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Taxus metabolomics: methyl jasmonate preferentially induces production of taxoids oxygenated at C-13 in *Taxus* x *media* cell cultures

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Abstract

Cells from suspension cultures of *Taxus cuspidata* were extracted with pentane as a source of relatively non-polar taxoids. Of the 13 taxoids identified in this fraction, eight were oxygenated at C-14 and two had not been previously described. These taxoids, along with existing taxoid standards, were employed to profile the metabolites of *Taxus* x *media* cv. *Hicksii* cell suspension cultures induced with methyl jasmonate to produce paclitaxel (Taxol®). The majority of the taxoid metabolites produced in these induced cultures were oxygenated at C-13, and not C-14.

Keywords: *Taxus* x *media* cv. *Hicksii*; *Taxus cuspidata*; Taxaceae; Yew; Metabolomics; Metabolic profiling; Taxane diterpenoids; Taxoids; Paclitaxel; Taxol®

1. Introduction

Plants of the genus *Taxus* (yews) produce a class of natural products known as taxane diterpenoids, or taxoids, characterized by the taxane (pentamethyl [9.3.1.0]^{3,8}tricyclopentadecane) skeleton. Over 380 taxoids and modified taxoids have been isolated and characterized (Baloglu and Kingston, 1999). The most economically and pharmaceutically important of these compounds is the anticancer drug paclitaxel (**1**; Fig. 1), known commercially as Taxol®. However, a significantly more abundant taxoid in *Taxus* is taxine B (**2**; Fig. 1), first described by Lucas (1856) as part of a mixture of compounds called "taxine." The taxines are responsible for the toxicity of yew tissues; particularly *T. baccata* L. and *T. cuspidata* L. in which taxine concentrations are highest (Wiegerinck et al., 1996). Crude "taxine" has been isolated in yields of 1.2% dry wt. of *T. baccata* L. leaves (Wiegerinck et al., 1996). In contrast, the highest paclitaxel yields are in the range of 0.01–0.04% dry wt. of the tissue (Ketchum et al., 1999c and references cited therein), although amounts up to 0.08% have been reported (Hoke et al., 1992).

The biochemical pathway that originates with taxadiene and culminates in the production of paclitaxel likely branches from the main pathway leading to the formation of the more abundant taxines. Deviations from this main pathway, the result of the variations in the pattern of cytochrome P450 oxygenations and subsequent acylations, in addition to the formation of paclitaxel (**1**, Fig. 1) and other taxoids with cytotoxic activity, are likely to lead to the production of taxoids oxygenated at C-14, and other classes of taxoids. An understanding of the enzymatic reactions that lead to paclitaxel requires an understanding of the relatively few intermediates of the 380 defined taxoid metabolites that are directly involved in paclitaxel biosynthesis. It is estimated that the biosynthesis of paclitaxel, from the universal diterpenoid precursor geranylgeranyl diphosphate, involves at least 20 distinct enzymatic steps with a similar number of taxoid intermediates (Hezari and Croteau, 1997).

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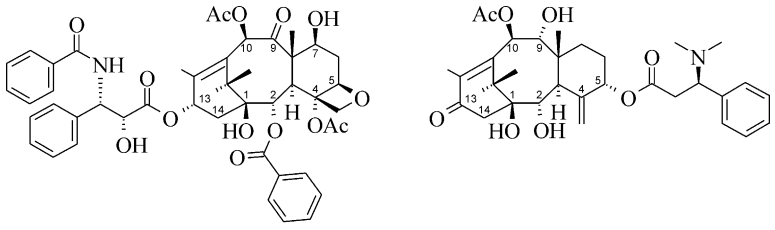
The metabolic profiling of taxoids produced by *Taxus* cell suspension cultures is an essential step in the identification of intermediates directly involved in paclitaxel biosynthesis. Equally important is the identification of metabolites that are intermediates of parallel or divergent taxoid pathways. Manipulation of the genes that encode these pathway steps, either by up- or down-regulation, requires an understanding of the identities and endogenous levels of these paclitaxel intermediates, as well as of those taxoids not involved in paclitaxel formation, and of the flux through the various pathways during the normal growth cycle and following elicitation.

Useful tools for investigating paclitaxel biosynthesis are plant cell suspension cultures of *Taxus* inducible by methyl jasmonate elicitation and capable of producing significant amounts of paclitaxel (Ketchum et al., 1999a; Yukimune et al., 1996; Mirjalili and Linden; 1996). Such cultures can allow biochemical and molecular elucidation of the paclitaxel biosynthetic pathway and can serve as a source of the relevant intermediates, enzymes and genes. In this report, we describe the principal taxoids produced by *Taxus* x *media* cv. *Hicksii* cell suspension cultures during normal growth and upon elicitation with methyl jasmonate.

2. Results and discussion

Cell suspension cultures of *Taxus cuspidata*, cell line P93AF, have been continuously maintained since October 1993 (Ketchum and Gibson, 1996). As part of continuing studies of paclitaxel production in *Taxus* sp.,



1 Paclitaxel (Taxol) **2** Taxine B

Fig. 1. Structures of paclitaxel (Taxol®; **1**) and taxine B (**2**).

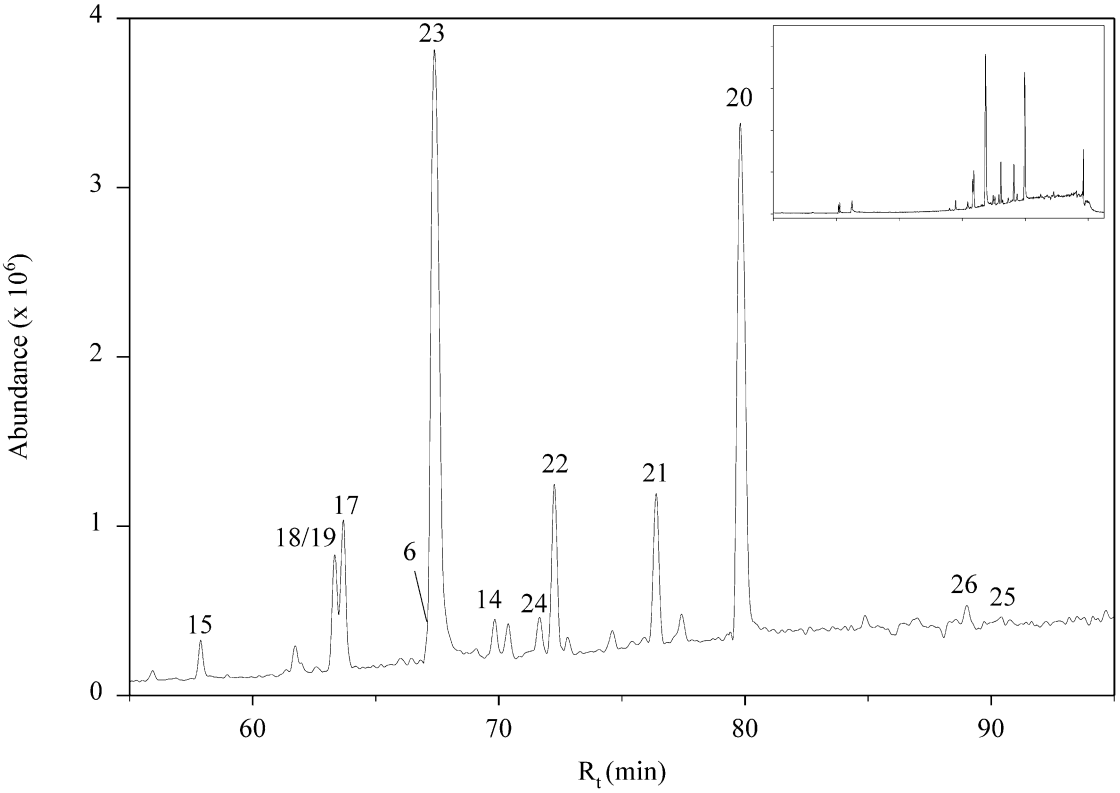
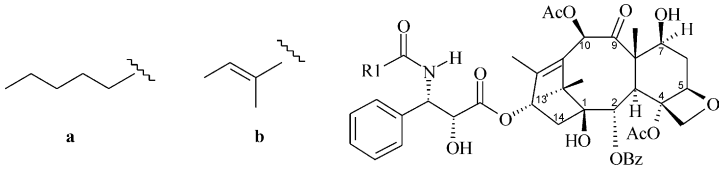


Fig. 2. Total ion mass chromatogram of the pentane extract of *Taxus cuspidata* cell line P93AF. For clarity, only the region from 20 to 85 min is shown. The full chromatogram is shown in the inset. Peak identities correspond to structures shown in Table 1.

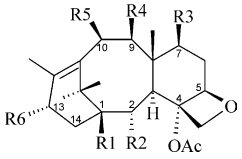
Table 1
Taxane diterpenoids (taxoids) isolated from suspension cell cultures of *Taxus cuspidata* and *Taxus* x *media*

Taxoids with an oxetane ring and C-13 phenylisoserine side chain



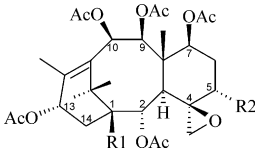
	Taxoid[a]	MW	R1
1	Paclitaxel (Taxol®)	853.33	Ph
3	Taxol C	847.38	a
4	Cephalomannine	831.35	b

Taxoids with an oxetane ring



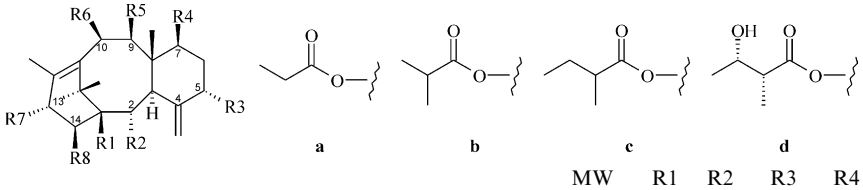
	Taxoid[a]	MW	R1	R2	R3	R4	R5	R6
5	Baccatin VI	714.29	OH	OBz	OAc	OAc	OAc	OAc
6	1β-Dehydroxybaccatin VI	698.29	H	OBz	OAc	OAc	OAc	OAc
7[b]	Baccatin IV	652.27	OH	OAc	OAc	OAc	OAc	OAc
8	9-Dihydro-13-acetyl-baccatin III	630.27	OH	OBz	OH	OH	OAc	OAc
9	9-Dihydro-baccatin III	588.26	OH	OBz	OH	OH	OAc	OH
10	Baccatin III	586.24	OH	OBz	OH	=O	OAc	OH
11	10-Deacetyl-baccatin III	544.23	OH	OBz	OH	=O	OH	OH

Taxoids with a C-4(20) epoxide



	Taxoid[a]	MW	R1	R2
12	Baccatin I	636.28	H	OAc
13	Taxuspin V	610.26	OH	OH

Taxoids with a C-4(20) double bond



	Taxoid[a]	MW	R1	R2	R3	R4	R5	R6	R7	R8
14	5α,7β,9α,10β,13α-Pentaacetoxy-2α-benzoyloxytaxa-4(20),11-diene[c]	682.30	H	OBz	OAc	OAc	OAc	OAc	OAc	H
15	2α,5α,7β,9α,10β,13α-Hexaacetoxytaxa-4(20),11-diene[d]	620.28	H	OAc	OAc	OAC	OAc	OAc	OAc	H
16[b]	5α-Hydroxy-2α,7β,9α,10β,13α-pentaacetoxytax-4(20),11-diene	578.27	H	OAc	OH	OAc	OAc	OAc	OAc	H
17	2α,5α,10β-Triacetoxy-14β-(3-hydroxy-2-methyl)butyryloxytaxa-4(20),11-diene	562.31	H	OAc	OAc	H	H	OAc	H	d
18	2α,5α,9α,10β,14β-Pentaacetoxytaxa-4(20),11-diene	562.28	H	OAc	OAc	H	OAc	OAc	H	OAc
19	5α,7β,9α,10β,13α-Pentaacetoxytaxa-4(20),11-diene	562.28	H	H	OAc	OAc	OAc	OAc	OAc	H
20	2α,5α,10β-Triacetoxy-14β-(2-methyl)butyryloxytaxa-4(20),11-diene	546.32	H	OAc	OAc	H	H	OAc	H	c
21	2α,5α,10β-Triacetoxy-14β-isobutyryloxytaxa-4(20),11-diene	532.30	H	OAc	OAc	H	H	OAc	H	b
22	2α,5α,10β-Triacetoxy-14β-propionyloxytaxa-4(20),11-diene	518.29	H	OAc	OAc	H	H	OAc	H	a
23	Taxuyunnanine C	504.27	H	OAc	OAc	H	H	OAc	H	OAc
24	2α,5α,10β-Triacetoxytaxa-4(20),11-diene[c]	446.27	H	OAc	OAc	H	H	OAc	H	H
25	5α-Acetoxytaxa-4(20),11-diene[e]	330.26	H	H	OAc	H	H	H	H	H
26	5α-Hydroxytaxa-4(20),11-diene[e]	288.25	H	H	OH	H	H	H	H	H

[a] All compounds are described and cited in Baloglu and Kingston (1999) unless otherwise noted.
[b] Compounds **7** and **16** have been tentatively identified by mass spectroscopy based on molecular ion and fragmentation pattern, but have not yet been confirmed by NMR.
[c] New compound described in this report.
[d] Kingston et al., 1993.
[e] Hefner et al., 1996.

cell suspension cultures are elicited with methyl jasmonate to induce paclitaxel production (Ketchum et al., 1999a), frozen in liquid nitrogen, and stored at −80 °C for subsequent analysis. Cell lines screened and selected for their ability to produce paclitaxel were examined for the presence of other taxoid metabolites as a source of standards and intermediates for subsequent enzymatic studies.

The diterpene olefin, taxadiene, is the first committed intermediate of taxoid biosynthesis (Koepp et al., 1995), and is formed via the cyclization of geranylgeranyl diphosphate by taxadiene synthase (Hezari et al., 1995). The next step of the pathway is oxygenation at C-5 of the olefin, by a cytochrome P450 monooxygenase, to form 5α-hydroxy-taxa-4(20),11-diene (Hefner et al., 1996). Subsequent steps involve the acylation and/or additional oxygenations; however, the exact sequence is unclear (Wheeler et al., 2001). To evaluate the presence of these early intermediates and other relatively non-polar taxoids, induced cells of *T. cuspidata*, cell line P93AF, were extracted with pentane.

The total ion mass chromatogram of the pentane extract of dried P93AF cells is presented in Fig. 2, with the corresponding peak identifications and compound structures listed in Table 1. Compounds in the pentane extract had retention times ranging from 55 to 90 min

Table 2
Taxoid production in 5 ml suspension cell cultures of *Taxus* x *media* cv. *Hicksii* with and without methyl jasmonate elicitation

Cell line + treatment	10-DAB		Baccatin III		Cephalomannine		Paclitaxel	
	(μg)	(mg l^{-1})[1]	(μg)	(mg l^{-1})[a]	(μg)	(mg l^{-1})[a]	(μg)	(mg l^{-1})[a]
Mh00W	9.07	2.59	2.91	0.83	2.36	0.67	3.92	1.12
Mh00W + MJ	37.1	10.6	7.21	2.06	5.37	1.53	25.0	7.14
Mh00D	2.86	0.82	10.4	2.97	5.88	1.68	43.7	12.5
Mh00D + MJ	3.82	1.09	13.2	3.77	1.37	0.39	14.8	4.23

[a] Concentrations are based on the 3.5 ml volume of the cultures at the time of harvest, and not on the 5 ml volume of the cultures when they are initially subcultured.

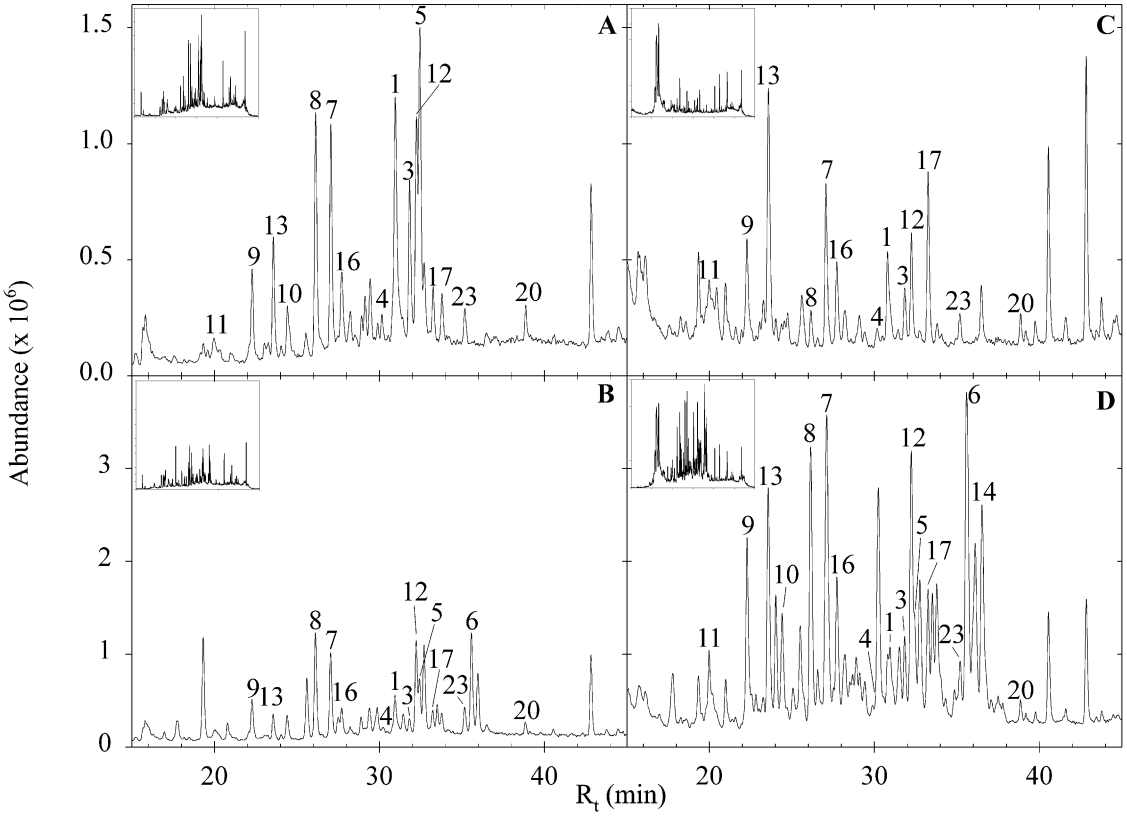


Fig. 3. Total ion mass chromatograms of acetonitrile extracts of dried *Taxus* x *media* cv. *Hicksii* cell suspension cultures. Cell lines Mh00D (A) and Mh00W (C) grown for 14 days without elicitation. Cell lines Mh00D (B) and Mh00W (D) grown for 7 days without elicitation, then grown for an additional 7 days following application of 200 μM methyl jasmonate. For clarity, only the "taxoid region" (Table 3) from 15 to 45 min is shown. The full chromatogram for each treatment is shown in the inset. Peak identities correspond to structures shown in Table 1.

when analyzed by method 3 (see Experimental section). The peaks identified comprised 90% of the integrated area of the total ion chromatogram in the region from 50 to 90 min, and 83% of the integrated area of the entire chromatogram from 0 to 105 min. Of the 13 compounds identified, one taxoid contained an oxetane ring while the other 12 were oxygenated taxa-4(20),11-dienes (Table 1). Two of these compounds have not been previously described. Compound **24** is an acetylated taxadiene with acetoxy-groups at C-2, C-5, and C-10. Compound **14** is an acetylated and benzoylated taxadiene with acetoxy-groups at C-5, C-7, C-9, C-10, and C-13, and a benzoxy-group at C-2. The only taxoid in the pentane extract that contained an oxetane ring was 1β-dehydroxybaccatin VI (**7**).

Eight of the 13 identified taxoids were oxygenated at C-14. This high proportion of C-14 oxygenated taxoids (relative to C-13 oxygenated taxoids) is similar to the distribution reported by Menhard et al. (1998) in methyl jasmonate elicited *T. chinensis* cell cultures. The major difference between this investigation and the earlier work with *T. chinensis* was the use of pentane as an extraction solvent; Menhard et al. (1998) employed methanol/diethyl ether. Undoubtedly, the proportion of C-13 to C-14 oxygenated compounds observed will vary with the polarity of the extraction solvent.

The prominence of C-14 oxygenated taxoids in induced P93AF cells, and the similar abundance of C-14 oxygenated taxoids reported by other investigators (Eisenreich et al., 1996; Menhard et al., 1998), prompted us to determine if methyl jasmonate preferentially induces the formation of C-14 oxygenated taxoids over other types. For this purpose, two differentially responsive cell lines of *Taxus* x *media* cv. *Hicksii,* derived from sibling embryos, were employed for metabolic profiling. Cell line Mh00D produces modest amounts of paclitaxel after 14 days in culture (up to 11.3 μg ml^{-1}) without methyl jasmonate elicitation. Attempts to elicit this cell line with methyl jasmonate usually result in a reduction in paclitaxel production (related to a general decline in the health of the cultures), or has no effect on paclitaxel production compared to control cultures. Cell line Mh00W produces little paclitaxel (~0.5 μg ml^{-1}) after 14 days of culture without methyl jasmonate elicitation, but produces up to 13.2 μg ml^{-1} paclitaxel when elicited with methyl jasmonate. The response to methyl jasmonate of cell line Mh00W is more typical of the results observed with cell cultures of other *Taxus* species

Table 3
Comparison of the distribution of taxoids in two cell lines of *T.* x *media* cv. *Hicksii*, with or without methyl jasmonate elicitation, based on the area of peaks (ion abundance X peak width X 10^6) in the total ion chromatograms of the acetonitrile extracts

Peak ID	R_t (min)	Mh00D	Mh00D + MJ	Change (%)	Mh00W	Mh00W + MJ	Change (%)
1	31.0	14.9	4.3	−71	1.1	6.3	472
3	31.8	6.1	1.3	−79	2.0	6.1	199
4	30.2	0.9	0.4	−55	1.0	0.0	−100
5	32.5	6.8	1.4	−79	0.0	0.0	0
6	35.6	0.0	10.0	∞	0.0	41.9	∞
7	27.1	9.6	9.2	−4	7.6	41.7	450
8	26.1	11.5	12.0	4	1.7	34.1	1937
9	22.3	4.5	5.1	14	5.4	23.6	337
10	24.4	2.9	0.0	−100	0.0	10.6	∞
11	20.0	1.0	0.0	−100	2.8	14.4	412
12	32.2	3.9	5.9	50	4.2	35.8	745
13	23.6	4.9	2.8	−42	11.5	27.3	138
14	36.5	0.0	0.0	0	0.0	28.1	∞
16	27.7	3.7	1.6	−57	3.8	12.7	233
C−14 oxygenated taxoids							
17	33.3	2.4	1.6	−32	7.5	9.2	23
20	38.9	1.3	1.5	9	1.2	2.1	80
23	35.2	1.8	2.8	59	1.7	3.9	128
Total area of chromatogram[a]		133.8	180.6	35	287.7	633.5	120
Total area of taxoid region[b]		102.0	115.9	14	102.7	481.2	369
Total area of identified taxoids		76.2	60.0	−21	51.5	297.8	478
% Identified (chromatogram[a])		57	33	−42	18	47	162
% Identified (taxoid region[b])		75	52	−31	50	62	23
Total area of C-14 taxoids		5	6	11	10	15	45
% C-14 Taxoids (chromatogram[a])		4	3	−18	4	2	−34
% C-14 Taxoids (taxoid region[b])		5	5	−2	10	3	−69
% C-14 Taxoids (identified taxoids)		7	10	41	20	5	−75

[a] Total area of all integrated peaks of the total ion mass chromatogram, omitting the solvent peaks at the beginning of the injection and the peaks following the re-equilibration of the column at 53.5 min.

[b] Total area of the integrated peaks in the region between the most polar taxoid identified, 10-deacetylbaccatin III (19.9 min), and the least polar taxoid, taxadiene (45.2 min).

(Ketchum et al., 1999a,b,c; Yukimune et al., 1996; Mirjalili and Linden, 1996). Five-millilitre cultures of Mh00D produced 44 μg of paclitaxel during non-elicited growth (12.5 mg l^{-1}), but paclitaxel amounts decreased by 66% when methyl jasmonate was added after 7 days of culture (Table 2) For cell line Mh00W, the response was the opposite, with paclitaxel amounts increasing by over 650%, to 25 μg (7.14 mg l^{-1}), following methyl jasmonate elicitation (Table 2).

Total ion mass chromatograms for both cell lines and both treatments are shown in Fig. 3 and values for the integrated peaks are presented in Table 3. For cell line Mh00D, the taxoids identified from non-elicited cells accounted for 57% of the area of the peaks in the full chromatogram, and 75% of the peaks in the taxoid region. The remaining area of the chromatogram was distributed between 54 small peaks in the full chromatogram (mean area 0.8% per peak; maximum 5.1%), and distributed between 29 small peaks in the taxoid region (mean area 0.9% per peak; maximum peak area 6.7%). The C-14 oxygenated taxoids account for a minor percentage of the taxoids identified, regardless of elicitation treatment, and ranged from 7% of the identified taxoids, to 4% of the peak area of the entire chromatogram.

Elicitation of Mh00D resulted in an increase in the integrated peak area by 35% over the entire chromatogram and 14% of the taxoid region (Table 3; Figs. 3A and B). The proportion of identified components decreased by about one-third, to 52%, when Mh00D cells were elicited with methyl jasmonate, indicating that elicitation increased the proportion of unknown compounds in these extracts. The remaining area of the chromatogram was distributed between 61 small peaks in the full chromatogram (mean area 1.6% per peak; maximum 9.1%), and distributed between 29 small peaks in the taxoid region (mean area 0.2% per peak; maximum peak area 15.1%). Elicitation resulted in a decline in nearly all taxoids containing the oxetane ring (compounds **1**–**13**) with two notable exceptions. Compound **6** increased to significant levels in Mh00D cells treated with methyl jasmonate, although this component was not detected in the control (Figs. 3A and B). Compound **12** also increased nearly 50% over the control (Table 3). Because of the decline in paclitaxel (**1**) and the increase in these oxetane-containing taxoids, it may be that compounds **6** and **12** are degradation products of more highly substituted oxetane-containing taxoids; or they may represent products of a divergent pathway induced upon elicitation. The proportion of C-14 oxygenated taxoids did not significantly change in these cells following elicitation, and ranged from 10% of the identified taxoids to 3% of the peak area of the entire chromatogram.

For uninduced cell line Mh00W, the total area of identified taxoids accounted for 51% of the entire chromatogram (Table 3; Figs. 3C and D). The proportion of C-14 oxygenated compounds was higher in uninduced Mh00W cells than in identically treated Mh00D cells, and ranged from 20% of the total identified taxoids to 4% of the area of the entire chromatogram (Table 3). Elicitation increased the integrated peak area by 120% over the entire chromatogram and by 369% over the taxoid region (Table 3; Figs. 3C and D). Taxoids identified in the non-elicited cells accounted for only 18% of the peak area of the full chromatogram, but 50% of the peak area in the taxoid region. The remaining peak area was distributed between 85 small peaks in the full chromatogram (mean area 1.5% per peak; maximum peak area 28.5%), and distributed between 33 small peaks in the taxoid region (mean area 1.5% per peak; maximum peak area 11.4%). The proportion of identified peaks increased substantially when Mh00W was elicited with methyl jasmonate, to 47% of the peak area in the entire chromatogram and to 62% of the peaks in the taxoid region. The remaining area of the chromatogram was distributed between 80 small peaks in the full chromatogram (mean area 1.3% per peak; maximum 14.6%), and distributed between 40 small peaks in the taxoid region (mean area 1% per peak; maximum peak area 6.1%). The proportion of C-14 oxygenated taxoids dropped significantly in extracts of elicited Mh00W cells, to 5% of the identified taxoids and to 2% of the area of the entire chromatogram.

Nearly all taxoids increased in total amount in cell line Mh00W following elicitation. The only exception was cephalomannine (**4**), which was present in small amounts in non-elicited cells, but was not detectable following elicitation. While **1** increased nearly 5-fold following elicitation, the greatest increase was in 13-acetyl-9-dihydro-baccatin III (**6**), with a greater than 19-fold increase following elicitation. This observation is consistent with previous work that demonstrated significant accumulation of **6** in elicited cultures of *T. canadensis* (Ketchum et al., 1999a, b). Significantly, the earlier work also showed a substantial amount of baccatin VI (**5**) to accumulate in *T. canadensis* cultures in response to methyl jasmonate elicitation; however that taxoid was not detected in elicited cultures of Mh00W (Ketchum et al., 1999b; Table 3; Fig. 3D).

In both cell lines of *Taxus* x *media* cv. *Hicksii*, elicitation with methyl jasmonate increases nearly all identifiable taxoids, but causes a proportionally greater increase in C-13 oxygenated taxoids than in C-14 oxygenated taxoids. These results are different from those of Menhard et al. (1998) who found that C-14 oxygenated taxoids accounted for a significant majority of the taxoids in induced and control *T. chinensis* cell cultures. With the cell cultures in the present investigation, there was a nearly even distribution of taxoids, with essentially all identified components within the same order of magnitude in ion abundance. Menhard et al. (1998)

found that a single compound, taxuyunnanine C (**23**), accounted for 85%, by mass, of the taxoids identified in a *Taxus chinensis* cell culture elicited with 30 μM methyl jasmonate.

There appear to be no known *naturally occurring* taxoids that contain the oxetane ring and that are oxygenated at C-14, although numerous compounds of this type have been prepared semi-synthetically (e.g. Appendino et al., 2002). While these P93AF cells had been screened previously, and were known to produce paclitaxel, they are amongst the lowest producing cultures of our present stocks, suggesting that oxygenation at C-14 may be incompatible with paclitaxel production. Taxadiene that is shunted into this pathway is diverted from the more pharmaceutically important taxoids, such as paclitaxel, baccatin III, and 10-deacetylbaccatin III. The cytochrome P450 oxygenase that catalyzes the hydroxylation at C-14 of the taxane skeleton has recently been cloned and characterized in this laboratory (Jennewein et al., submitted). This gene would be an important candidate for down-regulation in cell lines that produce large amounts of C-14 oxygenated taxoids but small amounts of the pharmaceutically important C-13 oxygenated taxoids.

The evolutionary advantage gained by *Taxus* in the production of taxoids, i.e., as a feeding deterrent, is almost certainly imparted by the much more abundant taxines than by the less abundant, but pharmaceutically significant taxoid, paclitaxel. In the current model for taxoid biosynthesis, the main pathway flux from taxadiene is directed to taxine B and related compounds, with one or more branches off this pathway leading to structurally similar taxoids bearing the oxetane function, such as paclitaxel. An understanding of the relationships of these, almost certainly, interconnected biosynthetic routes should reveal the targets for genetic engineering to divert taxadiene directly to paclitaxel, thereby maximizing the production of this important drug.

3. Experimental

3.1. Plant cell cultures

Plant cell cultures were initiated, maintained, and screened for paclitaxel production as previously described (Ketchum and Gibson, 1996; Hezari et al., 1997; Ketchum et al., 1999b). For extraction of taxoids, *Taxus cuspidata* cell line P93AF was grown for one week in B5NB suspension medium, elicited with 200 μM methyl jasmonate (Aldrich), and grown for another 7 days (Ketchum et al., 1999a). The cultures were then harvested by removing the medium, washing the cells in phosphate buffered saline, briefly drying on a 60 μm nylon filter in a Büchner funnel with vacuum, and then freezing by submersion in liquid nitrogen. Cells were stored at −35 °C prior to extraction.

For metabolic studies of actively growing cells, recently established cell cultures of *Taxus* x *media* were used. These cell lines were initiated, maintained, and elicited by similar protocols. Mh00D is a *T.* x *media* cell line that produces moderate amounts of paclitaxel and is insensitive to elicitation with methyl jasmonate. In contrast, Mh00W is a *T.* x *media* cell line that produces only small amounts of paclitaxel without methyl jasmonate elicitation. For metabolite abundance studies, 5 ml aliquots of a 7-day culture containing 1 ml packed cell volume were dispensed to individual wells of a 6-well plate (Falcon). Cells were elicited with 200 μM methyl jasmonate and grown for an additional 7 days. Control cells were grown for an additional 7 days without addition of methyl jasmonate.

3.2. Cell extraction

For extraction of the less polar taxoids, frozen *Taxus cuspidata* cells (cell line P93AF) were lyophilized, ground to a fine powder in a coffee grinder, and the powder extracted three times with rapid stirring for 1 h in a 50-fold (w/v) excess of pentane. The pentane extracts were combined, evaporated to dryness in a centrifugal vacuum concentrator, and the residue was dissolved in acetonitrile (40 mg ml^{-1}) prior to HPLC-MS analysis. The pentane extract yielded 1.84 g from 150.27 g of lyophilized cells, corresponding to 2.50 kg of frozen cells.

To obtain the total taxoid profile, an aliquot of suspension cultured cells and medium was removed and extracted by homogenization in an equal volume of methylene chloride in a ground glass tissue grinder; the process was repeated three times, the combined extracts were evaporated to dryness in a centrifugal vacuum concentrator, and the residue was dissolved in acetonitrile (40 mg ml^{-1}) prior to HPLC-MS analysis. For five-ml cultures set up in six-well plates, the entire culture (medium and cells) was harvested, dried in a centrifugal vacuum concentrator, finely ground and extracted in three ml acetonitrile (by placing the material and acetonitrile in a test tube in an ultrasonic bath for 30 min). Insoluble material was pelleted by brief centrifugation and the pellet was extracted a second time. The combined acetonitrile extracts were evaporated to dryness in a centrifugal vacuum concentrator and the residue was dissolved in 500 μl acetonitrile prior to HPLC-MS analysis.

3.3. HPLC conditions

HPLC/MSD instrumentation consisted of an Agilent Series 1100 HPLC with diode array and mass detectors, with Chemstation Software Rev. 8.03. Three methods of HPLC analysis were used in this study: Method 1:

extracts were eluted from a Discovery HS-F5 250 × 4.6 mm column (Supelco), 5 μm particle size, with guard column, 40–70% CH_3CN over 30 min, 100% CH_3CN for 5 min, and re-equilibration at 40% CH_3CN for 10 min. Method 2: extracts were eluted from a Prodigy ODS (3) 250 × 4.6 mm (Phenomenex), 5 μm particle size, analytical column with guard, by linear gradient with acetonitrile and water, 1 ml/min, from 50% CH_3CN to 100% CH_3CN over 50 min, with re-equilibration in 50% CH_3CN for 10 min. Method 3: extracts were eluted from a Prodigy ODS (3) 250 × 4.6 mm (Phenomenex), 5 μm particle size, analytical column with guard, by linear gradient with acetonitrile and water, 1 ml min^{-1}, from 5% CH_3CN to 100% CH_3CN over 95 min, returning to 5% over 1 min, and re-equilibration in 5% CH_3CN for 9 min.

Approximately 1 mg of residue in 25 μl acetonitrile was separated by Method 1 and the major components were collected. If these fractions did not contain chromatographically pure compounds, the material was dried and dissolved in acetonitrile, and not more than 1 mg solute was separated using Method 2, to yield pure, isolated compounds that were analyzed by NMR.

Mass detection of taxoids was by atmospheric pressure chemical ionization (APCI) in the positive ion mode. Drying gas was N_2 at 60 psi, 5 l min^{-1}, 350 °C. The vaporizer was set to 400 °C, fragmentor to 60 V, capillary to 3000 V, and corona current to 8 μA. For comparison of peak areas based on total ion abundance, identical integration parameters were used by the software for automatic integration of each treatment.

Identification of taxoids was accomplished by comparison of retention time and mass fragmentation pattern with authentic standards. In cases where standards were not available, mass fragmentation patterns were combined with NMR data of collected peaks for structure elucidation.

3.4. NMR measurements

NMR spectra were measured on a Varian Inova-500 (^1H 500 MHz, ^{13}C 125 MHz) and a Varian Inova-400 (^1H 400 MHz, ^{13}C 100 MHz) using C_6D_6 (δ 7.15 and 128.0) as the solvent. NMR measurements were made using the standard pulse sequences, TOCSY, ROESY, HSQC, and HMBC, from the Varian pulse program library. Cs-ion FAB MS data were determined on a Fisons-VG AutoSpec mass spectrometer operating at 8 kV.

3.5. 5α,7β,9α,10β,13α-Pentaacetoxy-2α-benzoyloxy-taxa-4(20),11-diene (**14**)

White amorphous powder; ^{13}C NMR spectral data (125.8 MHz, C_6D_6): δ 49.6 (*d,* C-1), δ 71.3 (*d,* C- 2), δ 43.8 (*d,* C-3), δ 140.6 (*s,* C-4), δ 76.3 (*d,* C-5), δ 35.3 (*t,* C-6), δ 70.2 (*d,* C-7), δ 47.7 (*s,* C-8), δ 71.7 (*d,* C-9), δ 76.2 (*d,* C-10), δ 137.9 (*s,* C-11), δ 133.6 (*s,* C-12), δ 70.4 (*d,* C-13), δ 28.4 (*t,* C-14), δ 38.0 (*s,* C-15), δ 27.8 (*q,* C-16), δ 31.4 (*q,* C-17), δ 15.7 (*q,* C-18), δ 13.9 (*q,* C-19), δ 119.6 (*t,* C-20), δ 20.3 (*q,* C–acetate-methyl), δ 20.4 (*q,* C–acetate-methyl), δ 20.7 (*q,* C–acetate-methyl), δ 20.9 (*q,* C–acetate- methyl), δ 21.2 (*q,* C–acetate-methyl), δ 164.7 (*s,* C–benzyl-CO), δ 168.5 (*s,* C–CO), 169.3 (*s,* C–CO), δ 169.4 (*s,* C–CO), δ 169.4 (*s,* C–CO), δ 169.8 (*s,* C–CO), δ 130.7 (*s,* C–benzyl-1′), δ 128.8 (*d,* C–benzyl-3′), δ 130.2 (*d,* C–benzyl-2′), δ 133.2 (*d,* C–benzyl-4). ^1H NMR spectra data (500.2 MHz, C_6D_6): δ 2.08 (1H, H-1), δ 6.17 (1H, *dd, J* = 2.0 Hz, *J* = 6.7 Hz, H-2), δ 3.56 (1H, *d, J* = 6.8 Hz, H-3), δ 5.38 (1H, *dd, J* = 2 Hz, H-5), δ 1.61 (1H, *m,* H-6β), δ 2.10 (1H, *m,* H-6α), δ 5.74 (1H, *m,* H-7), δ 6.56 (1H, *d, J* = 10.7 Hz, H-9), δ 6.33 (1H, *d, J* = 10.7 Hz, H-10), δ 6.14 (1H, *dd, J* = 6.7 Hz, H-13), δ 1.82 (1H, *m,* H-14β), δ 2.55 (1H, *m,* H-14α), δ 2.02 (3H, *s,* H-16), δ 1.04 (3H, *s,* H-17), δ 2.39 (3H, *s,* H- 18), δ 1.11 (3H, *s,* H-19), δ 5.12 (1H, *d, J* = 1.5 Hz, H-20), δ 4.81 (1H, *d*, *J* = 1.5 Hz, H-20), δ 1.71 (3H, *s,* H-acetate), δ 1.62 (3H, *s,* H-acetate), δ 1.48 (3H, *s,* H-acetate), δ 1.77 (3H, *s,* H-acetate), δ 1.80 (3H, *s,* H-acetate), δ 6.99 (2H, *dd, J* = 7.5 Hz, *J* = 8.3 Hz, H-benzyl-3′), δ 8.13 (2H, *d, J* = 8.3 Hz, H-benzyl-2′), δ 7.06 (1H, *dd, J* = 7.5 Hz, H-benzyl-4′). Cs-ion FABMS (probe) nominal mass mode, found *m/z* 682.31 (calc. for $C_{37}H_{46}O_{12}$ 682.30 $[M]^+$). Observed 623.2 [M-acetate (59)]$^+$, 550.5 [M-benzoate (131)]$^+$. Insufficient material to provide a sufficiently intense $[M]^+$, $[M+H]^+$, or [M-acetate (59)]$^+$ signal to measure the exact mass for Cs-ion FAB (probe) HRMS.

3.6. 2α,5α,10β-Triacetoxytaxa-4(20),11-diene (**24**)

White amorphous powder; ^{13}C NMR spectral data (125.8 MHz, C_6D_6, obtained from HSQC, insufficient material available for direct observation of $^{13}C\{^1H\}$ spectrum): δ 52.1 (*d*, C-1), δ 71.8 (*d*, C-2), δ 41.1 (*d*, C-3), δ 78.3 (*d*, C-5), δ 28.6 (*t*, C-6), δ 33.3 (*t*, C-7), δ 43.9 (*t*, C-9), δ 69.9 (*d*, C-10), δ 29.8 (*t*, C-13), δ 18.1 (*t*, C-14), δ 25.2 (*q*, C-16), δ 31.4 (*q*, C-17), δ 20.9 (*q*, C-18), δ 22.0 (*q*, C-19), δ 116.1 (*t*, C-20), δ 21.0 (*q*, C-acetate), δ 20.6 (*q*, C-acetate), δ 20.6 (*q*, C-acetate). ^1H NMR spectral data (500.2 MHz, C_6D_6): δ 1.87 (1H, *m*, H-1), δ 5.64 (1H, *dd*, *J* = 1.8 Hz, *J* = 5.6 Hz H-2), δ 3.12 (1H, *d*, *J* = 5.6 Hz, H-3), δ 5.40 (1H, *dd*, *J* = 3 Hz, *J* = 3 Hz, H-5), δ 1.64 (1H, *m*, H-6), δ 1.53 (1H, *m*, H-6), δ 1.90 (1H, *m*, H-7), δ 0.89 (1H, *m*, H-7), δ 2.42 (1H, *m*, H-9), δ 1.54 (1H, *m*, H-9), δ 6.27 (1H, *dd*, *J* = 5.4 Hz, *J* = 12.4 Hz, H-10), δ 2.21 (1H, *m*, H-13), δ 1.91 (1H, *m*, H-13), δ 1.80 (2H, *m*, H-14), δ 1.76 (3H, *s*, H-16), δ 1.07 (3H, *s*, H-17), δ 1.99 (3H, *s*, H-18), δ 0.77 (3H, *s*, H- 19), δ 5.17 (1H, *m*, H-20), δ 4.96 (1H, *m*, H-20), δ 1.67 (3H, *s*, H-acetate), δ 1.70 (3H, *s*, H-acetate), δ 1.64 (3H, *s*, H-acetate). Cs-ion FAB HRMS *m/z* 446.2661 (calc. for $C_{26}H_{38}O_6$, 446.2668).

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References

Appendino, G., Belloro, E., Del Grosso, E., Minassi, A., Bombardelli, E., 2002. Synthesis and evaluation of 14-*nor*-A-secotaxoids. Eur. J. Org. Chem. 2, 277–283.

Baloglu, E., Kingston, D.G.I., 1999. The taxane diterpenoids. Phytochemistry 62, 1448–1472.

Eisenreich, W., Menhard, B., Hylands, P.J., Zenk, M.H., Bacher, A., 1996. Studies on the biosynthesis of taxol: the taxane carbon skeleton is not of mevalonoid origin. Proc. Natl. Acad. Sci. USA 93, 6431–6436.

Hefner, J., Rubenstein, S.M., Ketchum, R.E.B., Gibson, D.M., Williams, R.M., Croteau, R., 1996. Cytochrome P450-catalyzed hydroxylation of taxa-4(5),11(12)-diene to taxa-4(20),11(12)-dien-5α-ol: the first oxygenation step in taxol biosynthesis. Chem. Biol. 3, 479–489.

Hezari, M., Lewis, N.G., Croteau, R., 1995. Purification and characterization of taxa-4(5),11(12)-diene synthase from Pacific yew (*Taxus brevifolia*) that catalyzes the first step of taxol biosynthesis. Arch. Biochem. Biophys. 322, 437–444.

Hezari, M., Croteau, R., 1997. Taxol biosynthesis: an update. Planta Med. 63, 291–295.

Hezari, M., Ketchum, R.E., Gibson, D.M., Croteau, R., 1997. Taxol production and taxadiene synthase activity in *Taxus canadensis* cell suspension cultures. Arch. Biochem. Biophys. 337, 185–190.

Hoke, S.H., Wood, J.M., Cooks, R.G., Li, X.H., Chang, C.J., 1992. Rapid screening for taxanes by tandem mass-spectrometry. Anal. Chem. 64, 2313–2315.

Jennewein, S., Rither, C.D., Williams, R.M., Croteau, R. Taxoid metabolism: taxoid 14B-hydroxylase is a cytochrome P450-dependent monooxygenase. Submitted.

Ketchum, R.E.B., Gibson, D.M., 1996. Paclitaxel production in cell suspension cultures of *Taxus*. Plant Cell Tissue Organ Cult. 46, 9–16.

Ketchum, R.E.B., Gibson, D.M., Croteau, R.B., Shuler, M.L., 1999a. The kinetics of taxoid accumulation in cell suspension cultures of *Taxus* following elicitation with methyl jasmonate. Biotechnol. Bioeng. 62, 97–105.

Ketchum, R.E.B., Tandon, M., Gibson, D.M., Begley, T., Shuler, M.L., 1999b. Isolation of labeled 9-dihydrobaccatin III and related taxoids from cell cultures of *Taxus canadensis* elicited with methyl jasmonate. J. Nat. Prod. 62, 1395–1398.

Ketchum, R.E.B., Luong, J.V., Gibson, D.M., 1999c. Efficient extraction of paclitaxel and related taxoids from leaf tissue of *Taxus* using a potable solvent system. J. Liq. Chromatogr. Relat. Technol. 22, 1715–1732.

Kingston, D.G.I., Molinero, A.A., Rimoldi, J.M., 1993. The taxane diterpenoids. In: Herz, W., Kirby, G.W., Moore, R.E., Steglich, W., Tamm, C. (Eds.), Progress in the Chemistry of Organic Natural Products, Vol. 61. Springer-Verlag, New York, pp. 1–206.

Koepp, A.E., Hezari, M., Zajicek, J., Stofer Vogel, B., LaFever, R.E., Lewis, N.G., Croteau, R., 1995. Cyclization of geranylgeranyl diphosphate to taxa-4(5),11(12)-diene is the first committed step of taxol biosynthesis in Pacific yew. J. Biol. Chem. 270, 8686–8690.

Lucas, H., 1856. Über ein in den Blättern von *Taxus baccata* L. enthaltenes Alkaloid (das Taxin). Arch. Pharm. 85, 145–149.

Menhard, B., Eisenreich, W., Hylands, P.J., Bacher, A., Zenk, M.H., 1998. Taxoids from cell cultures of *Taxus chinenesis*. Phytochemistry 49, 113–125.

Mirjalili, N., Linden, J.C., 1996. Methyl jasmonate induced production of taxol in suspension cultures of *Taxus cuspidata:* ethylene interaction and induction models. Biotechnol. Prog. 12, 110–118.

Wheeler, A.L., Long, R.M., Ketchum, R.E., Rithner, C.D., Williams, R.M., Croteau, R., 2001. Taxol biosynthesis: differential transformations of taxadien-5-alpha-ol and its acetate ester by cytochrome P450 hydroxylases from *Taxus* suspension cells. Arch. Biochem. Biophys. 390, 265–278.

Wiegerinck, P.H.G., Fluks, L., Hammink, J.B., Mulders, S.J.E., de Groot, F.M.H., van Rozendaal, H.L.M., Scheeren, H.W., 1996. Semisynthesis of some 7-deoxypaclitaxel analogs from taxine B. J. Org. Chem. 61, 7092–7100.

Yukimune, Y., Tabata, H., Higashi, H., Hara, Y., 1996. Methyl jasmonate-induced overproduction of paclitaxel and baccatin III in *Taxus* cell suspension cultures. Nat. Biotechnol. 14, 1129–1132.