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Short communication

Evaluation of paclitaxel rearrangement involving opening of the oxetane ring and migration of acetyl and benzoyl groups

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Abstract

The stability of drug is a critical factor in quality control, drug efficacy, safety, storage, and production conditions. The rearrangement of paclitaxel, which involves opening of the oxetane ring and migration of acetyl group occurred on heating a powder of purified paclitaxel. Subsequently, the unusual migration of benzoyl groups progressed rapidly in organic solvents. These rearrangement derivatives were isolated carefully. The structures of the intermediate derivative **A** and the product derivative **B** were confirmed using ^{1}H NMR, high performance liquid chromatography (HPLC), and mass spectrometry. We proposed the rearrangement pathway here for the first time. Neither derivative exhibited bioactivity in SKOV3 (ovarian cancer) or MDA-MB-435 (breast cancer) cell culture assays.

Keywords: Paclitaxel; Oxetane ring opening; Acetyl migration; Benzoyl migration; Rearrangement

1. Introduction

Paclitaxel (Genexol®, Taxol®), a compound originally isolated from the bark of the Pacific yew tree *Taxus brevifolia* in 1971 [1], has been one of the most important anticancer agents in recent decades owing to its unique cytotoxicity mechanism [2,3]. Since the yield of purified paclitaxel from *T. brevifolia* is very low (about 0.04% of the bark dry weight), and bark-stripping leads to the destruction of scarce plant material [4], many attempts have been made to develop new methods for the reliable production of paclitaxel from renewable resources including semi-synthesis, total synthesis, and plant cell culture strategies [5–8]. In the search for other bioactive compounds, many other taxane derivatives have been isolated or synthesized chemically for structure–activity relationship studies [4,9–11]. The degradation of the oxetaine ring of paclitaxel has been studied by treating paclitaxel with acid [12], and Meerwein's reagent [13,14]. The resulting degradation products, such as D-secopaclitaxel, were elucidated using liquid chromatography–mass spectrometry (LC–MS) profiling and NMR analysis. However, in these previous reports, the degradation conditions of paclitaxel were harsh and the chemical reaction methods or the resulting degradation products were not isolated.

The degradation of the final product has a very critical impact on quality control, drug efficacy, safety, storage, and production conditions. Moreover, the identification of degraded compounds and causes have an important role in the drug development process [15,16]. In this report, we describe a new evaluation of paclitaxel rearrangement that yielded two derivatives under heating and in solution (Figs. 1 and 2). Little is known the isolation and characteristics of these compounds, i.e., derivatives **A** and **B** (Fig. 2). The proposed rearrangement mechanism will provide useful knowledge to further the commercial production of paclitaxel.

2. Experimental

2.1. Material and chemicals

Paclitaxel was used as received from Samyang Genex Corporation (Daejeon, Korea). All other reagents and solvents were analytical or HPLC grade.

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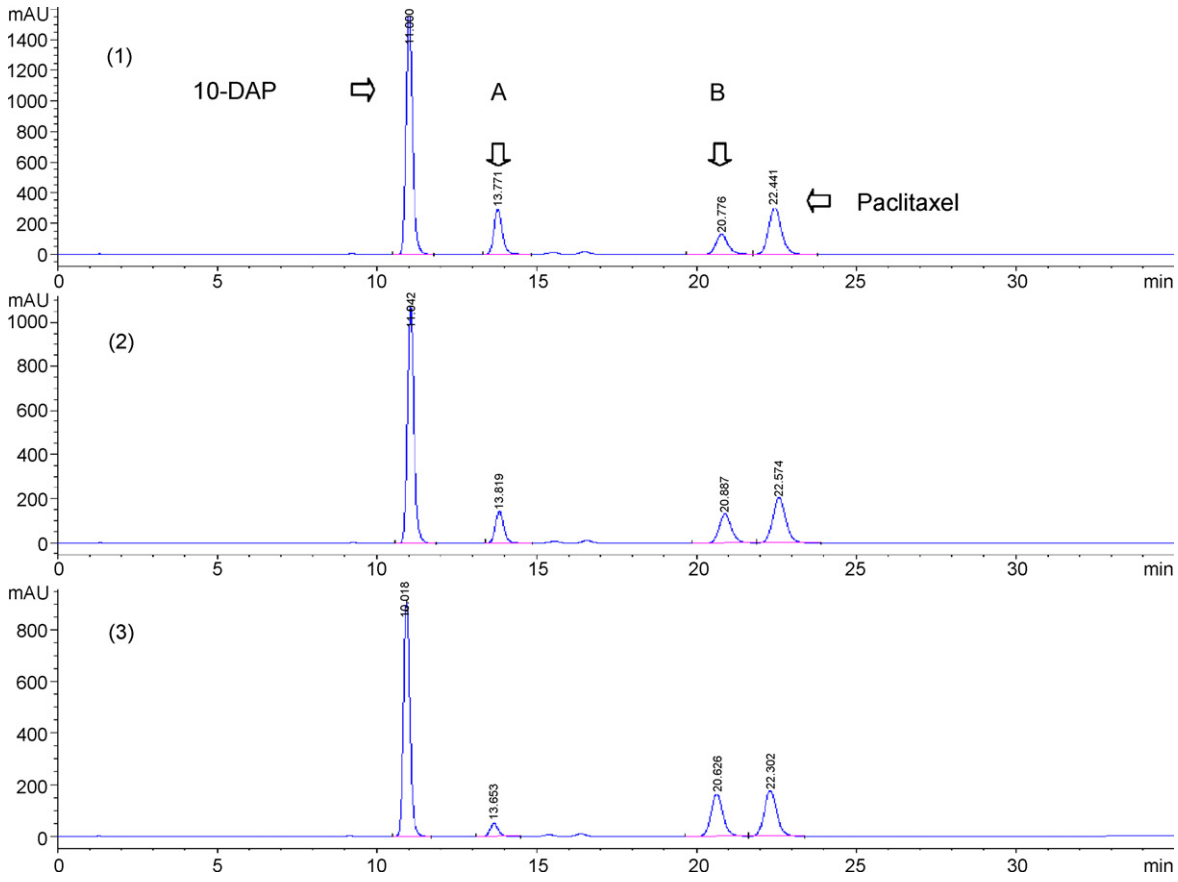

Fig. 1. Chromatograms for a mixture of **A**, **B**, and paclitaxel analyzed by HPLC. (1) Original chromatogram. (2) After 2.5 h at room temperature. (3) After 5.0 h at room temperature. 10-Deacetylpaclitaxel (10-DAP) and paclitaxel were used as internal references. (A) Derivative **A** and (B) derivative **B**.

2.2. Rearrangement of paclitaxel and separation of the resulting product

To prepare **A** and **B**, purified paclitaxel was dissolved in dichloromethane and evaporated under reduced pressure. To accelerate the rearrangement, the recovered paclitaxel was dried and stored in a vacuum oven at 100 °C for 3 days. The amount of **A** increased gradually during storage. A small amount (content 0.4%) of **A** was fractionated using semi-preparative HPLC on a Waters Delta Prep 3000 system fitted with a Curosil PFP column (20 mm × 250 mm, d_p = 5 μm; Phenomenex, Torrance, CA, USA). It was eluted with a gradient of water:acetonitrile from 65:35 to 35:65 over 30 min at a flow rate of 18.0 ml min^{-1} with monitoring at 227 nm. To isolate **A**, the fractionated solution was immediately extracted three times with dichloromethane, and then dried. After the fractionated solution was held for at least 12 h at room temperature, **B** converted from **A** was separated from the solution using dichloromethane extraction, followed by drying.

2.3. Chemical rearrangement of paclitaxel

The oxetane ring-opened derivatives **A** and **C** were prepared using a modification of the method of Chen et al. [17]. Paclitaxel (100 mg, 0.117 mmol) was dissolved in dry dichloromethane (5 ml), cooled to 0 °C, and treated with $SnCl_4$ (130 μl in 0.5 ml dichloromethane, 0.001 mmol) for 15 min (conversion < 40%). The mixture was applied to a silica gel column directly and eluted with 60% ethyl acetate in hexane, affording **A** and **C**.

2.4. High performance liquid chromatography (HPLC) analysis

The fractionated solution was analyzed using a HP1090 HPLC system (Hewlett-Packard, Palo Alto, CA, USA) fitted with an ODS-18 column (4.6 mm × 250 mm, d_p = 5 μm; Shiseido, Tokyo, Japan). HPLC was performed isocratically using solution **A** (water:acetonitrile = 2:3) for 20 min at a flow rate of 1.2 ml min^{-1} followed by a gradient from solution **A** to acetonitrile from 100:0 to 10:90 over 60 min. The elution was monitored at 227 nm.

2.5. Spectroscopy analysis

^{1}H NMR spectra were obtained on a JNM-AL400 (JEOL, Tokyo, Japan) spectrometer operating at 400 MHz. The chemical shifts are reported in ppm relative to that of tetramethylsilane (TMS, $\delta = 0$) as an internal standard, and the coupling constants are given in Hertz.

Liquid chromatography/mass (LC/ESI-MS) spectrometry was performed using an ABI MARINER (Applied Biosystems, Foster City, CA, USA) coupled to a LC-10AD

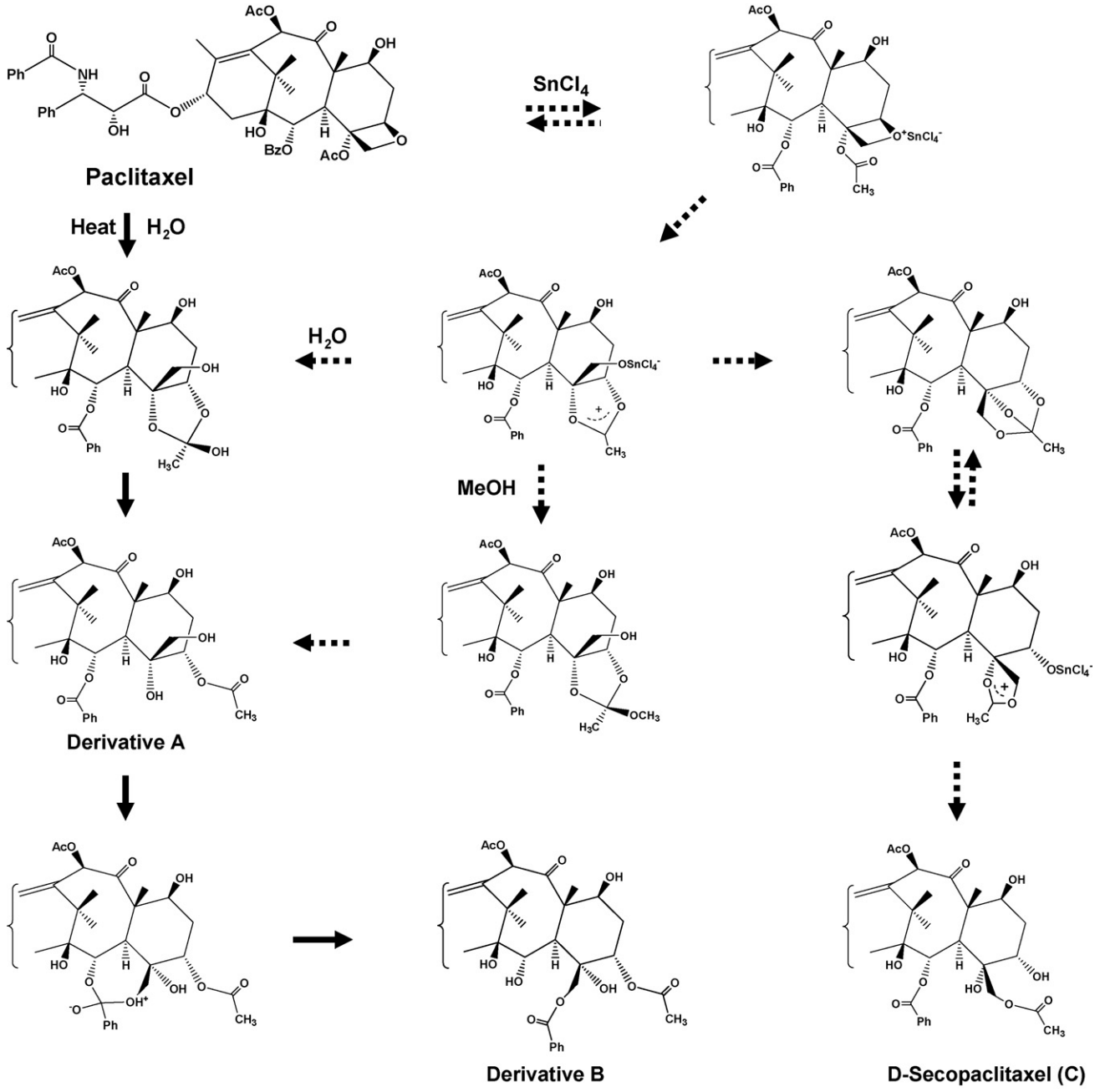

Fig. 2. Possible mechanism of oxetane ring opening and migration during paclitaxel rearrangement. Dotted arrow, previous reported mechanism; solid arrow, new proposed mechanism.

HPLC system (Shimadzu, Kyoto, Japan) with a C18 column (3.2 mm × 200 mm, d_p = 5 μm) at the Korea Basic Science Institute (Daejeon, Korea). The elution was performed using a gradient of water and acetonitrile from 65:35 to 35:65 within 30 min (flow rate = 1.0 ml min^{-1}). The eluent was monitored at 227 nm with a photo diode array detector.

2.6. Bioactivity assay of derivatives *A* and *B*

We also performed a bioactivity assay for derivatives **A** and **B** in SKOV3 (ovarian cancer) and MDA-MB-435 (breast cancer) cell culture assays using MTT [18].

3. Results and discussion

3.1. Isolation and identification of the rearranged products of paclitaxel

Derivative **A** was rapidly converted into derivative **B** in organic solvent (Fig. 1). Therefore, derivative **A** could be obtained by immediately extracting the fractionated solution with dichloromethane to avoid conversion to **B**. After the fractionated solution was held for at least 12 h at room temperature, **B** formed from **A** was easily obtained from the solution using dichloromethane extraction. After storing paclitaxel powder at

room temperature for 5 months, only a trace of derivative **A** was detected in the HPLC analysis. Moreover, derivative **A** did not form from paclitaxel powder treated with methanol instead of dichloromethane under the same conditions.

Rearranged derivatives **A** and **B** were isolated by preparative-HPLC, and their structures were determined by ^{1}H NMR and mass spectrometry (MS), as well as HPLC analysis. The results were confirmed by comparison with data from chemically synthesized samples. When HPLC was repeated over time, the peak corresponding to **A** rapidly diminished as the peak corresponding to **B** increased (Fig. 1). In these analyses, the relative retention times (RRTs) of the two peaks (relative to paclitaxel) were 0.612 and 0.925, respectively.

The molecular weights of **A** and **B**, as determined by MS, were 872.2466 and 872.2600 Da, respectively, both of which represent an 18 Da increase relative to paclitaxel (854.2190 Da). Derivative **B**, which we showed was produced from derivative **A**, is an important impurity that should be monitored closely during the commercial production and storage of paclitaxel. The origin of derivative **B** has not been previously reported.

Previous reports have described several possible paclitaxel derivatives with opened oxetane rings and molecular weights of 871 Da. Using only LC–MS and LC–tandem MS substructural techniques without isolation and further analysis, Volk et al. proposed that the acid degradation of paclitaxel leads to the formation of an oxetane ring-opened derivative (**A**) [12]. They proposed that the oxetane ring is opened through the acid-catalyzed addition of water (18 Da) to the paclitaxel core. Boge et al. performed a detailed NMR analysis of D-secopaclitaxel [13], in which the oxetane ring had been opened by treating paclitaxel with triethyloxonium tetrafluoroborate (Meerwein's reagent) [14].

On treatment with $SnCl_4$, a milder Lewis acid, 2′-OCO_2CH_2Ph-substituted paclitaxel was converted into the corresponding substituted derivative **A** and **C** analogues, while paclitaxel reacted very poorly with $SnCl_4$, and the taxane ring rearrangement could not be avoided as a side reaction. These compounds could be separated chromatographically, and were also found to be somewhat unstable in organic solvents by Chen et al. [17]. However, we were able to isolate small amounts of **A** and **C** using a modification of their method. HPLC, MS, and ^{1}H NMR studies of these compounds were performed to confirm the structure of derivative **A**.

^{1}H NMR analyses of paclitaxel, **A**, and **B** are compared with previously obtained data in Table 1. In **A**, the 20-Ha and 20-Hb peaks were shifted to 3.62 and 3.42 ppm from 4.29 and 4.19 ppm, respectively, due to opening of the oxetane ring. The 5-H peak was shifted from 4.95 to 5.32 ppm by migration of the acetyl group from the 4-position to the 5-position, and the 3-H peak of the acetyl group was shifted from 2.38 to 2.16 ppm.

All the data obtained for **A** were consistent with those obtained for a sample prepared on treatment with $SnCl_4$, and conversion properties of derivative **A** to **B** were in good agreement. Therefore, the sample obtained by heating and the sample obtained by $SnCl_4$ treatment were determined to be the same compound. The structure of this compound (**A**) was also confirmed by comparison with the ^{1}H NMR data obtained for 2′-OCO_2CH_2Ph-substituted paclitaxel [17].

In **B**, the 20-Ha and 20-Hb peaks were shifted to 4.61 and 4.38 ppm from 3.62 and 3.42 ppm, respectively, by migration of the benzoyl group in **A**. The 2-H peak was shifted from 5.67 to 5.43 ppm, and the 2-OH peak was at 5.11 ppm. These shifts provide solid evidence for an unusual event, i.e., the migration of the benzoyl group in the paclitaxel oxetane ring.

The ^{1}H NMR data for **B** were also compared with data from acid-catalyzed rearrangement and acyl group migration studies of 9-dihydro-13-acetylbaccatin-III [19]. The ^{1}H NMR data for the oxetane ring arrangement and acetyl and benzoyl group migration were in good agreement with our results.

3.2. Proposed rearrangement mechanism

A plausible mechanism for oxetane ring opening and acetyl group migration (the dotted arrow in Fig. 2), and an explanation for the bioactivity of D-secopaclitaxel, were reported by Kingston and co-workers in work with Meerwein's reagent [14]. Their proposed mechanism was later confirmed by Chen et al. [17].

As shown in Fig. 2, the oxetane oxygen, which is likely the most basic atom in the molecule, is activated by coordination with a Lewis acid. A backside attack of the acetoxy group at 4-C onto 5-C follows, and the resulting acetoxonium ion traps

Table 1
Comparison of the ^{1}H NMR data for selected oxetane ring-opened compounds

Proton	Paclitaxel	Derivative **A**	Derivative **B**	Analogue **A**[a]	Analogue **B**[b]	Derivative **C**[c]
2-H	5.67 (d, 6.8)	5.67 (d, 8.4)	5.43 (d, 12)	5.65 (d, J = 5.8)	4.46	5.57 (d 5.3)
3-H	3.79 (d, 6.8)	3.86 (d, 8.4)	3.80 (dd, 9.4)	3.88 (d, J = 5.8)	2.50	4.04 (d 5.3)
5-H	4.95 (dd, 9.2)	5.32 (bs)	5.55 (bs)	5.32 (br s)	5.34	3.90 (bs)
20-Ha	4.29 (d, 8.4)	3.62 (d)	4.61 (d, 9.6)	3.46 (AB, J = 11.3)	5.02	a. 4.05
20-Hb	4.19 (d, 8.8)	3.42 (d)	4.38 (d, 12)		4.67	b. 3.88
4-AcMe	2.38(s)					
5-AcMe		2.16 (s)	2.20 (s)	2.15 (s)	2.15 (or 2.04)	
20-AcMe						1.59 (s)
4-OH		4.38 (s)	3.69 (s)	4.11 (s)	3.35	
2-OH			5.11 (s)		4.18	

[a] 2′-OCO_2CH_2Ph substituted derivative **A** obtained by reaction with $SnCl_4$ [17].
[b] Rearranged product of 9-dihydro-13-acetylbaccatin-III [19].
[c] D-Secopaclitaxel [13].

water and methanol to yield the oxetane ring-opened, acetyl group-migrated derivative.

In this study, this ring-opening process could progress without activation of the oxetane ring system by a Lewis acid like $SnCl_4$ (the solid arrow in Fig. 2). Furthermore, in methanol solution at room temperature, the benzoyl group can migrate spontaneously via a 7-membered ring hemiacetal intermediate. This benzoyl group migration from 2-C to 20-C in paclitaxel has not been mentioned in previous reports. Although benzoyl migration in 9-dihydro-13-acetylbaccatin III was obtained with two other products via a chemical reaction (p-TSA·H_2O (3 eq), methanol, RT), the taxane skeleton (ring **A**) was not maintained, owing to a side reaction at 1-C and 15-C [19]. This unusual benzoyl shift is easily illustrated using the bent cup- or cage-shaped taxane skeleton [20,21], which provides an opportunity for reaction of the 20-C-OH and benzoyl carbonyl. In D-secopaclitaxel, the benzoyl shift cannot occur because the acetoxy group is already positioned at 20-C [9,13].

3.3. Bioactivity assay of derivatives *A* and *B*

D-Secopaclitaxel proved to be at least 20 times less bioactive than paclitaxel in a tubulin depolymerization assay, and it was inactive in a KB cell culture assay [14]. In this study, bioactivity assays were carried out at 0.1 μM in SKOV3 (ovarian cancer) and MDA-MB-435 (breast cancer) cell culture assays using MTT [18]. The resulting activities of saline solution (control), paclitaxel, and derivatives **A** and **B** for SKOV3 were 1.23 ± 0.064 (mean ± standard deviation), 0.73 ± 0.040, 1.16 ± 0.041, and 1.14 ± 0.042, respectively, and those for MDA-MB-435 were 0.93 ± 0.050, 0.40 ± 0.09, 0.92 ± 0.046, and 0.92 ± 0.032, respectively. Although they showed slight activity compared to the saline solution, the activities of derivative **A** and **B** were much less than those of paclitaxel. The importance of the oxetane ring structure in bioactivity has previously been reported using other derivatives, such as D-secopaclitaxel [6]. Although derivatives **A** and **B** displayed low bioactivity, we are the first to report on the activities of these derivatives, and to confirm the importance of the oxetane ring for bioactivity.

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