Supporting Information for

Synthesis of Stable Cholesteryl-PEG-Peptide Conjugates with Non-Disperse PEG Lengths.

Edgar Cristóbal-Lecina,[†] Daniel Pulido,[†] Pau Martín-Malpartida,[‡] Maria J. Macias,[‡,#] Fernando Albericio,[†,§] and Miriam Royo[†,*]

† Department of Surfactants and Nanobiotechnology, Institute for Advanced Chemistry of Catalonia (IQAC-CSIC), Jordi Girona 18-26, 08034 Barcelona (Spain) and Centro de Investigación Biomédica en Red Bioingeniería, Biomateriales y Nanomedicina (CIBER-BBN).

‡ Institute for Research in Biomedicine (IRB Barcelona) and The Barcelona Institute of Science and Technology (BIST), Baldiri i Reixach 10, 08028 Barcelona (Spain)

ICREA, Passeig Lluis Companys 23, 08010 Barcelona (Spain)

§ Department of Inorganic and Organic Chemistry, University of Barcelona, Marti i Franqués 1-11, 08028 Barcelona (Spain) and School of Chemistry and Physics University of KwaZulu-Natal, 4041 Durban, South Africa

Table of contents

1. General Methods

Reagents and solvents were purchased from commercial sources and were used without further purification. Thin layer chromatography (TLC) was performed on Merck 60F254 aluminum silica plates that were visualized by UV light (254 nm), or by potassium permanganate stain. Flash chromatography on silica gel was carried out on a Teledyne Isco Combiflash Rf instrument using Redisep Rf silica gel columns. HPLC-PDA-MS analysis were performed on a Waters Alliance 2795 with an automated injector and a photodiode array detector Waters 2996 coupled to an electrospray ion source (ESI-MS) Micromass ZQ mass detector, and the MassLynx 4.1 software. The instrument was operated in the positive ESI (+) ion mode. Analyses were carried out using diverse elution conditions depending on the different compounds: Elution condition 1: XSelect™ C_{18} column (4.6 mm×50 mm, 3.5 μm). Elution solvent system: A: 0.1% HCOOH in CH_3CN and B: CH_3OH. Gradient B: 5 to 100% B over 3.5 min at a flow rate of 1.6 mL/min. λ= 210 nm. Elution condition 2: XSelect™ C_{18} column (4.6 mm×50 mm, 3.5 μm). Elution solvent system: A: 0.1% HCOOH in CH_3CN and B: CH_3OH. Gradient B: 5 to 100% B over 4.5 min at a flow rate of 2.0 mL/min. λ= 210 nm. Elution condition 3: XSelect™ C_{18} column (4.6 mm×50 mm, 3.5 μm). Elution solvent system: A: 0.1% HCOOH in H_2O and B: 0.07% HCOOH in CH_3CN. Gradient B: 5 to 100% B over 4.5 min at a flow rate of 2.0 mL/min. λ= 210 nm. Elution condition 4: XBridge™ C_{18} column (4.6 mm×50 mm, 3.5μm). Elution solvent system: A: 20 mM NH_4HCO_3 in H_2O and B:CH_3CN. Gradient: 5-100% B over 3.5min at a flow rate of 1.6 mL/min. λ= 210 nm. Elution condition 5: XSelect™ C_{18} column (4.6 mm×50 mm, 3.5 μm). Elution solvent system: A: 0.1% HCOOH in H_2O and B: 0.07% HCOOH in CH_3CN. Gradient: 0 to 20% B over 4.5 min at a flow rate of 2 mL/min. λ= 210 nm. Elution condition 6: Symmetry™ C_4 column (4.6 mm×250 mm, 5μm). Elution solvent system: A: 0.01% HCOOH in H_2O and B: 0.07% HCOOH in CH_3CN; gradient: 5-100% B over 30 min at a flow rate of 1 mL/min. λ= 210 nm.

UPLC-PDA-ELSD-MS analysis were performed on a Waters Acquity system with a binary solvent manger, a sample manager, a photodiode array λ detector coupled to an electronic light scattering detector and an electrospray ion source (ESI-MS) SQ mass detector. Samples were

eluted using elution condition 7: ACQUITY UPLC® CSH™ C_{18} column (2.1 μm×50 mm, 1.7μm). Elution solvent system: A: 0.01% HCOOH in H_2O and B: 0.01% HCOOH in CH_3CN; gradient: 5-100% B over 3 min at a flow rate of 0.6 mL/min.

High-Resolution Mass Spectroscopy (HRMS) was carried out using an LC/MSD-TOF spectrometer from Agilent Technologies, at the Mass Spectrometry & Proteomics Core Facility of the Institute for Research in Biomedecine. Semi-preparative RP-HPLC purification was performed on a Waters system with a 2545 binary gradient module, a 2767 manager collector and a 2489 UV detector, coupled to an electrospray ion source (ESI-MS) Micromass ZQ mass detector, and the MassLynx 4.1 software. Crude peptides were purified using a BEH C18 column (19 mm × 150 mm 5 μm). Elution solvent system: A:0.1 %TFA in H_2O and B: 0.1%TFA in CH_3CN. Gradient: 10% to 20% B over 8 min at a flow rate of 16 mL/min.

Chol-PEGn and Chol-PEGn-OMe were characterized by ^{1}H-NMR (400 MHz) and ^{13}C-NMR (101 MHz) spectroscopy on a Varian Mercury 400 MHz instrument at the NMR unit of the Scientific and Technological Centers of the University of Barcelona (CCiTUB). Chemical shifts (δ) are expressed in ppm relative to tetramethylsilane (TMS). Coupling constants (*J*) are expressed in Hertz (Hz). The following abbreviations are used to indicate multiplicity: s: singlet; d: doublet, t: triplet, m: multiplet, and br: broad signal.

Solid-phase peptide synthesis. Peptide synthesis was carried out at the Peptide Synthesis Unit (U3) of the ICTS NANBIOSIS of the CIBER on Bioengineering, Biomaterials & Nanomedicine (CIBER-BBN) located at the IQAC-CSIC (http://www.nanbiosis.es/portfolio/u3-synthesis-of-peptides-unit/). The synthesis was performed manually following a Fmoc/tBu-based strategy using the adequate polymeric solid-support on each case. Prior to the synthesis, resins were carefully washed with DMF (3 × 1 min) and CH_2Cl_2 (3 × 1 min). Solvents and soluble reagents were removed by vacuum suction. The Fmoc group was eliminated by treatment with piperidine:DMF (20:80 v/v; 2 × 5 min) and coupling reactions were performed using the corresponding standard Fmoc-amino acids (3 equiv.), DIC (3 equiv.) and HOBt·H_2O (3 equiv.)

in DMF for 1 h at room temperature. The completion of the coupling was monitored with the Kaiser test. After every step of deprotection and coupling the resin was thoroughly washed with DMF (3 × 1 min), CH_2Cl_2 (3 × 1 min) and again with DMF (3 × 1 min).

2. Analysis data of Chol-PEGn derivatives (^{1}H and ^{13}C-NMR, UPLC-PDA-ELSD-MS, HPLC-PDA-MS and HRMS)

Figure S1. ^{1}H-NMR analysis of Chol-PEG4, **4a**

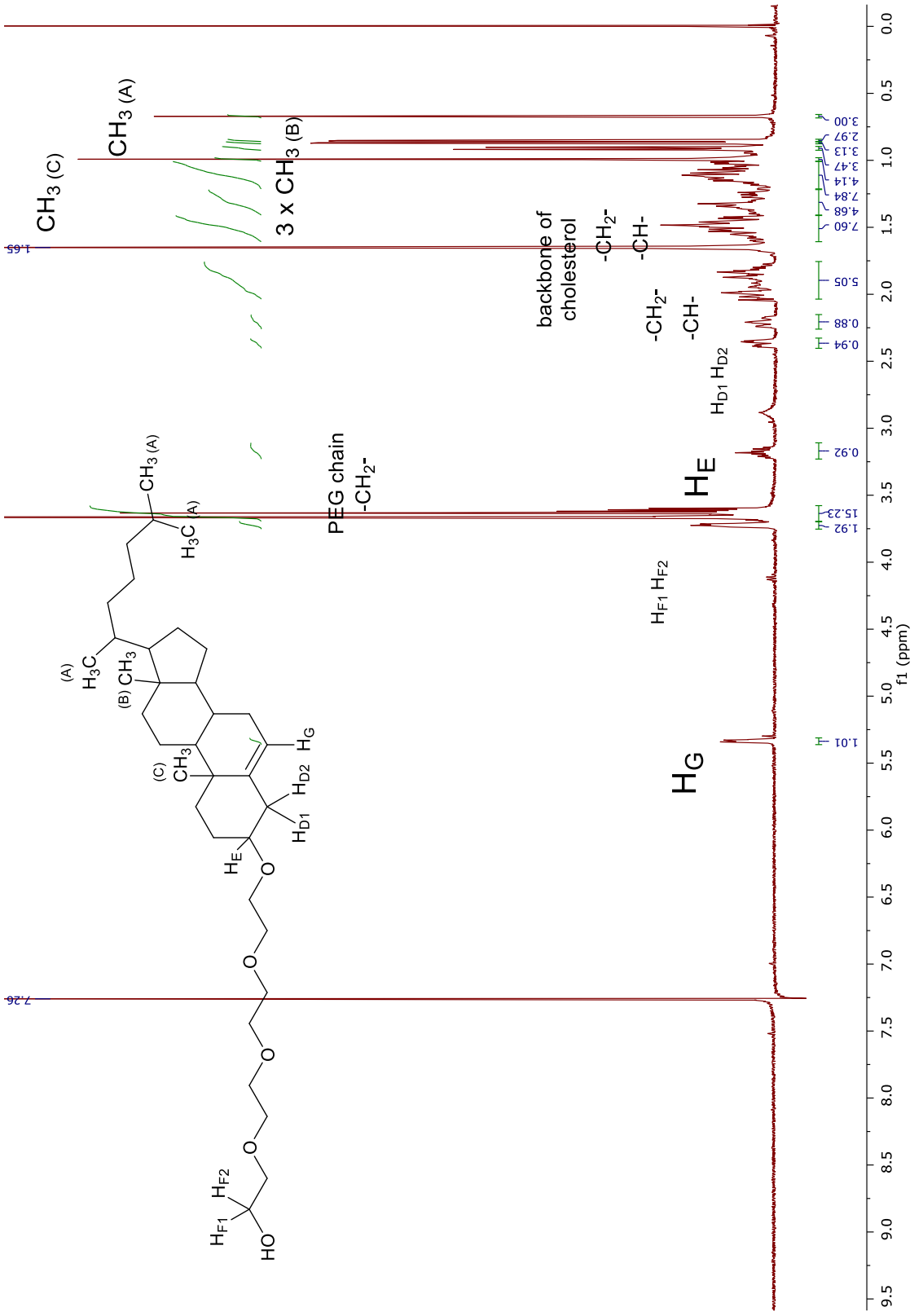


Figure S2. ^{13}C-NMR analysis of Chol-PEG4, **4a**

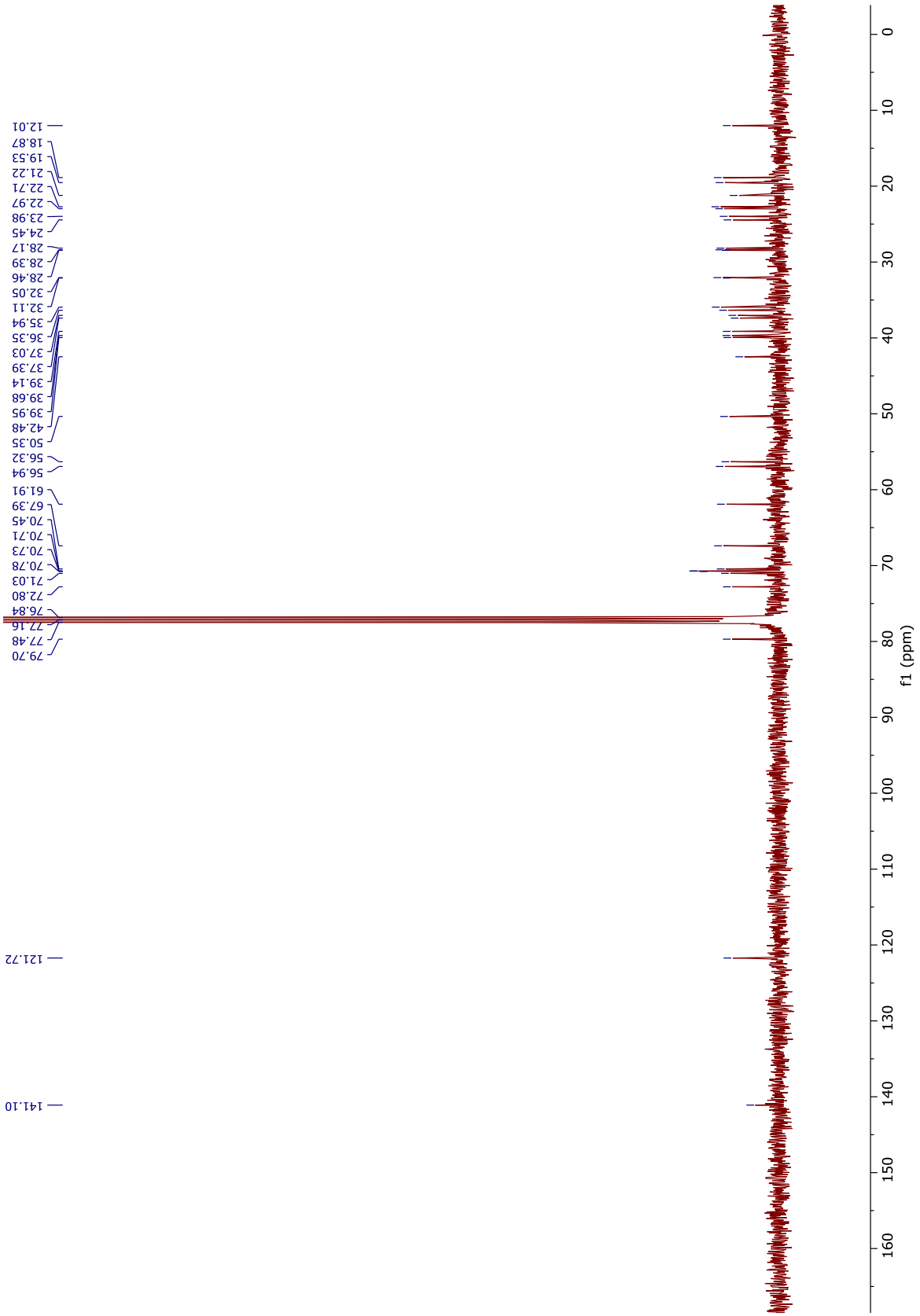


Figure S3. HRMS analysis of Chol-PEG4, **4a**

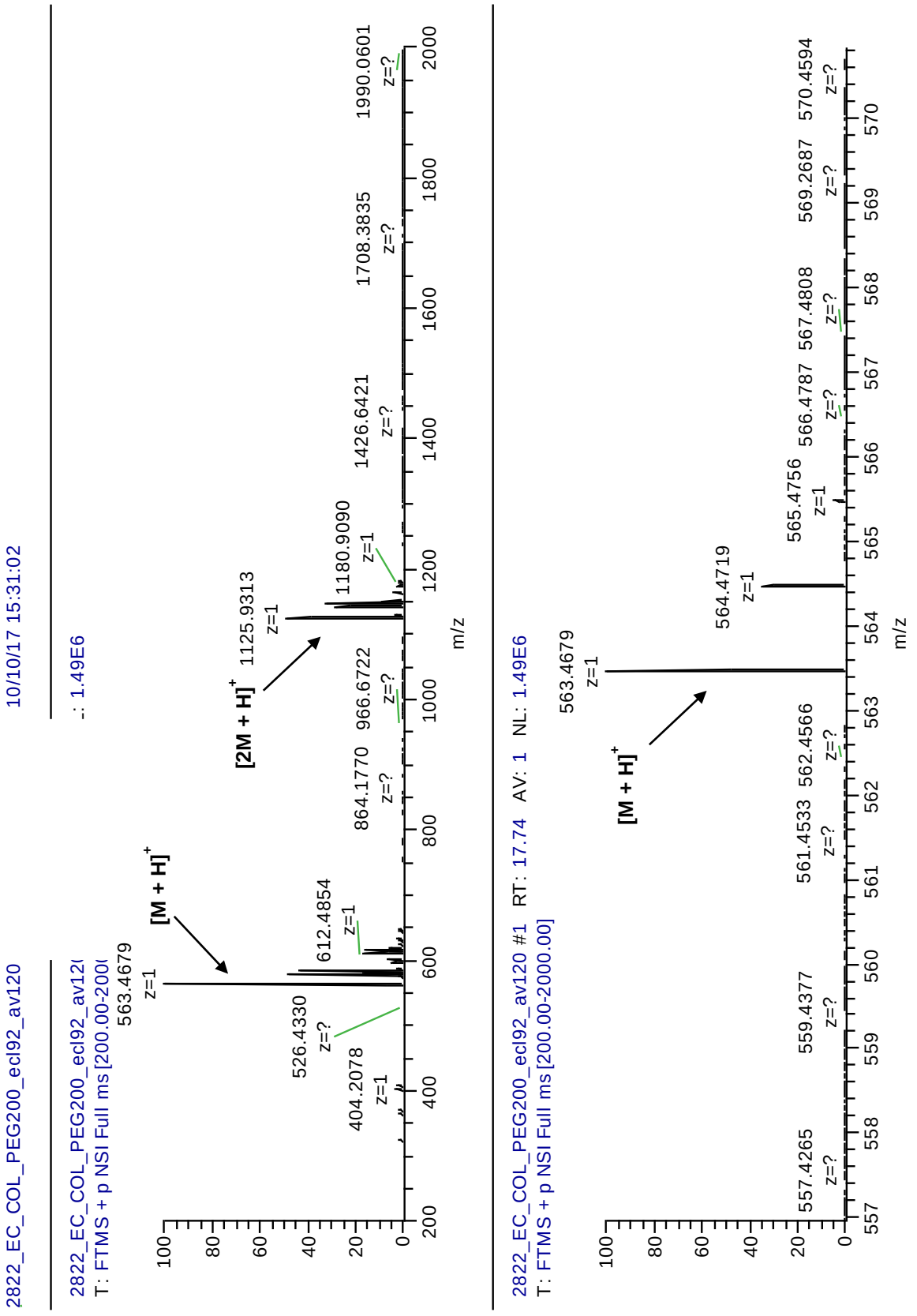


Figure S4. UPLC-PDA-ELSD-MS analysis of Chol-PEG4, **4a**

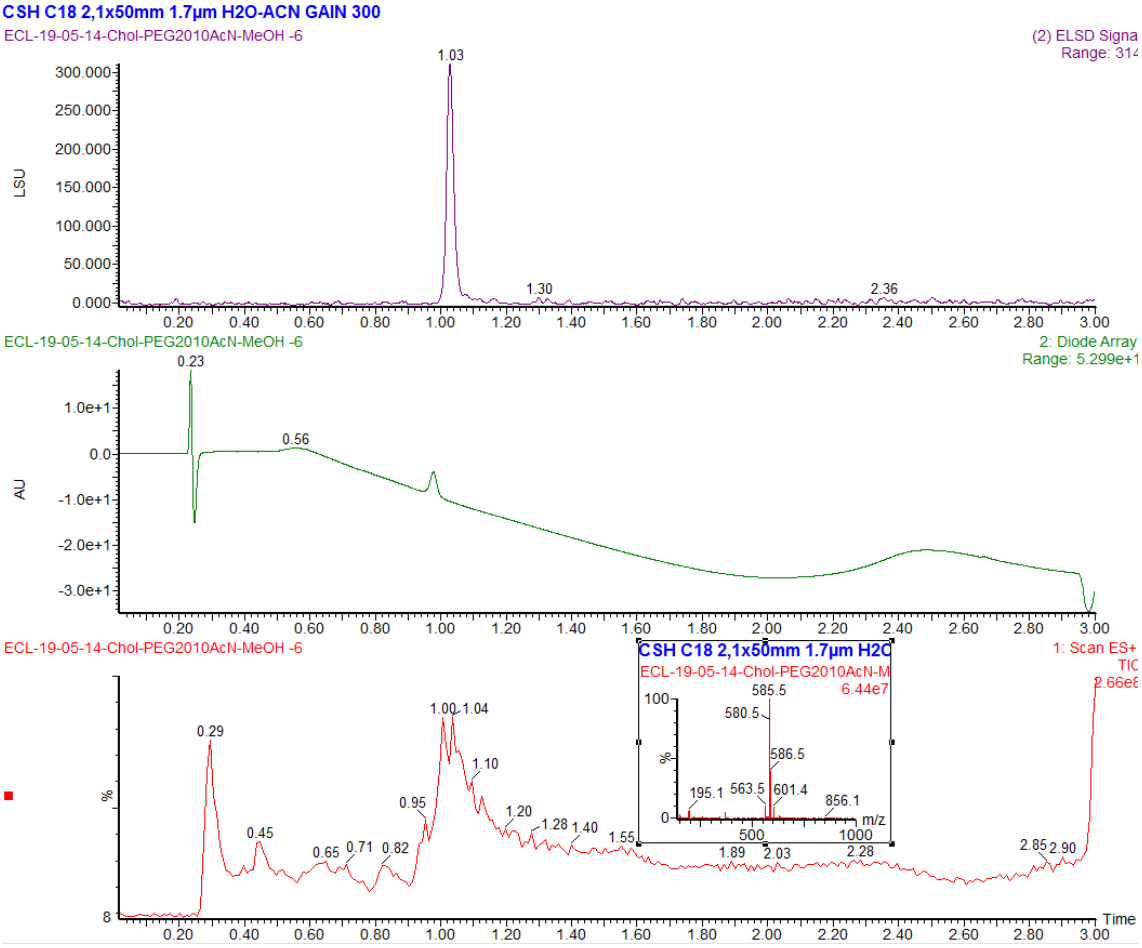


Figure S5. ^{1}H-NMR analysis of Chol-PEG8, **5**

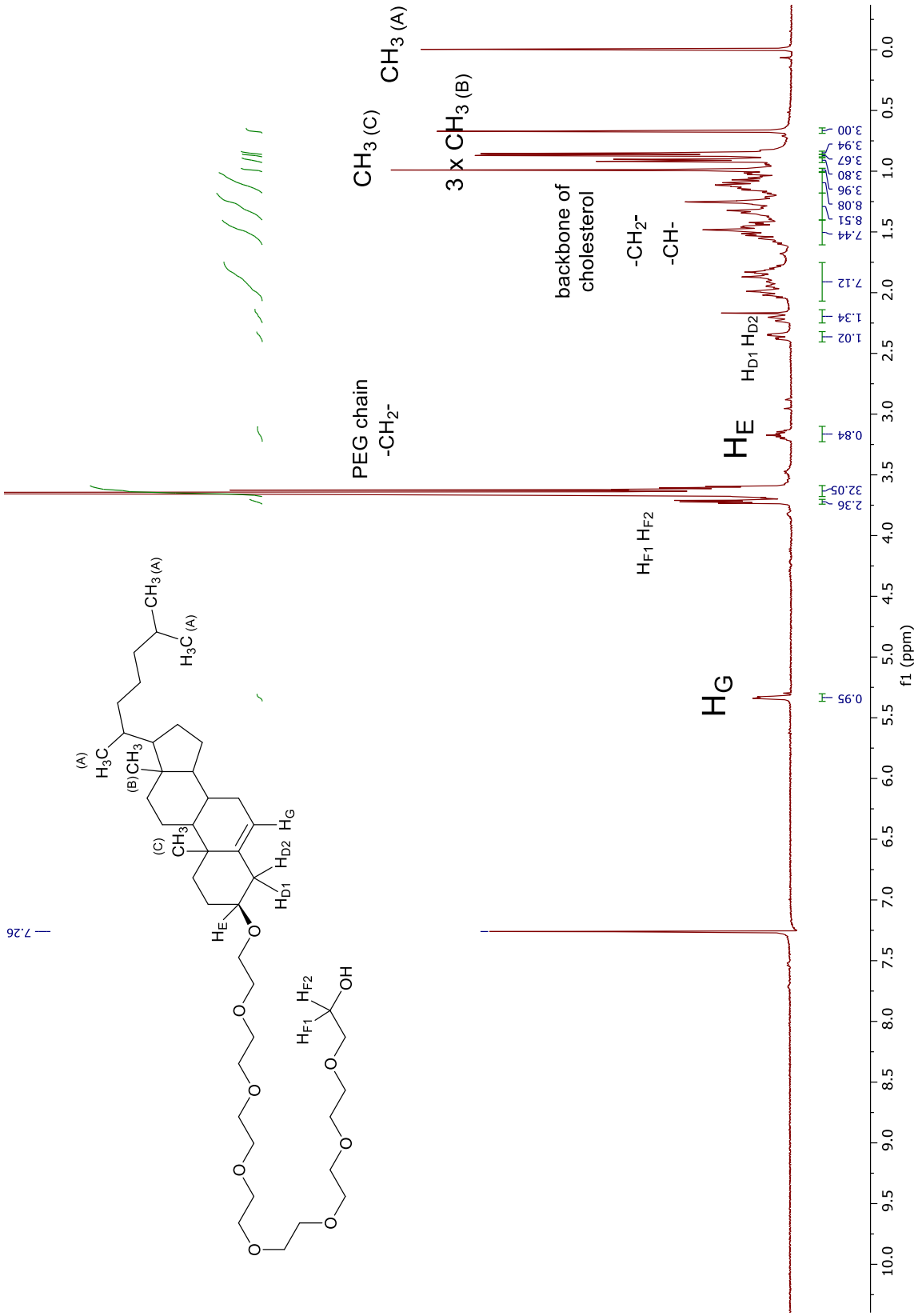


Figure S6. ^{13}C-NMR analysis of Chol-PEG8, **5**

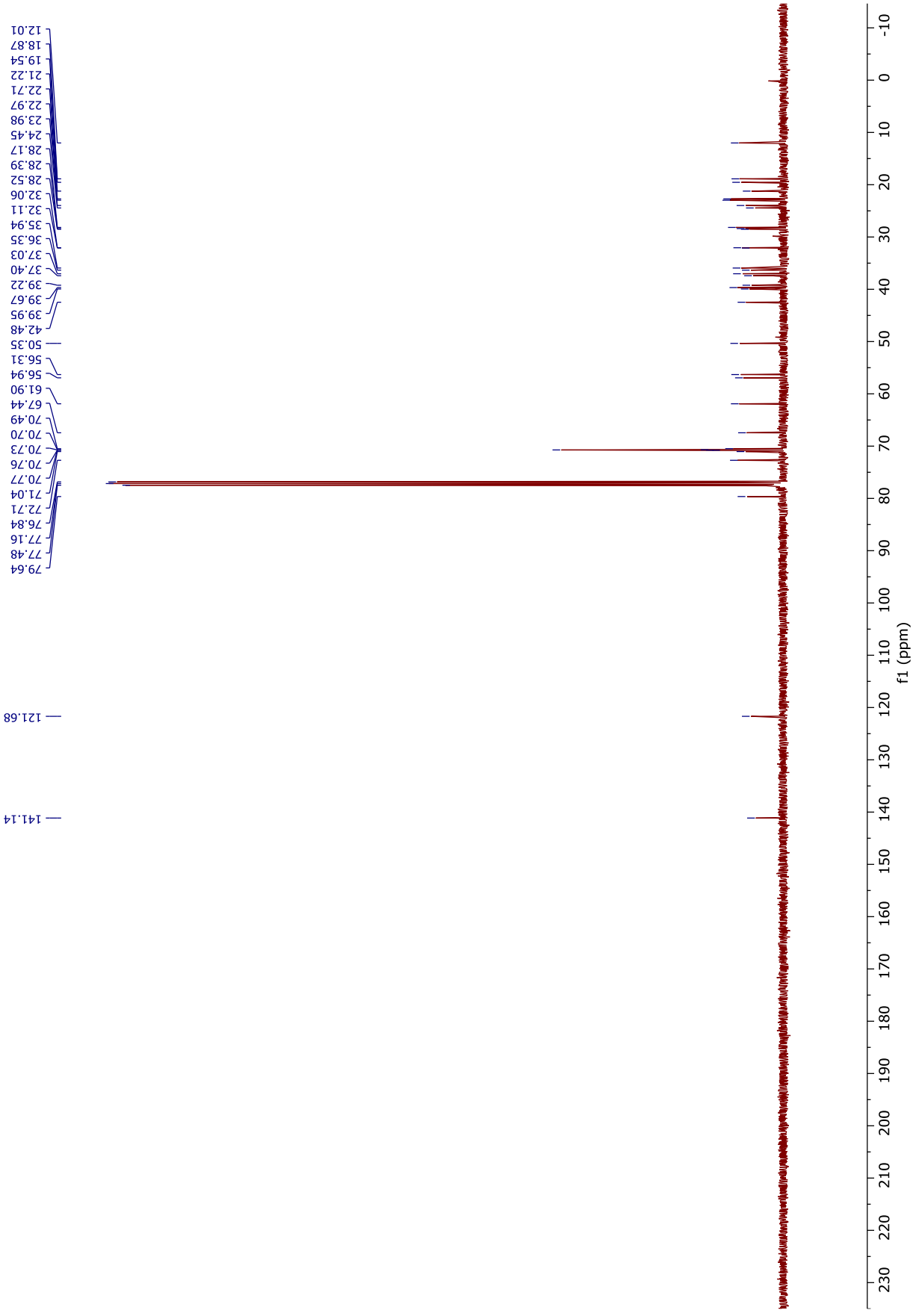


Figure S7. HRMS analysis of Chol-PEG8, **5**

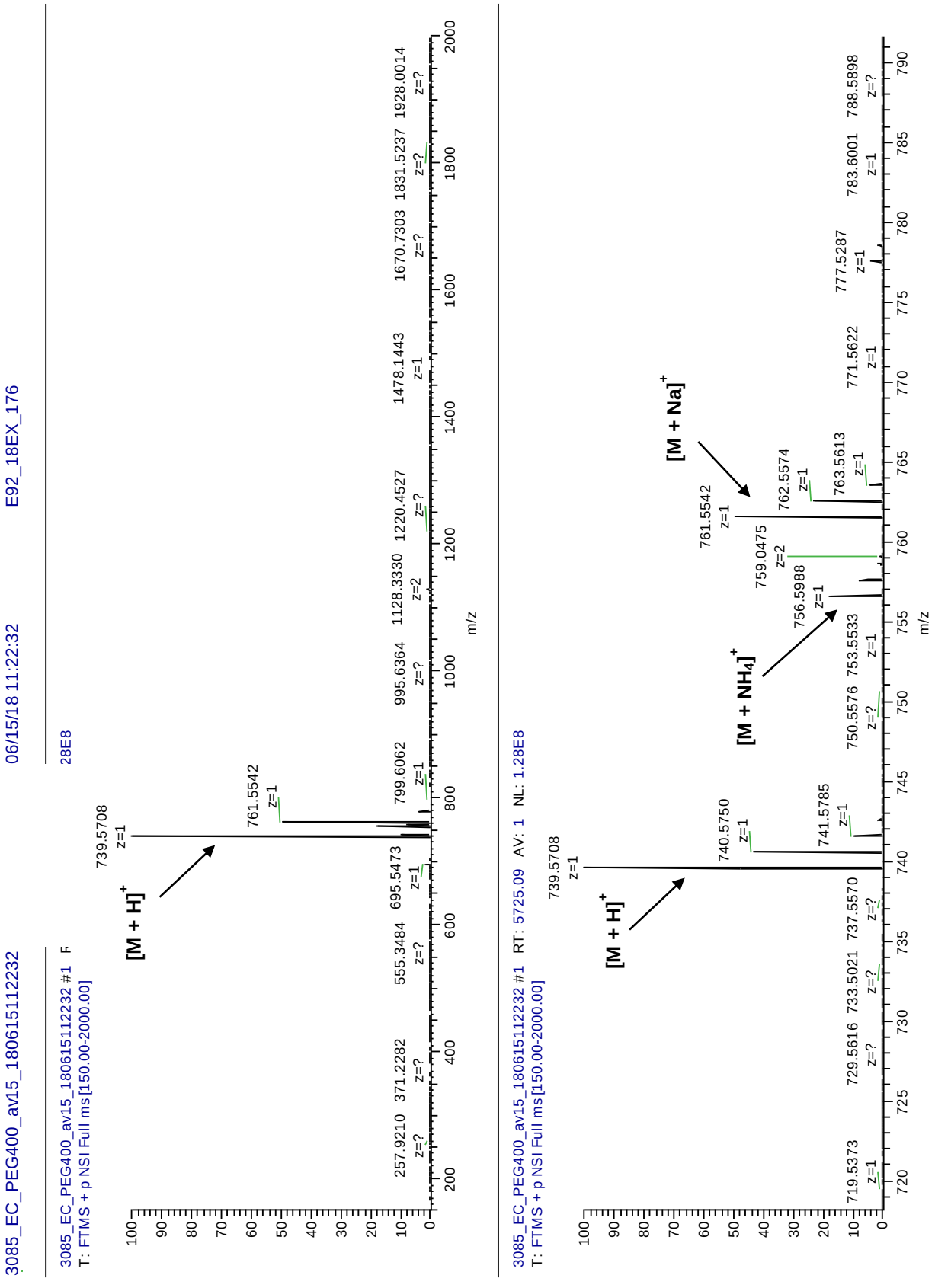


Figure S8. UPLC-PDA-ELSD-MS analysis of Chol-PEG8, **5**

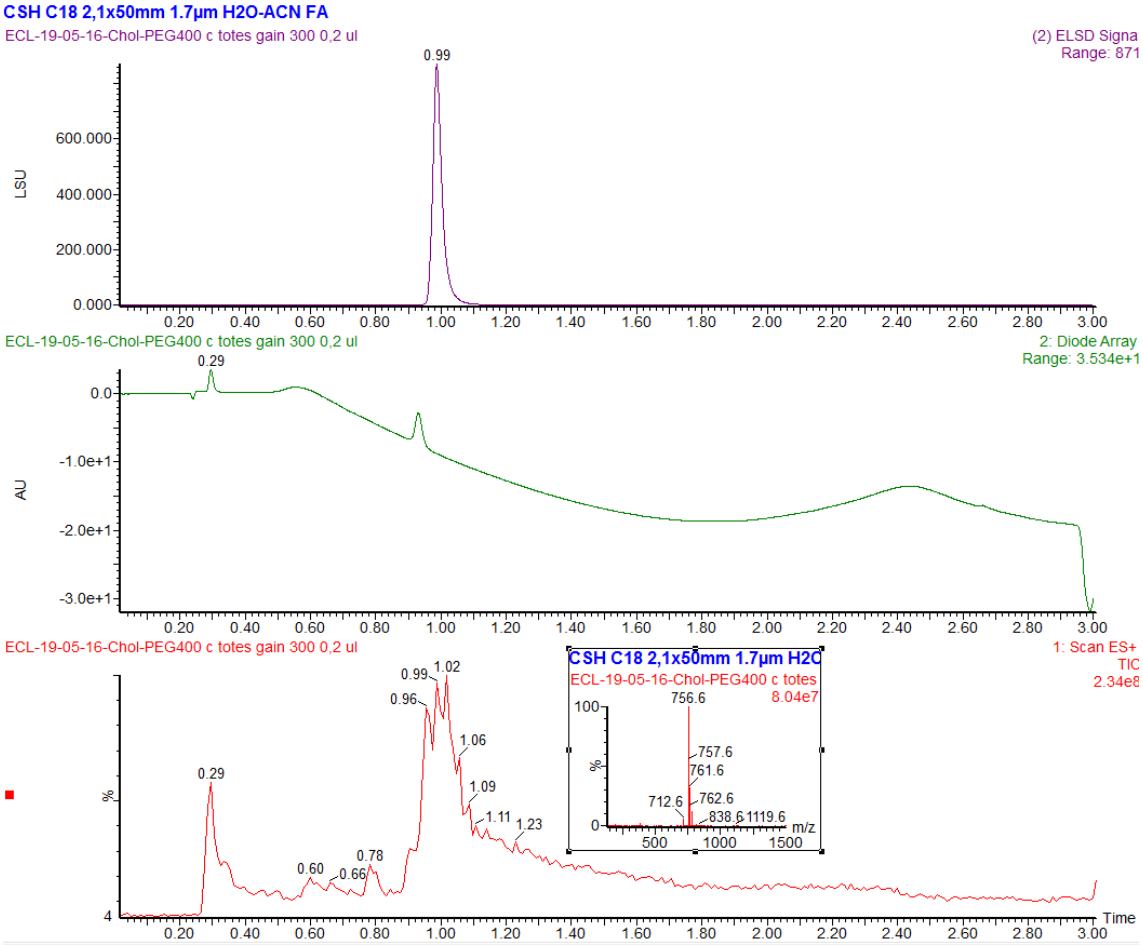


Figure S9. [1]H-NMR analysis of Chol-PEG12, **6**

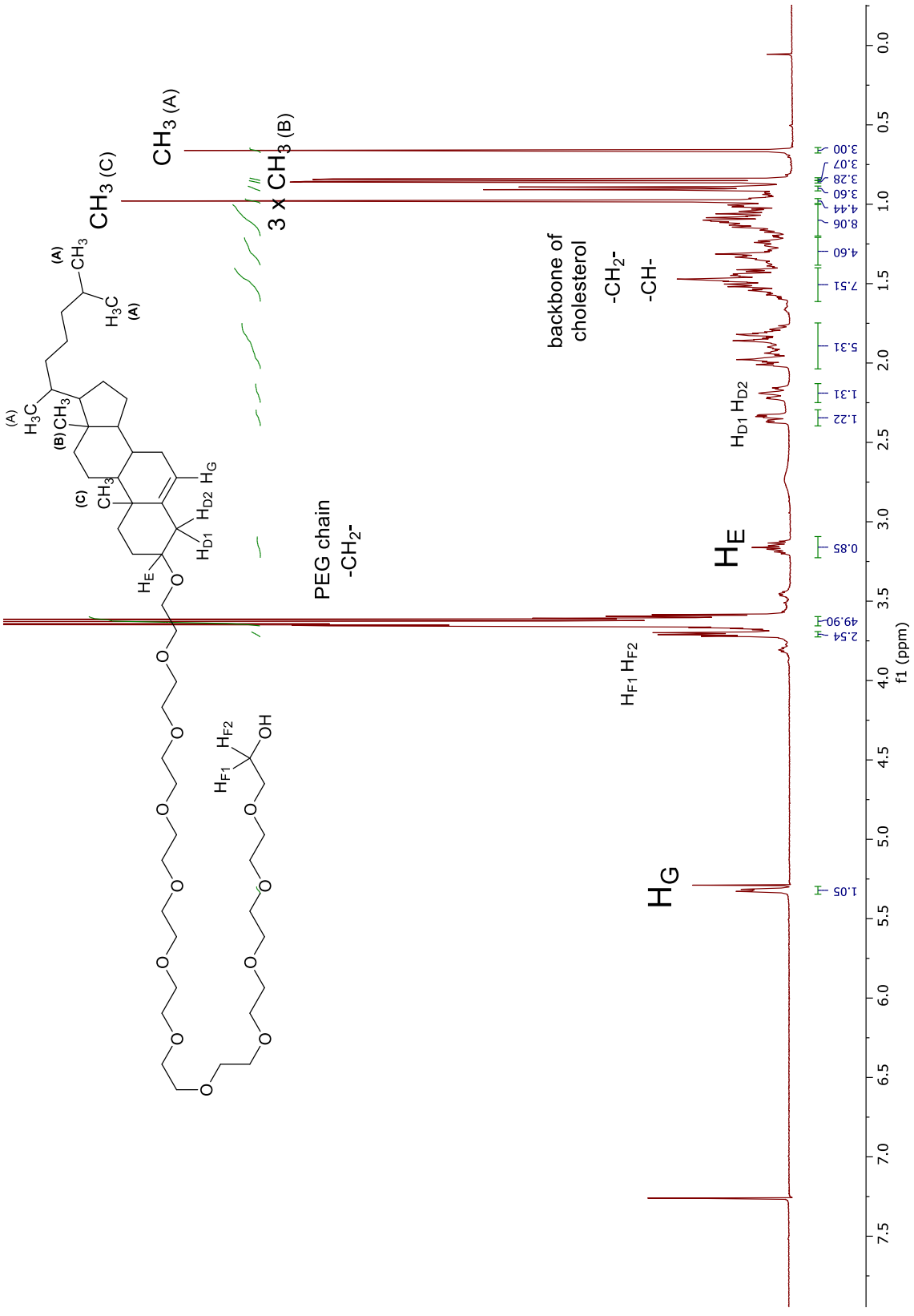


Figure S10. ^{13}C-NMR analysis of Chol-PEG12, **6**

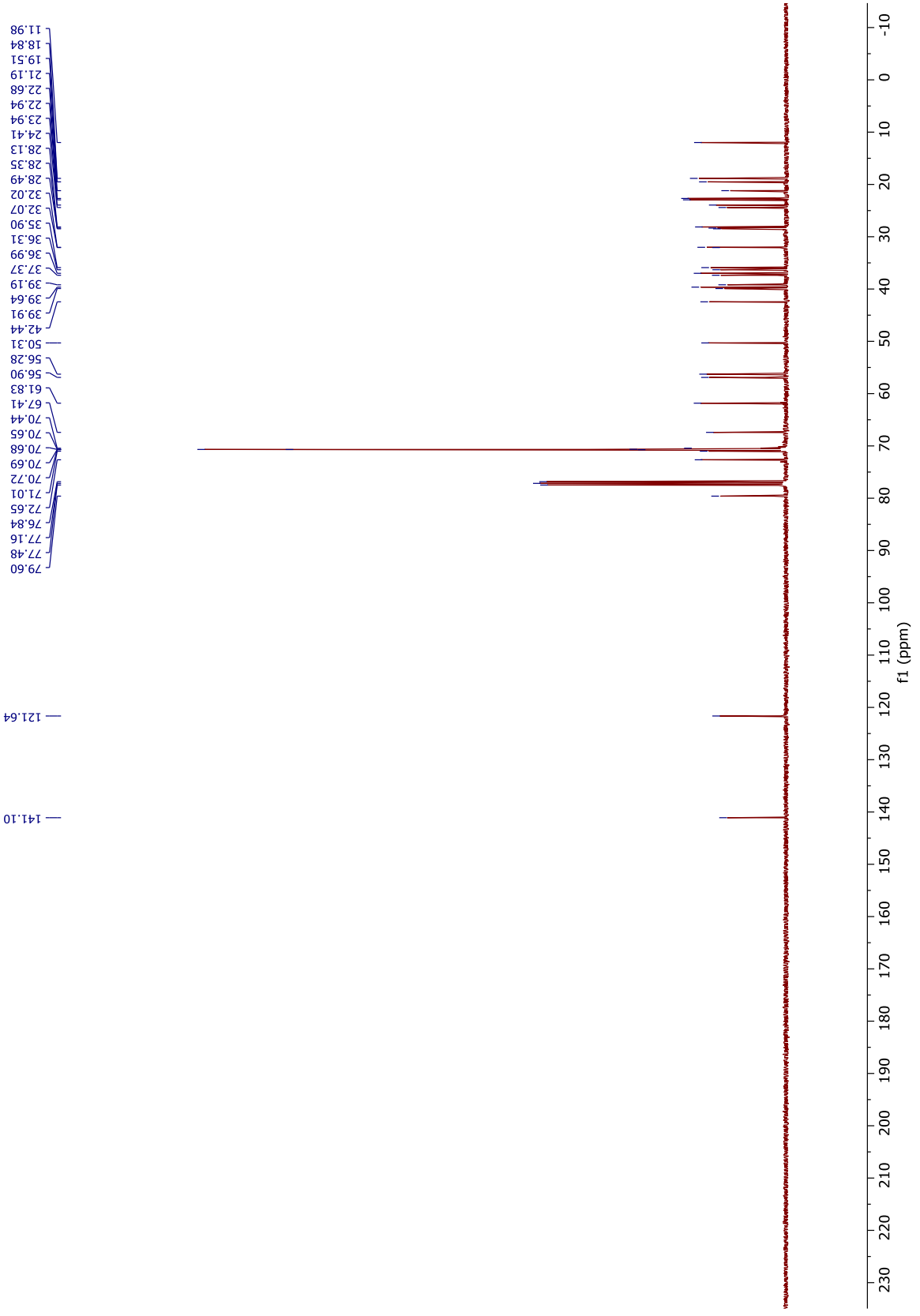


Figure S11. HRMS analysis of Chol-PEG12, **6**

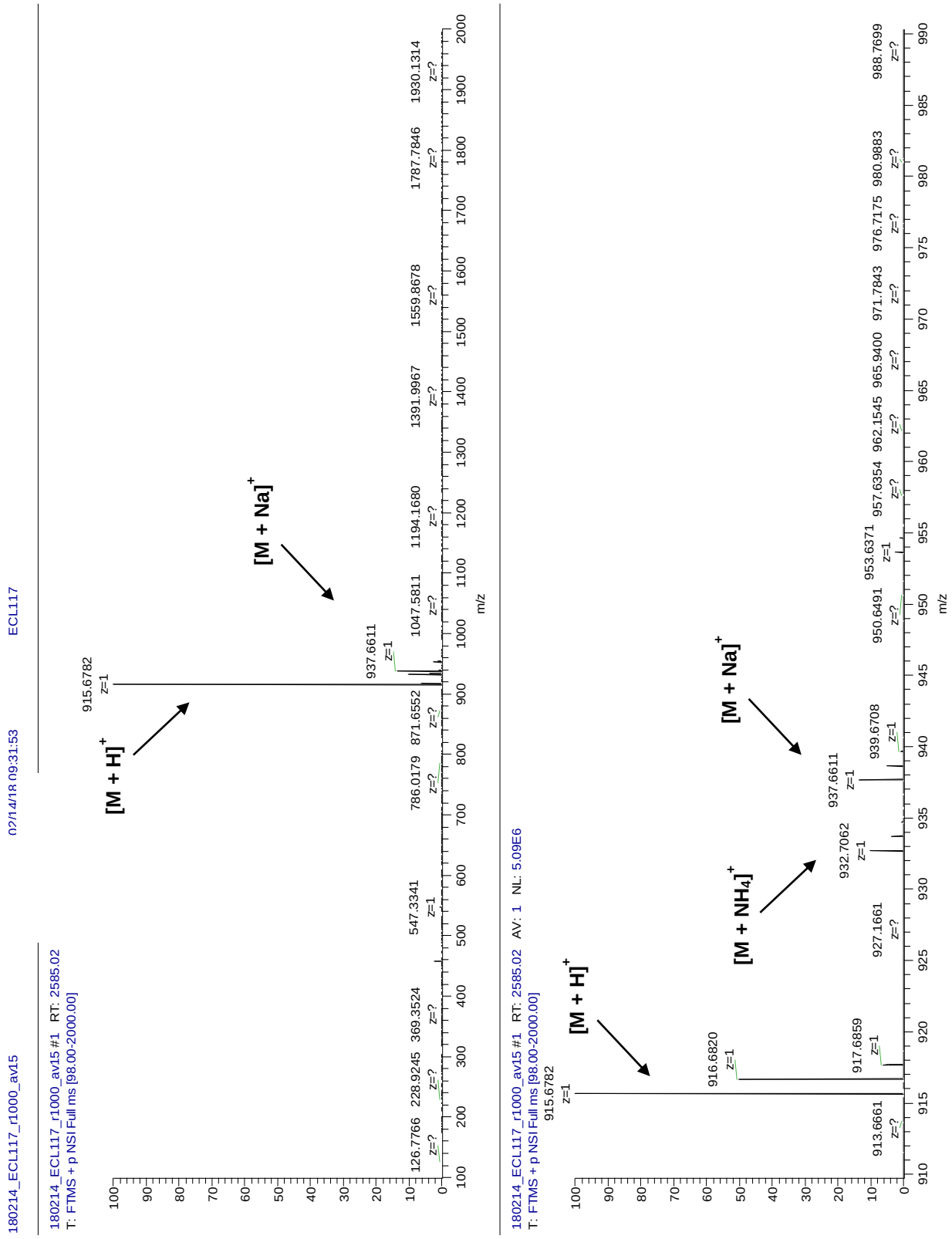


Figure S12. UPLC-PDA-ELSD-MS analysis of Chol-PEG12, **6**

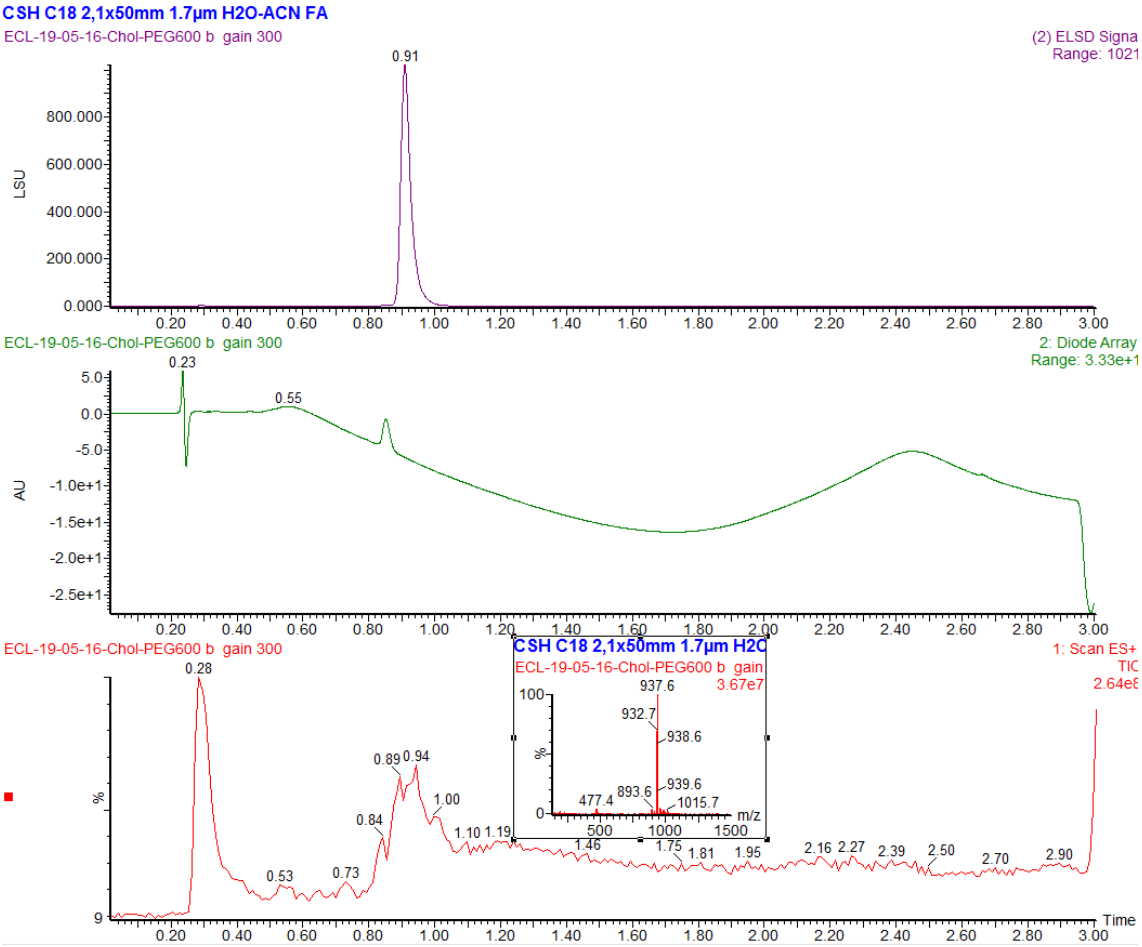


Figure S13. [1]H-NMR analysis of Chol-PEG16, **7**

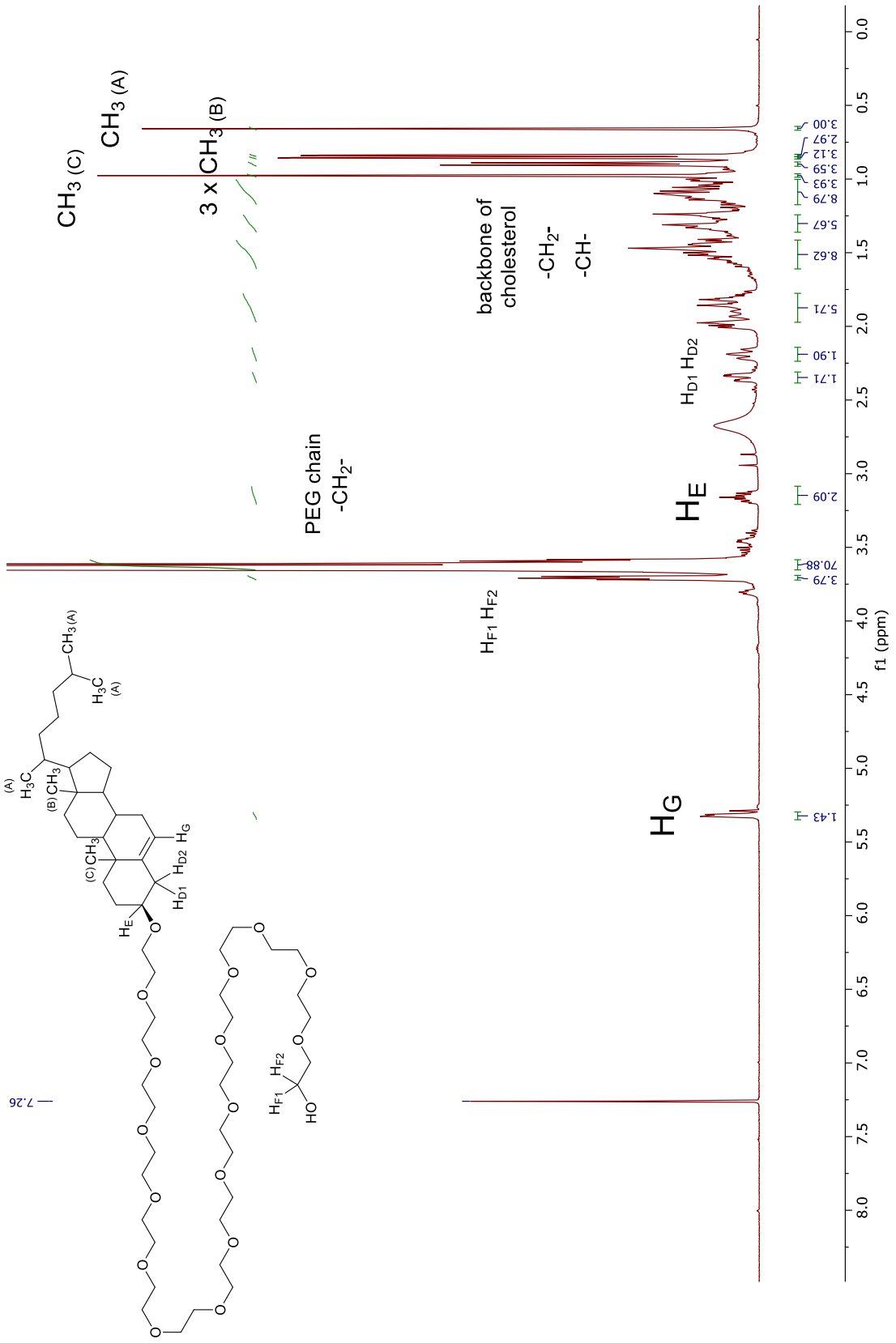


Figure S14. ^{13}C-NMR analysis of Chol-PEG16, **7**

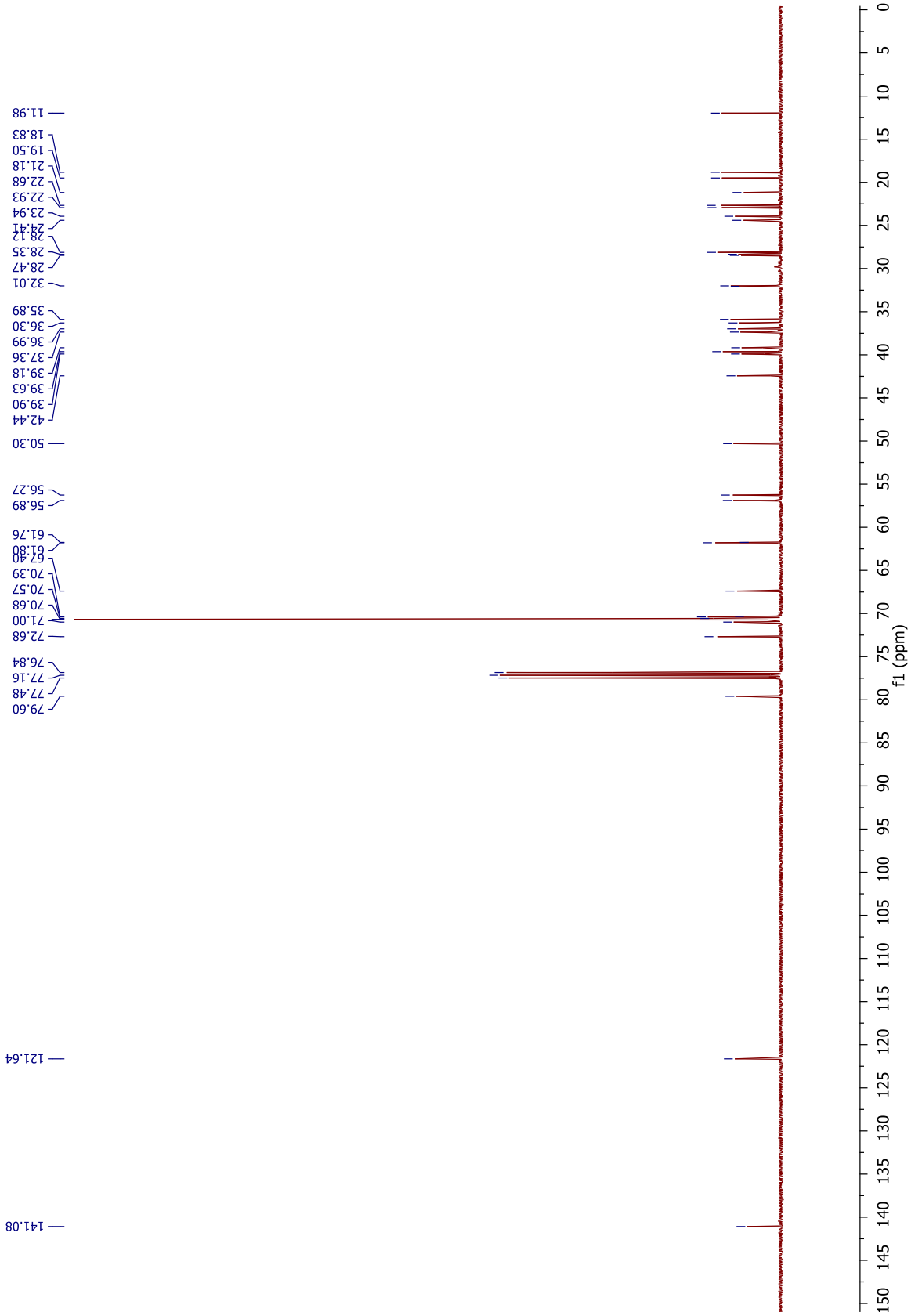


Figure S15. HRMS analysis of Chol-PEG16, **7**

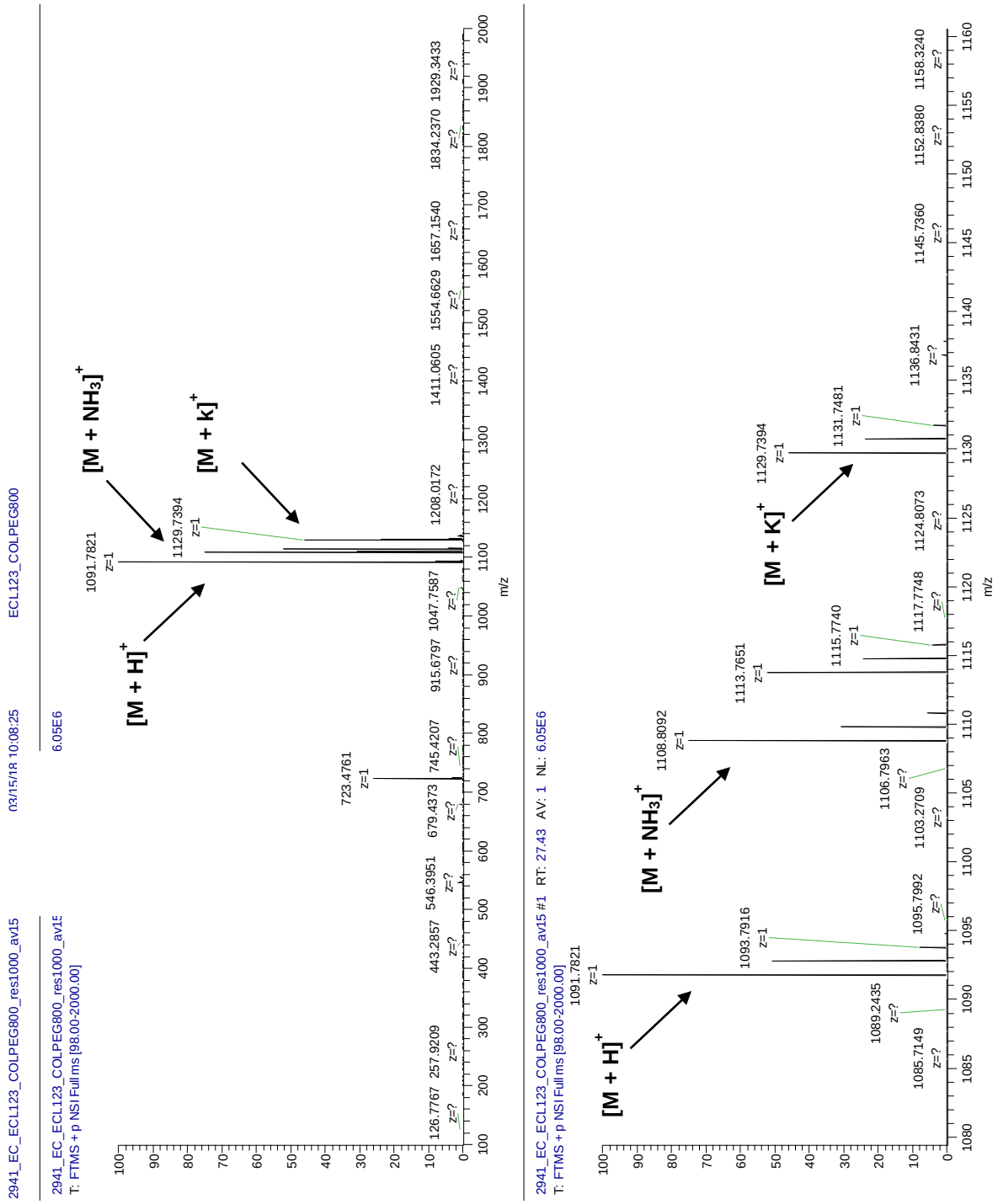


Figure S16. UPLC-PDA-ELSD-MS analysis of Chol-PEG16, **7**

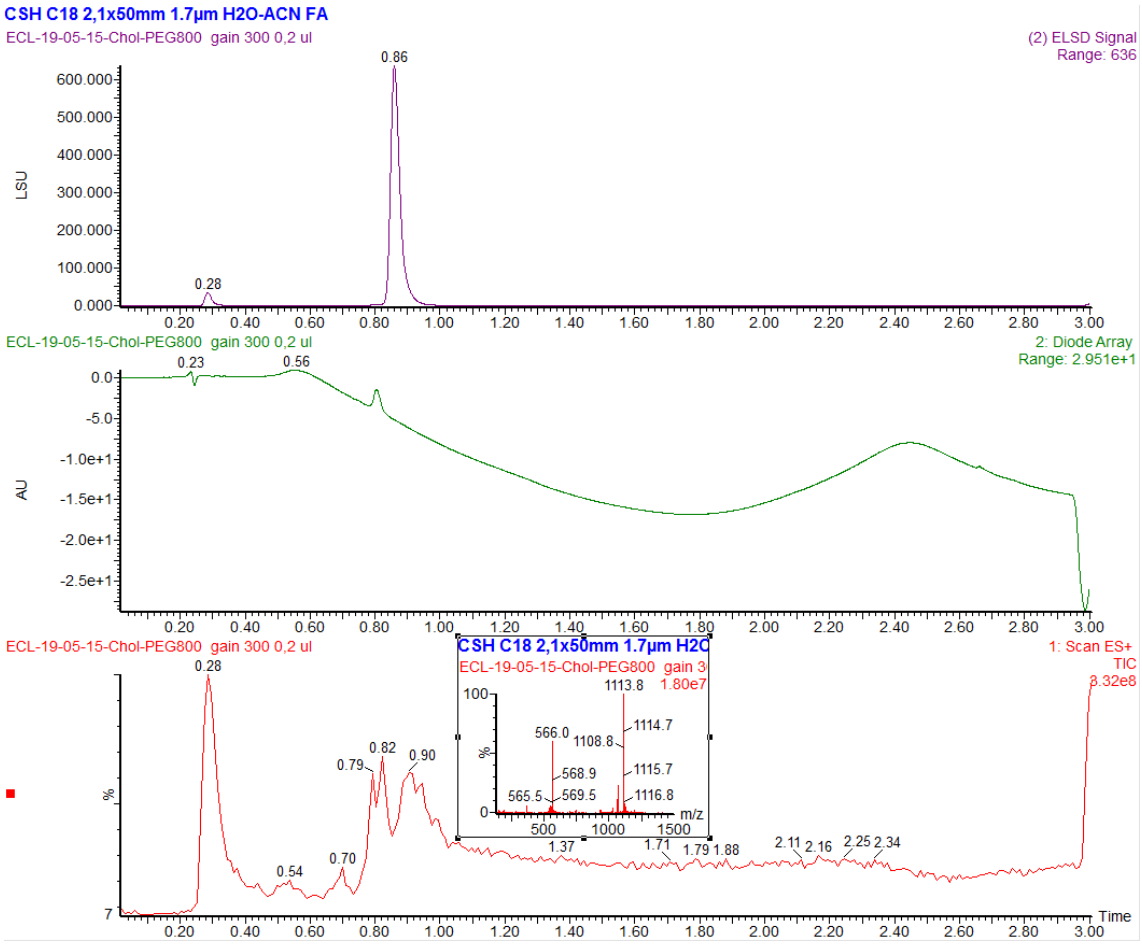


Figure S17. [1]H-NMR analysis of Chol-PEG20, **8**

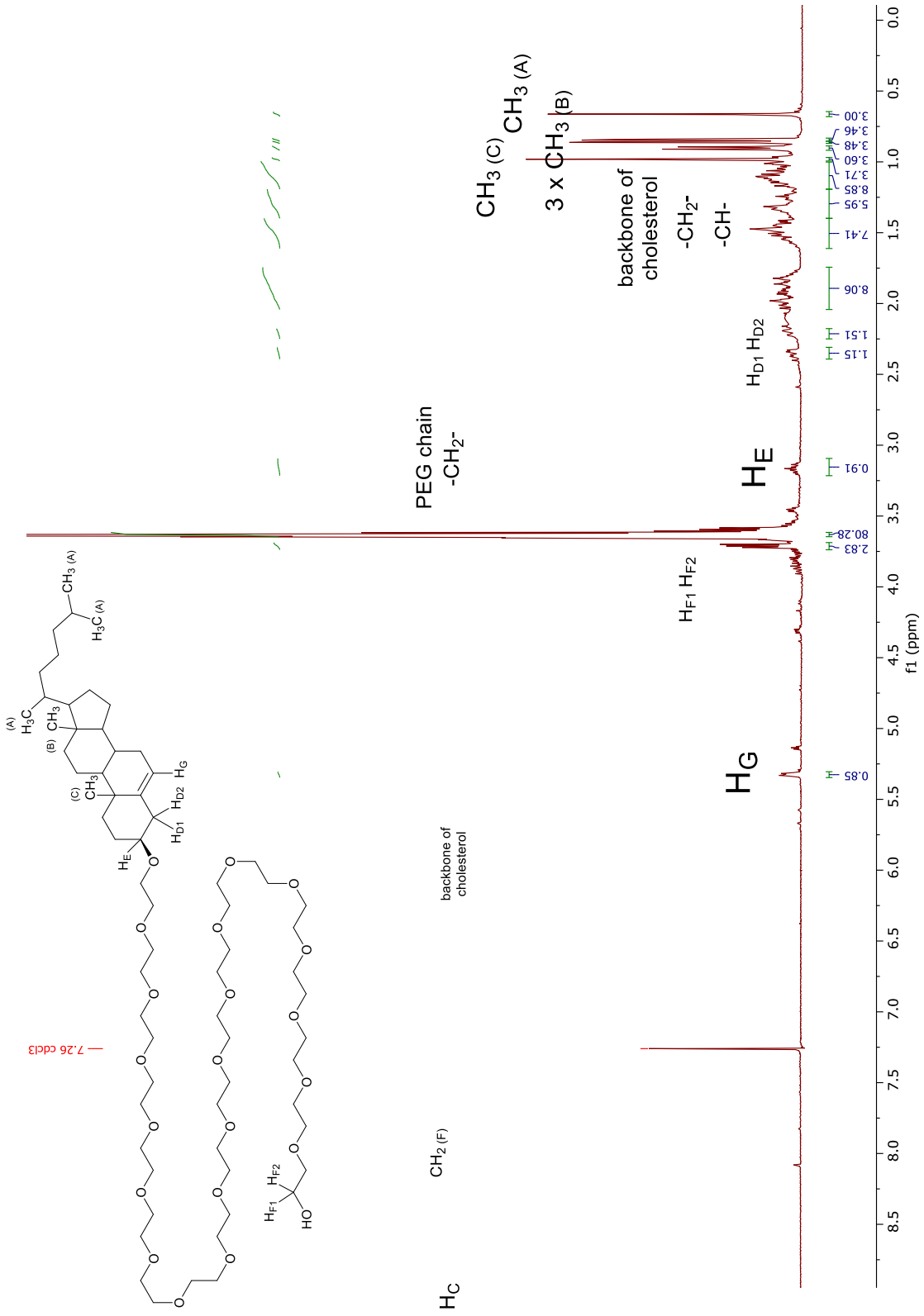


Figure S18. ^{13}C-NMR analysis of Chol-PEG20, **8**

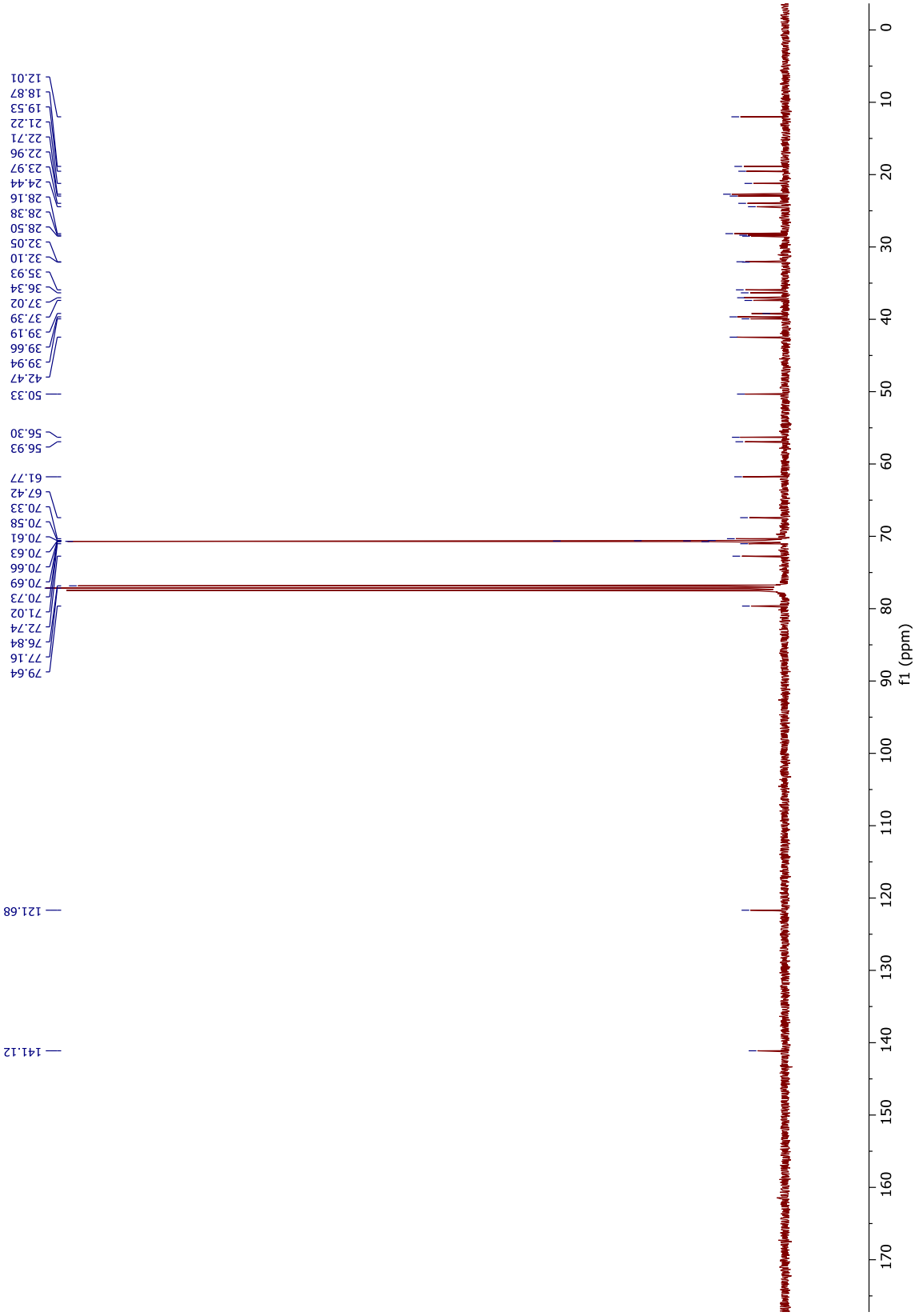


Figure S19. HRMS analysis of Chol-PEG20, **8**

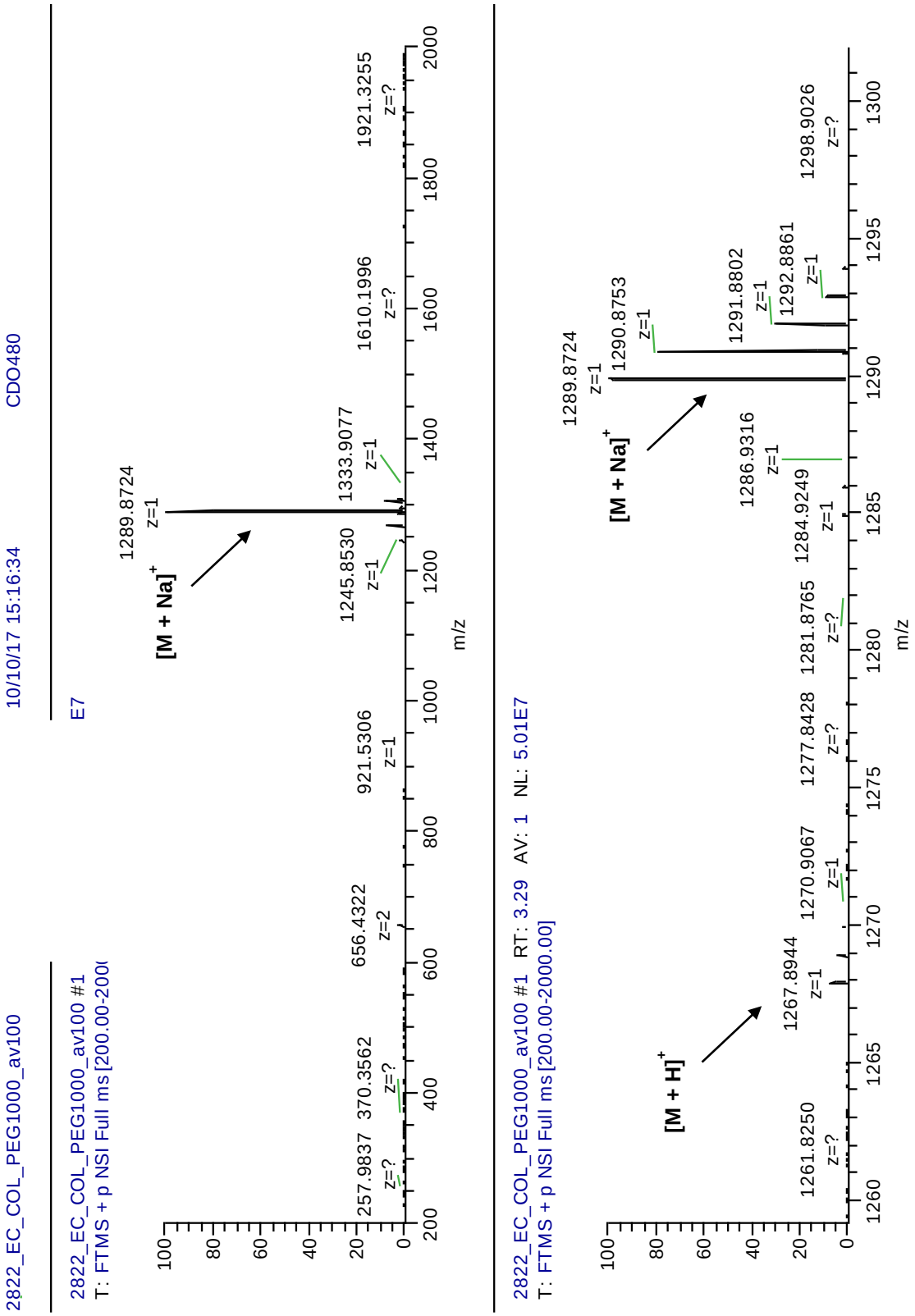


Figure S20. UPLC-PDA-ELSD-MS analysis of Chol-PEG20, **8**

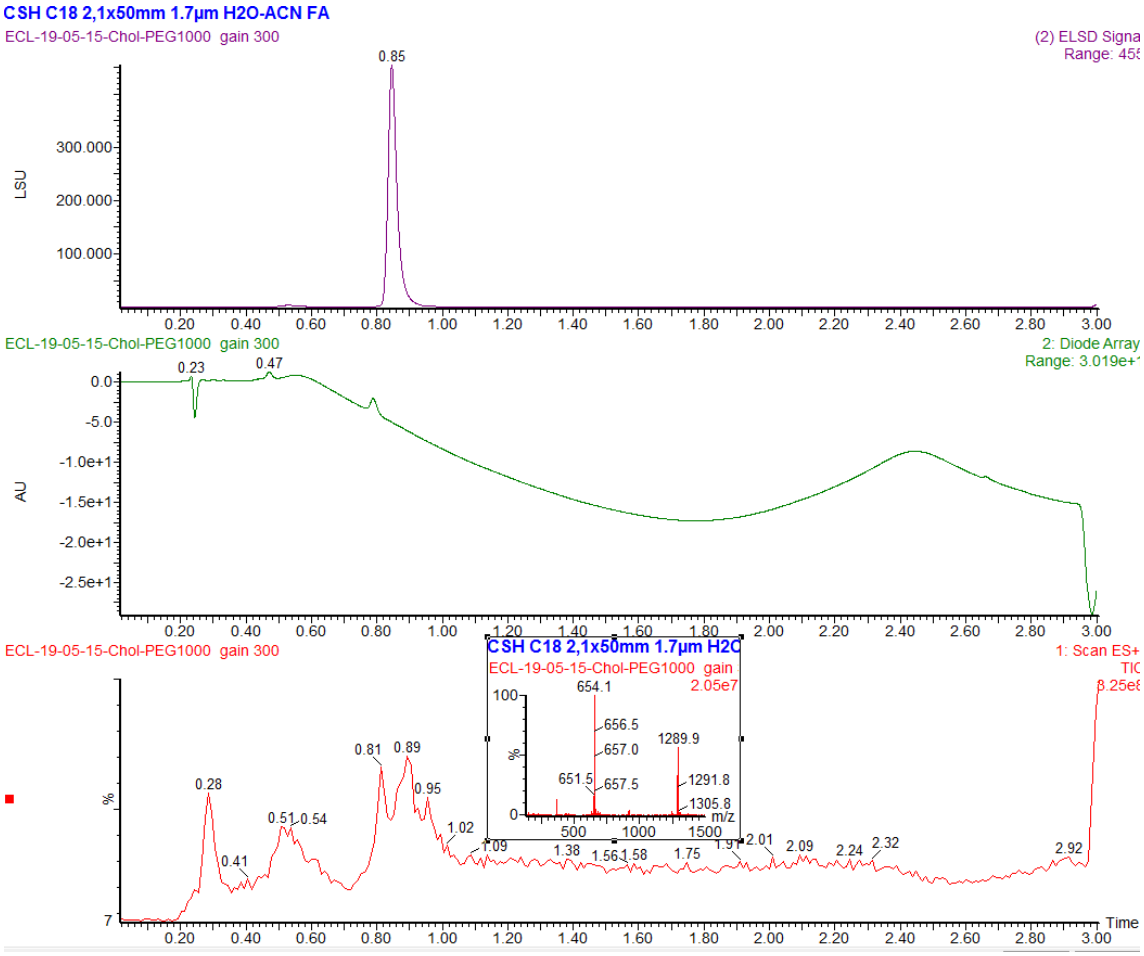


Figure S21. ^{1}H-NMR analysis of Chol-PEG4-OCH$_3$, **9**

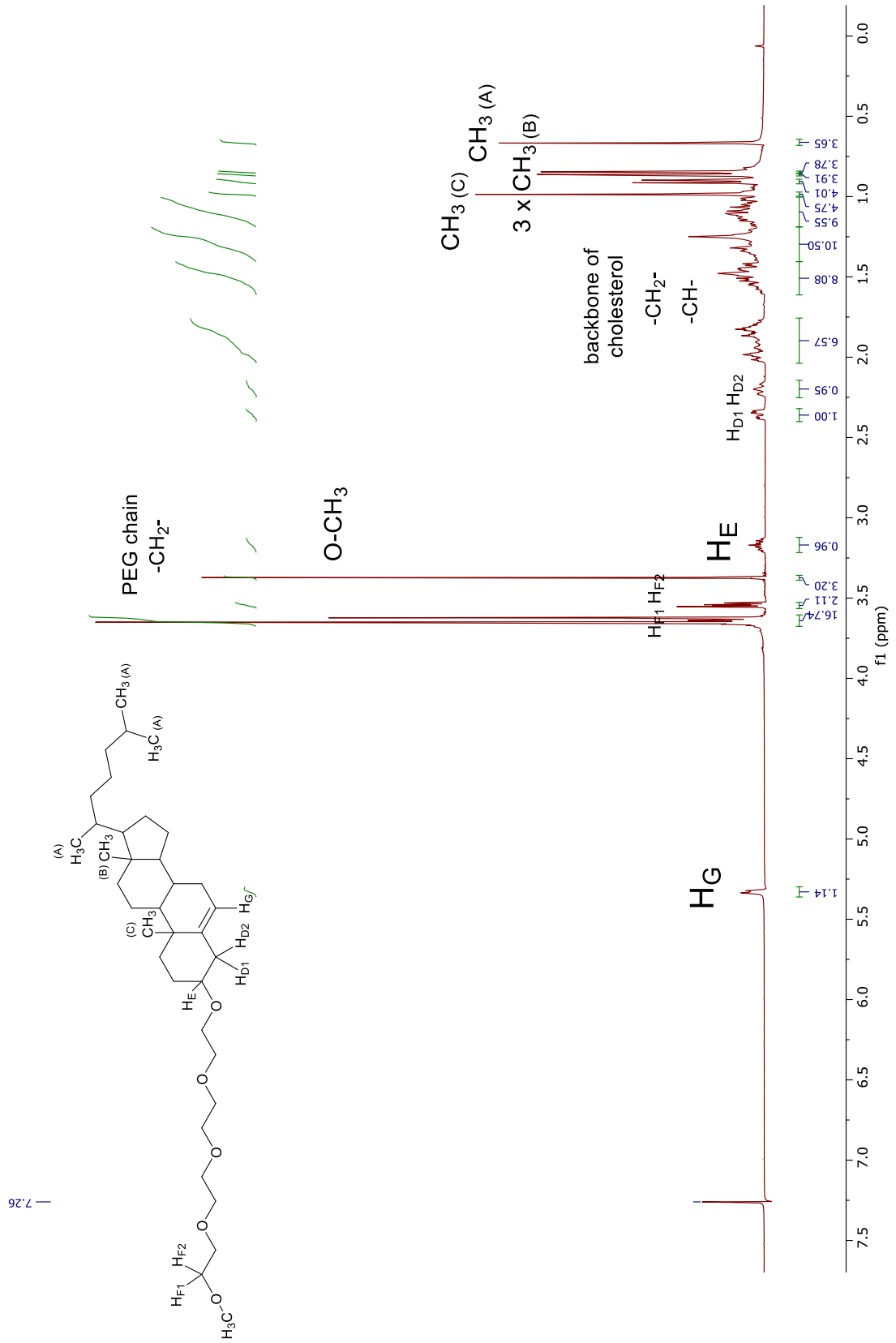


Figure S22. ^{13}C-NMR analysis of Chol-PEG4-OCH$_3$, **9**

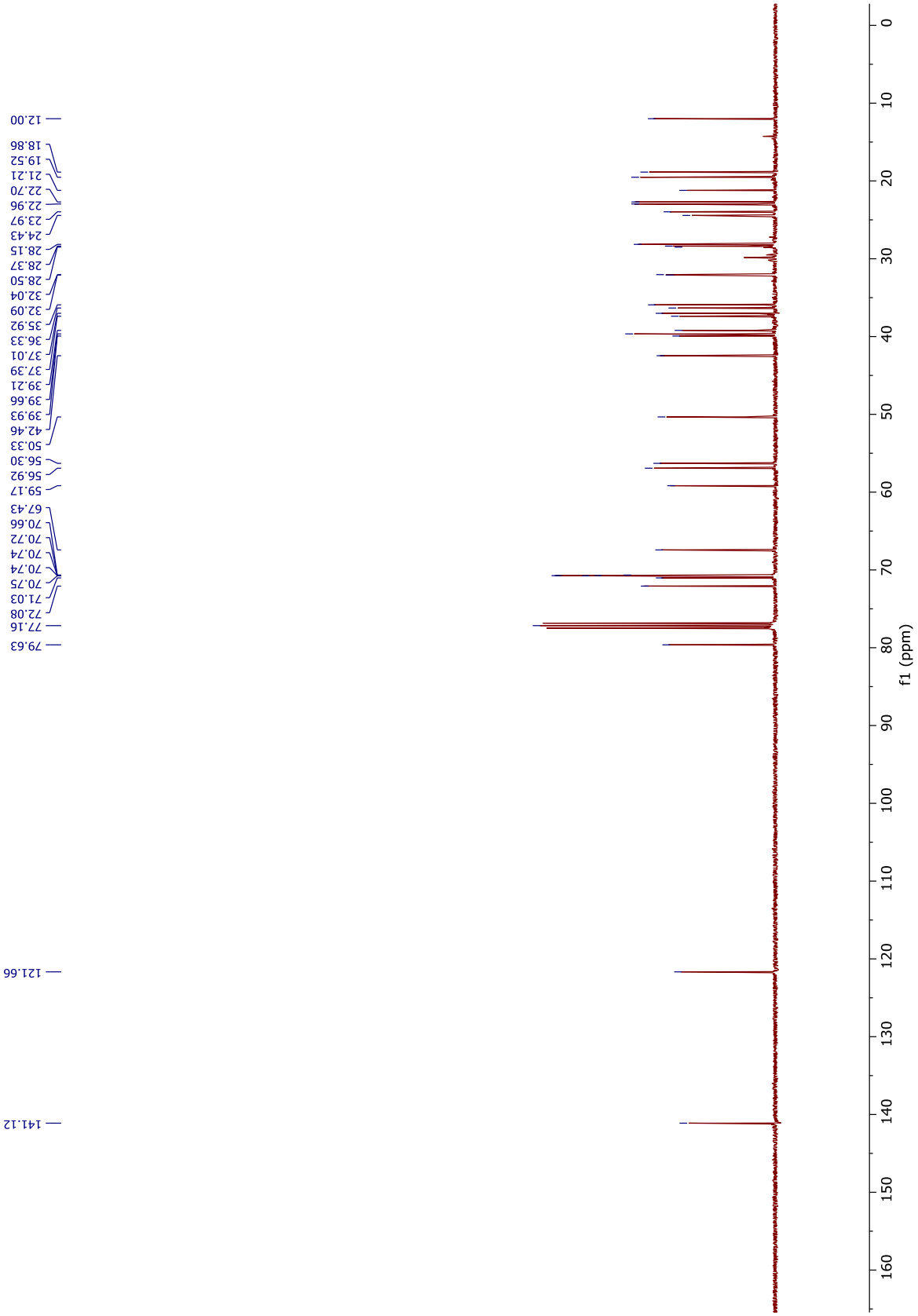


Figure S23. HRMS analysis of Chol-PEG4-OCH_3, **9**

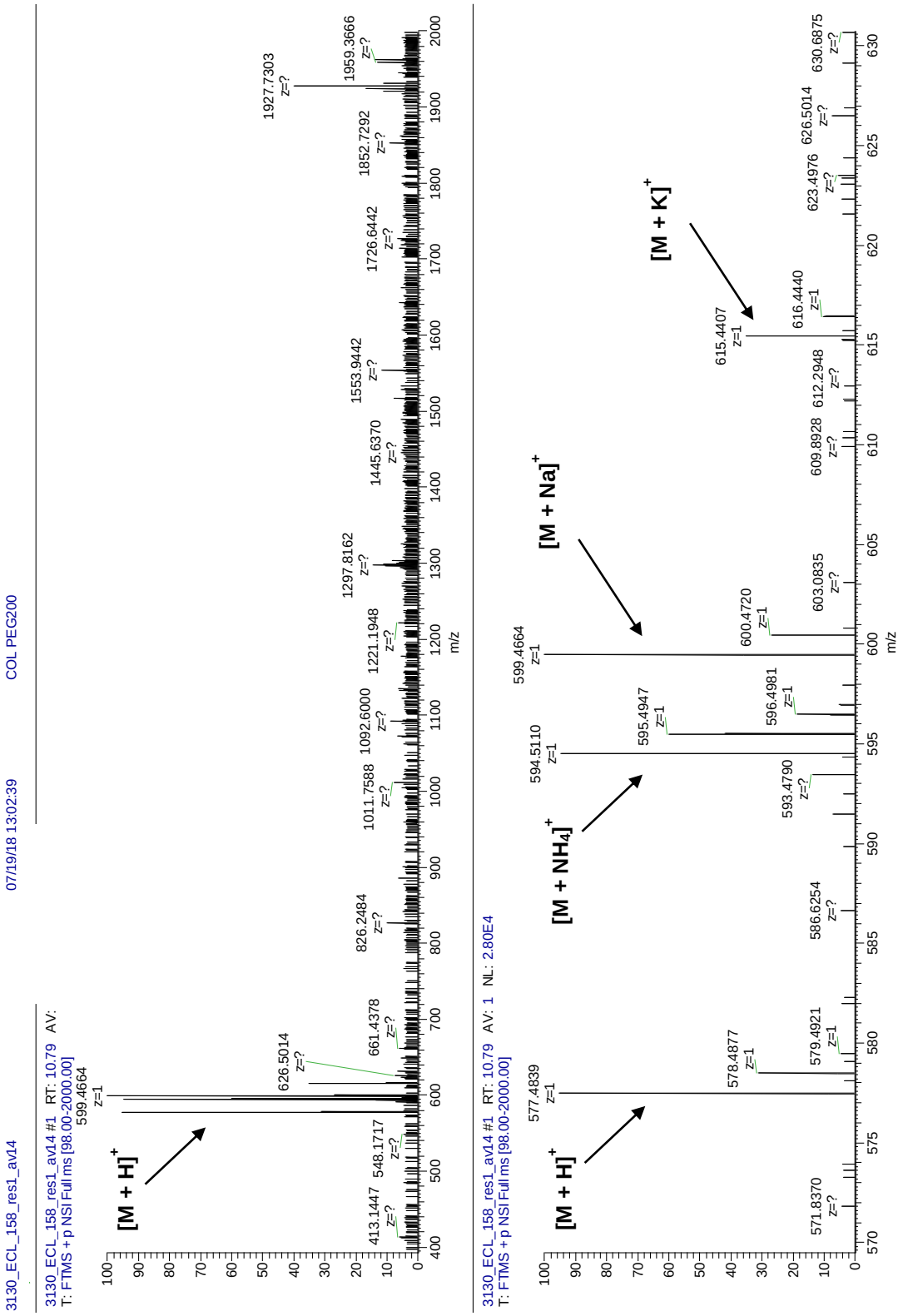


Figure S24. ^{1}H-NMR analysis of Chol-PEG8-OCH$_3$, **10**

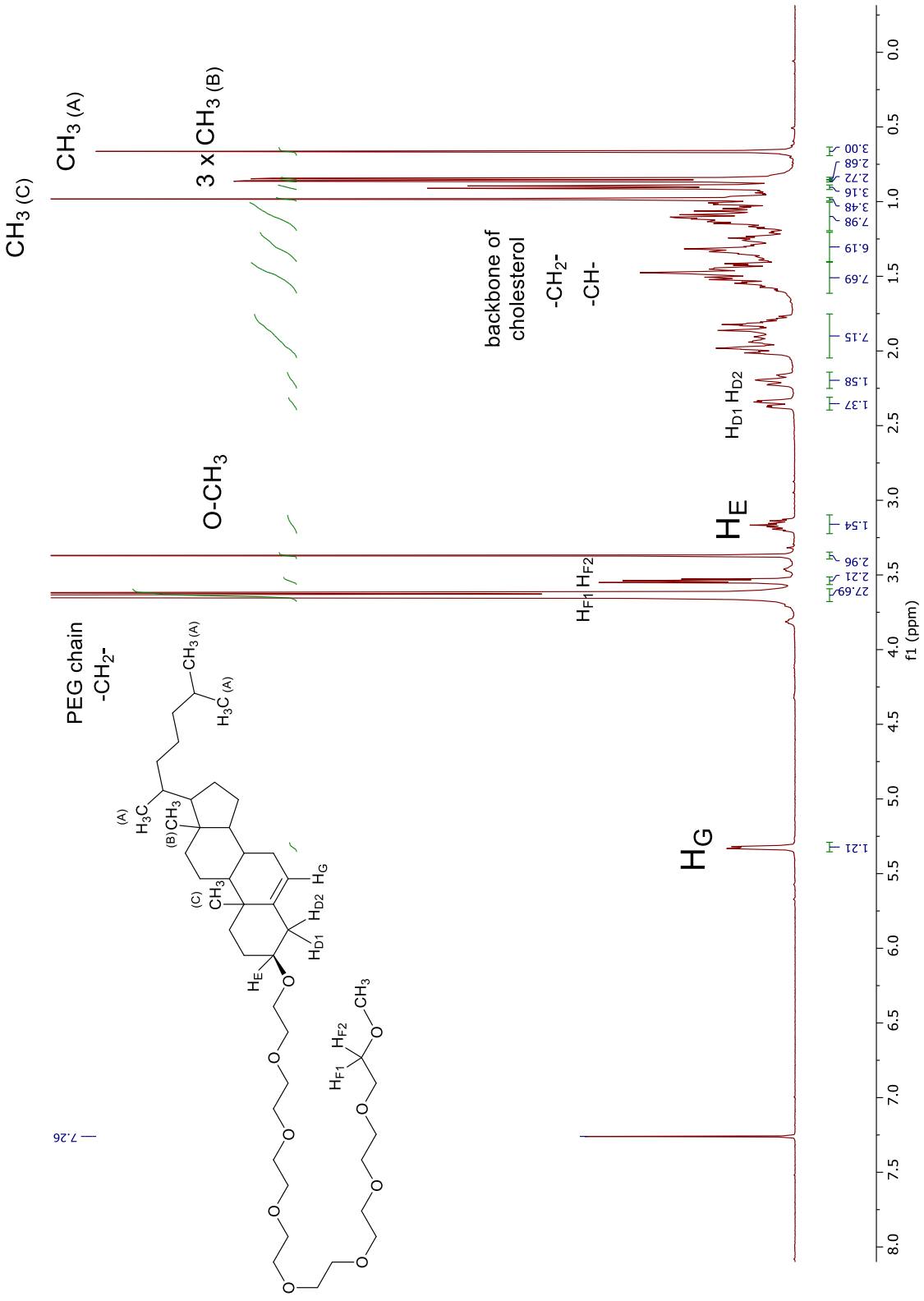


Figure S25. ^{13}C- NMR analysis of Chol-PEG8-OCH_3, **10**

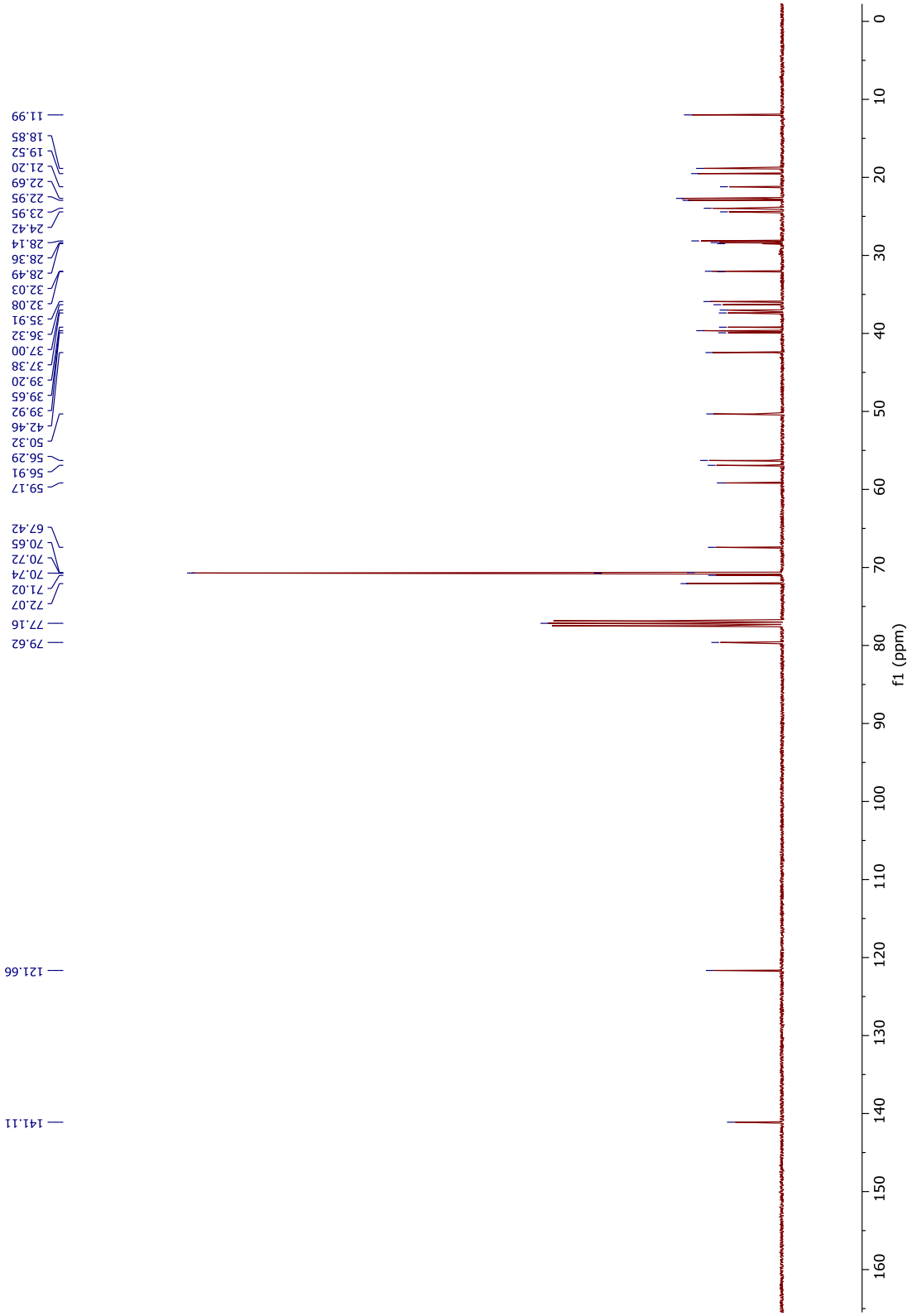


Figure S26. HRMS analysis of Chol-PEG8-OCH_3, **10**

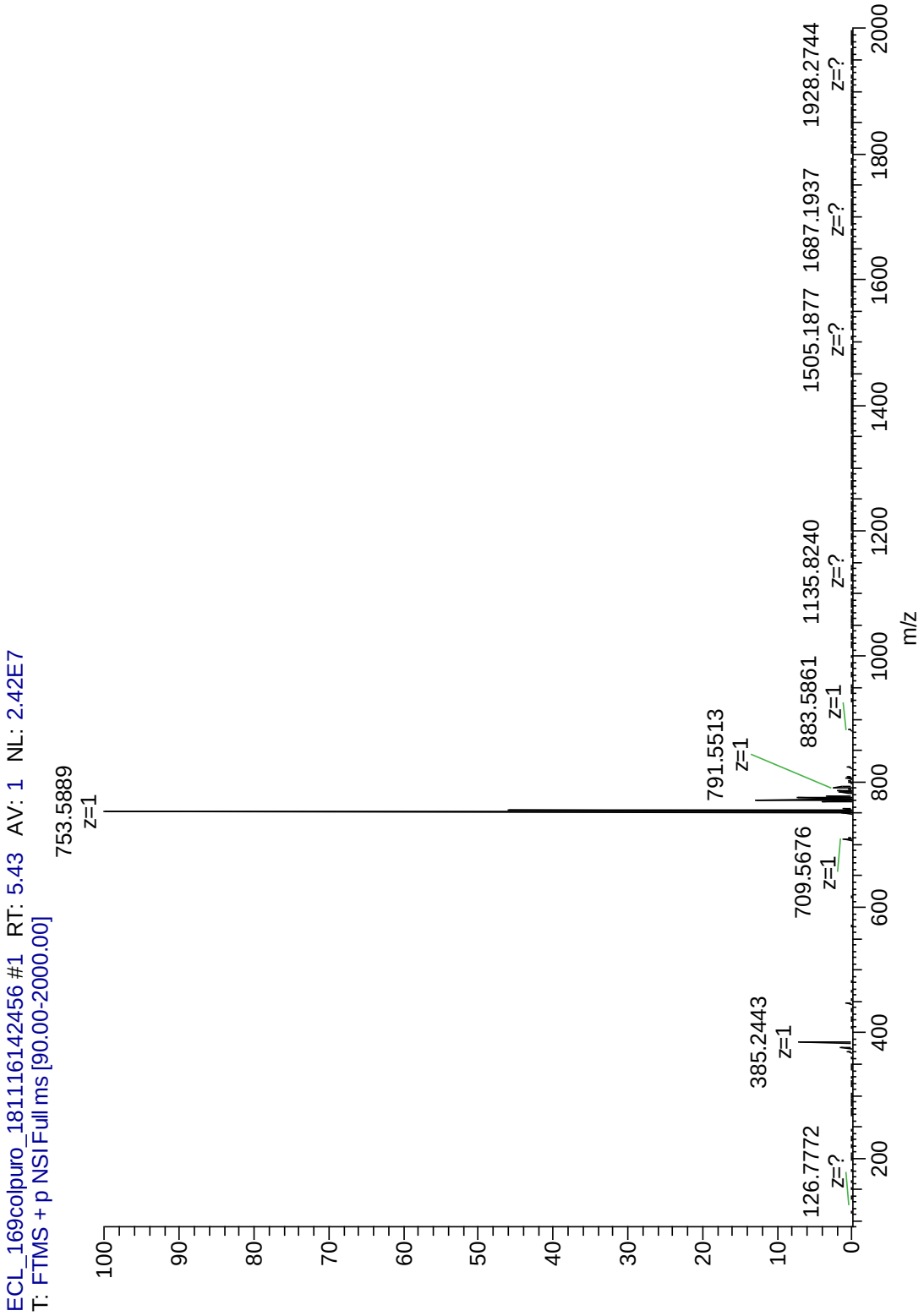


Figure S27. ^{1}H-NMR analysis of Chol-PEG12-OCH$_3$, **11**

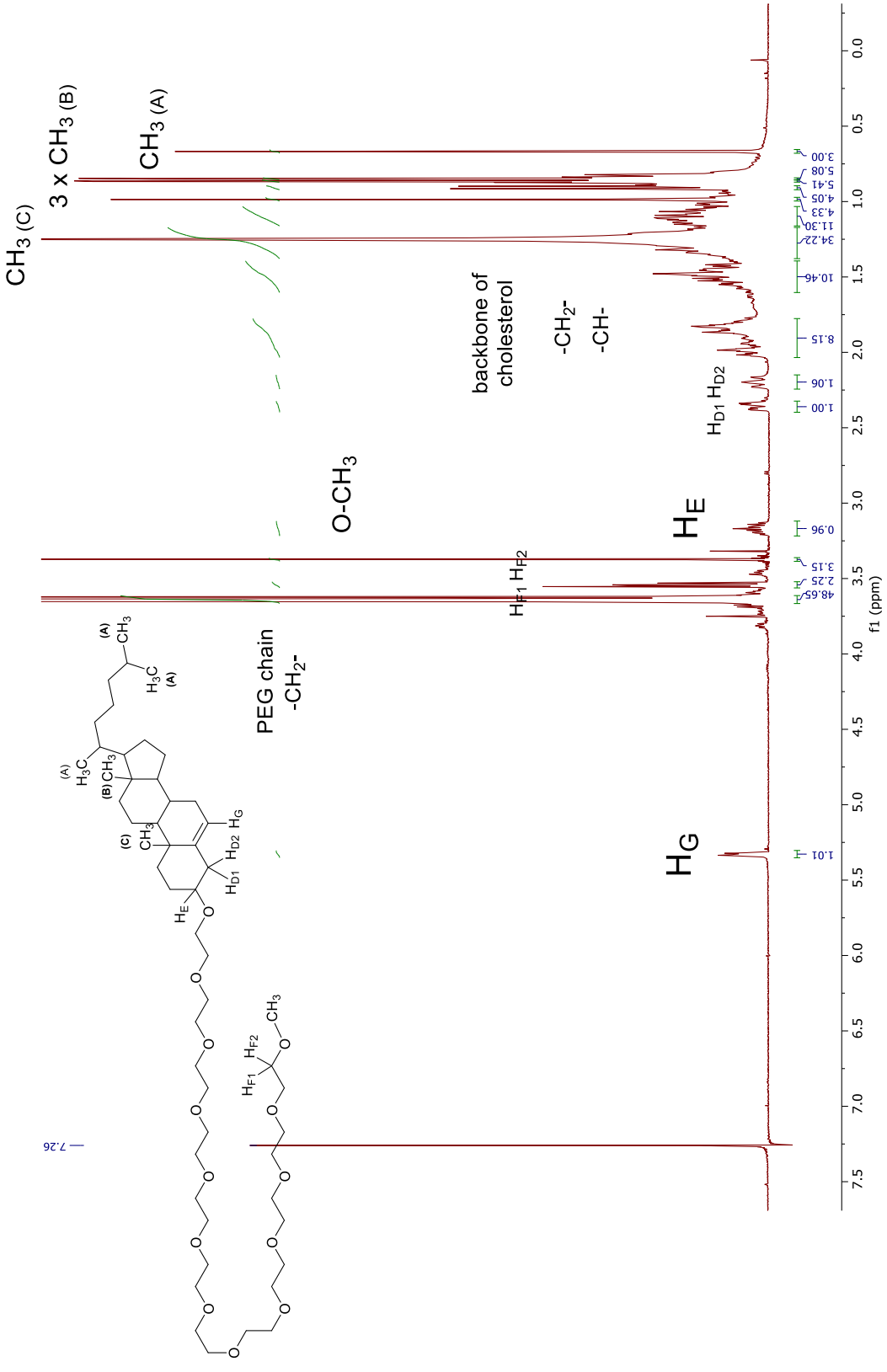


Figure S28. ^{13}C- NMR analysis of Chol-PEG12-OCH_3, **11**

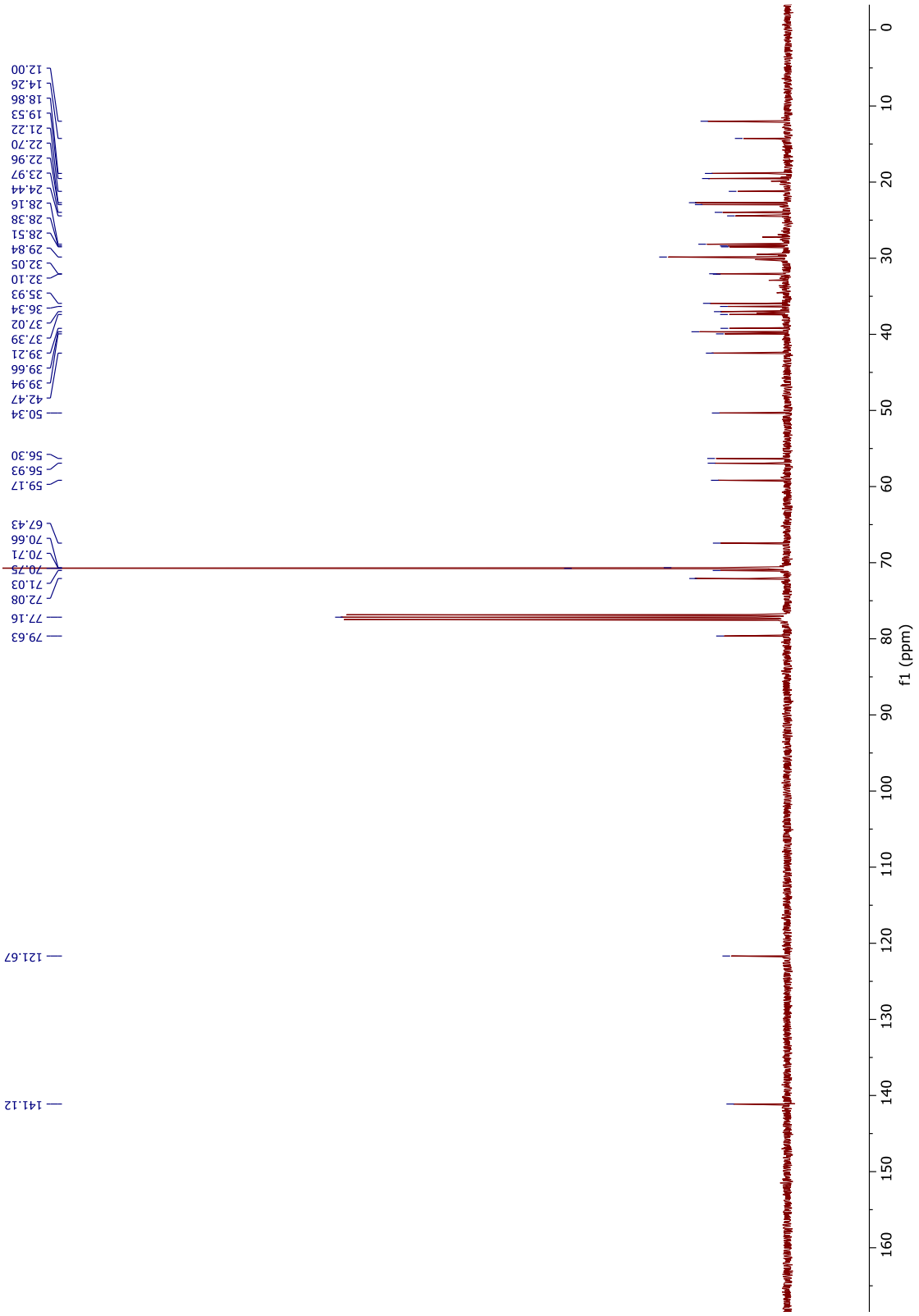


Figure S29. HRMS analysis of Chol-PEG12-OCH_3, **11**

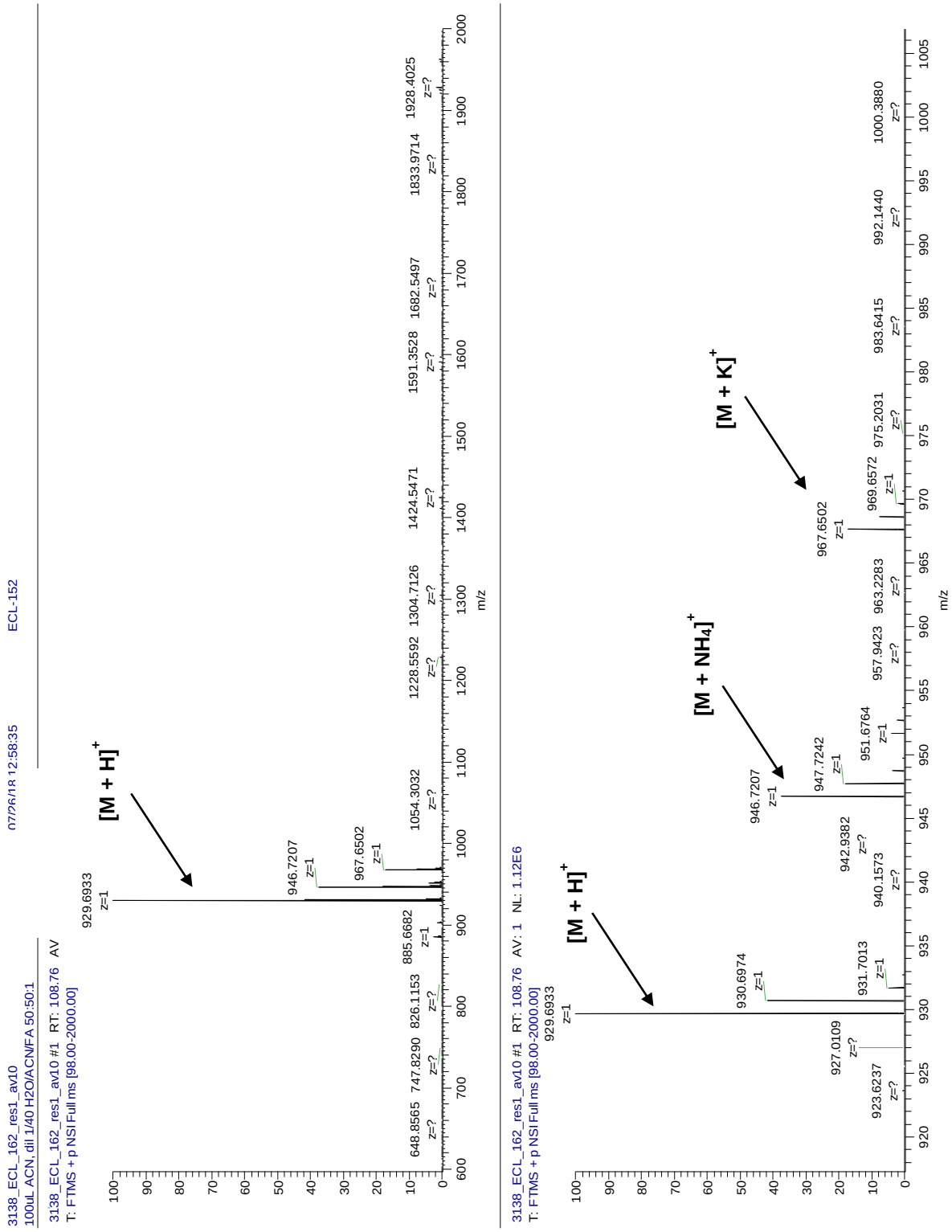


Figure S30. HRMS analysis of Chol-PEG4-RGD, **13**

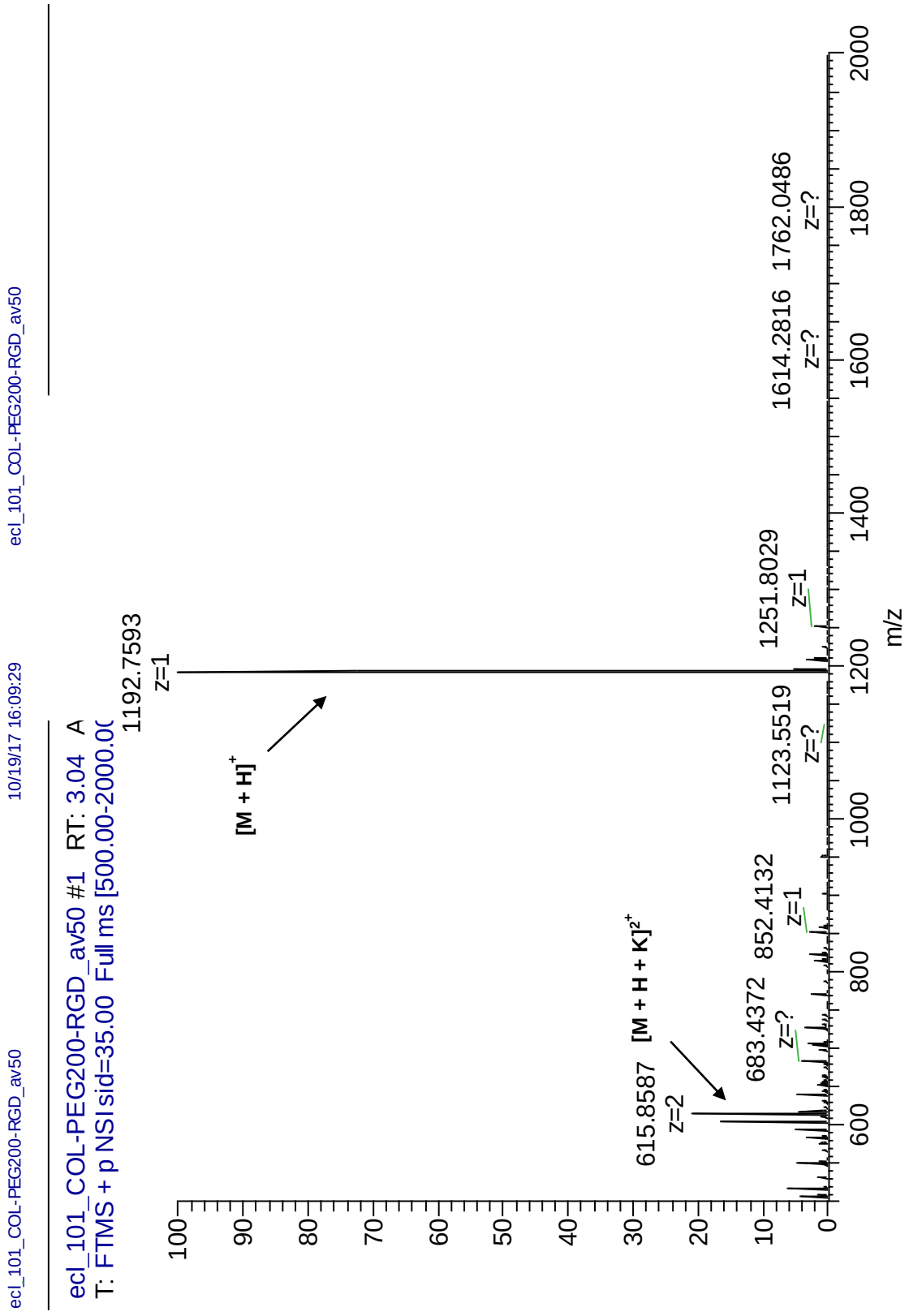


Figure S31. UPLC-PDA-ELSD-MS analysis of Chol-PEG4-RGD, **13**

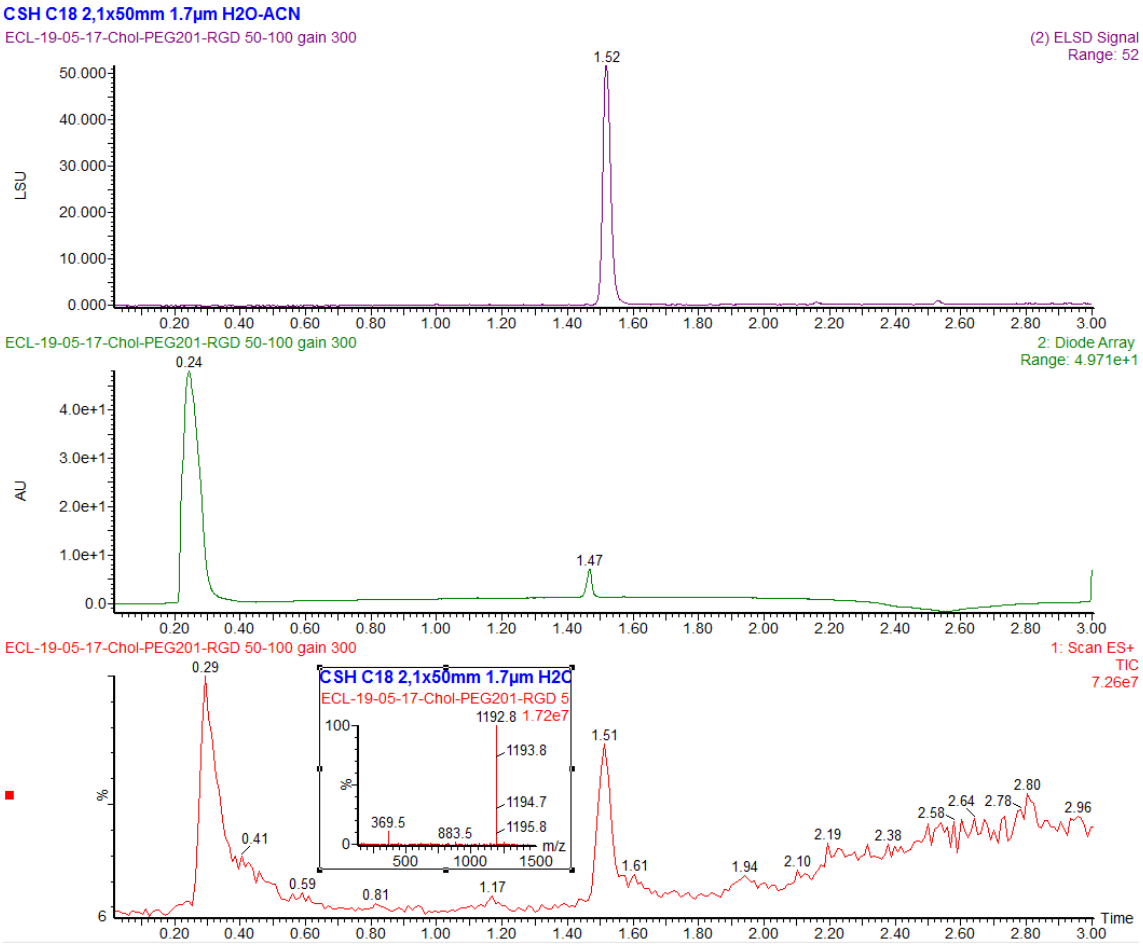


Figure S32. HPLC-PDA-MS analysis of Chol-PEG4-RGD, **13**

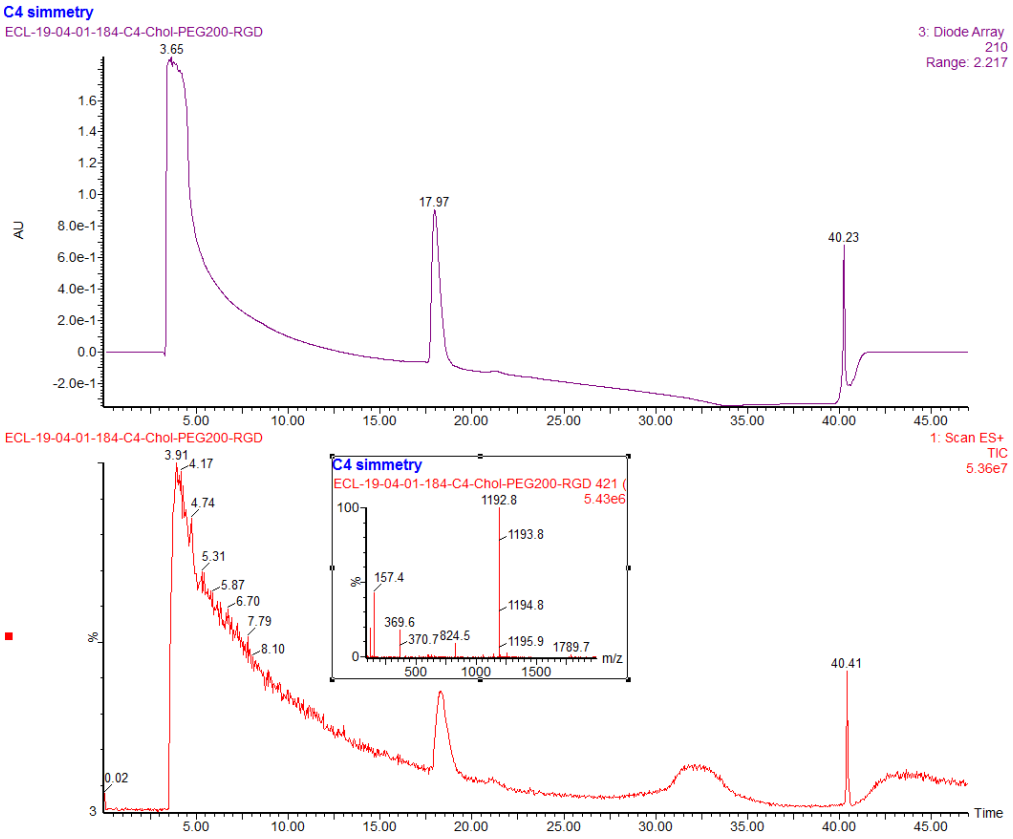


Figure S33. HRMS analysis of Chol-PEG8-RGD, **14**

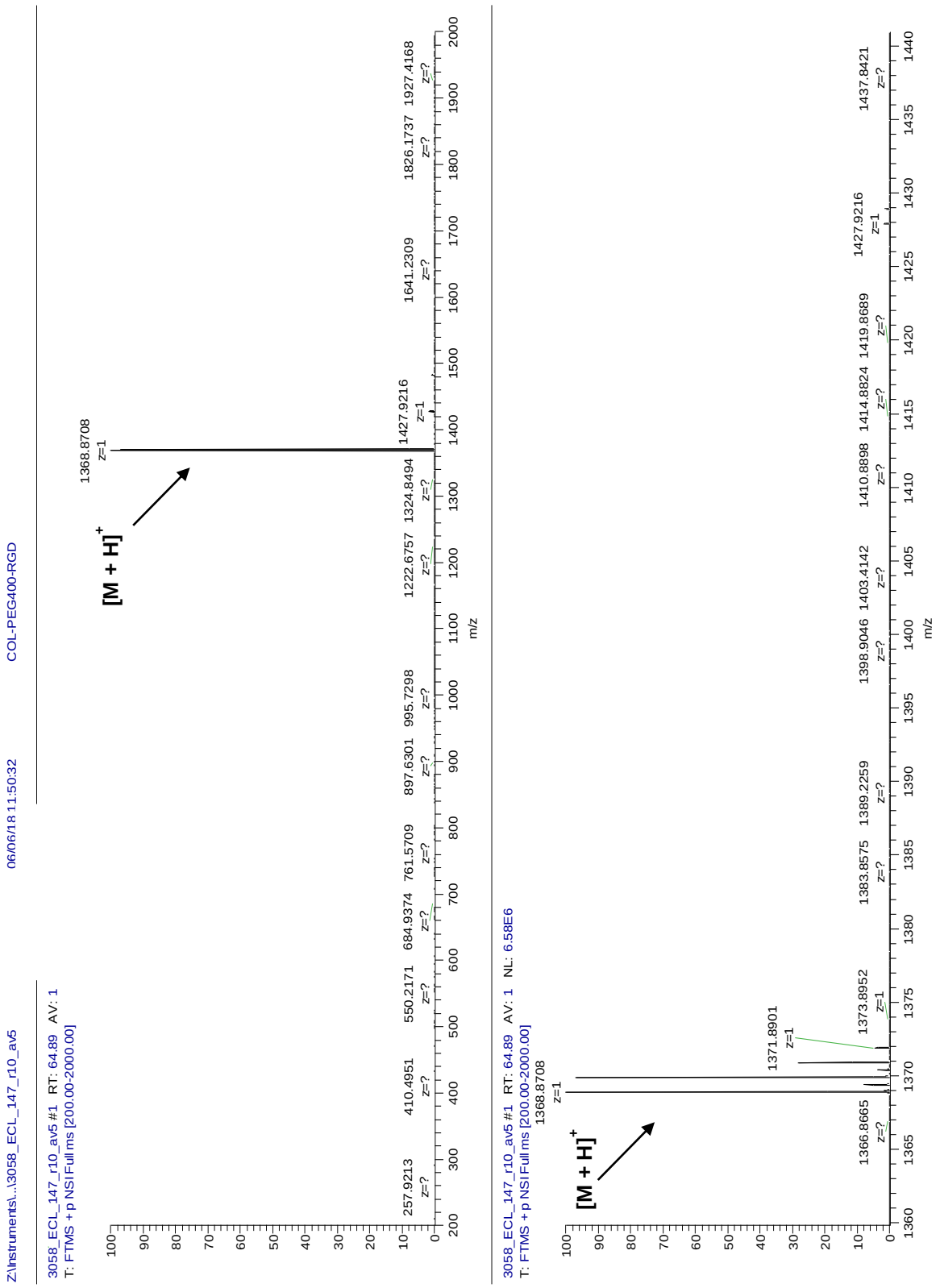


Figure S34. UPLC-PDA-ELSD-MS analysis of Chol-PEG8-RGD, **14**

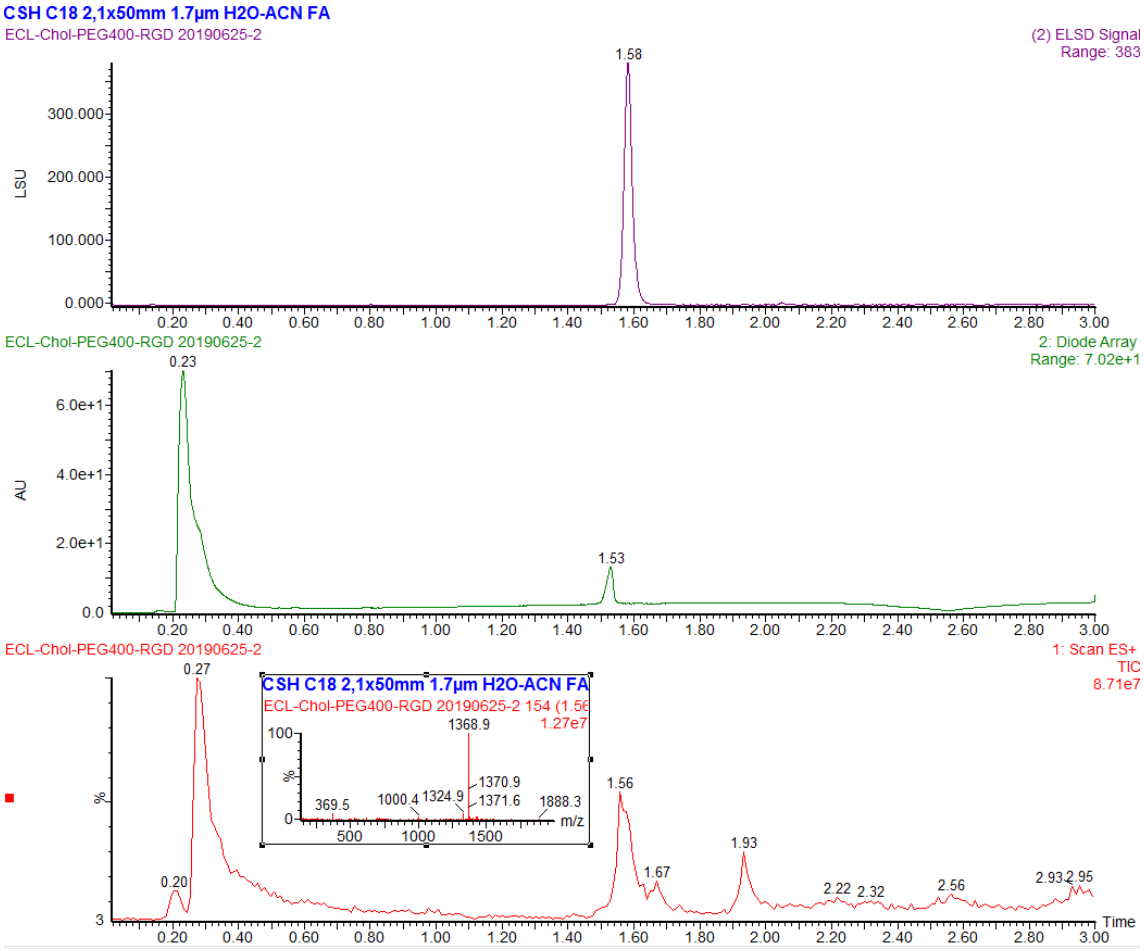


Figure S35. HPLC-PDA-MS analysis of Chol-PEG8-RGD, **14**

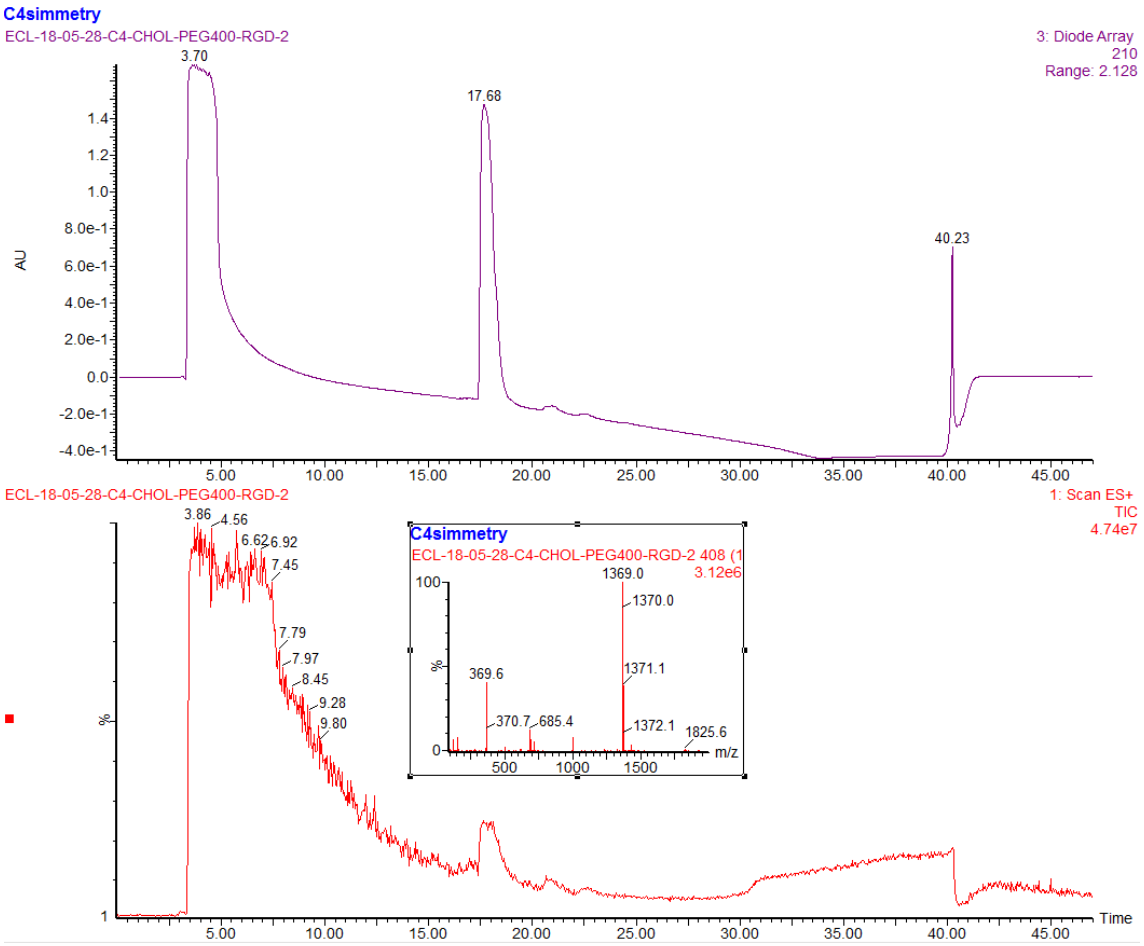


Figure S36. HRMS analysis of Chol-PEG12-RGD, **15**

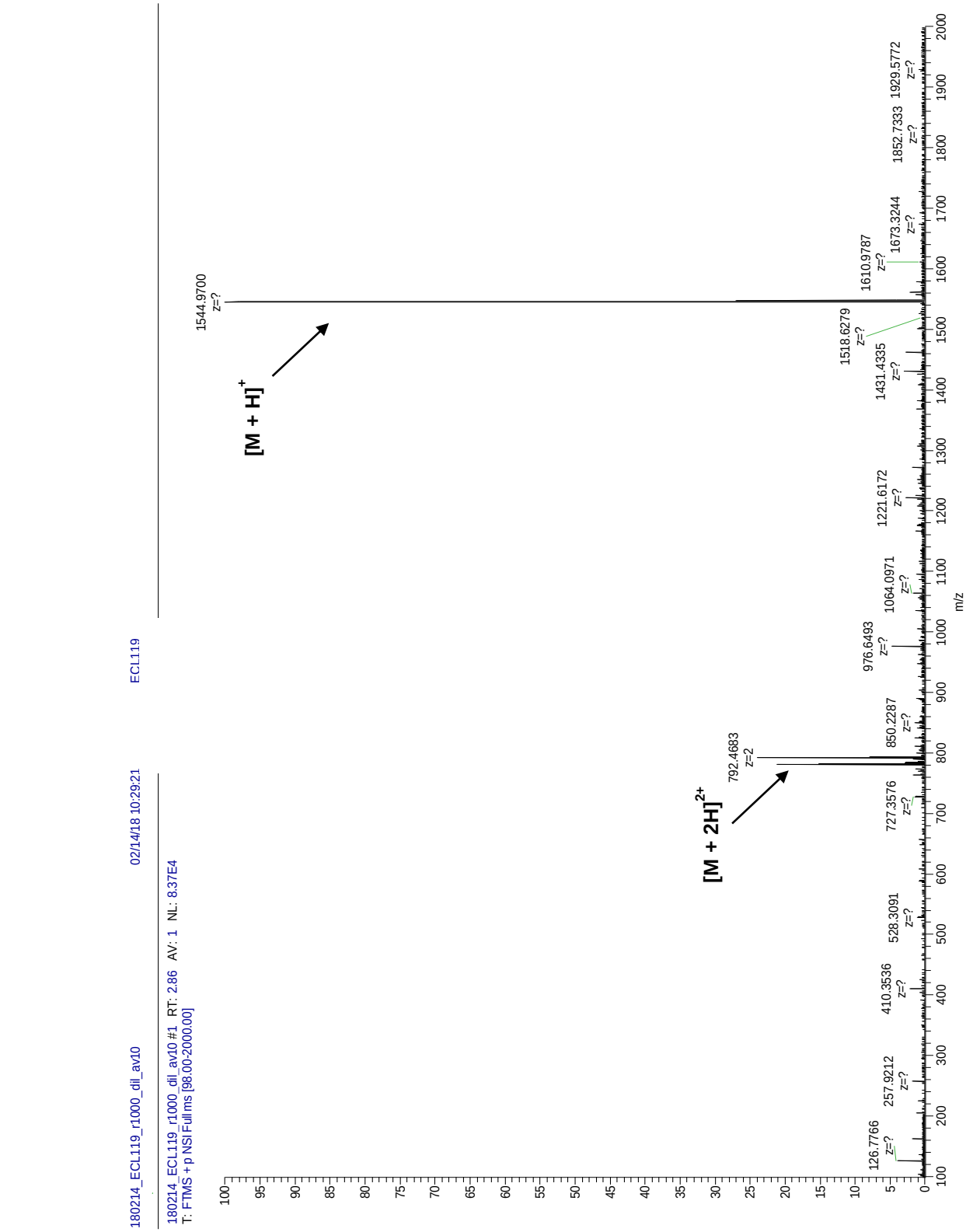


Figure S37. UPLC-PDA-ELSD-MS analysis of Chol-PEG12-RGD, **15**

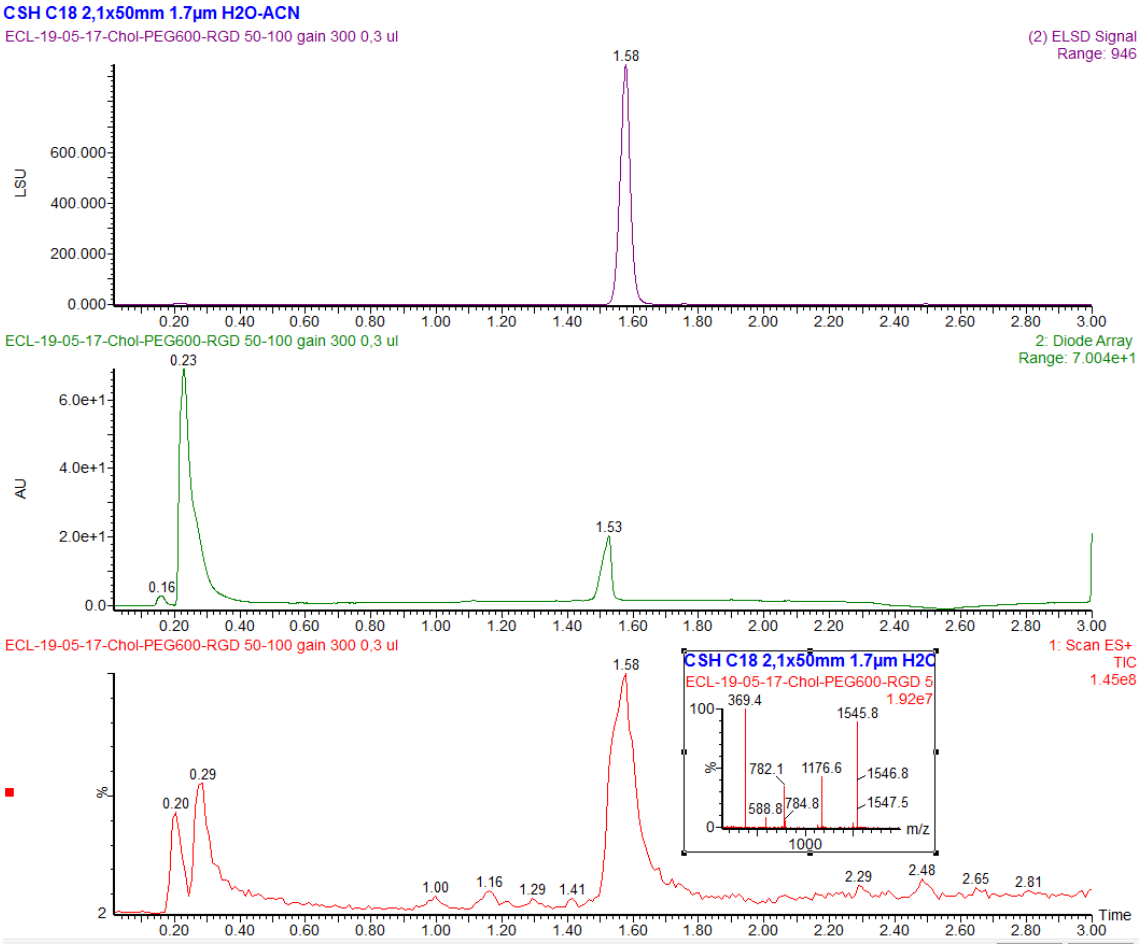


Figure S38. HPLC-PDA-MS analysis of Chol-PEG12-RGD, **15**

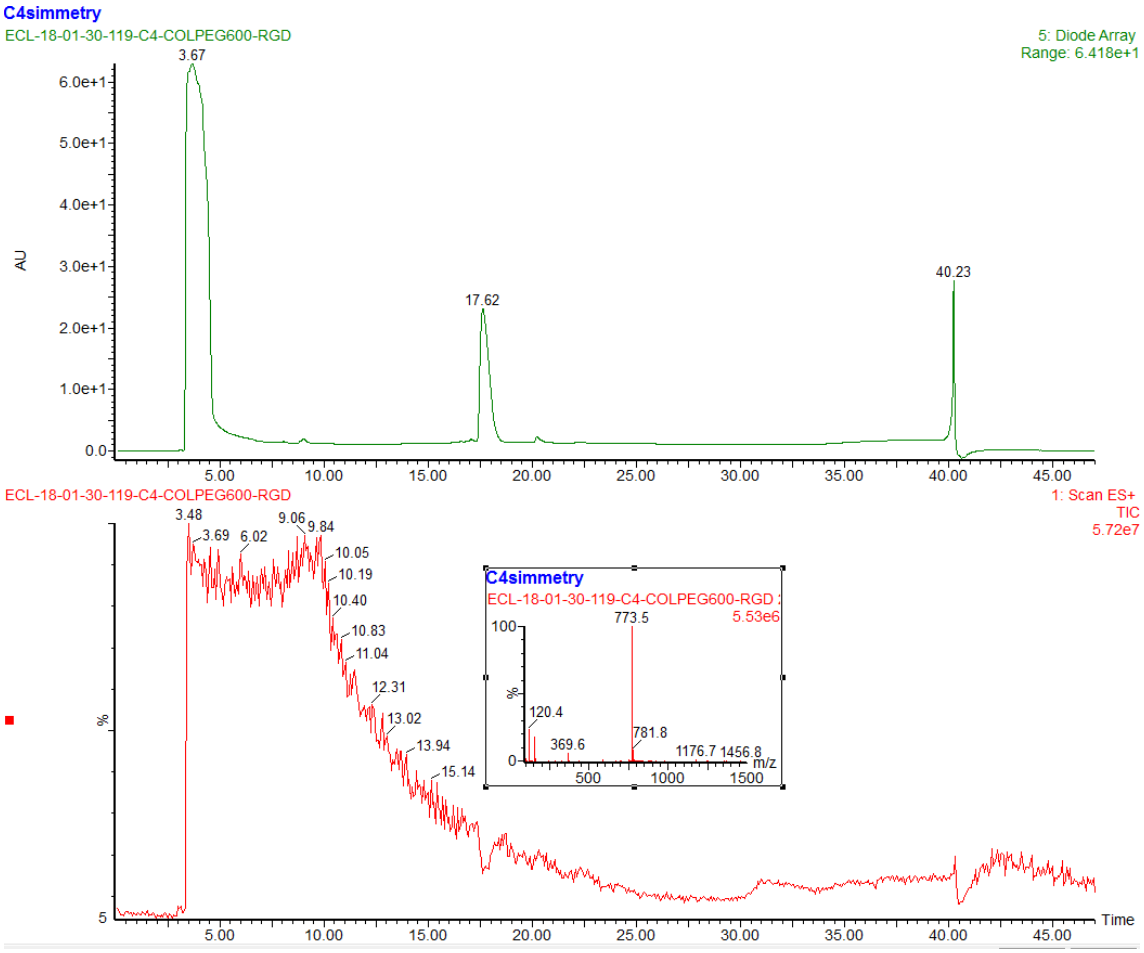


Figure S39. HRMS analysis of Chol-PEG16-RGD, **16**

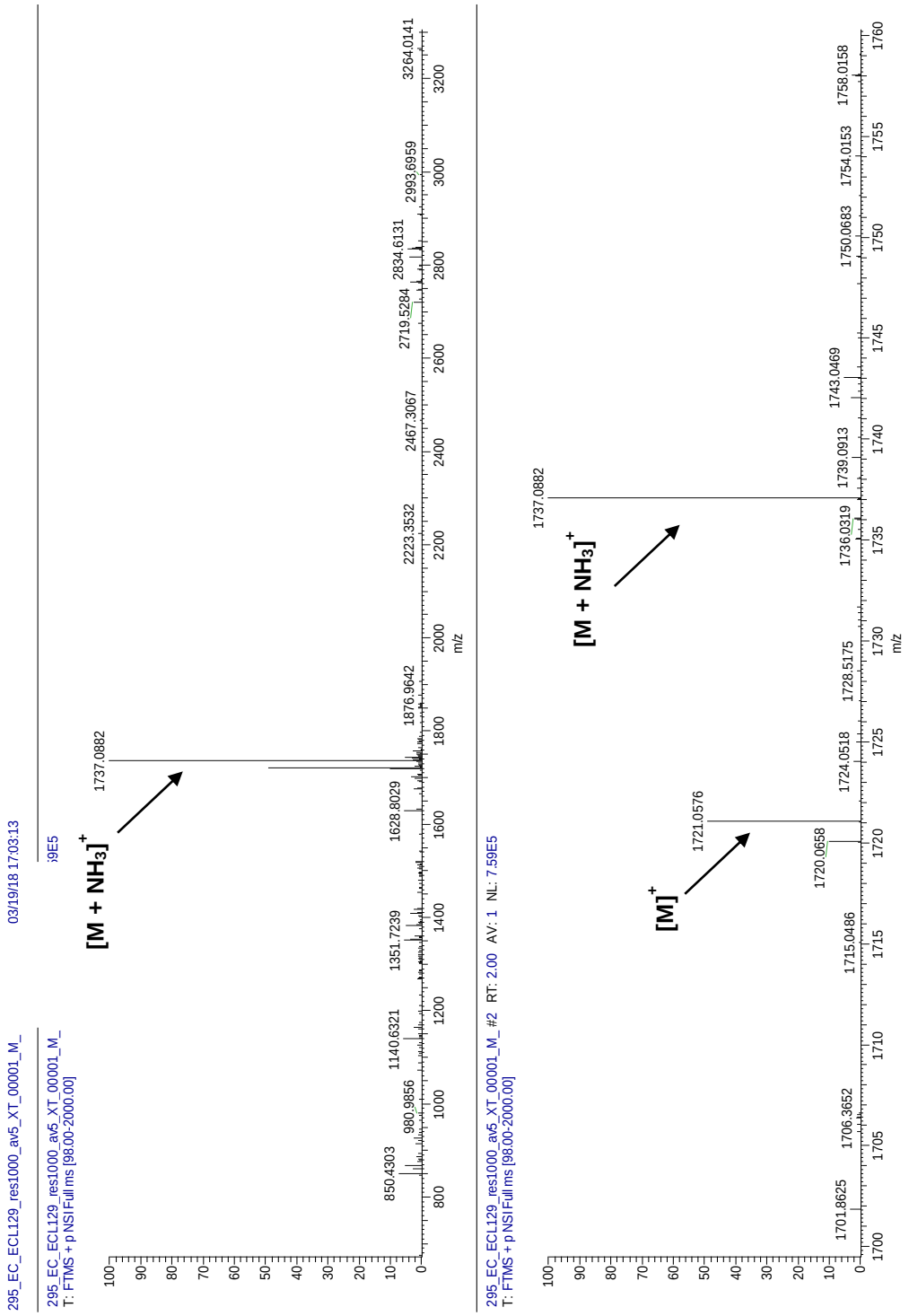


Figure S40. UPLC-PDA-ELSD-MS analysis of Chol-PEG16-RGD, **16**

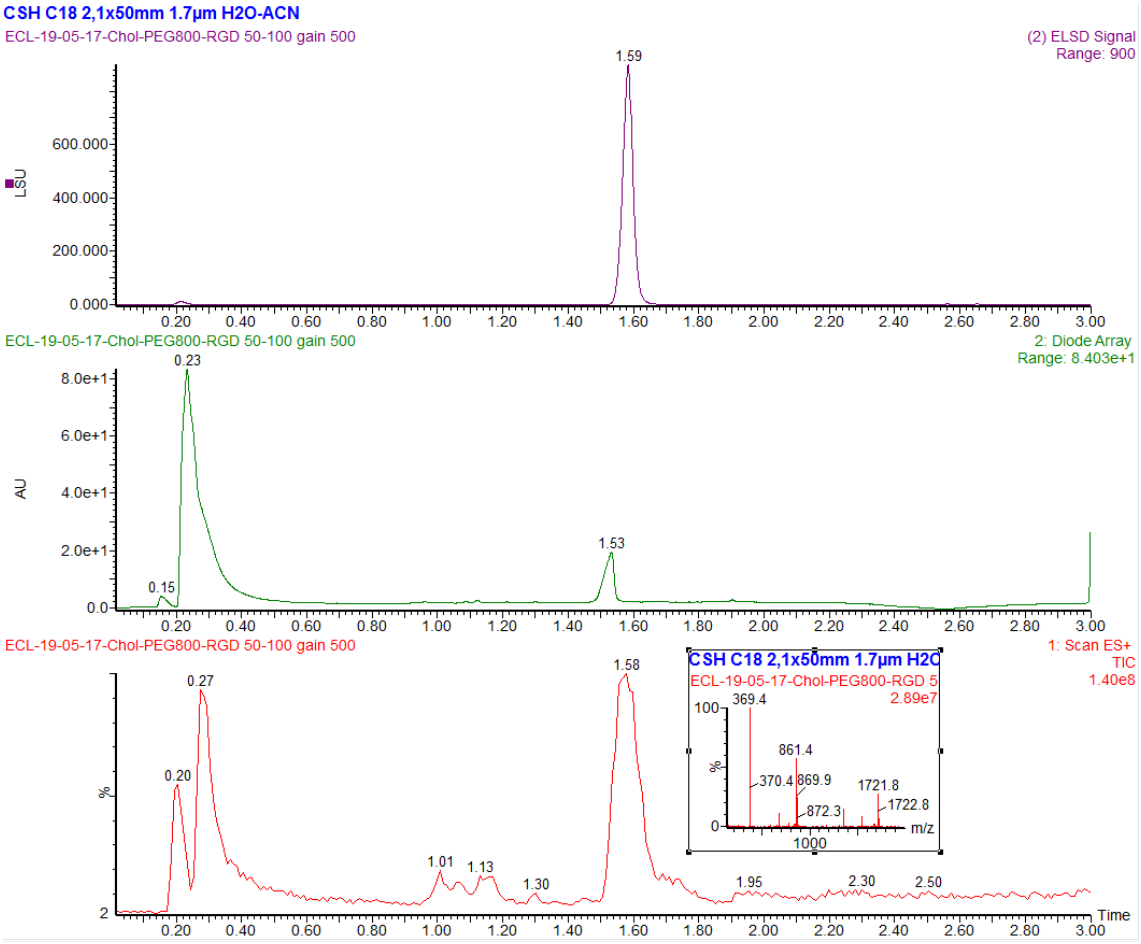


Figure S41. HPLC-PDA-MS analysis of Chol-PEG16-RGD, **16**

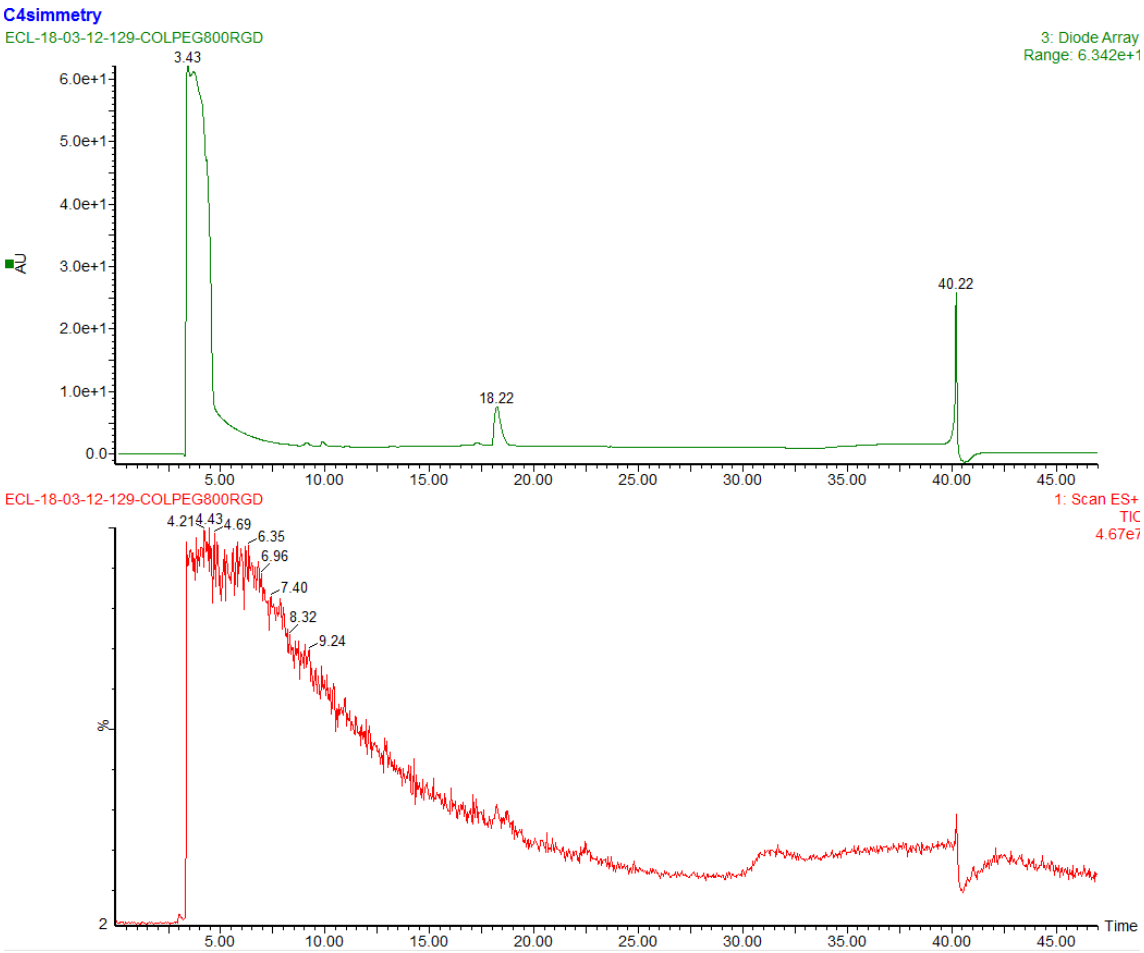


Figure S42. HRMS analysis of Chol-PEG20-RGD, **17**

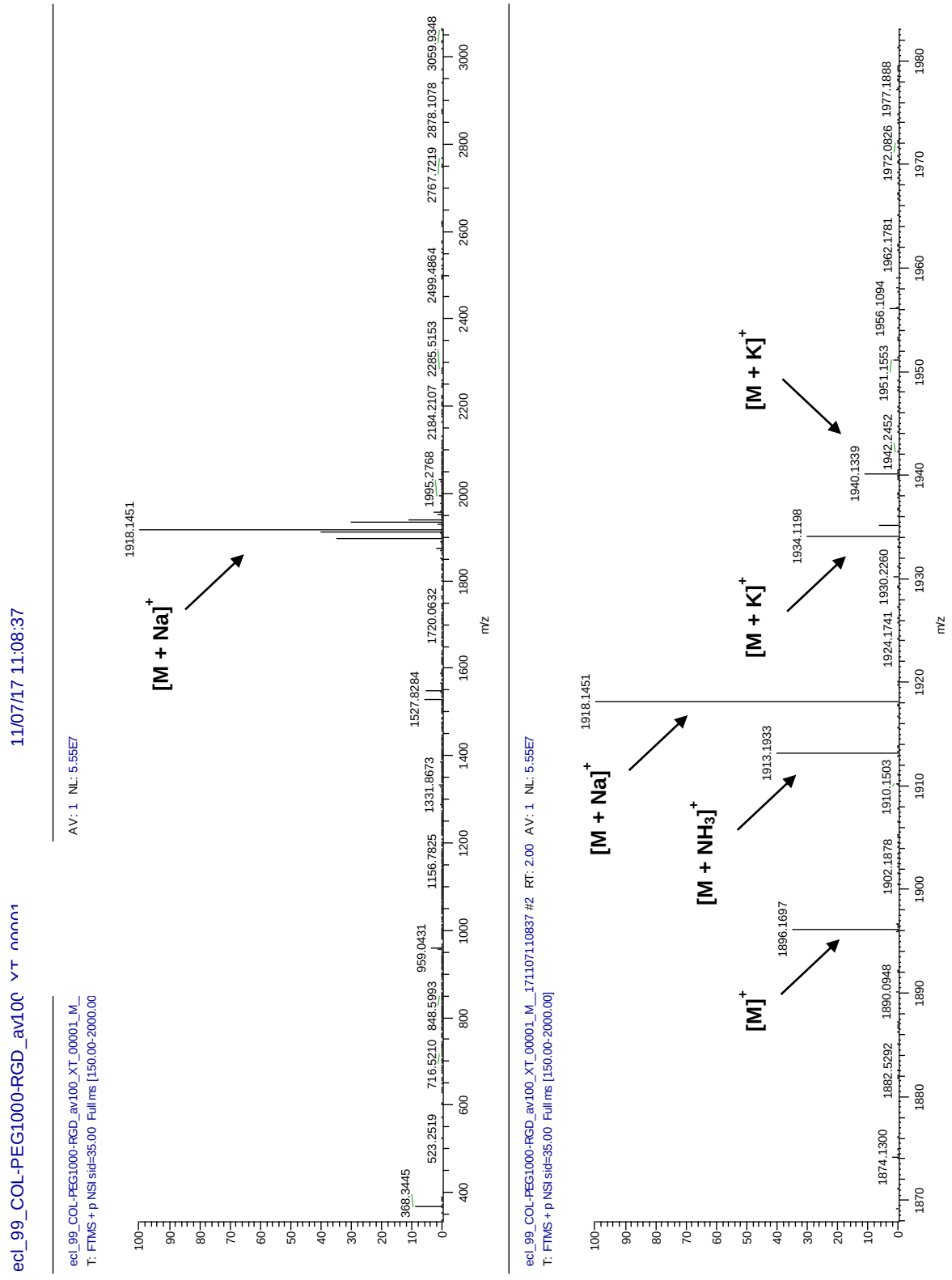


Figure S43. UPLC-PDA-ELSD-MS analysis of Chol-PEG20-RGD, **17**

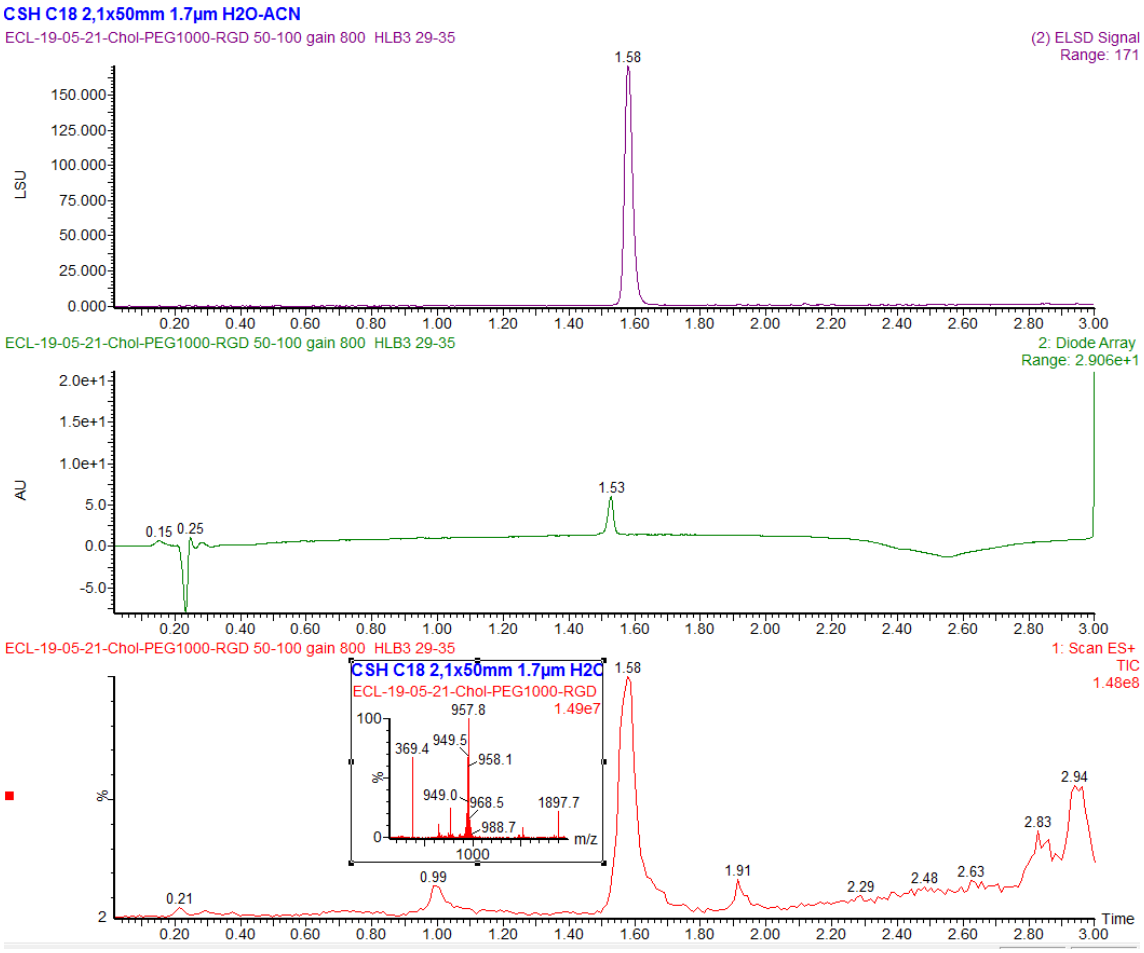


Figure S44. HPLC-PDA-MS analysis of Chol-PEG20-RGD, **17**

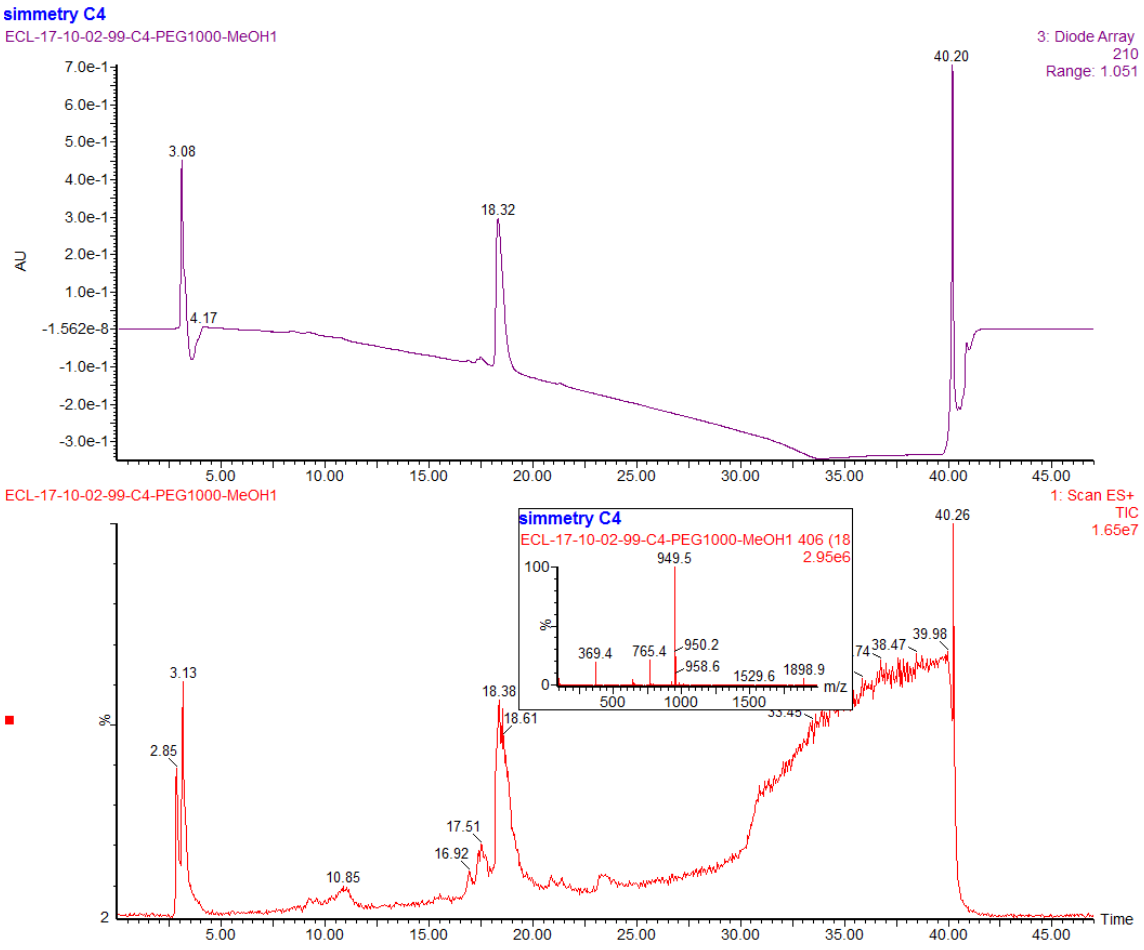


Figure S45. [1]H-NMR analysis of Chol-PEG4-maleimide, **20**

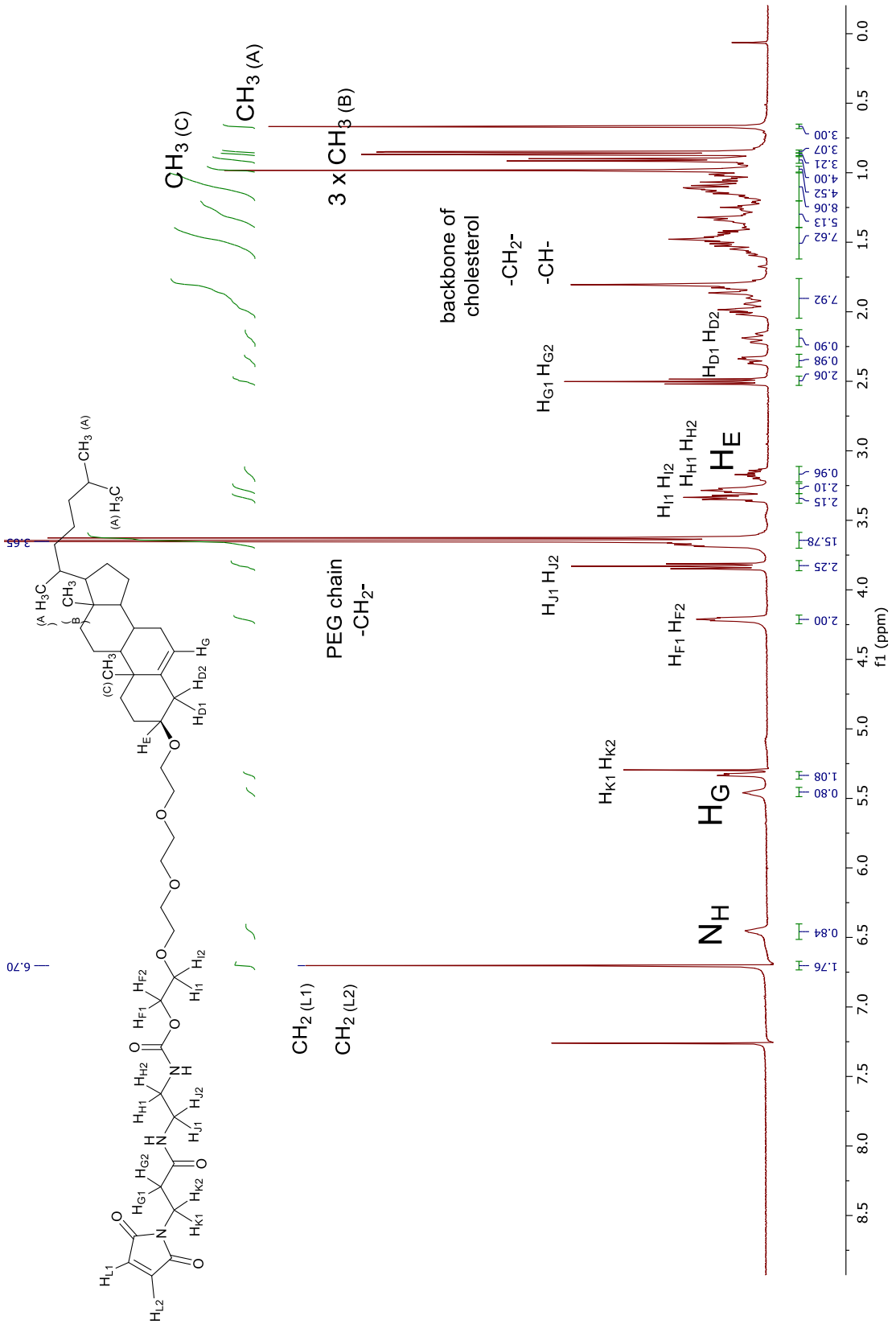


Figure S46. ^{13}C-NMR analysis of Chol-PEG4-maleimide, **20**

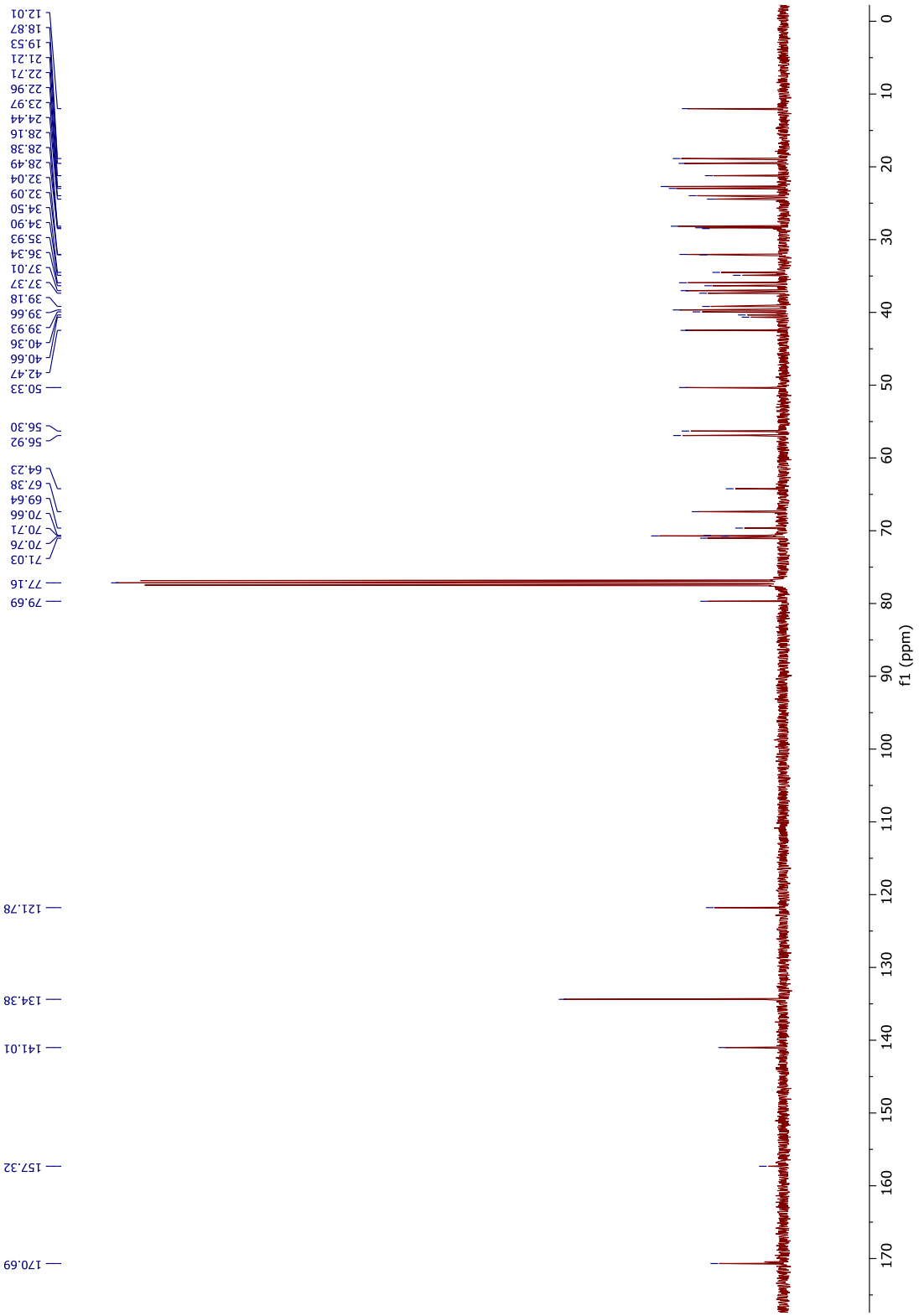


Figure S47. HRMS analysis of Chol-PEG4-maleimide, **20**

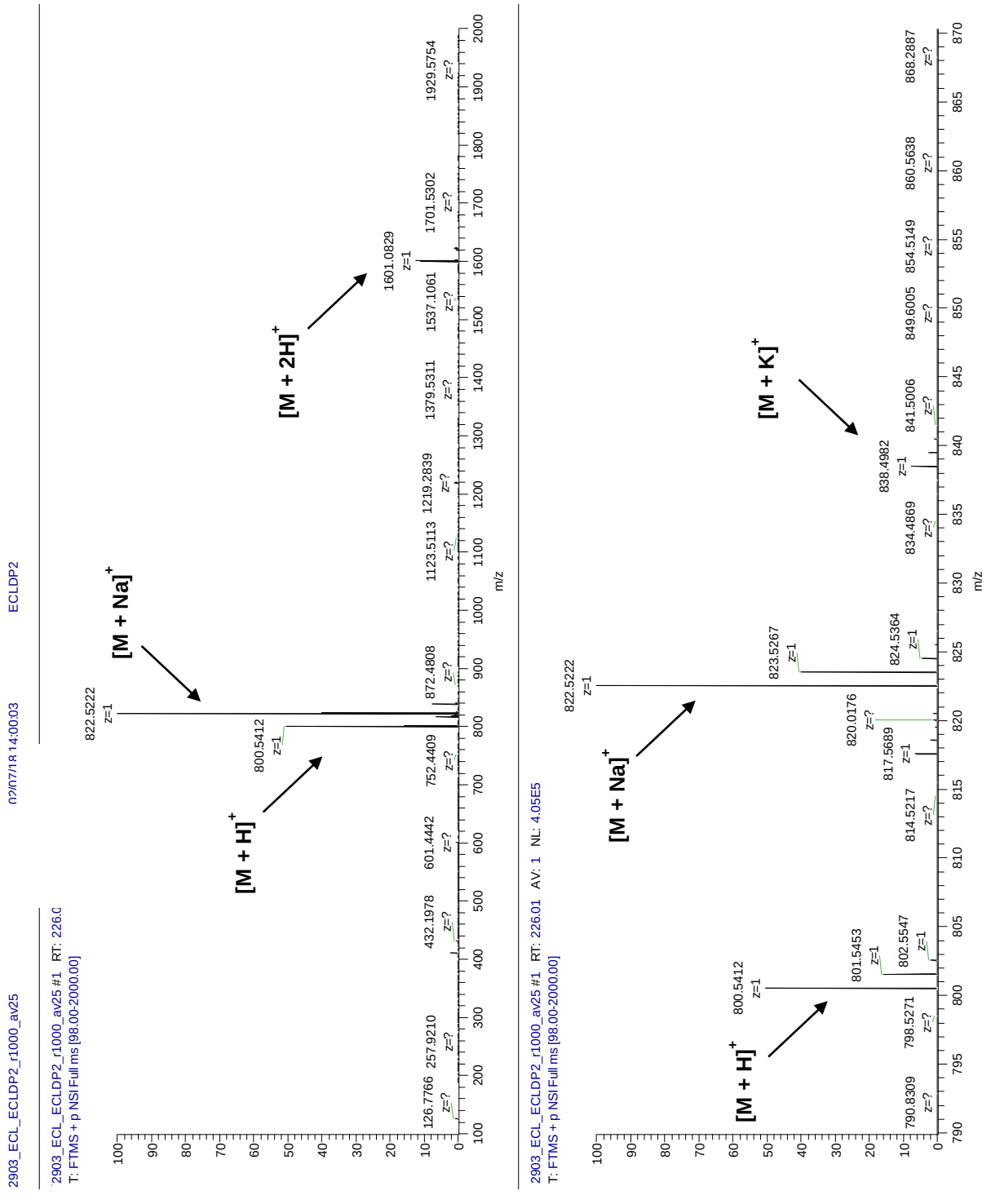


Figure S48. HRMS analysis of Chol-PEG4-Tf_1, **22**

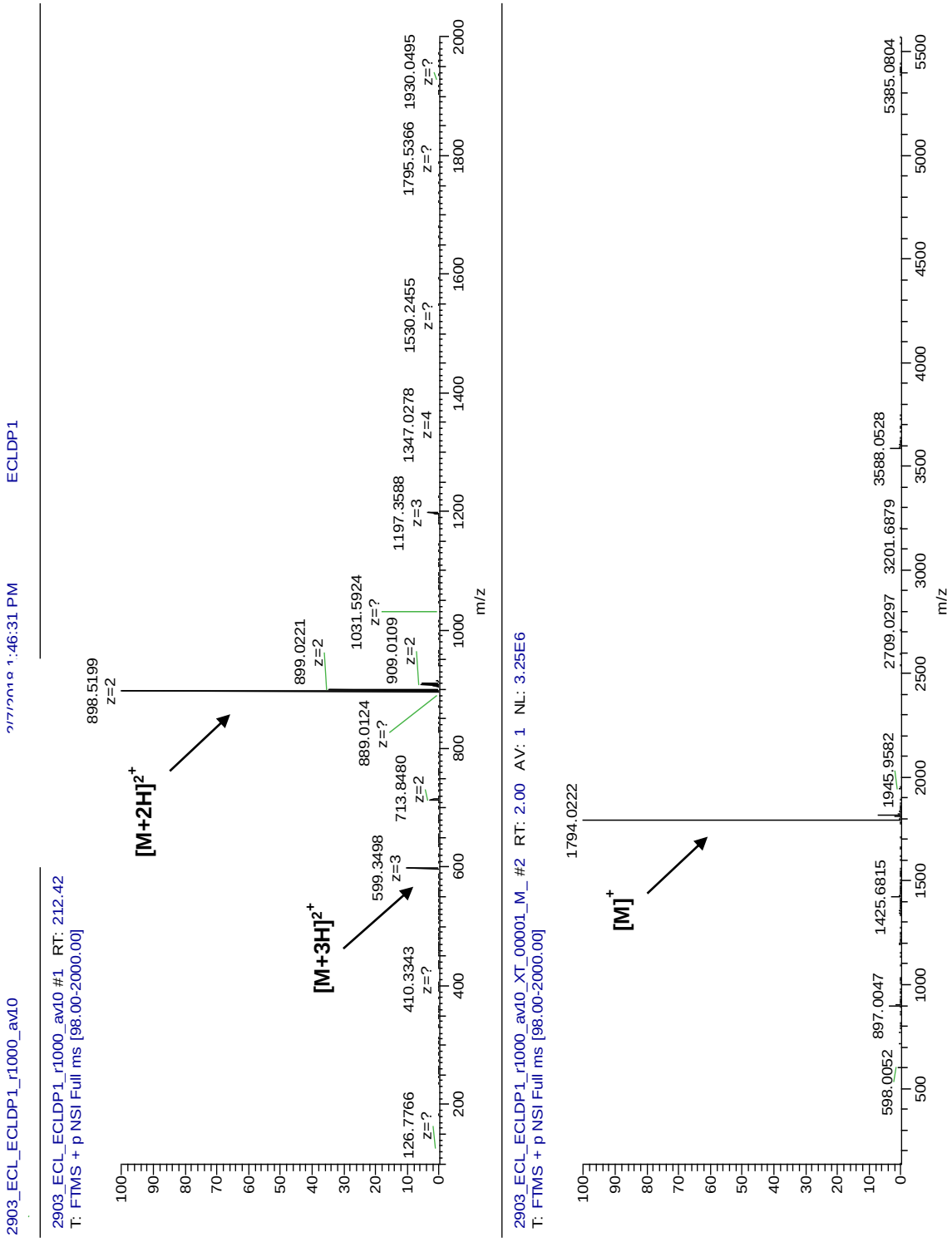
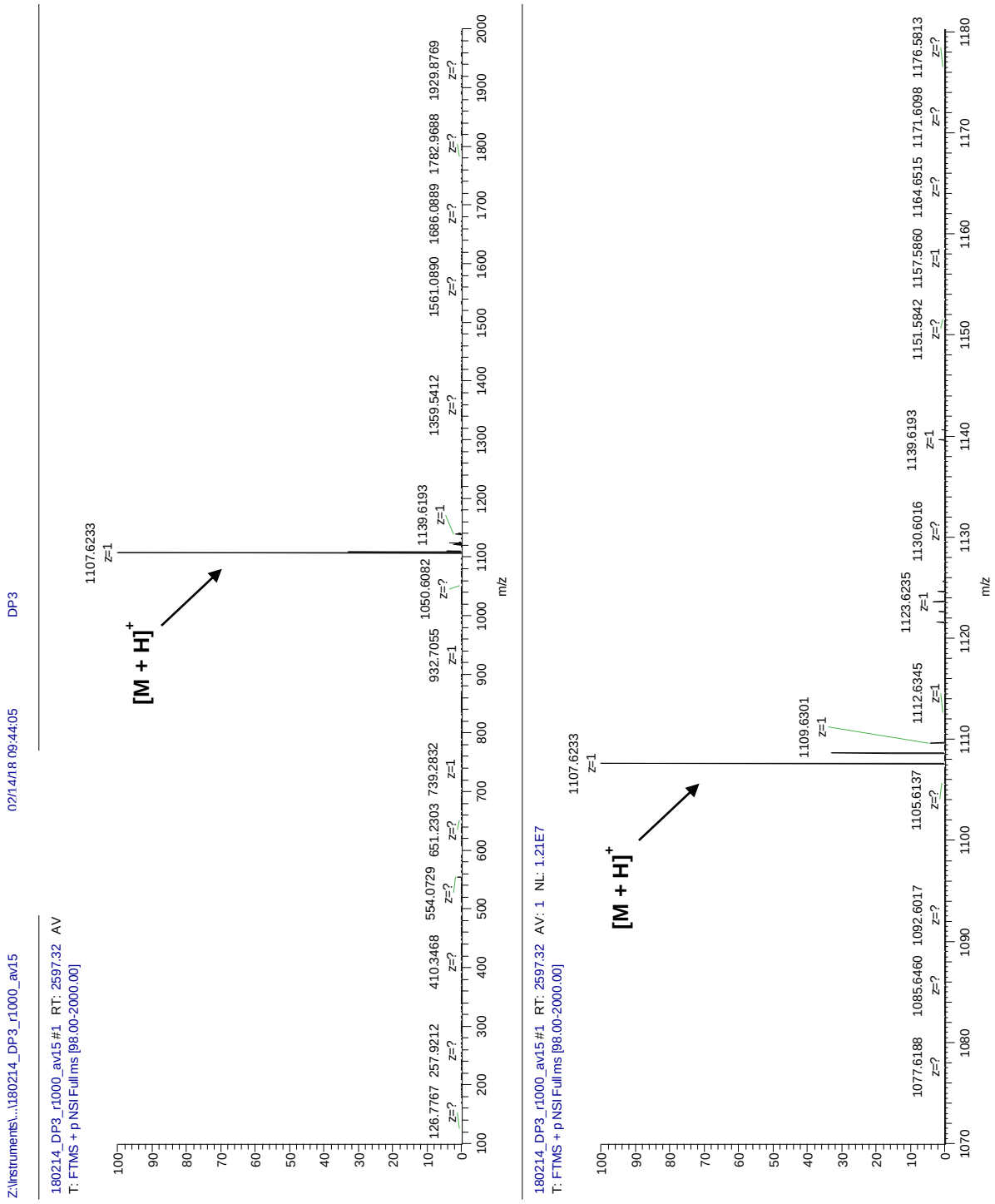


Figure S49. HRMS analysis of Chol-PEG4-gluthation, **23**



3.PDI estimation for Chol-PEGn and Chol-PEGn-RGD compounds

Polydispersity index (PDI)

The polydispersity indes (PDI) is a common measure of polymer or oligomer heterogeneity. It is defined as the ratio between the number averaged molecular weight and the weight averaged molecular weight of the sample (equation 1).

$$\text{PDI} \equiv \frac{Mw}{Mn} \equiv \frac{\sum_I NiMi^2 / \sum_I NiMi}{\sum_I Ni\text{Mi} \,/\, \sum_I Ni}$$

Equation 1: Polydispersity Index

Evaluation of Current PEG Sources

The PDIs values were calculated following the methodology described by Davis et al[1] from the MS.

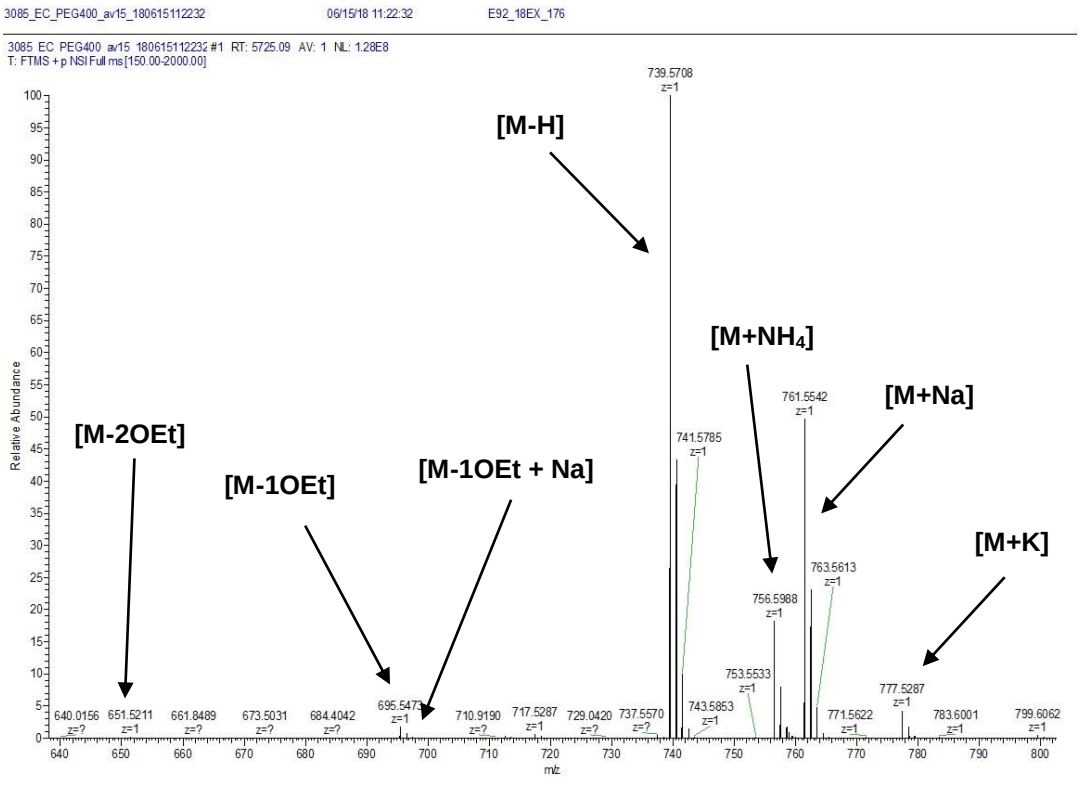


Figure S50. HR-ESI-MS analysis of Chol-PEG8, **5**: peaks **A** as H^+H^+, $H^+NH_4^+$, H^+Na^+, H^+K^+ dications; **B** (inset) is the H^+H^+, H^+Na^+ dication of Chol-PEG7 homologue; **C** (inset) is the H^+H^+, dication of Chol-PEG6 homologue. Heights of peaks A, B, C are in ratio 100:1.17:0.07 = 98.7% oligomer purity, PDI = 1.000052.

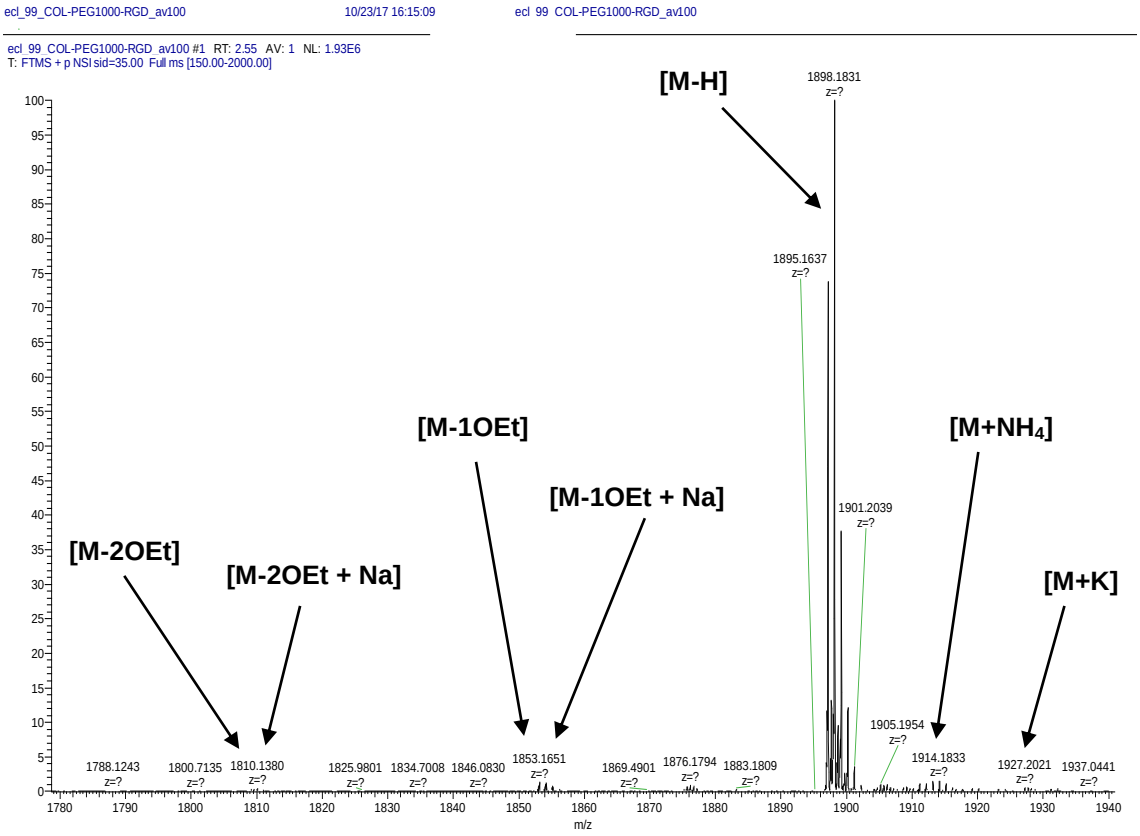


Figure S51. HR-ESI-MS analysis of Chol-PEG20-RGD, **17**: peaks **A** as H^+H^+, $H^+NH_4^+$, H^+K^+ dications; **B** (inset) is the H^+H^+, H^+Na^+ dication of Chol-PEG19-RGD homologue; **C** (inset) is the H^+H^+, H^+Na^+ dication of Chol-PEG18 homologue. Heights of peaks A, B, C are in ratio 100:2.02:0.42 = 97.6% oligomer purity, PDI = 1.00001936.

4. High resolution NMR experiments of Chol-PEGn-RGD and c(RGDfK) peptide. Experiments were recorded on a Bruker Avance III 600-MHz spectrometer equipped with a quadruple (^1H, ^{13}C, ^{15}N, ^{31}P) resonance cryogenic probe head and a z-pulse field gradient unit at 298 K or a Varian Mercury 400, equipped with a double resonance wide-band probe head and a z-pulse field gradient at 298K (IRB Barcelona).

c(RGDfK) peptide analysis: 2D TOCSY and NOESY experiments were recorded in DMSO and in H_2O/D_2O with 50ms and 300ms mixing times, with 2K points in the direct dimension and 600 FIDs. Spin systems corresponding to each amino acid were identified due to their characteristic signals. The correct sequence connectivity and the cyclic nature of the peptide was corroborated using the NOE patterns.

Chol-PEGn-RGD derivatives analysis: ^{1}H 1D experiments were acquired with 32768 points and 16 scans, and ^{13}C 1D experiments were acquired with 65535 points and 2048 scans. For PEG length verification, the ethylene peaks (67.43ppm) were integrated in the corresponding 1D ^{13}C spectra and compared in the different experiments. 2D ^{1}H-TOCSY and ^{1}H-NOESY experiments for Chol-PEGn-RGD **(13, 14, 15**, **16** and **17)** were acquired in 90 H_2O/10%D_2O (mixing times of 50ms and 300ms, respectively). Each experiment was acquired as 2K points in the direct dimension and 600 FIDs in the indirect one with 16 and 48 scans respectively.

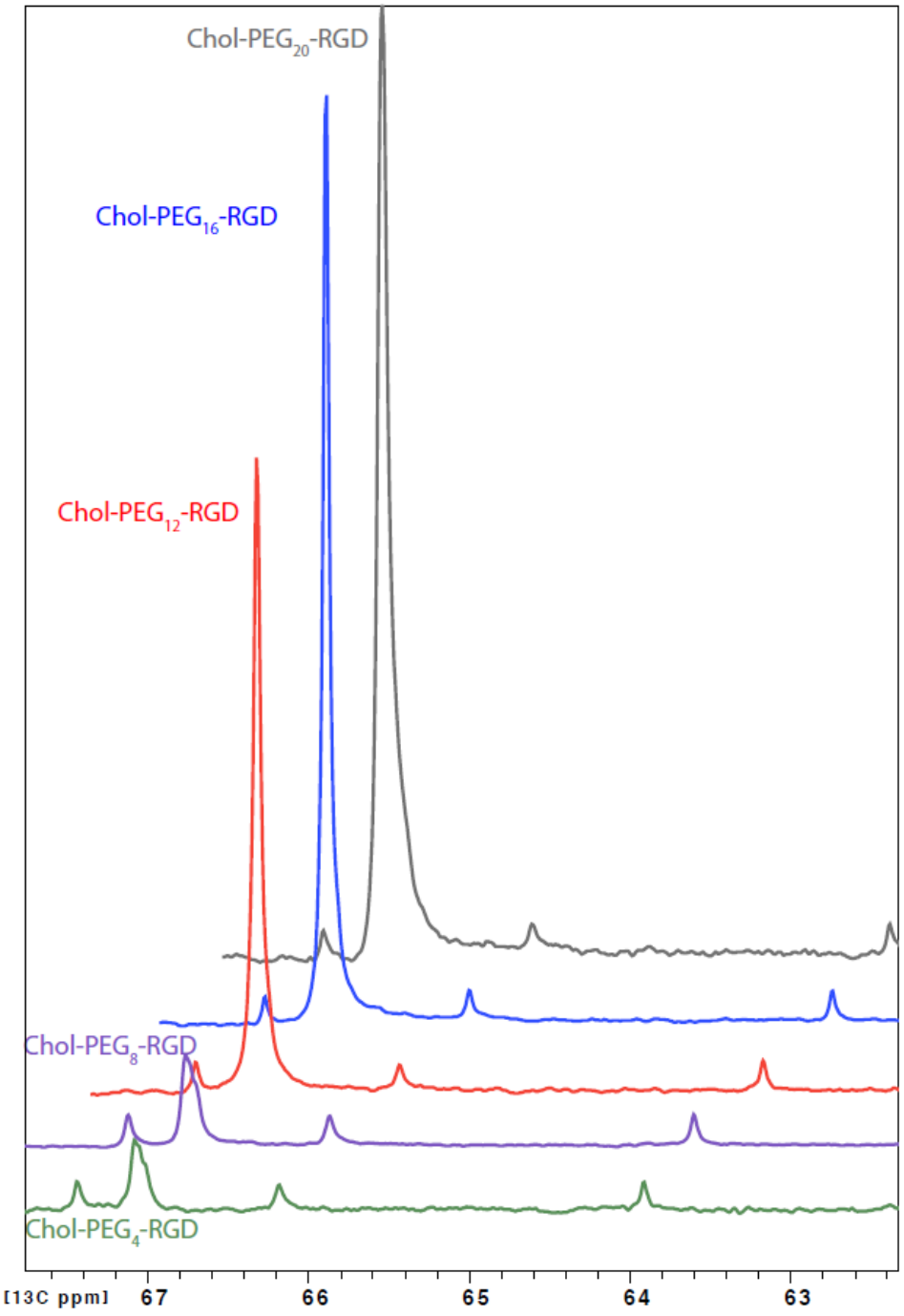


Figure S52: Comparison of the ^{13}**C**-NMR spectra for the different Chol-PEG moieties showing the carbon signals of the PEG chain (-O**C**H$_2$**C**H$_2$O-).

4.1 Assignment of RGD peptide in DMSO and H_2O

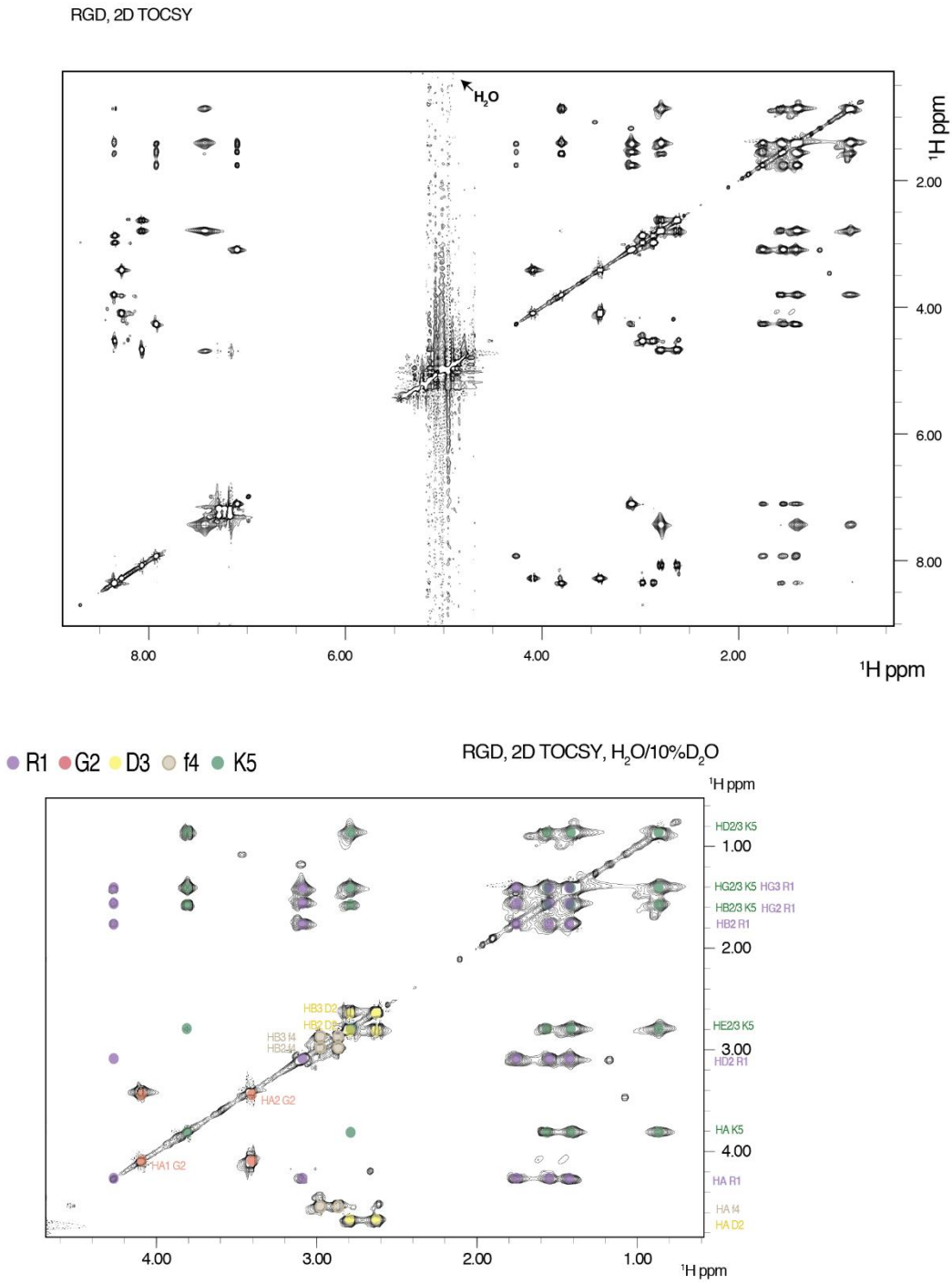


Figure S53: cyclic (RGDfK) peptide (**12**) 2D, TOCSY acquired in H_2O/D_2O (up). Side-chain region of the TOCSY (down). All resonances are indicated. Spin systems are colored as indicated in the figure, to facilitate the identification of the residues

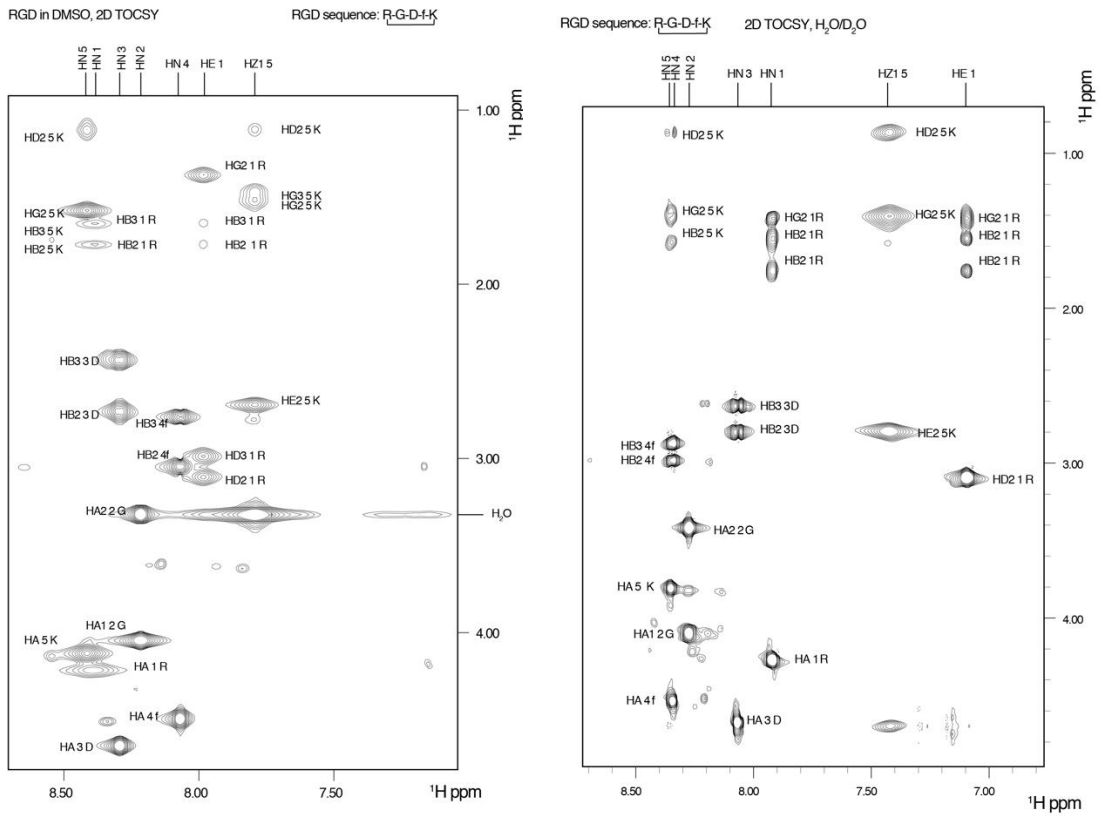


Figure S54: cyclic (RGDfK) peptide (**12**) 2D, TOCSY. Amide side-chain region of the peptide in DMSO (left) and H2O/D2O (right). Assignments are indicated for all residues.

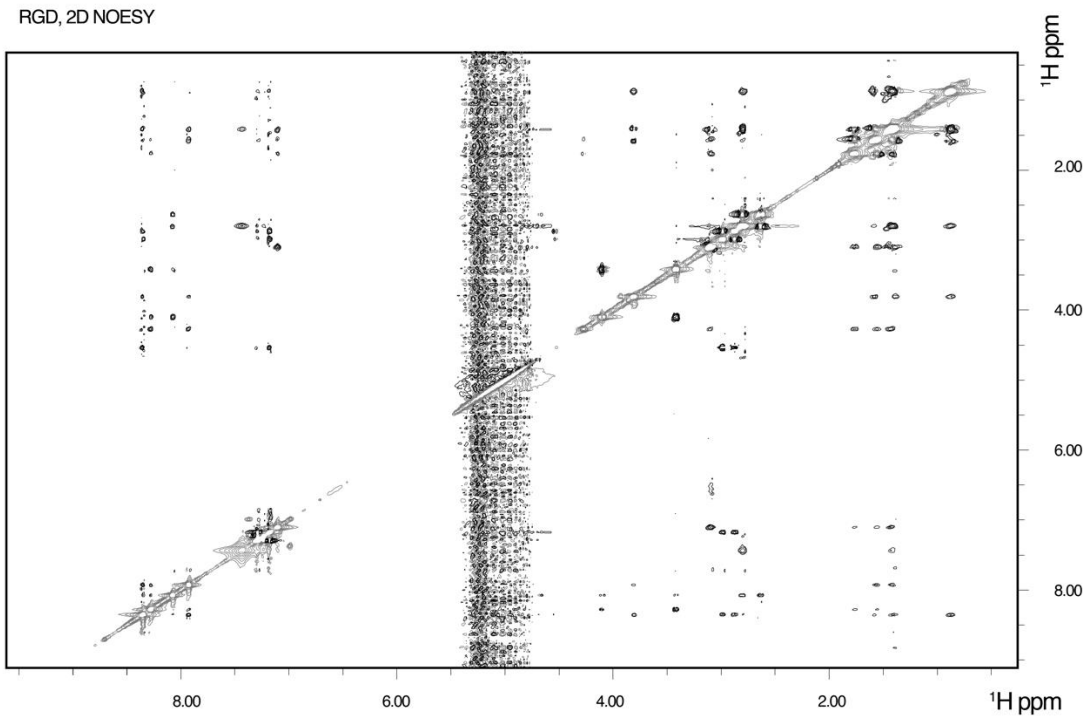


Figure S55: cyclic (RGDfK) peptide (**12**) 2D, NOESY.

4.2 Assignment of Chol-PEG12-RGD, 15

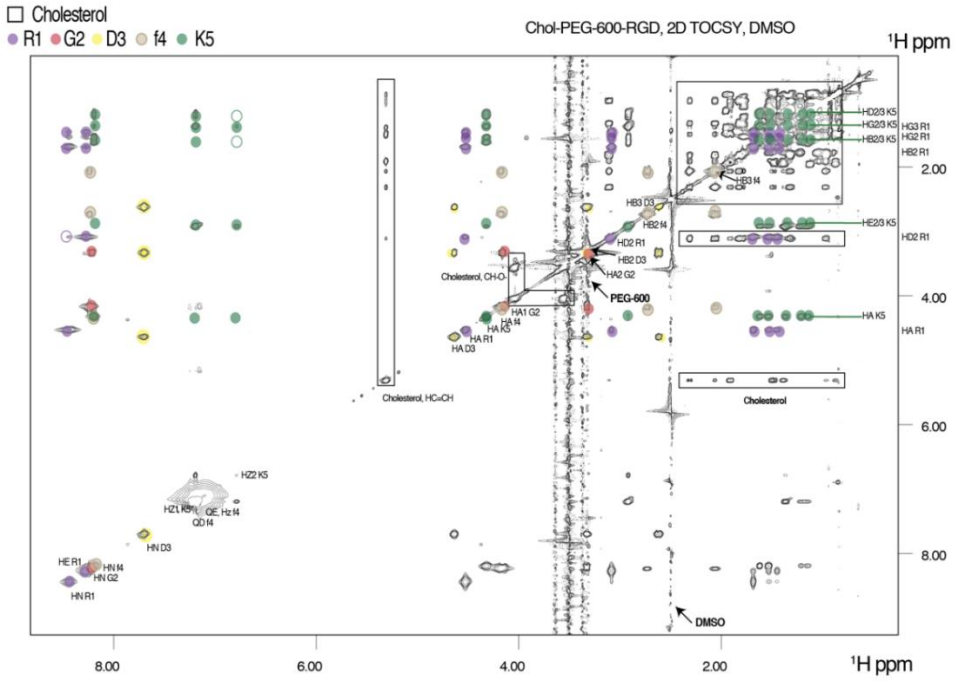


Figure S56. 2D TOCSY in DMSO, of Chol-PEG12-RGD (**15** or Chol-PEG600-RGD). Full spectrum.

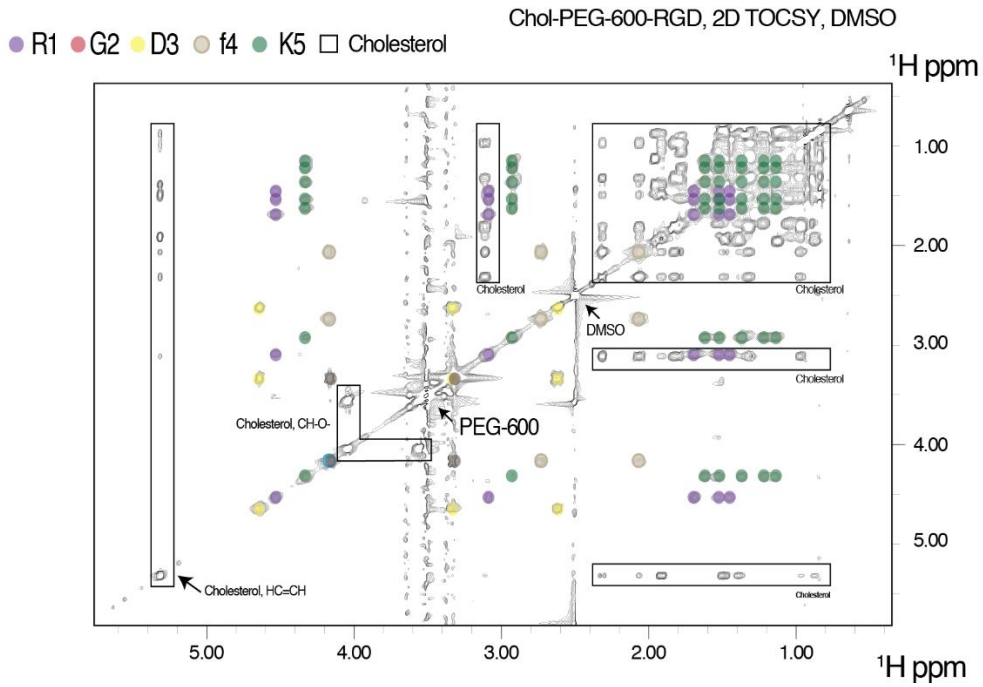


Figure S57. Expanded region of the TOCSY shown as Figure S56, displaying the region corresponding to the aliphatic resonances. Cholesterol resonances are indicated as boxes. Each of the five amino acids are indicated with colors (R1 in violet, G2 in red, D3 in yellow, f4 in beige and K5 in green). All protons present in the corresponding amino acid spin system are colored.

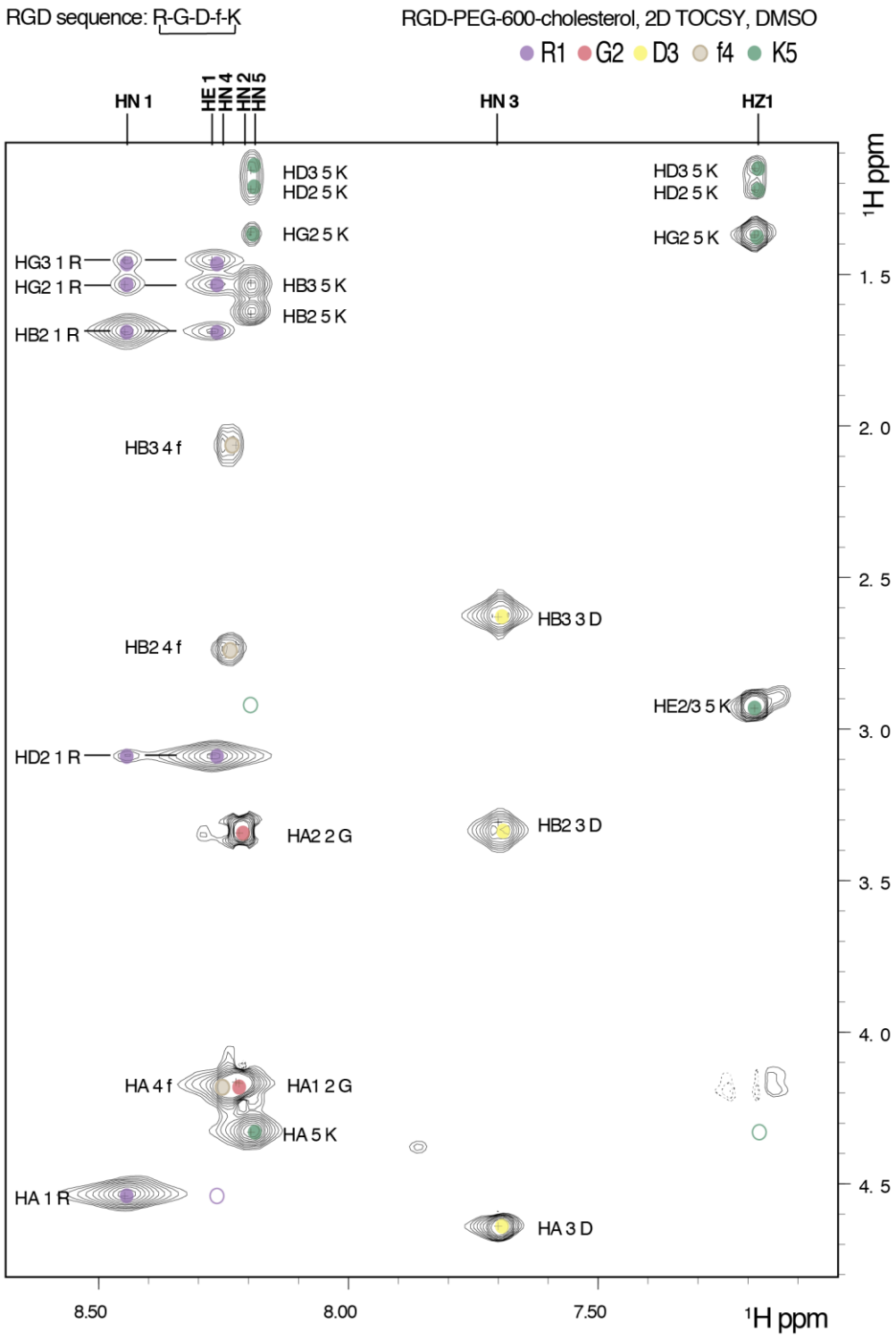


Figure S58: Amide-aliphatic region of the TOCSY shown in Figure S56. Only the amino acids and the R1 and K5 side chains are visible in this region. All peaks are colored as in Figure S57 and labeled with the corresponding assignments.

4.3 Assignment of Chol-PEG20-RGD, 17

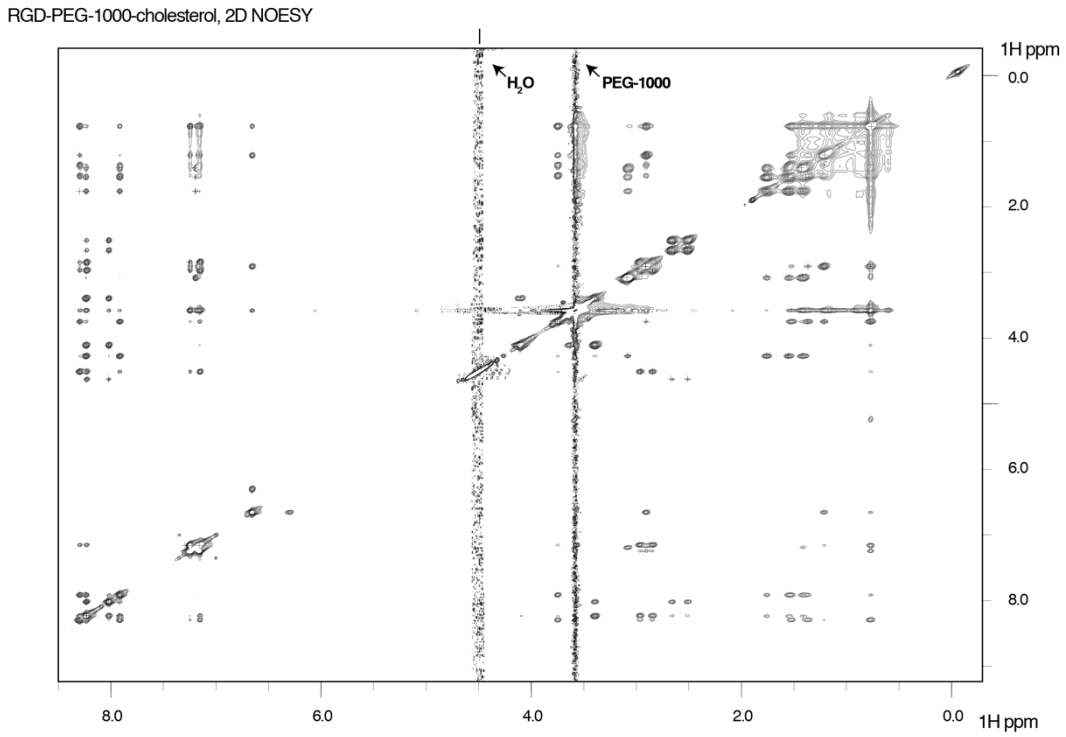


Figure S59. 2D NOESY of Chol-PEG20-RGD (**17**, also Chol-PEG1000-RGD) sample in 90% H_2O / 10% D_2O. Full spectrum. The resonances of the water (solvent) and PEG are indicated with arrows.

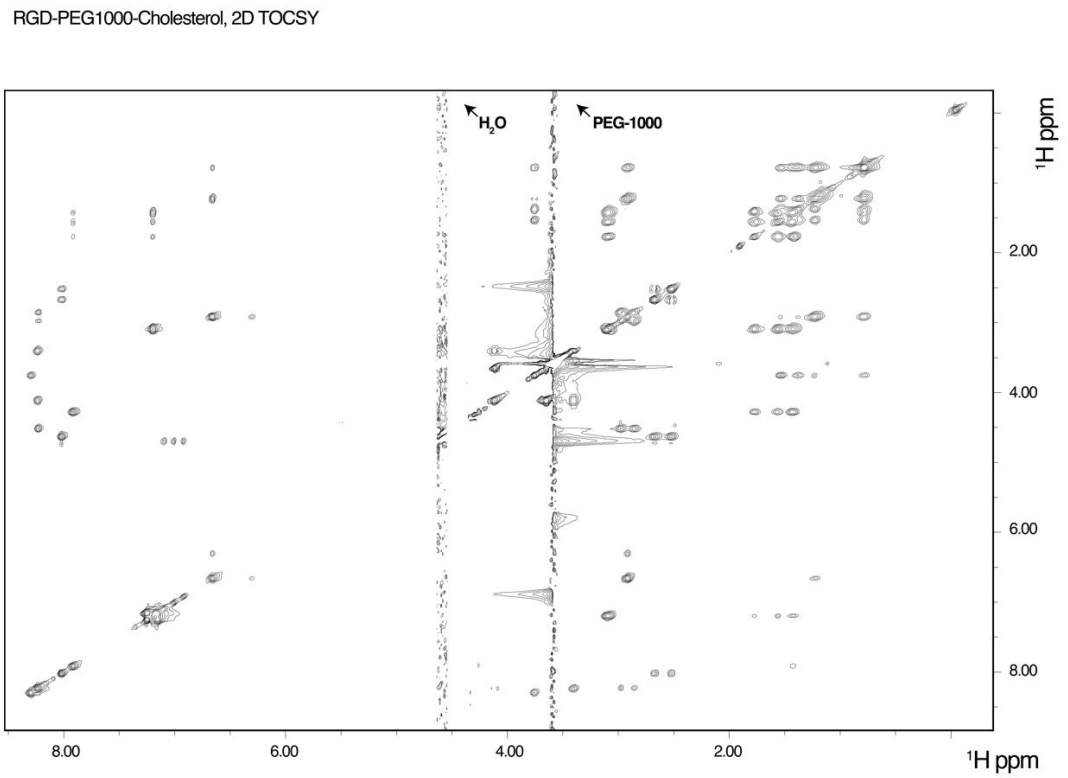


Figure S60. 2D TOCSY of Chol-PEG20-RGD (**17**, also Chol-PEG1000-RGD) sample in 90% H_2O / 10% D_2O. Full spectrum. Water (solvent) and PEG resonances are indicated with arrows.

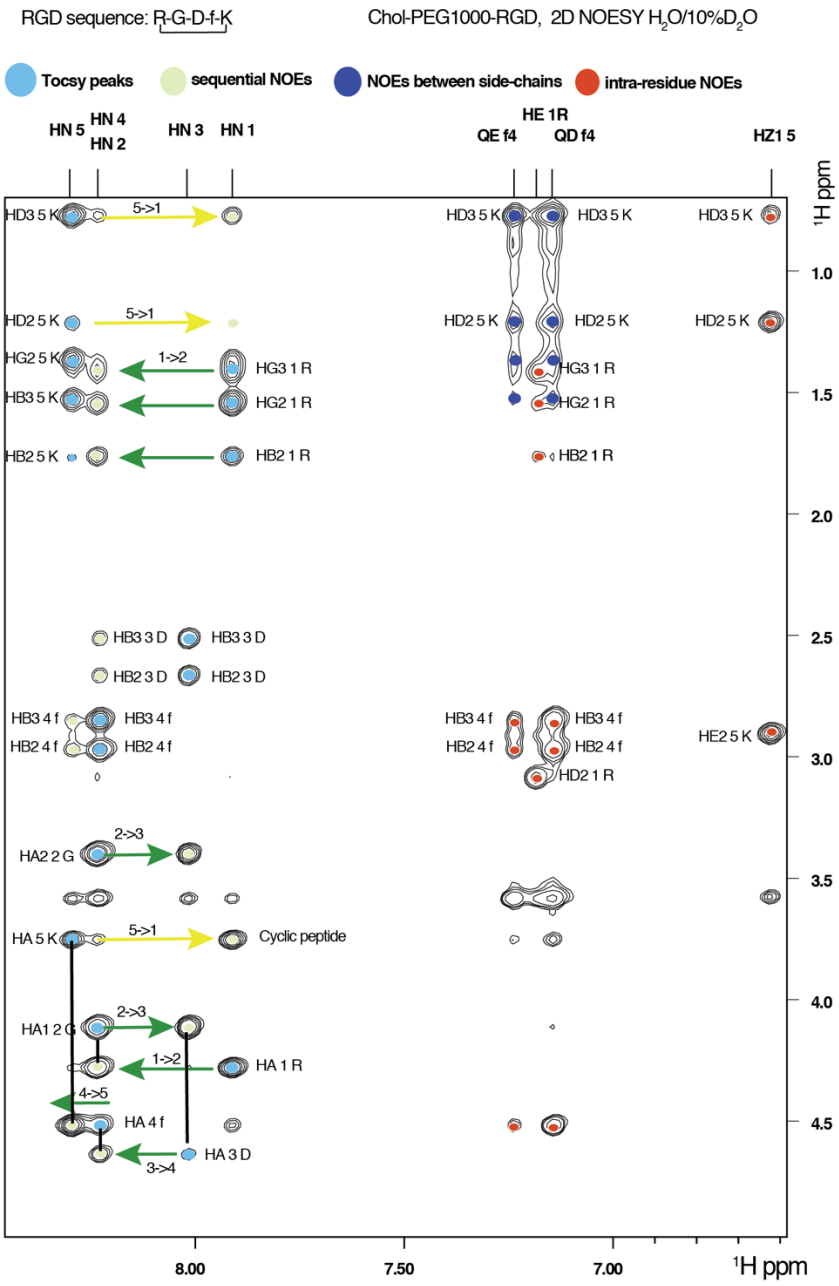


Figure S61. Amide-aliphatic region of the NOESY shown in Figure S59. All amino acids and the R1 and K5 side chains are visible in this region. All intra-residue peaks are colored in blue whereas the NOEs that allow us to perform the sequential assignment are indicated in light green. Sequential connectivity is indicated with arrows, Side/chain side-chain and aromatic side/chain NOEs are colored in royal blue and red respectively. All assignments are indicated. Amide chemical shifts are indicated on the top.

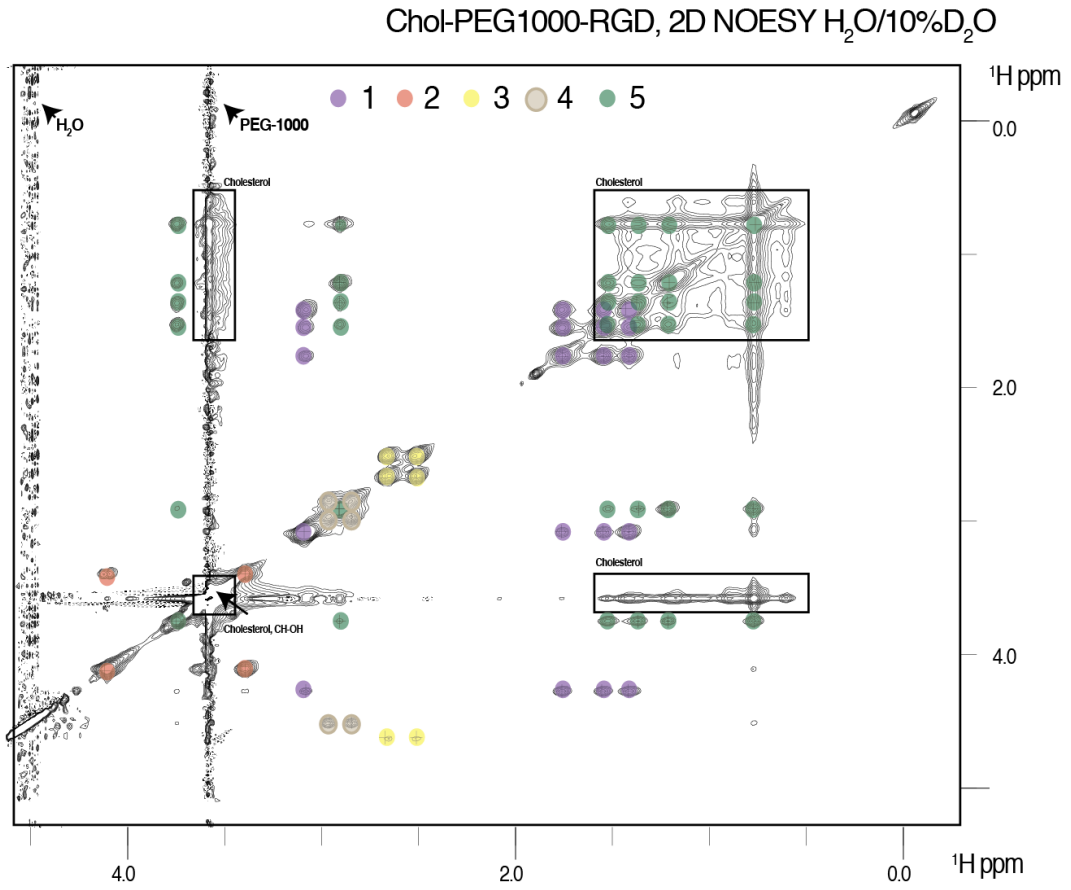


Figure S62. Expanded region of the TOCSY shown as Figure S60, displaying the region corresponding to the aliphatic resonances. Cholesterol resonances are indicated as boxes. Each of the five amino acids are indicated with colors (R1 in violet, G2 in red, D3 in yellow, f4 in beige and K5 in green). All protons present in the corresponding amino acid spin system are colored. Water (solvent) and PEG resonances are indicated with arrows.

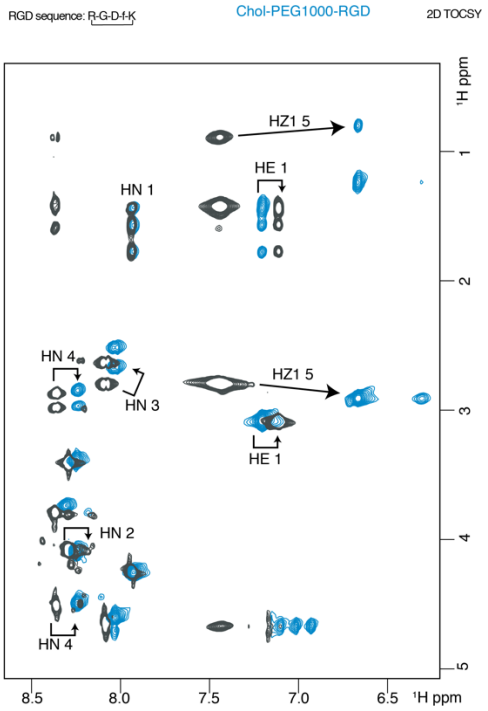


Figure S63. Superposition of the resonances corresponding to the cyclic peptide (black) to that of the peptide bound to the Chol-PEG20 moiety (light blue). Whereas small chemical shift changes are observed for all amide resonances, the chemical shift changes observed at Lys5 HZ1 proton are very large (indicated with arrows), corroborating that the specific attachment of the Chol-PEG moiety occurs at the side-chain of Lys5.

4.4 2D, TOCSY spectrums of Chol-PEG4-RGD (13), Chol-PEG8-RGD (14) and Chol-PEG16-RGD (15).

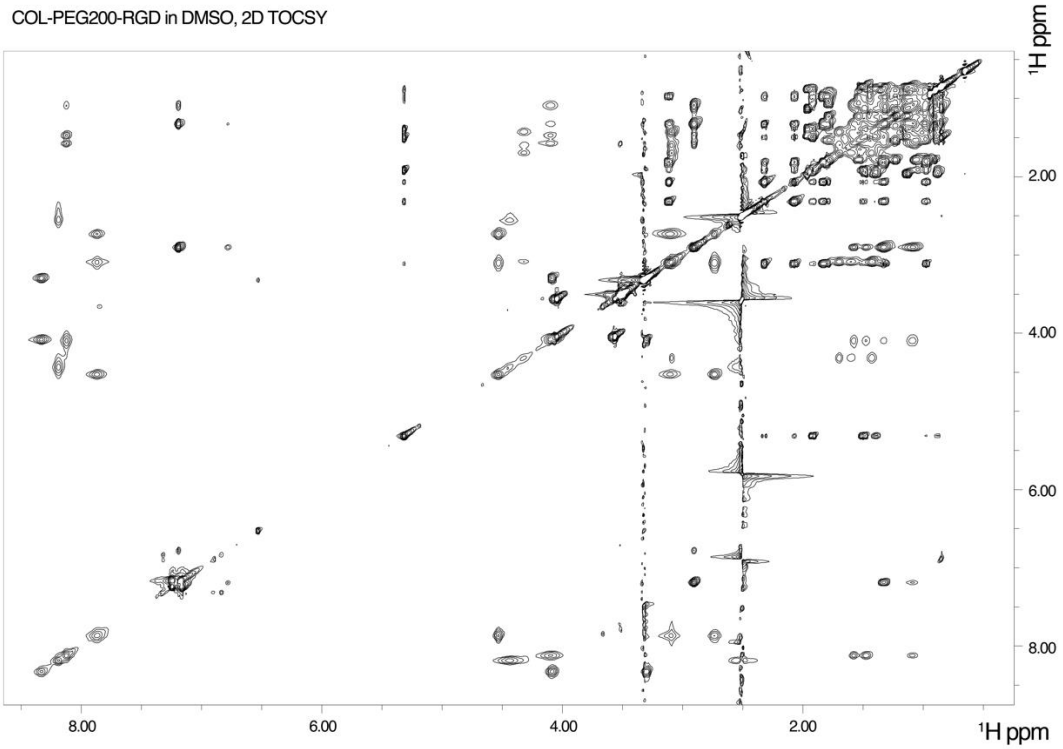


Figure S64. 2D TOCSY spectrum in DMSO corresponding to Chol-PEG4-RGD (**13**, also named as Chol-PEG200-RGD).

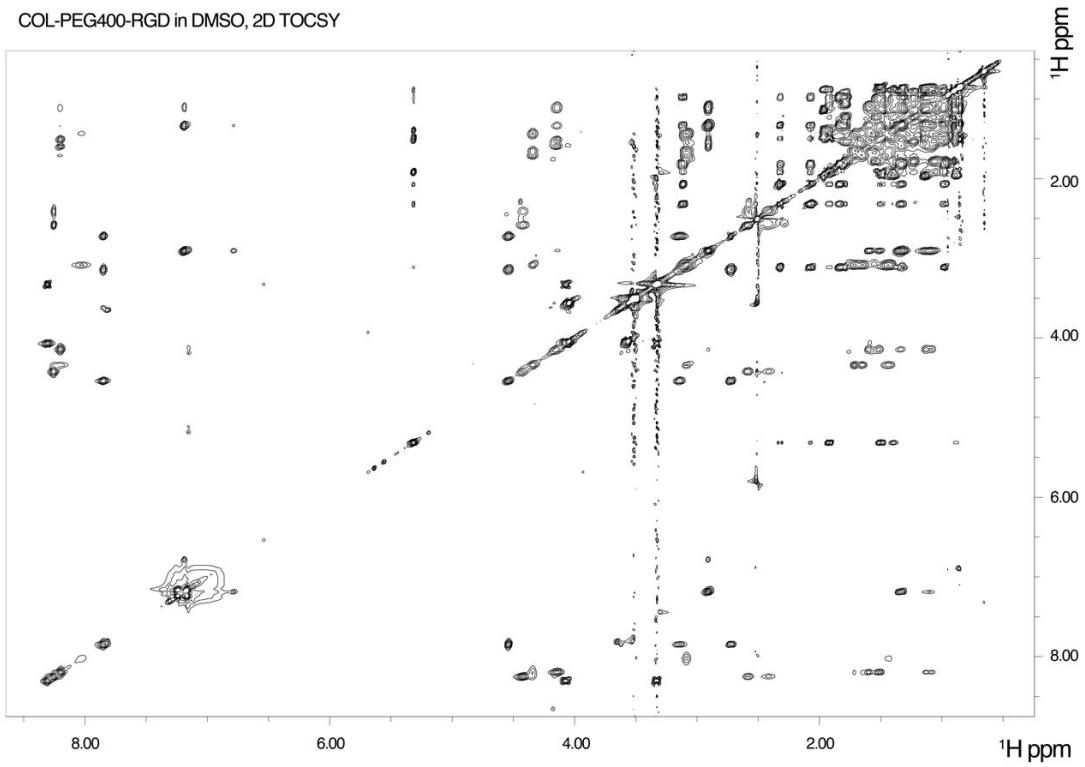


Figure S65. 2D TOCSY spectrum in DMSO corresponding to Chol-PEG8-RGD (**14**, also named as Chol-PEG400-RGD).

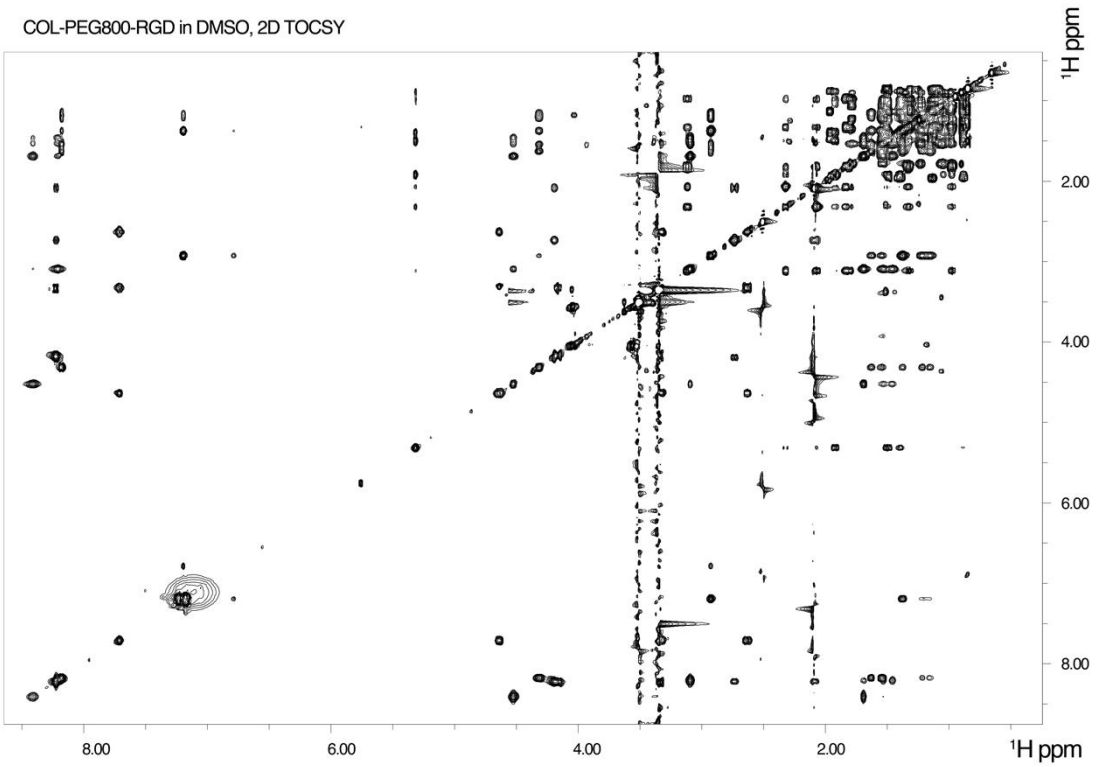


Figure S66. 2D TOCSY spectrum in DMSO corresponding to Chol-PEG16-RGD (**16**, also named as Chol-PEG800-RGD).

4.5 NMR assignment tables of c(RGDfK) (12) and Chol-PEG16-RGD (16) in DMSO.

Table S1. NMR assignment table of the RGD peptide in DMSO

ARG 1	HN	HA	HB2	HB3	HG2	HD2	HD3	HE	
	8.390	4.213	1.784	1.659	1.381	3.120	2.981	7.985	
GLY 2	HN	HA1	HA2						
	8.219	4.050	3.322						
ASP 3	HN	HA	HB2	HB3					
	8.073	4.500	3.042	2.760					
PHE 4	HN	HA	HB2	HB3	HZ	QD	QE		
	8.298	4.636	2.740	2.436	7.227	7.206	7.288		
LYS 5	HN	HA	HB2	HB3	HG2	HG3	HD2	HE2	HZ1
	8.418	4.108	1.770	1.666	1.566	1.509	1.130	2.696	7.793

Table S2. NMR assignment table of the RGD peptide coupled to the Chol-PEG16-RGD in DMSO

ARG 1	HN	HA	HB2	HG2	HG3	HD2	HE	
	8.407	4.518	1.691	1.538	1.457	3.109	8.216	
GLY 2	HN	HA1	HA2					
	8.225	4.166	3.337					
ASP 3	HN	HA	HB2	HB3				
	7.708	4.625	3.321	2.629				
PHE 4	HN	HA	HB2	HB3	HZ	QD	QE	
	8.222	4.176	2.722	2.068	7.227	7.206	7.288	
LYS 5	HN	HA	HB2	HB3	HG2	HD2	HD3	HE2
	8.177	4.311	1.634	1.532	1.368	1.225	1.159	5.061

4.6 NMR assignment tables of c(RGDfK) (12) and and Chol-PEG20-RGD (17) in 90% H_2O / 10 % D_2O.

Table S3. NMR assignment table of the RGD peptide in 90% H_2O / 10 % D_2O

ARG 1	HN	HA	HB2	HG2	HD2	HE	
	7.922	4.273	1.661	1.435	3.087	7.099	
GLY 2	HN	HA1	HA2				
	8.267	4.095	3.418				
ASP 3	HN	HA	HB2	HB3			
	8.066	4.669	2.806	2.630			
PHE 4	HN	HA	HB2	HB3	HZ	QD	QE
	8.347	4.534	2.994	2.887	7.227	7.170	7.288
LYS 5	HN	HA	HB2	HG2	HD2	HE2	HZ1
	8.351	3.807	1.582	1.411	0.873	2.798	7.430

Table S4. NMR assignment of the RGD peptide coupled to the Chol-PEG20-RGD in 90% H_2O / 10 % D_2O

ARG 1	HN	HA	HB2	HG2	HD2	HE		
	8.288	3.734	1.762	1.358	3.079	7.192		
GLY 2	HN	HA1	HA2					
	8.216	4.123	3.384					
ASP 3	HN	HA	HB2	HB3				
	8.018	4.621	2.676	2.514				
PHE 4	HN	HA	HB2	HB3	HZ	QD	QE	
	8.226	4.521	2.971	2.846	7.227	7.154	7.288	
LYS 5	HN	HA	HB2	HB3	HG2	HD2	HD3	HE2
	7.908	4.271	1.770	1.582	1.403	1.215	0.767	6.647

5. References

(1) French, A. C.; Thompson, A. L.; Davis, B. G. High-Purity Discrete PEG-Oligomer Crystals Allow Structural Insight. *Angew. Chem. Int. Ed.* **2009**, *48* (7), 1248–1252.