Synthesis and Cytotoxic Activities of New Fatty Acid Esters of 20(*S*)-Protopanaxadiol

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In order to find new lead compounds with antitumour activies, thirteen new fatty acid esters of 20(*S*)-protopanaxadiol (PPD) were synthesized using oleoyl chloride or fatty acids and *N*,*N*'-dicyclohexylcarbodiimide (DCC). Their cytotoxic activities were tested using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] method, and the structure-activity relationships between the fatty acid esters of PPD and their cytotoxic activities are discussed.

Key words: Protopanaxadiol, Cytotoxic Activities, Structure-Activity Relationships

Introduction

Natural ginsenoside-type saponins are widely distributed in *Panax* species of the Araliaceae family, such as *Panax ginseng*, *Panax pseudo-ginseng*, *Panax pseudo-ginseng* Wall. var. *notoginseng*, *Panax quinquefolius*, and *Gynostemma pentaphyllum* of the Cucurbitaceae family. *P. ginseng* has been used as one of the most valuable traditional medicines in the Orient for over 2000 years, and ginsenosides (secondary products) have been widely acknowledged for their good antitumour activity. Previous studies of the structure-acitvity relationship between the ginsenosides and their antitumour effects have shown that the protopanaxadiol (PPD)-type ginsenosides (such as Rg3, Rh2, C-K) are more effective than the protopanaxatriol (PPT)-type ginsenosides, and the aglycones (such as PPD) are more effective than the glycosides. Studies on the metabolism (Chen *et al*., 1999; Hasegawa, 2004; Wang *et al*., 2001) have demonstrated that orally administered ginsenosides pass through the stomach and small intestine without decomposition by either gastric juice or liver enzymes, but are rather metabolized in the large intestine by colonic bacteria. The PPD monoglucoside C-K is the major bacterial metabolite of PPD-type ginsenosides. C-K is further biotransformed to its fatty acid conjugates, which potentiate the antitumour activity of C-K through effective accumulation in the body and reduce the toxicity of C-K (Akao, 1992; Hasegawa *et al*., 2002; Zhang *et al*., 2005). This evidence allows us to hypothesize that fatty acid esters of PPD should be effective new lead compounds with antitumour activity.

In our previous papers (Gao *et al*., 2007; Huang *et al*., 2008; Li *et al*., 2008), we have reported the method of oxidative alkaline degradation to prepare PPD derivatives with high yield from the ginsenosides of stems and leaves of *Panax quinquefolium* L. Here, thirteen new fatty acids esters of PPD (**1**–**13**, Fig. 1) were synthesized using oleoyl chloride or fatty acids and *N*,*N*'-dicyclohexylcarbodiimide (DCC). Their antitumour activities were tested using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] method, and the preliminary structure-activity relationships between the fatty acid esters of PPD and their cytotoxic activities are discussed.

Material and Methods

General

NMR spectra were measured with Bruker ARX-300 and ARX-600 spectrometers (Bruker, Rheinstetten, Germany), using DMSO-d_6 as solvent and TMS as internal standard. ESI-MS was performed on a Finnigan LCQ mass spectrometer (Thermo Electron, San Jose, CA, USA). Silica gel (200 ~ 300 mesh) and silica gel G (Qingdao Marine Chemical Group Co. Ltd, Qingdao, China)

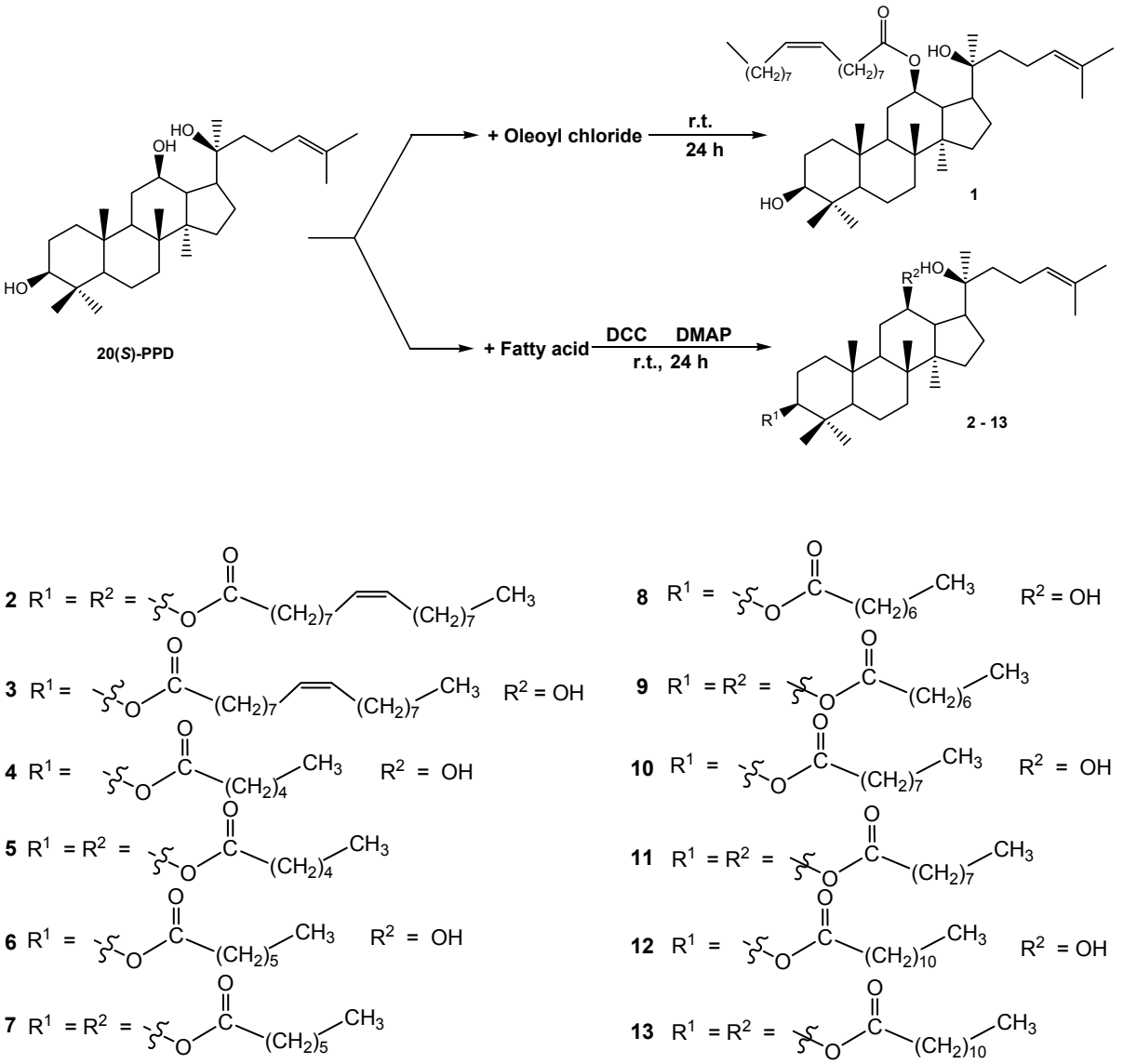

Fig. 1. Synthetic route and chemical structures of compounds **1**–**13**.

were used for column chromatography and TLC, respectively.

Preparation of 20(S)-PPD

Thirty g ginsenosides of leaves and stems of *Panax quinquefolium* L. were hydrolyzed by oxidative alkaline degradation according to the literature (Cui *et al*., 1994; Gao *et al*., 2007; Huang *et al*., 2008; Hui *et al*., 2005). The resulting product (13.6 g) was subjected to chromatography on a silica gel column. Elution was stepwise with a $CHCl_3$/MeOH gradient (from 100:2 to 100:3) to afford 20(*S*)-PPD (1.2 g).

*Preparation of compounds **1**–**13***

1 was obtained according to Hasegawa *et al.* (2002). Oleoyl chloride (25 mmol) was added dropwise to a solution of PPD (200 mg) in EtOAc (60 mL), then mixed with saturated $NaHCO_3$ solution (30 mL) in an ice bath, and stirred for 24 h

Table I. ^{13}C NMR data of compounds **1**–**13** (in ppm, measured in CD_3Cl).

C	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	PPD
1	38.6	38.4	38.5	38.6	38.4	38.6	38.3	38.5	38.1	38.5	38.5	38.6	38.4	38.9
2	27.2	23.6	23.7	23.7	23.5	23.7	23.4	23.6	23.2	23.6	23.6	23.7	23.5	27.3
3	78.7	80.2	80.4	80.4	80.4	80.5	80.0	80.4	79.8	80.4	80.2	80.4	80.1	78.8
4	38.9	37.9	37.9	37.9	37.9	37.9	37.7	37.8	37.5	37.8	37.9	37.9	37.9	38.9
5	55.8	55.8	55.9	55.9	55.9	55.9	55.7	55.8	55.5	55.8	55.9	55.8	56.1	55.8
6	18.2	18.1	18.0	18.1	18.1	18.1	17.9	18.0	17.7	18.1	18.0	18.2	18.0	18.2
7	34.6	34.5	34.7	34.7	34.8	34.7	34.6	34.6	34.3	34.6	34.8	34.7	34.8	34.4
8	39.7	39.7	39.7	39.7	39.7	39.7	39.5	39.7	39.3	39.7	39.7	39.8	39.7	39.7
9	49.9	49.8	49.9	49.9	49.6	49.9	49.6	49.9	49.6	49.9	49.7	49.9	49.6	50.0
10	37.1	37.0	36.9	37.0	37.0	37.0	36.8	36.9	36.6	36.9	37.1	37.0	37.0	37.0
11	28.1	28.2	31.2	31.2	28.2	31.2	28.0	31.1	27.8	31.1	28.1	31.2	28.1	31.2
12	76.6	76.4	70.9	70.9	76.5	70.8	76.1	70.8	75.8	70.8	76.6	70.8	76.4	70.8
13	44.8	44.8	47.8	47.7	44.7	47.7	44.6	47.6	44.5	47.6	44.8	47.8	44.8	47.7
14	52.8	52.9	51.6	51.6	52.9	51.6	52.7	51.5	52.6	51.5	52.9	51.6	52.9	51.6
15	31.5	31.4	30.9	30.9	31.4	31.0	31.4	30.9	31.3	30.9	31.4	31.0	31.4	30.9
16	26.4	26.4	26.4	26.4	26.3	26.5	26.1	26.4	25.8	26.4	26.4	26.5	26.3	26.4
17	52.9	52.7	53.4	53.4	52.6	53.5	52.4	53.4	52.2	53.4	52.6	53.4	52.6	53.4
18	15.6	15.6	15.7	15.7	15.6	15.7	15.4	15.6	15.2	15.6	15.6	15.7	15.6	15.7
19	16.2	16.2	16.1	16.1	16.2	16.2	16.0	16.1	16.1	16.1	16.2	16.2	16.2	16.1
20	73.6	73.6	74.4	74.4	73.6	74.2	73.3	74.2	73.1	74.3	73.6	74.5	73.5	74.3
21	26.2	26.1	26.8	26.9	26.1	26.8	26.1	26.8	25.7	26.7	26.1	27.0	26.1	26.9
22	36.1	36.0	34.4	34.4	36.1	34.5	35.9	34.4	35.7	34.4	36.1	34.4	36.1	34.8
23	22.3	25.3	22.3	22.3	25.3	22.3	25.3	22.3	25.1	22.3	25.3	22.4	25.3	22.3
24	125.2	125.2	124.8	124.9	124.9	125.0	125.0	125.0	124.9	124.9	125.2	124.8	125.2	124.9
25	131.3	131.3	131.8	131.8	131.2	131.7	131.0	131.6	130.5	131.6	131.3	131.9	131.2	131.8
26	25.8	25.8	25.7	25.7	25.7	25.8	25.5	25.7	25.3	25.7	25.8	25.7	25.7	25.7
27	17.7	17.7	17.7	17.7	17.6	17.8	17.4	17.7	17.2	17.7	17.6	17.7	17.6	17.7
28	28.0	28.0	28.0	28.0	27.9	28.0	27.8	27.9	27.9	27.9	28.0	28.0	27.9	28.0
29	15.3	16.5	16.5	16.5	16.5	16.5	16.3	16.5	15.8	16.5	16.5	16.5	16.5	15.3
30	17.3	17.3	16.8	16.8	17.2	16.8	17.0	16.7	16.8	16.7	17.3	16.8	17.2	16.8
Fatty acid part														
	172.4	173.6	173.6	173.7	173.4	173.8	173.3	173.8	173.0	173.7	173.6	173.7	173.5	
	129.7	172.4	129.7	13.9	172.3	14.0	172.2	14.0	171.9	14.0	172.3	14.1	172.4	
	130.0	129.7	130.0		14.1		13.8		13.6		14.1		14.1	
	14.1	130.0	14.1											
		14.1												

at room temperature (r.t.). The reaction mixture was extracted with EtOAc three times to get the crude product. The crude product was isolated by repeated column chromatography on silica gel eluted with a gradient of petroleum ether and acetone (from 100:4 to 100:6) to afford compound **1**.

Compounds **2**–**13** were obtained by adding different fatty acids (oleic acid, hexanoic acid, heptylic acid, octanoic acid, nonanoic acid, lauric acid, 3.3 mmol, respectively), *N*,*N*'-dicyclohexylcarbodiimide (DCC, 3.3 mmol), and 4-dimethylaminopyridine (DMAP, 0.3 mmol) to a solution of PPD (300 mg) in dry tetrahydrofuran. The mixture was stirred for 24 h at room temperature, then filtered, and washed with $CHCl_3$. The filtrate was recovered to get the crude product. The crude product was isolated by repeated column chromatography on silica gel eluted with a gradient of petroleum ether and acetone (from 100:0 to 100:8) to afford compounds **2**–**13**, respectively.

In vitro cytotoxicity bioassay

HepG2, A549, and HL-60 cells were used as the target cells in the cytotoxicity assay according to the method reported (Jin *et al*., 2008). For

Table II. Important ^{1}H NMR and MS data of compounds **1**–**13**.

No.	**1**	**2**	**3**	**4**	**5**
H-3	3.20, dd-like	4.49, dd, 5.4, 10.5	4.48, dd, 5.5, 10.5	4.40, dd, 5.5, 11.0	4.30, m
H-12	4.73, m	4.73, m	3.60, td, 5.5, 10.5	3.60, td, 5.5, 10.0	4.73, m
H-24	5.16, m	5.16, t, 7.0	5.16, t, 7.0	5.16, t, 7.0	5.15, t, 7.0
	5.34, m	5.34, m	5.34, m	2.29, t, 7.5	2.27, t, 7.5
Fatty acid part	0.88, br.s	0.88, br.s	0.88, br.s	0.89, br.s	0.84, br.s
		5.34, m			2.27, t, 7.5
		0.88, br.s			0.84, br.s
MS	724 $[M]^+$	1027 $[M+K]^+$	747 $[M+Na]^+$	581 $[M+Na]^+$	679 $[M+Na]^+$
No.	**6**	**7**	**8**	**9**	**10**
H-3	4.48, dd, 5.5, 10.5	4.45, m	4.48, dd, 5.5, 11.0	4.35, m	4.48, dd, 5.5, 10.5
H-12	3.60, td, 5.0, 10.5	4.75, m	3.60, td, 5.0, 10.5	4.60, m	3.60, td, 5.0, 10.0
H-24	5.15, t, 7.0	5.12, t, 7.0	5.15, t, 7.0	5.02, t, 7.0	5.15, t, 7.0
	2.29, t, 7.5	2.31, t, 7.5	2.29, t, 7.5	2.20, t, 7.5	2.30, t, 7.5
Fatty acid part	0.88, br.s	0.82, br.s	0.88, br.s	0.80, br.s	0.88, br.s
		2.31, t, 7.5		2.20, t, 7.5	
		0.82, br.s		0.80, br.s	
MS	595 $[M+Na]^+$	707 $[M+Na]^+$	609 $[M+Na]^+$	735 $[M+Na]^+$	623 $[M+Na]^+$
No.	**11**	**12**	**13**	PPD	
H-3	4.42, m	4.48, dd, 5.0, 11.0	4.40, m	3.20, dd, 5.3, 10.8	
H-12	4.65, m	3.60, td, 5.5, 10.5	4.79, m	3.58, td, 5.3, 10.3	
H-24	5.10, t, 7.0	5.16, t, 7.0	5.16, t, 7.0	5.16, t, 7.0	
	2.26, t, 7.5	2.29, t, 7.5	2.29, m		
Fatty acid part	0.85, br.s		2.40, t, 7.5		
	2.26, t, 7.5	0.89, br.s	0.88, br.s		
	0.85, br.s		0.85, br.s		
MS	763 $[M+Na]^+$	642 $[M]^+$	847 $[M+Na]^+$	483 $[M+Na]^+$	

drug exposure experiments, after contact of the cells with the drug for 72 h, 10 μL MTT solution (2.5 mg/mL) were added to each well, and the tumour cells were incubated at 37 °C with fetal calf serum in a humidified atmosphere of 5% CO_2 for 4 h. At the end of the incubation period, the growth medium was removed and replaced with 100 μL DMSO (at room temperature). After agitating (Vortex, model HYQ-212A, Crystal Technology and Industries Inc., Addison, TX, USA) for 10 min, the absorbance was determined at 492 nm on a Bio-Rad (model 550; Hercules, CA, USA) microplate reader to calculate the 50% inhibition concentration (IC_{50}). DMSO and MTT were purchased from Sigma Chemical (St. Louis, MO, USA).

Results and Discussion

The traditional acylation method using acyl chloride (Hasegawa *et al.*, 2002) was not suitable for the synthesis of PPD-3-monoesters, *i. e.* compounds **3**, **4**, **6**, **8**, **10**, **12**; these esters were obtained using DCC.

PPD derivatives were synthesized as described in the Materials and Methods section (Fig. 1). Yields of the compounds were 28% for **1**, 21% for **2**, 16% for **3**, 19% for **4**, 14% for **5**, 20% for **6**, 15% for **7**, 20% for **8**, 15% for **9**, 20% for **10**, 16% for **11**, 18% for **12**, 15% for **13**.

The chemical structures of compounds **1**–**13** (Fig. 1) were confirmed on the basis of NMR (^{1}H, ^{13}C, DEPT, HMQC, HMBC) and MS data, respectively (Tables I and II). All compounds were evaluated for their cytotoxic activities against three cell lines, *i.e.* HepG2, A549, and HL-60, using the MTT method, and the bioassay results are listed in Table III and Fig. 2. PPD, which is considered an effective *Panax ginseng* constituent with antitumour activity, was used as positive control. In comparison with PPD, compounds **3**, **10**, **12**, and **13** were found to possess higher cytotoxic activities in HepG2 cells; **3**, **8**, **10**, and **12** were more active against A549 cells, and **2**, **3**, and

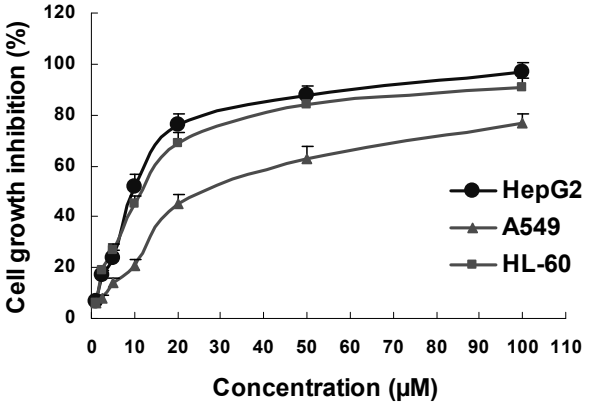

Fig. 2. Inhibition of the growth of three cell lines (HepG2, A549, HL-60) by compound **3**.

Table III. Cytotoxic activities of compounds **1**–**13**.

Compound	IC_{50} ± SD [μM]		
	HepG2	A549	HL-60
1	56.60 ± 3.21	> 100	32.46 ± 2.44
2	41.66 ± 3.99	> 100	20.25 ± 3.20
3	9.20 ± 1.15	29.65 ± 2.94	11.55 ± 2.73
4	> 100	> 100	50.40 ± 2.93
5	> 100	> 100	> 100
6	> 100	> 100	56.24 ± 4.72
7	> 100	> 100	> 100
8	49.68 ± 4.32	78.58 ± 4.35	32.56 ± 2.61
9	53.35 ± 3.35	> 100	36.11 ± 2.72
10	38.56 ± 3.78	58.21 ± 2.85	35.66 ± 1.97
11	42.43 ± 2.65	> 100	33.82 ± 2.41
12	19.28 ± 2.22	38.65 ± 3.19	22.34 ± 1.82
13	35.52 ± 2.43	> 100	34.39 ± 2.54
20(*S*)-PPD	39.42 ± 1.15	99.89 ± 1.59	27.06 ± 1.25

12 were more active against HL-60 cells. Compound **3** (PPD-3-monooleate) exhibited the highest cytotoxic activity in all three cell lines. The lines HepG2 and HL-60 were more sensitive to all compounds than A549, including the positive control PPD.

On the basis of these results, the following preliminary conclusions regarding structure-activity relationships were drawn:

1) The bioassay results depicted in Table III reveal that some of the synthesized new fatty acid esters of PPD possess better cytotoxic activities in comparison with PPD, such as compounds **2**, **3**, **8**, **10**, **12**, and **13**, among which compound **3** (PPD-3-monooleate), an unsaturated fatty acid ester of PPD, showed much better cytotoxic activities in all cell lines tested compared with PPD and other saturated fatty acid esters of PPD. The unsaturation of the fatty acid plays an important role.

2) Comparing the cytotoxic activities of compounds **1**–**3**, PPD-3-monoester **3** showed better cytotoxic activities than the PPD-3,12-diester **2** and the PPD-12-monoester **1**. The position of esterification is a key factor for cytotoxic activities.

3) Compounds **4**–**7** showed much lower cytotoxic activities against all three cell lines compared with PPD. The cytotoxic activity of the fatty acid esters of 20*(S)*-PPD increases with fatty acid chain length. When the carbon number of the fatty acid chain is lower than 8, the fatty acid esters of PPD show lower cytotoxic activities.

4) The PPD-3-monooleate showed the highest cytotoxic activities in the three cell lines (Fig. 2). Further structure modification work will be carried out on the basis of PPD-3-monooleate.

Acknowledgements

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