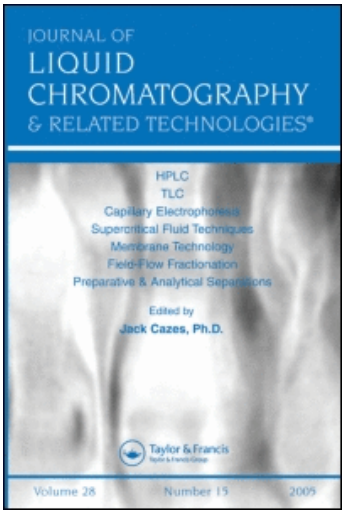

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A Large Scale Process for Paclitaxel and other Taxanes from the Needles of *Taxus* x *Media Hicksii* and *Taxus Floridana* Using Reverse Phase Column Chromatography

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A LARGE SCALE PROCESS FOR PACLITAXEL AND OTHER TAXANES FROM THE NEEDLES OF *TAXUS* X *MEDIA HICKSII* AND *TAXUS FLORIDANA* USING REVERSE PHASE COLUMN CHROMATOGRAPHY

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ABSTRACT

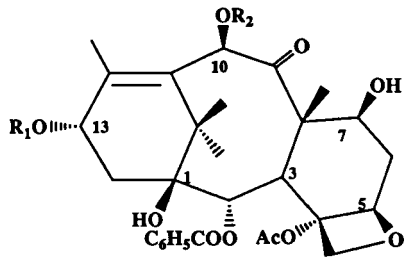
Although the renewable, needle bio-mass of *Taxus* x *media* Hicksii was proposed as the future source for paclitaxel in 1990, no details for the actual isolation of paclitaxel and other taxanes have been published, other than testing the extracts by HPLC analysis. Compared to the bark, the needle source poses additional problems, eg. chlorophylls, waxes and co-eluting taxanes, and thus, there is a need for an efficient method.

We have developed a new large-scale process based on a single reverse phase column, which takes the $CHCl_3$ extract of *T* x *media* Hicksii directly, and is eluted with acetonitrile (30-60%) in water. Paclitaxel and five other taxanes crystallize out from the fractions, and they are filtered and recrystallized. The taxanes that co-elute with paclitaxel are removed by ozonolysis and a short silica column to give paclitaxel in a yield of 0.012-0.015 % from the dry needles.

The needle mass of *T. floridana* was also processed by the same method on a pilot-plant scale. From the fresh needles, paclitaxel (0.01%) and 10-deacetyl baccatin III (0.06%) and two other taxanes are obtained. A discussion on the two plants as sources for paclitaxel is presented.

INTRODUCTION

On the basis of its demonstrated activity, paclitaxel **1** is currently viewed with much interest as an effective antitumor drug[1-3]. Paclitaxel is being produced at present by isolation from the bark of *Taxus brevifolia* (Pacific yew), although for future isolation, the bark will be replaced by a renewable source such as the needles of various *Taxus* spp., one such source being the cultivar, *Taxus* x *media* Hicksii (ornamental yew)[4,5]. In spite of such importance, there appear to be little or no published data, with details on the actual isolation of paclitaxel (and other taxanes) from these needles, other than testing of various plant samples and their parts by analytical HPLC.[6,7] In the extracts of this cultivar, paclitaxel co-elutes with several unrelated taxanes[8,9]. Minute amounts of four of these taxanes were isolated as two equilibrium (inseparable) mixtures and their structures elucidated by spectroscopic means[8].



1: $R_1 = C_6H_5CH(NHCOC_6H_5)CH(OH)CO$
$R_2 = CH_3CO$

7: $R_1 = R_2 = H$

The current large-scale process for paclitaxel from the bark of *T. brevifolia* is said to involve two or more chromatographic steps using silica and/ or Florisil columns. Isolation from the needles must pose additional challenges due to the

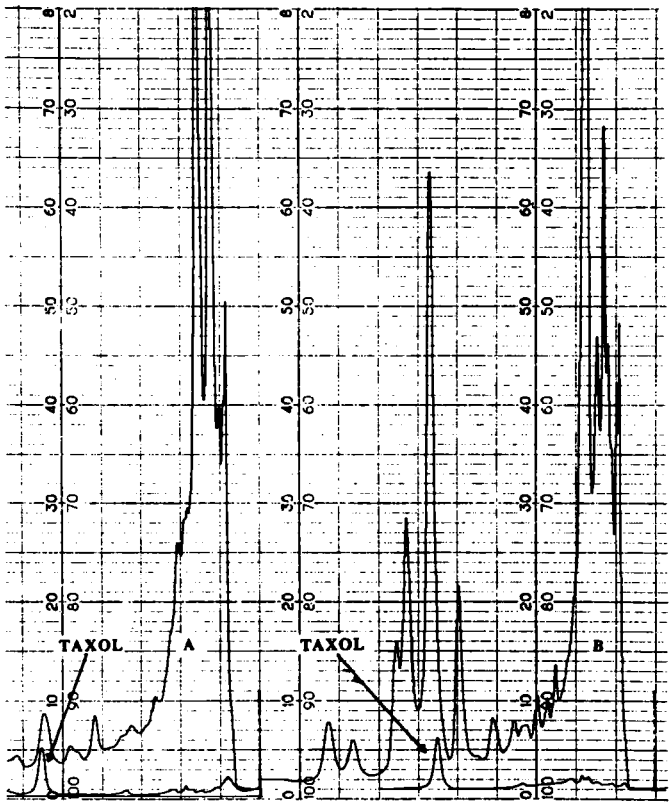


Figure 1. An analytical trace of the needle extract of A) *T.* Floridana, and B) *T. x media* Hicksii.

increased content of waxes, chlorophylls, carotenoids etc., as well as the co-eluting taxanes[8,9]. An analytical HPLC trace of the needle extract of *T.* x *media* Hicksii (Fig. 1A), shows the relatively large amounts of the co-eluting taxanes that accompany paclitaxel, and which must be separated from it.

We reported a new process for paclitaxel and related taxanes from the bark of *T. brevifolia* by the use of a single reverse phase column[10,11], both on a laboratory and pilot-plant scale, which was simpler and gave higher yields. The present paper deals with a pilot-plant scale chromatographic process for the needles of *Taxus* x

media Hicksii and of *Taxus floridana* (Florida yew) with details for the isolation of paclitaxel and several other taxanes, on a scale of 50-200 lb of plant material.

MATERIALS AND METHODS

Plant Material

The needles of *Taxus* x *media* Hicksii (200 lbs of the dried material) were kindly supplied by Hauser Inc., Boulder CO, during May/June 1993.

The needles of *Taxus floridana* were collected (50 lbs, fresh) from the campus of the University of Florida during April/May, 1993 and used fresh.

Step 1: Extraction and Concentration

The extraction was carried out using methanol, in stainless steel tanks of capacity 100 or 200 gal, equipped with lids that could be clamped tightly and with an outlet (1" dia) at the bottom for draining, fabricated by Pillsbury Alloy Fabrication, Inc. Jacksonville, FL. 200-250 Lbs of the needles could be extracted in the 200 gal tank and 70-90 lbs in the 100-gal tank. For several hours during the day, the extract was recycled by being pumped back into the tank as a rotating spray. Three to four such extractions, each running for a day, were carried out. The progress of the extraction was monitored by uv-absorbance at 275 nm.

The methanolic extract was concentrated under reduced pressure ($<30^0$ C) using a semi-continuously operated still with a receiving capacity of 220 gal. The extract was fed into a jacketed still-tank (100 gal) heated by steam, introduced into the jacket. The vapors were led into a vertical tube-type condenser (4 ft long and 1 foot in diameter with 1" pipes for the cooling water), where they were condensed by chilled water at 10^0 C and the condensate taken to a series of four receiving tanks, each of 55 gal capacity.

The system was kept under reduced pressure by using a vacuum pump, and the rate of distillation maintained by the steam introduced into the jacket as needed. Distillation was carried out until the volume of the concentrate reached 20-25 gal from the extract obtained from a 200 lb batch of the plant material.

Step 2: Solvent-Partition

Partition of the concentrated methanolic extract with chloroform ($CHCl_3$) was performed in 50 or 100 gal tanks, equipped with an air-driven stirrer. The concentrate from a 200 lb batch (25 gal) was stirred with water (20 gal) and $CHCl_3$ (or dichloromethane) (20 gal) for about 30 minutes and, after 2-14 hours to allow for any emulsion to clear, the solvent layer was drained off from the bottom into stainless steel containers. Two additional extractions were carried out by using 15 and 10 gallons respectively, of $CHCl_3$.

Concentration of the combined $CHCl_3$ extract under reduced pressure was carried out in an all-glass, steam heated, circulating evaporator (Ace Glass Co., Vineland, N.J.). The resulting concentrate (app. 2 gal) was further stripped of its solvent in a rotary evaporator to a thick syrup which was then poured into glass trays and converted to a brittle, dark green glassy solid, using a vacuum oven maintained at 35-40^0 C. This glassy solid represented a yield of 50-55 g per Kg of the dried needles of *Taxus* x *media* Hicksii and about 25 g per Kg of the fresh needles of *T. floridana*.

Optional Pretreatment of the Extract

In preliminary trials, attempts were made to "preclean" the $CHCl_3$ extract (remove chlorophylls, waxes etc.) through selective solvent partition methods, but they were not very satisfactory. The best method found to be was, passing a solution of the $CHCl_3$ extract in a mixture of methanol, acetone and water (7:1:2) through a column of C_{18} bonded reverse phase silica (15-35 micron particles, 3 g per gram of the sample).

The column was washed with the same solvent mixture until the uv absorbance became negligible ("Fraction I") and then with a mixture of methanol, ligroin and ethyl acetate (2:1:1) until the column and the wash became colorless ("Fraction II"). "Fraction I" (taxane-containing, 40% of the total) was concentrated to a syrup and applied to the column as described below. "Fraction II" (60% of the total) contained no paclitaxel but the lipophilic constituents: chlorophylls, carotenoids, waxes, steroids etc.

Subsequent trials showed that such pretreatment was not necessary and that the chloroform extract solid could be applied directly on to the column as described below.

Step 3: Chromatography

For the chromatography, stainless steel columns of two sizes were used: a 4"x 4' and a 6" x 6', both fabricated by Fluitron Inc. Ivyland, PA. The columns were rated for operation up to 200 psi. Each of the columns was equipped with a lid that could be sealed tightly and containing an inlet connection (0.5"). A three-way valve was attached to this inlet, to allow for feeding the column, for venting and for measuring the pressure. A circular stainless steel mesh was inserted securely at the lower end of the column to support a wad of glass wool. The lower, tapered end of the column was connected to a nipple (1/4 inch), to which was attached a rubber tube for collecting the column effluent into fraction bottles.

The columns were packed with C_{18} bonded silica (Spherisorb, 15-35 micron diameter, Phase Separations Inc., Norwalk CT) as a slurry in methanol. Approximately 3-4 Kg and 12-13 Kg of silica gel were required for the 4" and 6" columns respectively. After a thorough wash with methanol, the columns were equilibrated with 25% acetonitrile in water.

For running the 6" diameter column, the dark green glassy solid from the chloroform extract of the needles (2.3-2.7 kg) was dissolved in acetonitrile (AN, 5 L) and, while the mixture was being stirred with the equilibrated silica (1-2 L), diluted with water to make 20 L. After 15 minutes of stirring, the mixture was allowed to stand for 15-30 minutes and the clear supernatant siphoned off into another container. The slurry was applied to the column, followed by part of the supernatant, after which, the column was sealed. The remaining supernatant was pumped into the column using a diaphragm metering pump (Pulsa 680, Pulsafeeder Inc. Rochester, NY), maintaining a pressure of 30-80 psi. After the sample has been pumped, the column was eluted with a step gradient of 35, 40, 45, 50 and 60 % acetonitrile (AN) in water. The change of solvent was dictated by the results of TLC and HPLC analysis of the fractions, but generally, 40-50 L of each solvent was used. After this, the column was washed with methanol, followed by a mixture of ethyl acetate and ligroin (1:1) until the effluent was nearly colorless. Following this, the column was again washed with methanol and equilibrated with 25% acetonitrile in water, ready for reuse.

Fractions of approximately 2 L were collected and these were monitored by uv absorbance at 275 nm, TLC and analytical HPLC. The column fractions were allowed to stand at room temperature for 2-10 days, by which time, many of them showed a substantial degree of crystallization. Soon after, the crystals were filtered in groups, analyzed for purity and composition (TLC and analytical HPLC) and recrystallized from the appropriate solvent.

A similar procedure was used with the 4" diameter column, on which was applied approximately 500-700 g of the $CHCl_3$ extract solids dispersed in 5-6 l of the solvent mixture as was described above.

The chromatography was also run using aqueous methanol as the solvent, starting with 30 % methanol in water and continuing up to 65 %. The results obtained were comparable to those seen with the AN/ water system, except that the rate and extent of crystallization of the various components was less.

ANALYTICAL AND OTHER METHODS

Analytical HPLC was performed using two different units. For routine use, a combination of a Waters 501 pump, with a U6K injector, a 486 tunable absorbance detector and a Goerz Servogor 120 recorder was used. For determinations of purity and quantitative information on composition etc., a setup containing a Waters 600 E pump with gradient control system, a 996 photodiode array detector, a 717 autosampler, coupled with an NEC-386 computer and printer was used. Waters Millennium 1.1 program was used with the instrument. Standard columns (4.6 mm x 25 cm, Whatman, Partisil) packed with C_8 bonded silica, 5 micron diameter were used with either of the solvents: 50% AN/ water, or a 5:4:1 mixture of AN, water and methanol. The flow rate for both was 0.5 mL per min. For routine use, the detector was set at 254 nm, and for purity determinations using the photodiode array detector, the data were collected both at 254 and 220 nm.

Thin-layer chromatography was carried out using silica gel HF-60, 254+366 (EM Science/Fisher) and solvent systems consisting of acetone/$CHCl_3$ or MeOH/$CHCl_3$. Visualization was by a uv-lamp and by charring with 1 N H_2SO_4. Column chromatography was performed using silica gel (Fisher, 100-200 and 235-425 mesh) and Florisil (Fisher F-101, 100 mesh) were used, with a solvent sequence consisting of ligroin/$CHCl_3$, $CHCl_3$, 2-5% acetone and finally, 2-10% MeOH in $CHCl_3$.

Ozonolysis was carried out using the ozonizer made by Ozone Research and Equipment Company, Phoenix, AZ.

Melting points were determined on Fisher-Johns apparatus and are uncorrected. The following instruments were used to record the spectra described here: uv, Perkin-Elmer, Lambda 3B; ir, Perkin-Elmer, PE-1420; and nmr, Varian VXR-300, Varian Gemini-300 and General Electric QE-300 spectrometers. Mass spectra (FAB) were obtained on a Finnegan Mat 95Q spectrometer using a cesium gun operated at 15 Kev of energy.

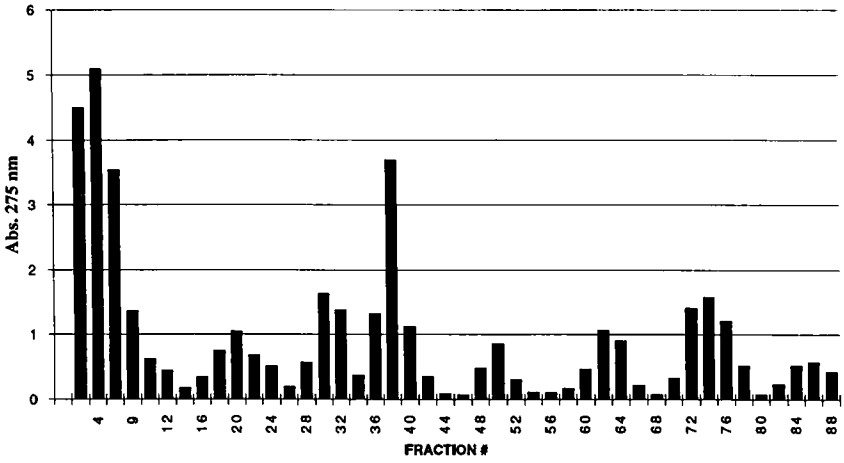


Figure 2. Column on *T. x media* Hicksii.

RESULTS

Elution Sequence for *Taxus* x *media* Hicksii:

The yield data given below are for the 4" columns, with 3 kg of the C_{18} silica and 600 g of the $CHCl_3$ extract obtained from 12 kg of the dry needles. Data for the 6" diameter column are presented below under the heading, paclitaxel.

After the sample was loaded, elution with 30-35% AN/ water gave large amounts of water-soluble, polar constituents, which accounted for the bulk of the uv absorbance at 275 nm. Elution with 35-40% solvent mixture gave a number of minor components, followed by a major component, identified as brevifoliol[12,13]. Washing with 45-50% AN/ water started the elution of taxanes I and II, followed by paclitaxel, all of which crystallized together from the fractions, as they stood for 2-8 days. From the eluates with 55-60% AN/ water, there was another region of crystallization, from which taxane III was isolated. As the column was being washed with MeOH, the fractions again deposited crystals, consisting of taxane IV. Further elution with methanol produced large amounts of crystals consisting of sitosterols. The progress of the column, followed by absorbance at 275 nm is shown in Fig. 2.

CHARACTERIZATION OF THE TAXANE CONSTITUENTS of *T.* x *MEDIA* HICKSII

Brevifoliol 2:

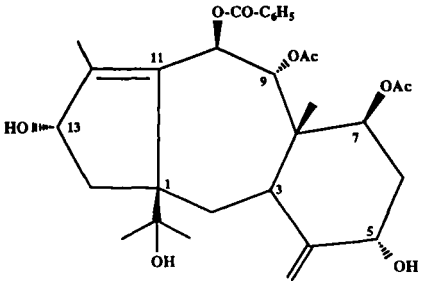
Fractions from the 40% AN/ water were partially concentrated, the solid which separated was filtered, and decolorized by dissolving in CH_2Cl_2 and passage through a column of Florisil (3 g per gram of the sample). The product was crystallized from acetone/ ligroin (2:1), yield, 2.5 g (0.02%). Brevifoliol **2** (also called brevitaxane[13]) is a colorless crystalline solid, m.p. 220-222^0 C (lit.[12], 200-205^0 C); specific rotation -27^0. Anal. calc for $C_{31}H_{40}O_9$: C, 66.89; H, 7.24. Fd. C, 67.13; H, 7.35.

Taxanes I and II 3 and 4:

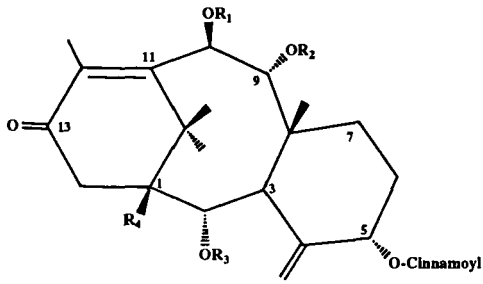
The crude crystals (25 g) consisting of **1**, **3** and **4** were processed by two methods. In one, a solution in $CHCl_3$/ ligroin (3:1, 250 mL) was chromatographed on silica (150 g), with the eluent changed to $CHCl_3$, 2% acetone, 5% acetone, 2% MeOH and 5% MeOH, all in $CHCl_3$. The mixture of **3** and **4** appeared first (2-5% acetone/ $CHCl_3$), followed by **1** (2% MeOH/ $CHCl_3$). Concentration of the appropriate fractions gave the mixture of **3** and **4** (12 g, 0.1%). (For the second method, see under paclitaxel)

A portion of this mixture of **3** and **4** (1 g) was applied to a C_{18} reverse phase column (25 g) in 40% AN/ water, and elution with 45 and 50% AN/ water. After one week, the fractions with the crystals were filtered in groups. Although **3** and **4** were separated, such that each contained the other to the extent of 10% or less, further recrystallizations gave worse mixtures, thus suggesting that isomerization was taking place in solution. Data on a crystalline 90:10 mixture of **3** and **4**: m.p. 136-138^0 C, specific rotation ($CHCl_3$): 214 (lit.[14], m.p. 163-165^0 C, rotation, 185, on a powder form of the sample); Anal. Calc. for $C_{31}H_{38}O_8$, H_2O: C, 66.89; H, 7.24. Fd. C, 66.51; H, 7.19.

The ^{1}[H] and the ^{13}[C] nmr spectra of the crystalline **3** and **4** gave evidence for mixtures of two compounds. From the spectral data, these two were identified as a mixture of 5-O-cinnamoyl-9-acetyltaxicin I **3** and 5-O-cinnamoyl-10-acetyltaxicin I **4**, as described by Chmurney et al.[9] from *Taxus* x *media* Hicksii, and by Appendino et al.,[14] from the needles of *Taxus baccata*. The latter authors obtained the two as amorphous powders by using HPLC and preparative TLC.



2: BREVIFOLIOL

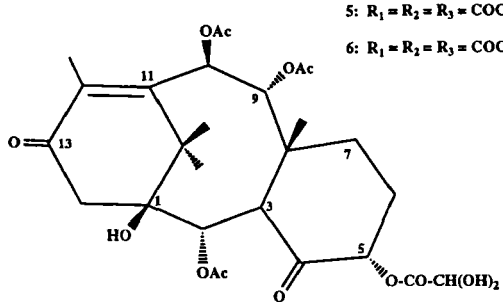


3: $R_1 = COCH_3$, $R_2 = R_3 = H$, $R_4 = OH$

4: $R_1 = R_3 = H$, $R_2 = COCH_3$, $R_4 = OH$

5: $R_1 = R_2 = R_3 = COCH_3$, $R_4 = OH$

6: $R_1 = R_2 = R_3 = COCH_3$, $R_4 = H$

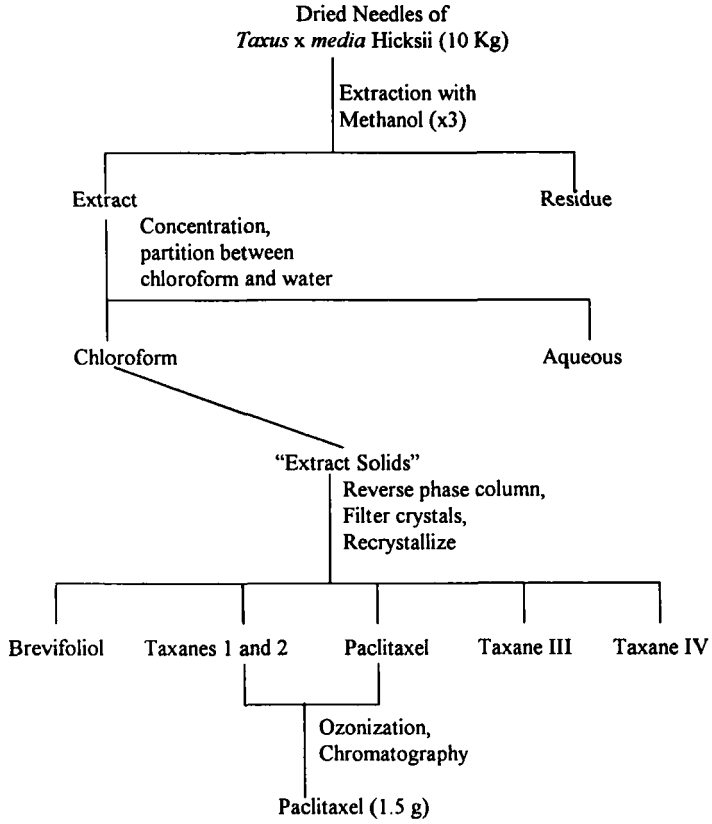


13

The mixture of **3** and **4** on acetylation (Ac_2O / pyridine, r.t. 6 h) gave the acetate, m.p. 238-241^0 C, the nmr spectrum of which showed it to be a single entity, unlike the starting mixture. It was identical with taxane III (**5** see below).

Taxane III 5:

Crude crystals of taxane III were filtered and recrystallized from acetone/hexane to give colorless needles, yield, 2.5 g (0.02%), m.p. 238-241^0 C; specific rotation ($CHCl_3$): 214 (lit[15]. 218); MS (FAB): 645 (M^+ + Na), 623 (M^+ + H), 475 [(MH^+)-148 (cinnamoyl)], 415 (475-AcOH), 355 (415-AcOH), 295 (355 - AcOH). The spectral data showed that it is the 5-O-cinnamoyl 2a,9a,10b-triacetyl taxicin I (Appendino et al.,[14] and Baxter et al.[15]. Anal. Calc. for $C_{35}H_{42}O_{10}$, H_2O: C, 65.61; H, 6.92. Fd. C, 66.00; H, 6.72.



Scheme 1

Taxane IV 6:

This compound was recrystallized from acetone/ligroin, yield, 2.6 g, (0.02) %, m.p. 265-267^0 C; specific rotation ($CHCl_3$): 133 (lit.[15], 137); MS (FAB): 607 (MH^+), 459 (607 - 148 (cinnamate), 399 (459 - HOAc), 339 (399 - HOAc), 279 (339 - HOAc); Anal. Calc for $C_{35}H_{42}O_9$: C, 69.02, H, 7.03. Fd. C, 69.29, H, 6.98.

The nmr spectral data that it is 5(O)-cinnamoyl taxicin II-2a,9a,10b-triacetate (Appendino et al.[14] and Baxter et al.[15]).

Paclitaxel

After the SiO_2 chromatography described under **3** and **4**, the paclitaxel still contained these two (<5%) and for their removal, the sample (2.5 g) was ozonized in $CHCl_3$/ MeOH (9:1, 30 mL) at -70° C for 10-15 min. After completion of the reaction (HPLC), the mixture was treated with $(CH_3)_2S$ and let stand for 2-3 h. After concentration, the mixture was chromatographed on silica gel (65 g) in $CHCl_3$ Elution with 2-5% acetone in $CHCl_3$ gave pure **1**, which was crystallized from acetone/ligroin, yield, 1.5 g, (0.012 %), m.p. 219-221° C. Its spectral and physical data were the same as those of an authentic sample.

In subsequent trials, a second method of processing was used, involving a 6"-diameter column run on 2.5 Kg of the extract from 50 Kg of dried needles. The crude mixture of **1**, **3** and **4** (95 g) was processed by direct ozonization (without the intermediate silica column) in 30 g portions (9:1 $CHCl_3$/MeOH, 400 mL) and the product chromatographed on silica as before. By this method, the yield of pure **1** was 7.5 g (0.015 %),

A summary of the process used in connection with *T.* x *media* Hicksii is given in Scheme **1**.

Ozonolysis of 5:

The ozonolysis converted **3**,**4** and **5** to other products and to characterize one of these, ozonolysis was carried out on pure **5** (1 g), as described above. The product was crystallized from acetone/ligroin to give colorless needles, yield, 0.8 g., m.p.168-170° C, specific rotation (pyridine), 130; HR/MS: 569.2239, Calc. for $C_{27}H_{36}O_{13}$, 569.2234; [H] nmr ($CDCl_3$:

Taxus Floridana

The 45 lb (20 kg) batch of fresh needles gave 500 g of the $CHCl_3$ extract which was applied on to a C_{18} silica column (4" diameter). The solvent sequence used for elution was the same as that given under *Taxus* x *media* Hicksii.

After the initial polar (phenolic) components, the first taxane to elute, 10-deacetyl baccatin III (**7**) appeared with the 35% AN/ water and crystallized almost immediately from the fractions forming glistening plates. The next area which showed significant crystallization was the 40-45 % AN/ water eluate, and it contained a new 11(15→1)-abeotaxane, which was designated as taxiflorine (**8**).

With the 50 % AN/ water was eluted paclitaxel **1**, accompanied by baccatin VI (**9**).

Characterization and yield data on each of the preceding compounds are given below.

10-Deacetyl baccatin III 7

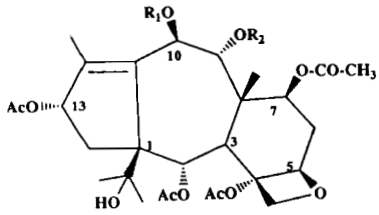
The crude crystals (17 g) after recrystallization gave 12 g (0.06 %) of pure **7**, m.p. 232-234^0 C. The spectral and HPLC data were identical with those of an authentic sample[10].

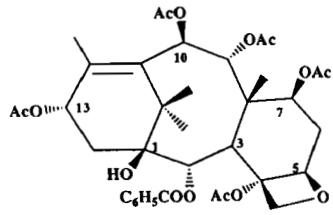
Taxiflorine 8

The crude crystals (2.5 g) were recrystallized from acetone/ligroin to yield colorless prisms, yield, 1.2 g (0.006 %), m.p. 254-255^0 C, specific rotation, [α]D23, -26.1. Anal. Calc. for $C_{35}H_{44}O_{13}$: C, 62.48; H, 6.59. Fd. C, 62.12; H, 6.63.

The ^{1}[H] nmr spectrum of pure crystalline (**8**) in $CDCl_3$ showed broad peaks with poor resolution, and double sets of peaks, The spectrum was sharper in DMSO, but it still had the extra peaks, ^{13}[C] spectrum also showed the extra peaks, which suggested the existence of either an equilibrium mixture[16], or a conformational inversion[17]. The spectrum gave evidence for one benzoate, four acetates, and an oxetane ring. The spectrum of the mono acetate (**10**) showed that it is a single compound. Although isomeric with baccatin VI (**9**), it was different from it. The striking difference between the ^{1}[H] spectra of the two was with the H-13 signal: in **10** at δ 5.60, and in **9**, at δ 6.3. A comparison with other related taxanes showed that in those with a 6-membered A-ring, the H-13 signal appears at 6.2-6.5[18], whereas in those with a 5-membered A-ring, as in the 11(15→1)-abeotaxanes, it appears at 5.4-5.7[19].

Positions 9 and 10 in **8** carry the free OH, and the benzoate. Of the signals at δ 6.30 and 5.90 in **8**, acetylation shifts one of these down-field from δ 5.9 to d 6.2, while the other stays essentially unchanged (δ 6.37). Since the allylic H-10 must be more down-field than H-9, the signal at δ 6.30 in **8** (and 6.37 in **10**) can be assigned to H-10 and the one at δ 5.9 in **8** (and 6.2 in **10**) to H-9, thus showing that the benzoate in **8** is at C-10 and the OH at C-9. This leaves positions 2 α,4 α,7 β and 13 α for the four acetate functions, which were confirmed by the COSY and HETCOR spectra.





8 TAXIFLORINE: $R_1 = C_6H_5CO$, $R_2 = H$

9 BACCATIN VI

10 $R_1 = C_6H_5CO$, $R_2 = CH_3CO$

11 $R_1 = R_2 = C_6H_5CO$

12 $R_1 = R_2 = CH_3CO$

Benzoylation of **8** gave the monobenzoate **11**, which was also a single entity as shown by the ^{1}[H] nmr spectrum, This structure **8** with the hydroxyl at 9, has the potential for intramolecular trans-esterification between the 10-benzoate as well as the 7-acetate[9,19]. The fact that both acetylation and benzoylation readily gave single products discounts the trans-esterification possibility as being responsible for the anomalous nmr spectrum of **8**. To verify if the appearance is due to an equilibrium between two rotamers, the spectrum was taken in DMSO-d_6 at temperatures ranging from -20° to 100°. At lower temperatures, the spectra were sharper and showed two sets of peaks. At higher temperatures the peaks coalesced into a single set, as well as became broad to the extent that some were barely seen. This behavior suggested that the conformational equilibrium between rotamers is responsible and that the presence of the 9-OH facilitates this process.

Baccatin VI 9

The fractions from the 50% AN/ water elution contained two components: **9** and **1**. The crude crystals from the first portion (3.5 g) containing mostly **9** were taken up in $CHCl_3$ (50 mL) and decolorized by passage through a column of Florisil (20 g). The solid was crystallized from acetone/ligroin to yield **9**, 1.6 g. With the additional amount from the purification of paclitaxel (see below), the yield was 1.95 g (0.01 %), m.p. 250-252° C, specific rotation, $[\alpha]^{D,23}$, -11°, MS (FAB), 714; Anal. Calc. for $C_{37}H_{46}O_{14}$ (714): C, 62.18; H, 6.49. Fd. C, 61.83; H, 6.49. The nmr spectral data agreed with those Senilh et al. [20]

Table 1

Protein NMR Spectra of Taxiflorine Esters

H AT	10	11	12
2	6,19, d, J=7,8	6.26, d, J=7.8	6.07, d, J=7.8
3	2.99, d, J=7.8	3.06, d, J=7.8	2.92, d, J=7.8
5	4.98, d, J=7.5	5.01, d, J=7.5	4.98, d, J=7.5
6	2.68, m	2.7, m	2.52, m
	1.84, m	1.84, m	1.84, m
7	5.52, m	5.64, m	5.49, t, J=7.8
9	6.32, d, J=10.8	6.48, d, J=10.8	6.04, d, J=10.8
10	6.44, d, J=10.8	6.72, d, J=10.8	6.27, d, J=10.8
13	5.62, t, J=7.8	5.64, m	5.61, t, J=7.8
14	2.30, dd, J=7.4, 14.2	2.34, dd, J=7.5, 14.7	2.30, m
	1.72, dd, 7.4, 14.2	1.78, m	1.72, m
16	1.16, s	1.24, s	1.15, s
17	1.19, s	1.21, s	1.13, s
18	1.72, s	1.72, s	1.83, s
19	1.64, s	1.95, s	1.66, s
20	4.5, 4.42, d, J=7.9	4.52, 4.44, d, J=7.2	4.47, 4.38, d, J=7.5
Ph(2', 6')	7.93, d	u	--
Ph(3', 5')	7.45, t	7.24, m	--
Ph(4')	7.62, t	7.37, m	--
Ph'(2",6")	--	7.63, d, J=7.2	--
Ph'(3",5")	--	7.24, m	--
Ph'(4")	--	7.37, m	--
OAc	2.02, s	2.14, s	2.11, s
OAc	2.14, s, (2x)	2.05, s	2.1, s
OAc	1.86, s	--	2.08, s
OAc	1.8, s	--	2.03, s
OAc	--	--	2.01, s
OAc	--	--	1.95, s

Paclitaxel 1

The crude crystals (4.5 g) from the second part of the peak, which was mostly **1** but had some **9**, were taken up in $CHCl_3$ (60 mL) and applied to a short column of Florisil (40 g). Elution with $CHCl_3$ gave **9** and subsequent elution with 2-5 %

acetone/ $CHCl_3$ gave **1** which was recovered and crystallized from acetone/ ligroin, yield, 1.98 g (0.01 %), m.p. 220-222^0 C. The spectral and HPLC data showed that it was identical with paclitaxel.

DISCUSSION

Taxus x *media* Hicksii

Isolation of paclitaxel and other taxanes has been carried out on a pilot-plant scale from the needles of *Taxus x media* Hicksii. Briefly, methanol extraction, concentration and partition of the concentrate between water and $CHCl_3$, followed by concentration of the $CHCl_3$ layer gave approximately 5% of the $CHCl_3$ extractives, from the dried needles. This extract is applied directly on to a reverse phase (C_{18}) silica column. For example, on a 6"x 6' column, 2.5 kg of the extract (from 50 kg of the dry needles) can be loaded using the 25% AN/ water medium. The column was then developed with a step gradient: 30-60% AN/ water (in increments of 5%), during which, most of the taxanes of interest were eluted. The column was then washed free of the chlorophyll, waxes etc., re-equilibrated and reused.

It is generally understood that, compared to the bark, large-scale processing of the needles of *T.* x *media* Hicksii would have more challenges because of the presence of the lipophilic chlorophylls, carotenoids and waxes, as well as unrelated taxanes that co-elute with paclitaxel[8,9] (Fig. 1A). For this reason, methods such as fractional extraction with nonpolar solvents[21], or extraction of the plant material with aqueous alcohol[22] have been proposed. If one must employ such a pretreatment step, the procedure given here: passage through a reverse phase column in a methanol/ acetone/ water mixture (see methods) appears to be the most efficient and convenient.

However, our experience has shown both with the bark extract (*T. brevifolia*)[10,11] and with the needle extract (3 species of *Taxus*), that the chlorophylls or waxes present in the needles pose no problem with the column performance. One explanation may be that the C_{18} bonded silica gel seems to behave as a long-chain hydrocarbon and thus, exhibits great affinity for these non-polar components, which appear to be taken up by the silica and almost "dissolved" in the gel matrix. Thus, the waxes are not present as an insoluble powder at the top of the column to block the column flow. Because of the apparently high distribution coefficients for the chlorophylls and waxes as compared to the taxanes, in favor of the silica and against the 30-60% AN/ water mixtures (or even a 7:1:2 methanol/acetone/water mixture as

seen above), they remain on the column until all of the taxanes are eluted. After the elution of the taxanes is over, the column can be completely stripped of these lipophilic components, then equilibrated with the AN/ water and made ready for reuse.

It is important to distinguish the process described here, which uses a reverse phase column, from being characterized as an "HPLC" process. Although reverse phase column chromatography is used extensively in analytical HPLC-methodology, its use in preparative work is relatively less frequent, and when it is used, the methods are carried out almost as an extension of the analytical technique. For example, the compounds to be separated have already been purified by other means, the columns are selected to reflect the sample / adsorbent ratio of 1:1000 or (much) more, the sample is applied in a minimum volume as a clear solution, and the columns are usually run at high pressures (500-1500 psi).

In contrast, applying a crude extract of the plant, which is essentially insoluble in the mobile phase, on the column; using a sample / adsorbent ratio of 1:5, and a large sample volume, all show that the method given here is clearly not an extension of the HPLC protocol. In spite of these unfavorable changes, the column appears to perform normally, i.e., giving acceptable resolution (Fig. 2, the compounds being eluted in the order of their retention times), a good flow rate at medium pressures (2-4 l per hour at 40-120 psi) and for many of the products, the purity increases from 1% or less to 50% or more, after this single reverse-phase column.

As an illustration of this last point, a very important advantage of the reverse phase column as described here is that when the fractions are allowed to stand for about a week, a number of the taxanes crystallize out directly, leaving many impurities in solution. Based on the HPLC analysis of the fractions before and after the crystallization, it appeared that the degree of crystallization was better than 75%. Thus, the crystals, although still relatively crude, are significantly purer than what one might obtain by concentration of the whole fraction to dryness, or extraction of the fraction with a solvent (eg. dichloromethane) and concentration. In many cases (eg. compounds **2**, **5**, **6** and **7** from *T.* x *media* Hicksii, and **8**, **9** and **1** from *T. floridana*), further purification of the crude crystals involves only a recrystallization, or a simple "filtration" type column and no additional chromatography. Thus, this advantage of crystallization is lacking in the normal phase silica column chromatography, with commonly used solvents such as dichloromethane or acetone.

Next to the chlorophyll/ wax problem, the problem of co-eluting taxanes is of importance as far as the yields of paclitaxel from the needles of *Taxus* x *media* Hicksii are concerned. These taxanes, primarily **3** and **4**, occur to the extent of 4-6

times as much as paclitaxel in these needles (Fig. 1A). Because of their higher uv-extinction values (due to the cinnamate moiety), even trace amounts of these appear as significant impurities in the usual uv-based HPLC analyses. Thus, to reach the 99+% level of purity for paclitaxel, at least two, if not three, successive columns are needed. Hence, as an alternative, ozonization of the crude mixture of **1**, **3** and **4** was used here for the final purification, in which **3** and **4** are converted to more polar products, which are more readily separable from **1**. The ozone also causes extensive bleaching of the sample, thus assisting further in the purification.

To learn of the identity of the product after ozonization, the reaction was carried out with pure **5** (acetate of **3** and **4**). The analytical and spectral data of the crystalline product indicated the presence of the C=O function at 4 and a hydrated glyoxalyl ester group at C_5.

Thus, the process described here for the needles of *Taxus* x *media* is simpler because, the extract does not require pretreatment, essentially a single column gives crystalline products and, because of fewer steps, the yields are higher than those reported earlier[4]. The reverse phase column can be run on a large scale economically, because of the high capacity loading of the column, re-usability of the adsorbent and the solvent(s).

T. floridana

In the earlier studies conducted to examine various species and cultivars of *Taxus* as sources for paclitaxel[4,5,23], the species *T. floridana* has received scant, if any attention. As a source of paclitaxel, this plant must be counted as one of the important ones because, in several tests by actual isolation, both on laboratory and pilot-plant scale, the yield of paclitaxel has been consistently 0.01% on a fresh basis, and hence, potentially 0.02% or more on a dry basis. To make the plant even more appealing, 10-deacetyl baccatin III can also be isolated, again consistently in yields of 0.05-0.06% from the fresh leaves.

Additionally, this plant yields two other compounds: baccatin VI (**9**) and taxiflorine (**8**), both of which contain the oxetane ring and hence can serve as precursors for active compounds; **9** to produce 9-dihydropaclitaxel[(24)] type analogues, and **8**, to produce analogues with the 11(15→1)-abeotaxane skeleton. This is not the case with *Taxus* x *media* Hicksii, where the unrelated (4/20 methylene) taxanes which have no declared use at present, exceed paclitaxel in yield by 4-6 fold. Finally, isolation of paclitaxel and the other useful taxanes is considerably easier from *T. floridana* than that of paclitaxel alone from *Taxus* x

media Hicksii, because there are no co-eluting taxanes in *T. floridana* (Fig. 1A and B) and paclitaxel and the other three components are readily obtained pure after a single reverse phase column.

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