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

Table of contents

Materials and methods

All manipulations requiring anhydrous conditions were carried out in flame-dried glassware, with magnetic stirring and under nitrogen atmosphere. All commercially available reagents were used as received. Anhydrous solvents were purchased from commercial sources and withdrawn from the container by syringe, under a slight positive pressure of nitrogen. The reactions were monitored by analytical thin-layer chromatography (TLC) using Macherey-Nagel 0.20 mm silica gel 60 with fluorescent indicator pre-coated polyester sheets (40 × 80 mm). Visualization was accomplished by irradiation with a UV lamp and/or staining with Cerium/Molibdate reagent, ninhydrin or cynnamaldehyde. Automated chromatography was performed with Teledyne Isco CombiFlash Rf 150. HPLC purifications were performed on Dionex Ultimate 3000 equipped with Dionex RS Variable Wavelenght Detector (semipreparative column: Atlantis Prep T3 OBD^{TM} 5 µm 19 × 100 mm; flow 15 mL/min unless stated otherwise) and preparative HPLC LaPrep∑ equipped with autosampler AS3950 and a Phenomenex Luna C-18(2) column, 10 µm, 250 × 21.2 mm, with precolumn at 30 mL/min flow rate. HPLC traces of final products were performed on Hitachi Chromaster (column oven Chromaster 5310, pump Chromaster 5110, autosampler Chromaster 5210, DAD Chromaster 5430) equipped with a Phenomenex Luna C-18(2) column, 10 µm, 250 × 4.6 mm, with precolumn at 1.4 mL/min flow rate were used. Peak integrations of HPLC traces were performed with software Chromeleon 6.80 SR11 Build 3161. Lyophilization of final products was performed by dissolving the sample in water and dioxane and freezing the solution in dry ice. The frozen product was kept in a 5Pascal Lio5P DGT lyophilizer for at least 48 h at -50 °C.

^{1}H-NMR spectra were recorded on a spectrometer operating at 400 or 500 MHz. Chemical shifts are reported in ppm (δ) with the solvent reference relative to tetramethylsilane (TMS) employed as the internal standard ($CDCl_3$ δ = 7.26 ppm). The following abbreviations are used to describe spin multiplicity: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, bs = broad signal, dd = doublet of doublet, ddd = doublet of doublet of doublet, ddt = doublet of doublet of triplet, td = triplet of doublet. ^{13}C-NMR spectra were recorded on a spectrometer operating at 100.63 MHz or 126 MHz, with complete proton decoupling. Chemical shifts are reported in ppm (δ) relative to TMS with the respective solvent resonance as the internal standard.

Low resolution mass spectra (MS) were recorded on Thermo Finnigan LCQ Advantage (ESI source), Micro Waters Q-Tof (ESI source) and Thermo Fisher linear ion trap LTQ XL mass spectrometer.

Biological assays

Solid-phase receptor binding assay

Recombinant human integrin $\alpha_V\beta_3$ receptor (R&D Systems, Minneapolis, MN, USA) was diluted to 0.5 µg/mL in coating buffer containing 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM $MnCl_2$, 2 mM $CaCl_2$, and 1 mM $MgCl_2$. An aliquot of diluted receptor (100 µL/well) was added to 96-well microtiter plates (Nunc MaxiSorp) and incubated overnight at 4 °C. The plates were then incubated with blocking solution (coating buffer plus 1% bovine serum albumin) for 2 h at r.t. to block nonspecific binding. After washing 2 times with blocking solution, plates were incubated shaking for 3 h at r.t., with various concentrations (10^{-5}-10^{-12} M) of test compounds in the presence of 1 µg/mL biotinylated vitronectin (Molecular Innovations, Novi, MI, USA). Biotinylation was performed using an EZ-Link Sulfo-NHS-Biotinylation kit (Pierce, Rockford, IL, USA). After washing 3 times, the plates were incubated shaking for 1 h at r.t., with streptavidin-biotinylated peroxidase complex (Amersham Biosciences, Uppsala, Sweden). After washing 3 times with blocking solution, plates were incubated in the dark with 100 µL/well of Substrate Reagent Solution (R&D Systems, Minneapolis, MN, USA) for 30 min with shaking. After stopping the reaction with the addition of 50 µL/well 2N H_2SO_4, absorbance at 415 nm was read in a SynergyTM HT Multi-Detection Microplate Reader (BioTek Instruments, Inc.). Each data point represents the average of triplicate wells; data analysis was carried out by nonlinear regression analysis with GraphPad Prism software (GraphPad Prism, San Diego, CA, USA). Each experiment was repeated in duplicate.

Flow cytometry analysis of cell-surface $\alpha_V\beta_3$ Integrin expression

For expression analysis of integrin $\alpha_V\beta_3$ on 786-O and U87MG cancer cell lines, mouse anti-human integrin $\alpha_V\beta_3$ AlexaFluor 488-conjugated monoclonal antibody (clone: 23C6; R&D systems, Minneapolis, USA) and as an isotype control mouse IgG1 antibody with AlexaFluor488-conjugate (clone: MOPC-21; ThermoFisher Scientific, Waltham, USA) were used. Cell binding was measured using FACSCalibur (BD Biosciences, San Jose, USA) and visualized with Flowing Software version 2.5 (flowingsoftware.btk.fi). Results of integrin expression are shown in Figure S1.

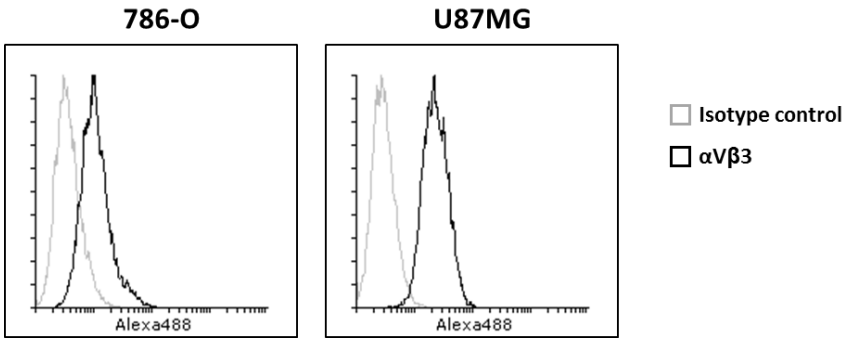


Figure S1. Flow cytometry analysis of 786-O and U87MG cells cells after incubation with AlexaFluor 488-labeled antibodies (anti $\alpha_V\beta_3$ mAb 23C6 and isotype control MOPC-21).

Cell culture

The integrin $\alpha_V\beta_3$+ cell lines U87MG and 786-O were obtained from ATCC (Manassas, USA) and CLS (Eppelheim, Germany), respectively. The U87MG cell line was cultured in Eagle's Minimum Essential medium (PAN, Aidenbach, Germany) with 10% fetal bovine serum (Biowest, Nuaillé, France) and penicillin-streptomycin (PAN). The 786-O cell line was cultured in RPMI 1640 (PAN) supplemented with 2 mM L-glutamine (PAN) and 10% fetal bovine serum (Biowest). All cell lines were cultured as recommended.

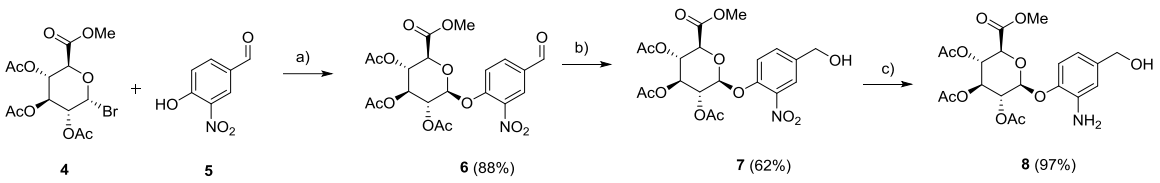
Cell lines were authenticated using Multiplex Cell Authentication by Multiplexion (Heidelberg, Germany) as described recently (*Int. J. Cancer* **2013**, *132*, 308-314). The SNP profiles matched known profiles or were unique. The purity of cell lines was validated using the Multiplex cell Contamination Test by Multiplexion (Heidelberg, Germany) as described recently (*Nucleic Acids Res.* **2009**, *37*, e119). No Mycoplasma, SMRV or interspecies contamination was detected.

Cell viability assays

Prior treatment, U87MG and 786-O cell lines were seeded in 96 well black clear plates (PerkinElmer, Waltham, USA) with 2000 cells per well and incubated at 37°C with 5% CO_2. After 24 hours, cells were treated with different concentrations of *cyclo*(DKP-RGD)-A-Gluc-MMAE (**3a**), *cyclo*(DKP-RGD)-B-Gluc-MMAE (**3b**)and free MMAE (Hycultec, Beutelsbach, Germany). Additionally, cells were treated with and without 40U per well β-glucuronidase Type IX-A dissolved in 75 mM phosphate buffer, pH 6.8 (Sigma-Aldrich, St. Louis, USA). After 96 hours, quantitative determination of cell viability was performed by CellTiter Glo 2.0 (Promega, Madison, USA) and measured by CLARIOstar (BMG Labtec, Ortenberg, Germany).

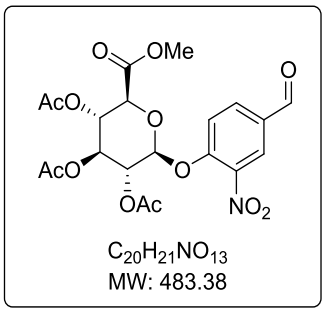
Synthesis of RGD-MMAE conjugates 3a and 3b

*Synthesis of amine **8***



Scheme S1. Synthesis of intermediate **8**. Reagents and conditions: a) Ag_2O, molecular sieves, CH_3CN, overnight; b) Silica gel, $NaBH_4$, *i*PrOH/$CHCl_3$, 2.5 h; c) H_2, Pd/C, EtOAc, EtOH, MeOH, overnight.

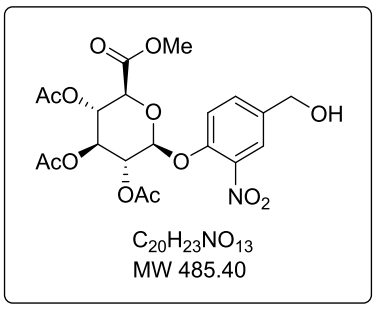
*(2S,3R,4S,5S,6S)-2-(4-formyl-2-nitrophenoxy)-6-(methoxycarbonyl)tetrahydro-2H-pyran-3,4,5-triyl triacetate (**6**)*



To a solution of 4-hydroxy-3-nitrobenzaldehyde **5** (5 g, 29.6 mmol, 1 equiv.) and acetobromo-α-D-glucuronic acid methyl ester **4** (8.5 g, 31.3 mmol, 1.1 equiv.) in CH_3CN (100 mL), molecular sieves (10 g) and Ag_2O (18 g) were added and the mixture was stirred under argon overnight. The crude was filtered and concentrated under vacuum. H_2O (100 mL) was added and the mixture was extracted with ethyl acetate (2 x 200 mL). The organic phase was dried over $MgSO_4$, concentrated and purified by automated chromatography (gradient: from 100% CH_2Cl_2/ 0% CH_2Cl_2:CH_3OH 9:1 to 50% CH_2Cl_2/ 50% CH_2Cl_2:CH_3OH 9:1 in 27 minutes) to give compound **6** (9.33 g, 88%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 9.97 (s, 1H), 8.31 (d, *J* = 2.0 Hz, 1H), 8.08 (dd, *J* = 8.7, 2.1 Hz, 1H), 7.50 (d, *J* = 8.6 Hz, 1H), 5.43 – 5.39 (m, 2H), 5.34 – 5.26 (m, 2H), 4.33 (d, *J* = 8.5 Hz, 1H), 3.70 (s, 3H), 2.12 (d, *J* = 6.8 Hz, 3H), 2.06 (t, *J* = 6.0 Hz, 6H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 188.73, 170.03, 169.37, 169.20, 166.76, 153.39, 141.20,

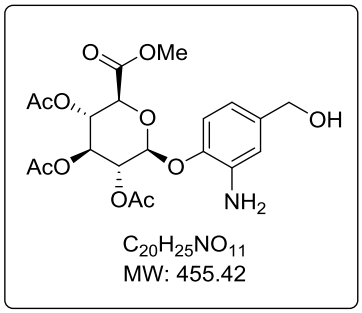
134.41, 131.54, 126.81, 118.81, 98.63, 72.75, 70.26, 69.82, 68.22, 53.22, 20.70, 20.66, 20.65.

*(2S,3R,4S,5S,6S)-2-(4-(hydroxymethyl)-2-nitrophenoxy)-6 (methoxycarbonyl)tetrahydro-2H-pyran-3,4,5-triyl triacetate (**7**)*



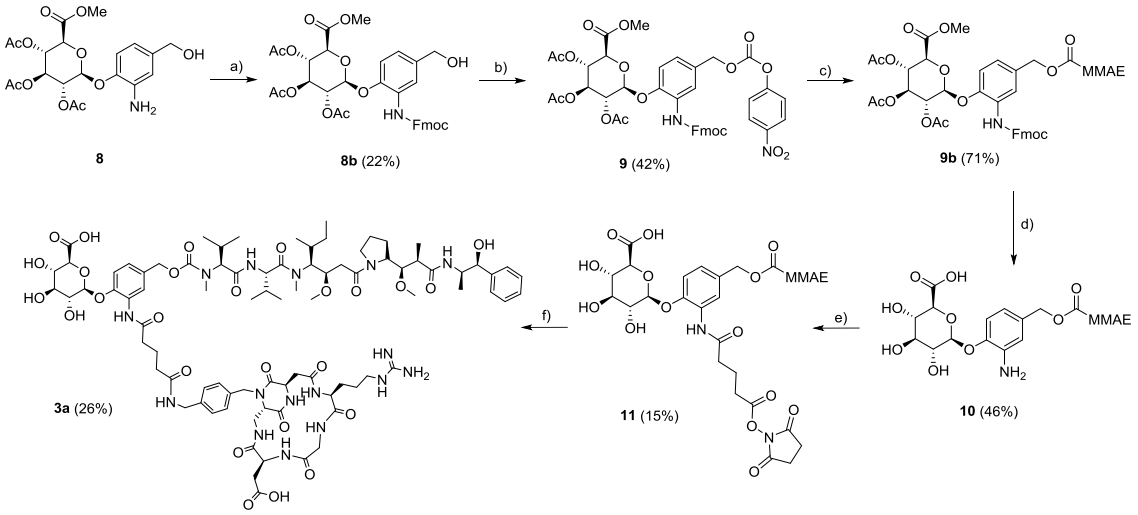
To a solution of **6** (9.21 g, 19 mmol, 1 equiv.) in *i*PrOH/$CHCl_3$ (37 mL/135 mL) under argon, $NaBH_4$ (1.23 g, 32.5 mmol, 1.7 equiv.) was added and the mixture was stirred at 0 ºC for 2.5 h. Excess of $NaBH_4$ was quenched with water. The mixture was diluted with AcOEt and washed with a 1 M solution of NH_4OH and brine. The organic phase was dried over $MgSO_4$, concentrated and purified by automated chromatography (gradient: from 100% CH_2Cl_2/0% CH_2Cl_2:CH_3OH 9:1 to 0% CH_2Cl_2/ 100% CH_2Cl_2:CH_3OH 9:1 in 27 minutes) to give compound **7** (5.71 g, 62%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.79 (d, *J* = 2.0 Hz, 1H), 7.52 (dd, *J* = 8.5, 2.1 Hz, 1H), 7.35 (d, *J* = 8.5 Hz, 1H), 5.35 – 5.25 (m, 3H), 5.18 (d, *J* = 6.9 Hz, 1H), 4.71 (d, *J* = 4.5 Hz, 2H), 4.22 – 4.19 (m, 1H), 3.73 (d, *J* = 6.4 Hz, 3H), 2.18 (d, *J* = 4.5 Hz, 1H), 2.11 (s, 3H), 2.05 (d, J = 3.4 Hz, 6H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 170.01, 169.32, 169.30, 166.70, 148.10, 141.22, 137.36, 131.90, 123.15, 120.16, 99.84, 72.46, 71.10, 70.15, 68.71, 63.37, 53.03, 20.55, 20.51, 20.47.

*(2S,3R,4S,5S,6S)-2-(2-amino-4-(hydroxymethyl)phenoxy)-6-(methoxycarbonyl)tetrahydro-2H-pyran-3,4,5-triyl triacetate (**8**)*



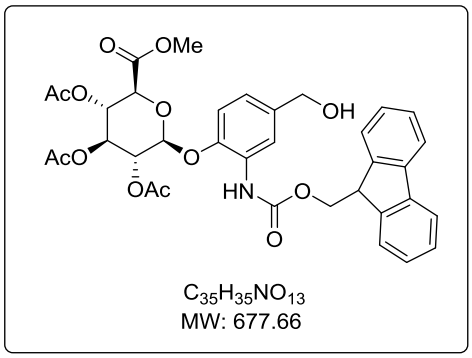
Compound **7** (5.71 g, 11.8 mmol, 1 equiv.) was dissolved in AcOEt (80 mL), EtOH (180 mL) and MeOH (40 mL). A tip of spatula of Pd/C was added under argon. H_2 was added and the reaction mixture was stirred overnight at room temperature. The catalyst was filtered over celite and concentrated under vacuum. The crude (compound **8**) was used without further purification. Yield: 5.2 g (97%). ^{1}H NMR (500 MHz, CDCL3) δ 6.88 (d, J = 8.2 Hz, 1H), 6.71 (d, J = 1.8 Hz, 1H), 6.63 (dd, J = 8.2, 1.9 Hz, 1H), 5.38 – 5.24 (m, 3H), 4.99 (t, J = 10.3 Hz, 1H), 4.53 (s, 2H), 4.13 (t, J = 12.0 Hz, 1H), 3.76 (d, J = 14.7 Hz, 3H), 3.04 (s, 2H), 2.11 – 1.99 (m, 9H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 169.99, 169.63, 169.37, 166.78, 143.67, 137.80, 137.30, 116.70, 116.50, 114.46, 100.52, 72.49, 71.59, 70.93, 69.22, 64.93, 52.98, 20.72, 20.57, 20.45.

*Synthesis of cyclo(DKP-RGD)-A-Gluc-MMAE (**3a**)*



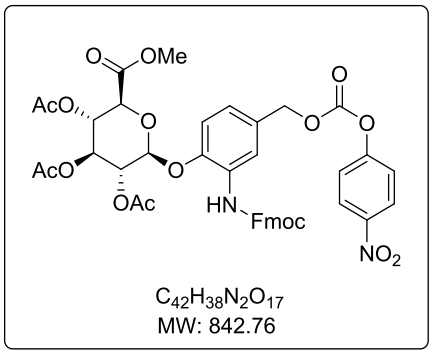
Scheme S2. a) Fmoc-OSu, *i*Pr_2NEt, DMAP, CH_2Cl_2, 3 h; b) 4-nitrophenylchloroformate, pyridine, THF, 3 h; c) MMAE, HOBt, *i*Pr_2NEt, pyridine, DMF, 2 h: d) LiOH, 1:1 MeOH/H_2O, 2 h; e) Di(succinimidyl)glutarate, *i*Pr_2NEt, DMAP, DMF, 3 h; f) *cyclo*[DKP-RGD] (**15**), PBS/DMF, 3 h.

*Fmoc-(2S,3R,4S,5S,6S)-2-(2-amino-4-(hydroxymethyl)phenoxy)-6-(methoxycarbonyl)tetrahydro-2H-pyran-3,4,5-triyl triacetate (**8b**)*



Compound **8** (200 mg, 0.44 mmol, 1 equiv.) was dissolved in CH_2Cl_2 (12 mL) and *i*Pr_2NEt (115 µL, 0.66 mmol, 1.5 equiv.) and DMAP (13.4 mg, 0.11 mmol, 0.25 equiv.) were added. Fmoc-OSu (178 mg, 0.53 mmol, 1.2 equiv.) was then added and the reaction was stirred under argon for 3 hours. The reaction mixture was washed with a 1 M aqueous solution of $KHSO_4$ (2 ×) and brine (1 ×). The organic phase was dried over $MgSO_4$, concentrated and purified by automatic chromatography (eluent: 9.8:0.2 CH_2Cl_2/MeOH) to give compound **8b**. Yield: 66 mg (22%). MS (ESI+): *m/z* calcd. for $[C_{35}H_{36}NO_{13}]^+$: 678.22 $[M + H]^+$; found: 678.17; *m/z* calcd. for $[C_{35}H_{35}NO_{13}Na]^+$: 700.20 $[M + Na]^+$; found: 700.25.

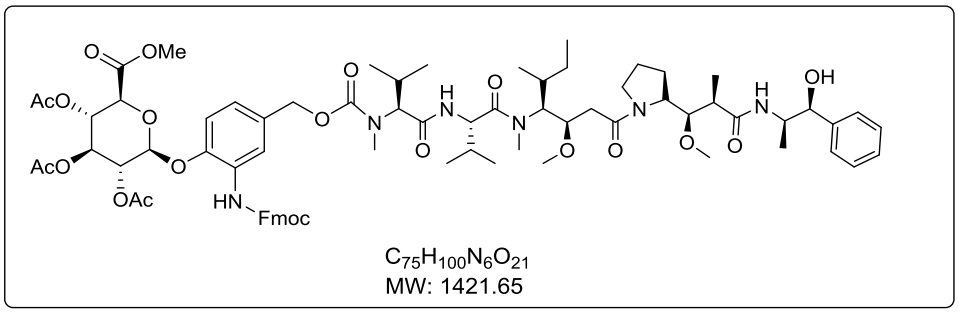
*Fmoc-(2S,3R,4S,5S,6S)-2-(2-amino-4-((((4-nitrophenoxy)carbonyl)oxy)methyl)phenoxy)-6-(methoxycarbonyl)tetrahydro-2H-pyran-3,4,5-triyl triacetate (**9**)*



$C_{42}H_{38}N_2O_{17}$
MW: 842.76

Compound **8b** (66 mg, 0.097 mmol, 1 equiv.) was dissolved in THF (2.9 mL) and then pyridine (19.6 µL, 0.242 mmol, 2.5 equiv.) was added. The reaction mixture was stirred at 0 ºC under argon, then 4-nitrophenylchloroformate (39.3 mg, 0.195 mmol, 2 equiv.) was added and the mixture was stirred at room temperature for 3 hours. The mixture was then diluted with CH_2Cl_2 (40 mL) and washed with an aqueous solution of citric acid (2 x 20 mL) and brine (1 x 20 mL). The organic phase was dried over $MgSO_4$, concentrated and purified by automatic chromatography (eluent: 4:6 hexane/ethyl acetate) to give compound **9**. Yield: 34 mg (42%). MS (ESI+): *m/z* calcd. for $[C_{42}H_{38}N_2O_{17}Na]^+$: 865.21 $[M + Na]^+$; found: 865.17.

*MMAE-Fmoc-(2S,3R,4S,5S,6S)-2-(2-amino-4-(hydroxymethyl)phenoxy)-6-(methoxycarbonyl)tetrahydro-2H-pyran-3,4,5-triyl triacetate (**9b**)*

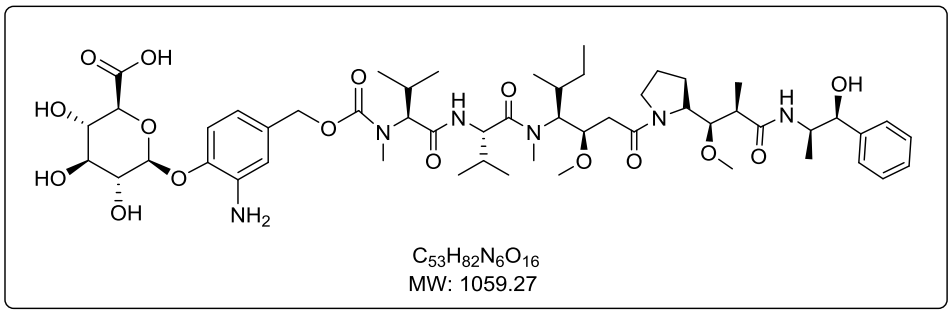


$C_{75}H_{100}N_6O_{21}$
MW: 1421.65

Compound **9** (15 mg, 0.0178 mmol, 1.5 equiv.), MMAE (8.5 mg, 0.0119 mmol, 1 equiv.) and HOBt (0.32 mg, 0.00238 mmol, 0.2 equiv.) were dissolved in DMF (340 µL) at 0 ºC. Pyridine (12 µL, 0.149 mmol, 12.5 equiv.) and *i*Pr_2NEt (4.1 µL, 0.0238 mmol, 2

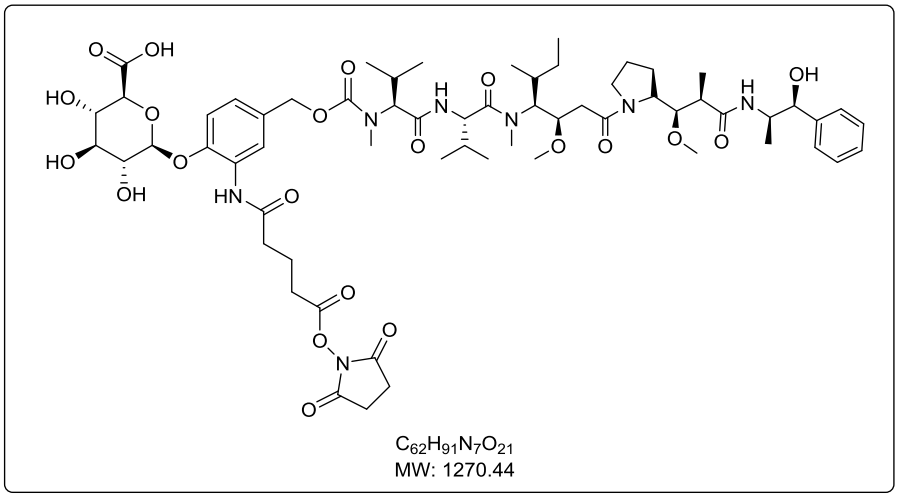
equiv.) were then added. The reaction mixture was stirred at room temperature under argon for 2 hours. The solvent was evaporated and a preparative HPLC purification was carried out [Gradient: 95% (H_2O + 0.05% CF_3COOH) / 5% CH_3CN to 50% (H_2O + 0.05% CF_3COOH) / 50% CH_3CN in 1 min and then from 50% (H_2O + 0.05% CF_3COOH) / 50% CH_3CN to 100% CH_3CN in 14 min] to give compound **9b**. Yield: 12.06 mg (71%). MS (ESI+): *m/z* calcd. for $[C_{75}H_{100}N_6O_{21}Na]^+$: 1443.68 $[M + Na]^+$; found: 1443.67.

*MMAE-β-glucuronide (**10**)*



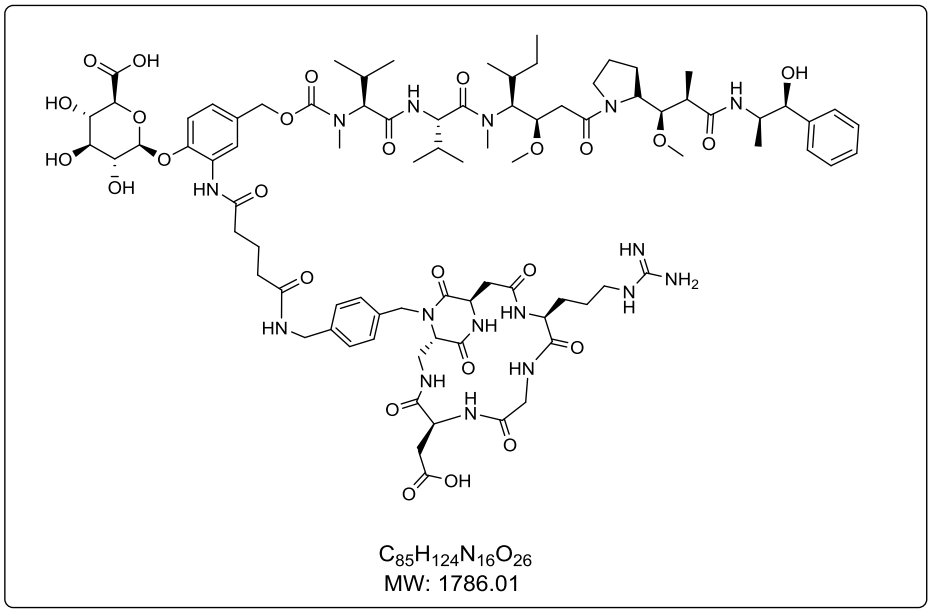
Compound **9b** (10 mg, 0.007 mmol, 1 equiv.) was dissolved in MeOH (250 µL) and cooled to – 20 ºC. A solution of LiOH (1.35 mg, 0.066 mmol, 8 equiv.) in H_2O (250 µL) was then added and the reaction mixture was stirred at 0 ºC for 2 hours. The crude was purified by preparative HPLC [Gradient: 95% (H_2O + 0.05% CF_3COOH) / 5% CH_3CN to 100% CH_3CN in 15 min] to give compound **10**. Yield: 3.42 mg (46%). MS (ESI+): *m/z* calcd. for $[C_{53}H_{82}N_6O_{16}Na]^+$: 1081.57 $[M + Na]^+$; found: 1081.58.

*MMAE-β-glucuronide-hemiglutarate-N-hydroxysuccinimidyl (**11**)*



Compound **10** (3.62 mg, 0.0034 mmol, 1 equiv.) was dissolved in DMF under argon. Di(succinimidyl)glutarate (5.58 mg, 0.0171mmol, 5 equiv.), *i*Pr_2NEt (pH 8-10) and DMAP (0.415 mg, 0.0034 mmol, 1 equiv.) were then added and the reaction mixture was stirred at 50 ºC for 6 hours. The crude was purified by preparative HPLC [Gradient: 95% (H_2O + 0.05% CF_3COOH) / 5% CH_3CN to 30% (H_2O + 0.05% CF_3COOH) / 70% CH_3CN in 15 min and 100% CH_3CN in 3 min] to give compound **11**. Yield: 1.44 mg (33%). MS (ESI+): *m/z* calcd. for $[C_{62}H_{92}N_7O_{21}]^+$: 1270.63 $[M + H]^+$; found: 1270.33; *m/z* calcd. for $[C_{62}H_{91}N_7O_{21}Na]^+$: 1292.62 $[M + Na]^+$; found: 1292.5.

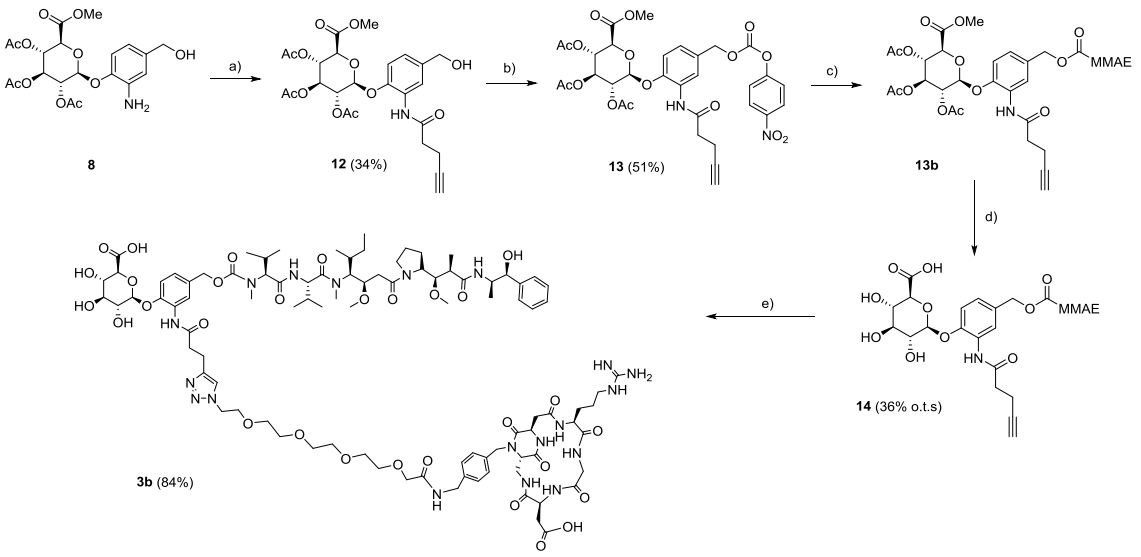
*cyclo(DKP-RGD)-A-Gluc-MMAE (**3a**)*



$C_{85}H_{124}N_{16}O_{26}$
MW: 1786.01

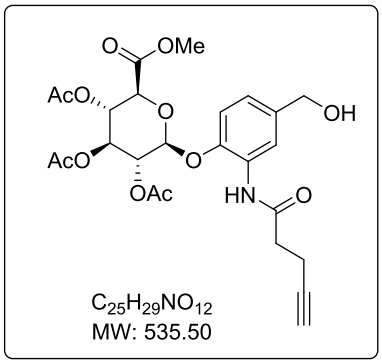
Cyclo[DKP-RGD]-CH_2NH_2 **15** (3.09 mg, 0.0036 mmol, 1 equiv.) was dissolved in phosphate buffer (100 µL, pH= 7.5) and the pH adjusted to 7.3-7.6 with an aqueous solution of sodium hydroxide. This mixture was then added to a solution of compound **11** in DMF (100 µL) at 0 ºC and the reaction mixture was stirred for 3 hours. The pH was kept between 7.3 and 7.6 all the time. The crude was purified by preparative HPLC [Gradient: 95% (H_2O + 0.05% CF_3COOH) / 5% CH_3CN to 100% CH_3CN in 15 min] to give compound **3a**. Yield: 0.84 mg (26%). MS (ESI+): *m/z* calcd. for $[C_{85}H_{125}N_{16}O_{26}Na]^{2+}$: 904.44 $[M + H + Na]^{2+}$; found: 904.50; *m/z* calcd. for $[C_{85}H_{124}N_{16}O_{26}Na_2]^{2+}$: 915.44 $[M + 2Na]^{2+}$; found: 915.92; *m/z* calcd. for $[C_{85}H_{123}N_{16}O_{26}]^-$: 1783.88 $[M - H]^-$; found: 1784.83.

*Synthesis of cyclo(DKP-RGD)-B-Gluc-MMAE (**3b**)*



Scheme S3. a) 4-pentynoic acid, HATU, HOBt, *i*Pr_2NEt, DMF, overnight; b) 4-nitrophenylchloroformate, pyridine, THF, 3 h; c) MMAE, HOBt, *i*Pr_2NEt, pyridine, DMF, 2 h: d) LiOH, 1:1 MeOH/H_2O, 2 h; e) N_3-PEG-4-*cyclo*[DKP-RGD] (**16**), $CuSO_4$ • $5H_2O$, sodium ascorbate, DMF/H_2O.

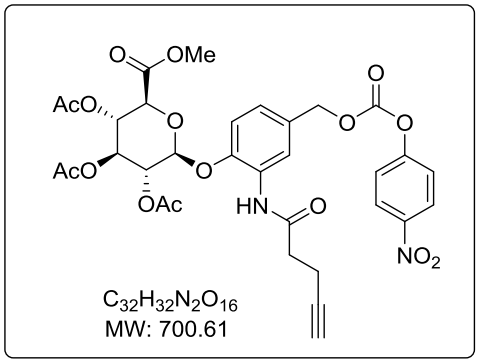
*4-pentyonyl-(2S,3R,4S,5S,6S)-2-(2-amino-4-(hydroxymethyl)phenoxy)-6-(methoxycarbonyl)tetrahydro-2H-pyran-3,4,5-triyl triacetate (**12**)*



4-Pentynoic acid (32.4 mg, 0.33 mmol, 1.5 equiv.) was dissolved in DMF (7.5 mL) and preactivated with HATU (142.2 mg, 0.374 mmol, 1.7 equiv.), HOBt (50.53 mg, 0.374 mmol, 1.7 equiv.) and *i*Pr_2NEt (153 µL, 0.88 mmol, 4 equiv.) for 20 minutes. A solution of compound **8** (100 mg, 0.22 mmol, 1 equiv.) in DMF (3 mL) was then added and the pH adjusted to 8-9 with *i*Pr_2NEt. The reaction mixture was washed with a 1 M aqueous

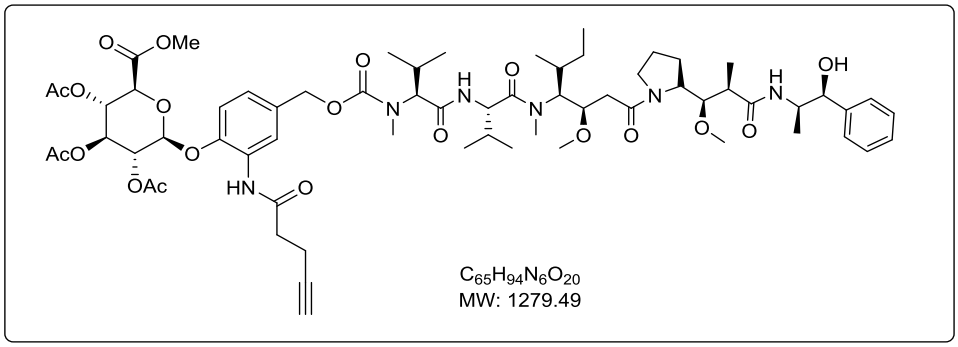
solution of $KHSO_4$ (2 ×) and brine (1 ×). The organic phase was dried over $MgSO_4$, concentrated and purified by automatic chromatography (gradient: from 100% CH_2Cl_2/ 0% CH_3OH to 95% CH_2Cl_2/ 5% CH_3OH in 12 minutes) to give compound **12**. Yield: 40 mg (34%). MS (ESI+): *m/z* calcd. for $[C_{25}H_{29}NO_2Na]^+$: 558.16 $[M + Na]^+$; found: 558.17; MS (ESI-): *m/z* calcd. for $[C_{25}H_{28}NO_2]^-$: 534.14 $[M - 1H]^-$; found: 533.92.

*4-pentyonyl-(2S,3R,4S,5S,6S)-2-(2-amino-4-((((4-nitrophenoxy)carbonyl)oxy)methyl)phenoxy)-6-(methoxycarbonyl)tetrahydro-2H-pyran-3,4,5-triyl triacetate (**13**)*



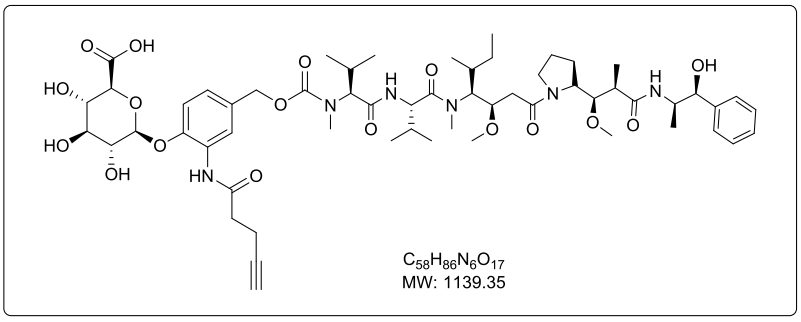
Compound **12** (30 mg. 0.056 mmol, 1 equiv.) was dissolved in THF (1.6 mL) and cooled to 0 ºC. Pyridine (11.3 µL, 0.14 mmol, 2.5 equiv.) and 4-nitrophenyl chloroformate (22.6 mg, 0.112 mmol, 2 equiv.) were then added. The reaction mixture was stirred under argon at room temperature for 2 hours. The reaction mixture was washed with a 1 M aqueous solution of $KHSO_4$ (2 ×) and brine (1 ×). The organic phase was dried over $MgSO_4$, concentrated and purified by automatic chromatography (gradient: from 100% hexane/ 0% ethyl acetate to 45% hexane/ 55% ethyl acetate in 12 minutes) to give compound **13**. Yield: 20 mg (51%). MS (ESI+): *m/z* calcd. for $[C_{32}H_{32}N_2O_{16}Na]^+$: 723.16 $[M + Na]^+$; found: 723.17 ; MS (ESI-): *m/z* calcd. for $[C_{32}H_{31}N_2O_{16}]^-$: 699.17 $[M - 1H]^-$; found: 698.92.

*MMAE-4-pentyonyl-(2S,3R,4S,5S,6S)-2-(2-amino-4-(hydroxymethyl)phenoxy)-6-(methoxycarbonyl)tetrahydro-2H-pyran-3,4,5-triyl triacetate (**13b**)*



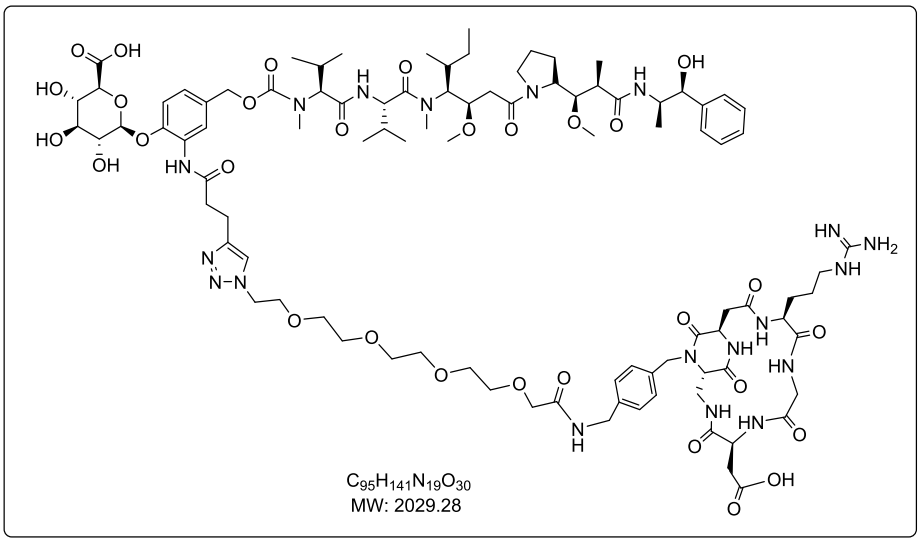
Compound **13** (20 mg, 0.029 mmol, 1.5 equiv.), MMAE (13.7 mg, 0.019 mmol, 1 equiv.) and HOBt (0.5 mg, 0.0038 mmol, 0.2 equiv.) were dissolved in DMF (550 µL) at 0 ºC. Pyridine (19.3 µL, 0.24 mmol, 12.7 equiv.) and *i*Pr_2NEt (6.6 µL, 0.038 mmol, 2 equiv.) were then added and the reaction mixture was stirred at room temperature under argon for 2 hours. The solvent was evaporated and the mixture was purified by automatic chromatography (gradient: from 100% CH_2Cl_2/ 0% CH_3OH to 95% CH_2Cl_2/ 5% CH_3OH in 13 minutes) to give compound **13b**. Yield: 14 mg (58%). MS (ESI+): *m/z* calcd. for $[C_{65}H_{94}N_6O_{20}Na]^+$: 1301.64 $[M + Na]^+$; found: 1301.58.

*MMAE-β-glucuronide-alkyne (**14**)*



Compound **13b** (3 mg, 0.0023 mmol, 1 equiv.) was dissolved in MeOH (100 µL) and cooled to – 20 ºC. A solution of LiOH (0.45 mg, 0.019 mmol, 8 equiv.) in H_2O (100 µL) was then added and the reaction mixture was stirred at 0 ºC for 2 hours. The crude was purified by preparative HPLC [Gradient: 95% (H_2O + 0.05% CF_3COOH) / 5% CH_3CN to 100% CH_3CN in 15 min] to give compound **14**. Yield: 6.06 mg (62 %). MS (ESI+): *m/z* calcd. for $[C_{58}H_{86}N_6O_{20}Na]^+$: 1161.59 $[M + Na]^+$; found: 1161.89; MS (ESI-): *m/z* calcd. for $[C_{58}H_{85}N_6O_{20}]^-$: 1137.6 $[M - 1H]^-$; found: 1137.95.

*cyclo(DKP-RGD)-B-Gluc-MMAE (**3b**)*



Compound **14** (5.24 mg, 0.0046 mmol, 1.5 equiv.) and N_3-PEG-4-*cyclo*[DKP-RGD] **16** (3.08 mg, 0.0031 mmol, 1 equiv.) were dissolved in a degassed mixture of H_2O/DMF (1:1). A degassed solution of $CuSO_4 \cdot 5\ H_2O$ (0.39 mg, 0.0016, 0.5 equiv.) and sodium ascorbate (0.37 mg, 0.0019 mmol, 0.6 equiv.) were then added at room temperature and stirred overnight at 30 ºC. The solvents were evaporated and the mixture was purified by semipreparative HPLC [Gradient: 100% (H_2O + 0.1% CF_3COOH) / 0% (CH_3CN + 0.1% CF_3COOH) to 0% (H_2O + 0.1% CF_3COOH) / 100% (CH_3CN + 0.1% CF_3COOH) in 20 min] to give compound **3b**. Yield: 5.27 mg (84%). MS (ESI+): *m/z* calcd. for $[C_{95}H_{143}N_{19}O_{30}]^{2+}$: 1015.01 $[M + 2H]^{2+}$; found: 1015.81; *m/z* calcd. for $[C_{95}H_{144}N_{19}O_{30}]^{3+}$: 677.01 $[M + 3H]^{3+}$; found: 677.75.

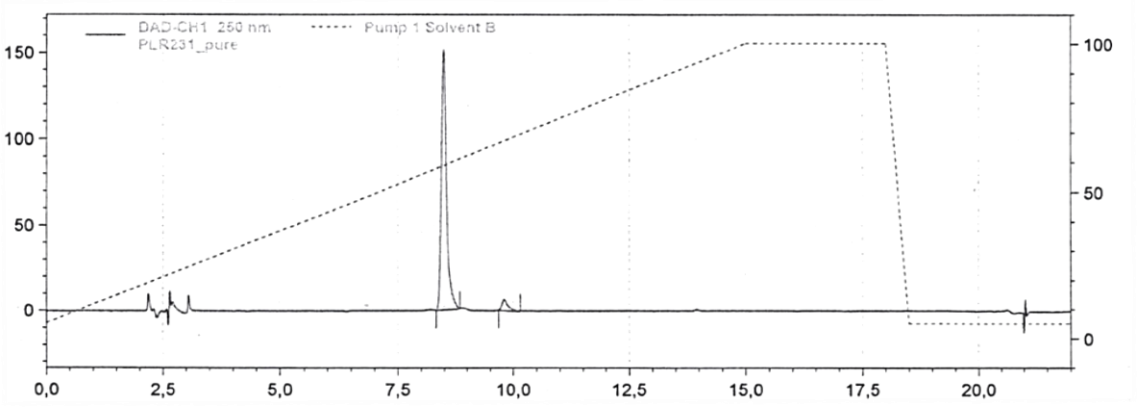
HPLC traces of final compounds

The purity of *cyclo*(DKP-RGD)-A-Gluc-MMAE **3a** was analyzed by HPLC using a Phenomenex Luna C-18(2) 10 µm, 250 × 21.2 mm column (with precolumn) at 30 mL/min flow rate. For *cyclo*(DKP-RGD)-B-Gluc-MMAE **3b**, the purity was analyzed by HPLC using a Atlantis Prep T3 OBDTM 5 µm 19 × 100 mm column (flow 15 ml/min).

*cyclo(DKP-RGD)-A-Gluc-MMAE (**3a**)*

Gradient: 95% (H_2O + 0.05 % CF_3COOH)/5% CH_3CN to 0% (H_2O + 0.05 % CF_3COOH)/100% CH_3CN in 15 min, t_R (product): 8.5 min

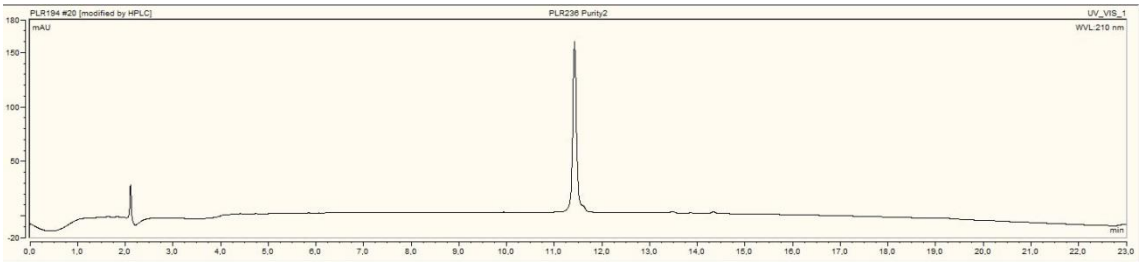
Purity: 96%.



*cyclo(DKP-RGD)-B-Gluc-MMAE (**3b**)*

Gradient: 100% (H_2O + 0.1% CF_3COOH) / 0% (CH_3CN + 0.1% CF_3COOH) to 0% (H_2O + 0.1% CF_3COOH) / 100% (CH_3CN + 0.1% CF_3COOH) in 20 min; t_R (product): 11.4 min.

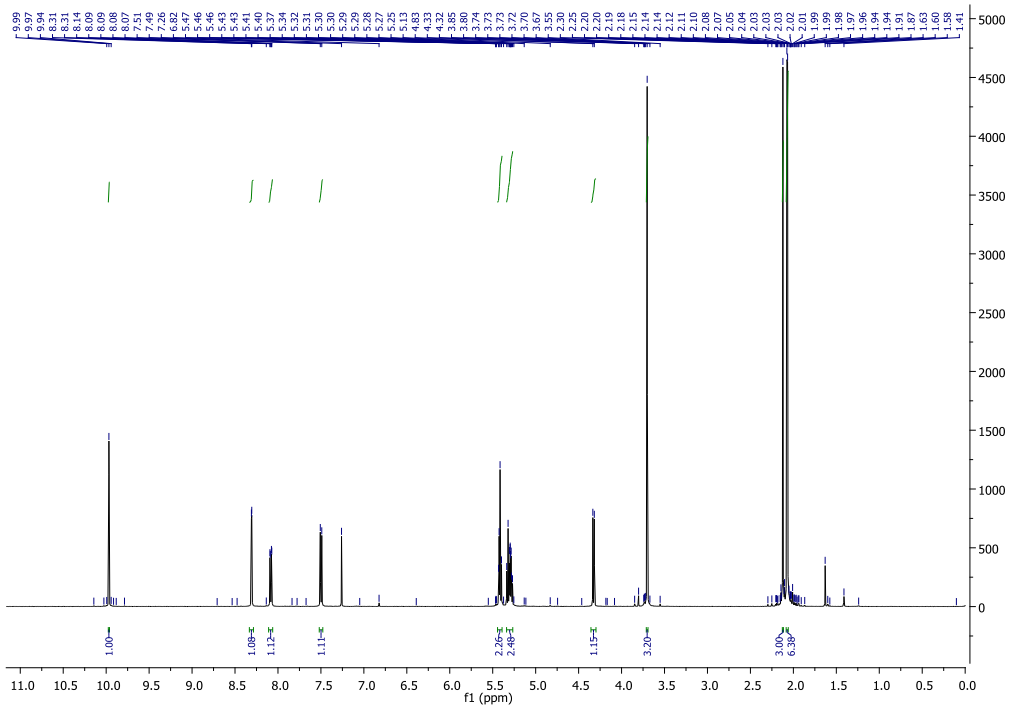
Purity: 94%.



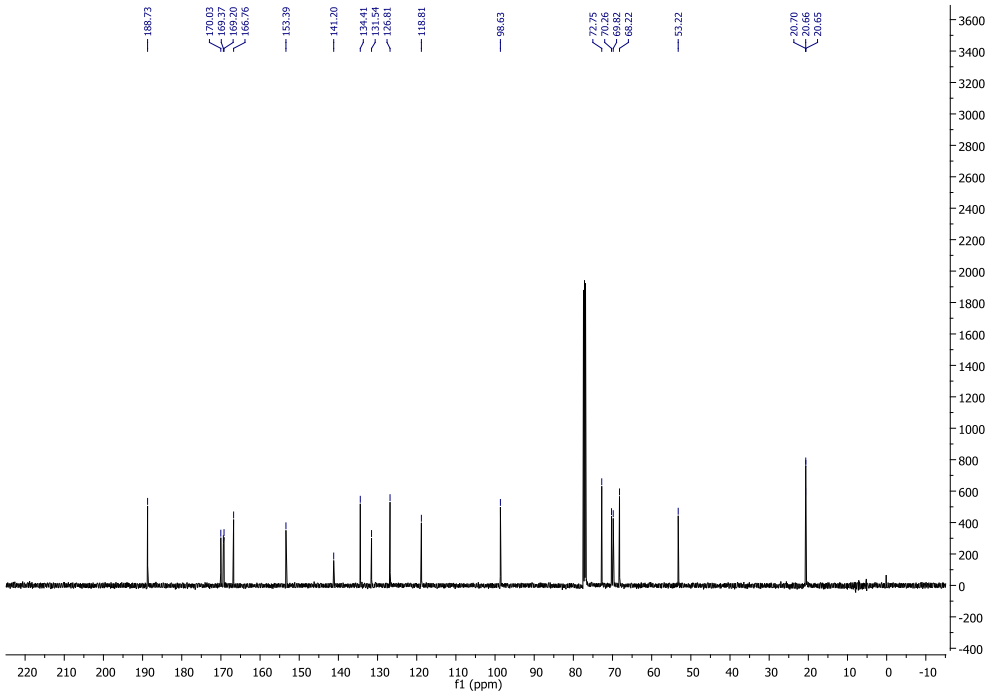
^{1}H NMR and ^{13}C NMR spectra

*(2S,3R,4S,5S,6S)-2-(4-formyl-2-nitrophenoxy)-6-(methoxycarbonyl)tetrahydro-2H-pyran-3,4,5-triyl triacetate (**6**)*

^{1}H NMR (500 MHz, $CDCl_3$)

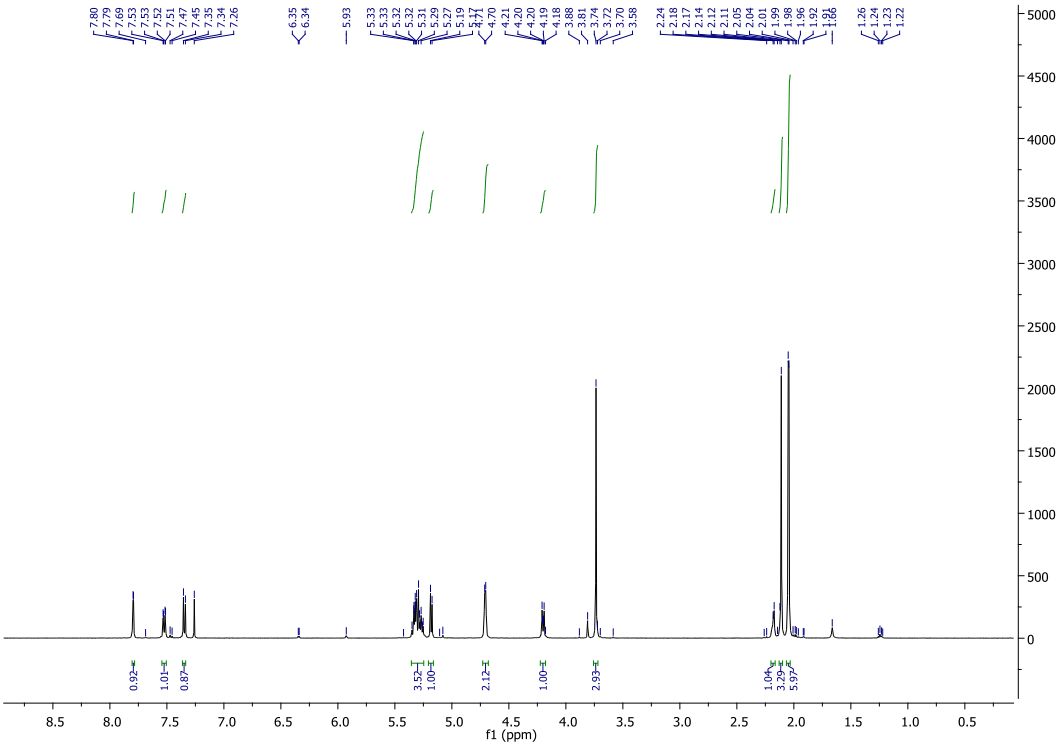


^{13}C NMR (126 MHz, $CDCl_3$)

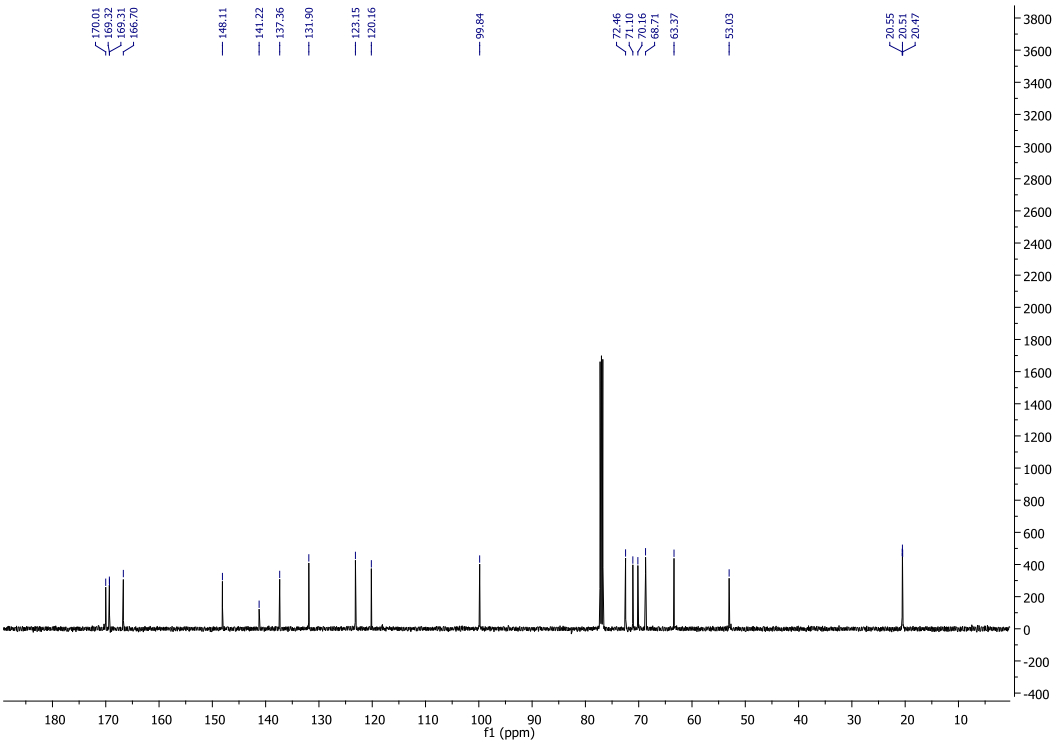


*(2S,3R,4S,5S,6S)-2-(4-(hydroxymethyl)-2-nitrophenoxy)-6 (methoxycarbonyl)tetrahydro-2H-pyran-3,4,5-triyl triacetate (**7**)*

^{1}H NMR (500 MHz, $CDCl_3$)

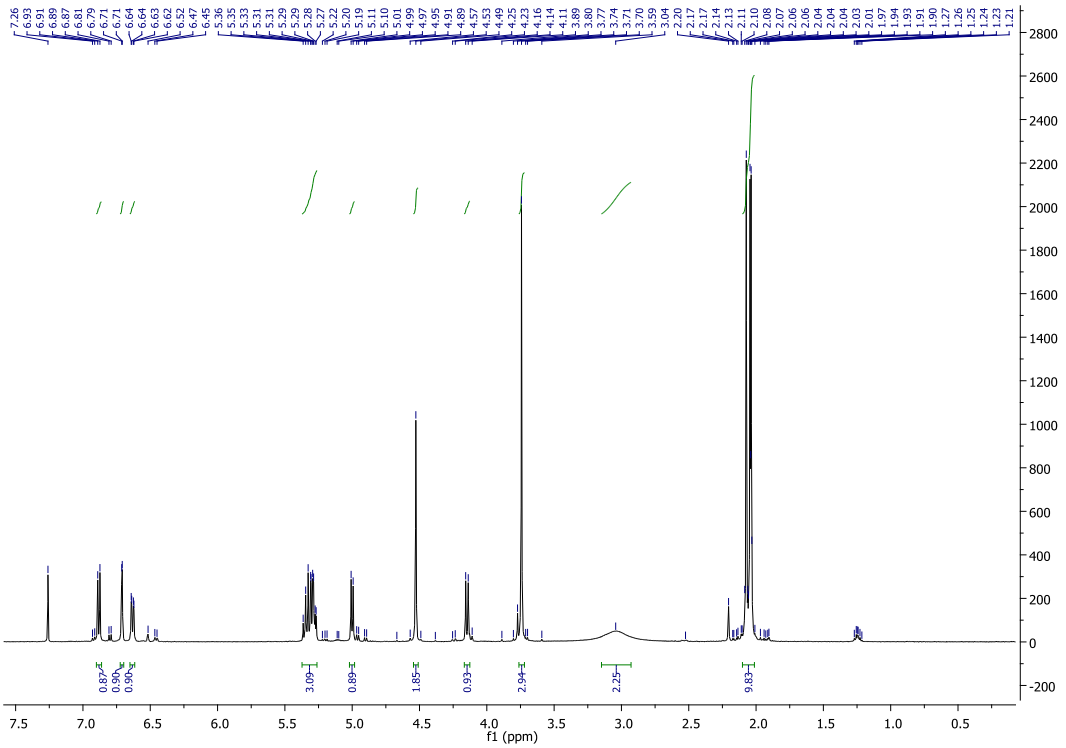


^{13}C NMR (126 MHz, $CDCl_3$)

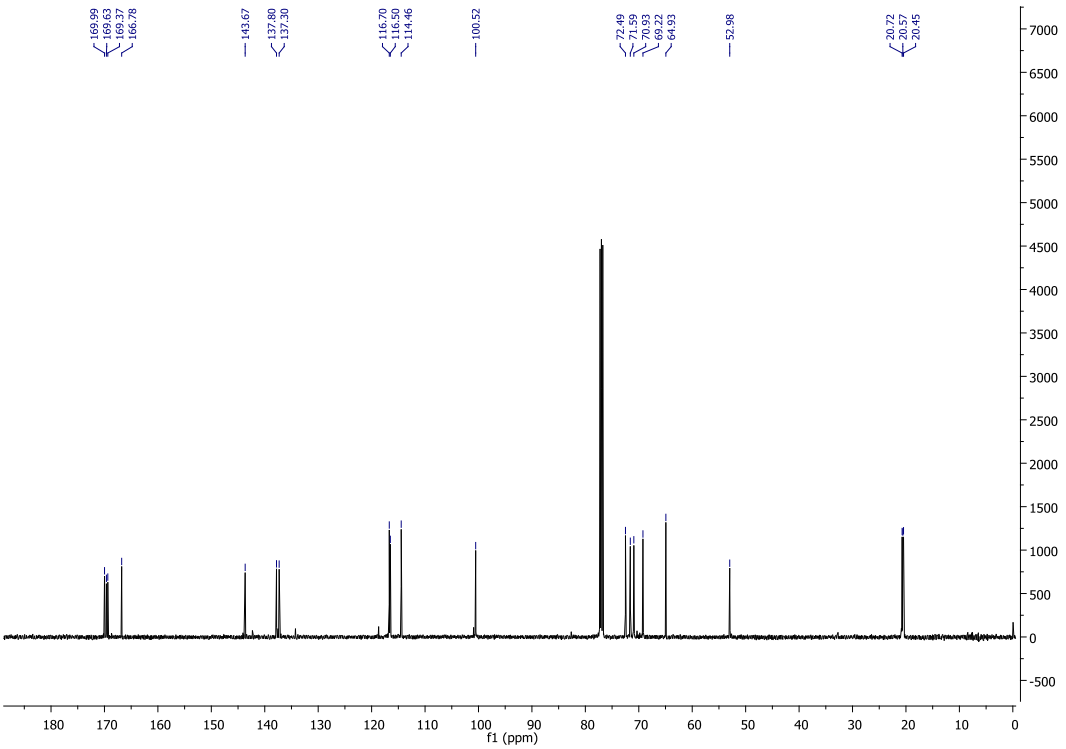


*(2S,3R,4S,5S,6S)-2-(2-amino-4-(hydroxymethyl)phenoxy)-6-(methoxycarbonyl)tetrahydro-2H-pyran-3,4,5-triyl triacetate (**8**)*

^{1}H NMR (500 MHz, $CDCl_3$)

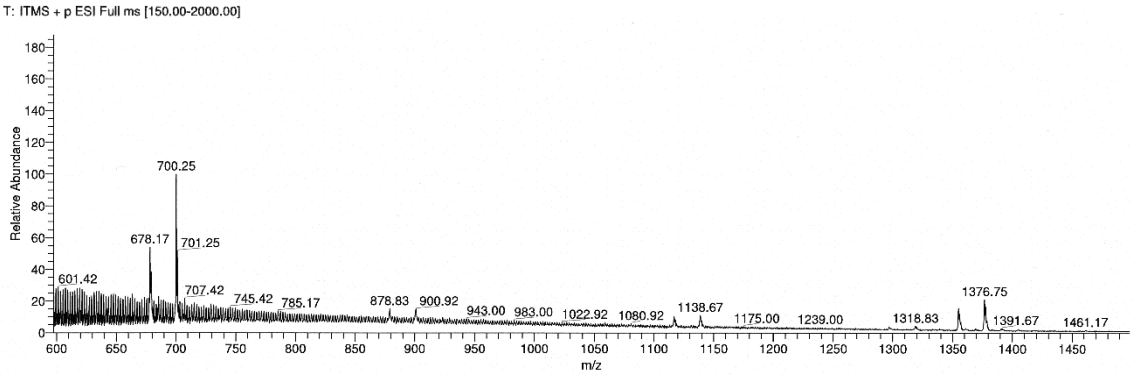


^{13}C NMR (126 MHz, $CDCl_3$)

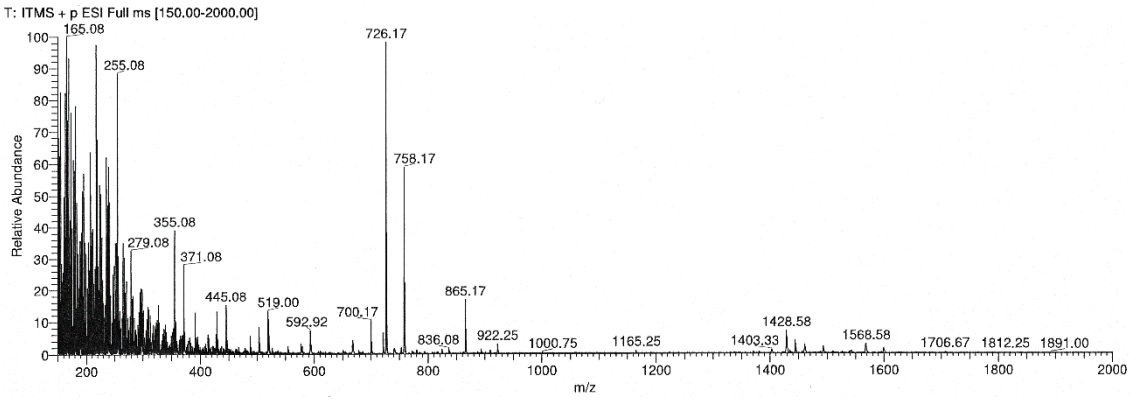


Mass Spectrometry data

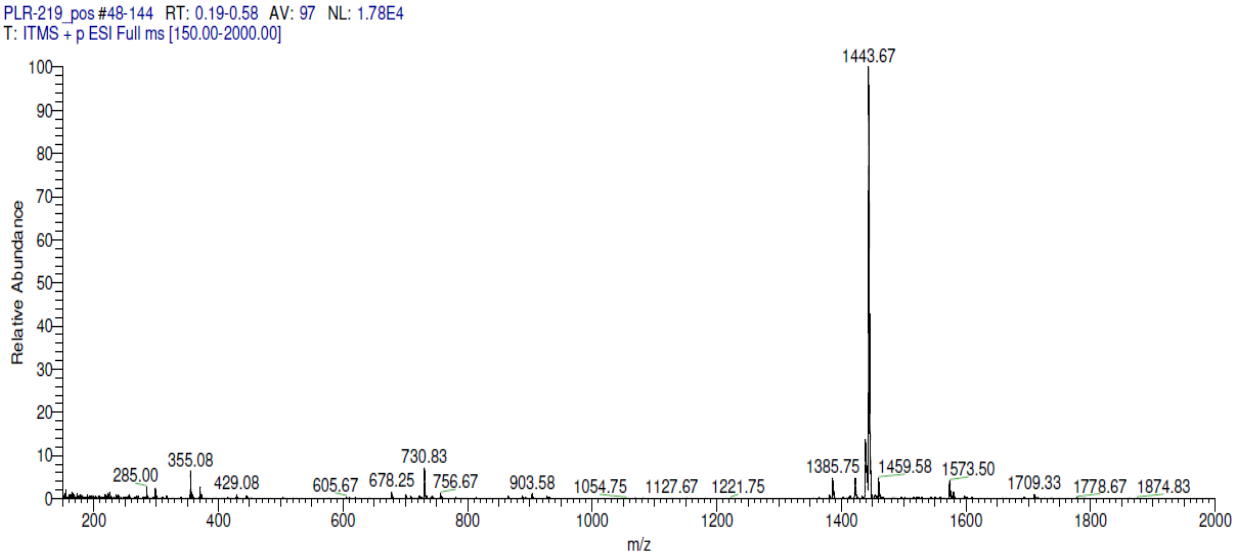
Fmoc-(2S,3R,4S,5S,6S)-2-(2-amino-4-(hydroxymethyl)phenoxy)-6-(methoxycarbonyl)tetrahydro-2H-pyran-3,4,5-triyl triacetate (**8b**)



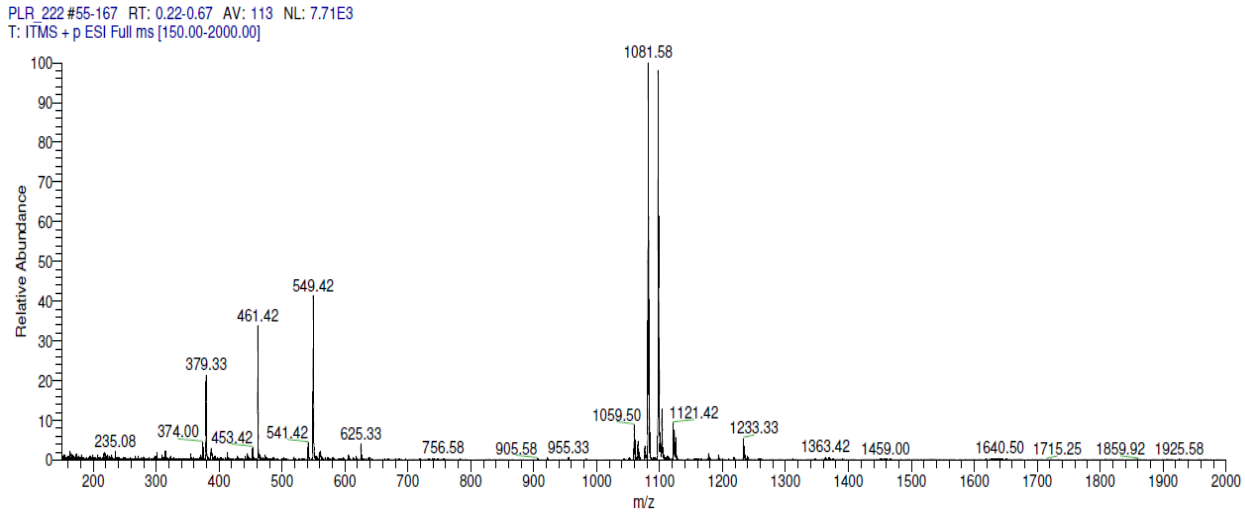
Fmoc-(2S,3R,4S,5S,6S)-2-(2-amino-4-((((4-nitrophenoxy)carbonyl)oxy)methyl)phenoxy)-6-(methoxycarbonyl)tetrahydro-2H-pyran-3,4,5-triyl triacetate (**9**)



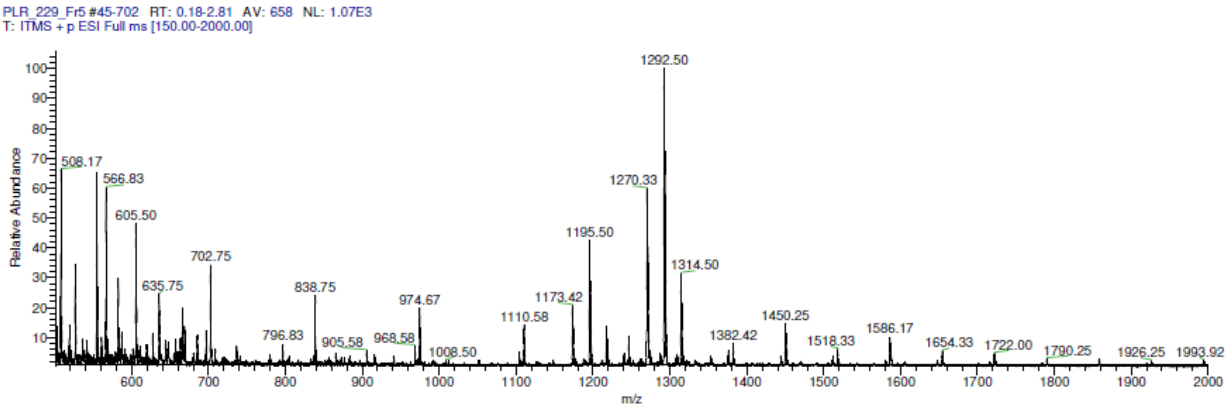
MMAE-Fmoc-(2S,3R,4S,5S,6S)-2-(2-amino-4-(hydroxymethyl)phenoxy)-6-(methoxycarbonyl)tetrahydro-2H-pyran-3,4,5-triyl triacetate (**9b**)



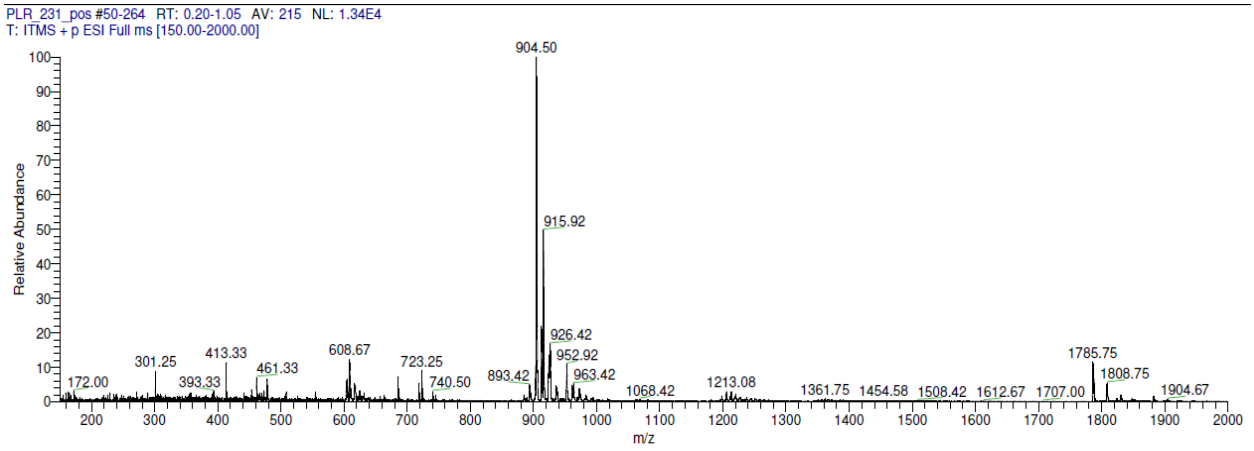
MMAE-β-glucuronide (**10**)

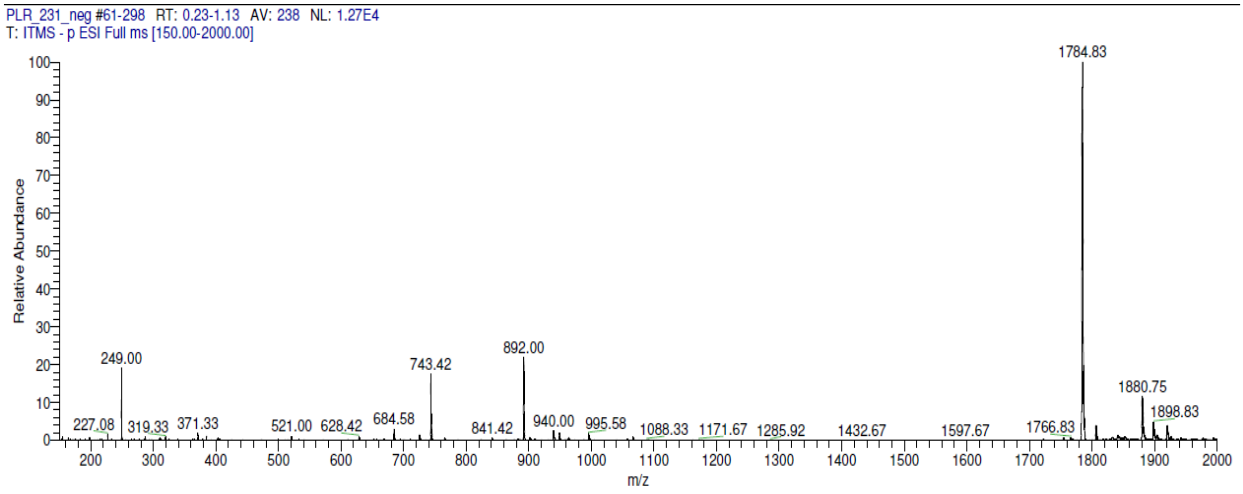


MMAE-β-glucuronide-hemiglutarate-N-hydroxysuccinimidyl (**11**)

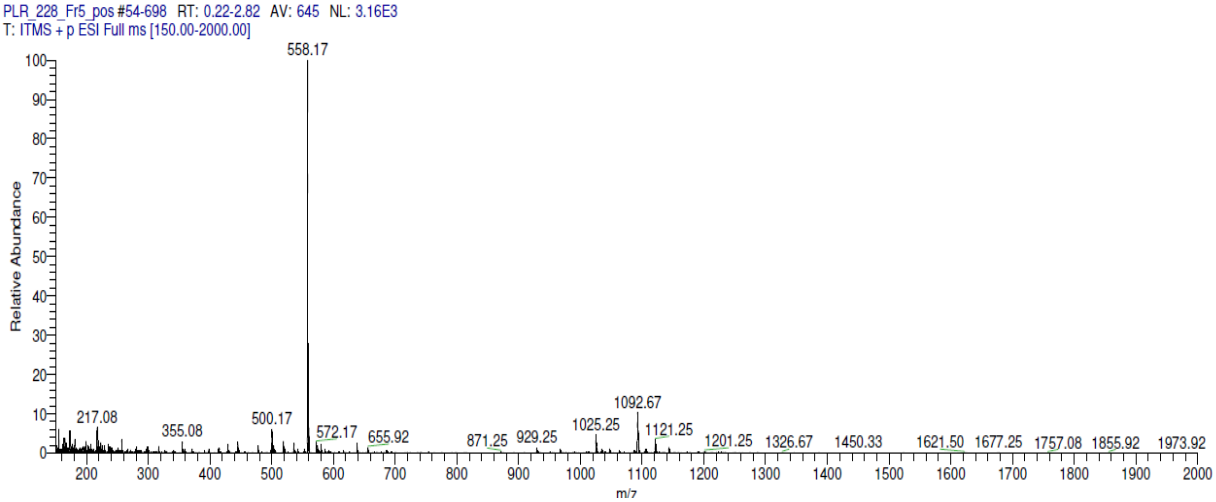


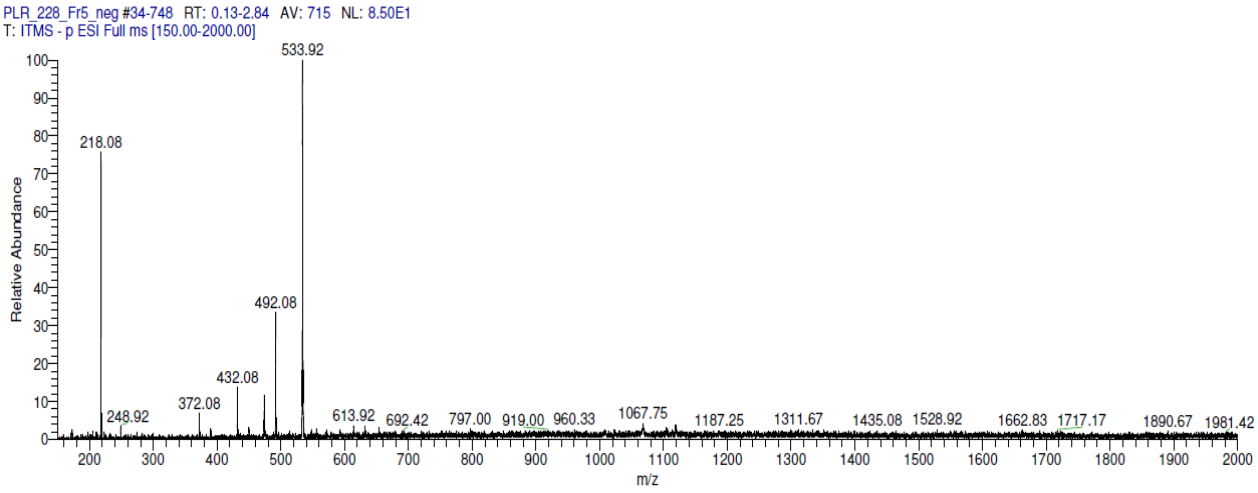
cyclo(DKP-RGD)-A-Gluc-MMAE (**3a**)



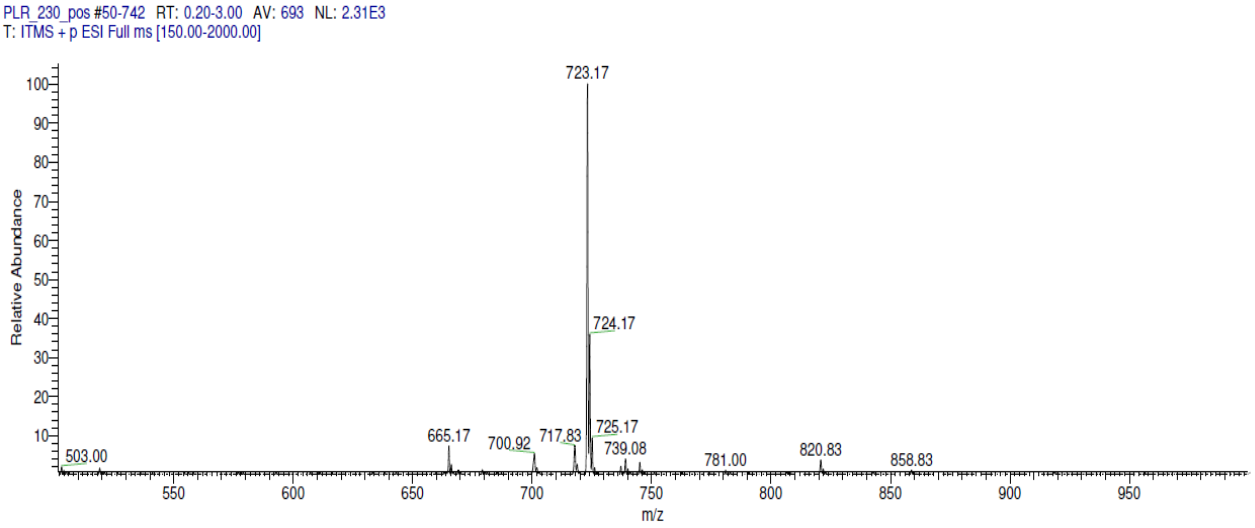


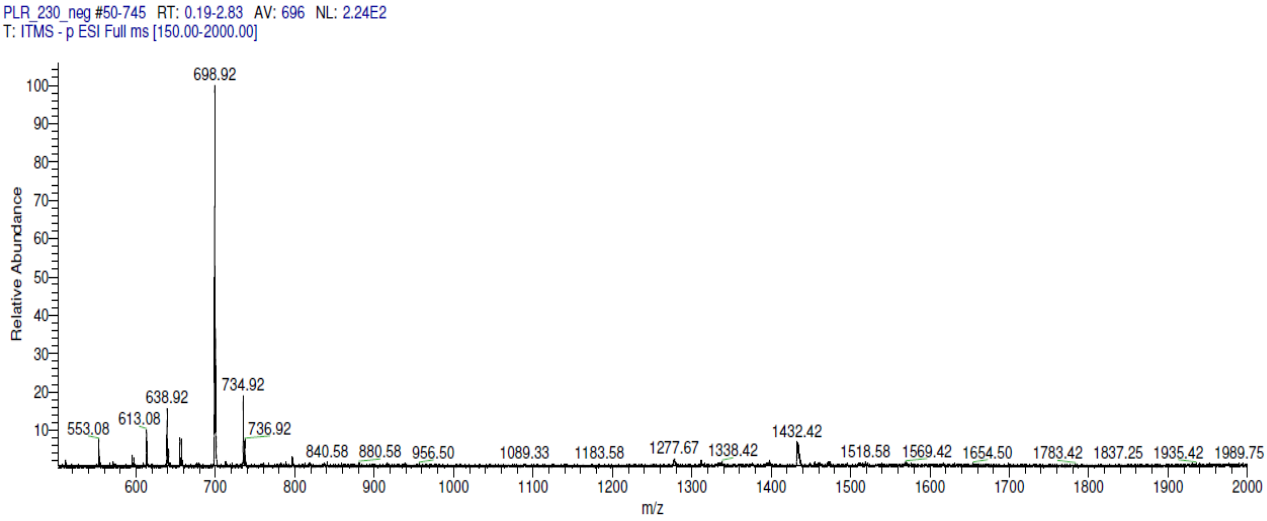
4-pentyonyl-(2S,3R,4S,5S,6S)-2-(2-amino-4-(hydroxymethyl)phenoxy)-6-(methoxycarbonyl)tetrahydro-2H-pyran-3,4,5-triyl triacetate (**12**)



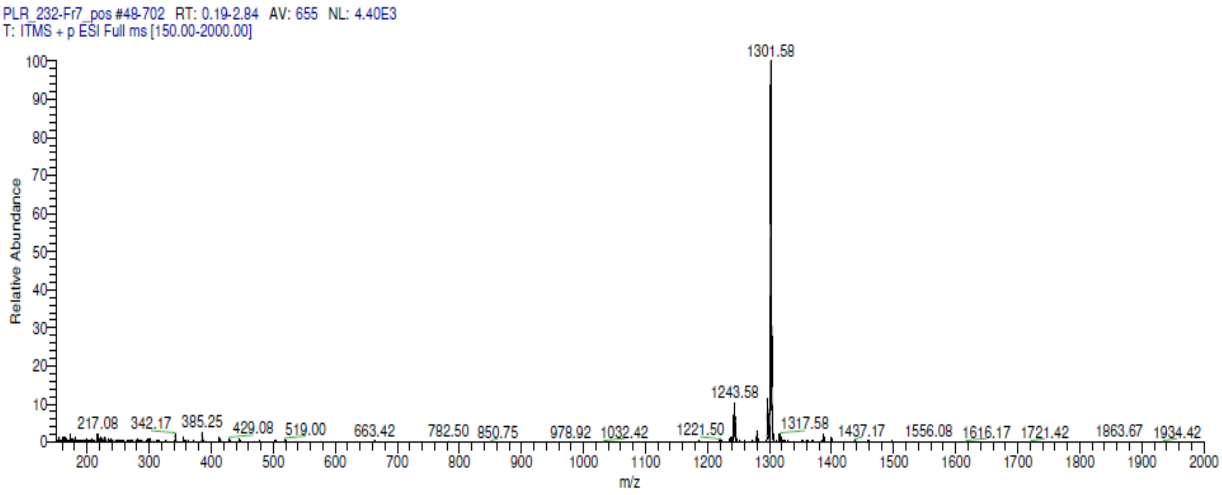


4-pentyonyl-(2S,3R,4S,5S,6S)-2-(2-amino-4-((((4-nitrophenoxy)carbonyl)oxy)methyl)phenoxy)-6-(methoxycarbonyl)tetrahydro-2H-pyran-3,4,5-triyl triacetate (**13**)

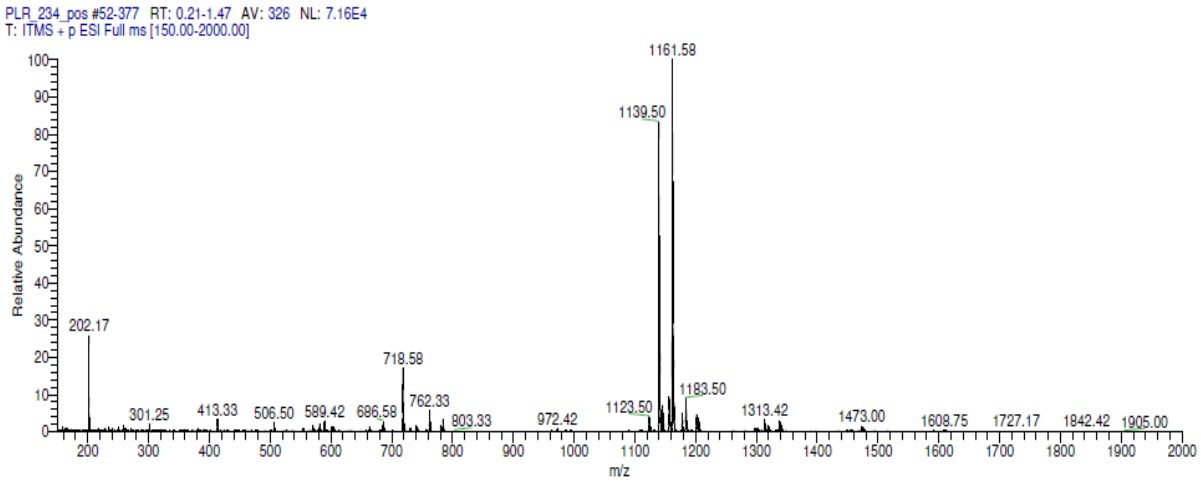


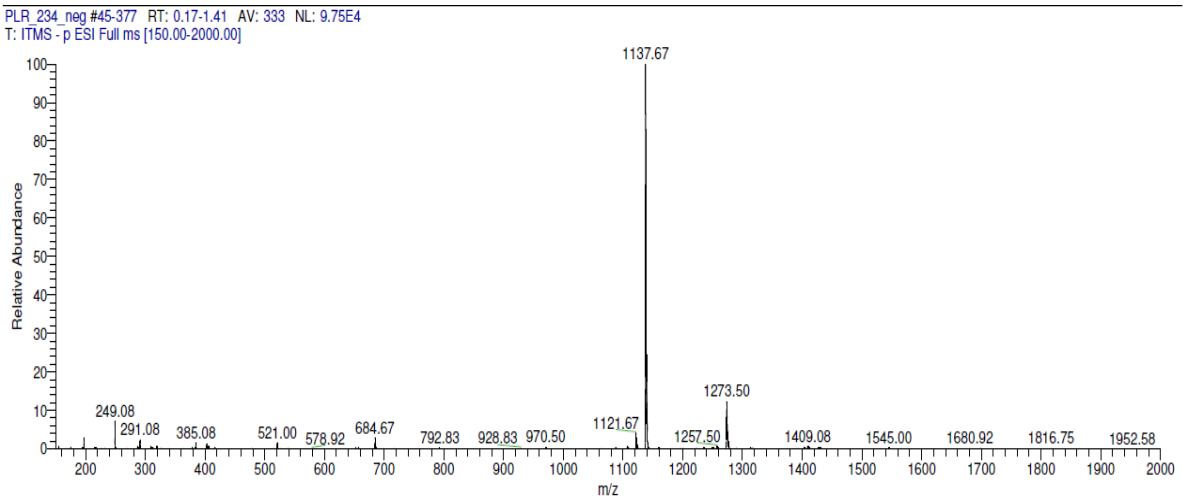


MMAE-4-pentyonyl-(2S,3R,4S,5S,6S)-2-(2-amino-4-(hydroxymethyl)phenoxy)-6-(methoxycarbonyl)tetrahydro-2H-pyran-3,4,5-triyl triacetate (**13b**)



MMAE-β-glucuronide-alkyne (**14**)





cyclo(DKP-RGD)-B-Gluc-MMAE (**3b**)