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


# Supporting Information

## Enzyme-triggered delivery of chlorambucil from conjugates based on the cell-penetrating peptide BP16

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## 1. Materials and methods

Unless otherwise stated, common chemicals and solvents (HPLC-grade or reagent-grade quality) were purchased from commercial sources and used without further purification. The 9-fluorenylmethoxycarbonyl (Fmoc) derivatives and Fmoc-Rink-4-methylbenzhydrylamine (MBHA) resin (0.56 mmol/g) were obtained from Senn Chemicals International (Gentilly, France), NovaBiochem (Schwalbach, Germany) or IRIS Biotech GmbH (Marktredwitz, Germany). Ethyl 2-cyano-2-(hydroxyimino)acetate (Oxyma) was purchased from Novabiochem (Nottingham, UK). Trifluoroacetic acid (TFA), triisopropylsilane (TIS), dimethyl sulfoxide (DMSO), *N,N*'-diisopropylcarbodiimide (DIPCDI), chlorambucil (CLB), 5(6)-carboxyfluorescein (CF), hydrazine monohydrate and ethylenediaminetetraacetic acid (EDTA) were from Sigma–Aldrich (St. Louis, MO, USA). Cathepsin B (bovine spleen) was purchased from EMD chemicals (San Diego, CA, USA). L-(+)-Cysteine was obtained from Fischer Scientific (Waltham, MA USA). Piperidine and pyridine were purchased from Fluka (Buchs, Switzerland). Acetic anhydride ($Ac_2O$) was purchased from Panreac (Barcelona, Spain). *N*-Methyl-2-pyrrolidinone (NMP), *N,N*-dimethylformamide (DMF), $CH_3OH$, $CH_2Cl_2$, diethyl ether and solvents for high performance liquid chromatography (HPLC) were obtained from Scharlau (Sentmenat, Spain). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), paraformaldehyde and bisbenzimide trihydrochloroacetic acid (Hoechst 33258) were purchased from Sigma-Aldrich (St. Louis, MO, USA). MitoTracker(R)Red CMXRos was from Molecular Probes, Life Technologies (Eugene, Oregon, USA). Dulbecco's modified Eagle's medium (DMEM), phosphate buffered saline (PBS), fetal bovine serum (FBS), penicillin-streptomycin and trypsin were obtained from GIBCO BRL (Grand Island, NY, USA).

Compounds were analyzed under standard analytical HPLC conditions with a Dionex liquid chromatography instrument composed of an UV/Vis Dionex UVD170U detector, a P680 Dionex bomb, an ASI-100 Dionex automatic injector, and CHROMELEON 6.60 software. Detection was performed at 220 nm. Solvent A was 0.1% aq. TFA and solvent B was 0.1% TFA in $CH_3CN$. Analysis was carried out with a Kromasil 100 $C_{18}$ (4.6 mm × 40 mm, 3.5 µm) column with a 2-100% B linear gradient over 7 min at a flow rate of 1.0 mL $min^{-1}$.

Electrospray ionization mass spectrometry (ESI-MS) analyses were performed with an Esquire 6000 ESI ion Trap LC/MS (Bruker Daltonics) instrument equipped with an electrospray ion source (University of Girona). The instrument was operated in the positive ESI(+) ion mode. Samples (5 µL) were introduced into the mass spectrometer ion source directly through an HPLC autosampler. The mobile phase (80:20 $CH_3CN/H_2O$ at a flow rate of 100 µL $min^{-1}$) was delivered by a 1200 Series HPLC pump (Agilent). Nitrogen was employed as both the drying and nebulizing gas.

High-resolution mass spectra (HRMS) were recorded under conditions of ESI with a Bruker MicrOTOF-Q IITM instrument using a hybrid quadrupole time-of-flight mass spectrometer (University of Girona). Samples were introduced into the mass spectrometer ion source by direct infusion through a syringe pump and were externally calibrated using sodium formate. The instrument was operated in the positive ESI(+) ion mode.

HPLC-MS analyses were performed under standard analytical HPLC conditions described above with an Agilent Technologies 1200 HPLC system equipped with a VWD detector and coupled with an Esquire 6000 ESI ion Trap LC/MS (Bruker Daltonics) instrument with an electrospray ion source (University of Girona). The instrument was operated in the positive ESI(+) ion mode.

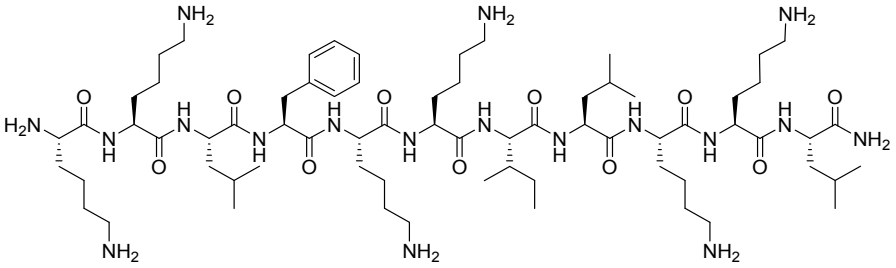
## 2. Cell lines

The human breast cancer MCF-7, melanoma SKMEL-28, prostate cancer PC-3 and pancreas cancer CAPAN-1 cell lines were obtained from the American Tissue Culture Collection (ATCC, Rockville, MD, USA). The human skin fibroblasts 1BR3G were obtained from EucellBank (University of Barcelona, Barcelona, Spain). Cells were maintained in DMEM supplemented with 10% FBS and 100 U $ml^{-1}$ penicillin-streptomycin at 37 ºC under a humidified atmosphere containing 5% $CO_2$. Cells were passaged two times per week.

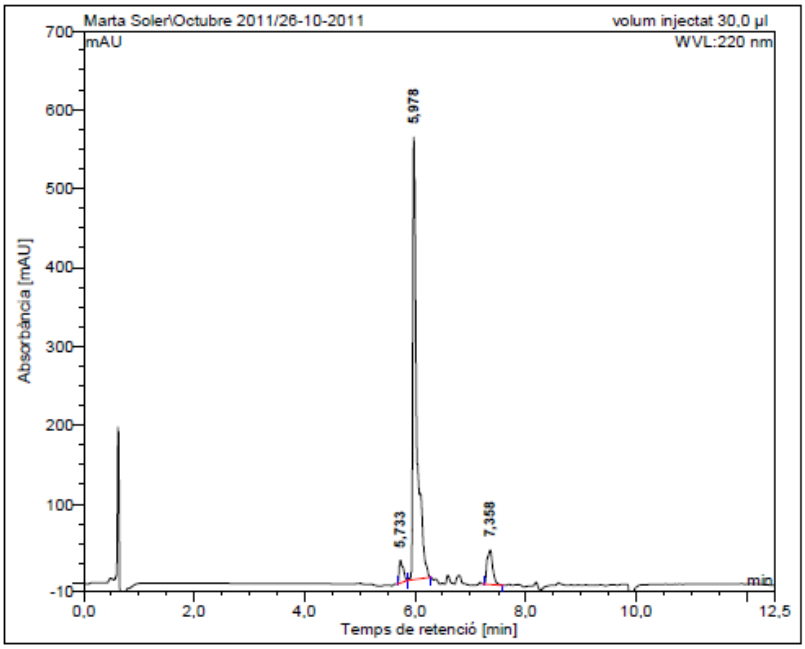
## 3. HPLC, ESI-MS and HRMS of peptides and CLB-peptide conjugates

**Figure S1**: a) HPLC chromatogram (λ= 220 nm), b) HRMS spectrum (*m/z*).

**BP16**

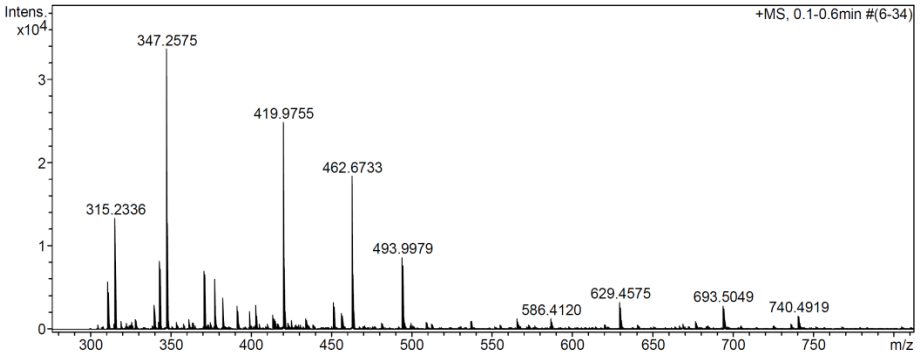


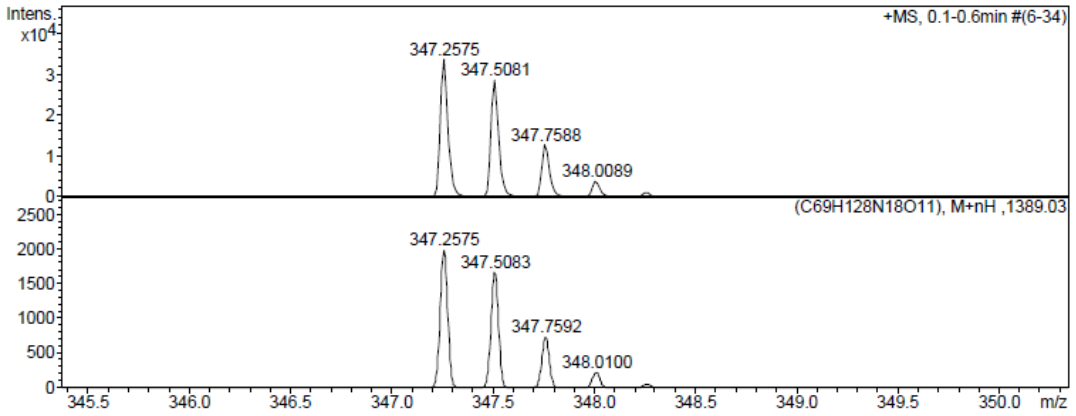
**a)**



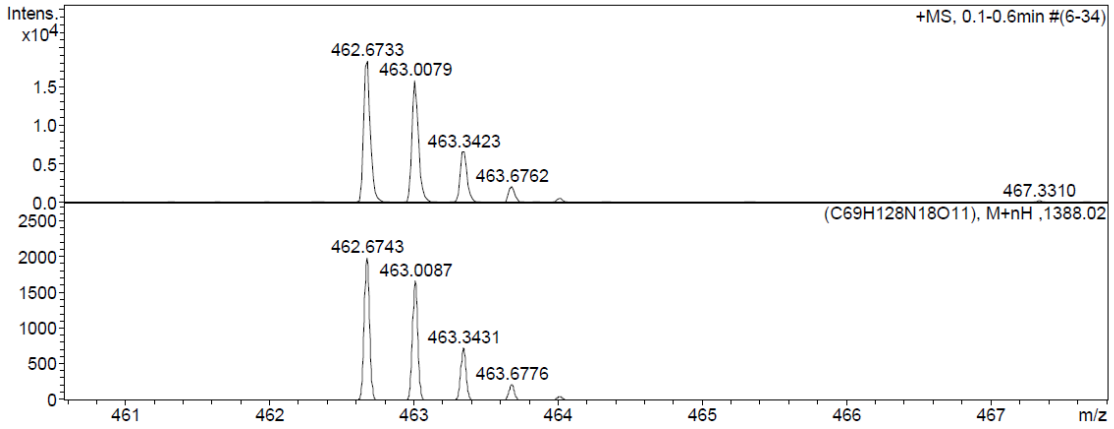

| No. | mps retenc min | alçada mAU | Area mAU*min | Area relativa % |
|---|---|---|---|---|
| 1 | 5,73 | 29,200 | 2,392 | 4,34 |
| 2 | 5,98 | 560,424 | 47,628 | 86,44 |
| 3 | 7,36 | 43,883 | 5,076 | 9,21 |
| Total: | | 633,507 | 55,097 | 100,00 |

**b)**





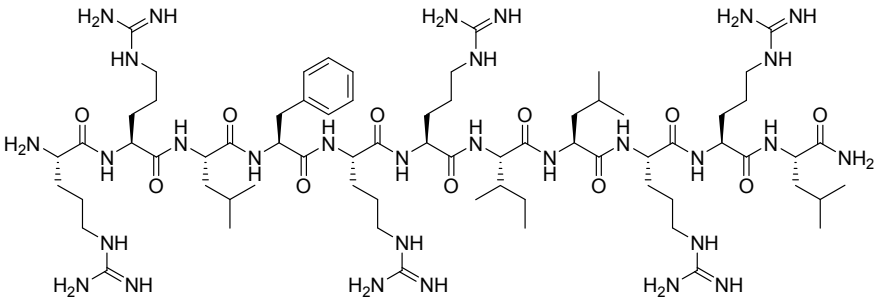

Observed HRMS (top) with the theoretical isotope prediction (bottom).



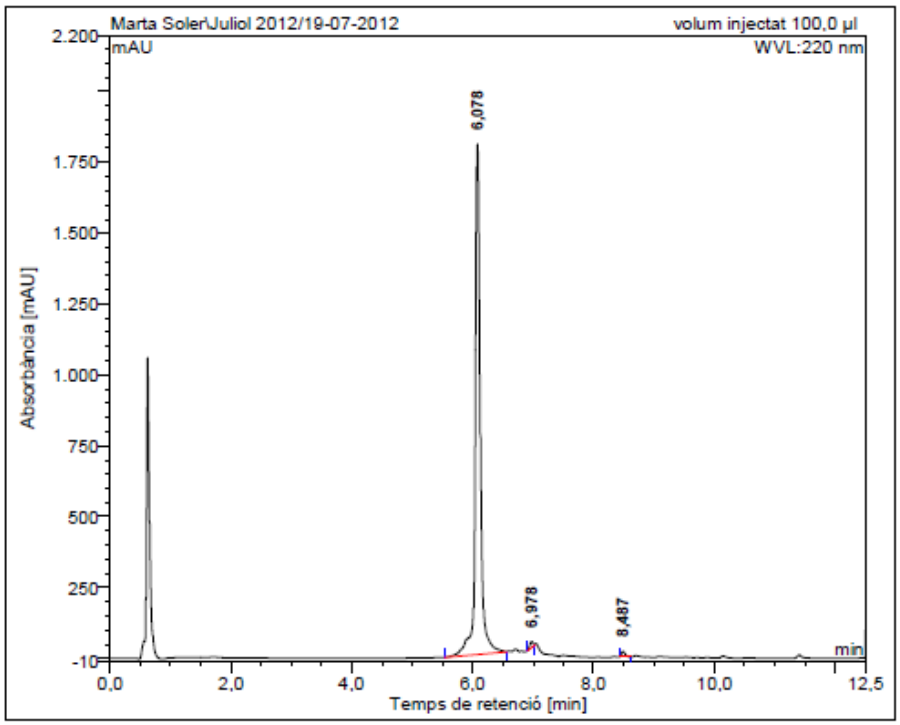

Observed HRMS (top) with the theoretical isotope prediction (bottom).

**Figure S2**: a) HPLC chromatogram (λ= 220 nm), b) HRMS spectrum (*m/z*).

**BP308**

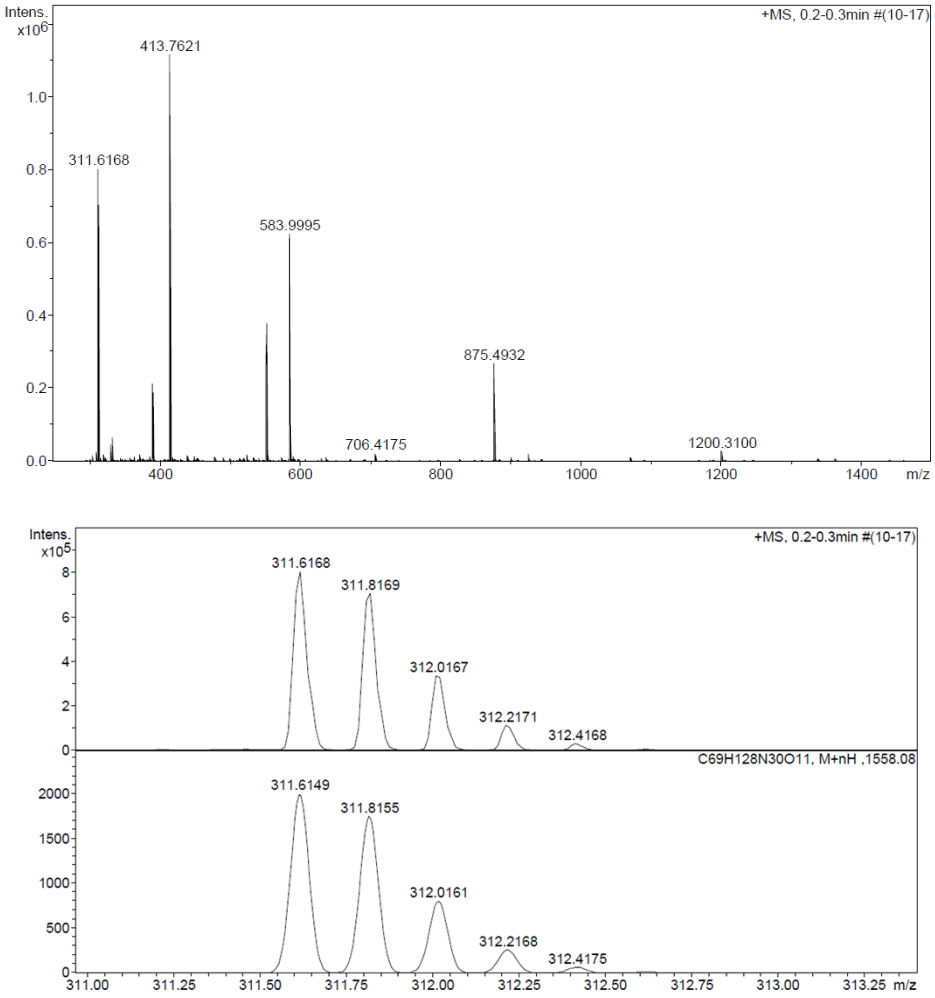


**a)**

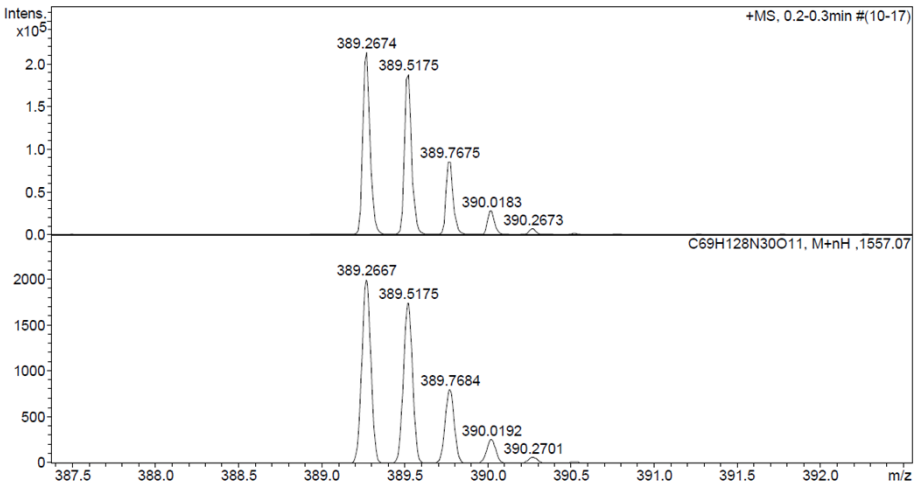



| No. | mps retenc min | alçada mAU | Area mAU*min | Area relativa % |
|---|---|---|---|---|
| 1 | 6,08 | 1801,317 | 177,418 | 98,51 |
| 2 | 6,98 | 19,947 | 1,208 | 0,67 |
| 3 | 8,49 | 20,643 | 1,470 | 0,82 |
| Total: | | 1841,907 | 180,096 | 100,00 |

**b)**



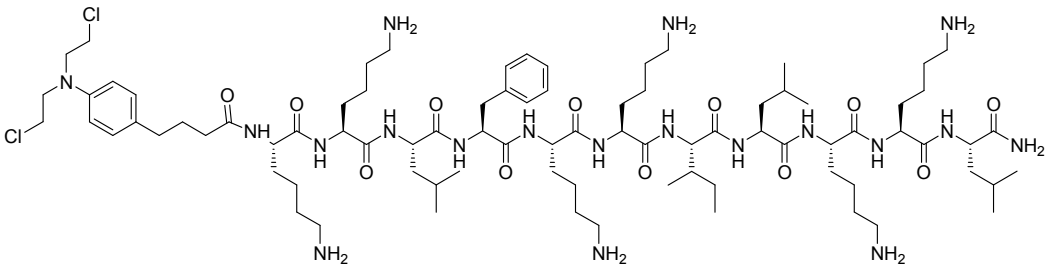

Observed HRMS (top) with the theoretical isotope prediction (bottom).



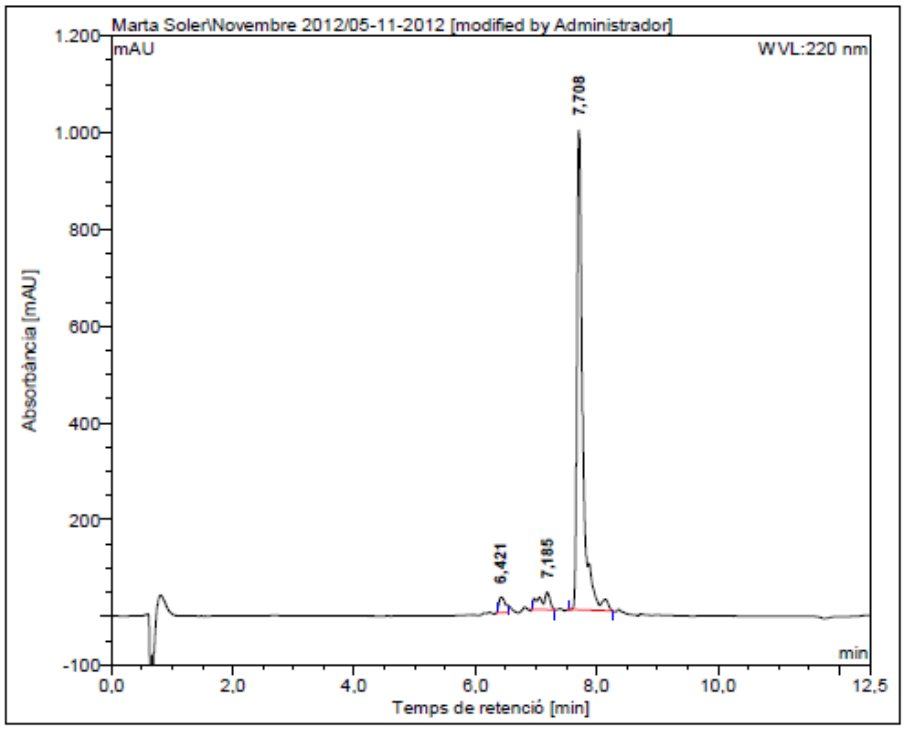

Observed HRMS (top) with the theoretical isotope prediction (bottom).

**Figure S3**: a) HPLC chromatogram (λ= 220 nm), b) HRMS spectrum (*m/z*).

**BP325**

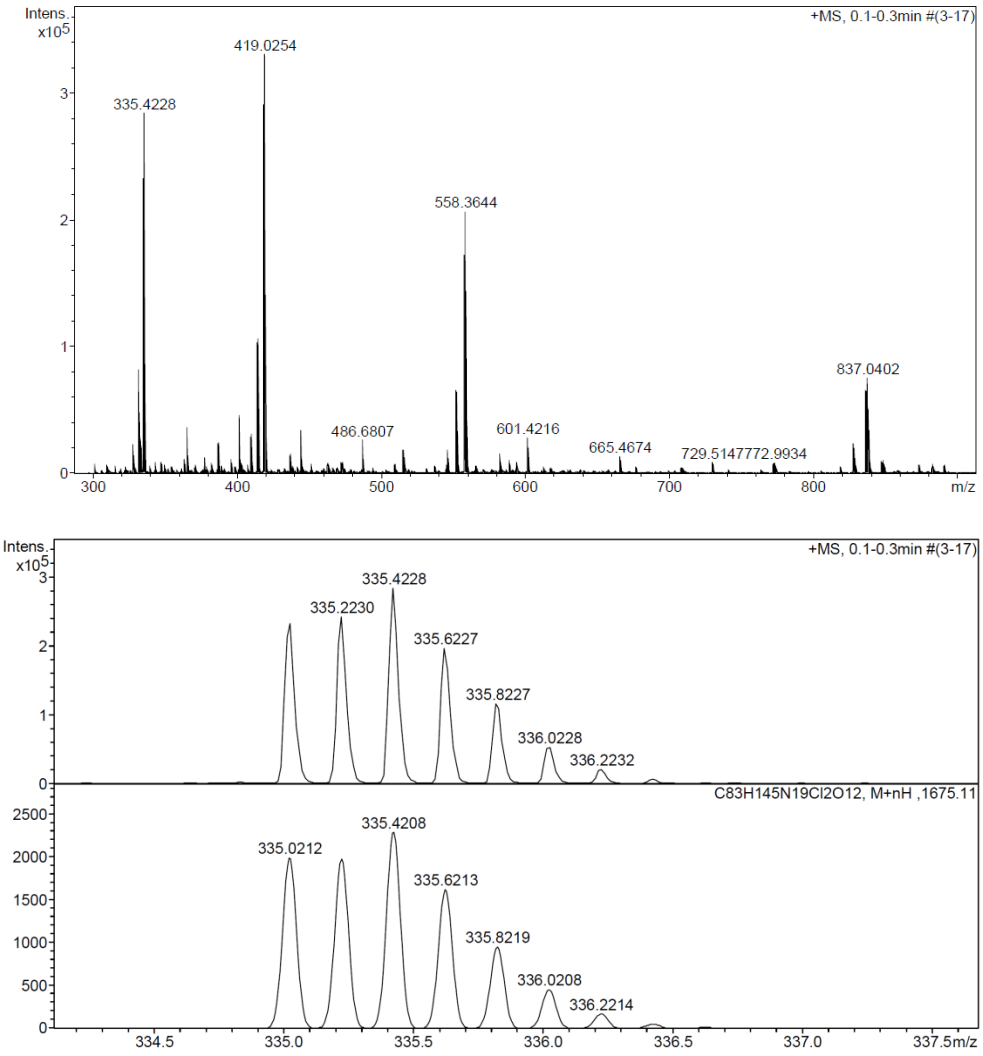


**a)**

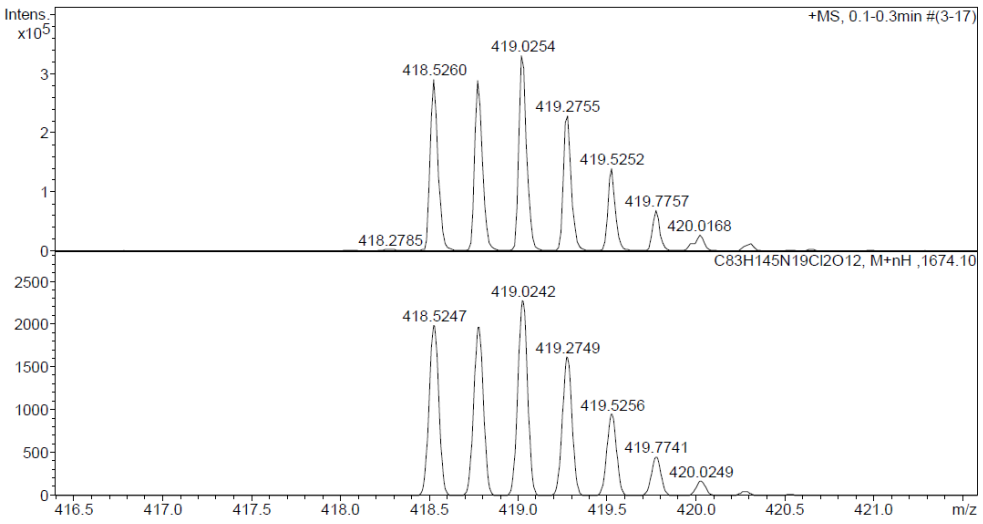



| No. | mps retenc min | alçada mAU | Area mAU*min | Area relativa % |
|---|---|---|---|---|
| 1 | 6,42 | 31,278 | 3,951 | 3,28 |
| 2 | 7,19 | 35,817 | 7,126 | 5,91 |
| 3 | 7,71 | 991,334 | 109,512 | 90,81 |
| Total: | | 1058,429 | 120,589 | 100,00 |

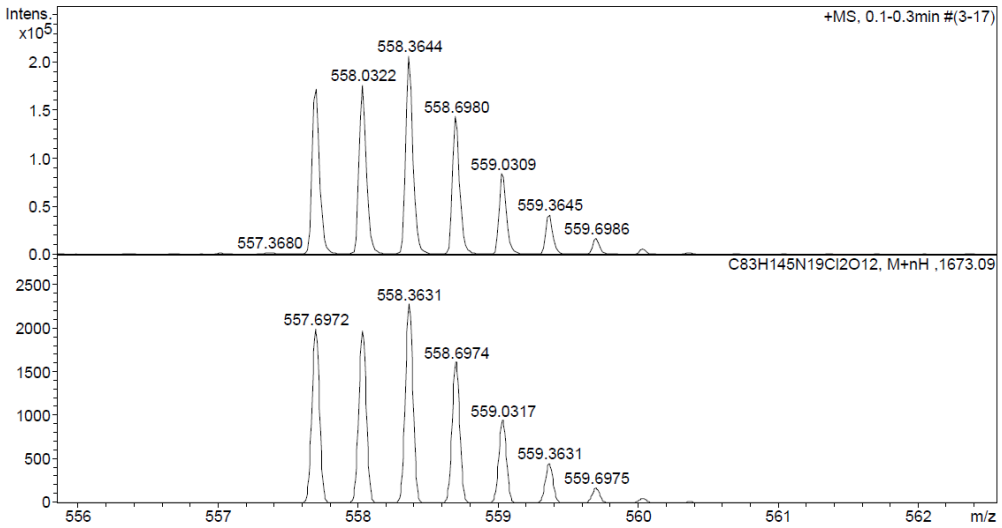
**b)**



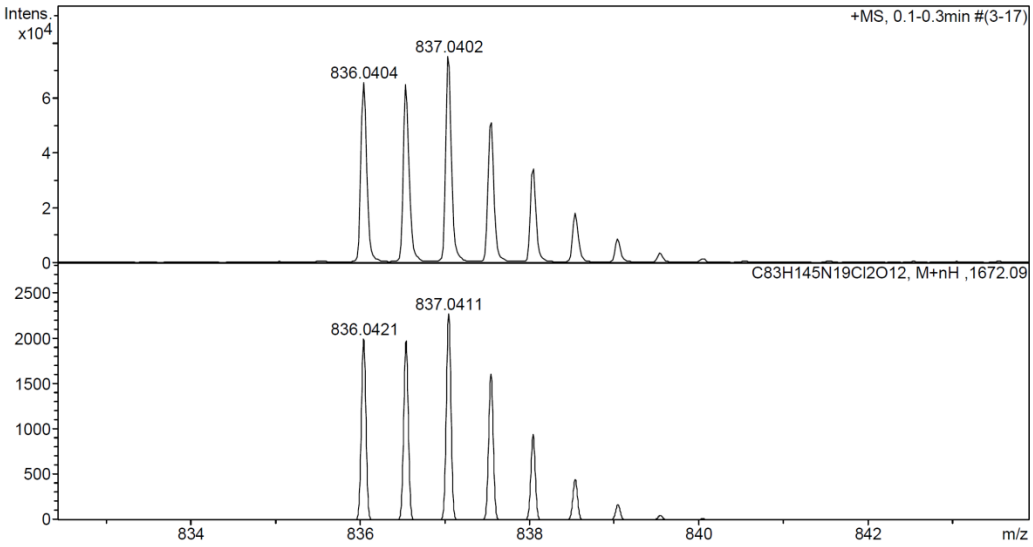

Observed HRMS (top) with the theoretical isotope prediction (bottom).




Observed HRMS (top) with the theoretical isotope prediction (bottom).



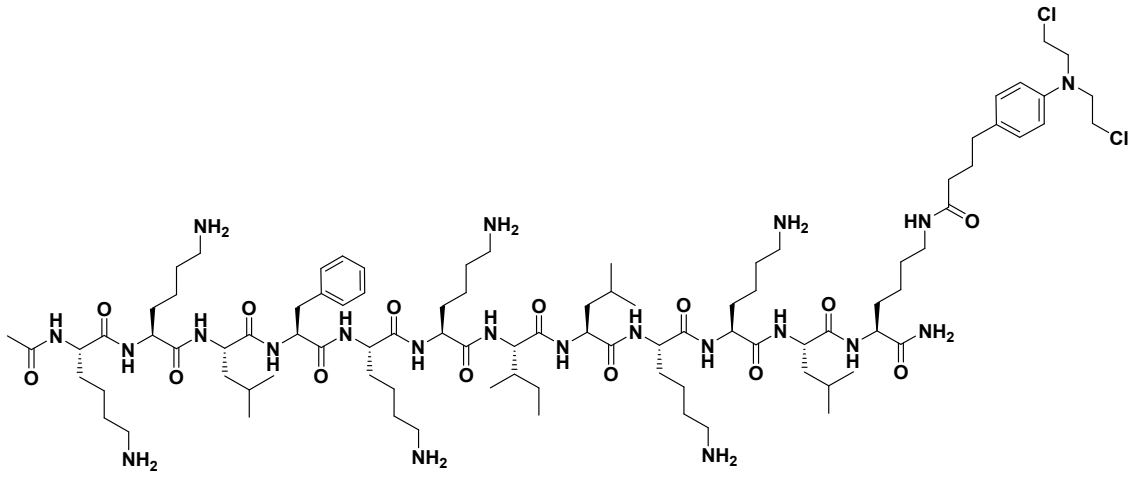

Observed HRMS (top) with the theoretical isotope prediction (bottom).




Observed HRMS (top) with the theoretical isotope prediction (bottom).

**Figure S4**: a) HPLC chromatogram (λ= 220 nm), b) HRMS spectrum (*m/z*).

**BP331**



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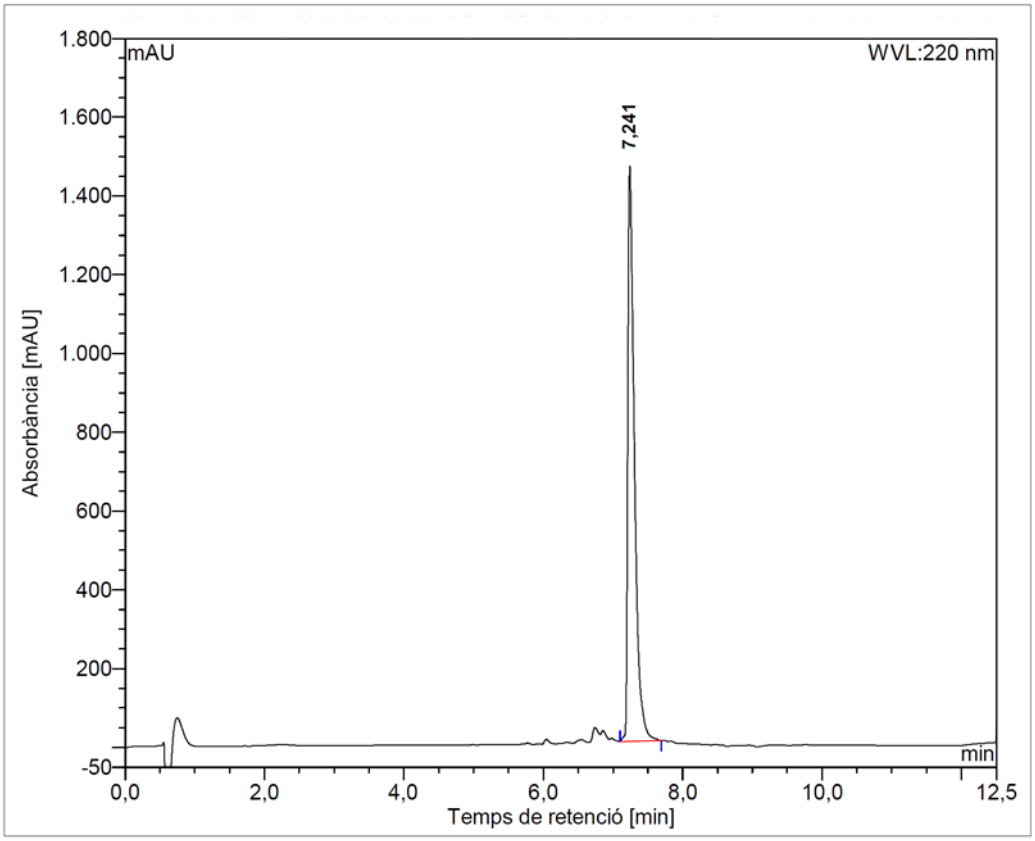
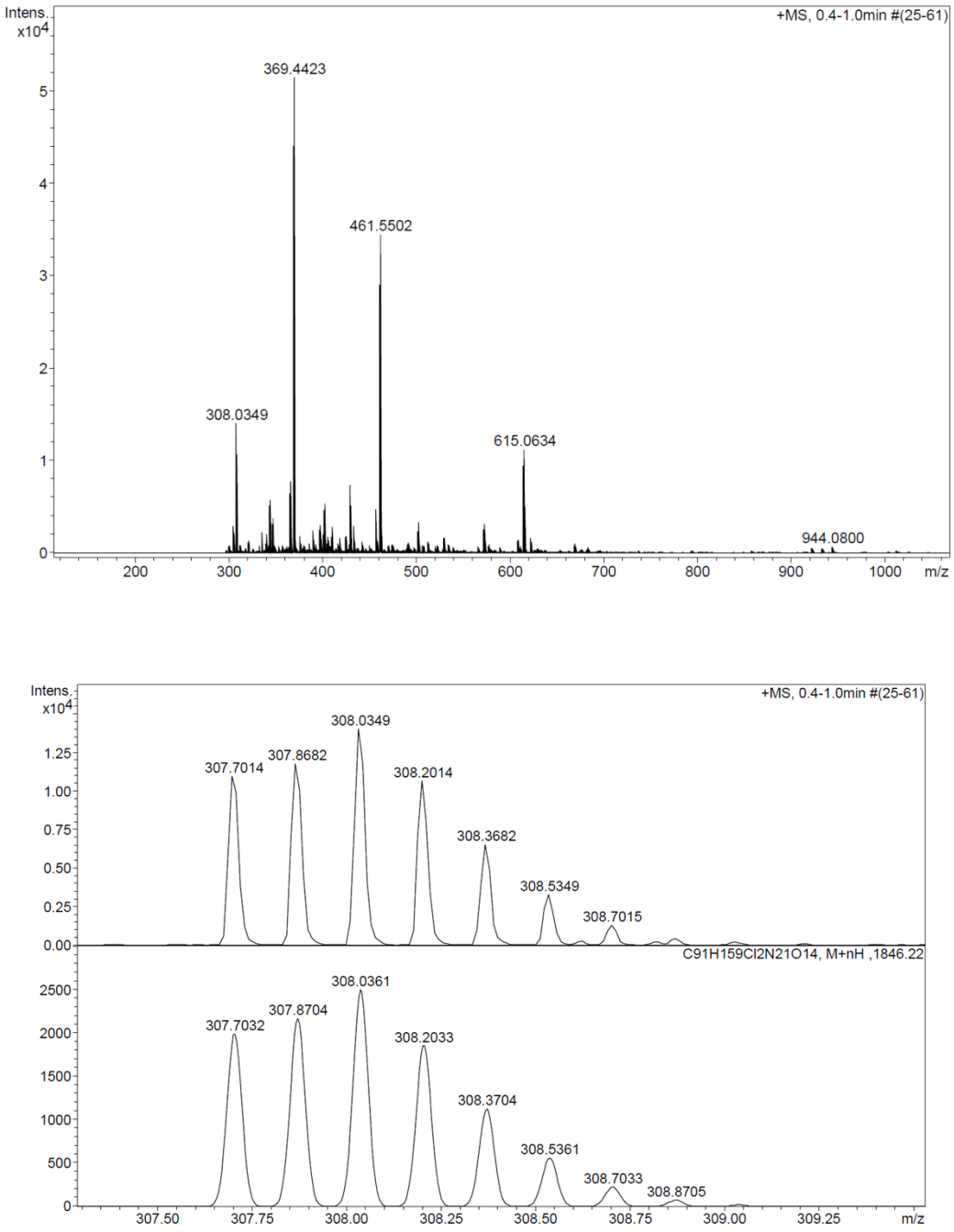
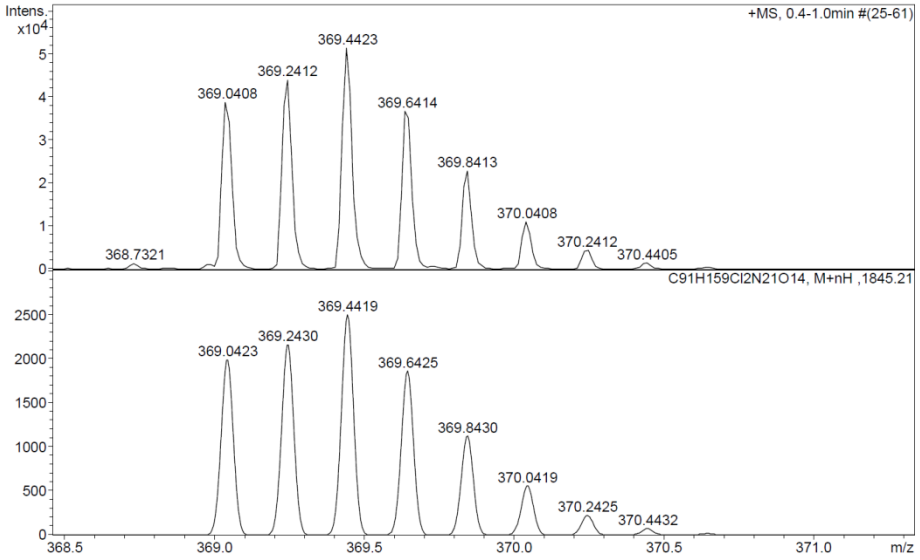



| No. | mps retenc min | alçada mAU | Area mAU*min | Area relativa % |
|---|---|---|---|---|
| 1 | 7,24 | 1460,388 | 161,177 | 100,00 |
| Total: | | 1460,388 | 161,177 | 100,00 |

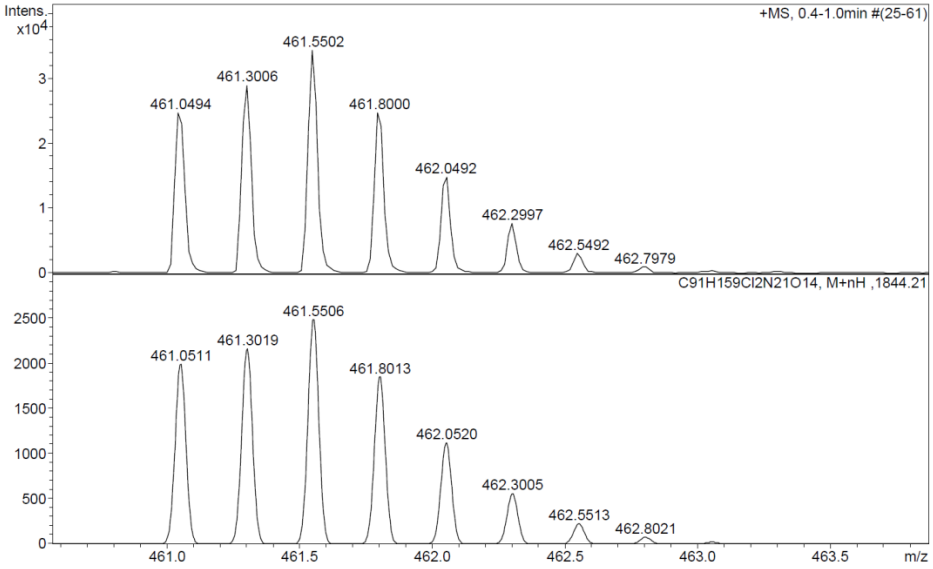
**b)**



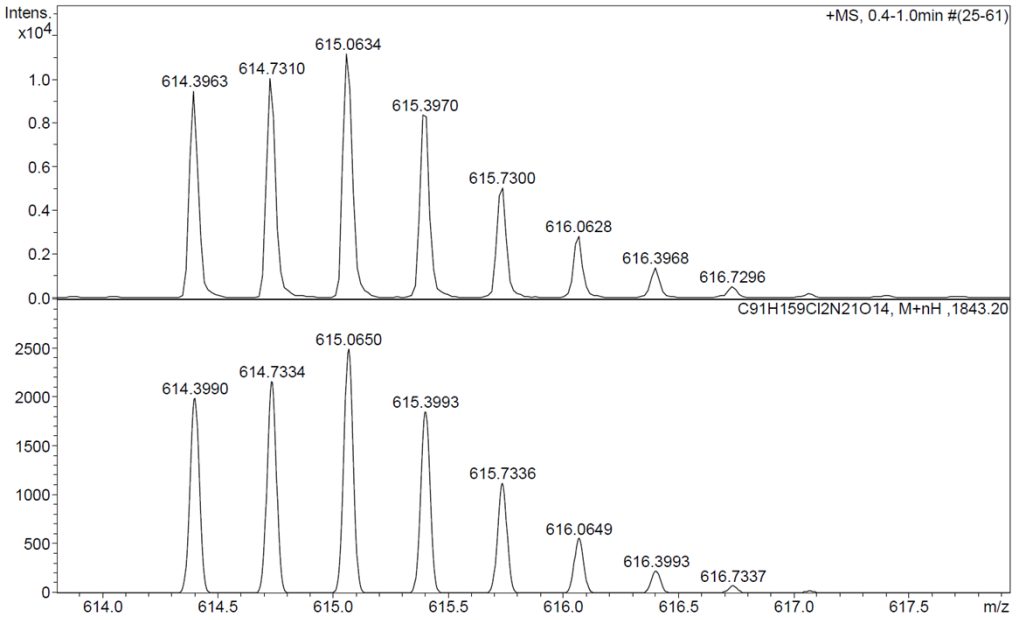

Observed HRMS (top) with the theoretical isotope prediction (bottom).




Observed HRMS (top) with the theoretical isotope prediction (bottom).



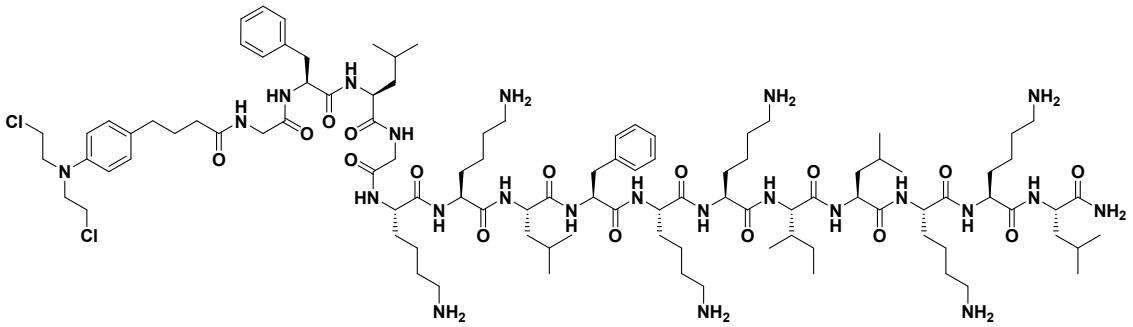

Observed HRMS (top) with the theoretical isotope prediction (bottom).



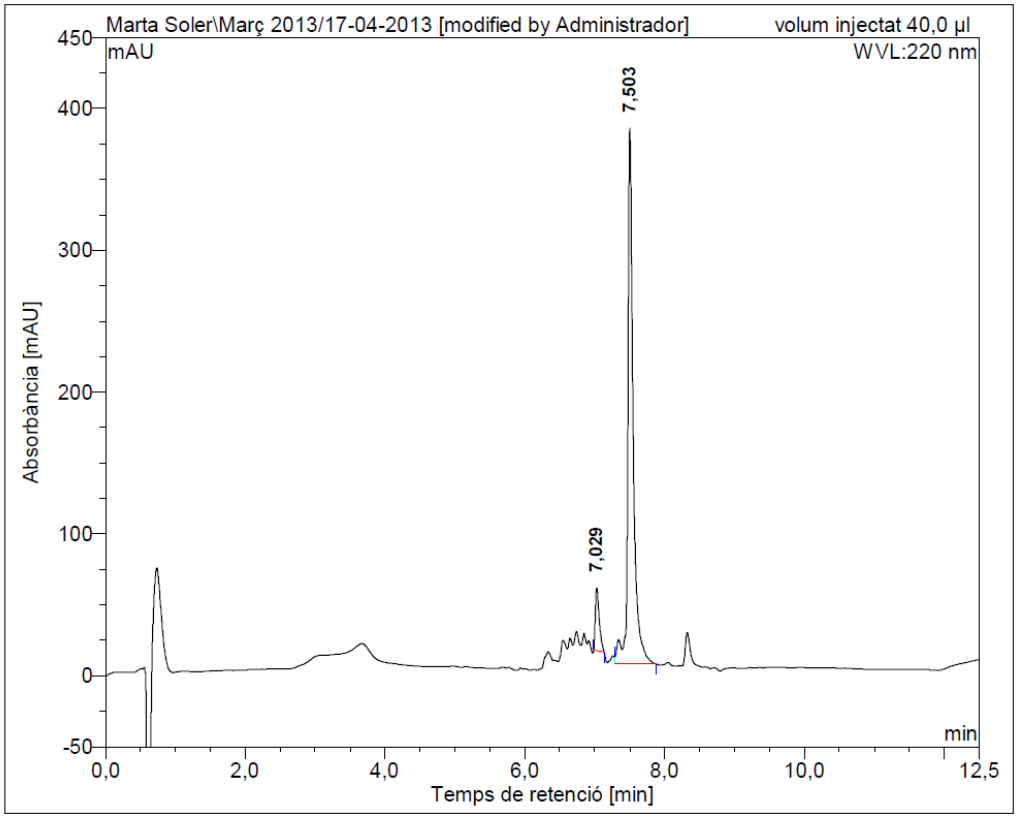

Observed HRMS (top) with the theoretical isotope prediction (bottom).

**Figure S5**: a) HPLC chromatogram (λ= 220 nm), b) HRMS spectrum (*m/z*).

**BP332**

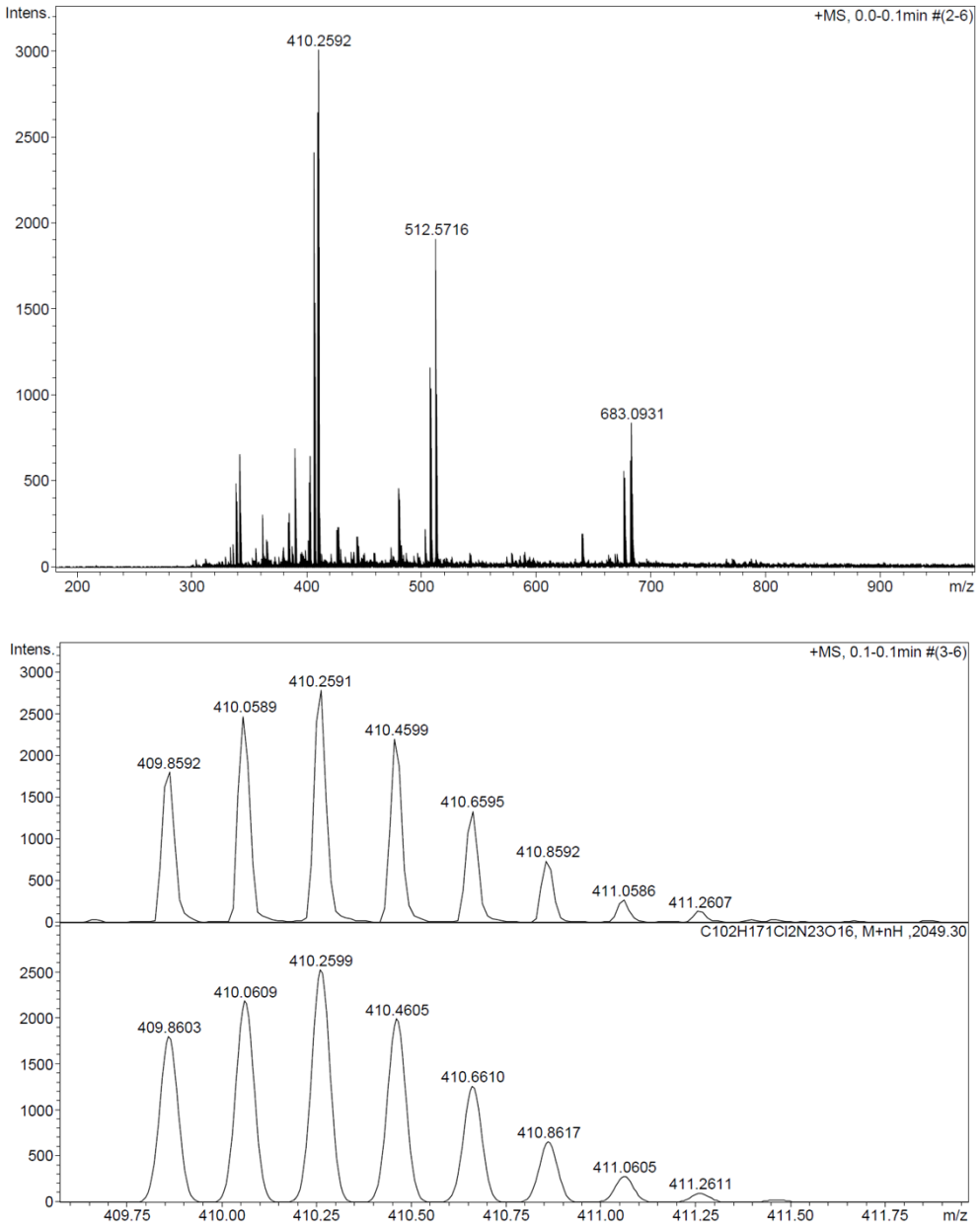


**a)**

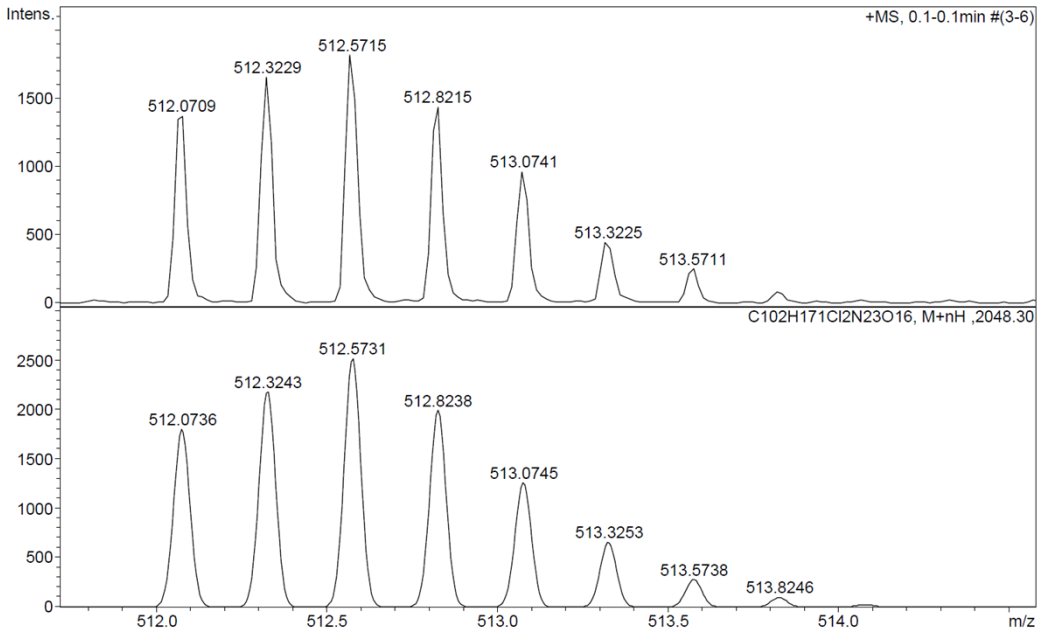



| No. | mps retenc min | alçada mAU | Area mAU*min | Area relativa % |
|---|---|---|---|---|
| 1 | 7,03 | 44,244 | 2,866 | 7,83 |
| 2 | 7,50 | 377,981 | 33,741 | 92,17 |
| Total: | | 422,225 | 36,607 | 100,00 |

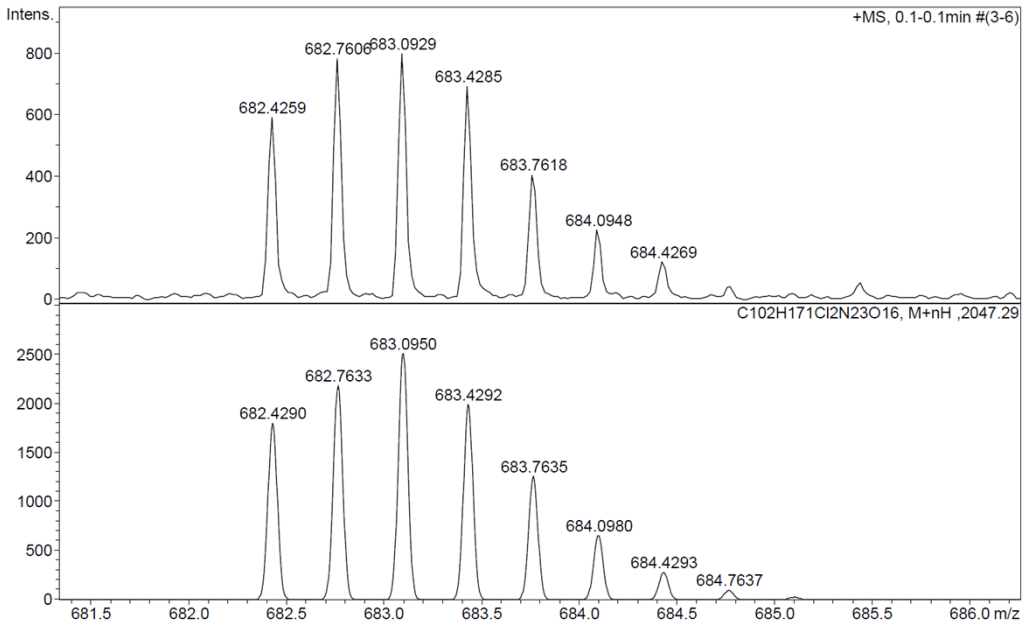
**b)**




Observed HRMS (top) with the theoretical isotope prediction (bottom).



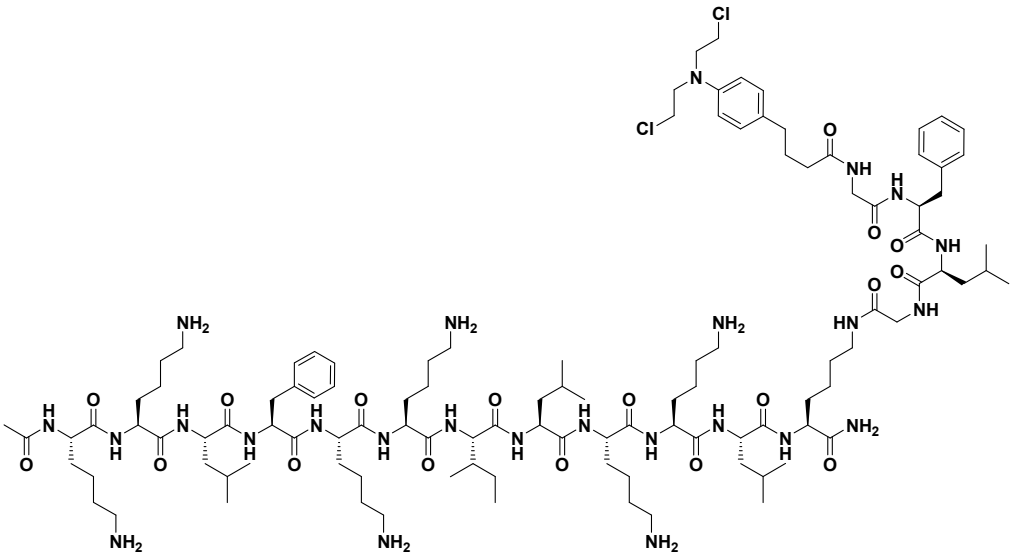

Observed HRMS (top) with the theoretical isotope prediction (bottom).



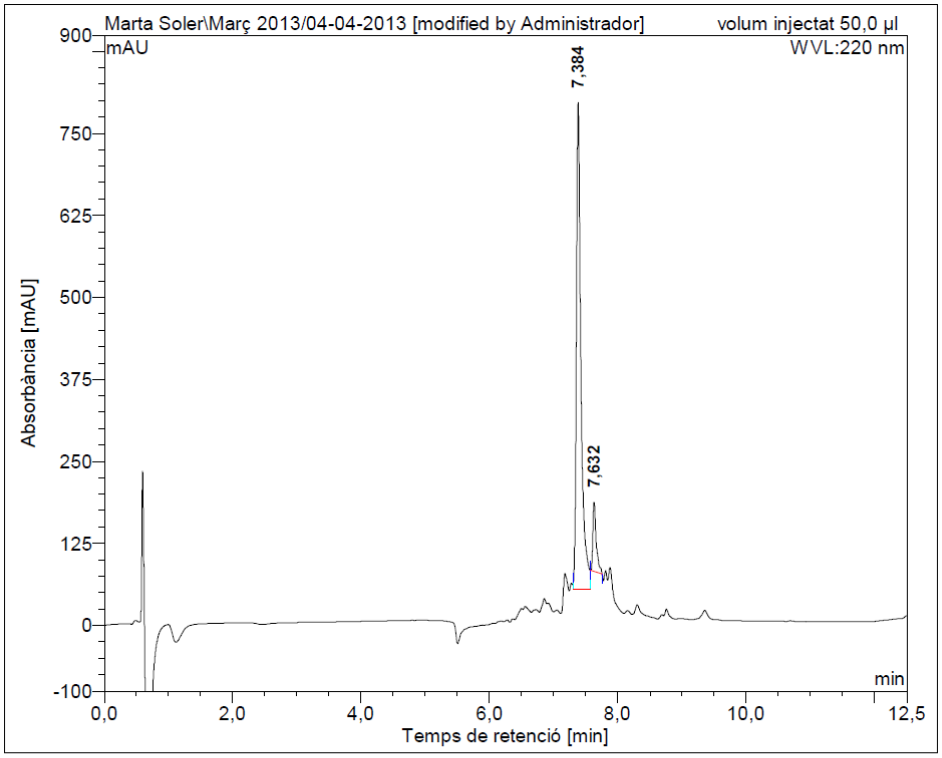

Observed HRMS (top) with the theoretical isotope prediction (bottom).

**Figure S6**: a) HPLC chromatogram (λ= 220 nm), b) HRMS spectrum (*m/z*).

**BP333**

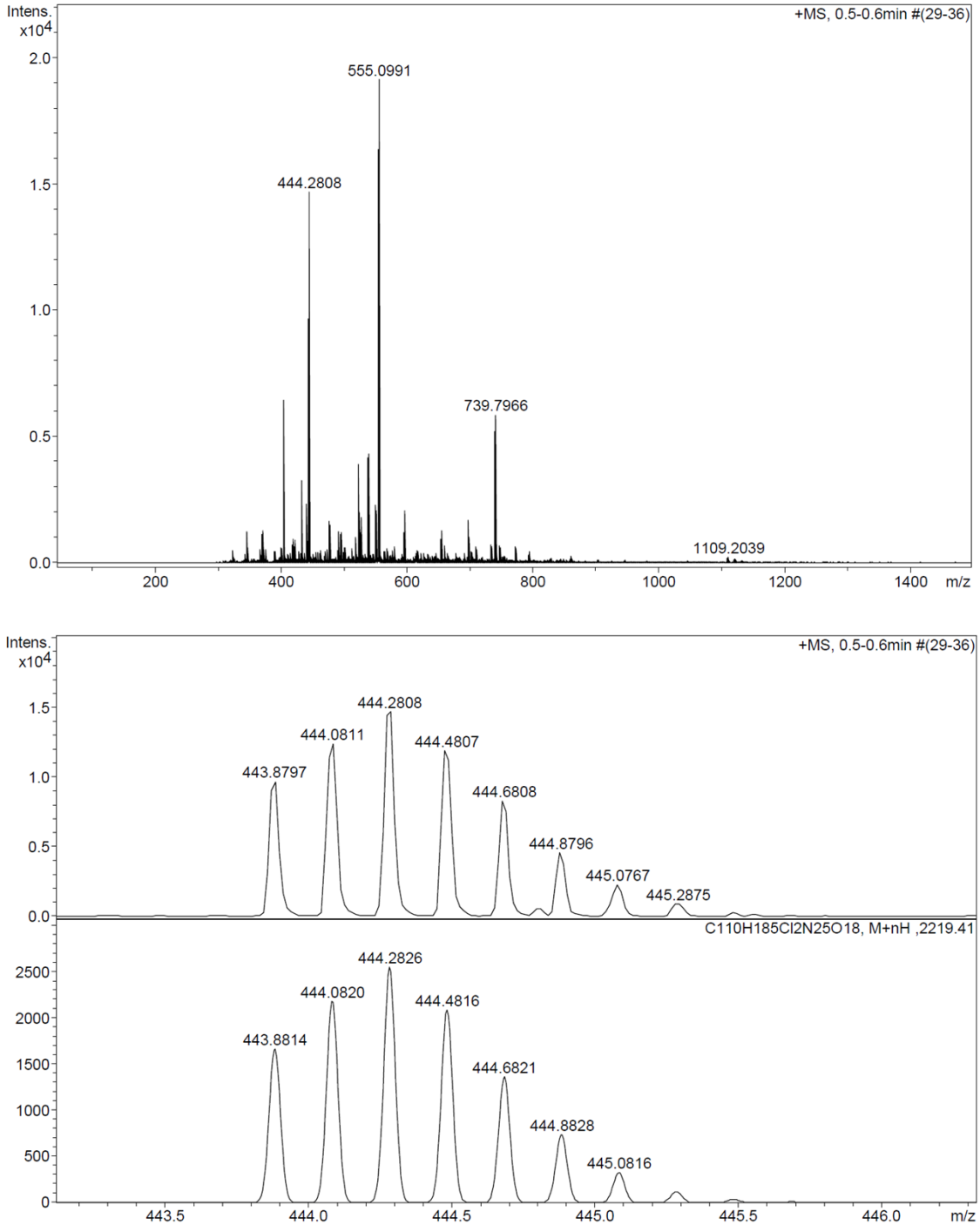


**a)**

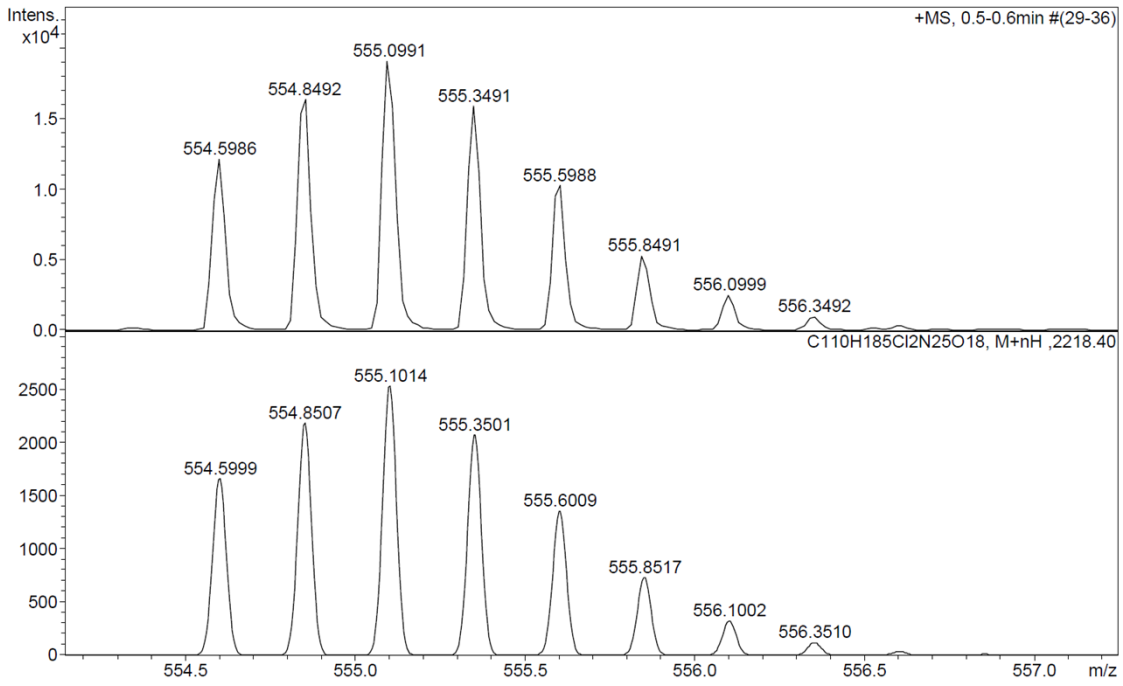



| No. | mps retenc min | alçada mAU | Area mAU*min | Area relativa % |
|---|---|---|---|---|
| 1 | 7,38 | 742,124 | 61,018 | 90,51 |
| 2 | 7,63 | 104,960 | 6,398 | 9,49 |
| Total: | | 847,085 | 67,416 | 100,00 |

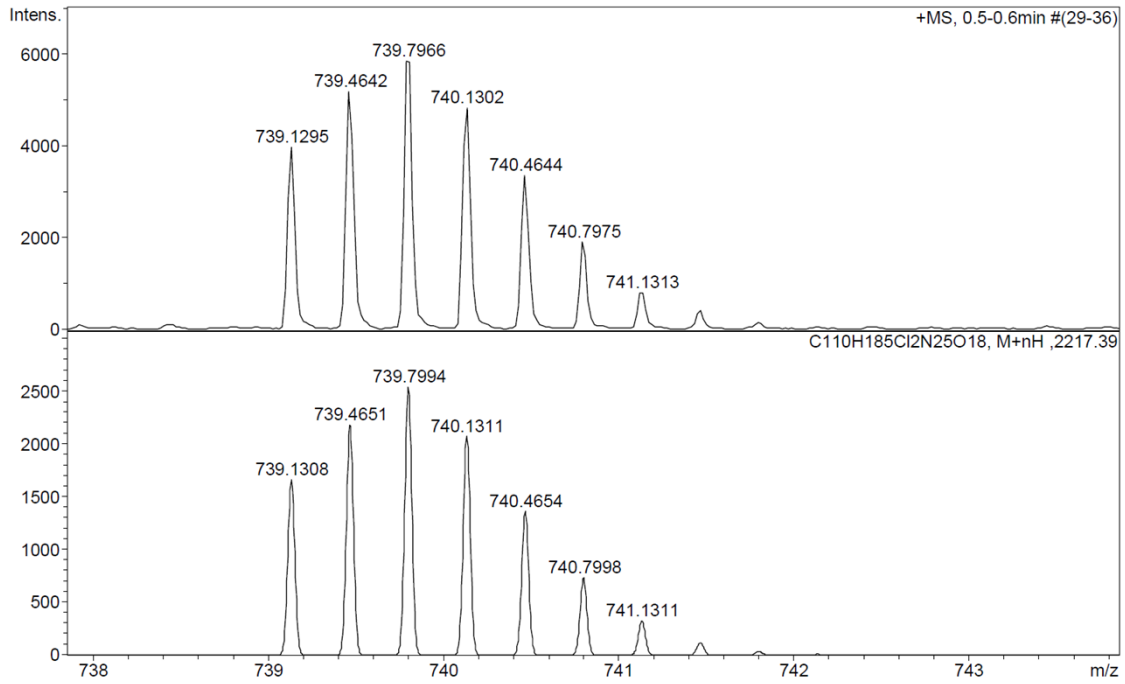
**b)**




Observed HRMS (top) with the theoretical isotope prediction (bottom).



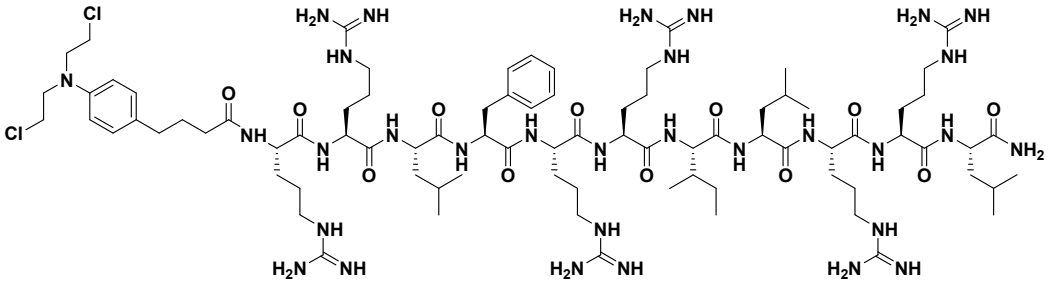

Observed HRMS (top) with the theoretical isotope prediction (bottom).



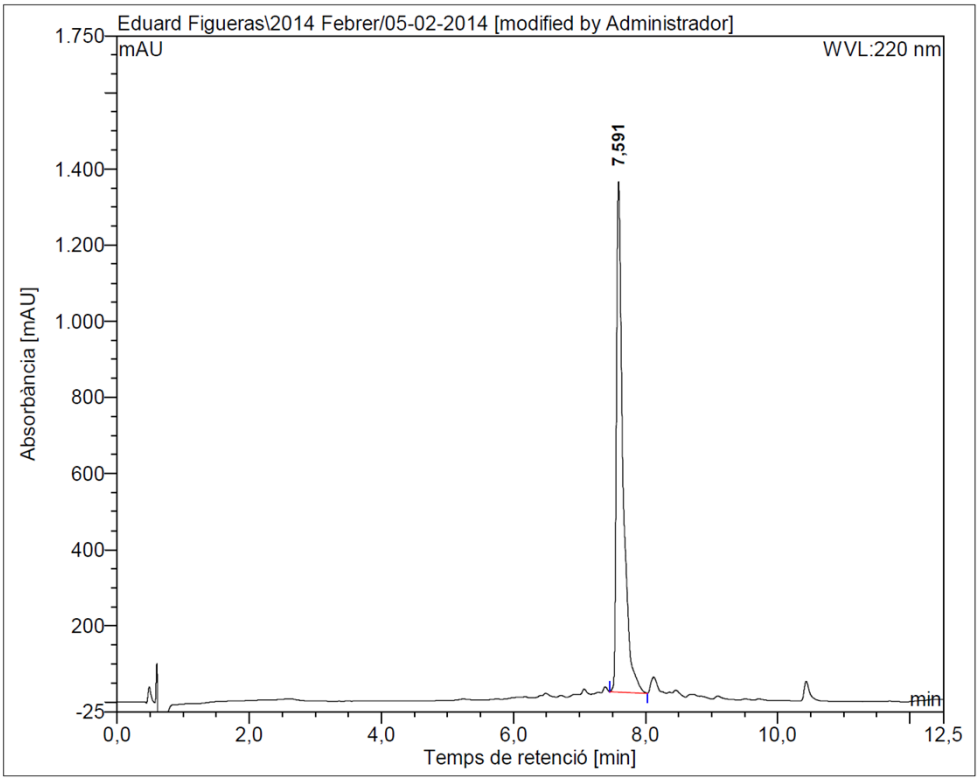

Observed HRMS (top) with the theoretical isotope prediction (bottom).

**Figure S7**: a) HPLC chromatogram (λ= 220 nm), b) HRMS spectrum (*m/z*).

**BP334**

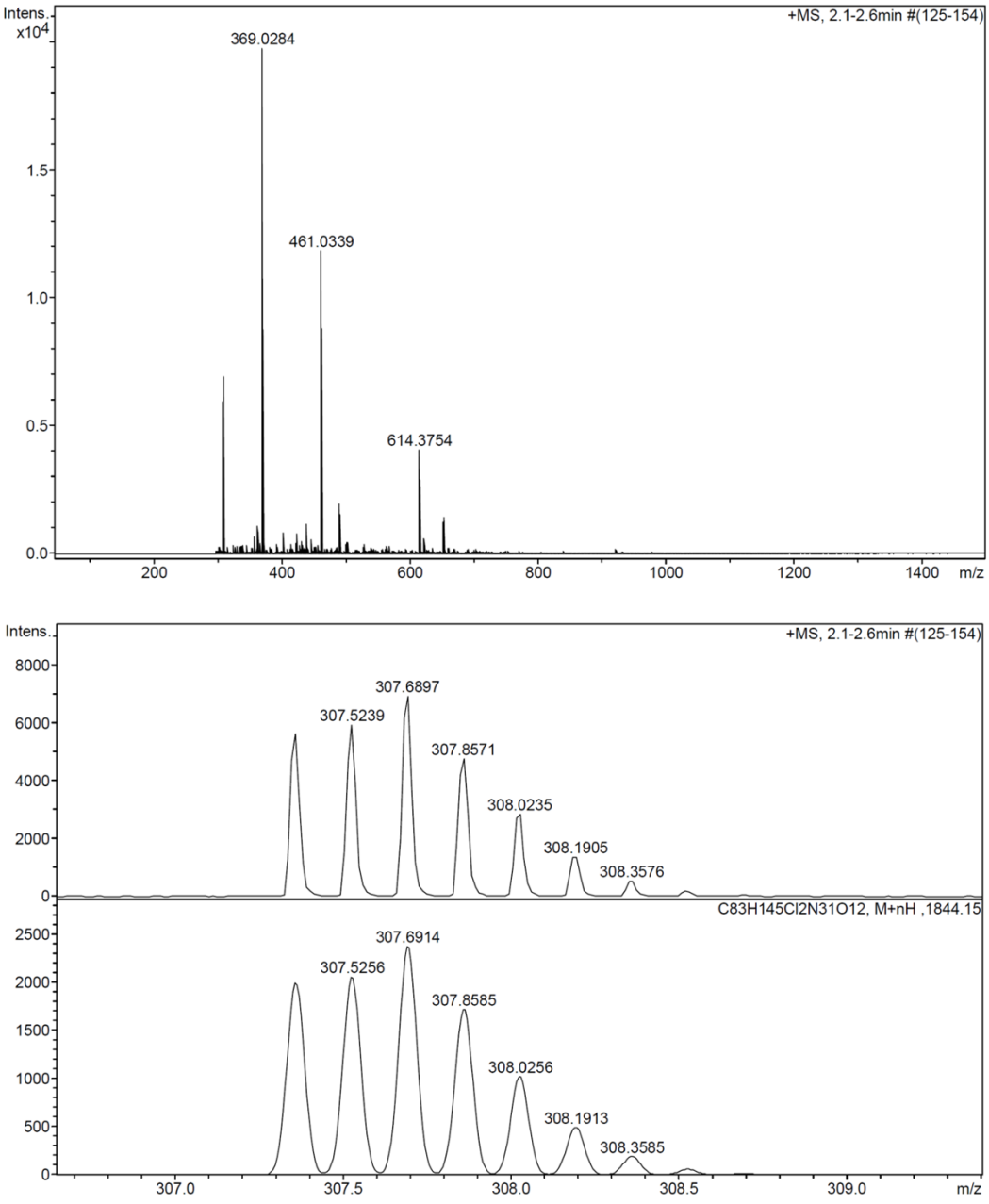


**a)**

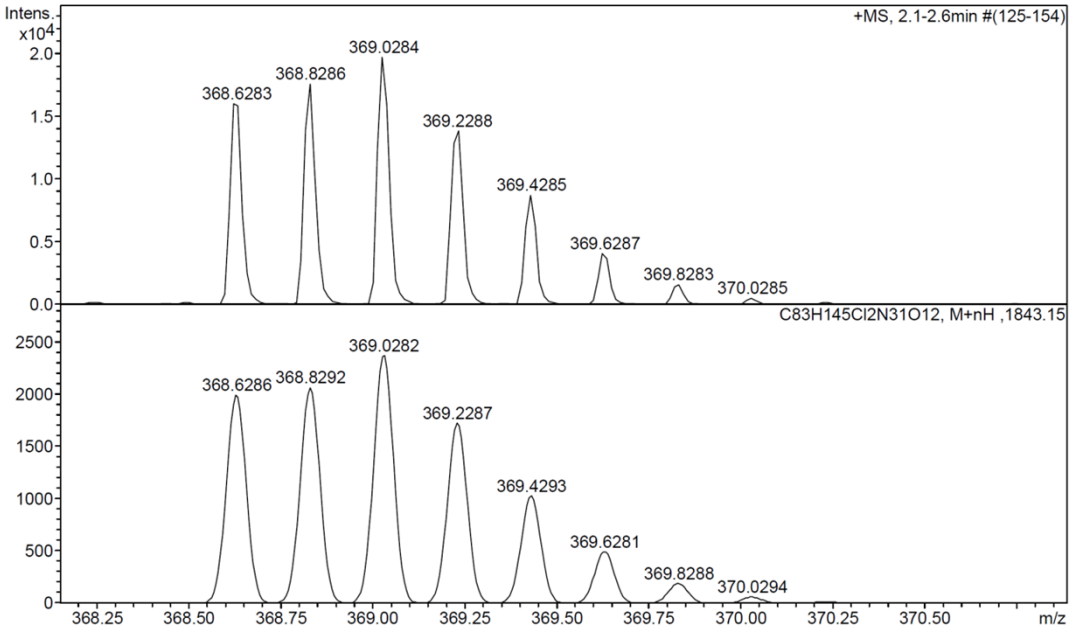



| No. | Temps retenció min | alçada mAU | Area mAU*min | Area relativa % |
|---|---|---|---|---|
| 1 | 7,59 | 1340,298 | 155,670 | 100,00 |
| Total: | | 1340,298 | 155,670 | 100,00 |

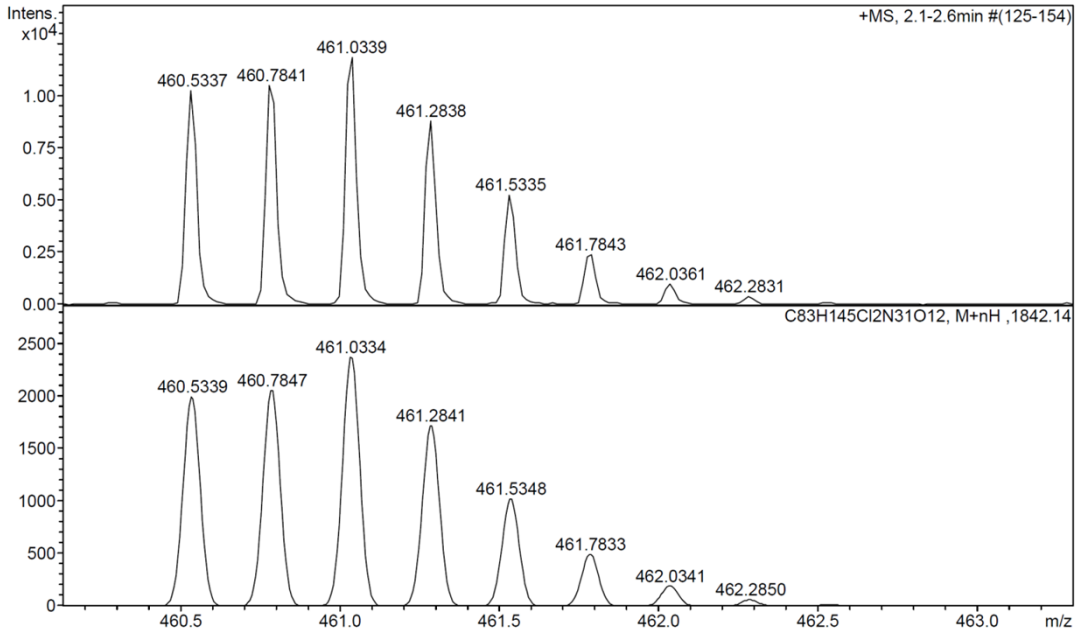
**b)**



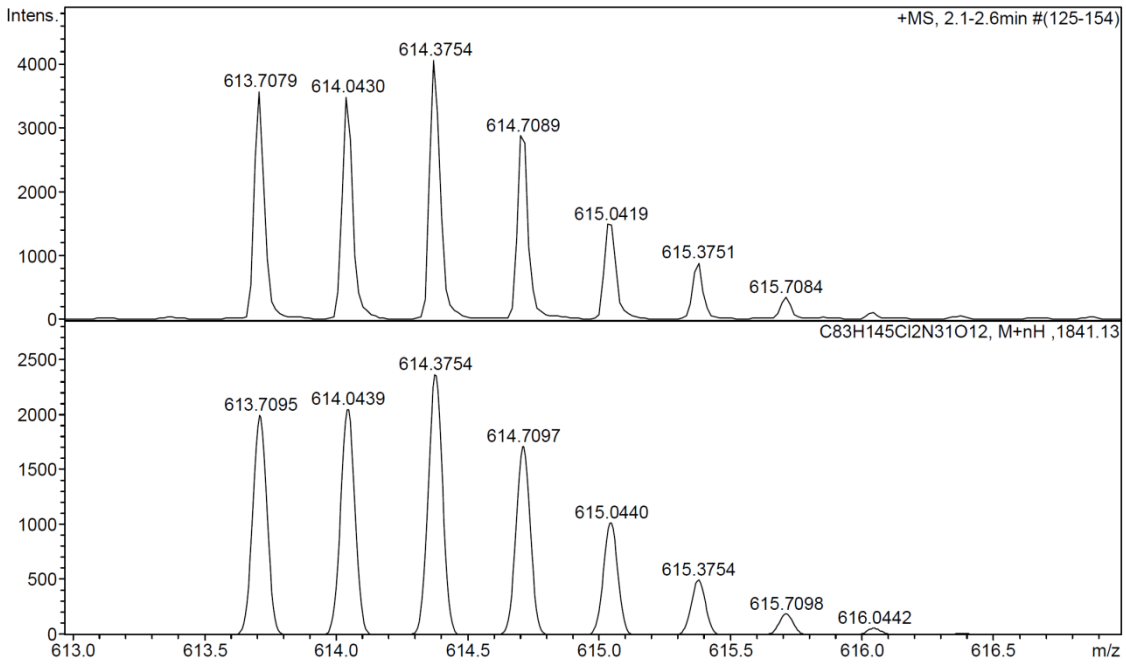

Observed HRMS (top) with the theoretical isotope prediction (bottom).




Observed HRMS (top) with the theoretical isotope prediction (bottom).



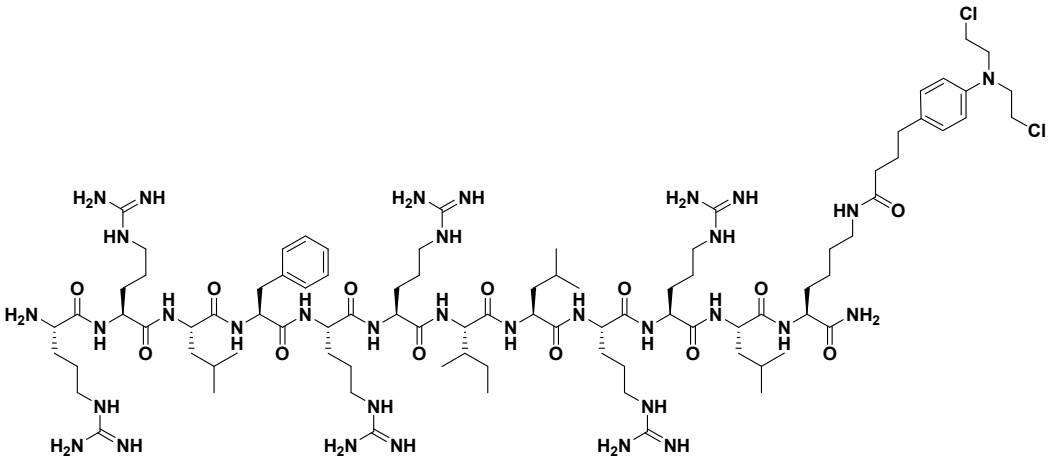

Observed HRMS (top) with the theoretical isotope prediction (bottom).




Observed HRMS (top) with the theoretical isotope prediction (bottom).

**Figure S8**: a) HPLC chromatogram (λ= 220 nm), b) HRMS spectrum (*m/z*).

**BP335**



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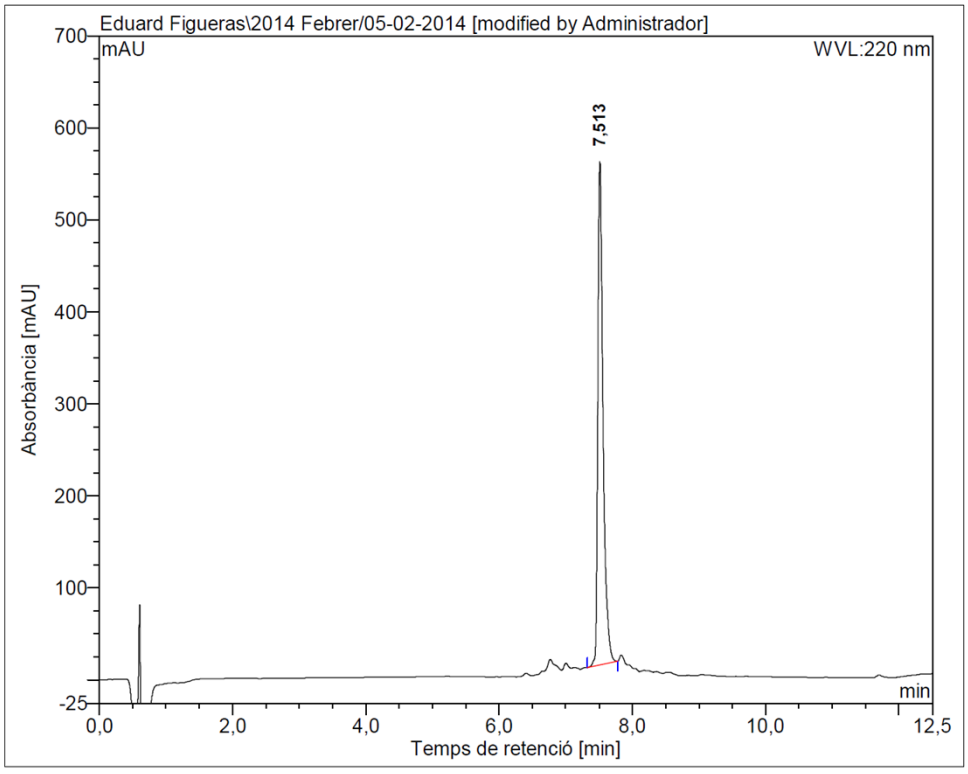
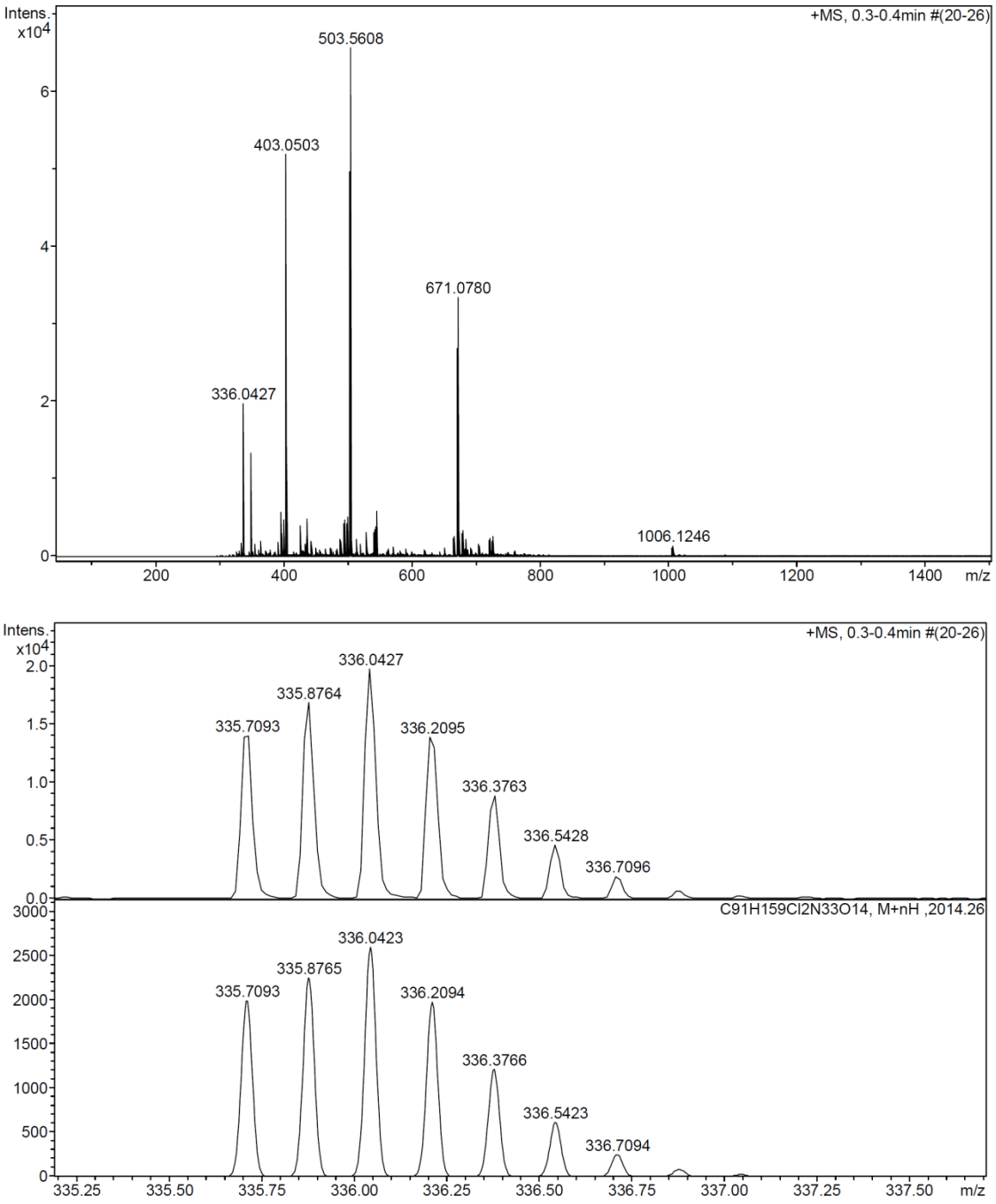
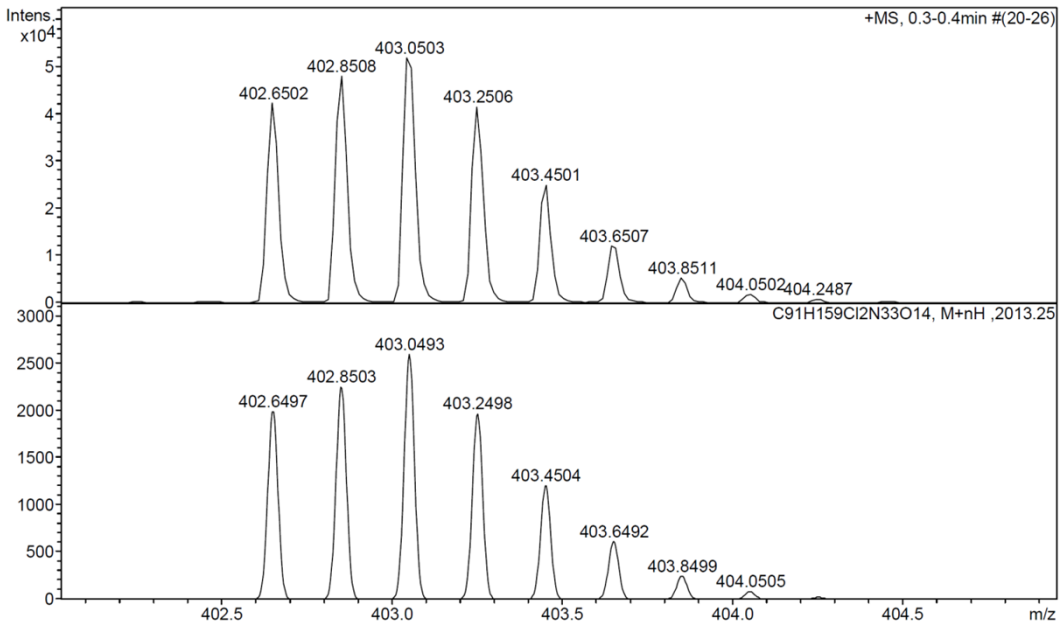



| No. | Temps retenció min | alçada mAU | Area mAU*min | Area relativa % |
|---|---|---|---|---|
| 1 | 7,51 | 546,652 | 49,784 | 100,00 |
| Total: | | 546,652 | 49,784 | 100,00 |

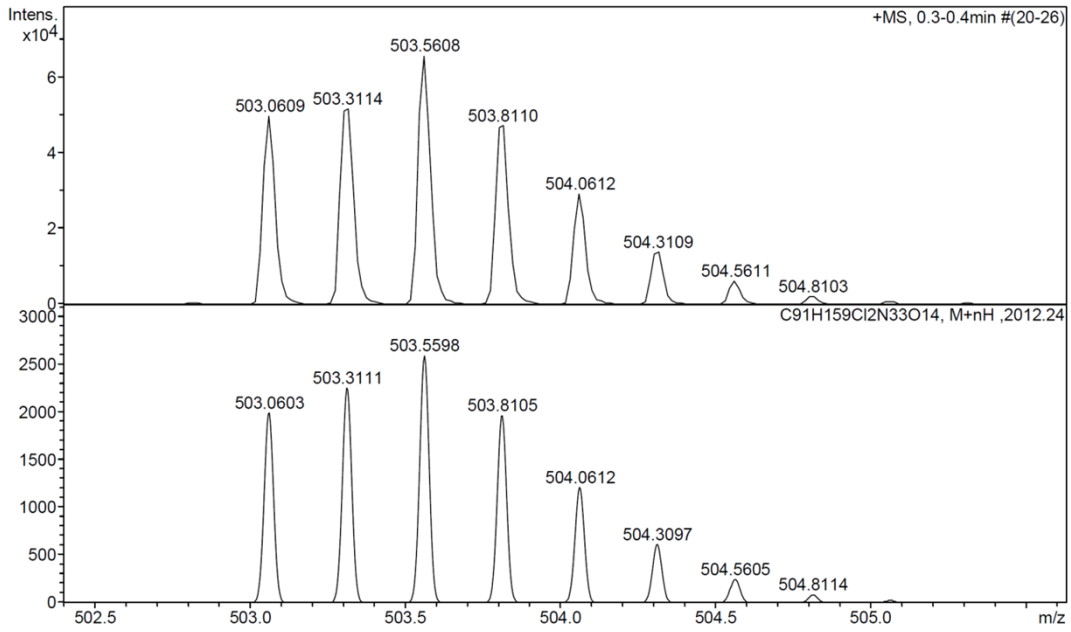
**b)**



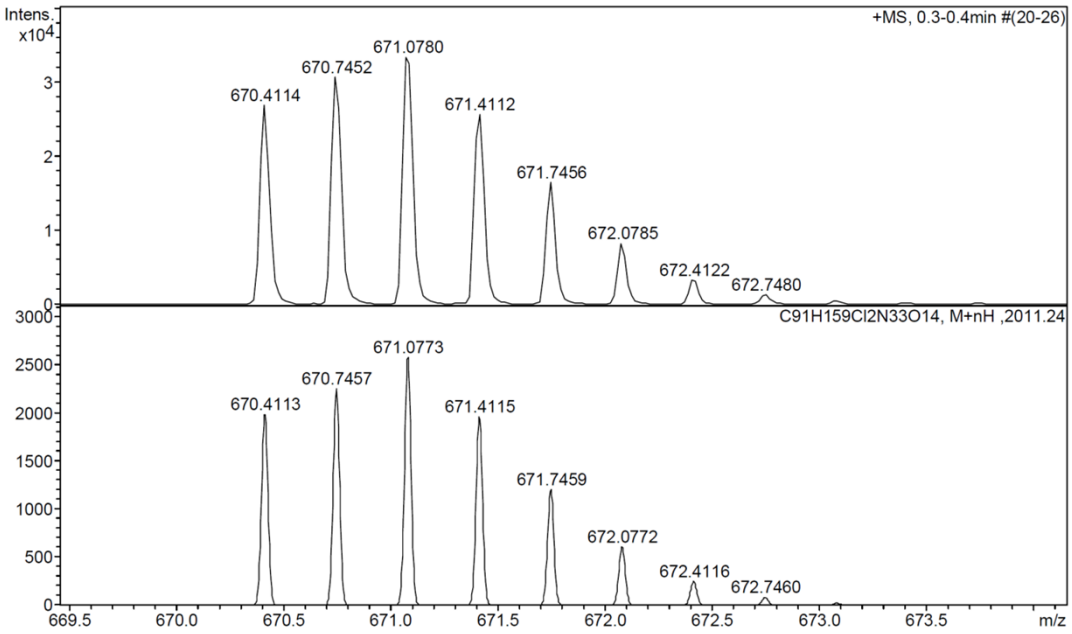

Observed HRMS (top) with the theoretical isotope prediction (bottom).



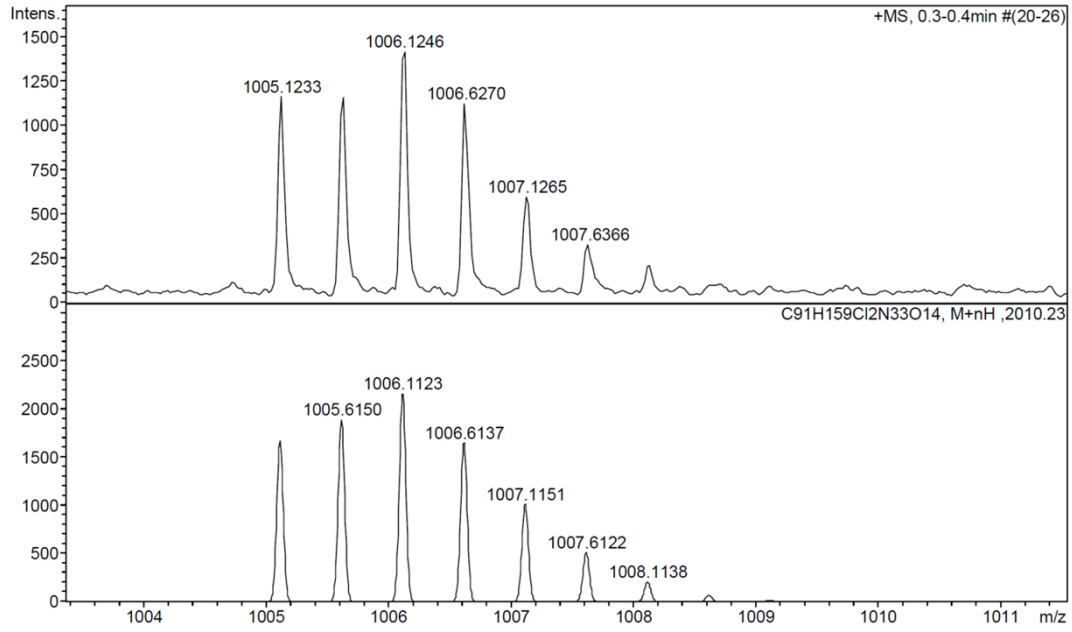

Observed HRMS (top) with the theoretical isotope prediction (bottom).




Observed HRMS (top) with the theoretical isotope prediction (bottom).



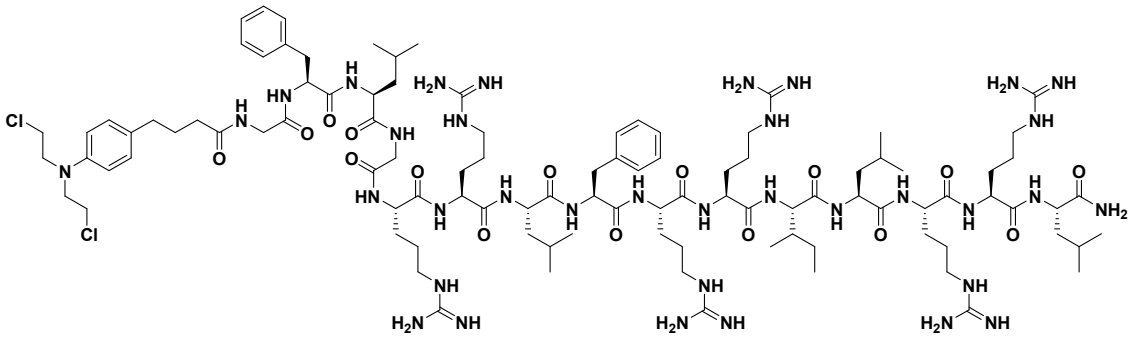

Observed HRMS (top) with the theoretical isotope prediction (bottom).



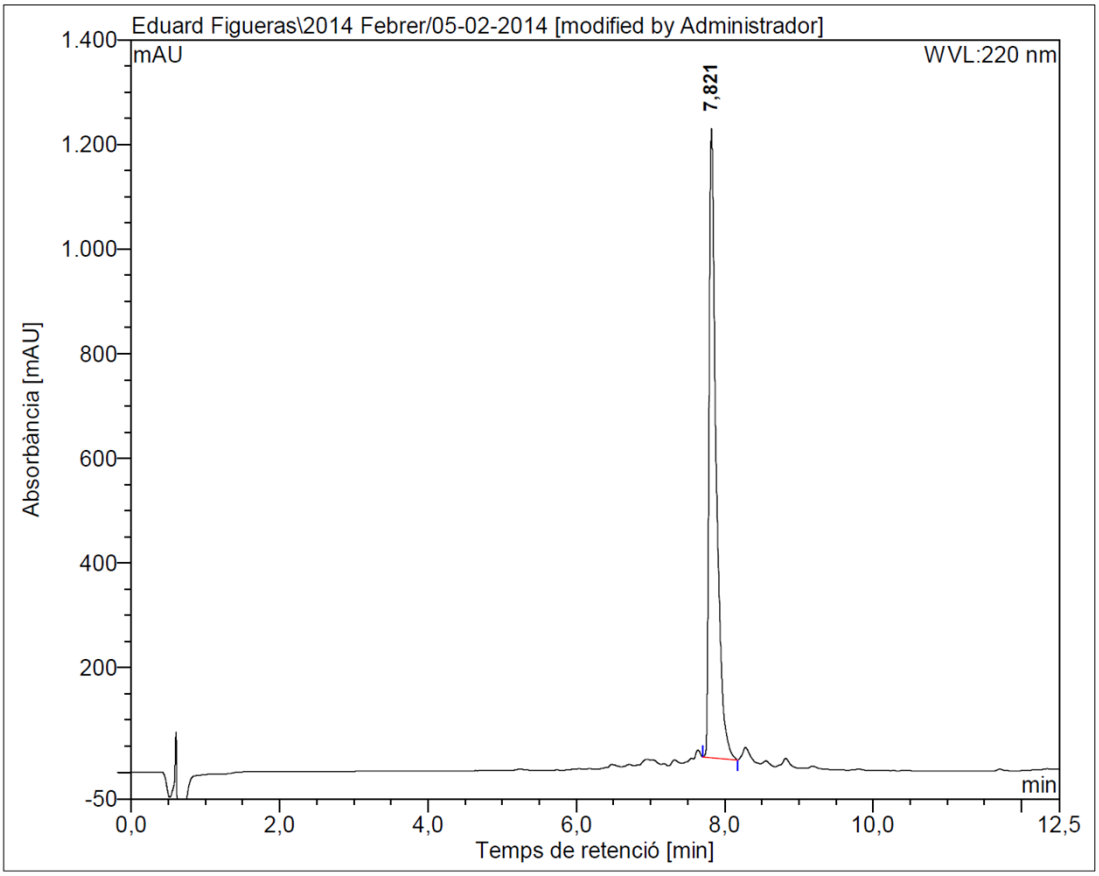

Observed HRMS (top) with the theoretical isotope prediction (bottom).

**Figure S9**: a) HPLC chromatogram (λ= 220 nm), b) HRMS spectrum (*m/z*).

**BP336**

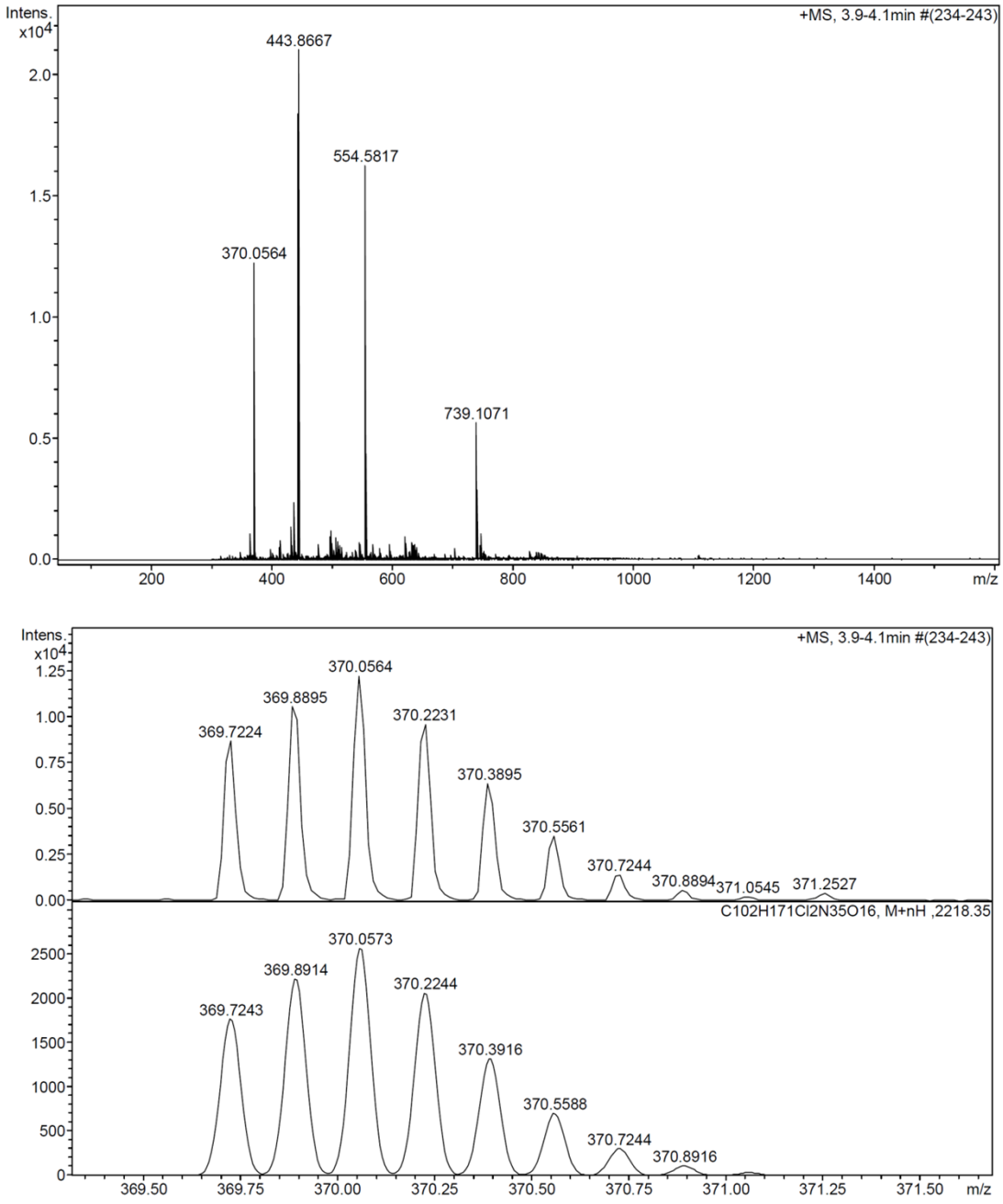


**a)**

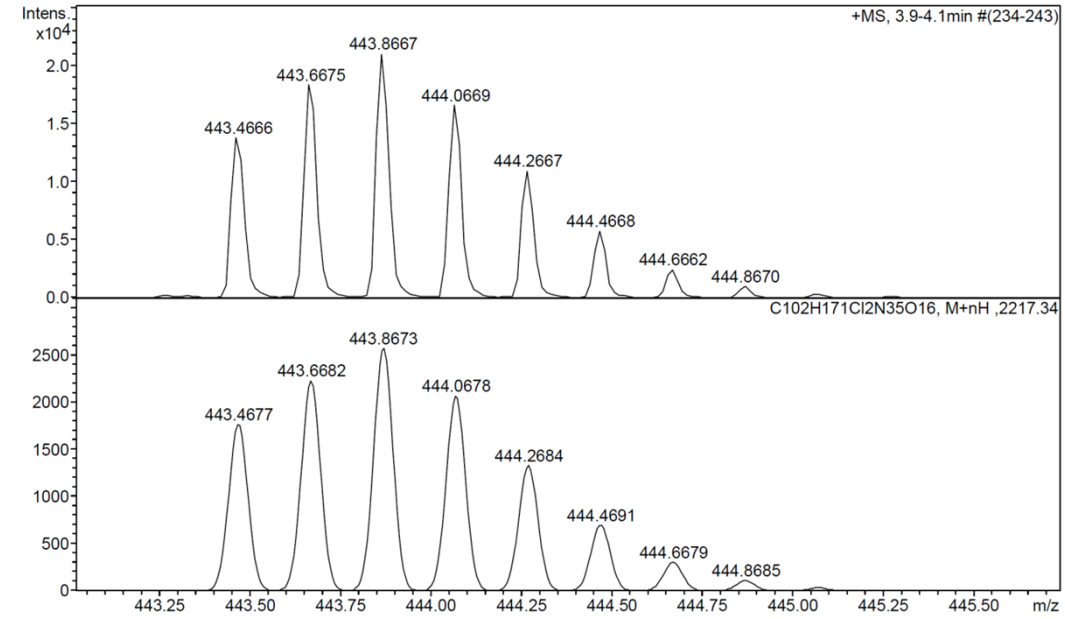



| No. | Temps retenció min | alçada mAU | Area mAU*min | Area relativa % |
|---|---|---|---|---|
| 1 | 7,82 | 1201,956 | 133,830 | 100,00 |
| Total: | | 1201,956 | 133,830 | 100,00 |

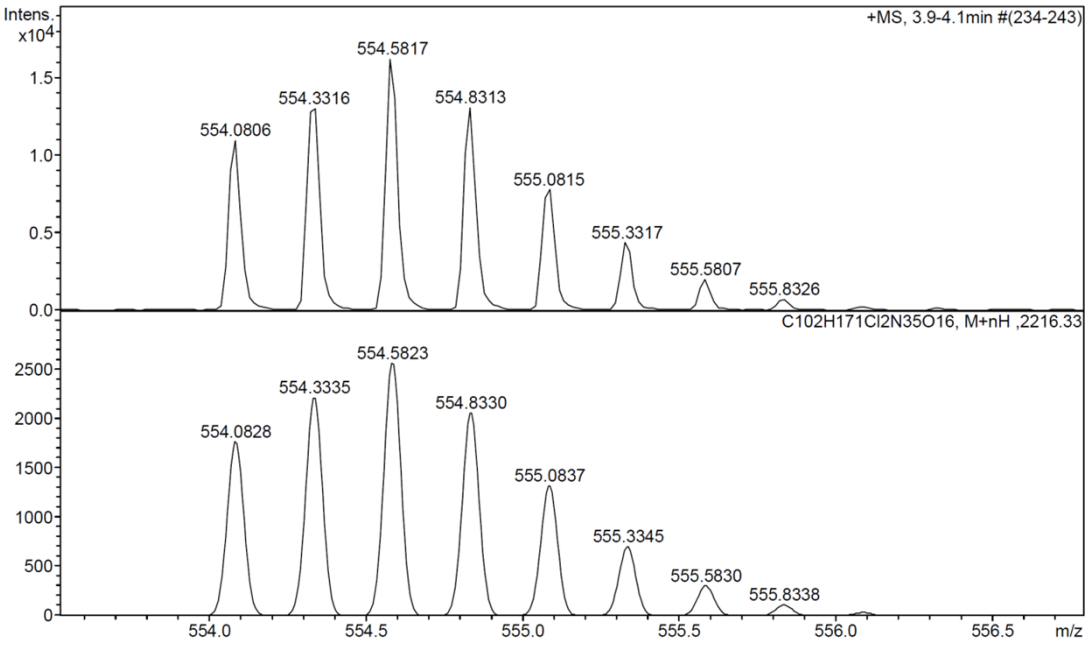
**b)**



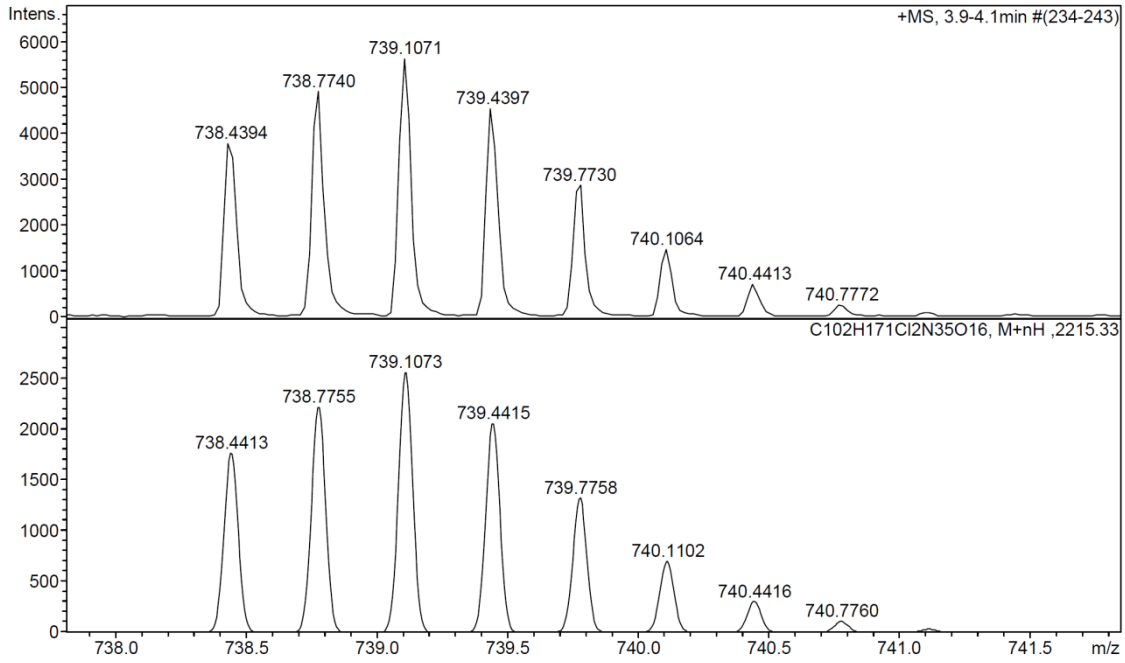

Observed HRMS (top) with the theoretical isotope prediction (bottom).




Observed HRMS (top) with the theoretical isotope prediction (bottom).



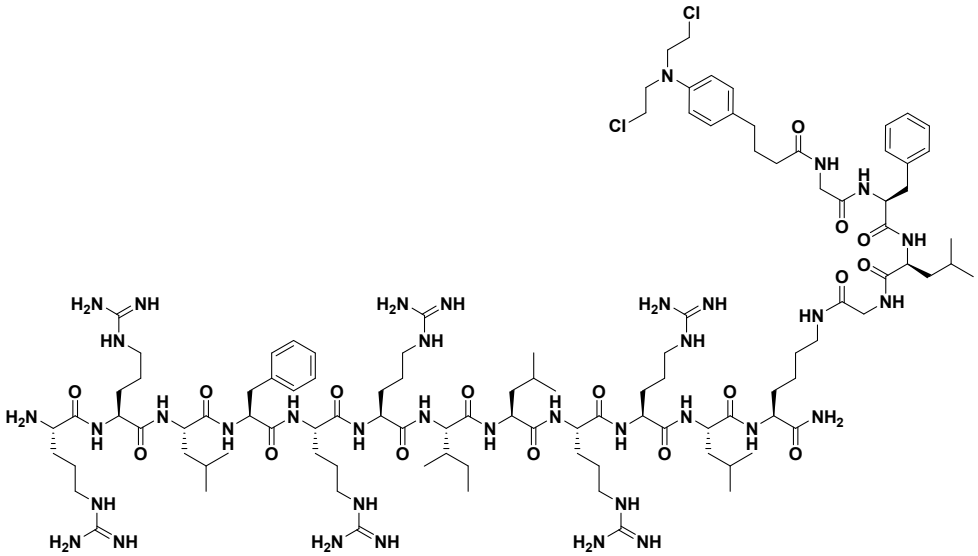

Observed HRMS (top) with the theoretical isotope prediction (bottom).



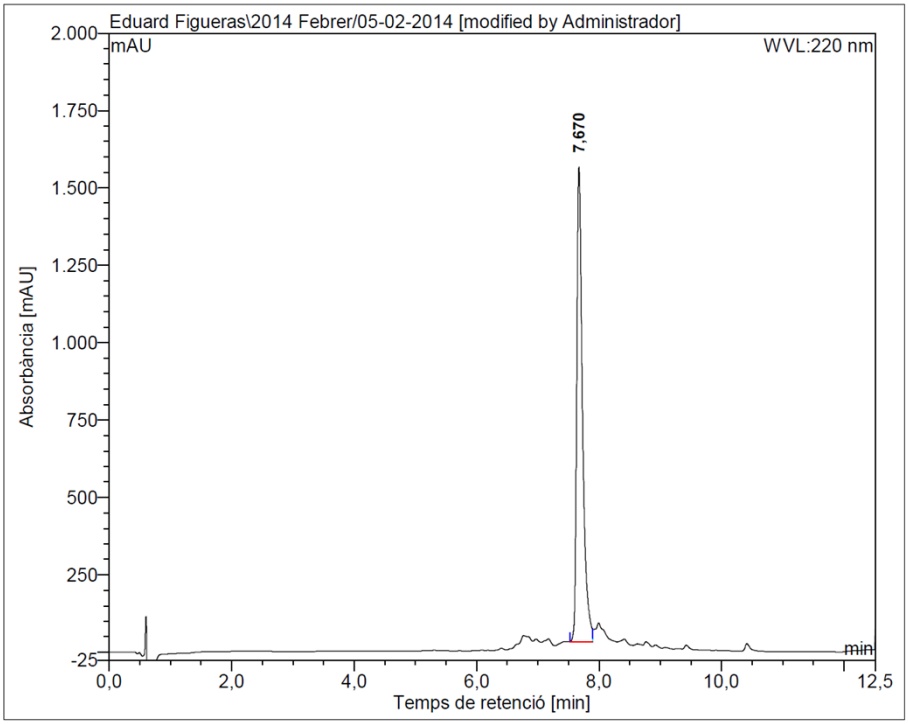

Observed HRMS (top) with the theoretical isotope prediction (bottom).

**Figure S10**: a) HPLC chromatogram (λ= 220 nm), b) HRMS spectrum (*m/z*).

**BP337**

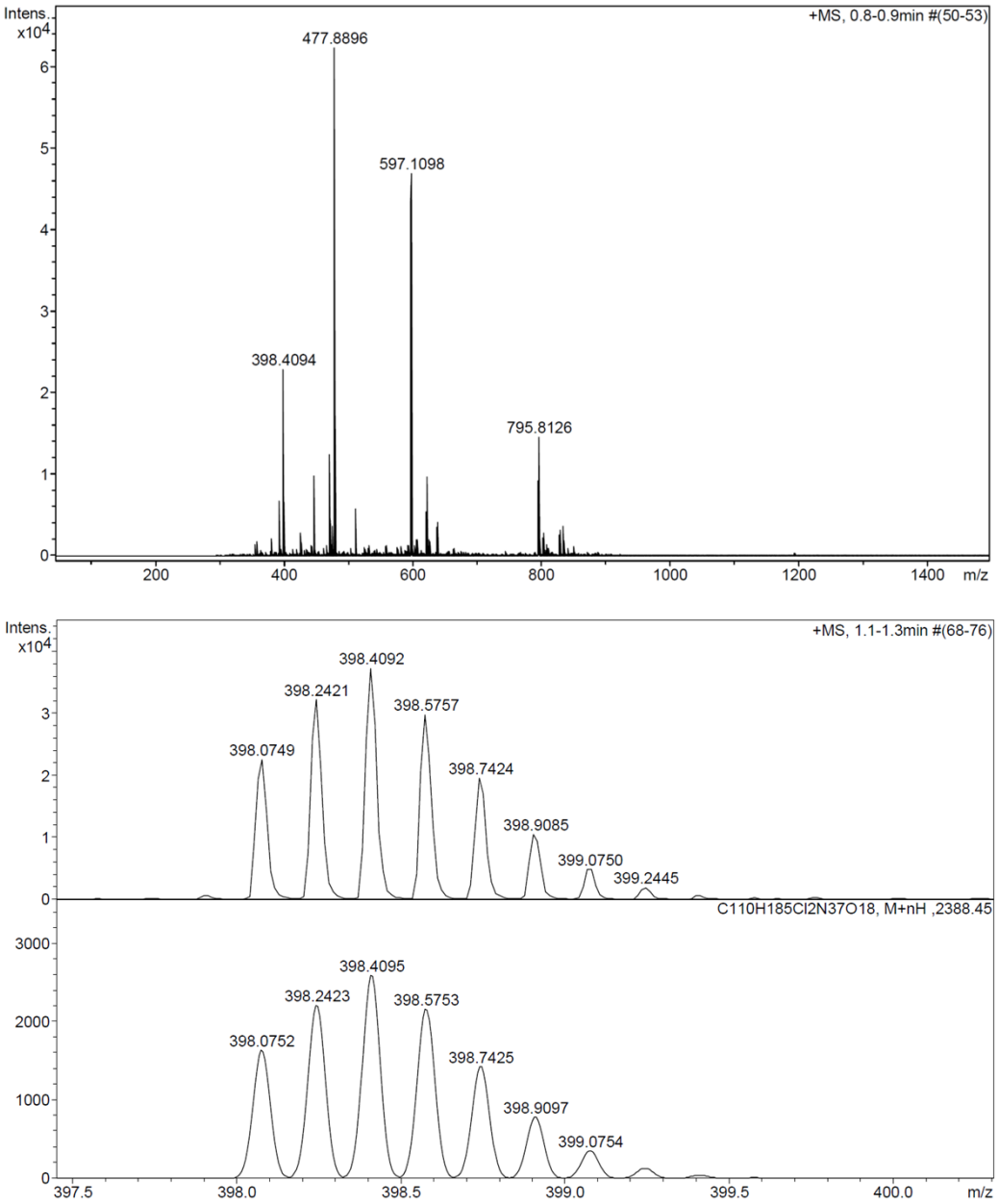


**a)**

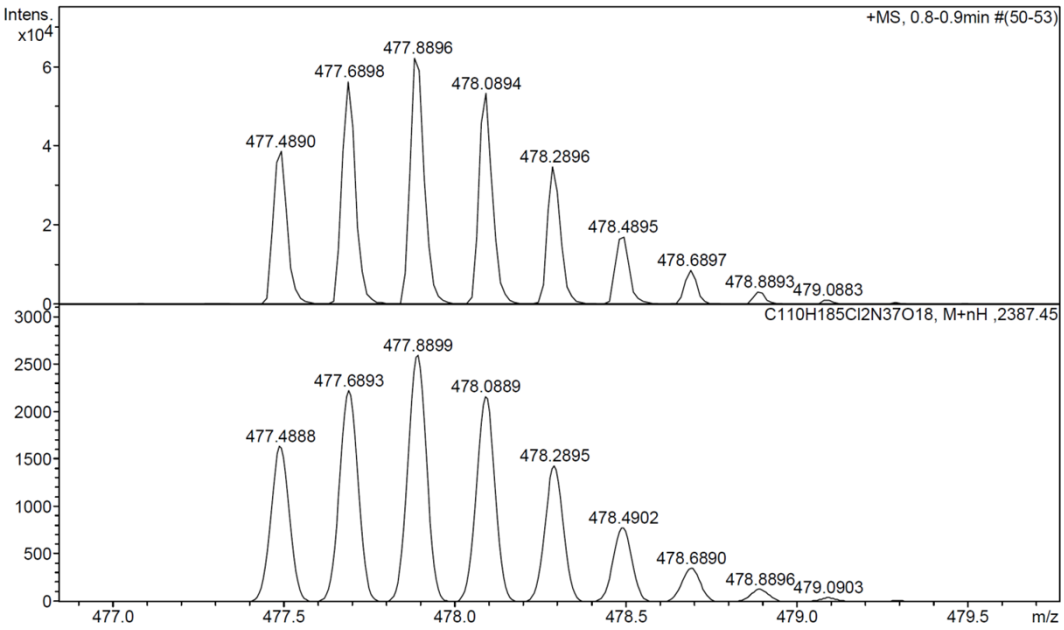



| No. | Temps retenció min | alçada mAU | Area mAU*min | Area relativa % |
|---|---|---|---|---|
| 1 | 7,67 | 1534,141 | 168,935 | 100,00 |
| Total: | | 1534,141 | 168,935 | 100,00 |

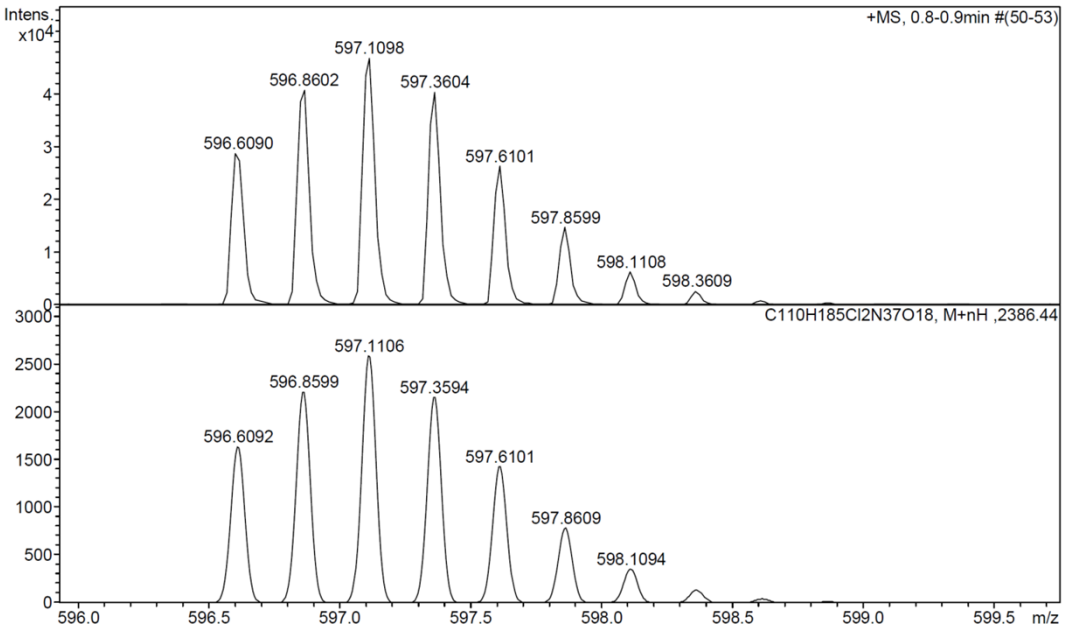
**b)**



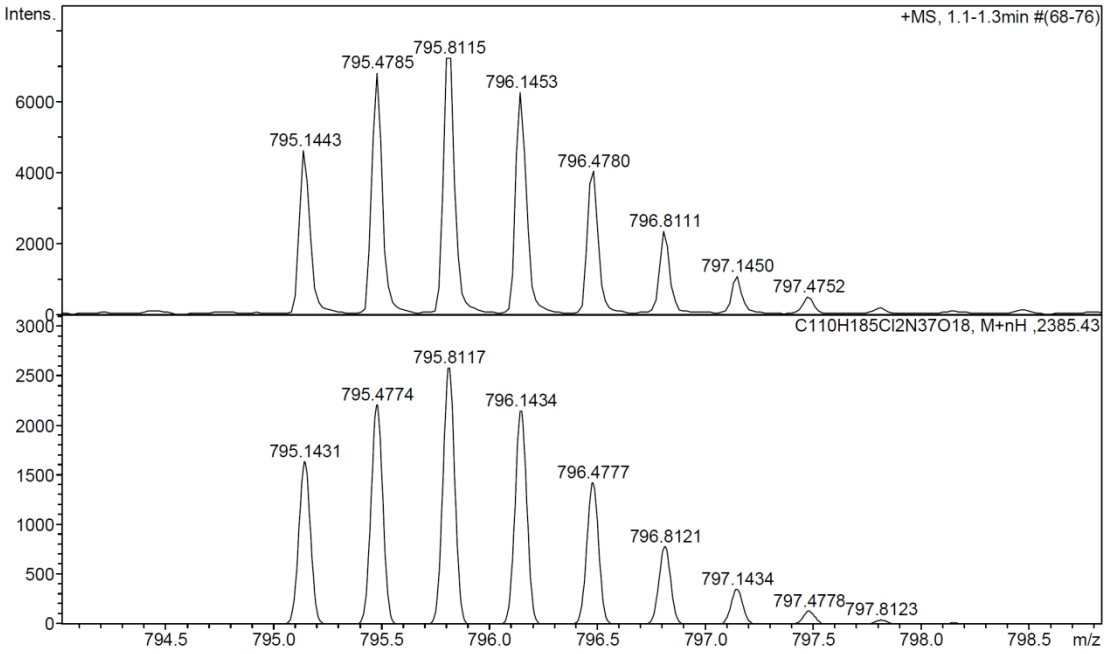

Observed HRMS (top) with the theoretical isotope prediction (bottom).




Observed HRMS (top) with the theoretical isotope prediction (bottom).




Observed HRMS (top) with the theoretical isotope prediction (bottom).

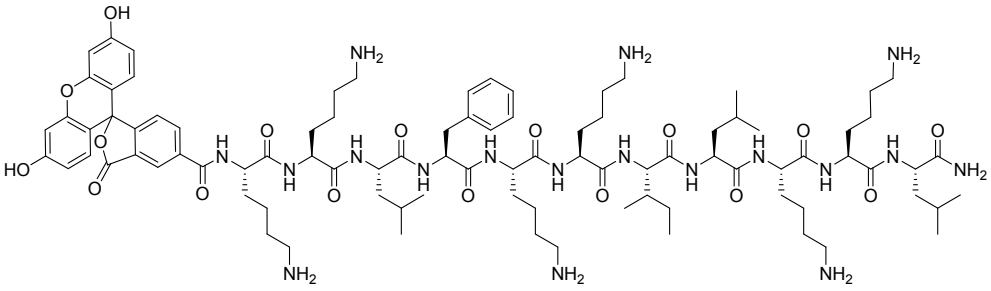



Observed HRMS (top) with the theoretical isotope prediction (bottom).

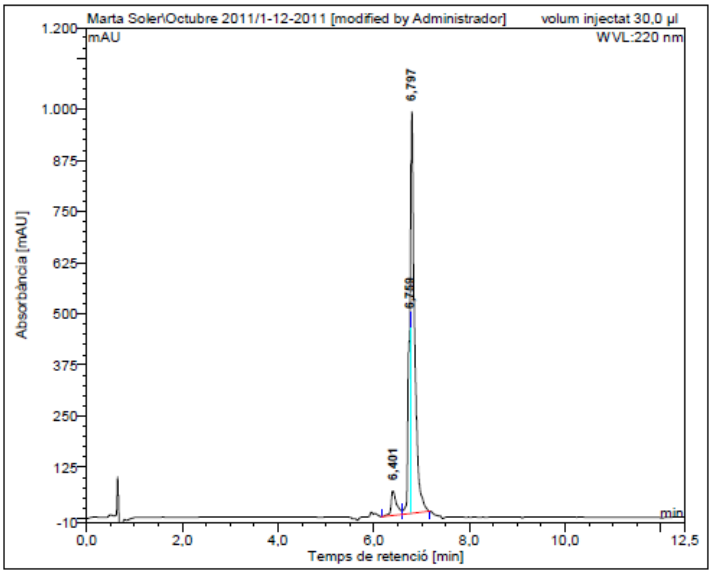
## 4. HPLC, ESI-MS and HRMS of 5(6)-carboxyfluorescein labeled peptides

**Figure S11**: a) HPLC chromatogram (λ= 220 nm), b) HRMS spectrum (*m/z*).

**CF-BP16**

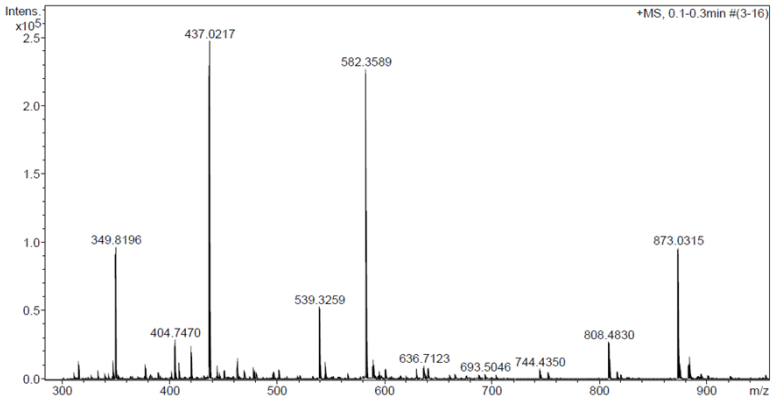


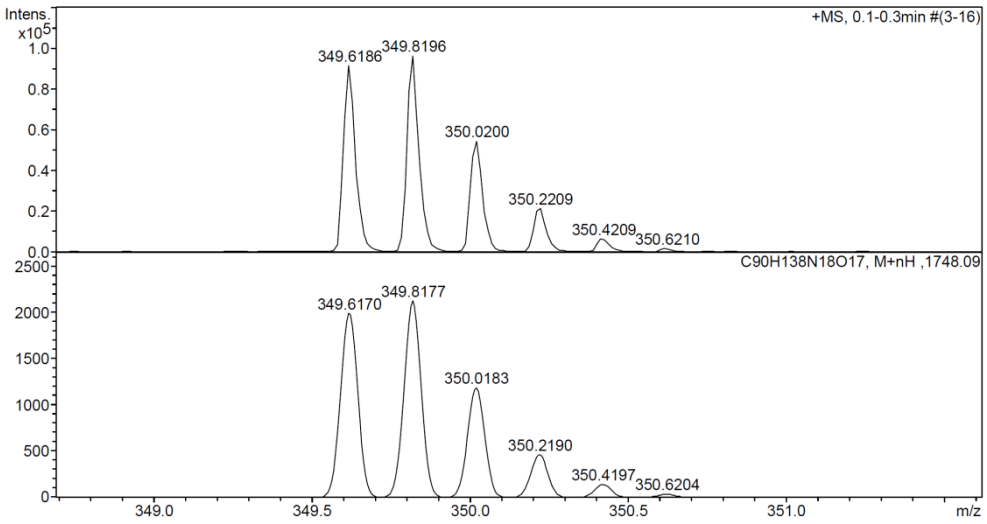
a)



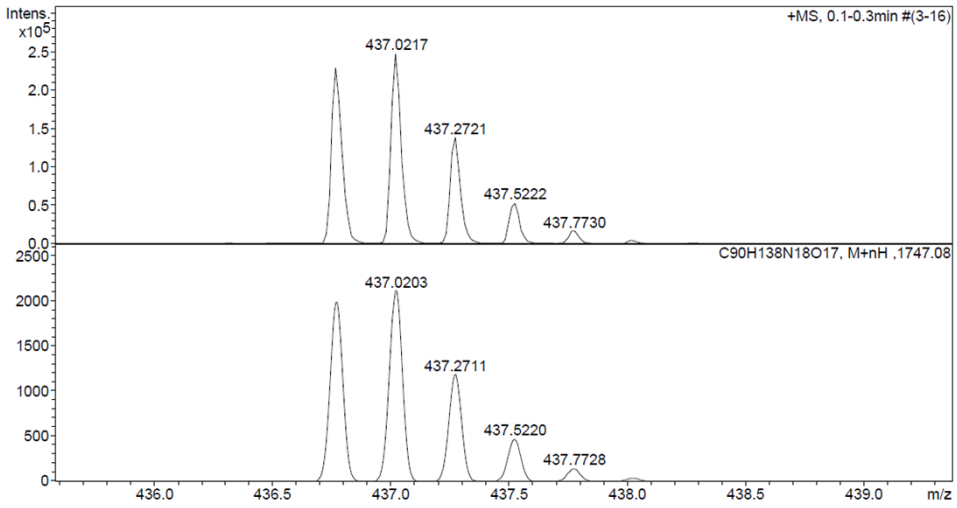

| No. | mps retenc min | alçada mAU | Area mAU*min | Area relativa % |
|---|---|---|---|---|
| 1 | 6,40 | 60,473 | 8,138 | 6,40 |
| 2 | 6,76 | 473,768 | 22,057 | 17,34 |
| 3 | 6,80 | 981,748 | 97,024 | 76,26 |
| Total: | | 1515,990 | 127,220 | 100,00 |

**b)**

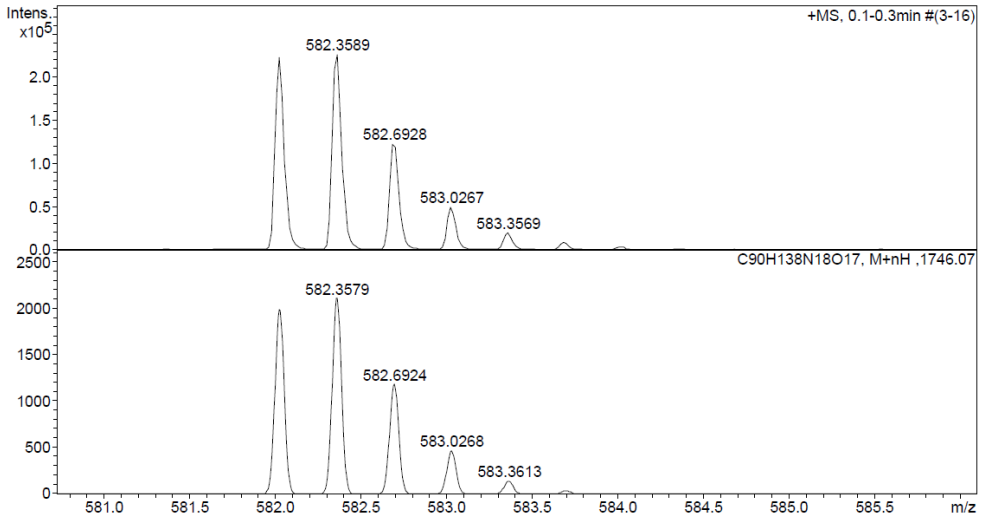





Observed HRMS (top) with the theoretical isotope prediction (bottom).



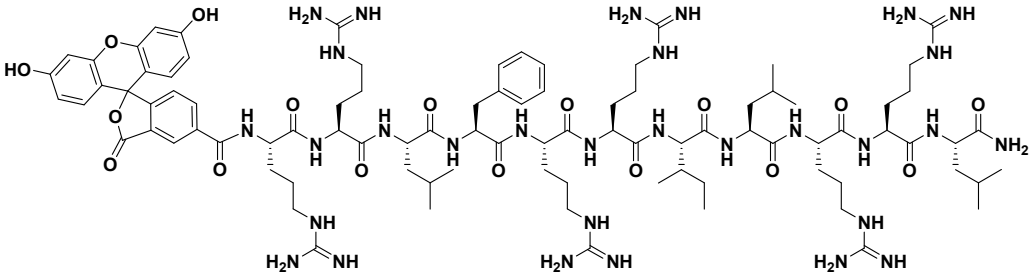

Observed HRMS (top) with the theoretical isotope prediction (bottom).



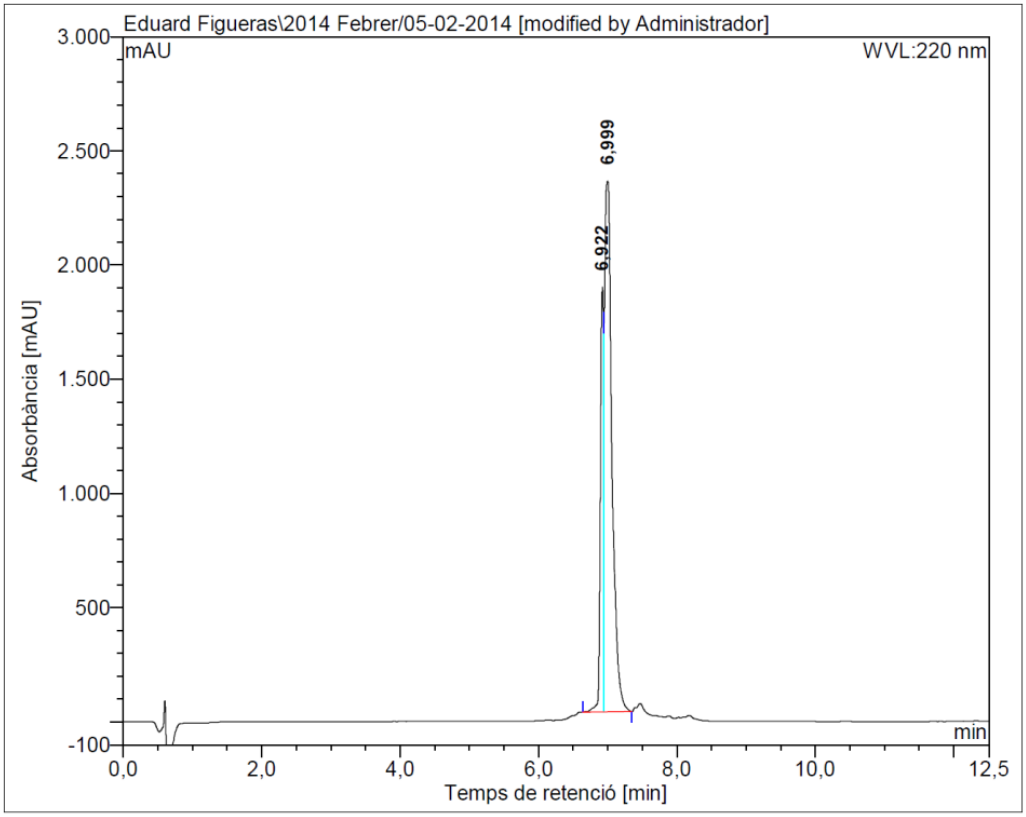

Observed HRMS (top) with the theoretical isotope prediction (bottom).

**Figure S12**: a) HPLC chromatogram (λ= 220 nm), b) HRMS spectrum (*m/z*).

**CF-BP308**

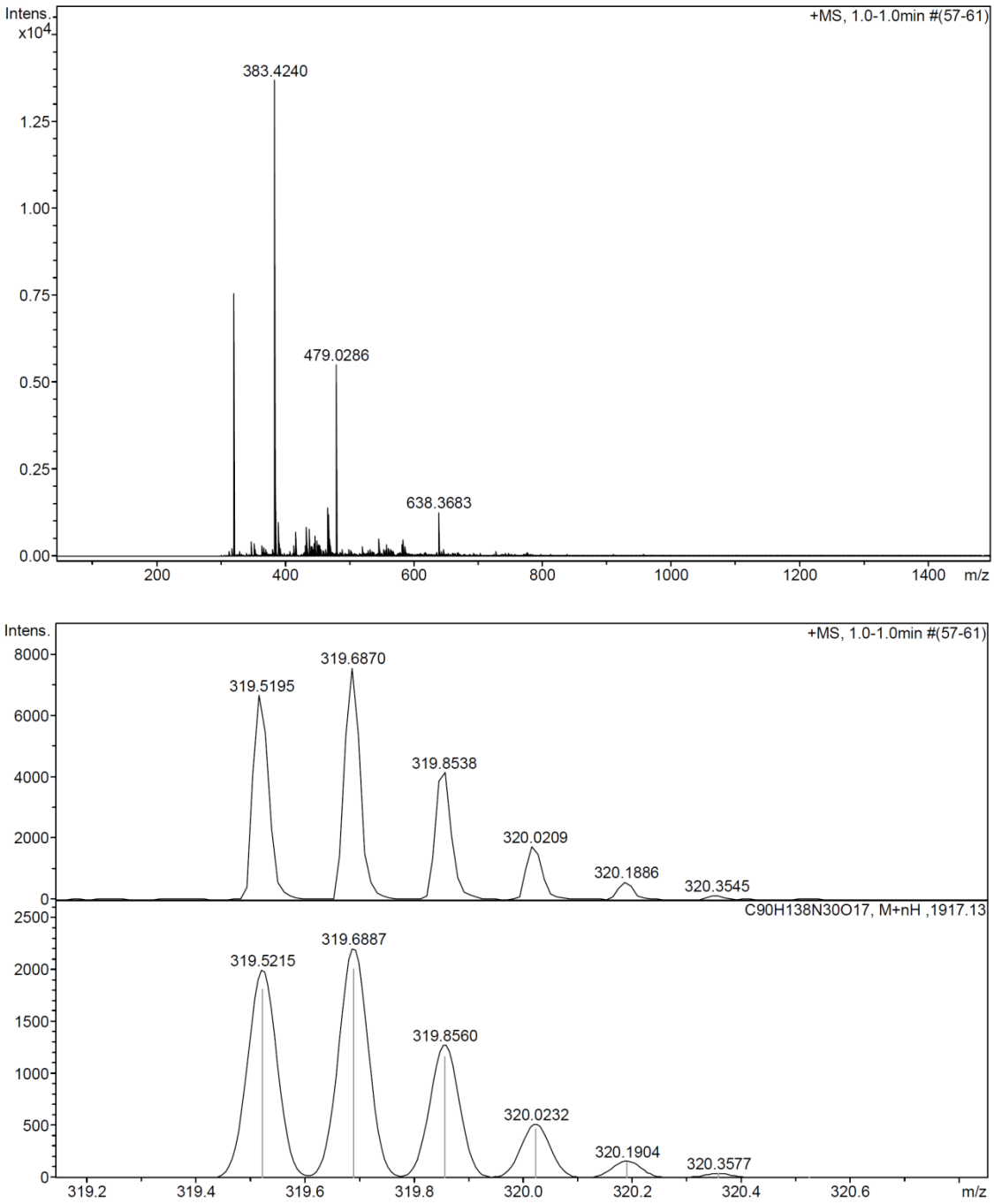


**a)**

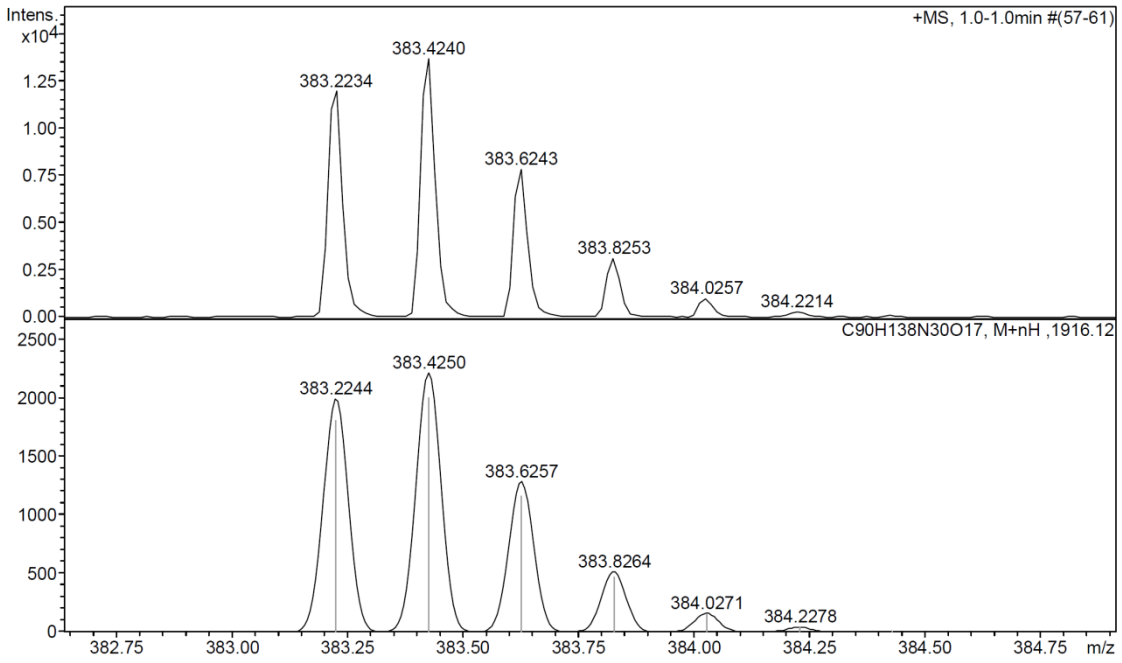



| No. | Temps retenció min | alçada mAU | Area mAU*min | Area relativa % |
|---|---|---|---|---|
| 1 | 6,92 | 1859,025 | 98,247 | 25,25 |
| 2 | 7,00 | 2323,905 | 290,924 | 74,75 |
| Total: | | 4182,930 | 389,171 | 100,00 |

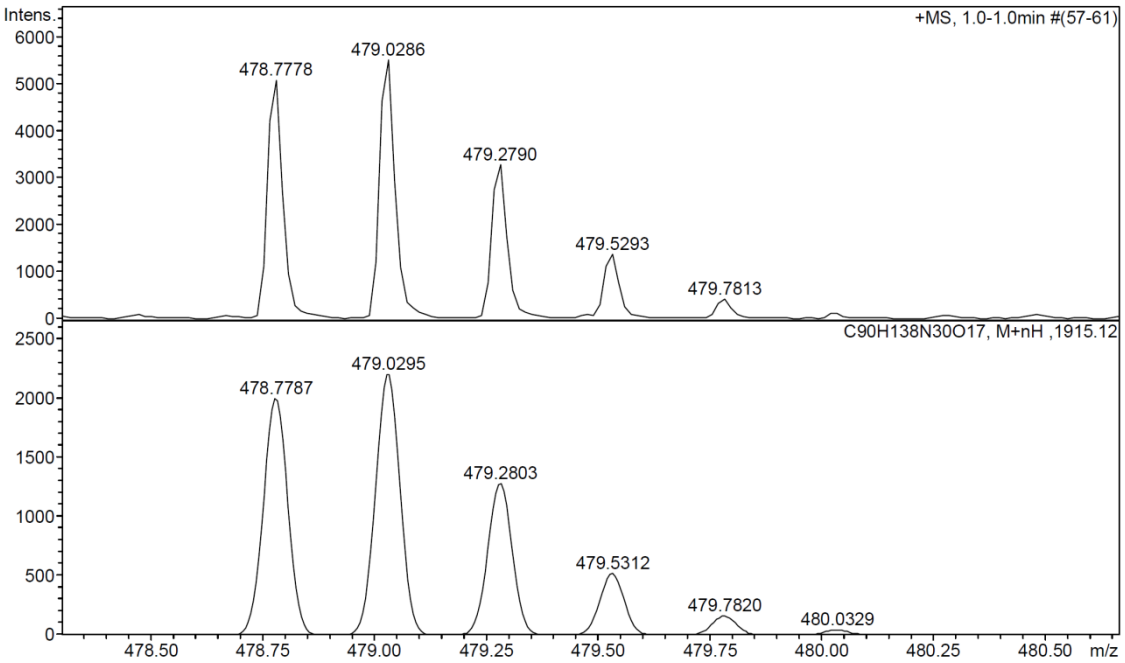
**b)**



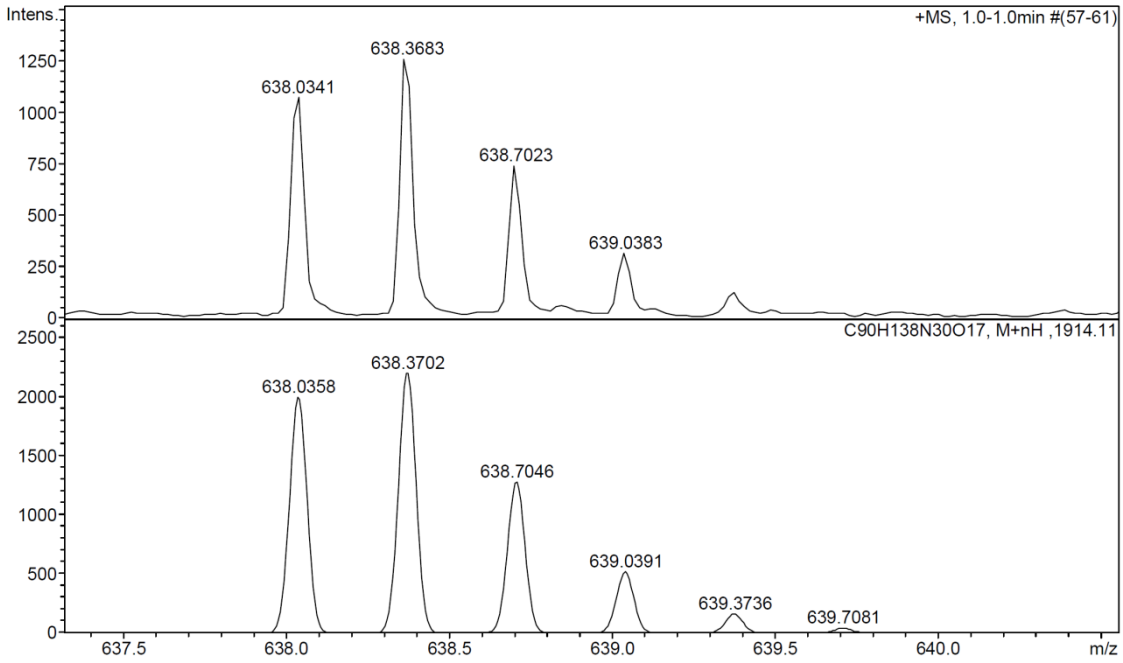

Observed HRMS (top) with the theoretical isotope prediction (bottom).




Observed HRMS (top) with the theoretical isotope prediction (bottom).



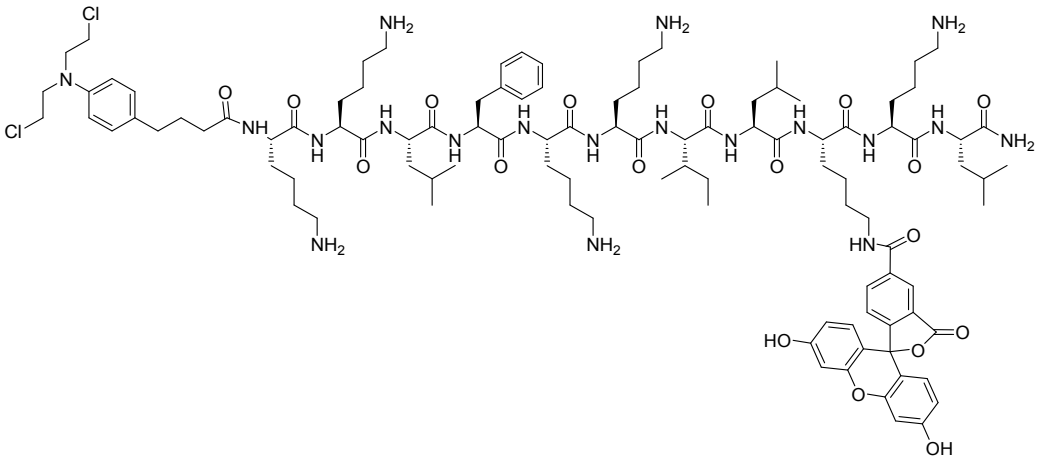

Observed HRMS (top) with the theoretical isotope prediction (bottom).



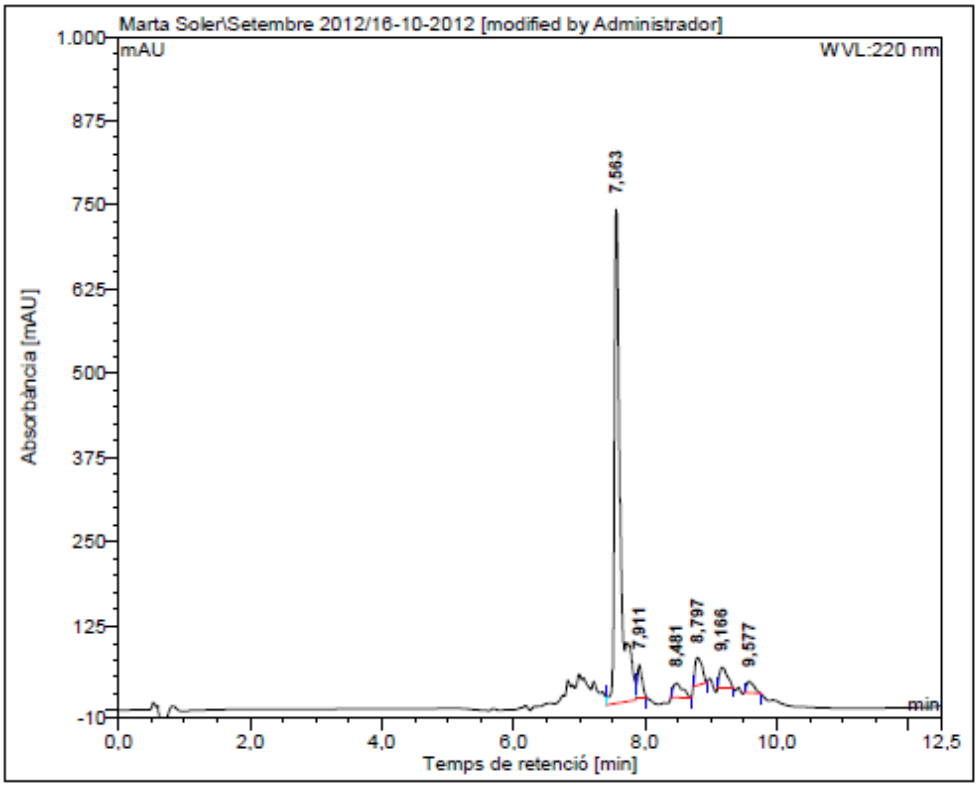

Observed HRMS (top) with the theoretical isotope prediction (bottom).

**Figure S13**: a) HPLC chromatogram (λ= 220 nm), b) HRMS spectrum (*m/z*).

**BP326**

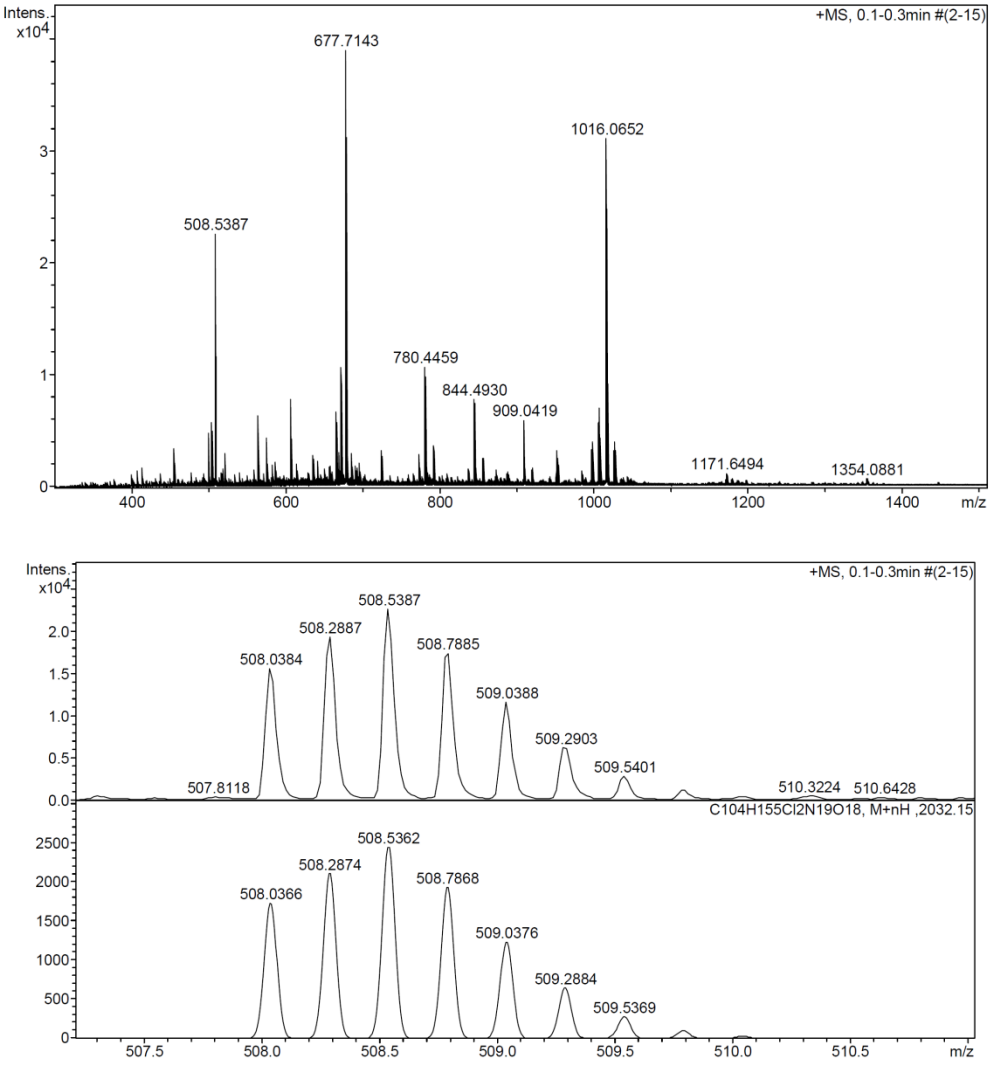


**a)**

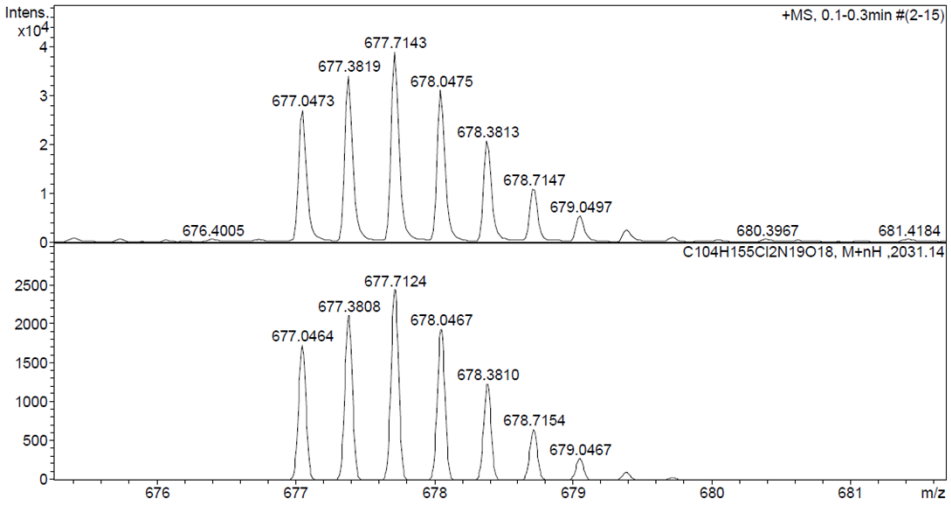



| No. | mps retenc min | alçada mAU | Area mAU*min | Area relativa % |
|---|---|---|---|---|
| 1 | 7,56 | 732,423 | 77,055 | 80,59 |
| 2 | 7,91 | 48,005 | 3,811 | 3,99 |
| 3 | 8,48 | 20,782 | 3,596 | 3,76 |
| 4 | 8,80 | 40,837 | 4,961 | 5,19 |
| 5 | 9,17 | 29,235 | 3,969 | 4,15 |
| 6 | 9,58 | 17,041 | 2,219 | 2,32 |
| Total: | | 888,322 | 95,611 | 100,00 |

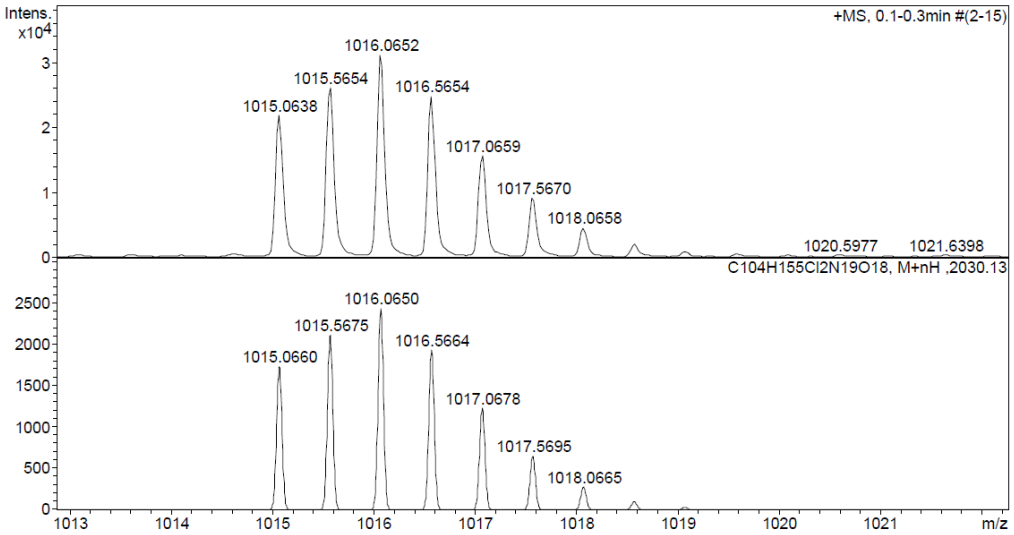
**b)**




Observed HRMS (top) with the theoretical isotope prediction (bottom).



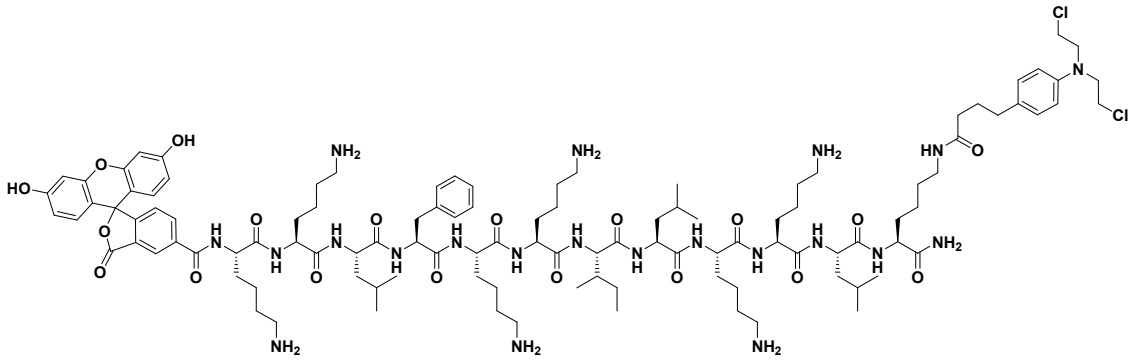

Observed HRMS (top) with the theoretical isotope prediction (bottom).



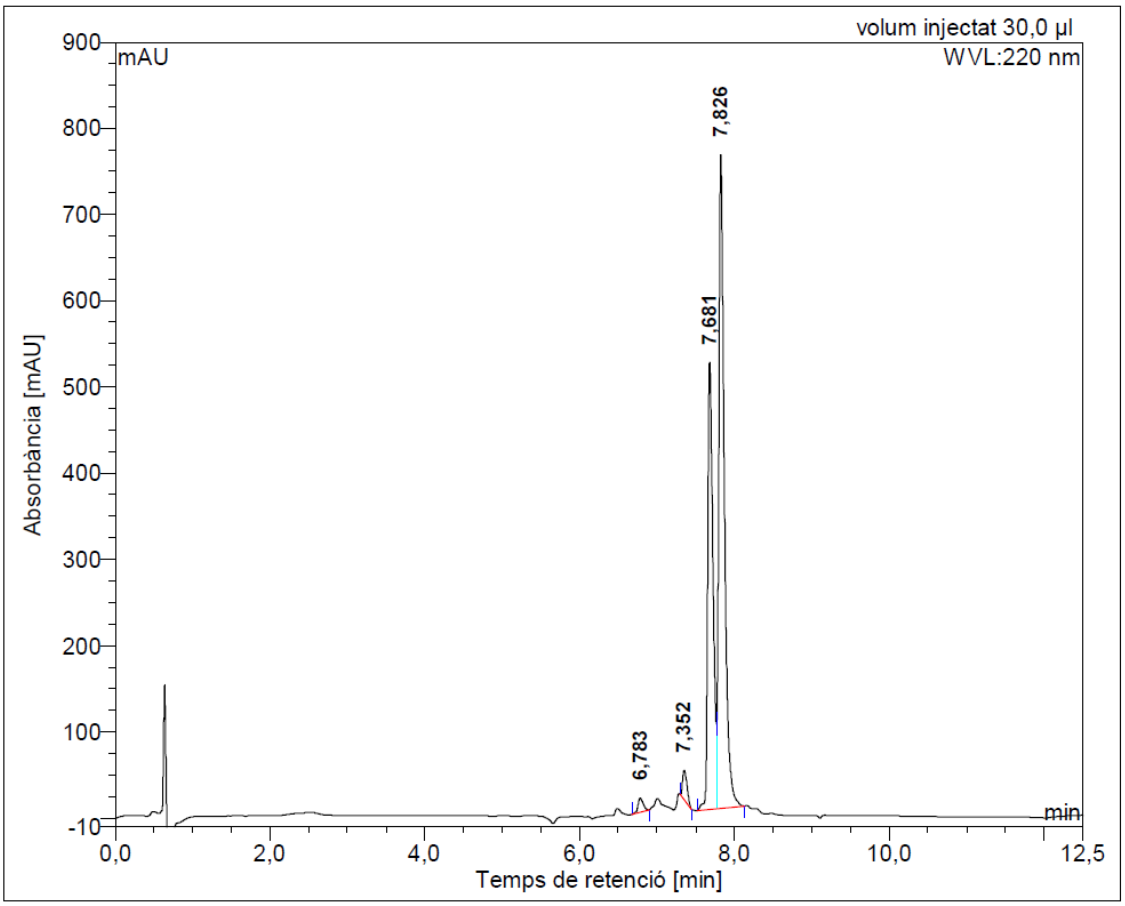

Observed HRMS (top) with the theoretical isotope prediction (bottom).

**Figure S14**: a) HPLC chromatogram (λ= 220 nm), b) HRMS spectrum (*m/z*).

**BP338**

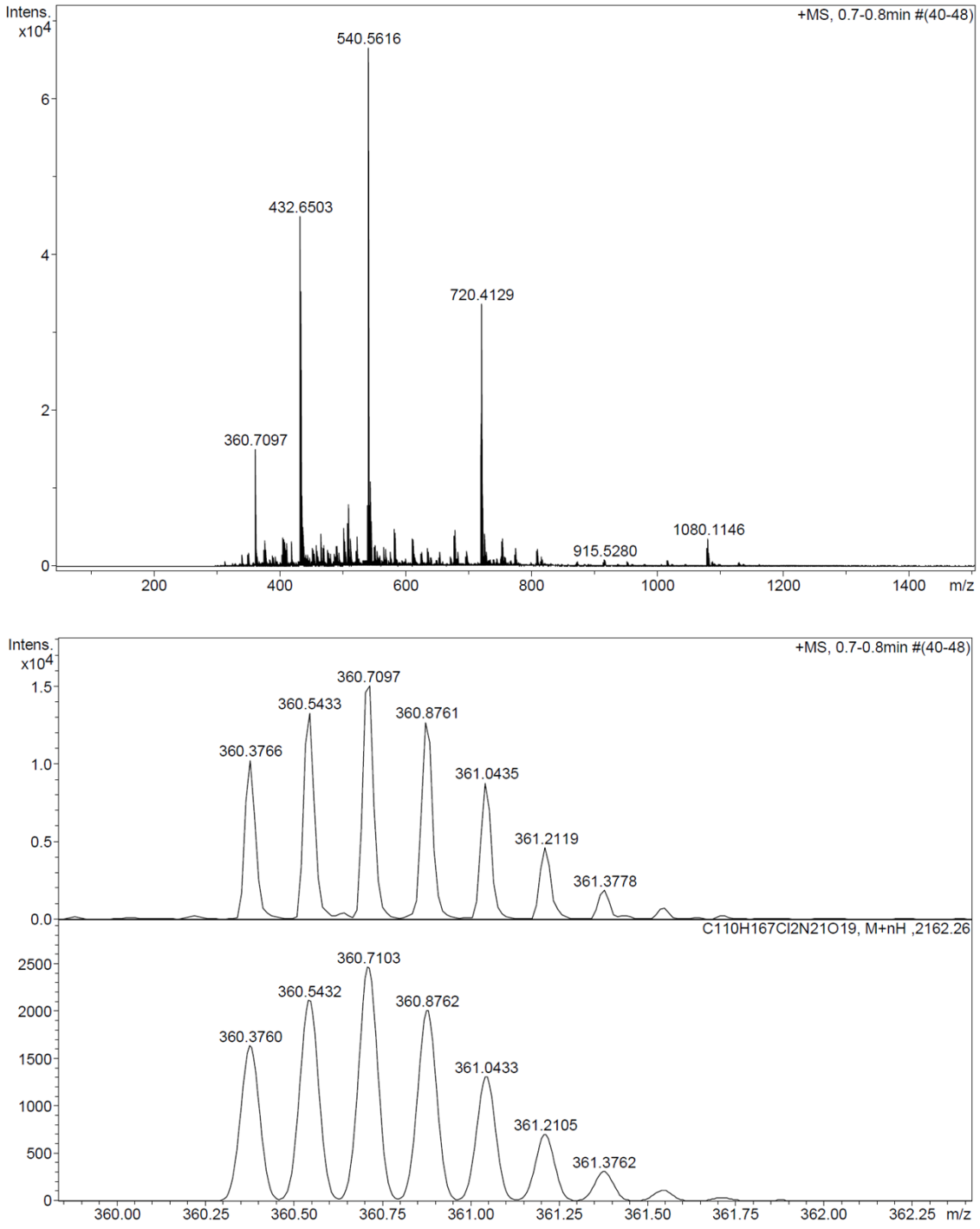


**a)**

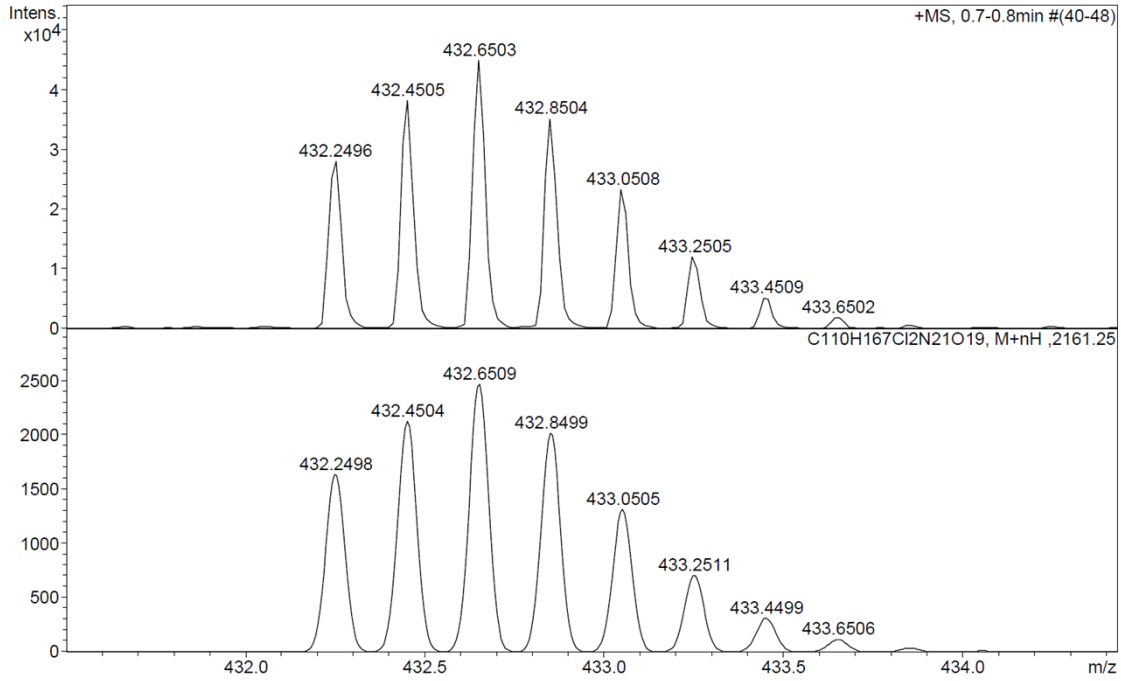



| No. | mps retenc min | alçada mAU | Area mAU*min | Area relativa % |
|---|---|---|---|---|
| 1 | 6,78 | 16,216 | 1,282 | 1,19 |
| 2 | 7,35 | 34,135 | 2,255 | 2,09 |
| 3 | 7,68 | 518,041 | 40,429 | 37,41 |
| 4 | 7,83 | 758,095 | 64,094 | 59,31 |
| Total: | | 1326,487 | 108,061 | 100,00 |

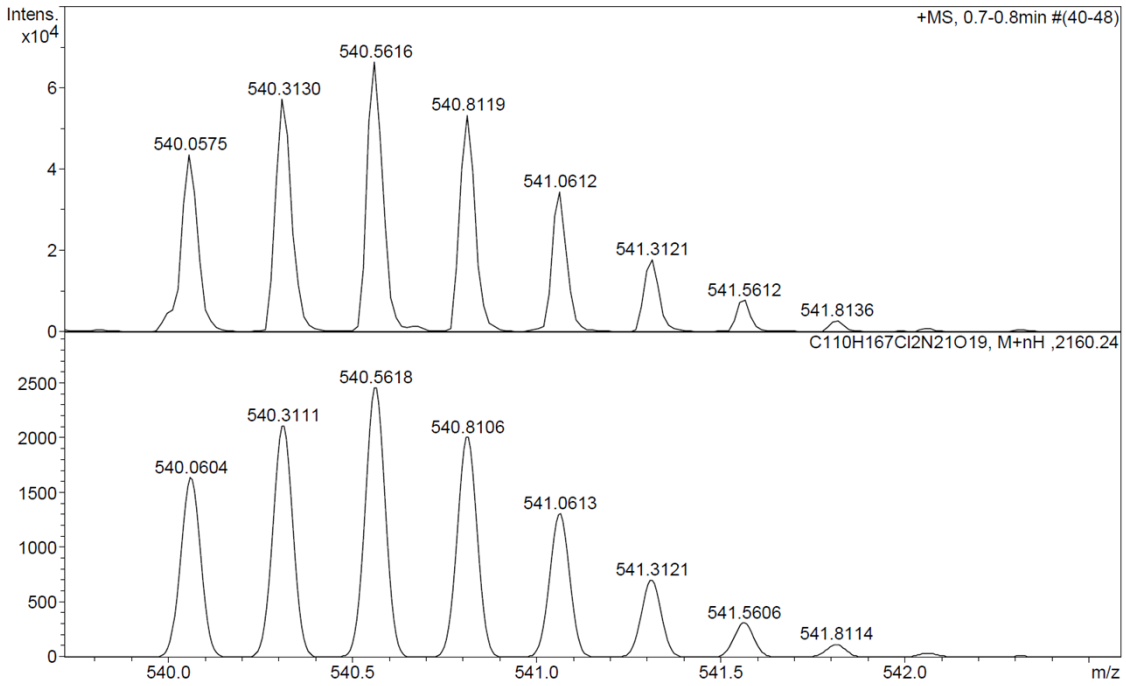
**b)**



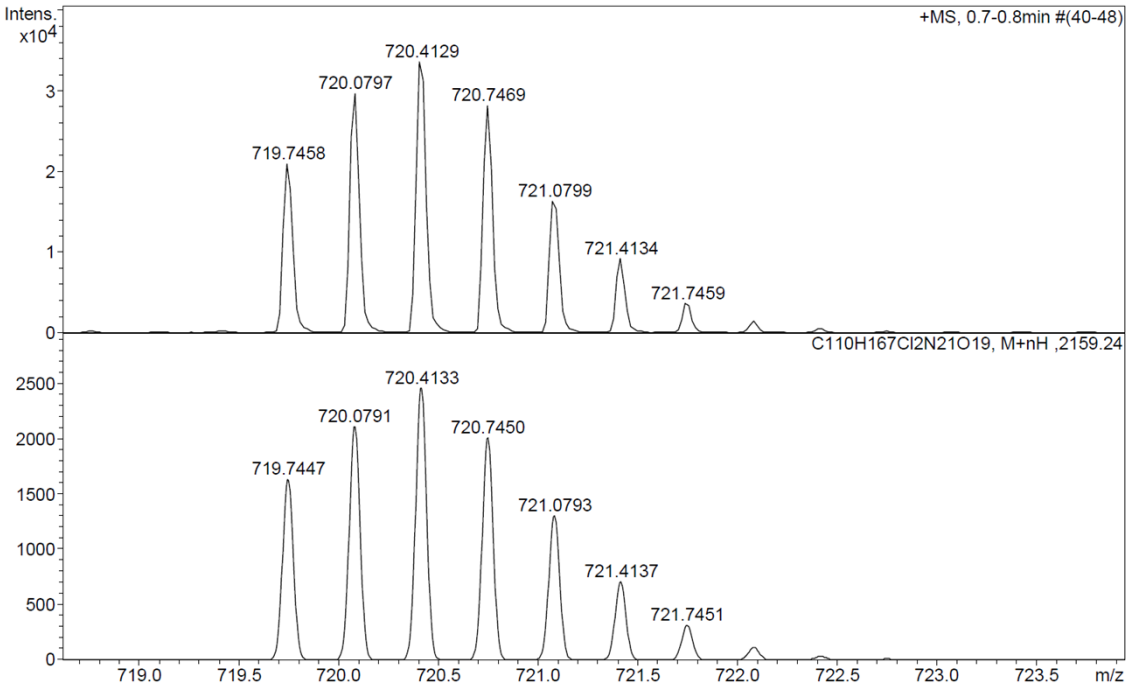

Observed HRMS (top) with the theoretical isotope prediction (bottom).




Observed HRMS (top) with the theoretical isotope prediction (bottom).



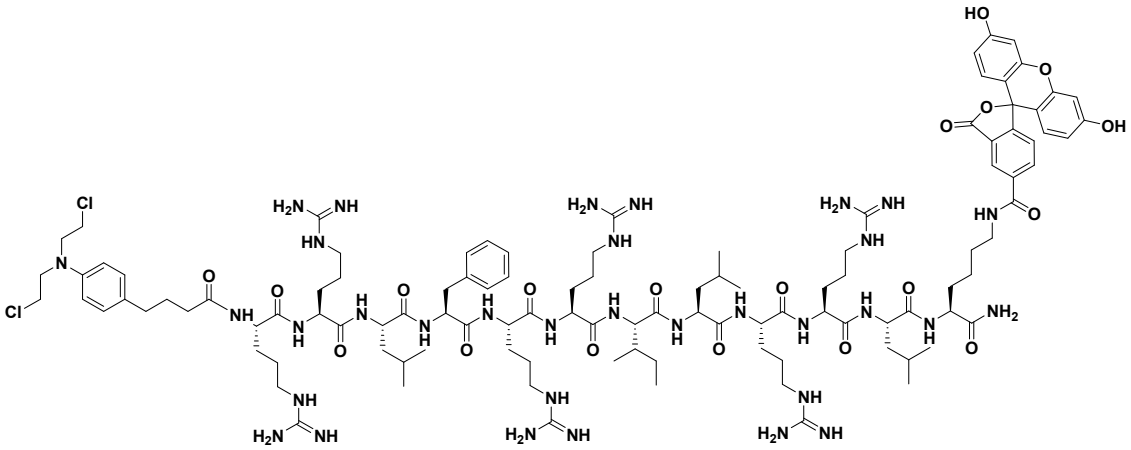

Observed HRMS (top) with the theoretical isotope prediction (bottom).



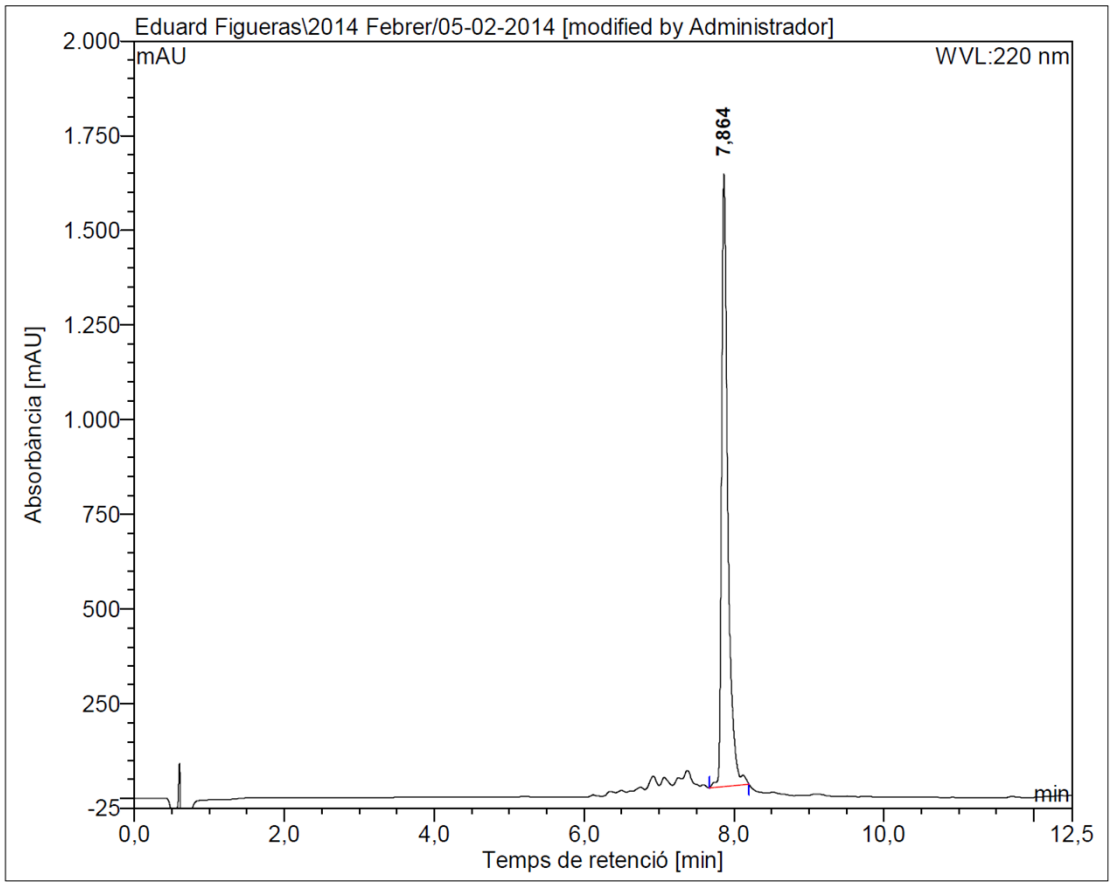

Observed HRMS (top) with the theoretical isotope prediction (bottom).

**Figure S15**: a) HPLC chromatogram (λ= 220 nm), b) HRMS spectrum (*m/z*).

**BP339**

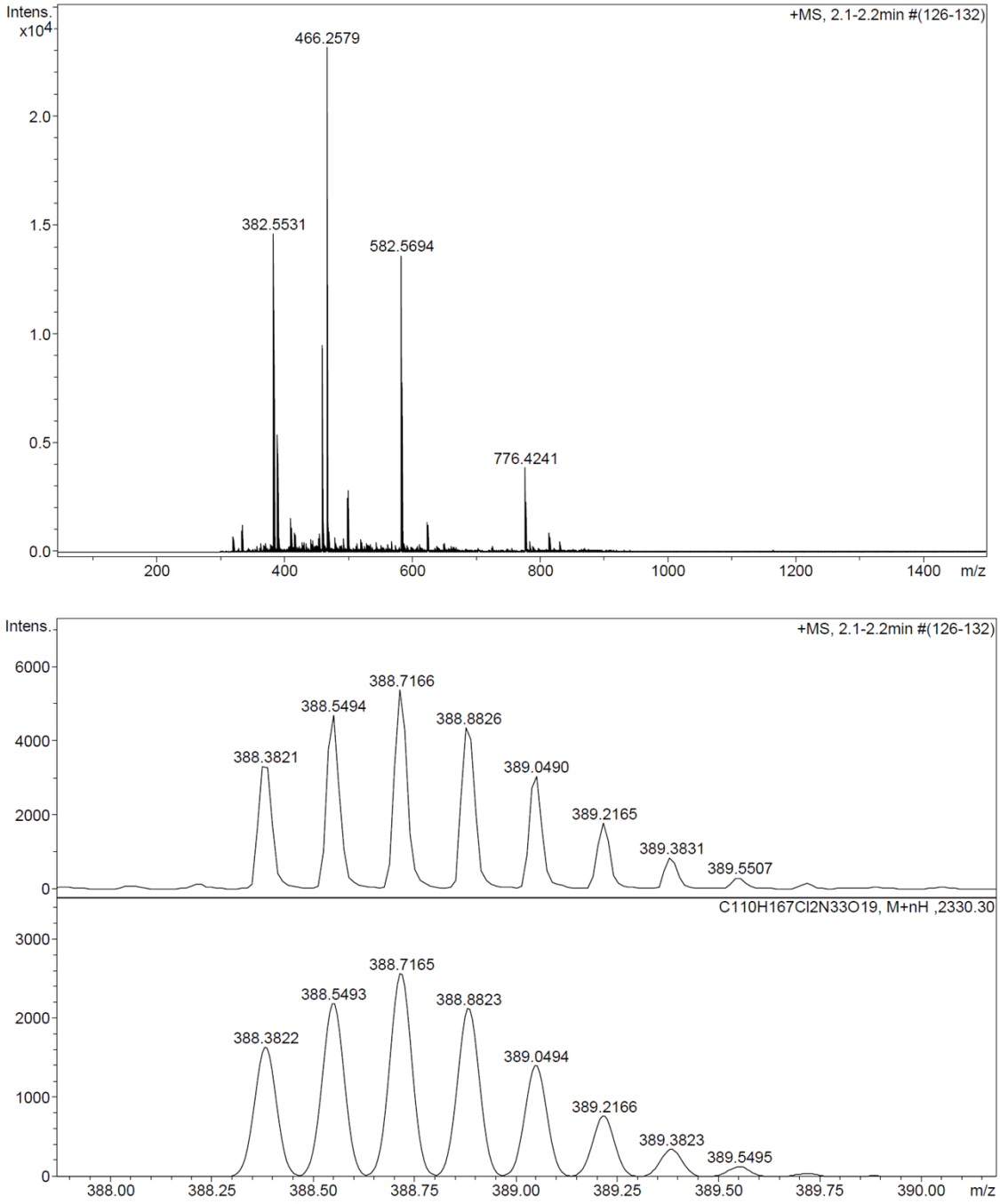


**a)**

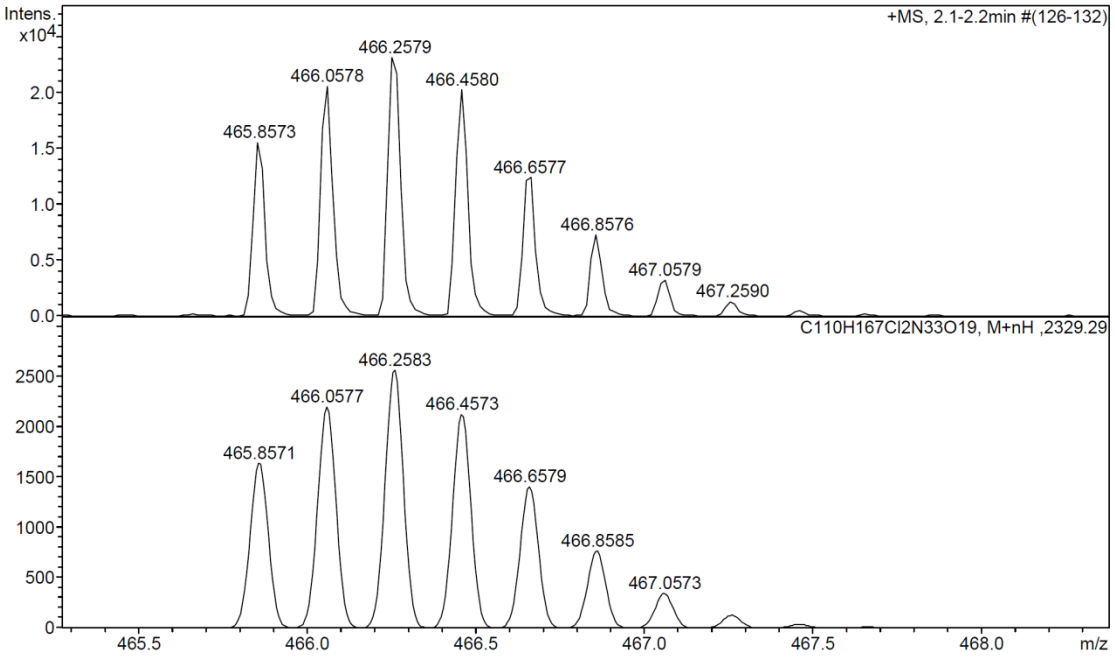



| No. | Temps retenció min | alçada mAU | Area mAU*min | Area relativa % |
|---|---|---|---|---|
| 1 | 7,86 | 1616,497 | 156,827 | 100,00 |
| Total: | | 1616,497 | 156,827 | 100,00 |

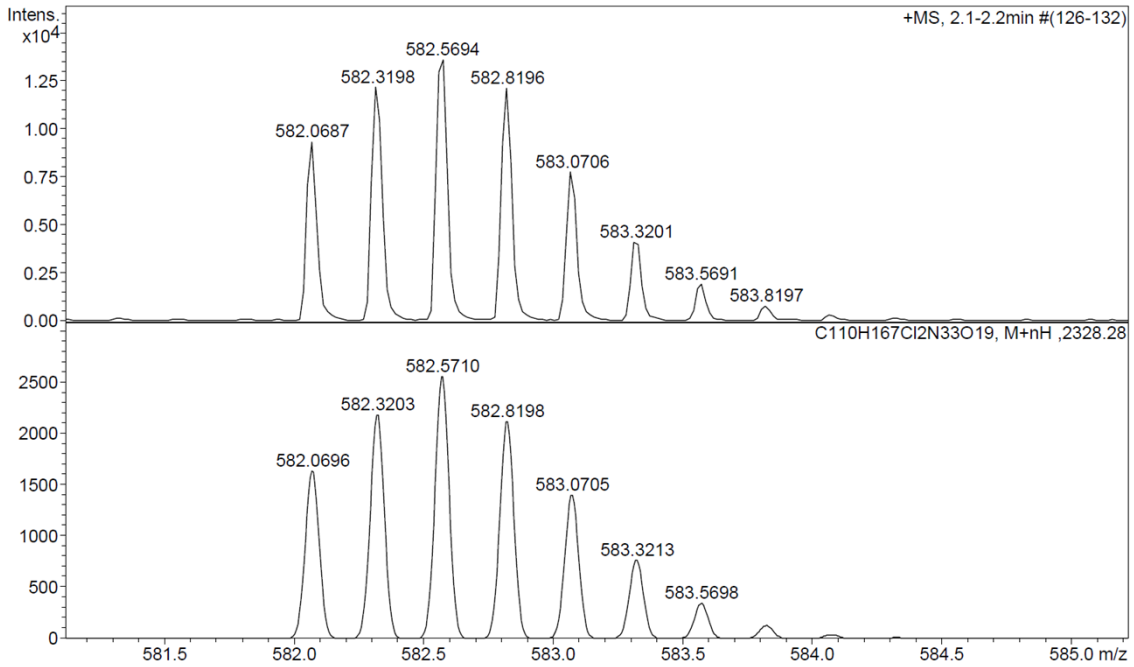
**b)**



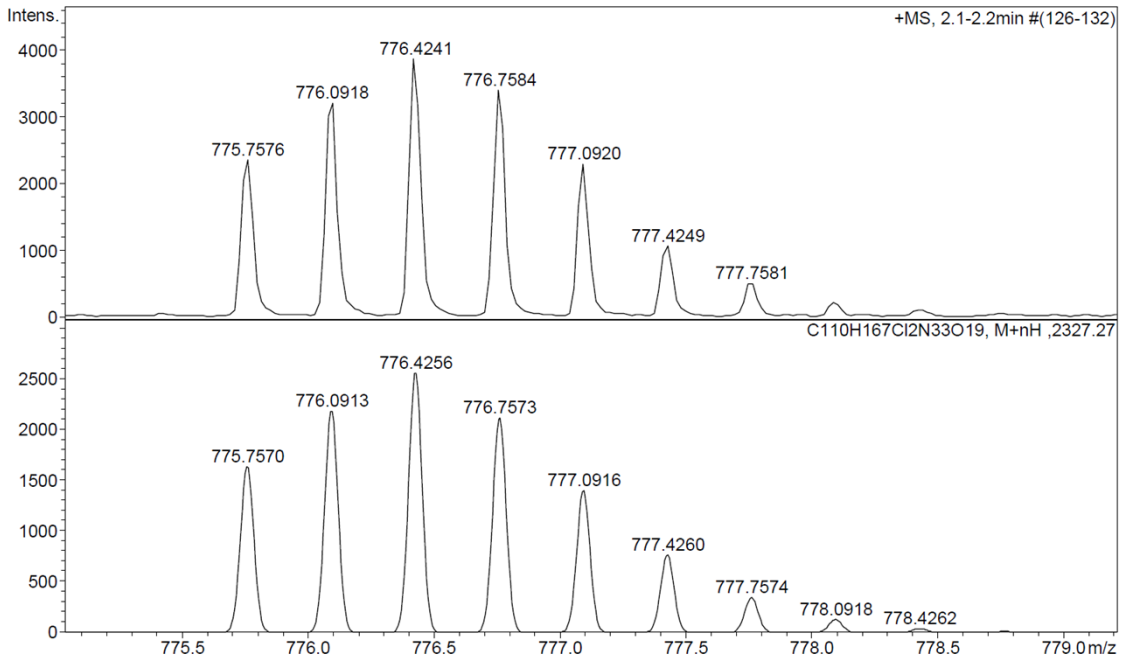

Observed HRMS (top) with the theoretical isotope prediction (bottom).




Observed HRMS (top) with the theoretical isotope prediction (bottom).



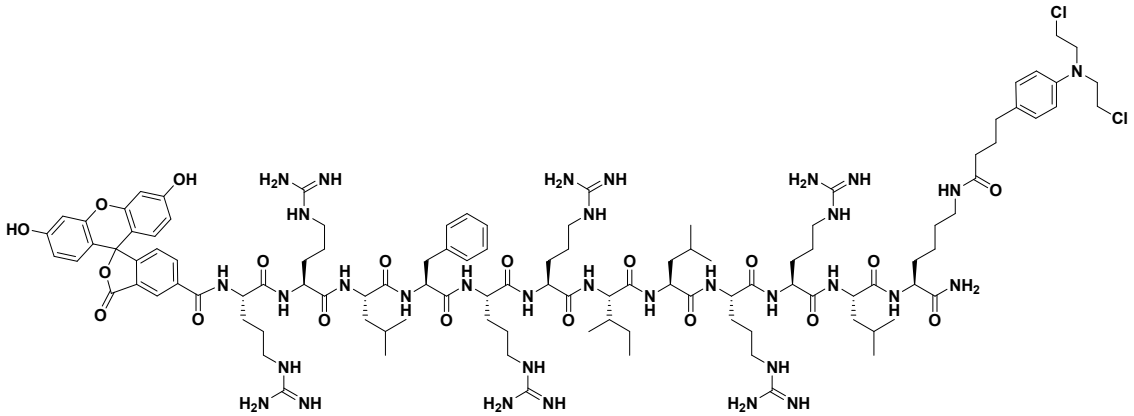

Observed HRMS (top) with the theoretical isotope prediction (bottom).



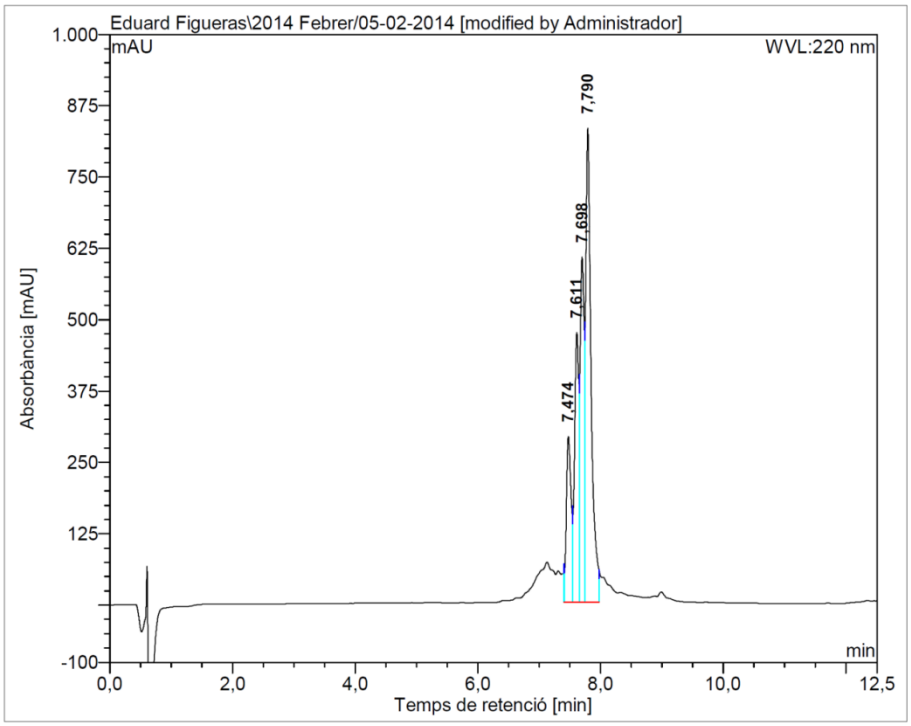

Observed HRMS (top) with the theoretical isotope prediction (bottom).

**Figure S16**: a) HPLC chromatogram (λ= 220 nm), b) HRMS spectrum (*m/z*).

**BP340**

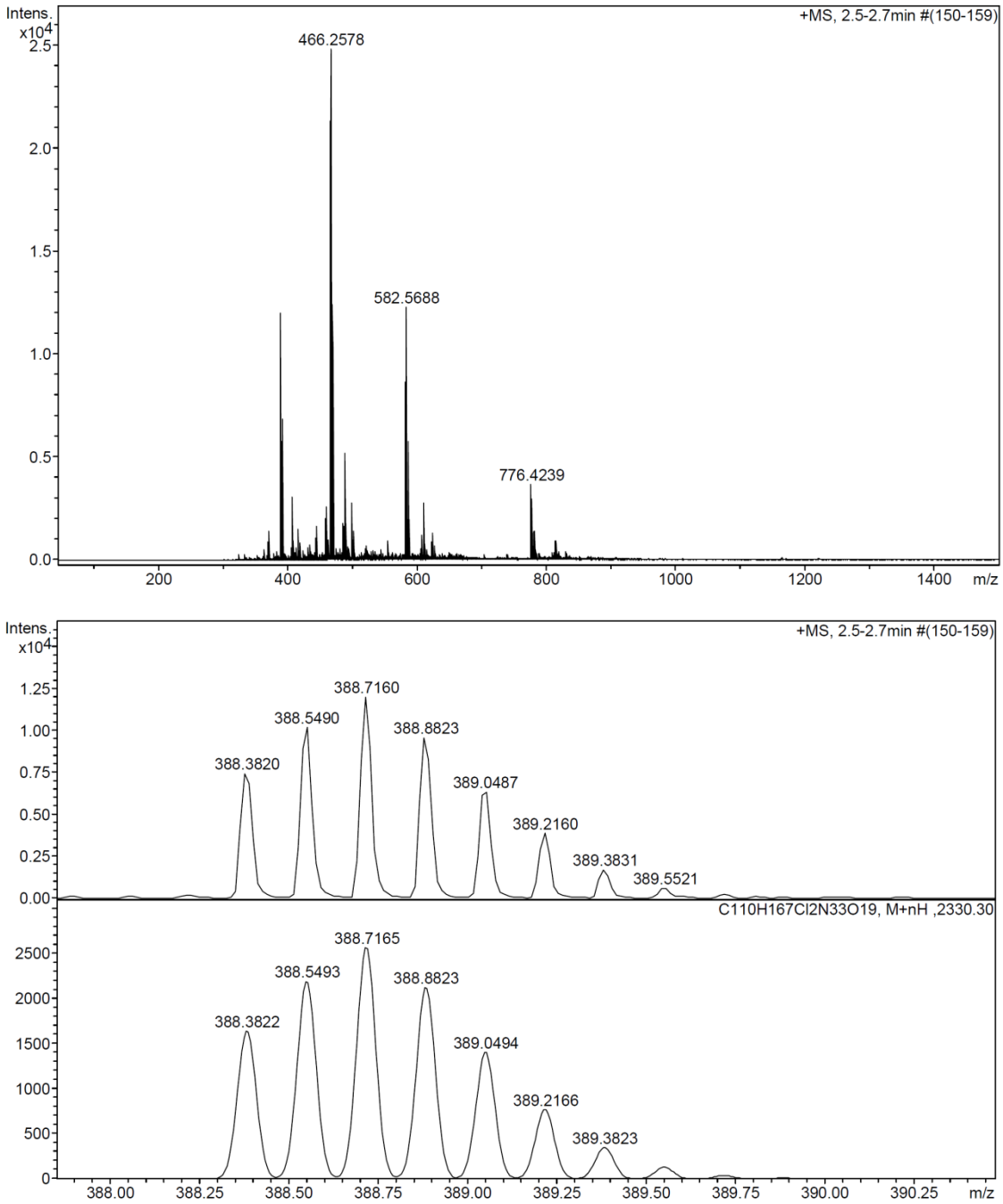


**a)**

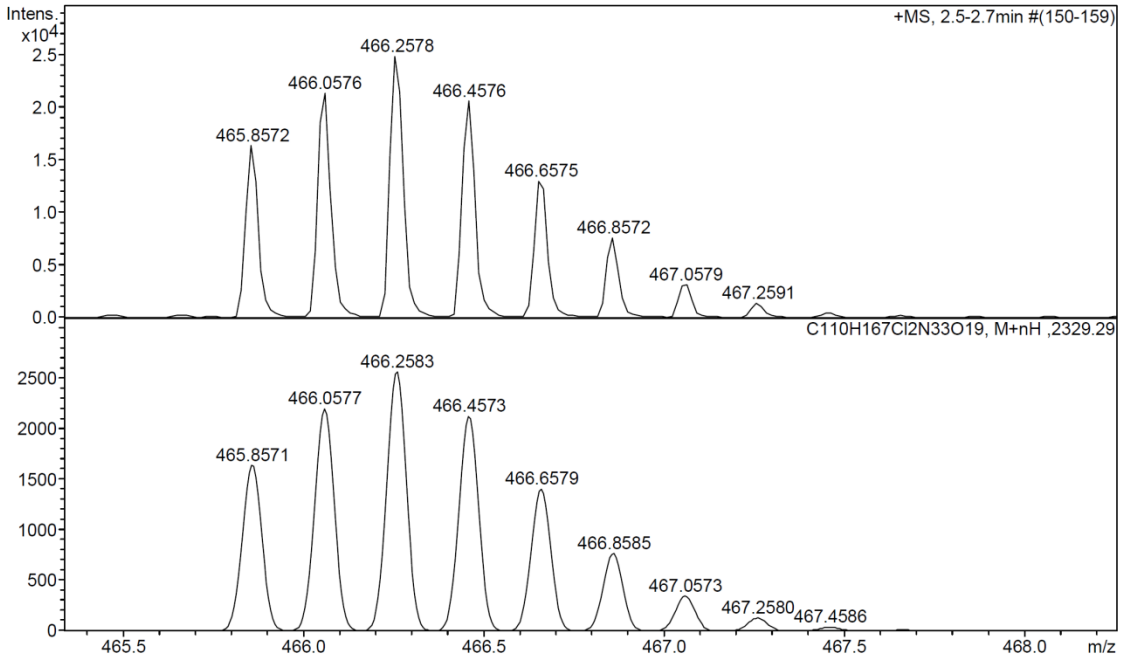



| No. | Temps retenció min | alçada mAU | Area mAU*min | Area relativa % |
|---|---|---|---|---|
| 1 | 7,47 | 290,082 | 25,776 | 13,11 |
| 2 | 7,61 | 472,827 | 37,592 | 19,13 |
| 3 | 7,70 | 604,560 | 47,196 | 24,01 |
| 4 | 7,79 | 830,108 | 85,978 | 43,75 |
| Total: | | 2197,577 | 196,541 | 100,00 |

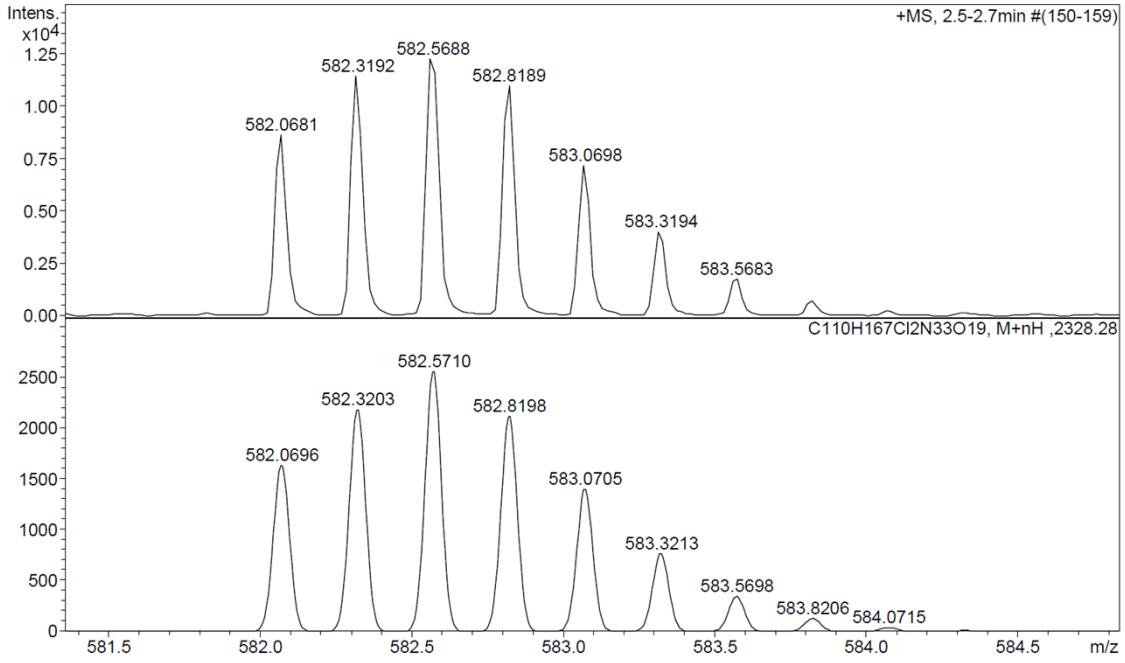
**b)**



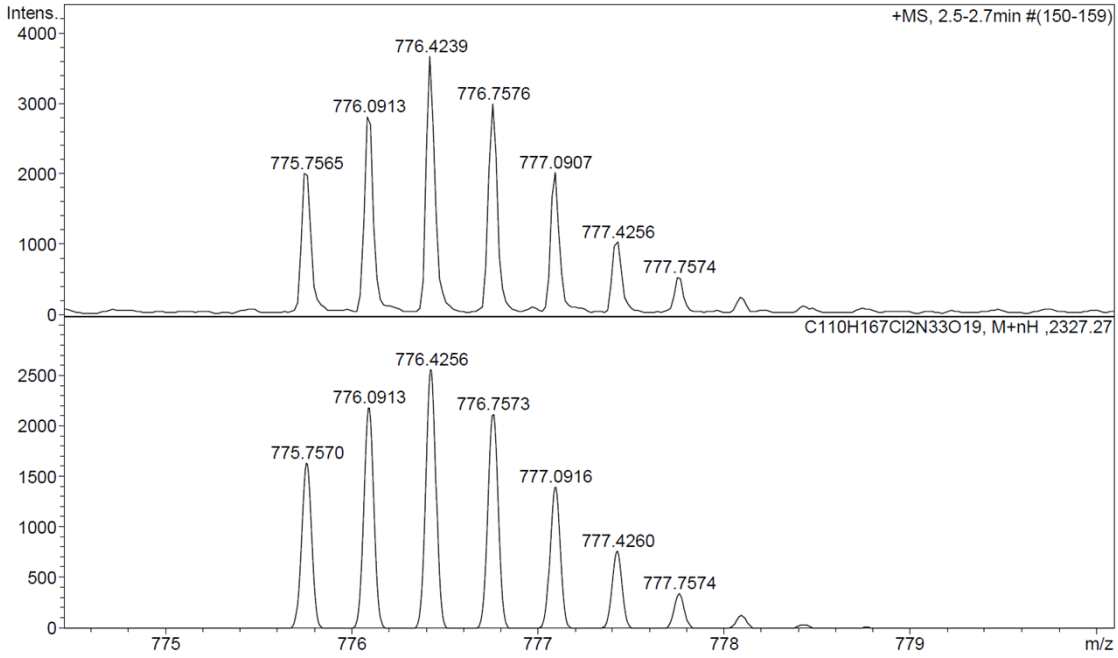

Observed HRMS (top) with the theoretical isotope prediction (bottom).




Observed HRMS (top) with the theoretical isotope prediction (bottom).




Observed HRMS (top) with the theoretical isotope prediction (bottom).




Observed HRMS (top) with the theoretical isotope prediction (bottom).