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Synthesis, isolation, stereostructure and cytotoxicity of paclitaxel analogs from cephalomannine

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ABSTRACT

Four paclitaxel derivatives were afforded by preparative HPLC separation of two pairs of diastereoisomers, which were obtained by catalytic hydrogenation and epoxidation of the C-13 side-chain double bond of cephalomannine, a naturally occurring paclitaxel analog. The four paclitaxel derivatives were analyzed using NMR, CD spectroscopy, and side-chain hydrolysis in order to measure their optical rotations and GC characteristics. In this way, the stereoconfigurations of the products were determined. Evaluation of the compounds' activity indicated that they had differing cytotoxic activities: compound **5** had superior activity in BCG-823 tumor cells compared to paclitaxel, while compound **7** had superior activity in HCT-8 and A549 tumor cells compared to paclitaxel. These results indicate that the stereoconfiguration of the paclitaxel *N*-acyl side chain has a significant impact on its activity.

1. Introduction

Paclitaxel, or Taxol® (**1**, Fig. 1), is a structurally peculiar diterpenoid compound that was isolated from the bark of the North American *Taxus brevifolia* by Wani et al. in 1971 [1]. Because of its significant anticancer activity, unique mechanism of action, complex molecular structure, and multiple chiral centers, research on its chemical synthesis, structural modification, and structure–activity relationship has long been a focus in the field of natural product chemistry [2]. At present, paclitaxel and its semisynthetic derivative, docetaxel (**2**, Fig. 1), are mainly used as clinical treatments for breast, ovarian, and non-small-cell lung cancer [3].

As can be seen from its name, cephalomannine (**3**, Fig. 1) was initially considered to have been isolated from *Cephalotaxus mannii*, but it was later discovered that there was an error in the plant classification and that cephalomannine was actually from *Taxus wallichiana*, a species of yew [4]. In later phytochemical studies, cephalomannine was found in several other yew species [5–7]. The structure of cephalomannine is very similar to that of paclitaxel, differing only in its C-13 side chain: paclitaxel has an *N*-benzoyl group in its C-13 side chain, while cephalomannine has an *N*-tigloyl group. Because of these structural similarities, the separation of cephalomannine from paclitaxel is very difficult, and several methods for effectively separating the two compounds have been developed [8–10]. Since the amount of cephalomannine might even be higher than that of paclitaxel in certain *Taxus* plants, it can also be a useful semi-synthetic raw material that can be converted into paclitaxel or docetaxel [11,12]. Furthermore, there have been reports of interesting structural modifications and biotransformations of cephalomannine [13–15]. Therefore, structural modification of readily available cephalomannine can contribute to a better understanding of the structure–activity relationship in the anticancer effects of taxanes.

The epoxidation of cephalomannine has been reported in a patent on the preparation of cephalomannine analogs, but the diastereomers were not separated in this report [16]. There is no stereoselectivity of hydrogenation and epoxidation reactions on the double bond of cephalomannine's side-chain and the afforded compounds in the patent were a pair of diastereoisomers. Therefore, in the current study, catalytic hydrogenation and epoxidation of cephalomannine were carried out separately on the side-chain *N*-tigloyl double bond to obtain two different diastereoisomers, which were then

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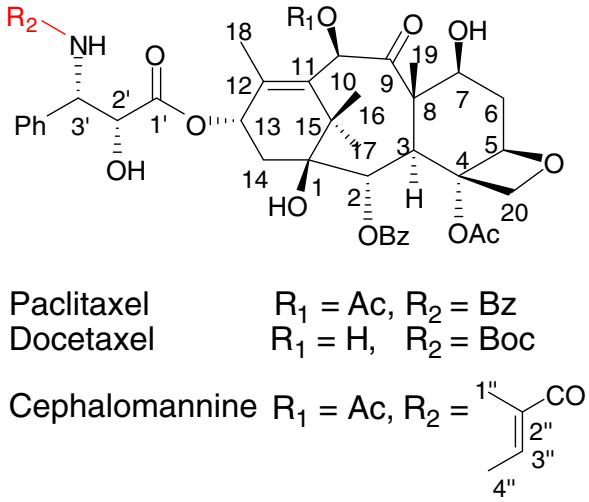

Fig. 1. Structures of paclitaxel (**1**), docetaxel (**2**) and cephalomannine (**3**).

separated using preparative high-performance liquid chromatography (HPLC). In addition, the three-dimensional structures as well as anticancer activities of the obtained diastereoisomers were studied.

2. Experimental

2.1. General

^{1}H and ^{13}C NMR spectra were obtained on a Varian Unity INOVA 400/54 and Bruker ARX-500 spectrometer in $CDCl_3$ with TMS as the internal standard. Mass spectra were obtained on a VG Auto spec 3000 or on a Finnigan MAT90 instrument. Optical rotations were measured on a Perkin-Elmer 341, and CD spectra were measured on a JASCO J-810 spectropolarimeter. Preparative HPLC was performed on a Shimadzu LC-8A liquid chromatograph equipped with a Zorbax PrepHT GF (21.2 mm × 25 cm, 7 μm) or Venusil MP C18 (20 mm × 25 cm, 5 μm) column. GC–MS analysis was performed on a GC (Agilent, 6890N) interfaced with a mass selective detector (Agilent, 5973B). Silica gel H (Qingdao Sea Chemical Factory, Qingdao, People's Republic of China) was used for column chromatography.

Spots on TLC (silica gel G) were detected by spraying with H_2SO_4–EtOH. Commercially available reagents and solvents were directly used without further purification.

2.2. Substrates

Cephalomannine (**3**) was purchased from Kunming Wuyi Biotechnology Co., Ltd., China. The structures were characterized on the basis of ^{1}H NMR and ^{13}C NMR spectra, and the purities were determined to be >95% by HPLC analyses.

2.3. Preparation of compounds **4** and **5**

A solution of cephalomannine (200 mg, 0.24 mmol) and Pd–C (10 mg, 5%) under H_2 atmosphere was strongly stirred for 24 h. Then the reaction solution was filtered and evaporated to give a mixture of compounds **4** and **5**. The mixture was further subjected to preparative reversed-phase HPLC eluting with CH_3CN–H_2O (60/40, v/v) to give compounds **4** (85 mg, t_R = 16.7 min) and **5** (82 mg, t_R = 18.6 min).

2.3.1. N-debenzoyl-N-(S-2-methylbutanoyl)paclitaxel (**4**)

White powder, $[a]_D^{25}$ − 49 ° (c 0.10, MeOH); IR ν_{max} 3438, 2961, 1730, 1714, 1638 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) and ^{13}C NMR (125 MHz, $CDCl_3$): see Table 1. MS (ESI, MeOH) *m/z* 834 $[M + H]^+$.

2.3.2. N-debenzoyl-N-(R-2-methylbutanoyl)paclitaxel (**5**)

White powder, $[a]_D^{25}$ − 25 ° (c 0.10, MeOH); IR ν_{max} 3438, 2962, 1730, 1716, 1638 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) and ^{13}C NMR (125 MHz, $CDCl_3$): see Table 1; MS (ESI, MeOH) *m/z* 834 $[M + H]^+$. HR-ESI-MS: 834.3632 $[M + H]^+$ (calcd. for $C_{45}H_{56}NO_{14}$ 834.3622).

2.4. Preparation of compounds **6** and **7**

The solution of cephalomannine (250 mg, 0.30 mmol) in CH_3Cl_3 (50 mL) was added in *m*CPBA (3-chloroperbenzoic acid, 62 mg, 0.36 mol, 1.2 equiv.). The reaction mixture was stirred for 8 h under argon prior to being quenched with aqueous solution of $Na_2S_2O_3$. The organic phase was separated, washed with brine and dried over anhydrous Na_2SO_4. Then the organic solvent was removed under reduced pressure to give the mixture of compounds **6** and **7**. The mixture was further subjected to preparative reversed-phase HPLC eluting with CH_3CN–H_2O (60/40, v/v) to give compounds **6** (76 mg, t_R = 12.7 min) and **7** (72 mg, t_R = 15.2 min).

2.4.1. N-debenzoyl-N-[(2R,3S)-2,3-epoxy-2-methylbutanoyl] paclitaxel (**6**)

White powder, $[a]_D^{25}$ − 38 ° (c 0.10, MeOH); IR ν_{max} 3422, 2988, 1710, 1632 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) and ^{13}C NMR (125 MHz, $CDCl_3$): see Table 1; MS (ESI, MeOH) *m/z* 848 $[M + H]^+$. HR-ESI-MS: 848.3466 $[M + H]^+$ (calcd. for $C_{45}H_{54}NO_{15}$ 848.3415).

2.4.2. N-debenzoyl-N-[(2S,3R)-2,3-epoxy-2-methylbutanoyl] paclitaxel (**7**)

White powder, $[a]_D^{25}$ − 66 ° (c 0.10, MeOH); IR ν_{max} 3422, 2988, 1712, 1632 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) and ^{13}C NMR (125 MHz, $CDCl_3$): see Table 1; MS (ESI, MeOH) *m/z* 848 $[M + H]^+$. HR-ESI-MS: 848.3446 $[M + H]^+$ (calcd. for $C_{45}H_{54}NO_{15}$ 848.3415).

2.5. Complete alkaline hydrolysis of compounds **4**–**7**

A solution of **4** (or **5**, **6**, **7**) (50 mg) and KOH (100 mg) in MeOH (15 mL) was stirred overnight at room temperature. The reaction mixture was neutralized with 1 N HCl and extracted with EtOAc (10 mL × 3). Then the combined organic phase was dried over anhydrous Na_2SO_4 and evaporated to give the residue. The two residues, from compounds **4** and **5**, were dissolved in MeOH (5 mL) and treated with excess CH_2N_2. After evaporation, the residues were dissolved in $CHCl_3$ (5 mL) and filtered. An aliquot of the two filtrates was analyzed by GC using a chiral column (Astec Chiraldex G-TA G0012-08, 30 m, 50 °C), to give peaks at t_R 12.5 or 11.4 min, respectively. The standard methyl ester of (*R*)-or (*S*)-2-methylbutyric

Table 1
^{1}H and ^{13}C NMR data for compounds **3**, **4**, **5**, **6**, **7** (δ in ppm, in $CDCl_3$).

Position	Cephalomannine (**3**)		Compounds **4**/**5**[a]		Compounds **6**/**7**[b]	
	δ_H (*J* in Hz)	δ_C	δ_H (*J* in Hz)	δ_C	δ_H (*J* in Hz)	δ_C
1	–	79.0 s	–	78.7 s	–	79.0/78.9 s
2	5.67 d (7.2)	74.9 d	5.68 d (7.2)	74.9 d	5.67 d (6.8)	74.9/74.8 d
3	3.78 d (7.2)	45.5 d	3.78 d (7.2)	45.5 d	3.77 t (6.8)	45.5/45.6 d
4	–	81.1 s	–	81.0 s	–	81.0/81.1 s
5	4.94 d (8.0)	84.4 d	4.94 d (8.0)	84.3 d	4.93 d (10.0)	84.3 d
6	1.88 m	35.5 t	1.86 m	35.5 t	1.81 m	35.4/35.5 t
	2.54 m		2.53 m		2.50 m	
7	4.40 m	72.2 d	4.40 m	72.0 d	4.39 m	72.1 d
8	–	58.5 s	–	58.4 s	–	58.5 s
9	–	203.8 s	–	203.6 s	–	203.5 s
10	6.27 s	75.6 d	6.28 s	75.5 d	6.28 d (3.6)	75.6/75.5 s
11	–	133.1 s	–	133.5 s	–	133.7 s
12	–	142.2 s	–	141.9 s	–	141.9 s
13	6.21 t (8.4)	72.2 d	6.18 t (8.8)	72.4 d	6.18 q (8.8)	72.2/72.3 d
14	2.34 m	35.5 t	2.33 m	35.5 t	2.36 m	35.5 t
	2.28 m		2.29 m		2.24 m	
15	–	43.1 s	–	43.1 s	–	43.1 s
16	1.15 s	21.8 q	1.15 s	21.8 q	1.15 s	21.8/21.7 q
17	1.26 s	26.8 q	1.26 s	26.6 q	1.26 s	26.8 q
18	1.80 s	14.7 q	1.82 s	14.7 q	1.79 s	14.7/14.8 q
19	1.68 s	9.5 q	1.68 s	9.5 1q	1.67 s	9.5 q
20	4.29 d (8.4)	76.5 t	4.29 d (8.4)	76.4 d	4.28 d (8.4)	76.4 t
	4.19 d (8.4)		4.18 d (8.4)		4.17 d (8.4)	
1′	–	172.9 s	–	172.8 s	–	172.7 s
2′		73.3 d		73.0 d		73.0/73.3 d
3′	–	54.8 d	–	54.1 d	–	54.1/54.3 d
4-OAc	–	170.4 s	–	171.2 s	–	170.1 s
	2.36 s	22.5 q	2.24 s	22.5 q	2.23 s	22.5 q
10-OAc	–	171.4 s	–	172.8 s	–	171.2 s
	2.25 s	20.8 q	2.17 s	20.8 q	2.16 s	20.8 q
2-OBz	–	167.1 s	–	166.8 s	–	166.9 s
Ph-1	–	129.2 s	–	129.1 s	–	129.2 s
Ph-2,6	8.13 d (7.2)	130.3 d	8.10 d (7.2)	130.1 d	8.10 d (7.2)	130.3 d
Ph-3,5	7.51 m	128.3 d	7.50 m	128.8 d	7.50 m	128.3 d
Ph-4	7.62 m	133.8 d	7.61 m	133.0 d	7.60 m	133.8 d
3′-Ph-1	–	131.3 s	–	138.0 s	–	131.3 s
3′-Ph-2,6	7.40 br. s	127.0 d	7.39 m	128.4 d	7.38 br. s	127.0 d
3′-Ph-3,5	7.42 br. s	128.8 d	7.40 m	126.7 d	7.31 m	128.8 d
3′-Ph-4	7.34 m	129.0 d	7.33 m	129.1 d	7.21 m	129.0 d
NH	6.51 d (8.8)	–	6.28 d (8.8)	–	–	–
NH-CO	–	169.1 s	–	176.5 s	–	171.5 s
1″	1.62 s	12.2 q	1.38 m	17.5 q	1.80 s	13.5/13.4 q
2″	–	138.2 s	2.34 m	43.1 q	–	60.0/59.9 s
3″	6.43 q (6.8)	132.0 d	1.08 m	27.1 q	2.98/3.00 q (4.4)	59.6/59.3 s
4″	1.72 d (6.8)	13.9 q	0.86 t (7.2)	11.8 q	1.42 d (4.4)	12.6/12.7 q

[a] The ^{1}H and ^{13}C NMR data of the diastereoisomers 4 and 5 were completely identical.
[b] The diastereoisomers 6 and 7 had a weak difference in ^{13}C NMR data.

Table 2
Cytotoxicity of paclitaxel and compounds **4**, **5**, **6** and **7**.

Compd.	IC_{50} (nM)				
	HCT-8	Bel-7402	BGC-823	A549	A2780
Paclitaxel	51	6.0	3.29	16	7.86
4	460	12.5	16.5	240	18.6
5	680	7.2	2.14	22	9.45
6	220	10.5	9.45	86	12.8
7	25	5.2	2.66	5.0	7.05

acid gave a peak at t_R 11.4 and 12.5 min. The other two residues, from compounds **6** and **7**, were separated via column chromatography over silica gel eluting with EtOAc followed by $CHCl_3$–MeOH (1:2) to yield (2R, 3S)- or (2S, 3R)-2,3-epoxy-2-methylbutanoic acid, respectively, for measuring optical rotations.

2.6. Cytotoxicity bioassays

The cytotoxicity assays against the HCT-8 human colorectal adenocarcinoma cell line, Bel-7402 human liver cancer cell line, BGC-823 human gastric cancer cell line, A549 human lung carcinoma cell line and the A2780 human ovarian were accessed using MTT method.

Cells were plated in the RPMI 1640 with 10% fetal calf serum media on 96-well plates in a total volume of 100 μL with a density of 1×10^{-4} cells mL^{-1}. Triplicate wells were treated with media and tested compounds. The plates were incubated at 37 °C in 5% CO_2 for 72 h. Cell viability was determined based on mitochondrial conversion of 3[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT, Sigma) to formazan. The amount of MTT converted to formazan is a sign of the number of viable cells. Each well was supplemented with 50 μL of a 1 mg mL^{-1} solution of MTT in uncompleted media. The plates were incubated in 37 °C, 5% CO_2 for an additional 4 h. The media was carefully removed from each well and then 200 μL of DMSO was added. The plates were gently agitated until the reaction color was uniform and the OD_{570} was determined using a micro-plate reader (Wellscan MK3, Labsystems Dragon). Microsoft® Excel 2000 was used to analyze data. Media-only treated cells served as the indicator of 100% cell viability. The 50% inhibitory concentration (IC_{50}) was defined as the concentration that reduced the absorbance of the untreated wells by 50% of the control in the MTT assay.

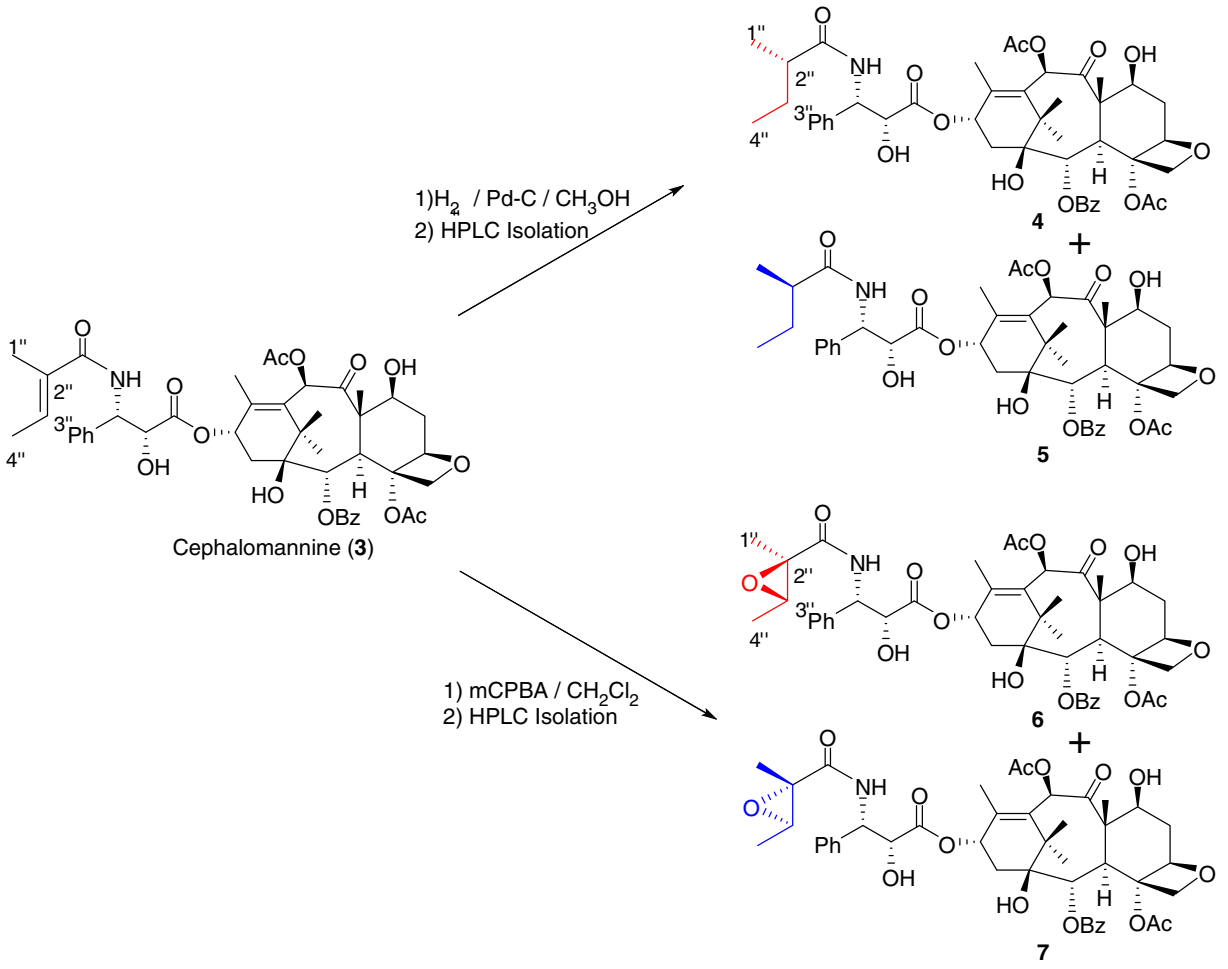

Fig. 2. Synthesis of paclitaxel analogs from cephalomannine.

3. Results and discussion

Compounds **4** and **5** were produced by catalytic hydrogenation of the N-tigloyl double bond of cephalomannine (Fig. 2). The ^{1}H-NMR results showed that the product of the hydrogenation appeared to be a single compound. In particular, the signal for the vinyl proton on the cephalomannine side-chain double bond disappeared (δ_H, 6.43, 1H, q, J = 6.8 Hz, Table 1) and the signal for the 4′-methyl group appeared as a triplet signal (δ_H, 0.86, 3H, t, J = 7.2 Hz, Table 1). However, HPLC results showed that there was a pair of products present in a 1:1 ratio, which indicates that there was no stereoselectivity of the hydrogenation of the cephalomannine $\Delta^{2'',3''}$ double bonds, and a pair of diastereoisomers was formed. Therefore, optically pure compounds **4** and **5** were obtained by separating the hydrogenation products using preparative HPLC.

Next, analysis was performed to confirm the stereoconfiguration of the newly-formed C-2″ chiral carbon in compounds **4** and **5**. There were no significant differences between the circular dichroism (CD) spectra of compounds **4** and **5**, and hence, the absolute configurations of these two compounds could not be determined using CD spectroscopy. Gabetta et al. isolated *N*-debenzoyl-*N*-(2-methylbutanoyl) paclitaxel from *Taxus media* cv. Hicksii and confirmed that the stereoconfiguration of its 2-methylbutanoyl structural unit was *S*, and the optical rotation of this compound is $[a]_D^{25}$ − 48 ° (c 0.10, MeOH) [17]. In this study, the optical rotations of **4** and **5** are $[a]_D^{25}$ − 49 ° (c 0.10, MeOH) and $[a]_D^{25}$ − 25 ° (c 0.10, MeOH), respectively. Hence, it can be deduced that compound **4** and the *N*-debenzoyl-*N*-(2-methylbutanoyl) paclitaxel isolated by Gabetta et al. are the same. Therefore, the 2-methylbutanoyl structural unit in compound **4** has the *S* configuration, while C2″ in compound **5** has the *R* configuration. In order to further confirm the stereoconfigurations of compounds **4** and **5**, gas chromatography (GC) was used to analyze the hydrolyzates of both compounds in comparison to a standard control. The t_R values of the 2-methylbutyric acids obtained from complete hydrolysis of compounds **4** and **5** were 12.5 min and 11.4 min, respectively, which correspond to (*S*)- and (*R*)-2-methylbutyric acid. Thus, the structures of compounds **4** and **5** were *N*-debenzoyl-*N*-(*S*-2-methylbutanoyl)paclitaxel and *N*-debenzoyl-*N*-(*R*-2-methylbutanoyl)paclitaxel, respectively. All ^{1}H and ^{13}C NMR data for compounds **4** and **5** were assigned by comparison with cephalomannine and paclitaxel NMR data (Table 1).

Compounds **6** and **7** were produced by the epoxidation of cephalomannine (Fig. 2). The ^{1}H NMR results showed that the products were a pair of diastereomers, in which the signal of the proton on the double bond of the side chain of cephalomannine (δ_H, 6.43, 1H, q, J = 6.8 Hz, Table 1) was replaced with a proton on an epoxy carbon (δ_H, 2.98/3.00, 1H, q, J = 4.4 Hz). The HPLC results also showed that the epoxidation products were a pair of compounds with a ratio of 1:1, which again indicates that that there was no stereoselectivity of the epoxidation of the double bonds. Optically pure compounds **6** and **7** were obtained using preparative HPLC, and there were negligible differences between the ^{1}H and ^{13}C NMR signals of the two compounds (Table 1).

In order to determine the stereo configuration of the newly formed epoxy group in compounds **6** and **7**, complete hydrolysis was carried out. A pair of 2,3-epoxy-2-methylbutanoic acid enantiomers with rotation values of $[a]_D^{25}$ − 5 ° and + 5°(c 0.10, $CHCl_3$) was obtained, respectively, which is consistent with the optical rotation values of (2*R*,3*S*)- and (2*S*,3*R*)-2,3-epoxy-2-methylbutanoic acid obtained from an asymmetric synthesis [18]. Thus, it can be deduced that the structures of compounds **6** and **7** were *N*-debenzoyl-*N*-[(2*R*,3*S*)-2,3-epoxy-2-methylbutanoyl]paclitaxel and *N*-debenzoyl-*N*-[(2*S*,3*R*)-2,3-epoxy-2-methylbutanoyl]paclitaxel, respectively. All ^{1}H and ^{13}C NMR data for compounds **4** and **5** were assigned by comparison with cephalomannine, paclitaxel, and 2,3-epoxy-2-methylbutanoic acid NMR data (Table 1).

With paclitaxel and as a positive control, the cytotoxic activities of compounds **4**, **5**, **6**, and **7** were evaluated in five types of tumor cells, and the results are shown in Table 2. The activities of compounds **4** and **6** are weaker than that of paclitaxel, while the activities of compounds **5** and **7** are comparable to those of paclitaxel. Furthermore, the activity of compound **5** in the BGC-823 tumor cell line was superior to those of paclitaxel, and the activities of compound **7** in HCT-8 and A549 cells were 2 and 3 times higher than those of paclitaxel, respectively.

In summary, four paclitaxel derivatives were obtained by preparative HPLC separation of two pairs of diastereoisomers, which were obtained from catalytic hydrogenation and epoxidation of the C-13 side-chain double bond of cephalomannine and their stereostructures were confirmed by chemical and spectral methods. Various cytotoxic activities of these two pairs of diastereoisomers indicated that the stereoconfiguration of the *N*-acyl in the paclitaxel side chain has a significant impact on its activity, and increasing the oxygenation of *N*-acyl side chains improves the activity. This result can further enrich our understanding on the structure–activity relationship in paclitaxel.

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Appendix A. Supplementary data

Supplementary data associated with this article, including ^{1}H and ^{13}C NMR spectra, CD and HPLC spectra, can be found in the online version, at doi: http://dx.doi.org/10.1016/j.fitote.2013.07.011.

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