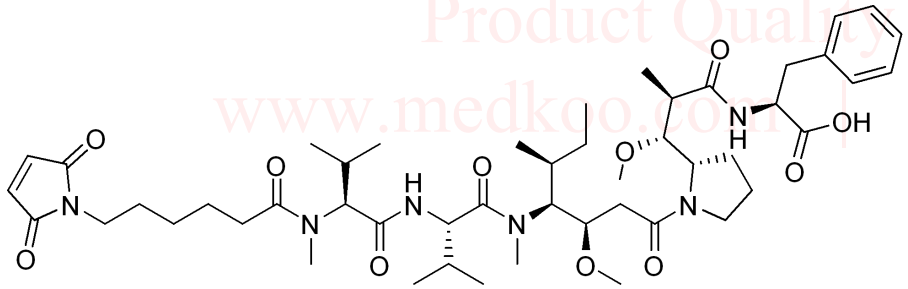
Mafodotin NMR analysis. Solvent =DMSO-d6



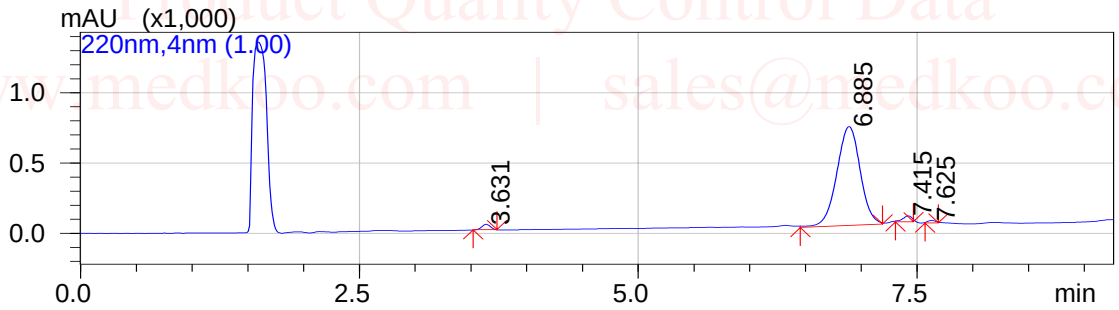
MedKoo Cat#: 206584
Name: Mafodotin
CAS#: 863971-19-1
Lot#YXM61003
Chemical Formula: C49H76N6O11
Exact Mass: 924.5572
Molecular Weight: 925.18

Mafodotin HPLC analysis

==== Shimadzu LCMSsolution Analysis Report ====

Acquired by	: Admin
Sample Name	: Mafodotin
Sample ID	:
Vail #	: 128
Injection Volume	: 10 uL
Data File Name	: Mafodotin007.lcd
Method File Name	: 80ACN-0.2Flow.lcm
Batch File Name	:
Report File Name	: DefaultLCMS.lcr
Data Acquired	: 10/3/2016 5:01:00 PM
Data Processed	: 10/3/2016 5:10:19 PM

<Chromatogram>



Ret. Time	Conc.	Area	Height	Peak Start	Peak End	Area%
3.631	1.72442	186384	37913	3.520	3.733	1.7244
6.885	95.23295	10293289	704402	6.453	7.189	95.2330
7.415	2.22931	240956	42925	7.307	7.467	2.2293
7.625	0.81332	87908	17833	7.573	7.691	0.8133

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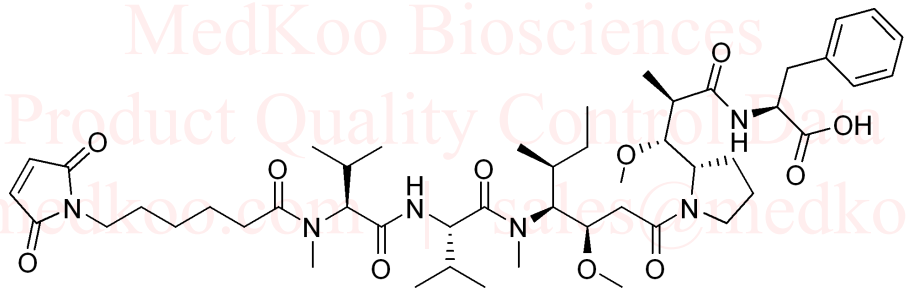
MS of Mafodotin: Positive model:

20161003_161003110700 #30 RT: 0.35 AV: 1 NL: 7.66E8
T: + c ESI ms [300.00-1000.00]

Mafodotin MS analysis



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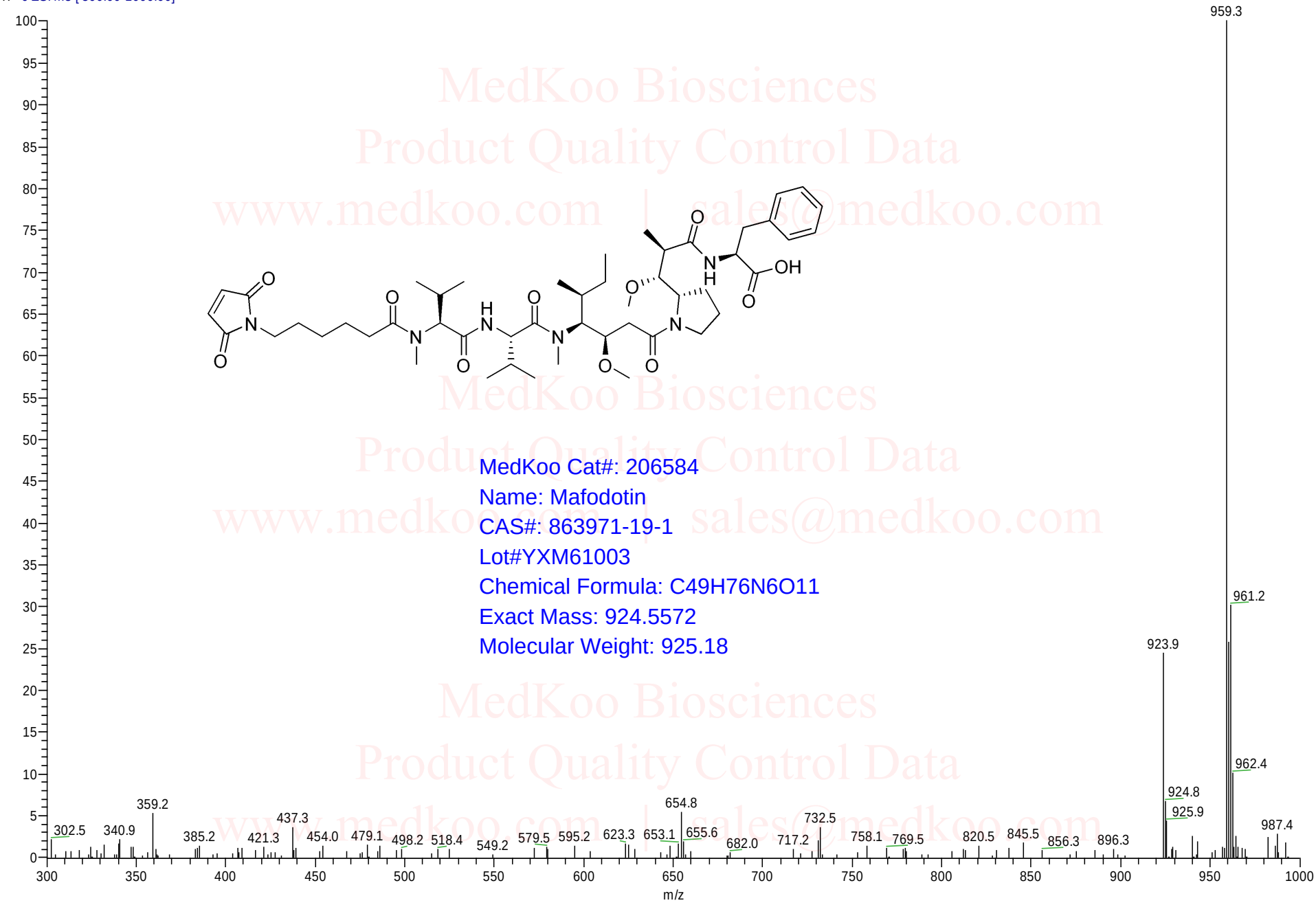
MS of Mafotodin: Negative model:

Mafodotin MS analysis



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was agitated for 2 h. The resin was then filtered, washed with DMF (6 × 10 mL), CH_2Cl_2 (6 × 10 mL), and ethyl ether (6 × 10 mL), and dried in vacuo.

MMAF (MeVal-Val-Dil-Dap-Phe). MeVal-Val-Dil-Dap-Phe-2-chlorotrityl resin (100 mg, 0.044 mmol) in a 5 mL syringe was treated with 2% TFA in CH_2Cl_2 (4 mL) for 5 min at ambient temperature. Preparative HPLC purification provided 27 mg (73%) of white solid. Reversed-phase HPLC analysis: 95% at 5.74 min. ^{1}H NMR (DMF-d_7): δ 0.78 (3H, t), 0.90−1.15 (19H, m), 1.28−1.44 (m), 1.46−1.62 (m), 1.75−2.00 (m), 2.03−2.12 (m), 2.14−2.23 (m), 2.24−2.48 (m), 2.50−2.61 (m), 2.78−2.82 (m), 2.97−3.09 (m), 3.12 (s), 3.18−3.21 (m), 3.26 (d), 3.30 (s), 3.34 (s), 3.41−3.46 (m), 3.49 (d), 3.53−3.60 (m), 3.61−3.69 (m), 3.72−3.78 (m), 3.93 (d), 4.10 (2H, br d), 4.12−4.38 (1H, br), 4.64−4.92 (3H, m), 7.21−7.35 (5H, m), 8.12 (0.5H, d), 8.40 (0.5H, d), 8.78−8.83 (1H, m), 9.03 (1H, br s), 9.63 (1H, br s). HRMS (ESI) calcd for $C_{39}H_{66}N_5O_8$ $(MH)^+$ 732.4911; found, *m*/*z* 732.4890.

Maleimidocaproyl-Val-Cit-PABC−MMAF (L1−MMAF). MeVal-Val-Dil-Dap-Phe-2-chlorotrityl resin (120 mg, 0.053 mmol) in a 5 mL syringe was treated with a solution of maleimidocaproyl-Val-Cit-PAB-OCOpNP (313 mg, 0.42 mmol, 8 equiv), 1-hydroxy-7-azabenzotriazole (HOAt, 2 mg, 0.015 mmol, 0.3 equiv), and DIEA (148 μL, 0.84 mmol, 16 equiv) in DMF (4 mL) for 16 h. The resin was then filtered and washed with DMF (6 × 3 mL), CH_2Cl_2 (6 × 3 mL) and ethyl ether (6 × 3 mL). Cleavage from resin by treatment with 2% TFA in CH_2Cl_2 (4 mL) for 5 min and preparative HPLC purification of the released material generated 15 mg (21%) of white solid that was 98% pure by reversed-phase HPLC analysis (retention time 7.20 min).^{1}H NMR (DMSO-d_6): δ 0.75−0.95 (28H, m), 1.05 (3H, t), 1.14 (m), 1.22−1.53 (m), 1.53−1.80 (m), 1.80−2.40 (m), 2.80 (br s), 2.96 (d), 3.11 (s), 3.13 (s), 3.20 (s), 3.62−3.70 (d), 3.83−3.94 (m), 4.08−4.24 (m), 4.28−4.5 (m), 4.50−4.68 (m), 4.90−5.10 (m), 5.35 (s), 5.96 (1H, t), 7.02 (2H, s), 7.08−7.32 (9H, m), 7.54 (2H, d), 7.80 (d), 8.04−8.12 (m), 8.16−8.33 (m), 10.0 (1H, br s). HRMS (ESI) calcd for $C_{68}H_{104}N_{11}O_{16}$ $(MH)^+$ 1330.7663; found, *m*/*z* 1330.7665.

Maleimidocaproyl-Val-Cit−MMAF (L2−MMAF). Fmoc-Cit (140 mg, 0.352 mmol) and Fmoc-Val (60 mg, 0.176 mmol) were coupled sequentially to MeVal-Val-Dil-Dap-Phe-2-chlorotrityl resin (200 mg, 0.088 mmol) using HATU coupling chemistry as described above for MeVal-Val-Dil-Dap-Phe-2-chlorotrityl resin. Then, to the dry Val-Cit-MeVal-Val-Dil-Dap-Phe-2-chlorotrityl resin a solution of 6-maleimidocaproic acid *N*-hydroxysuccinimide ester (54 mg, 0.176 mmol) in DMF (3 mL) was added. The mixture was shaken at room temperature for 4 h. Cleavage from resin by treatment with 2% TFA in CH_2Cl_2 (4 mL) for 5 min provided 28 mg (27%) of white solid after preparative HPLC purification. Reversed-phase HPLC analysis: 98% at 6.16 min. ^{1}H NMR (DMF-d_7): δ 0.69−0.92 (28H, m), 1.01−1.06 (3H, m), 1.17 (m), 1.42−1.53 (m), 1.53−1.68 (m), 1.70−2.28 (m), 2.28−2.46 (m), 2.74−2.88 (m), 2.89−3.00 (m), 3.04−3.06 (m), 3.15 (s), 3.18 (s), 3.24 (s), 3.40−3.47 (m), 3.48−3.58 (m), 3.59−3.68 (m), 3.71−3.76 (d), 3.96 (m), 4.14 (t), 4.23 (m), 4.38−4.52 (m), 4.58−4.76 (m), 4.89 (m), 5.95 (m), 7.01 (2H, s), 7.17−7.28 (5H, m), 7.77 (m), 7.89 (m), 7.96 (m), 8.04 (m), 8.17 (m), 8.28 (m), 8.38 (d), 8.47 (m). HRMS (ESI) calcd for $C_{60}H_{97}N_{10}O_{14}$ $(MH)^+$ 1181.7186; found, *m*/*z* 1181.7164.

Maleimidocaproyl-PABC−MMAF (L3−MMAF). MeVal-Val-Dil-Dap-Phe-2-chlorotrityl resin (100 mg, 0.044 mmol) was treated with a solution of maleimidocaproyl-PAB-OCO-pNP (42 mg, 0.088 mmol, 2 equiv), 1-hydroxybenzotriazole (HOBt, 1 mg, 9 μmol, 0.1 equiv), and DIEA (30 μL, 0.176 mmol, 4 equiv) in DMF (3 mL) for 16 h. The resin was then filtered and washed with DMF (6 × 3 mL), CH_2Cl_2 (6 × 3 mL), and ethyl ether (6 × 3 mL). Cleavage from resin by treatment with 2% TFA in CH_2Cl_2 (4 mL) for 5 min followed by preparative HPLC purification provided 31 mg (66%) of white solid which was 99% pure by reversed-phase HPLC analysis (retention time 7.14 min).^{1}H NMR (DMF-d_7): δ 0.76−0.98 (18H, m), 1.04 (2H, d), 1.12 (3H, m), 1.25−1.40 (m), 1.51−1.61 (m), 1.63−1.69 (m), 1.76−1.90 (m), 1.94−2.40 (m), 1.35−2.38 (m), 2.50−2.60 (m), 2.96 (s), 3.10 (s), 3.23 (s), 3.27 (s), 3.30 (s), 3.34 (s), 3.41−3.50 (m), 3.50−3.80 (m), 3.90−4.20 (m), 4.33−4.48 (m), 4.54−4.62 (m), 4.63−4.77 (m), 4.82−4.93 (m), 5.06−5.20 (m), 7.02 (2H, s), 7.36−7.21 (9H, m), 7.70 (2H, d), 8.11 (0.5H, d), 8.36 (0.5H, d), 10.00 (1H, br s). HRMS (ESI) calcd for $C_{57}H_{84}N_7O_{13}$ $(MH)^+$ 1074.6127; found, *m*/*z* 1074.6095.

Maleimidocaproyl−MMAF (L4−MMAF). 6-Maleimidocaproic acid (37 mg, 0.176 mmol, 2 equiv) and HATU (67 mg, 0.176 mmol, 2 equiv) were dissolved in anhydrous DMF (3 mL), followed by the addition of DIEA (62 μL, 0.35 mmol, 4 equiv). The solution was then transferred to a 5 mL syringe containing MeVal-Val-Dil-Dap-Phe-2-chlorotrityl resin (200 mg, 0.088 mmol), and the mixture was agitated for 3 h. The resin was filtered, washed with DMF (6 × 3 mL), CH_2Cl_2 (6 × 3 mL), and ethyl ether (6 × 3 mL), and dried in vacuo. Drug−linker was cleaved off the resin by treatment with 2% TFA in CH_2Cl_2 (4 mL) for 5 min and then purified by preparative HPLC to give 57 mg (70%) of white solid. Reversed-phase HPLC analysis: 98% at 6.72 min. ^{1}H NMR (DMF-d_7): δ 0.78−0.96 (18H, m), 1.04 (2H, t), 1.12 (3H, m), 1.25−1.36 (m), 1.41 (2H, d), 1.46−1.65 (m), 1.73−1.87 (m), 1.88−2.07 (m), 2.08−2.26 (m), 2.32−2.48 (m), 2.48−2.67 (m), 2.79−2.84 (m), 2.96 (d), 3.02 (d), 3.10 (d), 3.23 (d), 3.27 (s), 3.30 (s), 3.34 (s), 3.45 (t), 3.52−3.61 (m), 3.62−3.80 (m), 3.93 (d), 4.09−4.28 (m), 4.54−4.78 (m), 4.82−4.91 (2H, m), 7.03 (2H, s), 7.21−7.35- (5H, m), 7.51 (0.5H, d), 8.11 (0.5H, d), 8.36 (0.5H, d), 8.48 (0.5H, d), 13.0 (br s). HRMS (ESI) calcd for $C_{49}H_{77}N_6O_{11}$ $(MH)^+$ 925.5650; found, *m*/*z* 925.5636.

Maleimidocaproyl−[^{13}C]MMAF (L4−[^{13}C]MMAF) was prepared as described above starting from *N*-Fmoc-L-[ring-$^{13}C_6$]-phenylalanine-2-chlorotrityl resin. LCMS (ESI): *m*/*z* 931.62 $(MH)^+$.

Preparation of Antibody−Drug Conjugates. The conjugates with eight drug molecules per antibody were prepared as described previously (*12*, *19*). Briefly, cBR96 or cAC10 (5−15 mg/mL) were mixed with DTT (10 mM, final) at 37 °C for 30 min, and the buffer was exchanged by elution through Sephadex G-25 resin with PBS containing 1 mmol/L diethylenetriaminepentaacetic acid. PBS containing 1 mmol/L diethylenetriaminepentaacetic acid was added to the reduced mAb bringing its final concentration to 2.5 mg/mL. The thiol concentration was ∼8.4 thiols/mAb as measured by Ellman's reagent, DTNB. A 9.5-fold molar excess of linker−drug (L1−MMAF, L1−Dox (*10*), L1−MMAE (*12*), or L4−MMAE) was added to the reduced antibody at 4 °C for 1 h, and the conjugation reaction was quenched by adding a 20-fold excess of cysteine. The reaction mixture was concentrated by centrifugal ultrafiltration and buffer-exchanged through Sephadex G-25 equilibrated with PBS at 4 °C. The conjugate was then sterile filtered through a 0.2-μm filter.

cAC10 antibody−drug conjugates with four drugs per antibody were prepared by partial reduction of the mAb (*14*, *20*) followed by reaction with desired linker−drug (L1−MMAF, L2−MMAF, L3−MMAF, or L4−MMAF). The antibody cAC10 (10 mg/mL) was partially reduced by addition of 3.0 molar equivalents of DTT at pH 8.0, followed by incubation at 37 °C for ∼2 h. The reduction reaction was then chilled to ∼10 °C and the excess DTT removed via diafiltration. The thiol concentration was determined by DTNB, and the SH/Ab ratio was found to be in the range of 3.8−4.5. The linker−drug was