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Full Length Research Paper

Production of two intermediate taxoids, 2-hydroxy-5α,10β-diacetoxytaxadiene and 2-hydroxy-5α,10β,14β-triacetoxytaxadiene, from *Taxus chinensis* cell culture

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The production of taxuyunnanine C (Tc) (2α,5α,10β,14β-tetraacetoxytaxa-4(20),11(12)-diene) was significantly enhanced when *Taxus chinensis* cell cultures were treated with methyl jasmonate (MJA) or 2,3-dihydroxypropyl jasmonate (DHPJA), a newly synthesized jasmonate analog, in combination with *in situ* absorption. Along with the production of Tc, two intermediate taxoids were produced and purified. On the basis of liquid chromatography-mass spectrometry (LC-MS) and nuclear magnetic resonance (NMR) spectral data, the chemical structures of the two intermediate taxoids were found to be 2-hydroxy-5α,10β-diacetoxytaxadiene and 2-hydroxy-5α,10β,14β-triacetoxytaxadiene. This is the first study to report the production of these two taxoids using plant cell culture.

Key words: *Taxus chinensis*, taxuyunnanine C, methyl jasmonate, 2,3-dihydroxypropyl jasmonate, *in situ* absorption, 2-hydroxy-5α,10β-diacetoxytaxadiene, 2-hydroxy-5α,10β,14β-triacetoxytaxadiene.

INTRODUCTION

Over 350 taxoids are produced by the genus Taxus (Baloglu and Kingston, 1999; Itokawa, 2003) and new taxoids are being discovered (Arjun et al., 2002; Shen et al., 2002; Wang and Shi, 2009; Zhang et al., 2010) among them. Taxol® (generic name paclitaxel) is the most well known because of its anticancer activity. Paclitaxel is a cyclized diterpenoid with 8 oxygen substituents and 11 chiral centers. All hypotheses on paclitaxel biosynthesis are based on the cyclization of the universal diterpenoid precursor geranylgeranyl diphosphate (GGPP) to form taxadiene, the skeleton upon which paclitaxel is built. At least 20 steps are involved in the production of paclitaxel from GGPP, with bifurcations occurring in the taxoid synthetic pathway (Hezari and Croteau, 1997; Croteau et al., 2006; Nims et al., 2006; Engels et al., 2008).

The main structure types of the taxane diterpenoids include a six-eight-six cycle with C-4(20) double bonds and a six-eight-six-four cycle with an epoxy pentane cycle. Paclitaxel, with epoxy pentane and C-13 side chains, exhibits good antitumor activity. Most of the

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Abbreviations: Tc, Taxuyunnanine C; **MJA**, methyl jasmonate; **DHPJA**, 2,3-dihydroxypropyl jasmonate; **LC-MS**, liquid chromatography-mass spectrometry; **NMR**, nuclear magnetic resonance; **GGPP**, geranylgeranyl diphosphate; **IPP**, isoprenyl diphosphate; **DAPP**, dimethylallyl diphosphate; **GGPPS**, geranylgeranyl pyrophosphate synthase; **TASY**, taxadiene synthase; **T5αH**, taxadiene 5α-hydroxylase; **HPLC**, high performance liquid chromatography; **HR-ESI-MS**, high resolution electrospray ionization mass spectra; **6-BA**, 6-benzyladenine; **Vc**, ascorbic acid; **NAA**, naphthaleneacetic acid; **2,4-D**, 2,4-dichlorophenoxy-acetic acid; **PVDF**, polyvinylidenedifluoride; **TLC**, thin layer chromatography; **TDAT**, taxadiene acetyltransferase.

taxanes are irrelevant intermediates of the paclitaxel biosynthetic pathway and are mere by-products. Any effort to improve the production yields of paclitaxel and its immediate precursors should take the auxiliary taxane biosynthetic pathways into consideration (Ketchum et al., 2007). Furthermore, either up- or down regulation of the genes that encode the enzymes involved in the paclitaxel or taxoid pathway requires a clear understanding of the metabolic pathways (Ketchum et al., 2003).

The first 3 steps of paclitaxel biosynthesis have been elucidated. Derived from isoprenyl diphosphate (IPP) and dimethylallyl diphosphate (DAPP), GGPP is synthesized by geranylgeranyl pyrophosphate synthase (GGPPS) (Hefner et al., 1998). GGPP is converted to taxa 4(5), 11(12)-diene by taxadiene synthase (TASY) to establish the taxane ring (Koepp et al., 1995; Wildung and Croteau, 1996) and then to taxa 4(20), 11(12)-dien-5α-ol by taxadiene 5α-hydroxylase (T5αH) (Hefner et al., 1996; Jennewein et al., 2004). This is the first hydroxylation at the C-5α position involving allylic migration of the double bond. Taxa 4 (5), 11 (12) - diene have already been synthesized in *Escherichia coli* (Huang et al., 2001; Wang, 2002).

Whether acetylation at the C-5α position is followed by hydroxylation(s), or whether hydroxylation is followed by acetylation, presently remains unclear. A considerable precursor flux to taxoid, rather than to paclitaxel, has been reported for both intact Taxus tissues (Kikuchi and Yatagai, 2003) and derived cell cultures (Lan et al., 2002; Parc et al., 2002; Takeya, 2003). Among the taxoids, 14-hydroxy taxoids (taxuyunnanine C (Tc) and its relatives) are the most common. C-14 hydroxylation is probably an early side-pathway of taxane synthesis. Paclitaxel has no oxygen substituent at C-14. Only taxanes with a C-5 acetyl oxygen substituent and C-10 hydroxylation can be catalyzed by taxane 14β-hydroxylase to form a C-14 hydroxyl substituent. The cytochrome P450 taxoid 14β-hydroxylase principally utilizes the C5-acetate esters of 5α-hydroxytaxadiene and 5α,10β-dihydroxytaxadiene as preferred substrates (Ketchum et al., 2007). Pathway bifurcations towards 13α-hydroxy taxoids or towards 14β-hydroxy taxoids occur very early, following 5α- and 10β-hydroxylation of the taxane core.

Menhard et al. (1998) reported that a high proportion of C-14 oxygenated taxoids (relative to C-13 oxygenated taxoids) were distributed in methyl jasmonate (MJA)-treated *T. chinensis* cell cultures. Similar abundances of C-14 oxygenated taxoids were also reported by Eisenreich et al., (1996). In the current study, we observed a similar phenomenon: the production yield of Tc is markedly improved by elicitation and *in situ* absorption. After elicitation with MJA (or its derivative) and *in situ* absorption, we purified two taxoids; 2-hydroxy-5α,10β-diacetoxytaxadiene and 2-hydroxy-5α,10β,14β-triacetoxytaxadiene for the first time from a plant cell culture. 2-hydroxy-5α,10β-diacetoxytaxadiene is a newly found intermediate taxoid and is reported here for the first time. These findings further illustrated the Tc biosynthetic pathway.

MATERIALS AND METHODS

Cell line and subcultures

T. chinensis cell suspension culture was kindly supplied by Prof. JJ Zhong and was continuously maintained in our laboratory since October 2005. The cell culture was maintained in Murashige and Skoog medium (Murashige, 1962), supplemented with 0.5 mg/L 6-benzyladenine (6-BA), 0.2 mg/L 2,4-dichlorophenoxy-acetic acid (2,4-D), 0.5 mg/L naphthaleneacetic acid (NAA), 100 mg/L ascorbic acid (Vc), and 30 g/l sucrose. The pH value was adjusted to 5.8 before autoclaving. The cells were subcultured every 2 weeks in a 500 ml Erlenmeyer flask containing 100 ml of medium on a rotary shaker at 110 rpm, at 25°C in the dark. The inoculum density was 100 g fresh cells/L medium.

Elicitation

For shake-flask cultures, 2 g of fresh cells were inoculated in a 100 ml Erlenmeyer flask containing 20 ml of medium with the same culture conditions as those used for the subcultures. On day 7, MJA or DHPJA (a newly reported jasmonate analog, synthesized and kindly supplied by Prof. XH Qian) was added to the cultures at 1 µl of ethanol per ml of culture medium at 100 µM, after being sterilized by filtering through 0.22 µm polyvinyllidenedifluoride (PVDF) syringe filters (Millipore). The same volume of ethanol was added to the control.

Pretreatment and addition of absorbents

Before use, the absorbent XAD-7 was soaked in methanol overnight on a rotary shaker at 110 rpm and then washed with distilled water. After being filter-dried, the absorbents were autoclaved for 15 min at 115°C for sterilization. Because the combination of 100 µM MJA or its derivative and 100 g/l absorbent on day 7 efficiently enhanced Tc production in our previous research, 100 g/l absorbents were added to the cultures on day 7 in all the experiments.

Separation of cells and absorbents

Flasks with absorbents were kept on a platform before sampling. Cells and absorbents naturally separated due to different densities and the cells remained in the supernatant. Then, the cells were carefully transferred into another clean flask, leaving the absorbents. This procedure was repeated several times with the addition of distilled water to completely separate the cells from the absorbent.

Extraction and analysis of taxanes

As the methodology proposed by Qian et al. (2004) for taxane extraction is time-consuming, we adopted a faster method (Zhang et al., 2000). The results demonstrated that the new method yielded an equivalent extraction efficiency (data not shown).

For taxane extraction, 100 mg of powdered dry cells (or 250 mg of absorbents) were soaked in 4 ml of methanol and dichloromethane (1:1, v/v) and then ultrasonicated for 30 min (6 times). After centrifugation (4,000 rpm for 10 min), the extract was completely dried at 25°C using a rotary evaporator. The residue was dissolved in 4 ml of dichloromethane and 1 ml of distilled

water and extracted 4 times. After sufficient mixing, the mixture was centrifuged (4,000 rpm for 10 min). The organic phase (bottom layer) was collected and completely dried at 25°C by using a rotary evaporator. The residue was dissolved in 1 ml of chromatography-pure methanol and filtered through a 0.22 µm PVDF syringe filter (Millipore).

High performance liquid chromatography (HPLC) conditions

A volume of 10 µl was analyzed by reverse-phase HPLC, using a Waters Alliance 2695 HPLC system. An alkyl phenyl column (250 × 4.6 mm, 5 µm) was used at 25°C. The mobile phase consisted of acetonitrile and water. The acetonitrile content was 40% (v/v), increasing to 100% in 30 min. The flow rate was adjusted to 1 ml/min. Taxane was monitored at 227 nm using an authentic standard as the reference.

Preparation and purification of new taxoids

As new taxoids were produced when absorbents were combined to elicit *T. chinensis* cultures, all of the absorbents used to absorb secondary metabolites in *T. chinensis* cell cultures were collected and soaked in eight-fold volumes of methanol and dichloromethane (1:1, v/v) and then ultrasonicated for 3 h. The extract was then completely dried at 25°C by using a rotary evaporator. The residue was dissolved in chromatography-pure methanol and filtered through a 0.22 µm PVDF syringe filter (Millipore).

Preparation of the taxoids was completed with the help of the Medicinal Chemistry group, DICP, CAS. After several analytical HPLC verifications, preparative HPLC conditions were determined (Waters DP4000 system, CAPCELL PAK C_{18} column (250 × 20 mm, 5 µm)). The mobile phase consisted of methanol and water (60:40, v/v) or acetonitrile and water (60:40, v/v) (used when methanol and water did not work). The flow rate was 15 ml/min. Taxoids were monitored at 201 and 227 nm. The preparative component was then analyzed in a Waters 2695 system with a WAT052840 C_{18} column (250 × 4.6 mm, 4 µm) to determine the component purity. The analytical mobile phase consisted of methanol and water (75:25, v/v) and the flow rate was 1 ml/min.

After preparation, the separated components were analyzed again by using thin layer chromatography (TLC). TLC was completed with chloroform and methanol (96:4, v/v) and petroleum ether and propanone (70:30, v/v). All the components containing impurities were eliminated.

To improve the purity of the compounds for NMR, the compounds were further purified by column chromatography. First, the compounds were dissolved in petroleum ether and propanone. When petroleum ether and propanone (90:10, v/v) were used, the Rf value of compound 1 was 0.12 in TLC. Petroleum ether and propanone (90:10, v/v) were selected as eluting agents for compound 1. The ingrain agent was 12% H_2SO_4 with vanillin to saturation. A glass column of 35 cm × 1.2 mm was fixed with cotton at the bottom; about 15 g of silica gel with a mesh size range of 200 to 300 was used to pack the column. Then, petroleum ether and propanone (90:10, v/v) were poured to immerse the column. After volatilization of the organic solvent, the samples mixed with silica gel in the eluent were scattered evenly on the bed of silica gel in the column. Eluent (2 ml) was poured onto the samples, then a small amount of cotton was placed onto the eluent, and 300 ml of eluent was poured into the column, with the eluent-storing flask upside-down. Using pressurized elution, 3 ml aliquots of eluent were collected. TLC was performed for every 5~10 test tubes. The outspread agent was petroleum ether and propanone (80:20, v/v). The same samples were combined, and the organic solvent was volatilized until white amorphous powders were acquired. The same method was applied to compound 2, but with petroleum ether and propanone (95:5, v/v) as the eluent.

NMR measurements

NMR spectra were measured on a Varian Mercury 400 spectrometer (^{1}H 400 MHz, ^{13}C 100 MHz) using $CDCl_3$ (δ7.26 and 77.0) as the solvent. NMR measurements were made using the standard pulse sequences, HSQC, HMBC, ^{1}H-^{1}H COSY, and ROESY from the Varian pulse program library. HR-MS data were determined on a HR-ESI-MS: Q-TOF Micro LC-MS-MS spectrometer in m/z. ^{1}H (400 MHz) and ^{13}C NMR (100 MHz) spectral data of compounds 1 (2-hydroxy-5α,10β-diacetoxy-taxadiene) and 2 (2-hydroxy-5α,10β,14β-triacetoxytaxadiene) in $CDCl_3$ are shown in Table 1.

RESULTS

Cell suspension cultures of *T. chinensis* have been continuously maintained in our laboratory since October 2005. The cultures were elicited with MJA or its newly synthesized analog, DHPJA, in combination with *in situ* absorption to improve taxane production. MJA or its derivative has been proved to be very effective in producing taxoids (Wang and Zhong, 2002; Zhong 2002). Resins were usually used to extract taxoids outside the cell culture (Sun et al., 2009). We developed a technology to combine elicitation and *in situ* absorption to improve taxoid production.

Following elicitation and *in situ* absorption, Tc production was greatly enhanced (data not shown). Other intermediate taxanes were produced, 2 of which were purified and characterized.

Absorbents were separated and collected from the culture medium and then extracted. The extracts were prepared and purified using the method described above. The HPLC eluting profiles of the extracts from both the cells and the absorbents are shown in Figure 1. The fraction indicated in Figure 1B was a mixture of 2 compounds (compounds 1 and 2), which were separated, collected, purified, and subjected to HR-ESI-MS and NMR analysis (HSQC, HMBC, ^{1}H-^{1}H COSY and ROESY). As previously reported, the most abundant compound in the cultured strain was Tc (2α,5α,10β,14β-tetraacetoxytaxa-4(20),11(12)-diene), which is characterized by acetyl groups at C-14, C-2, C-5, and C-10; an unsubstituted methylene C-13; and a 4(20) exocyclic methylene function.

Compound 1 was purified as a white amorphous solid. ESI-MS displayed ion peaks at m/z 427.1 ([M+Na]$^+$), revealing its molecular weight to be 404. Combined analysis by ESI-MS, ^{1}H NMR, and ^{13}C NMR lead to a proposed molecular formula of $C_{24}H_{36}O_5$. HR-ESI-MS also suggested the same formula. The compound was identified as 2-hydroxy-5α,10β-diacetoxytaxadiene and its structure is shown in Figure 2 A.

Compound 2 was also obtained as a white amorphous solid. ESI-MS yielded ion peaks at m/z 485 ([M+Na]$^+$), revealing that its molecular weight was 462. Following combined analysis of ESI-MS, ^{1}H NMR, and

Table 1. ^{1}H (400 MHz) and ^{13}C NMR (100 MHz) spectral data of compounds 1 and 2 in $CDCl_3$.

Position	Compound 1		Compound 2	
	^{1}H	^{13}C	^{1}H	^{13}C
1	1.96	54.8	1.96	63.3
2	4.05	70.2	4.08	70.2
3	2.93	43.2	2.70	43.7
4		144.8		143.2
5		78.6	5.24	79.0
6	5.24	29.4	1.82	28.9
7	1.83	33.9	1.22 (1.90)	33.9
8	1.26 (1.98)	39.9		39.6
9		43.8	1.60 (2.24)	43.8
10	1.60 (2.29)	70.6	6.03	70.1
11		133.9		136.9
12		136.4		135.7
13		30.1	2.58 (2.70)	38.1
14	1.98 (2.42)	17.8	4.84	71.2
15	1.50 (1.94)	36.8		37.6
16		31.9	1.15	31.4
17	1.09	25.5	1.56	25.8
18	1.56	21.1	2.10	21.0
19	2.12	22.7	0.89	22.3
20	0.89	117.4	5.30 (5.78)	118.8
2-OH	5.35 (5.45)		3.58	

^{13}C NMR data, its molecular formula was proposed to be $C_{26}H_{38}O_7$. HR-ESI-MS also gave the same formula. It was identified as 2-hydroxy-5α,10β,14β-triacetoxy-taxadiene, and its structure is shown in Figure 2B.

DISCUSSION

In summary, studies were carried out with *T. chinensis* suspension-cultured cells under control (non-induced) and induced conditions (with MJA or its derivative) with *in situ* absorption. Two new abundant intermediate taxoids were isolated, purified, and characterized by NMR. Being intracellular, Tc was the main component of taxoids obtained from the non-induced cells. Following elicitation and absorption, the production of Tc was significantly reduced in cells, but it was still secreted and accumulated in the absorbents. Simultaneously, several other intermediate taxoids that did not exist in the control accumulated in the absorbents. This observation clearly indicates that acetylation of 5α-hydroxytaxadiene preferentially diverts pathway flux away from 13α-hydroxy taxoids and towards 14β-hydroxy taxoids, and this is consistent with previous biochemical studies reporting a 14β-hydroxylase preference for 5α-acetylated derivatives as substrates (Ketchum et al., 2007). A major bifurcation of the taxoid biosynthetic pathway occurs at the level of 5α-hydroxytaxadiene, leading to the production of 14β-hydroxy taxoids.

Our work demonstrated the previously undetermined hydroxylation and acetylation steps between taxa-4(20), 11(12)-dien-5α-OH and Tc. The steps involved in taxoid synthesis are most likely to be as follows: Taxa-4(20), 11(12)-dien-5α-OH is esterified by taxadiene acetyltransferase (TDAT) to form taxa-4(20),11(12)-dien-5α-yl acetate, followed by 10β-hydroxylation and esterification, and 2α-hydroxylation. 2-hydroxy-5α,10β-diacetoxytaxadiene is thus synthesized. After 14β-hydroxylation and esterification, 2-hydroxy-5α,10β,14β-triacetoxytaxadiene is synthesized. With 2α-esterification, Tc is formed.

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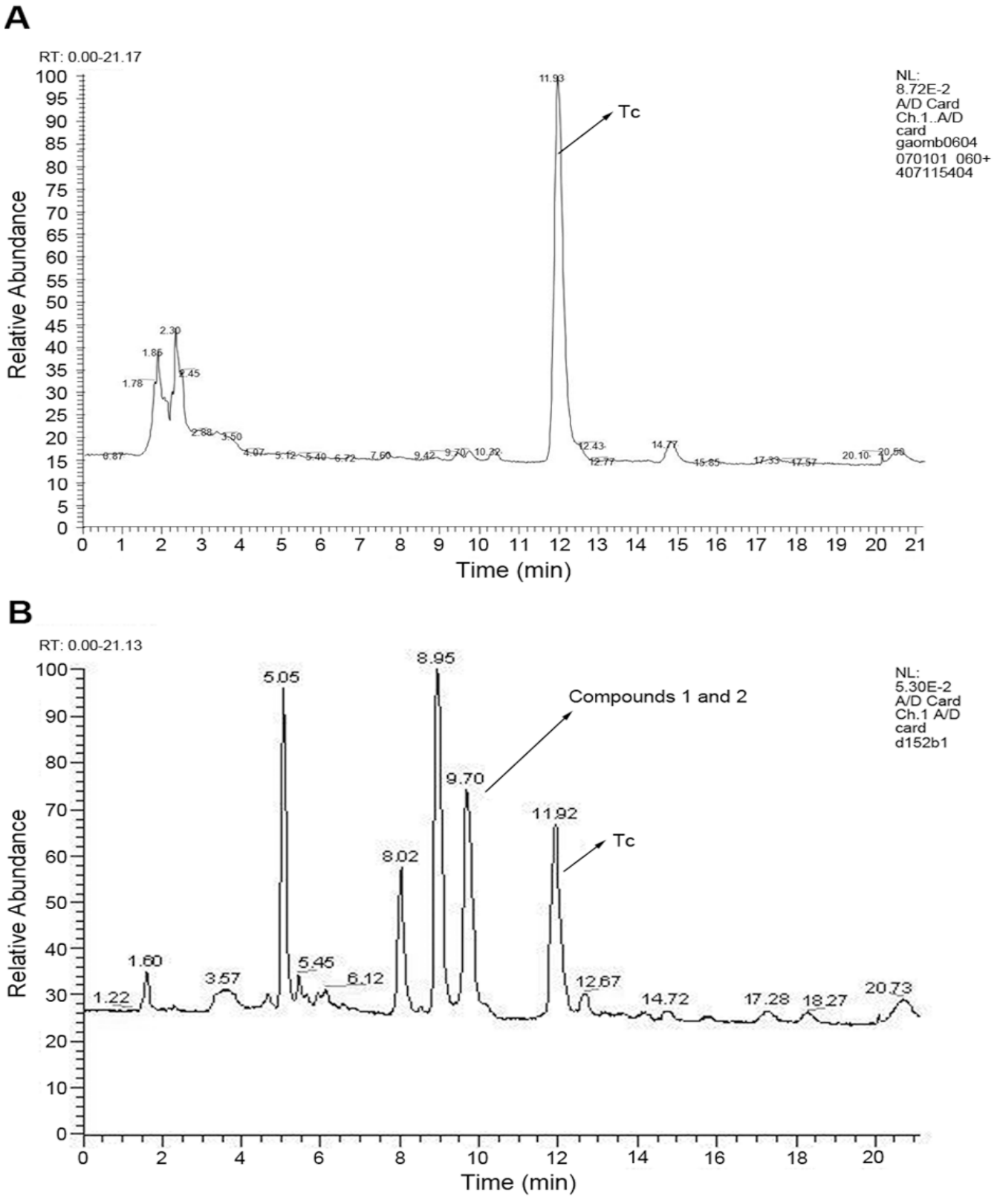

Figure 1. HPLC profiles of extracts from *T. chinensis* cell cultures in 100 µM methyl jasmonate and 100 µM methyl jasmonate in combination with 100 g/l absorbents. (A) Cell extracts treated with 100 µM methyl jasmonate for 8 days; (B) absorbent extracts treated with 100 µM methyl jasmonate in combination with 100 g/l absorbents for 8 days. Cultures were carried out in Murashige and Skoog medium, supplemented with 30 g/l sucrose, 100 mg/l ascorbic acid (Vc), 0.5 mg/l 6-benzyladenine (6-BA), 0.2 mg/l 2,4-dichlorophenoxy-acetic acid (2,4-D), and 0.5 mg/l naphthaleneacetic acid (NAA) on a reciprocating shaker (100 strokes/min) at 25°C. The inoculum size was 2.0 g wet cells per 20 ml medium.

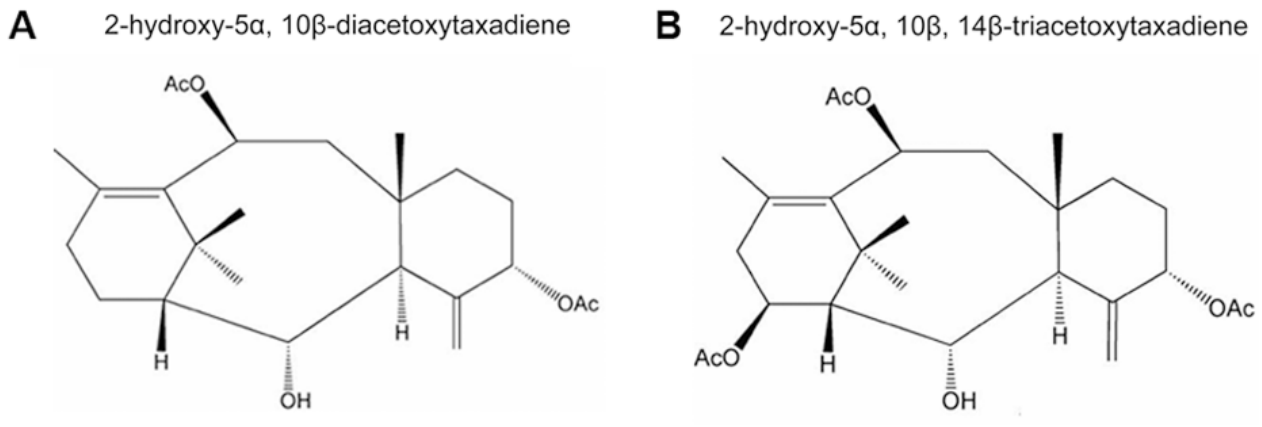

Figure 2. Structures of 2-hydroxy-5α,10β-diacetoxytaxadiene (A) and 2-hydroxy-5α,10β,14β-triacetoxytaxadiene (B).

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