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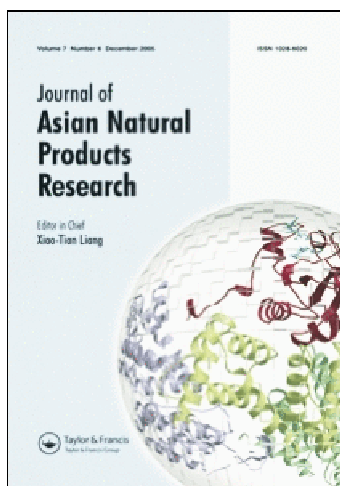

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Four new minor taxanes from cell cultures of *Taxus chinensis*

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Four new minor taxanes (**1**–**4**) have been isolated from Ts-19 cell cultures of *Taxus chinensis* together with five known taxanes (**5**–**9**) by silica gel chromatography combined with semi-preparative HPLC chromatography. On the basis of the analyses of the chemical and spectroscopic (IR, MS, 1D, and 2D NMR) data, the structures of new compounds were elucidated as 5α-hydroxy-2α,10β-diacetoxy-14β-(3-hydroxy-2-methyl-butyryl)oxytaxa-4(20),11-diene (**1**), 2α,5α,10β-triacetoxy-14β-(2-hydroxy-propionyl)oxytaxa-4(20),11-diene (**2**), 2α,5α,10β-triacetoxy-14β-(2-hydroxy-3-methyl-butyryl)oxytaxa-4(20),11-diene (**3**), and 2α-benzoxy-4α,9α,10β,13α-tetraacetoxytax-11-ene (**4**), respectively. Compounds **1**–**5** were pharmacologically evaluated for their cytotoxicities against five human cancer cell lines (HCT-8, Bel-7402, BGC-823, A549, and A2780) and their reversing activity towards multi-drug resistance A549/taxol tumor cell line, and the results showed that all of the tested compounds exhibited very low cytotoxicities, while compound **4** possessed twice the reversing activity as that of verapamil at 10 μM.

Keywords: taxane; MDR reversing activity; cell cultures; *Taxus chinensis*

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1. Introduction

Paclitaxel (Taxol®), a diterpene originally isolated from *Taxus brevifolia* [1], and the semi-synthetic-related substance, Taxotere® [2] have attracted considerable interest all over the world to conduct research work during the last 20 years for their use in the chemotherapy of various tumors. The supply crisis for taxol or taxotere has stirred the interest to produce these compounds by means of isolation from other *Taxus* species, semi-synthesis, total synthesis, as well as plant cell culture, etc. However, due to the low content of paclitaxel in natural sources and the lack of a commercially viable total synthesis, much attention has been directed to cell culture as a renewable resource. There were a number of reports on the successful production of paclitaxel and its analogues with C-13 oxygenated group and oxetane moiety, but their yields were too low to be produced commercially [3–6]. Interestingly, most of the research groups in the *Taxus* cell culture reached a very similar result that the cell cultures of *Taxus* sp. were able to produce a large amount of one type of taxanes – 4(20),11(12)-taxadienes with C-14 oxygenated functional groups (Figure 1, compounds **6**–**9**) rather than C-13 oxygenated functional groups [4–9].

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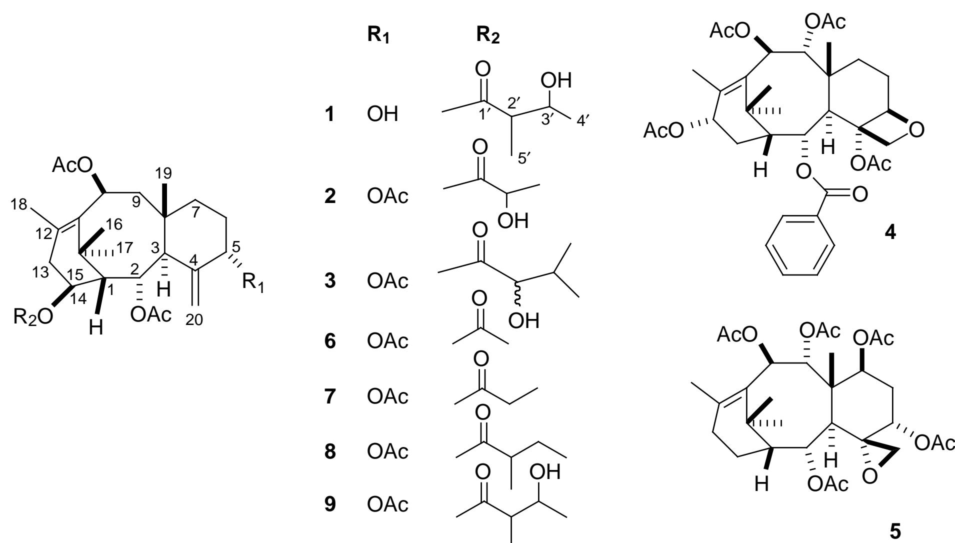

Figure 1. The structures of compounds **1**–**9**.

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This type of taxanes possesses potent reversing activity towards multi-drug-resistant (MDR) tumor cells [6], and can be used as starting materials for the semi-synthesis of more effective reversal agents through chemical and/or enzymatic modification [10–13]. In this context, we accumulated about 10 kg of dry cell cultures to obtain compounds **6**–**9** for the structural optimization. However, in the course of this study, except for the major metabolites **6**–**9**, other five minor taxanes (Figure 1, compounds **1**–**5**) were obtained by the way. Among them, compounds **1**–**4** are four new compounds. Herein, their structural elucidation and reversing activity against MDR tumor cell line A549/taxol are reported.

2. Results and discussion

Compound **1** was isolated as colorless needle crystals. The ESI-MS/HR-ESI-MS showed a potassiated molecular ion peak at *m/z* 559 and a sodiated molecular ion peak at *m/z* 543, suggesting the molecular formula to be $C_{29}H_{44}O_8$. The ^{1}H, ^{13}C NMR, and DEPT spectra displayed the existence of taxane skeleton with four C-Me groups at δ_H 2.08, 1.65, 1.11, 0.81 and δ_C 21.5, 25.4, 31.8, 22.2; two acetoxyl moieties at δ_H 2.04, 2.02; δ_C 170.2, 169.9; and 21.5, 20.8; together with five oxygenated methines at δ_H 5.35 (dd, $J = 6.4$, 2.0 Hz), δ_C 70.8; δ_H 6.10 (dd, $J = 12.0$, 5.6 Hz), δ_C 70.3; δ_H 4.18 (s), δ_C 76.2; δ_H 5.08 (dd, $J = 9.6$, 4.8 Hz), δ_C 70.9; δ_H 3.84 (dq, $J = 6.4$, 7.2 Hz), δ_C 69.4. The signals at δ_H 5.10 and 4.74 (brs), δ_C 147.6 (s) and 113.4 (t) typically indicated the presence of C-4(20) exocyclic double bond in **1**. The signals at δ_H 2.40 (1H, q, $J = 7.2$ Hz, H-2′), 3.84 (1H, dq, $J = 6.4$, 7.2 Hz, H-3′), 1.20 (1H, d, $J = 6.4$ Hz, H-4′), 1.15 (1H, d, $J = 7.2$ Hz, H-5′) and correlations in the HMBC spectrum revealed a 3-hydroxy-2-methyl-butyryloxyl group in **1**. These data are very similar to those of yunnanxane (**9**) [14] except for the absence of one acetyl group, and H-5 signal at upfield (δ 4.18) instead of a signal at δ 5.30 in **9**, indicating the existence of an OH group at C-5 position in **1** rather than an acetoxyl group. It was confirmed by the molecular weight being 42 amu less than that of **9**, and an IR absorption bond at

$3448\,cm^{-1}$. Thus, the structure of **1** was elucidated as 5α-hydroxy-2α,10β-diacetoxy-14β-(3-hydroxy-2-methyl-butyryl)-oxytaxa-4(20),11-diene(5-deacetyl yunnanxane), and the assignments of its NMR spectroscopic data are listed in Tables 1 and 2.

Compound **2** was obtained as colorless needle crystals, which had the elemental composition of $C_{29}H_{42}O_9$ determined by the combination of ESI-MS/HR-ESI-MS and ^{1}H and ^{13}C NMR spectra. The ^{1}H and ^{13}C NMR spectra of **2** were very similar to those of compound **7** [7], except for the appearance of one oxygen-bearing methine signals at δ_H 4.22 (q, $J = 7.2$ Hz) and δ_C 66.7 and the disappearance of one methylene signals at δ_H 2.27 (q, $J = 7.2$ Hz) and δ_C 20.3 assigned to H-2′ and C-2′ (see Table 1). According to these data, one OH group might be introduced at C-2′ position of **2** by comparison with **7**, and it was further confirmed by the IR absorption bond at $3454\,cm^{-1}$. Therefore, the structure of **2** was concluded to be 2α,5α,10β-triacetoxy-14β-(2-hydroxy-propionyl)oxytaxa-4(20),11-diene, a 2′-hydroxylated analogue of **7**.

Compound **3** was afforded as a colorless amorphous powder, its elemental composition of $C_{31}H_{46}O_9$ was established by the combination of ESI-MS/HR-ESI-MS and ^{1}H and ^{13}C NMR spectra. The ^{1}H and ^{13}C NMR spectra of **3** were very similar to those of **9** [14], except that one oxygen-bearing

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Table 1. The ^{13}C NMR spectroscopic data for compounds **1**–**4** (100 MHz, $CDCl_3$, δ in ppm).

Position	**1**	**2**	**3**	**4**
1	59.1	59.2	60.0	45.7
2	70.8	70.4	70.4	71.0
3	40.0	42.1	42.1	43.5
4	147.7	142.2	142.2	81.6
5	76.2	78.3	78.2	83.9
6	30.9	28.9	28.8	31.5
7	33.0	33.8	33.8	34.6
8	40.0	39.7	39.7	49.9
9	43.8	43.9	43.8	75.6
10	70.3	70.0	70.0	71.3
11	135.7	135.5	135.6	129.9
12	134.5	134.4	134.4	133.4
13	39.3	39.1	39.4	71.8
14	70.9	72.2	72.2	21.0
15	37.3	37.2	37.2	37.1
16	25.4	25.4	25.3	29.1
17	31.8	31.7	31.6	12.5
18	21.5	21.8	21.9	25.8
19	22.2	22.4	22.4	17.3
20	113.4	117.0	117.0	76.5
1′	175.1	174.8	173.9	
2′	46.9	66.7	74.7	
3′	69.4	20.3	32.0	
4′	20.7		18.8	
5′	14.0		15.8	
$OCOCH_3$	21.5; 20.8	21.4; 21.4; 20.9	21.4; 21.4; 20.9	22.1; 21.4; 21.1; 20.8
$OCOCH_3$	170.2; 169.9	170.2; 169.9; 169.7	170.2; 169.9; 169.7	170.1; 169.8; 169.8; 169.2
Bz*C*O				164.8
q-Bz				141.1
p-Bz				131.6
o-Bz				129.8
m-Bz				128.6

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Table 2. The ^{1}H NMR spectroscopic data for compounds **1–4** (400 MHz, $CDCl_3$, δ in ppm, J in Hz).

Position	**1**	**2**	**3**	**4**
1	1.85 (d, 2.0)	1.88 (d, 2.0)	1.92 (d, 2.0)	1.88 (d, 2.0)
2	5.35 (dd, 2.0, 6.4)	5.36 (dd, 2.0, 6.4)	5.38 (dd, 2.0, 6.4)	5.85 (d, 5.2)
3	3.21 (d, 6.4)	2.92 (d, 6.4)	2.92 (d, 6.4)	3.02 (d, 5.2)
4				
5	4.18 (s)	5.30 (s)	5.30 (s)	4.98 (d, 8.8)
6	1.73 (m)	1.82 (m)	1.80 (m)	1.85 (m)
7	Hα, 2.28 (m); Hβ, 2.10 (m)	Hα, 1.95 (m); Hβ, 1.24 (brd)	Hα, 1.97 (m); Hβ, 1.25 (m)	Hα, 1.93 (s); Hβ, 1.71 (brs)
8				
9	Hα, 1.62 (dd, 5.6, 14.8); Hβ, 2.32 (m)	Hα, 1.62 (brd); Hβ, 2.37 (dd, 13.6, 13.2)	Hα, 1.63 (dd, 5.6, 14.8); Hβ, 2.39 (m)	5.94 (d, 11.2)
10	6.10 (dd, 5.6, 12.0)	6.06 (dd, 5.6, 12.0)	6.05 (dd, 5.6, 12.0)	6.16 (d, 11.2)
11				
12				
13	Hα, 2.81 (dd, 9.6, 18.8); Hβ, 2.31 (m)	Hα, 2.49 (dd, 4.4, 19.2); Hβ, 2.38 (dd, 9.2, 19.2)	Hα, 2.89 (dd, 9.2, 19.2); Hβ, 2.43 (m)	5.57 (d, 8.8)
14	5.08 (dd, 4.8, 9.6)	5.08 (dd, 4.4, 9.2)	5.07 (dd, 4.8, 9.6)	2.55 (m); 2.37 (m)
15				
16	1.65 (s)	1.67 (s)	1.67 (s)	1.77 (s)
17	1.11 (s)	1.11 (s)	1.11 (s)	1.55 (s)
18	2.08 (s)	2.10 (s)	2.11 (s)	2.08 (s)
19	0.81 (s)	0.85 (s)	0.85 (s)	1.06 (s)
20	5.10 (s); 4.74 (s)	5.29 (s); 4.85 (s)	5.28 (s); 4.84 (s)	4.41 (d, 8.4); 4.16 (d, 8.4)
1′				
2′	2.40 (q, 7.2)	4.22 (q, 7.2)	3.98 (d, 2.4)	
3′	3.84 (dq, 6.4, 7.2)	1.37 (d, 7.2)	2.02 (m)	
4′	1.20 (d, 6.4)		1.03 (d, 7.2)	
5′	1.15 (d, 7.2)		0.86 (d, 7.2)	
OAc	2.04 (s); 2.02 (s)	2.18 (s); 2.06 (s); 2.04 (s)	2.19 (s); 2.06 (s); 2.04 (s)	2.27 (s); 2.10 (s); 2.08 (s); 1.98 (s)
p-Bz				7.60 (t, 7.6)
o-Bz				8.07 (d, 7.6)
m-Bz				7.47 (t, 7.6)

methine signals were observed at δ_H 3.98 (d, $J = 2.4$ Hz) and δ_C 74.7, instead of being observed at δ_H 3.86 (dq, $J = 7.0, 6.9$ Hz) and δ_C 69.5 in **9**. Besides, a HMBC experiment was used for the assignments of the quaternary carbons and attachment of the functional groups. The correlations of the signals indicated the presence of 2-hydroxyl-3-methyl-butyryloxy group, including an isopropyl function at δ_H 1.03 (3H, d, $J = 7.2$ Hz, H-4′), 0.85 (3H, d, $J = 7.5$ Hz, H-5′), 2.02 (1H, m, H-3′) and δ_C 32.0 (C-3′), 18.8 (C-4′), 15.8 (C-5′). It was supported by the observation of loss of correlations between the oxygen-bearing methine proton and methyl proton in ^{1}H–^{1}H COSY spectrum of **9**, while it was supported by the observation of correlations between oxygen-bearing methine proton (δ_H 3.98) and carbonyl carbon (δ_C 173.9) in HMBC spectrum. The correlation between H-14 at δ 5.10 (dd, $J = 4.4, 9.2$ Hz) with a carbon signal at δ 72.0 and C-1′ at δ 173.9 in the HMBC spectrum of **3** suggested the attachment of the 2-hydroxyl-3-methyl-butyryloxyl group at C-14. Thus, the structure of **3** was deduced as 2α,5α,10β-triacetoxy-14β-(2-hydroxyl-3-methyl-butyryl)oxytaxa-4(20),11-diene. However, the configurations of C-3′ need further confirmation.

Compound **4** was obtained as a colorless amorphous solid. The molecular formula $C_{35}H_{44}O_{11}$ was established by the analyses of HR-ESI-MS and ^{1}H and ^{13}C NMR spectroscopic data. The ^{1}H NMR spectrum indicated the presence of a taxane skeleton with four C-Me groups at δ 1.77, 1.55, 2.08, and 1.06, four acetyl Me groups at δ 2.27, 2.10, 2.08, and 1.98, and a benzoyl group at δ 7.60 (1H, t, $J = 7.6$ Hz), 8.07 (2H, d, $J = 7.6$ Hz), and 7.47 (2H, t, $J = 7.6$ Hz). The proton signals at δ 4.41 (1H, d, $J = 8.4$ Hz, H-20) and 4.16 (1H, d, $J = 8.4$ Hz, H-20) in the ^{1}H NMR spectrum together with the carbon signals at δ 83.9 (C-5) and 76.5 (C-20) in the ^{13}C NMR spectrum characteristically indicated the presence of a 4(20) oxetane ring in **4**. Combined with the analysis of the 2D NMR spectroscopic data (^{1}H–H COSY, HSQC, and HMBC), the four acetoxyl groups were assigned to C-4, C-9, C-10, and C-13, respectively, and the benzoxyl group to C-2 (see Tables 1 and 2). Thus, the structure of **4** was determined as 2α-benzoxy-4α,9α,10β,13α-tetraacetoxytax-11-ene.

The cytotoxicities against five human cancer cell lines (HCT-8, Bel-7402, BGC-823, A549, and A2780), as well as the reversing activity towards MDR tumor cell line – lung adenocarcinoma cell line A549/taxol of compounds **1**–**5** were preliminarily evaluated. The results showed that their cytotoxicities were very low (data not shown). This is presumably due to their lack of the C-13 side-chain and/or oxetane ring which are necessary for the cytotoxicity of taxoids. Compounds **2**, **3**, and **5** exhibited substantially lower potent reversing activity towards A549/taxol MDR tumor cells compared with verapamil – a positive control, and compound **1** showed moderate reversing activity. While compound **4** displayed significant reversing activity, about twice as that of verapamil at 10 μM (Table 3).

3. Experimental

3.1 General experimental procedures

Optical rotations in Me_2CO were carried out on a Perkin-Elmer Model-343 digital polarimeter. IR spectra were measured on

Table 3. MDR reversal activity of compounds **1**–**5** against A549/taxol MDR subline of human lung adenocarcinoma cell lines *in vitro*.

Compounds	Fold of reversal (FR)	Equivalent verapamil (%)
Verapamil	75.38	100
1	60.20	79.6
2	1.23	1.6
3	0.90	1.2
4	155.48	206.3
5	1.40	1.9

FR: the ratio of the IC_{50} of resistant cells only in the presence of anticancer drug to the IC_{50} of resistant cells only in the presence of both anticancer drug and reversal agents at 10 μM.

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an IMPACT-400 FT-IR microscope spectrometer. The ^{1}H and ^{13}C NMR spectra were recorded in $CDCl_3$ on Varian-400 and Bruker ARX-500 spectrometers using TMS as an internal standard. Chemical shifts (δ) are given in ppm and coupling constants (J) are given in Hz. ESI-MS and HR-ESI-MS were obtained on a Q-trap ESI mass spectrometer. Semi-preparative normal-phase HPLC was performed on a Shimadzu LC-6AD instrument with an Apollo silica gel column (5 μm, 250 mm × 10 mm i.d., flow rate 4 ml/min) and a Shimadzu RID-10A detector. Semi-preparative reversed-phase HPLC was carried out on the same instrument with a YMC-Pack ODS-A (5 μm, 250 mm × 10 mm i.d., flow rate 2 ml/min; YMC Co., Ltd, Tokyo, Japan). Silica gel (200–300 mesh; Qingdao Marine Chemical Industry, Qingdao, China) was used for column chromatography and analytical TLC was carried out on pre-coated silica gel GF-254 plates (Qingdao Marine Chemical Industry), and the visualization of TLC plates was performed by spraying with 5% H_2SO_4 in EtOH followed by heating at 105°C.

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3.2 Materials

The origin and cultural conditions of Ts-19 cell cultures of *Taxus chinensis* were described previously [9].

3.3 Extraction and isolation

A 10 kg of dry Ts-19 cell cultures of *T. chinensis* was obtained from several batch cultures, and was ground into a powder. The resulting powder was extracted with ether under reflux for 72 h three times to yield an ether extract weighing 200 g (2%, containing four major known C-14 oxygenated taxanes **6–9**). After which the dried residue was macerated in 95% EtOH (20 l) for 1 month to give 200 g (2%) crude extract. The crude extract was suspended with 1 l water, and extracted with EtOAc to afford an EtOAc-soluble fraction (29.0 g). This fraction was chromatographed on a silica gel column eluting gradiently with the mixtures of Me_2CO and petroleum ether (5% Me_2CO/95% petroleum ether to 100% Me_2CO) to give four fractions monitored by TLC (FA, 0.5 g; FB, 2.5 g; FC, 22.2 g; and FD, 5.5 g). The major part FC was subsectioned to five sub-fractions (FC1: 1.26 g, 5.6%; FC2: 1.20 g, 5.4%; FC3: 7.4 g, 33.3%; FC4: 2.8 g, 12.6%; and FC5: 0.58 g, 2.6%) by silica gel column chromatography (600 g, 300–400 mesh), eluted starting with 5% Me_2CO/95% petroleum ether up until 100% Me_2CO. Each portion of FC (FC1–FC5) was then performed by semi-preparative HPLC. A 12.3 mg of compound **1** was obtained from fractions FC4 and FC5 ($t_R = 9.73$ min, mobile phase: *n*-hexane/ EtOAc = 4:1). A 21.5 mg of compound **2** was obtained from fractions FC3 ($t_R = 14.73$ min, mobile phase: *n*-hexane/ EtOAc = 4:1). From FC1 and FC2, 80 mg of compound **3**, 24.5 mg of compound **4**, and 10.2 mg of compound **5** were obtained as yellowish substances (mobile phase: *n*-hexane/EtOAc = 6:1; $t_{R\mathbf{3}} = 16.59$ min; $t_{R\mathbf{4}} = 18.77$ min; and $t_{R\mathbf{5}} = 19.90$ min). Then these three yellowish substances were individually subjected to semi-preparative reversed-phase HPLC repeatedly to yield colorless crystals **3–5** (mobile phase: MeOH/H_2O = 78:22; **3**, 54.3 mg, $t_{R\mathbf{3}} = 27.69$ min; **4**, 12.3 mg, $t_{R\mathbf{4}} = 23.77$ min; and **5**, 6.4 mg, $t_{R\mathbf{5}} = 19.90$ min).

*3.3.1 5α-Hydroxy-2α,10β-diacetoxy-14β-(3-hydroxy-2-methyl-butyryl)oxytaxa-4(20),11-diene (**1**)*

Colorless needle crystals; $[\alpha]_D^{25}$ +41.9 ($c = 0.105$, Me_2CO); IR (KBr) ν_{max} cm^{-1}: 3448, 2981, 2927, 1736, 1246, 1018, 953; ^{1}H and ^{13}C NMR spectral data, see Tables 1 and 2; ESI-MS *m/z*: 559 $[M+K]^+$, 543 $[M+Na]^+$, 343, 283, 265 (100). HR-ESI-MS *m/z*: 543.2904 $[M+Na]^+$ (calcd for $C_{29}H_{44}O_8Na$, 543.2928).

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*3.3.2 2α,5α,10β-Triacetoxy-14β-(2-hydroxy-propionyl)oxytaxa-4(20),11-diene (**2**)*

Colorless needle crystals; $[\alpha]_D^{25}$ +34.3 ($c = 0.105$, Me_2CO); IR (KBr) ν_{max} cm^{-1}: 3454, 2925, 2858, 1741, 1730, 1246, 1018; ^1H and ^{13}C NMR spectral data, see Tables 1 and 2; ESI-MS *m/z*: 573 $[M+K]^+$, 557 $[M+Na]^+$ (100), 385, 283, 265. HR-ESI-MS *m/z*: 557.2700 $[M+Na]^+$ (calcd for $C_{29}H_{42}O_9Na$, 557.2721).

*3.3.3 2α,5α,10β-Triacetoxy-14β-(2-hydroxyl-3-methyl-butyryl)oxytaxa-4(20),11-diene (**3**)*

Colorless amorphous powder; $[\alpha]_D^{25}$ +39.0 ($c = 0.10$, Me_2CO); IR (KBr) ν_{max} cm^{-1}: 3520, 2962, 1736, 1236, 1018, 953; ^1H and ^{13}C NMR spectral data, see Tables 1 and 2; ESI-MS *m/z*: 601 $[M+K]^+$, 585 $[M+Na]^+$ (100), 385, 343, 283, 265. HR-ESI-MS *m/z*: 585.3014 $[M+Na]^+$ (calcd for $C_{31}H_{46}O_9Na$, 585.3034).

*3.3.4 2α-Benzoxy-4α,9α,10β,13α-tetraacetoxytax-11-ene (**4**)*

Colorless amorphous powder; $[\alpha]_D^{25}$ +50.6 ($c = 0.102$, MeOH); IR (KBr) ν_{max} cm^{-1}: 3193, 2918, 2849, 1741, 1716, 1458, 1376, 1166, 997, 973, 840; ^1H and ^{13}C NMR spectral data, see Tables 1 and 2; HR-ESI-MS *m/z*: 663.2756 $[M+Na]^+$ (calcd for $C_{35}H_{44}O_{11}Na$, 663.2782).

3.4 Evaluation of MDR reversal activity in vitro

The human non-small cell lung cancer – lung adenocarcinoma cell line A549 was maintained in the Department of Pharmacology, Institute of Materia Medica, Chinese Academy of Medical Sciences and Peking Union Medical College. The drug-resistant subline of A549/taxol was established by culturing the cells with gradually increasing concentrations of taxol [15]. The MDR tumor cells were incubated in the RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 U/ml of penicillin, and 100 μg/ml of streptomycin at 37°C in a humidified atmosphere of 5% CO_2 in air. Cells were subcultured twice every week by digesting with mixture of 0.025% trypsin and 0.01% EDTA solution. Cell proliferation was measured by the 3-(4,5-dimethylthiazo-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye reduction method [16]. Briefly, 1×10^4 viable cells (100 μl) were plated into each well of the 96-well plates and left to attach to the plate for 24 h, after which the medium was changed to one containing or lacking test reversal agents or paclitaxel (dissolved in 100 μl of dimethyl sulfoxide, DMSO). The medium was removed after 72 h of incubation, and 100 μl of fresh serum-free medium with 0.5 mg/ml of MTT and incubated for 4 h. The medium was then removed and 150 μl of DMSO was added to each well to dissolve the dark blue crystals by shaking in a mini-shaker. Absorbances were measured with a Wellscan MK3 microtitre plate reader (Labsystems Dragon, Helsinki, Finland) at test and reference wavelengths of 570 and 450 nm, respectively. The median drug concentration for 50% inhibition (IC_{50}) of tumor cell-growth was determined by plotting the logarithm of the drug concentration against the growth rate (percentage of control) of the treated cells.

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