Minireview

Simultaneous Identification and Determination of Major Taxoids from Extracts of *Taxus chinensis* Cell Cultures

Chun F. Zhao, Long J. Yu*, Li Q. Li, and Fu Xiang

College of Life Science and Technology, Huazhong University of Science and Technology, Wuhan 430074, Hubei, China. Fax: +86-27-87792265. E-mail: yulongjiang@hust.edu.cn

* Author for correspondence and reprint requests

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Liquid chromatography electrospray ionization tandem mass spectrometry (LC/ESI-MS/MS) and high-performance liquid chromatography (HPLC) methods have been used to identify and quantify the major taxoids from extracts of *Taxus chinensis* cell cultures. Chromatography was carried out on a reverse phase C18 column with isocratic-mode elution. By analytically comparing LC/ESI-MS/MS of the extracts with that of the available reference substances and literature, six taxoids were identified as taxuyunnanine C (Tc, **1**), yunnanxane (**2**), 2α,5α10β-triacetoxy-14β-propionyloxytaxa-4(20),11-diene (**3**), 2α,5α,10β-triacetoxy-14β-(2-methyl)butyryloxytaxa-4(20),11-diene (**4**), taxol (**5**), and baccatin III (B III, **6**), respectively. Among them, **2**, **3** and **4** were assigned in the absence of the corresponding reference substances, and **3** and **4** were detected in this cell line for the first time. The identification was validated by NMR spectra. The precise quantification of **1** and **5** was made using HPLC. The limit of detection (LOD), 0.5 μg/ml for **5**, 1.5 μg/ml for **1**, and the linearity and accuracy of the quantitative method were evaluated, indicating a wide linear range and satisfactory accuracy. The amounts of other identified taxoids were calculated on the basis of comparison of the absolute response factors of similar structural substances. The proposed method provides a rapid, conventional and reliable tool to characterize and study cell lines for elucidating the taxane biosynthesis.

Key words: Taxoid Analysis, *Taxus chinensis* Cell Cultures, LC/ESI-MS/MS

Introduction

Taxoids are a group of specific diterpenoid compounds with a pentamethyl [9.3.1.0]^{3,8} tricyclopentadecane taxane skeleton including taxol (**5**) (Fig. 1) and taxotere, a type of the most effective anticancer drugs. There are over 350 members of this family, classified according to their structural differences (Baloglu and Kingston, 1999). Each class of the taxoids has different polar and pharmaceutical properties and special biological activities. For example, 10-deacetylbaccatin III (10-DAB) and baccatin III (B III, **6**), which share the same taxane bone with taxol, are used as important start materials for the semisynthesis of taxol and taxotere (Guenard *et al*., 1993); the taxoids with an acyloxy group at C-14 such as taxuyunnanine C (Tc, **1**) and its analogues, which are the major metabolites of *Taxus chinensis* cell cultures (yields to 0.2% of dried cells), showed potential officinal values that increased the cellular accumulation of vincristine in multidrug-resistant tumor cells (Bai *et al*., 2004). The increasing clinical demands for active taxoids and the exhausted supply of natural *Taxus*, the initial source of taxoids, have spurred to produce these drugs or useful precursors by means of *Taxus* cell culture. Presently, the manipulation and regulation of the metabolic pathway at molecular level for the *Taxus* spp. cell culture system are believed to be a promising approach to increase the yields of taxol and related taxoids (Hezari *et al*., 1997; Ketchum *et al*., 2003). To regulate and manipulate the biosynthesis, the metabolic profiling of taxoids is a necessary step to provide fundamental chemical information directly involved in the taxol biosynthesis.

Metabolic profiling analysis of taxoids requires quick identification and measurement of major compounds present in the *Taxus* cell culture system. Reversed-phase column liquid chromatography is predominantly used for separating and determining taxoids in both plant materials and cell cultures. Detection is usually carried out by ultra-

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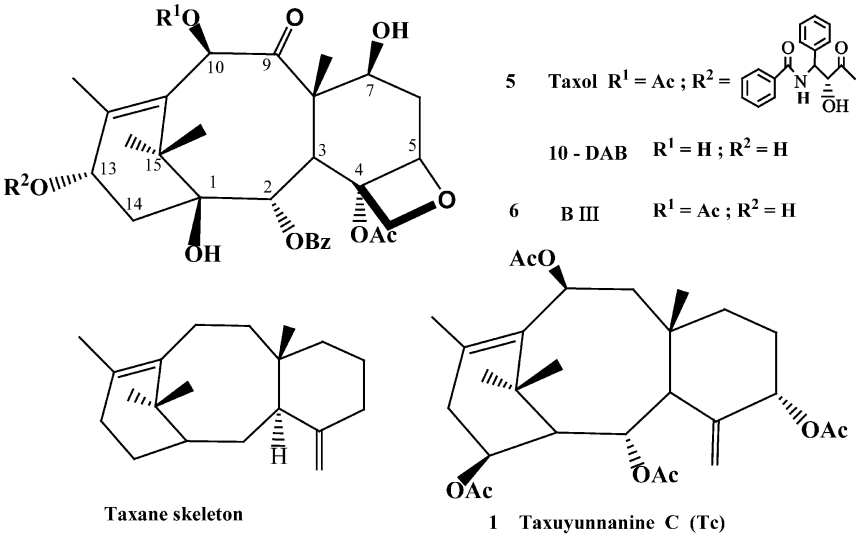

Fig. 1. The structures of taxoids.

violet (UV) detection at 227 nm after chromatographic separation. In order to tackle the problems arising from the complexity of biological samples, special taxane column, mobile phase, analytical parameters, solid-phase extraction (SPE) for sample pretreatment and quantitative methodology have been developed and studied (Shao and Locke, 1997; Theodoeidis *et al.*, 1998). Nevertheless, in all of these methods taxoids are determined comparing the retention time (t_R) with that of authentic substances that are not always available. Although a resolution of a standard mixture containing 13 taxoids assisted by photodiode array detection (PAD) was subsequently developed for this purpose (Bala *et al.*, 1999; Dolfinger and Locke, 2003), it is not suitable for the analysis of the components contained in other species of *Taxus* due to the difference in taxoid contents between various yew species (Parc *et al.*, 2002). Identification of metabolites is generally achieved by nuclear magnetic resonance (NMR) (Wani *et al.*, 1971), and this is not considered to be feasible for the routine study of metabolic profiling because of the time-consuming procedure and the requirement to produce a large amount of the samples for the preparation of the pure compounds. Thus, the attempt of metabolic profiling analysis of taxoids was often hampered by the identification of the analytes.

Recently, high-performance liquid chromatography (HPLC) coupled with mass spectrometry (MS) has become a powerful tool in the analysis of known as well as unknown metabolites from the complex matrix (Zhang *et al.*, 2004). This technology has also been applied to the analysis of taxoids in extracts of plants (Hoffmann *et al.*, 1998). The study of fragmentation behavior of taxanes through tandem mass spectrometry (MS/MS) provided a beneficial basis to analyze their structural characteristic features by means of this technology (Zeper *et al.*, 2000). Madhusudanan *et al.* (2002a, b) reported MS/MS profiling of more than 50 taxoids from different tissues of *T. wallichiana* based on LC/ESI-MS analysis. It has to be noticed, however, that analytical methods for simultaneous determination of multiple taxane constituents in extracts of plants or cell cultures of *Taxus chinensis* are still scarce.

As part of the desire to increase the yield of taxol production, the technologies of combined elicitation improving taxol production in the *Taxus chinensis* cell culture system have been established in our laboratory (Mei *et al.*, 1996; Yu *et al.*, 2002; Wang and Mei, 2001). The aim of the present study is to develop a simple, convenient and reliable HPLC and LC/ESI-MS method to achieve simultaneous determination and identification of major taxoids in extracts of *Taxus chinensis* cell cultures. This method should make the most out of LC, and the taxoid structural library and their mass spectra data published by literatures for the structural elucidation of major taxoids that coexist in methanol extracts of *Taxus* cell cultures with taxol. Further, the quantitative measurements of those taxoids are investigated by HPLC analysis.

Experimental

Chemicals

Standards of taxol, 10-DAB and B III were presented by NCI (USA); the reference standard of Tc was previously isolated with a purity of 98% by HPLC according to Eisenreich *et al.* (1996) and identified by NMR. Methanol and acetonitrile used for HPLC analysis were of chromatographic grade; other reagents used were of analytical grade.

Instrumentation

The LC/ESI-MS system consisted of a LC system with a TSP P4000 pump separation module, a 991 photodiode array detector (PAD), and a quadrupole ion-trap mass spectrometer equipped with an ESI interface (Finnigan ThermoQuest, USA). All the LC/MS operations and acquiring of data were controlled by LCQ software. The quantifications of taxoids were conducted on the HPLC system (Waters Co., USA) consisting of two 510 pumps, a 486 UV detector and a 746 data module.

Preparation of samples

Taxus plant cell line was established as previously described (Mei *et al.*, 1996). After subculturing for less than 15 d, the cell suspension cultures were harvested upon removal of the growth medium by filtration. The harvested cultures were lyophilized and placed at −70 °C as sample for the experiments. In addition, the frozen fresh cells were dried at 50 °C to a constant weight for the measurement of cell dry weight (D.W.), which was about 3.3%. 5.00 g samples of lyophilized cell cultures were soaked by ethyl acetate (3×5 ml) under sonication for 30 min. Then the organic solvent was evaporated to dryness. Residue was dissolved by 1 ml of methanol. 0.5 ml of the crude extract solution was employed for further separation by solid phase extraction (SPE). The procedure of SPE was done using an improved method followed by a published protocol (Theodoeidis *et al.*, 1998). A C18 SPE cartridge (500 mg/3 ml; Hanbon, Jiangsu, China) was used for the sample pretreatment. Prior to loading, the cartridge was conditioned ordinally using ethyl acetate, methanol and water washing. After loading, it was washed with 4×4 ml of water, 4 ml of 40% methanol in water, and 4 ml of 50% methanol in water. The compounds of interest were eluted with 5 ml of methanol and the collected fraction was evaporated to dryness. The residue was reconstituted in the solution with 2×200 μl of the mobile phase used by the present LC separation.

HPLC and LC/ESI-MS conditions

The conditions of HPLC have been previously optimized and will not be covered by this paper. The separation was completed on a 250 mm × 4.6 mm i.d. Cromasil C18 column (Elite, Dalian, China), which was packed with 5 μm C18 silica. The operating temperature was maintained at 25 °C. The LC mobile phase of acetonitrile/methanol/1 mM ammonium acetate in water (30:25:45) was isocratically eluted at the flow rate of 1 ml/min. UV detector of HPLC was set at 227 nm. The total analysis time was 35 min, including 30 min for the mobile phase elution and 5 min for 100% methanol elution to stabilize the column. The LC effluent was split in the ratio 10:1 to pass through the PAD before the ESI probe was introduced at 50 μl/min into the mass spectrometer. The chromatographic conditions of LC/MS were modified from the conditions of HPLC. The PAD was set at 190–280 nm and the chromatogram detected at 227 nm. The ESI source was operated at 200 °C in both positive and negative mode for the selection of MS operating manners by injecting a solution containing 10-DAB, B III, taxol, and Tc. The desolvation temperature was set at 250 °C, extract voltage was 4 V, desolvation gas and cone gas were set at 250 l/h and 40 l/h, respectively. The full-scan mass spectra were acquired over the range 100–1000 amu. The capillary voltage of the ESI source was set at 4.5 kV, cone voltage at 45 V. In the MS/MS experiments, the precursor ions $[M+H]^+$ for 10-DAB and taxol, and $[M+NH_4]^+$ for B III and Tc were selected and fragmented by helium gas in the ion trap at an ion collision energy of 25 eV.

Determination of taxoids

Stock solutions of taxol and Tc with a concentration of 10 mg/ml were prepared by dissolving weighed quantities of standards in methanol. An amount of volumes of the above stock solution was transferred and further diluted with the mobile phase solution to yield standard solutions with six final concentrations of 1.0, 10, 60, 100, 150, 250 μg/ml for taxol, and 6, 10, 60, 100, 200, 300 μg/ml for Tc, respectively. Three 10 μl injections were made at each concentration point. Peak areas

were plotted against the corresponding concentrations using linear regression to generate the standard curves. The amounts of taxol and Tc in samples were calculated using the corresponding calibration curve. The quantities of the related taxoids in samples were measured with taxol or Tc as the reference standard, depending upon their structures. Three injections were made for each sample. The limit of detection (LOD) was evaluated by the UV detector giving a signal equal to three times the noise (S/N = 3). The limit of quantification (LOQ) was defined as three times the LOD (Shao and Locke, 1997).

Method validation

The precision was evaluated from the triplicate determinations performed on the same day for a random sample. The relative standard deviation (R.S.D.) upon quantification showed less than 3.1%. The accuracy was assessed by a recovery experiment. A known quantity of taxol and Tc standard was added to 5.00 g samples of lyophilized cell cultures at low, medium and high levels of final concentrations of 10.0, 100 and 200 μg/ml, respectively.

NMR measurement

In order to confirm the identification of compounds **3** and **4** in the investigation, 0.5 mg of residue in 25 μl methanol, which was obtained from 400 g samples of lyophilized cell cultures, was prepared. The preparative separation was made by a Waters HPLC system consisting of a preparing column of 250 mm × 4.6 mm i.d. with 5 μm particle size. After the corresponding fractions were collected, the pure and isolated compound solutions were maintained and then analyzed by NMR. NMR spectra were measured on a Varian Inova-500 (^{1}H, 500 MHz; ^{13}C, 125 MHz) using $CDCl_3$ as the solvent and TMS as internal standard.

*2α,5α,10β-Triacetoxy-14β-propionyloxytaxa-4(20),11-diene (**3**)*

White amorphous powder. – ^{13}C NMR (125 MHz, $CDCl_3$): δ = 20.79 (q, C-18), 21.34 (q, Ac10-Me or Ac2 –Me), 21.76 (q, Ac5-Me), 22.31 (q, C-19), 27.88 (q, C-2′), 28.78 (t, C-6), 31.59 (q, C-17), 33.73 (t, C-7), 37.21 (s, C-15), 39.50 (t, C-13), 39.58 (s, C-8), 42.05 (d, C-3), 43.79 (t, C-9), 59.01 (d, C-1), 70.09 (d, C-10), 70.51 (d, C-14 or C-2), 70.56 (d, C-2 or C-14), 78.21 (d, C-5), 116.81 (t, C-20), 134.75 (s, C-12), 135.21 (s, C-11), 142.11 (s, C-4), 169.70 (s, Ac5-CO), 170.11 (s, Ac2-CO), 170.35 (s, Ac10-CO), 173.46 (s, C-1′). – ^{1}H NMR (500 MHz, $CDCl_3$) : δ = 0.80 (3H, s, H-19), 1.08 (3H, t, J = 7.7 Hz, H-3′ or 3H, s, H-17), 1.20 (1H, dt, J = 14.7, 3.4 Hz, H-7β), 1.59 (1H, dd, J = 15.3, 5.5 Hz, H-9α), 1.61 (3H, s, H-16), 1.74 (2H, m, H-6), 1.83 (1H, J = 2.2 Hz, H-1), 1.93 (1H, dt, J = 13.3, 7.1 Hz, H-7α), 2.00 (3H, s, H-2′), 2.06 (3H, s, Ac10-Me or Ac2-Me), 2.09 (3H, s, H-18), 2.13 (3H, s, Ac5-Me), 2.23 (2H, q, J = 7.6 Hz, H-2′), 2.34 (1H, m, H-9β), 2.38 (1H, dd, J = 19.0, 4.7 Hz, H-13β), 2.81 (1H, dd, J = 19.2, 8.6 Hz, H-13α), 2.90 (1H, d, J = 6.7 Hz, H-3), 4.80 (1H, s, H-20β), 5.00 (1H, dd, J = 9.2, 15.0 Hz, H-14), 5.24 (1H, s, H-20α), 5.31 (1H, dd, J = 6.6, 2.3 Hz, H-2), 6.01 (1H, dd, J =12.0, 5.8 Hz, H-10).

*2α,5α,10β-Triacetoxy-14β-(2-methyl)-butyryloxytaxa-4(20),11-diene (**4**)*

White amorphous powder. – ^{13}C NMR (125 MHz, $CDCl_3$): δ = 11.53 (q, C-4′), 16.51 (q, C-5′), 20.77 (q, C-18), 21.32 (q, Ac10-Me or Ac2–Me), 21.74 (q, Ac5-Me), 22.30 (q, C-19), 25.31 (q, C-16), 26.67 (t, C-3′), 27.88 (q, C-2′), 28.78 (t, C-6), 31.59 (q, C-17), 33.73 (t, C-7), 37.21 (s, C-15), 39.50 (t, C-13), 39.58 (s, C-8), 41.01 (d, C-2′), 42.05 (d, C-3), 43.77 (t, C-9), 59.00 (d, C-1), 70.06 (d, C-10), 70.49 (d, C-14 or C-2), 70.53 (d, C-2 or C-14), 78.18 (d, C-5), 116.78 (t, C-20), 134.72 (s, C-12), 135.18 (s, C-11), 142.08 (s, C-4), 169.66 (s, Ac5-CO), 170.08 (s, Ac2-CO), 170.31 (s, Ac10-CO), 173.43 (s, C-1′). – ^{1}H NMR (500 MHz, $CDCl_3$) : δ = 0.80 (3H, s, H-19), 1.07 (3H, t, J = 7.7 Hz, H-3′ or 3H, s, H-17), 1.20 (1H, dt, J = 14.7, 3.4 Hz, H-7β), 1.42 (1H, m, H-3′α), 1.59 (1H, dd, J = 15.3, 5.5 Hz, H-9α), 1.61 (3H, s, H-16), 1.74 (2H, m, H-6), 1.83 (1H, J = 2.2 Hz, H-1), 1.93 (1H, dt, J = 13.3, 7.1 Hz, H-7α), 2.00 (3H, s, H-2′), 2.06 (3H, s, Ac10-Me or Ac2-Me), 2.31 (1H, hp, H-2′), 2.34 (1H, m, H-9β), 2.38 (1H, dd, J = 19.0, 4.7 Hz, H-13β), 2.80 (1H, dd, J = 19.2, 8.6 Hz, H-13α), 2.79 (1H, d, J = 6.7 Hz, H-3), 4.78 (1H, s, H-20β), 4.99 (1H, dd, J = 9.2, 15.0 Hz, H-14), 5.21 (1H, s, H-20α), 5.30 (1H, dd, J = 6.6, 2.3 Hz, H-2), 6.03 (1H, dd, J = 12.0, 5.8 Hz, H-10).

Results and Discussion

LC/ESI-MS analyses of several taxoid standards

The solution, containing taxol, 10-DAB, B III and Tc standards (Fig. 1), was chromatographed

Table I. LC/ESI-MS data of taxoid standards.

Standard	Molecular formula	M_r	t_R [min]	MS data	MS data (major *m/z* of fragment ions and relative abundance)
10-DAB	$C_{29}H_{35}O_{10}$	544	5.64	562(70), 545(40)	545(10), 509(25), 405(40), 345 (50), 327(20), 287(30)
B III	$C_{31}H_{38}O_{11}$	586	8.20	604(85), 587(20)	604(10), 475(20), 465(40), 415(100), 387(30), 345(40), 327(20), 287(25)
Taxol	$C_{47}H_{51}NO_{14}$	853	16.90	854(100)	854(5), 569(16), 509(20), 327(15), 286(100), 268(20)
Tc	$C_{28}H_{40}O_8$	504	19.78	522(100)	522(10), 385(100), 343(12), 283(16), 265(55), 161(20)

to determine the retention time (t_R), UV trace and MS data for comparing with that of *Taxus* cell culture extracts. The scanning of mass spectra was conducted with both positive and negative ion mode. The positive ion mode was found to be more sensitive than the negative ion one, and thus the positive ion mode was applied. The corresponding t_R values in the chromatogram, *m/z* data in ESI-MS and tandem mass (MS/MS) data of the precursor ions at *m/z* 854, 545, 604 and 522, respectively, are listed in Table I.

As shown in Table I, the main molecular-related ion peaks given in the ESI-MS spectrum were $[M+H]^+$ for taxol, $[M+NH_4]^+$ for Tc, and both $[M+H]^+$ and $[M+NH_4]^+$ of two adduct ion types for 10-DAB and B III, indicating that the adduct ion type in ESI-MS spectra partly depends on the structural features of the taxoids. Ammonium cationization normally leads to the formation of ammonium adduct ions. However, if the sample is basic enough to combine with a proton from ammonium, protonated species may be observed (Nakata *et al.*, 1983). We deduce that taxol yields the strong protonized ions in ESI-MS spectra due to its nitrogen groups in the molecular structure, which readily capture protons in ESI-MS spectra. While for Tc with four acetyl groups protonation seems to be difficult, it predominantly gives $[M+NH_4]^+$ in the ammonium acetate atmosphere. These typical mass spectra by ESI are consistent with those of the taxoids in *Taxus wallichiana* described by Madhusudanan *et al.* (2002b) and could provide useful information on identification of the molecular ion peaks in ESI-MS spectra of cell samples.

In order to investigate the fragmentation patterns of the taxoids under the given conditions, MS/MS analysis of precursor ions at *m/z* 854, 545, 604 and 522 was performed. As shown in Table I, the MS/MS of the $[M+H]^+$ ion of taxol gave main characteristic fragments at *m/z* 286 and 327, which should correspond to the fragment ion of the C-13 side chain and taxane skeleton fragments with 6/8/6 type, respectively. These results agree with many related reports (Zeper *et al.*, 2000; Madhusudanan *et al.*, 2002a, b). Relationships between taxane structural types, number and nature of substituents, and MS/MS spectra have been partly summarized in the published literature (Madhusudanan *et al.*, 2002a). For example, normal taxanes containing a C-4 (20) double bond give characteristic fragments at *m/z* 265 accompanied by a peak at *m/z* 283, 263/281 or 261/279 depending on whether the taxane skeleton has four, five or six substituents, respectively. The present study also demonstrated some of these results. The precursor ion spectrum of the ammonium adduct of Tc at *m/z* 522 gave rise to sequential eliminations of acetic acid units and the indicative fragment ions at *m/z* 265/283. It is observed from the MS/MS spectra of precursor ions that the most intensive product ions are at *m/z* 286 for the component taxol and 385 for the component Tc, repectively. These fragmentation behaviors could be utilized consequently for deducing unknown taxane structure types.

Identification of major taxoids of sample

The peak identification of the extract sample was made on the basis of bellows: (i) comparison of the t_R values in the chromatograms and molecular weight values given by ESI-MS with those of available standards; (ii) comparison of the corresponding information with what had been described in published documents; (iii) the biogenetic backgrounds. The components of the sample solution were firstly separated by LC, and then analyzed by simultaneous UV trace and ESI-MS monitoring. ESI-MS was carried out in total ion scanning with positive ion mode. The simultaneous LC-UV chromatogram and total ion chromatogram (TIC) with positive ESI spectrum of the sample are shown in Fig. 2.

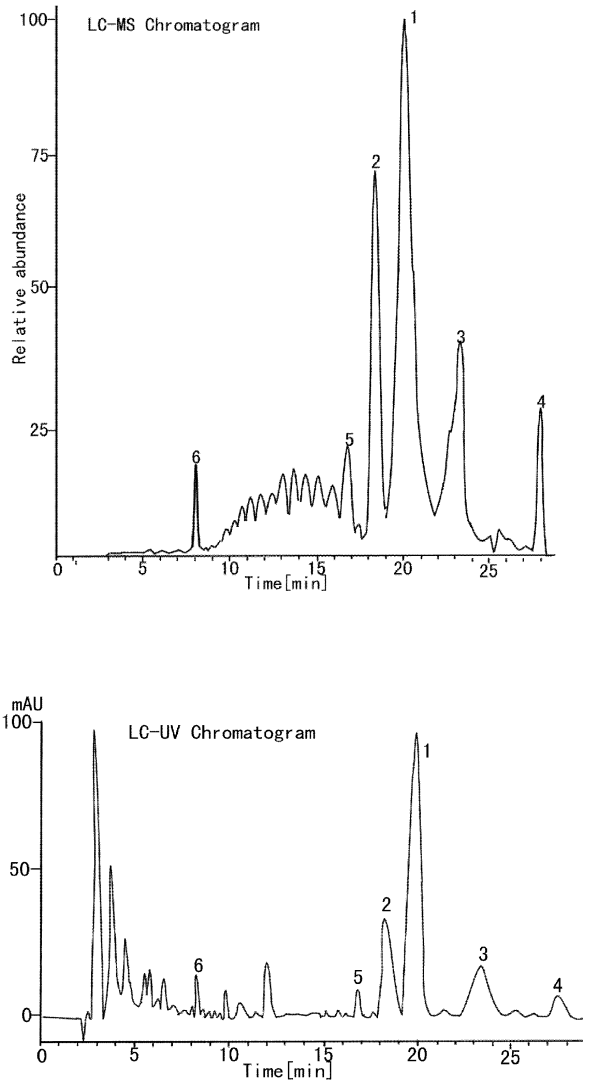

Fig. 2. Simultaneous LC-UV and total ion ESI(+)-mass chromatograms (TIC) of extracts of *Taxus chinensis* cell cultures. Chromatographic conditions are described in Experimental.

Six main peaks can be obviously observed in the LC-MS chromatogram and the simultaneous LC-UV chromatogram. According to the relative abundance from high to low in TIC, the peaks were numbered as 1–6, and their t_R values were 19.81, 18.12, 23.23, 27.90, 16.92, and 8.21 min, respectively. The ESI-MS data gave major peaks at *m/z* 522, 580, 536, 854, 546 and 604, respectively, corresponding to the above t_R values. Among the six peaks, peaks 1, 5, 6 can be easily identified from the sample as Tc, taxol and B III by comparison of the t_R values and the ESI-MS data of the peaks with those of the standards. In terms of that the ammoniated ion peak ($[M+NH_4]^+$) is a predominantly molecular-related ion peak under the experimental conditions of ESI-MS spectra. The molecular weight of the unknown components corresponding to peaks 2, 3, and 4 can been calculated as 562, 518 and 546, respectively.

The ESI-MS/MS experiment was carried out in the next run by selecting precursor ions ($[M+NH_4]^+$) *m/z* at 522, 580, 536 and 564 for their structural elucidation. The retention time (t_R), partly ESI-MS spectrum, MS/MS spectrum and the identification of peaks 1, 2, 3, 4 are demonstrated in Fig. 3. We started with manual searches of the taxoids with the given M_r from related published reviews (Baloglu and Kingston, 1999; Shigemori and Kobayashi, 2004), and followed by the interpretation of the experimental fragmentation pattern and the analysis of the biogenetic background of taxoid derivation. Specifically, the results of the literature search showed that there were at least six taxoids with M_r 562, four with 518 and three with 546 in the publication of Baloglu and Kingston (1999) and Shigemori and Kobayashi (2004). From the six isomeric taxoids with M_r 562, only four were isolated from *Taxus mairei*, a mutation of *Taxus chinensis*. Moreover, the ESI-MS/MS spectrum of the precursor ion at *m/z* 580 showed the presence of a fragment ion at *m/z* 283/265 and the subsequent loss of four acetic acid units from the parent ion, indicating that the taxane skeleton of the compound belongs to a normal taxane with four substituents and an exocyclic C-4 (20) double bond. The comparison of the ESI-MS/MS data with that of yunnanxane in the sample of bark of *Taxus wallichiana* (Madhusudanan *et al.*, 2002a) indicated that both molecules possessed the similar fragment patterns. Based on the above consideration and the fact that there is a very close genetic relation among *T. chinensis*, *T. yunnanesis* and *T. wallichiana*, we conclude that compound **2** is considered a yunnanxane.

Similarly, it is noticed that compounds **3** and **4** showed similar fragment patterns to those of **1** and **2**, indicating their structural similarity. Considering the biogenetic background, compounds **3** and **4** should correspond to 2α,5α,10β-triacetoxy-14β-propionyloxytaxa-4(20),11-diene (sinenxan B) and 2α,5α,10β-triacetoxy-14β-(2-methyl)butyryloxytaxa-4(20),11-diene (sinenxan C), respectively.

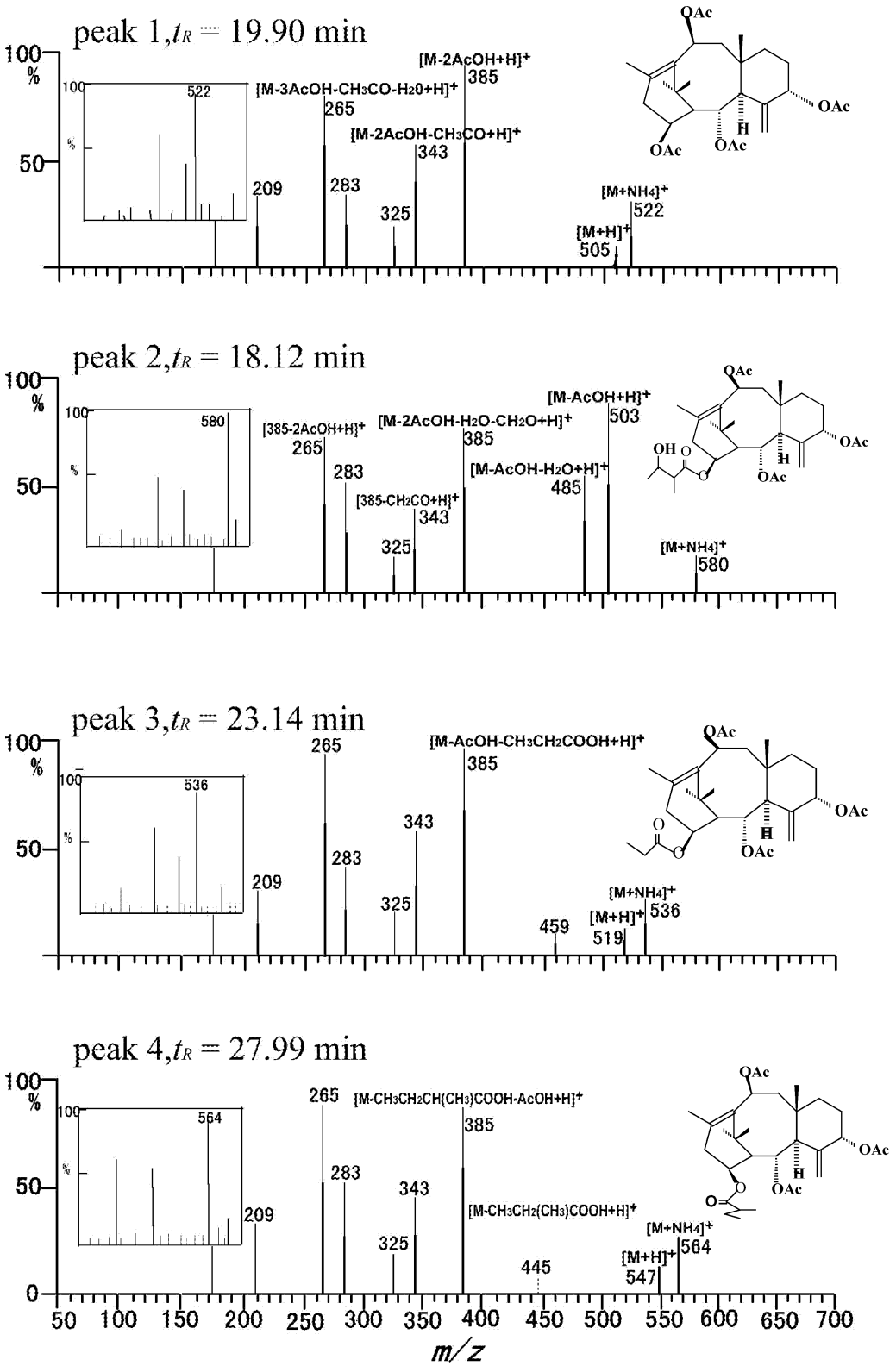

Fig. 3. ESI-MS spectra of peaks **1**, **2**, **3**, **4** (in the left frames) in LC/MS chromatograms, and MS/MS analyses of the precursor $[M+NH_4]^+$ ions at *m/z* 522, 580, 536 and 564 of extracts from *Taxus chinensis* cell cultures. The calculated molecular structures are shown on the right sides.

These taxoids were previously reported in cell cultures of *T. chinensis* but were not detected in the cell line established by our group. Their chromatographic retention orders in the present study are consistent with other previous reports (Wu *et al*., 1998). Other main ion peaks at *m/z* 536 and 564 in the MS/MS can be reasonably explained if they are sinenxan B and sinenxan C. Hence, compounds **3** and **4** were identified**.** These identifications were further confirmed by ^{1}H and ^{13}C NMR spectra (see Experimental). Thus, the qualitative analysis of the compounds was achieved, despite the absence of available standards for compounds **2**, **3** and **4**.

It is noticed that 10-DAB was not detected in the sample by UV and MS detector. This is probably due to the genetic or regulative factors, which can lead to the paucity of the taxoid. This also may reflect the characteristics of taxane biosynthesis of the cell line.

Speediness and convenience are the most obvious advantages when comparing the LC/MS/MS method of taxoid identification with the NMR method. By scanning the available taxane structural library, the separation and identification of major taxanes in chromatograms can be completed only in two parallel runs without large-scale sample preparation and purification. In particular, in the case of studying the capability of producing taxanes of cell lines, it is often difficult, even impossible to obtain the NMR spectrum data from each chromatographic constituent. Nevertheless, the method used in this investigation makes a quick identification of the metabolites within half an hour. Moreover, in terms of isocratic-mode HPLC used in this investigation it is more feasible and less time-consuming than any other method of detection. Simultaneous LC-UV and LC/ESI-MS detection facilitates the quantification of the identified analytes in the routine isocratic-mode HPLC because the chromatographic retention time of the components can be smoothly producible in another HPLC system.

Determination of the major taxoids in sample

Quantitative analyses of the identified taxoids in sample were conducted with the HPLC system. Visual comparison (Fig. 2) and the purity factor calculated using the PAD system related software (the result is not listed) indicated that the individual peak of components **1**–**6** contained only a single component. Therefore, it is possible to quantify these taxoids by comparison of the peak areas given by HPLC. The contents of taxol and Tc in samples were determined accurately by their standard curves. The quantification of the other identified taxoids was made by a computational method based on investigating the absolute response factors (ARF) of taxoid molecules toward UV detection. Because the UV spectrum of taxoid is mainly derived from the structural character of the taxane skeleton, the taxoids whose structures are similar to each other should have a close relationship of signal responses under the same detection conditions (Theodoeidis *et al.*, 1998). Although the use of the corresponding pure standard is commonly necessary for quantitative HPLC analysis, the studies by Ciutaru *et al*. (2004) revealed that it is possible to directly employ ARF

Table II. The arrangement of the results of the determined major taxoids in extracts of *Taxus chinensis*.

Peak	t_R^a [min]	M_r	Molecular formula	Identification	Content [μg/g]	Reference
1	19.81	504	$C_{28}H_{40}O_8$	Taxuyunnanine C (**1**)	1235.0 ± 28.4	Ma *et al.* (1994)
2	18.12	562	$C_{31}H_{46}O_9$	yunnanxane (**2**)	425.0	Eisenreich *et al.* (1998)
3	23.23	518	$C_{29}H_{42}O_8$	2α,5α,10β-Triacetoxy-14β-propionyloxyt = axa-4(20),11-diene or sinenxane B (**3**)	261.0	Ma *et al.* (1994)
4	27.91	546	$C_{31}H_{46}O_8$	2α,5α,10β-Triacetoxy-14β-(2-methyl)buty = ryloxytaxa-4(20),11-diene or sinenxane C (**4**)	45.2	Ma *et al.* (1994)
5	16.92	853	$C_{47}H_{51}NO_{14}$	Taxol (**5**)	5.5 ± 0.8	Wani *et al.* (1971)
6	8.21	586	$C_{31}H_{38}O_{11}$	Baccatin III (**6**)	18.4	Senilh *et al.* (1984)

[a] Retention time.
Contents were calculated according to cell dry weight.
Reference documents refer to the original literature in which the isolation, identification and name of a taxoid was first reported.

for the putative quantification of the taxoids that possess similar structures. From the ARF point of view, the related taxoids in the present study can be divided into two groups: one contains a normal taxane skeleton with the oxetane ring, an other possess a taxane skeleton with an opened oxetane ring. **5**, **6** and 10-DAB belong to the former, **1** and the identified components **2**, **3** and **4** belong to the latter. Thus, we approximately calculated the amount of the taxoids **2**, **3**, **4** and **6** by comparing the peak area ratios of the analyte with corresponding standards (**6** *vs* taxol, the others *vs* Tc). The results of identification and determination of the major taxoids in the sample are summarized in Table II.

Linearity, limit of detection (LOD), limit of quantification (LOQ) and accuracy

Six-point calibration curves were constructed over the concentration range 1.0–220 μg/ml for taxol, and 6.0–300 μg/ml for Tc. These concentration ranges were selected on the basis of an anticipated dynamic range of the ingredients in the *Taxus chinensis* cell culture system (Menhard *et al.*, 1998). It can be seen from Table III that they displayed good linear correlations in the ranges.

	Taxol	Tc
Linearity (r)	0.9993	0.9936
Range [μg/ml]	1.0–220	6.0–300
LOD [μg/ml]	0.5	1.5
LOQ [μg/ml]	1.6	4.5

Table III. Linearity, LOD and LOQ of taxol and Tc according to HPLC analysis.

LOD and LOQ for both taxol and Tc were evaluated. According to 10 μl injected sample solution, the corresponding mass to 0.5 μg/ml of the LOD of taxol should be 5 ng injected, which is similar to the result of 0.37 μg/ml (5.6 ng) in the bulk drug reported by Shao and Locke (1997).

The recovery of taxane was measured by adding taxol and Tc standard solution to lyophilized cell cultures of *Taxus chinensis*, each level was analyzed three-fold, the mean recoveries varied from 88.5–102.2% at low level, 95.7–103.4% at medium level, and 97.5–102.8% at high level, indicating a satisfiable accuracy and repeatability within a deviation of 3.4–6.5% (R.S.D.)

Conclusion

An application of LC/ESI-MS/MS and HPLC analysis has been achieved that enables simultaneous identification and determination of major taxoids in exacts of *T. chinensis* cell culture. With the help of an available taxoid structural library, the method allows rapid identification of taxoids in case of lack of the corresponding reference substances. It is also suited for taxoid routine analysis in plant or other complex matrix without complicated sampling. More significantly, all determinations and identifications can be completed only in several parallel runs. The analytical methodology provids a rapid, conventional and reliable tool to profile a group of taxol-polarity-related taxoids produced by *Taxus* cell culture for elucidating taxol biosynthesis.

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