One-pot synthesis, anti-tumor evaluation and structure-activity relationships of novel 25-OCH$_3$-PPD derivatives

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Section A: Chemistry

$^1$H NMR and $^{13}$C NMR spectra were measured on a Bruker ARX-300 spectrometer in the appropriate solution (CDCl$_3$) using tetramethylsilane as internal standard. HR-ESI-MS were recorded on an Agilent 1100 LC-MSD TOF (time-of-flight) system. Solvents were dried with the appropriate drying agents and distilled under reduced pressure before use. All commercially available starting materials were of commercial quality and were used without further purification. All described reactions were monitored by TLC (HSGF254 silica gel plates). Silica gel (200-300 mesh, China) was used for column chromatography. And 5-Fluorouracil (5-Fu) was purchased from sigma-Aldrich (St. Louis, MO, USA).
Section B: The synthesis of compounds a1-a8 and b1-b7

To a solution of AD-1 (5.0 g) in dichloromethane (150 mL), chloroacetyl chloride (1.6 mL) or bromoacetyl bromide (1.8 mL) was dropwise added slowly, and the mixture was stirred for 2 h. Pure water was added to quench the reaction. Then mixture was poured into a separatory funnel to remove the aqueous layer. The rest organic layer was washed with 5% aqueous NaHCO$_3$ and dried (MgSO$_4$). After solvent was removed under reduced pressure, residue (yellowish solid) was chromatographed by silica gel columns to give a1-a8 (chloroacetyl products) or b1-b7 (bromoacetyl products). Ethyl acetate and petroleum ether (v/v = 1:10-1:1) were used as eluting solvent.

**(20R)-25-Methoxyl-3β, 12β-O-di-(L-chloracetyl)-dammarane-20-ol (a1)**

White solid, Mp: 205-207 °C; $^1$H NMR (400 MHz, CDCl$_3$, δ, ppm): 5.04 (td, 1H, $J = 10.8, 5.2$ Hz, H-12), 4.51 (dd, 1H, $J = 10.9, 5.2$ Hz, H-3), 3.17 (s, 3H, OCH$_3$), 2.57 (m, 1H), 1.14 (s, 6H), 1.07 (s, 3H), 0.98 (s, 3H), 0.94 (s, 3H), 0.90 (s, 3H), 0.87 (s, 6H); $^{13}$C NMR (100 MHz, CDCl$_3$, δ, ppm): 167.3, 166.2, 83.1, 76.4, 74.5, 74.1, 55.8, 53.5, 51.8, 50.0, 49.0, 47.8, 45.6, 41.4, 40.5, 39.8, 38.9, 38.6, 37.1, 35.2, 34.8, 31.2, 31.0, 28.0, 27.4, 26.8, 26.7, 25.1, 25.0, 18.2, 17.7, 16.9, 16.2, 15.7, 15.3. HR-ESIMS m/z 645.3695 [M+H]$^+$ (calcd for C$_{35}$H$_{59}$Cl$_2$O$_6$, 645.3689).

**(20R)-25-Methoxyl-3β-O-(L-chloracetyl)-dammarane-12β, 20-diol (a2)**

White solid, Mp: 219-221 °C; $^1$H NMR (400 MHz, CDCl$_3$, δ, ppm): 4.62 (dd, 1H, $J = 10.8, 5.2$ Hz, H-3), 3.90 (td, 1H, $J = 10.6, 5.4$ Hz), 3.15 (s, 3H, OCH$_3$), 2.57 (m, 1H), 1.14 (s, 6H), 1.07 (s, 3H), 1.03 (s, 3H), 0.98 (s, 3H), 0.90 (s, 3H), 0.88 (s, 6H); $^{13}$C NMR (100 MHz, CDCl$_3$, δ, ppm): 166.4, 83.0, 74.3, 74.1, 70.9, 56.0, 51.8, 53.5, 50.4, 50.0, 49.9, 41.1, 41.1, 40.1, 38.8, 38.3, 37.4, 35.0, 34.7, 30.7, 30.7, 28.1, 27.4, 27.4, 26.6, 25.2, 25.0, 18.2, 17.3, 16.9, 16.5, 15.8, 15.8. HR-ESIMS m/z 569.3978 [M+H]$^+$ (calcd for C$_{33}$H$_{58}$ClO$_5$, 569.3973).

**(20R)-25-Methoxyl-12β-O-(L-chloracetyl)-dammarane-3β, 20-diol (a3)**

White solid, Mp: 221-223 °C; $^1$H NMR (400 MHz, CDCl$_3$, δ, ppm): 5.04 (td, 1H, $J = 10.8, 5.6$ Hz, H-12), 3.45 (m, 1H, H-3), 3.18 (s, 3H, OCH$_3$), 1.15 (s, 6H), 1.07 (s, 3H), 1.01 (s, 6H), 0.96 (s, 6H), 0.90 (s, 3H), 0.88 (s, 3H); $^{13}$C NMR (100 MHz, CDCl$_3$, δ, ppm): 166.5, 78.1, 77.2, 74.5, 73.7, 55.6, 53.3, 52.3, 50.2, 49.4, 48.8, 45.4, 40.9, 39.7, 38.7, 38.6, 37.0, 36.9, 34.6, 32.5, 30.9, 29.0, 28.0, 28.0, 27.0, 26.6, 24.8, 18.0, 17.4, 17.1, 16.0, 15.8, 15.3. HR-
ESIMS m/z 569.3980 [M+H]+ (calcd for C\textsubscript{33}H\textsubscript{58}ClO\textsubscript{5}, 569.3973).

(20R)-25-Methoxyl-20-O-(L-chloracetyl)-dammarane-3β, 12β-diol (a4)

White solid, Mp: 214-216 °C; \textsuperscript{1}H NMR (400 MHz, CDCl\textsubscript{3}, δ, ppm): 3.51 (td, 1H, J = 10.3, 5.2 Hz, H-12), 3.23 (m, 1H, H-3), 3.15 (s, 3H, OCH\textsubscript{3}), 2.16 (m, 1H), 1.15 (s, 6H), 1.10 (s, 3H), 0.97 (s, 3H), 0.89 (s, 3H), 0.88 (s, 6H), 0.78 (s, 3H); \textsuperscript{13}C NMR (100 MHz, CDCl\textsubscript{3}, δ, ppm): 167.1, 80.1, 79.1, 74.7, 70.3, 55.6, 53.5, 51.8, 50.1, 49.3, 49.2, 46.9, 40.5, 39.7, 39.1, 38.9, 37.3, 35.0, 34.9, 31.2, 30.7, 28.2, 27.6, 27.2, 26.6, 26.2, 25.1, 18.5, 17.4, 17.0, 16.3, 15.8, 15.8. HR-ESIMS m/z 569.3982 [M+H]+ (calcd for C\textsubscript{33}H\textsubscript{58}ClO\textsubscript{5}, 569.3973).

25-Methoxyl-3β, 12β-O-di-(L-chloracetyl)-dammar-20(22)-ene (a5)

White solid, Mp: 211-213 °C; \textsuperscript{1}H NMR (400 MHz, CDCl\textsubscript{3}, δ, ppm): 5.05 (m, 1H, H-12), 4.55 (dd, 1H, J = 10.9, 5.5 Hz, H-3), 3.18 (s, 3H, OCH\textsubscript{3}), 2.50 (td, 1H, J = 10.7, 6.3 Hz), 1.13 (s, 6H), 1.03 (s, 3H, s), 0.98 (s, 3H), 0.94 (s, 3H), 0.89 (s, 3H), 0.87 (s, 6H); \textsuperscript{13}C NMR (100 MHz, CDCl\textsubscript{3}, δ, ppm): 167.1, 166.4, 137.5, 124.8, 83.0, 76.9, 74.6, 55.9, 51.4, 51.4, 50.4, 49.2, 49.2, 47.0, 41.3, 40.1, 39.6, 39.6, 38.7, 37.3, 34.8, 32.2, 29.8, 28.1, 28.1, 27.5, 25.2, 25.2, 25.1, 18.2, 18.2, 16.8, 16.4, 15.8, 15.7. HR-ESIMS m/z 627.3590 [M+H]+ (calcd for C\textsubscript{35}H\textsubscript{57}Cl\textsubscript{2}O\textsubscript{5}, 627.3583).

25-Methoxyl-dammar-20(22)-ene-3β, 12β-diol (a6)

White solid, Mp: 202-204 °C; \textsuperscript{1}H NMR (400 MHz, CDCl\textsubscript{3}, δ, ppm): 5.42 (m, 1H, H-22), 3.71 (td, 1H, J = 10.5, 5.2 Hz, H-12), 3.19 (m, 1H, H-3), 3.16 (s, 3H, OCH\textsubscript{3}), 1.65 (s, 3H), 1.13 (s, 6H), 1.02 (s, 3H), 0.97 (s, 3H), 0.88 (s, 6H), 0.78 (s, 3H); \textsuperscript{13}C NMR (100 MHz, CDCl\textsubscript{3}, δ, ppm): 140.5, 126.1, 79.1, 74.5, 71.5, 56.1, 50.7, 50.5, 50.4, 49.3, 40.3, 39.2, 39.2, 37.4, 35.1, 32.6, 30.6, 28.2, 27.6, 27.5, 25.2, 22.6, 18.4, 18.4, 16.9, 16.4, 15.8, 15.5. HR-ESIMS m/z 475.4157 [M+H]+ (calcd for C\textsubscript{31}H\textsubscript{55}O\textsubscript{3}, 475.4151).

25-Methoxyl-3β, 12β-O-di-(L-chloracetyl)-dammar-20(21)-ene (a7)

White solid, Mp: 208-210 °C; \textsuperscript{1}H NMR (400 MHz, CDCl\textsubscript{3}, δ, ppm): 5.04 (m, 1H, H-12), 4.51 (dd, 1H, J = 10.9, 4.8 Hz, H-3), 3.18 (s, 3H, OCH\textsubscript{3}), 1.12 (s, 6H), 1.08 (s, 3H), 1.02 (s, 3H), 0.96 (s, 6H), 0.90 (s, 3H); \textsuperscript{13}C NMR (100 MHz, CDCl\textsubscript{3}, δ, ppm): 166.5, 166.2, 152.6, 107.7, 82.7, 78.1, 74.5, 55.7, 53.4, 51.1, 50.3, 49.1, 47.7, 47.1, 41.0, 40.9, 40.9, 39.7, 38.9, 38.9, 37.1, 34.7, 31.8, 31.8, 28.0, 27.1, 27.1, 25.0, 25.0, 18.1, 18.0, 16.5, 16.1, 15.5, 15.3. HR-ESIMS m/z 627.3587 [M+H]+ (calcd for C\textsubscript{35}H\textsubscript{57}Cl\textsubscript{2}O\textsubscript{5}, 627.3583).
25-Methoxyl-12β-O-(L-chloracetyl)-dammar-20(21)-ene-3β-ol (a8)

White solid, Mp: 217-219 °C; 1H NMR (400 MHz, CDCl3, δ, ppm): 5.03 (td, 1H, J = 10.9, 5.4 Hz, H-12), 3.52 (dd, 1H, J = 10.8, 4.8 Hz, H-3), 3.14 (s, 3H, OCH3), 2.57 (m, 1H), 1.14 (s, 3H), 1.05 (s, 3H), 0.95 (s, 6H), 0.92 (s, 3H), 0.87 (s, 6H); 13C NMR (100 MHz, CDCl3, δ, ppm): 167.2, 150.6, 115.1, 78.8, 76.3, 74.7, 55.9, 51.8, 51.8, 50.4, 49.2, 48.3, 47.8, 43.6, 41.3, 41.3, 39.0, 38.2, 37.2, 34.9, 33.6, 32.1, 29.8, 29.5, 29.5, 28.3, 28.1, 28.1, 18.3, 18.2, 16.6, 16.6, 16.4, 15.5. HR-ESIMS m/z 551.3873 [M+H]+ (calcd for C33H56ClO4, 551.3867).

(20R)-25-Methoxyl-3β,12β-O-di-(L-bromoacetyl)-dammarane-20-ol (b1)

White solid, Mp: 198-200 °C; 1H NMR (400 MHz, CDCl3, δ, ppm): 5.04 (td, 1H, J = 10.8, 5.2 Hz, H-12), 4.48 (dd, 1H, J = 10.6, 5.4 Hz, H-3), 3.16 (s, 3H, OCH3), 1.24 (s, 6H), 1.10 (s, 3H), 1.03 (s, 3H), 0.98 (s, 3H), 0.95 (s, 3H), 0.86 (s, 6H); 13C NMR (100 MHz, CDCl3, δ, ppm): 171.0, 167.1, 83.2, 76.7, 73.2, 74.1, 56.0, 53.3, 51.3, 49.9, 49.2, 47.6, 46.3, 43.7, 40.5, 39.9, 38.7, 38.7, 37.1, 35.8, 34.9, 32.7, 31.2, 28.0, 27.2, 26.5, 26.5, 25.2, 25.0, 18.3, 17.7, 16.8, 16.3, 15.7, 15.5. HR-ESIMS m/z 733.2685 [M+H]+ (calcd for C35H59Br2O6, 733.2678).

(20R)-25-Methoxyl-3β-O-(L-bromoacetyl)-dammarane-12β,20-diol (b2)

White solid, Mp: 213-215 °C; 1H NMR (400 MHz, CDCl3, δ, ppm): 4.65 (dd, 1H, J = 10.9, 5.2 Hz, H-3), 3.91 (m, 1H, H-12), 3.15 (s, 3H, OCH3), 1.17 (s, 6H), 1.05 (s, 3H), 0.98 (s, 3H), 0.95 (s, 3H), 0.90 (s, 3H), 0.88 (s, 6H); 13C NMR (100 MHz, CDCl3, δ, ppm): 167.9, 83.0, 74.6, 74.1, 72.0, 55.9, 53.5, 51.7, 50.5, 49.0, 47.8, 43.8, 40.5, 39.7, 38.9, 38.8, 37.1, 35.2, 34.8, 31.4, 31.0, 28.1, 27.3, 26.8, 26.6, 25.1, 25.0, 18.2, 17.7, 16.9, 16.1, 15.5, 15.5. HR-ESIMS m/z 613.3475 [M+H]+ (calcd for C33H58Br2O5, 613.3468).

(20R)-25-Methoxyl-12β-O-(L-bromoacetyl)-dammarane-3β,20-diol (b3)

White solid, Mp: 227-229 °C; 1H NMR (400 MHz, CDCl3, δ, ppm): 5.00 (m, 1H, H-12), 3.49 (m, 1H, H-3), 3.18 (s, 3H, OCH3), 1.25 (s, 6H), 1.15 (s, 3H), 1.07 (s, 3H), 1.04 (s, 3H), 0.98 (s, 6H), 0.78 (s, 3H); 13C NMR (100 MHz, CDCl3, δ, ppm): 167.0, 78.9, 77.2, 74.7, 74.1, 56.0, 53.3, 51.3, 50.0, 49.1, 47.8, 43.7, 40.5, 39.5, 39.1, 39.1, 37.0, 35.8, 34.9, 32.5, 31.1, 28.2, 27.4, 26.9, 26.9, 25.7, 25.0, 18.4, 17.4, 16.8, 16.1, 15.5, 15.5. HR-ESIMS m/z 613.3473 [M+H]+ (calcd for C33H58BrO5, 613.3468).

(20R)-25-Methoxyl-12β-O-(L-bromoacetyl)-dammarane-3β, 12β-diol (b4)

White solid, Mp: 206-208 °C; 1H NMR (400 MHz, CDCl3, δ, ppm): 3.54 (td, 1H, J = 10.4,
5.2 Hz, H-12), 3.21 (dd, 1H, J = 11.3, 5.1 Hz, H-3), 1.27 (s, 3H), 1.22 (s, 3H), 1.18 (s, 3H), 0.98 (s, 3H), 0.97 (s, 3H), 0.88 (s, 6H), 0.77 (s, 3H); 13C NMR (100 MHz, CDCl3, δ, ppm): 166.8, 79.2, 79.1, 73.3, 70.2, 56.0, 54.8, 51.4, 50.1, 49.3, 49.2, 43.7, 40.0, 39.9, 39.1, 39.0, 37.3, 35.0, 35.0, 31.3, 30.7, 28.2, 27.3, 27.3, 27.2, 26.6, 25.3, 18.5, 17.2, 16.4, 16.3, 15.8, 15.8. HR-ESIMS m/z 613.3477 [M+H]+ (calcd for C33H58BrO5, 613.3468).

25-Methoxyl-3β, 12β-O-di-(L-bromoacetyl)-dammar-20(22)-ene (b5)

White solid, Mp: 228-230 °C; 1H NMR (400 MHz, CDCl3, δ, ppm): 5.03 (m, 1H, H-12), 3.91 (m, 1H, H-3), 3.15 (s, 3H, OCH3), 1.23 (s, 6H), 1.12 (s, 3H), 1.02 (s, 3H), 0.96 (s, 6H), 0.86 (s, 3H), 0.76 (s, 3H); 13C NMR (100 MHz, CDCl3, δ, ppm): 167.2, 166.2, 137.6, 125.7, 83.2, 77.2, 74.7, 55.9, 52.1, 51.4, 50.5, 49.2, 48.1, 47.4, 43.7, 40.0, 39.9, 39.0, 38.9, 37.4, 35.1, 34.9, 32.3, 31.2, 28.1, 27.3, 26.7, 25.1, 25.0, 18.3, 17.7, 16.9, 16.3, 15.7, 15.5. HR-ESIMS m/z 715.2579 [M+H]+ (calcd for C35H57Br2O5, 715.2573).

25-Methoxyl-3β-O-(L-bromoacetyl)-dammar-20(22)-ene-12β-ol (b6)

White solid, Mp: 201-203 °C; 1H NMR (400 MHz, CDCl3, δ, ppm): 4.53 (dd, 1H, J = 10.9, 5.2 Hz, H-12), 3.82 (m, 1H, H-12), 3.16 (s, 3H, OCH3), 1.24 (s, 6H), 1.13 (s, 3H), 1.03 (s, 3H), 0.97 (s, 3H), 0.93 (s, 3H), 0.87 (s, 3H); 13C NMR (100 MHz, CDCl3, δ, ppm): 167.1, 137.6, 125.9, 82.9, 74.6, 70.8, 55.9, 51.4, 50.4, 49.2, 47.0, 46.9, 40.1, 39.6, 38.9, 38.7, 37.3, 35.1, 34.8, 32.0, 31.4, 28.1, 27.3, 26.8, 25.2, 25.0, 18.1, 17.7, 16.8, 16.3, 15.7, 15.7. HR-ESIMS m/z 595.3370 [M+H]+ (calcd for C33H56Br2O4, 595.3362).

25-Methoxyl-3β, 12β-O-di-(L-bromoacetyl)-dammar-20(21)-ene (b7)

White solid, Mp: 224-226 °C; 1H NMR (400 MHz, CDCl3, δ, ppm): 4.99 (m, 1H, H-12), 4.51 (m, 1H, H-3), 3.16 (s, 3H, OCH3), 1.22 (s, 6H), 1.19 (s, 3H), 1.14 (s, 3H), 0.94 (s, 3H), 0.86 (s, 6H); 13C NMR (100 MHz, CDCl3, δ, ppm): 167.2, 166.2, 152.7, 107.8, 83.2, 77.2, 74.7, 55.9, 52.1, 51.4, 50.5, 49.2, 48.1, 47.4, 43.7, 40.0, 39.9, 39.0, 38.9, 37.4, 35.4, 34.9, 32.3, 31.2, 28.1, 27.3, 26.7, 25.1, 25.0, 18.3, 17.7, 16.9, 16.3, 15.7, 15.5. HR-ESIMS m/z 715.2581 [M+H]+ (calcd for C35H57Br2O5, 715.2573).
Section C: Cytotoxicity assay

The cancer cells (HCT-116, A549, α-2, BGC-823, SGC-7901, C4-2B and MCF-7) were obtained from the Cell Bank of Chinese Academy of Sciences (Shanghai, China). *In vitro* inhibitory activities were determined by the MTT assay. Briefly, cells were seeded into 96-well plates for 24 h in 5% CO\textsubscript{2} air, then exposed in designated various concentration of compounds (1-100 µM). After incubated for another 48 h, 15 µL of MTT solution (5 mg/mL) was added to each well, and incubated for an additional 4 h at 37 °C. After removing the supernatant, 100 µL of DMSO was added and incubated at 37 °C for 10 minutes. The absorbance at 490 nm was read on a Bio-Rad (model 550) microplate reader to obtain the IC\textsubscript{50} values.
Section D: Determination of morphological changes of cells

α-2 cells were seeded at a concentration of $1 \times 10^5$ cells/well in a six-well plate and grown for 24 h. The cells were then treated with compound a7 or 0.1% DMSO for 12 h. The cellular morphology was observed with a phase contrast microscope (Leica, Nussloch, Germany).

After the same experiment described above, cells were washed once with PBS, fixed with 4% paraformaldehyde, and washed three times with PBS. Then these cells were stained with DAPI in dark for 30 min, and then examined under a fluorescent microscope (IX51, OLYMPUS, Japan).
Section E: Cell cycle analysis by flow cytometry

α-2 cells exposed to compound a7 were trypsinized, harvested, washed once with PBS. Then cells were re-suspended in ice-cold 70% ethanol, and stored at -20 °C overnight. After PBS washing, the cells were treated with 1 mg/mL RNase for 30 min, then stained with Propidium Iodide (PI) solution in the dark. Cell cycle distribution was analyzed using FACScan (Becton-Dickinson, USA).