Taxol and taxane formation in plant cell culture

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The production of taxol, cephalomannine and baccatin III from a suspension cell culture of *Taxus brevifolia* has been investigated using HPLC and tandem mass spectrometry (MS/MS). Interference from other products in the cell culture medium led to development of an MS/MS assay based on negative ion desorption chemical ionization using [$^2H_3$]-labelled internal standards for measurements of these compounds at the level of 2–8 ng cm^{-3} of medium. The use of parent scans allowed the detection of a number of new taxanes for further study.

Many natural antitumour agents induce G_2/M phase cell cycle arrest of tumour cells. Among these antimitotic natural products, taxol isolated from the bark of *Taxus brevifolia*[1,2] is the first compound to cause cell cycle arrest by promoting the assembly of microtubules *in vitro*.[3] Taxol (paclitaxel) has recently been approved for the treatment of ovarian and breast cancers.[4] Since, currently, the bark of *T. brevifolia* is the only approved source for clinically used taxol, the increasing demand for this compound has stimulated intensive efforts in searching for alternative sources and approaches to produce it.[2] A total synthesis of taxol has been elegantly achieved[5] although the overall yield is still too low to be a viable approach for commercial production. Successful semisynthesis of taxol from synthetic phenylisoserine and natural baccatin III or its precursors isolated from the needles of yew trees will be an immediate alternative source for providing urgently needed taxol.[6] Another attractive alternative is the culturing of plant tissues. Several reports[7,8] and patents[9] have disclosed the production of taxol and other taxanes by callus tissues or suspension culture of cells. The purpose of the present study was to investigate the biochemical production of taxol, cephalomannine and baccatin III from the suspension culture of *T. brevifolia* callus derived from the stem segments. A highly compound-specific and sensitive tandem mass spectrometric method has been developed for quantitative determination of taxanes.

Results and discussion

Determination of taxol and related taxanes

The concentration of taxol in various species of *Taxus* trees is very low.[10–13] We expected that its concentration in the initial plant cells in culture would be even lower still. Therefore, it was critical to develop a highly sensitive analytical method to monitor the biosynthesis of taxol and related taxanes in cell culture and thereby allow modification of the growth conditions (media, pH, hormone, light cycle, chemical elicitation and biological elicitation) for further optimization of the production yield.

Specific antibodies for the detection of taxol have been developed.[14] The indirect competitive inhibition enzyme immunoassay used in our studies utilized a commercially available monoclonal antibody with broad specificity for taxanes with the C_{13}-oxygen functional group and oxetane ring. This immunoassay was used to determine three key taxanes at a concentration of approximately 10 nmol dm^{-3} (EC_{50}: taxol, 7 nmol dm^{-3}; cephalomannine, 6 nmol dm^{-3}; baccatin III, 12 nmol dm^{-3}). However, this assay could not be used for the measurement of the concentration of a specific taxane. Therefore, a specific analytical method was sought.

Table 1 MS/MS and HPLC quantitation of taxanes in plant tissue culture extracts

	ng taxane cm^{-3} of medium		
Method[a]	Taxol	Cephalomannine	Baccatin III
MS/MS	2.0×10^2 (4.5)	3.7×10^2 (6.6)	7.2×10^1 (3.8)
HPLC	1.7×10^2	3.1×10^2	1.3×10^2

[a] MS/MS percent relative standard deviations are displayed in parentheses.

Several HPLC methods have been developed for the analysis of taxanes in crude extracts of *Taxus* plants[8] or cells in culture.[7] In general, reverse phase separations with a gradient solvent system containing methanol–acetonitrile have been used. In order to eliminate column re-equilibration and increase throughput, we developed a flow rate gradient system using tetrahydrofuran instead of acetonitrile to maximize the separation of taxol from cephalomannine (Fig. 1A). This separation system allows the complete separation of taxol, cephalomannine, baccatin III and their 10-deacetyl analogues. The chromatogram of the methanol extract (Fig. 1B) of the medium of *T. brevifolia* suspension cell culture clearly shows the presence of taxol and cephalomannine at a concentration of approximately 200 ng cm^{-3} (Table 1). The quantitation of baccatin III, however, was hampered by other constituents (Fig. 1B). A similar limitation for the quantitation of baccatin III and O^{10}-deacetylbaccatin III was also observed in the HPLC analysis of numerous extracts of various *Taxus* needles. It thus becomes evident that a more compound-specific analytical method is essential for the simultaneous quantitation of taxol, cephalomannine and baccatin III in a crude extract.

Mass spectrometry

Mass spectral analyses have been used for structural identification of taxol and related taxanes using fast-atom bombardment,[15] matrix-assisted laser desorption,[16] thermospray[13] and electrospray[8b,17] ionization techniques. Our laboratories[10,11] and other groups[18] recently investigated the mass spectrometry of taxanes using desorption chemical ionization (DCI). This is a soft ionization method suitable for studying non-volatile, thermally unstable compounds, such as taxol. In addition, it is a matrix-free ionization method, completely devoid of matrix interference, which facilitates the analysis of ng–pg amounts of substances in a complex mixture.

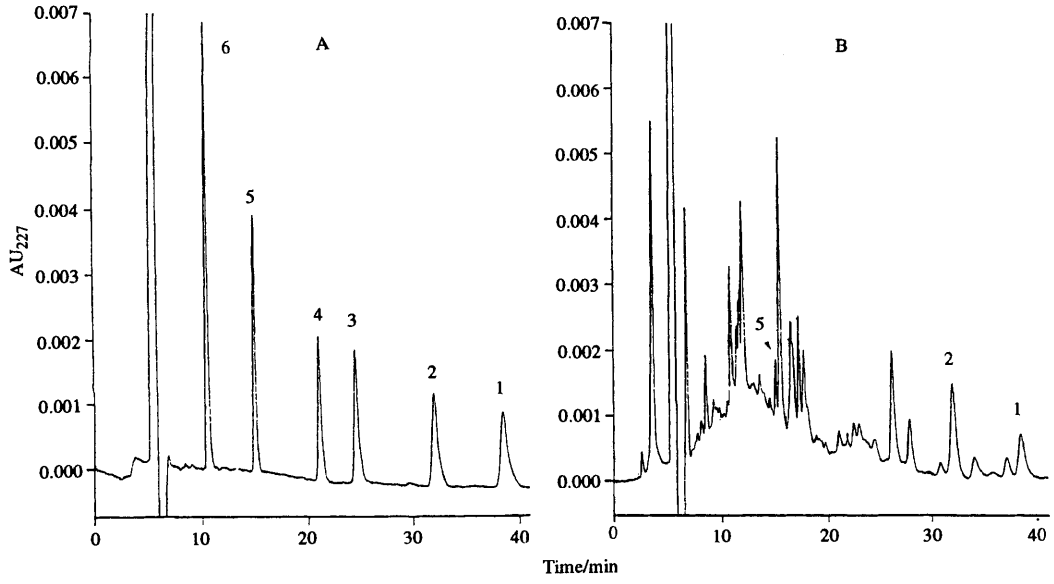


Fig. 1 C_{18}-reverse phase HPLC chromatograms of taxanes. (A) Standard compounds, and (B) MeOH extract of *Taxus brevifolia* cell suspension cultures. 1, taxol; 2, cephalomannine; 3, O^{10}-deacetyltaxol; 4, O^{10}-deacetylcephalomannine; 5, baccatin III; 6, O^{10}-deacetylbaccatin III.

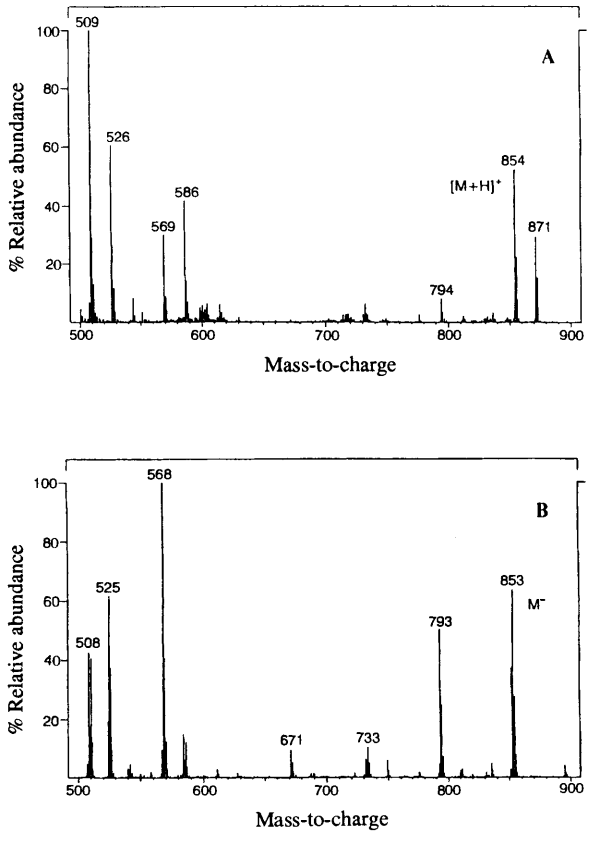


Fig. 2 Desorption chemical ionization spectra of taxol: (A) positive ion and (B) negative ion. Both spectra were taken with ammonia as the reagent gas

For the development of a mass spectrometric method, different reagent gases were evaluated based on the absolute intensity of molecular or pseudo molecular ion produced. Ammonia was initially investigated as a reagent gas for the ionization of the desorbed neutral taxanes. In the positive ion spectrum, Fig. 2A, the protonated molecular cation (*m/z* 854) along with the ammoniated cation (*m/z* 871) are observed in addition to several structurally characteristic fragment ions, which were also observed by other groups.[18] In the negative ion ammonia spectrum, the radical anion (*m/z* 853) is observed along with [M − H]. (*m/z* 852) and several fragment ions which are comparable to the fragments observed in the positive ion spectrum. However, under similar conditions, the absolute intensity of the radical anion is nearly an order of magnitude greater than that of the protonated cation. Other reagent gases were investigated including isobutane and ethylamine; however, none of these gases produced a molecular or pseudo molecular ion which was significantly more intense than the radical anion produced with ammonia. With this mode of ionization, single stage mass spectrometry was unable to provide the compound specificity for the quantitation of taxanes in crude extracts. For these reasons, a mass spectrometric method was developed using negative ion ammonia chemical ionization tandem mass spectrometry.

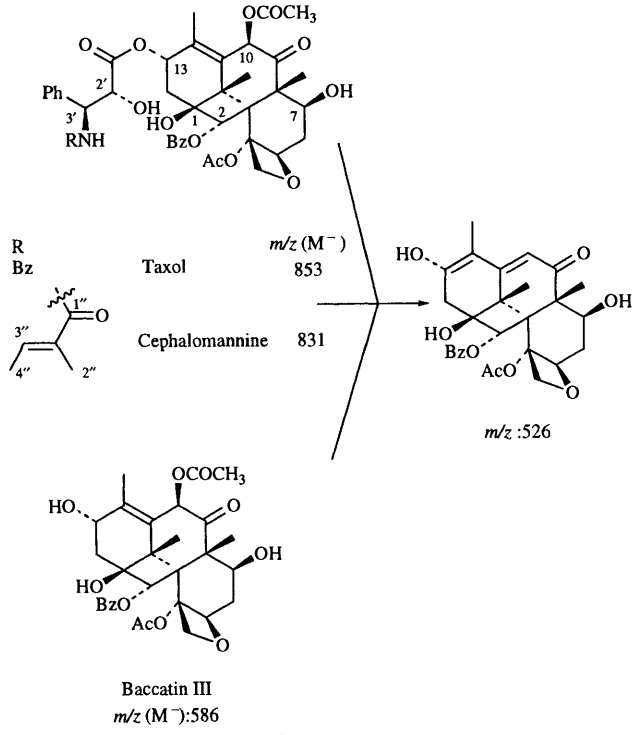
Tandem mass spectrometry (MS/MS) combines high separation power with the unique capability for compound characterization.[8b,11,13,17,19,20] Two different MS/MS modes were used to identify (essential) taxanes of complex mixtures.[11] In each of these scans, the general reaction $m_1^{\bullet-} \longrightarrow m_2^{\bullet-} + m_3$ is monitored. In the product ion scan, $m_1^{\bullet-}$ is held constant and the induced fragments ($m_2^{\bullet-}$) are used to characterize the parent molecular ion. In the parent scan, $m_2^{\bullet-}$ is held constant and the set of parents ($m_1^{\bullet-}$) which give rise to the particular product ion ($m_2^{\bullet-}$) are determined. All the product ion spectra of taxol, cephalomannine and baccatin III were characterized by a common fragment of *m/z* 526 (Scheme 1). Therefore, taxol, cephalomannine and baccatin III could be simultaneously monitored by scanning the parent ions of *m/z* 526 and quantification was possible with the method of external standard addition. This quantitation method requires 4–6 measurements at different concentrations of external standards to yield adequate reproducibility (relative standard deviation = *ca.* ± 30%).[11]

Internal standard

The reproducibility and throughput of the MS/MS parent scan can be improved by using stable isotope-labelled internal standards. A preliminary synthetic scheme was designed for the preparation of [$^2H_3$]taxol from O^{10}-deacetyltaxol, which was utilized to determine taxol content in crude extracts of different *T. brevifolia* needles and bark samples.[10] This trideuteriotaxol sample also contains [$^2H_2$]taxol (28%). A new synthetic scheme was thus designed to prepare the trideuterio standard compounds of taxol, cephalomannine and baccatin III (Scheme 2). The O^{10}-acetyl group of taxol and cephalomannine was selectively removed using zinc acetate. The [$^2H_3$]acetyl group was attached to the O^{10}-deacetyl compounds, and then separated by thin-layer chromatography to yield pure internal

references for taxol and cephalomannine, respectively. The [$^2H_3$]baccatin III was then obtained from reductive cleavage of the phenylisoserine side chain.

The product ion spectra of all three internal standard compounds showed the loss of the O^{10}-[$^2H_3$]-acetyl group in conjunction with the cleavage of the phenylisoserine side chain to produce the peak at *m/z* 526. This result confirms that loss of acetic acid primarily occurs at the 10-position and not at the 4-position, under these activation conditions (Scheme 1). The

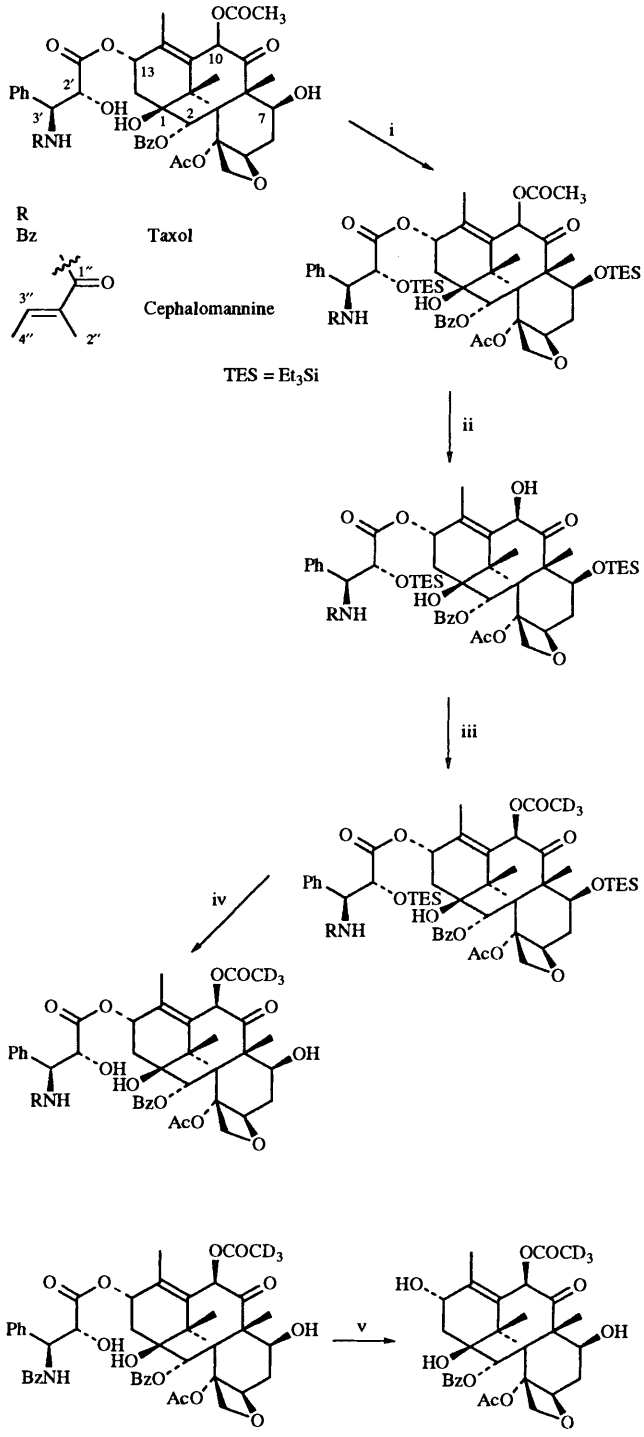


Scheme 1

mass spectrometer's response to the internal standards was evaluated by integrating the reconstructed ion chromatograms generated by multiple reaction monitoring of taxol, cephalomannine and baccatin III and the corresponding [$^2H_3$]-compounds. Calibration curves were constructed for the trideuteriated internal standards by plotting the ratio of integrated ion chromatograms of the undeuteriated to trideuteriated compounds *versus* the mass ratio of the same compound.

Quantitative determination of taxanes in plant suspension cultures

Suspension culture of cells initiated from callus tissue of *T. brevifolia* young stems was grown in a semicontinuous manner. At the time of transfer, one-third of the cells and medium was removed and replenished with fresh medium. The cells were removed from the medium and the medium was extracted (see Experimental section). The MeOH fraction (see Experimental section) was subjected to direct HPLC or MS analysis. A single-stage mass spectrum of the crude MeOH fraction is shown in Fig. 3A. It displays the presence of three targeted taxanes at *m/z* 853, 831 and 586. The parent ion MS/MS spectrum of *m/z* 526 remarkably improves the signal-to-noise ratio (Fig. 3B). The product ion MS/MS spectra of these peaks (not shown) verified the structural identity of taxol, cephalomannine and baccatin III, respectively. Using internal trideuterio standards, quantitation of these taxanes was performed by recording the MS/MS parent ion spectrum of *m/z* 526. The results of the HPLC and MS/MS analyses are displayed in Table 1 and compare favourably. The higher value for baccatin III determined by the HPLC is attributed to the overlap with other impurities (Fig. 1B). This study clearly illustrates the superior specificity of the MS/MS method.



Scheme 2 *Reagents and conditions:* i, TESCl, pyridine, room temp., 24 h; ii, $Zn(OAc)_2$, MeOH–$CHCl_3$, 55 °C, 5 days; iii, CD_3COCl, pyridine, 3 h; iv, 1% HCl–MeOH, room temp., 2 h; v, Bu_4NBH_4, room temp., 34 h

Other taxanes

The parent ion spectrum of *m/z* 526 of the tissue culture also discloses the presence of other unidentified taxanes with the same diterpenoid skeleton (Fig. 3B). The parent ion spectrum of *m/z* 284 (phenylisoserine fragment) is shown in Fig. 3C. This spectrum also reveals the existence of a new phenylisoserine-containing taxane with a different diterpenoid moiety (*m/z* 795). Meanwhile, the single-stage mass spectrum also suggests the presence of two abundant taxanes (*m/z* 652 and 610) (Fig. 3A) with neither the phenylisoserine side-chain nor the baccatin III fragment. Further isolation and structure elucidation of these new taxanes are in progress.

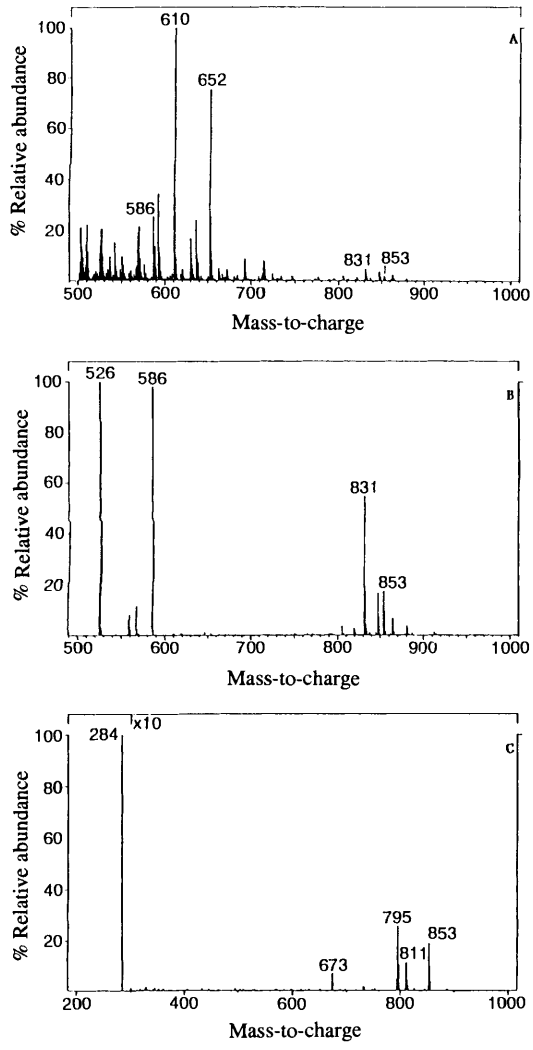


Fig. 3 Desorption negative chemical ionization mass spectra of the MeOH extract of *Taxus brevifolia* cell suspension cultures. (A) Single-stage mass spectrum, (B) parent ion spectrum of *m/z* 526, and (C) parent ion spectrum of *m/z* 284.

Experimental

Materials

Taxol, cephalomannine, O^{10}-deacetyltaxol, O^{10}-deacetylcephalomannine, baccatin III and O^{10}-deacetylbaccatin III were provided by Professor Qizhen Fang, Institute of Meteria Medica, Chinese Academy of Medical Sciences, Beijing, China, and by Drs Kenneth M. Snader and Matthew Suffness of the National Cancer Institute. The standard mixture containing 16.7 μg cm^{-3} of each reference compound was prepared in MeOH. Gamborg's B5 basal medium with minimal organics, 2,4-dichlorophenoxyacetic acid, 1-naphthalylacetic acid, *p*-nitrophenyl phosphate, and goat anti-mouse IgG alkaline phosphatase conjugate were purchased from Sigma Chemical Co.

Initiation of callus tissue

Taxus brevifolia callus cultures were obtained from Dr Paula Chee (Upjohn Company, Kalamazoo, MI), or initiated from young shoots of *T. brevifolia* plantlets obtained from Dr Richard Spjut (World Botanical Associates, Laural, MD) collected in Oregon. Ex-plant tissue, young needles and stem sections were sterilized by immersion in 70% ethanol for 30 s, washed with sterile distilled water and treated with 1.75% aqueous sodium hypochlorite for 5–10 min with shaking. After thorough washing with sterile distilled water, 3–5 mm segments were placed on B5 basal medium [21] supplemented with 2.5 mg dm^{-3} 2,4-dichlorophenoxyacetic acid (2,4-D), 1 mg dm^{-3} 1-naphthalylacetic acid (NAA), 0.8% agar, and 3% sucrose. The pH of the medium was adjusted to 5.6–5.7 before autoclaving for 15 min at 121 °C and 17 lb in^2 (psi). Undifferentiated growth appeared after 6 weeks of incubation in the dark at 25–26 °C.

Suspension culture of plant cells

Cell suspension cultures were initiated from callus tissue. Cultures were incubated in the dark at 25–26 °C in B5 medium,[21] composition as indicated in the callus tissue section except that agar was omitted, and placed on a rotary shaker at 110 rpm. Cultures were maintained in a semicontinuous mode [22] by removing one third of the cells and used medium every 5 days and replacing them with the same volume of fresh medium.

Cells were removed from medium through vacuum filtration and the medium (50 cm^3) was extracted three times with an equal volume of methylene dichloride. After evaporation of the combined methylene dichloride extracts under reduced pressure, the residue was partitioned between equal volumes of 90% aqueous MeOH and hexane. The MeOH layer was removed and evaporated to dryness. The residue was taken up in MeOH (0.5 cm^3 for each 50 cm^3 original medium) and the partitioning repeated once more. Aliquots of the final methanol fraction were used for the immunological and HPLC determination of taxanes.

Immunoassay

The total amount of taxanes was determined with an enzyme-linked immunoassay purchased from Hawaii Biotechnology Group, Aiea, Hawaii. Briefly, a 1% taxol-protein coating antigen solution was used to coat the wells of a 96 well microtitre plate. The monoclonal primary antibody (TA04) supplied, was diluted 1:1000. Standard curves were obtained with baccatin III at concentrations from 0 to 270 ng cm^{-3}. Goat anti-mouse IgG alkaline phosphatase conjugated was used as the secondary antibody and *p*-nitrophenyl phosphate as the alkaline phosphatase substrate. Samples were diluted to yield values within the linear portion of the standard curve. After the recommended washing and incubation steps, the absorbance of each well was determined on a dual wavelength ELISA reader at 414 nm (reference wavelength = 690 nm). In a typical immunoassay, 8.1 mg of extract from 50 cm^3 of medium yielded a total taxane concentration of 472.4 μg (two independent determinations, each in duplicates).

HPLC Analysis

The HPLC system consists of a Rheodyne 7125 injector, Hitachi L-6200 pump, Hitachi L-4000 UV detector and Hitachi D-2500 integrator. An Alltech Econosphere C-18, 5 μ cartridge (250 × 4.6 mm) with C-18 guard cartridge was used for analysis. Solvents (MeCN, THF and MeOH) are HPLC grade, and filtered through 0.2 μm Nylon 66 filter prior to use. Analysis was performed using a mobile phase H_2O–MeCN–THF (58:33:9) and monitored by UV absorption at 227 nm. The flow rate gradient elution was 0.4–1.4 cm^3 min^{-1} within 25 min, and after 25 min the flow rate was 1.4 cm^3 min^{-1}. Six mm^3 of the MeOH solution (equivalent to 600 mm^3 of the original cell culture medium) was used in each injection.† Quantitation of taxanes was performed by addition of the mixture of standards into the MeOH solution of the cell culture extract.

Mass spectrometry

Taxane-containing solutions were prepared for analysis by pipetting onto a rhenium wire filament of the direct evaporation probe. This probe was then introduced into the source of a

† 1 mm^3 = 1 μl.

Finnigan Triple Stage Quadrupole (TSQ) 700 mass spectrometer. The temperature of the filament was stepped from ambient to 150 °C and then ramped to 800 °C at a rate of 22 °C s^{-1} while data were acquired. The temperature was finally stepped to 1200 °C to pyrolyse any remaining material. Radical anions of taxol, cephalomannine, baccatin III and the corresponding [$^2H_3$]-internal standards were produced by electron attachment using ammonia as the reagent gas at an indicated pressure of 7000 mTorr. Under these ionization conditions, some $[M - H]^-$ is also produced but this form of molecular ion was not utilized in the MS/MS quantitation procedure.

Quantitative analysis of taxol, cephalomannine and baccatin III was performed using a parent ion scan. Quadrupole 3 was set to pass the common product ion of *m/z* 526 while scanning quadrupole 1. Collisional activation was achieved in all experiments by accelerating ions to a nominal 30 eV and colliding them with an argon target at 0.5 mTorr. To optimize sensitivity, a modified form of the parent ion scanning procedure was utilized in which a 10 dalton window was scanned in 0.05 s around each of the parent ions of the standard taxanes. The instrument response was obtained by integrating the reconstructed ion chromatogram for each of the ions corresponding to the radical species of taxol, cephalomannine, baccatin III and the corresponding [10-$^2H_3$]-compounds. The desorption time for the compound of interest typically ranged between 3 and 10 s.

Prior to quantitation, the instrument response was calibrated for all three internal standards *versus* the corresponding standard compounds. Solutions of standards and internal standards were prepared in methylene dichloride. A single filament was used for the simultaneous calibration of all three internal standards at a given level. The instrument response to [O^{10}-$^2H_3$]taxol was established by analysing 0.4, 1.0, 2.0, 3.0 and 4.0 ng of taxol; each sample was spiked with 1.0 ng of [O^{10}-$^2H_3$]taxol. The corresponding amounts of cephalomannine and [O^{10}-$^2H_3$]cephalomannine were loaded, respectively. Baccatin III was calibrated at a slightly lower level, 0.08, 0.2, 0.4, 0.6 and 0.8 ng aliquots of baccatin III were loaded onto filaments along with 0.2 ng of [O^{10}-$^2H_3$]baccatin III. The lowest level of each taxane was loaded on the same filament and the ion currents for all taxanes were simultaneously recorded. The instrument response to the [$^2H_3$]taxanes and each level of taxane standard was recorded five times before moving to the next amount of standard. A plot of the ratio of instrument responses to each taxane and [O^{10}-$^2H_3$]taxane *versus* the mass ratio of the same compounds was then constructed for instrument calibration.

Plant tissue culture extracts were prepared for MS/MS analysis by two separate procedures. The first involved spiking 97.2 μg of the MeOH extract with 83.3 ng of [O^{10}-$^2H_3$]taxol, 200.0 ng of [O^{10}-$^2H_3$]cephalomannine and 36.0 ng of [O^{10}-$^2H_3$]baccatin III in MeOH. This sample was then evaporated to dryness and reconstituted in 100 mm^3 of methylene dichloride in preparation for MS/MS analysis. All samples were prepared by dissolution in 100 mm^3 of methylene dichloride; 1 mm^3 of the resulting solution was analysed corresponding to a trideuteriated and hence a standard taxane level in the high fmol to low pmol range. Each of these determinations was performed in triplicate and the concentration of each taxane in a given sample was determined from the calibration curve. The concentration of each taxane in the cell culture was then calculated and is shown in Table 1.

$O^{2'}$,O^7-Bis(triethylsilyl)-taxol and $O^{2'}$,O^7-bis(triethylsilyl)-cephalomannine

To the cold solution of taxol and cephalomannine (3.2:1, 100 mg, 0.11 mmol) in pyridine (1 cm^3) was added triethylsilyl chloride (0.4 cm^3, 2.4 mmol), dropwise. The reaction mixture was stirred at room temperature for 24 h. The solvent was removed under reduced pressure and the residue was diluted with ethyl acetate (6 cm^3). The organic solution was washed with 0.1 mol dm^{-3} hydrochloric acid (2 cm^3 × 2) and brine (2 cm^3), dried (Na_2SO_4) and concentrated under reduced pressure. The residue was separated by silica gel chromatography with CH_2Cl_2–CH_3OH (20:1) as eluent. The products, a mixture of $O^{2'}$,O^7 bis(triethylsilyl)taxol and $O^{2'}$,O^7-bis(triethylsilyl)cephalomannine (110 mg, 91%) were obtained as white amorphous solid; δ_H(500 MHz, $CDCl_3$) 0.3–0.5 (m, CH_3CH_2Si), 0.5–0.6 (m, CH_3CH_2Si), 0.77 (t, *J* 4.0, CH_3CH_2Si),‡ 0.79 (t, *J* 8, CH_3CH_2Si), 0.90 (t, *J* 8, CH_3CH_2Si), 0.92 (t, *J* 8, CH_3CH_2Si), 1.78 (d, *J* 1.5, cephalomannine 4″-H), 2.15 (d, *J* 1.5, cephalomannine 2″-H), 5.53 (dd, *J* 2.0, 9.0, cephalomannine 3′-H), 5.68 (dd, *J* 2.0, 9.0, taxol 3′-H), 6.41 (dq, *J* 1.5, 7.5, cephalomannine 3″-H), 6.66 (d, *J*, 9.0 cephalomannine NH), 7.10 (d, *J* 9.0, taxol NH) and 7.73 (d, *J* 3, taxol *o*-H of NBz).

$O^{2'}$,O^7-Bis(triethylsilyl)-O^{10}-deacetyltaxol and $O^{2'}$,O^7-bis(triethylsilyl)-O^{10}-deacetylcephalomannine

To a solution of a mixture of $O^{2'}$,O^7-bis(triethylsilyl)taxol and $O^{2'}$,O^7-bis(triethylsilyl)cephalomannine (100 mg, 0.09 mmol) in methylene dichloride–methanol (1:3; 15 cm^3) was added zinc acetate (60 mg, 3.5 equiv.). The reaction mixture was stirred at 55 °C for 5 days after which it was evaporated under reduced pressure, and the residue was diluted with ethyl acetate (5 cm^3). The resulting solution was washed with brine (2 cm^3 × 2), dried (Na_2SO_4) and evaporated under reduced pressure. The residue was separated by TLC [developed twice with CH_2Cl_2–acetone (20:1)] to give $O^{2'}$,O^7-bis(triethylsilyl)-O^{10}-deacetyltaxol [34 mg, 76% based on recovered unchanged $O^{2'}$,O^7-bis(triethylsily)taxol] and $O^{2'}$,O^7-bis(triethylsilyl)-O^{10}-deacetylcephalomannine [12 mg, 73% based on recovered unchanged $O^{2'}$,O^7-bis(triethylsilyl)cephalomannine] as white powder.

$O^{2'}$,O^7-bis(triethylsilyl)-O^{10}-deacetyltaxol δ_H(500 MHz, $CDCl_3$) 0.3–0.5 (m, 4 H, CH_3CH_2Si), 0.5–0.6 (m, 8 H, CH_3CH_2Si), 0.75 (t, 9 H, *J* 8, CH_3CH_2Si), 0.87 (t, 9 H, *J* 8, CH_3CH_2Si), 1.03 (s, 3 H, 17-H), 1.46 (s, 3 H, 16-H), 1.68 (s, 3 H, 19-H), 1.83 (ddd, 1 H, *J* 2.5, 11.0, 15.0, 6-H), 1.87 (s, 3 H, 18-H), 2.07 (dd, 1 H, *J* 9.5, 15.0, 14-H), 2.34 (dd, 1 H, *J* 9.5, 15.0, 14-H), 2.45 (ddd, 1 H, *J* 7.0, 9.5, 15.0, 6-H), 2.48 (s, 3 H, 4-OAc), 3.82 (d, 1 H, *J* 7, 3-H), 4.16 (d, 1 H, *J* 8.5, 20-H), 4.22 (br s, 1 H, 10-OH), 4.26 (d, 1 H, *J* 8.5, 20-H), 4.32 (dd, 1 H, *J* 7.0, 11.0, 7-H), 4.62 (d, 1 H, *J* 2.0, 2′-H), 4.88 (dd, 1 H, *J* 2.5, 9.5, 5-H), 5.04 (s, 1 H, 10-H), 5.60 (d, 1 H, *J* 7, 2-H), 5.64 (dd, 1 H, *J* 2.0, 9.5, 3′-H), 6.23 (t, 1 H, *J* 9.5, 13-H), 7.08 (d, 1 H, *J* 9.5, NH), 7.28 (tt, 1 H, *J* 1.5, 7.0, *p*-H of phenyl), 7.31 (dd, 2 H, *J* 8.0, 8.5, *m*-H of NBz), 7.36 (dd, 2 H, *J* 7.0, 8.5, *m*-H of phenyl), 7.38 (tt, 1 H, *J* 1.5, 8.0 Hz, *p*-H of NBz), 7.47 (dd, 2 H, *J* 1.5, 7.5 Hz, *o*-H of phenyl), 7.50 (dd, 2 H, *J* 7.5, 8.0 Hz, *m*-H of OBz), 7.58 (tt, 1 H, *J* 1.0, 7.5, *p*-H of OBz), 7.68 (dd, 2 H, *J* 1.5, 8.5, *o*-H of NBz), 8.07 (dd, 2 H, *J* 1.0, 8.0, *o*-H of OBz). $O^{2'}$,O^7-Bis(triethylsilyl)-O^{10}-deacetylcephalomannine δ_H(500 MHz, $CDCl_3$) 0.3–0.5 (m, 4 H, CH_3CH_2Si), 0.5–0.6 (m, 8 H, CH_3CH_2Si), 0.78 (t, 9 H, *J* 7.0, CH_3CH_2Si), 0.92 (t, 9 H, *J* 7.0, CH_3CH_2Si), 1.09 (s, 3 H, 17-H), 1.23 (s, 3 H, 16-H), 1.69 (dq, 1 H, *J* 1.0, 7.0, 4″-H), 1.73 (s, 3 H, 19-H), 1.78 (d, 3 H, *J* 1.0, 18-H), 1.89 (ddd, 1 H, 1.5, 10.5, 15.0, 6-H), 1.90 (d, 3 H, *J* 1.0, 2″-H), 2.11 (dd, 1 H, *J* 8.5, 15.0, 14-H), 2.22 (dd, 1 H, *J* 8.5, 15.0, 14-H), 2.44 (ddd, 1 H, *J* 5.0, 6.5, 15.0, 6-H), 2.49 (s, 3 H, 4-OAc), 3.82 (d, 1 H, *J* 7.0, 3-H), 4.20 (d, 1 H, *J* 8.5, 20-H), 4.26 (s, 1 H, 10-OH), 4.30 (d, 1 H, *J* 8.5, 20-H), 4.37 (dd, 1 H, *J* 6.5, 10.5, 7-H), 4.60 (d, 1 H, *J* 2.0, 2′-H), 4.92 (dd, 1 H, *J* 1.5, 5.0, 5-H), 5.10 (s, 1 H, 10-H), 5.53 (dd, 1 H, *J* 2.0, 9.0, 3′-H), 5.65 (d, 1 H, *J* 7.0, H-2), 6.26 (t, 1 H, *J* 8.5, 13-H), 6.41 (qd, 1 H, *J* 1.0, 7.0, 3″-H), 6.69 (d, 1 H, *J* 9.0, NH), 7.26 (tt, 1 H, *J* 1.0, 7.5, *p*-H of phenyl), 7.27 (t, 2 H, *J* 7.5, *m*-H of phenyl), 7.35 (tt, 2 H, *J* 1.0, 7.5, *o*-H of phenyl), 7.49 (dd, 2 H, *J* 7.5, 8.5, *m*-H of OBz), 7.57

‡ *J* Values recorded in Hz.

(tt, 1 H, *J* 1.5, 7.5, 2-H, *p*-H of OBz) and 8.11 (dd, 2 H, *J* 1.5, 8.5, *o*-H of OBz).

$O^{2'}$,O^{7}-Bis(triethylsilyl)[O^{10}-*acetyl*-$^{2}H_3$]taxol

To a solution of $O^{2'}$,O^{7}-bis(triethylsilyl)-O^{10}-deacetyltaxol (pre-equilibrated with D_2O, 24 mg, 0.023 mmol) in pyridine was added [$^{2}H_3$]acetyl chloride (0.016 cm^3, 0.22 mmol). After the reaction mixture had been stirred at room temperature for 3 h it was evaporated under reduced pressure. The residue was diluted with ethyl acetate and the solution washed with D_2O, 0.1 mol dm^{-3} hydrochloric acid, sat. aqueous $NaHCO_3$ and brine, dried (Na_2SO_4) and evaporated under reduced pressure. The residue was separated by TLC [developed with CH_2Cl_2–CH_3OH (40:1)], to give the product (16 mg, 64%) as white powder; δ_H(500 MHz, $CDCl_3$) 0.3–0.5 (m, 4 H, CH_3CH_2Si), 0.5–0.56 (m, 4 H, CH_3CH_2Si), 0.77 (t, 9 H, *J* 9.0, CH_3CH_2Si), 0.84 (t, 9 H, *J* 9.0, CH_3CH_2Si), 1.11 (s, 3 H, 17-H), 1.16 (s, 3 H, 16-H), 1.64 (s, 3 H, 19-H), 1.87 (ddd, 1 H, *J* 1.5, 10.5, 15.0, 6-H), 1.96 (d, 3 H, *J* 1, 18-H), 2.10 (dd, 1 H, *J* 10.0, 15.5, 14-H), 2.38 (dd, 1 H, *J* 10.0, 15.5, 14-H), 2.49 (ddd, 1 H, *J* 6.5, 9.5, 15.0, 6-H), 2.48 (s, 3 H, 4-OAc), 3.76 (d, 1 H, *J* 7.0, 3-H), 4.15 (d, 1 H, *J* 8.5, 20-H), 4.27 (d, 1 H, *J* 8.5, 20-H), 4.41 (dd, 1 H, *J* 6.5, 10.5, 7-H), 4.64 (d, 1 H, *J* 2.0, 2′-H), 4.89 (dd, 1 H, *J* 1.5, 9.5, 5-H), 5.60 (d, *J* 7.0, 2-H), 5.64 (dd, 1 H, *J* 2.0, 9.0, 1 H, 3′-H), 6.17 (t, 1 H, *J* 10.0, 13-H), 6.39 (s, 1 H, 10-H), 7.06 (d, 1 H, *J* 9.0, NH), 7.28 (tt, 1 H, *J* 1.0, 7.0, *p*-H of phenyl), 7.31 (dd, 2 H, *J* 7.5, 8.5, *m*-H of NBz), 7.36 (dd, 2 H, *J* 7.0, 5.5, *m*-H of phenyl), 7.38 (tt, 2 H, *J* 1.0, 7.5, *o*-H of phenyl), 7.47 (dd, 1 H, *J* 1.5, 7.5, *p*-H of NBz), 7.50 (dd, 2 H, *J* 7.5, 8.5, *m*-H of OBz), 7.58 (tt, 1 H, *J* 1.0, 7.0, *p*-H of OBz), 7.73 (dd, 2 H, *J* 1.5, 8.5, *o*-H of NBz) and 8.13 (dd, 2 H, *J* 1.0, 8.0, *o*-H of OBz).

$O^{2'}$,O^{7}-Bis(triethylsilyl)[O^{10}-*acetyl*-$^{2}H_3$]cephalomannine

Treatment of $O^{2'}$,O^{7}-di(triethylsilyl)-O^{10}-deacetylcephalomannine (pre-equilibrated with D_2O, 10 mg, 0.0096 mmol) as described above for the taxol analogue gave the trideuteriated product (8 mg 76%); δ_H(500 MHz, $CDCl_3$) 0.34–0.46 (m, 4 H, CH_3CH_2Si), 0.53–0.58 (m, 8 H, CH_3CH_2Si), 0.78 (t, 9 H, *J* 8.0, CH_3CH_2Si), 0.90 (t, 9 H, *J* 8.0, CH_3CH_2Si), 1.19, (s, 3 H, 17-H), 1.22 (s, 3 H, 16-H), 1.68 (s, 3 H, 19-H), 1.69 (dd, 3 H, *J* 1.0, 7.0, 4″-H), 1.79 (q, 1 H, *J* 1.0, 2″-H), 1.89 (ddd, 1 H, *J* 2.0, 10.5, 15.0, 6-H), 1.99 (d, 3 H, *J* 1.0, 18-H), 2.11 (dd, 1 H, *J* 9.0, 15.0 14-H), 2.21 (dd, 1 H, *J* 9.0, 15.0, 14-H), 2.35 (s, 3 H, 4-OAc), 2.45 (ddd, 1 H, *J* 7.0, 10.0, 15.0, 6-H), 3.80 (d, 1 H, *J* 7.0, 3-H), 4.18 (d, 1 H, *J* 8.5, 20-H), 4.29 (d, 1 H, *J* 8.5, 20-H), 4.45 (dd, 1 H, *J* 7.0, 10.5, 7-H), 4.61 (d, 1 H, *J* 2.0, 2′-H), 4.92 (dd, 1 H, *J* 2.0, 10.0, 5-H), 5.54 (dd, 1 H, *J* 2.0, 9.0, 3′-H), 5.69 (d, 1 H, *J* 7.0, 2-H), 6.21 (t, 1 H, *J* 9.0, 13-H), 6.40 (tt, 1 H, *J* 1.0, 7.0, 3″-H), 6.45 (s, 1 H, 10-H), 6.67 (d, 1 H, *J* 9.0, NH), 7.27 (tt, 1 H, *J* 1.0, 7.0, *p*-H of phenyl), 7.28 (dd, 2 H, *J* 7.0, 7.5, *m*-H of phenyl), 7.35 (tt, 2 H, *J* 1.0, 7.5, *o*-H of phenyl), 7.49 (dd, 2 H, *J* 7.5, 8.5, *m*-H of OBz), 7.58 (tt, 1 H, *J* 1.5, 7.5, *p*-H of OBz) and 8.11 (dd, 2 H, *J* 1.5, 8.5, *o*-H of OBz).

[O^{10}-*acetyl*-$^{2}H_3$]Taxol

To the solution of $O^{2'}$,O^{7}-di(triethylsilyl)[O^{10}-*acetyl*-$^{2}H_3$]taxol (15 mg, 0.014 mmol) in methanol (2 cm^3) was added dropwise 1% aq. HCl (1 cm^3). The reaction mixture was stirred at room temperature for 2 h after which it was concentrated under reduced pressure (< 35 °C). The residue was diluted with ethyl acetate and the solution washed with sat. aqueous $NaHCO_3$ and brine, dried (Na_2SO_4) and evaporated under reduced pressure. The residue was applied to silica gel TLC and developed with CH_2Cl_2–CH_3OH (20:1) to give product (10.2 mg, 87%) as a white powder; δ_H(500 MHz $CDCl_3$) identical with that of taxol except for the absence of the O^{10}-acetyl signal (δ 2.37, 3 H).

[O^{10}-*acetyl*-$^{2}H_3$]Cephalomannine

Treatment of $O^{2'}$,O^{7}-di(triethylsilyl)[O^{10}-*acetyl*-$^{2}H_3$]cephalomannine (3.9 mg, 0.0084 mmol) as described above for the taxol analogue gave the trideuteriated product (1.82 mg, 65%) as white powder; δ_H(500 MHz, $CDCl_3$) identical with that of cephalomannine except for the absence of the O^{10}-acetyl signal (δ 2.34, 3 H).

[O^{10}-*acetyl*-$^{2}H_3$]Baccatin III

The procedure followed was that developed by Kingston *et al.*[23] To the solution of [O^{10}-*acetyl*-$^{2}H_3$]taxol (4.3 mg, 0.005 mmol) in anh. CH_2Cl_2 (0.1 cm^3) was added tetrabutylammonium borohydride (2 mg). After the reaction mixture had been stirred at room temperature for 24 h, additional tetrabutylammonium borohydride (1 mg) was added to it followed after continued stirring for 10 h, by acetic acid (0.003 cm^3). After being stirred for 15 min, the reaction mixture was subjected to silica gel TLC [developed with CH_2Cl_2–CH_3OH (20:1)]. The product (2.5 mg, 85%) was collected as white powder; δ_H(500 MHz, $CDCl_3$) identical with that of baccatin III except for the absence of the O^{10}-acetyl signal (δ 2.24, 3 H).

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