Pergamon

PII: S0031-9422(96)00183-5

Phytochemistry, Vol. 43, No. 1, pp. 95–98, 1996

0031-9422/96 $15.00 + 0.00

TAXANES EXPORTED FROM *TAXUS* × *MEDIA* HICKSII CUTTINGS INTO LIQUID MEDIUM OVER TIME

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(*Received in revised form* 12 *February* 1996)

Key Word Index—*Taxus* × *media* Hicksii; Taxaceae; yew; *in vitro* culture; taxane; taxol; diterpene.

Abstract—Cuttings from *Taxus* × *media* Hicksii were maintained in Gamborg's B5 liquid culture medium, where 10-deacetyl baccatin III, baccatin III, 10-deacetyl taxol, cephalomannine, 7-epi-10-deacetyl taxol and taxol accumulated in the incubation medium over time. Greater amounts of each taxane were recovered from plant material as compared with liquid medium. Medium was removed and replaced weekly, biweekly or triweekly for up to nine weeks. The sum of all taxol recovered from the liquid that had been harvested from a yew cutting and extracted from the medium weekly over eight weeks was approximately equal to the amount of taxol which would be recovered from the fresh plant cutting, and the cutting still contained taxol. This suggests that *in vitro* cultures of yew cuttings may be a re-usable source of taxol and related taxanes. Copyright © 1996 Elsevier Science Ltd

INTRODUCTION

Taxol (Paclitaxel) (**1**), an important anti-cancer drug, is present in the bark and other tissues of the Pacific Yew tree (*Taxus brevifolia* Nutt., Taxaceae) at 0.001 to 0.1 percent of the dry weight of the plant [1–3]. Several other taxane diterpenes related to taxol normally found in yew plants must be separated from taxol during its purification. These compounds include baccatin III (**2**), cephalomannine (**3**) and derivatives of taxol or baccatin III, (**4**, **5**, **6**). Although many of these taxanes are without antineoplastic activity, there is hope that semi-synthetic taxol derivatives from compounds such as 10-deacetylbaccatin III can be made more effective, more stable, or more soluble in water than taxol [4–5].

A number of research groups have measured taxol recovered from yew cells. Fett-Neto *et al.* [6] found that more taxol was produced by *T. cuspidata* suspension cultures in the stationary growth phase as compared to log growth phase. Similar results were obtained by Srinivasan *et al.* [7] using *T. baccata* suspension cultures. Strobel *et al.* [8] demonstrated that finely cut pieces of fresh inner bark from *T. brevifolia* could synthesize taxol from radiolabeled acetate or other terpenoid precursors. To date, no studies have reported on the synthesis or export of taxanes from yew stem explants.

We have shown that taxanes, including taxol, are exported from yew cuttings incubated in liquid growth medium. The taxanes are more easily extracted from the growth medium than from the plant, but are present at lower concentrations in the medium. The procedure described in this report provides an additional source for taxol which takes advantage of easily harvested and renewable yew foliage.

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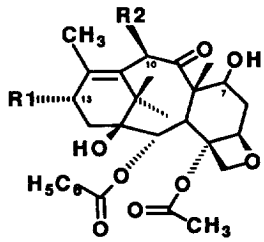
RESULTS AND DISCUSSION

At least six different taxanes were recovered from plant cuttings and medium samples in which the cuttings were incubated. Depending on the taxane, between four and 120 times more compound was detected in plant extracts as compared with incubation medium. Detectable levels of these taxanes appeared in the incubation medium after four to six days of incubation. Table 1 compares the amounts of each of the six taxanes extracted from the medium and from plant cuttings after one, two or three weeks of incubation. Significantly more baccatin III (**2**) and 10-deacetyl taxol (**5**) were recovered from the plant after one week of incubation as compared with two or three weeks. In contrast, more taxol (**1**) was recovered from plant material after two and three weeks in culture than after one week. More cephalomannine (**3**) and taxol (**1**) were detected in the medium after one week of incubation as compared with two or three weeks. More 10-deacetyl baccatin III (**4**) was recovered from the medium after two or three weeks in culture than after one week.

Fresh untreated yew cuttings yielded 59.12 μg of taxol per gram dry weight of plant $\pm$8.73 (standard deviation, $n = 3$). This was significantly less than the amount of taxol recovered from cuttings after one week's incubation. Apparently, incubation in the cul-



4	10 Deacetyl baccatin III	R1 = OH	R2 = OH
2	Baccatin III	R1 = OH	R2 = OAc
5	10 Deacetyl taxol	R1 = COCH(OH)CH(Ph)NHCOPh	R2 = OH
3	Cephalomannine	R1 = COCH(OH)CH(Ph)NHCOC$_4$H$_7$	R2 = OAc
6	7 epi-10-10 Deacetyl taxol	R1 = COCH(OH)CH(Ph)NHCOPh	R2 = OH
1	Taxol	R1 = COCH(OH)CH(Ph)NHCOPh	R2 = OAc

ture medium promoted the increase of taxol in the plant cutting. Studying the effects of postharvest storage conditions, Schutzki *et al.* [9] recovered more taxol from yew tissues that had been stored for three to nine days after harvest, as compared with immediately after harvest, but found that taxol dropped back to harvest levels after 27 days of storage. If the increased concentration of taxanes in our experiment was due solely to storage, they would most likely have declined between day seven and day 21 of incubation. The amount of taxol recovered from the plant cuttings actually increased after seven days of incubation. As evidence that they were alive, cuttings incubated in Warburg flasks continued to produce oxygen after two weeks and some cuttings developed callus.

The amount of taxol exported into the medium declined somewhat over the three weeks of the experiment, while the amount retained in the plant increased. Taxol was present in the cuttings at approximately 0.01% of the dry weight of the plant, but the amount in the medium was only 0.0001–0.0002% of the dry weight of the plant material incubated. The amount of most taxanes extracted from the maintenance medium did not change appreciably through the three weeks of the experiment. The suggests that there may be a limit on the amount of taxane that could be exported into a given volume of medium.

Although the cuttings used in each experiment were generally taken from the same group of yew plants, differences in taxane measurements may have been due to clonal variation, the time of year [10], environmental conditions or experimental error. The HPLC method employed completely separated taxol from the chemically similar cephalomannine and 7-epi-10 deacetyl taxol.

In additional experiments, yew cuttings were continuously incubated in the liquid medium for up to nine weeks. The medium was aseptically removed from the culture vials and replaced with fresh solution weekly, biweekly or triweekly (Table 2). When the medium was harvested weekly, (**4**) and (**5**) were present in the greatest average amount per week (at 95% confidence),

Table 1. Average amount* of six taxanes extracted from *T.* × *media* Hicksii cuttings or the medium in which the cuttings were placed after 1, 2 or 3 weeks of incubation

	Week 1		Week 2		Week 3	
Taxane	Plant	Medium	Plant	Medium	Plant	Medium
10-Deacetyl baccatin III	77.58±21.03	4.25±0.62	91.97±23.3	7.97±1.67†	80.33±10.2	8.10±2.73†
Baccatin III	50.51±5.62†	7.00±0.81	30.87±1.82	7.16±1.09	35.73±1.73	6.20±2.05
10-Deacetyl taxol	52.98±4.81†	2.90±1.05	36.43±2.74	1.74±0.72	30.06±3.05	1.75±0.59
Cephalomannine	52.50±10.31	2.24±0.53†	58.97±6.94	1.08±0.27	47.71±7.80	1.26±0.34
7-epi-10-Deacetyl taxol	6.97±3.70	0.63±0.18	6.16±1.86	0.54±0.18	6.30±3.06	0.73±0.12
Taxol	83.47±6.47	2.25±0.44†	105.12±5.50†	1.03±0.32	115.09±9.28†	0.95±0.23

*Taxane concentrations were calculated as μg per g dry weight of plant material±s.e. Two to four cuttings or medium from two to four cuttings were pooled for each sample, $n = 4$ pooled samples. Cuttings and culture medium were harvested at the same time.

†These plant or medium samples contained more of the specified taxane than those of comparable samples from other weeks (at 95% confidence).

Table 2. Average amount of six taxanes extracted from culture medium in which *T.* × *media* Hicksii cuttings were incubated. Harvested medium was replaced with fresh liquid weekly, biweekly or triweekly for 9 weeks

Taxane	Weekly Harvest $n = 60$ μg^{-1} g dry wt	Bi-weekly Harvest $n = 22$ μg^{-1} g dry wt	Tri-weekly Harvest $n = 16$ μg^{-1} g dry wt
10-Deacetyl baccatin III	8.18±0.41*	4.37±0.51 (3.87)†	7.20±0.32 (2.40)
Baccatin III	6.07±0.12	6.80±0.22 (3.40)	5.90±0.47 (1.97)
10-Deacetyl taxol	9.45±1.00	3.44±0.83 (1.72)	4.80±0.32 (1.60)
Cephalomannine	5.16±0.39	3.98±0.70 (1.99)	5.57±0.99 (1.86)
7-epi-10-Deacetyl taxol	1.39±0.05	0.49±0.007 (0.25)	0.51±0.03 (0.17)
Taxol	6.74±0.46	2.87±0.47 (1.44)	3.17±0.79 (1.06)

*Values are $\mu g\ g^{-1}$ dry wt of the plant cutting placed in the culture medium. Each value is taken from two or three pooled samples±s.e. for each harvest period.

†Average per week.

followed by (**1**)–(**3**), and (**6**). For most taxanes, the average weekly harvest yield was greater than or equal to the amount recovered biweekly or triweekly. Bi- and triweekly harvest of taxanes were similar in amount. When the recovery was calculated on a 'per week' basis, however, weekly recovery was always significantly greater than either bi- or tri-weekly recovery to greater than 99% confidence.

Greater total amounts of baccatin derivatives (**2**, **4**) were recovered from medium harvested bi- and triweekly as compared with taxol derivatives (**1**, **5**, **6**). It is unknown whether baccatin III or 10-deacetyl baccatin III are synthesized before cephalomannine or taxol derivatives, but it is logical to assume that the less derivatized compounds would be synthesized first. Koepp *et al.* [11] have shown that taxa-4(5),11(12)-diene is the product of the first committed step in synthesis of all the taxanes.

All data reported here was recovered from uncontaminated cultures. It is therefore unlikely that the taxanes were produced by a yew endophyte or other symbiont. Cultures that developed mould did not export as much taxane into the culture medium, but taxanes were present in the plant cuttings at higher concentrations. This suggests that specific environmental or internal conditions are required for taxane export.

This study provides indirect evidence that taxanes were synthesized anew by the cuttings in culture. The sum of all the taxol extracted weekly from medium over a nine week period of time in culture was 53.93 μg per gram dry weight of plant, approximately equal to the amount obtained from a fresh cutting. This implies that taxanes were being synthesized over the duration of the experiment and not merely leached out of dead or dying cells. A major advantage of the system described here is that the plant material can be reused continuously, providing an efficient use for yew plant cuttings. With further manipulation of incubation conditions, more taxol may be harvested in a given amount of time. Culture conditions for several yew species and cultivars have been optimized for suspension or tissue culture [12–14], but they have not yet been optimized for the incubation of cuttings.

EXPERIMENTAL

Plant material and experimental set-up. Cuttings from *T.* × *media* Hicksii were obtained from the Carlson Nursery Inc., Gresham, Oregon. The cuttings were taken from tips of branches of the present and previous growth seasons from 3- to 6-year-old plants in May and July, 1993, and in January and May, 1994.

The cuttings were thoroughly scrubbed with Ivory soap and soaked alternately in 20% commercial bleach and 70% EtOH 4×, followed by a thorough rinse with sterile distilled H_2O. Cuttings of 3–5 cm were randomly placed in sterilized 8 dram shell vials containing 5 ml Gamborg's B-5 liquid medium [15] supplemented with 5 μM 2,4-dichlorophenoxyacetic acid and 3% sucrose. The capped vials were incubated in the dark. Incubation medium was harvested and replaced as specified.

Extraction and sample preparation. Plant cuttings were ground using a coffee grinder and extracted 3× with MeOH. After centifugation, the supernatant was concd under reduced pressure and further treated as described for incubation medium. Medium samples were extracted immediately or stored at −77°. Medium was extracted 3× with an equal volume of hexane. The aq. fr. was extracted 3× using a C-18 Sep Pak. Taxanes and related compounds were removed from the Sep Pak with MeOH. After the MeOH was removed, the residue was dissolved in a known volume of MeOH and filtered through a 0.2 μm filter before sepn by HPLC.

Analytical methods. Sepn was accomplished using a Curosil B, 5 μm 250 × 3.2 mm column and guard column. Isocratic HPLC conditions: 55%A (10 mM NH_4OAc, pH 4.0) and 45%B (HPLC grade MeCN); flow rate, 1 ml min^{-1}, 228 nm. Three 10-μl injections were made for each sample. To aid in peak identification, one injection for each sample was spiked with a mixture of *ca* 0.6 μg of each of the 6 taxanes. Taxane calibration curves (range = 0.16–3.3 μg) were made from authentic taxane samples.

Retention times for taxanes: **4** (5.22 min), **2** (7.40 min), **5** (12.99 min), **3** (16.93 min) **6** (19.29 min), **1** (20.54 min). Variation was reported as s.e. or s.d. Significance was assessed by t test. Taxanes were reported as $\mu g\ g^{-1}$ dry wt of plant sample or plant

sample from which the incubation medium was recovered.

Frs containing putative taxanes were collected after HPLC from plant samples. The presence of the taxanes was verified by FAB-MS at 70 eV on a VG Analytical 7070E mass spectrometer (probe) using a thioglycerol matrix. The products were identified by comparison with standards.

Acknowledgements—Taxane standards were provided by Dr K. Snader, Drug Synthesis & Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute Bethesda, MD. Yew cuttings were supplied by Carlson Nursery, Inc, Gresham, Oregon. Mass spectroscopy was performed by Lorne Isabelle of Oregon Graduate Institute of Science and Technology, Beaverton, OR. Support for this work by the M. J. Murdock Charitable Trust and the University of Portland is gratefully acknowledged.

REFERENCES

1. Wani, M. C., Taylor, W. H., Wall, M. E., Coggon, P. and McPhail, A. T. (1971) *J. Am. Chem. Soc.* **93**, 2325.
2. Witherup, K. M., Look, S. A., Stasko, M. W., Ghiorzi, T. J. and Muschik, G. M. (1990) *J. Nat. Prod.* **53**, 1249.
3. Kingston, D. G. I. (1991) *Pharmac. Ther.* **52**, 1.
4. Guénard, D., Guéritte-Voegelein, F. and Potier, P. (1993) *Acc. Chem. Res.* **26**, 160.
5. Kingston, D. G. I. (1994) *TIBTEC* **12**, 222.
6. Fett-Neto, A. G., Zhang, W. Y. and DiCosmo F. (1994) *Biotech. Bioeng.* **44**, 205.
7. Srinivasan, V., Pestchanter, L., Moser, S., Hirasuna, T. J., Taticek, R. A. and Shuler, M. L. (1995) *Biotech. Bioeng.* **47**, 666.
8. Strobel, G. A., Stierle, A. and van Kuijk, F. J. G. M. (1992) *Plant Science* **84**, 65.
9. Schutzki, R. E., Chandra, A. and Nair, M. G. (1994) *Phytochemistry* **37**, 405.
10. Kelsey, R. G. and Vance, N. C. (1992) *J. Nat. Prod.* **55**, 912.
11. Koepp, A. E., Hezari, M., Zajicek, J., Vogel, B. S., LaFever, R. E., Lewis, N. G. and Croteau, R. (1995) *J. Biol. Chem.* **270**, 8686.
12. Fett-Neto, A. G. and DiCosmo, F. (1992) *Planta Med.* **58**, 464.
13. Gibson, D. M., Ketchum, R. E. B., Vance, N. C. and Christen, A. A. (1993) *Plant Cell Rep.* **12**, 479.
14. Wickremesinhe, E. R. M. and Arteca, R. N. (1994) *J. Plant Physiol.* **144**, 183.
15. Gamborg, O. L., Miller, R. A. and Okima, K. (1968) *Exp. Cell Res.* **50**, 151.