 100% (v/v) water
Mobile Phase B: 0.1% (v/v) formic acid, 100% (v/v) acetonitrile

Gradient:	Time (min)	Profile %A	Profile %B
	0	80	20
	4.5	10	90
	4.6	10	90
	7.0	85	15

Flow Rate: 1.0 ml/min
Injection: 4µL
Solvent: 100% Methanol

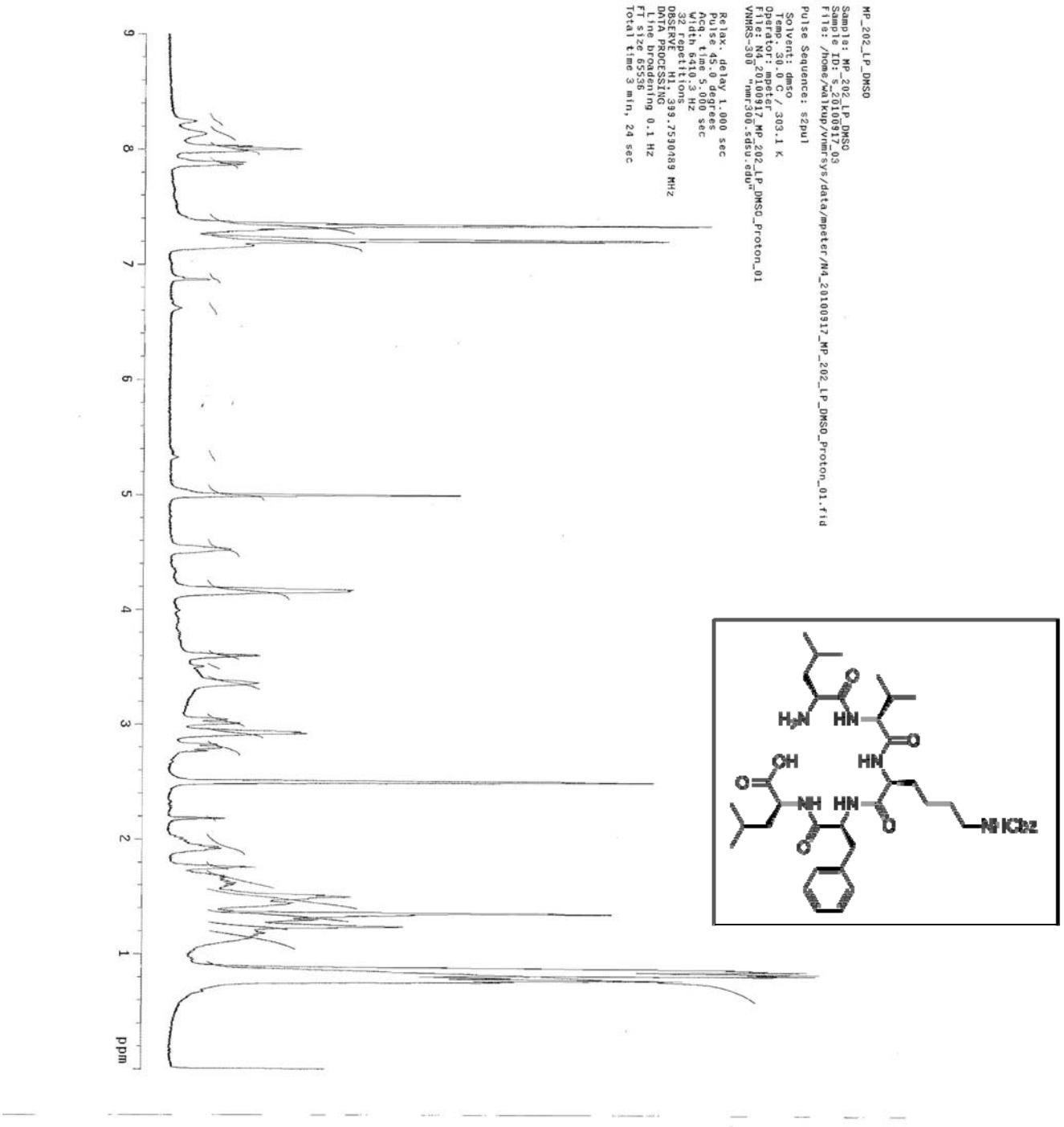
Method B

Instrument: Waters Flex Inject
Waters 2487 Dual λ Absorbance Detector
Column: Symmetry C_{18} 3.5µm
4.6x75mm Column

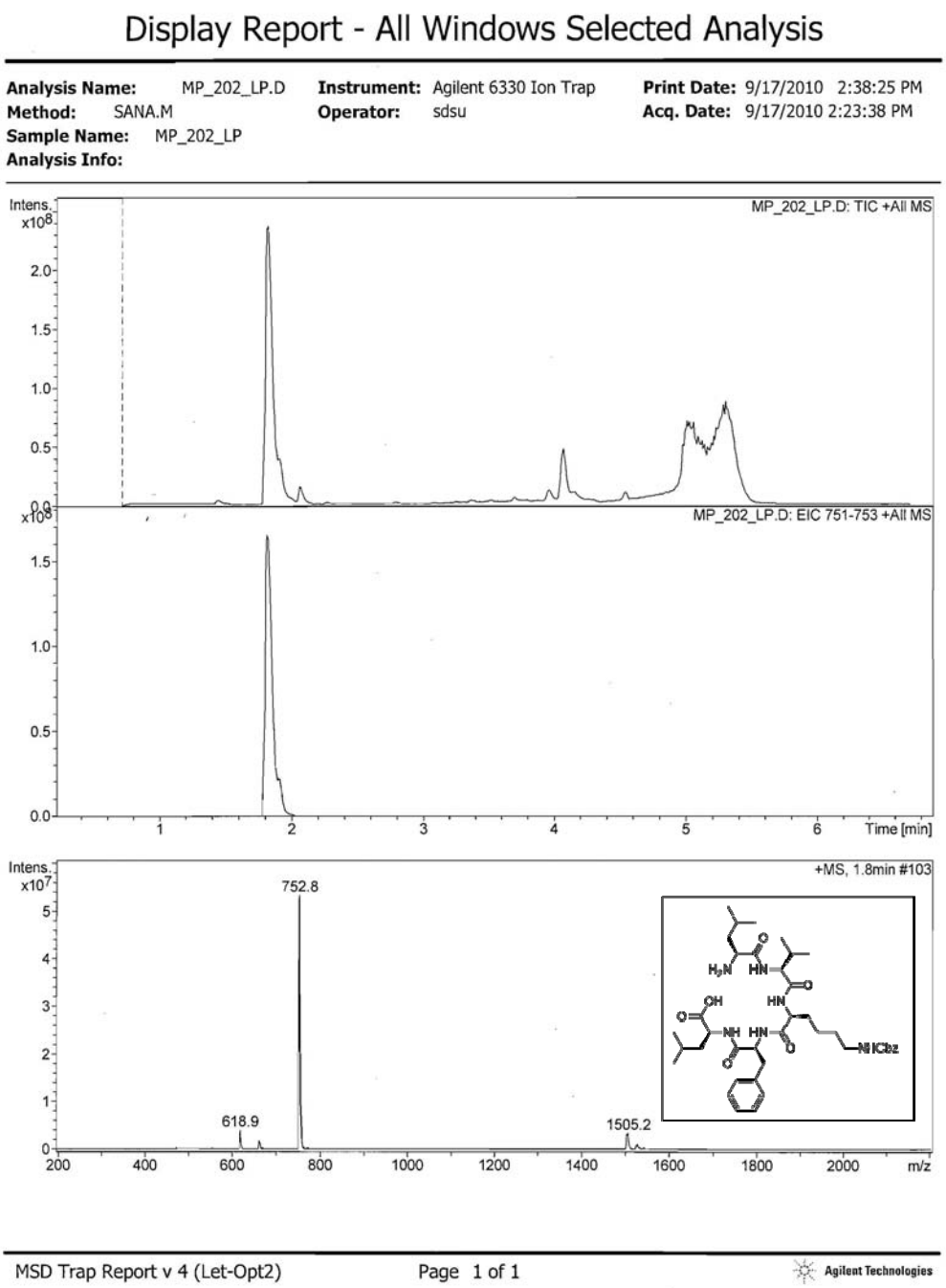
Mobile Phase A: 0.1% (v/v) Trifluoroacetic acid, 100% (v/v) water
Mobile Phase B: 0.1% (v/v) Trifluoroacetic acid, 100% (v/v) acetonitrile
λ_1: 215nm
λ_2: 222nm

Gradient:	Time (min)	Profile %A	Profile %B
	0	70	30
	4.00	0	100
	13.00	0	100
	15.00	70	30
	16.00	70	30

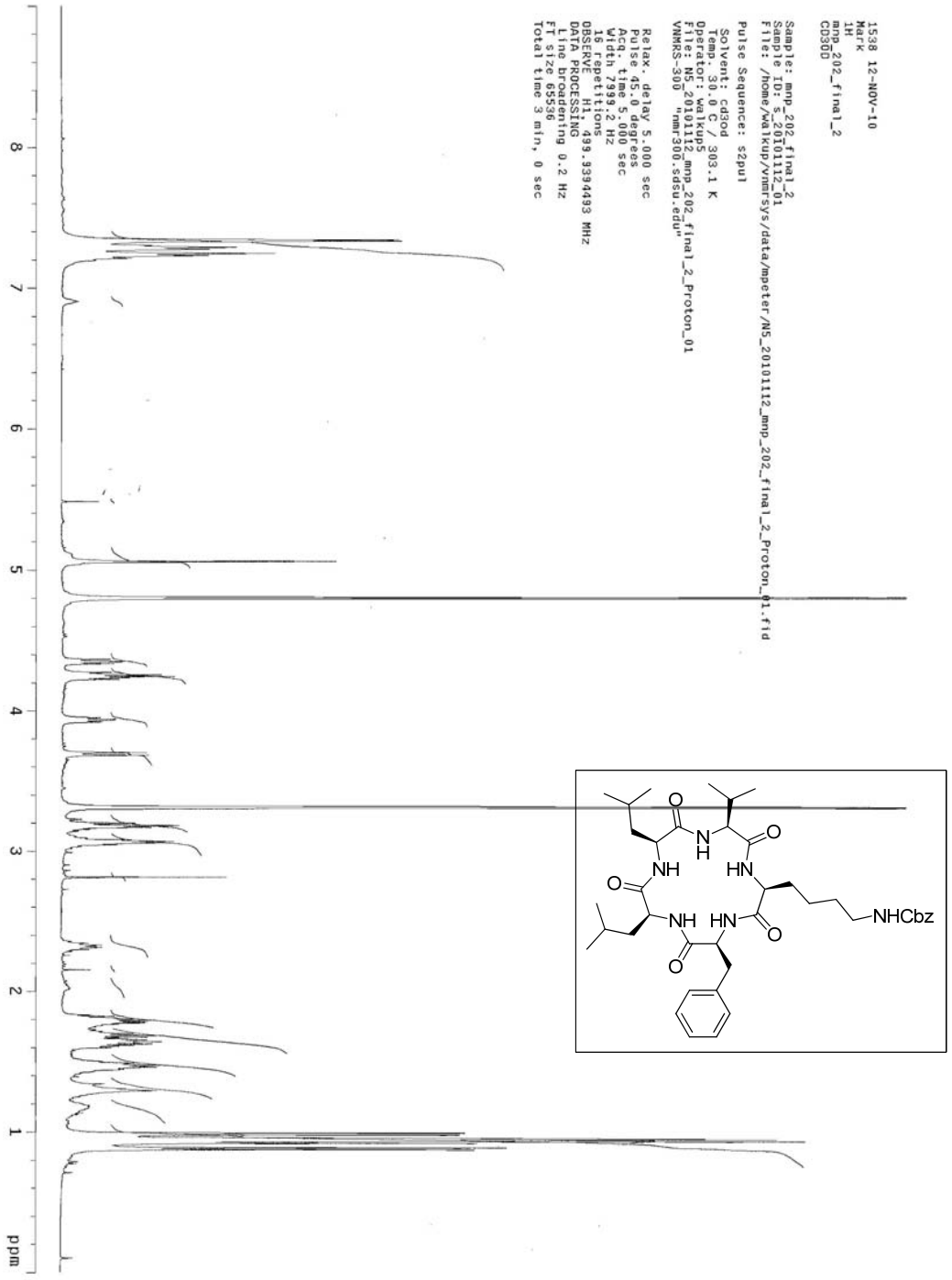
Flow rate: 0.50 ml/min
Injection: 20µL
Solvent: 100% Methanol



^{1}H NMR Double Deprotected Pentapeptide 1-T-II: *HO-Leu-Phe-Lys(Cbz)-Val-Leu-NH$_2$*



LCMS Double Deprotected Pentapeptide 1-T-II: *HO-Leu-Phe-Lys(Cbz)-Val-Leu-NH$_2$*



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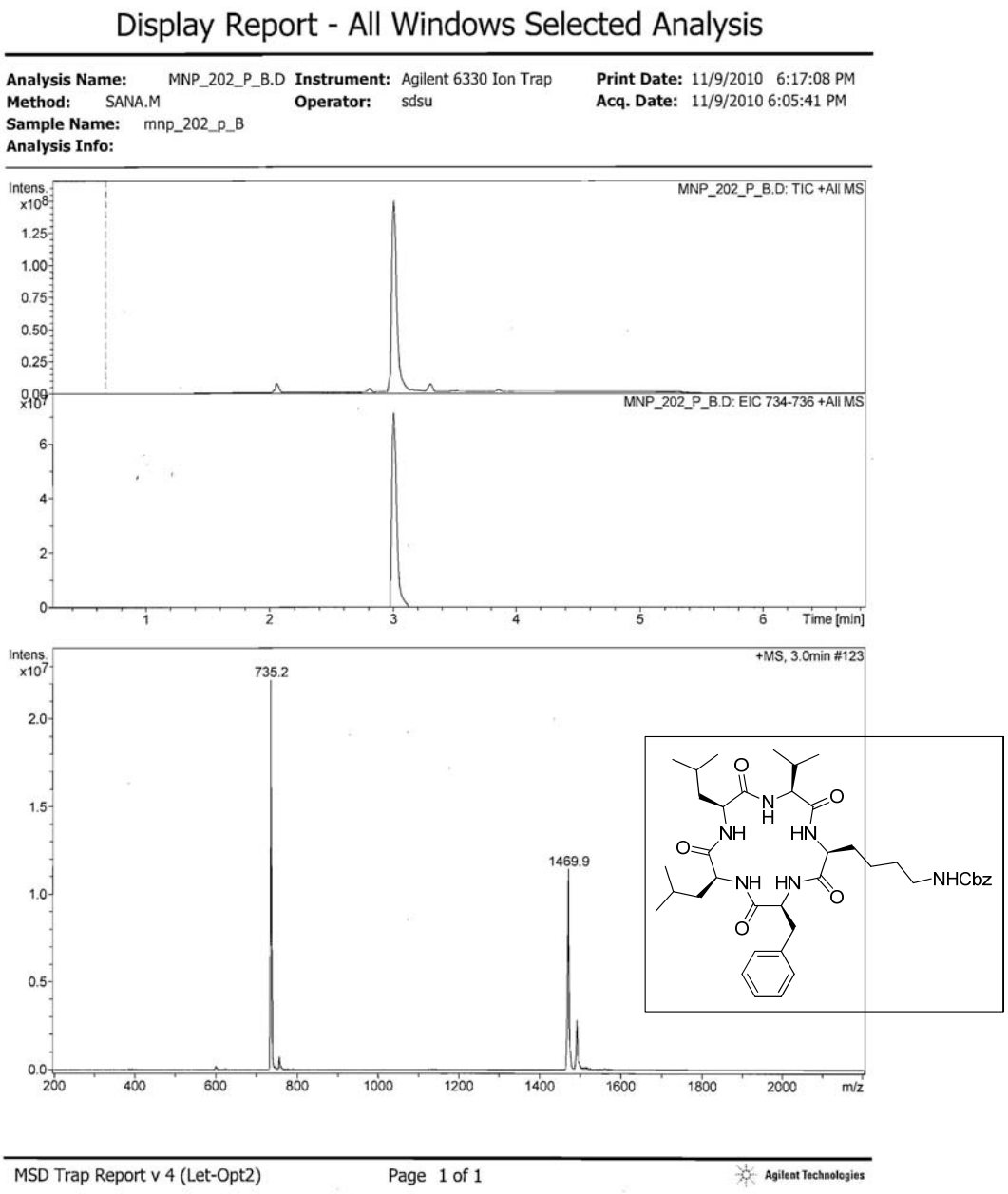

LCMS Cyclized Pentapeptide 1-T-II: *Phe-Lys(Cbz)-Val-Leu-Leu*

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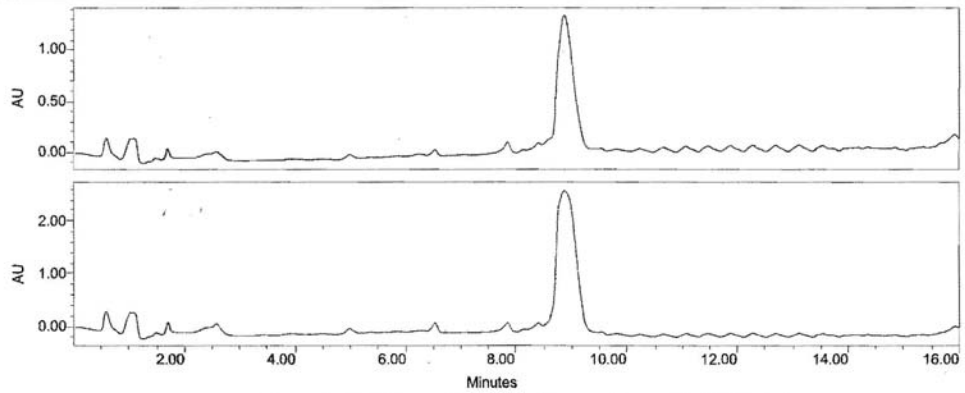

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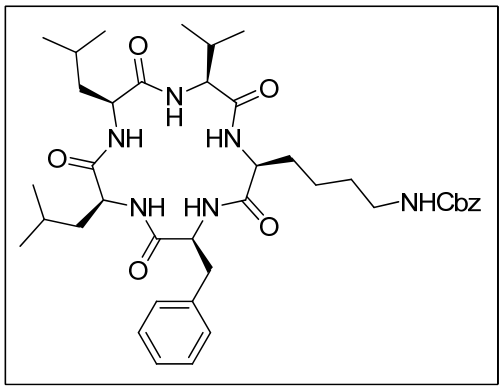

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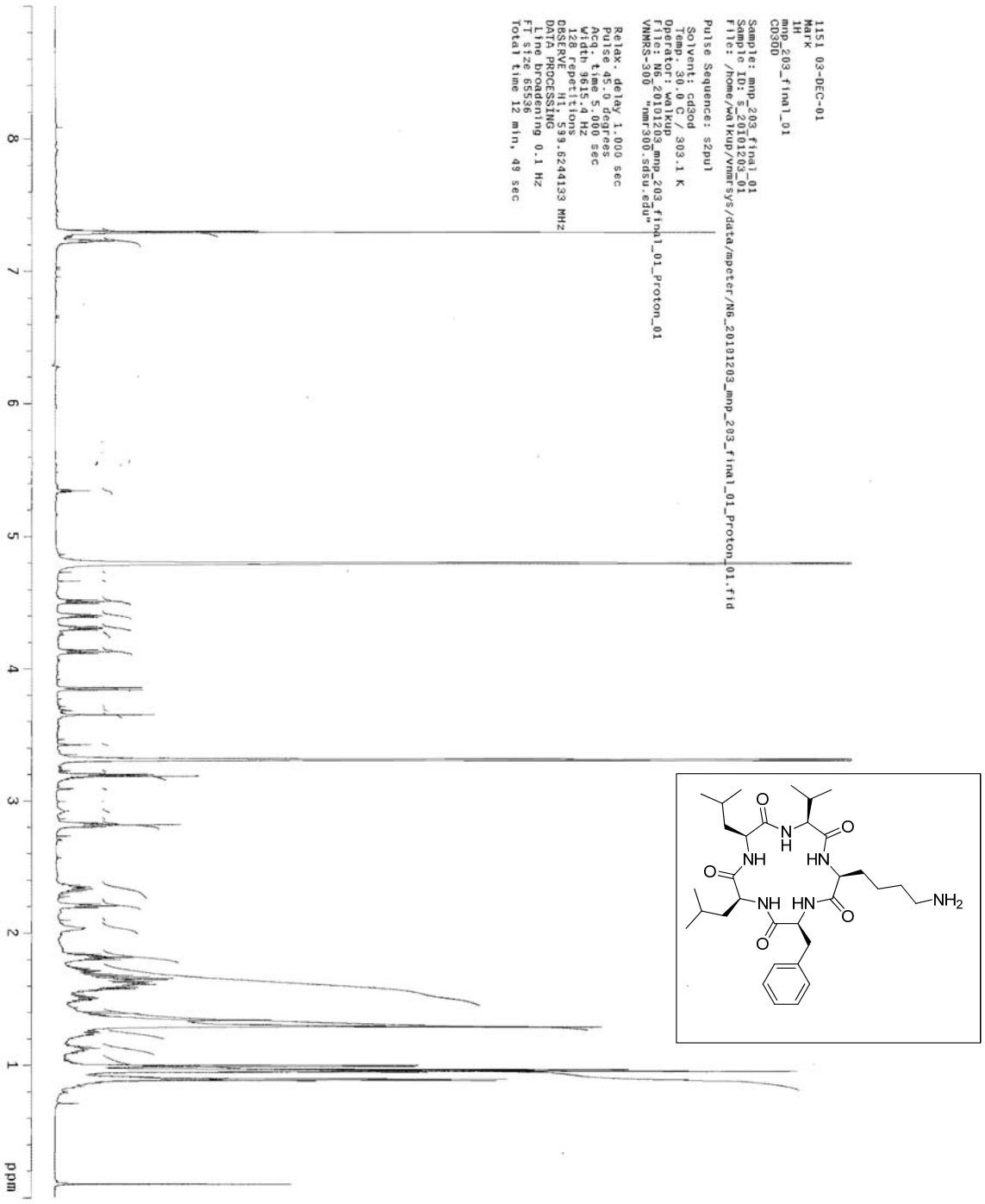

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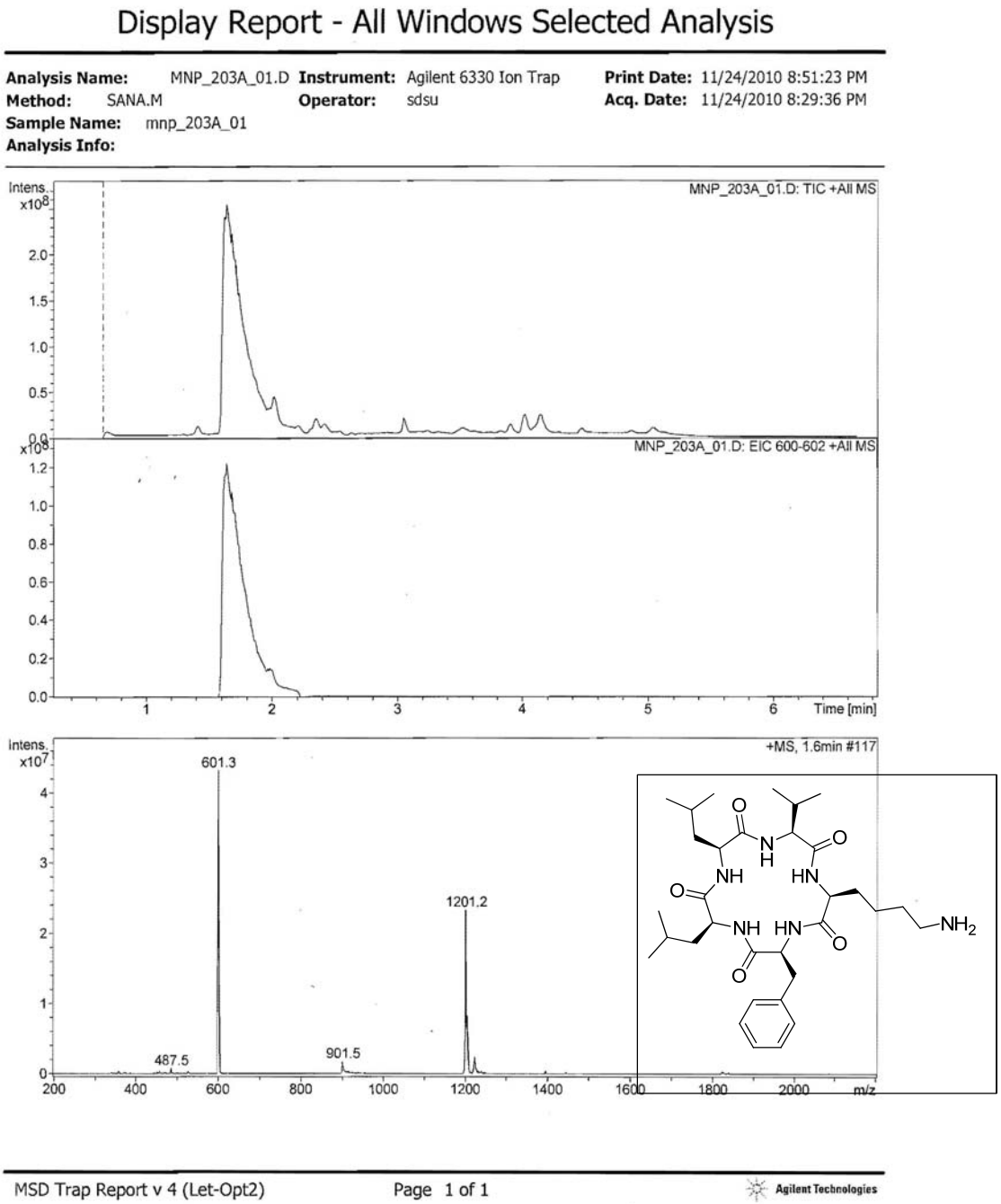

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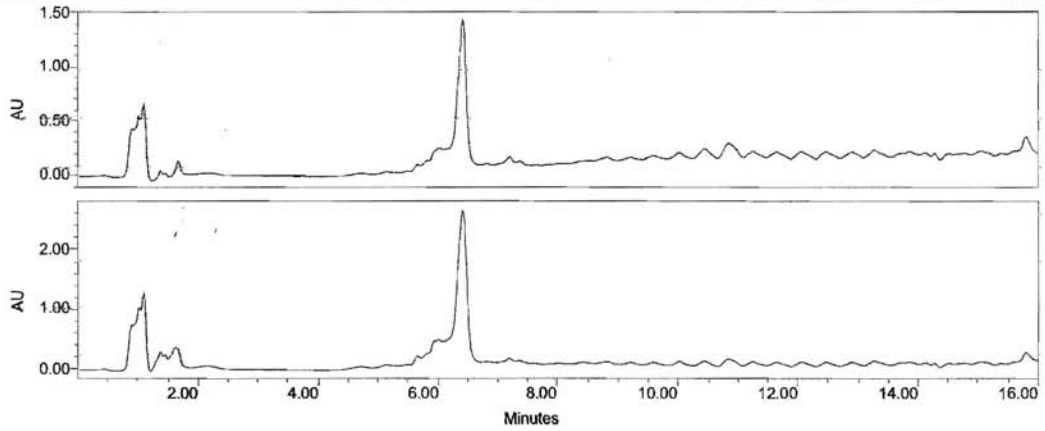

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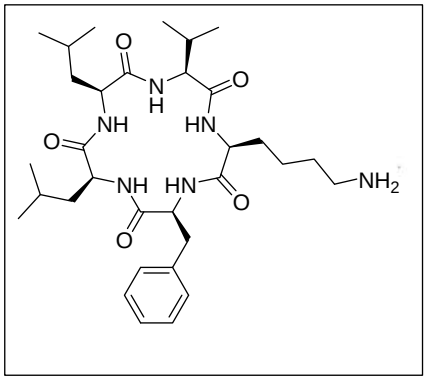

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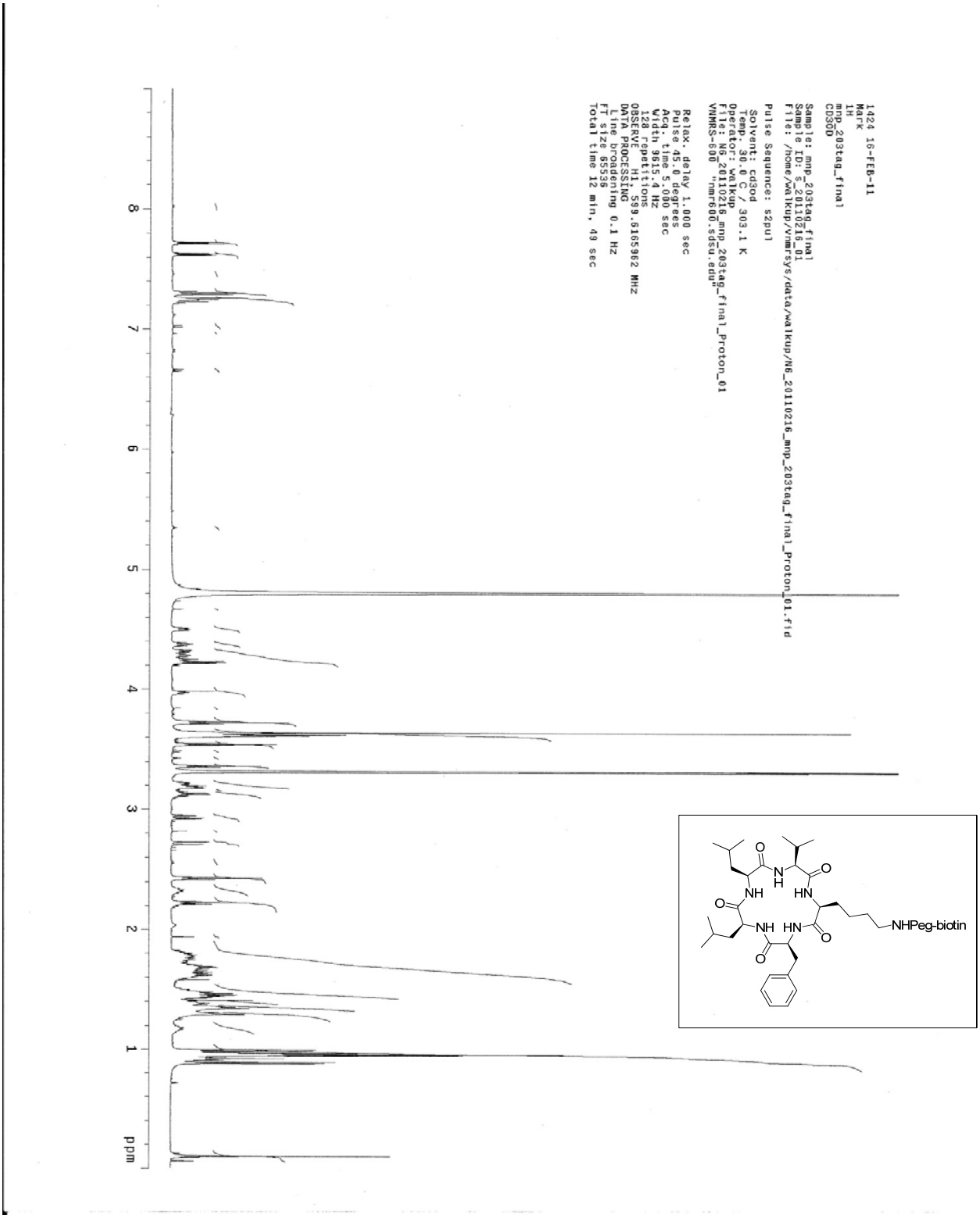

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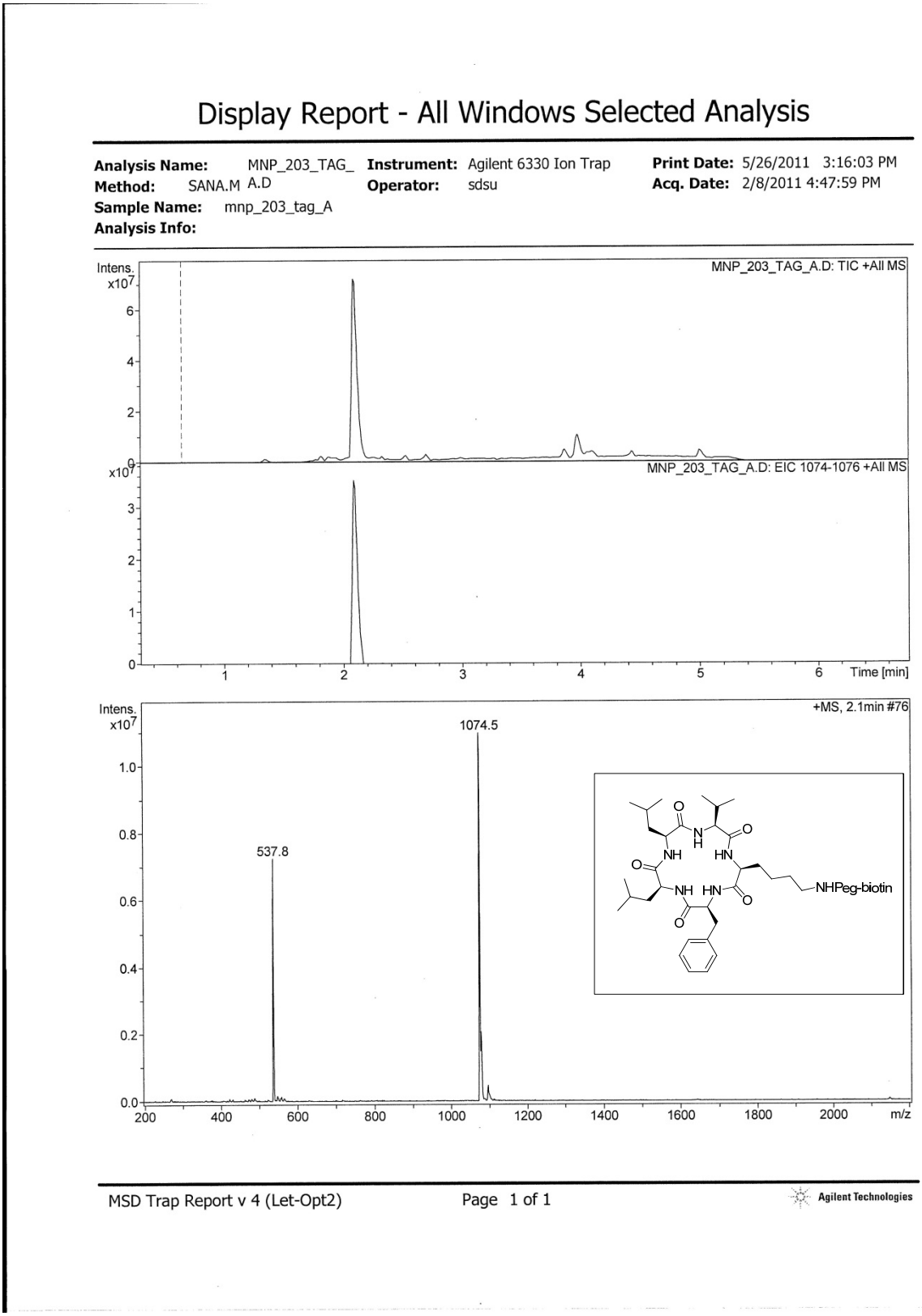

LCMS Biotinylated Pentapeptide 1-T-II: *Phe-Lys(Peg-biotin)-Val-Leu-Leu*

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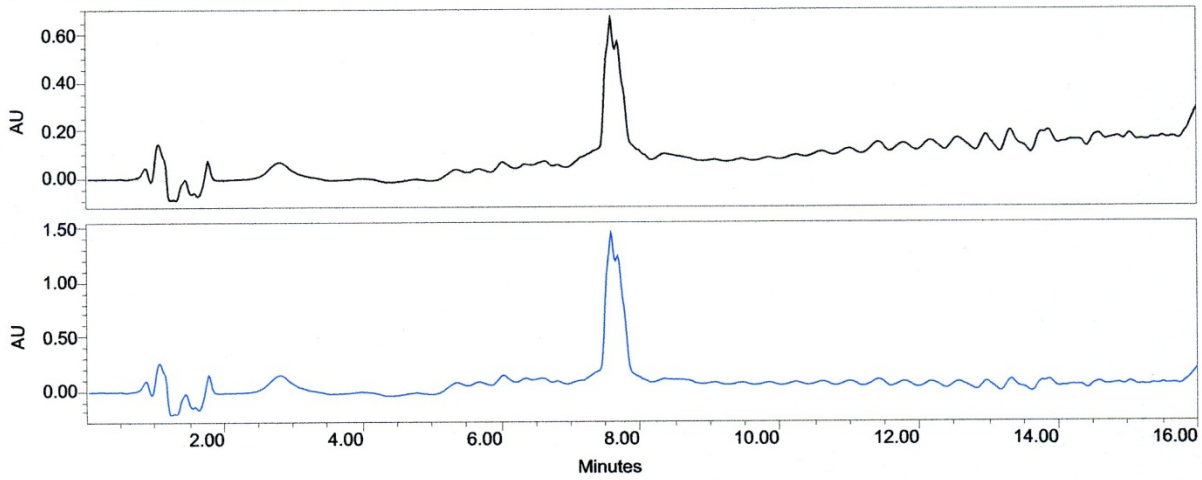

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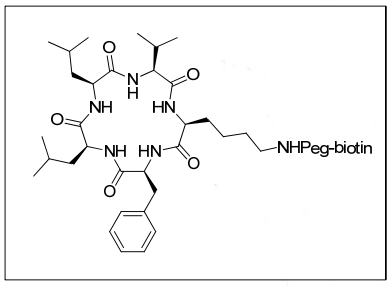

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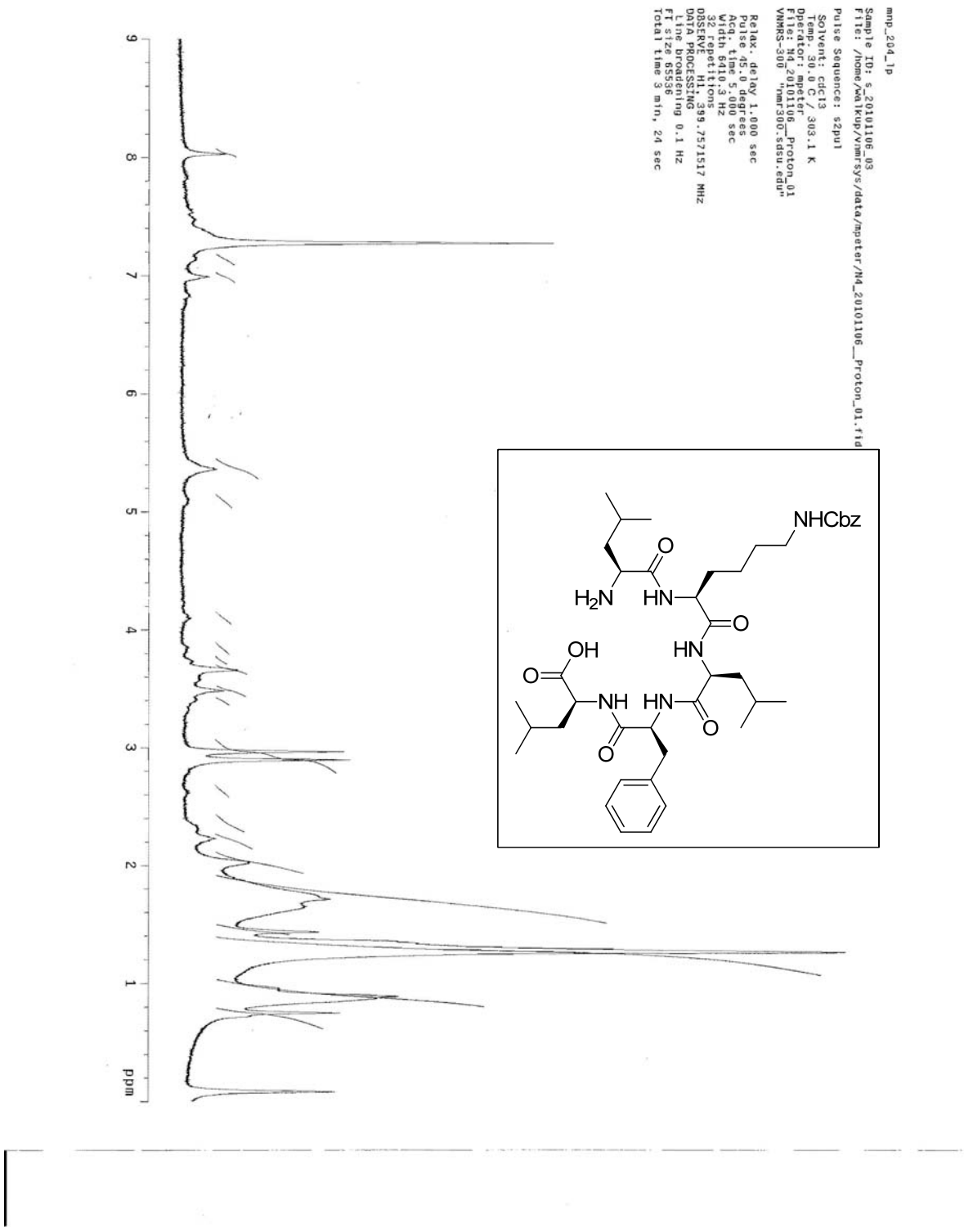
HPLC Biotinylated Pentapeptide 1-T-II: *Phe-Lys(Peg-biotin)-Val-Leu-Leu*



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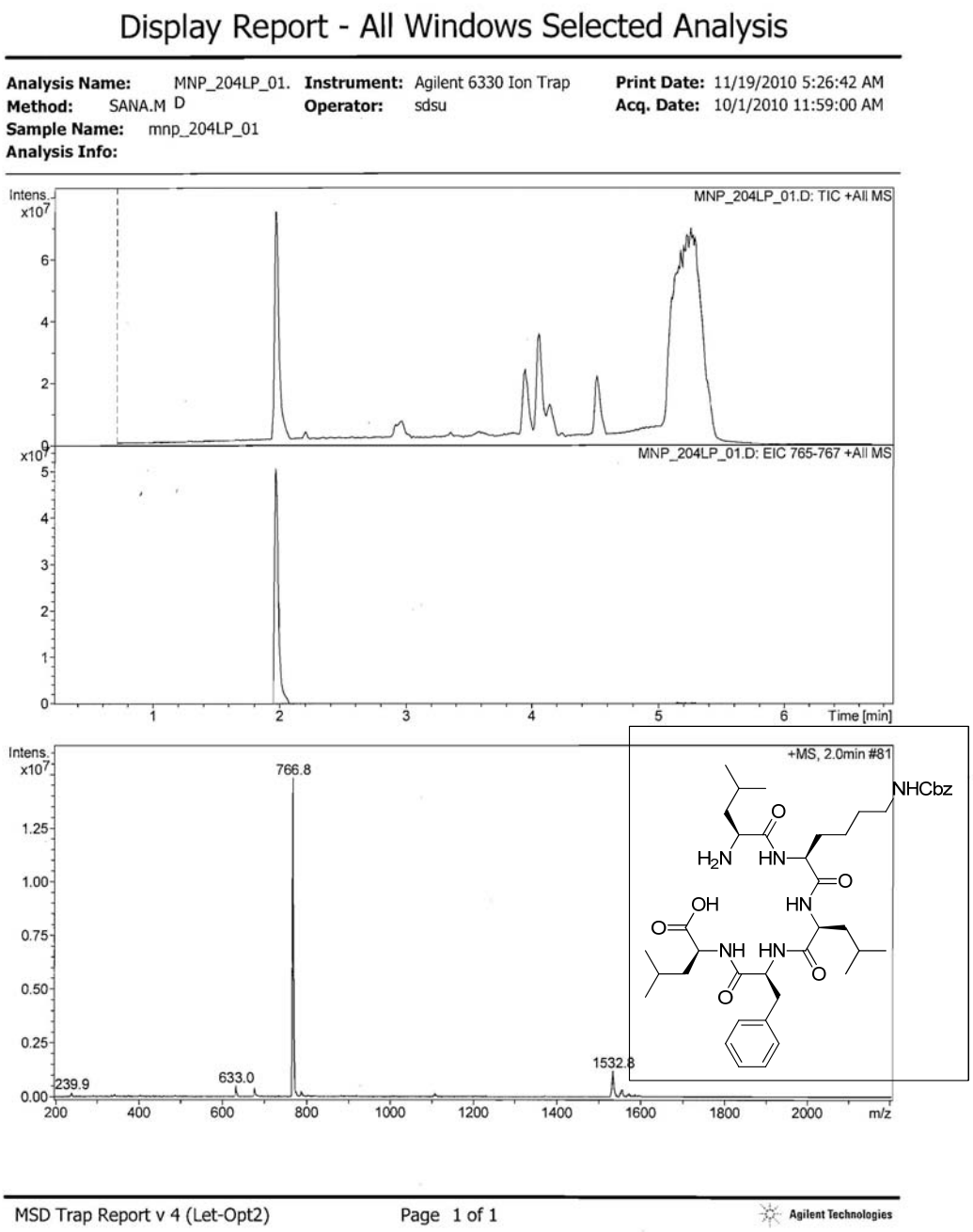
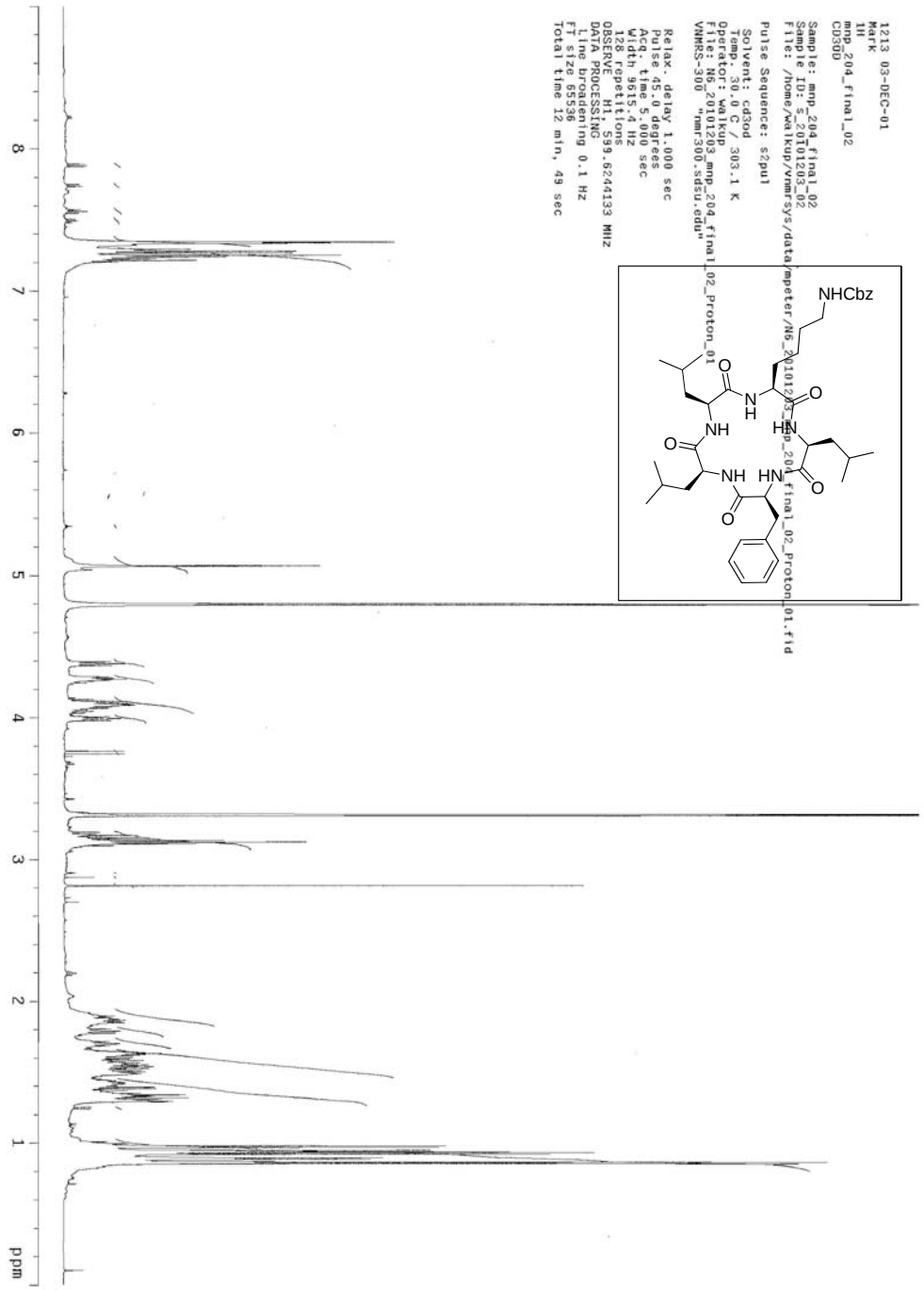


LCMS Double Deprotected Pentapeptide 1-T-III: *HO-Leu-Phe-Leu-Lys(Cbz)-Leu-NH$_2$*



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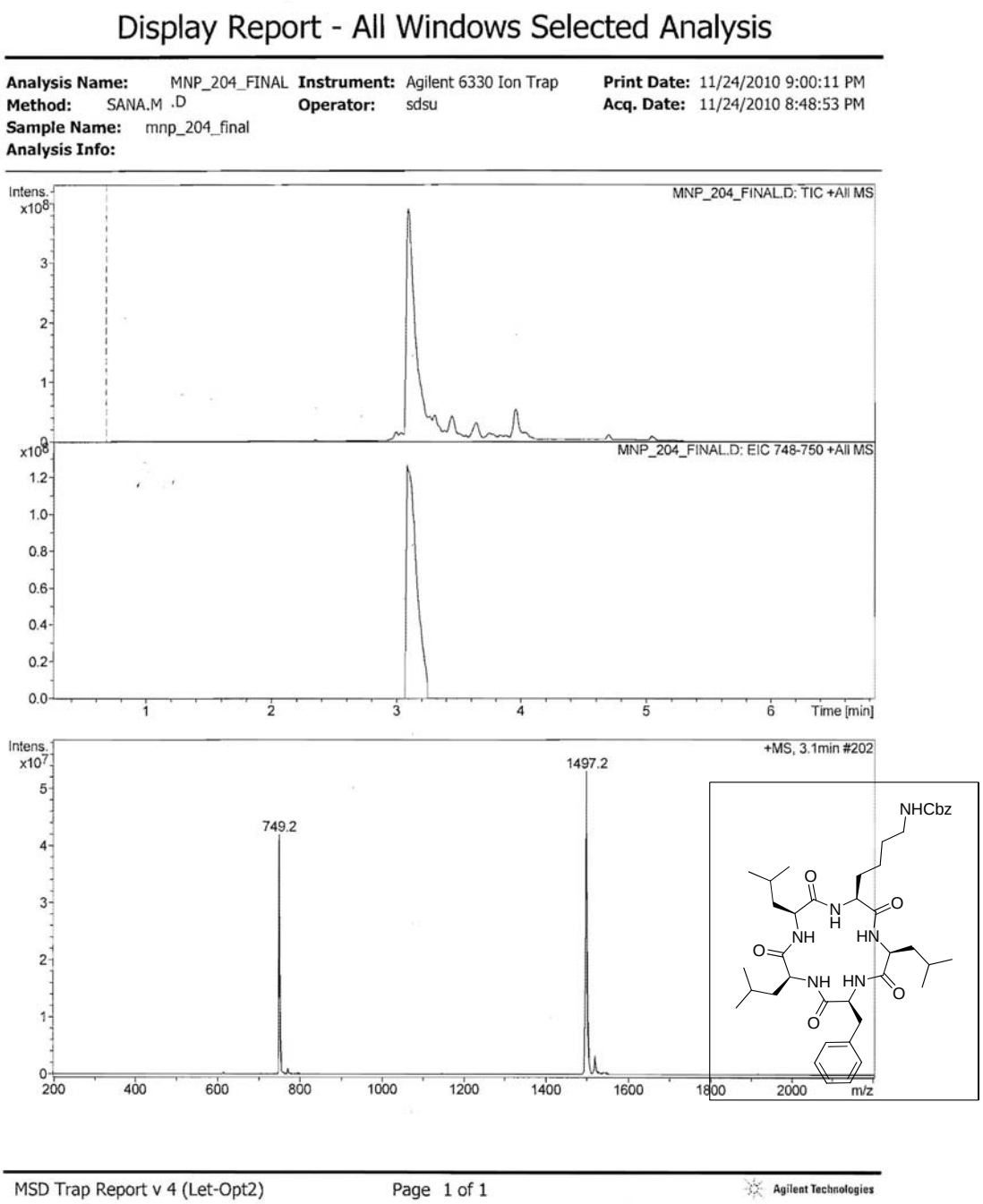

LCMS Cyclized Pentapeptide 1-T-III: *Phe-Leu-Lys(Cbz)-Leu-Leu*

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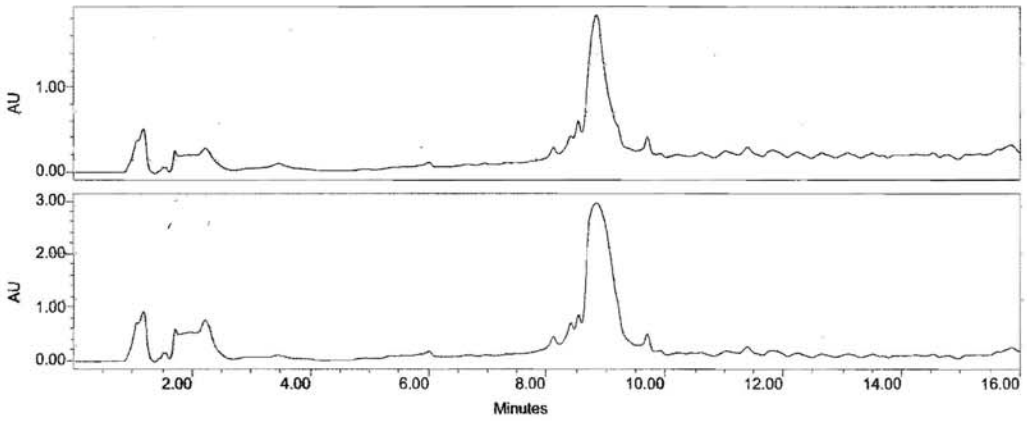

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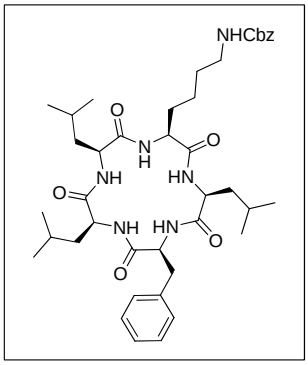

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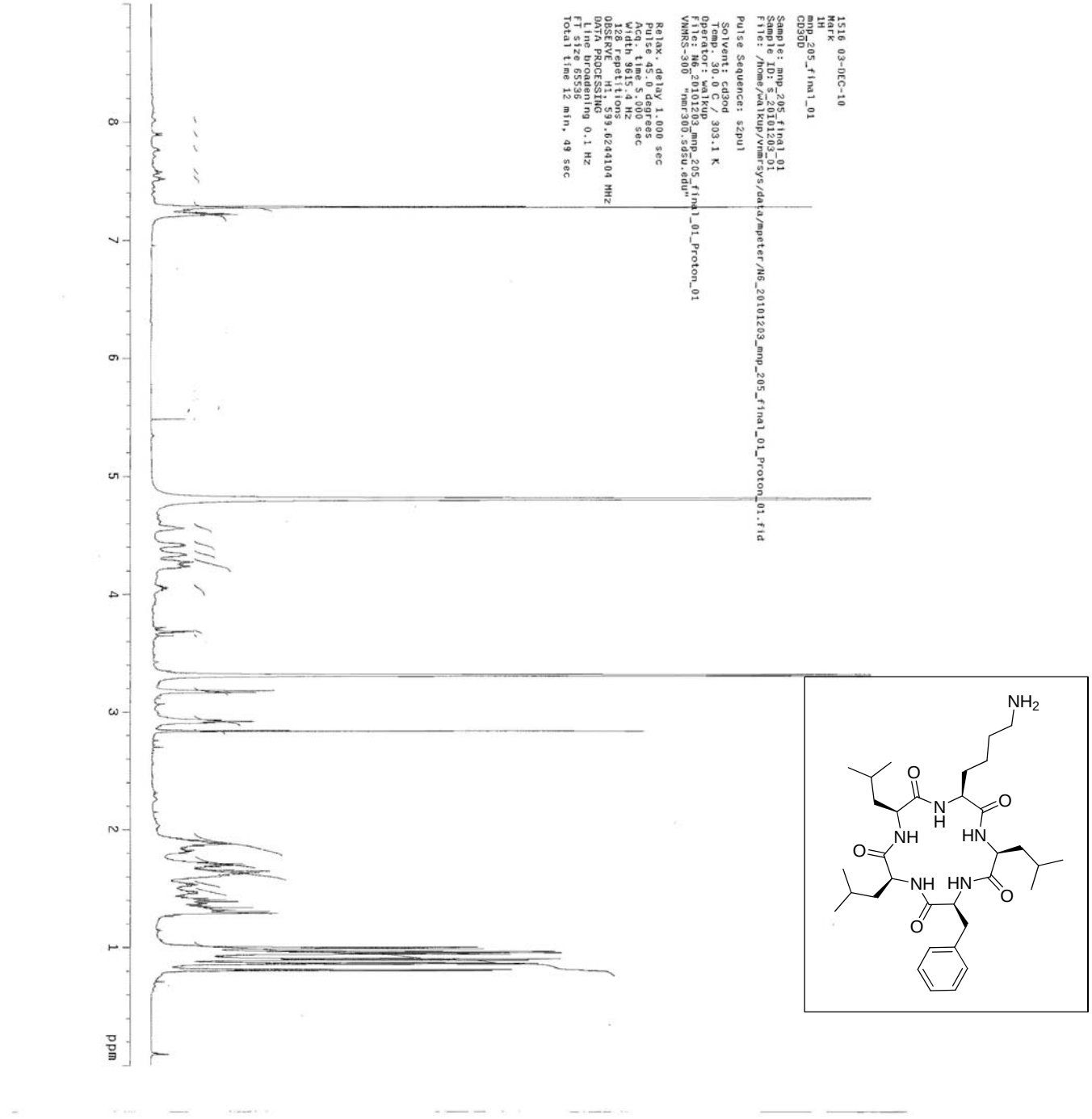

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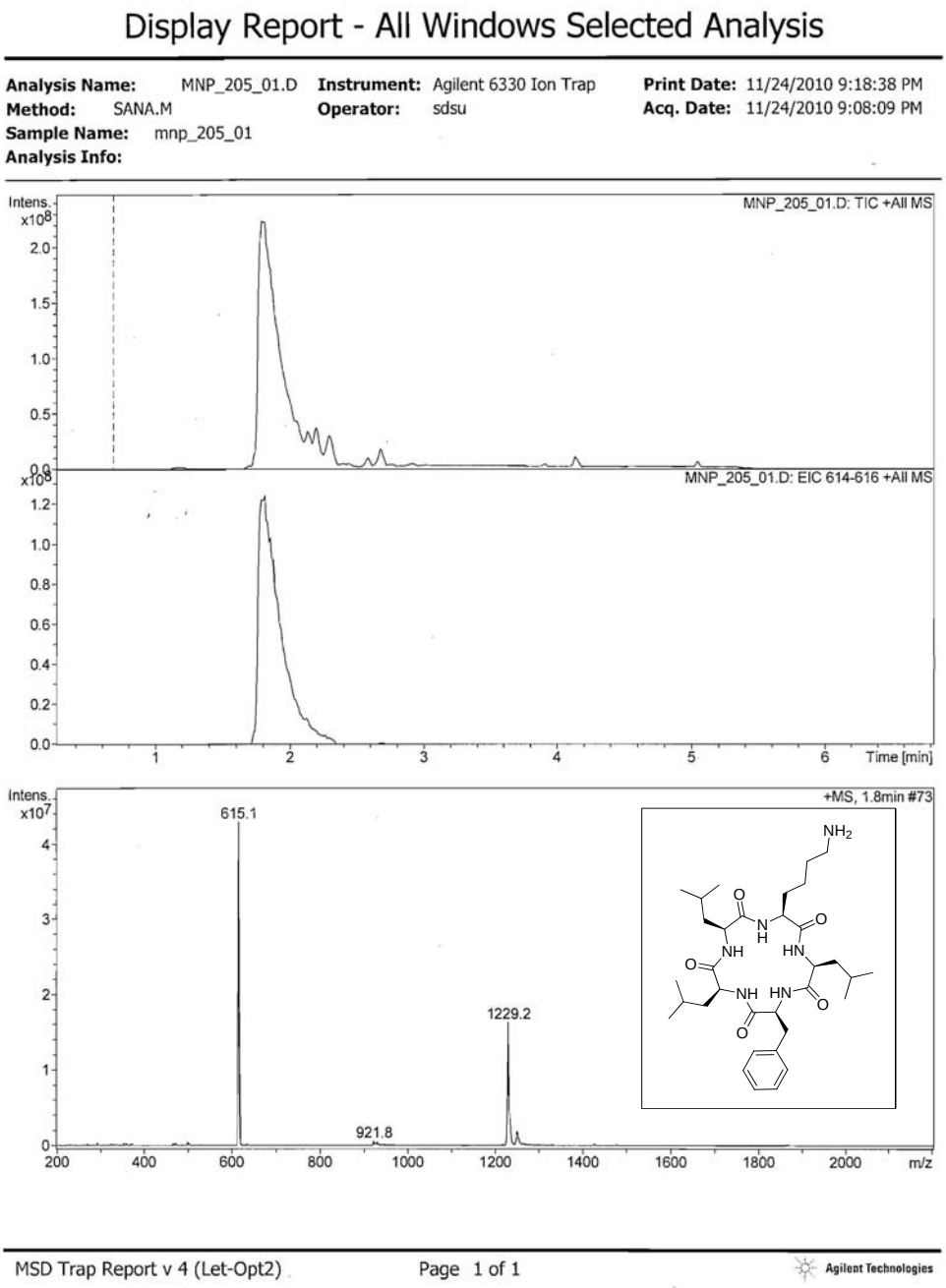

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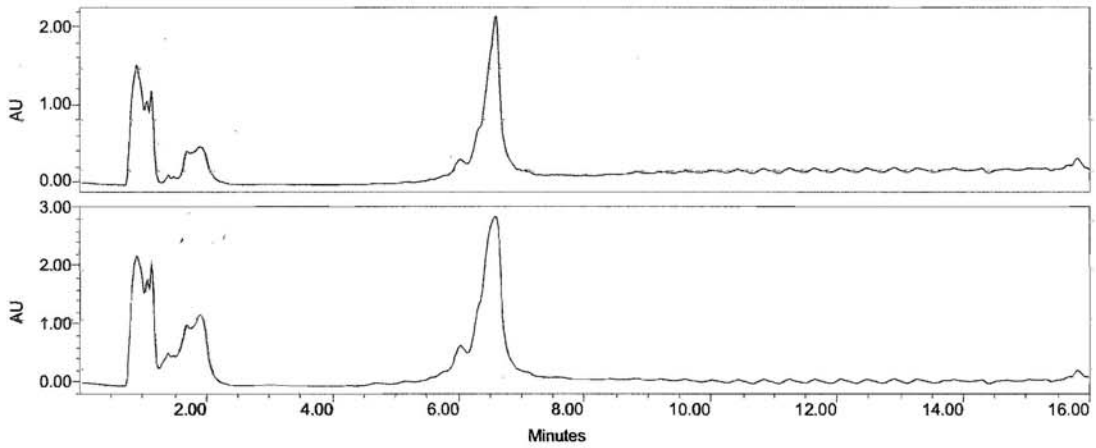

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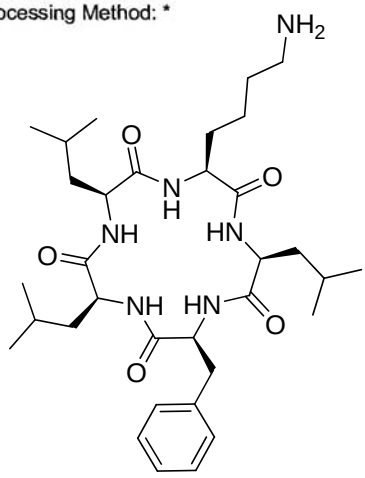

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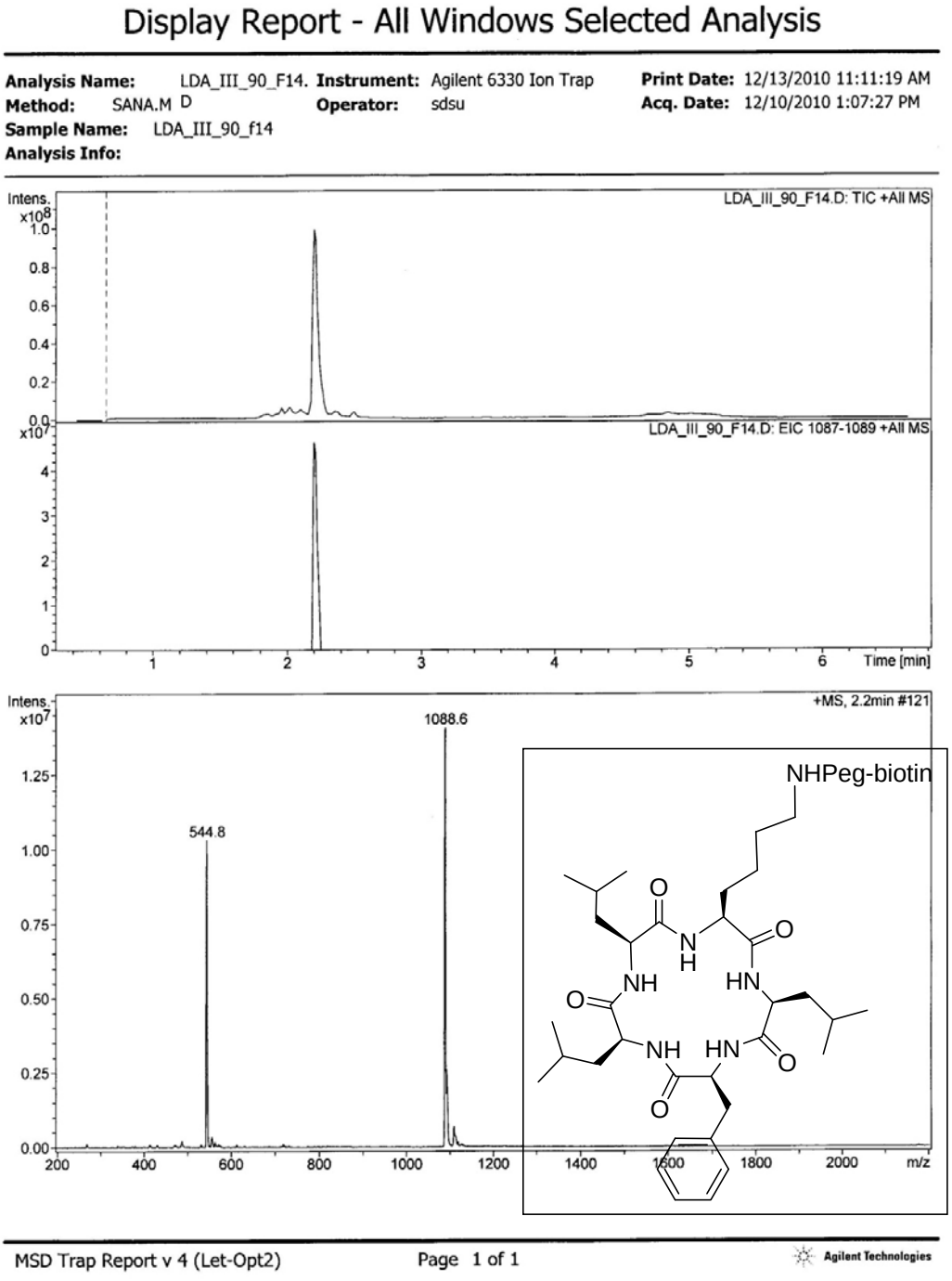
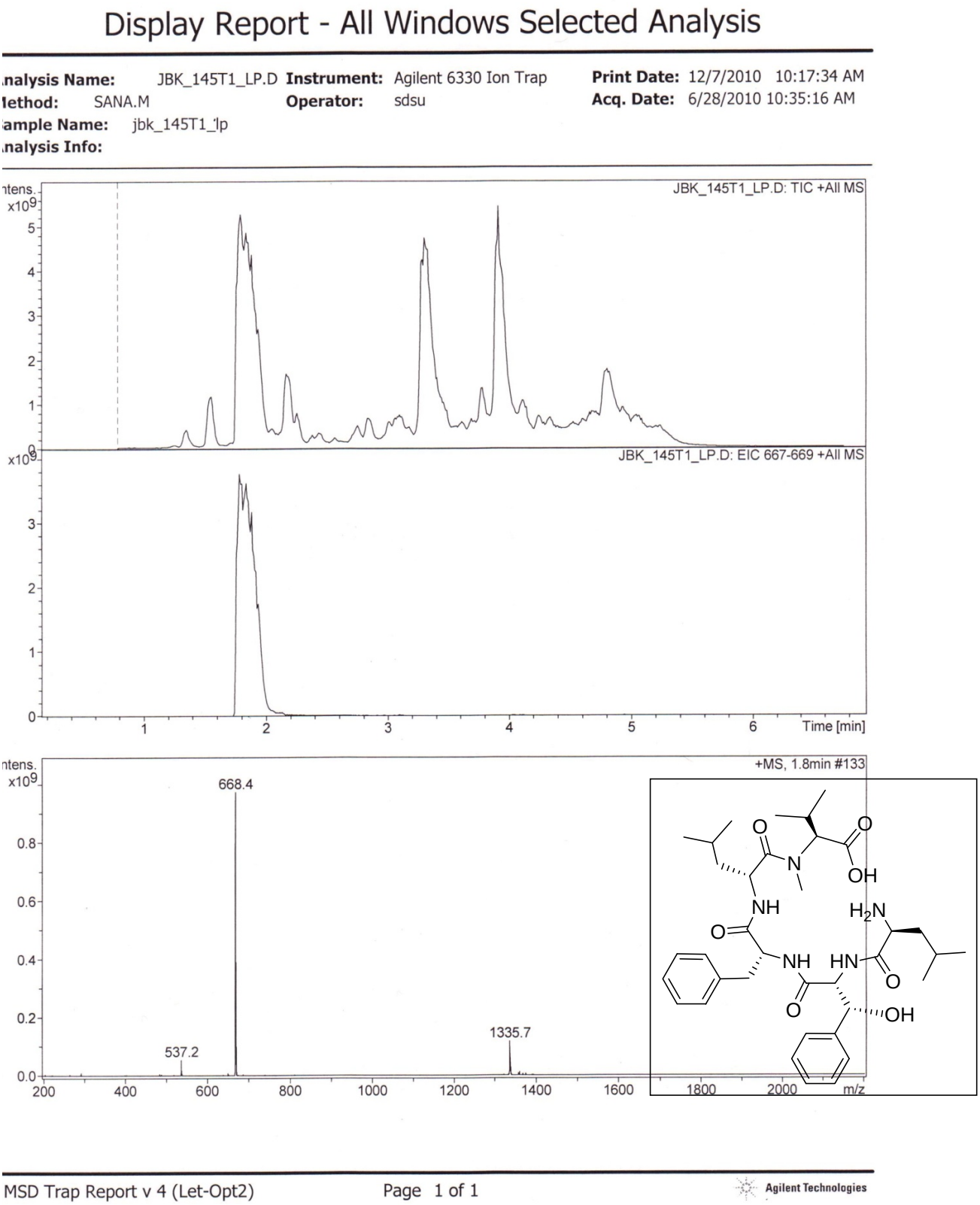
HPLC Cyclized Pentapeptide 1-T-III: *Phe-Leu-Lys-Leu-*

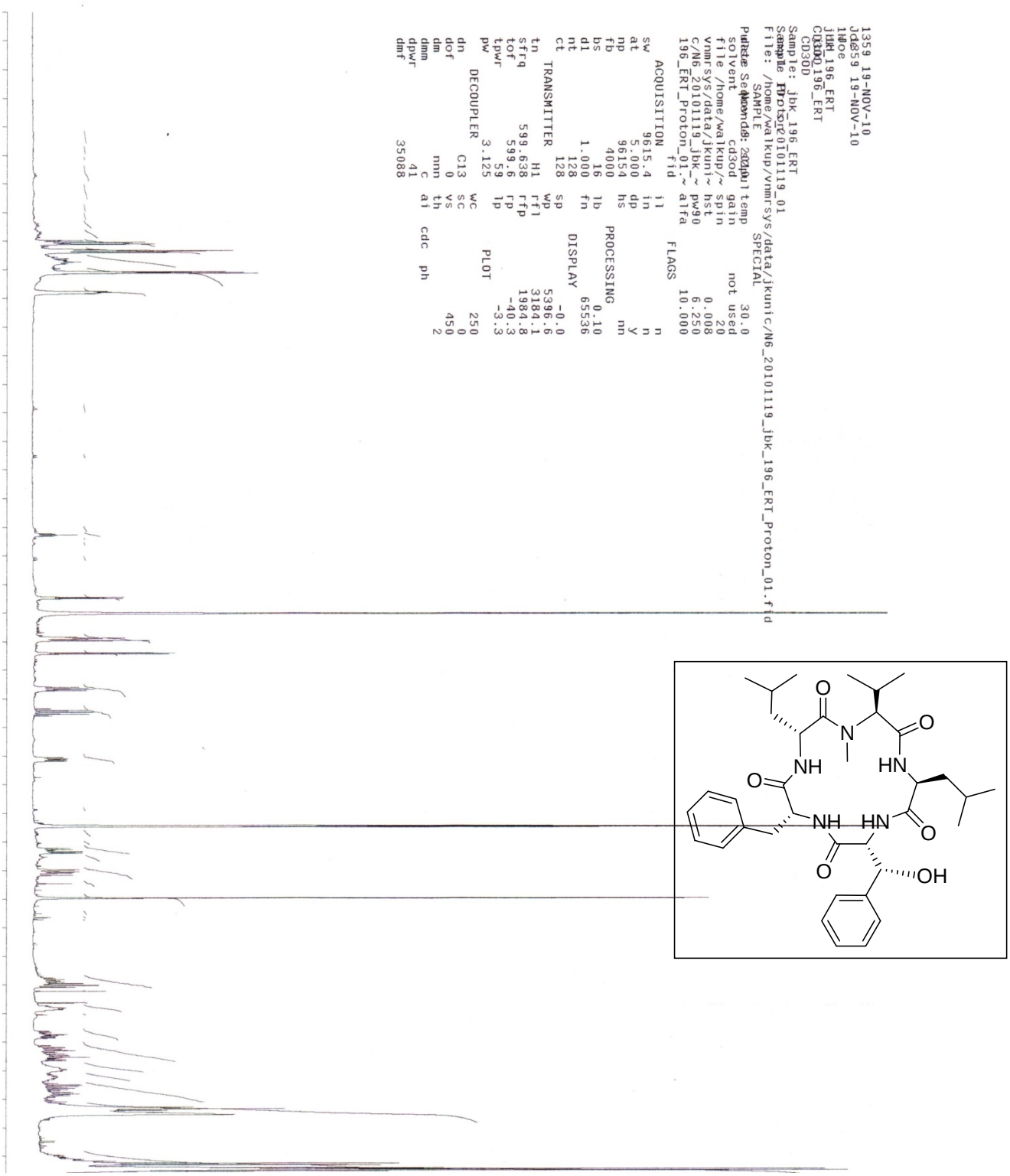


Leu

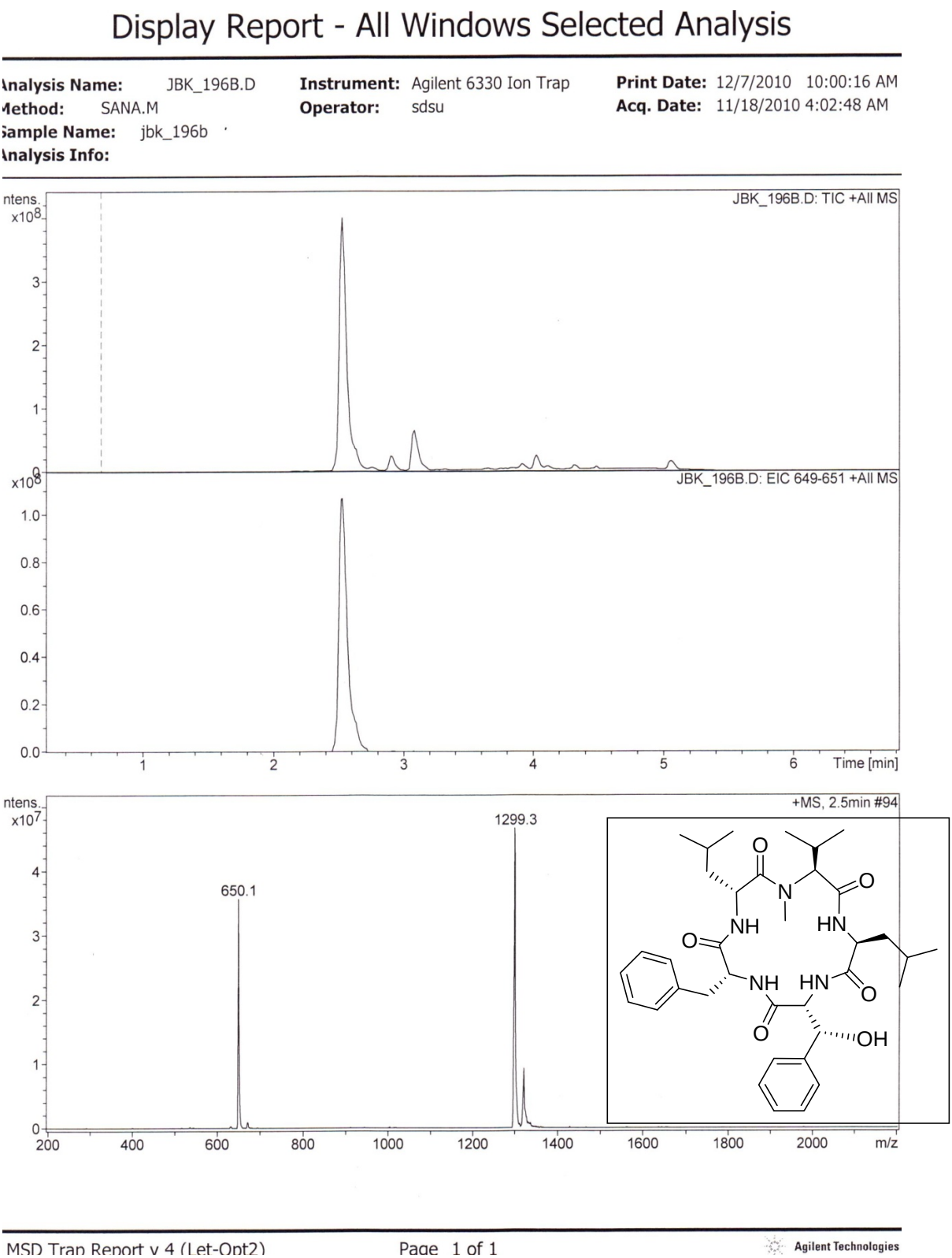
^{1}H NMR Biotinylated Pentapeptide 1-T-III: *Phe-Leu-Lys(Peg-biotin)-Leu-Leu*



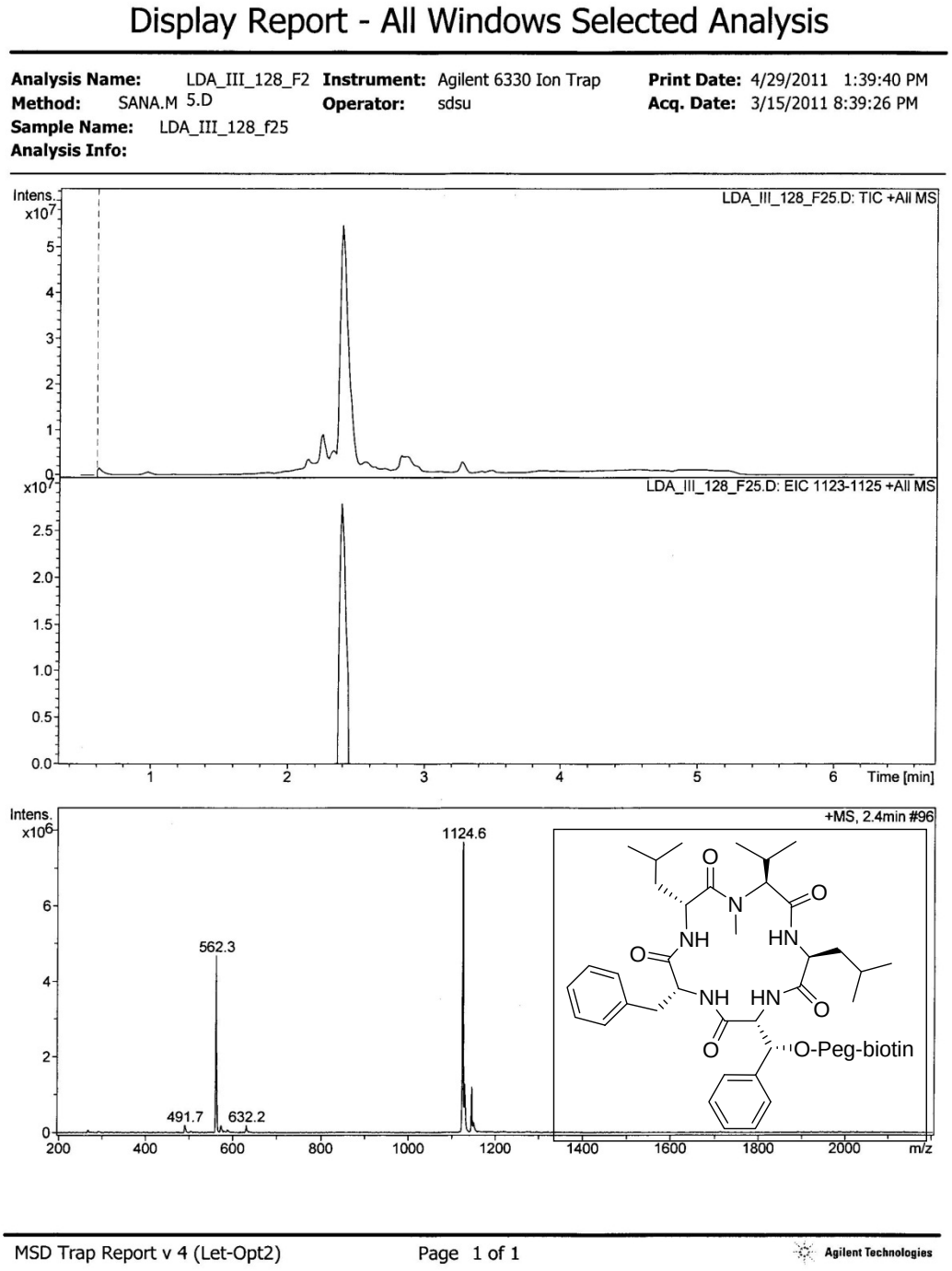
LCMS Double Deprotected Pentapeptide 2-T-I: *(2R, 3R)(2S,3S) β-OH-Phe-Leu-N-Me-Val-D-Leu-D-Phe*



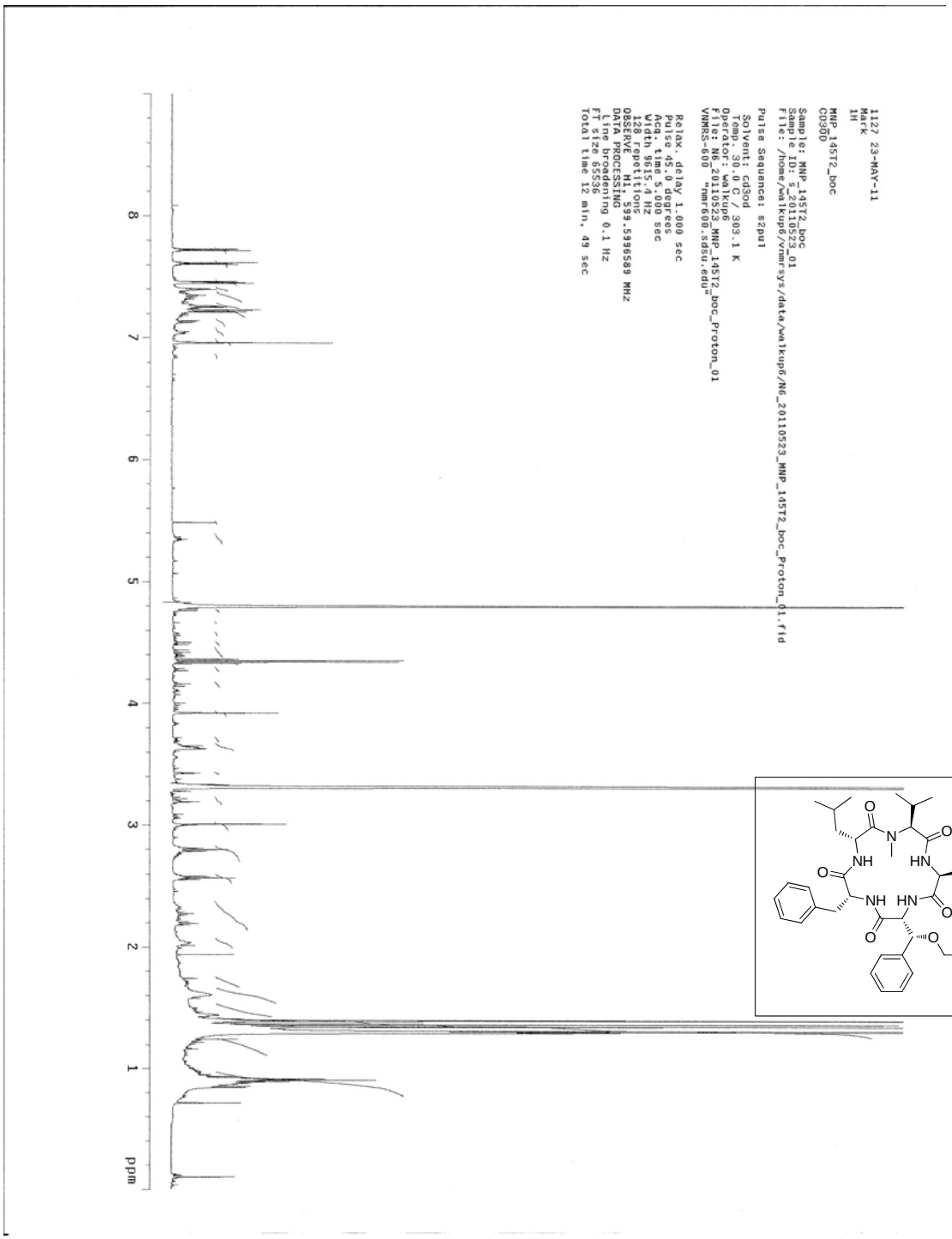
^{1}H NMR Cyclized Pentapeptide 2-T-I: *(2R, 3R) β-OH-Phe-Leu-N-Me-Val-D-Leu-D-Phe*



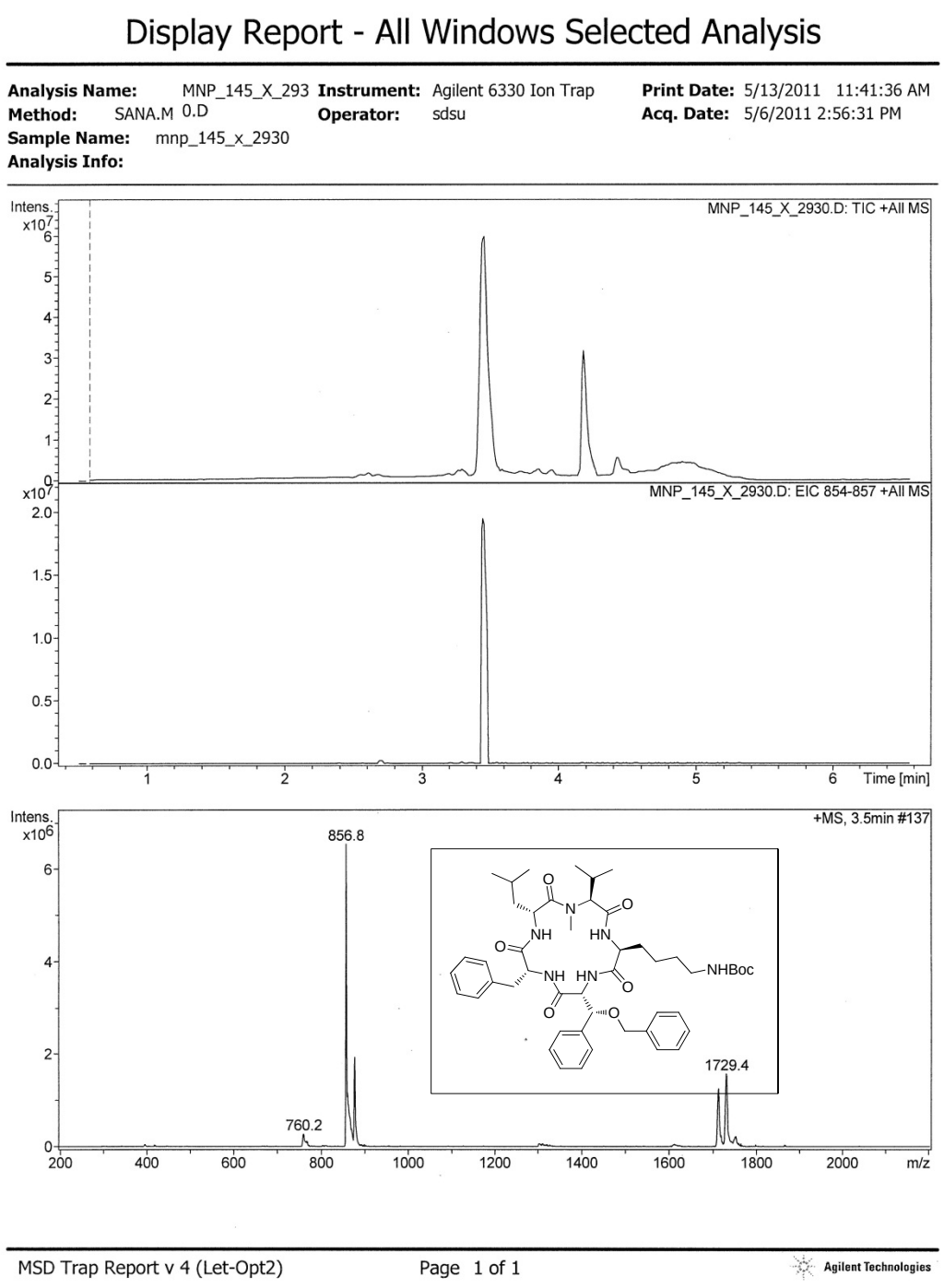
LCMS Cyclized Pentapeptide 2-T-I: *(2R, 3R) β-OH-Phe-Leu-N-Me-Val-D-Leu-D-Phe*



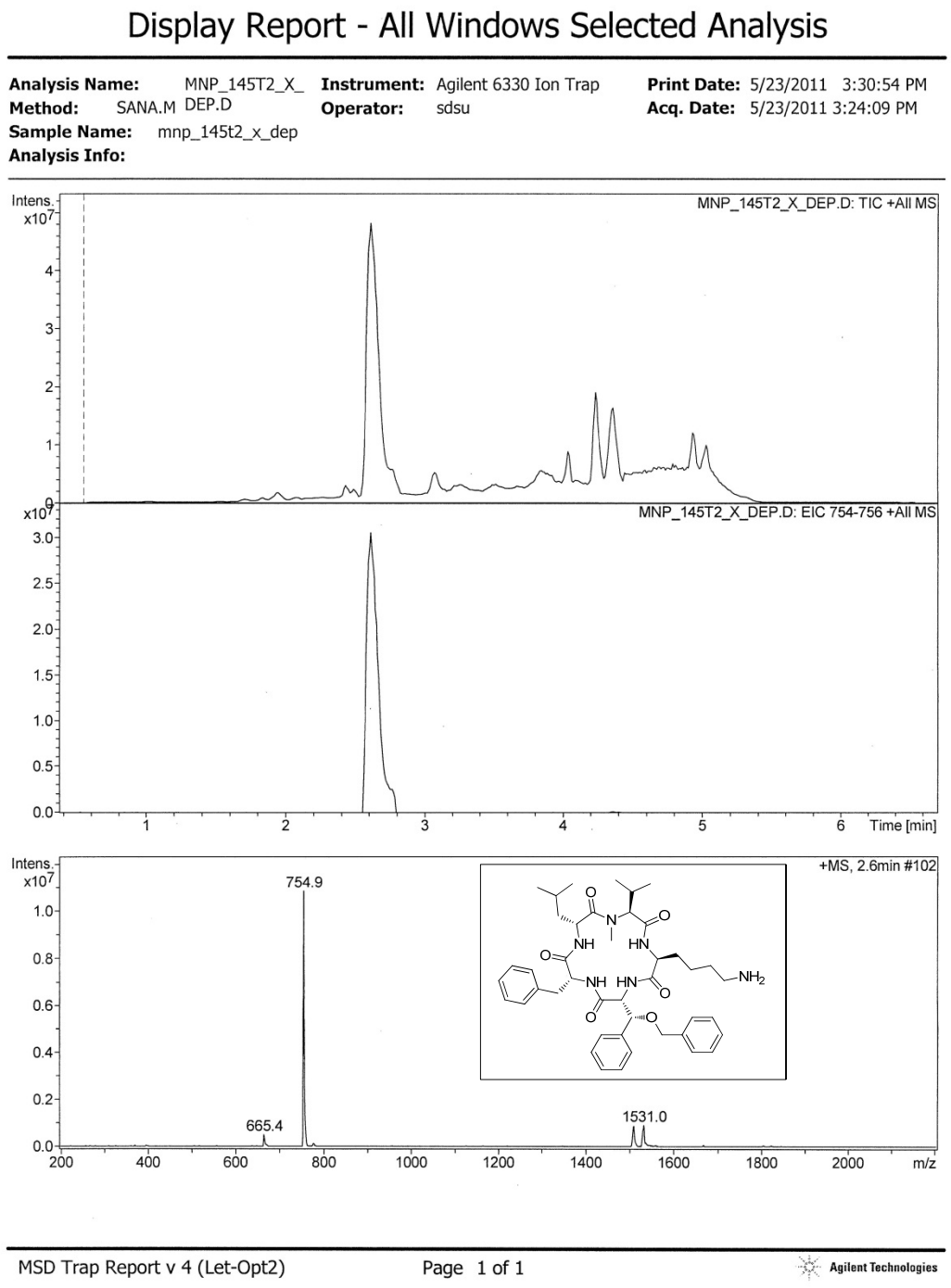
LCMS Biotinylated Pentapeptide 2-T-I: *(2R, 3R) β-O(Peg-biotin)-Phe-Leu-N-Me-Val-D-Leu-D-Phe*



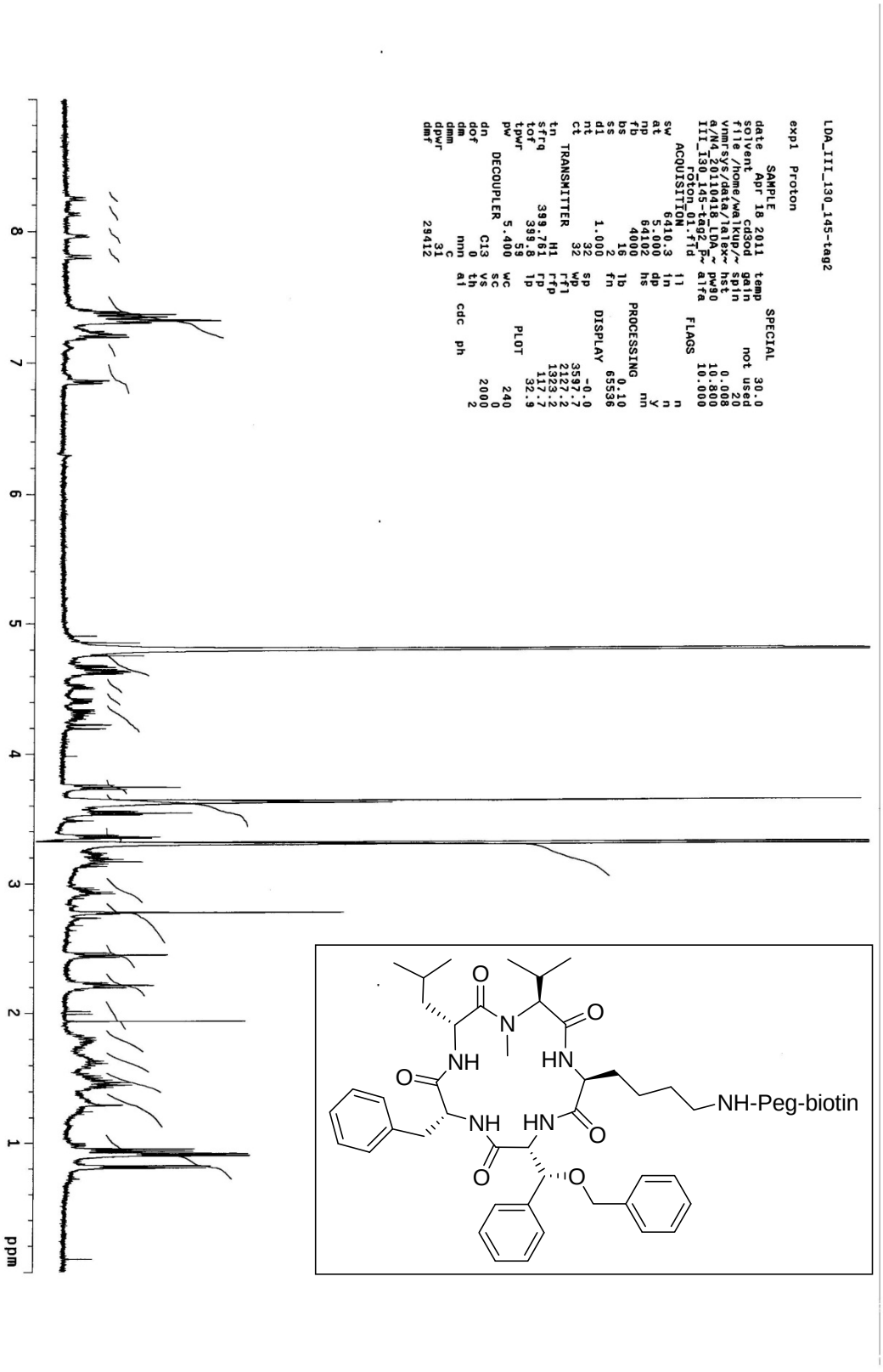
^{1}H NMR Cyclized Pentapeptide 2-T-II: *(2R, 3R)(2S,3S) β-OBn-Phe-Lys(Boc)-N-Me-Val-D-Leu-D-Phe*



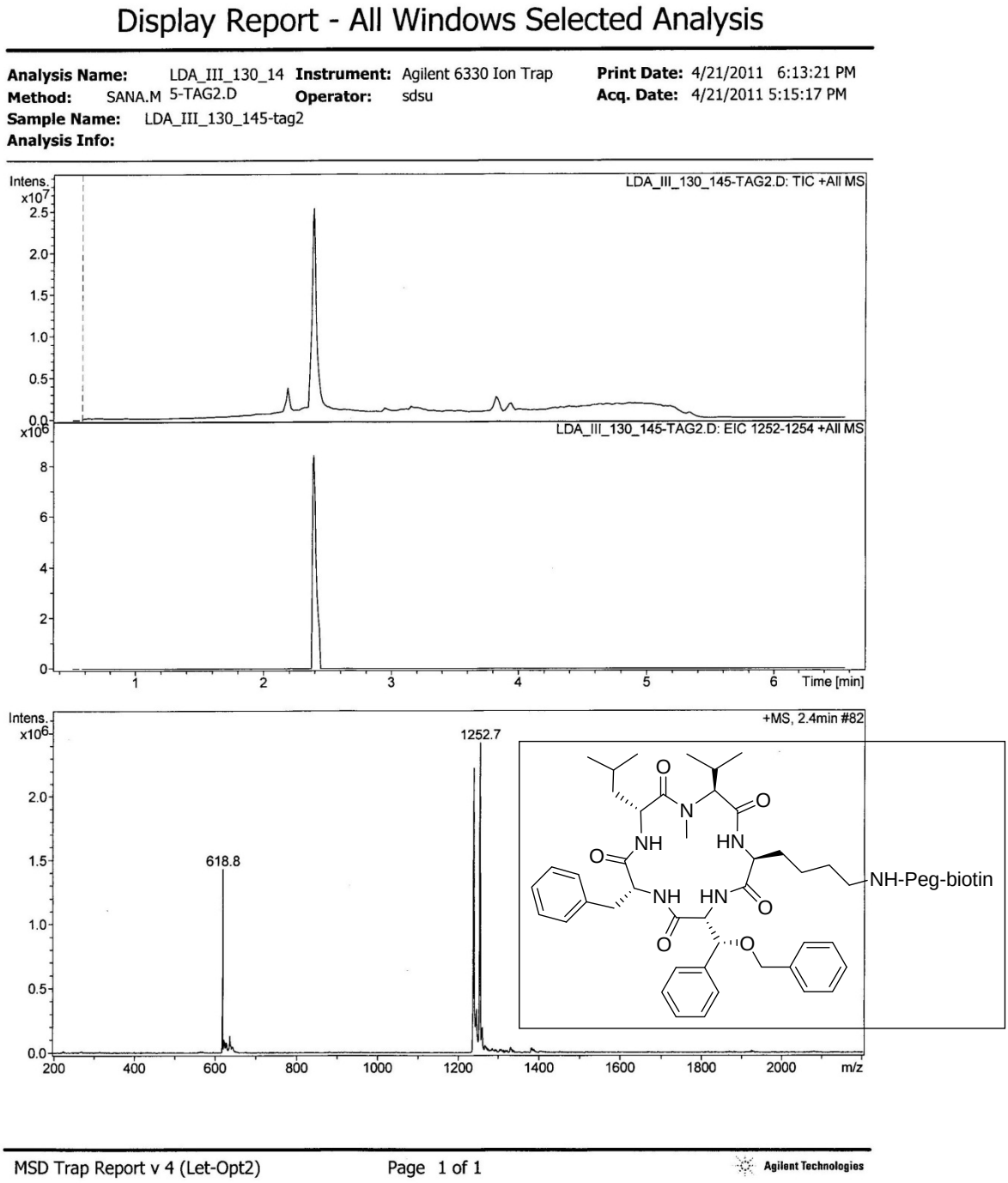
LCMS Cyclized Pentapeptide 2-T-II: *(2R, 3R)(2S,3S) β-OBn-Phe-Lys(Boc)-N-Me-Val-D-Leu-D-Phe*



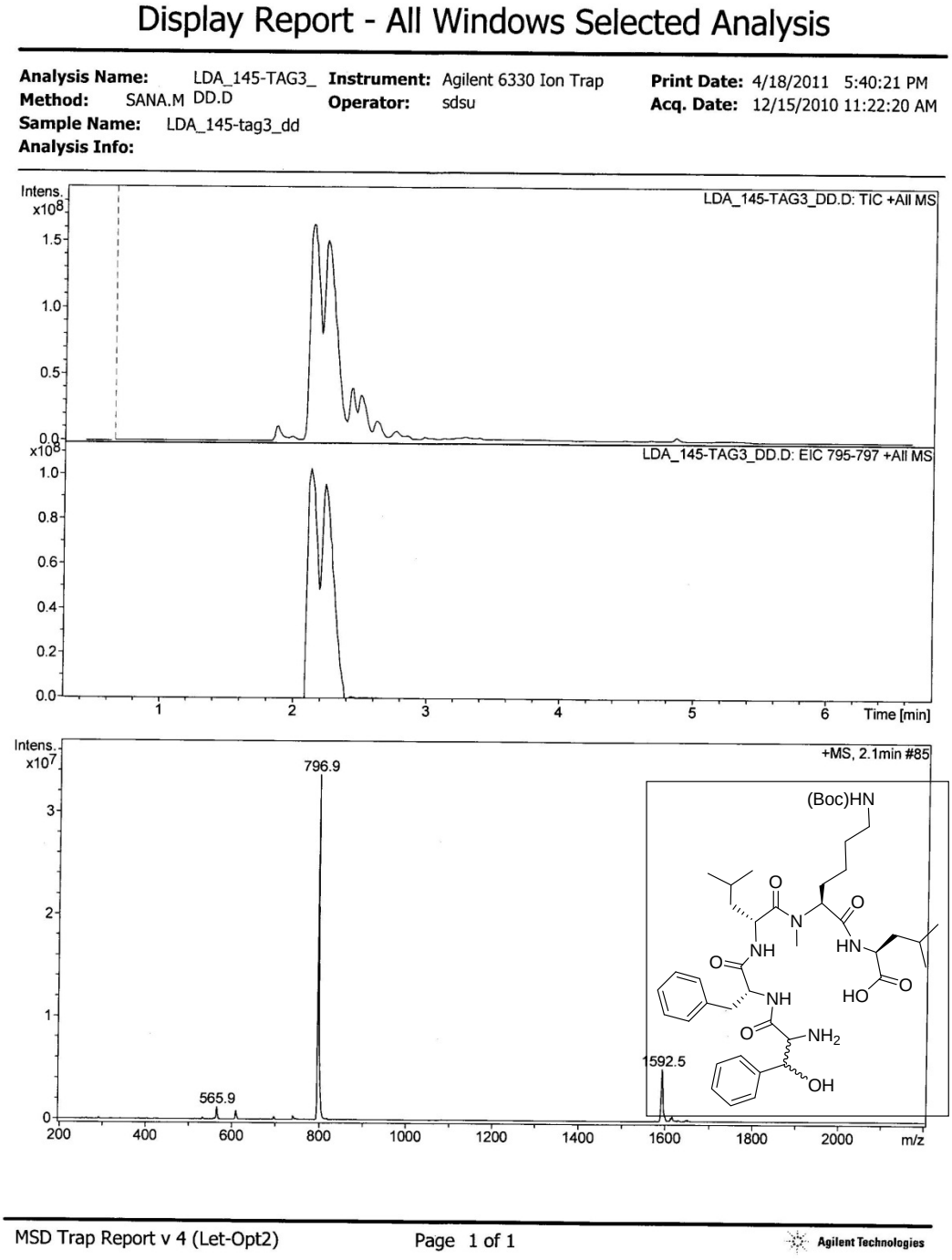
LCMS Cyclized Pentapeptide 2-T-II: *(2R, 3R)(2S,3S) β-OBn-Phe-Lys-N-Me-Val-D-Leu-D-Phe*



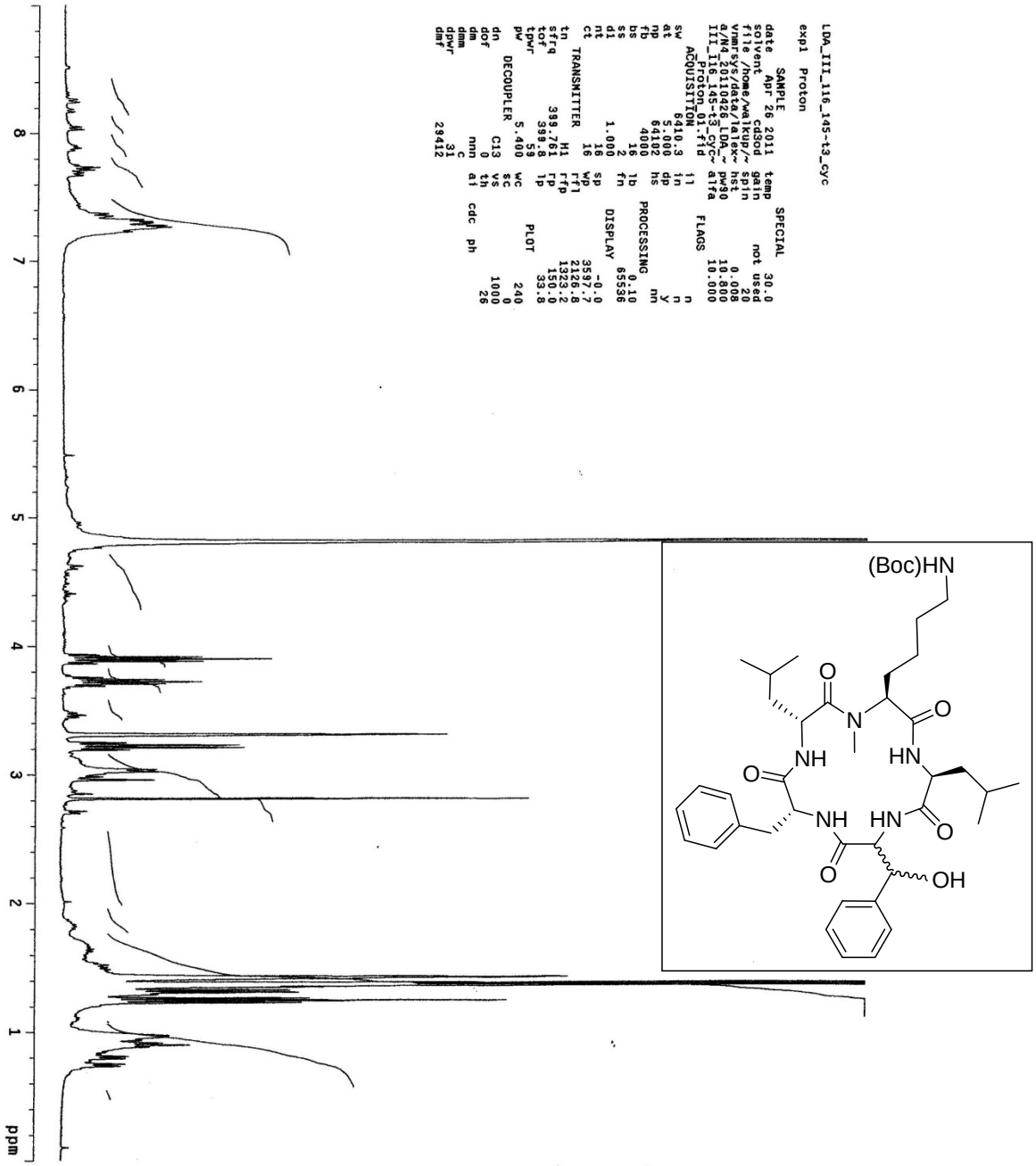
^{1}H NMR Biotinylated Pentapeptide 2-T-II: *(2R, 3R)(2S,3S) β-OBn-Phe-Lys(peg-biotin)-N-Me-Val-D-Leu-D-Phe*



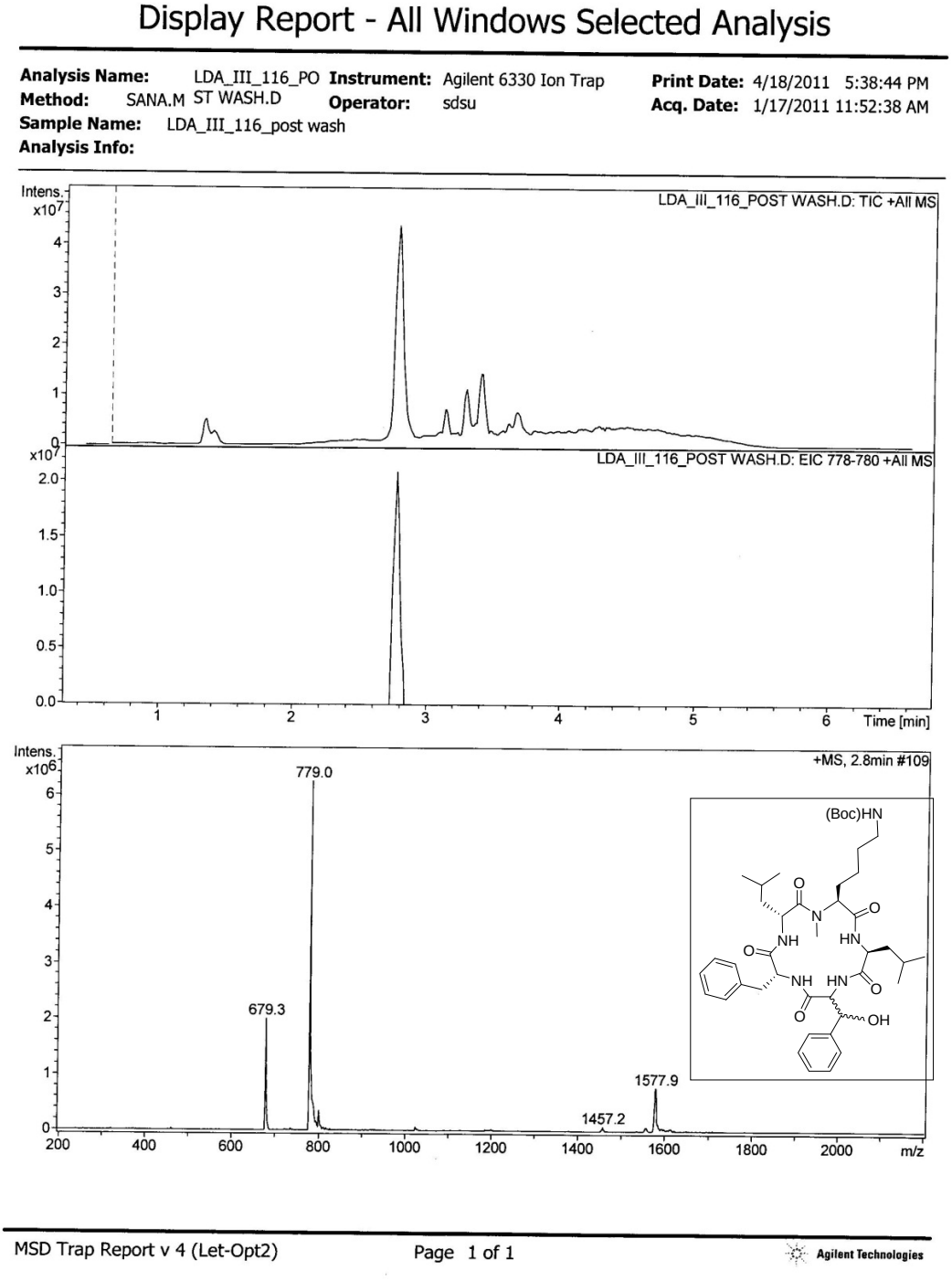
LCMS Biotinylated Pentapeptide 2-T-II: *(2R, 3R)(2S,3S) β-OBn-Phe-Lys(peg-biotin)-N-Me-Val-D-Leu-D-Phe*



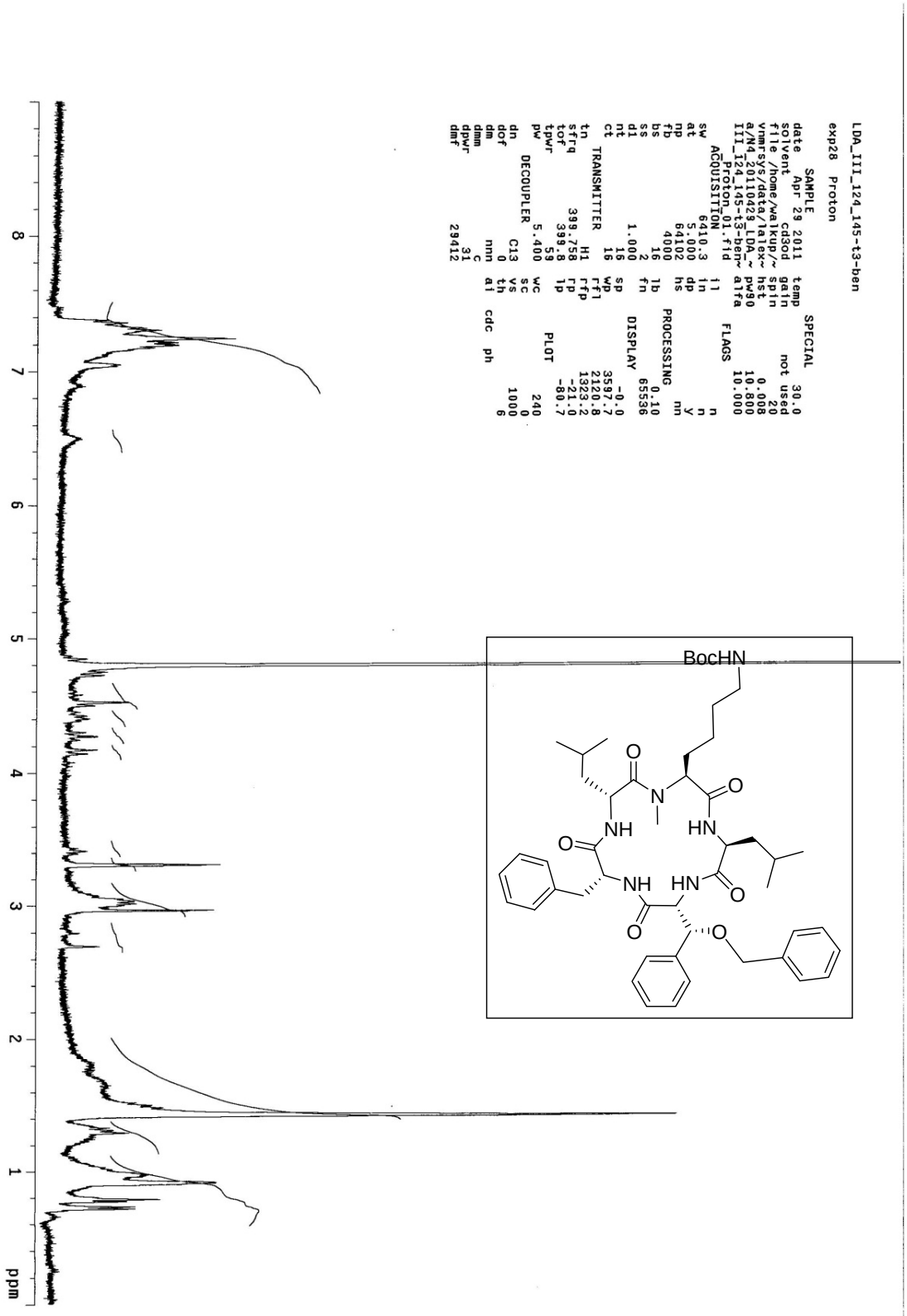
LCMS Double Deprotected Pentapeptide 2-T-III: *OH-Leu-N-Me-Lys(Boc)-D-Leu-D-Phe-(2R,3R)(2S,3S)-β-OH-Phe-NH$_2$*



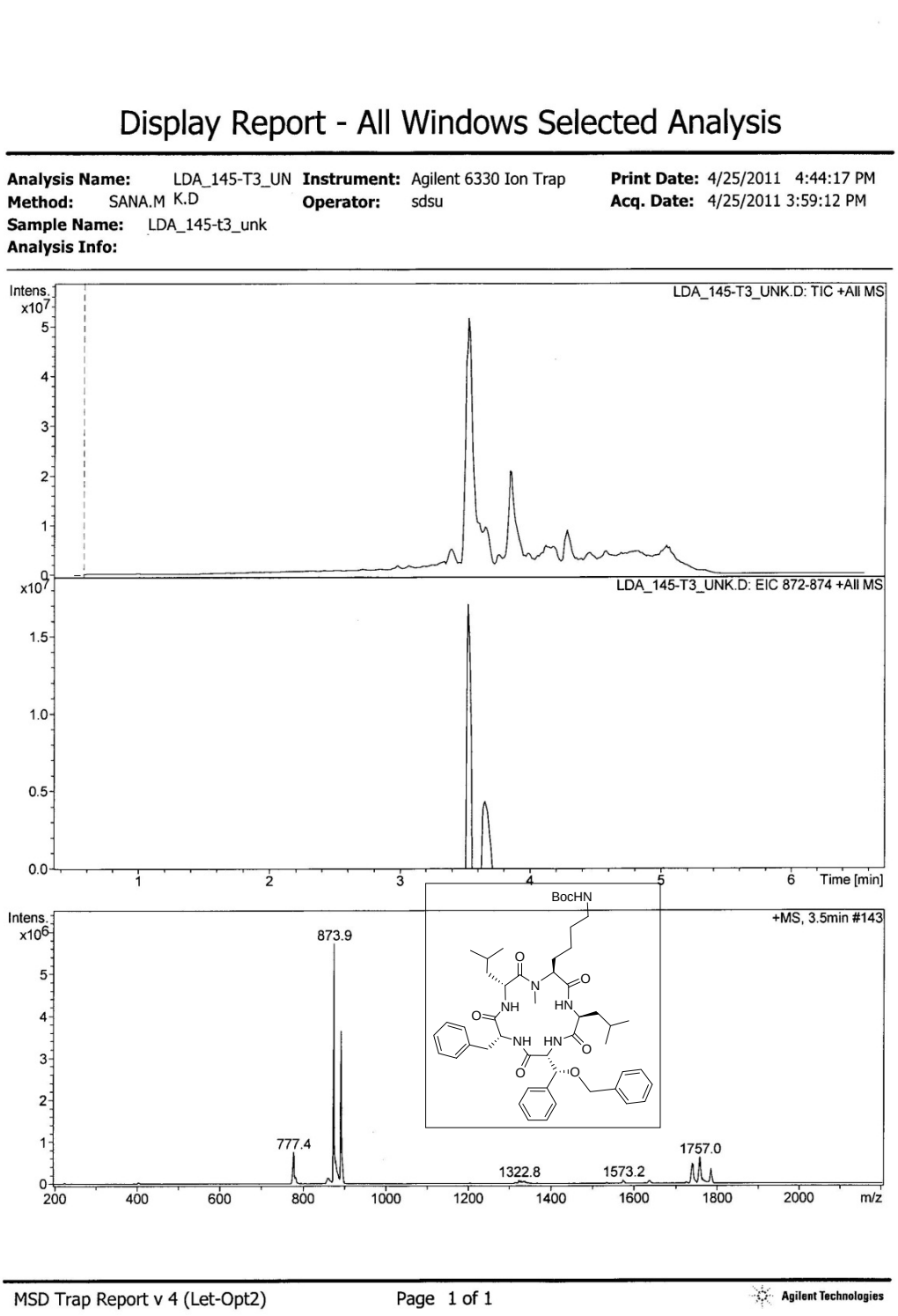
[1]H NMR Cyclized Pentapeptide 2-T-III: *(2R,3R),(2S,3S) β-OH-Phe-Leu-N-Me-Lys(Boc)-D-Leu-D-Phe*



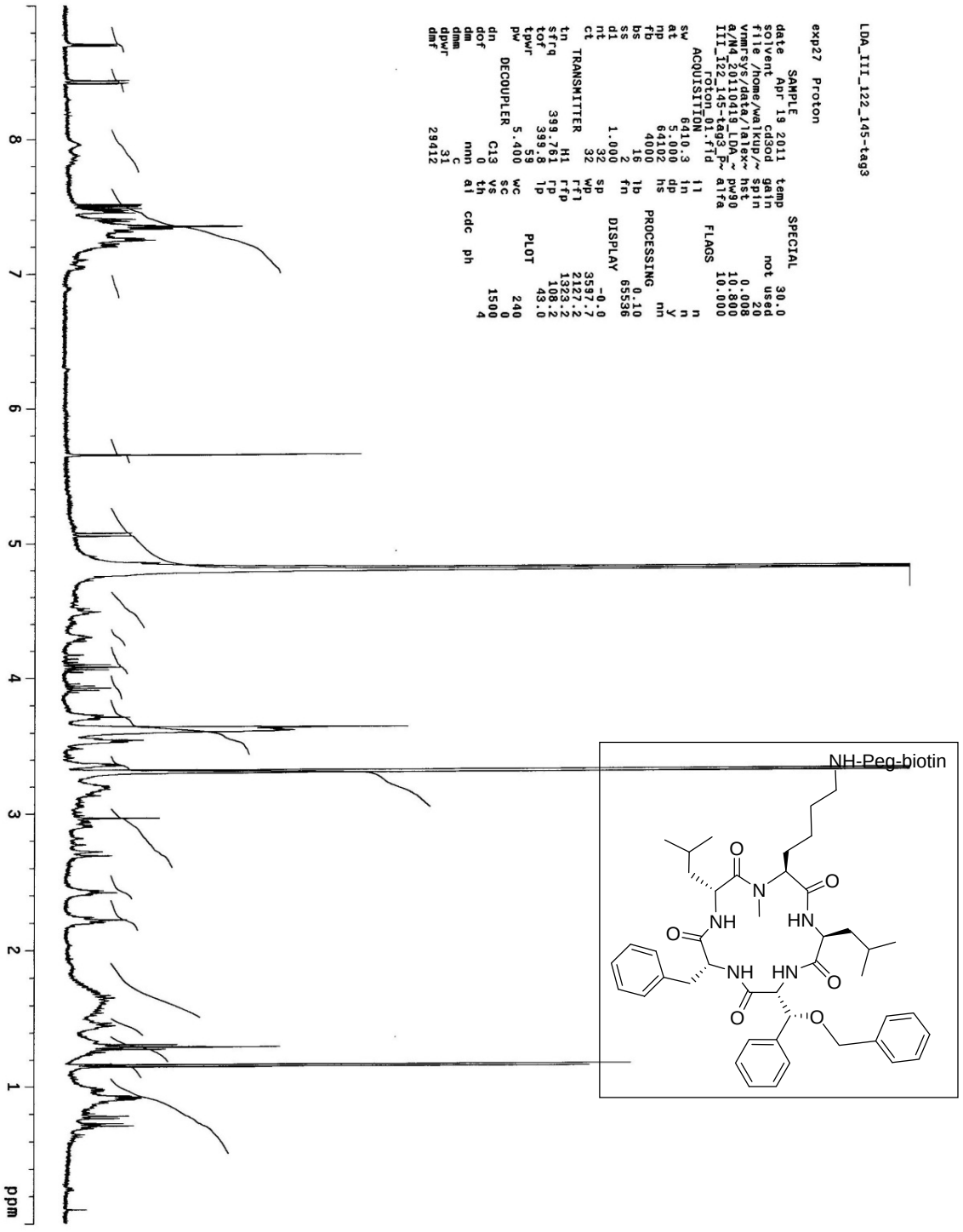
LCMS Cyclized Pentapeptide 2-T-III: *(2R,3R),(2S,3S) β-OH-Phe-Leu-N-Me-Lys(Boc)-D-Leu-D-Phe*



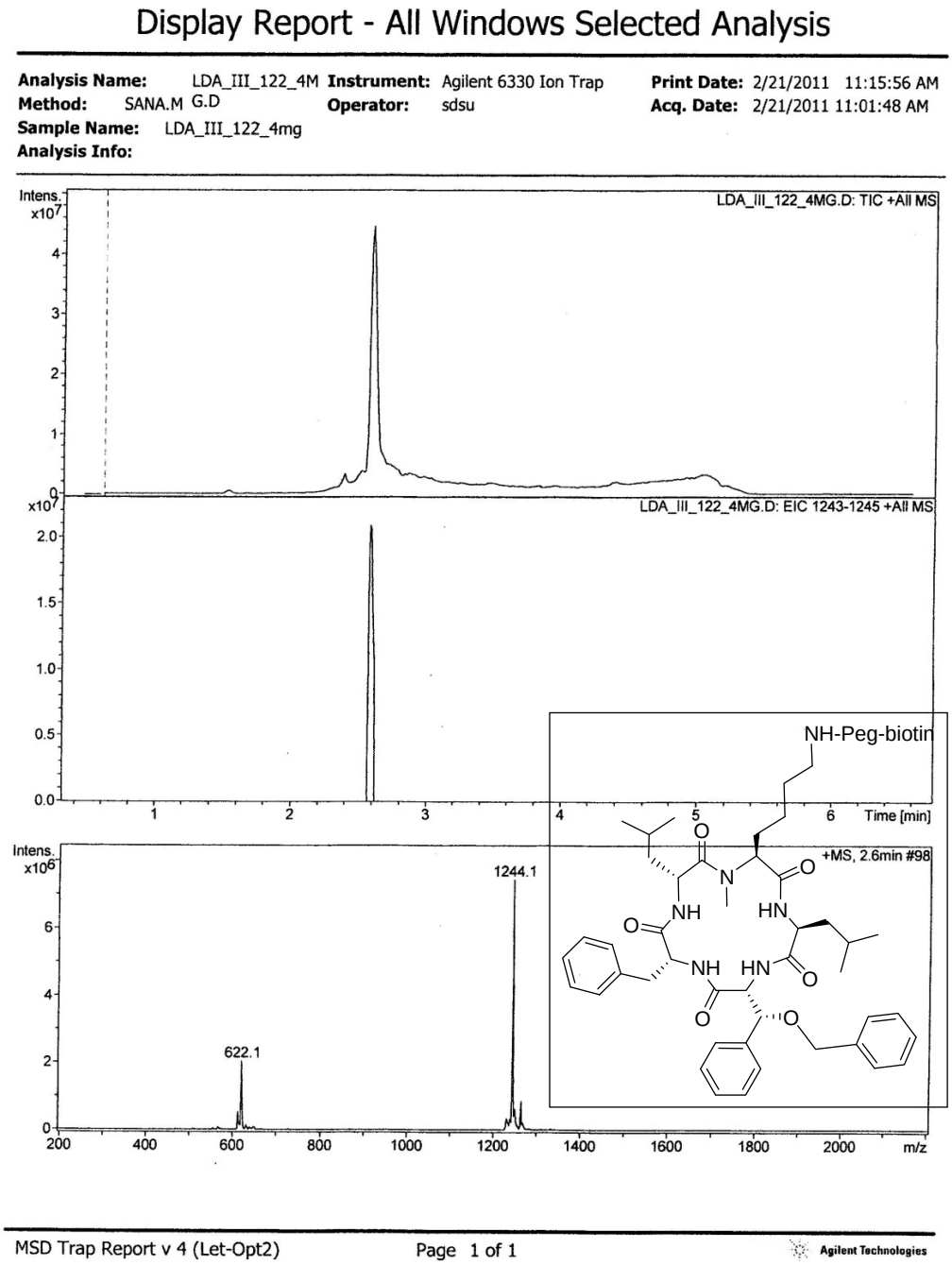
[1]H NMR Cyclized Pentapeptide 2-T-III: *(2R,3R),(2S,3S) β-OBn-Phe-Leu-N-Me-Lys(Boc)-D-Leu-D-Phe*



LCMS Cyclized Pentapeptide 2-T-III: *(2R,3R),(2S,3S) β-OBn-Phe-Leu-N-Me-Lys(Boc)-D-Leu-D-Phe*



^{1}H NMR Biotinylated Pentapeptide 2-T-III: *(2R,3R)β-OBn-Phe-Leu-NMe-Lys(Biotin)-*



DLeu

LCMS Biotinylated Pentapeptide 2-T-III: *(2R,3R)β-OBn-Phe-Leu-N-Me-Lys(Peg-biotin)-D-Leu-D-Phe*

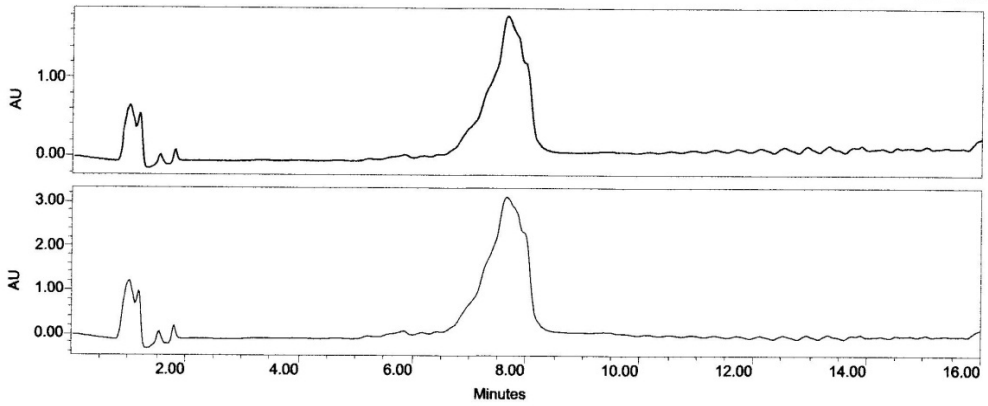
SDSU

Project Name: Defaults
Reported by User: System

'Breeze

SAMPLE INFORMATION

Sample Name:	LDA_145-t3_peg-biotin	Acquired By:	System
Sample Type:	Unknown	Sample Set Name:	
Vial:	1	Acq. Method:	primary_sanA_ss_ACN
Injection #:	177	Date Acquired:	3/2/2011 2:47:17 PM
Run Time:	16.00 Minutes	Injection Volume:	25.00 ul



Channel: 2487Channel 1 Channel Desc.: Processing Method: *
Channel: 2487Channel 2 Channel Desc.: Processing Method: *

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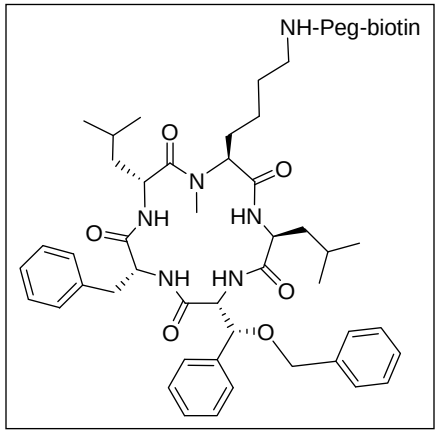
2	****	****	****	****	****	****	****



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HPLC Biotinylated Pentapeptide 2-T-III: *(2R,3R)β-OBn-Phe-Leu-N-Me-Lys(Peg-biotin)-D-Leu-D-Phe*