**Synthesis of novel 25-hydroxyprotopanaxadiol derivatives by
methylation and methoxycarbonylation using dimethyl carbonate as
a environment-friendly reagent and their anti-tumor evaluation**

Junhui Guo^{a,b}, Zhe Xu^{a,b}, Yafei Liu^{a,b}, Xude Wang^{a,b}, Yuqing Zhao^{a,b,*}

^a *School of Traditional Chinese Medicine Chemical, Shenyang Pharmaceutical University,*

Shenyang 110016, PR China

^b *Key Laboratory of Structure-based Drug Design & Discovery, Ministry of Education, Shenyang*

Pharmaceutical University, Shenyang 110016, PR China

*Corresponding author. Y. ZHAO

Tel.: +86 24 23986521.; fax: +86 24 23986521

E-mail address: zyq4885@126.com

Abstract

A previous study involving 25-hydroxyprotopanaxadiol (25-OH-PPD) illustrated that the anti-cancer activity increased by 1-3 times after C-3/C-12-OH was substituted by short-chain fatty acids. In addition, 25-OCH₃-PPD was also one of our research interests; the unique difference in structure between 25-OH-PPD and 25-OCH₃-PPD is that in C-25, the latter activity was 2-5 times higher than that of 25-OH-PPD. These data serves as the scientific basis of our continuing research. To further confirm the effect of short chain acylated and methylated products on the activity and to identify more potent, higher selectivity compounds, we modified 25-OH-PPD with a green environment-friendly and non-toxic chemical dimethyl carbonate (DMC), which plays the role of both solvent and reagent. This experiment yielded 14 derivatives. Their in vitro anti-tumor activities were tested on two different human tumor cell lines (HeLa and DU145) and one normal cell line (IOSE144) by standard MTT assay. The results showed that compounds **3**, **5**, **6**, **10**, **11**, **12**, and **13** exhibited higher cytotoxic activity on two cell lines, with IC₅₀ values within the range of 1.1-12 μM. Compounds **12** and **13** exhibited the highest potent activity, with IC₅₀ values of 1.1 and 1.2 μM, respectively, on HeLa cells. Antitumor activity significantly increased after the hydroxyl groups are substituted by methyl. The results of the present study may provide useful data for evaluating the structure-activity relationships of other dammarane-type sapogenins and developing new antitumor agents.

Key words: 25-hydroxyprotopanaxadiol; dimethyl carbonate; derivatives; anti-tumor activity

Panax ginseng is a famous traditional Chinese herbal medicine that has been used for various medical purposes for thousands of years. In recent years, there has been a rapid increase in developing ginseng products as cancer preventive or therapeutic agents¹⁻³. Ginsenosides are considered to be the main bioactive constituents of *Panax ginseng*, and most of saponins and aglycones have shown to possess anti-cancer activity, with the aglycones being more effective than the saponins^{4,5}. 25-hydroxyprotopanaxadiol [20(*R*)-dammarane-3 β , 12 β , 20, 25-tetrol, 25-OH-PPD Figure 1], a promising antitumor natural product, was isolated from the fruit of *P. ginseng*⁶. In our previous studies, we found that 25-OH-PPD can inhibit cancer cell proliferation, cause cell cycle arrest in vitro, and inhibit the growth of xenograft tumors without any detectable host toxicity in vivo. Its IC₅₀ values were within the range of 10-60 μ M for most cell lines, demonstrating a 5-15-fold greater growth inhibition than ginsenoside-Rg₃⁷ (an agent already being marketed for cancer therapy). Pharmacokinetic studies have shown that 25-OH-PPD has a higher absolute oral bioavailability, and its tissue distribution order was liver > kidney > tumor > spleen⁸⁻

11.

Figure 1. Chemical structures of 25-OH-PPD and 25-OCH₃-PPD

The results of previous studies on the structure-activity relationships of ginsenosides suggest that the anti-tumor activities were affected by structural types of dammarane saponin, which is a type of side group and number of sugar moieties¹¹⁻¹³. Most saponins and aglycones possess fine anti-tumor activity, whereas solubility, bioavailability, and toxicity shortcomings affect their metabolism and clinical applications. Studies have shown that structural modification for saponins and aglycones can significantly improve physicochemical property, solubility, bioavailability, and

reduced toxicity. Moreover, it can also enhance its biological activity¹⁴⁻¹⁸. In the present study, 25-OH-PPD has been modified with different types of amino acids and saturated fatty acids; thus, generating a series of C-3 and C-12-OH substituted products. Most fatty acids and amino acids derivatives exhibited better cytotoxicity to cancer cells and no cytotoxicity to normal cells; some compounds showed a 2-10-fold higher anticancer activity compared to that observed in 25-OH-PPD. The structure-activity relationships of 25-OH-PPD saturated fatty acids derivatives suggest that a longer fatty acid chain was associated with a lower activity, which was contrary to previous findings that the shorter fatty acid chain conferred higher activity^{19,20}. 25-methoxylprotopanaxadiol (25-OCH₃-PPD, Figure 1), a structurally similar compound to that of 25-OH-PPD has recently gained our interest; its difference mainly lies on C-25. The biological activities of 25-OCH₃-PPD, 25-OH-PPD and some known analogs were evaluated in 12 human cancer cell lines, and the order of anti-tumor activities for the test compounds were 25-OCH₃-PPD > 25-OH-PPD > PPD > Rg₃ > saponins. The results showed that anti-tumor activity notably increased 1-3-fold after C-25-OH was substituted with -OCH₃^{21,22}. These findings indicate that cytotoxicity was significantly enhanced when C-3/C-12-OH or C-25-OH was substituted with short chain-fatty acids or methoxy. This report is to describe the methylation of C-3/C-12/C-20-OH and methoxycarbonylation of C-20/C-25-OH short-chain in 25-OH-PPD.

As part of our continuing research to develop novel compounds with high potency and high selectivity, as well as to obtain C-20 and C-25-OH substituted products to explore the structure-activity relationships, we focus on methylation and short-chain acylation. Traditional reagents for acylation and alkylation reactions such as diazomethane, dimethyl sulfate, and halogenated methane are hazardous and highly toxic. These reagents not only pose a great harm to human health but also require high amount of strong bases during the reaction, resulting in equipment corrosion and a high amount of salt waste^{23,24}. In the last few years, dimethyl carbonate (DMC), a new non-toxic and ecofriendly compound, has emerged as a safe and efficient alternative reagent for methylation and methoxycarbonylation reactions²⁵. The reactivity of DMC is regulative. At T

= 90 °C, methoxycarbonylation reactions occur, in which the nucleophilic anion attacks the acyl carbon to produce the transesterification product and the methoxide anion as the leaving group (BAc2 mechanism). By contrast, methylation reactions are observed at higher reaction temperatures ($T \geq 160$ °C), in which the nucleophilic anion attacks the methyl group of the organic carbonate (BA12 mechanism); under such conditions, the leaving group (methoxycarbonate anion, CH_3OCOO^-) is unstable and it rapidly decomposes into methoxide anion and CO_2 ^{26–28}.

25-OH-PPD was modified with DMC, and 14 compounds including methoxycarbonylated derivatives (**C1–C9**) and methylated derivatives (**C10–C14**) were synthesized²⁹ (Scheme 1), these compounds were characterized by MS, ¹H-NMR, ¹³C-NMR, and IR spectroscopy³⁰. Compound 3 was obtained as a white tufted crystals (ethyl acetate). The molecular formula was determined as $\text{C}_{33}\text{H}_{58}\text{O}_6$ by HR-ESIMS m/z 550.4391[M+H]⁺ (Calcd for $\text{C}_{33}\text{H}_{59}\text{O}_6$, 551.4382). Positive Liebermann-Burchard and negative Molish reactions suggested C3 to be a sapogenin. The IR spectrum (cm^{-1}): the IR spectrum displayed strong absorption bands at 3374 and 1062, suggestive of the presence of hydroxyl groups, the 1741 suggested the presence of carbonyl group in the structure. The ¹³C NMR spectrum presented all 33 carbon resonances and confirmed the presence of a carbonyl carbon signal at δ 155.8. And based on the previous work, we have summarized chemical shift change rules when the hydroxyl of parent compound (25-OH-PPD) was replaced. In the ¹³C NMR spectrum, signals will change from δ_c 78.9 (C-3), 70.9 (C-12), 74.7 (C-20), 70.5 (C-25) to δ_c 85.2, 79.2, 81.1, 84.5, respectively. So C10 was a disubstituted derivative at C-3 and C-20 with the signals at δ_c 85.3 and 81.3. δ_c 18.0 (C-6) and 55.9 (C-5) are characteristic carbon signal of glycol type ginseng saponin. By comparing ¹³C-NMR data with C3 and 25-OH-PPD, both the nucleus data are basically the same, the δ_c 72.4 and 71.1 was C-12 and C-25 hydroxy carbon signal, respectively. Δ_c 155.8, 85.2 and 54.5 showed that C-3-OH was derivatized into methoxycarbonylation, δ_c 81.1 and 55.5 showed that C-20-OH was derivatized into methylation. Its ¹H NMR spectrum showed signals for eight tertiary methyl groups at δ 1.23 (6H, s; 2 × CH₃), δ 1.05 (3H, s; CH₃), δ 0.98 (3H, s; CH₃), δ 0.93 (3H, s; CH₃), δ 0.91 (3H, s; CH₃), δ 0.89 (3H, s;

CH₃), δ 0.87 (3H, s; CH₃), two methoxyl protons at δ 3.78 (3H, s; -OCH₃) and δ 3.37 (3H, s; -OCH₃), two protons of oxygen-bearing carbons at δ 4.38 (1H, t; J =8.1 Hz; H-3), δ 3.60 (1H, td; J =5.1, 10.6 Hz; H-12). By a comparison of the NMR data of C3 with that of 25-OH-PPD, C3 was defined as 20-methoxy-3 β -O-methoxycarbonyl-dammar-12 β , 25-diol. The other compounds could be confirmed on the basis of C3.

Cytotoxicity of these derivatives was evaluated on two different human tumor cell lines (Hela and DU145) and one normal cell line (IOSE144) using 3-(4,5dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) assay³¹. 25-OCH₃-PPD, 25-OH-PPD, and 5-Fu were used as references. Results of the MTT assay were expressed in IC₅₀ values (Table 1). All of the methylated derivatives displayed stronger anti-tumor activity, and most of the methoxycarbonylated derivatives had potent or similar cytotoxic activity in vitro compared with 25-OCH₃-PPD, 25-OH-PPD, and 5-Fu. The compounds **3**, **5**, **6**, **10**, **11**, **12** and **13** showed a higher anti-tumor activity, their IC₅₀ values on DU145 and Hela cell lines are shown in Figure 2.

Scheme 1. Synthesis of compounds 1-14.

Figure 2. The IC₅₀ values of the strongest compounds for DU145 and Hela tumor cell lines

Results of the MTT assay of the tested compounds on DU145 cell line (Table 1, Figure 3) show that compounds **6** and **12** exhibited the most potent activity, with IC₅₀ values of 1.2 and 2.6 μM, respectively, a 20-fold to 22-fold increase over 5-Fu; meanwhile, these compounds showed low toxicity on IOSE144 cell (normal cell). Compounds **3**, **6**, **10**, **11** and **12** exhibited stronger activities than the standard drug 25-OH-PPD and 5-Fu, with IC₅₀ values of 3.9, 1.2, 5.6, 3.2 and 2.6 μM, respectively. Moreover, the proliferation of DU145 cells was suppressed in a dose-dependent manner after treatment with compounds 1-14. At 30 μM, the cell growth inhibition ability of compounds **3**, **5**, **6**, **10**, **11** and **12** was 18.6, 19.7, 38.4, 32.5, 20.4 and 20.6 μM, respectively (Figure 3).

Table 1. Anti-proliferative activity (IC₅₀ ± SD values, μmol/L, 48 h) of 25-OH-PPD and synthetic derivatives substituted with DMC in two human tumor cell lines.

Compound	HeLa IC ₅₀ (μmol/L)	DU145 IC ₅₀ (μmol/L)	IOSE144 IC ₅₀ (μmol/L)
1	59.9	87.1	>100
2	26.9	27.2	>100
3	7.1	3.9	>100
4	34.6	29.2	>100
5	15.9	10.9	>100
6	10.4	1.2	>100
7	38.2	32.6	>100
8	30.2	41.1	>100
9	>100	80.3	>100
10	6.7	5.6	>100

11	1.3	3.2	>100
12	1.1	2.6	>100
13	1.2	12.1	>100
14	21.8	18.7	>100
5-Fu	28.2	26.1	36.10
25-OCH ₃ -PPD	22.5	5.4	>100
25-OH-PPD	46.4	57.9	>100

Data on Hela cell line (Table 1, Figure 3) showed similar results. Compounds **12** and **13** exhibited remarkable anti-tumor activity, with IC₅₀ values of 1.1 and 1.2 μM, respectively, and cell inhibition activity of 95% and 97% at 30 μM, respectively. The results indicated that methylated derivatives showed stronger activity than the methoxycarbonylated derivatives against DU145 and, especially, Hela cell line. These results may provide useful data for evaluating structure-activity relationship of other dammarane-type sapogenins in developing new antitumor agents.

Figure 3. Derivatives inhibit the proliferation of human cancer cells DU145, Hela in vitro.

Current data suggests that the introduction of micromolecular nonpolar groups to the side chains

and double bond at C-20/C-22 or C-24/C-25 of 25-OH-PPD increased anti-proliferative activity. For 25-OH-PPD, the anti-cancer activities can excessively increase when hydroxyl groups are replaced by -OCH₃, compared to -C(O)OCH₃, and the derivatives that contain a double bond at C-20/C-22 or C-24/C-25 exhibited higher activity than in the absence of double-bond compounds. Moreover, the substitution position and number of methoxycarbonylation and methylation sites also play an important role in cytotoxic activities. Mono-substituted compounds that showed the highest activity included **C6**, **C11**, **C12**, whereas a significant reduction in anti-cancer activity was observed in substituted groups such as **C4**, **C7**, **C8**, **C9** and **C14**. Antitumor activity of esters was reduced when C-20 and C-25-OH were simultaneously substituted with methoxyl group or methoxycarbonylation; therefore resulting in the decreasing order of activity as follows: monosubstituted derivatives > disubstituted derivatives > multisubstituted derivatives. In our previous study, we only obtained C-3 and C-12-OH substituted derivatives of 25-OH-PPD, and evaluation of structure-activity relationships was not comprehensive enough. The **C1-C14** included C-3, C-12, C-20, and C-25-OH substituted derivatives, and we found that C-20 and C-25-OH showed a greater biological effect than C-3 and C-12-OH. Our activity data showed that the order of methoxycarbonylation and methylation of differently hydroxyl-substituted positions derivatives active were as follows: C-20 > C-25 > C-3 > C-12.

Since few studies on the modification or SAR in dammarane-type sapogenins have been reported, and as the derivatives of 25-OH-PPD in this investigation show very low cytotoxicity against human normal cells, this work might provide a basis for the development of new candidates with potent antitumor activities. Further studies concerning the structure modification and the anti-proliferation mechanism are currently under investigation in our laboratory.

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References and notes

1. Mochizuki, M.; Yoo, C.Y.; Mtsuzawa, K.; Sato, K.; Saiki, I.; Tonooda, S.; Samukiwa, K.; Azuma, I. *Biol. Pharm. Bull.* **1995**, 18, 9.
2. Lee, J.Y.; Shin, J.W.; Chun, K.S.; Park, K.K.; Chung, W.Y.; Bang, Y.J.; Sung, J.H.; Surh, Y.J. *Carcinogenesis*. **2005**, 26, 2.
3. Li, G.; Wang, Z.; Sun, Y.; Liu, K.; Wang, Z. *Basic.Clin. Pharmacol. Toxicol.* **2006**, 98, 6.
4. Attele, A.S.; Wu, J.A.; Yuan, C.S. *Biochem Pharmacol.* **1999**, 58, 11.
5. Gillis.C.N. *Biochem. Pharmacol.* **1997**, 54, 1.
6. Wang, W.; Rayburn, E.R.; Zhao, Y.Q. *Cancer Letter.* **2009**, 278, 2.
7. Wang, W.; Rayburn, E.R.; Zhao, Y. *Cancer Chemother Pharmacol.* **2007**, 59, 5.
8. Zhao, J.; Li, N.; Zhang, H.; Wu, C.F; Piao, H.; Zhao, Y.Q. *J.Bioorg. Med. Chem.L.* **2011**, 21, 3.
9. Hao, M.; Wang, W.; Zhao, Y.; Zhang, R.; Wang, H. *Eur. J. Drug. Metab Pharmacokinet.* **2011**, 35, 3-4.
10. Zhang, X.D.; Xu, J.; Gu, J.; Zhao, Y.Q. *J. Chromatogr. B.* **2007**, 858, 1-2.
11. Wang, W.; Rayburn, E.R.; Hao, M.; Zhao, Y.Q.; Hill, D.L.; Zhang, R.; Wang, H. *Prostata.* **2008**, 68, 8.
12. Akao, T.; Kida, H.; Kanaoka, M.; Hattori, M.; Kobashi, K. *J. Pharm. Pharmacol.* **1998**, 50, 10.
13. Wakabayashi, C.; Murakami, K.; Hasegawa, H.; Murata, J.; Saiki, I. *Biochem. Biophys. Res. Commun.* **1998**, 246, 3.
14. Wang, T.; Meng, Q.G.; Zhang, J.F. *Fitoterapia*, 2010, 81,7.
15. Liu, X K.; Ye, B.J.; Zhao Y.Q. *European Journal of Medicinal Chemistry*, 2011,46, 6.
16. Wang, P.; Yuan, H.N.; Zhao, Y.Q. *Steroids*. 2012, 78, 115-586.
17. Liu, Y.F.; Yuan, H.N.; Zhao, Y.Q. *Steroids*. 2013, 21, 2013.
18. Qu, F.Z.; Liu, Y.F.; Zhao, Y.Q. *Bioorganic & Medicinal Chemistry Letters*. 2013, 24, 23.
19. Wang, P.; Bi, X.L.; Zhao, Y.Q. *Eur.J.Med.Chem.* **2012**, 55, 137-45.

20. Wang, P.; Bi, X.L.; Zhao, Y.Q. *Steroids*. **2013**, 78, 2.
21. Zhao, Y.Q.; Wang, W.; Rayburn, E.R.; *Med Chem*. **2007**, 3, 1.
22. Bi, X.L.; Zhao, Y.Q.; Yang, W. *Clin Exp Pharmacol Physiol*. **2009**, 36, 11.
23. Pacheco M.A.; Marshall C.L. *Energy Fuels*. **1997**, 11, 2–29.
24. Guo, X.C.; Qin, Z.F.; Wang, G.F.; Wang, J.G. *Chem. Lett*. **2008**, 249, 6.
25. Pietro, T.; Laura, R.; Alessandro, J. *Org. Chem*. **2005**, 70, 6.
26. Pietro, T.; Maurizio, S. ; *Acc. Chem. Res*. **2002**, 35, 9.
27. Roberta, B.; Fernanda, C.; Maria, C. *Molecules*. **2011**, 16, 2.
28. Memoli, S.; Selva, M.; Tundo, P. *Chemosphere*. **2001**, 43, 1.
29. The synthesis method of compounds **1-14**: The reaction was carried out at 90-165°C under atmospheric pressure in a reactor equipped with a stirrer and a reflux condensor for DMC. 25-OH-PPD (18 g) was added to a solution of TBAB (30.3 g), K₂CO₃ (17.85 g) in dry DMC (264.6 mL), the mixture was allowed to stir at 90°C for 5 h and 165°C for 3 h. The solvent was removed under reduced pressure to give a faint yellow solid. The solid dissolved in ethyl ether and washed with H₂O, dried (MgSO₄) and concentrated under reduced pressure to give the crude product (Scheme 1.). The crude products were isolated by silica gel chromatography and eluted with petroleum ether- ethyl acetate (4:1,2:1,1:1) to give the pure products **C1-6**. (**1**. 6.69 g, **2**. 0.022 g, **3**. 4.55 g, **4**. 0.32 g, **5**. 0.06 g, **6**. 0.07 g), eluted with petroleum ether-ethyl acetate (15:1,10:1,6:1,4:1) to give the pure products **C7-14** (**7**. 0.05 g, **8**. 0.02 g, **9**. 0.155 g, **10**. 0.13g, **11**. 0.45g, **12**. 0.52 g, **13**. 0.61 g, **14**. 0.22 g).
30. 20(*R*)-3- β -O-methoxycarbonyl-dammar-12 β , 20, 25-triol (**1**) Mp: 266-268°C. [α]_D²⁵ +28.0(c 0.5, CH₂Cl₂); ¹HNMR (300 MHz, CDCl₃): δ 4.34 (1H, t, J =8.1 Hz; H-3), δ 3.77 (3H, s, -OCH₃), δ 3.60 (1H, td, J =5.1, 10.1 Hz; H-12), δ 1.23 (6H, s; 2 $\times$ CH₃), δ 1.13 (3H, s; CH₃), δ 0.99 (3H, s, CH₃), δ 0.93 (3H, s, CH₃), δ 0.88 (3H, s, CH₃), δ 0.86 (6H, s, 2 $\times$ CH₃); ¹³CNMR (75 MHz, CDCl₃): 38.6 (C-1), 23.6 (C-2), 85.3 (C-3), 38.1 (C-4), 55.9(C-5), 18.1 (C-6), 34.7 (C-7), 39.7 (C-8), 49.9 (C-9), 37.0 (C-10), 31.3 (C-11), 71.0 (C-12), 48.4 (C-13), 51.6 (C-14), 31.0 (C-15), 26.3 (C-16), 49.9 (C-17), 16.2 (C-18), 16.3 (C-19), 74.2 (C-20), 21.8 (C-21), 42.9 (C-22), 17.6 (C-23), 44.1 (C-24), 70.0 (C-25), 29.4 (C-26), 29.4 (C-27), 27.9 (C-28), 15.6 (C-29), 17.1 (C-30),

155.8 (carbonyl group). IR (cm⁻¹): 3276(OH), 2954(CH₃), 1392(CH₃), 1724 (carbonyl group). HR-ESIMS: m/z [M+H]⁺ 537.4166. (Calcd for C₃₂H₅₆O₆, 536.4159).

20(*R*)-25-*O*-methoxycarbonyl-dammar-3 β , 12 β , 20-triol (**2**) Mp: 189-191 °C. $[\alpha]_D^{25} +26.1$ (c 0.5, CH₂Cl₂); ¹HNMR (300 MHz, CDCl₃): δ 3.91 (1H, td, J =5.0, 9.8 Hz; H-12), δ 3.46 (1H, dd; J =5.1, 10.2 Hz; H-3), δ 3.38 (3H, s, -OCH₃), δ 1.23 (6H, s; 2 $\times$ CH₃), δ 1.12 (3H, s; CH₃), δ 0.97 (3H, s, CH₃), δ 0.92 (3H, s, CH₃), δ 0.87 (3H, s, CH₃), δ 0.85 (6H, s, 2 $\times$ CH₃); ¹³CNMR (75 MHz, CDCl₃): 38.9 (C-1), 22.0 (C-2), 78.2 (C-3), 37.1 (C-4), 55.8 (C-5), 18.2 (C-6), 34.7 (C-7), 39.7 (C-8), 49.9 (C-9), 37.1 (C-10), 31.4 (C-11), 71.0 (C-12), 48.4 (C-13), 51.6(C-14), 31.0 (C-15), 26.3 (C-16), 49.9 (C-17), 15.6 (C-18), 16.1 (C-19), 74.2(C-20), 22.0 (C-21), 42.8 (C-22), 17.4 (C-23), 41.0 (C-24), 84.2 (C-25), 25.7 (C-26), 25.7 (C-27), 27.3 (C-28), 15.3 (C-29), 17.1 (C-30), 154.1 (carbonyl group). IR (cm⁻¹): 3384 (OH), 2948 (CH₃), 1386(CH₃), 1745(carbonyl group). HR-ESIMS: m/z [M+H]⁺ 537.4243. (Calcd for C₃₂H₅₆O₆, 536.4251).

20(*R*)-20-methoxy-3 β -*O*-methoxycarbonyl-dammar-12 β , 25-diol (**3**) Mp: 169-170 °C. $[\alpha]_D^{25} +15.0$ (c 0.1, CH₂Cl₂); ¹HNMR (300 MHz, CDCl₃): δ 4.38 (1H, t; J =8.1 Hz; H-3), δ 3.78 (3H, s, -OCH₃), δ 3.60 (1H, td, J =5.1, 10.6 Hz; H-12), δ 3.37 (3H, s, -OCH₃), δ 1.23 (6H, s; 2 $\times$ CH₃), δ 1.05 (3H, s; CH₃), δ 0.98 (3H, s, CH₃), δ 0.93 (3H, s, CH₃), δ 0.91 (3H, s, CH₃), δ 0.89 (3H, s, CH₃), δ 0.87 (3H, s, CH₃); ¹³CNMR (75 MHz, CDCl₃): 38.6 (C-1), 23.5 (C-2), 85.3 (C-3), 38.1 (C-4), 55.9(C-5), 18.0 (C-6), 34.5 (C-7), 39.7 (C-8), 49.8 (C-9), 37.1 (C-10), 31.0 (C-11), 72.4 (C-12), 46.8 (C-13), 51.8 (C-14), 31.0 (C-15), 26.4 (C-16),50.9 (C-17),16.2 (C-18),16.3 (C-19), 81.3(C-20), 22.1 (C-21), 42.5 (C-22), 18.0 (C-23), 44.6 (C-24), 71.1 (C-25), 29.2 (C-26), 29.2 (C-27),27.9 (C-28), 15.5 (C-29), 17.1 (C-30), 155.8 (carbonyl group). IR (cm⁻¹): 3366 (OH), 2956 (CH₃), 1380(CH₃), 1741(carbonyl group). HR-ESIMS: m/z [M+H]⁺ 551.4391.(Calcd for C₃₃H₅₈O₆, 550.4382).

20(*R*)-3 β , 25-di-(*O*-methoxycarbonyl)-dammar-12 β , 20-diol (**4**) Mp: 267-268 °C. $[\alpha]_D^{25} +21.0$ (c 0.5, CH₂Cl₂); ¹HNMR (300 MHz, CDCl₃): δ 4.38 (1H, t; J =7.7Hz; H-3), δ 3.63 (3H, s, -OCH₃), δ 3.61 (3H, s, -OCH₃), δ 3.60 (1H, td, J =5.0, 10.2 Hz; H-12), δ 1.29 (6H, s; 2 $\times$ CH₃), δ 1.12 (3H, s; CH₃), δ 1.07 (3H, s, CH₃), δ 1.04 (3H, s, CH₃), δ 0.99 (3H, s, CH₃), δ 0.92 (3H, s, CH₃), δ 0.90 (3H, s, CH₃); ¹³CNMR (75 MHz, CDCl₃): 38.5 (C-1), 23.6 (C-2), 85.2 (C-3), 38.0 (C-4), 55.9(C-5), 18.1 (C-6), 34.7 (C-7), 39.7 (C-8), 49.9 (C-9), 37.0 (C-10), 31.3 (C-11), 70.8 (C-12), 48.4 (C-13), 51.6(C-14), 31.0 (C-15), 26.3 (C-16), 49.9 (C-17), 16.1 (C-18), 16.3 (C-19), 74.1(C-

20), 21.9 (C-21), 42.8 (C-22), 17.4 (C-23), 41.0 (C-24), 84.2 (C-25), 25.7 (C-26), 25.7 (C-27), 27.9 (C-28), 15.6 (C-29), 17.1 (C-30), 155.8, 154.1 (carbonyl group). IR (cm⁻¹): 3416 (OH), 2964 (CH₃), 1393(CH₃), 1736 (carbonyl group). HR-ESIMS: m/z [M+H]⁺ 595.4247.(Calcd for C₃₄H₅₈O₈, 594.4241).

(20*E*)-3β-*O*-methoxycarbonyl-dammar-20(22)-en-12β, 25-diol (**5**) Mp: 132-135 °C. $[\alpha]_D^{25} +20.5$ (c 0.1, CH₂Cl₂); ¹HNMR (300 MHz, CDCl₃): δ5.40 (1H, m; H-22), 4.28 (1H, t; J =8.6Hz; H-3), δ3.75 (3H, s, -OCH₃), δ3.69 (1H, td, J =4.8, 10.1 Hz; H-12), δ1.64 (3H, s; CH₃), δ1.21 (3H, s; CH₃), δ1.19 (3H, s; CH₃), δ1.01 (3H, s, CH₃), δ0.91 (3H, s, CH₃), δ0.89 (3H, s, CH₃), δ0.87 (3H, s, CH₃), δ0.86 (3H, s, CH₃); ¹³CNMR (75 MHz, CDCl₃): 38.6 (C-1), 23.5 (C-2), 85.2 (C-3), 38.0 (C-4), 55.9(C-5), 18.0 (C-6), 34.8 (C-7), 40.0 (C-8), 50.1 (C-9), 37.0 (C-10), 32.3 (C-11), 73.1 (C-12), 50.0 (C-13), 50.4(C-14), 30.5 (C-15), 27.3 (C-16), 55.9 (C-17), 16.2 (C-18), 16.3 (C-19), 140.1 (C-20), 12.4 (C-21), 125.8 (C-22), 22.9 (C-23), 42.9 (C-24), 70.8 (C-25), 29.4 (C-26), 29.1 (C-27), 27.8 (C-28), 15.6 (C-29), 16.7 (C-30), 155.7(carbonyl group). IR (cm⁻¹): 3419(OH), 2960(CH₃), 1387(CH₃), 1741(carbonyl group). HR-ESIMS: m/z [M+H]⁺ 519.4123.(Calcd for C₃₂H₅₄O₅, 518.4118).

20(*R*)-3β-*O*-methoxycarbonyl-dammar-24-en-12β, 20-diol (**6**) Mp: 249-250 °C. $[\alpha]_D^{25} +27.1$ (c 0.100, CH₂Cl₂); ¹HNMR (300 MHz, CDCl₃): δ5.10 (1H, m; H-24), δ4.30(1H, t; J =8.4Hz; H-3), δ3.75 (3H, s, -OCH₃), δ3.61 (1H, td, J =5.1, 10.2 Hz; H-12), δ1.69 (3H, s; CH₃), δ1.64 (3H, s; CH₃), δ1.19 (3H, s; CH₃), δ1.22 (3H, s, CH₃), δ0.92 (3H, s, CH₃), δ0.90 (3H, s, CH₃), δ0.89 (3H, s, CH₃), δ0.86 (3H, s, CH₃); ¹³CNMR (75 MHz, CDCl₃): 38.6 (C-1), 23.6 (C-2), 85.3 (C-3), 38.1 (C-4), 55.9(C-5), 18.1 (C-6), 34.7 (C-7), 39.8 (C-8), 49.9 (C-9), 37.0 (C-10), 31.3 (C-11), 70.7 (C-12), 48.6 (C-13), 51.6(C-14), 31.0 (C-15), 26.3 (C-16), 49.9 (C-17), 16.2 (C-18), 16.4 (C-19), 74.8(C-20), 21.9 (C-21), 42.3 (C-22), 17.8(C-23), 124.5 (C-24), 70.0 (C-25), 132.3 (C-26), 25.8 (C-27), 27.9 (C-28), 15.6 (C-29), 17.1 (C-30), 155.8 (carbonyl group). IR (cm⁻¹): 3411(OH), 2952(CH₃), 1388(CH₃), 1741(carbonyl group). HR-ESIMS: m/z [M+H]⁺ 519.4109.(Calcd for C₃₂H₅₄O₅, 518.4118).

(20*R*)-20-methoxy-3β, 25-di-(-*O*-methoxycarbonyl)-dammar-12β-ol (**7**) Mp: 149-151 °C. $[\alpha]_D^{25} +5.0$ (c 0.100, CH₂Cl₂); ¹HNMR (300 MHz, CDCl₃): δ4.21 (1H, t; J =8.1Hz; H-3), δ3.77 (3H, s, -OCH₃), δ3.69 (3H, s, -OCH₃), δ3.60 (1H, td, J =5.1, 10.2 Hz; H-12), δ3.36 (3H, s, -OCH₃), δ1.47 (6H, s; 2 ×CH₃), δ1.04 (3H, s; CH₃), δ0.98 (3H, s, CH₃), δ0.93 (3H, s, CH₃), δ0.91 (3H, s, CH₃), δ0.89 (3H, s, CH₃), δ0.87 (3H, s, CH₃); ¹³CNMR (75 MHz, CDCl₃): 38.6 (C-1), 23.5 (C-2), 85.1 (C-3), 38.1 (C-4), 55.9(C-5), 18.0 (C-6), 34.5 (C-7), 39.7 (C-8), 49.8 (C-9),

37.1 (C-10), 31.0 (C-11), 72.3 (C-12), 46.8 (C-13), 51.7(C-14), 31.0 (C-15), 26.4 (C-16),49.8 (C-17),16.2 (C-18),16.3 (C-19), 81.3(C-20), 22.0 (C-21), 42.4 (C-22), 17.7 (C-23), 41.2 (C-24), 84.5 (C-25), 25.6 (C-26), 25.7 (C-27),27.9 (C-28), 15.5 (C-29), 17.1 (C-30), 155.8, 154.0 (carbonyl group). IR (cm⁻¹): 3359(OH), 2955(CH₃), 1441(CH₃), 1741(carbonyl group). HR-ESIMS: m/z [M+H]⁺ 609.5412.(Calcd for C₃₅H₆₀O₈, 608.5423).

20(*R*)-20-methoxy-3 β -*O*-methoxycarbonyl-dammar-24-en-12 β -ol (**8**) Mp: 125-127 °C. [α]_D²⁵ +27.5 (c 0.100, CH₂Cl₂); ¹HNMR (300 MHz, CDCl₃): δ 5.12 (1H, m; H-24), δ 4.34 (1H, t; J=7.8Hz; H-3), δ 3.79 (3H, s, -OCH₃), δ 3.21 (1H, td, J=4.6, 9.8Hz; H-12), δ 3.16 (3H, s, -OCH₃), δ 1.62 (3H, s; CH₃), δ 1.25 (3H, s; CH₃), δ 1.09 (3H, s; CH₃), δ 1.00 (3H, s, CH₃), δ 0.96 (3H, s, CH₃), δ 0.93 (3H, s, CH₃), δ 0.90 (3H, s, CH₃), δ 0.88 (3H, s, CH₃); ¹³CNMR (75 MHz, CDCl₃): 38.6 (C-1), 23.5 (C-2), 85.1 (C-3), 38.1 (C-4), 55.9(C-5), 18.1 (C-6), 34.5 (C-7), 39.7 (C-8), 49.8 (C-9), 37.1 (C-10), 31.0 (C-11), 72.2 (C-12), 46.8 (C-13), 51.7(C-14), 31.0 (C-15), 26.5 (C-16), 50.8 (C-17), 16.2 (C-18), 16.3 (C-19), 81.3(C-20), 22.1 (C-21), 42.1 (C-22), 17.6 (C-23), 125.3 (C-24), 130.9 (C-25), 25.7 (C-26), 25.7 (C-27), 27.9 (C-28), 15.5 (C-29), 17.1 (C-30), 155.8 (carbonyl group). IR (cm⁻¹): 3321(OH), 2953(CH₃), 1390(CH₃), 1741(carbonyl group). HR-ESIMS: m/z [M+H]⁺ 483.5438.(Calcd for C₃₅H₅₆O₅, 482.5431).

20(*E*)-3 β , 12 β , 25-tri-(-*O*-methoxycarbonyl)-dammar-20(22)-ene (**9**) Mp: 99-101 °C. [α]_D²⁵ +25.0(c 0.100, CH₂Cl₂); ¹HNMR (300 MHz, CDCl₃): δ 5.28 (1H, m; H-21), δ 4.21(1H, td, J=4.9,10.2 Hz; H-12), δ 4.16(1H, t; J=8.0 Hz; H-3), δ 3.75 (3H, s, -OCH₃), δ 3.61 (3H, s, -OCH₃), δ 3.38 (3H, s, -OCH₃), δ 1.64 (3H, s; CH₃), δ 1.21 (3H, s; CH₃), δ 1.19 (3H, s; CH₃), δ 1.01 (3H, s, CH₃), δ 0.92 (3H, s, CH₃), δ 0.91 (3H, s, CH₃), δ 0.89 (3H, s, CH₃), δ 0.88 (3H, s, CH₃); ¹³CNMR (75 MHz, CDCl₃): 38.6 (C-1), 23.5 (C-2), 85.1 (C-3), 38.1 (C-4), 55.8(C-5), 18.0 (C-6), 34.6 (C-7), 39.7 (C-8), 50.2 (C-9), 37.1 (C-10), 32.1 (C-11), 79.2 (C-12), 47.4 (C-13), 51.1(C-14), 29.7 (C-15), 25.6 (C-16), 55.8 (C-17), 16.1 (C-18), 16.3 (C-19), 137.9(C-20), 12.2 (C-21), 123.4 (C-22), 22.3 (C-23), 40.3 (C-24), 83.9 (C-25), 25.6 (C-26), 25.6 (C-27), 27.9 (C-28), 15.6 (C-29), 16.6 (C-30), 155.7,154.0,155.3 (carbonyl group). IR (cm⁻¹): 3463(OH), 2955(CH₃), 1389(CH₃), 1741(carbonyl group). HR-ESIMS: m/z [M+H]⁺ 635.4003.(Calcd for C₃₆H₅₈O₉, 634.4001).

20(*R*)-3 β , 20-dimethoxy-dammar-12 β , 25-diol (**10**) [α]_D²⁵ +28.5(c 0.100, CH₂Cl₂); ¹³CNMR (75 MHz, CDCl₃): 38.9 (C-1), 22.2 (C-2), 88.5 (C-3), 37.3 (C-4), 55.4 (C-5), 18.0 (C-6), 34.7 (C-7), 39.8 (C-8), 49.9 (C-9), 37.3

(C-10), 31.1 (C-11), 72.8 (C-12), 46.8 (C-13), 51.8 (C-14), 31.1 (C-15), 26.4 (C-16), 50.8 (C-17), 16.1 (C-18), 16.1 (C-19), 81.4 (C-20), 22.1 (C-21), 42.4 (C-22), 18.4 (C-23), 44.5 (C-24), 71.2 (C-25), 29.2 (C-26), 29.2 (C-27), 28.0 (C-28), 15.6 (C-29), 17.1 (C-30), -OCH₃ (55.5, 54.8). ESI-MS: m/z [M+H]⁺ 507.5 (Calcd for C₃₂H₅₈O₄, 506.5).

20(*E*)-25-methoxy-dammar-20(22)-en-3 β , 12 β -diol (**11**)_{[α]_D²⁵ +20.5(c 0.100, CH₂Cl₂); ¹³CNMR (75 MHz, CDCl₃): 38.8 (C-1), 22.8 (C-2), 78.2 (C-3), 38.6 (C-4), 55.7 (C-5), 18.0 (C-6), 34.5 (C-7), 39.5 (C-8), 50.1 (C-9), 37.0 (C-10), 31.8 (C-11), 73.8 (C-12), 48.7 (C-13), 51.5 (C-14), 31.8 (C-15), 26.8 (C-16), 55.7 (C-17), 15.1 (C-18), 15.8 (C-19), 138.4 (C-20), 12.6 (C-21), 122.9 (C-22), 22.8 (C-23), 43.1 (C-24), 84.1 (C-25), 28.6 (C-26), 28.7 (C-27), 27.7 (C-28), 15.3 (C-29), 16.6 (C-30), -OCH₃ (56.7). ESI-MS: m/z [M+H]⁺ 475.5 (Calcd for C₃₁H₅₄O₃, 474.5).}

20(*R*)-20-methoxy-dammar-24(25)-en-3 β , 12 β -diol (**12**)_{[α]_D²⁵ +33.5(c 0.100, CH₂Cl₂); ¹³CNMR (75 MHz, CDCl₃): 38.9 (C-1), 22.6 (C-2), 78.6 (C-3), 38.9 (C-4), 55.8 (C-5), 18.1 (C-6), 34.6 (C-7), 39.7 (C-8), 49.8 (C-9), 37.2 (C-10), 30.9 (C-11), 72.3 (C-12), 46.8 (C-13), 51.7 (C-14), 29.6 (C-15), 25.6 (C-16), 50.5 (C-17), 15.3 (C-18), 15.5 (C-19), 81.3 (C-20), 22.1 (C-21), 42.0 (C-22), 17.6 (C-23), 125.2 (C-24), 130.7 (C-25), 26.3 (C-26), 26.4 (C-27), 27.9 (C-28), 15.5 (C-29), 16.1 (C-30), -OCH₃ (55.3). ESI-MS: m/z [M+H]⁺ 475.4 (Calcd for C₃₁H₅₄O₃, 474.4).}

20(*E*)-3 β , 25-dimethoxy-dammar-20(22)-en-12 β -ol (**13**)_{[α]_D²⁵ +18.0(c 0.100, CH₂Cl₂); ¹³CNMR (75 MHz, CDCl₃): 38.0 (C-1), 22.2 (C-2), 87.5 (C-3), 37.9 (C-4), 55.9 (C-5), 18.1 (C-6), 33.9 (C-7), 39.0 (C-8), 49.9 (C-9), 36.4 (C-10), 31.1 (C-11), 70.0 (C-12), 48.8 (C-13), 49.5 (C-14), 30.9 (C-15), 27.0 (C-16), 55.9 (C-17), 15.1 (C-18), 15.6 (C-19), 138.1 (C-20), 12.1 (C-21), 122.0 (C-22), 21.7 (C-23), 42.2 (C-24), 81.3 (C-25), 28.2 (C-26), 28.3 (C-27), 28.0 (C-28), 14.6 (C-29), 17.2 (C-30), -OCH₃ (55.5). ESI-MS: m/z [M+H]⁺ 489.5 (Calcd for C₃₁H₅₆O₃, 488.5).}

20(*R*)-3 β , 12 β , 20, 25-tetramethoxy-dammarane (**14**)_{[α]_D²⁵ +25.0(c 0.100, CH₂Cl₂); ¹³CNMR (75 MHz, CDCl₃): 38.9 (C-1), 26.0 (C-2), 87.4 (C-3), 37.9 (C-4), 55.5 (C-5), 17.3 (C-6), 34.2 (C-7), 39.1 (C-8), 49.5 (C-9), 36.4 (C-10), 29.9 (C-11), 77.9 (C-12), 48.8 (C-13), 51.6 (C-14), 31.6 (C-15), 26.0 (C-16), 50.8 (C-17), 15.3 (C-18), 15.9 (C-19), 80.3 (C-20), 21.2 (C-21), 45.9 (C-22), 17.1 (C-23), 45.9 (C-24), 79.8 (C-25), 28.4 (C-26), 28.7 (C-}

27), 27.0 (C-28), 14.9 (C-29), 16.5 (C-30), -OCH₃ (55.5, 54.9). ESI-MS: *m/z* [M+H]⁺ 535.3 (Calcd for C₃₄H₆₂O₄, 534.3).

31. Cytotoxicities of these derivatives were evaluated in prostatic carcinoma cell line DU145, human carcinoma of uterine cervix cell line Hela and human ovarian surface epithelial cell IOSE144 by 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT; Sigma Chemical Co., Ltd, USA) assay, using 25OCH₃-PPD, 25-OH-PPD and 5-Fu as references. In short, 5×10³ cells /well were seeded into 96-well plates; 24 h later, the cells were treated with serial dilutions of the compounds (0-100 μM) for another 48 h. 10μL of the MTT solution (5 mg/mL) was added to each well, and the tumor cells were incubated at 37 °C in a humidified atmosphere of 5% CO₂ air for 4 h. At the end of the incubation period, the growth medium was removed and replaced with 100 μL of DMSO (Sigma Chemical Co., Ltd, USA) at room temperature. The solution was agitated with a vortex mixer for 10 min, and the absorbance was measured at 492 nm on a Bio-Rad (model 550) microplate reader to calculate 50% inhibition concentration (IC₅₀). Results of cytotoxic studies were expressed by the IC₅₀ values (Table 1.).