Taxol and Related Taxanes. I. Taxanes of *Taxus brevifolia* Bark

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The published procedures for the isolation of taxol from the Pacific yew (*Taxus brevifolia*) and other species of *Taxus* are cumbersome, and the yields of taxol are in the range of 0.0075–0.01%. This paper describes a simple and efficient procedure for the isolation of taxol and its major natural analogues from the bark of *T. brevifolia* consisting of a single chromatographic column (using silica gel, Florisil, or a reverse-phase C18-silica), followed by crystallization. Isolated yields of taxol from five "pooled" bark samples (blended from many different batches by the supplier) were in the range of 0.02–0.04%, and from bark collected from a more restricted locale, yields reached 0.06%. The procedure also yielded taxol analogues, such as 10-deacetylbaccatin III (0.02–0.04%), 10-deacetyltaxol-7-xyloside (0.06–0.1%), taxol-7-xyloside (0.005–0.01%), 10-deacetyltaxol (0.01–0.02%), 10-deacetylcephalomannine-7-xyloside (0.006–0.01%), and cephalomannine (0.005–0.007%). Of these, 10-deacetyltaxol-7-xyloside is the most abundant taxane in the Pacific yew bark.

KEY WORDS: *Taxus brevifolia;* taxol; taxane-7-xylosides; 10-deacetylbaccatin III; 10-deacetyltaxol; cephalomannine.

INTRODUCTION

Taxol (1; Fig. 1a) is a diterpene ester isolated from the bark of *Taxus brevifolia* by Wani *et al.* in 1971 (1). Its unique mechanism of action (2) and the promising results in clinical trials in ovarian (and possibly breast) carcinomas (3,4) have made it currently the most important new antitumor agent under test and procurement of taxol has assumed a high priority.

The bark of *T. brevifolia* is currently considered to be the best source of taxol, although taxol occurs widely in various parts of several species of *Taxus;* however, the needles of one of these species may become the preferred source (5–7). Semisynthesis from natural taxanes (8,9) and total synthesis are also being actively pursued.

While the bark of *T. brevifolia* will serve as the major source of taxol for the short term, no publications on the isolation of taxanes from the bark of *T. brevifolia* have appeared since 1986. Two previous papers (10,11) described the isolation of 10-deacetylbaccatin III, 7-epi-taxol, 10-deacetyl-10-oxotaxol, and decinnamoyl taxinine J in low yields (0.003%) (from side fractions of a large-scale taxol isolation (of the bark) process. Miller *et al.* (12,13) obtained taxol (0.0108%), cephalomannine (0.0163%), and 10-deacetyltaxol from *T. wallichiana.* Senilh *et al.* (14) isolated, from the bark of *T. baccata,* taxol (0.0165%), cephalomannine (0.0064%), 10-deacetyltaxol (0.0029%), 10-deacetyl cephalomannine (0.0034%), taxol-7-xyloside (0.0058%), cephalomannine-7-xyloside (0.0039%), 10-deacetyltaxol-7-xyloside (0.022%), and 10-deacetylcephalomannine-7-xyloside (0.0096%). Isolation of taxol/taxanes required multiple chromatographic columns, countercurrent distribution, and preparative thin-layer chromatography (tlc). A large-scale taxol process from the bark of *T. brevifolia* was developed by Polysciences Laboratories Inc., but only sketchy details were published (11), with a reported yield of 0.0075–0.01%.

The present work was begun with the objective of developing a simpler and more efficient procedure for the isolation of taxol and its natural analogues from the bark of *T. brevifolia.* Results described here indicate that a simplified procedure consisting essentially of one chromatographic column, followed by direct crystallization of the major components, is possible. Since the steps involved are fewer, there is the possibility of realizing higher yields.

MATERIALS AND METHODS

General. Melting points were determined on a 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, Nicolet NY-300. Thin-layer chromatography (tlc) was performed with silica gel (Merck H60-P254/366), using acetone and/or methanol in chloroform. Visualization was by uv and/or spraying with 1 *N* H_2SO_4, followed by charring.

Plant Material. The plant materials (bark) supplied as *Taxus brevifolia* were purchased or obtained from a variety of sources. Two samples of bark collected from a limited area (habitat) were purchased from Mr. Charles Edson, Research Resources Inc., Medford, OR 97501, during May–September 1990, and are designated E-1 and E-2. One other similarly collected (restricted-area) sample (PB-1) was received from Pacific Biotechnology Inc., 17627 N.E. 65th, Redmond, WA 98052. During September 1990 to September 1991, three pooled samples, designated NC-1, NC-2, and NC-3 (pooled from many collections and similar to what is being used in the large-scale isolation), were received from the National Cancer Institute. Another such pooled sample (MM) was received from Medimolecule Inc., 4620 Starboard Drive, Boulder, CO 80301. During January 1992, still another pooled batch (WP) was purchased from Mr. Walt Phillips, 12108 S.E. Boise, Portland, OR 97226. All of the bark samples were reduced to <0.5-cm mesh, air-dried at ambient temperature before extraction.

Extraction Method. Scheme I shows the extraction and purification steps used in the isolation. The ground bark (3 kg) was immersed in methanol for 24 hr at ~23°C and drained. The process was repeated two or three more times, based on the uv absorbance (275 nm) as an indicator of extraction. The combined extract was concentrated (<45°C) to a syrup (~500 mL), diluted with water (to 1.5 L), and partitioned with chloroform (3×; 1, 0.8, and 0.8 L). Each of the organic layers was washed once with water and concentrated to a syrup, which was then dried *in vacuo.* The solid, designated the "chloroform-extract solids" (approx 60 g) was kept in a tightly stoppered bottle until use.

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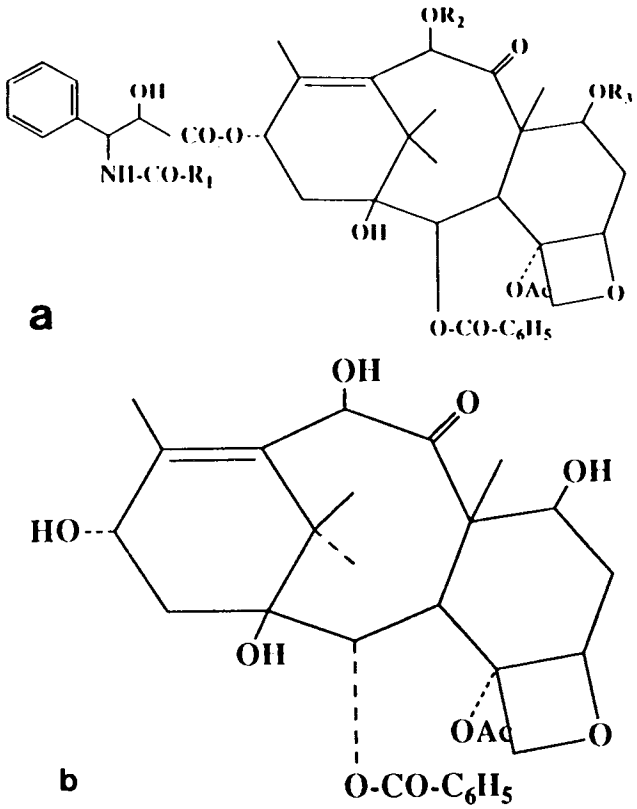

Fig. 1. (a) (1) Taxol: R_1 = benzoyl; R_2 = acetyl; R_3 = H. (2) Cephalomannine: R_1 = tiglyl; R_2 = acetyl; R_3 = H. (4) 10-Deacetyltaxol-7-xyloside: R_1 = benzoyl; R_2 = H; R_3 = D-xylosyl. (5) 10-Deacetylcephalomannine-7-xyloside: R_1 = tiglyl; R_2 = H; R_3 = D-xylosyl. (6) Taxol-7-xyloside: R_1 = benzoyl; R_2 = acetyl; R_3 = D-xylosyl. (7) 10-Deacetyltaxol: R_1 = benzoyl; R_2 = H; R_3 = H. (b) (3) 10-Deacetylbaccatin III.

Column Chromatography. This was performed using three adsorbents: Si gel (Fisher A-743-1, 230–425 mesh), Florisil (Fisher F 101-3), and a reverse phase-type adsorbent such as C8- or C18-bonded silica (Lichroprep, 15- to 25-μm size; E. M. Science). Possible variations from batch to batch of these adsorbents must be recognized and corrected for.

High-performance liquid chromatography (HPLC) was carried out using Waters analytical/semiprep system, consisting of one each of Nos. 510 and 6000A pumps, a No. 680 gradient controller, a No. U6K injector, a No. 440 uv detector set at 254 nm, and a Fisher Recordall 5000 recorder. Standard analytical columns (4.6 × 250 mm) containing silica gel or C8-, C18-, or CN-bonded silica gel columns were purchased from Fisher. Semiprep columns were prepared using 1-in.-diameter stainless-steel columns (Waters) or glass columns (Ace Glass Co.) and packing with the various materials listed above. In general, samples were applied as either solutions or fine suspensions at a concentration of approximately 10%. The columns were run either by gravity flow or with pressure (10–100 psi) as needed.

To a column prepared from the reverse-phase adsorbent (C18-silica, 15 to 35-μm size, 100–125 g) in 25% acetonitrile/water was applied the chloroform extract solid (10 g) in the same solvent (100 mL), and the column developed using a step gradient of acetonitrile in water (in increments of 5 until 50%, then in increments of 10%) until a concentration of 80% acetonitrile was reached. The change of solvent was based on the monitoring data, i.e., when the concentration of the substances being eluted became low, which happened generally within 500–1000 mL of the eluant. Fractions (10–20 mL) were collected and monitored by tlc, analytical HPLC, and uv absorption (275 nm). Based on these data, the appropriate fractions were combined and concentrated. The three major components, 10-deacetylbaccatin III, 10-deacetyltaxol-7-xyloside, and taxol, appeared in that order, being eluted, respectively, with 25–30, 30–40, and 40–50% acetonitrile/water. Individual components were obtained by concentration of the appropriate fractions and crystallization from acetone/ligroin or acetonitrile/water.

Alternatively, to a silica column prepared from 100 g of the adsorbent in chloroform/ligroin (1:1), the sample (chloroform-extract solids, 10 g) in the same solvent (100 MI) was added. The column was developed with the same solvent, which was changed by step gradient to chloroform, 2 and 5% acetone in chloroform, and subsequently 2, 5, and 10% methanol in chloroform. Collection of fractions and monitoring were carried out as described above. Generally, the three major compounds—taxol, 10-deacetylbaccatin III, and 10-deacetyltaxol-7-xyloside—emerged in this order, in the chloroform/acetone and the chloroform/methanol eluates. The solids were crystallized using acetone/ligroin or acetonitrile/water.

The same procedure was used with Florisil instead of silica gel, following the same elution sequence.

Scheme I

Methanol extract (4×) of the bark of *Taxus brevifolia*

Concentration, partition between water and chloroform

- Water
- Chloroform
  - Concentration
  - Concentrate solids
    - Chromatography
    - Fractions
      - Crystallization

RESULTS

The chloroform-extractable solids represented approximately 2% of the weight of the bark. Of the taxanes found in the extract, the following were recognized and isolated (for structures, see Figs. 1a and b): taxol (1), cephalomannine (2), 10-deacetylbaccatin III (3), 10-deacetyltaxol-7-xyloside (4), 10-deacetylcephalomannine-7-xyloside (5), taxol-7-xyloside (6), and 10-deacetyltaxol (7). The last four compounds are isolated from the bark of *T. brevifolia* for the first time, although these were originally reported from the bark of *T. baccata* (14), and cephalomannine and 10-deacetyltaxol from *T. wallichiana* (12,13).

The isolated yields of taxol and two of its major analogues from the various batches of the bark are shown in

Table I. Yields of Taxol and its Analogues from the Bark of *T. brevifolia*

Source	Quality	Method	Percentage[a]		
			Taxol	A	B
1. ED-1	3.8 kg	Silica	0.04	0.03	0.08
2. ED-2	1 kg	C-18	0.06	0.03	0.1
3. ED-2	1 lb	Florisil	0.05	0.03	0.08
4. PB-1	100 g	C-18	0.06		
5. NC-1	1 kg	C-18	0.02	0.03	0.06
6. NC-2	1 kg	C-18	0.04		
7. NC-3	1 lb	C-18	0.03	0.03	0.055
8. MM	1 lb	C-18	0.02		
9. WP	1 kg	C-18	0.03	0.03	0.07

[a] A, 10-deactylbaccatin III; B, 10-deacetyltaxol-7-xyloside.

Table I. Since these studies were carried out using eight batches of the bark, the overall yields quoted here represent the actual range. The yield data were mostly obtained from the reverse-phase column technique, followed by direct crystallization. In one or two comparative runs, the silica column was found to give comparable yields.

Taxol and its analogues described here were also tested in an L-1210 cell culture assay using the protocols described by Thayer *et al.* (15) and the results are shown in Table II.

In the following section, the physical and spectral properties of the various taxanes are described. The ir and ^{13}C-NMR data were obtained and are available upon request.

Taxol (1). Taxol was crystallized first from acetonitrile/water and then from acetone/hexane to form colorless needles, m.p. 219–222°C with decomposition [lit., 213–216°C (1)]; [α], two batches: −51.2, −51.2 (methanol) [lit., −49, methanol (1)], −23.8, −24.0 (pyridine) [lit., −21, pyridine (14)]; and $E_{228\ nm}$, 30,300, compared to that of a reference sample of pure taxol, 30,200. The proton and ^{13}C-NMR spectra were identical to those recorded from an authentic sample of taxol obtained from the NCI.

Analytical HPLC (6), RP-CN, 42% acetonitrile in water, and monitored at 254 nm, showed a single peak (Fig. 2). The chromatogram was further analyzed in detail at both 254 and 220 nm using a photodiode array detector and NEC 386/33i computer. Peak purity determination showed that the taxol peak was 99.2% homogeneous. The area of the taxol peak accounted for essentially 100% of the total area of all the peaks when seen at 254 nm and 98.8% of the total peak area as seen at 220 nm. Two of the samples were submitted for elemental analysis. *Anal.* Calcd for $C_{47}H_{51}NO_{14}$: C, 66.10; H, 6.02; N, 1.64. Found (two batches): C, 66.00, 66.21; H, 6.04, 6.09; N, 1.60, 1.63.

Table II. Cytotoxic Activity of Taxol and its Analogues

Compound	IC_{50} (mEq/ml)
1. Taxol	0.02
2. Cephalomannine	0.04
3. 10-Deacetyltaxol-7-xyloside	0.6
4. 10-Deacetylcephalomannine-7-xyloside	0.8
5. Taxol-7-xyloside	0.07
6. 10-Deacetyltaxol	0.02
7. 10-deacetylbaccatin III	>10

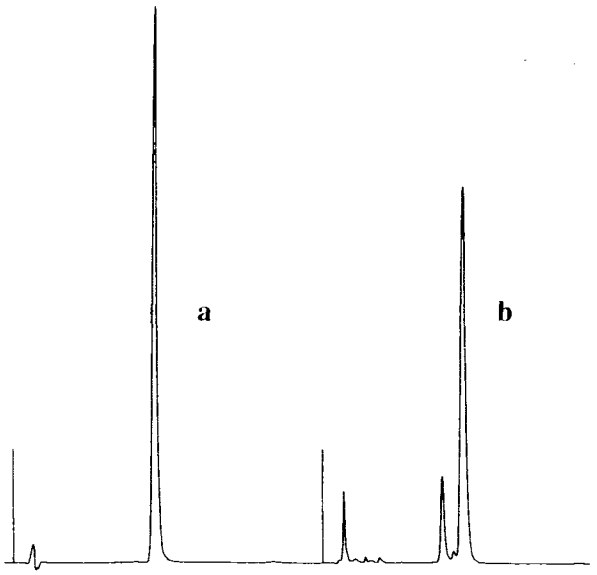

Fig. 2. HPLC profile of (a) taxol and (b) cephalomannine/taxol mixture.

10-Deacetylbaccatin III (3). The compound crystallized as colorless glistening plates, m.p. 232–234°C [lit., 234–236°C (12)]; [α], −40.7 (methanol). The proton NMR spectrum was identical to that recorded earlier (8). *Anal.* Calcd for $C_{29}H_{36}O_{10}$: C, 63.96; H, 6.59. Found: C, 63.82; H, 6.59.

10-Deacetyltaxol-7-xyloside (4). This crystallized from acetone or methanol/water as colorless needles, m.p. 245–248°C; [α], −1 (pyridine), −14 (methanol) [lit. (14), 246–248°C and −2 (pyridine)]. Its ^1H-NMR was identical to that recorded earlier (14). *Anal.* Calcd for $C_{50}H_{57}NO_{17}$, H_2O: C, 62.42; H, 6.18; N, 1.46. Found: 62.66, 62.64; H, 6.24, 6.29; N, 1.58, 1.54.

Acetylation of the compound (acetic anhydride/pyridine, 100°C, 30 min) gave an acetate crystallized from ether, m.p. 216–218°C [lit., 219–220°C (14)]. The ^1H-NMR spectrum was identical to that described (14). *Anal.* Calc. for $C_{60}H_{67}NO_{22}$, H_2O: C, 61.47; H, 5.93. Found: C, 61.35; H, 5.93.

10-Deacetylcephalomannine-7-xyloside (5). Crystallization from acetone gave colorless needles, yield 0.004–006%, m.p. 245–249°C [lit., 250–252°C (14)]. The ^1H-NMR spectrum was identical to that recorded earlier (14). *Anal.* Calcd for $C_{48}H_{59}NO_{17}$, H_2O: C, 61.35; H, 6.54; N, 1.49. Found: 61.54, 61.62; H, 6.84, 6.80; N, 1.42, 1.44.

Taxol-7-xyloside (6). Crystallization from acetone/hexane gave colorless plates, yield 0.004–0.006%, m.p. 233–237°C [lit., 236–238°C (14)]. The ^1H-NMR spectrum was identical to that recorded earlier (14). *Anal.* Calcd for $C_{52}H_{59}NO_{18}$: C, 63.34; H, 6.03; N, 1.42. Found: C, 63.08; H, 6.11; N, 1.42.

10-Deacetyltaxol (7). This crystallized for the first time from aqueous acetonitrile as colorless needles, yield 0.01–0.02%, m.p. 192–196°C. The ^1H-NMR spectrum was identical to that recorded earlier (13,14). *Anal.* Calcd. for $C_{45}H_{49}NO_{13}$: C, 66.57; H, 6.08; N, 1.73. Found: C, 66.42; H, 6.14; N, 1.78.

Cephalomannine (2). Crystallization from acetone/ligroin gave colorless silky needles, yield 0.005–0.007%, m.p. 185–188°C [lit., 186–188°C (12)], identical to an authentic sample of cephalomannine by HPLC and ^{1}H-NMR spectra (12,14).

DISCUSSION

A simple and practical scheme suitable for the isolation of taxol and several of its important natural analogues is described here, consisting of a single chromatographic column followed by crystallization(s), starting from the "chloroform extract solids." In contrast, the methods described in the literature for taxol and related taxanes (10–14), including the large-scale process developed by Polysciences Inc. (11), consist of multiple column and various other manipulative steps. For the operation of the column described here, three adsorbents were studied, and all three were found to give comparable yields of the key components, such as taxol, 10-deacetylbaccatin III, and 10-deacetyltaxol-7-xyloside. The reverse-phase column is more convenient, and most compounds listed here can be purified by direct crystallization. When this does not happen with the minor components because of their low content, the combined fractions and the mother liquors from the crystallizations may be rechromatographed on a silica column for successful crystallization.

Bark samples collected from narrow habitat gave isolated yields of taxol in the range of 0.04–0.06%. On the other hand, the yields of taxol from five "pooled" samples of the bark, which are comparable to those being used in the large-scale isolation of taxol, ranged from 0.02 to 0.04%, in comparison to the generally reported yield of 0.01%.

The purity of the taxol was determined by a number of criteria. Elemental analysis, molecular extinction coefficient, optical rotation, and proton and ^{13}C-NMR spectra all indicated excellent purity. HPLC analysis showed a purity range of 98.5–99% for the taxol samples (Fig. 2). Finally, the cytotoxic IC_{50} value in the L-1210 cell culture assay was in the same range as that of an authentic sample of taxol.

The isolation yield of taxol from the bark of *T. brevifolia* is generally accepted as 0.01% (dry wt basis). The yield of taxol on large-scale extraction (Polysciences Inc.) is quoted as 0.004–0.016% (11). In a congressional hearing (16) conducted by Rep. Ron Weiden (Oregon), it was stated that the yields of taxol by the Hauser process have been improved from 0.0067 to 0.01% (20,000 lb being needed, instead of 30,000 lb per kg of taxol). This number (0.01%) is extensively quoted as a standard in both the scientific and the lay press. This paper documents a substantially improved isolation procedure.

Taxol may also be prepared by semisynthesis from 10-deacetyl baccatin III (8,9), which is present to the extent of 0.01–0.1% in the needles of *T. baccata* and others (6,8,17) and is being isolated on a large scale in France. However, since large amounts of the bark are being processed in the United States for taxol, it would be prudent to isolate this important analogue also at the same time. The procedure outlined here makes this possible, and the bark is shown to be a good source for this compound.

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