**General Information.** All commercially available anhydrous solvents were used without further purification. Commercially available aldehydes were purchased from MilliporeSigma and used without further purification. 4-Methylpentanal was synthesized by the oxidation of 4-methylpentanol (Meyer et al., *J. Org. Chem.* **1994**, 59, 7549-7552). Auristatins and drug linkers were synthesized according to our previous reports (Doronina et al WO2009117531A1; Doronina et al., *Bioconjugate Chem.* **2006**, 17, 114-124 and Doronina et al., *Bioconjugate Chem.* **2008**, 19, 1960-1963). UPLC-MS system 1 consisted of a Waters SQ mass detector interfaced to an Acquity Ultra Performance LC equipped with an Acquity UPLC BEH C18 2.1 x 50 mm, 1.7µm reverse phase column. The acidic mobile phase (0.1% formic acid) consisted of a gradient of 3% acetonitrile/97% water to 100% acetonitrile (flow rate = 0.5 mL/min). UPLC-MS system 2 consisted of a Waters Xevo G2 ToF mass spectrometer interfaced to a Waters Acquity H-Class Ultra Performance LC equipped with an Acquity UPLC BEH C18 2.1 x 50 mm, 1.7 µm reverse phase column (Column 1) or CORTECS UPLC C18 2.1 x 50 mm, 1.6 µm reverse phase column (Column 2). Preparative HPLC was carried out on a Waters 2545 Binary Gradient Module with a Waters 2998 Photodiode Array Detector. Products were purified over a C12 Phenomenex Synergi 250 x 10.0 mm, 4 μm, 80 Å reverse phase column (Column 1) or a C12 Phenomenex Synergi 250 x 50 mm, 10 μm, 80 Å reverse phase column (Column 2) eluting with 0.1% trifluoroacetic acid in water (solvent A) and 0.1% trifluoroacetic acid in acetonitrile (solvent B). The purification methods generally consisted of linear gradients of solvent A to solvent B, ramping from 90% aqueous solvent A to 10% solvent A. The flow rate was 4.6 mL/min with monitoring at 254 nm.

**Table S1:** Data collection and refinement statistics for auristatin **17** bound to tubulin. Statistics for the highest-resolution shell are shown in parentheses.

|  |  |  |  |
| --- | --- | --- | --- |
| **Wavelength (Å…)** | 0.97 | **Number of non-hydrogen atoms** | 17953 |
| **Resolution range (Å…)** | 62.29 - 2.352 (2.436 - 2.352) | **macromolecules** | 17568 |
| **Space group** | P 21 21 21 | **ligands** | 219 |
| **Unit cell** | 104.491 155.148 181.885 90 90 90 | **water** | 166 |
| **Total reflections** | 864868 (115513) | **Protein residues** | 2196 |
| **Unique reflections** | 122884 (12034) | **RMS(bonds)** | 0.005 |
| **Completeness (%)** | 97.16 (75.19) | **RMS(angles)** | 0.95 |
| **Mean I/sigma(I)** | 9.98 (0.37) | **Ramachandran favored (%)** | 96.46 |
| **Wilson B-factor** | 56.73 | **Ramachandran allowed (%)** | 3.36 |
| **Reflections used in refinement** | 119575 (9170) | **Ramachandran outliers (%)** | 1.70 |
| **Reflections used for R-free** | 1954 (160) | **Clashscore** | 1.91 |
| **R-work** | 0.2508 (0.3793) | **Average B-factor** | 68.75 |
| **R-free** | 0.2671 (0.3940) | **macromolecules** | 68.88 |
|  |  | **ligands** | 63.09 |
|  |  | **solvent** | 62.47 |

**Experimental Procedure**



**General scheme for solid phase auristatin synthesis.**

**General Procedure for lantern loading.**

A D-series trityl alcohol lantern (8 mmol/lantern) was treated with 0.5 mL solution of 10% (V/V) acetyl chloride in dry DCM at RT for 3 h. The solution was filtered, and the lanterns were washed with dry DCM (3 × 3 mL) and used immediately without drying.

The lantern was treated with 0.5 mL of a solution of Fmoc-amino acid (0.14 M, 70 mmol, 8.75 equiv.) and DIPEA (0.5 M, 260 mmol, 33 equiv.) in DCM at rt for 2 h. The solution was filtered, and the lanterns were washed with DMF (3 × 3 mL) and DCM (3 × 3 mL) and vacuum-dried in a desiccator.

**General procedure for Fmoc deprotection**

The lantern was treated with a 0.5 mL solution of 20% (V/V) piperidine in DMF and shaken for 30 min. The solution was removed, and the lantern was subjected to the same deprotection conditions. The solution was filtered, and the lanterns are washed with DMF (3 × 3 mL) and DCM (3 × 3 mL) and vacuum-dried in a desiccator.

**General procedure for amide coupling**

Fmoc-amino acid (128 mmol, 16 equiv.) was dissolved in dry DMF (0.6 mL, 0.2 M final concentration) and DIPEA (217 mmol, 27 equiv.), and HATU (124 mmol, 15.5 equiv.) were added successively and the reaction was stirred for 5 min. The lantern was treated with the solution of activated Fmoc-amino acid and shaken for 2 h. The solution was filtered, and the lanterns were washed with DMF (3 × 3 mL) and DCM (3 × 3 mL) and vacuum-dried in a desiccator.

**General procedure for reductive amination.**

Aldehyde (40 mmol, 5 equiv.) was dissolved in a 0.6 mL solution of 1% (V/V) AcOH in DMF, followed by the addition of NaBH3CN (32 mmol, 4 equiv.). The lantern was treated with the solution and shaken for 2 h. The solution was filtered, and the lanterns were washed with DMF (3 × 3 mL) and DCM (3 × 3 mL) and vacuum-dried in a desiccator.

**General Procedure for cleavage of lantern.**

Lanterns are placed individually in 96-well plates and treated with 0.5 mL solution of 20% (V/V) HFIP in DCM for 1 h. Lanterns are removed and the cleaved products are concentrated using a stream of N2. Samples were dissolved in DMSO for UPLC analysis and preparative HPLC.

Auristatin **6**:



Prepared by reductive amination with acetaldehyde. Yield: 3.2 mg (52 %) Analytical UPLC-MS (UPLC 1): tr = 1.37 min, *m/z* (ES+) calculated 760.52 (M+H)+, found 760.47.

Auristatin **7**:



Prepared by reductive amination with propionaldehyde. Yield: 2.4 mg (38 %) Analytical UPLC-MS (UPLC 1): tr = 1.38 min, *m/z* (ES+) calculated 774.53 (M)+, found 774.54.

Auristatin **8**:



Prepared by reductive amination with butyraldehyde. Yield: 2.9 mg (46 %) Analytical UPLC-MS (UPLC 1): tr = 1.43 min, *m/z* (ES+) calculated 788.55 (M+H)+, found 788.51.

Auristatin **9**:



Prepared by reductive amination with valeraldehyde. Yield: 2.2 mg (34 %) Analytical UPLC-MS (UPLC 1): tr = 1.50 min, *m/z* (ES+) calculated 802.56 (M+H)+, found 802.19.

Auristatin **10**:



Prepared by reductive amination with 3-methylbutanal. Yield: 4.1 mg (64 %) Analytical UPLC-MS (UPLC 1): tr = 1.48 min, *m/z* (ES+) calculated 802.56 (M+H)+, found 802.48.

Auristatin **11**:



Prepared by reductive amination with hexanal. Yield: 3.0 mg (46 %) Analytical UPLC-MS (UPLC 1): tr = 1.55 min, *m/z* (ES+) calculated 816.58 (M+H)+, found 816.45.

Auristatin **12**:



Prepared by reductive amination with 4-methylpentanal. Yield: 3.7 mg (57 %) Analytical UPLC-MS (UPLC 1): tr = 1.55 min, *m/z* (ES+) calculated 816.58 (M+H)+, found 816.45.

Auristatin **13**:



Prepared by reductive amination with heptanal. Yield: 3.8 mg (57 %) Analytical UPLC-MS (UPLC 1): tr = 1.65 min, *m/z* (ES+) calculated 830.60 (M+H)+, found 830.67.

Auristatin **14**:



Prepared by reductive amination with octanal. Yield: 1.2 mg (17 %) Analytical UPLC-MS (UPLC 1): tr = 1.72 min, *m/z* (ES+) calculated 844.61 (M+H)+, found 844.45.

Auristatin **15**:



Prepared by reductive amination with nonanal. Yield: 1.5 mg (22 %) Analytical UPLC-MS (UPLC 1): tr = 1.80 min, *m/z* (ES+) calculated 858.63 (M+H)+, found 858.35.

Auristatin **16**:



Prepared by reductive amination with 3,5,5-trimethylhexanal. Yield: 2.8 mg (41 %) Analytical UPLC-MS (UPLC 1): tr = 1.71 min, *m/z* (ES+) calculated 858.63 (M+H)+, found 858.64.

Auristatin **17**:



Prepared by reductive amination with 3,5,5-trimethylhexanal. Yield: 2.9 mg (42 %) Analytical UPLC-MS (UPLC 1): tr = 1.71 min, *m/z* (ES+) calculated 872.65 (M+H)+, found 872.64.

Auristatin **18**:



Prepared by reductive amination with decanal. Yield: 4.0 mg (57 %) Analytical UPLC-MS (UPLC 1): tr = 1.87 min, *m/z* (ES+) calculated 872.64 (M+H)+, found 872.51.

Auristatin **19**:



Prepared by reductive amination with undecanal. Yield: 2.7 mg (38 %) Analytical UPLC-MS (UPLC 1): tr = 1.96 min, *m/z* (ES+) calculated 886.66 (M+H)+, found 886.58.

Auristatin **20**:



Prepared by reductive amination with dodecanal. Yield: 2.4mg (33 %) Analytical UPLC-MS (UPLC 1): tr = 2.06 min, *m/z* (ES+) calculated 900.67 (M+H)+, found 900.65.

Auristatin **21**:



Prepared by reductive amination with *N*-methyl-*N*-(4-oxoethyl)pivalamide. Yield: 4 mg (32 %) Analytical UPLC-MS (UPLC 2, Column 1): tr = 1.28 min, *m/z* (ES+) calculated 889.60 (M+H)+, found 889.66.

Auristatin **22**:



Prepared by reductive amination with *N*-methyl-*N*-(4-oxopropyl)pivalamide. Yield: 7 mg (55 %) Analytical UPLC-MS (UPLC 2, Column 1): tr = 1.23 min, *m/z* (ES+) calculated 903.61 (M+H)+, found 903.68.

Auristatin **23**:



Prepared was prepared by reductive amination with *N*-methyl-*N*-(4-oxobutyl)pivalamide. Yield: 17 mg (68 %) Analytical UPLC-MS (UPLC 2, Column 1): tr = 1.21 min, *m/z* (ES+) calculated 917.63 (M+H)+, found 917.67.

Auristatin **24**:



Boc-*N*-methyl-pentyl-AF was prepared by reductive amination with *N*-methyl-*N*-(4-oxopentyl)pivalamide. Yield: 3 mg (25 %) Analytical UPLC-MS (UPLC 2, Column 1): tr = 1.29 min, *m/z* (ES+) calculated 931.64 (M+H)+, found 931.71.

**General Scheme for Drug Linkers**



**General Procedure for reductive amination of auristatins on resin.**

A general peptide coupling with Fmoc-amino acids and HATU, and the intermediate auristatin on Cl-trityl resin was prepared as previously described (WO 2009117531A1).

Aldehyde (0.14 mmol, 2 equiv.) was dissolved in a 10 mL solution of 1% (V/V) AcOH in DMF, followed by the addition of NaBH3CN (0.12 mmol, 1.8 equiv.). The solution was added to a syringe with a PET frit containing resin (0.1 g, 0.07 mmol/g), and the mixture was agitated for about 2 h. The resin was filtered, washed with DMF, DCM and ethyl ether, and dried in a vacuum desiccator.

**General Procedure for removing allylic protecting groups.**

Phenylsilane (0.7 mmol, 10 equiv.) was dissolved in 1.4 mL of DCM, and the solution was added to a syringe with a PET frit containing resin (0.1 g, 0.07 mmol/g), and the mixture was agitated for 5 min. Pd(PPh)3 (14 umol, 0.2 equiv.) was dissolved in 0.3 mL of DCM and added to the resin mixture. The resin was agitated for 2 h, filtered, washed with DMF, DCM and ethyl ether, and dried in a vacuum desiccator.

**General Procedure for maleimide coupling and resin cleavage.**

3-(Maleimido)propionic acid *N*-hydroxysuccinimide ester (0.09 mmol, 1.2 equiv.) and DIPEA (0.14 mmol, 1.7 equiv.) were dissolved in 1.0 mL DMF, and the solution was added to a syringe with a PET frit containing resin (0.1 g, 0.07 mmol/g). The mixture was agitated for 2 h, filtered, washed with DMF, DCM and ethyl ether, and dried in a vacuum desiccator. A solution of 20% (V/V) HFIP in DCM was added to the resin for 1 h and filtered. Resin was washed with DCM and the combined organic layers were dried in vacuo. Samples were dissolved in ACN for UPLC analysis and DMSO for preparative HPLC.

Drug linker **25**:



Yield: 27 mg (29 %) Analytical UPLC-MS (UPLC 2, Column 1): tr = 1.19 min, *m/z* (ES+) calculated 1325.79 (M+H)+, found 1325.87.

Drug linker **26**:



Yield: 11 mg (12 %) Analytical UPLC-MS (UPLC 1): tr = 1.69 min, *m/z* (ES+) calculated 1224.74 (M+H)+, found 1224.55.

Drug linker **27**:



Yield: 33 mg (79 %) Analytical UPLC-MS (UPLC 2, Column 1): tr = 1.26 min, *m/z* (ES+) calculated 1266.79 (M+H)+, found 1266.96.