Supporting Information

Chemical Modification of Linkers Provide Stable Linker-payloads for the Generation of Antibody-drug Conjugates

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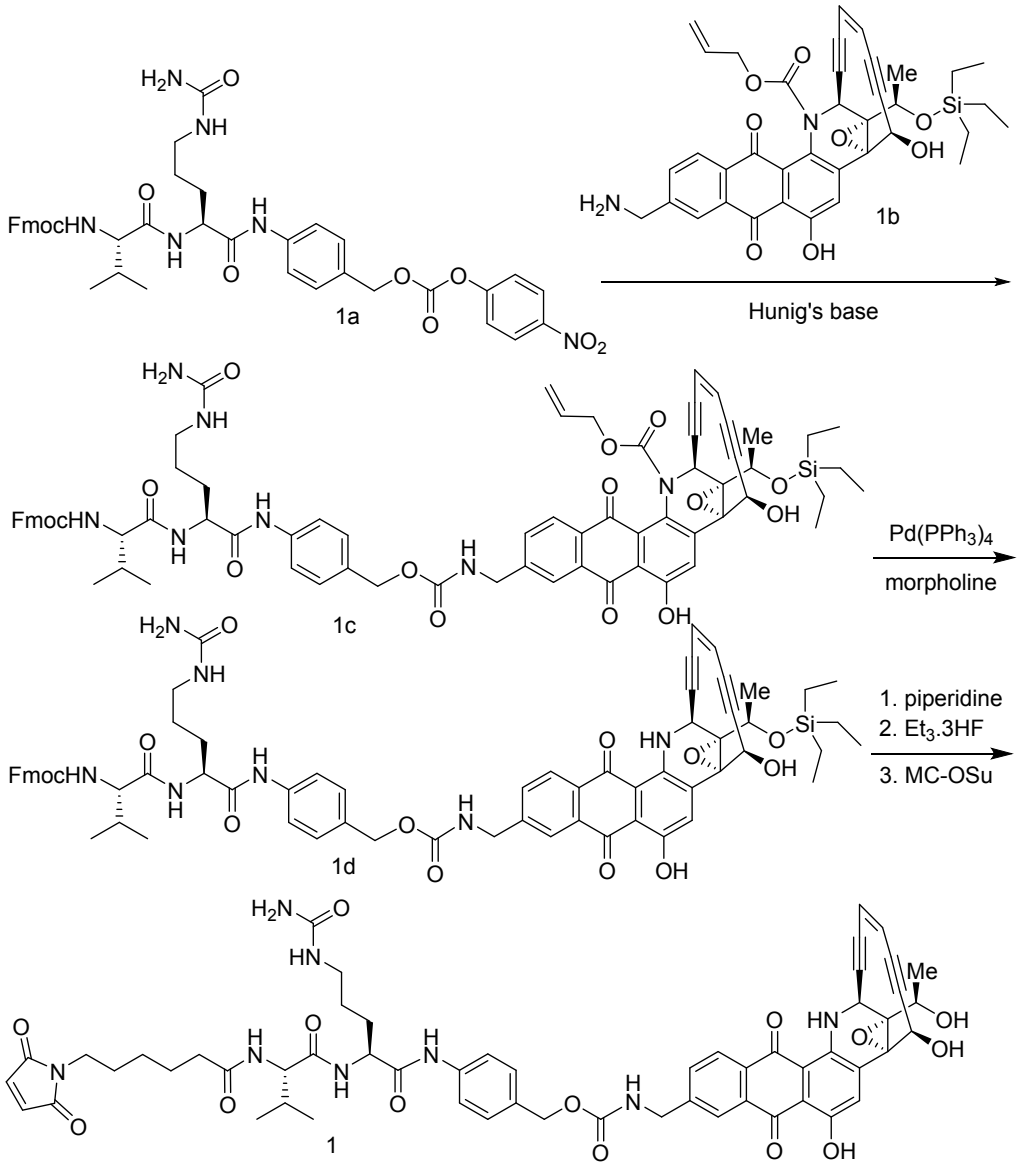
General Methods and Materials

Commercially available anhydrous solvents were used without further purification. Analytical thin layer chromatography was performed on silica gel 60 F254 aluminum sheets (EMD Chemicals, Gibbstown, NJ). Analytical HPLC was performed on a Varian ProStar 210 solvent delivery system configured with a Varian ProStar 330 PDA detector. Samples were eluted over a C12 Phenomenex Synergi 2.0 × 150 mm, 4 μm, 80 Å reversed-phase column. The acidic mobile phase consisted of acetonitrile and water, both containing 0.1% formic acid. Compounds were eluted with a linear gradient of acidic acetonitrile from 5% at 1 min post injection, to 95% at 11 min, followed by isocratic 95% acetonitrile to 15 min (flow rate = 1.0 mL/min). LC-MS was performed on a ZMD Micromass mass spectrometer interfaced to an HP Agilent 1100 HPLC instrument equipped with a C12 Phenomenex Synergi 2.0 × 150 mm, 4 μm, 80 Å reversed-phase column and method of ionization was electrospray. The acidic eluent consisted of a linear gradient of acetonitrile from 5% to 95% in 0.1% aqueous formic acid over 10 min, followed by isocratic 95% acetonitrile for 5 min (flow rate = 0.4 mL/min). Proton NMR was run on 400 Mhz Varian instrument. Purity of compounds were accessed using analytical HPLC using the same condition used in LC-MS.

Synthetic Procedure

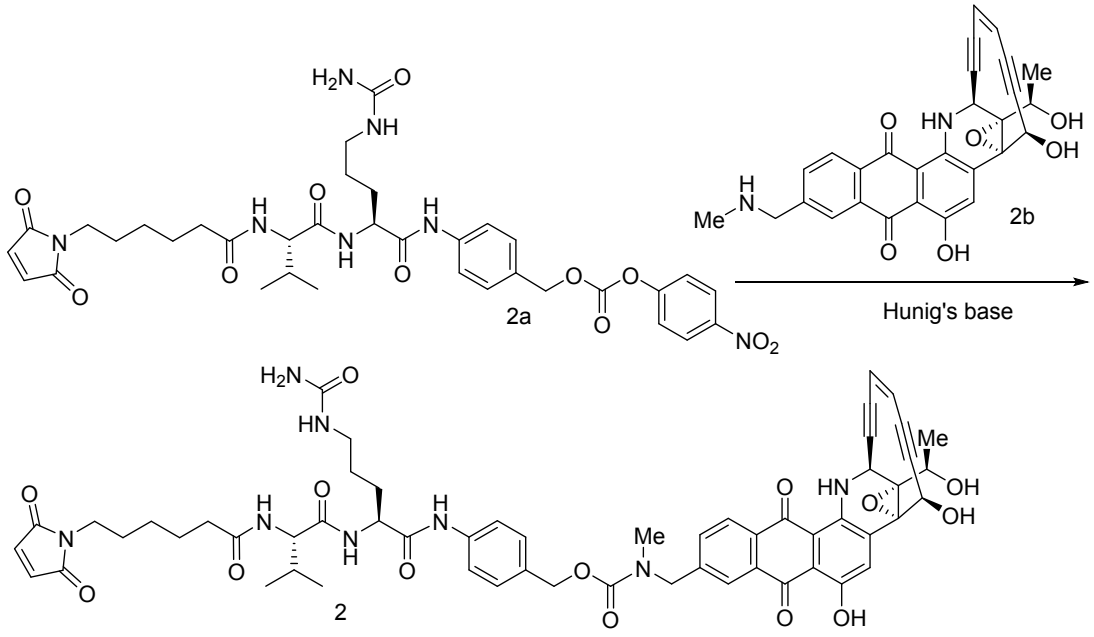


Synthesis of compound 1: To a solution of compound **1b**[1] (0.041 g, 0.061 mmol) in DMF (1 mL), Hunig's base (0.032 mL, mmol) and (9H-fluoren-9-yl)methyl ((S)-3-methyl-1-(((S)-1-((4-((((4-nitrophenoxy) carbonyl) oxy)methyl)phenyl) amino)-1-oxo-5-ureido-pentan-2-yl) amino)-1-oxobutan-2- yl)carbamate (0.056 g, 0.073 mmol) **1b** (commercially available, CAS:863971-53-3) was added at rt. After 3 h, LCMS analysis showed 85% conversion to compound **1c** ([M+H]: 1295.6). The crude reaction mixture was used in the next step without further purification.

Crude compound **1c** was dissolved in THF (1 mL) and the mixture was cooled to 0 °C. To this solution, $Pd(PPh_3)_4$ (7.05 mg, 6.10 μmol) was added followed by morpholine (0.013 mL, 0.146 mmol) . The color changed from brown to purple within 5 sec. The reaction was stirred at 0 °C for 2 h, after which LCMS showed the disappearance of starting material and the appearance of desired product **1d** ([M+H]: 1210.5). The reaction mixture was diluted with EtOAc (10 mL) and washed with pH 6.8 phosphate buffer (10 mL). The aqueous layer was extracted with EtOAc (3 x 10 mL) and the combined organic layers were dried over Na_2SO_4, filtered, and concentrated. The crude product was purified using a COMBIFLASH liquid chromatography (40 g silica gel) with 0-20% MeOH in CH_2Cl_2 gradient to give 74 mg of compound **1d** as a purple solid. ^{1}H-NMR (400 MHz, DMSO-d6) 6 13.17 (s, 1H), 10.07 (s, 1H), 10.00 (d, J=4.4 Hz, 1H), 8.52 (s, 1H), 8.21 (d, J=8 Hz, 1H), 8.13-8.10 (m, 2H), 8.01 (t, J=5.6 Hz, 1H), 7.89 (d, J=7.2 Hz, 2H), 7.81 (d, J=7.2 Hz, 1H), 7.74 (t, J=7.6 Hz, 2H, 7.60 (d, J=8.4 Hz, 2H), 7.44-7.39 (m, 3H), 7.32 (t, J=7.2 Hz, 3H), 6.71(d, J=5.2 Hz, 1H), 6.10-5.96 (m, 3H), 5.40 (s, 2H), 5.11 (dd, J=4.8, 1.2 Hz, 1H), 5.05-5.01 (m, 2H), 4.51 (dd, J=12.8, 6.4 Hz, 1H), 4.41-4.40 (m, 3H), 4.31-4.23 (m, 3H), 3.93 (t, J=8.8 Hz, 1H), 3.03-2.93 (m, 2H), 2.00-1.99 (m, 1H), 1.69-1.58 (m, 2H), 1.35 (d, J=6.4 Hz, 6H), 0.97-0.85 (m, 15H), 0.62 (q, J=8.0 Hz, 6H).

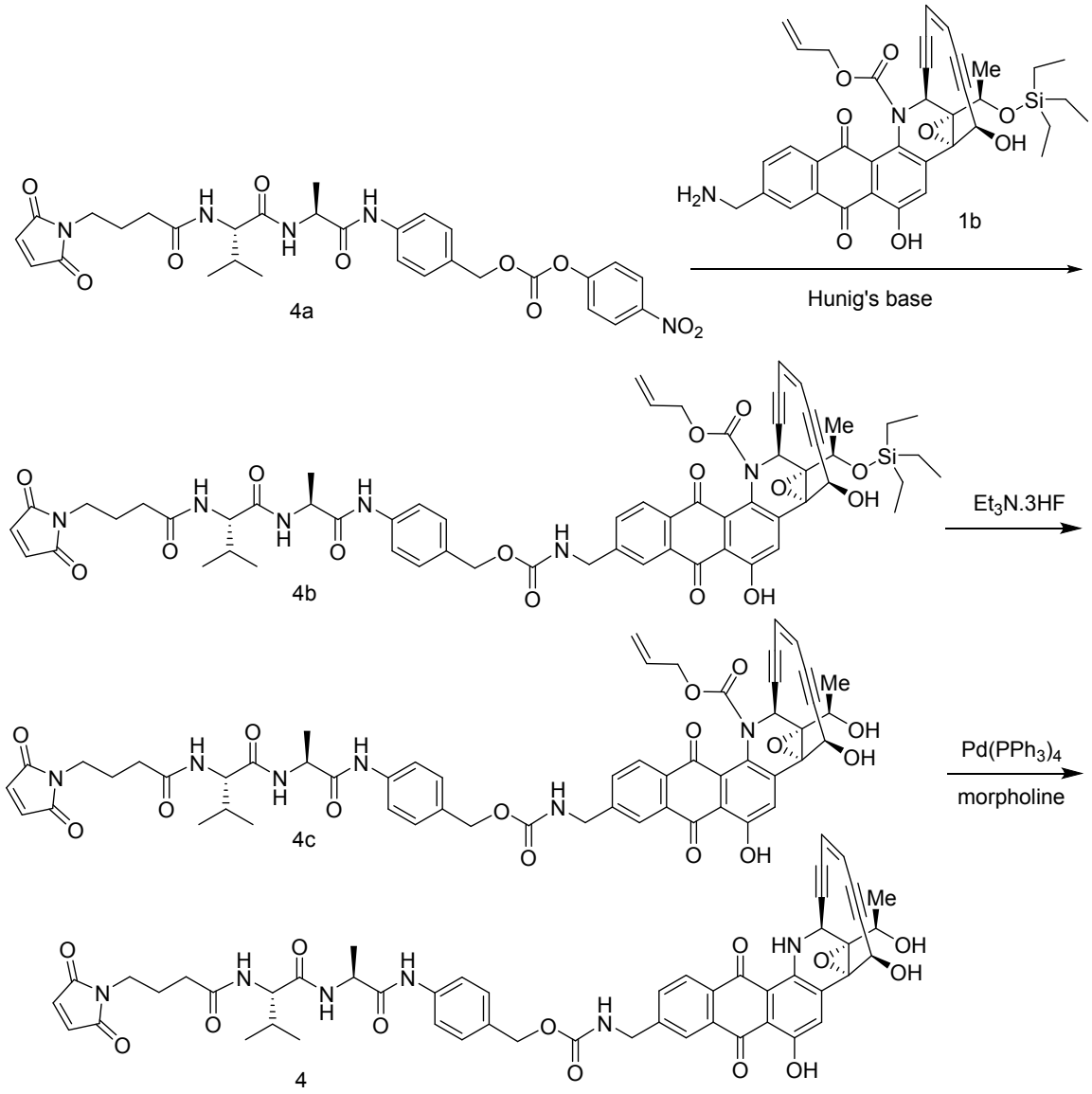
To a solution of compound **1d** (0.074 g, 0.061 mmol) in DMF (3 mL), piperidine (50 μL, 0.506 mmol) was added. The reaction mixture was stirred at RT for 1 h. LCMS ([M+H]: 988.4) showed deprotection of Fmoc group The reaction mixture was cooled to 0 °C and triethylamine (85 μL, 0.610 mmol) was added, followed by triethylamine trihydrofluoride (99 μL, 0.610 mmol). After 5 min, the cold bath was removed and the reaction mixture was stirred for lh. LCMS 115 ([M+H]:874.4) showed deprotection of TES group. The reaction mixture was diluted with EtOAc (50 mL) and washed with brine (50 mL). The organic layer was dried over Na_2SO_4, filtered, and concentrated. The crude compound 115 was dissolved in NMP (3 mL) and Hunig's base (0.032 mL, 0.183 mmol) was added, followed by 2,5-dioxopyrrolidin-l-yl 6-(2,5-dioxo-2, 5-dihydro-lH-pyrrol-l-yl)hexanoate (22.57 mg, 0.073 mmol). After 2 h, LCMS ([M+H]: 1068.5) showed the formation of product **1**. Another 1.2 equiv. of 6-maleimidocaproic acid N-hydroxysuccinimide ester (MC-OSu) and 3 equiv. of Hunig's base (0.032 mL, 0.183 mmol) were added. The reaction flask was

covered with aluminum foil and kept at 0 °C overnight. LCMS showed the completion of reaction. The reaction was diluted with 2 mL DMSO and purified on a Waters Delta Prep 4000 with an X Bridge prep C18 column (30 x 250 mm, 5 mm OBD) using 5-95% water/acetonitrile (0.05% formic acid) gradient over 40 min. A fraction collected at 20.2 min was found to contain the desired product. It was lyophilized to provide 6.3 mg (8.4% overall yield, LCMS purity >99%) of **1** as a purple solid. LCMS calculated for $C_{56}H_{59}N_8O_{14}$: 1068.1 (M+H); found 1068.3. ^{1}H-NMR (400 MHz, DMSO-d6) 6 13.11 (s, IH), 9.93 (s, 2H), 8.45 (s, IH), 8.42 (s, IH), 8.22-8. 13 (m, 2H), 8.08 (d, J=7.6 Hz, IH), 8.00 (t, J=6.0 Hz, IH), 7.80 (d, J=8.4 Hz, 2H), 7.60 (d, J=8.4 Hz, 2H), 7.30 (d, J=8.4 Hz, 2H), 6.99 (s, 2H), 6.67 (d, J=4.4 Hz, 2H), 6.07-5.96 (m, 3H), 5.40 (bs, 3H), 5.14 (d, J=3.6 Hz, IH), 5.06 (dd, J=4.8, 1.6 Hz, IH), 5.00 (s, 2H), 4.40-4.31 (m, 4H), 4.17 (dd, J=8.4, 6.8 Hz, IH), 3.07-2.91 (m, 2H), 2.20-2.09 (m, 2H), 1.99-193 (m, 2H), 1.70-1.45 (m, 5H), 1.31-1.14 (m, 7H), 0.85 (d, J=6.8 Hz, 3H), 0.77 (d, J=6.8 Hz, 3H).



Synthesis of compound 2: To a solution of **2b**[1] (Caution: compound 2b is extremely potent cytotoxic agent and should be handled in well ventilated fume hood. It is advised to prepare a solution than using as s powder) (1.7 mg, 3.52 µmol) in DMF (500 µL) was added **2a** 4-((S)-2-((S)-2-(6-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)hexanamido)-3-methylbutanamido)-5-ureidopentanamido)benzyl (4-nitrophenyl) carbonate (commercially available, CAS:159857-81-5) (3.12 mg, 4.23 µmol) and Hunig's base (0.615

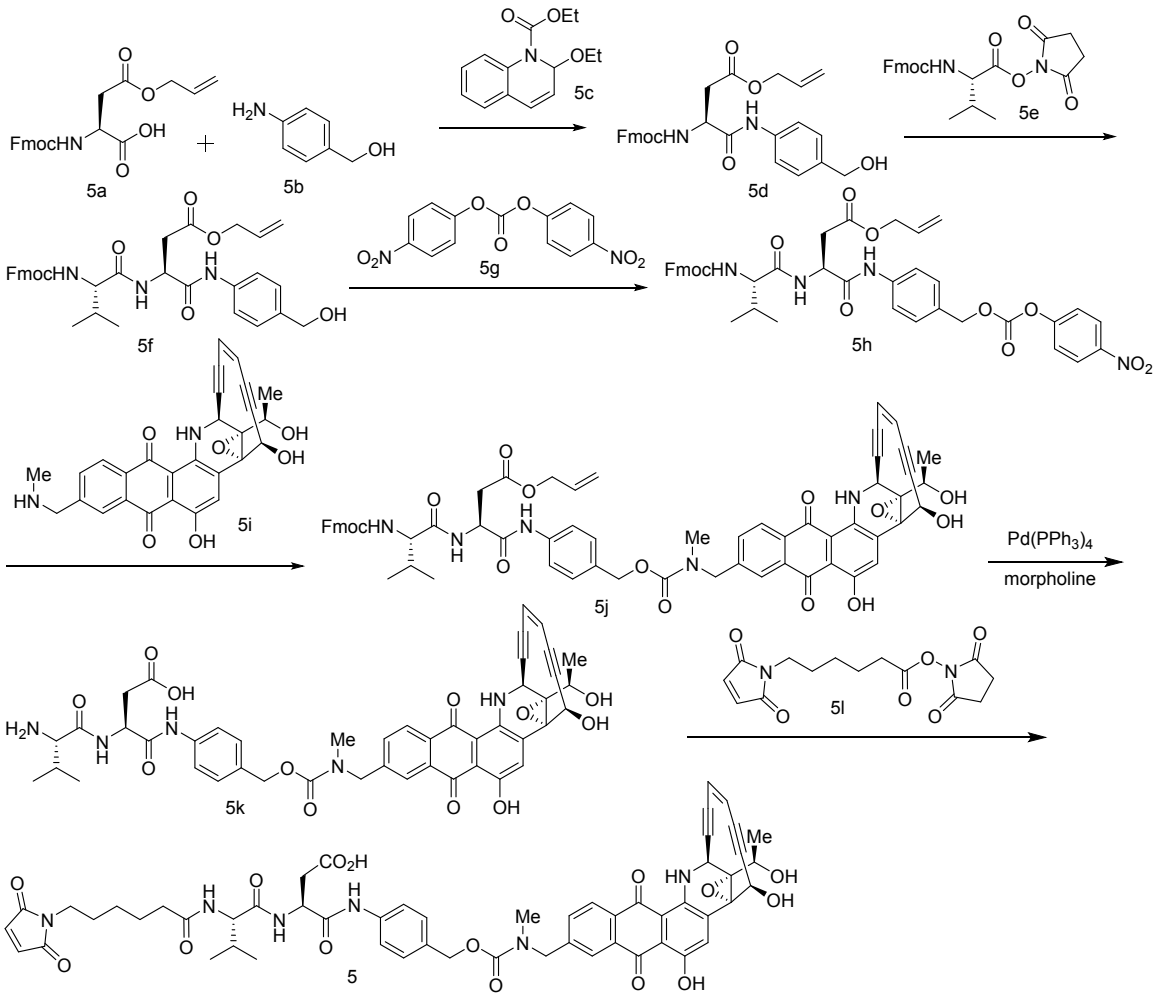
μL, 3.52 μmol) and stirred at rt overnight. LCMS (M+H=1081.2) show the completion of reaction. The reaction was diluted with dmso (2 mL) and purified on Shimadzu preparative HPLC using XBridge Prep C18 5 mm OBD 10x150 mm column eluting with 0-95% H_2O/MeCN (0.05% formic acid). The product containing fractions at 15 min was lyophilized to obtain 1.2 mg (LCMS purity = 95.7%) of **2** as purple solid. LCMS calculated for $C_{57}H_{60}N_8O_{14}$: 1081.4 (M+H); found 1081.2.



Synthesis of compound 4: To a solution of compound **1b**[1] (28 mg, 0.038 mmol) in DMF (0.5 mL) was added **4a** (25.3 mg, 0.038 mmol) followed by Hunig's base (0.020 mL, 0.114 mmol). The reaction was stirred at rt for 1h. LCMS (M+H=1151.4) shows >90%

conversion. The reaction was diluted with 5 mL dmso and purified directly on Simadzu prep. HPLC with X-Bridge C18 column (30x250 mm, 5 mm OBD) using 50-95% water/acetonitrile (0.05% formic acid) over 40 min. A fraction collected at 20.2 min was found to contain desired product and was lyophilized to provide 13 mg of desired product **4b** as brick red solid. LCMS calculated for $C_{61}H_{67}N_6O_{15}Si$: 1152.2 (M+H); found 1152.4.

To a solution of **4b** (13 mg, 0.011 mmol) in 0.5 mL THF was added $Et_3N.3HF$ (0.037 mL, 0.226 mmol) and the reaction was stirred at rt for 2h during which LCMS (M+H=1037.3) shows the completion of reaction. The mixture was dilute with EtOAc (10 mL) and quenched by pouring into sat. aq. NaHCO3 solution (10 mL). The layers were separated and the organic layer was washed with brine (10 mL), dried over Na_2SO_4, filtered and concentrated. The crude product **4c** was taken to the next step without further purification. To a solution of intermediate **4c** from above step in DMF (1 mL) at 0 °C was added $Pd(PPh_3)_4$ (1.305 mg, 1.129 µmol) followed by morpholine (1.476 µl, 0.017 mmol). Within 5 min, the reaction turned purple. LCMS (M+H=953.3) shows the completion of the reaction. The reaction was quenched by pouring into pH 6.8 phposphate buffer solution (10 mL) and extracted with EtOAc (10 mL). The organic layer was dried over Na_2SO_4, filtered and concentrated. The crude product was dissolved in 1 mL dmso and purified (3 injections) on Simadzu prep. HPLC with X Bridge prep C18 column (30x250 mm, 5 mm OBD) using 50-95% water/acetonitrile (0.05% formic acid) over 30 min. A fraction collected at 14.5 min was found to contain desired product and was lyophilized to obtain 1.4 mg (LCMS purity >90%) of **4** as purple solid. LCMS calculated for $C_{51}H_{49}N_6O_{13}$: 954.3 (M+H); found 954.4.



Synthesis of compound 5: To a solution of (S)-2-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)-4-(allyloxy)-4-oxobutanoic acid **5a** (1.0 g, 2.53 mmol) and (4-aminophenyl)methanol **5b** (0.623 g, 5.06 mmol) was added ethyl 2-ethoxyquinoline-1(2H)-carboxylate **5c** (1.251 g, 5.06 mmol) and the mixture was stirred at rt for 3h. LCMS shows product (M+H-H_2O=483.4.4). The reaction was diluted with EtOAc (50 ml) and was washed with sat. aq. $NaHCO_3$ (2x50 mL), brine (50 mL), dried over Na_2SO_4, filtered, concentrated and taken crude to next step.

To a solution of (S)-allyl 3-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)-4-((4-(hydroxymethyl)phenyl)amino)-4-oxobutanoate **5d** (2.53 mmol) in THF (10 mL) was added diethylamine (1.322 mL, 12.65 mmol) and stirred for 1h. LCMS shows deprotection of Fmoc group. The solvent and base was evaporated and the crude product was dissolved in THF (2 mL)/Water (2 mL) and added (S)-2,5-dioxopyrrolidin-1-yl-2-((((9H-fluoren-9-

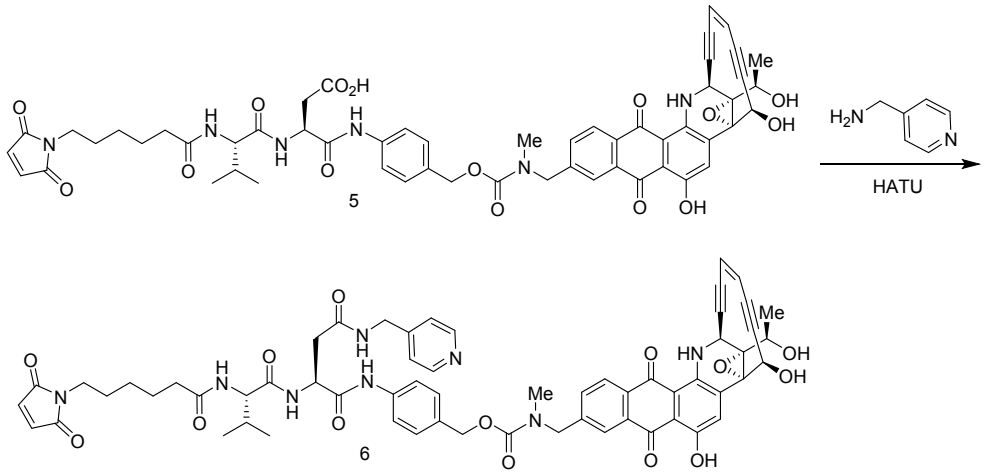
yl)methoxy)carbonyl)amino)-3-methylbutanoate (1104 mg, 2.53 mmol) followed by sodium bicarbonate (425 mg, 5.06 mmol). The reaction was stirred overnight at which LCMS (M+H=600.2) shows formation of product. The solvent was evaporated and the crude product was purified on combiflash (40 g silical gel) eluting with 0-100% MeOH/CH_2Cl_2 to yield desired alcohol **5f** as white solid.

The alcohol intermediate **5f** was dissolved in THF (2 mL) and was treated with bis(4-nitrophenyl) carbonate (1539 mg, 5.06 mmol) followed by Hunig's base (1.326 mL, 7.59 mmol) and stirred at rt for 3h. The solvent was evaporated and the crude product was purified on combiflash (40 g silica gel) eluting with 0-100% MeOH/CH2Cl2 to yield 587 mg of desired product **5h** as yellowish solid. LCMS calculated for $C_{41}H_{40}N_4O_{11}$: 964.3 (M+H); found 765.0.

To a solution of (S)-allyl 3-((S)-2-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)-3-methylbutanamido)-4-((4-((((4-nitrophenoxy)carbonyl)oxy)methyl)phenyl)amino)-4-oxobutanoate **5h** (20.92 mg, 0.027 mmol) and N-methyl aminomethyl uncialamycin **5i**[1] (11 mg, 0.023 mmol) in DMF (0.5 ml) was added 2,6-lutidine (5.31 µl, 0.046 mmol) and stirred for 3h at which LCMS (M+H=1108.3) shows the completion of reaction. The reaction was quenched by the addition of sat. aq. $NaHCO_3$ solution (20 mL) and extracted with EtOAc 2x20 mL). Dried over Na_2SO_4 and solvent was evaporated. Take crude to next step.

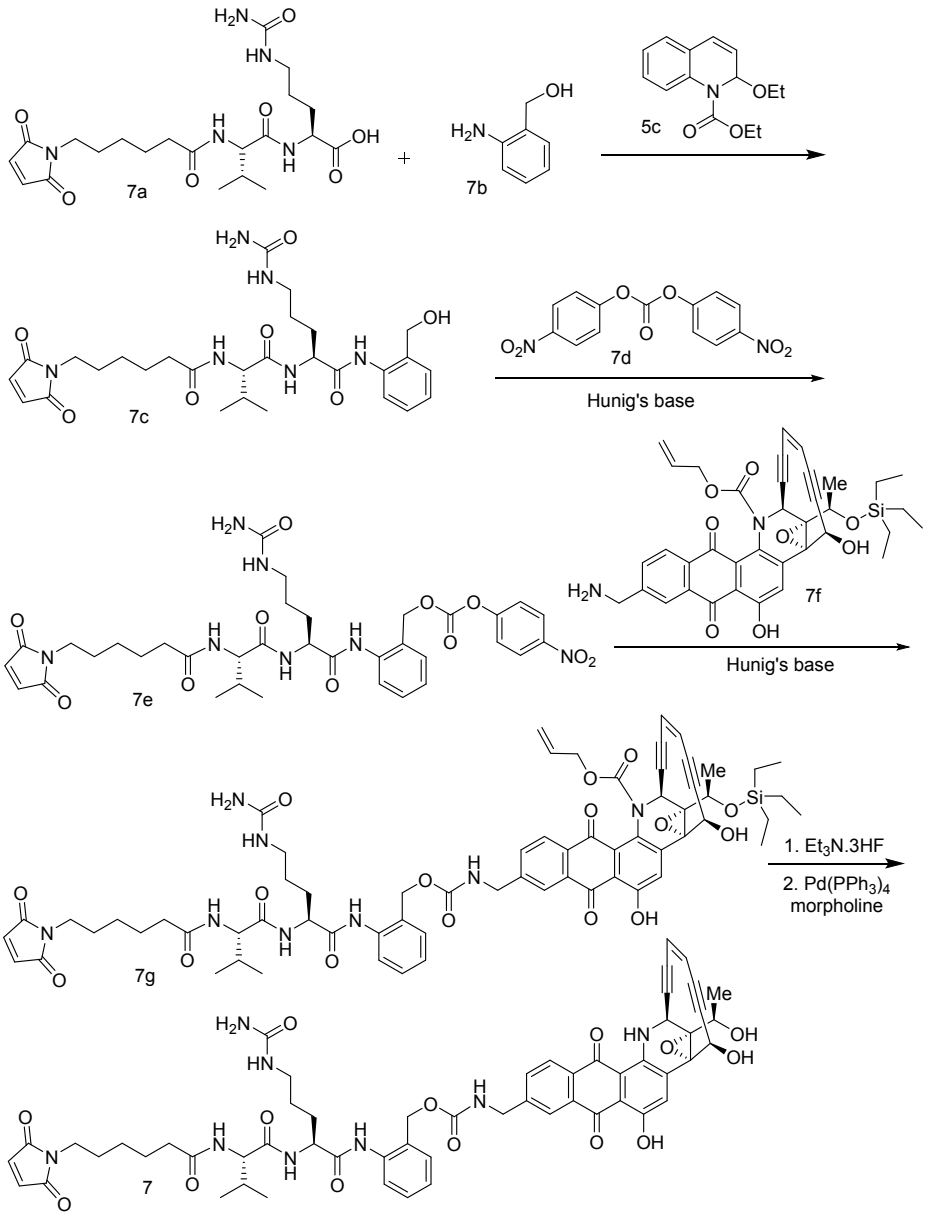
To a solution of **5j** (0.023 mmol) in DMF (1 mL) was added morpholine (4.01 µl, 0.046 mmol) and $Pd(PPh_3)_4$ (2.66 mg, 2.300 µmol) and stirred for 1h. LCMS (M+H=1068.3) shows the deprotection of alloc group. To this mixture was added diethylamine (12.02 µl, 0.115 mmol) and stirred for 1h at which LCMS (M+H=846.2) shows the completion of reaction. The reaction was diluted with dmso (2 mL) purified on Shimadzu preparative HPLC using XBridge Prep C18 5mm OBD 10x150 mm column eluting with 0-95% H_2O/MeCN (0.05% formic acid). The product containing fractions at 12 min was lyophilized to obtain desired 5.1 mg of product **5k** as purple solid. LCMS calculated for $C_{45}H_{44}N_5O_{12}$: 846.2 (M+H); found 846.1.

To a solution of **5k** (5.1 mg, 6.03 µmol) in DMF (0.5 mL) was added 2,5-dioxopyrrolidin-1-yl 6-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)hexanoate (2.045 mg, 6.63 µmol) and 2,6-LUTIDINE (2.107 µl, 0.018 mmol) and the mixture was stirred at rt for 3h at which LCMS (M+H=1039.2) shows the completion of reaction. The reaction was diluted with dmso (2 mL) purified on Shimadzu preparative HPLC using XBridge Prep C18 5mm OBD 10x150 mm column eluting with 0-95% H2O/MeCN (0.05% formic acid). The product containing fraction at 12.2 min was collected and lyophilized to obtain desired product **5** (LCMS purity = 90.1%) as purple solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.53 (s, 1H), 8.31 (s, 1H), 8.22 (d, J = 6.3 Hz, 1H), 8.09 (s, 1H), 7.88 (s, 1H), 7.77 (s, 1H), 7.64 (s, 2H), 7.34 (s, 1H), 7.27 (s, 1H), 6.99 (s, 2H), 6.69 (s, 1H), 6.45 (dd, J = 18.3, 6.7 Hz, 3H), 6.15 – 5.91 (m, 2H), 5.86 (s, 1H), 5.59 (ddd, J = 27.1, 6.2, 2.1 Hz, 3H), 5.38 (dd, J = 6.0, 2.2 Hz, 2H), 5.32 (s, 1H), 5.17 (s, 1H), 5.07 (dt, J = 11.4, 6.0 Hz, 3H), 4.66 (s, 3H), 4.39 (s, 3H), 4.09 (t, J = 7.1 Hz, 1H), 3.93 – 3.69 (m, 2H), 1.97 (dt, J = 6.3, 3.2 Hz, 2H), 1.84 (ddd, J = 9.2, 4.5, 2.0 Hz, 2H), 1.72 (dddd, J = 14.5, 9.2, 7.0, 2.1 Hz, 2H), 1.45 (ddd, J = 8.6, 5.9, 3.6 Hz, 4H), 1.32 (d, J = 6.4 Hz, 3H), 0.85 (t, J = 8.0 Hz, 3H). LCMS calculated for $C_{55}H_{54}N_6O_{15}$: 1039.0 (M+H); found 1039.3.



Synthesis of 6: To a 1.5 mmolar solution of **5** (400 µL, 0.600 µmol) in dmso was added pyridin-4-ylmethanamine (120 µL, 1.200 µmol), 2,6-lutidine (0.349 µL, 3.00 µmol), HATU (0.684 mg, 1.800 µmol) and stirred at rt for 1h at which LCMS (M+H=1129.4) shows the formation of product. The reaction was dilute with dmso (2 mL) purified on Shimadzu preparative HPLC using XBridge Prep C18 5mm OBD 10x150 mm column eluting with 0-95% H2O/MeCN (0.05% formic acid). The product containing fractions at

13 min was lyophilized to obtain 0.3 mg (LCMS purity> 99%) of **6** as purple solid. LCMS calculated for $C_{61}H_{60}N_8O_{14}$: 1129.4 (M+H); found 1129.3.



Synthesis of 7: To a solution of (S)-2-((R)-2-(6-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)hexanamido)-3-methylbutanamido)-5-ureidopentanoic acid **7a** (202 mg, 0.432 mmol) and (2-aminophenyl)methanol **7b** (106 mg, 0.864 mmol) in DMF (2.0 mL) was added ethyl 2-ethoxyquinoline-1(2H)-carboxylate 5**c** (214 mg, 0.864 mmol) and the vial was wrapped with aluminum foil and the milky solution was stirred at rt overnight. Reaction turned into clear yellowish solution and LCMS (M+H=573.2) shows the completion of the reaction.

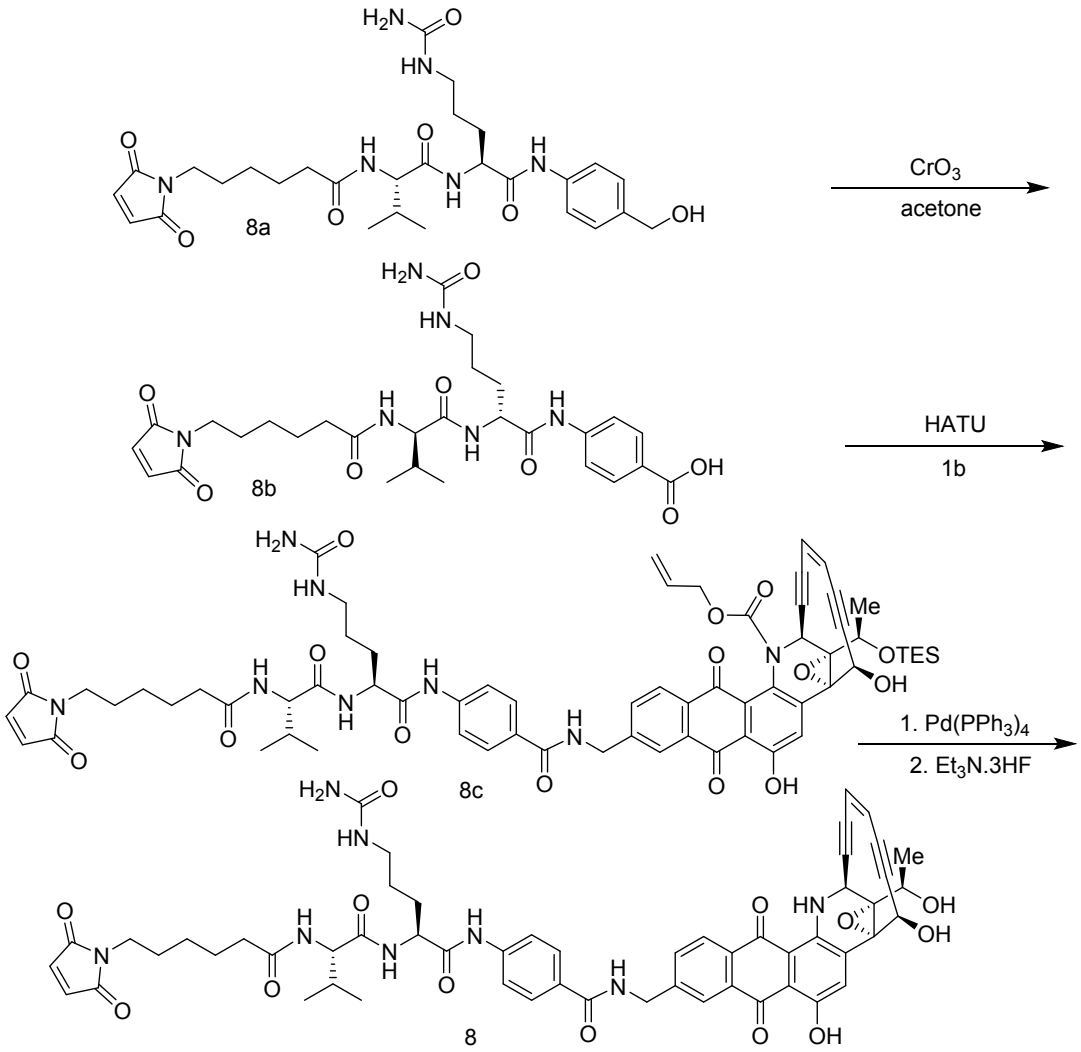
The reaction was diluted with 1 mL dmso and purified (3 injections) on Simadzu prep. HPLC with X Bridge prep C18 column (30x250 mm, 5 mm OBD) using 0-95% water/acetonitrile (0.1% TFA) over 40 min. A fraction collected at 15.5 min was found to contain desired product and was lyophilized to obtain 58 mg of **7c**. LCMS calculated for $C_{28}H_{40}N_6O_7$: 573.3 (M+H); found 573.3.

To a solution of 6-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)-N-((S)-1-(((S)-1-((2-(hydroxymethyl)phenyl)amino)-1-oxo-5-ureidopentan-2-yl)amino)-3-methyl-1-oxobutan-2-yl)hexanamide **7c** (28 mg, 0.049 mmol) in DMF (1 mL) was added bis(4-nitrophenyl) carbonate (29.7 mg, 0.098 mmol) followed by Hunig's base (0.026 mL, 0.147 mmol). The reaction was stirred at rt for 2h during which LCMS (M+H=738.31) showed the completion of the reaction. After the evaporation of base, the crude reaction was directly subjected to combiflash column chromatography on 12 g silica gel eluting with 0-50 % MeOH/CH_2Cl_2 to yield 38 mg of desired product **7d** as off white solid. LCMS calculated for $C_{35}H_{43}N_7O_{11}$: 738.3 (M+H); found 738.3.

To a solution of 2-((S)-2-((S)-2-(6-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)hexanamido)-3-methylbutanamido)-5-ureidopentanamido)benzyl (4-nitrophenyl) carbonate **7e** (28.0 mg, 0.038 mmol) in DMF (1 mL) was added **7f** (25.3 mg, 0.038 mmol) followed by Hunig's base (0.020 mL, 0.114 mmol). The reaction was stirred at rt for 3h and left in 0 °C freezer over the weekend. LCMS (M+H=1265.5) shows >90% conversion. The reaction was diluted with EtOAc (20 mL) and was washed with sat. aq. NaHCO3 (20 mL) and brine (20 mL). The combined organic layers were dried over Na_2SO_4, filtered and concentrated. The brick red residue was diluted with 5 mL dmso and purified on Simadzu prep. HPLC with X Bridge prep C18 column (30x250 mm, 5 mm OBD) using 5-95% water/acetonitrile (0.05% formic acid) over 40 min. A fraction collected at 17 min was found to contain desired product and was lyophilized to obtain 10.4 mg of **7g**. LCMS calculated for $C_{66}H_{76}N_8O_{16}Si$: 1265.5 (M+H); found 1265.5.

To a solution of **7g** (10.4 mg, 8.22 μmol) in DMF (1 mL) was added $Et_3N.3HF$ (0.027 mL, 0.164 mmol) and the reaction was stirred at rt for 1h during which HPLC analysis shows

the completion of reaction. The mixture was dilute with EtOAc (10 mL) and quenched by pouring into sat. aq. $NaHCO_3$ solution (10 mL). The layers were separated and the organic layer was washed with brine (10 mL), dried over Na2SO4, filtered and concentrated. The crude product was taken to the next step without further purification. To the crude mixture from previous step in DMF (1 mL) at 0 °C was added $Pd(PPh_3)_4$ (0.950 mg, 0.822 μmol) followed by morpholine (1.074 μl, 12.33 μmol). Within 5 min, the reaction turned purple. HPLC shows the completion of the reaction. The reaction was quenched by pouring into pH 6.8 phposphate buffer solution (10 mL) and extracted with EtOAc (10 mL). The organic layer was dried over Na_2SO_4, filtered and concentrated. The crude product was dissolved in 1 mL dmso and purified (3 injections) on Simadzu prep. HPLC with X Bridge prep C18 column (30x250 mm, 5 mm OBD) using 50-95% water/acetonitrile (0.05% formic acid) over 40 min. A fraction collected at 9.5 min was found to contain desired product and was lyophilized to obtain 1.2 mg (LCMS purity >90%) of **7** as purple solid. LCMS calculated for $C_{56}H_{58}N_8O_{14}$: 1067.4 (M+H); found 1067.5.



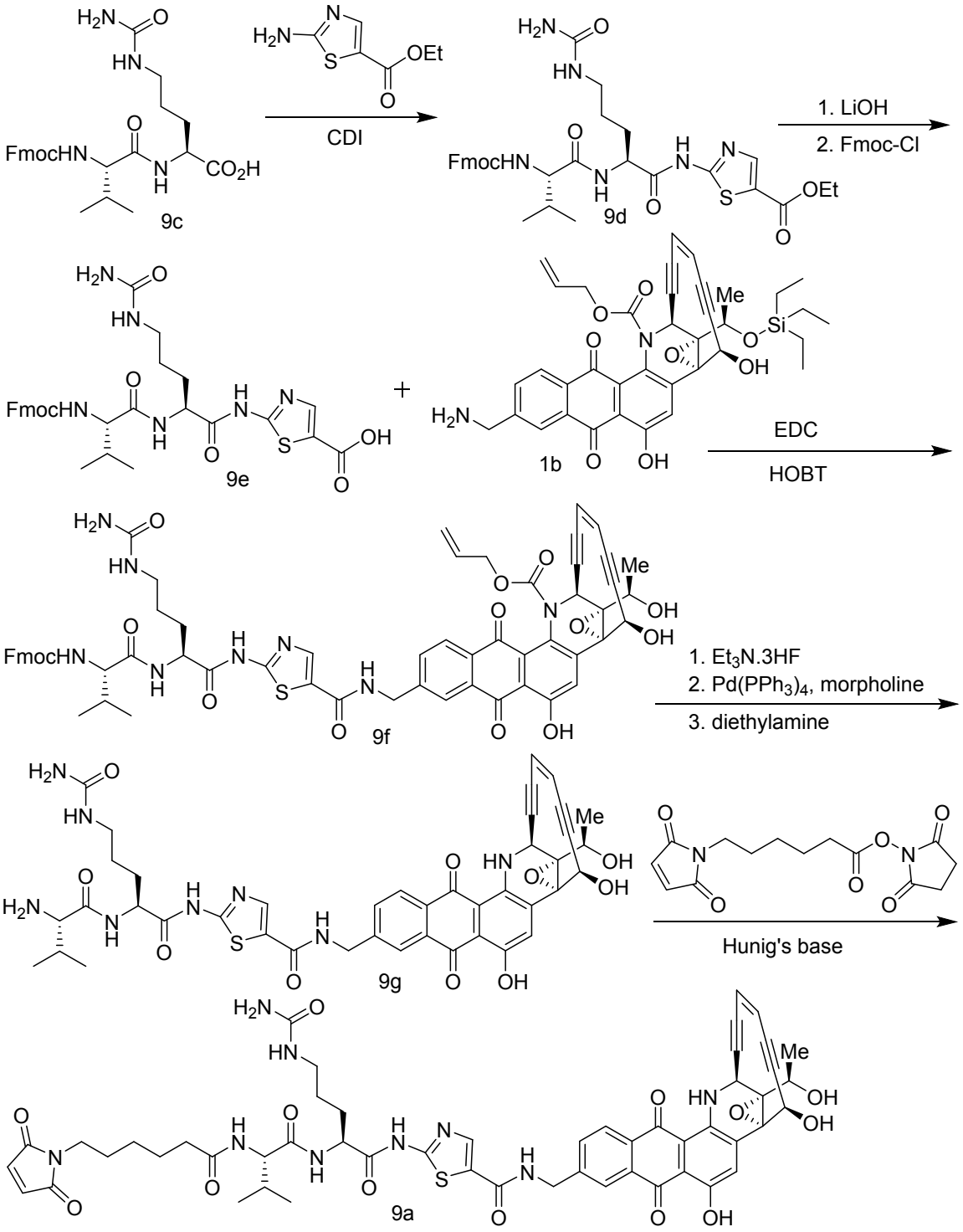
Synthesis of compound 8: To a solution of 6-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)-N-((S)-1-(((S)-1-((4-(hydroxymethyl)phenyl)amino)-1-oxo-5-ureidopentan-2-yl)amino)-3-methyl-1-oxobutan-2-yl)hexanamide **8a** (commerrcially available CAS: 159857-80-4) (1g, 1.746 mmol) in acetone (10 mL) was added CrO_3 in dil. H_2SO_4 (1.746 mL, 3.49 mmol) dropwise via syringe. LCMS (M+H=587.3) at 1h shows the desired carboxylic acid. The reaction was decanted and the supernatant liquid was separated from the green precipitate. The precipitate was washed with MeOH (10 mL). The combined organic layer was dried and the crude product was purified on combiflash on 40 g silical gel eluting with 0-100% MeOH/CH_2Cl_2 to yield 107 mg of desired product **8b** as white solid. LCMS calculated for $C_{28}H_{38}N_6O_8$: 587.6 (M+H); found 587.3.

To a solution of 4-((S)-2-((S)-2-(6-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)hexanamido)-3-methylbutanamido)-5-ureidopentanamido)benzoic acid **8b** (4.60 mg, 7.84 µmol) in DMF (0.5 mL) was added HATU (3.72 mg, 9.80 µmol) and Hunig's base (3.42 µl, 19.59 µmol) After 30 min, LCMS (M+H=705.4) shows the presence of acid-HATU complex. This solution was added to **1b** (4.35 mg, 6.53 µmol) and stirred at RT for 30 min and stored at -20 °C freezer over the weekend. LCMS (M+H=1235.5) shows complete conversion. The reaction was diluted with dmso (1 mL) and was injected to prep HPLC with Waters Delta Prep 4000 with X Bridge prep C18 column (30x250 mm, 5 mm OBD) using 5-95% water/acetonitrile (0.05% formic acid) over 30 min. A fraction collected at 19 min was found to contain the desired product which was lyophilized to obtain 5.2 mg of **8c** as yellowish powder.

To the solution of **8c** (5.2 mg, 4.21 µmol) was added $Et_3N.3HF$ (70 µl, 0.430 mmol) and the mixture was stirred at rt for 30 min. LCMS (M+H=1121.5) shows conversion to desired desilylated product. The reaction was diluted with EtOAc (10 mL) and washed with sat. aq. $NaHCO_3$ (10 mL), brine (10 mL), dried over Na_2SO_4, filtered, concentrated and subjected to vacuum for 1h. The crude product was taken to the next step without further purification.

The solution of crude product from previous reaction in DMF (1 mL) was cooled to 0 °C and $Pd(PPh_3)_4$ (0.7 mg, 0.627 µmol) was added followed by morpholine (1.3 µl, 15.05 µmol). The reaction turned purple in 10 min indicating the alloc deprotection has begun. The reaction was allowed to proceed for 20 min more in which LCMS (M+H=1037.5) shows the completion of reaction. The reaction was diluted with EtOAc (20 mL) and was washed with pH 6.8 phosphate buffer (10 mL), water (10 mL) and brine (10 ml). Combined organic layers were dried over Na2SO4, filtered and concentrated. The crude product was dissolved in 2 mL dmso and purified on Waters Delta Prep 4000 with X Bridge prep C18 column (30x250 mm, 5 mm OBD) using 5-95% water/acetonitrile (0.05% formic acid) over 40 min. A fraction collected at 29.2 min was found to contain desired product and was lyophilized to obtain 1.2 mg (LCMS purity >90%) of **8** as purple solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.22 (s, 1H), 10.00 (s, 1H), 9.14 (t, J = 6.0 Hz, 1H), 8.52 (d, J = 7.8

Hz, 1H), 8.36 – 8.03 (m, 3H), 8.00 – 7.77 (m, 5H), 7.71 (d, *J* = 8.6 Hz, 2H), 6.99 (s, 2H), 6.68 (d, *J* = 5.0 Hz, 1H), 6.22 – 5.72 (m, 3H), 5.40 (d, *J* = 20.8 Hz, 3H), 5.15 (d, *J* = 4.7 Hz, 1H), 5.10 – 4.99 (m, 1H), 4.65 (d, *J* = 5.8 Hz, 2H), 4.43 – 4.36 (m, 1H), 4.33 (q, *J* = 6.2 Hz, 1H), 4.20 (dd, *J* = 8.6, 6.8 Hz, 1H), 4.09 (q, *J* = 5.3 Hz, 4H), 2.26 – 2.08 (m, 2H), 1.76 – 1.54 (m, 1H), 1.48 (q, *J* = 7.9, 7.5 Hz, 4H), 1.26 – 1.06 (m, 4H), 0.84 (dd, *J* = 14.1, 6.7 Hz, 8H). LCMS calculated for $C_{55}H_{56}N_8O_{13}$: 1037.3 (M+H); found 1037.3.



Synthesis of compound 9a: After stirring a solution of (S)-2-((S)-2-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)-3-methylbutanamido)-5-ureidopentanoic acid **9c** (2.88 g, 5.81 mmol), CDI (1.036 g, 6.39 mmol) and Hunig's base (2.028 mL, 11.61 mmol) for 30 min was added ethyl 2-aminothiazole-5-carboxylate (1g, 5.81 mmol) and stirred for 3h.

Diluted with 50 ml of water and extracted with 3x50 mL EtOAc. Combined organic layers were evaporated to dryness and purified on combiflash (80g sillica gel), 0-50% MeOH/CH_2Cl_2 to yield 531 mg of **9d**. LCMS calculated for $C_{32}H_{38}N_6O_7S$: 651.2 (M+H); found 651.2.

To a solution of **9d** (531 mg) in MeCN (1 mL)/H_2O (1 mL) for 3h was added LiOH (75 mg, 3.15 mmol) and stirred for 3h at which LCMS (M+H=401.3) shows deprotection of Fmoc and hydorlysis of ethyl ester. pH was adjusted to pH 7 with 1M aq. HCl and lyophilized the mixture to dryness to provide crude amino carboxylic acid.

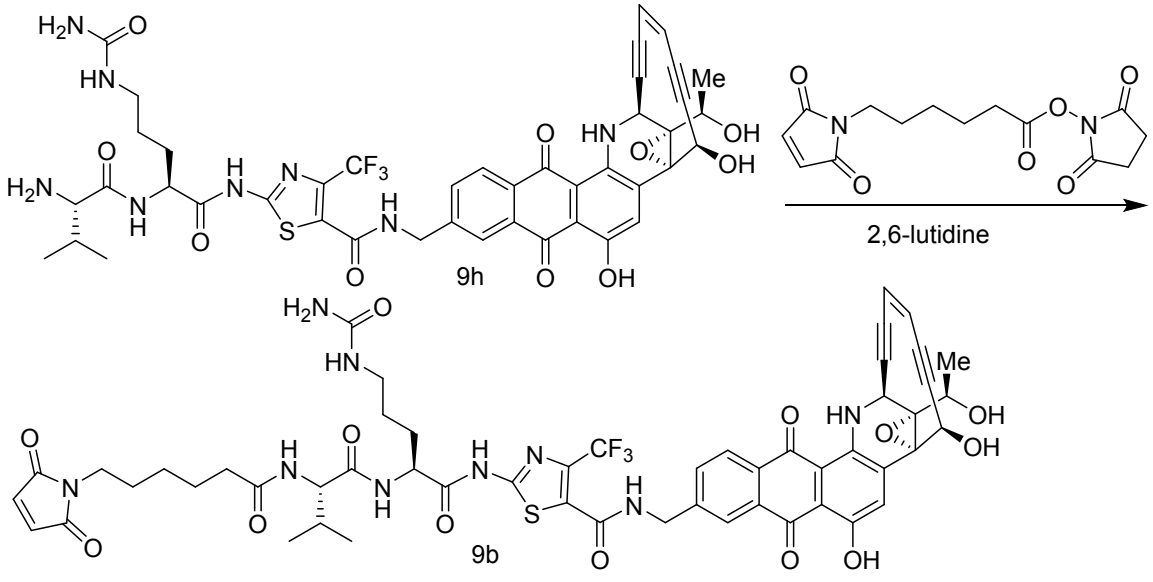
To a mixure of crude amino-carboxylic acid (126 mg, 0.315 mmol) and Hunig's base (0.110 mL, 0.630 mmol) in DMF (2 mL) at 0 °C was added (9H-fluoren-9-yl)methyl carbonochloridate (81 mg, 0.315 mmol) and stirred for 1h. Solvent was evaporated and the crude product was purified on combiflash eluting with 0-100% MeOH/CH_2Cl_2 to yield 185 mg of **9e**. LCMS calculated for $C_{30}H3_4N_6O_7S$: 623.2 (M+H); found 623.0.

To a solution of 2-((S)-2-((S)-2-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)-3-methylbutanamido)-5-ureidopentanamido)thiazole-5-carboxylic acid **9e** (85 mg, 0.137 mmol) and **1b** (91 mg, 0.137 mmol) in DMF (2 mL) was added EDC (52.3 mg, 0.273 mmol), HOBT (41.8 mg, 0.273 mmol) and Hunig's base (0.095 mL, 0.546 mmol) and stirred for 3h at which LCMS (M+H=1271.2) shows the completion of reaction. The reaction was worked up with EtOAc/water and purified on silica gel combiflash using 0-100% MeOH/CH_2Cl_2 to provide 73 mg of desired product **9f**. LCMS calculated for $C_{67}H_{70}N_8O_{14}SSi$: 1271.4 (M+H); found 1271.4.

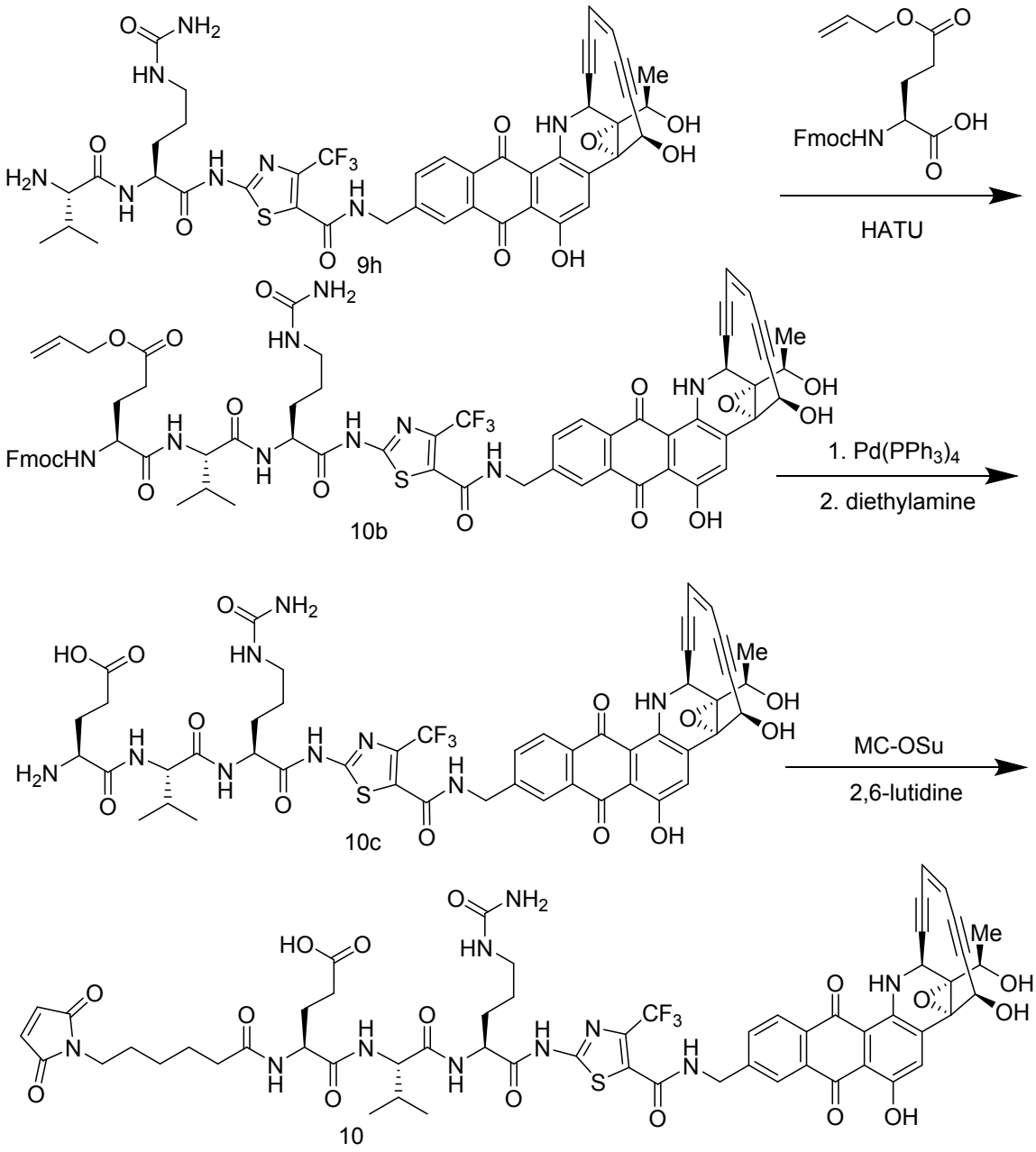
To a solution of **9f** (73 mg, 0.057 mmol) in THF (1 mL) was added $Et_3N.3HF$ (0.093 mL, 0.574 mmol) and stirred at rt for 1h at which LCMS (M+H=1158.1) shows completion of reaction. The reaction was worked up with sat. aq. $NaHCO_3$/EtOAc and taken crude to next step. At 0 °C, to a crude solution of product from previous step (0.066 g, 0.057 mmol) in DMF (1 mL) was added morpholine (9.93 µl, 0.114 mmol) and $Pd(PPh_3)_4$ (6.59 mg, 5.70 µmol). After 2h, LCMS (M+H=1074.2) shows the completion of reaction. Worked up with

sat. aq,. $NaHCO_3$/EtOAc and taken crude to next step. To a solution of crude product from previous step (61.2 mg, 0.057 mmol) in THF (1 mL) was added diethylamine (0.060 mL, 0.570 mmol) and stirred for 30 min. LCMS (M+H=851.1) shows the completion of reaction. The solvent was evaporated and the crude product was diluted with dmso (3 mL) and purified (3 injections) on Shimadzu preparative HPLC using XBridge Prep C18 5mm OBD 10x150 mm column eluting with 0-100% H_2O/MeCN (0.05% formic acid). The product containing fractions at 9.5 min was collected and lyophilized to obtain 9 mg of **9g** as firebrick red solid. LCMS calculated for $C_{42}H_{42}N_8O_{10}S$: 851.2 (M+H); found 851.2.

To a solution of **9g** (9 mg, 10.58 µmol) in DMF (0.5 mL) was added 2,6lutidine (2.464 µl, 0.021 mmol) and 2,5-dioxopyrrolidin-1-yl 6-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)hexanoate (6.52 mg, 0.021 mmol). The reaction was stirred at rt for 5h in which LCMS (M+H=1044.2) shows the formation of product. The reaction was diluted with dmso (3 ml) and purified (3 injections) on Shimadzu preparative HPLC using XBridge Prep C18 5mm OBD 10x150 mm column eluting with 0-100% H_2O/MeCN (0.05% formic acid). The product containing fractions at 15.5 min was collected and lyophilized to obtain 2.3 mg (LCMS purity >90%) of **9a** as firebrick red solid. LCMS calculated for $C_{52}H_{53}N_9O_{13}S$: 1044.3 (M+H); found 1044.3.



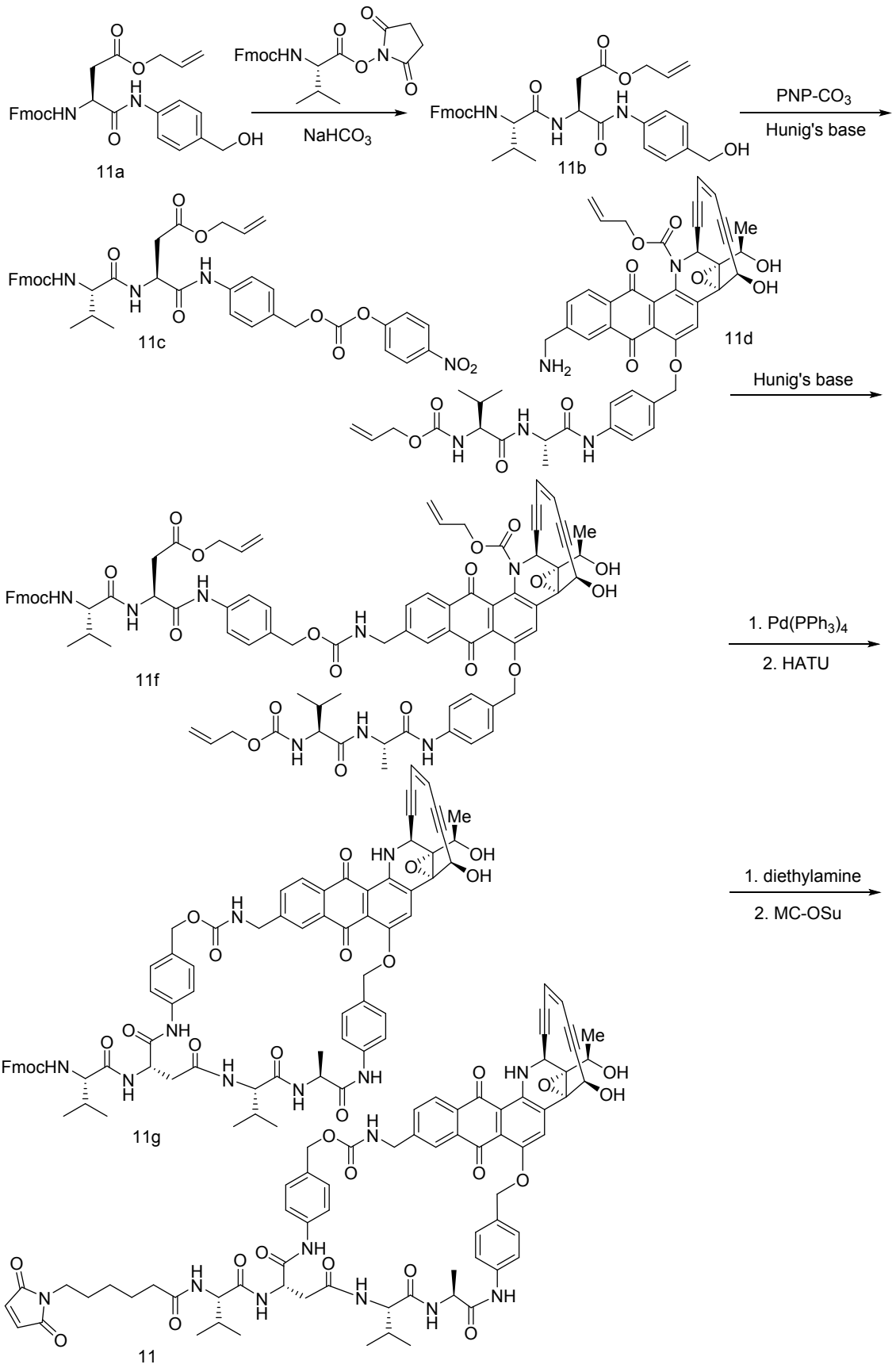
Synthesis of compound 9b: Compound **9h** was prepared in a manner analogues to **9g** using correspinding ethyl 2-amino-4-(trifluoromethyl)thiazole-5-carboxylate. To a solution of 2,5-dioxopyrrolidin-1-yl 6-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)hexanoate (15.10 mg, 0.049 mmol) in DMF (1 mL) was added **9h** (22.5 mg, 0.024 mmol) and 2,6-dimethylpyridine (8.26 µl, 0.073 mmol). The reactiopn was stired at rt for 2h during which LCMS (M+H=1112.3) shows the completion of reaction. The solvent and base was evaporated in evaporator and the crude product was dissolved in 4 ml dmso. The crude prodct was purified (5 injections) on Shimadzu preparative HPLC using XBridge Prep C18 5mm OBD 10x150 mm column eluting with 0-95% H_2O/MeCN (0.05% formic acid). The product containing fractions at 12.5 min were collected and lyophilized to obtain 16 mg (LCMS purity >95%) **9b** as purple solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ 13.11 (s, 1H), 9.94 (d, J = 4.6 Hz, 1H), 9.26 (s, 1H), 8.46 (s, 1H), 8.33 – 7.95 (m, 3H), 7.88 – 7.59 (m, 2H), 6.92 (s, 2H), 6.61 (d, J = 5.1 Hz, 1H), 6.15 – 5.65 (m, 3H), 5.46 – 5.23 (m, 3H), 5.09 (d, J = 4.7 Hz, 1H), 5.00 (dd, J = 4.6, 1.6 Hz, 1H), 4.54 (d, J = 5.9 Hz, 2H), 4.42 – 4.22 (m, 2H), 4.13 (dd, J = 8.8, 6.9 Hz, 1H), 3.61 – 3.44 (m, 7H), 2.88 (q, J = 6.6 Hz, 2H), 2.08 (dtt, J = 20.6, 13.6, 7.1 Hz, 2H), 1.89 (dq, J = 14.2, 7.1 Hz, 1H), 1.77 – 1.57 (m, 2H), 1.49 – 1.34 (m, 3H), 1.32 – 1.17 (m, 1H), 1.12 (q, J = 7.7 Hz, 2H), 0.76 (dd, J = 15.0, 6.7 Hz, 6H). LCMS calculated for $C_{53}H_{52}F_3N_9O_{13}S$: 1112.3 (M+H); found 1112.2.



Synthesis of compound 10: To a solution of (S)-2-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)-5-(allyloxy)-5-oxopentanoic acid (15.37 mg, 0.038 mmol) and **9h** in DMF (0.5 mL) was treated with 2,6-lutidine (8.75 µl, 0.075 mmol) and HATU (19.03 mg, 0.050 mmol) and the mixture was sirred at rt for 1h. LCMS (M+H=1311.2) shows formation of product. The reaction was worked up with EtOAc/sat. sq. $NaHCO_3$ and taken crude to next step. A solution of **10b** (32.8 mg, 0.025 mmol) in DMF (0.5 mL) was treated with $Pd(PPh_3)_4$ (2.89 mg, 2.500 µmol) and morpholine (4.36 µl, 0.050 mmol). After 1h LCMS shows new peak (pdt does not ionize). This solution was treated with

diethylamine (0.131 mL, 1.250 mmol) and LCMS at 30 min showed the formation of product (M+H=1046.3). The reaction was diluted with dmso (0.5 ml) and purified on Shimadzu LC-20AP preparative HPLC with XBridge Prep C18 5mm OBD 10x150 mm column eluting with 0-95% H_2O/MeCN (0.05% formic acid). The product containing fraction at 9.2 min was lyophilized to obtain the 7 mg of desired product **10c** as purple solid.

A solution of **10c** (7 mg, 6.68 μmol) in DMF (0.5 mL) was treated with 2,5-dioxopyrrolidin-1-yl 6-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)hexanoate (3.09 mg, 10.02 μmol) and stirred for 3h at which LCMS (M+H=1241.3) shows the completion of reaction. The reaction was diluted with dmso (0.5 ml) and purified on Shimadzu LC-20AP preparative HPLC with XBridge Prep C18 5mm OBD 10x150 mm column eluting with 0-95% H2O/MeCN (0.05% formic acid). The product containing fraction at 14 min was lyophilized to obtain 4 mg (1H-NMR purity >90%) of desired product **10** as purple solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ 13.19 (s, 1H), 11.69 (s, 1H), 10.01 (d, J = 4.6 Hz, 1H), 9.32 (s, 1H), 8.53 (s, 1H), 8.34 (s, 1H), 8.32 – 8.10 (m, 2H), 8.00 (d, J = 8.0 Hz, 1H), 7.92 – 7.66 (m, 2H), 6.99 (s, 2H), 6.68 (d, J = 5.1 Hz, 1H), 6.20 – 5.86 (m, 3H), 5.55 – 5.28 (m, 4H), 5.16 (d, J = 4.9 Hz, 1H), 5.07 (dd, J = 4.6, 1.7 Hz, 1H), 4.61 (d, J = 5.8 Hz, 2H), 4.49 – 4.10 (m, 3H), 3.94 – 3.71 (m, 3H), 2.96 (q, J = 6.5 Hz, 2H), 2.35 – 2.16 (m, 2H), 2.10 (td, J = 7.3, 4.1 Hz, 2H), 2.05 – 1.92 (m, 2H), 1.85 (dddd, J = 15.7, 8.8, 5.8, 3.4 Hz, 2H), 1.55 – 1.39 (m, 4H), 1.32 (d, J = 6.4 Hz, 3H), 1.20 (dt, J = 15.1, 7.0 Hz, 2H), 0.84 (dd, J = 16.2, 6.8 Hz, 6H). LCMS calculated for $C_{58}H_{59}F_3N_{10}O_{16}S$: 1241.3 (M+H); found 1241.3.


FmocHN
OH
11a
NaHCO3
11b
PNP-CO3
Hunig's base
11c
NO2
11d
NH2
Me
OH
Hunig's base
11f
1. Pd(PPh3)4
2. HATU
11g
1. diethylamine
2. MC-OSu
11

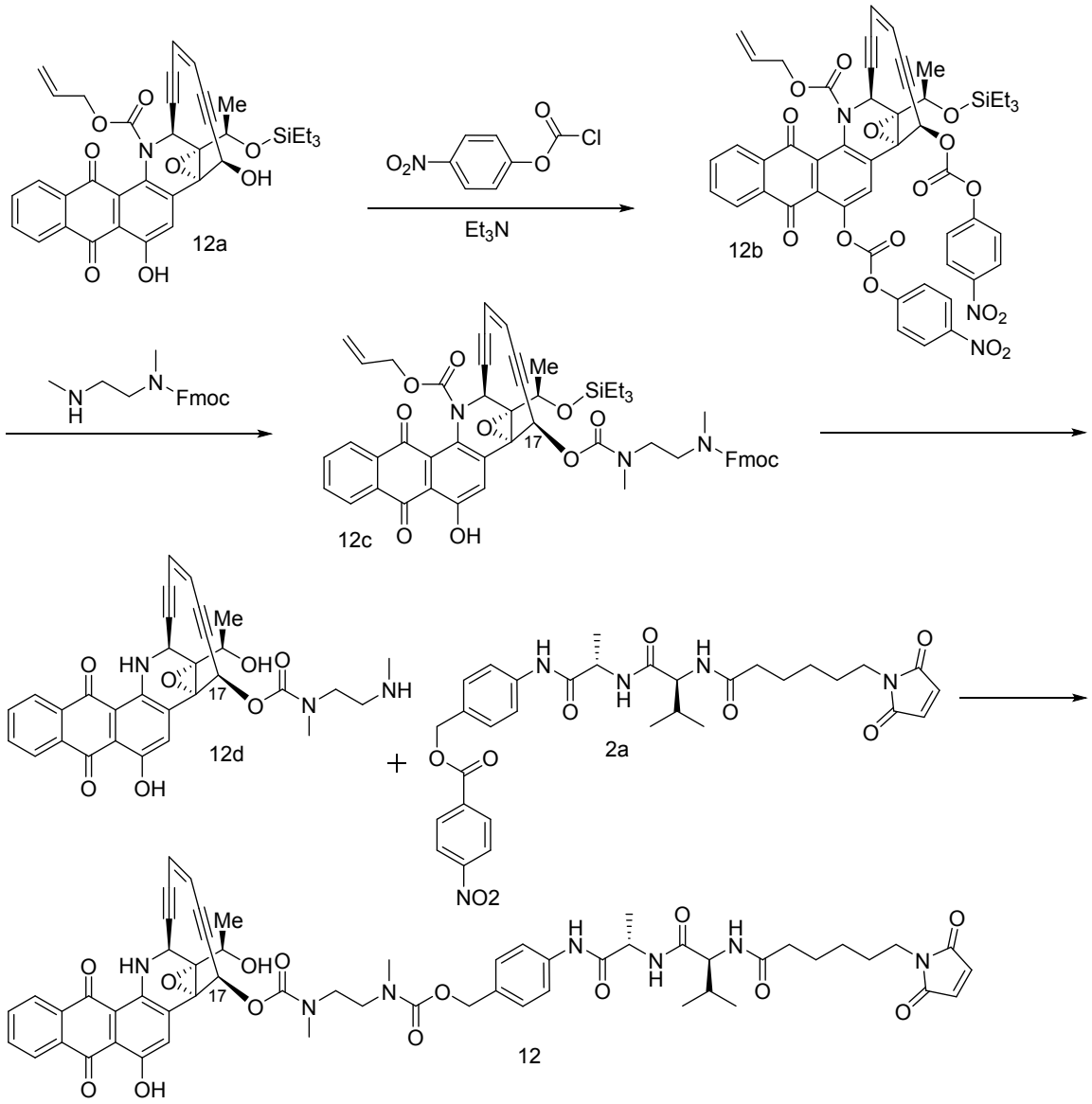
Synthesis of compound 11: To a solution of (S)-allyl 3-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)-4-((4-(hydroxymethyl)phenyl)amino)-4-oxobutanoate **11a** (1266 mg, 2.53 mmol) in THF (2 mL) was added diethylamine (1.322 mL, 12.65 mmol) and stirred for 1h. The solvent and base was evaporated and the crude product was dissolved in THF (2 mL)/Water (2 mL) and added (S)-2,5-dioxopyrrolidin-1-yl 2-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)-3-methylbutanoate (1104 mg, 2.53 mmol) followed by sodium bicarbonate (425 mg, 5.06 mmol). The reaction was stirred overnight. The solvent was evaporated and the crude product was purified on combiflash (40 g silical gel) eluting with 0-100% MeOH/CH_2Cl_2 to yield desired alcohol **11b** (M+H=600.2) as white solid.

The alcohol intermedaite **11b** was dissolved in THF (2 mL) and was treated with bis(4-nitrophenyl) carbonate (1539 mg, 5.06 mmol) followed by Hunig's base (1.326 mL, 7.59 mmol) and stirred at rt for 3h. The solvent was evaporated and the crude product was purified on combiflash (40 g silical gel) eluting with 0-100% MeOH/CH_2Cl_2 to yield desired product **11c** as yellowish solid. LCMS calculated for $C_{41}H_{40}N_4O_{11}$: 765.2 (M+H); found 765.0.

To a solution of (S)-allyl 3-((S)-2-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)-3-methylbutanamido)-4-((4-((((4-nitrophenoxy)carbonyl)oxy)methyl)phenyl)amino)-4-oxobutanoate **11c** (20.92 mg, 0.027 mmol) and **11d** (11 mg, 0.023 mmol) in DMF (0.5 ml) was added 2,6-lutidine (5.31 µl, 0.046 mmol) and stirred for 3h at which LCMS (M+H=1108.3) shows the completion of reaction. The reaction was quenched by the addition of sat.aq. $NaHCO_3$ solution and extracted with EtOAc (3x10 mL). Dried over Na_2SO_4 and solvent was evaporated and taken crude to next step.

A mixture of **11f** (0.039 mmol), morpholine (6.80 µl, 0.078 mmol) and $Pd(PPH_3)_4$ (18.03 mg, 0.016 mmol) was stirred at 0 °C for one hour at rt for 1h. LCMS (M+H=1329.5) shows removal of all 3 alloc groups. The reaction was diluted with 2 ml dmso and purified on Shimadzu preparative HPLC using XBridge Prep C18 5mm OBD 10x150 mm column eluting with 0-95% H2O/MeCN (0.05% formic acid). The product containing fractions

were collected and passed through $NaHCO_3$ resin, extracted with CH_2CH_2, dried over Na_2SO_4 and concentrated to obtain 8.4 mg of amino carboxylic aci intermediate. To a solution of seco-uncialamycin linker analogue (8.4 mg, 6.32 μmol) in DMF (3 mL) was added Hunig's base (0.011 mL, 0.063 mmol) followed by HATU (12.01 mg, 0.032 mmol). LCMS (M+H=1311.4) after 5 min shows the completion of reaction. The reaction was worked up with sat.aq. $NaHCO_3$ solution/EtOAc and taken crude to next step. To a crude solution of mcarocycle was added diethylamine (1.0 ml, 9571 μmol) and stirred for 1h. LCMS (M+H=1089.4) shows the completion of reaction. The base was evaporated and the crude product was purified on Shimadzu preparative HPLC using XBridge Prep C18 5mm OBD 10x150 mm column eluting with 0-95% H_2O/MeCN (0.05% formic acid). The product containing fractions were collected to obtail 1.5 mg solid. To a solution of macrocyclic uncialamycin linker analogue (1.5 mg, 1.377 μmol) in DMF (250 μL) was added 2,5-dioxopyrrolidin-1-yl 6-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)hexanoate (0.849 mg, 2.75 μmol) and 2,6-lutidine (0.481 μL, 4.13 μmol) and stirred for 1h. LCMS (M+H=1282.5) shows the completion of reaction. The reaction was diluted to 1 ml dmso and the crude product was purified on Shimadzu preparative HPLC using XBridge Prep C18 5mm OBD 10x150 mm column eluting with 0-95% H2O/MeCN (0.05% formic acid). The product containing fractions were collected to obtail 0.9 mg (LCMS purity = 95.5%) of **11** as purole solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.70 (s, 3H), 9.93 – 9.38 (m, 1H), 8.88 (s, 1H), 8.53 (s, 1H), 8.33 (s, 1H), 8.16 – 7.92 (m, 2H), 7.82 – 7.69 (m, 1H), 7.63 (d, J = 8.3 Hz, 1H), 7.59 – 7.51 (m, 2H), 7.30 (d, J = 8.2 Hz, 1H), 6.99 (s, 1H), 6.71 (d, J = 11.6 Hz, 1H), 6.12 – 5.84 (m, 1H), 5.38 (dd, J = 6.0, 2.1 Hz, 3H), 5.18 (s, 0H), 5.08 – 4.92 (m, 3H), 4.42 (s, 1H), 4.33 (s, 1H), 4.08 (d, J = 7.6 Hz, 1H), 3.91 – 3.68 (m, 6H), 2.68 (s, 1H), 2.36 – 2.29 (m, 1H), 2.16 (q, J = 7.4 Hz, 1H), 2.08 (s, 1H), 2.03 – 1.91 (m, 5H), 1.89 – 1.80 (m, 5H), 1.70 (tddd, J = 16.3, 8.0, 6.5, 3.9 Hz, 7H), 1.53 – 1.42 (m, 3H), 1.34 (dd, J = 13.1, 6.7 Hz, 4H), 1.24 (s, 1H), 1.21 – 1.10 (m, 1H), 0.86 (dd, J = 23.0, 6.8 Hz, 9H).LCMS calculated for $C_{69}H_{72}N_9O_{16}$: 1283.3 (M+H); found 1283.5.



Synthesis of compound 12: A solution of 4-nitrophenyl carbonochloridate (0.126 g, 0.627 mmol) in THF (1 mL) was added to a cooled solution of **12a** (0.1 g, 0.157 mmol) and triethylamine (0.131 ml, 0.941 mmol) in THF/DCM 2:1 (6 mL) at 0 °C. The cloudy orange mixture was stirred for 3 h while warming to rt. The reaction mixture was concentrated. The solid was purified on silica 80 g ISCO column using 0- 45 % ethyl acetate in hexanes. The desired fractions were concentrated to give **12b** bright golden yellow solid. LCMS calculated for $C_{50}H_{41}N_3O_{16}Si$: 968.9 (M+H); found 968.8. ^{1}H NMR (500 MHz, Chloroform-*d*) δ 8.44 – 8.33 (m, 2H), 8.23 – 8.17 (m, 1H), 7.85 – 7.78 (m, 1H), 7.72 – 7.65 (m, 3H), 7.52 – 7.45 (m, 3H), 6.96 – 6.90 (m, 1H), 6.44 (t, *J* = 1.0 Hz, 1H), 6.04 (d, *J* = 1.7 Hz, 1H), 5.90 – 5.74 (m, 3H), 5.73 – 5.61 (m, 2H), 5.08 (dq, *J* = 17.2, 1.5 Hz, 1H), 5.01

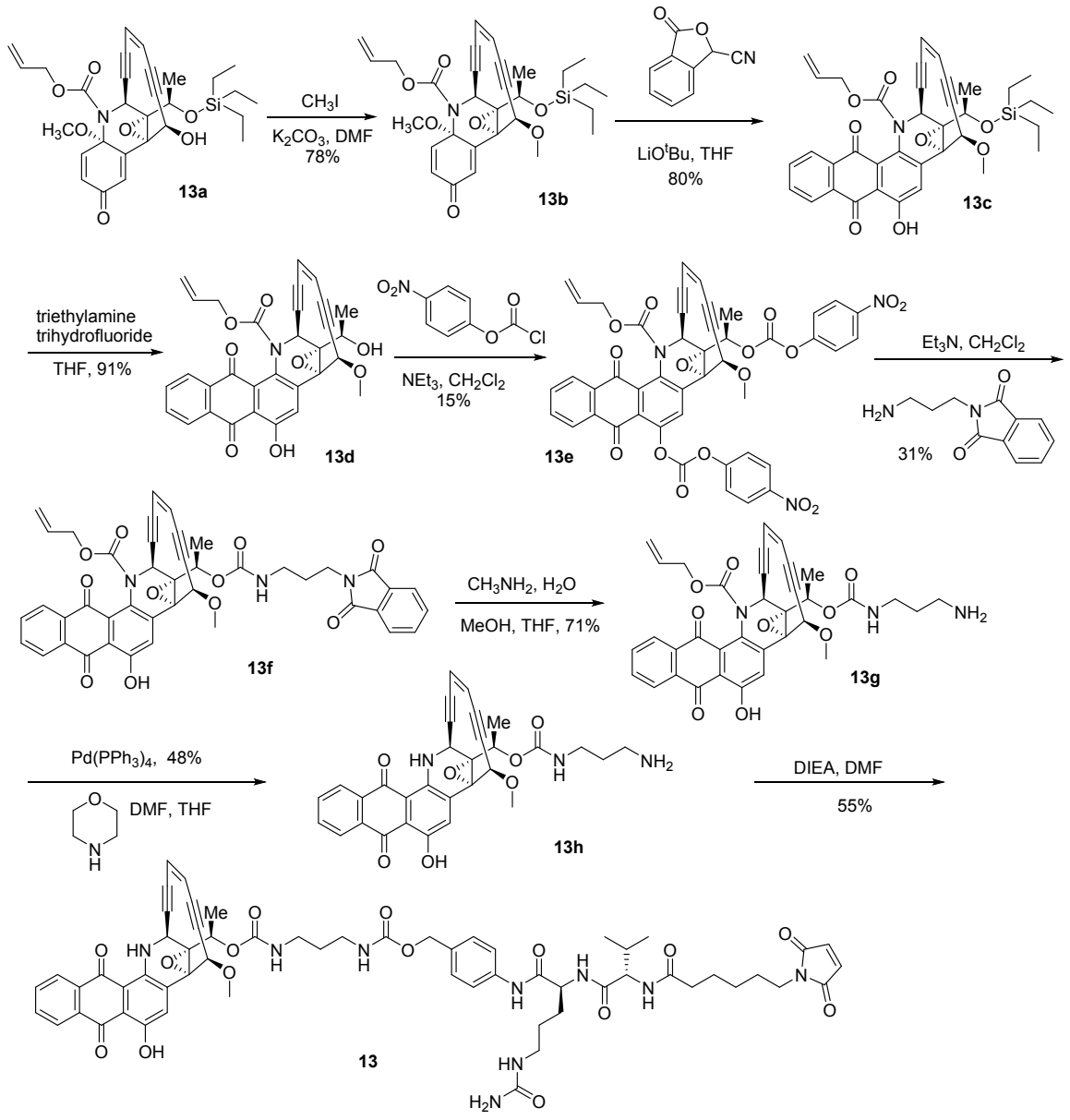
(dq, *J* = 10.4, 1.2 Hz, 1H), 4.85 – 4.70 (m, 1H), 4.67 – 4.48 (m, 3H), 4.15 (q, *J* = 7.2 Hz, 1H), 1.32 – 1.25 (m, 2H), 1.01 (td, *J* = 7.9, 3.3 Hz, 5H), 0.73 – 0.63 (m, 9H).

Hunig's base (0.038 mL, 0.217 mmol) was added to a mixture of **12b** (0.035 g, 0.036 mmol) and (9H-fluoren-9-yl)methyl methyl(2-(methylamino)ethyl)carbamate trifluoromethyl carbonate (0.035 g, 0.080 mmol) in THF for 2 h and concentrated. The orange oily residue was purified on silica gel (24 g ISCO column) using 0-45 % ethyl acetate in hexanes. The desired fractions were concentrated to give **12c** as orange oil. LCMS calculated for $C_{56}H_{56}N_3O_{11}Si$: 974.3 (M+H); found 974.8. ^{1}H NMR (500 MHz, Chloroform-*d*) δ 8.21 (dd, *J* = 27.2, 8.2 Hz, 4H), 7.84 – 7.75 (m, 6H), 7.65 – 7.58 (m, 3H), 7.41 (t, *J* = 7.2 Hz, 3H), 7.34 (d, *J* = 7.4 Hz, 3H), 6.92 (d, *J* = 8.6 Hz, 1H), 5.67 (s, 2H), 4.69 (s, 1H), 4.61 – 4.54 (m, 2H), 4.49 – 4.41 (m, 2H), 3.60 (s, 1H), 3.52 (p, *J* = 6.6 Hz, 1H), 3.20 (s, 1H), 3.16 – 3.07 (m, 3H), 3.06 – 2.97 (m, 4H), 2.90 (d, *J* = 13.7 Hz, 2H), 2.73 – 2.64 (m, 2H), 1.83 – 1.70 (m, 2H), 1.69 – 1.56 (m, 2H), 1.55 – 1.43 (m, 2H), 0.98 (t, *J* = 7.8 Hz, 6H).

Triethylamine trihydrofluoride (0.074 ml, 0.452 mmol) added to a solution of **12c** (0.022 g, 0.023 mmol) in THF (2mL) and the reaction mixture was stirred at rt for 5 h. LCMS (860.3) show the completion of reaction. The reaction was quenched with sat. $NaHCO_3$ and extracted with ethyl acetate (10 mL), dried over Na_2SO_4 and concentrated to give a golden yellow film which was taken crude to next step.

Pyrrolidine (0.663 ml, 0.066 mmol) and $Pd(PPh_3)_4$ (1.277 mg, 1.105 µmol) were added to a solution of crude product from previous step (0.019 g, 0.022 mmol) in DMF (1 ml) and the purple mixture was stirred at rt for 5 h. LCMS (554.2, M+H) shows the completion of reaction. The mixture was purified on Sunfire PREP OBD^{TM} 5 µm (19 x 100 mm) reversed phase column using 25-100% of B (95% acetonitrilel/ 5% water / 0.05% Formic acid) in A (5% aetonitrile / 95% water /0.05% Formic acid) over 15 min. The desired fraction was lyophylized down to give 5 mg of **12d** as purple solid.

Hunig's base (3.94 µl, 0.023 mmol) was added to a mixture of **12d** (0.005 g, 9.03 µmol) and 4-((S)-2-((S)-2-(6-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)hexanamido)-3-methylbutanamido)-5-ureidopentanamido)benzyl (4-nitrophenyl) carbonate (6.66 mg, 9.03 µmol) in DMF (0.4 mL) was stirred at rt for 1 h under nitrogen atmosphere and monitored by mass spec. The mixture was purified on Sunfire PREP OBD^{TM} 5 µm (19 x 100 mm) reversed phase column using 25-100% of B (95% acetonitrilel/ 5% water / 0.05% Formic acid) in A (5% aetonitrile / 95% water /0.05% Formic acid) over 25 min. The desired fraction was lyophylized down to give 2.2 mg (LCMS purity = 87%) of **12** as pink solid. LCMS calculated for $C_{57}H_{60}N_7O_{14}$: 1066.4 (M+H); found 1066.4.



Preparation of compound **13:** K_2CO_3 (0.771 g, 5.58 mmol) was added to a mixture of compound **13a**[1] (1 g, 1.860 mmol) and methyl iodide (0.347 mL, 5.58 mmol) in DMF (7 mL) at rt. The reaction mixture was stirred at rt over the weekend. The reaction was quenched by addition of $NaHCO_3$ solution. The aqueous solution was extracted with EtOAc. The combined organic layers were dried, filtered, and concentrated. The crude product was purified on a silica gel column with 0-100% EtOAc in hexanes to provide compound **2** as a white solid in 78% yield. LC-MS *m/z* 574.1 $[M+Na]^+$.

LiOtBu (1M in THF, 3.53 mL, 3.53 mmol) was added to a solution of 3-oxo-1,3-dihydroisobenzofuran-1-carbonitrile (0.562 g, 3.53 mmol) in THF (16 mL) at -78 °C. After the reaction mixture was stirred at -78 °C for 20 mim, compound **13b** (0.65 g, 1.178 mmol) in THF (16 mL) was added to the reaction mixture at -78 °C. The reaction mixture was allowed to warm up to rt, and stirred at rt for 1h. The reaction was quenched by addition of $NaHCO_3$ solution. The aqueous solution was extracted with EtOAc. The combined organic layers were dried, filtered, and concentrated. The crude product was purified on a silica gel column with 0-100% EtOAc in hexanes to provide compound **13c** as a brick red solid in 80% yield. LC-MS *m/z* 652.2 $[M+H]^+$.

Triethylamine trihydrohydride (4 mL, 24.54 mmol) in THF (4 mL) was added to a solution of compound **13c** (0.613 g, 0.941 mmol) in THF (12 mL) at rt. After the reaction mixture was stirred at rt for 1.5 h, it was poured to sat. $NaHCO_3$ solution. The aqueous solution was extracted with EtOAc. The combined organic layers were dried, filtered, and concentrated. The crude product was purified on a silica gel column with 0-100% EtOAc in hexanes to provide compound **13d** as a yellow solid in 91% yield. LC-MS *m/z* 538.0 $[M+H]^+$.

Triethylamine (0.130 mL, 0.932 mmol) was added to a solution of 4-nitrophenyl carbonochloridate (0.188 g, 0.932 mmol) and compound **13d** (0.167 g, 0.311 mmol) in CH_2Cl_2 (5 mL) at 0 °C. After the reaction mixture was stirred at at 0 °C for 1 h, the cooling bath was removed and the reaction mixture was stirred at rt for 4 h. The reaction was quenched by addition of the $NaHCO_3$ solution. The aqueous solution was extracted with EtOAc, and the combined organic layers were dried, filtered, and concentrated. The crude

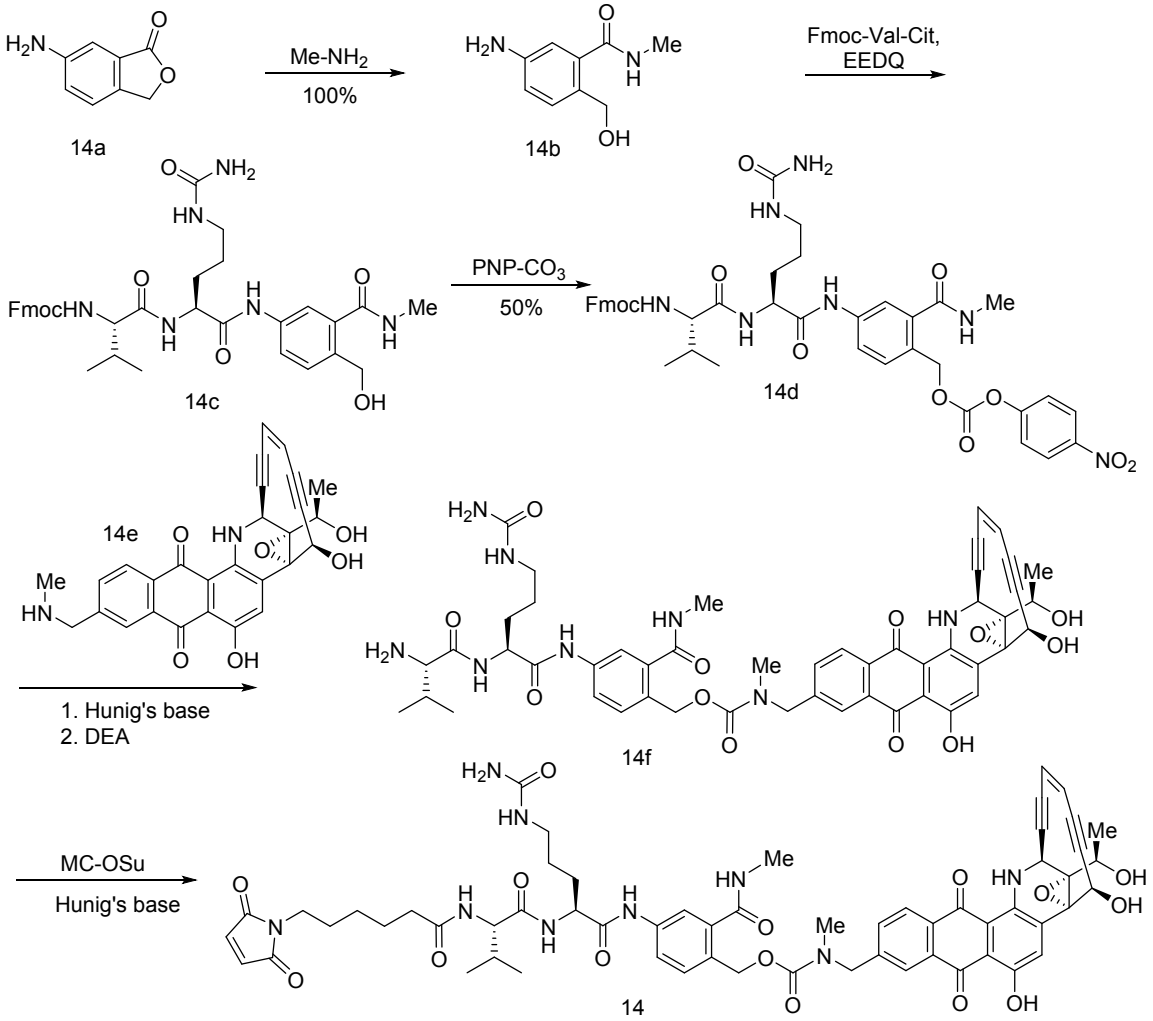
product was purified on a silica gel column with 0-100% EtOAc in hexanes to provide compound **13e** as a yellow solid in 15% yield. LC-MS *m/z* 868.1 $[M+H]^+$.

Triethylamine (0.019 mL, 0.137 mmol) was added to a mixture of compound **13e** (39.7 mg, 0.046 mmol) and *N*-(3-aminopropyl)phthalimide hydrochloride (33.0 mg, 0.137 mmol) in CH_2Cl_2 (4 mL) at rt. After the reaction mixture was stirred at rt for 1h, The reaction was quenched by addition of water. The aqueous solution was extracted with EtOAc. The combined organic layers were dried, filtered, and concentrated. The crude product was purified on a silica gel column with 0-100% EtOAc in hexanes to provide compound **13f** as a red solid in 31% yield. LC-MS *m/z* 768.0 $[M+H]^+$.

To a solution of compound **13f** (10.7 mg, 0.014 mmol) in MeOH (1 mL) and THF (0.5 ml) at 0 °C was added CH_3NH_2 in water (40wt%, 0.040 mL, 0.460 mmol). The reaction was allowed to proceed at 0 °C for 30 min. The cold bath was removed and the reaction was stirred for 2 h. The reaction was quenched by addition of 10 mL 1:1 (v/v) mixture of water containing 0.05% formic acid and acetonitrile. The crude product was purified by preparative HPLC (C18 column) using acetonitrile and water containing 0.05% formic acid as the mobile phases. The product-containing fractions were pooled, frozen, and left on the lyophilizer. The compound **13g** was obtained as a red solid in 71% yield. LC-MS *m/z* 638.1 $[M+H]^+$.

Morpholine (5.83 µl, 0.067 mmol) was added to a mixture of compound **13g** (15.00 mg, 0.028 mmol) and $Pd(PPh_3)_4$ (3.22 mg, 2.79 µmol) in DMF (0.5 mL) and THF (0.5 mL) at 0 °C. After the reaction mixture was stirred at 0 °C for 1 h, the reaction mixture was allowed to warm to rt, and stirred at rt for 1h. The reaction was quenched by addition of 4 mL 1:1 (v/v) mixture of water (0.05% formic acid) and acetonitrile. The crude product was purified by preparative HPLC (on C18 reverse phase column) using acetonitrile and water containing 0.05% formic acid as the mobile phases. The product-containing fractions were pooled, frozen, and left on the lyophilizer. Compound **13h** was obtained as a purple solid in 48% yield. LC-MS *m/z* 454.0 $[M+H]^+$.

DIEA (0.692 µl, 3.97 µmol) was added to a solution of compound **13h** (2.2 mg, 3.97 µmol) and compound **2a** (2.93 mg, 3.97 µmol) in DMF (0.5 mL) at rt. After the reaction mixture was stirred at rt for 20 min, the reaction was quenched by addition of 4 mL 1:1 (v/v) mixture of water (0.05% formic acid) and acetonitrile. The crude product was purified by preparative HPLC (on C18 reverse phase column) using acetonitrile and water containing 0.05% formic acid as the mobile phases. The product-containing fractions were pooled, frozen, and left on the lyophilizer. Compound **13** was obtained as a purple solid (LCMS purity = 95.5%) in 55% yield. LC-MS *m/z* 1152.3 $[M+H]^+$.



Synthesis of compound 14: A mixture of 6-aminoisobenzofuran-1(3H)-one **14a** (1.0 g, 6.70 mmol) and methanamine (2M MeOH) (16.76 mL, 33.5 mmol) in CH_2Cl_2 (5 mL) was stirred for 3 days. LCMS ($M+H-H_2O$=163.0) shows the presence of eliminated product.

The solvent was evaporated to obtain colorless paste. LCMS calculated for $C_9H_{12}N_2O_2$: (M+H-H_2O=163.0); found 163.0.

To a solution of (S)-2-((S)-2-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)-3-methylbutanamido)-5-ureidopentanoic acid (1.681 g, 3.39 mmol) and 5-amino-2-(hydroxymethyl)-N-methylbenzamide **14b** (1.22 g, 6.77 mmol) in THF (5 mL) was added ethyl 2-ethoxyquinoline-1(2H)-carboxylate (1.674 g, 6.77 mmol) and the mixture was stirred at rt overnight. LCMS (M+H-H_2O= 659.3) shows product. The reaction was directly purified on combiflash using 40 g silical gel eluting with 0-100% MeOH/CH_2Cl_2 to yield 1.2g of **14c** as white solid. LCMS calculated for $C_{35}H_{42}N_6O_7$: (M+H-H_2O= 659.2); found 659.3.

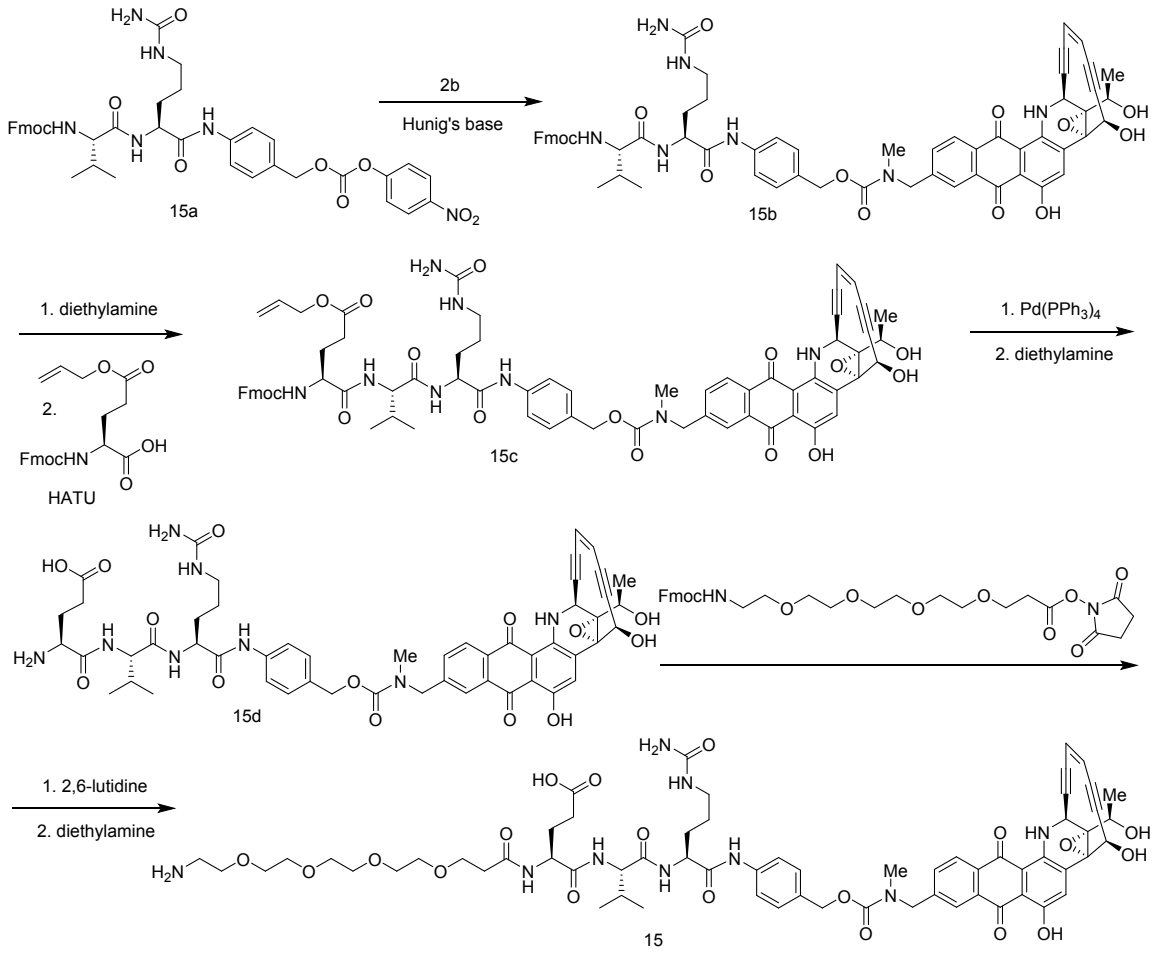
To a solution of (9H-fluoren-9-yl)methyl ((S)-1-(((S)-1-((4-(hydroxymethyl)-3-(methylcarbamoyl)phenyl)amino)-1-oxo-5-ureidopentan-2-yl)amino)-3-methyl-1-oxobutan-2-yl)carbamate **14c** (206 mg, 0.313 mmol) in DMF (1 mL) was added bis(4-nitrophenyl) carbonate (190 mg, 0.625 mmol) followed by Hunig's base (0.164 mL, 0.938 mmol). The reaction was stirred at rt for 3h at which LCMS (M+H = 824.3) shows product formation. It was directly injected to combiflash (40 g silica gel) and eluted with 0-100% MeOH/CH_2Cl_2 to yield 130 mg of **14d** as white solid.

To a solution of **14e** (26 mg, 0.054 mmol) in DMF (0.5 mL) was added (9H-fluoren-9-yl)methyl ((S)-3-methyl-1-(((S)-1-((3-(methylcarbamoyl)-4-((((4-nitrophenoxy)carbonyl)oxy)methyl)phenyl)amino)-1-oxo-5-ureidopentan-2-yl)amino)-1-oxobutan-2-yl)carbamate **14d** (66.6 mg, 0.081 mmol) follwed by 2,6-lutidine (0.013 mL, 0.108 mmol). The reaction was stirred at rt for 3h at which LCMS (M+H=1167.3) shows the completion of reaction. The reaction was worked up with sat. aq. $NaHCO_3$/EtOAc and taken crude to next step.

To the crude material from above (63.0 mg, 0.054 mmol) in DMF (0.5 mL) was added diethylamine (0.056 mL, 0.540 mmol). LCMS (M+H= 945.4) after 30 min shows the completion of reaction. The reaction was diluted with dmso (0.5 ml) and purified on

Shimadzu LC-20AP preparative HPLC with XBridge Prep C18 5mm OBD 10x150 mm column eluting with 0-95% H_2O/MeCN (0.05% formic acid). The product containing fraction at 11 min was lyophilized to obtain14 mg of **14f** as purple solid.

Stirred a solution of **14f** (14 mg, 0.015 mmol), 2,5-dioxopyrrolidin-1-yl 6-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)hexanoate (6.85 mg, 0.022 mmol) and 2,6-lutidine (5.18 µl, 0.044 mmol) for 3h at which LCMS (M+H=1138.4) shows the completion of reaction. The reaction was diluted with dmso (0.5 ml) and purified on Shimadzu LC-20AP preparative HPLC with XBridge Prep C18 5mm OBD 10x150 mm column eluting with 0-95% H_2O/MeCN (0.05% formic acid). The product containing fraction at 13.5 min was lyophilized to obtain 7 mg (1H-NMR purity >90%) of **14** as purple solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ 13.16 (s, 1H), 11.70 (s, 0H), 10.15 (s, 1H), 10.01 (d, J = 4.5 Hz, 1H), 8.52 (d, J = 8.6 Hz, 1H), 8.32 (s, 1H), 8.24 (d, J = 8.0 Hz, 1H), 8.09 (t, J = 12.0 Hz, 2H), 7.81 (d, J = 8.6 Hz, 1H), 7.69 – 7.49 (m, 1H), 7.45 – 7.20 (m, 1H), 7.00 (s, 2H), 6.71 (d, J = 4.8 Hz, 1H), 6.36 – 5.89 (m, 3H), 5.57 – 5.31 (m, 3H), 5.17 (t, J = 6.7 Hz, 3H), 5.07 (dd, J = 4.6, 1.6 Hz, 1H), 4.65 (s, 2H), 4.34 (dd, J = 13.6, 7.5 Hz, 2H), 4.19 (t, J = 7.7 Hz, 1H), 3.88 – 3.70 (m, 1H), 3.71 – 3.49 (m, 1H), 2.97 (d, J = 13.8 Hz, 2H), 2.87 (s, 3H), 2.72 (s, 3H), 2.15 (qt, J = 14.2, 7.3 Hz, 2H), 2.02 – 1.89 (m, 1H), 1.84 – 1.73 (m, 1H), 1.73 – 1.64 (m, 0H), 1.58 – 1.39 (m, 4H), 1.32 (d, J = 6.4 Hz, 3H), 1.19 (q, J = 7.7 Hz, 2H), 0.84 (dd, J = 13.8, 6.6 Hz, 6H). LCMS calculated for $C_{59}H_{63}N_9O_{15}$: (M+H= 1138.4); found 1138.4.



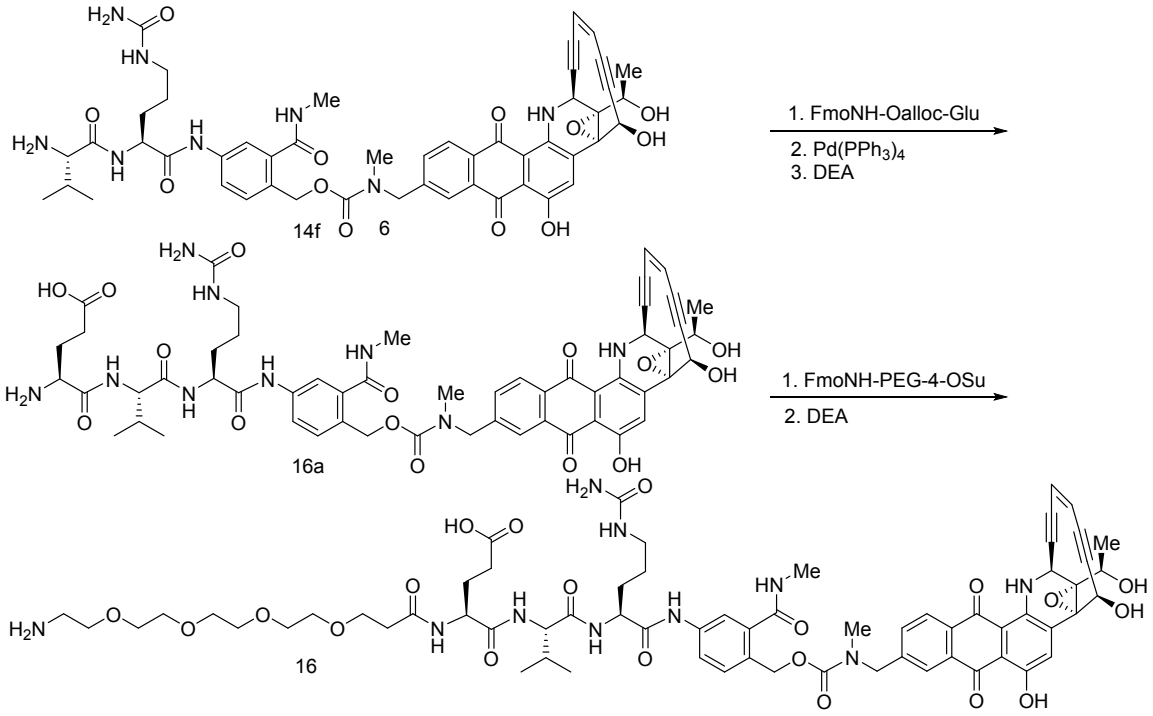
Synthesis of 15: Stirred a mixture of (9H-fluoren-9-yl)methyl ((S)-3-methyl-1-(((S)-1-((4-((((4-nitrophenoxy)carbonyl)oxy)methyl)phenyl)amino)-1-oxo-5-ureidopentan-2-yl)amino)-1-oxobutan-2-yl)carbamate **15a** (124 mg, 0.162 mmol), **2b** (78 mg, 0.162 mmol) and 2,6-lutidine (94 microliter, 0.8 mmol) in 0.5 mL of DMF was stirred overnight. LCMS (M+H=1110.4) showed the completion of reaction which was worked with sat. aq. $NaHCO_3$/EtOAc. Layers separated, organic layer dried over Na_2SO_4, filtered, concentrated and taken crude to next step.

To a solution of **15b** (180 mg, 0.162 mmol) in DMF (1 mL) was added diethylamine (0.085 mL, 0.810 mmol) and stirred for 30 min. LCMS (M+H=888.4) showed the completion of reaction. The crude product was purified on reverse phase colbiflash using 50 g C-18 column eluting with 0-7-% MeCN/water with 0.05% formic acid. The desired fractions were collected and lyophilized to obtain 60 mg of free amine. A mixture of (S)-2-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)-5-(allyloxy)-5-oxopentanoic acid (27.7 mg, 0.068 mmol) and free amine from previous step (60 mg, 0.068 mmol) was treated with 2,6-

lutidine (0.024 mL, 0.203 mmol) and 2-(3H-[1,2,3]triazolo[4,5-b]pyridin-3-yl)-1,1,3,3-tetramethylisouronium hexafluorophosphate(V) (51.4 mg, 0.135 mmol) in 0.5 mL DMF and stirred for 1h. LCMS (M+H=1279.6) shows the formation of product. The reaction was worked up with EtOAc/Sat. sq. $NaHCO_3$ and taken crude to next step.

To the reaction from above step was added morpholine (0.012 mL, 0.135 mmol) followed by Pd $(PPh_3)_4$ (7.81 mg, 6.76 μmol) and stirred for 30 min. LCMS (M+H=1240.0) shows the deprotection of alloc group. To this mixture was added diethylamine (0.035 mL, 0.338 mmol) and stirred for 30 min at which LCMS (M+H=1017.6) shows the completion of reaction. The reaction was purified on reverse phase combiflash using 30 g C-18 column eluting with 0-50% MeCN/Water (0.05% formic acid). The desired fractions were collected and lyophilized to obtain 21 mg of **15d** as purple solid.

To a mixture **15d** (21 mg, 0.021 mmol) and 2,3,5,6-tetrafluorophenyl 1-(9H-fluoren-9-yl)-3-oxo-2,7,10,13,16,19,22,25,28-nonaoxa-4-azahentriacontan-31-oate **15d** (16.76 mg, 0.021 mmol) was added 2,6-lutidine (7.21 μl, 0.062 mmol) in 0.5 mL DMF and stirred for 1h. LCMS (M/2+H= 829.5) shows the completion of reaction. To this reaction was added diethylamine (10.79 μl, 0.103 mmol) and stirred for 30min at whic LCMS (M/2+H=721.3) shows the completion of reaction. The reaction was diluted with dmso (0.5 ml) and purified on Shimadzu LC-20AP preparative HPLC with XBridge Prep C18 5mm OBD 10x150 mm column eluting with 0-95% H2O/MeCN (0.05% formic acid). The product containing fraction at 12.0 min was lyophilized to obtain 9 mg (LCMS purity >90%) of **15** as purple solid. LCMS calculated for $C_{63}H_{78}N_9O_{19}$: (M+H= 1264.3); found 1264.3.

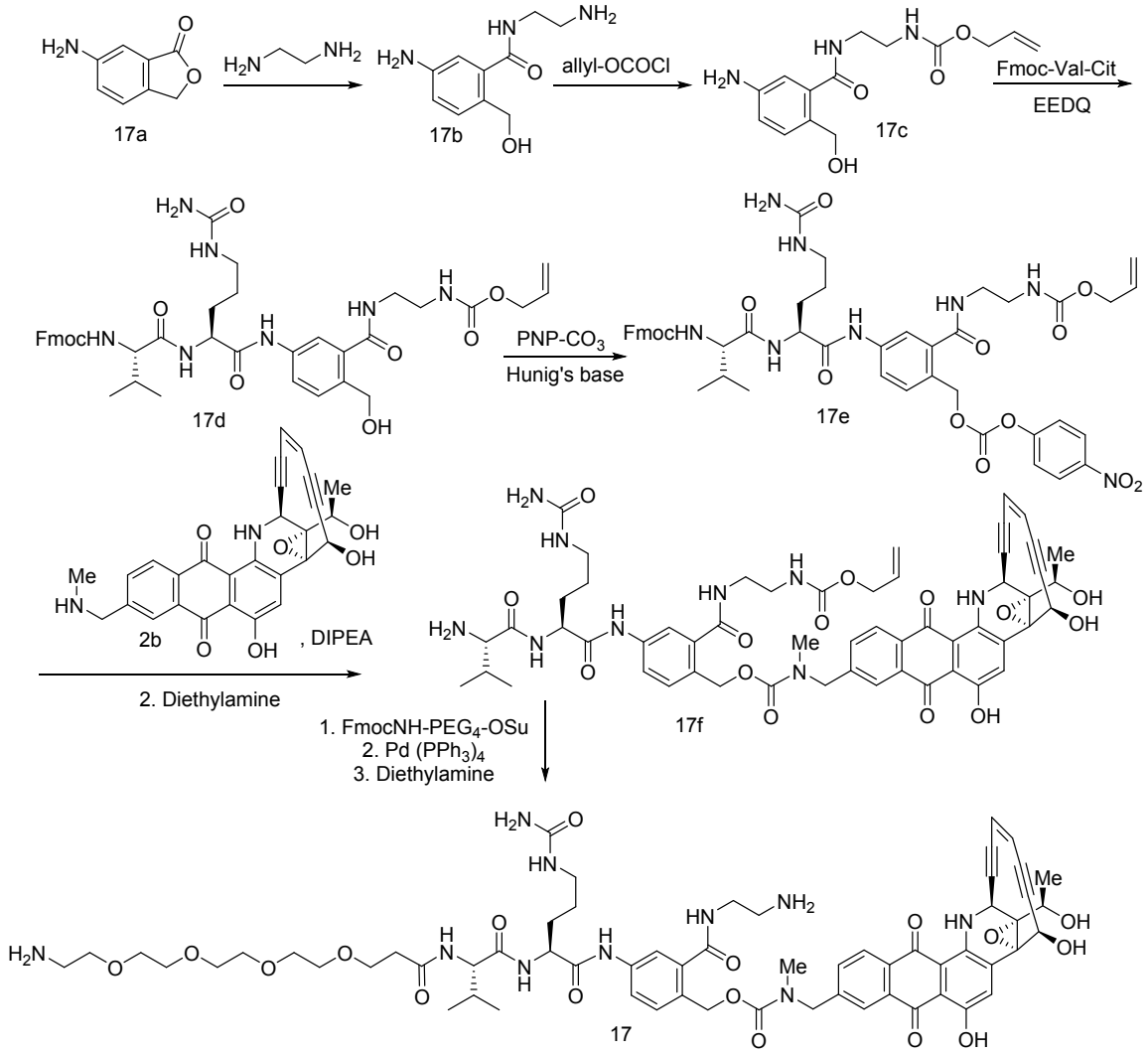


Synthesis of compound 16: A mixture of (S)-2-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)-5-(allyloxy)-5-oxopentanoic acid (38.1 mg, 0.093 mmol) and **14f** (88 mg, 0.093 mmol) was treated with 2,6-lutidine (0.033 mL, 0.279 mmol) and 2-(3H-[1,2,3]triazolo[4,5-b]pyridin-3-yl)-1,1,3,3-tetramethylisouronium hexafluorophosphate(V) (70.8 mg, 0.186 mmol) and stirred for 1h. LCMS (M+H=1279.6) shows the formation of product. The reaction was worked up with EtOAc/Sat. sq. $NaHCO_3$ and taken crude to next step.

To the reaction from above step was added morpholine (0.016 mL, 0.186 mmol) followed by $Pd(PPh_3)_4$ (10.76 mg, 9.31 μmol) and stirred for 30 min. LCMS (M+H=1240.0) shows the deprotection of alloc group. To this mixture was added diethylamine (0.049 mL, 0.466 mmol) and stirred for 30 min at which LCMS (M+H=1017.6) shows the completion of reaction. The reaction was purified on reverse-phase combiflash (40 g c-18 column) eluting with 0-100% water in acetonitrile (0.05% formic acid) to provide 54 mg of **16a** as purple solid.

A solution of **16a** (10.2 mg, 9.50 μmol) and 2,5-dioxopyrrolidin-1-yl 1-(9H-fluoren-9-yl)-3-oxo-2,7,10,13,16-pentaoxa-4-azanonadecan-19-oate (5.55 mg, 9.50 μmol) in DMF (0.5

mL) was treated with 2,6-lutidine (3.32 µl, 0.028 mmol) and stirred for 3h. LCMS (M+H=1357.5) shows the completion of reaction. To this reaction was added diethylamine (0.020 mL, 0.190 mmol) and stirred for 30 min at which LCMS (M+H=1135.4) shows the completion of reaction. The reaction was diluted with dmso (0.5 ml) and purified on Shimadzu LC-20AP preparative HPLC with XBridge Prep C18 5mm OBD 10x150 mm column eluting with 0-95% H_2O/MeCN (0.05% formic acid). The product containing fraction at 11.5 min was lyophilized to obtain 2.6 mg (LCMS purity = 83%) of **16** as purple solid. LCMS calculated for $C_{65}H_{80}N_{10}O_{20}$: (M+H= 1321.3); found 1321.3.



Preparation of 17: To a solution of 6-aminoisobenzofuran-1(3H)-one **17a** (1 g, 6.70 mmol) in methanol (10 mL) was added ethane-1,2-diamine (2.246 mL, 33.5 mmol) and stirred the reaction at rt for 3h. LCMS (M+H-H_2O=192.2) shows the disappearance of SM and presence of product. The solvent and the excess of reagent was removed by evaporation and the crude product was taken to next step.

To a solution of 5-amino-N-(2-aminoethyl)-2-(hydroxymethyl)benzamide **17b** (1402 mg, 6.70 mmol) in DMF (5 mL) at 0 °C was added a solution of allyl chloroformate (0.715 mL, 6.70 mmol) in THF (1 mL). The reaction was stirred for 1h at which LCMS (M+H-H_2O=276.2) showed the formation of product. Quenched by the addition of sat. aq. $NaHCO_3$ and extracted with EtOAc. The crude product was purified on 40 g silica gel eluting with 0-100% MeOH in CH_2Cl_2 to yield 286 mg of **17c** as white solid.

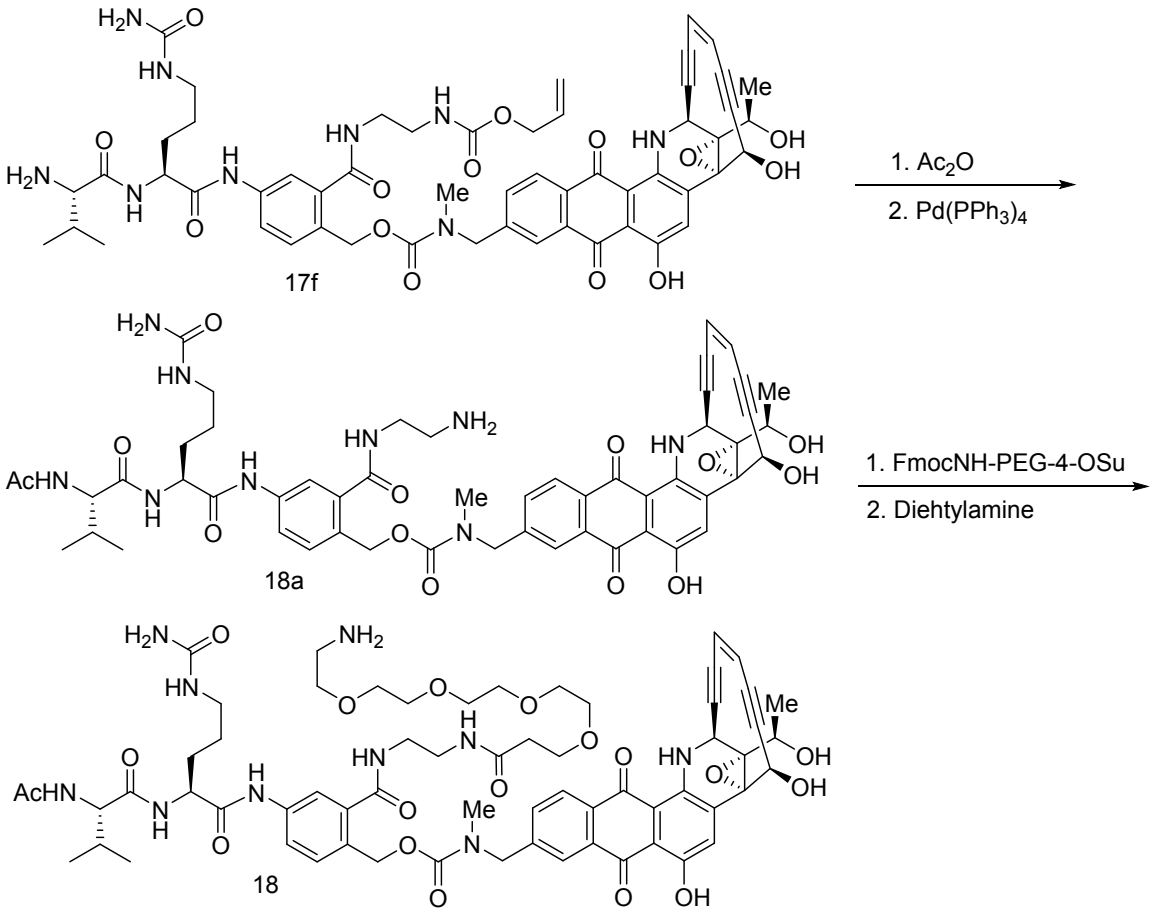
To a solution of (S)-2-((S)-2-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)-3-methylbutanamido)-5-ureidopentanoic acid (242 mg, 0.488 mmol) and allyl (2-(5-amino-2-(hydroxymethyl)benzamido)ethyl)carbamate **17c** (286 mg, 0.975 mmol) in MeOH (2 mL) was added ethyl 2-ethoxyquinoline-1(2H)-carboxylate (241 mg, 0.975 mmol) and the mixture was stirred at rt overnight. LCMS (M+H=772.5) shows new peak. The reaction was directly purified on combiflash using 40 g silica gel eluting with 0-100% MeOH/CH_2Cl_2 to yield 102 mg of **17d** as white solid.

To a solution of **17d** (102 mg, 0.132 mmol) in DMF (1 mL) was added bis(4-nitrophenyl) carbonate (121 mg, 0.396 mmol) and Hunig's base (0.046 mL, 0.264 mmol). The reaction was stirred at rt for 3h at which LCMS (M+H=937.3) shows the completion of reaction and directly purified on combiflash on a 40 g silica gel column eluting with 0-50% MeOH in CH_2Cl_2 to yield 103 mg of **17e** as white solid.

Stirred a mixture of **17e** (103 mg, 0.11 mmol) and **2b** (53.0 mg, 0.110 mmol) in 5 mL DMF was added 2,6-lutidine (38 microliter, 0.33 mmol) and stirred overnight. LCMS (M+H=1110.4) showed the completion of reaction which was worked with sat. aq. $NaHCO_3$/EtOAc and dried. To this crude mixture in DMF (5 mL) was added diethylamine

(0.057 mL, 0.550 mmol) and stirred for 30 min. LCMS (M+H=1058.4) shows the completion of reaction. The product was directly injected to reverse phase combifhash (150g C-18) and eluted with 0-50% water in MeCN (0.05% formic acid) to get 65 mg of **17f** as purple solid. LCMS calculated for $C_{54}H_{59}N_9O_{14}$: (M+H= 1058.4); found 1058.4.

To a solution of **17f** (150 mg, 0.142 mmol) and 2,5-dioxopyrrolidin-1-yl 1-(9H-fluoren-9-yl)-3-oxo-2,7,10,13,16-pentaoxa-4-azanonadecan-19-oate (83 mg, 0.142 mmol) in DMF (0.5 mL) was added 2,6-lutidine (0.050 mL, 0.425 mmol) and stirred for 2h. LCMS (M+H=1527.1) shows formaton of product. The crude product was purified on reverse phase combiflash eluting with 0-100% MeCN/water (0.05% formic acid) to yield 178 mg of product as purple solid. To a solution of product from previous step (70 mg, 0.046 mmol) in DMF (2 mL) was added triethylsilane (0.015 mL, 0.092 mmol) followed by $Pd(PPh_3)_4$ (26.5 mg, 0.023 mmol) and the reactionwa s stirred for 1h. LCMS (M+H=1221.2) shows the deprotection of alloc and Fmoc. Another peak LCMS (M+H=1444.2) with only deprotection of alloc group was also seen. The crude reaction was purified on 28 g reverse phase silica gel combiflash eluting 0-95% Water in MeCN to yield 2.3 mg (LCMS purity = 81.8%) of **17** and 16 mg of Fmoc protected product as purple solid. LCMS calculated for **17** $C_{61}H_{76}N_{10}O_{17}$: (M+H= 1221.2); found 1221.3.

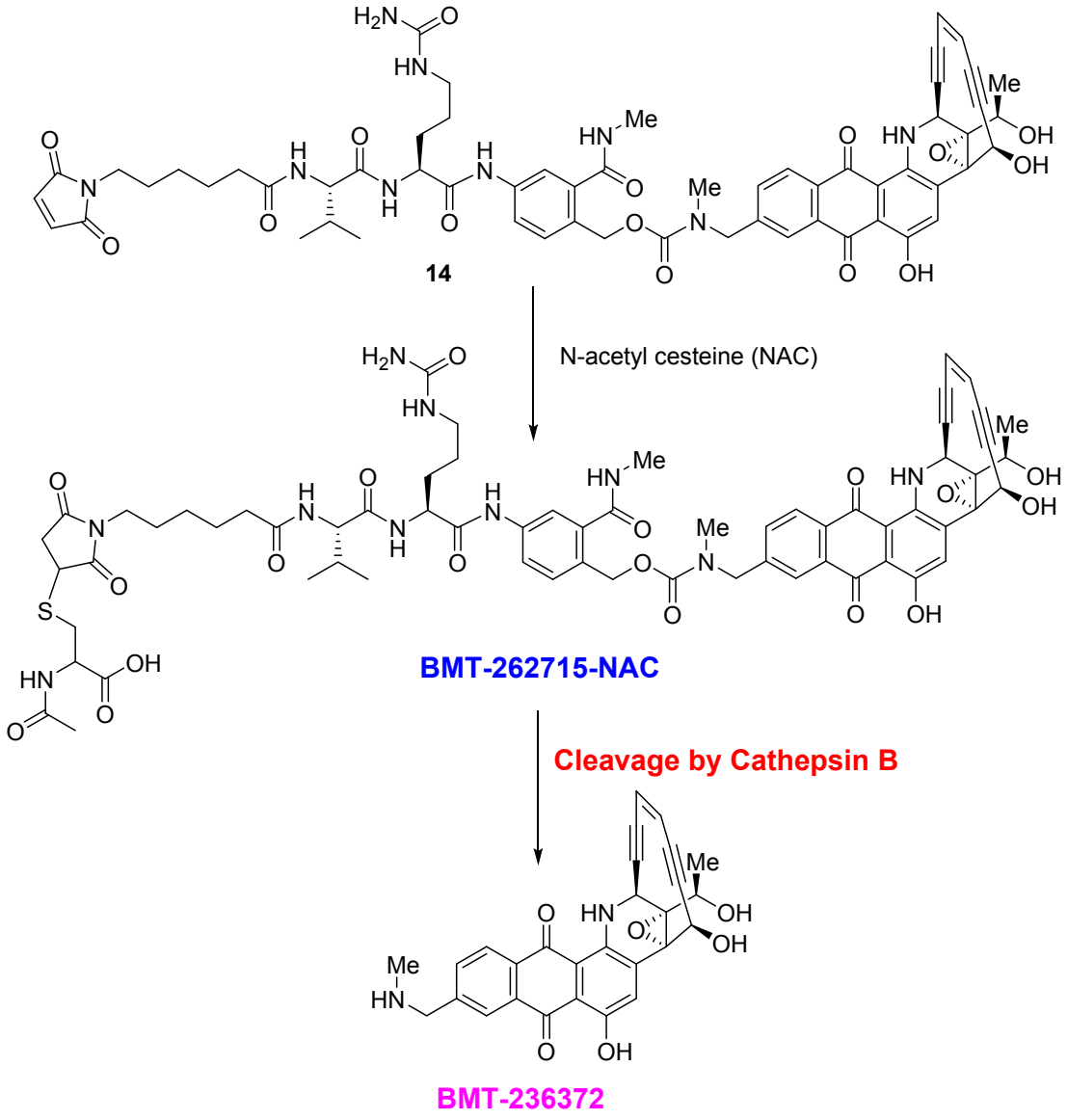


Preparation of compound 18: A solution of branched linker **17f** in DMF (0.5 mL) was treated with 2,6-lutidine (10.24 µl, 0.088 mmol) followed by acetic anhydride (2.76 µl, 0.029 mmol). LCMS (M+H=1100.3) after 5 min shows the completion of acetylation. To this mixture was added morpholine (5.10 µl, 0.059 mmol) followed by $Pd(PPh_3)_4$ (6.77 mg, 5.86 µmol) and stirred for 1h. LCMS (M+H=1016.3) shows the completion of reaction. The reaction was directly purified on a reverse phase combiflash using 50 g C-18 column eluting with 0-50% water/MeCN (0.05% formic acid) to yield 16.4 mg of **18a** as purple solid. LCMS calculated for **18a** $C_{52}H_{57}N_9O_{13}$: (M+H= 1016.4); found 1016.3.

To a solution of **18a** (22 mg, 0.022 mmol) in DMF (0.5 mL) was added 2,5-dioxopyrrolidin-1-yl 1-(9H-fluoren-9-yl)-3-oxo-2,7,10,13,16-pentaoxa-4-azanonadecan-19-oate (12.66 mg, 0.022 mmol) and 2,6-lutidine (7.57 µl, 0.065 mmol). The reaction was stirred for 1h at which LCMS (M+H=1486.3) showed the completion of reaction. To this reaction was added diethylamine (0.011 mL, 0.108 mmol) and stirred for 30 min at which

LCMS (M+H=1363.3) shows the formation of product. The reaction was diluted with dmso (0.5 ml) and purified on Shimadzu LC-20AP preparative HPLC with XBridge Prep C18 5mm OBD 10x150 mm column eluting with 0-95% H_2O/MeCN (0.05% formic acid). The product containing fractions were lyophilized to obtain 3.9 mg (LCMS purity = 86.4%) of **18** as purple solid. LCMS calculated for **18** $C_{63}H_{78}N_{10}O_{18}$: (M+H=1263.5); found 1263.3.

General Procedure for Cathepsin B Enzyme Digestion and Serum Stability Study:



Scheme 1: Release of free payload BMT-236372 from **14** (BMT-262715)

Experimental procedure:

1. **Blocking maleimide group with N-Acetyl Cysteine (NAC)**: Compound **14** (BMT-262715) was diluted to 1 mM in DMSO. A 30 µl of 1mM of **14** was incubated with 30 µl of 15 mM NAC (in 50 mM Potassium phosphate buffer pH 7.0) at room temperature for one hour.

2. **Cathepsin-B Digestion:** 7.5 µL of the BMT-262715-NAC (0.5 mM) was transferred to individual tube containing 135 µl of Cathepsin B buffer (25 mM Sodium Acetate, 1 mM EDTA, 1 mM DTT, pH 5.5) and the digestion was started by the addition of 7.5 µl of the diluted Cathepsin B enzyme (Activated, 1.45 µM). The samples were incubated at 37 ^{0}C for 4 hours. After 4 hours, 20 µl aliquot was taken and quenched with 80 µl of 0.1% formic in 3:1 MeOH:ACN. Negative controls were included for **14** (BMT-262715) without the addition of Cathepsin B and with/without addition of **2b** (BMT-236372). After quenching, all samples were held at -20 ^{0}C for 1 hour and further centrifuged at 14000 rpm for 5 mins. The supernatant was transferred to a fresh tube and put in the UPLC autosampler for analysis.
3. **Stability of 14** (BMT-262715)**:** 6 µL of the BMT-262715-NAC (0.5 mM) was transferred separately to individual tubes containing 144 µl of 1X PBS, mouse, rat or human serum. The samples were incubated at 37 ^{0}C for 0, 4, and 24 hours. After each of the time points, an aliquot of 20 µl was taken from the samples and quenched with 80 µl of of 0.1% formic in 3:1 MeOH:ACN. After quenching, all samples were held at -20 ^{0}C for 1 hour and further centrifuged at 14000 rpm for 15 mins. The supernatant was transferred to a fresh vial and stored at -20 ^{0}C until analysis.

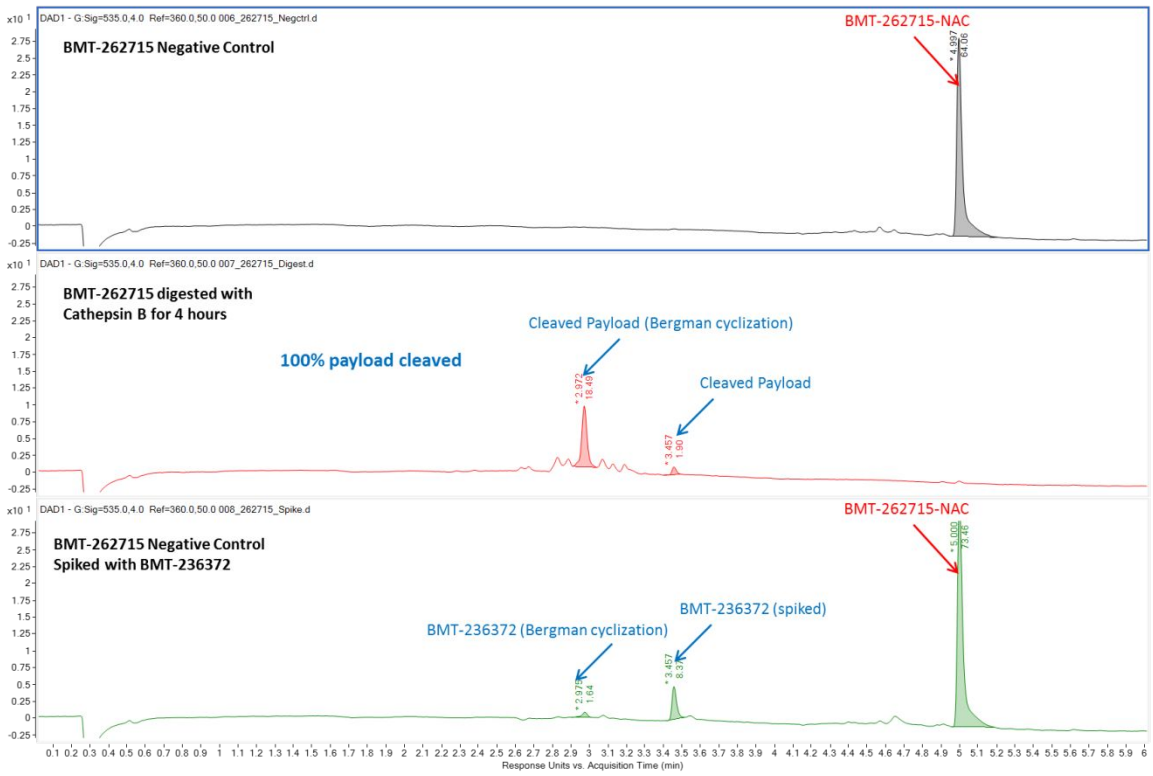


Fig. 2 Comparison of DAD-535 profiles of **14** (BMT-262715) in 1X PBS (0-24 hrs)

Fig. 3 Comparison of DAD-535 profiles of **14** (BMT-262715) in mouse serum (0-24 hrs)

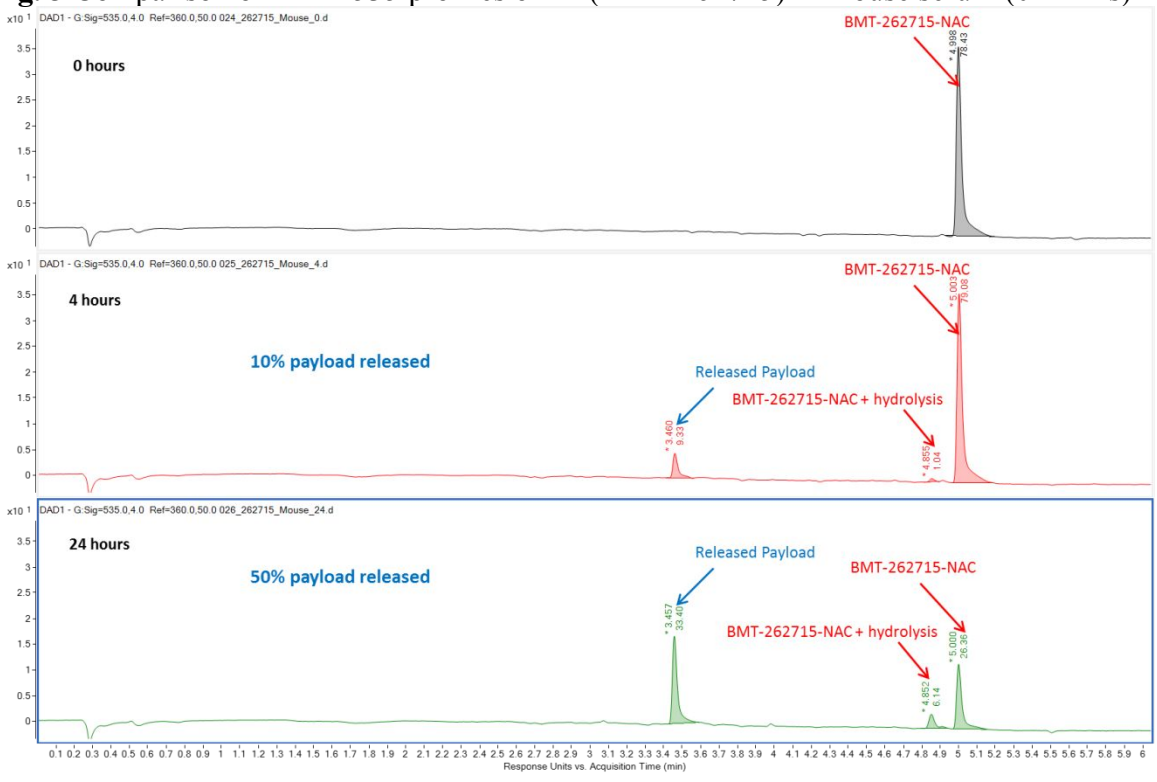


Fig. 4 Comparison of DAD-535 profiles of **14** (BMT-262715) in rat serum (0-24 hrs)

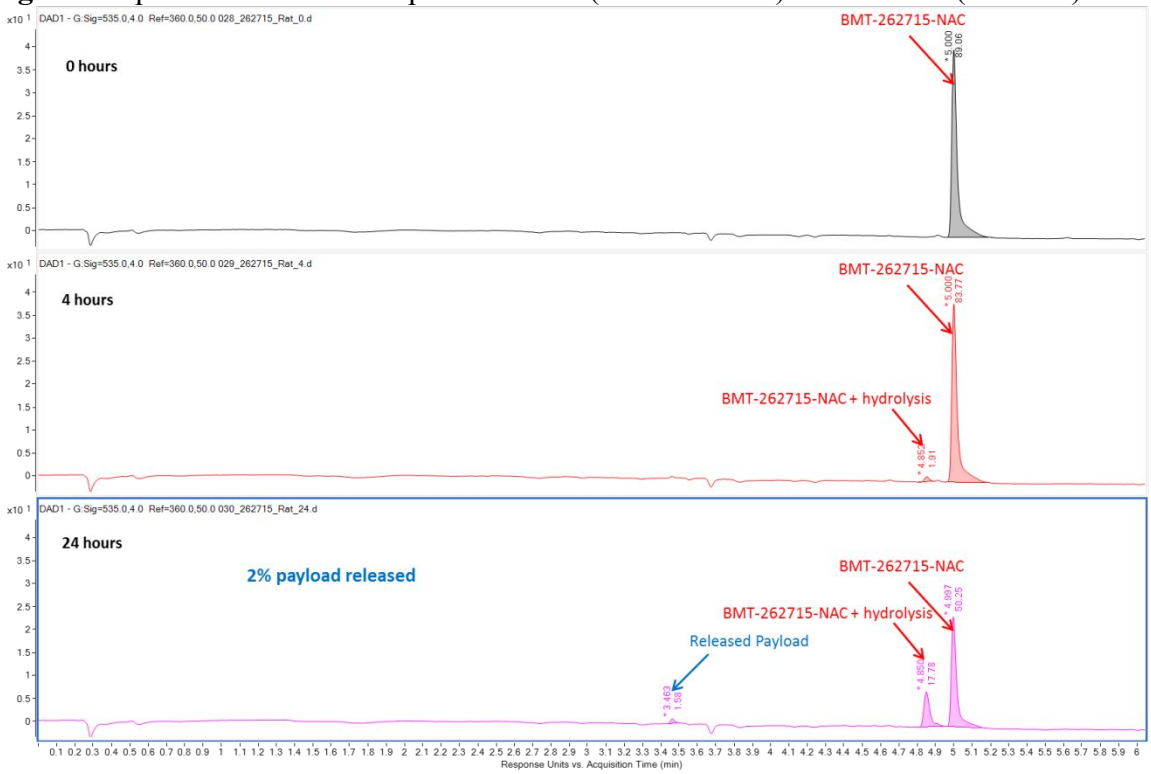


Fig. 5 Comparison of DAD-535 profiles of **14** (BMT-262715) in human serum (0-24 hrs)

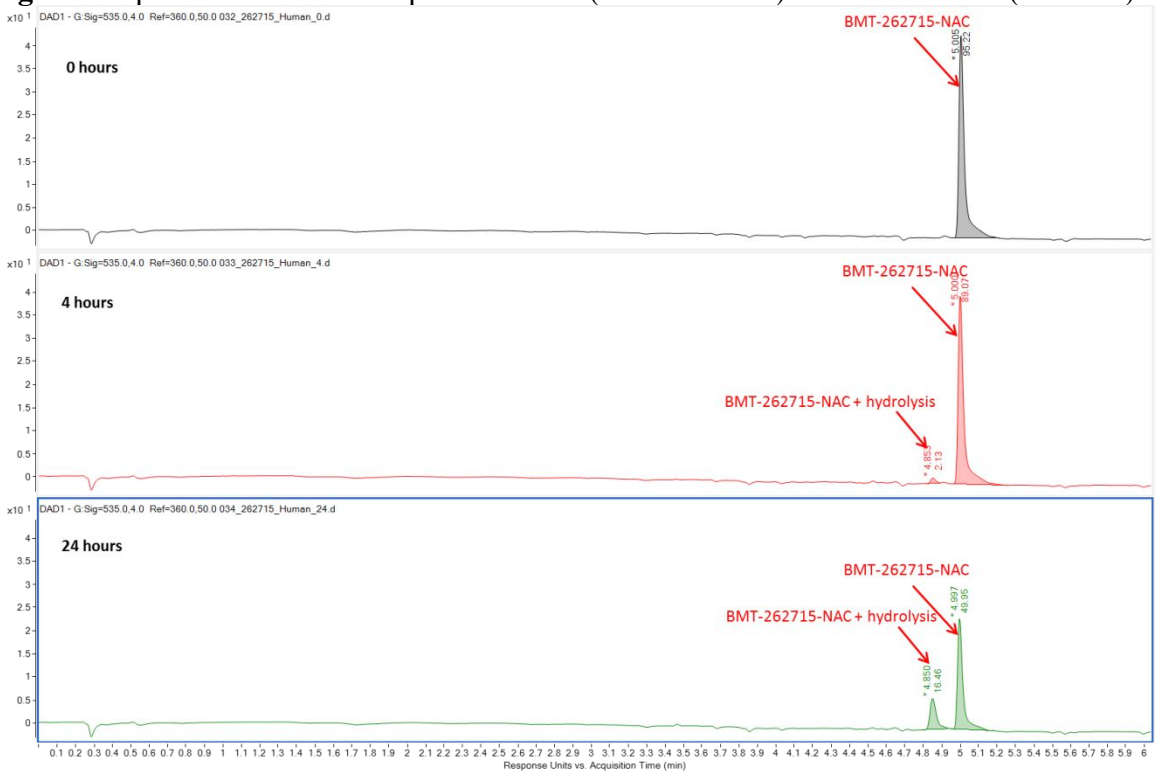
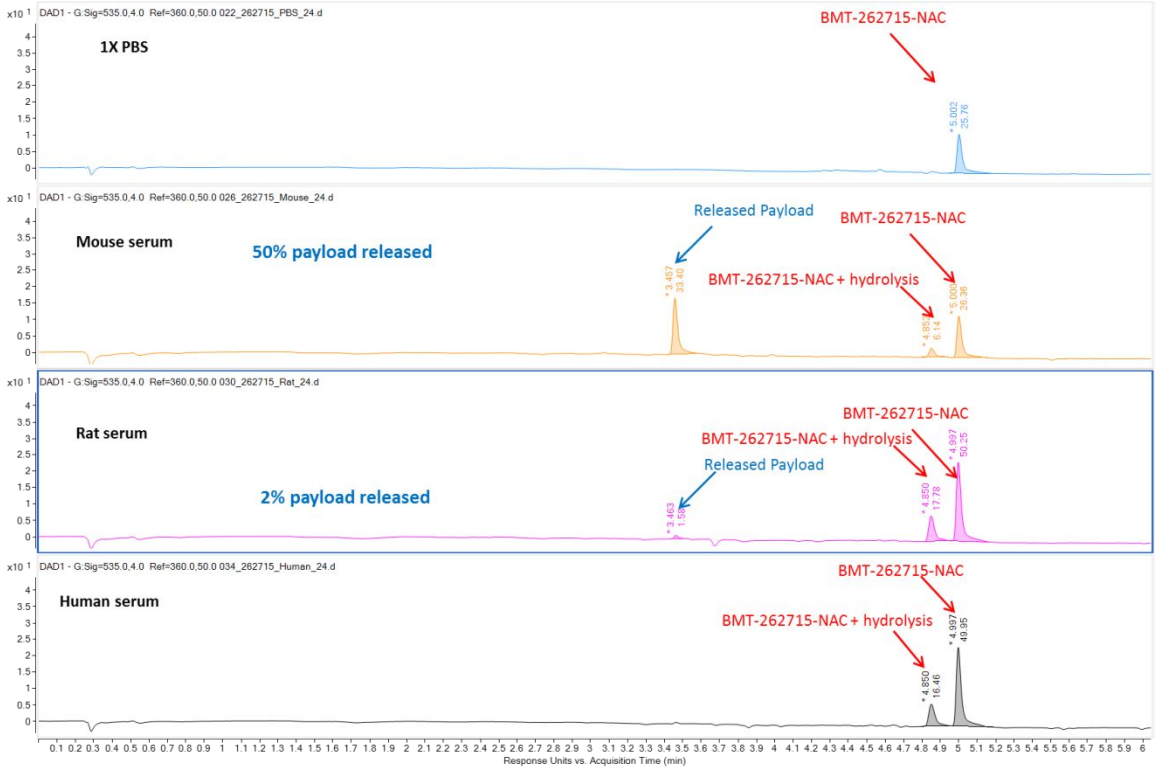


Fig. 6 Comparison of DAD profiles of **14** (BMT-262715) in mouse, rat and human serum at 24 hrs



Conclusions

Assay/Sera	% payload cleaved/Released		Result
	4 hrs	24 hrs	
Cathepsin B	100	100	Cleaved by Cat B (Fig. 1)
1X PBS	0	0	Stable (Fig. 2)
Mouse Serum	10	50	NOT Stable (Fig. 3)
Rat Serum	0	2	Stable (Fig. 4)
Human Serum	0	0	Stable (Fig. 5)

References

1. Nicolaou, K. C., Wang, Y. Lu, M., Mandal, D., Pattanayak, M. R., Yu, R., Shah, A. A., Chen, J. S., Zhang, H., Crawford, J. J., Pasunoori, L., Poudel, Y. B., Chowdari, N. S., Pan, C., Nazeer, A., Gangwar, S., Vite, G. D., Pitsinos, E. N. *J. Am. Chem. Soc.* **2016**, *138*, 8235-8246.

LCMS compound 1

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Method Info     : test transfer method
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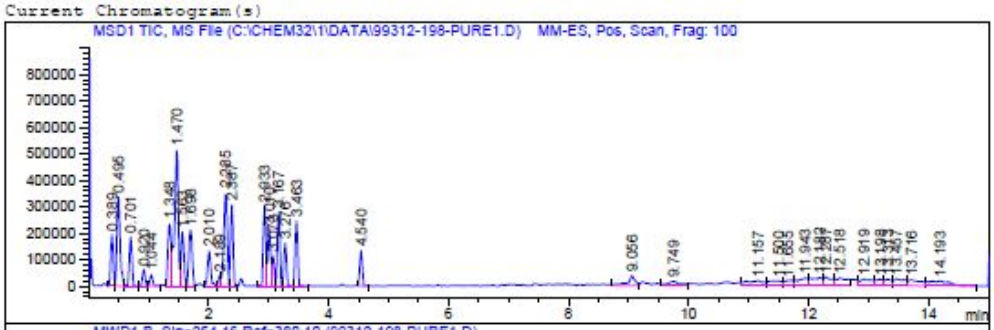
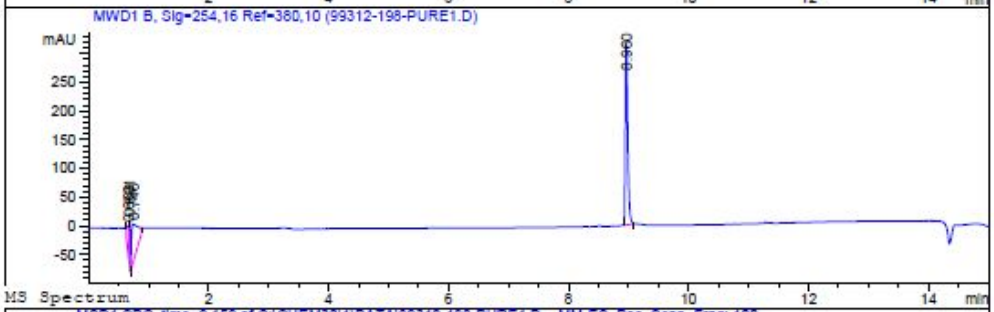
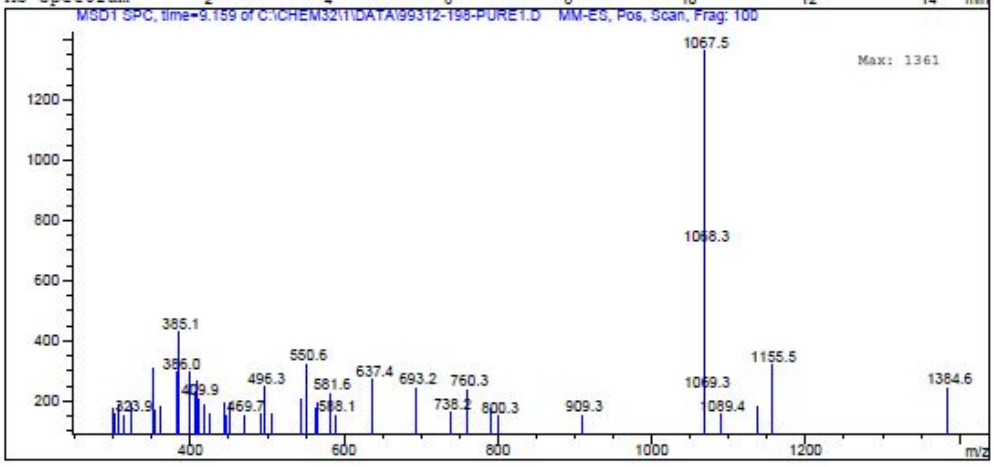




MS Spectrum



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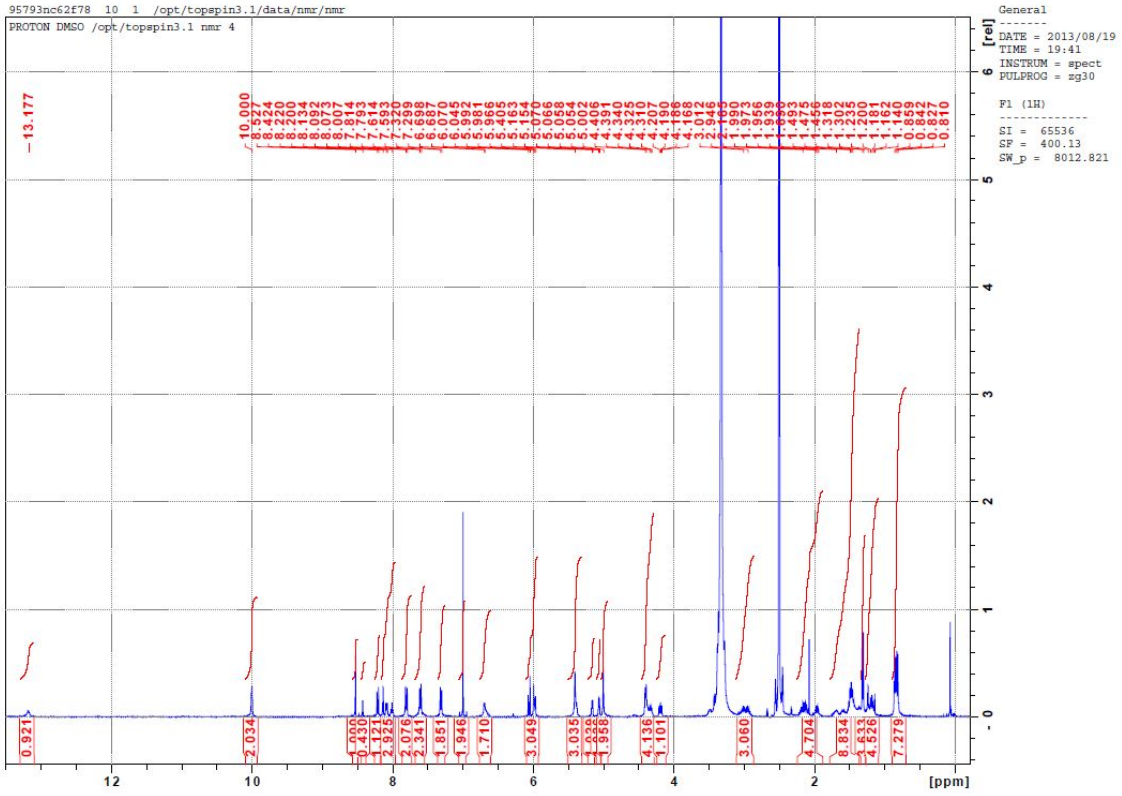
^{1}H-NMR compound 1



LCMS compound 2

Notebook_page 99312-380 user_ poudely project_ 99010 Intensity of peak from (1000-1700) is 1000x as it should be for mass

Sample Set Name	99312	Vial:	1:F,2
Sample Name:	99312-380-pure	Injection #:	1
Channel Name:	PDA Single 254.0 nm	Injection Volume:	2.00 ul
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Acq. Method Set:	3 Min method	Date Processed:	4/6/2015 3:26:46 PM PDT
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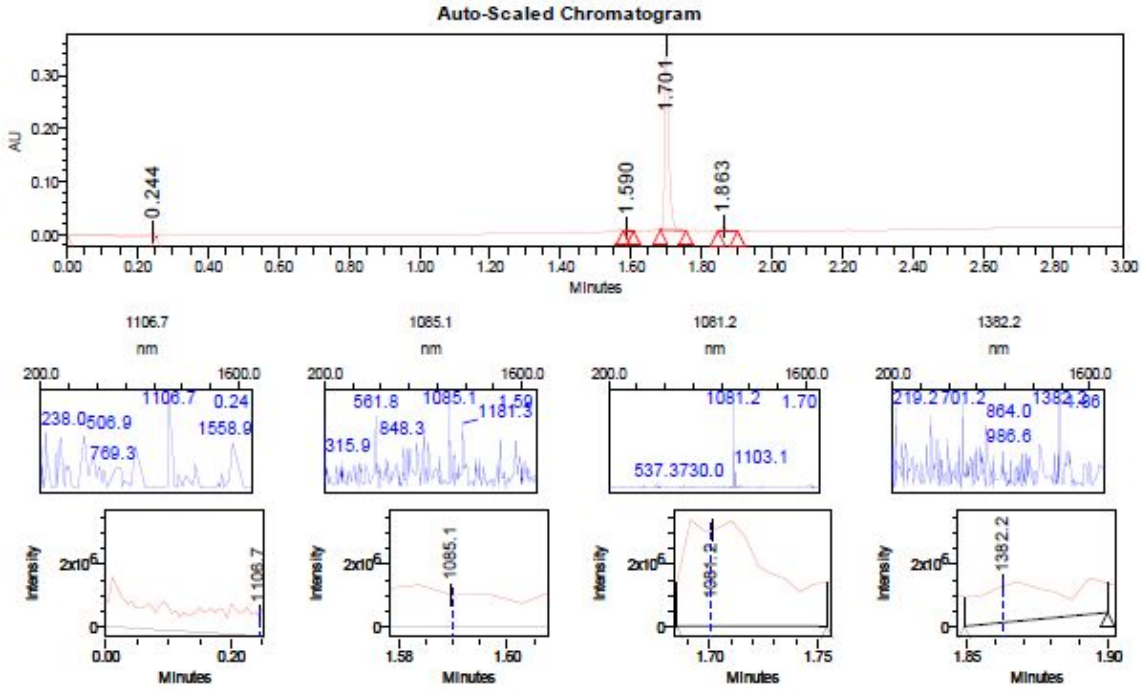

Processed Channel: PDA 254.0 nm (200-600)nm

	Processed Channel	Retention Time (min)	Area	% Area	Height
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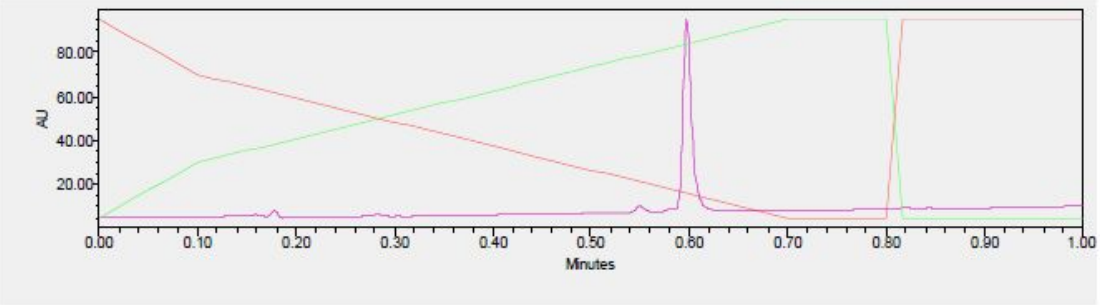
2	PDA 254.0 nm (200-600)nm	1.590	3951	1.17	5486
3	PDA 254.0 nm (200-600)nm	1.701	289256	85.82	351511
4	PDA 254.0 nm (200-600)nm	1.863	8746	2.59	8622

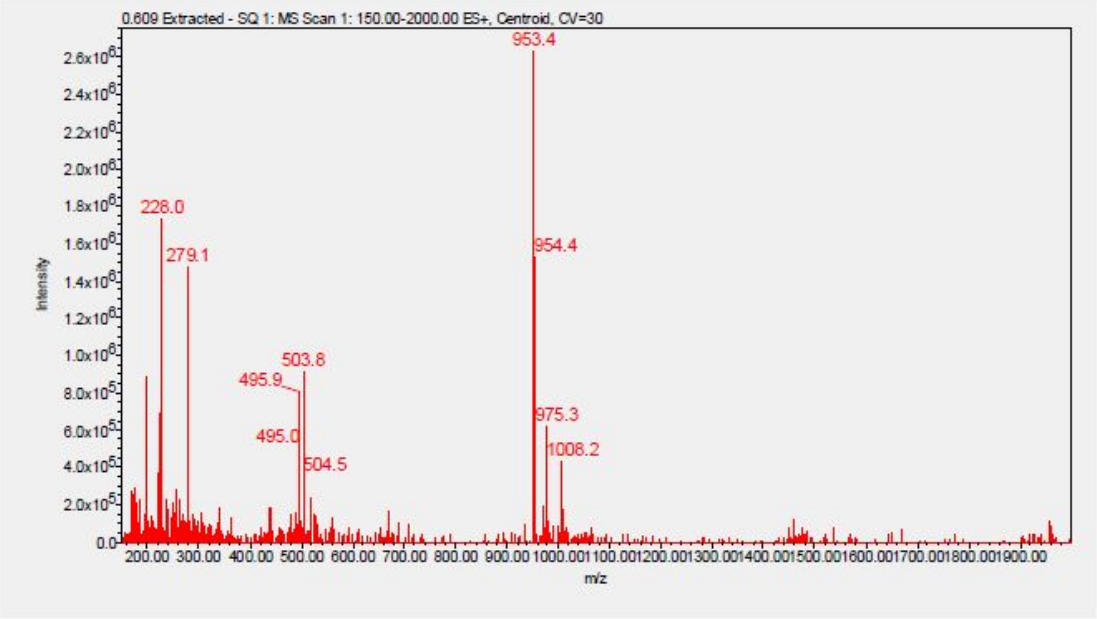
LCMS compound 4

99312-245-pure

UV



Mass



LCMS compound 5

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Date Acquired: 6/1/2015 1:33:42 PM PDT

Injection #: 1
Injection Volume: 1.00 ul
Run Time: 3.0 Minutes
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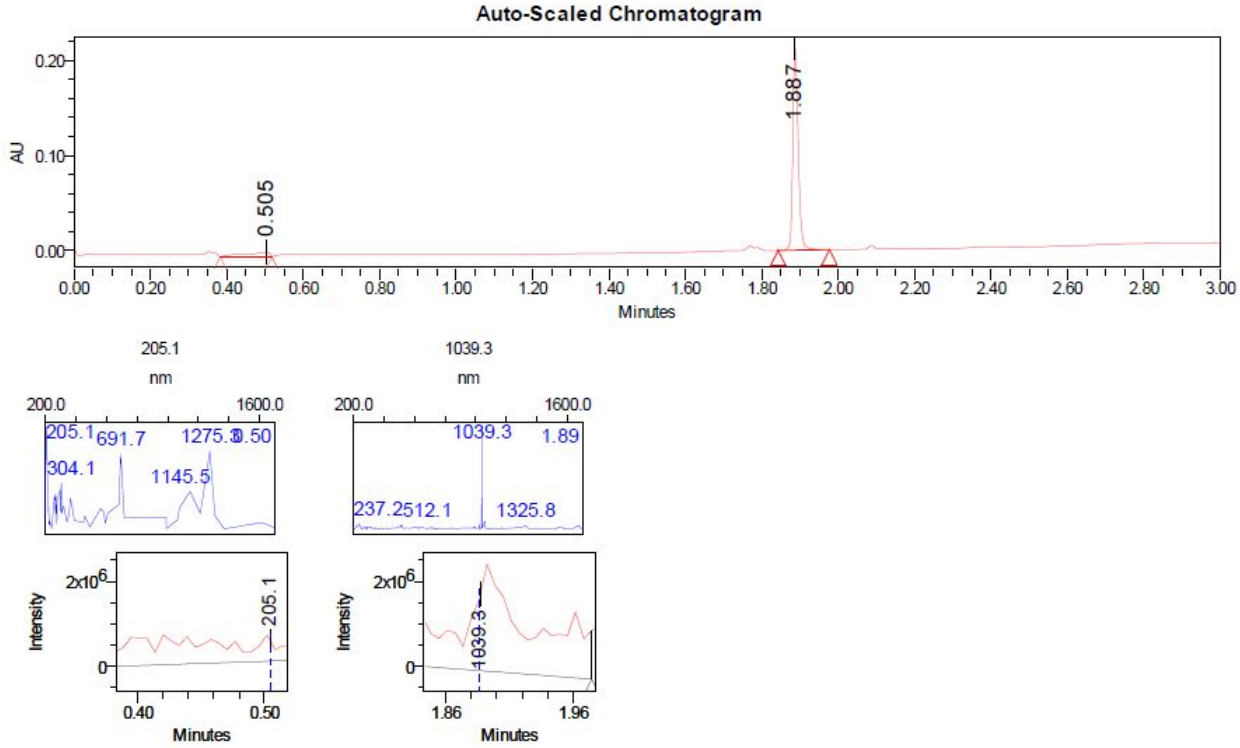

Processed Channel: PDA 254.0 nm (200-600)nm

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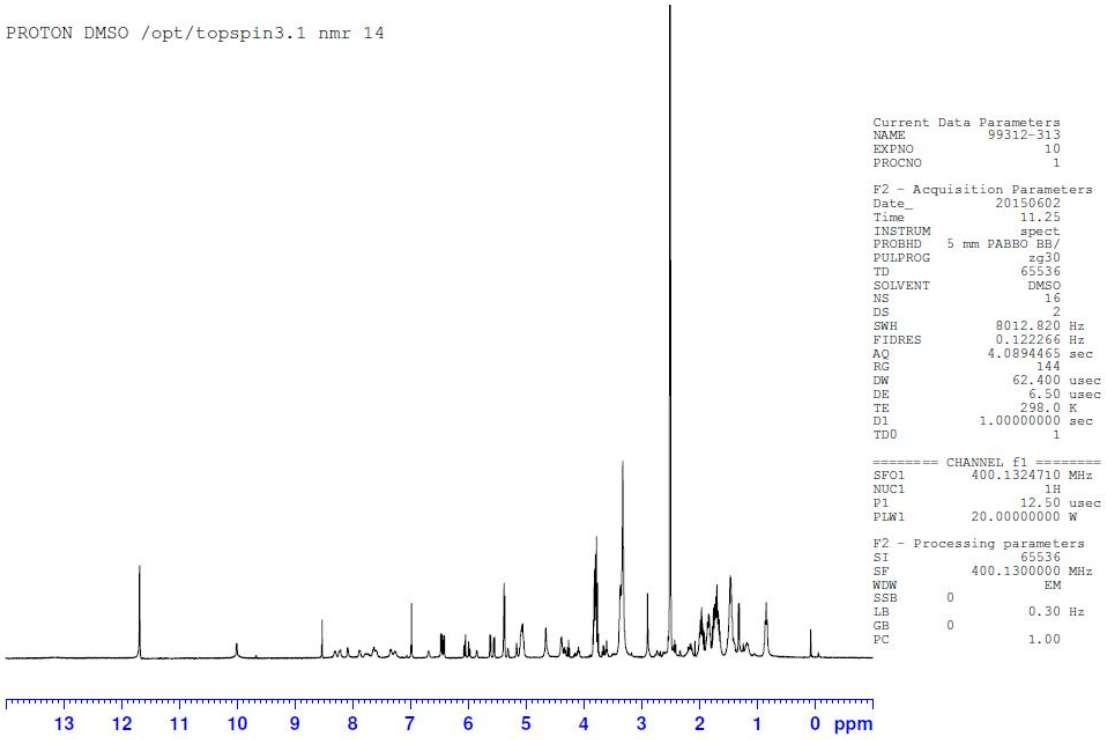
1H-NMR compound 5



LCMS compound 6

Notebook_page 99312-417 user_ poudely project_ 99010 Intensity of peak from (1000-1700) is 1000x as it should be for mass

Sample Set Name	aa_Yam	Vial:	1:F,1
Sample Name:	99312-417-pure	Injection #:	1
Channel Name:	PDA Single 254.0 nm	Injection Volume:	10.00 ul
Proc. Chnl. Descr.:	PDA 254.0 nm (200-600)nm	Run Time:	3.0 Minutes
Acq. Method Set:	3 Min method	Date Processed:	6/5/2015 3:39:42 PM PDT
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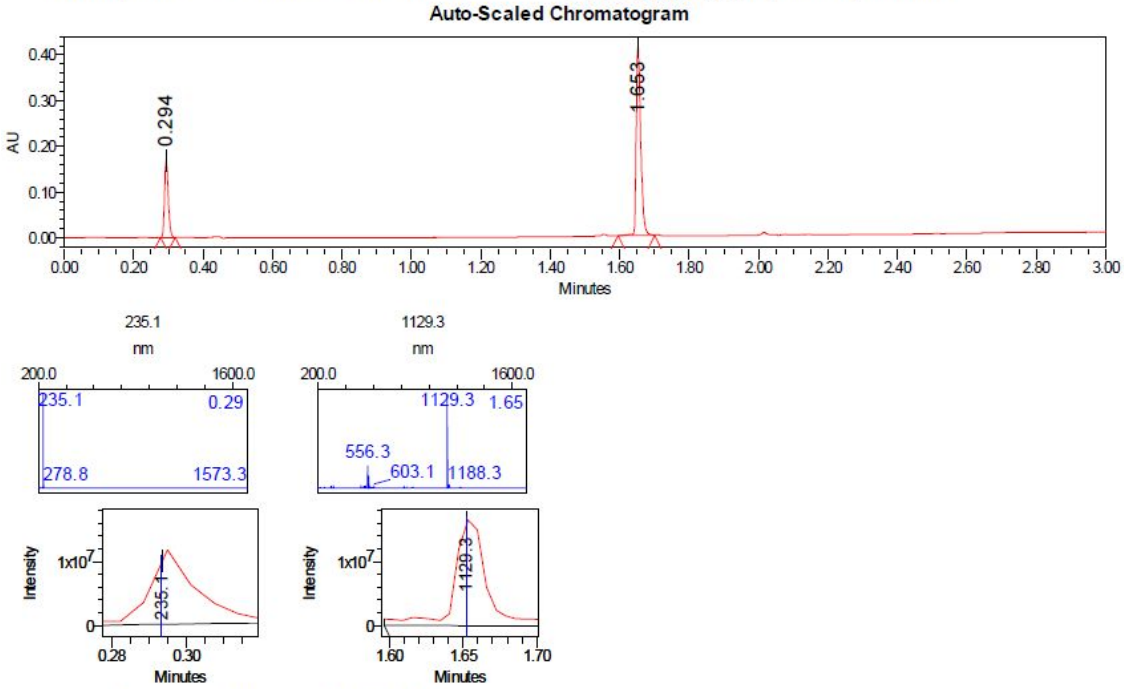

Processed Channel: PDA 254.0 nm (200-600)nm

	Processed Channel	Retention Time (min)	Area	% Area	Height
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2	PDA 254.0 nm (200-600)nm	1.653	376243	74.90	414766

LCMS compound 7

Basic_Report Report

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Injection #:	1	Date Processed:	7/7/2014 3:11:59 PM PDT
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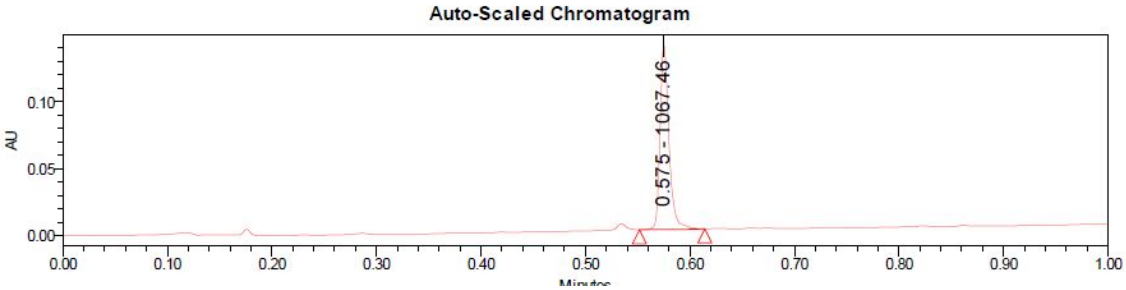
Sample Set Name	99312	Proc. Chnl. Descr.:	PDA 254.0 nm (210-600)nm



LCMS compound 8

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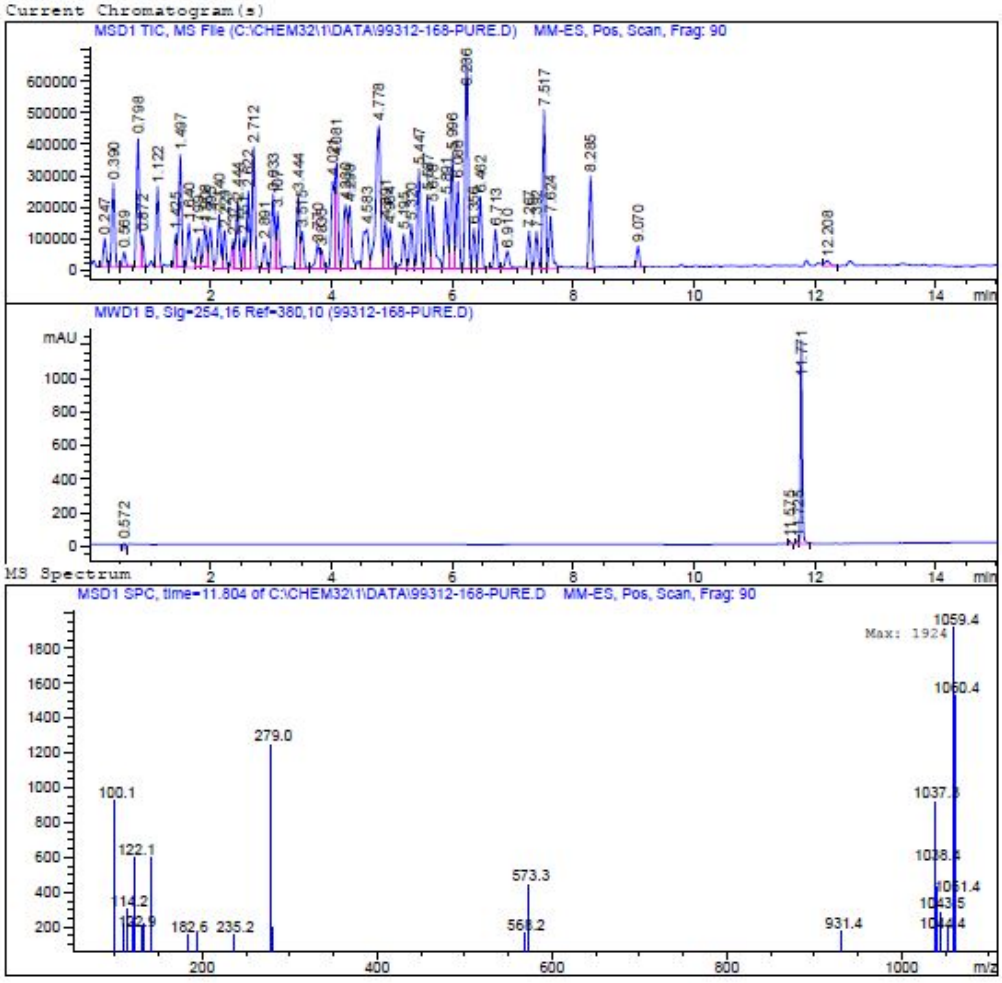


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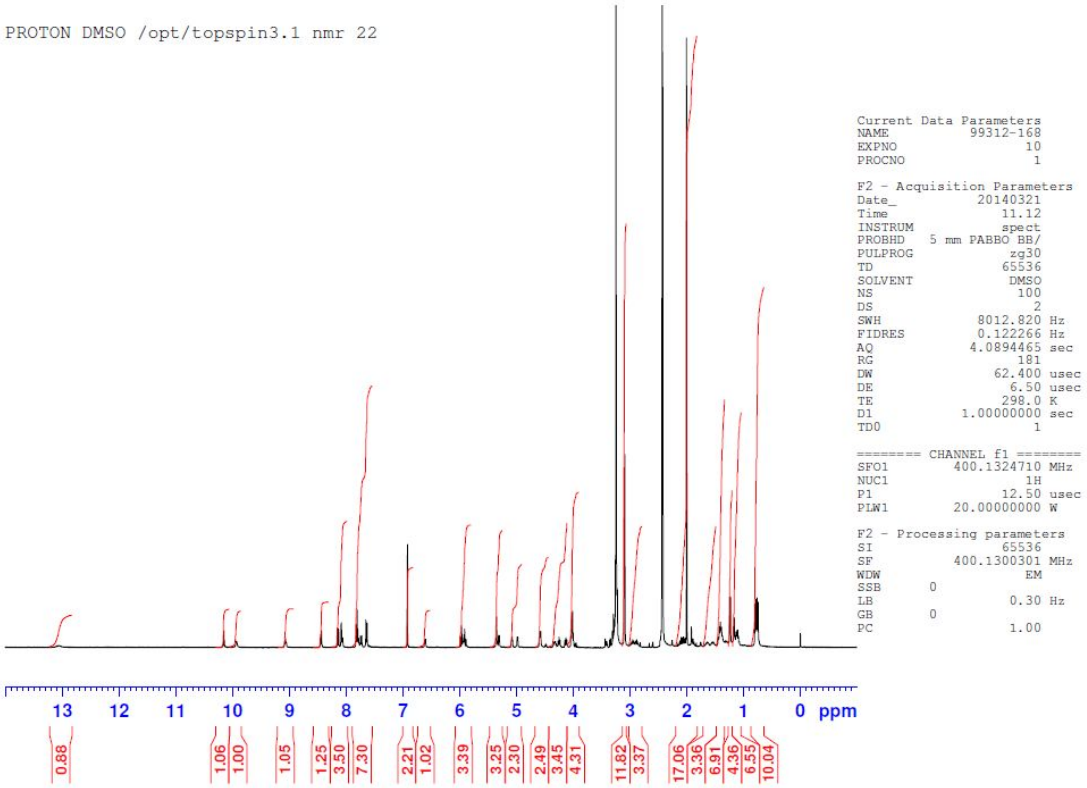
1H-NMR compound 8



LCMS compound 9a

Basic_Report Report

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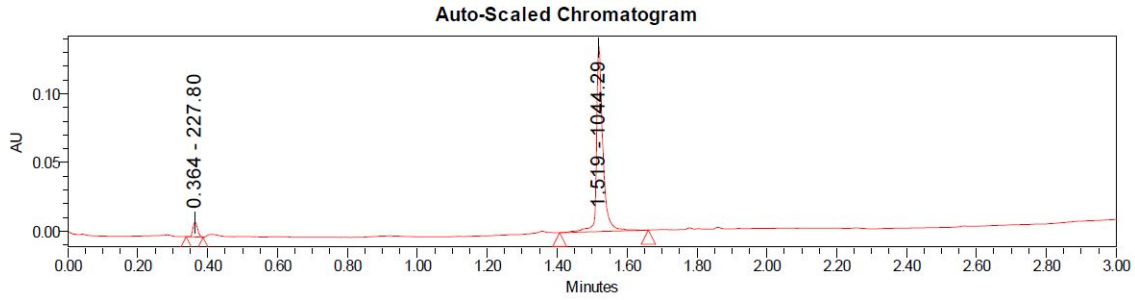
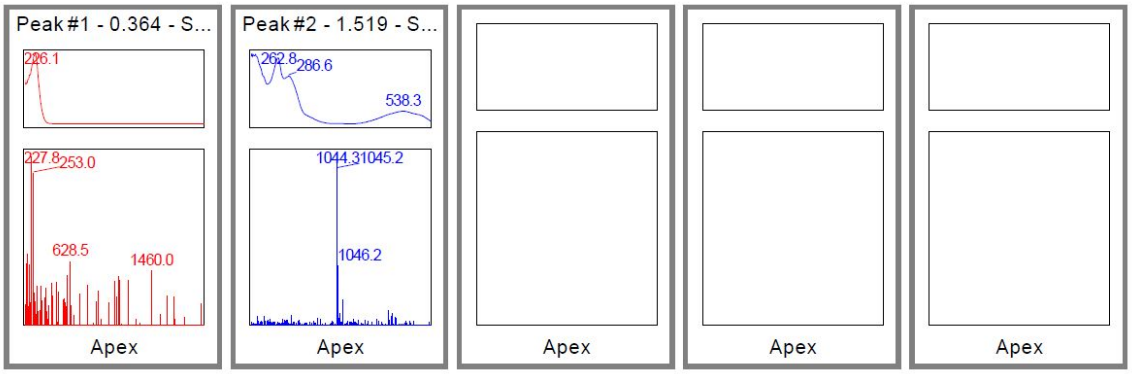
Sample Set Name	aaa	Proc. Chnl. Descr.:	PDA 254.0 nm (200-600)nm





LCMS compound 9b

Basic_Report Report

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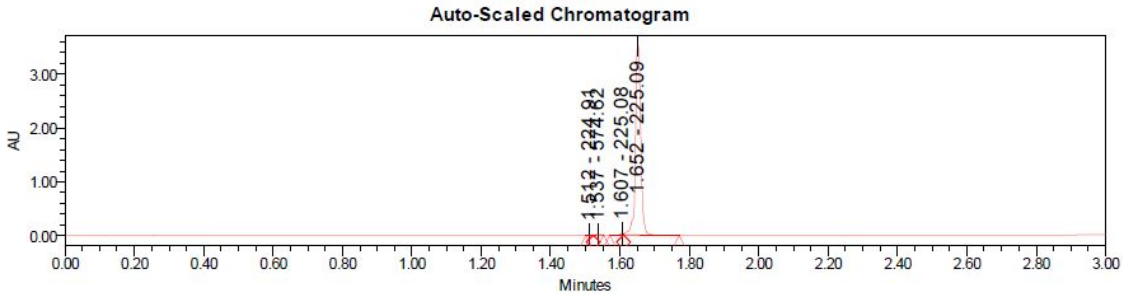
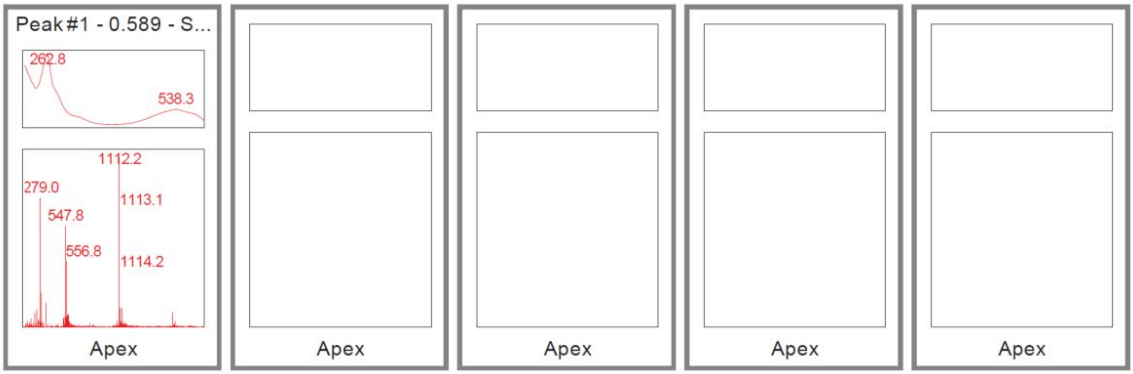
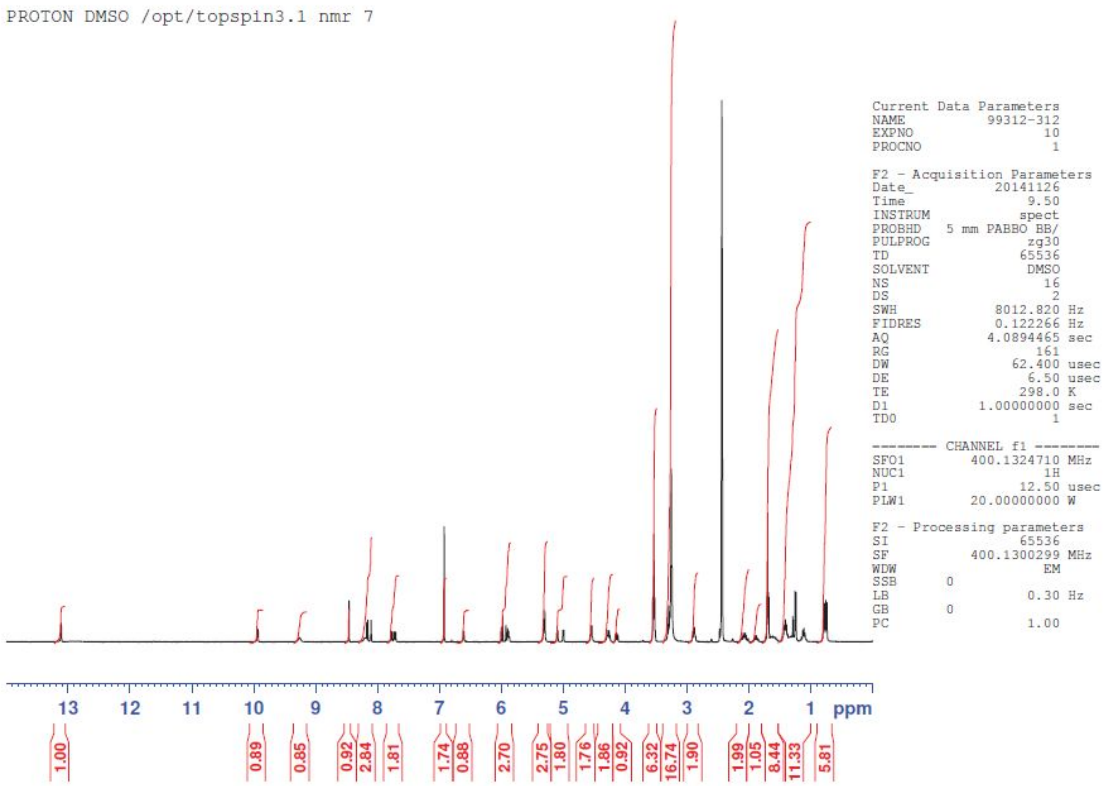




^{1}H-NMR 9b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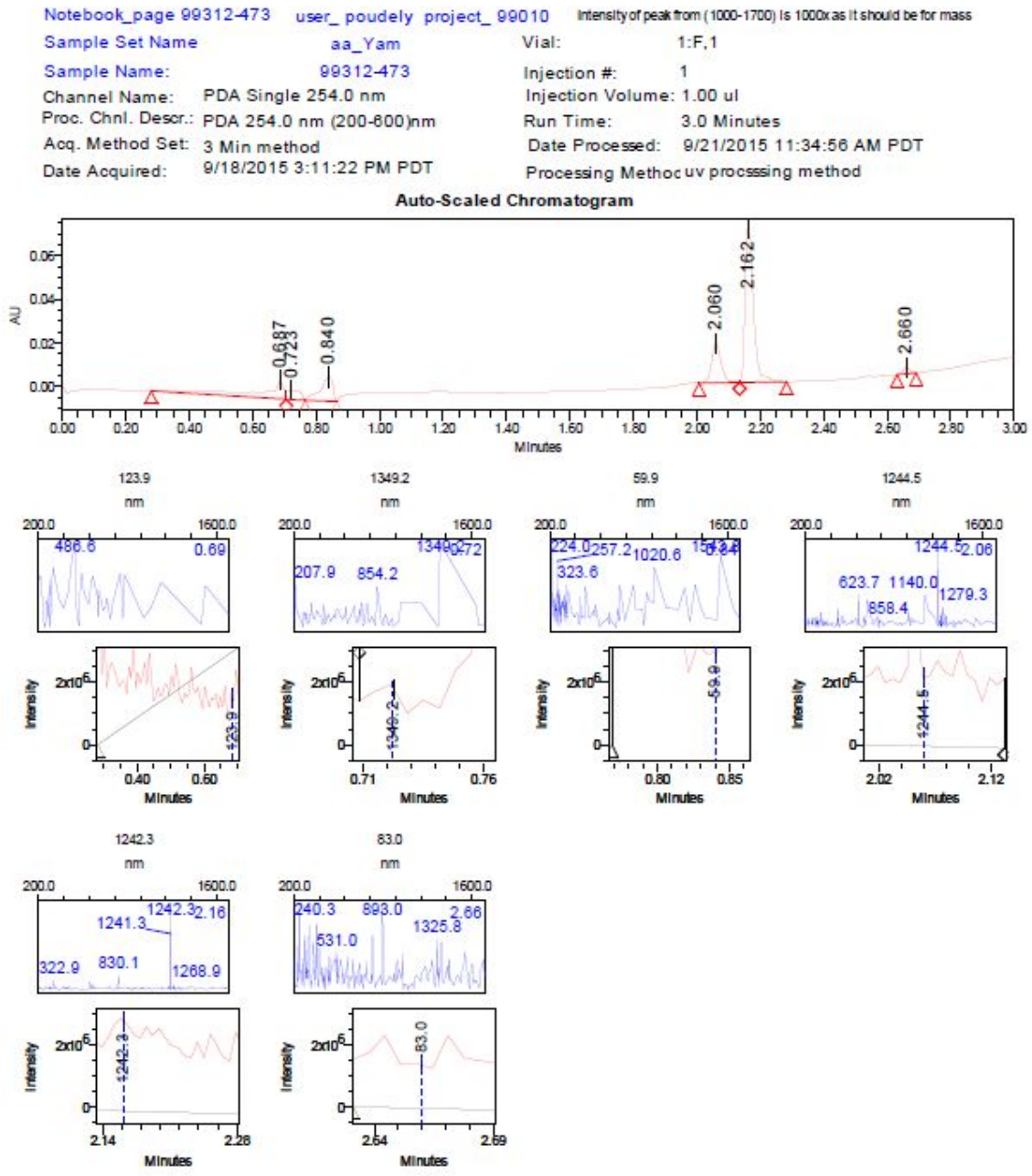
LCMS compound 10



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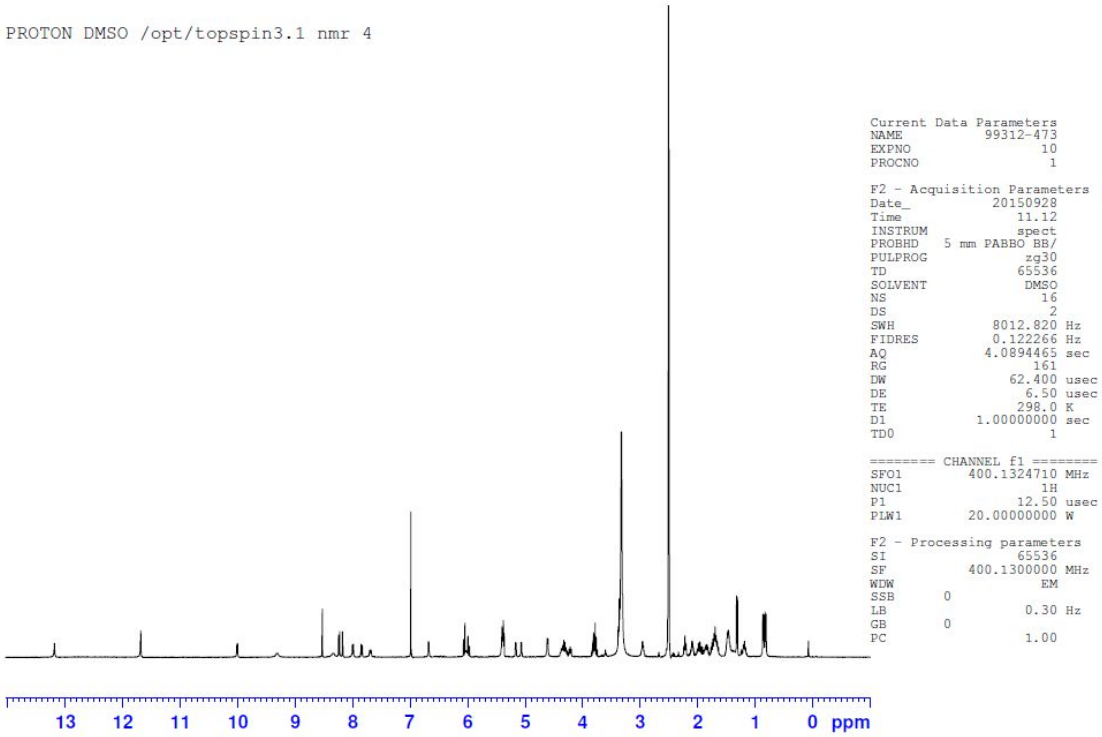
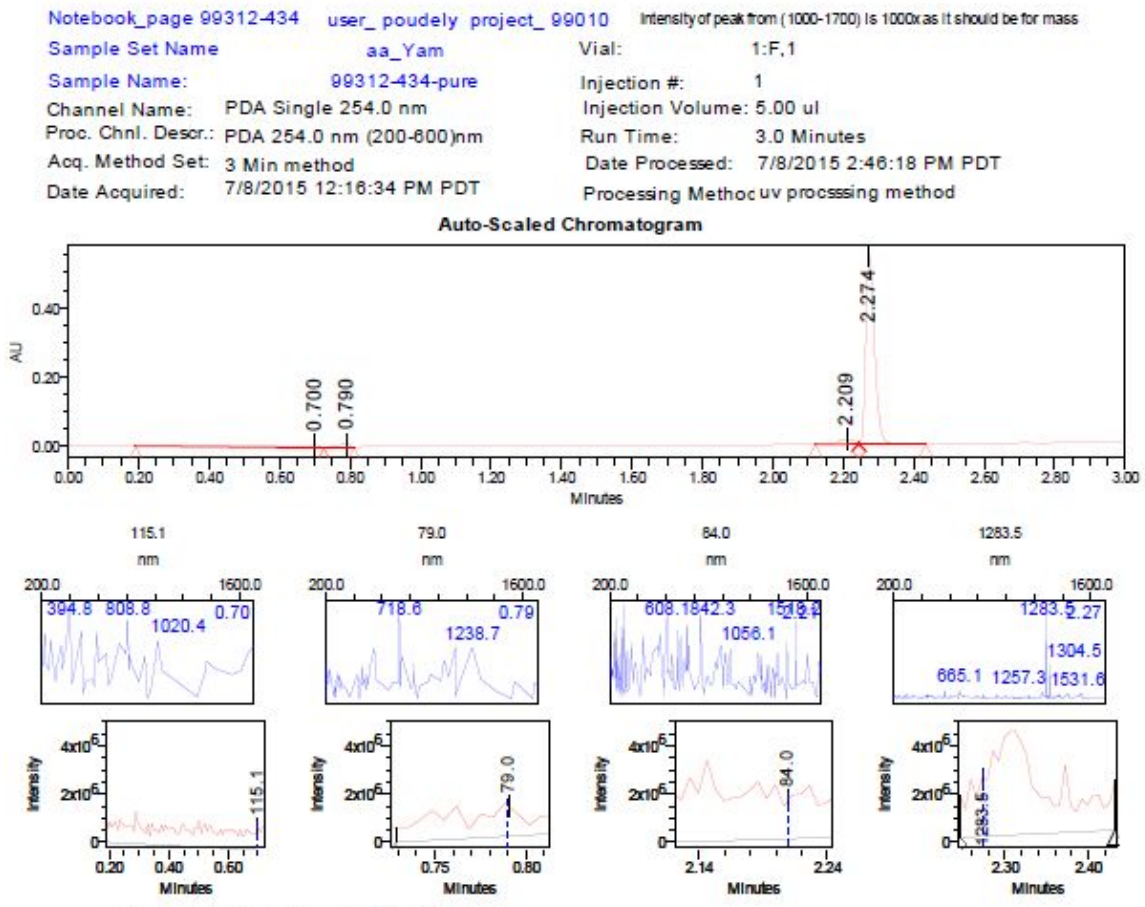


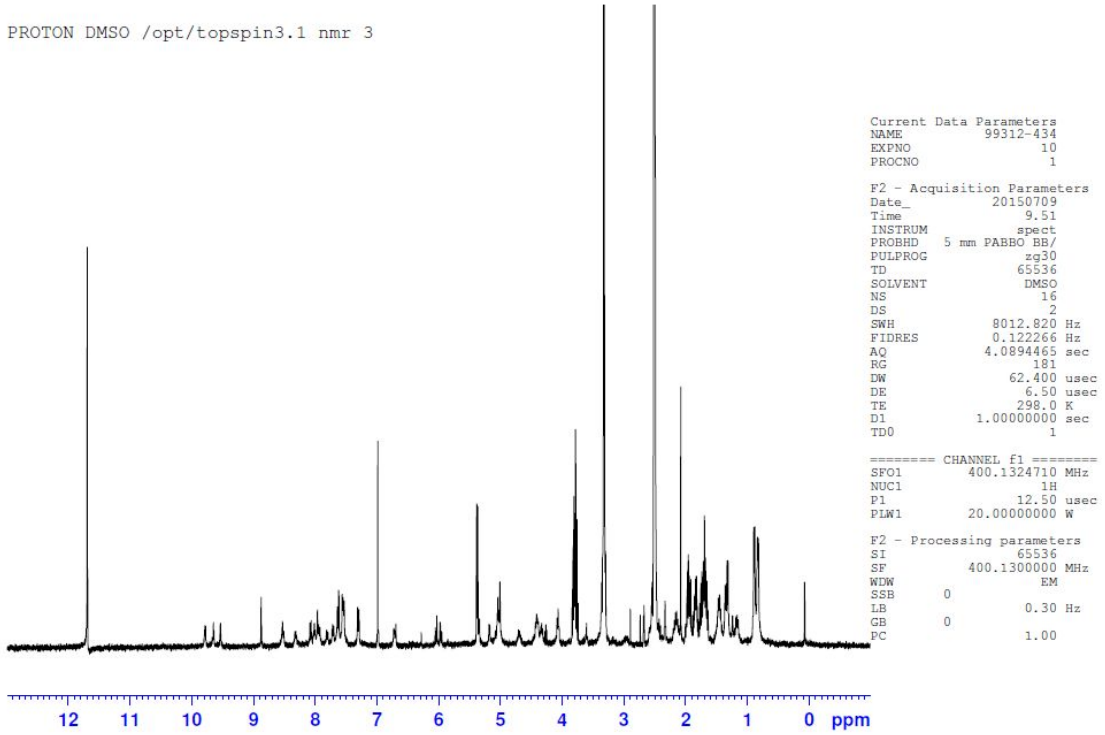
LCMS compound 11



Processed Channel: PDA 254.0 nm (200-600)nm

	Processed Channel	Retention Time (min)	Area	% Area	Height
1	PDA 254.0 nm (200-600)nm	0.700	85512	7.74	8801
2	PDA 254.0 nm (200-600)nm	0.790	30106	2.72	10204
3	PDA 254.0 nm (200-600)nm	2.209	44449	4.02	14348
4	PDA 254.0 nm (200-600)nm	2.274	944888	85.51	545312

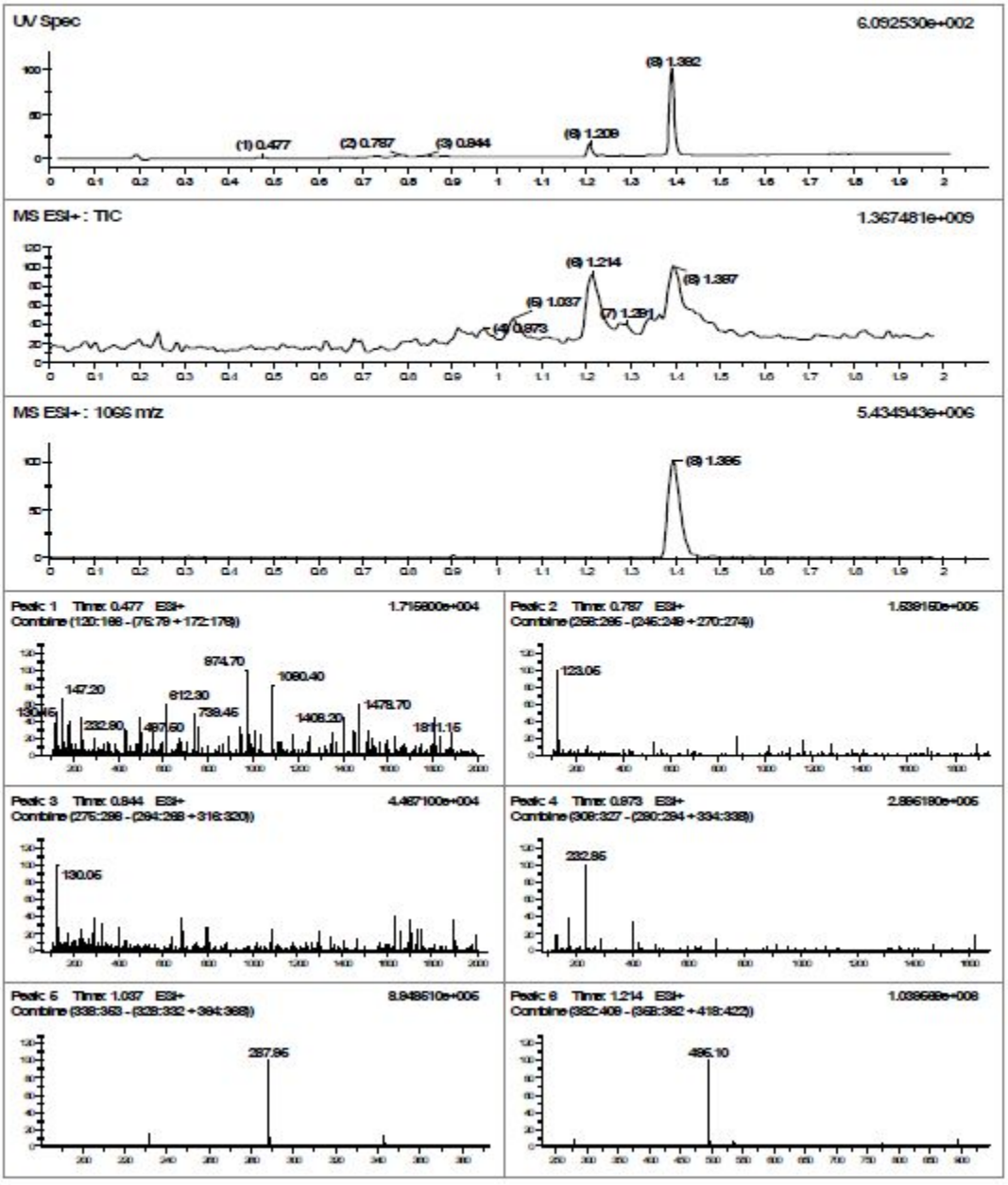
1H-NMR compound 11



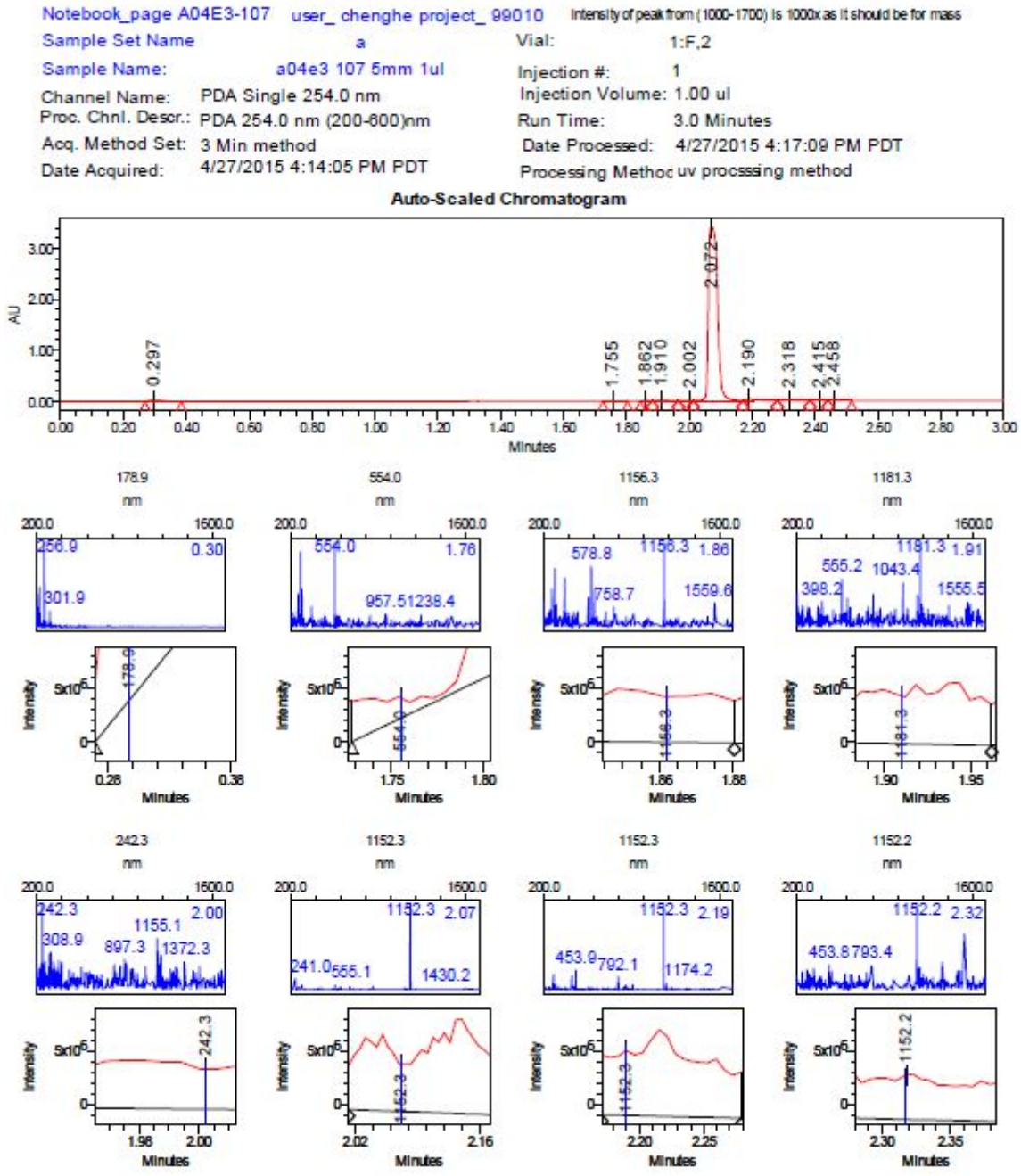
LCMS compound 12

Sample ID: 99440-607 FRAC12
Acquired: 05-15-2015 11:05:17
File Name: 2015_0515_1100.031
LCMSMW1: 1065 +

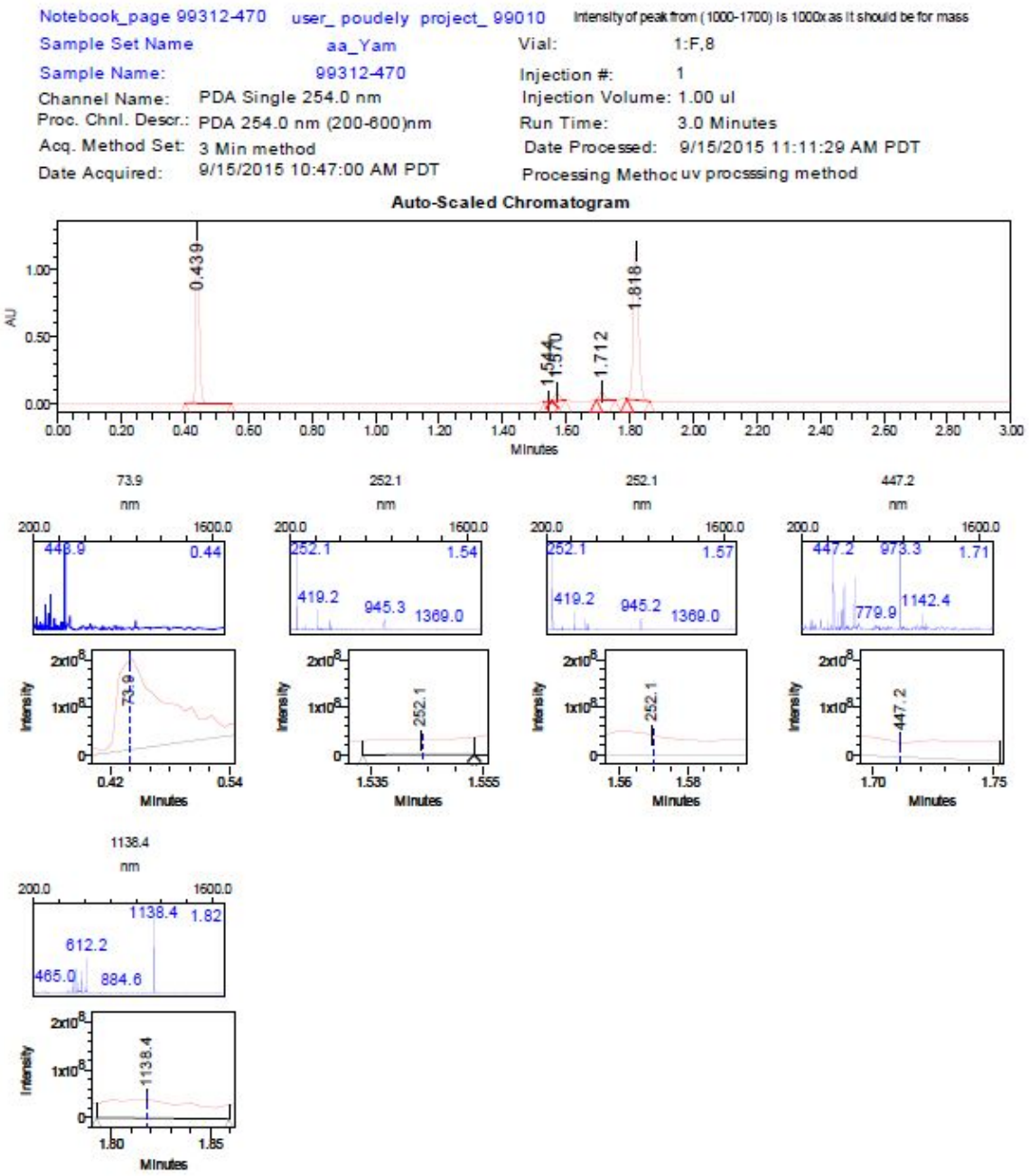
Instrument: WFD-409D-LCMS1
User: christiana.iwuagwu



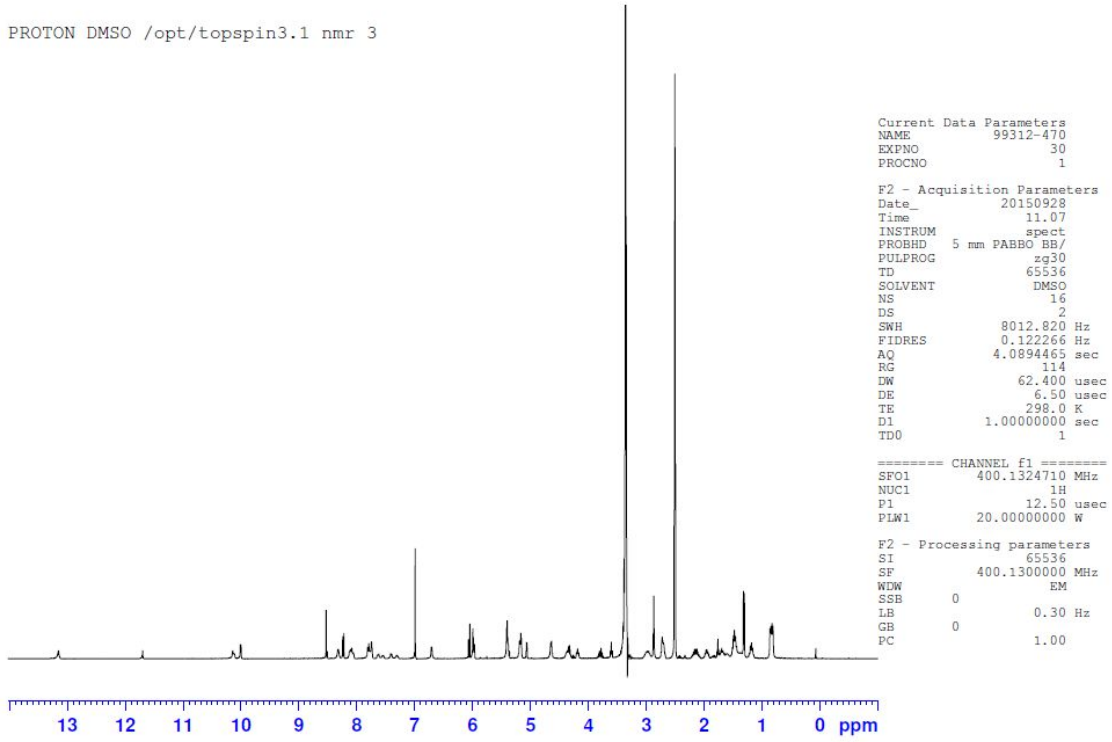
LCMS compound 13



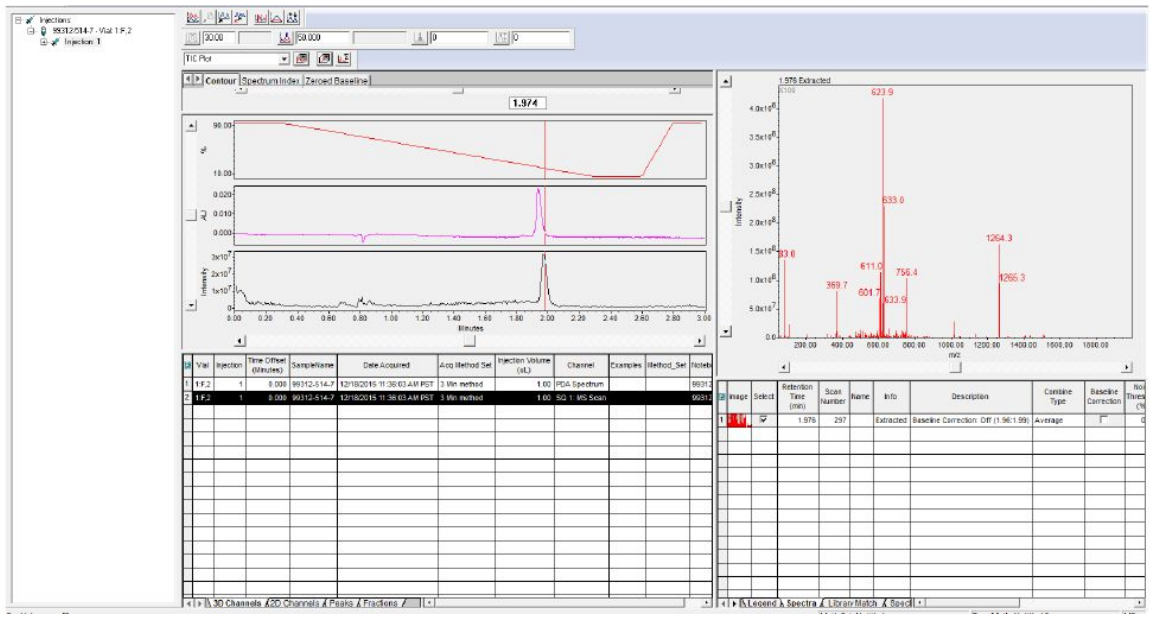
LCMS compound 14



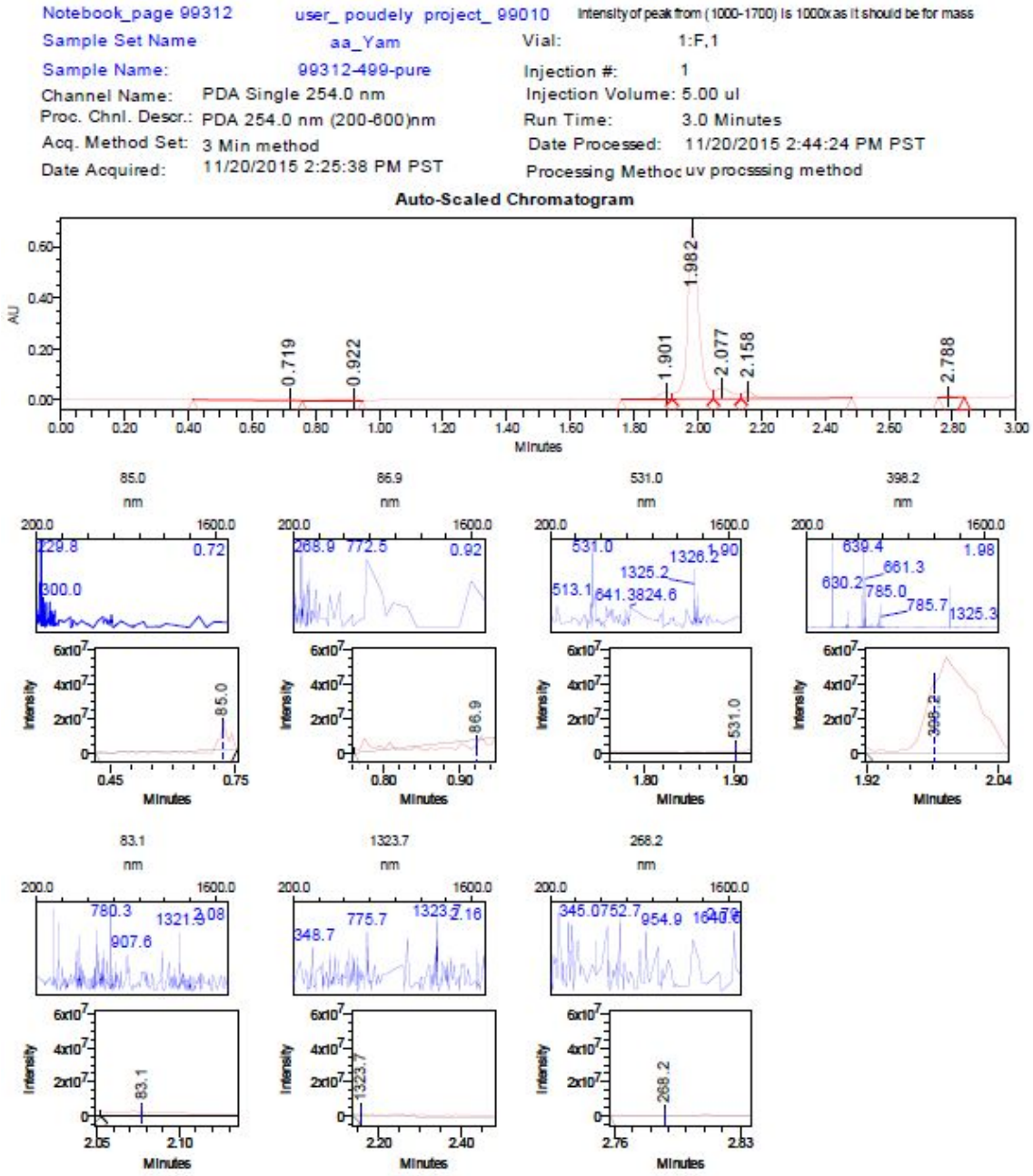
1H-NMR compound 14



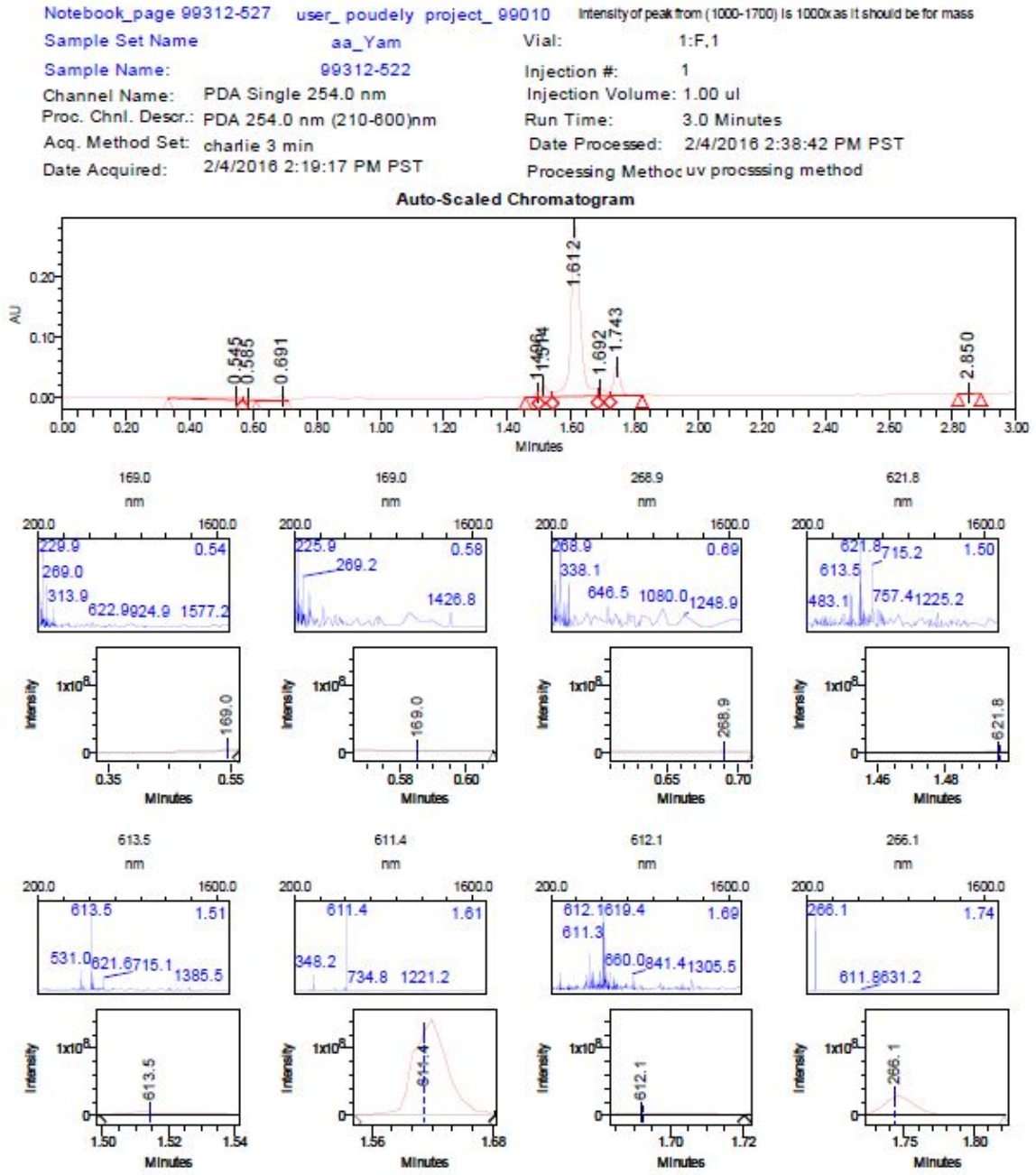
LCMS compound 15



LCMS compound 16



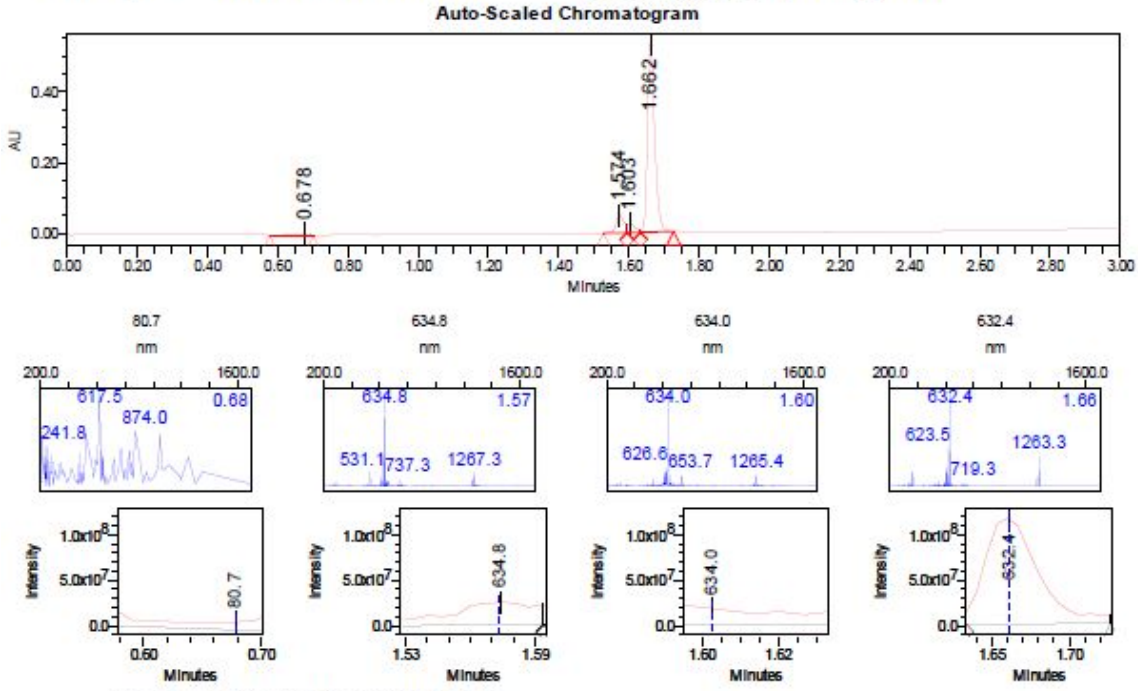
LCMS compound 17



LCMS compound 18

Notebook_page 99312-449 user_ poudely project_ 99010 Intensity of peak from (1000-1700) is 1000x as it should be for mass

Sample Set Name	aa_Yam	Vial:	1:F,3
Sample Name:	99312-511-24	Injection #:	1
Channel Name:	PDA Single 254.0 nm	Injection Volume:	1.00 ul
Proc. Chnl. Descr.:	PDA 254.0 nm (200-600)nm	Run Time:	3.0 Minutes
Acq. Method Set:	3 Min method	Date Processed:	12/11/2015 3:53:26 PM PST
Date Acquired:	12/11/2015 3:36:14 PM PST	Processing Metho	uv procsssing method



Processed Channel: PDA 254.0 nm (200-600)nm

	Processed Channel	Retention Time (min)	Area	% Area	Height
1	PDA 254.0 nm (200-600)nm	0.678	28123	3.08	7255
2	PDA 254.0 nm (200-600)nm	1.574	80239	8.80	50082
3	PDA 254.0 nm (200-600)nm	1.603	39690	4.35	25978
4	PDA 254.0 nm (200-600)nm	1.662	764028	83.77	532677