Toward Homogeneous Erythropoietin: Non-NCL Based Chemical Synthesis of the Gln^{78}–Arg^{166} Glycopeptide Domain

Zhongping Tan, [1] *Shiying Shang,* [1] *Tamara Halkina,* [1] *Yu Yuan,* [1] *Samuel J. Danishefsky*[1,2,]*

[1]Laboratory for Bioorganic Chemistry, Sloan-Kettering Institute for Cancer Research,

1275 York Avenue, New York, New York 10021,

[2]Department of Chemistry, Columbia University, Havemeyer Hall, 3000 Broadway,

New York, New York 10027

E-mail: s-danishefsky@ski.mskcc.org

Materials and Methods

All commercial materials (Aldrich, Fluka, EMD Biosciences) were used without further purification. All solvents were reagent grade or HPLC grade (Fisher). Anhydrous THF, diethyl ether, CH_2Cl_2 (DCM), toluene, and benzene were obtained from a dry solvent system (passed through column of alumina) and used without further drying. All reactions were carried out under air atmosphere unless otherwise is specified in the text. NMR spectra (^1H and ^{13}C) were recorded on a Bruker Advance DRX-500 MHz, referenced to TMS or residual solvent and were reported in ppm. NMR data are reported as follows: s, singlet; d, doublet; t, triplet; q, quartet; quin, quintet; m, multiplet; dd, doublet of doublets. Low-resolution mass spectral analyses were performed with a JOEL JMS-DX-303-HF mass spectrometer. Analytical TLC was performed on E. Merck silica gel 60 F254 plates and flash column chromatography was performed on E. Merck silica gel 60 (40–63 mm). Yields refer to chromatographically pure compounds.

HPLC

All separations involved a mobile phase of 0.05% TFA (v/v) in water (solvent A)/0.04% TFA in acetonitrile (solvent B). LC-MS analyses were performed using a Waters 2695 Separations Module and a Waters 996 Photodiode Array Detector equipped with Varian Microsorb 100-5, C18 150x2.0mm and Varian Microsorb 300-5, C4 250x2.0mm columns at a flow rate of 0.2 mL/min. Preparative separations were performed using a Ranin HPLC solvent delivery system equipped with a Rainin UV-1 detector and Varian Dynamax using Varian Microsorb 100-5, C18 250x21.4mm and Varian Microsorb 300-5, C4 250x21.4mm columns at a flow rate of 16.0 mL/min.

Solid-phase Peptide Synthesis

Automated peptide synthesis was performed on an Applied Biosystems Pioneer continuous flow peptide synthesizer. Peptides were synthesized under standard automated Fmoc/^{t}Bu protocols. The deblocking solution was a mixture of 100/2/2 of DMF/piperidine/DBU. During SPPS, the side chain of Glu and Asp are protected with either Dmab [4-{1-(4,4-dimethyl-2,6-dioxocyclohexylidene)-3-methylbutyl}amino benzyl] or ^{t}Bu (t-butyl). The side chain of Lys protected with either ivDde [1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)-3-methylbutyl] or Alloc (allyloxycarbonyl). All the other amino acids are protected with protecting groups most commonly used as follows: Ser and Thr, with ^{t}Bu; Arg, with Pbf (2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl); Gln, Asn and His, with trt (trityl); Trp, with Boc.Most of the natural amino acids building blocks are commercially available. Fmoc-Lys(Alloc)-OH was purchased from Fluka. All the other building blocks, including the pseudoproline dipeptides, were purchased from EMD Biosciences. Upon completion of automated synthesis on a 0.05 mmol scale, the peptide resin was washed with DCM. Cleavage was carried out using AcOH/TFE/DCM (1:1:8). The resin was removed by filtration, and the resulting solution was concentrated. The residue was precipitated with ether and centrifuged. The pellet was resuspended in MeCN/H_2O (1:1) and lyophilized.

General Procedure for Peptide Thioester and Phenolic Ester Preparartion

1.0 eq. of fully protected peptide and 6.0 eq. of phenol derivative or thio-alcohol were mixed with 0.28 eq. of 26 mM DMAP in DCM. 3.0 eq. of 29 mM DCC in DCM was then added and the reaction mixture was stirred at room temperature. After 2 hr, the solvent was removed and the residue was precipitated with ether and the supernatant was discarded. The fully protected peptide obtained above was subjected to 1 ml cocktail B to remove the protecting groups. After 45 min, TFA was removed with air-flow. The residue was precipitated with ether and centrifuged. The pellet was dissolved in MeCN/H_2O (1:1) for HPLC analysis and purification.

Compound 4: Fmoc-Asp(ODmab)-(Dmb)Lys(ivDde)-OH

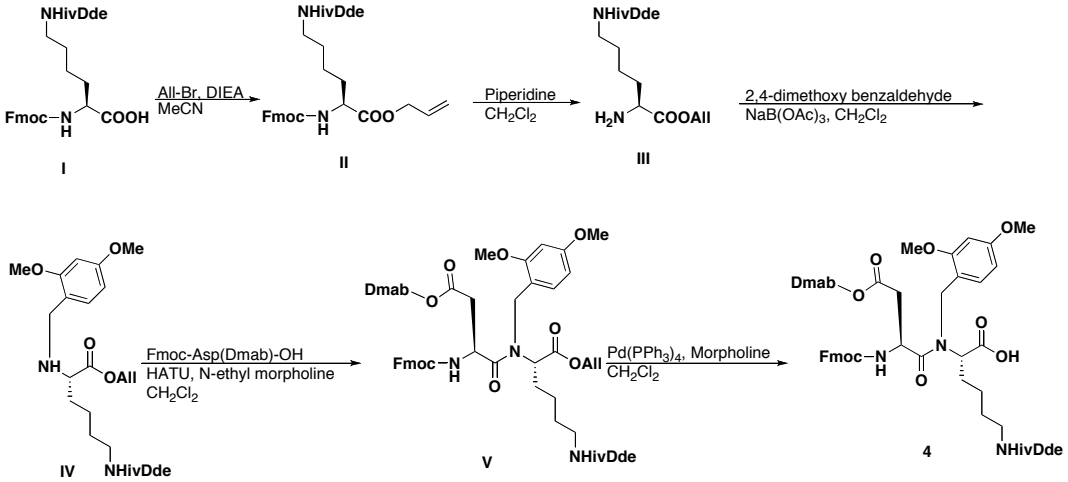
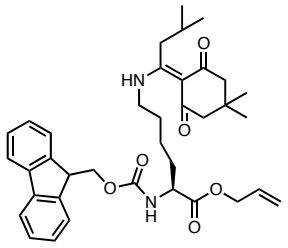


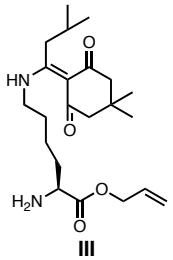
Figure 1 Synthesis of Fmoc-Asp(ODmab)-(Dmb)Lys(ivDde)-OH **4**.



II

II: Fmoc-Lys(ivDde)-OAll In a 100 mL round bottom flask, Fmoc–Lys(ivDde)–OH (0.7425 g, 1.29 mmol, 1.0 equiv) was dissolved in 2.6 mL CH_3CN. Allyl bromide (3.06 mL, 35.8 mmol, 28 equiv) was added dropwise. Then diisopropylethyl amine (2.7 mL, 15.5 mmol, 12 equiv) was added slowly and the mixture was stirred at 40 ˚C for 1.5 hr. EtOAc (80 mL) was added and the organic extracts were washed with satd NH_4Cl (25 mL), water (25 mL), satd $NaHCO_3$ (25 mL), and brine (50 mL). The mixture was dried (Na_2SO_4), filtered, and concentrated by rotary evaporation to afford the crude **II** (1.0428 g), which was used in next step without further purification.

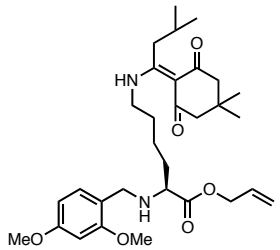
TLC: *Rf* 0.48 (1:1 Hexanes/EtOAc). $[\alpha]_D^{19}$: +5.24° (*c* 1.0, $CDCl_3$). **IR** (ZnSe, film): 3305, 2955, 2867, 1724, 1636, 1568, 1465, 1450, 1421, 1335, 1248, 1106, 937, 759, 740. **^{1}H-NMR** (500 MHz): δ 13.77 (s, 1H), 7.75 (d, 2H, *J* = 7.5), 7.58 (m, 2H), 7.38 (t, 2H, *J* = 7.5), 7.29 (t, 2H, *J* = 7.5), 5.89 (m, 1H), 5.41 (d, 1H, *J* = 8.1), 5.32 (d, 1H, *J* = 17.2), 5.24 (d, 1H, *J* = 10.4), 4.64 (d, 2H, *J* = 5.3), 4.38 (m, 3H), 4.21 (t, 1H, *J* = 7.0), 3.40 (m, 2H), 2.95 (br s, 2H), 2.34 (s, 2H), 2.29 (s, 2H), 1.92 (m, 2H), 1.68 (m, 3H), 1.49 (m, 2H), 0.98 (s, 6H), 0.96 (d, 6H, *J* = 6.7). **^{13}C-NMR** (125 MHz): δ 199.8, 196.3, 176.6, 172.1, 156.2, 144.1, 143.9, 141.5, 127.9, 127.3, 125.3, 120.2, 119.4, 107.3, 67.3, 66.4, 54.0, 53.8, 47.3, 43.5, 37.6, 30.1, 29.3, 29.1, 28.9, 28.5, 22.8. **ESI-MS** *m/z* (rel int): (pos) 615.5 ($[M+H]^+$, 45), 637.3 ($[M+Na]^+$, 100); (neg) 613.6 ($[M-H]^-$, 32), 649.5 ($[M+Cl]^-$, 100).



III

IIi: H-Lys(ivDde)-OAll In a 100 mL round bottom flask, crude **II** (1.29 mmol) was dissolved in 20% piperidine in CH_2Cl_2 (16 mL). After 30 min at rt, toluene (40 mL) was added. The mixture was concentrated by rotary evaporation. Purification by silica flash chromatography (2.6% MeOH in EtOAc) yielded **III** (444.9 mg, 88% for 2 steps).

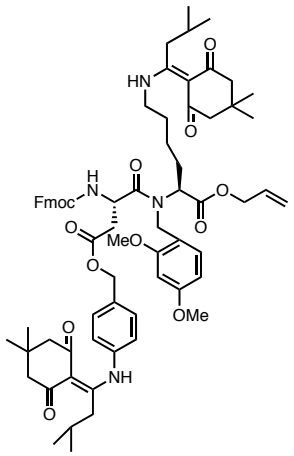
TLC: *Rf* 0.23 (4% MeOH in EtOAc). $[\alpha]_D^{20}$: –1.86° (*c* 1.0, $CDCl_3$). **IR** (ZnSe, film): 2956, 2868, 1735, 1681, 1637, 1569, 1465, 1422, 1366, 1321, 1174, 1100, 990, 919, 732. **^{1}H-NMR** (500 MHz): δ 13.73 (s, 1H), 5.89 (m, 1H), 5.27 (m, 3H), 4.59 (d, 2H, *J* = 5.8), 3.42 (m, 4H), 2.94 (br s, 2H), 2.35 (s, 2H), 2.29 (s, 2H), 1.92 (m, 1H), 1.75 (m, 1H), 1.68 (m, 3H), 1.52 (m, 2H), 0.99 (s, 6H), 0.94 (d, 6H, *J* = 6.7). **^{13}C-NMR** (125 MHz): δ 176.6, 175.8, 132.1, 119.0, 107.2, 65.8, 54.4, 43.7, 37.6, 34.5, 30.1, 29.3, 29.2, 28.4, 23.3, 22.8. **ESI-MS** *m/z* (rel int): (pos) 393.2 ($[M+H]^+$, 100), 415.4 ($[M+Na]^+$, 83); (neg) 391.5 ($[M-H]^-$, 28), 427.4 ($[M+Cl]^-$, 100).



IV

IV: Dmb-Lys-OAll In a 200 mL round bottom flask, **III** (1.99 g, 5.07 mmol, 1.0 equiv) was dissolved in 50 mL CH_2Cl_2. Dimetgoxybenzylaldehyde (0.927 g, 5.58 mmol, 1.1 equiv) was added, followed by addition of sodium triacetoxyborohydride (1.61 g, 7.60 mmol, 1.5 equiv). The mixtured was stirred at rt for 4 hrs. satd $NaHCO_3$ (30 mL) was then added and stirred at rt for 1 hr. The layers were separated, and the aq layer was extracted with CH_2Cl_2. The combined organic extracts were washed with brine (50 mL), dried (Na_2SO_4), and concentrated by rotary evaporation. Purification by silica flash chromatography (5:1 → 2:1 → 1.5:1 → 1:1 hexanes/EtOAc) yielded **IV** (1.7849 g, 65%) as a yellow oil.

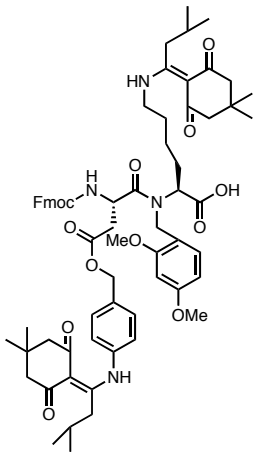
TLC: *Rf* 0.18 (1:1 Hexanes/EtOAc). $[\alpha]_D^{20}$: +2.53° (*c* 1.0, $CDCl_3$). **IR** (ZnSe, film): 2955, 2867, 1732, 1637, 1570, 1507, 1464, 1420, 1366, 1321, 1288, 1208, 1157, 1037, 987, 921, 833, 732. **^{1}H-NMR** (500 MHz): δ 13.70 (s, 1H), 7.24–7.11 (m, 1H), 7.09 (d, 1H, *J* = 7.9), 6.39 (s, 1H), 6.38 (m, 1H), 5.86 (m, 1H), 5.30 (dt, 1H, *J* = 15.9, 1.3), 5.21 (dd, 1H, *J* = 10.4, 1.2), 4.51 (d, 2H, *J* = 5.8), 3.79 (s, 3H), 3.76 (s, 3H), 3.64 (q, 2H, *J* = 13.1), 3.36 (q, 2H, *J* = 7.0), 3.23 (t, 1H, *J* = 6.7), 2.94 (br s, 2H), 2.32 (m, 5H), 1.92 (m, 2H), 1.62 (m, 4H), 1.45 (m, 2H), 0.99 (s, 6H), 0.94 (d, 6H, *J* = 6.8). **^{13}C-NMR** (125 MHz): δ 176.5, 175.1, 160.4, 158.8, 132.3, 130.6, 129.2, 128.4, 125.5, 120.6, 118.7, 107.2, 103.9, 98.7, 65.5, 60.7, 55.5, 47.4, 43.7, 37.6, 33.3, 30.1, 29.4, 29.1, 28.4, 23.4, 22.8. **ESI-MS** *m/z* (rel int): (pos) 543.3 ($[M+H]^+$, 80), 565.3 ($[M+Na]^+$, 100), 581.3 ($[M+K]^+$, 6); (neg) 541.6 ($[M-H]^-$, 30), 577.5 ($[M+Cl]^-$, 100).



V

V: Fmoc-Asp(Dmab)-(Dmb)-Lys-OAll In a 200 mL round bottom flask, azeotroped **IV** (1.7849 g, 3.29 mmol, 1.0 equiv) and Fmoc–Asp(Dmab)–OH (3.29 g, 4.93 mmol, 1.5 equiv) were dissolved in CH_2Cl_2 (47 mL). N-Ethyl-morpholine (0.624 mL, 4.93 mmol, 1.5 equiv) and HATU (1.88 g, 4.93 mmol, 1.5 equiv) were then added. After 12 hrs at rt, water (48 mL) was added, the layers were separated, and the aq layer was extracted with CH_2Cl_2. The combined organic extracts were washed with brine (100 mL), dried (Na_2SO_4), and concentrated by rotary evaporation. Purification by silica flash chromatography (1.5:1 → 1:1 hexanes/EtOAc) yielded **V** (2.80 g, 72%).

TLC: *Rf* 0.50 (1:2 Hexanes/EtOAc). $[\alpha]_D^{20}$: –16.5° (*c* 1.0, $CDCl_3$). **IR** (ZnSe, film): 2957, 2868, 1737, 1643, 1588, 1509, 1452, 1322, 1248, 1210, 1161, 1039, 920, 739. **^{1}H-NMR** (500 MHz): δ 15.23 (s, 1H), 13.64 (s, 1H), 7.73 (d, 2H, *J* = 7.2), 7.54 (d, 2H, *J* = 6.6), 7.35 (d, 4H, *J* = 8.1), 7.26 (m, 2H), 7.15 (d, 1H, *J* = 7.8), 7.04 (d, 2H, *J* = 7.8), 6.37 (m, 2H), 5.95 (d, 1H, *J* = 9.0), 5.77 (m, 1H), 5.34 (m, 1H), 5.14 (m, 4H), 4.58 (d, 1H, *J* =15.4), 4.48, (d, 1H, *J* = 15.9), 4.43 (d, 2H, *J* = 5.6), 4.37 (m, 2H), 4.17 (m, 1H), 3.72 (s, 3H), 3.70 (s, 3H), 3.28 (m, 2H), 2.90 (m, 5H), 2.74 (dd, 1H, *J* = 15.7, 6.1), 2.46 (s, 2H), 2.37 (s, 2H), 2.31 (br s, 3H), 1.92 (m, 1H), 1.79 (m, 2H), 1.50 (m, 2H), 1.24 (m, 1H), 1.05 (s, 6H), 0.97 (s, 6H), 0.93 (d, 6H, *J* = 6.6), 0.73 (d, 6H, *J* = 5.4). **^{13}C-NMR** (125 MHz): δ 200.4, 196.6, 176.6, 176.5, 170.8, 170.6, 170.4, 161.3, 159.0, 155.7, 143.9, 141.5, 136.9, 135.5, 132.1, 130.9, 129.1, 127.9, 127.3, 126.8, 125.3, 120.2, 118.5, 116.1, 107.9, 107.2, 104.0, 98.8, 67.3, 65.9, 58.9, 55.5, 55.4, 53.9, 52.5, 48.9, 47.3, 43.7, 38.5, 38.1, 37.6, 30.2, 30.1, 29.7, 29.1, 28.7, 28.5, 28.4, 24.0, 22.8, 22.7. **ESI-MS** *m/z* (rel int): (pos) 1191.8 ($[M+H]^+$, 7), 1213.9 ($[M+Na]^+$, 100), 1229.9 ($[M+K]^+$, 4); (neg) 1189.9 ($[M–H]^–$, 100).



4

4: Fmoc-Asp(ODmab)-(Dmb)Lys(ivDde)-OH In a 250 mL round bottom flask, **V** (1.7526 g, 1.47 mmol, 1.0 equiv) was dissolved in 7 mL CH_2Cl_2. Morpholine (0.24 mL, 2.79 mmol, 1.9 equiv) and $Pd(Ph_3P)_4$ (17 mg, 0.0147 mmol, 0.01 equiv) were added. The solution was stirred at rt for 1 hr. The mixture was diluted with CH_2Cl_2 (160 mL), washed with 5% citric acid (40 mL × 2), and brine. The organic extract was dried (Na_2SO_4), filtered, and concentrated by rotary evaporation to afford the crude **4** (1.8668 g) as a yellow foam, which was used in next step without further purification.

TLC: *Rf* 0.33 (15:1 CH_2Cl_2/MeOH). $[\alpha]_D^{20}$: –14.6° (*c* 1.0, $CDCl_3$). **IR** (ZnSe, film): 2957, 2868, 1730, 1642, 1557, 1509, 1452, 1418, 1385, 1323, 1248, 1210, 1160, 1039, 916, 833, 759, 733. **^{1}H-NMR** (500 MHz): δ 15.23 (s, 1H), 13.60 (s, 1H), 7.71 (d, 2H, *J* = 7.2), 7.55 (m, 2H), 7.34 (m, 4H), 7.24 (m, 4H), 7.13 (m, 4H), 7.03 (m, 2H), 6.38 (s, 1H), 6.32 (d, 1H, *J* = 6.1), 5.38 (m, 1H), 5.10 (m, 2H), 4.64 (d, 1H, *J* = 15.7), 4.52 (d, 1H, *J* = 15.9), 4.38 (m, 1H), 4.27 (m, 2H), 4.14 (m, 1H), 4.02 (t, 1H, *J* = 6.5), 3.73 (s, 3H), 3.67 (s, 3H), 3.25 (m, 2H), 3.00 (m, 5H), 2.77 (dd, 1H, *J* = 15.9, 6.0), 2.46 (s, 2H), 2.39 (s, 2H), 2.32 (m, 6H), 2.03 (m, 1H), 1.90 (m, 1H), 1.81 (m, 1H), 1.73 (m, 1H), 1.47 (m, 2H), 1.24 (m, 2H), 1.05 (s, 6H), 0.98 (s. 6H), 0.93 (m, 6H), 0.86 (d, 1H, *J* = 6.0), 0.73 (d, 6H), *J* = 6.5). **^{13}C-NMR** (125 MHz): δ 200.6, 196.9, 176.9, 176.8, 173.3, 172.1, 170.8, 161.7, 159.2, 156.0, 144.1, 141.7, 138.3, 137.2, 135.6, 129.4, 129.3, 128.6, 128.2, 127.5, 127.0 125.7, 125.6, 120.4, 116.0, 108.2, 107.4, 104.4, 99.1, 67.6, 66.2, 60.1, 55.8, 55.7, 54.1, 52.7, 49.1, 48.6, 47.5, 43.9, 38.8, 38.2, 37.2, 30.4, 30.3, 30.0, 29.4, 29.3, 28.8, 28.7, 24.2, 230.0, 21.9. **ESI-MS** *m/z* (rel int): (pos) 1151.8 ($[M+H]^+$, 12), 1173.9 ($[M+Na]^+$, 100), 1189.9 ($[M+K]^+$, 5); (neg) 1149.8 ($[M–H]^–$, 100).

Compound 7 was purified by C4 column with linear gradients from 45% solvent B to 65% solvent B over 30 min. The flow rate was 16 ml/min and detection was by UV-absorbance at 256 nm.

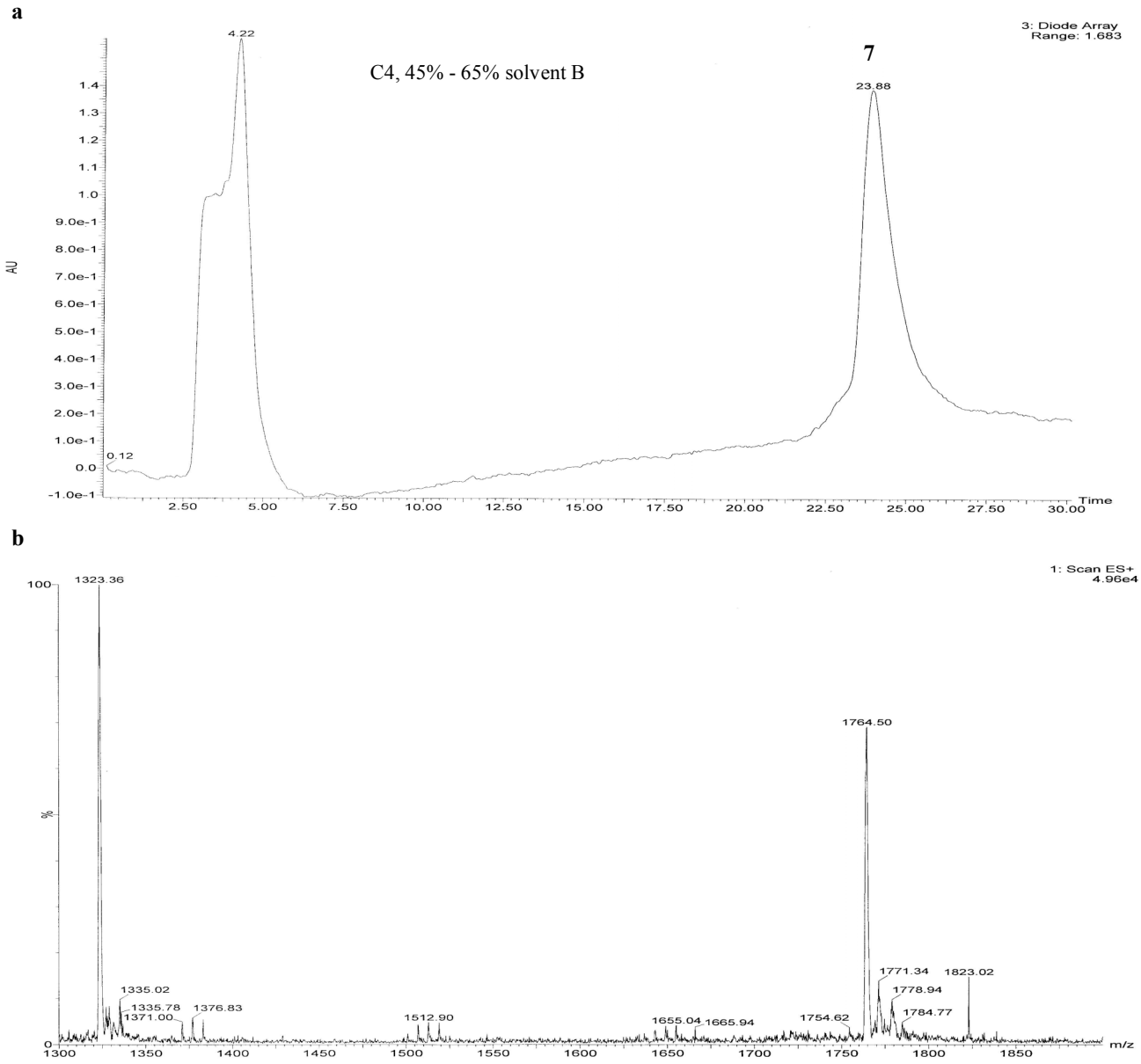


Figure 2 (**a**) LC-UV trace of purified compound **7**. (**b**) ESI-MS of compound **7**. MS (ESI) calcd. for $C_{252}H_{381}N_{53}O_{65}S_3$ $[M+3H]^{3+}$ *m/z* = 1762.92, $[M+4H]^{4+}$ *m/z* = 1233.44.

Compound 9 was purified by C18 column with linear gradients from 45% solvent B to 55% solvent B over 30 min. The flow rate was 16 ml/min and detection was by UV-absorbance at 248 nm.

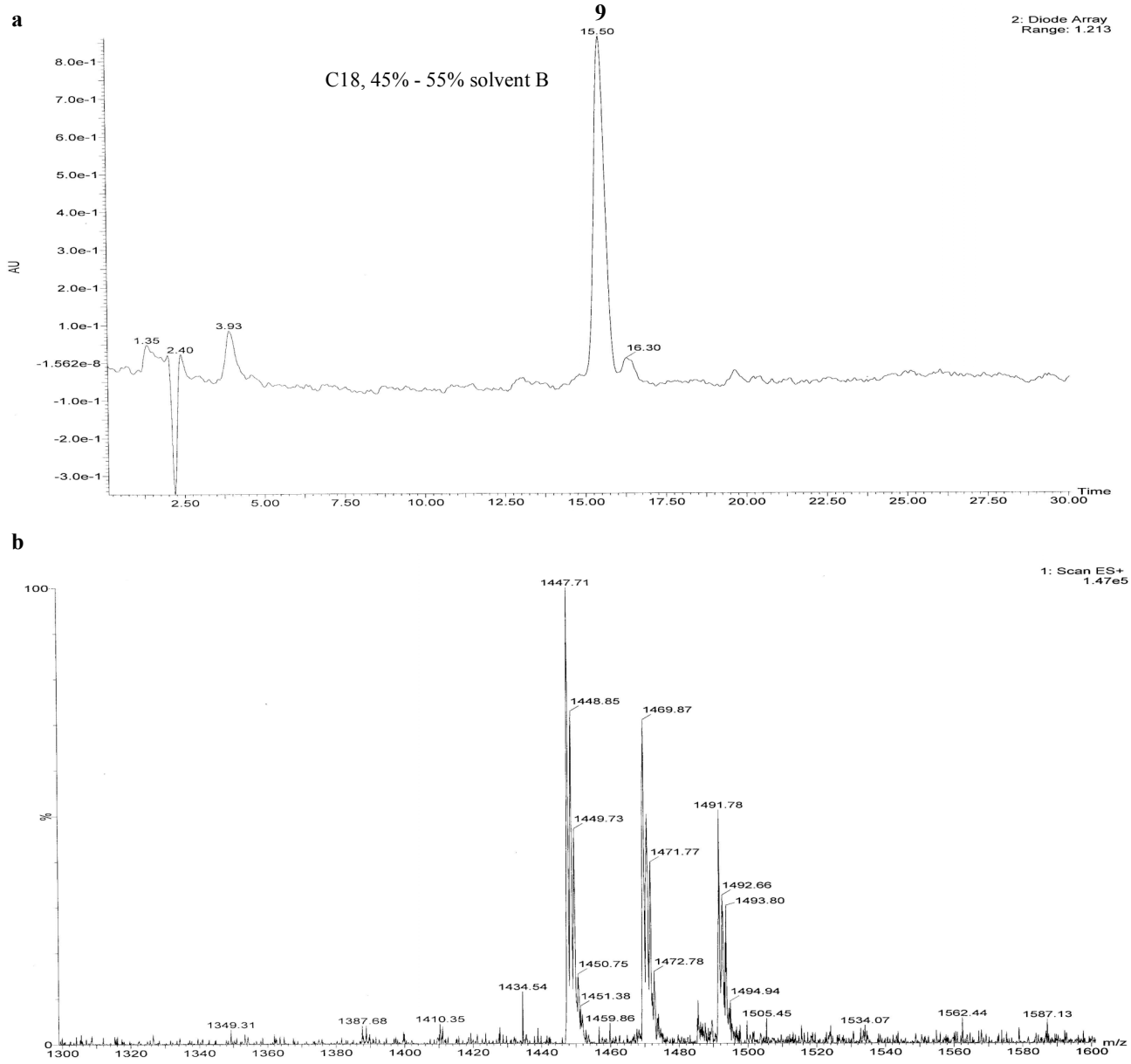
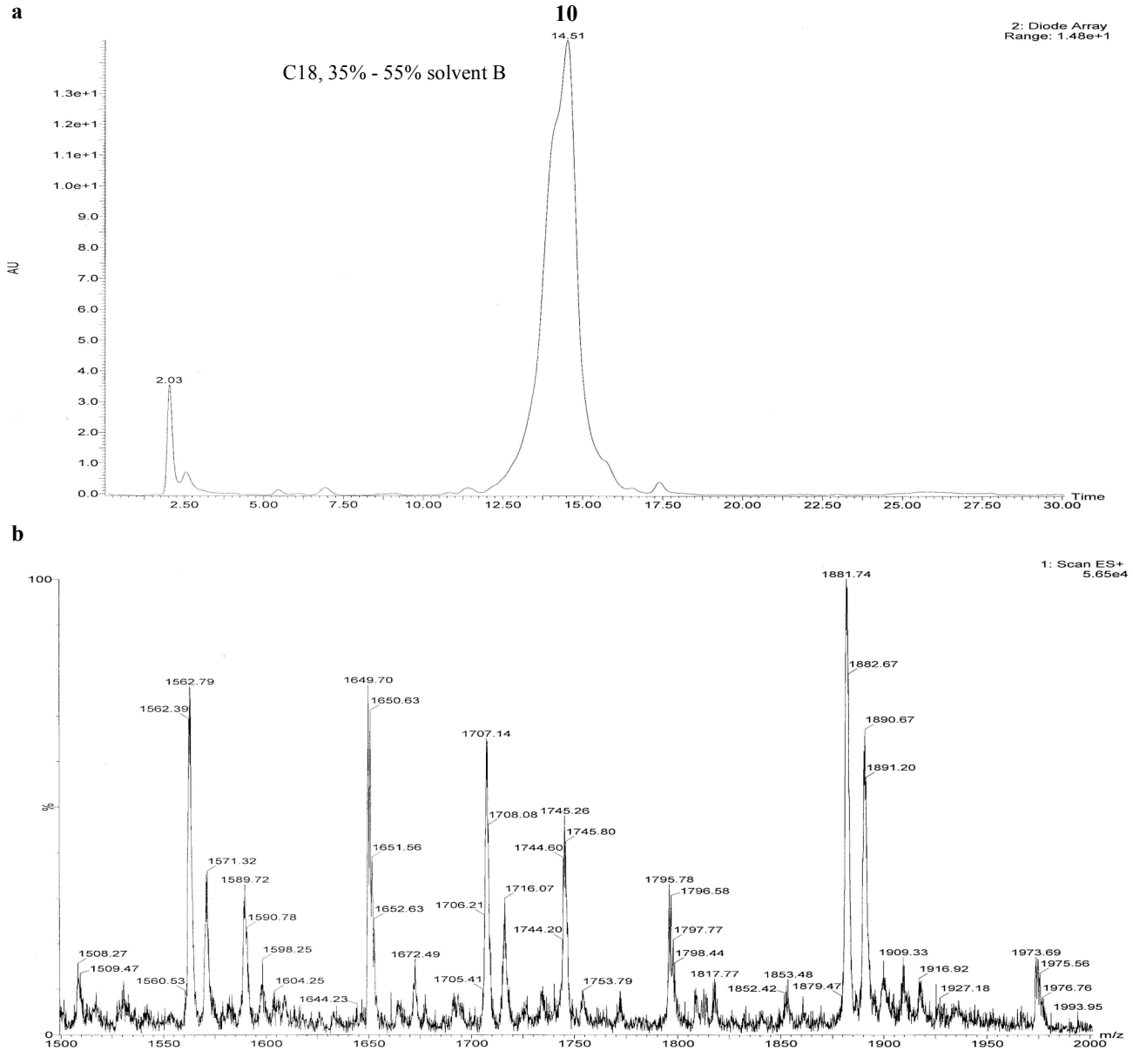


Figure 3 (**a**) LC-UV trace of purified compound **9**. (**b**) ESI-MS of compound **9**. MS (ESI) calcd. for $C_{68}H_{94}N_{12}O_{19}S_2$ $[M+H]^+$ *m/z* = 1447.63.

Compound 10 Peptide **9** (1.0 mg, 0.69 µmol), dodecamer sugar **6** (1.1 mg, 0.46 µmol) and HATU (1.4 mg, 3.7 µmol) were dissolved in 30 µl DMSO and DIEA (0.24 mg, 1.8 µmol) was added. The reaction was stirred at rt for 15 min and was acidified by the addition of 200 µl of MeCN/H_2O (1:1) containing 5% AcOH. The mixture was purified by C18 column with linear gradients from 37% solvent B to 52% solvent B over 30 min. The flow rate was 16 ml/min and detection was by UV-absorbance at 265 nm. A white solid (0.5 mg, 29%) was obtained after lyophilization. It did not escape our notice that the proximity-accelerated lactone formation reaction also occurred under the conditions employed for our aspartylation reaction.



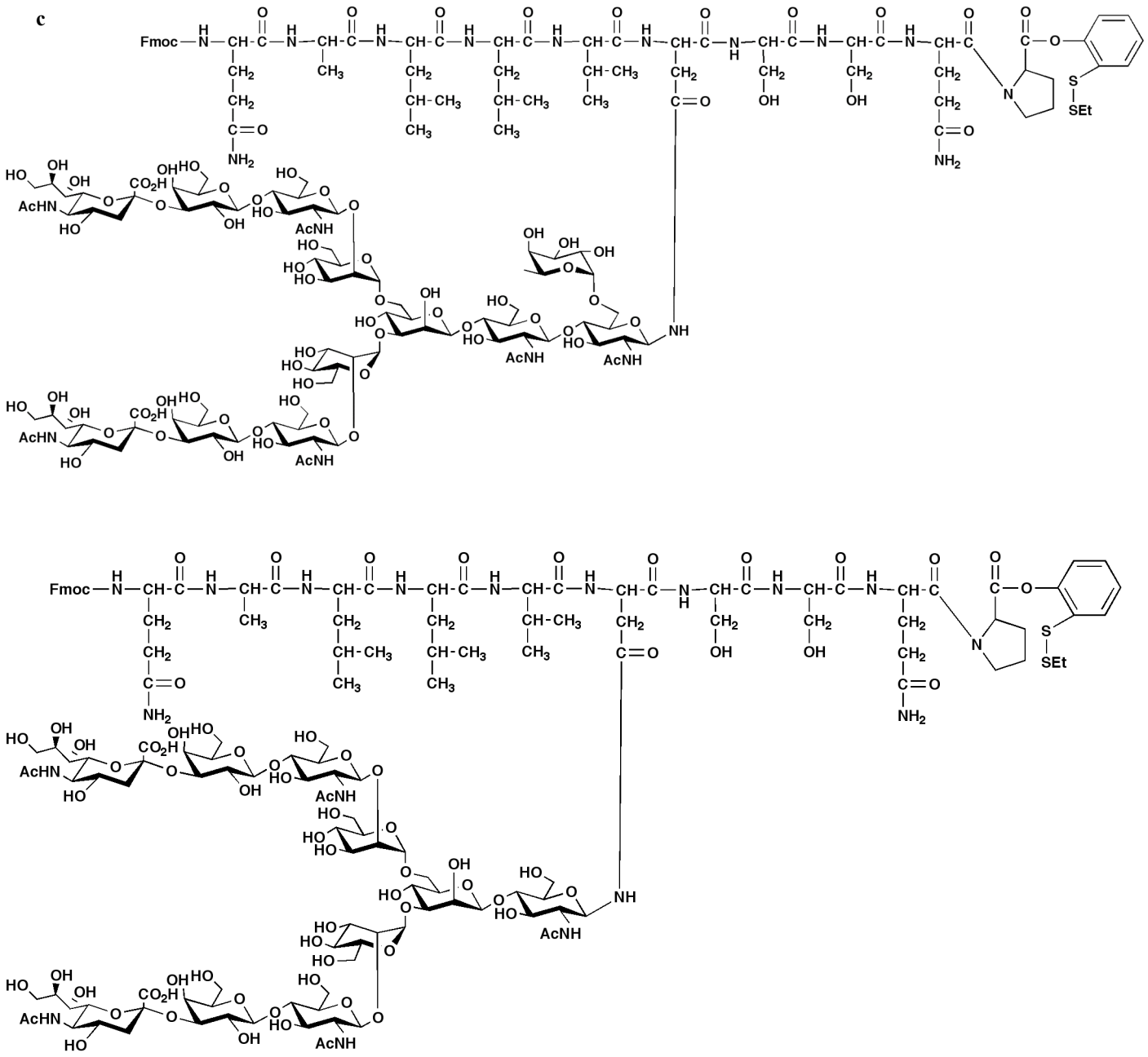


Figure 4 (**a**) LC-UV trace of purified compound **10**. The purified product with different degrees of lactone formation forms a peak with shoulders. (**b**) ESI-MS of compound **10**. MS (ESI) calcd. for $C_{158}H_{241}N_{19}O_{83}S_2$ (product without lactone) $[M+2H]^{2+}$ *m/z* = 1899.24; $C_{158}H_{239}N_{19}O_{82}S_2$ (product with one lactone) $[M+2H]^{2+}$ *m/z* = 1890.23; $C_{158}H_{237}N_{19}O_{81}S_2$ (product with two lactone) $[M+2H]^{2+}$ *m/z* = 1881.23. Compound **10** showed a fragmentation peak of $[M-292]^{2+}$, corresponding to the loss of one monosaccharide, sialic acid, during ionization. The peak, which is about 349 Da less than compound **10**, could be assigned to be a side product resulting from the decamer sugar, a truncated N-linked glycan that lacks two monosaccharides, fucose and N-acetylglucosamine. MS (ESI) calcd. for $C_{144}H_{218}N_{18}O_{74}S_2$ (product without lactone) $[M+2H]^{2+}$ *m/z* = 1724.67; $C_{144}H_{216}N_{18}O_{73}S_2$ (product with one lactone) $[M+2H]^{2+}$ *m/z* = 1715.66; $C_{144}H_{214}N_{18}O_{72}S_2$ (product with two lactones) $[M+2H]^{2+}$ *m/z* = 1706.65. (**c**) The structure of glycopeptide **10** with the N-linked dodecamer sugar and the side product with the N-linked decamer sugar.

Compound 11 was purified by C4 column with linear gradients from 49% solvent B to 58% solvent B over 30 min. The flow rate was 16 ml/min and detection was by UV-absorbance at 260 nm.

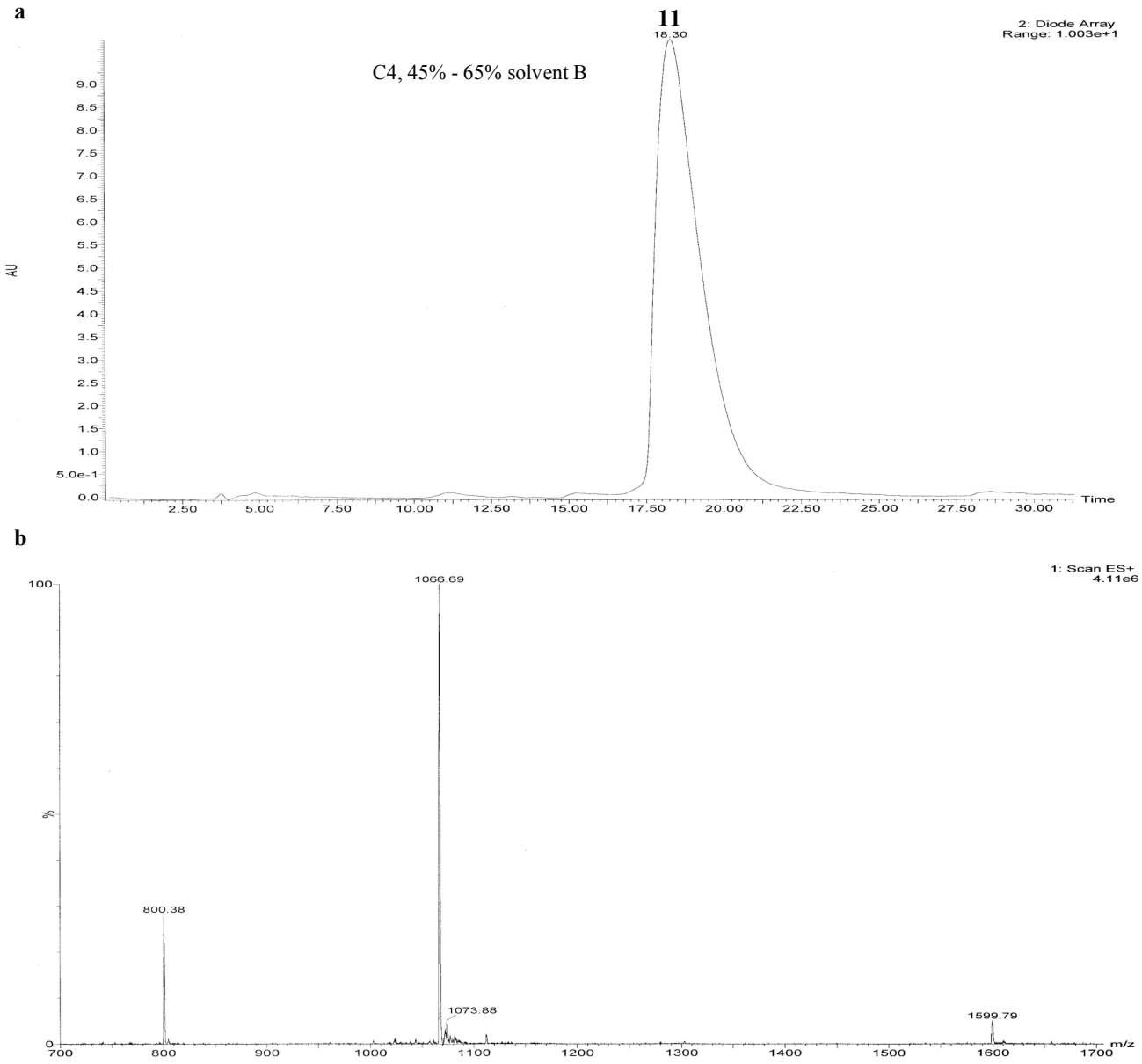
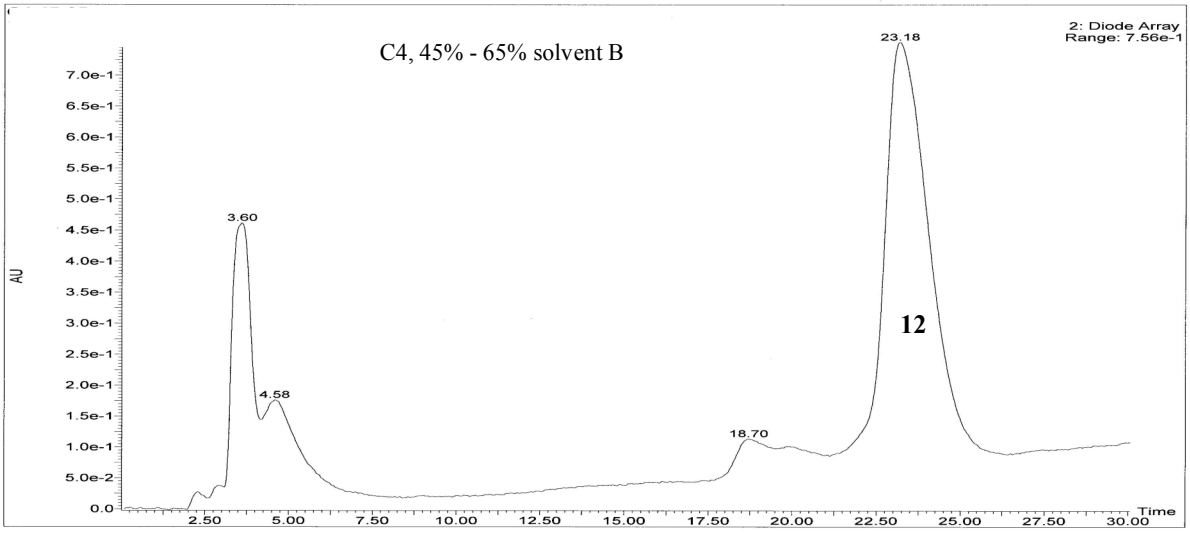


Figure 5 (**a**) LC-UV trace of purified compound **11**. (**b**) ESI-MS of compound **11**. MS (ESI) calcd. for $C_{148}H_{243}N_{37}O_{39}S$ $[M+2H]^{2+}$ *m/z* = 1598.40, $[M+3H]^{3+}$ *m/z* = 1065.94, $[M+4H]^{4+}$ *m/z* = 799.71.

Compound 12 Glycopeptide **10** (1.0 mg, 0.26 µmol), peptide **11** (2.0 mg, 0.63 µmol), TCEP•HCl (0.2 mg, 0.87 µmol) and HOOBt (1.4 mg, 8.6 µmol) were dissolved in 20 µl DMSO and DIEA (0.75 mg, 5.8 µmol) was added. The reaction was stirred at rt for 21.5 hr and was acidified by the addition of 500 µl of MeCN/H_2O (1:1) containing 5% AcOH. The mixture was purified by C4 column with linear gradients from 45% solvent B to 65% solvent B over 30 min. The flow rate was 16 ml/min and detection was by UV-absorbance at 262 nm. A white solid (0.6 mg, 33%) was obtained after lyophilization.

a



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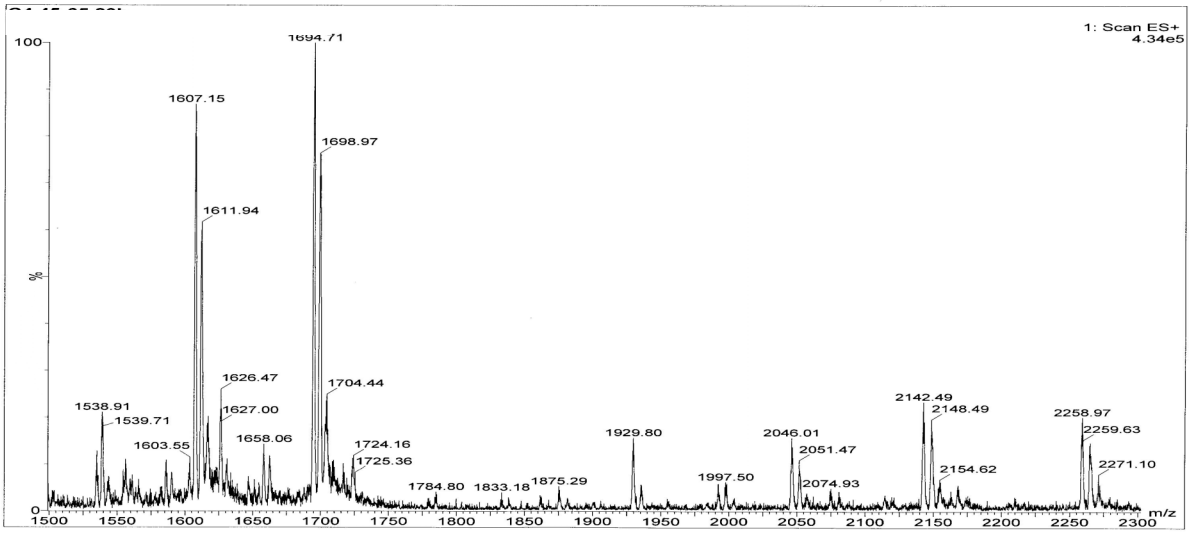
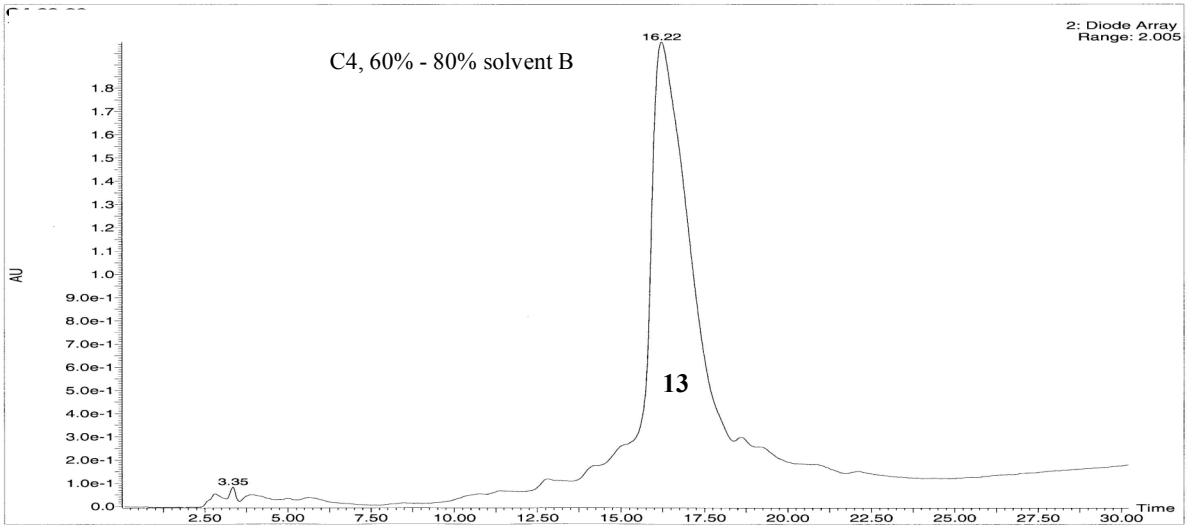


Figure 6 (**a**) LC-UV trace of purified compound **12**. (**b**) ESI-MS of compound **12**. MS (ESI) calcd. for $C_{298}H_{474}N_{56}O_{121}S$ (product without lactone) $[M+3H]^{3+}$ *m/z* = 2269.41, $[M+4H]^{4+}$ *m/z* = 1702.31; $C_{298}H_{472}N_{56}O_{120}S$ (product with one lactone) $[M+3H]^{3+}$ *m/z* = 2263.41, $[M+4H]^{4+}$ *m/z* = 1697.81; $C_{298}H_{470}N_{56}O_{119}S$ (product with two lactone) $[M+3H]^{3+}$ *m/z* = 2257.41, $[M+4H]^{4+}$ *m/z* = 1693.31.

Compound 13 was purified by C4 column with linear gradients from 55% solvent B to 75% solvent B over 30 min. The flow rate was 16 ml/min and detection was by UV-absorbance at 257 nm.

a



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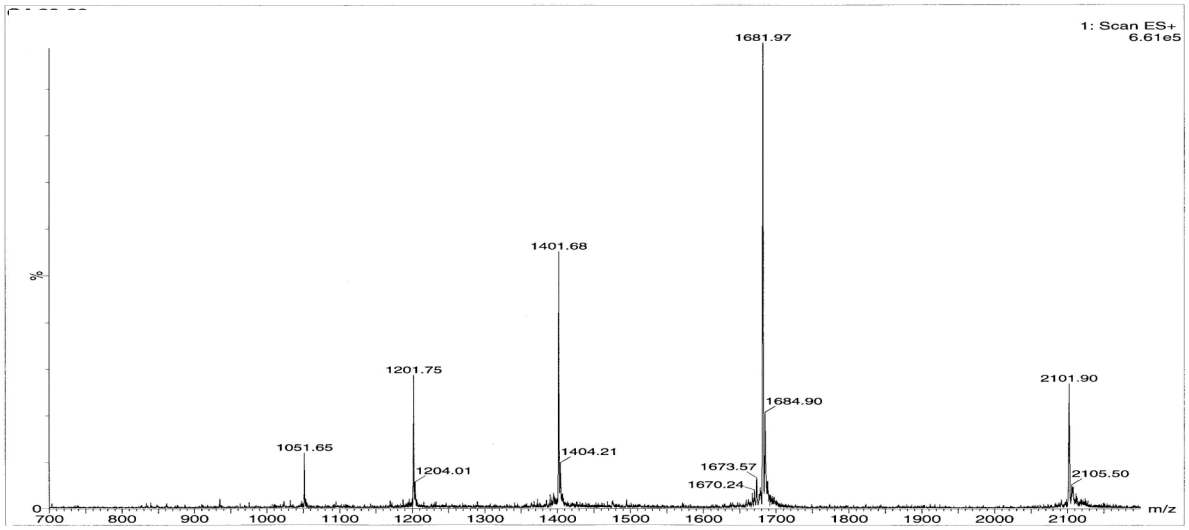
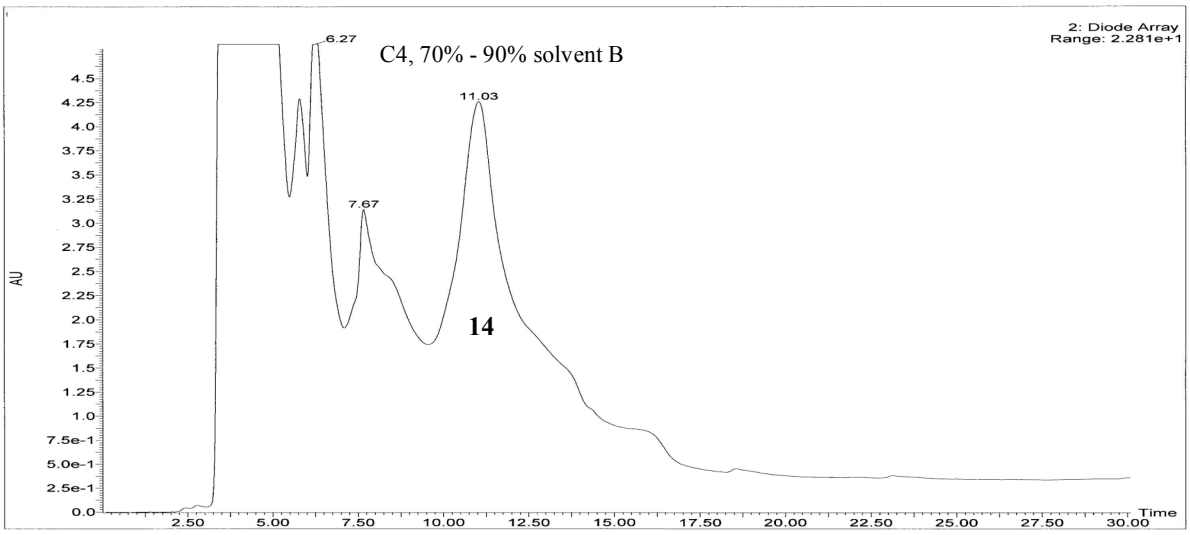


Figure 7 (**a**) LC-UV trace of purified compound **13**. (**b**) ESI-MS of compound **13**. MS (ESI) calcd. for $C_{385}H_{591}N_{81}O_{24}S_2$ $[M+4H]^{4+}$ *m/z* = 2100.30, $[M+5H]^{5+}$ *m/z* = 1680.44, $[M+6H]^{6+}$ *m/z* = 1400.53, $[M+7H]^{7+}$ *m/z* = 1200.60, $[M+8H]^{8+}$ *m/z* = 1050.65.

Compound 14 Glycopeptide **12** (0.5 mg, 0.07 µmol), glycopeptide **13** (0.6 mg, 0.07 µmol), AgCl (1.0 mg, 7 µmol) and HOOBt (0.7 mg, 4.3 µmol) were dissolved in 20 µl DMSO and DIEA (0.37 mg, 2.9 µmol) was added. The reaction was stirred at rt for 4 hr and was acidified by the addition of 500 µl of MeCN/H_2O (1:1) containing 5% AcOH. The mixture was purified by C4 column with linear gradients from 60% solvent B to 80% solvent B over 30 min. The flow rate was 16 ml/min and detection was by UV-absorbance at 258 nm. A white solid (0.3 mg, 30%) was obtained after lyophilization.

a



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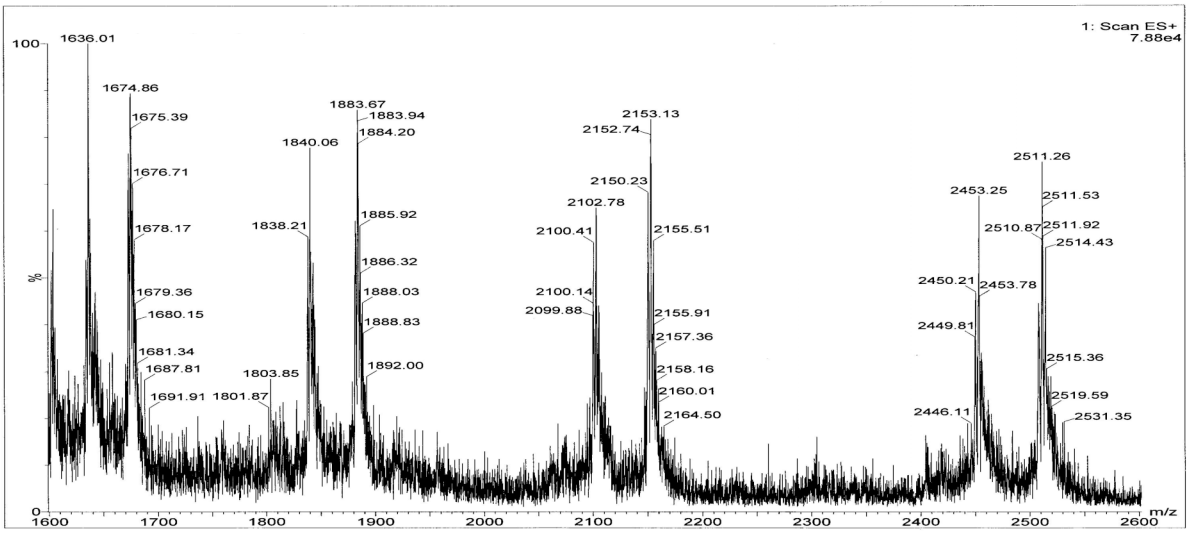


Figure 8 (**a**) LC-UV trace of purified compound **14**. (**b**) ESI-MS of compound **14**. MS (ESI) calcd. for $C_{678}H_{1055}N_{137}O_{243}S_2$ (product without lactone) $[M+6H]^{6+}$ *m/z* = 2512.40, $[M+7H]^{7+}$ *m/z* =2153.63, $[M+8H]^{8+}$ *m/z* = 1884.55, $[M+9H]^{9+}$ *m/z* = 1675.26; $C_{678}H_{1053}N_{137}O_{242}S_2$ (product with one lactone) $[M+6H]^{6+}$ *m/z* = 2509.40, $[M+7H]^{7+}$ *m/z* =2151.05, $[M+8H]^{8+}$ *m/z* = 1882.30, $[M+9H]^{9+}$ *m/z* = 1673.26; $C_{678}H_{1051}N_{137}O_{241}S_2$ (product with two lactone) $[M+6H]^{6+}$ *m/z* = 2506.39, $[M+7H]^{7+}$ *m/z* =2148.48, $[M+8H]^{8+}$ *m/z* = 1880.05, $[M+9H]^{9+}$ *m/z* = 1671.26.

Compound 15 was purified by C4 column with linear gradients from 35% solvent B to 55% solvent B over 30 min. The flow rate was 16 ml/min and detection was by UV-absorbance at 258 nm.

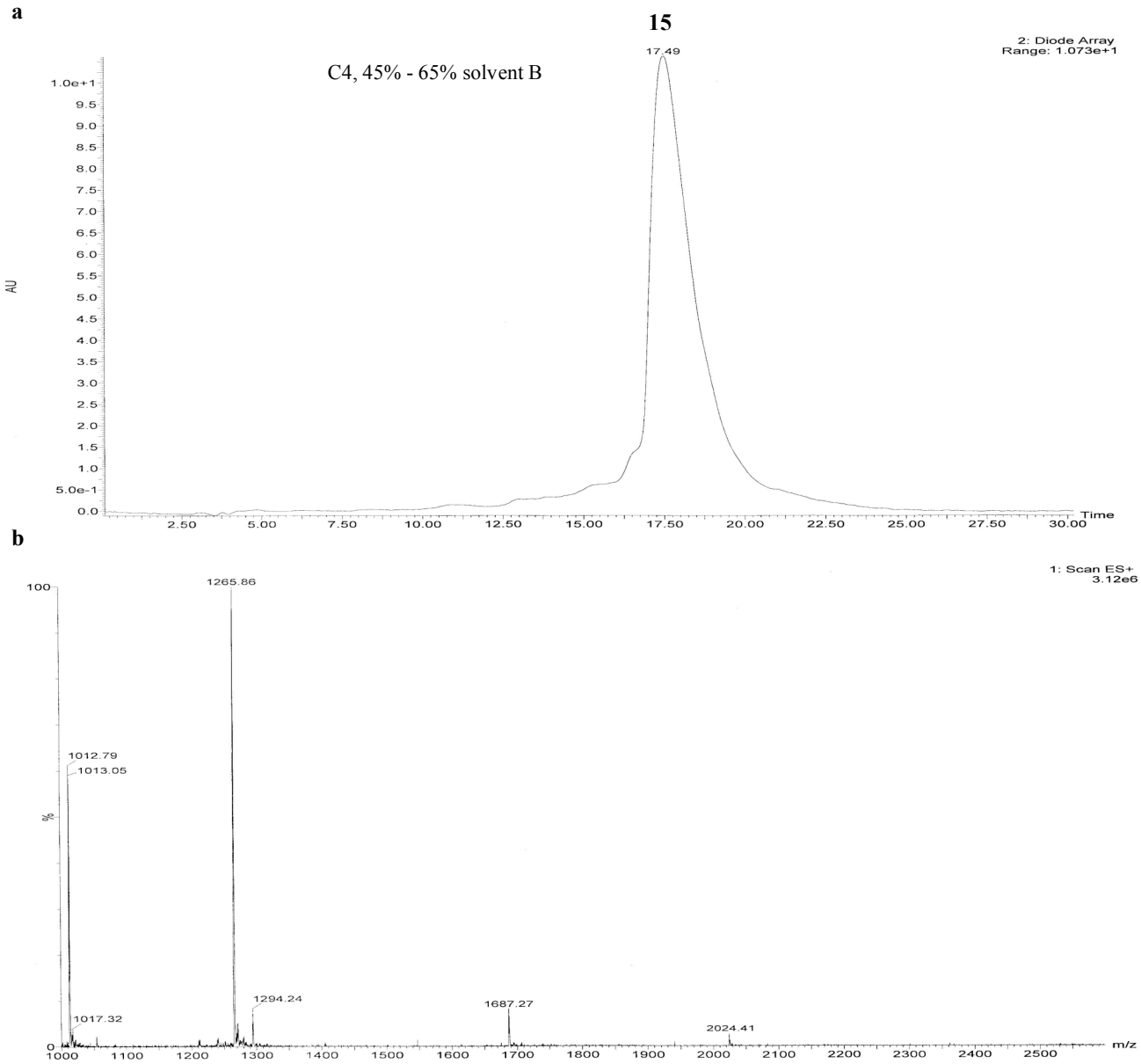


Figure 9 (**a**) LC-UV trace of purified compound **15**. (**b**) ESI-MS of compound **15**. MS (ESI) calcd. for $C_{237}H_{376}N_{60}O_{60}S$ $[M+2H]^{2+}$ *m/z* = 2528.40, $[M+3H]^{3+}$ *m/z* = 1685.93, $[M+4H]^{4+}$ *m/z* = 1264.70, $[M+5H]^{5+}$ *m/z* = 1011.96.

Compound 16 was purified by C18 column with linear gradients from 55% solvent B to 75% solvent B over 30 min. The flow rate was 16 ml/min and detection was by UV-absorbance at 265 nm.

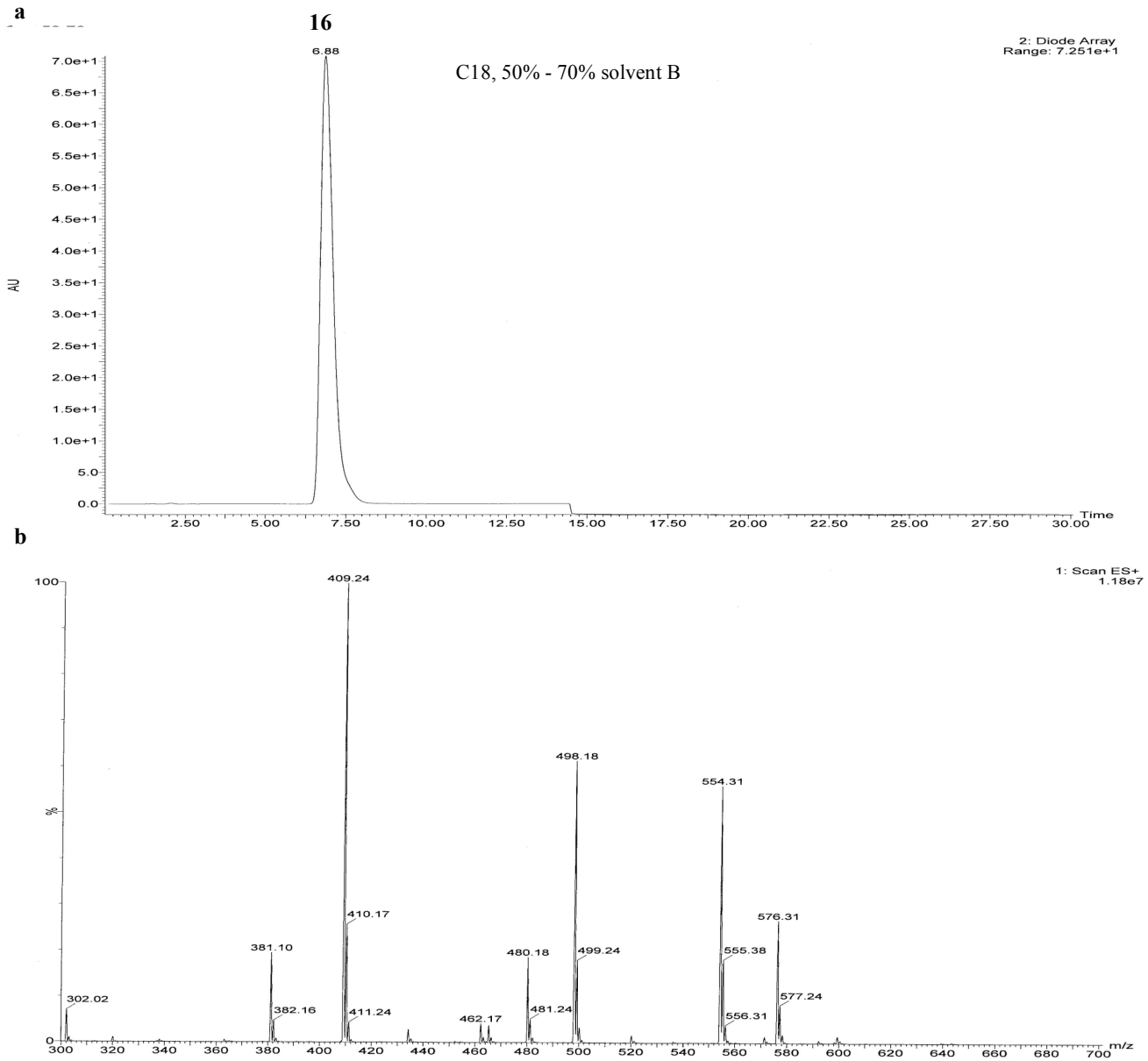


Figure 10 (**a**) LC-UV trace of purified compound **16**. (**b**) ESI-MS of compound **16**. MS (ESI) calcd. for $C_{29}H_{35}N_3O_8$ $[M+H]^+$ *m/z* = 554.24.

Compound 17 was purified by C18 column with linear gradients from 40% solvent B to 60% solvent B over 30 min. The flow rate was 16 ml/min and detection was by UV-absorbance at 220 nm.

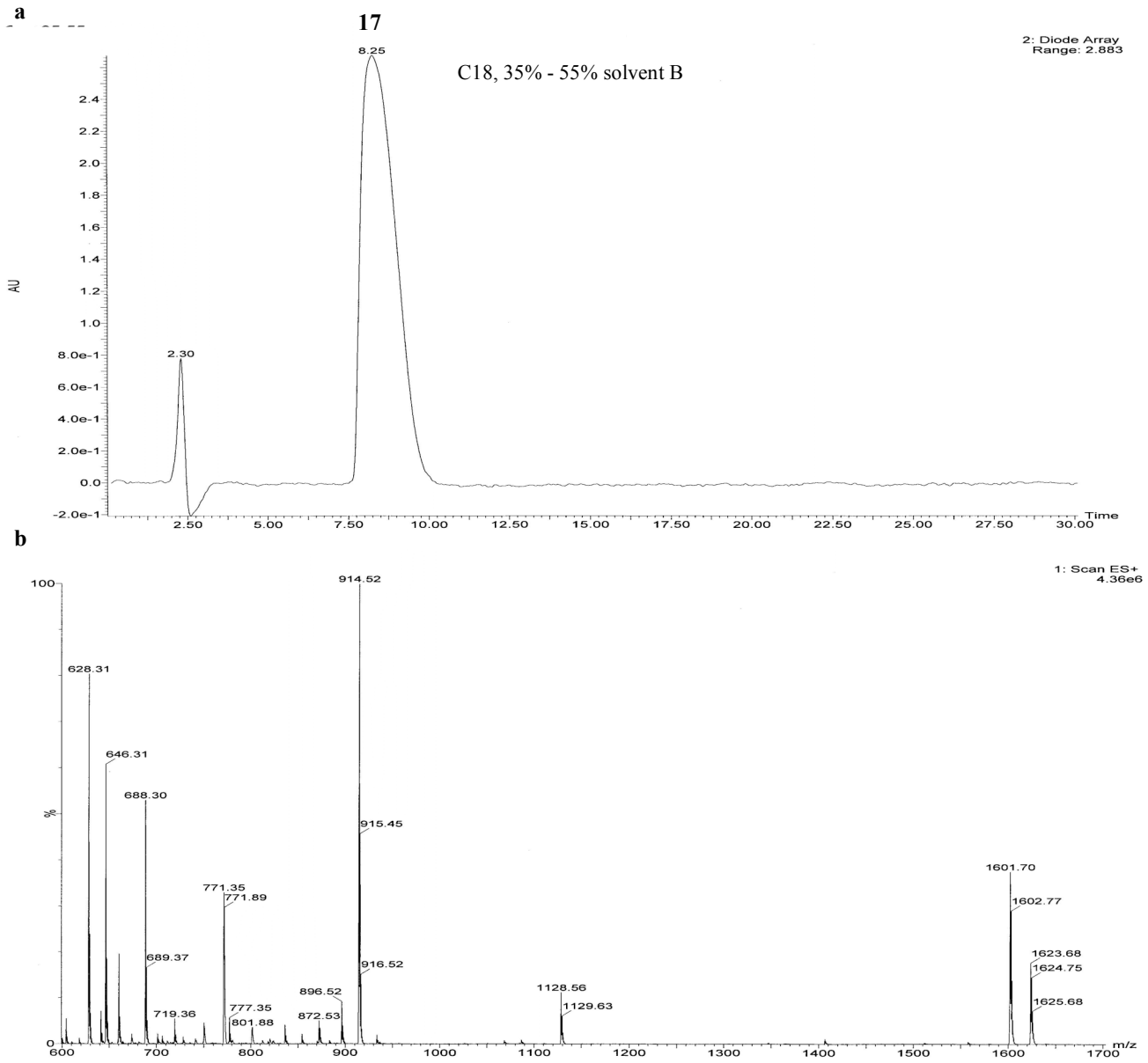


Figure 11 (**a**) LC-UV trace of purified compound **17**. (**b**) ESI-MS of compound **17**. MS (ESI) calcd. for $C_{69}H_{92}N_4O_{39}$ $[M+H]^+$ *m/z* = 1601.53.

Compound 18 was purified by C18 column with linear gradients from 35% solvent B to 55% solvent B over 30 min. The flow rate was 16 ml/min and detection was by UV-absorbance at 235 nm.

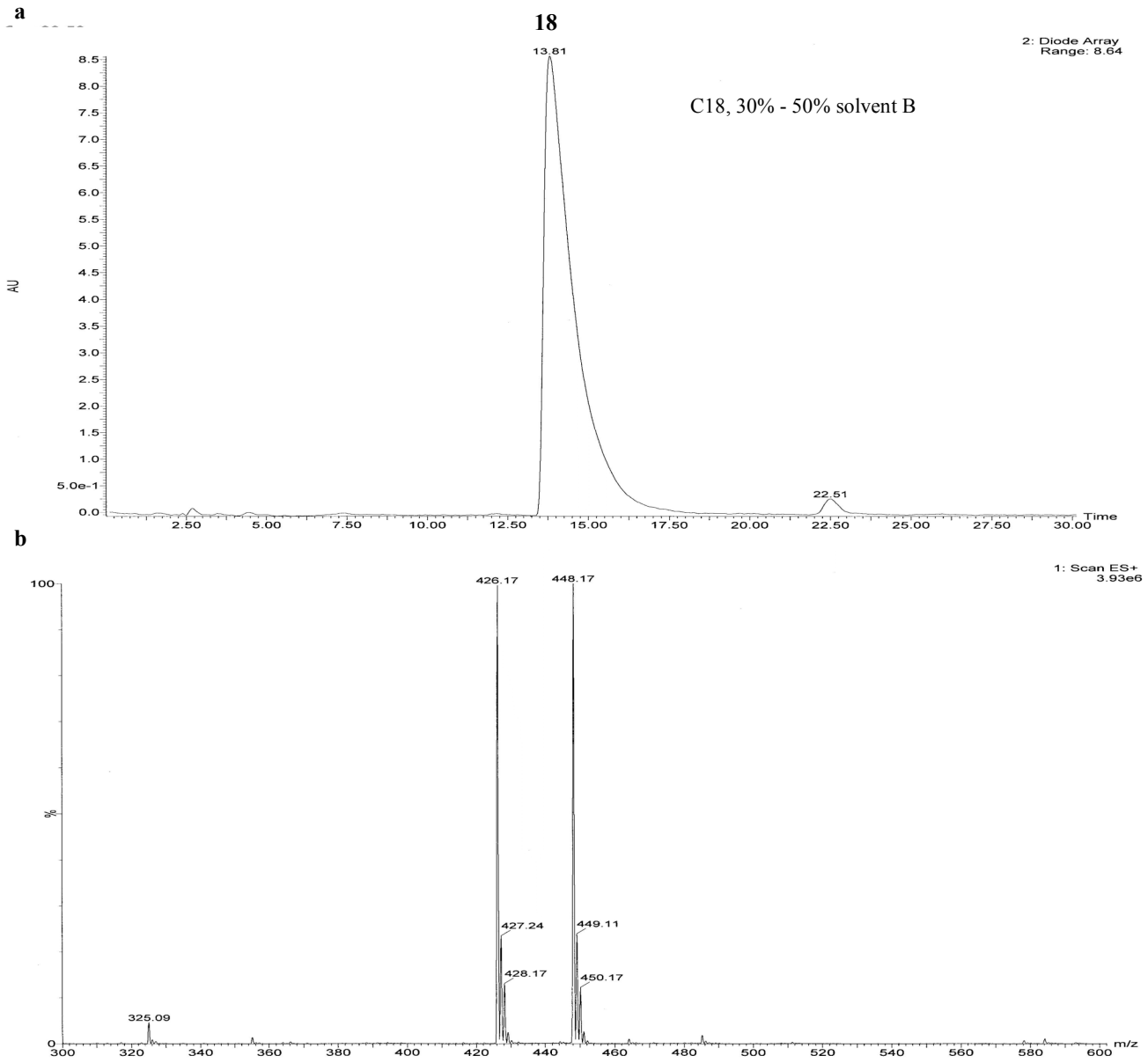


Figure 12 (**a**) LC-UV trace of purified compound **18**. (**b**) ESI-MS of compound **18**. MS (ESI) calcd. for $C_{69}H_{92}N_4O_{39}$ $[M+H]^+$ *m/z* = 426.14.

Compound 19 Peptide **16** (8.0 mg, 15 µmol), protected glycophorin **17** (18.0 mg, 10 µmol) and DMAP (0.2 mg, 1.5 µmol) were dissolved in 200 µl DCM and DCC (3.0 mg, 15 µmol) was added. The reaction was stirred at rt for 30 min and was filtered. Purification of the residue by chromatography on silica gel (50:1→15:1 DCM:MeOH) gave pure product 15 mg (70%).

The product of compound **16** and **17** (50 mg, 23 µmol) was dissolved in 0.6 ml MeOH. Palladium on carbon (10 wt. % loading, 0.4 mg) was added followed by 0.15 ml AcOH/MeOH (1 drop AcOH in 2 ml MeOH). The mixture was stirred at rt under H_2 for 35 min. The mixture was filtered through Celite with MeOH and concentrated to give pure product 44.6mg (95%).

The product of hydrogenolysis (44.6 mg, 21 µmol) was coupled to peptide **18** (30.0 mg, 70 µmol) in the same way as described above for the coupling of **16** and **17**. The resulting mixture was concentrated and the t-butyl ester was deprotected in 2 ml cocktail B. The reaction mixture was purified by C18 column with isocratic gradients 50% solvent B over 35 min. The flow rate was 16 ml/min and detection was by UV-absorbance at 265 nm. Lyophilization of the HPLC fractions gave pure **19** as a white powder (14.6 mg, 26%)

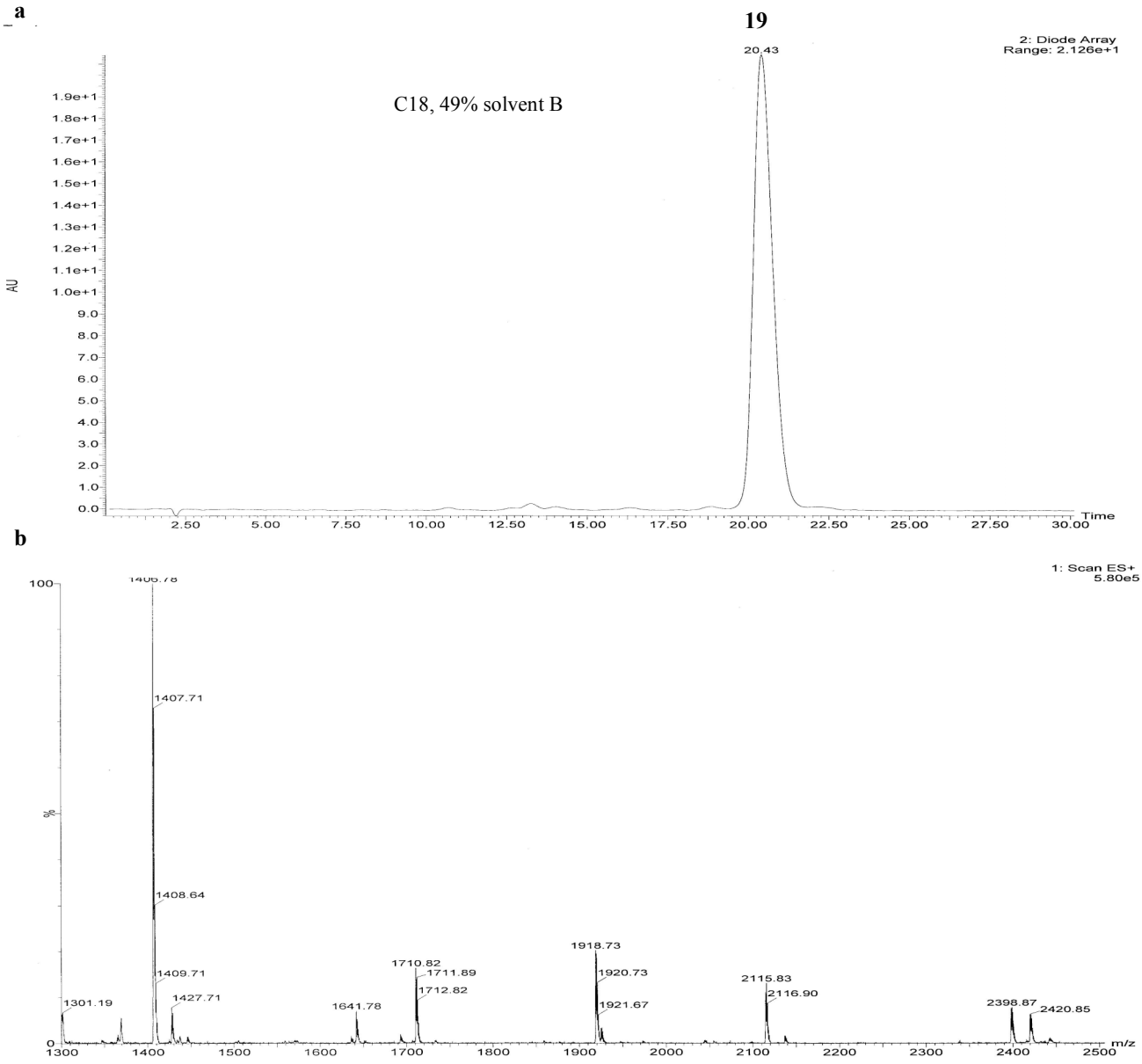


Figure 13 (**a**) LC-UV trace of purified compound **19**. (**b**) ESI-MS of compound **19**. MS (ESI) calcd. for $C_{106}H_{137}N_{10}O_{49}S_2$ $[M+H]^+$ *m/z* = 2396.79.

Product of 15+19 Glycopeptide **19** (1.9 mg, 0.79 µmol), peptide **15** (4.8 mg, 0.95 µmol), TCEP•HCl (0.2 mg, 0.87 µmol) and HOOBt (4.3 mg, 26 µmol) were dissolved in 38 µl DMSO and DIEA (2.2 mg, 17 µmol) was added. The reaction was stirred at rt for 40 hr. 4 µl piperidine was added. The reaction was stirred at rt for 10 min and was acidified by the addition of 2 ml of MeCN/H_2O (1:1) containing 5% AcOH. The mixture was purified by C4 column with linear gradients from 55% solvent B to 75% solvent B over 30 min. The flow rate was 16 ml/min and detection was by UV-absorbance at 258 nm. A white solid (2.4 mg, 43%) was obtained after lyophilization.

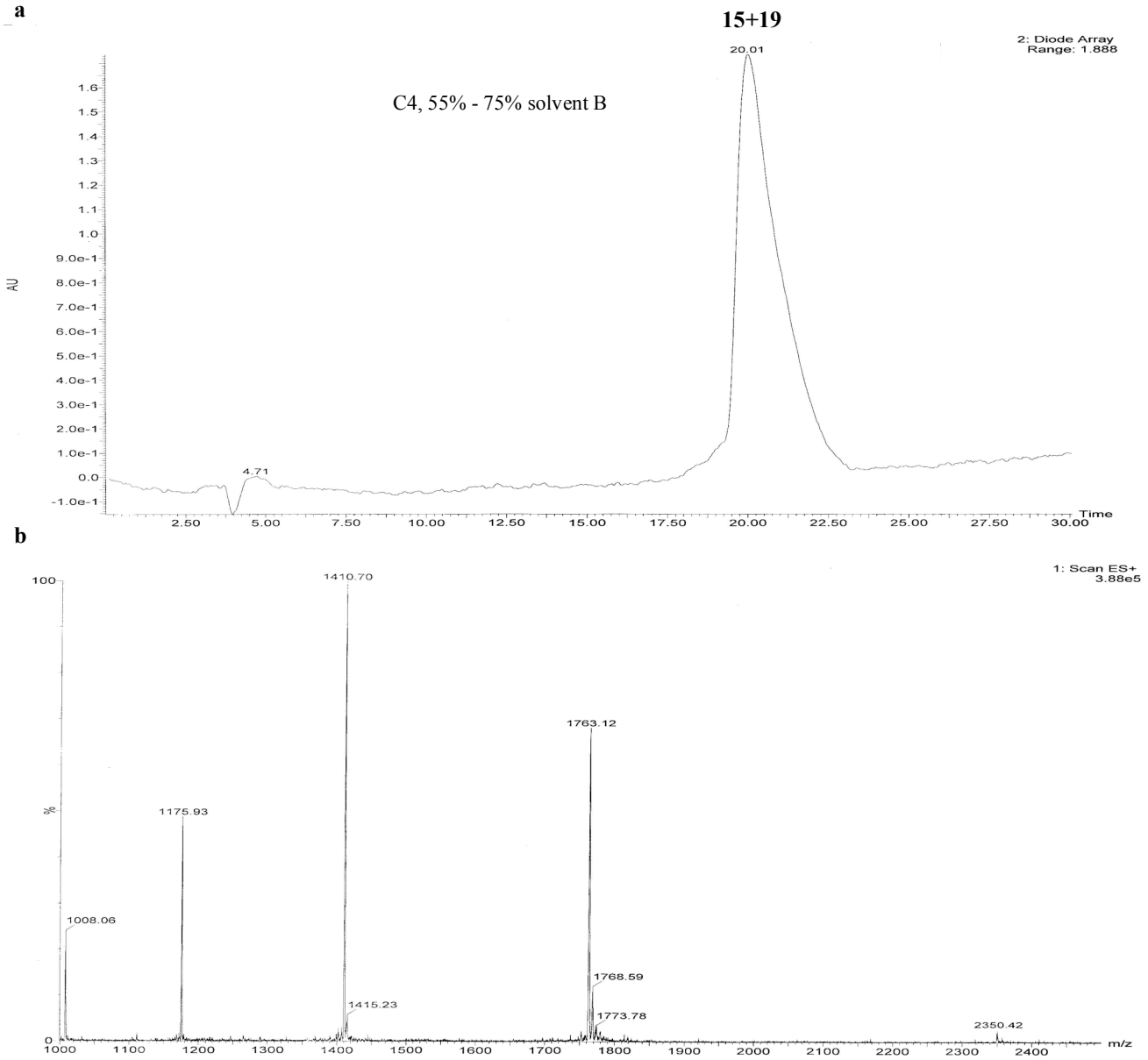
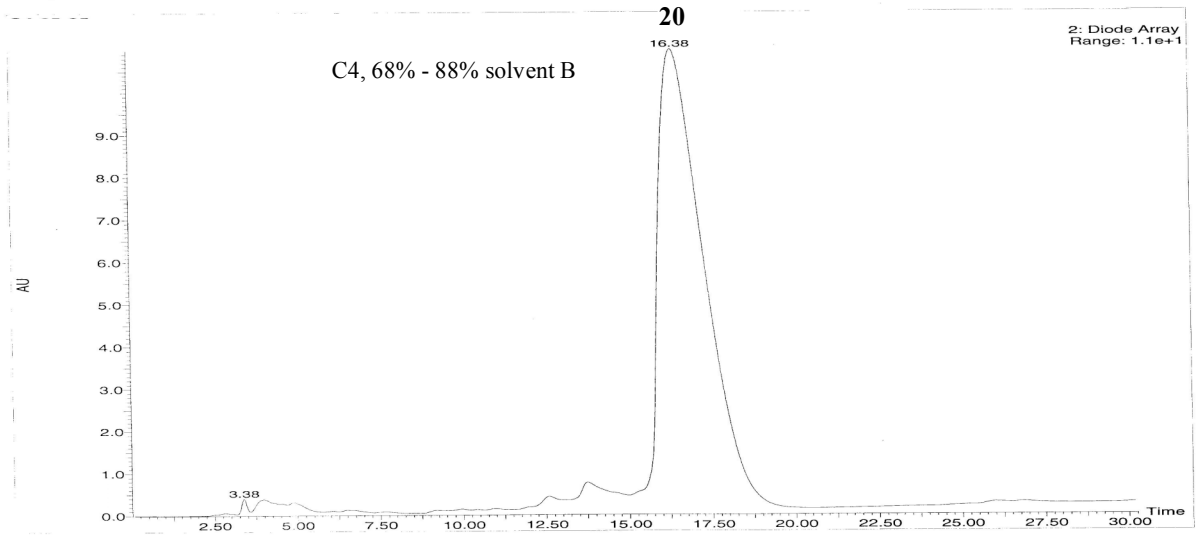


Figure 14 (**a**) LC-UV trace of purified **15**+**19**. (**b**) ESI-MS of compound **15**+**19**. MS (ESI) calcd. for $C_{320}H_{492}N_{70}O_{106}S$ $[M+3H]^{3+}$ *m/z* = 2348.83, $[M+4H]^{4+}$ *m/z* = 1761.88, $[M+5H]^{5+}$ *m/z* = 1409.70, $[M+6H]^{6+}$ *m/z* = 1174.92, $[M+7H]^{7+}$ *m/z* = 1007.21.

Compound 20 was purified by C4 column with linear gradients from 65% solvent B to 85% solvent B over 30 min. The flow rate was 16 ml/min and detection was by UV-absorbance at 260 nm.

a



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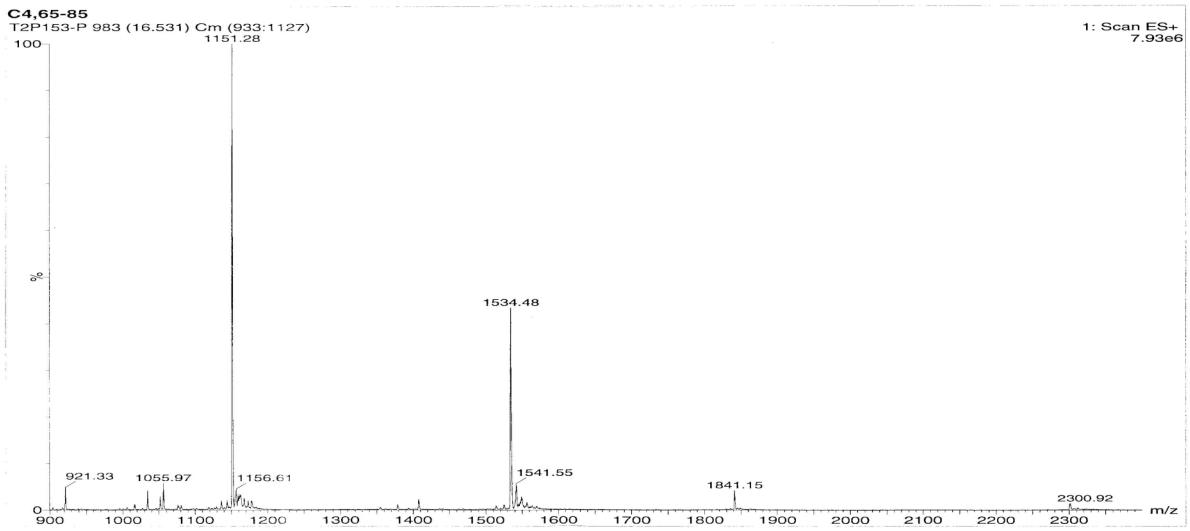


Figure 15 (**a**) LC-UV trace of purified **20**. (**b**) ESI-MS of compound **20**. MS (ESI) calcd. for $C_{220}H_{338}N_{48}O_{55}S_2$ $[M+2H]^{2+}$ *m/z* = 2299.23, $[M+3H]^{3+}$ *m/z* = 1533.15, $[M+4H]^{4+}$ *m/z* = 1150.12, $[M+5H]^{5+}$ *m/z* = 920.29.

Product of 15+19+20 The product of **15**+**19** (2.0 mg, 0.28 μmol) and peptide **20** (1.8 mg, 0.39 μmol) was coupled and deprotected in the same way as for **15**+**19**. The mixture was purified by C4 column with linear gradients from 65% solvent B to 85% solvent B over 30 min. The flow rate was 16 ml/min and detection was by UV-absorbance at 257 nm. A white solid (1.7 mg, 53%) was obtained after lyophilization.

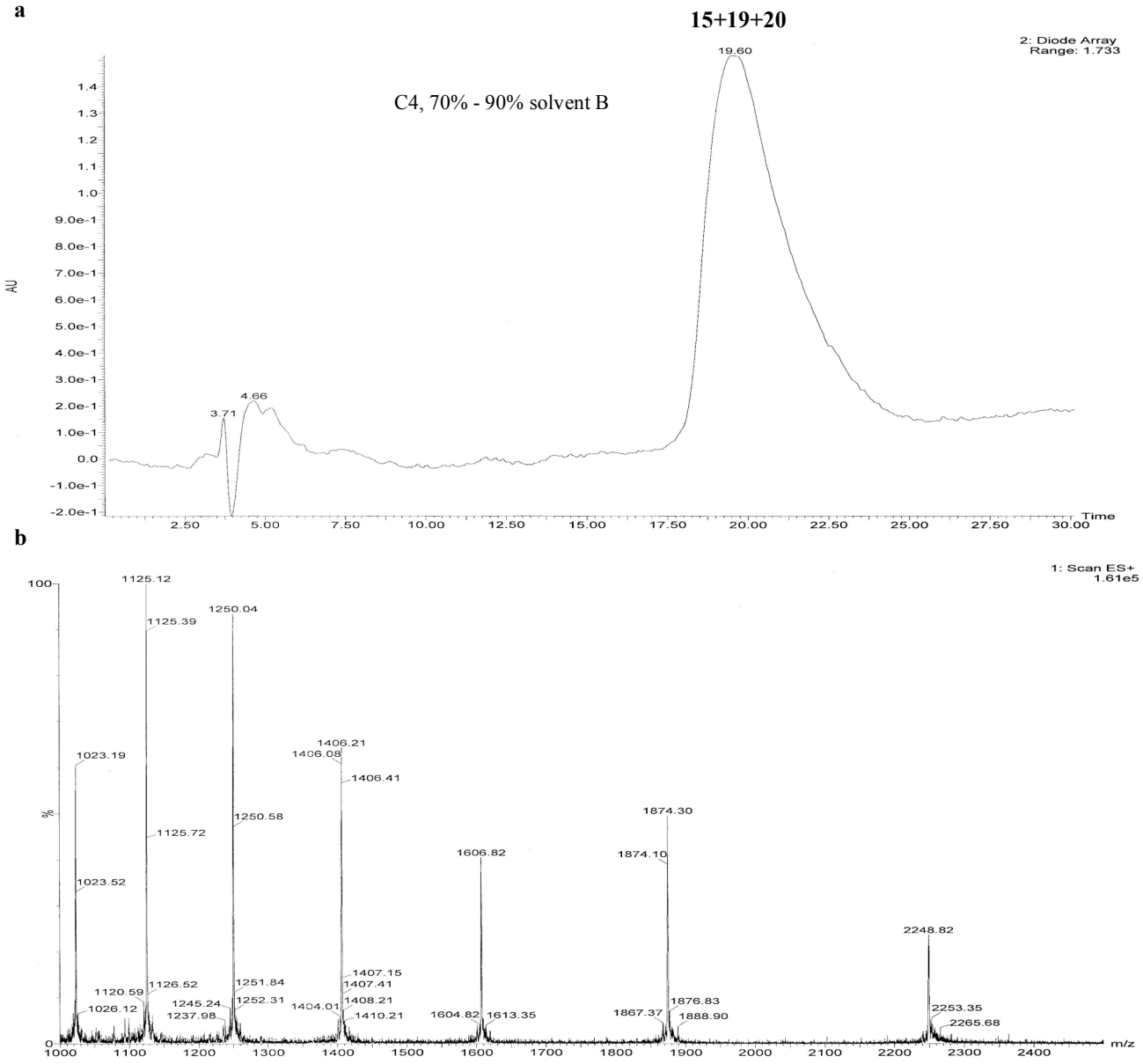


Figure 16 (**a**) LC-UV trace of purified **15**+**19**+**20**. (**b**) ESI-MS of compound **15**+**19**+**20**. MS (ESI) calcd. for $C_{517}H_{810}N_{118}O_{158}S$ $[M+5H]^{5+}$ *m/z* = 2247.37, $[M+6H]^{6+}$ *m/z* = 1872.98, $[M+7H]^{7+}$ *m/z* = 1605.55, $[M+8H]^{8+}$ *m/z* = 1404.98, $[M+9H]^{9+}$ *m/z* = 1248.99, $[M+10H]^{10+}$ *m/z* = 1124.19, $[M+11H]^{11+}$ *m/z* = 1022.08.

Compound 21 The product of **15**+**19**+**20** (0.4 mg, 0.036 µmol) and glycopeptide **10** (0.14 mg, 0.036 µmol) was coupled and deprotected in the same way as for **15**+**19**+**20**. The mixture was purified by C4 column with linear gradients from 62% solvent B to 82% solvent B over 30 min. The flow rate was 16 ml/min and detection was by UV-absorbance at 257 nm. A white solid (0.2 mg, 39%) was obtained after lyophilization.

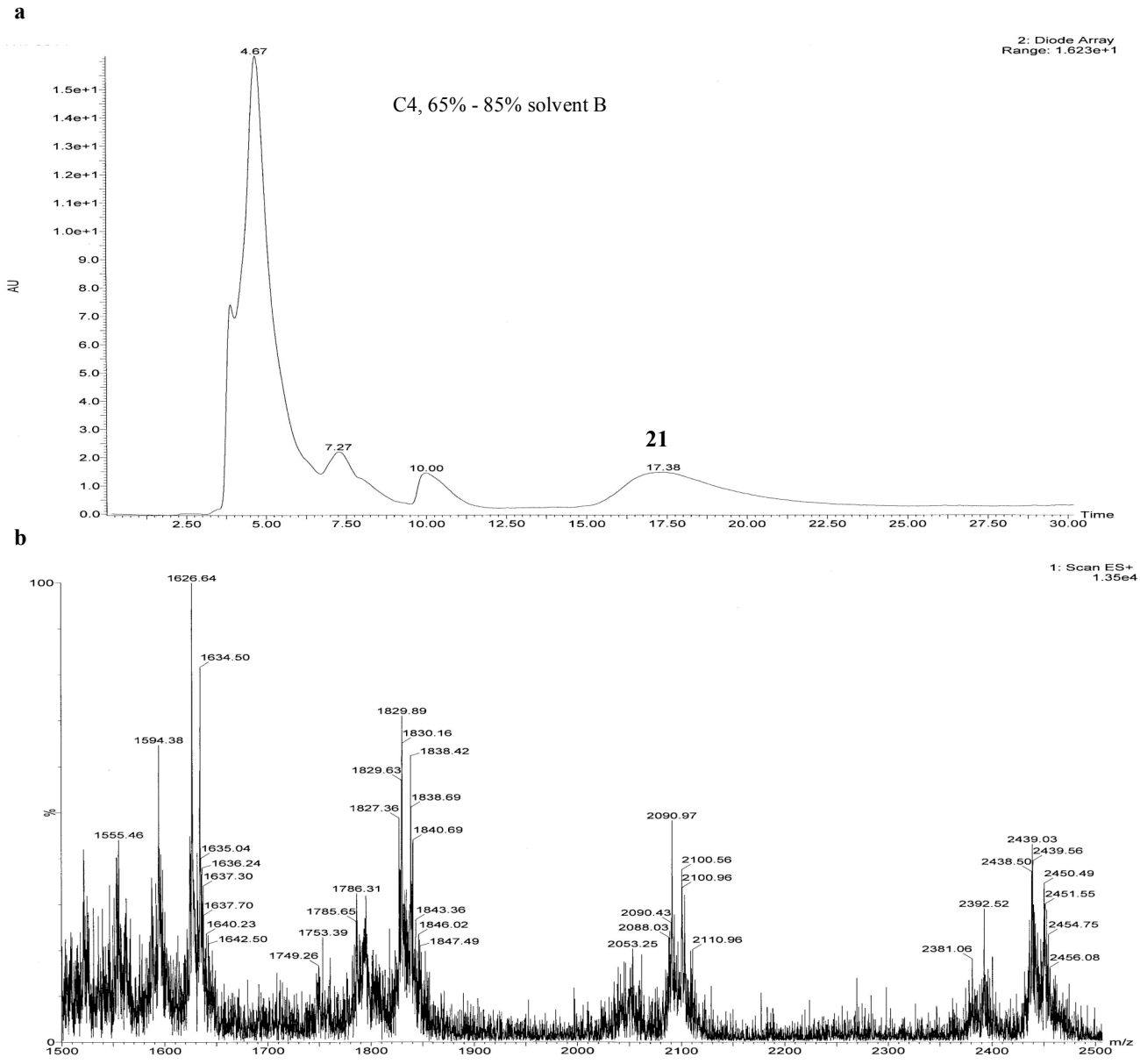


Figure 17 (**a**) LC-UV trace of purified **21**. (**b**) ESI-MS of compound **21**. MS (ESI) calcd. for $C_{652}H_{1027}N_{137}O_{236}S$ (Dilactone) $[M+6H]^{6+}$ *m/z* = 2431.71, $[M+7H]^{7+}$ *m/z* = 2084.46, $[M+8H]^{8+}$ *m/z* = 1824.03, $[M+9H]^{9+}$ *m/z* = 1621.47.

Compound 22 The fully protected glycophorin (20.0 mg, 11 µmol) were dissolved in 750 µl MeOH and 500 µl 1 M NaOH was added. The reaction was stirred at rt for 2hr, neutralized by 380 µl 1M HCl and concentrated.

The fully deprotected glycophorin and 11 mg Na_2CO_3 was dissolved in 100 µl H_2O. Fmoc-OSu in 100 µl ethylene glycol dimethyl ether was added. The mixture was stirred at rt for 2.5 hr, acidified by 15 µl AcOH and purified by C18 column with linear gradients from 15% solvent B to 35% solvent B over 30 min. The flow rate was 16 ml/min and detection was by UV-absorbance at 265 nm. A white solid (5.0 mg, 36%) was obtained after lyophilization.

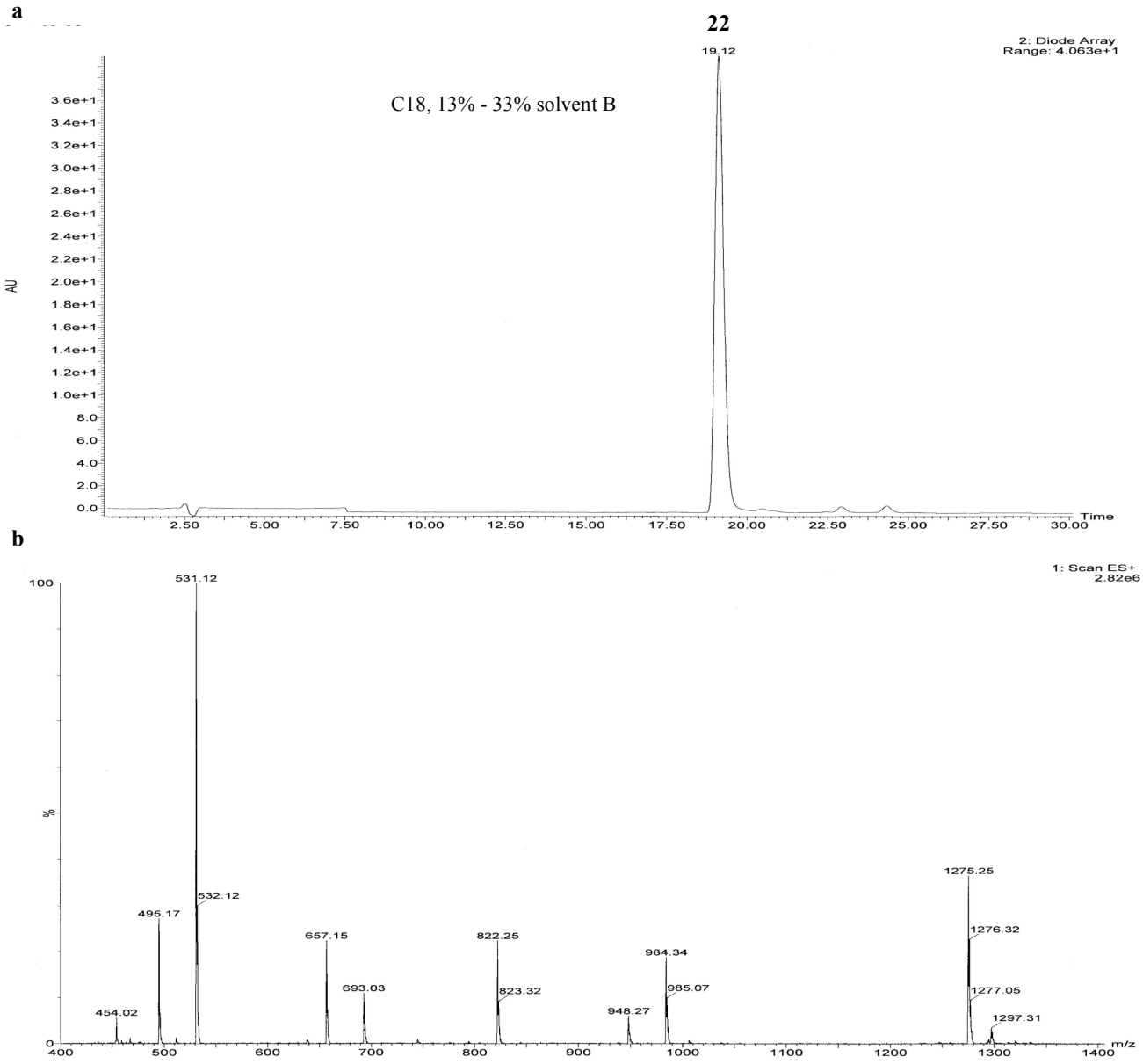


Figure 18 (**a**) LC-UV trace of purified compound **22**. (**b**) ESI-MS of compound **22**. MS (ESI) calcd. for $C_{54}H_{74}N_4O_{31}$ $[M+H]^+$ *m/z* = 1275.43.

Compound 23 Glycophorin **22** (2.1 mg, 1.6 µmol), peptide **18** (0.53 mg, 1.2 µmol) and HATU (1.9 mg, 5.0 µmol) were dissolved in 70 µl DMSO and DIEA (1.0 mg, 7.6 µmol) was added. The reaction was stirred at rt for 1 min and was acidified by the addition of 1 ml of MeCN/H_2O (1:2) containing 5% AcOH. The mixture was purified by C18 column with linear gradients from 42% solvent B to 52% solvent B over 30 min. The flow rate was 16 ml/min and detection was by UV-absorbance at 265 nm. A white solid (0.7 mg, 35%) was obtained after lyophilization.

The product of **22**+**18** (2.1 mg, 1.3 µmol) dissolved in 50 µl DMSO and 5 µl piperidine was added. The reaction was stirred at rt for 1 min and was acidified by the addition of 1 ml of MeCN/H_2O (2:1) containing 5% AcOH. The mixture was purified by C18 column with linear gradients from 22% solvent B to 42% solvent B over 30 min. The flow rate was 16 ml/min and detection was by UV-absorbance at 240 nm. A white solid **23** + **24** (1.5 mg, 88%) was obtained after lyophilization.

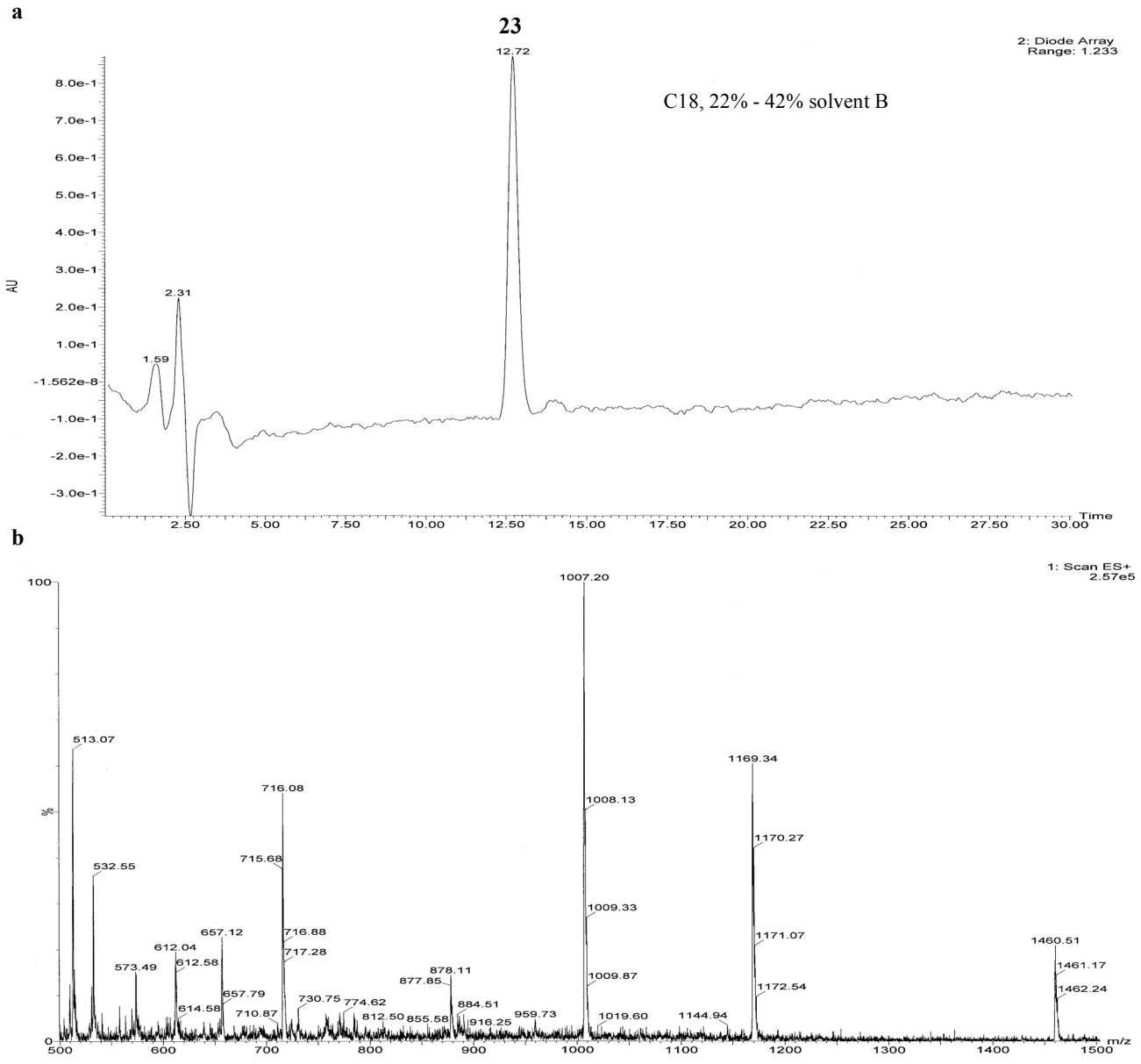


Figure 19 (**a**) LC-UV trace of purified compound **23**. (**b**) ESI-MS of compound **23**. MS (ESI) calcd. for $C_{58}H_{89}N_7O_{32}S_2$ $[M+H]^+$ *m/z* = 1460.50.

Compound 24

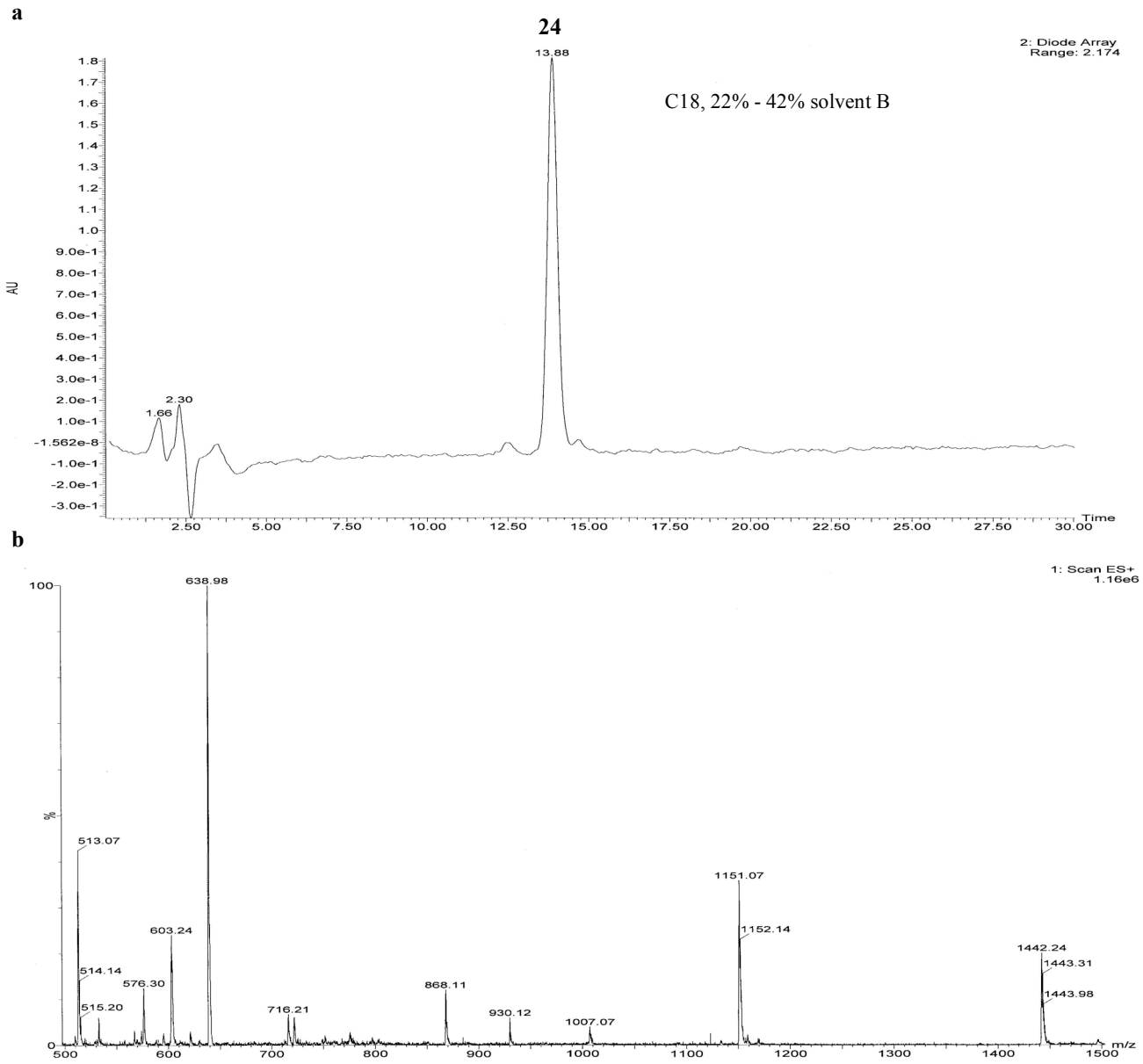


Figure 20 (**a**) LC-UV trace of purified compound **24**. (**b**) ESI-MS of compound **24**. MS (ESI) calcd. for $C_{58}H_{87}N_7O_{31}S_2$ $[M+H]^+$ *m/z* = 1442.49.

Compound 25 was purified by C18 column with linear gradients from 58% solvent A to 78% solvent B over 30 min. The flow rate was 16 ml/min and detection was by UV-absorbance at 265 nm.

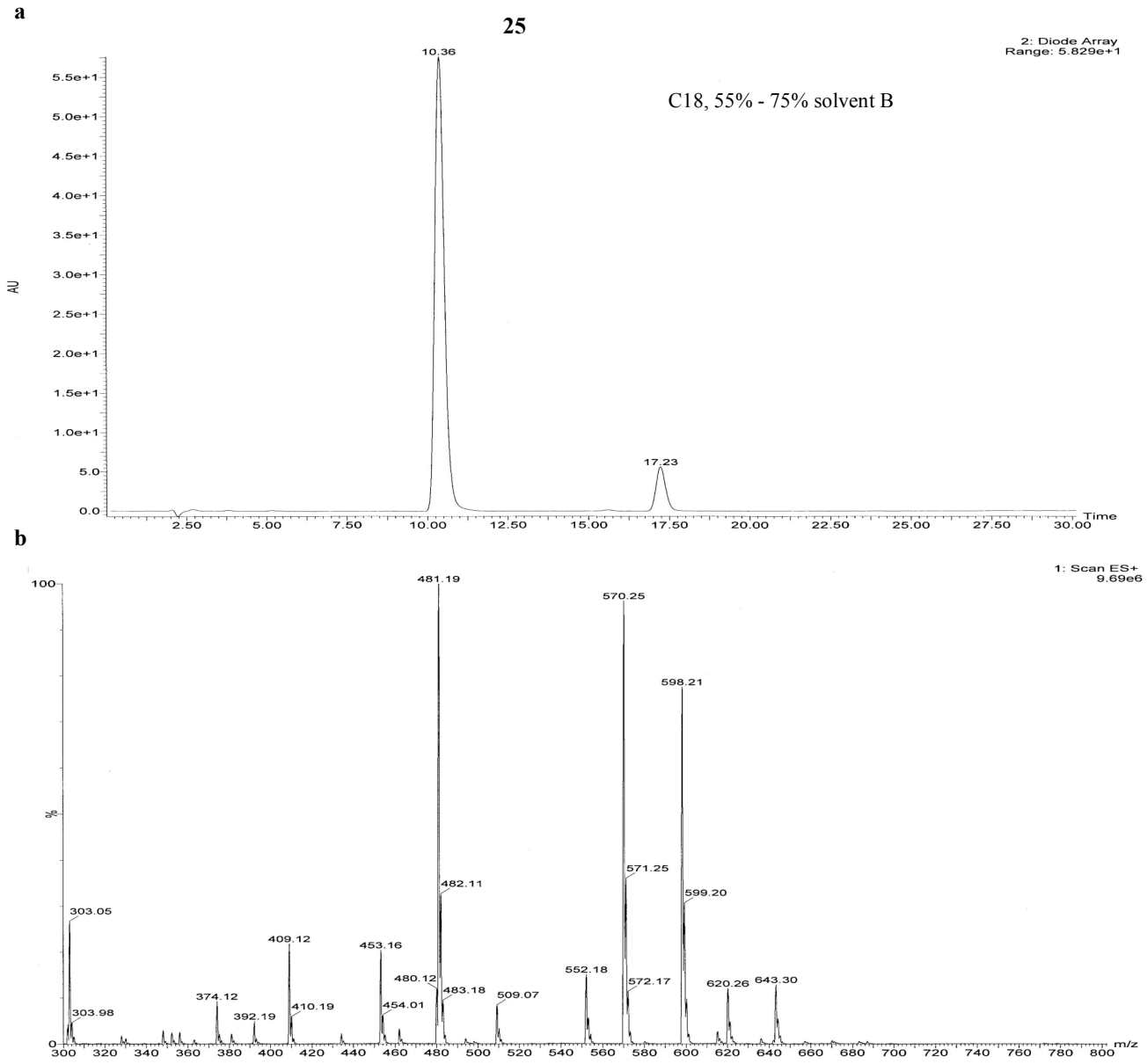


Figure 21 (**a**) LC-UV trace of purified compound **25**. (**b**) ESI-MS of compound **25**. MS (ESI) calcd. for $C_{30}H_{39}N_3O_8Si$ $[M+H]^+$ *m/z* = 598.25.

Compound 26 was purified by C18 column with linear gradients from 63% solvent B to 83% solvent B over 30 min. The flow rate was 16 ml/min and detection was by UV-absorbance at 265 nm.

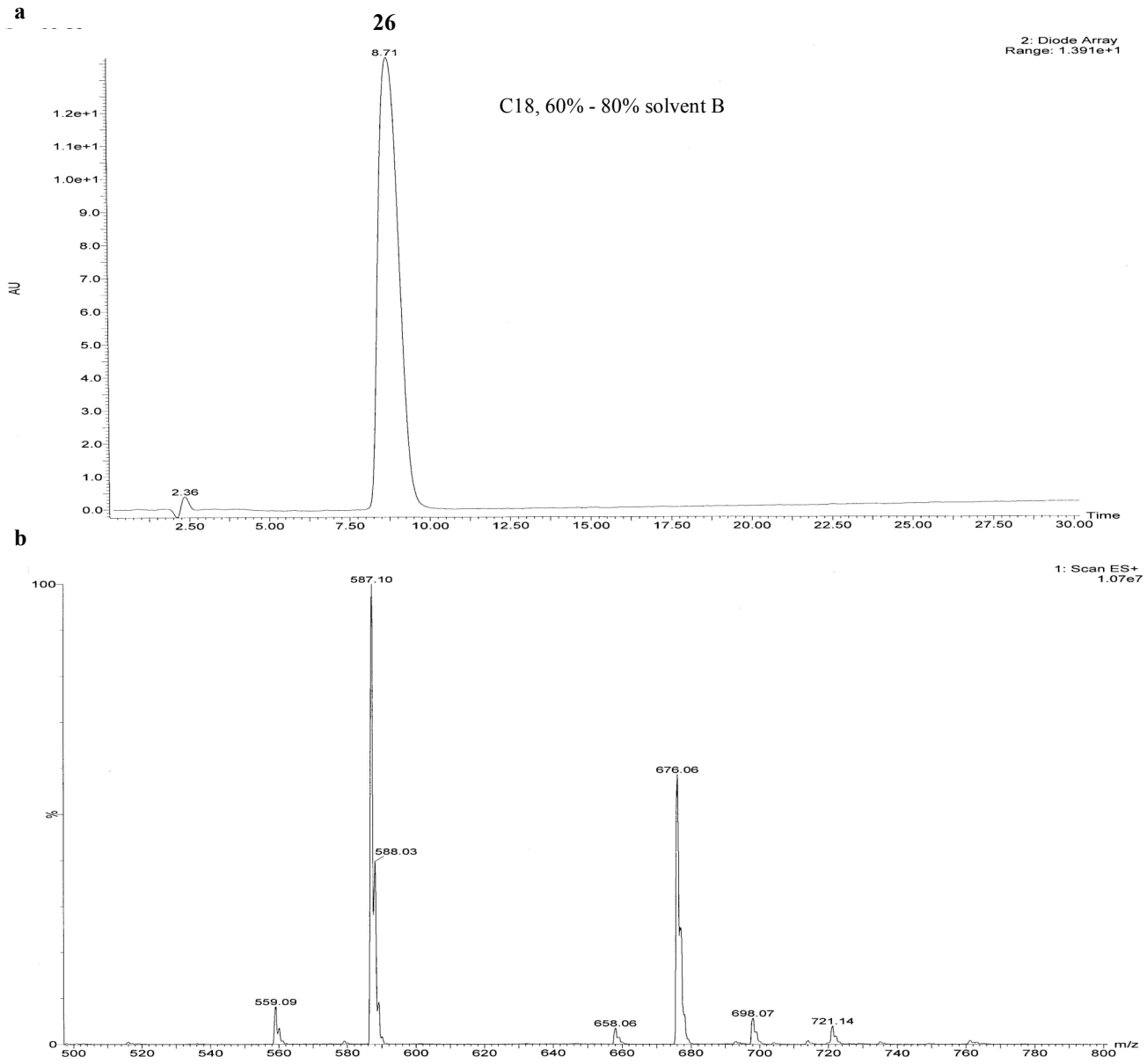


Figure 22 (**a**) LC-UV trace of purified compound **26**. (**b**) ESI-MS of compound **26**. MS (ESI) calcd. for $C_{39}H_{37}N_3O_8$ $[M+H]^+$ *m/z* = 676.26.

Compound 27 Glycopeptide **23**, **24** (1.0 mg, 0.68 µmol), peptide **25** (1.2 mg, 2.0 µmol) and HATU (3.0 mg, 8.0 µmol) were dissolved in 25 µl DMSO and DIEA (1.0 mg, 7.6 µmol) was added. The reaction was stirred at rt for 1 min and was acidified by the addition of 1 ml of MeCN/H_2O (1:1) containing 5% AcOH. The mixture was purified by C18 column with linear gradients from 55% solvent B to 75% solvent B over 30 min. The flow rate was 16 ml/min and detection was by UV-absorbance at 265 nm. A white solid (0.7 mg, 51%) was obtained after lyophilization.

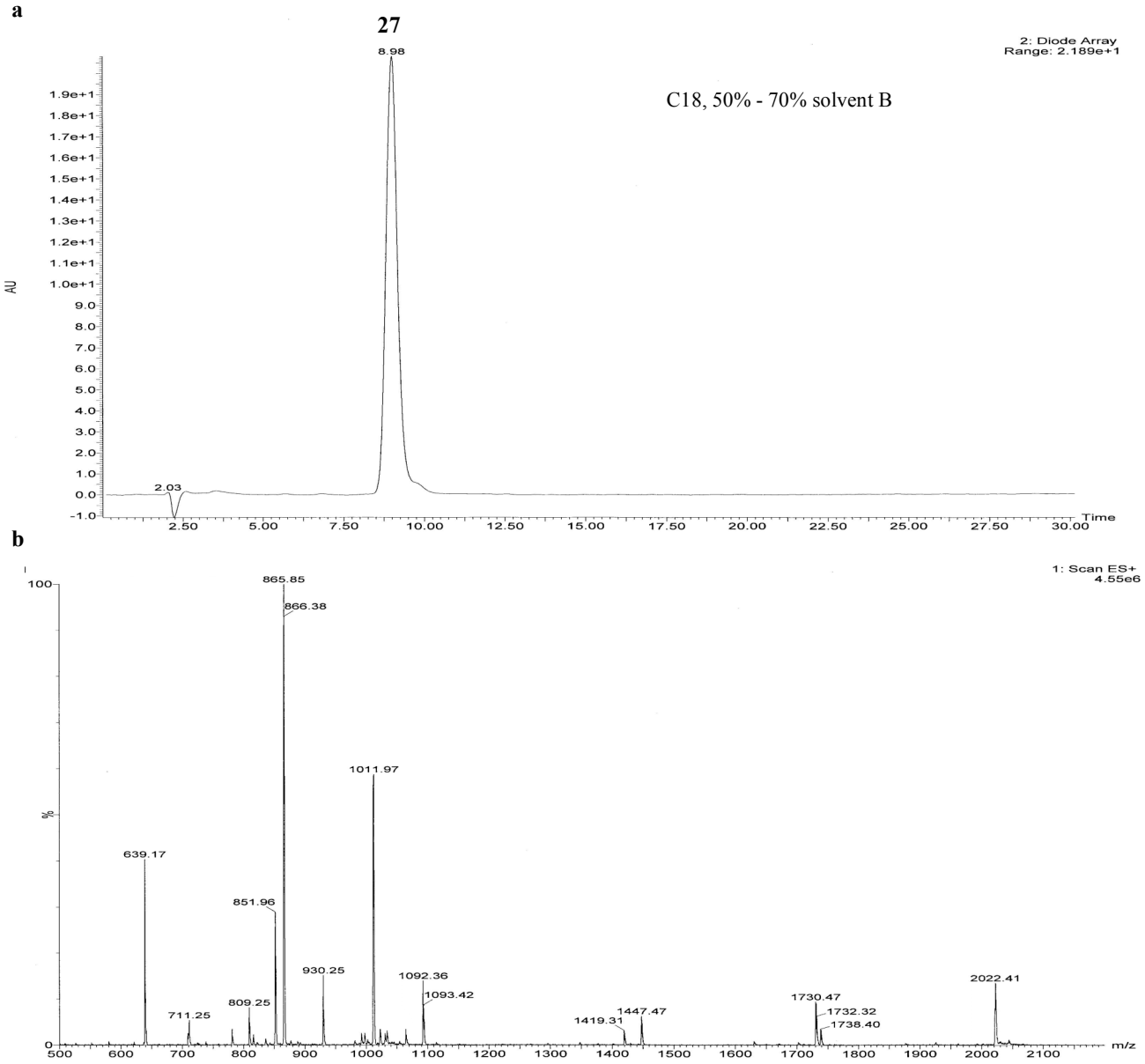


Figure 23 (**a**) LC-UV trace of purified compound **27**. (**b**) ESI-MS of compound **27**. MS (ESI) calcd. for $C_{88}H_{124}N_{10}O_{38}S_2Si$ $[M+H]^+$ *m/z* = 2021.73.

Compound 28 Glycopeptide **23**, **24** (0.8 mg, 0.55 µmol), peptide **26** (1.1 mg, 1.6 µmol) and HATU (3.0 mg, 8.0 µmol) were dissolved in 25 µl DMSO and DIEA (1.0 mg, 7.6 µmol) was added. The reaction was stirred at rt for 1 min and was acidified by the addition of 1 ml of MeCN/H_2O (1:1) containing 5% AcOH. The mixture was purified by C18 column with linear gradients from 60% solvent B to 80% solvent B over 30 min. The flow rate was 16 ml/min and detection was by UV-absorbance at 265 nm. A white solid (0.4 mg, 35%) was obtained after lyophilization.

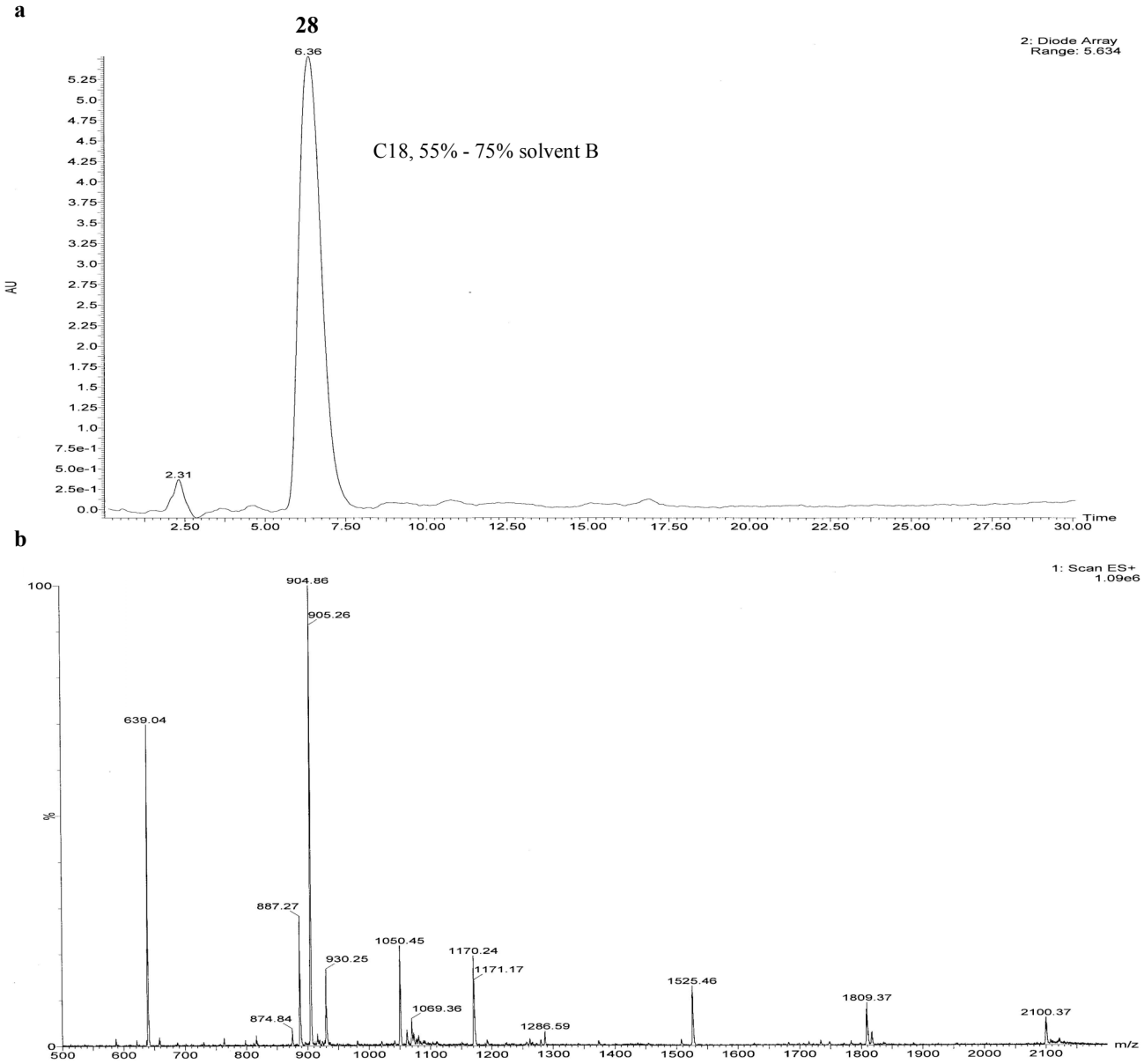


Figure 24 (**a**) LC-UV trace of purified compound **28**. (**b**) ESI-MS of compound **28**. MS (ESI) calcd. for $C_{97}H_{122}N_{10}O_{38}S_2$ $[M+H]^+$ *m/z* = 2099.74.

Compound 29 was synthesized by SPPS on a 0.05 mmol scale. After being cleaved from the resin support by 10 ml cocktail B, half of the peptide mixture was purified by C4 column with linear gradients from 44% solvent B to 49% solvent B over 30 min. The flow rate was 16 ml/min and detection was by UV-absorbance at 230 nm. A white solid (17 mg, 10%) was obtained after lyophilization.

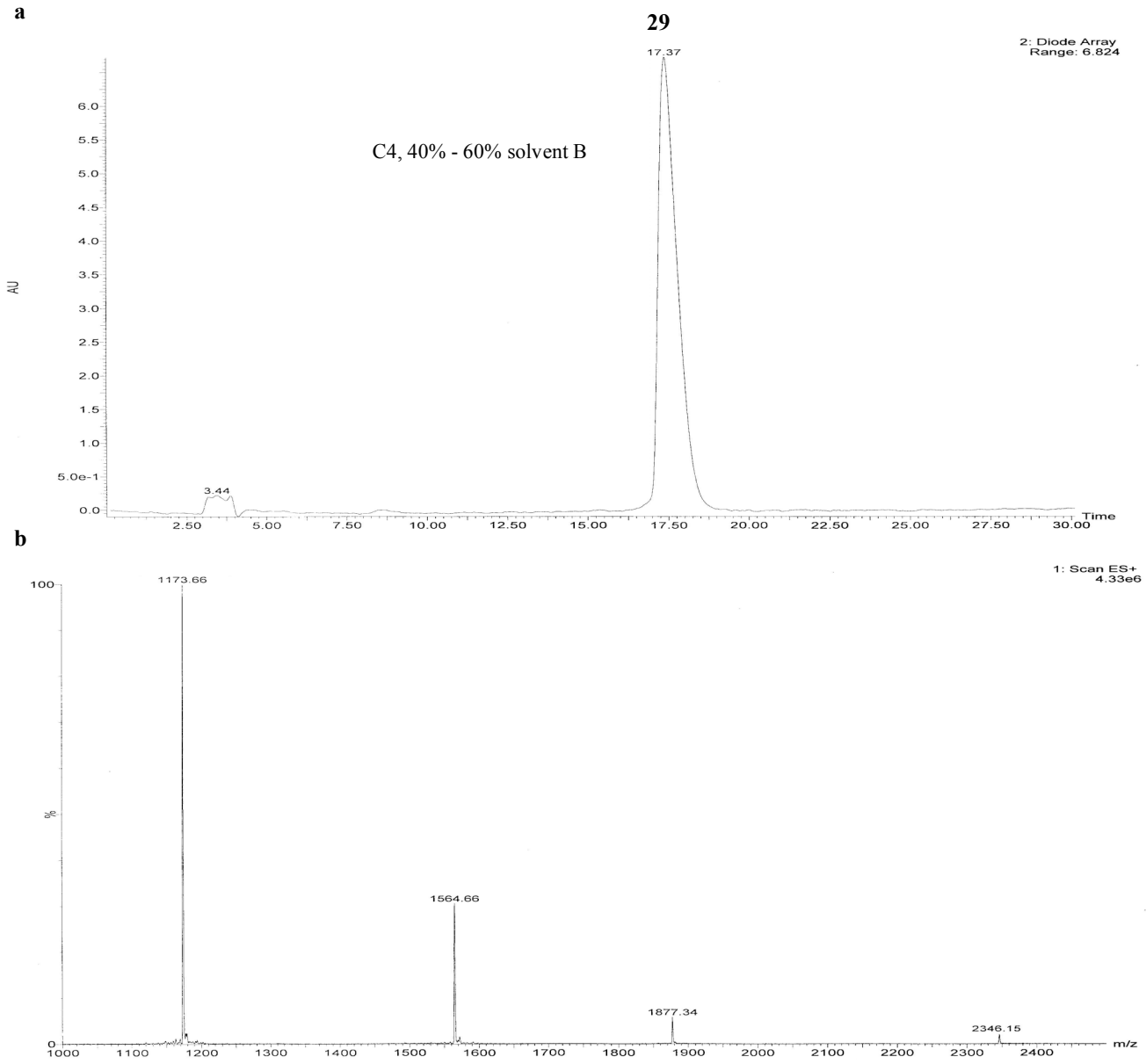


Figure 25 (**a**) LC-UV trace of purified compound **29**. (**b**) ESI-MS of compound **29**. MS (ESI) calcd. for $C_{210}H_{334}N_{60}O_{60}S$ $[M+2H]^{2+}$ *m/z* = 2345.23, $[M+3H]^{3+}$ *m/z* = 1563.82, $[M+4H]^{4+}$ *m/z* = 1173.12.

Product of 28+29 Glycopeptide **28** (0.4 mg, 0.10 µmol), peptide **29** (1.1 mg, 0.12 µmol), TCEP•HCl (0.058 mg, 0.20 µmol) and HOOBt (0.5 mg, 3.3 µmol) were dissolved in 33 µl DMSO and DIEA (0.3 mg, 2.2 µmol) was added. The reaction was stirred at rt for 22 hr. Piperidine (4.6 µl) was added. The reaction was stirred at rt for 1 min and was acidified by the addition of 1 ml of MeCN/H_2O (1:1) containing 5% AcOH. The mixture was purified by C4 column with linear gradients from 45% solvent B to 65% solvent B over 30 min. The flow rate was 16 ml/min and detection was by UV-absorbance at 277 nm. A white solid (0.4 mg, 59%) was obtained after lyophilization.

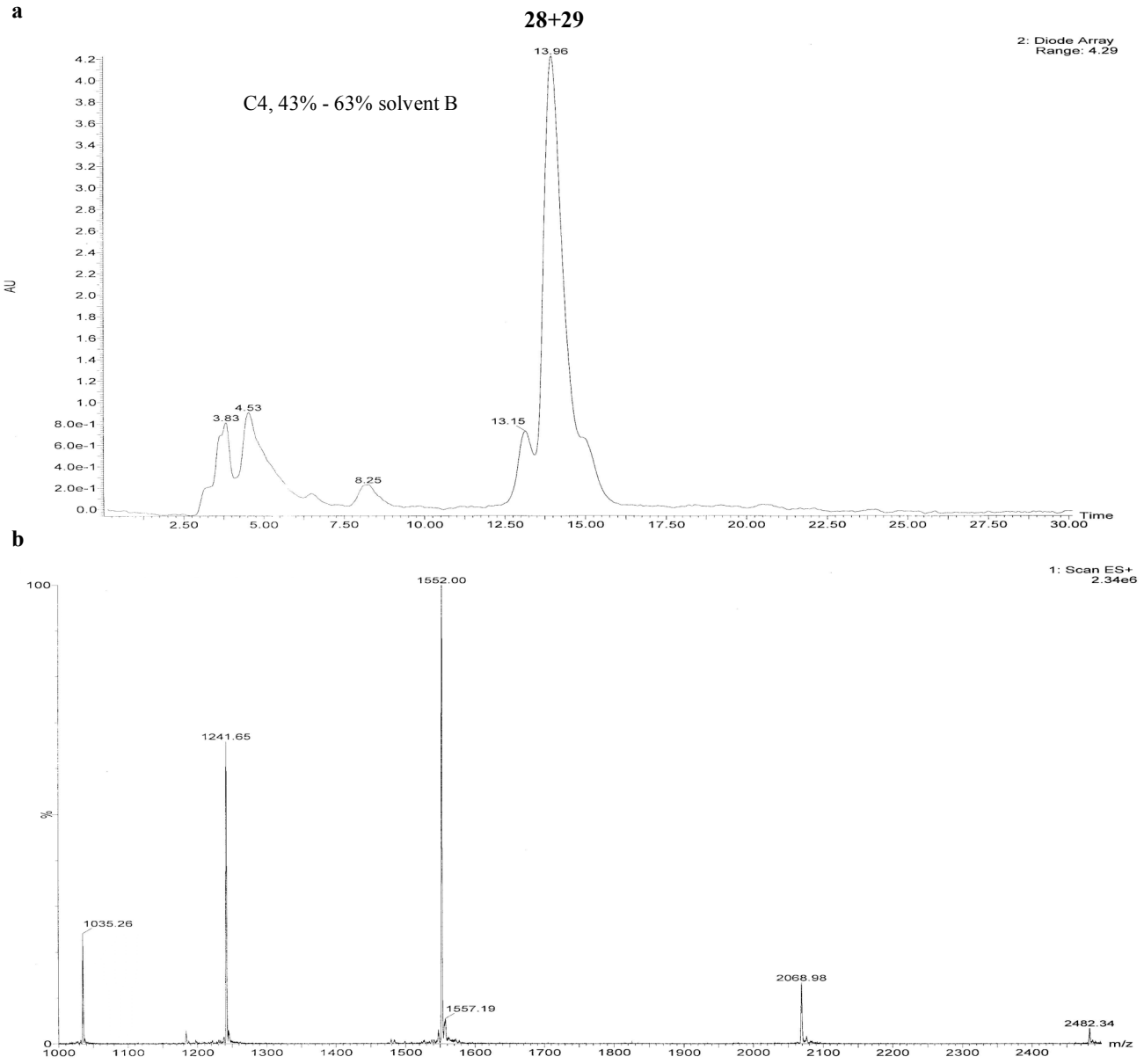


Figure 26 (**a**) LC-UV trace of purified compound **28**+**29**. (**b**) ESI-MS of compound **28**+**29**. MS (ESI) calcd. for $C_{270}H_{426}N_{70}O_{95}S$ $[M+3H]^{3+}$ *m/z* = 2068.01, $[M+4H]^{4+}$ *m/z* = 1551.26, $[M+5H]^{5+}$ *m/z* = 1241.21, $[M+6H]^{6+}$ *m/z* = 1034.51.

Compound 30 Fully-protected peptide EPO(88-122) was synthesized by SPPS on a 0.05 mmol scale (105 mg, 36%). Fully-protected peptide EPO(88-122) (34 mg, 5.7 μmol), 2-(ethyldithio)-phenol (8.0 mg, 43 μmol), DMAP(0.3 mg, 2.5 μmol) and DCC (5.2 mg, 25 μmol) were dissolved in 400 μl CH_2Cl_2. The reaction was stirred at rt for 2 h. The product was deprotected by 2 ml cocktail B. The mixture was purified by C4 column with linear gradients from 62% solvent B to 82% solvent B over 30 min. The flow rate was 16 ml/min and detection was by UV-absorbance at 265 nm. A white solid (3.2 mg, 13%) was obtained after lyophilization.

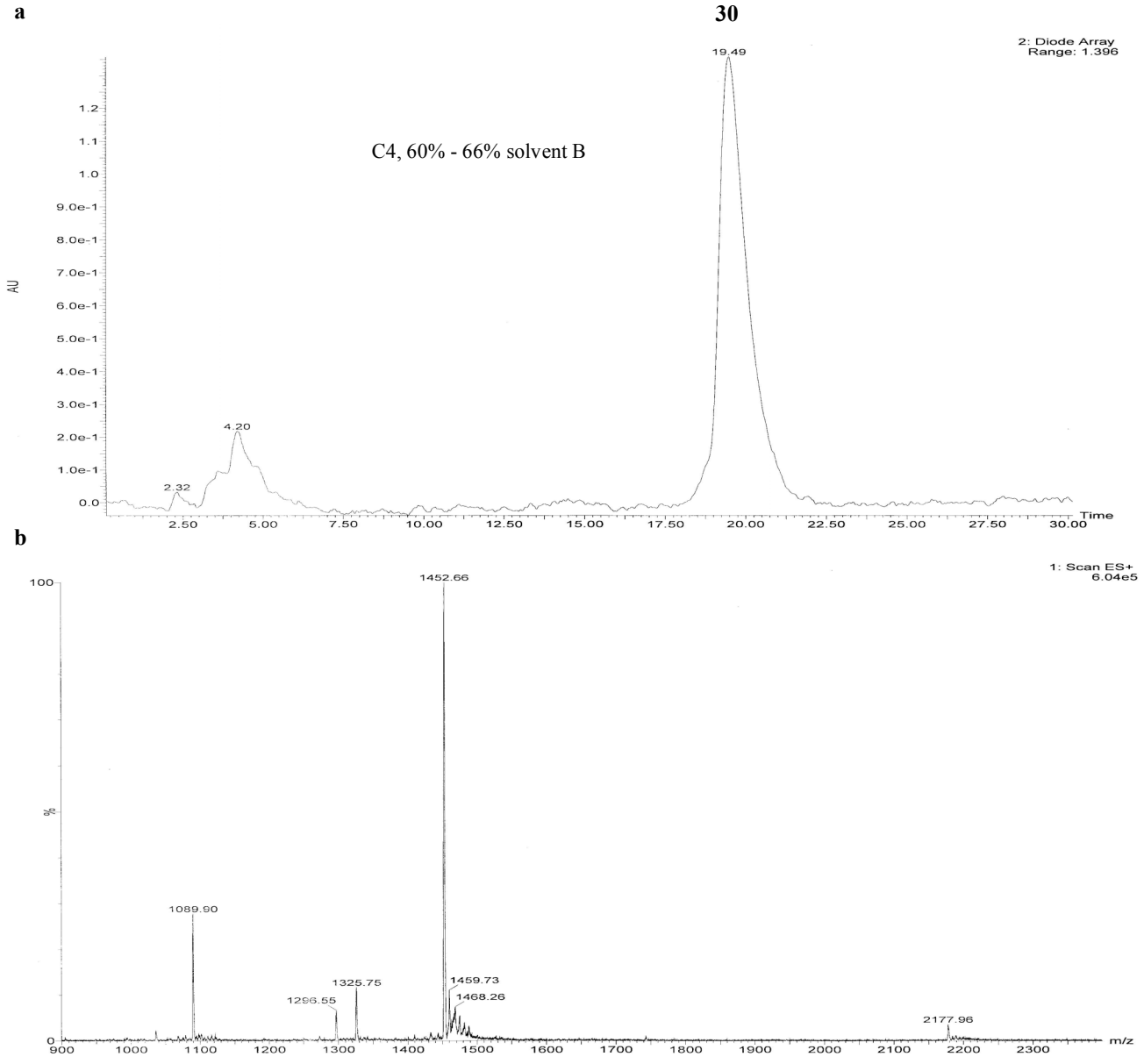
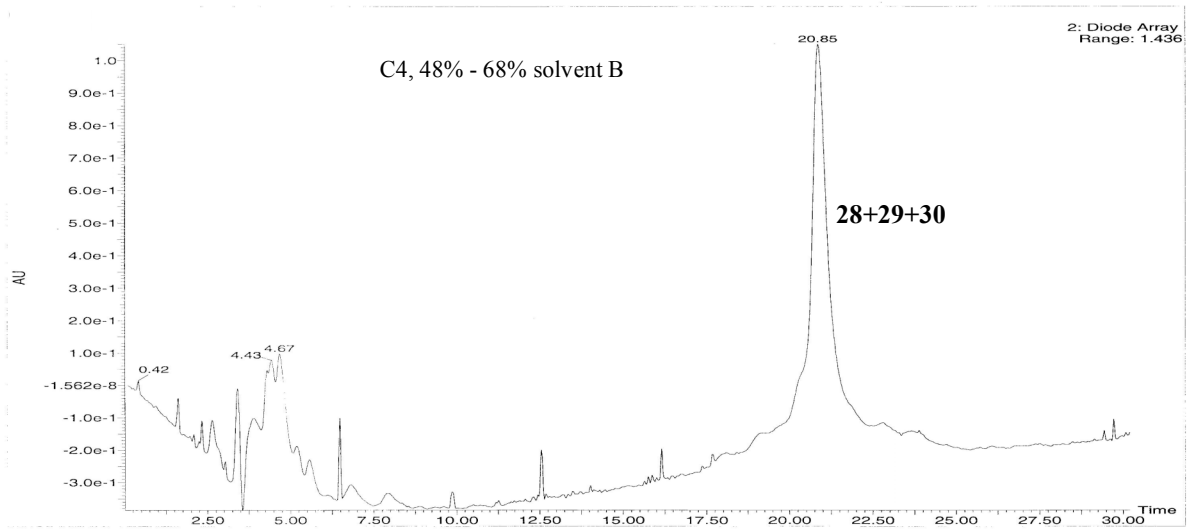


Figure 27 (**a**) LC-UV trace of purified compound **30**. (**b**) ESI-MS of compound **30**. MS (ESI) calcd. for $C_{202}H_{310}N_{48}O_{55}S_2$ $[M+2H]^{2+}$ *m/z* = 2177.12, $[M+3H]^{3+}$ *m/z* = 1451.75, $[M+4H]^{4+}$ *m/z* = 1089.06.

Product of 28+29+30 Glycopeptide **28**+**29** (0.7 mg, 0.11 µmol), peptide **30** (1.0 mg, 0.23 µmol), TCEP•HCl (0.064 mg, 0.22 µmol) and HOOBt (0.5 mg, 3.3 µmol) were dissolved in 35 µl DMSO and DIEA (0.3 mg, 2.2 µmol) was added. The reaction was stirred at rt for 30.5 hr. Piperidine (5.0 µl) was added. The reaction was stirred at rt for 1 min and was acidified by the addition of 1 ml of MeCN/H_2O (1:1) containing 5% AcOH. The mixture was purified by C4 column with linear gradients from 50% solvent B to 70% solvent B over 30 min. The flow rate was 16 ml/min and detection was by UV-absorbance at 280 nm. A white solid (0.6 mg, 52%) was obtained after lyophilization.

a



b

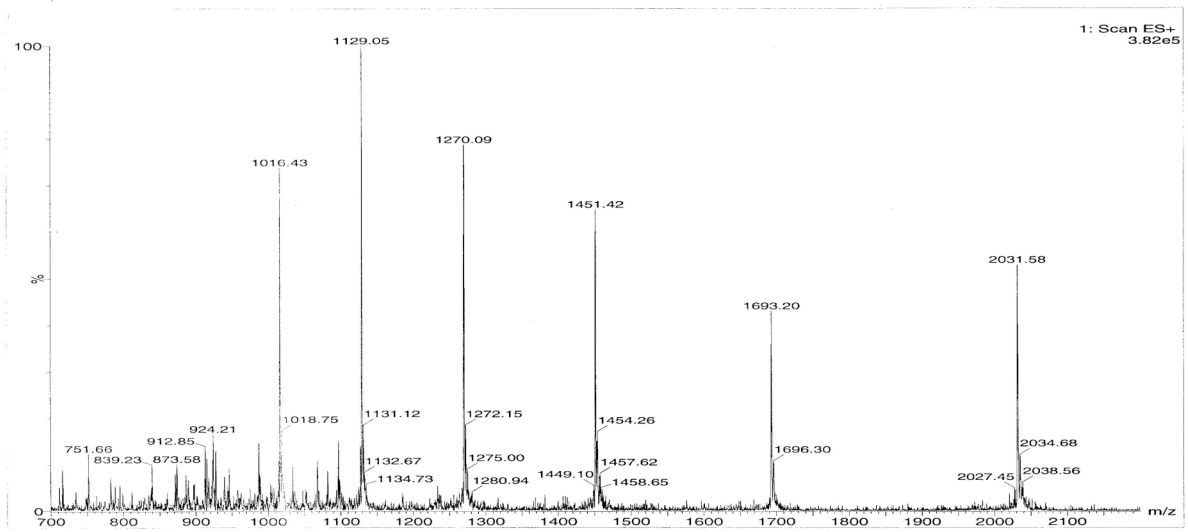
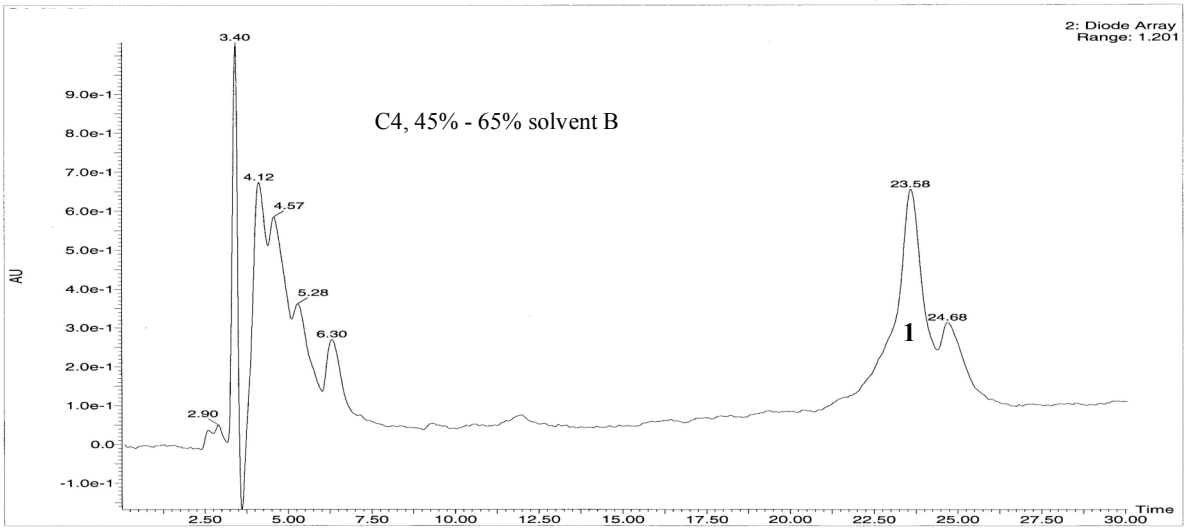


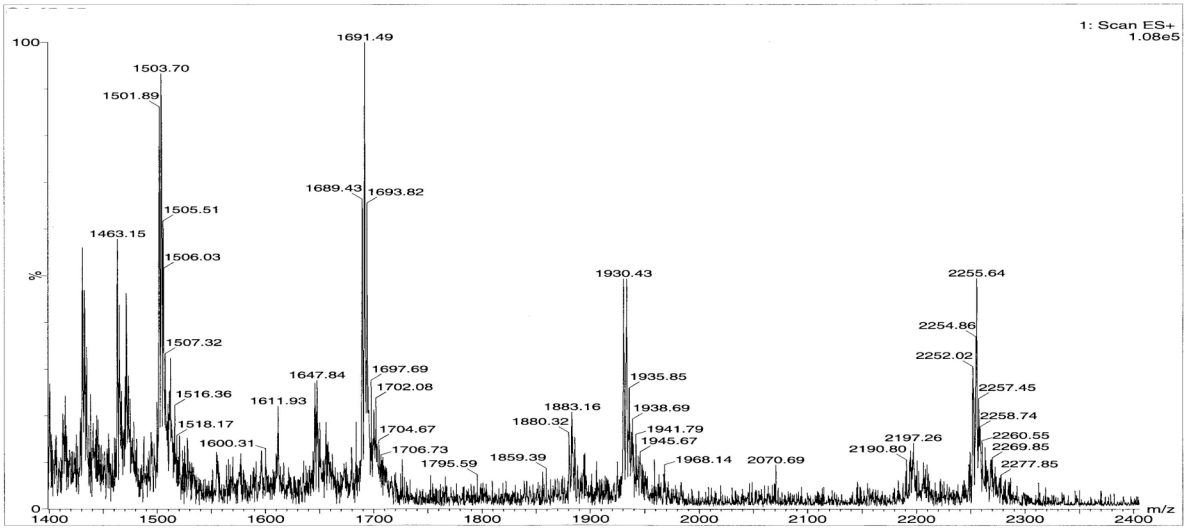
Figure 28 (**a**) LC-UV trace of purified compound **28**+**29**+**30**. (**b**) ESI-MS of compound **28**+**29**+**30**. MS (ESI) calcd. for $C_{449}H_{716}N_{118}O_{147}S$ $[M+5H]^{5+}$ *m/z* = 2030.04, $[M+6H]^{6+}$ *m/z* = 1691.87, $[M+7H]^{7+}$ *m/z* = 1450.31, $[M+8H]^{8+}$ *m/z* = 1269.15, $[M+9H]^{9+}$ *m/z* = 1128.24, $[M+10H]^{10+}$ *m/z* = 1015.52, $[M+11H]^{11+}$ *m/z* = 923.29, $[M+12H]^{12+}$ *m/z* = 846.43, $[M+13H]^{13+}$ *m/z* = 781.40.

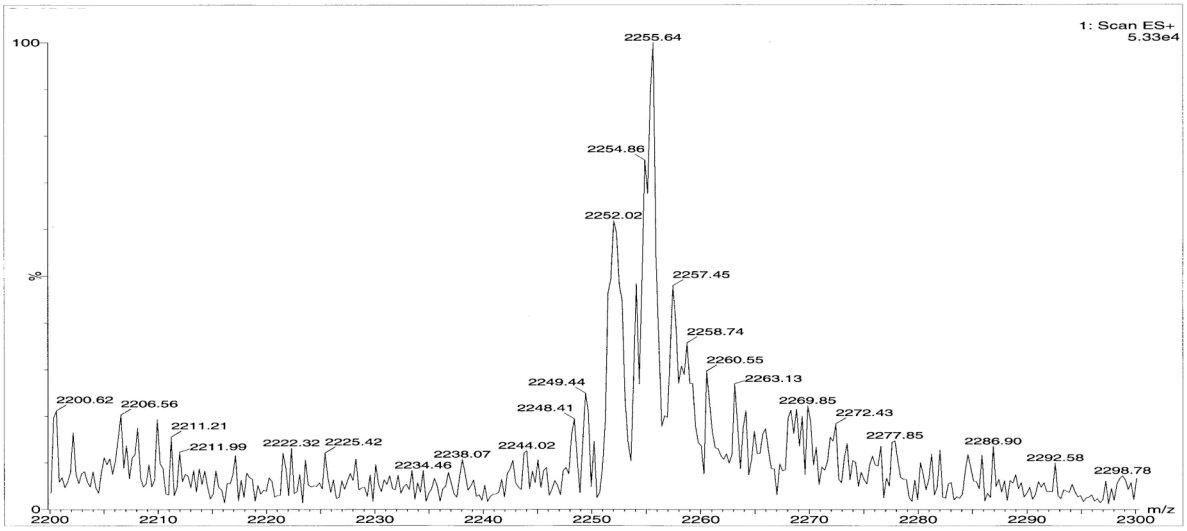
Compound 1 Glycopeptide **28**+**29**+**30** (0.3 mg, 0.03 µmol), glycopeptide **10** (0.3 mg, 0.12 µmol), TCEP•HCl (0.034 mg, 0.12 µmol) and HOOBt (0.5 mg, 3.3 µmol) were dissolved in 10 µl DMSO and DIEA (0.15 mg, 1.1 µmol) was added. The reaction was stirred at rt for 22 hr. Piperidine (1.2 µl) was added. The reaction was stirred at rt for 3 min and was acidified by the addition of 0.4 ml of MeCN/H_2O (1:1) containing 5% AcOH. The mixture was purified by C4 column with linear gradients from 47% solvent B to 67% solvent B over 30 min. The flow rate was 16 ml/min and detection was by UV-absorbance at 277 nm. A white solid (0.2 mg, 50%) was obtained after lyophilization.

a



b

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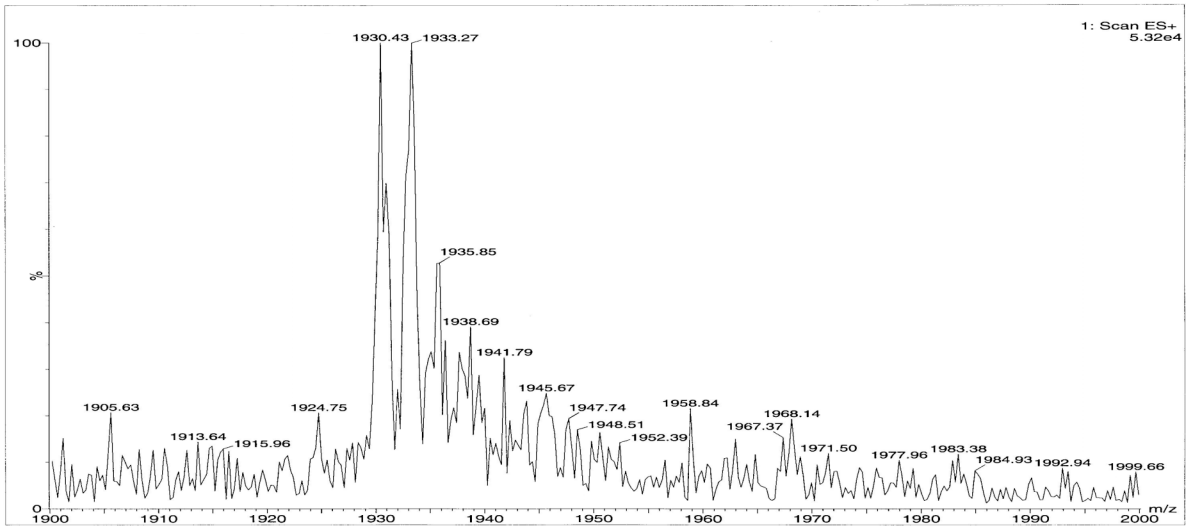
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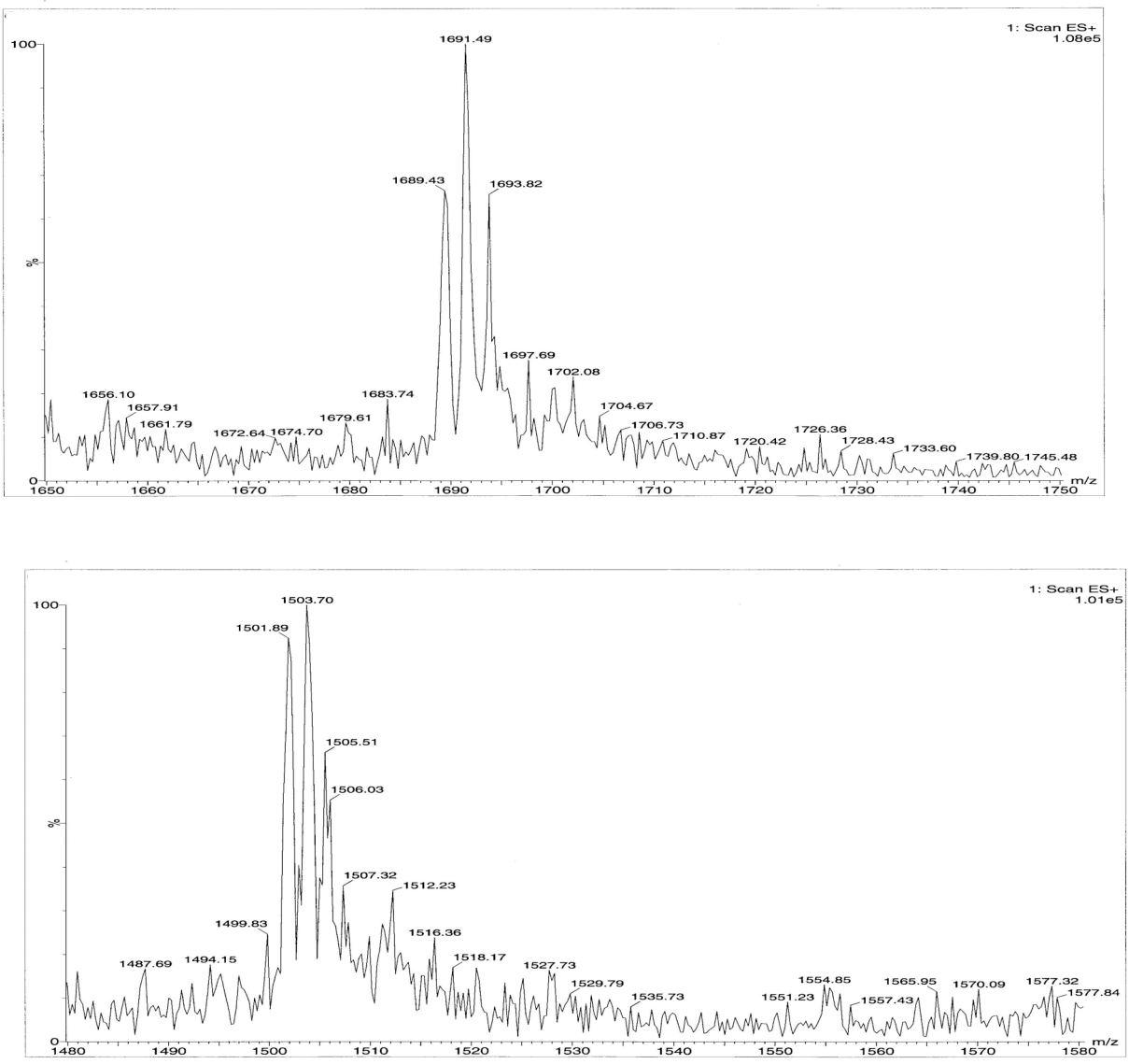

Figure 29 (**a**) LC-UV trace of purified compound **1**. (**b**) ESI-MS of compound **1**. MS (ESI) calcd. for $C_{584}H_{937}N_{137}O_{227}S$ $[M+6H]^{6+}$ (Monolactone) *m/z* = 2256.60, $[M+7H]^{7+}$ *m/z* = 1934.37, $[M+8H]^{8+}$ *m/z* = 1692.70, $[M+9H]^{9+}$ *m/z* = 1504.73; MS (ESI) calcd. for $C_{584}H_{935}N_{137}O_{226}S$ (Dilactone) $[M+6H]^{6+}$ *m/z* = 2253.59, $[M+7H]^{7+}$ *m/z* = 1931.79, $[M+8H]^{8+}$ *m/z* = 1690.20, $[M+9H]^{9+}$ *m/z* = 1502.51; MS (ESI) calcd. for $C_{584}H_{933}N_{137}O_{225}S$ (Trilactone) $[M+6H]^{6+}$ *m/z* = 2250.59, $[M+7H]^{7+}$ *m/z* = 1929.22, $[M+8H]^{8+}$ *m/z* = 1688.19, $[M+9H]^{9+}$ *m/z* = 1500.73. The small peaks, which are about 349 Da less than compound **1**, could also be assigned to be a side product resulting from the decamer sugar (**Figure 4c**), a truncated N-linked glycan that lacks two monosaccharides, fucose and N-acetylglucosamine. ESI-MS of compound this side product. MS (ESI) calcd. for $C_{570}H_{914}N_{136}O_{218}S$ $[M+6H]^{6+}$ (Monolactone) *m/z* = 2198.41, $[M+7H]^{7+}$ *m/z* = 1884.49, $[M+8H]^{8+}$ *m/z* = 1649.05, $[M+9H]^{9+}$ *m/z* = 1465.94; MS (ESI) calcd. for $C_{570}H_{912}N_{136}O_{217}S$ (Dilactone) $[M+6H]^{6+}$ *m/z* = 2195.40, $[M+7H]^{7+}$ *m/z* = 1881.92, $[M+8H]^{8+}$ *m/z* = 1646.80, $[M+9H]^{9+}$ *m/z* = 1463.94; MS (ESI) calcd. for $C_{570}H_{910}N_{136}O_{216}S$ (Trilactone) $[M+6H]^{6+}$ *m/z* = 2192.40, $[M+7H]^{7+}$ *m/z* = 1879.34, $[M+8H]^{8+}$ *m/z* = 1644.55, $[M+9H]^{9+}$ *m/z* = 1462.93.