A New Large-Scale Process for Taxol and Related Taxanes from *Taxus brevifolia*

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Purpose. In view of the demonstrated antitumor activity of taxol, ready availability of the drug is important. The current isolation methods starting from the bark of *Taxus brevifolia* involve multiple manipulations, leading to only taxol and in a yield of 0.01%. A new process consisting of a single reverse phase column is introduced here, and the present purpose is to determine its large scale applicability. ***Methods.*** The chloroform extractable fraction of the bark of *T. brevifolia* is applied directly on to a C-18 bonded silica column in 25% acetonitrile/water, with elution using a step gradient: 30-50% acetonitrile/water. On standing, eight different taxanes, including taxol, crystallize out directly from different fractions. The crystals are filtered and purified further by recrystallization. Taxol and four other taxanes are purified this way. The other three require a short silica column. Taxol is freed from cephalomannine by selective ozonolysis. ***Results.*** The large scale process gave taxol (0.04%), 10-deacetylbaccatin III (0.02%), 10-deacetyl taxol-7-xyloside (0.1%), 10-deacetyl taxol-C-7-xyloside (0.04%), 10-deacetyl cephalomannine-7-xyloside (0.006%), taxol-7-xyloside (0.008%), 10-deacetyl taxol (0.008%) and cephalomannine (0.004%). Processing of the needles of *T. brevifolia* gave brevifoliol (0.17%), and that of the wood, 10-deacetyl taxol-C-7-xyloside (0.01%) and 10-deacetyl taxol-C. ***Conclusions.*** The reverse phase column process is simpler (one column, direct crystallization), more efficient (eight taxanes obtained simultaneously) and also gives higher yields.

KEY WORDS: taxol; 10-deacetyltaxol; 10-deacetyltaxol-7-xyloside; taxol-7-xyloside; 10-deacetyltaxol-C-7-xyloside; reverse-phase; C-18-silica.

INTRODUCTION

Taxol (Fig. 1) is currently an important antitumor drug, originally discovered in 1971 from the bark of *Taxus brevifolia* (1). Based on its activity in human clinical trials, taxol has been approved by the Food and Drug Administration for use in ovarian carcinoma. Production of the drug required for the clinical use still poses some problems and challenges, and there is apparently some room for improvement.

The original and still the major source of the drug has been the bark of the Pacific yew (*Taxus brevifolia*), from which taxol is being isolated in a yield of 0.01-0.013%. Although several related taxanes that can serve as precursors for the semi-synthesis of taxol, as for example, 10-deacetylbaccatin III (Fig. 1), co-occur in the bark with taxol (2), there are no reports to indicate that these are being isolated from the bark on a large scale. Thus, the low yields of taxol realized by the current process, apparent unavailability of other useful taxane analogues, and the environmental concerns raised by the need to cut the slow-growing yew trees for harvesting the bark, are some of the reasons why the bark is no longer considered an attractive source for the large scale production of taxol.

Among the alternatives that are being actively studied are the following: 1) isolation of 10-deacetylbaccatin III from the European yew (*Taxus baccata*) and its semi-synthetic conversion to taxol (3,4) and 2) large-scale cultivation of the ornamental yew (*Taxus x media* Hicksii) and isolation of taxol from its needles/twigs (5). Among the future alternatives, are the total synthesis, of which two schemes have been published (6,7), and large-scale plant cell culture.

In an earlier publication from our laboratory, a simplified isolation procedure for taxol, using a single reverse-phase chromatographic column was described (2). The advantages of this process are simplicity, increased yields of taxol (0.02-0.04%) and simultaneous isolation of several important analogues of taxol. In spite of these crucial advantages, because the procedure used aqueous/organic solvents and a reverse phase type adsorbent with a fine particle size (15-35 microns), the question remained as to whether the process could be adapted to function effectively on a large scale. This paper describes some details of the process as it was successfully run on a pilot-plant scale for the production of pure taxol and some of its important analogues.

MATERIALS AND METHODS

Plant Material

The bark, wood and the dried needles of *Taxus brevifolia* were purchased from Mr. Patrick Connolly, Yew Wood Industries Company, 6928 North Interstate Avenue, Portland OR 97217. The bark and the wood were received after being dried and ground to a coarse mesh (approximately 5-15 mm). The initial shipment of 2000 lbs of the bark came from relatively young trees (app. 4″ diam) growing on Government lands, while another 5000 pounds came from more mature (app. 8–10″ diam) growing on private lands. The needles were received after a "quick-drying" process. Thus, several thousand pounds of the bark and a few hundred pounds of the needles and the wood were processed in this study.

Step 1: Extraction and Concentration

The extraction was carried out as a batch process, using methanol, in stainless steel tanks of capacity 200-300 gallons, equipped with lids that could be clamped tightly and with an outlet (1″ dia) at the bottom for draining. These were fabricated by Pillsbury Alloy Fabrication, Inc. Jacksonville, FL. Approximately 200-260 lbs of the bark could be extracted at a time in the 200 gallon tank and 300-400 lbs of bark in the 300 gallon tank. To facilitate the extraction, for several hours during the day, the extract was recycled by being pumped back into the tank through an opening in the lid, using a device to provide a gently rotating spray. Three to four such extractions, each running for a day were carried out. The

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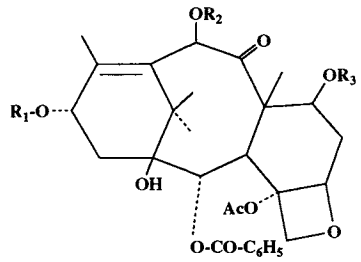


$R_1 = R_2 = R_3 = H$: 10-DEACETYL BACCATIN III

$R_1 = C_6H_5\text{-}CH(NH\text{-}CO\text{-}C_6H_5)\text{-}CH(OH)\text{-}CO$; $R_2 = CH_3CO$; $R_3 = H$: TAXOL

$R_1 = C_6H_5\text{-}CH(NH\text{-}CO\text{-}C_6H_5)\text{-}CH(OH)$; $R_2 = H$; $R_3 = H$: 10-DEACETYL TAXOL

$R_1 = C_6H_5\text{-}CH(NH\text{-}CO\text{-}C_6H_5)$; $R_2 = H$, R_3 = XYLOSYL: 10-DEACETYL TAXOL-7-XYLOSIDE

$R_1 = C_6H_5\text{-}CH(NH\text{-}CO\text{-}C_6H_5)$; $R_2 = CH_3CO$; R_3 = XYLOSYL: TAXOL-7-XYLOSIDE

$R_1 = C_6H_5\text{-}CH(NH\text{-}COC(CH_3){=}CH\text{-}CH_3)\text{-}CH(OH)\text{-}CO$; $R_2 = COCH_3$, $R_3 = H$: CEPHALOMANNINE

$R_1 = C_6H_5\text{-}CH(NH\text{-}COC(CH_3){=}CH\text{-}CH_3)$; $R_2 = H$, R_3 = XYLOSYL: 10-DEAC. CEPHALOMANNINE-7-XYLOSIDE

$R_1 = C_6H_5\text{-}CH(NH\text{-}CO\text{-}C_5H_{11})$; $R_2 = H$, R_3 = XYLOSYL: 10-DEACETYL TAXOL-C-7-XYLOSIDE

$R_1 = C_6H_5\text{-}CH(NH\text{-}CO\text{-}C_5H_{11})$; $R_2 = R_3 = H$: 10-DEACETYL TAXOL-C

Fig. 1. Structures of taxol and its analogues.

progress of the extraction was monitored by uv-absorbance values at 275 nm.

The methanolic extract was concentrated under reduced pressure (<30° C) using a semi-continuously operated still with a receiving capacity of 220 gallons. The extract was fed into a jacketed still-tank (100 gallons) 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), where they were condensed by chilled water at 10° C and the condensate taken to a series of four receiving tanks, each of 55 gallon capacity. The system was kept under reduced pressure 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 gallons from the extract obtained from a 200-250 lb batch of the plant material.

Step 2: Solvent-Partition

Extraction of the concentrated methanolic extract with chloroform was performed in 50-100 gallon tanks, equipped with an air-driven stirrer. The concentrate was stirred with water (10 gallons) and chloroform (20 gallons) 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 chloroform.

Concentration of the chloroform 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. 1-2 gallons) was further stripped of solvent in a rotary evaporator to a thick syrup which was then poured into glass trays and converted to a powder form, using a vacuum oven maintained at 35-40° C. The powder, obtained in a yield of 18-26 g per kilogram of the bark, was stored in tightly stoppered bottles at room temperature.

The extraction of the needles and wood followed the same procedure, with the yield of the extract, also being in the same range. The needle extract remained as a thick green glassy material.

Step 3: Chromatography

For 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 at 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 were required for the 4″ and 6″ columns re-

spectively. After a thorough wash with methanol, the columns were equilibrated with 25% acetonitrile in water.

For running the 6" diameter column, the solid from the chloroform extract of the bark (2-2.5 kg) was dissolved in acetonitrile (5 l) and while the mixture was being stirred with the equilibrated silica (1-2 l), diluted with water to make 20 l. The mixture was then 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 and 50% acetonitrile in water. The change of solvent was dictated by the results of the tlc and hplc of the fractions, but generally, 40-50 liters 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.

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 2-8 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 chloroform 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 acetonitrile/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 × 25 cm, Whatman, Partisil) packed with C-8-bonded silica, 5 micron diameter were used with either of the solvents: 50% acetonitrile/water, or a 5:4:1 mixture of acetonitrile, 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/chloroform or methanol/chloroform. Visualization was by a uv-lamp and by charring with 1 N sulfuric acid. 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/chloroform, chloroform, 2-5% acetone and finally, 2-10% methanol in chloroform.

UV-Spectra were obtained using Perkin Elmer Lambda 3B spectrophotometer.

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

RESULTS

In general, the first few fractions from the column contained chiefly the polyphenols (eg. condensed tannins), accounting for most of the uv-absorbance at 275 nm. The earliest taxane to appear was 10-deacetylbaccatin III (Fig. 1), which crystallized almost immediately from the fractions from 35% acetonitrile/water. A number of other taxanes were eluted next, with the major one being brevifoliol (8). However, most of these remained in solution.

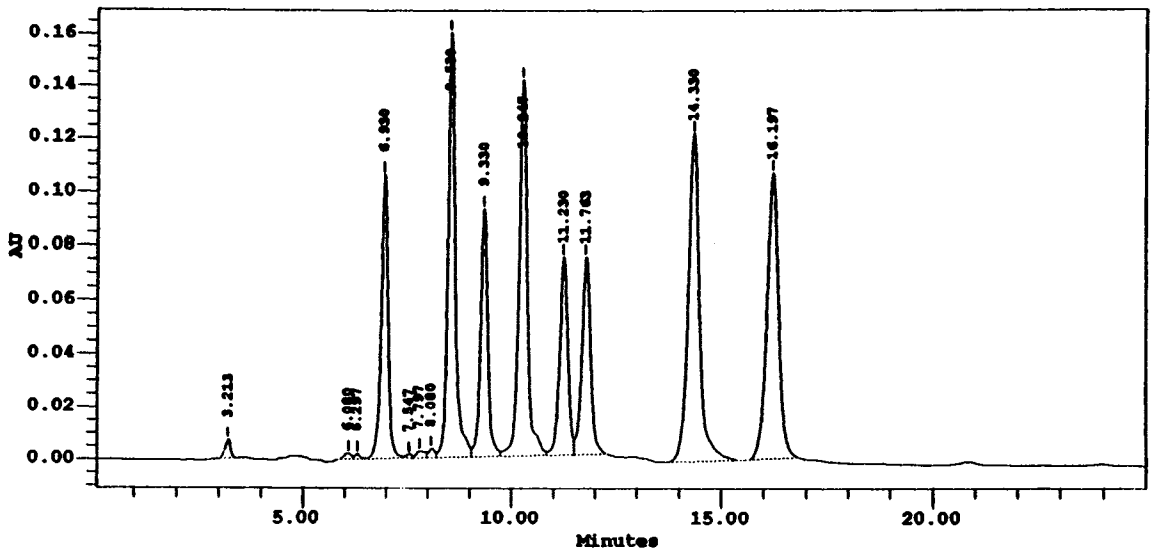
The next group of taxanes to be eluted were the various xylosidic taxanes (Fig. 1): 10-deacetyl cephalomannine-7-xyloside, 10-deacetyl taxol-7-xyloside, 10-deacetyl taxol-C-7-xyloside and taxol-7-xyloside, together with 10-deacetyl taxol. Of these, the first-two were well separated from each other. As the elution of 10-deacetyl taxol-7-xyloside was nearing completion, 10-deacetyl taxol-C-7-xyloside started to elute. Halfway through its elution, taxol-7-xyloside and 10-deacetyl taxol started to co-elute. These last three compounds also crystallized together. (For separation of these three components, see below.)

Continued elution of the column with 50% acetonitrile/water gave cephalomannine, followed by taxol. The earlier part of the band contained mixtures of the two, but the later fractions contained mostly taxol. Further elution of the column provided other, less polar taxanes, followed by two steroidal components: β sitosterol and its β-D glucoside which crystallized in large amounts on standing.

Characterization, yields and other data on each of the preceding compounds are given below. In calculating the yields, it is to be noted that 100 kg of the bark generally gave 2.5 kg of the chloroform extract solids, which was applied to the column. Where relevant, the amount of the crude crystals obtained directly from the fractions, the amount after recrystallizations, and what was obtained by processing of the filtrates (if applicable) are given. The yields are based on the purified samples (analytical hplc, uv-detection, 97-99%). Fig 2 shows the hplc trace of the eight crystalline taxanes obtained from the column, applied as a mixture.

10-Deacetyl Baccatin-III. This crystallized out as glistening plates from the fractions of the eluent containing 25-35 % acetonitrile and water. It was recrystallized from acetone twice to obtain as colorless plates, m.p. 232-234° C.

Yield data: crude crystals, 26 g; after recrystallization, 18 g; the material from the filtrates, after a short, normal phase silica column with chloroform, 2-5% acetone and 2-5% methanol in chloroform as the solvent system, followed by crystallization, gave 3 g. Total, 21 g; yield, 0.02%. The pro-



1. *10-DEACETYL BACCATIN III*
2. *10-DEACETYL CEPHAL.-7-XYLOSIDE*
3. *10-DEACETYL TAXOL-7-XYLOSIDE*
4. *10-DEACETYL TAXOL-C-7-XYLOSIDE*
5. *TAXOL-7-XYLOSIDE*
6. *10-DEACETYLTAXOL*
7. *CEPHALOMANNINE*
8. *TAXOL*

Fig. 2. HPLC pattern of taxol and seven of its analogues. Column used: Whatman C_8-bonded silica; solvent: 5:4:1 acetonitrile/water/methanol; flow rate: 0.5 ml per min.

ton and the carbon nmr spectra as well as the hplc elution pattern were identical with those seen with an authentic sample (2).

10-Deacetyl Cephalomannine-7-xyloside. Eluent fractions from 35-40 % acetonitrile/water gave this as a crystalline solid. After filtration and recrystallization twice from acetone (charcoal), it was obtained as a colorless crystalline solid, m.p. 250-252° C. The identity was confirmed by a comparison of the spectral data with those of a sample isolated and characterized earlier in our laboratory (2).

Yield data: crude crystals, 20 g, after recrystallization, 6 g; yield, 0.006 %.

10-Deacetyl Taxol-7-xyloside: A number of fractions from the 40-45 % acetonitrile/water showed a heavy degree of crystallization. (Fig. 3) Using analytical hplc, those fractions containing this xyloside as the major constituent were filtered and the combined solid dried. Recrystallization was carried out from a mixture of methanol and chloroform (1:1) with water (5-10 %) and using decolorizing charcoal, to yield 10-deacetyl taxol-7-xyloside as colorless rectangular plates, m.p. 247-249° C, and identical with an authentic sample (2).

Yield data: crude crystals, 180 g; after recrystallization, 99 g, yield 0.1 %. Its identity was confirmed by comparison with an authentic sample (2). 10-Deacetyl taxol-7-xyloside is the major taxane component of the bark and was isolated in yields of 0.06-0.1%. The bark from the more mature trees approached the 0.1% mark, while the bark from younger trees was closer to the 0.06% range. Quantitative hplc analysis of the bark extract showed that the content of this component was in the range of 0.12-0.14%, thus indicating a 70-80% efficiency of recovery.

10-Deacetyl Taxol-C-7 xyloside. This was also obtained from the fractions eluted with 45 % acetonitrile. Using analytical hplc, those fractions showing the highest ratio (8 or higher) for 10-deacetyl taxol-C-7-xyloside / 10-deacetyl taxol-7-xyloside were filtered and the combined solid dried. After two crystallizations (charcoal) from the solvents described above, 10-deacetyl taxol-C-7-xyloside was obtained as a colorless crystalline solid, m.p. 218-220° C (lit. 215-217, (9)).

Yield data: Crude crystals, 98 g; after recrystallization, 40 g; yield, 0.04 %. The proton nmr spectral data also agreed with those described by Senilh et al., (9).

Taxol-7-xyloside and *10-Deacetyl taxol.* The filtrates from the crystallizations of the 10-deacetyl taxol-C-7-xyloside contained taxol-7-xyloside and 10-deacetyl taxol as the major components. These filtrates were combined with the other column fractions containing the same components, and concentrated to dryness. The solid (55 g) was applied to a silica column (500 g., 5 x 75 cm) in chloroform. The elution sequence was 2% acetone (1 L), 5% acetone (1.5 L), 2% methanol (1.5 L), 5% methanol (1.5 L) and 10% methanol in chloroform (2 L). The major components, 10-deacetyl taxol and taxol-7-xyloside, appeared in the 2% methanol and 5% methanol/chloroform eluates respectively. After concentration of the appropriate fractions to dryness, taxol-7-xyloside

Fig. 3. Crystallization of taxanes from the column fractions.

was obtained from acetone as a colorless crystalline solid, m.p. 235-237° C; Yield, 7.8 g, 0.008 %. The spectral data agreed with those described in references (2) and (9).

10-Deacetyl taxol obtained from the above silica column was purified by crystallization from aqueous acetonitrile to give a colorless crystalline solid, m.p. 194-196° C, yield, 8 g; 0.008%. The spectral data were identical to those described in (2) and (9).

Cephalomannine. The fractions from the taxol/ cephalomannine band (obtained by using 45-50% acetonitrile/water) were analyzed by analytical hplc to determine the relative composition. Crystals from those fractions that contained 10% or higher of cephalomannine were filtered separately and the solid (25 g) was purified by a repeat of the reverse phase column chromatography using C-8 silica gel (25g per gram of the solid) as the adsorbent and 40-45% acetonitrile/water as the eluent. After monitoring the fractions by hplc, those crystalline solids that separated out which were essentially cephalomannine, were filtered and recrystallized once from acetonitrile/water and once more from acetone/ligroin. Cephalomannine was obtained as colorless needles, m.p.185-188° C, yield, 4 g, 0.004 %. The analytical, physical and spectral data were identical with those described (10).

Taxol. The crude crystalline solid from the fractions that contained less than 5% of cephalomannine was filtered (110 g) and subjected to one of three alternative procedures. In one, it was recrystallized using decolorizing charcoal, two or three times to reach final purity. Alternatively, the decolorization was carried out by filtration through a short column of Florisil (50 g for 10 g of the sample) in chloroform. Washing with 1-2% methanol in chloroform gave the taxol which was recovered and crystallized twice from acetone/ligroin.

The taxol so obtained was essentially free from cephalomannine (0.3% or less), as shown by analytical hplc (Fig. 4a). In the third alternative, for further purification, i.e. to remove the residual cephalomannine completely, the sample was subjected to ozonolysis in chloroform/ methanol (9:1) at -70°C (acetone-dry ice).

Thus, in a typical experiment, 30 g of the crude crystalline solid, obtained directly from the reverse phase column was dissolved in chloroform/ methanol mixture (9:1, 300 ml), cooled to -70°C and saturated with ozone over a period of 45 minutes. After checking for completion of the reaction by hplc, the reaction mixture was treated with dimethyl sulfide (10 ml) to decompose the ozonide(s) and let stand at room temperature overnight. After concentration to remove most of the solvent, the concentrate was partitioned between water and chloroform; the organic layer was separated and the extraction repeated twice more. The combined chloroform layer was concentrated to dryness and applied on to a silica column (300 g, 235-425 mesh) in chloroform. Elution with 5% acetone in chloroform gave the bulk of the taxol which was crystallized from acetone/ ligroin, to give 12 g of pure taxol.

Yield data: Crude crystals; a) high cephalomannine-containing, 25 g; low cephalomannine-containing, 110 g. The latter, after ozonolysis, short column (normal phase) and crystallization gave 40 g, 0.04 %. As shown by an analytical hplc trace (Fig 4b), this sample had a purity of 99% or better, with respect to taxol and was free from cephalomannine or 7-epi-taxol. Its physical, analytical and spectral properties were identical with an authentic sample obtained from the National Cancer Institute.

β-Sitosterol and Its 3-B-D-glucoside. The methanol eluates from the column rapidly deposited a heavy crystalline solid. The earlier fractions contained mainly the glucoside of β-sitosterol and the later fractions, mainly the sitosterol. The crystalline mixtures found in the fractions in between, were filtered and separated by digestion with acetone/ ligroin (1:1) in which the β-sitosterol was soluble. β-Sitosterol-D-glucoside was obtained as colorless plates from acetone, yield, 0.05%, m.p. 295-298° C, $[\alpha]_D$-40°, (lit. 298° C, -40.1° (11).

β-Sitosterol was obtained as colorless plates from acetone/ligroin, yield, 0.1%, m.p. 140-141° C, $[\alpha]_D$ -38°, (lit. 137-38° C, -38.2°, (11). The spectral data were in agreement with those recorded by Koizumi et al., (12).

Needle Extract of T. brevifolia. The steps of extraction of the dried needles with methanol, followed by concentration, partition between water and chloroform and concentration of the organic layer to a thick green syrup were similar to those described under the extraction of the bark.

The extract obtained from 50 lbs of the needles (600 g) was applied on to the reverse phase column (4" diameter, containing 3 kg of the C-18 bonded silica) using 30% methanol in water, as described under the "methods". Stepwise elution with 40, 45, 50, 55 and 60% methanol in water gave the successive components, which were recovered by crystallization, filtration and recrystallization.

Brevifoliol. Although this is present to some extent in the bark, the best source is the needles of the Pacific yew. Fractions from 40-45% methanol/ water containing this component were partially concentrated, whereby a crystalline solid separated, which was filtered (85 g). It was recrystallized from acetone/ligroin (1:1), yield, 41 g, 0.17 %. Brevifoliol was obtained as a colorless crystalline solid, m.p. 220-

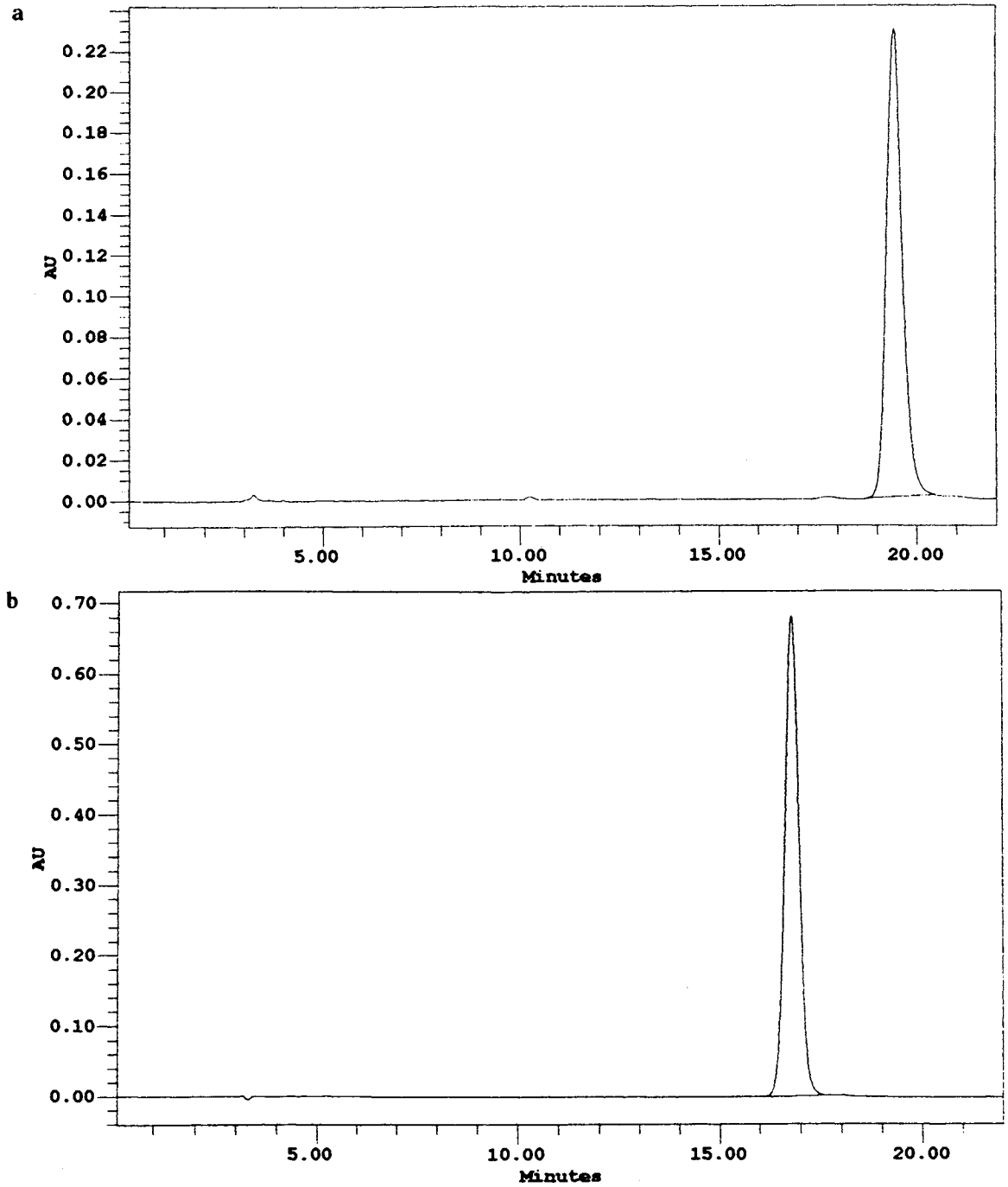


Fig. 4. (a) Column, C-8 bonded silica (Partisil); solvent, 1:1 acetonitrile/water; flow rate, 0.5 ml per minute. (b) Column, same as above; solvent, 5:4:1 acetonitrile, water and methanol; flow rate, 0.5 ml per min.

222° C (lit. (8): 200-205° C). Anal. Calc. for $C_{30}H_{40}O_9$: C, 66.16; H, 7.40. Found: C, 66.06, H, 7.25. The spectral data agreed with those described in (8).

Wood Extract of T. brevifolia. The chloroform extract (500 g) prepared from 50 lbs of the wood was subjected to reverse phase column chromatography on a 4″ diameter column using 25% acetonitrile/ water, as the initial solvent, as described above. The eluates from 40-45% acetonitrile/water showed a significant degree of crystallization. The crystalline material was filtered and recrystallized twice from acetone using charcoal. The product, a colorless crystalline solid, was identified as 10-deacetyl taxol-C-7-xyloside by analytical and spectral data (9). The yield was 0.01%.

The filtrates from the crystallization of the above xyloside were concentrated and applied on to a column of silica gel in chloroform and the column eluted with the solvent sequence described above under taxol-7-xyloside. The major component which was eluted with 2% methanol in chloroform was isolated and purified by crystallization from aqueous acetonitrile. The product was identified as 10-deacetyl

taxol-C, m.p. 168-70° C, $[\alpha]_D$ -45.6° (c = 1%, chloroform), ^1H nmr spectral data, ($CDCl_3$): 0.84, t(7 Hz), CH_3 of the hexanoyl; 1.125, s, CH_3-16; 1.25, s, CH_3-17; 1.56, t(7.2 Hz); CH_2 of the hexanoyl; 1.74, s, CH_3-19; 1.81, s, CH_3-18; 1.82, m, H-14 beta; 2.25, H-14 alpha; 2.34, s, Ac-4; 1.95 and 2.25, m, 6-H; 3.72, d(5.1 Hz), 2-OH; 3.88, d(7.2), H-3; 4.27, m, H-7; 4.2, 4.3, H-20, 4.68, dd (2.5, 5.1 Hz), H-2′; 4.92, d(7.8 Hz), H-5; 5.21, s, 10-H, 5.57, dd(9.3, 2.5 Hz), H-3′; 5.68, d(6.9 Hz), H-2; 6.18, t(9 Hz), H-13; 6.38, d(9.3 Hz), NH; 7.5-8.11, H-arom., ^{13}C spectral data: 9.9, 13.9, 14.3, 20.7, 22.3, 22.5, 25.4, 26.5, 31.3, 35.8, 36.6, 36.9, 43.1, 46.4, 54.4, 57.6, 72.0, 73.1, 74.5, 74.8, 78.6, 81.1, 84.1, 126.9, 128.2, 128.7, 128.9, 129.2, 130.2, 133.7, 136.1, 138.1, 138.2, 166.9, 170.3, 172.8, 173.1, 211., yield: 0.006%. Anal. calc. for $C_{44}H_{55}NO_{13}$: C, 65.57; H, 6.88; N, 1.74. Found: C, 65.65; H, 6.78; N, 1.75.

DISCUSSION

The use of reverse phase column chromatography for the isolation of taxol and its analogues was studied on a pilot plant scale and found to proceed in a manner comparable to or better than the laboratory-scale runs described earlier (2).

The initial few steps such as: extraction of the plant material with methanol, concentration of the extract, partition with a halocarbon solvent and concentration of this extract to dryness to yield the extract solids, are common to any process. Some have required additional steps such as fractional solvent-partitions for defatting purposes or for removal of insolubles from the extract solids etc. before the sample was ready for a column. In the current process, no defatting extractions were necessary and the extract solid, was directly applied on to the reverse phase column without any further pretreatment. This point is very significant, especially with the extract from the needles of *T. brevifolia,* which is high with respect to waxes, chlorophylls etc., and which generally has needed such pretreatments.

The process described here is simpler than those in the literature (9, 10, 13) because of the shorter extraction, absence of pretreatments, use of a single column and the fact that taxol and seven other taxanes crystallize out directly from the fractions, thereby making their purification easier. It must be noted that although the column fractions did contain lesser amounts of other taxane and nontaxane components, most of these remained in solution, whereas the eight taxanes listed above readily crystallized out and hence, could be separated from the others and purified easily. Also, this crystallization was found to be 75-90 % complete, based on a comparison of the hplc traces of the fractions as they were being collected, and after the crystals were removed by filtration. This is a distinct advantage because such an ease and extent of crystallization is not commonly seen with taxanes obtained in the normal phase chromatography, where solvents such as acetone or dichloromethane are used, and hence the entire fraction has to be concentrated and applied to another column to obtain the desired separation.

As a result of this direct crystallization from the fractions, taxol and four other taxanes: 10-deacetyl baccatin III, 10-deacetyl taxol-7-xyloside, 10-deacetyl cephalomannine-7-xyloside and 10-deacetyl taxol-C-7-xyloside can be taken to final purity by one or two recrystallizations. Depending on the choice of the three alternatives given for the purification of taxol, a short, second column is required, only if complete removal of cephalomannine by ozonolysis is desired. The remaining three: taxol-7-xyloside, 10-deacetyl taxol and cephalomannine require a small column for their final separation and purification.

Another point to be brought out is that the taxol, isolated in a significantly higher yield showed little, or no 7-epimer, in spite of the fact that the sample was in contact with the aqueous solvents for over two weeks during the chromatography and crystallization. Perhaps, the fact that the contact was mostly at ambient temperatures may have minimized this process.

Fewer steps are also generally more conducive for obtaining higher yields. This point is well reflected in the yield of chromatographically pure taxol, isolated in 0.04%, which is significantly higher than the one normally given for taxol from the bark. Quantitative analytical hplc run on the bark showed that the taxol content was 0.05-0.055%, thereby indicating a 75-80% recovery.

One of the reasons for the lower yields of taxol in the existing processes is the second large column required for the separation of cephalomannine from the taxol. Generally, because of only partial separation, one requires recycling of the mixtures. Use of ozone for the removal of the cephalomannine significantly improves this step and the resulting taxol is completely freed from cephalomannine. A chemical method using osmium tetroxide was described earlier by Kingston and associates (14) for this purpose. The advantages of the ozone procedure are that the reagent is much cheaper and is not likely to leave toxic residues in taxol. An additional advantage which is particularly important for the reverse phase column process is that the crude crystals that separate out directly from the fractions trap some of the dark-colored impurities and ozone bleaches these, thereby making the purification easier and without having to use any charcoal.

The current process is also more efficient because it provides not only taxol but also seven other important taxanes simultaneously. Some of these may serve as precursors for the semi-synthesis of taxol, eg. 10-deacetyl baccatin III and 10-deacetyl taxol. In contrast, in the earlier process (13), only taxol was obtained and in a yield of 0.01 %; and essentially no other useful analogues could be isolated, other than 10-deacetyl baccatin III, obtained in very low yields.

The process described here is also more versatile because without any changes, it can be applied to the extract from the needles and the wood as shown above. For example, one of the future sources for taxol will be the needles of *Taxus* x *media* Hicksii (ornamental yew) and currently, processes for large scale operations from this extract are being developed. There are indications that the process may involve more steps than the one developed for the bark, because of the additional constituents such as the chlorophylls, waxes and co-eluting taxanes (15, 16). However, from our experience with the needles of *Taxus brevifolia,* described here, one can expect that the same simple process will be quite applicable to the extract from the ornamental yew. Work along these lines with other species of *Taxus* is under way and the results will be published soon.

The current process is also more economical for the

following reasons. First, the single column approach is an important factor. Secondly, obtaining several other useful taxanes simultaneously is another point to consider. Thirdly, although the adsorbent requires a greater initial investment, the column can be regenerated and used over and over again, perhaps, dozens of times. Finally, the use of aqueous solvents, from which the organic portion can be recovered and reused is another factor.

The use of methanol in place of acetonitrile is still an important alternative. Although the resolution was nearly the same, crystallization was less than complete and took a longer time. However, introducing a slight change, in which the appropriate fractions are combined and partially concentrated to allow the crystallization to take place more rapidly and completely, will make this a very viable, cost-cutting alternative.

With regard to some general comments, although the columns were rated for operation at 200 psi, the usual operating pressure was in the range of 20-80 psi. As expected, as the organic solvent concentration of the eluent increased, the pressure reached the lower point, and the flow rate also steadily increased. For the 6" diameter column, the flow rate started with 2.5 l per hour and gradually increased to 4.5-6 l per hour. The total time for the completion of the column was 40-60 hours, followed by washing, which required another 20 hours. In our study, the columns were in operation for 8-10 hours a day, stopped for the night and restarted the next day.

DEDICATION

This publication is respectfully dedicated to the memory of Dr. Kenneth F. Finger, Associate Vice President of Health Affairs, J. Hillis Miller Health Center, University of Florida, who passed away on July 11, 1994. His interest and support for the project are greatly appreciated.

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