Supporting Information
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Supporting Information

Synthesis and Antiproliferative Properties of Bifunctional Allocolchicine Derivatives

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General information

Chemistry

Commercially available reagents («Aldrich», «Alfa Aesar», «Acros») were used without additional purification. Column chromatography was performed using Macherey-Nagel Kieselgel 60 (70-230 mesh). All ¹H and ¹³C NMR spectra were recorded at room temperature in DMSO-d₆ or CD₃OD on Agilent DD2 400 instruments. Chemical shifts (δ) are reported in parts per million (ppm) from tetramethylsilane (TMS) using the residual solvent resonance (DMSO-d₆: 2.50 ppm for ¹H NMR, 39.52 ppm for ¹³C NMR; CD₃OD: 3.31 ppm for ¹H NMR, 49.00 ppm for ¹³CNMR). Multiplicities are abbreviated as follows: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet; dd = doublet of doublets; dt = doublet of triplets; td = triplet of doublets). Colchicine and allocolchicine atom numbering was used for signals assignment. EI mass spectra (70 eV) were obtained on a DSQ II mass-spectrometer (Thermo Electron Corporation) with a quadrupole mass-analyzer. Combustion analysis was performed using an Elementar (Vario Micro Cube) apparatus. Solvents were purified according to the standard procedures. Petroleum ether used was of bp 40-70 °C.

Confocal analysis

For confocal analysis, PANC-1 cells were grown overnight on sterile cover slips in 200 µL of complete culture medium in 6-well plates (Costar). Compound 6b (5 µM) was dissolved in 4 mL of complete medium and added to the wells. Cells were cultivated for 72 h. After incubation, cells were fixed with 1% paraformaldehyde, permeabilized by 0.1% Triton X100 in PBS, washed, and treated with Mowiol 4.88 medium (Calbiochem, Germany). Tubulin was identified by anti-tubulin antibody (SantaCruz, USA) followed by anti-mouse IgG-AlexaFluor555 (Molecular Probes, Invitrogen, USA). Hoechst 33342 (Sigma) was used to visualize nuclei. Slides were analyzed using Eclipse TE2000 confocal microscope (Nikon, Japan).

Cell cycle analysis by flow cytometry

Cell cycle was analyzed using PI-stained DNA. PANK-1 cells were collected at indicated time, trypsinized, washed in ice-cold PBS, fixed by the addition of 70% ethanol and left for 2 h at -20 °C. Thereafter, the cells were washed twice in PBS, stained with 50 µg/mL of propidium iodide (Sigma Chemical Co) in PBS, 10 µg/mL of DNAse and analyzed by flow cytometry using FACScan device (BD, USA). Total 2000 events were collected. The results were analyzed using Flowing 2.5.1 software (Finland).
Procedures and analysis data for the synthesis of compound 16 (Scheme 1)

\[
\begin{align*}
&\text{(-)-}(aR, 7S) \text{ colchicine 1} \\
&\xrightarrow{\text{a}} \text{MeO} &\xrightarrow{\text{b}} \text{MeO} &\xrightarrow{\text{c}} \text{MeO}
\end{align*}
\]

**Scheme 1 Reagents and conditions:** (a) Boc₂O, DMAP, Et₃N, CH₃CN, reflux, 3 h; (b) MeONa, MeOH, 40 °C, 1 h; (c) TFA, CH₂Cl₂, r.t., 1 h.

**Synthesis of N-(tert-butoxycarbonyl)colchicine**

A half of the solution of Boc₂O (7.5 g, 34.55 mmol) in CH₃CN (42 mL) and 33.2 mL of TEA were added to a mixture of colchicine 1 (3.0 g, 7.51 mmol) and DMAP (0.9 g, 7.51 mmol) in CH₃CN (27 mL) under an inert atmosphere. The resulting solution was stirred at 100 °C for 1 h and then the second half of the solution of Boc₂O was added. After the solvent removal under reduced pressure, the product was purified by column chromatography, eluent: ethyl acetate/acetone (4:1), to afford N-(tert-butoxycarbonyl)colchicine as a redish foam (3.7 g, 7.36 mmol, 98 %), mp 105-107 °C. ¹H NMR (400 MHz, DMSO-d₆): δ = 7.26 (s, 1H, C4-H), 7.11 (d, J = 10.7 Hz, 1H, C12-H), 7.02 (d, J = 10.7 Hz, 1H, C11-H), 6.78 (s, 1H, C8-H), 4.90 (dd, J = 12.3, 5.8 Hz, 1H, C7-H), 3.87 (s, 3H, OMe), 3.83 (s, 3H, OMe), 3.77 (s, 3H, OMe), 3.54 (s, 3H, OMe), 2.69 (dd, J = 13.5, 6.0 Hz, 2H, C5-CH₂), 2.33 - 2.26 (m, 1H, C6-CH), 2.23 (s, 3H, CH₃C(O)), 1.96 - 1.85 (m, 1H, C6-CH), 1.49 (s, 9H, (CH₃)₃C) ppm. ¹³C NMR (101 MHz, DMSO-d₆): δ = 177.84, 163.50, 153.10, 152.97, 150.47, 150.01, 148.20, 140.82, 134.73, 134.48, 133.83, 131.72, 125.60, 112.12, 107.72, 84.34, 60.83, 60.64, 56.04, 55.83, 31.79, 29.24, 27.28 ppm. MS (EI): m/z (%) = 499 (2), 399 (40), 371 (16), 340 (18), 328 (26), 312 (82), 311 (46), 281 (62), 280 (40), 254 (34), 239 (19), 195 (18), 181 (15). Anal. Calcd for C₂₇H₃₃NO₈: C, 64.92; H, 6.66. Found: C, 65.21; H, 6.91.

**Synthesis of N-(tert-butoxycarbonyl)deacetylallocolchicine**

N-(tert-butoxycarbonyl)colchicine (4.5 g, 9.01 mmol) was dissolved in dry methanol (36 mL) under an inert atmosphere. MeONa (1.9 g, 36.05 mmol) was added and the mixture was stirred for 1 h at 40 °C. The reaction was stopped by addition of saturated NH₄Cl solution (55 mL). The organic solvent was removed under reduced
pressure. The aqueous phase was extracted with EtOAc (3 × 60 mL). The combined organic extracts were dried over Na₂SO₄ and concentrated. The product was purified by column chromatography, eluent: petroleum ether/ethyl acetate (3:1), to afford \textit{N}-(\textit{tert}-butoxycarbonyl)deacetylallocolchicine as an orange foam (2.5 g, 5.42 mmol, 60 %), mp 152-154 °C. ¹H NMR (400 MHz, DMSO-d₆): δ = 7.69 (d, J = 7.8 Hz, 1H, NH), 7.21 (s, 1H, C8-H), 7.10 (d, J = 10.6 Hz, 1H, C10-H), 7.02 (d, J = 10.8 Hz, 1H, C11-H), 6.76 (s, 1H, C4-H), 4.11 - 4.04 (m, 1H, C7-H), 3.87 (s, 3H, OMe), 3.83 (s, 3H, OMe), 3.79 (s, 3H, OMe), 3.54 (s, 3H, OMe), 2.55 (dd, J = 13.2, 5.9 Hz, 1H, C5-H), 2.17 (td, J = 13.0, 6.9 Hz, 1H, C5-H), 2.04 - 1.95 (m, 1H, C6-H), 1.86 - 1.77 (m, 1H, C6-H), 1.32 (s, 9H, (CH₃)₃C) ppm. ¹³C NMR (101 MHz, DMSO-d₆): δ = 177.99, 163.49, 154.49, 152.93, 151.01, 150.34, 140.68, 135.08, 134.37, 134.25, 130.50, 125.30, 112.11, 107.65, 78.13, 60.82, 60.63, 56.00, 55.84, 52.86, 35.79, 29.26, 28.13 ppm. MS (EI): m/z (%) = 457 (2), 401 (33), 373 (67), 339 (23), 312 (80), 297 (96), 281 (100), 266 (57), 254 (48), 224 (35), 211 (50), 181 (42), 153 (48), 152 (62), 141 (33). Anal. Calcd for C₂₅H₃₁NO₇: C, 65.63; H, 6.83. Found: C, 65.93; H, 7.11.

**Synthesis of deacetylallocolchicine (12)**

\[ \text{MeO} \quad \text{MeO} \quad \text{NH}_2 \quad \text{C}_{20}\text{H}_{23}\text{NO}_5 \quad \text{MM} = 367,4060 \]

\textit{N}-(\textit{tert}-butoxycarbonyl)deacetylallocolchicine (1.2 g, 2.72 mmol) was dissolved in CH₂Cl₂ (11 mL) and TFA (2.3 mL) was added dropwise. The reaction mixture was stirred for 1 h at room temperature after which a saturated sodium carbonate solution (29 mL) was added to quench the reaction. The organic solvent was removed under reduced pressure. The aqueous phase was extracted with EtOAc (4 × 50 mL). The combined organic extracts were dried over Na₂SO₄ and concentrated. The product was purified by column chromatography, eluent: petroleum ether - ethyl acetate - ethanol (1:1:1), to afford deacetylallocolchicine 12 as a yellow powder (0.9 g, 2.61 mmol, 96 %), mp 136-138 °C. ¹H NMR (400 MHz, DMSO-d₆): δ = 7.64 (s, 1H, C8-H), 7.05 (d, J = 10.6 Hz, 1H, C10-H), 6.99 (d, J = 10.8 Hz, 1H, C11-H), 6.74 (s, 1H, C4-H), 3.86 (s, 3H, OMe), 3.83 (s, 3H, OMe), 3.76 (s, 3H, OMe), 3.55 (s, 3H, OMe), 3.45 (dd, J = 10.4, 5.9 Hz, 1H, C7-H), 2.14 (m, 3H, C5-CH₂, C6-H), 1.49 (td, J = 10.0, 4.8 Hz, 1H, C6-H) ppm. ¹³C NMR (101 MHz, DMSO-d₆): δ = 178.16, 163.29, 153.25, 152.76, 150.16, 140.49, 135.59, 134.79, 133.86, 131.76, 125.35, 111.87, 107.37, 60.67, 60.53, 55.92, 55.85, 53.08, 29.79 ppm. MS (EI): m/z (%) = 357 (50), 328 (18), 312 (64), 298 (100), 297 (64), 281 (43), 267 (30), 254 (36), 253 (18), 239 (16), 195 (14), 181 (20), 149 (19). Anal. Calcd for C₂₀H₂₃NO₅: C, 67.21; H, 6.49. Found: C, 67.49; H, 6.73.
NMR spectra of the synthesized compounds

*N-(tert-Butoxycarbonyl)colchicine*

$^1$H NMR (400 MHz, DMSO-$d_6$)

$^{13}$C NMR (101 MHz, DMSO-$d_6$)
$N$-(tert-Butoxycarbonyl)deacetylallocolchicine

$^1$H NMR (400 MHz, DMSO-$d_6$)

$^{13}$C NMR (101 MHz, DMSO-$d_6$)
Deacetylallocolchicine (16)

$^1$H NMR (400 MHz, DMSO-$d_6$)

$^{13}$C NMR (101 MHz, DMSO-$d_6$)
Allocolchifoline (10a)

$^1$H NMR (400 MHz, DMSO-$d_6$)

$^{13}$C NMR (101 MHz, DMSO-$d_6$)
(5S)-5-(2-Hydroxyacetamido)-9,10,11-trimethoxy-6,7-dihydro-5H-dibenzo[a,c]cyclohepten-3-carboxylic acid (10b)

$^1$H NMR (400 MHz, DMSO-d$_6$)

$^{13}$C NMR (101 MHz, DMSO-d$_6$)
Methyl (5S)-5-{2-((tert-butoxycarbonyl)amino)acetamido}-9,10,11-trimethoxy-6,7-dihydro-5H-dibenzo[a,c]cyclohepten-3-carboxylate (11a)

$^1$H NMR (400 MHz, DMSO-d$_6$)

$^{13}$C NMR (101 MHz, DMSO-d$_6$)
(5S)-5-((tert-Butoxycarbonyl)amino)acetamido)-9,10,11-trimethoxy-6,7-dihydro-5H-dibenz[a,c][7]cyclohepten-3-carboxylic acid (11b)

$^1$H NMR (400 MHz, DMSO-d$_6$)

$^{13}$C NMR (101 MHz, DMSO-d$_6$)
Methyl (5S)-5-(2-aminoacetamido)-9,10,11-trimethoxy-6,7-dihydro-5H-dibenzo[a,c]cyclohepten-3-carboxylate (11c)

$^1$H NMR (400 MHz, DMSO-d$_6$)

$^{13}$C NMR (101 MHz, DMSO-d$_6$)
(5S)-5-(2-aminoacetamido)-9,10,11-trimethoxy-6,7-dihydro-5H-dibenzo[a,c]cyclohepten-3-carboxylic acid (11d)

$^1$H NMR (400 MHz, DMSO-d$_6$)

$^{13}$C NMR (101 MHz, DMSO-d$_6$)
2-Hydroxy-N-((5S)-3-hydroxy-2-iodo-9,10,11-trimethoxy-6,7-dihydro-5H-dibenzo[a,c]cyclohepten-5-yl)acetamide (18)

$^1$H NMR (400 MHz, DMSO-d$_6$)

$^{13}$C NMR (101 MHz, DMSO-d$_6$)
tert-Butyl (2-(((5S,3S)-3-hydroxy-2-iodo-9,10,11-trimethoxy-6,7-dihydro-5H-dibenzo[a,c]cyclohepten-5-yl)amino)-2-oxoethyl)carbamate (19)

$^1$H NMR (400 MHz, DMSO-d$_6$)

$^{13}$C NMR (101 MHz, DMSO-d$_6$)
1',2',3'-trimethoxybenzo[5',6':5,4]1H-(aR, 1S)-1-hydroxyacetamido 6,7-dihydrocyclohepta[3,2:f]-2'-hydroxymethylbenzofurane (12)

$^1$H NMR (400 MHz, DMSO-d$_6$)

$^{13}$C NMR (101 MHz, DMSO-d$_6$)
1',2',3'-trimethoxybenzo[5',6':5,4]1H-(aR, 1S)-1-tert-butoxycarbonylaminoacetamido-6,7-dihydrocyclohepta[3,2:f]-2''-hydroxymethylbenzofurane (13a)

$^1$H NMR (400 MHz, DMSO-d$_6$)

$^{13}$C NMR (101 MHz, DMSO-d$_6$)

$^1$H NMR (400 MHz, CD$_3$OD)

$^{13}$C NMR (101 MHz, CD$_3$OD)
Effect of 6a and 6b on cell cycle and tubulin binding

To check whether the low activity of compounds 6b and 7b-d is associated with the different profile of cellular activity, cell cycle analysis and tubulin binding assays were conducted in PANC-1 cells. Both control 6a and low active compound 6b with a free carboxylic group were used in the same concentrations. It was shown that both compounds effectively induced accumulation of the cells in G2/M phase, which steadily resulted in apoptosis (Figure 1 a, b). The only difference was found in the percentage of cells in G2/M phase.

The effect of low active compound 6b with a free carboxylic group on β-tubulin assembly and mitotic spindle formation was studied by confocal microscopy. Incubation of PANC-1 cell with 6b effectively disrupted the mitotic spindle, which led to the chromosome scattering and the inability of the cells to divide (Figure 1 c, d) as was previously shown for highly active allocolchicinoids 3, 4.18

![Figure 1](image_url)  
**Figure 1** Effect of 6a and 6b on cell cycle (a, b) and tubulin binding (c, d) in PANC-1 cells. a, b. Cells treated for 24 h by 5 µM 6a (a) or 6b (b). Markers 1-3 show cell in G2/M, G1, and apoptosis phases accordingly. Tables show percentage of cells in each phase. c, d. Organization of tubulin (red) in PANC-1 cells before (c) and after (d) the incubation for 24 h with 5 µM of 6b. Nuclei are stained with Hoechst 33342 (blue). Mean fluorescence intensity (MFI) was counted on several fields by red pixel numbers using ImageJ soft. Statistical difference (p<0.05) is pointed by asterisk. Scale bar corresponds to 9.7 µM.