Electronic Supplementary Material (ESI) for ChemComm.

Supporting Information

Iron-Dependent Lysosomal Dysfunction Mediated by a Natural Products Hybrid

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Contents

- **Chemistry**

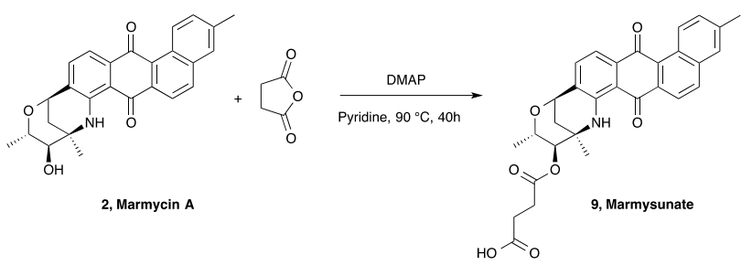
1. General experimental procedures

All starting materials were purchased from commercial sources and used without any further purification. Solvents were dried under standard conditions. NMR spectroscopy was performed on Bruker 500MHz apparatus, using deuterated solvents as detailed and at room temperature (300 K). Notation for the ^{1}H NMR spectral splitting patterns includes: singlet (s), doublet (d), triplet (t), quartet (q), broad (br) and multiplet/overlapping peaks (m). Chemical shifts (δ) are quoted in ppm and coupling constants (J) are quoted in Hertz. ^{1}H NMR spectra are reported using the residual non deuterated solvent as internal standard ($CDCl_3$ ^{1}H, 7.26 ppm). ^{13}C NMR spectra are reported relative to solvent ($CDCl_3$ ^{13}C, 77.0 ppm). High-resolution mass analysis (HRMS) was performed on a Waters LCT Premier XE, under electron spray ionization (ESI). Optical rotation measurements were performed on an Anton Paar Polarimeter MCP 300. The following parameters used were: temperature 20 °C, 100 mm path-length quartz cuvette, wavelength 589 nm. Reactions were monitored by thin-layer chromatography (TLC) using silica gel coated aluminium plates 60 F254 (Merck) and spots were visualized under UV light. Isolated yields were calculated following compounds purification. Flash chromatography was performed using Gerduran® silica gel 60 (40 – 63 μm, Merck) at room temperature, under a positive pressure of air. Preparative TLC was carried out using 0.50 mm Merck silica gel plates (60F-254). UPLC-MS analysis was performed on an Acquity apparatus (Waters) coupled with a triple quadrupole mass spectrometer detector. HPLC analysis was performed on all final compounds on a HPLC Alliance 2695 (Waters) apparatus, equipped with a PDA 996 detector. For the final compounds the purity was determined to be >95%.

2. Synthesis

Artesunate (**1**) was purchased from Sigma Aldrich. Marmycin A (**2**) and artesumycin (**3**) were synthesized according to modified procedures previously reported by us.[1]

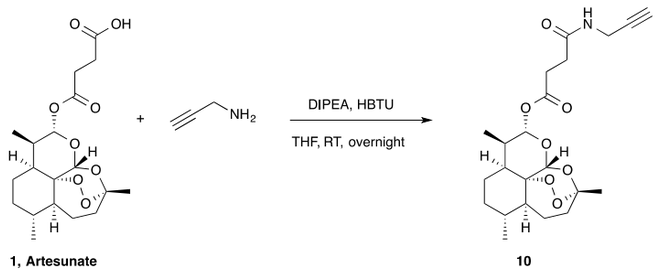
Synthesis of marmysunate (9)



Scheme S1: Synthesis of marmysunate from marmycin A and succinic anhydride.

A mixture of marmycin A (**2**, 4.3 mg, 0.0104 mmol, 1 equiv), DMAP (0.63 mg, 0,0052 mmol, 50 mol%) and succinic anhydride (13.4 mg, 0.104 mmol, 10 equiv) in dry pyridine (1 mL) was stirred at 90°C for 40 h. After this time the reaction was cooled to room temperature, diluted with chloroform and washed with aqueous HCl (pH ≈ 2). The organic layer was dried over $MgSO_4$ and concentrated to dryness under reduced pressure. The crude residue was purified by preparative TLC (ethyl acetate), to afford **9** as a red solid (1.9 mg, 0.0037 mmol, 36%). $[\alpha]_D^{20}$ +142 (*c* 0.015, THF). ^{1}H NMR (500 MHz, $CDCl_3$) δ 9.57 (1H, d, *J* = 9.0 Hz), 9.54 (1H, s), 8.37 (1H, d, *J* = 8.5 Hz), 8.10 (1H, d, *J* = 8.5 Hz), 7.68 (1H, s), 7.60-7.57 (2H, m), 7.51 (1H, d, *J* = 7.5 Hz), 4.83 (1H, s), 4.80 (1H, d, *J* = 10.0 Hz), 3.48-3.43 (1H, m), 2.86-2.73 (2H, m), 2.71-2.64 (2H, m), 2.56 (3H, s), 2.25 (1H, d, *J* = 13.0 Hz), 1.87 (1H, d, *J* = 13.0 Hz), 1.42 (3H, s), 1.08 (3H, d, *J* = 6.0 Hz). ^{13}C NMR (125 MHz, $CDCl_3$) δ 186.5, 186.2, 174.4, 171.5, 148.9, 139.0, 136.82, 136.76, 136.2, 134.9, 134.7, 132.3, 129.1, 128.7, 128.5, 127.8, 127.0, 122.5, 116.2, 111.2, 79.9, 69.4, 66.4, 51.5, 35.2, 29.5, 28.6, 25.1, 21.8, 18.0. HRMS (ESI-TOF) calcd. for $C_{30}H_{28}NO_7^+$ $[M+H]^+$ 514.1866, found: 514.1868.

Synthesis of artesunate-alkyne (10)



Scheme S2: Synthesis of artesunate-alkyne from artesunate and propargylamine.

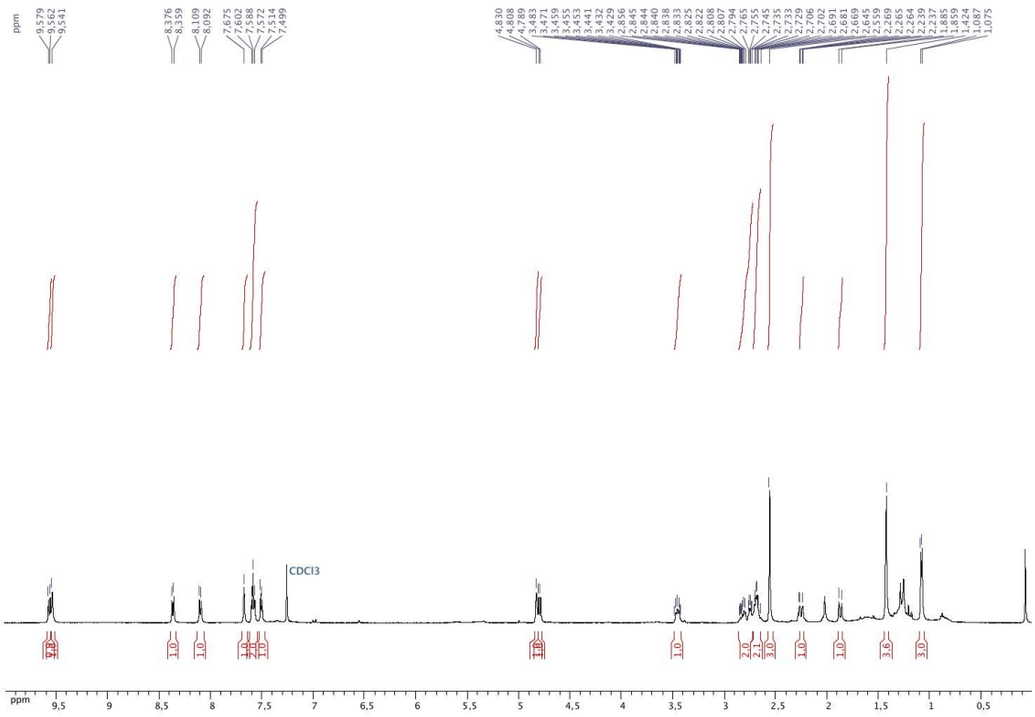
A solution of artesunate (**1**, 20 mg, 0.052 mmol, 1 equiv.), DIPEA (18 μL, 0.104 mmol, 2 equiv.) and HBTU (39.5 mg, 0.104 mmol, 2 equiv.) in dry THF (1 mL) was stirred at room temperature (RT) for 3 h. Then propargylamine (3.5 μL, 0.104 mmol, 2 equiv.) was added and the reaction was stirred overnight. After this time, the mixture was diluted with ethyl acetate and washed with water. The organic layer was dried over $MgSO_4$ and concentrated to dryness under reduced pressure. The crude residue was purified by flash chromatography (heptane: ethyl acetate, 1:1) to afford **10** (16 mg, 0.038 mmol, 73%) as a white solid. ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.91 (1H, br s), 5.78 (1H, d, *J* = 10.0 Hz), 5.43 (1H, s), 4.09-3.99 (2H, m), 2.83-2.71 (2H, m), 2.58-2.53 (2H, m), 2.50-2.44 (1H, m), 2.37 (1H, td, *J* = 14.0, 4.0 Hz), 2.22 (1H, t, *J* = 2.5 Hz), 2.03 (1H, dt, *J* = 13.5, 4.0 Hz), 1.92-1.86 (1H, m), 1.77 (1H, dq, *J* = 13.5, 4.0 Hz), 1.71 (1H, dq, *J* = 13.5, 3.5 Hz), 1.62 (1H, dt, *J* = 13.5, 4.5 Hz), 1.52-1.46 (1H, m), 1.43 (3H, s), 1.41-1.27 (3H, m), 1.02 (1H, td, *J* = 13.0, 3.5 Hz), 0.96 (3H, d, *J* = 6.0 Hz), 0.85 (3H, d, *J* = 7.0 Hz). ^{13}C NMR (75 MHz, $CDCl_3$) δ 171.8, 171.0, 104.6, 92.4, 91.6, 80.2, 79.6, 71.7, 51.7, 45.4, 37.4, 36.4, 34.2, 31.9, 30.8, 29.8, 29.4, 26.1, 24.7, 22.1, 20.3, 12.2. HRMS (ESI-TOF) calcd. for $C_{22}H_{31}NNaO_7^+$ $[M+Na]^+$ 444.1998, found: 444.2004.

3. Fragmentation study

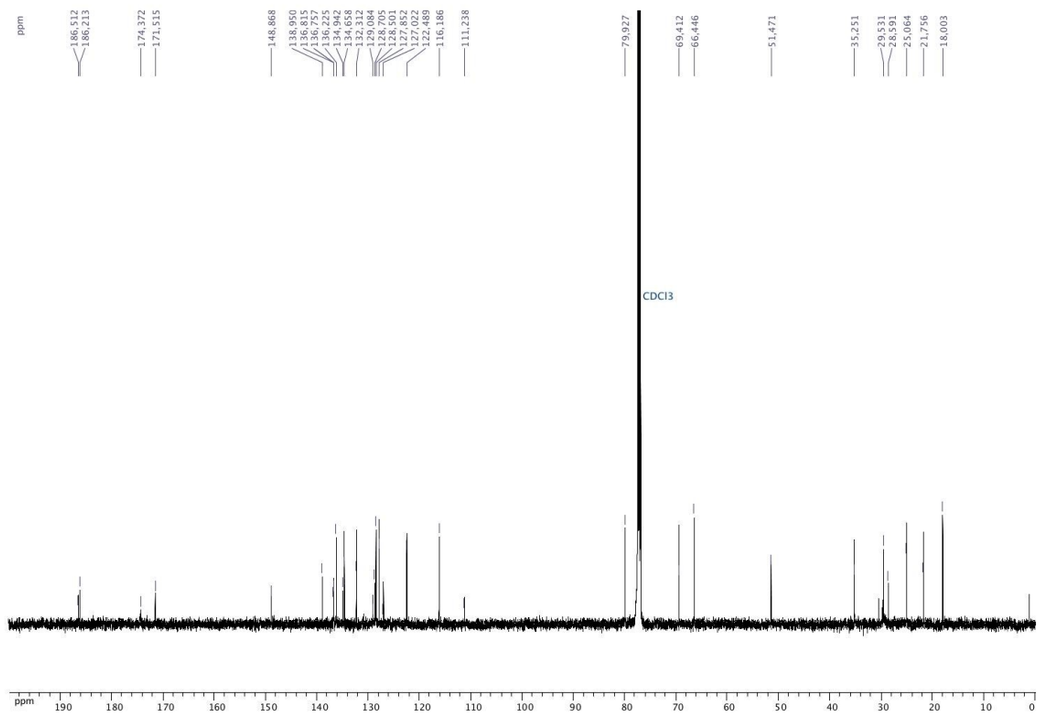
A degased 8 mM solution of $FeCl_2$•$4H_2O$ in acetonitrile (165 □l) was added to artesumycin **3** (1.1 mg, 0.0014 mmol) and the mixture was stirred at RT under argon. After 1.5 h, water (25 □l) was added and the reaction mixture was stirred for another 22 h at RT. The course of the reaction was monitored by UPLC-MS (HSS C18 2.1 x 50 mm, 1.7 μm; 0.6 ml/min; H_2O + 0.1% HCOOH: ACN + 0.1% HCOOH, 95: 5 – 5: 95)

4. NMR spectra

Marmysunate (9) - ^{1}H NMR (500 MHz, $CDCl_3$, 283 K)



Marmysunate (9) - ^{13}C NMR (125 MHz, $CDCl_3$, 283 K)



- **Biology**

Cell culture and proliferation assays. U2OS cell line was purchased from ATCC and was maintained in McCoy's 5A supplemented with 10% fetal bovine serum (FBS) and 1 × Antibiotic-Antimycotic (100× Gibco®) and incubated at 37 °C with 5% CO_2. Cell viability assay was carried out by plating 2,000 cells per well in 96-well plate (Corning). After 24 h, cells were treated with the indicated drugs at various concentrations for 72 hours, then incubated with CellTiter-Blue® (G8081, Promega, 20 µL/100 µL medium) for 1 h before recording fluorescence (560(20)Ex/590(10)Em) using a PerkinElmer Wallac 1420 Victor^2 Microplate Reader.

Drugs and inhibitors. Artesunate (**1**) was purchased from Sigma Aldrich (Sigma A3731). Marmycin A (**2**) and artesumycin (**3**) were synthesized as previously described.[1] Marmysunate (**9**) and artesunate alkyne (**10**) were prepared accordingly to **Scheme S1** and **S2**, respectively. Deferoxamine mesylate (DFO, Sigma D9533, 2 µM for iron chelation experiments, 2 h pre-treatment then 72 h co-treament) and ferric ammonium citrate (FAC, Sigma F5879, 50 µg/mL, for iron supplementation experiments, 2 h pre-treatment then 72 h co-treamen) were freshly prepared prior treatment.

Chemical labeling of clickable molecules and immunofluorescence. U2OS cells were cultured at 80% confluence and were treated with the indicated drugs at different concentrations. Lysotracker® Red DND99 (L7528, Invitrogen) was added 1-2 hours prior cell fixation. Chemical introduction of a fluorophore onto artesunate-alkyne in cells was performed as follow: cells were fixed for 12 min with 2% formaldehyde/PBS, washed with PBS and permeabilized for 10 min with 0.2% Triton X-100/PBS then washed three times with 1% Bovine Serum Albumin/PBS. The click reaction cocktail was prepared from Click-iT® EdU Imaging Kits (C10337, Life Technologies) according to the manufacturer protocol. Briefly, 430 µL of 1× Click-iT reaction buffer was mixed with 20 µL of CuSO4 solution, 1.2 µL Alexa Fluor® azide and 50 µL click reaction additive (sodium ascorbate) to reach a final volume of about 500 µL per coverslip. Next, coverslips were incubated with the click reaction cocktail in the dark for 30 mins at room temperature, then washed three times with 1% BSA/PBS. Coverslips were then washed one more time with PBS and mounted using VECTASHIELD HardSet Antifade Mounting Medium with DAPI (H-1500, 10 mL, Vector Laboratories). High resolution fluorescence images were acquired using a Deltavision real-time microscope (Applied Precision). 100x/1.4NA objective was used for 2D acquisitions that was deconvoluted using SoftWorx software (Ratio conservative – 15 iterations, Applied Presision). ImageJ was used for further image processing.

GFP-Lamp1 transduction. GFP-Lamp1 was transiently expressed in U2OS cells following the manufacturer instructions. Briefly, 10 µL of CellLight® Lysosomes- GFP BacMam 2.0 (C10596, Life Technologies) was mixed well with 500 µM U2OS culture medium and added to µ-dish (ibidi). After 16 h incubation, cells were washed with fresh medium and treated with Artesunate (5 µM, 4 h). Cells were then imaged using a 60x APO TIRF oil immersion objective of Nipkow Spinning Disk confocal system.

- **Supplementary figures**

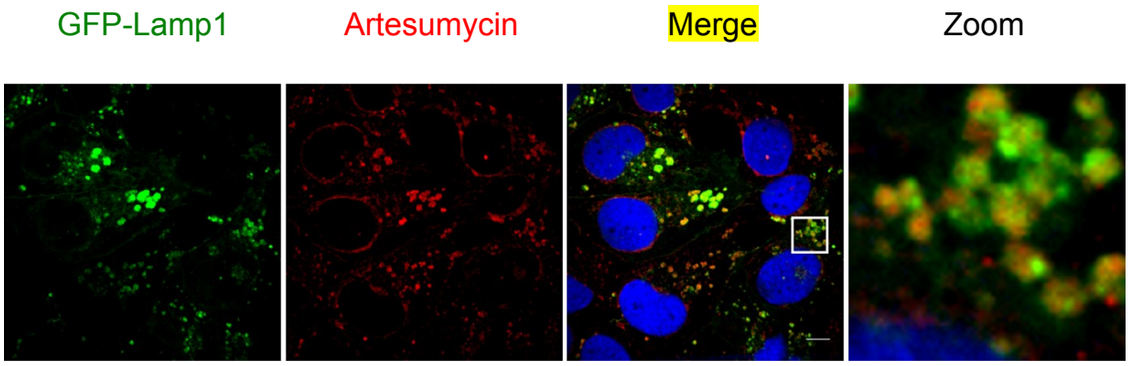


Figure S1: Fluorescence microscopy images of U2OS cells showing artesumycin (red) accumulation in lysosomes (GFP-Lamp1, green). The white box indicates the area of magnification of the main image. Zoom image is ×8. Scale bar 10 µm.

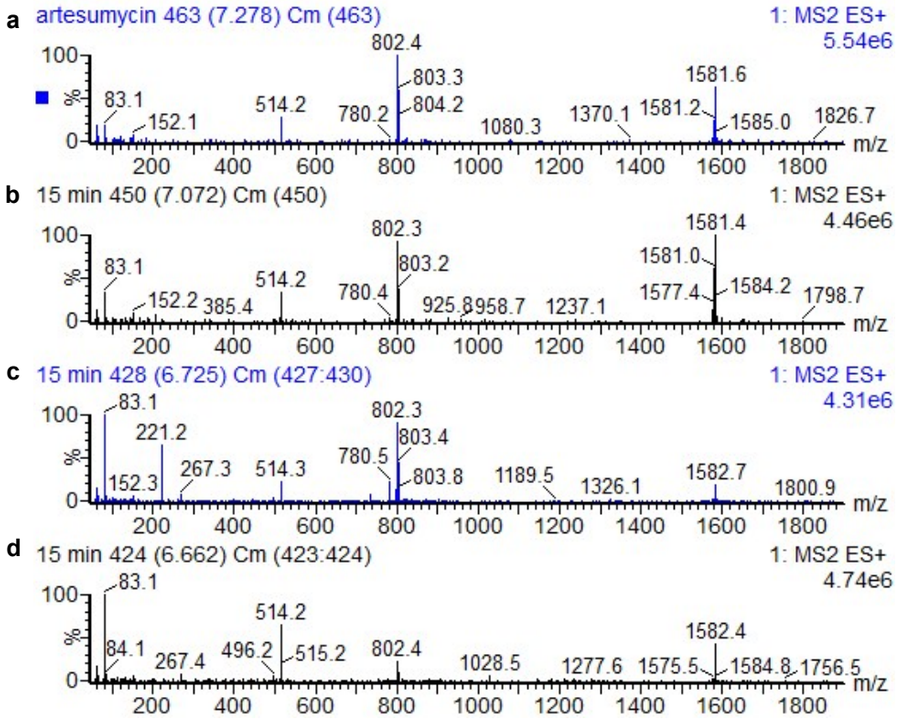


Figure S2: Comparison between the mass spectra of (**a**) artesumycin and (**b**) reaction product at 7.07 mins, (c) reaction product at 6.72 mins and (**d**) reaction product at 6.66 mins, obtained after 15 mintutes of reaction.

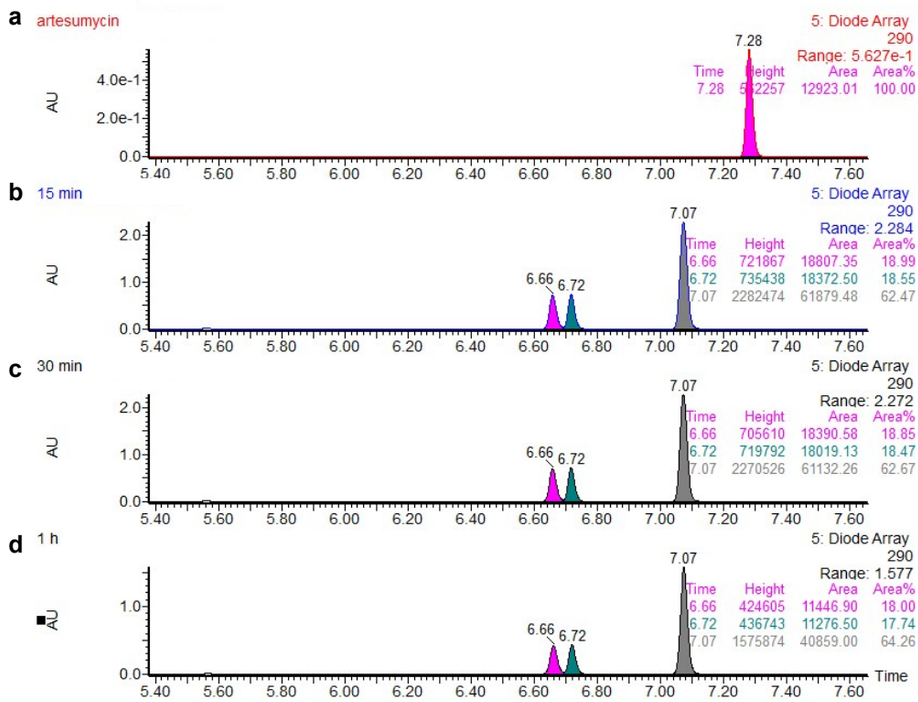


Figure S3: Iron-mediated fragmentation of artesumycin. Chromatograms of artesumycin (**a**) and artesumycin after (**b**) 15 mins, (**c**) 30 mins and (**d**) 1 h of reaction.

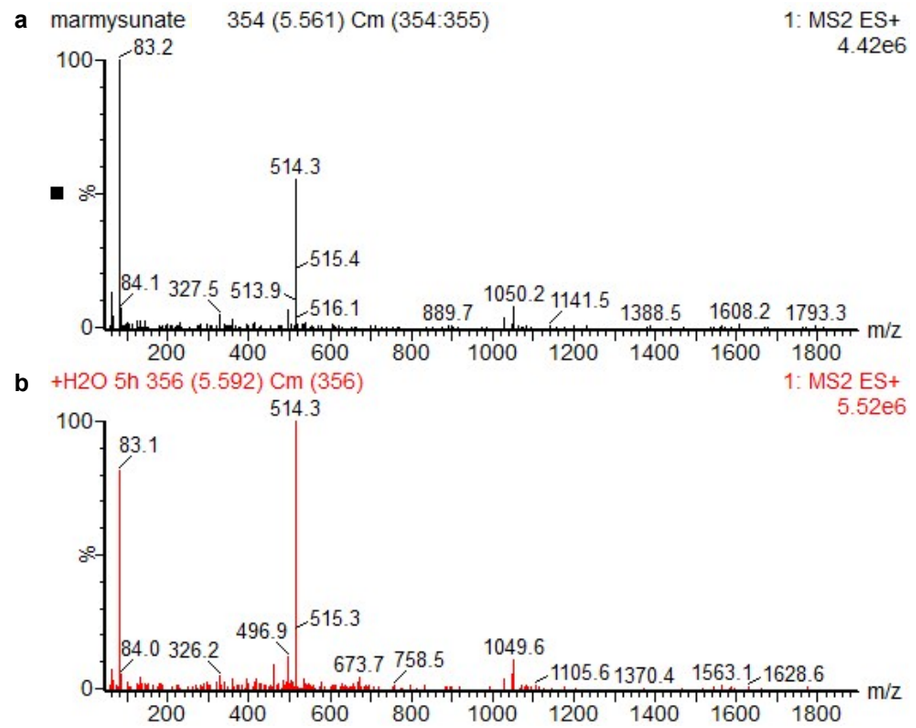


Figure S4: Comparison between the mass spectra of (**a**) synthetic marmysunate and (**b**) intermediate **9** obtained from the fragmentation of artesumycin.

• Reference

1. T. Cañeque, F. Gomes, T. T. Mai, G. Maestri, M. Malacria, R. Rodriguez, *Nat. Chem.* **2015**, *7*, 744 – 751.