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TAXUS SPP. NEEDLES CONTAIN AMOUNTS OF TAXOL COMPARABLE TO THE BARK OF *TAXUS BREVIFOLIA*: ANALYSIS AND ISOLATION

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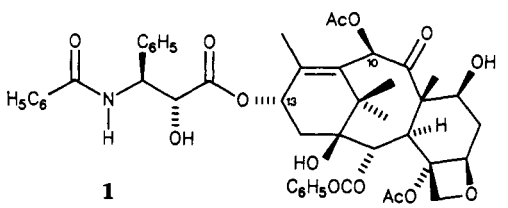
ABSTRACT.—New sources for the antitumor natural product taxol [**1**] are needed as demands for this promising cancer chemotherapeutic agent increase. Presently, supplies of taxol for clinical studies are obtained from the bark of *Taxus brevifolia*, a potentially limited source. Using analytical methods, the needles and stems of six *Taxus* species have been examined for taxol [**1**] and 10-deacetylbaccatin III [**5**], a related compound that can be converted to taxol through a semi-synthetic route. Amounts of taxol comparable to quantities reported from the bark of *T. brevifolia* were found in the needles of four of the *Taxus* species investigated. In addition, taxol was isolated from the needles of one *Taxus* species. Thus, *Taxus* needles may provide a renewable source of this valuable compound.

As part of an investigation into new sources for the promising cancer chemotherapeutic agent taxol [**1**] (1), we have examined the needles and stems of six *Taxus* species (Taxaceae) using analytical methods developed in our laboratory (2). Here we report the quantities of **1** and 10-deacetylbaccatin III [**5**] (3,4), a precursor for the synthesis of taxol (5), found in the six species. Our results indicate that needles from four of the *Taxus* species investigated may provide practical renewable sources of raw material for production of taxol. In addition, we demonstrate the rapid and efficient isolation of taxol from the needles of one species of *Taxus*.



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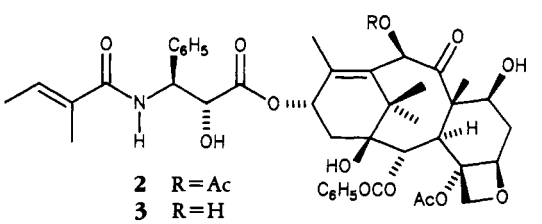

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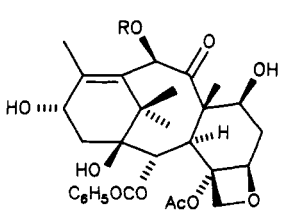

4 R=Ac
5 R=H

Taxol, a highly functionalized diterpene amide presently in Phase II clinical trials, has shown activity against the B16 melanoma and SRC MX-1 mammary and CX-1 colon xenografts. Promising results have also been obtained in the clinical treatment of advanced ovarian cancer (6). While the demand for taxol is steadily increasing, availability of the drug is limited due to the restricted nature of the source. Supplies of **1** are currently obtained from the bark of the slow-growing Pacific yew, *Taxus brevifolia* Nutt., found in the Pacific northwest from northern California to British Columbia. Present as approximately 0.01% of the bark dry wt, taxol is isolated in low yield from these evergreen trees (1,3,7,8). In order to isolate the kilogram quantities of compound needed for further clinical investigations, large numbers of trees need to be harvested, depleting the source of this valuable compound.

The promising antineoplastic potential of taxol has sparked keen interest in the search for alternate sources. Although the total synthesis of **1** has not been achieved, an efficient semi-synthetic method starting from 10-deacetylbaccatin III [**5**] has been reported (5). Compound **5** is reported to be present in relatively high yield in the needles (1 g/kg fresh needles or 0.1%) of *Taxus baccata* L. (5). Because the needles are a renewable source of precursor, this semi-synthetic method may prove to be a practical source of taxol.

Our approach to the problem of taxol supply has been to determine the amount of **1** and **5** in the needles of *T. brevifolia* and in the stems and needles of five additional species of *Taxus*: *T. baccata* cv. Repandens, *Taxus canadensis* Marsh., *Taxus cuspidata* Siebold & Zucc. cv. Capitata, *Taxus* × *media* Rehd. cv. Densiformis, and *T.* × *media* cv. Hicksii. After the segregation of plant parts, the vacuum-dried needles and vacuum-dried stems were ground and extracted overnight in a mixture of MeOH-CH_2Cl_2 (1:1). Following evaporation of solvents, the dried crude organic extracts were partitioned between CH_2Cl_2 and H_2O. The organic layer was then dried in vacuo to yield CH_2Cl_2-soluble fractions. The CH_2Cl_2 solubles were examined using an analytical hplc method described in detail elsewhere (2).

Figure 1A shows the baseline separation of **1** and four related taxanes, cephalomannine [**2**] (3,9), 10-deacetylcephalomannine [**3**] (10), baccatin III [**4**] (11), and 10-

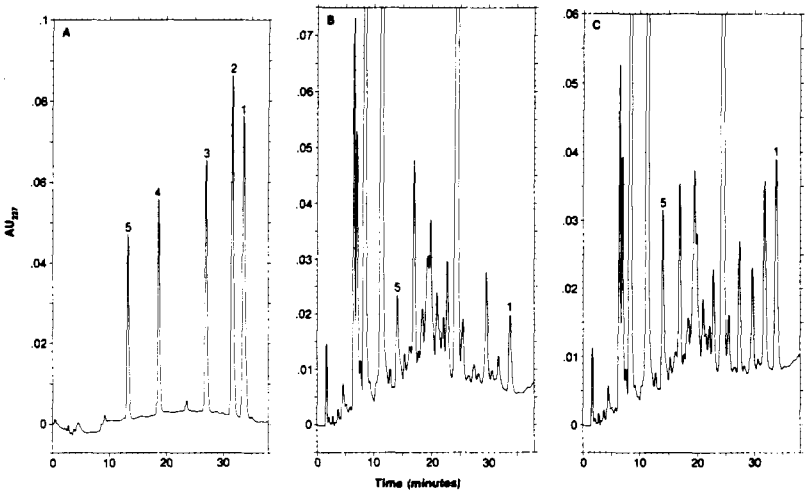

FIGURE 1. A. Hplc separation of *Taxus* standards: taxol [**1**], cephalomannine [**2**], 10-deacetylcephalomannine [**3**], baccatin III [**4**], and 10-deacetylbaccatin III [**5**]. B. Hplc separation of the CH_2Cl_2 solubles from the needles of *Taxus brevifolia*. C. Hplc separation of the CH_2Cl_2 solubles from the needles of *T. brevifolia* with spiking.

deacetylbaccatin III [**5**]. Identical conditions were used to analyze the CH_2Cl_2 partition fractions of the extracts. Figure 1B shows the trace obtained from analysis of the CH_2Cl_2 solubles from the needles of *T. brevifolia*. The chromatogram obtained when the same sample (Figure 1B) is spiked with a solution containing standards **1–5** is shown in Figure 1C. Following spiking, the identity of peaks associated with the five standards may be cautiously assigned. In Figures 1B and 1C, we have identified peaks corresponding to compounds **1** and **5** only. Results are shown in Figure 2 for chromatographic analysis of the CH_2Cl_2 partition fraction from *T. baccata* cv. Repandens needles (Figure 2A) and the CH_2Cl_2 partition fraction spiked with compounds **1–5** (Figure 2B).

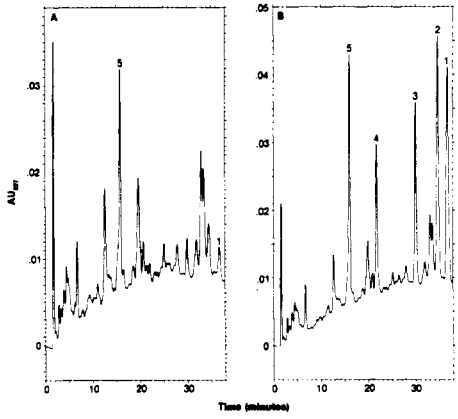

FIGURE 2. A. Hplc separation of the CH_2Cl_2 solubles from the needles of *Taxus baccata* cv. Repandens. B. Hplc separation of the MC solubles from the needles of *T. baccata* cv. Repandens with spiking.

Standard addition experiments were performed to quantitate **1** and **5** in each extract [see Witherup *et al.* (2) for method]. Data from analyses of all the CH_2Cl_2 solubles for the presence of taxol are summarized in Table 1. As seen in Table 1, the CH_2Cl_2 solubles derived from the stems of each species contained approximately the same amount of taxol (0.1 to 0.3 wt percent). These values are contrasted to approximately 2% taxol (by wt) reported for the taxol content in the CH_2Cl_2 solubles derived from the bark of *T. brevifolia* (2). The contrast in these values may reflect qualitative and quantitative differences in the extractable organics that stem and bark material yield.

Data for the taxol content of the CH_2Cl_2 solubles derived from the needles of each species show wider interspecies variation (Table 1). When comparing the data in terms of dry wt percent, the highest amount of taxol was observed in the needles of *T.* × *media* cv. Hicksii (0.01%) and the least amount was found in *T.* × *media* cv. Densiformis (0.002%), a range of approximately one order of magnitude. Relatively high amounts of taxol were also observed in the needles of *T. canadensis*, *T. cuspidata* cv. Capitata, and *T. brevifolia*. In fact, the dry wt percent of taxol in the needles of the four species, *T. brevifolia*, *T. canadensis*, *T. cuspidata* cv. Capitata, and *T.* × *media* cv. Hicksii, is roughly equivalent to the amount of taxol found in the bark of *T. brevifolia* (0.01% dry wt). The wt percent of taxol observed in the needle CH_2Cl_2 partition fractions derived from these four species (Table 1) is approximately 0.2% (versus 2% for the bark of *T. brevifolia*). This result suggests that the early CH_2Cl_2/H_2O partitioning step is considerably less

TABLE 1. Summary of Percent Taxol in Six *Taxus* spp.

Taxus spp.	Taxol content			
	Plant part[a]	Dry wt (%)	Crude residue (%)	CH_2Cl_2 solubles (%)
baccata cv. Repandens	N	0.003	0.01	0.05
	S	0.001	0.01	0.1
brevifolia	N	0.006	0.06	0.2
canadensis	N	0.009	0.05	0.3
	S	0.002	0.04	0.3
cuspidata cv. Capitata	N	0.008	0.05	0.2
	S	0.004	0.07	0.3
× *media* cv. Densiformis	N	0.002	0.009	0.03
	S	0.003	0.04	0.2
× *media* cv. Hicksii	N	0.01	0.04	0.2
	S	0.005	0.06	0.3

[a]N = needles, S = stems.

efficient at concentrating taxol in needle extracts compared to bark extracts, due to higher amounts of waxy, non-polar components in the needles.

In Table 2, data from analyses of the CH_2Cl_2 solubles from the six *Taxus* species for the presence of 10-deacetylbaccatin III [**5**] are summarized. The needles of *T. baccata* cv. Repandens contained the highest amount of **5** (0.02% dry wt). Significant amounts of **5** were also found in the needles of *T. brevifolia* (0.01% dry wt), *T.* × *media* cv. Hicksii (0.009% dry wt), and *T.* × *media* cv. Densiformis (0.007% dry wt). Chauviere *et al.* (12) and Colin *et al.* (13) reported the yield of 10-deacetylbaccatin III [**5**] from fresh needles of *T. baccata* ranged from 200 to 300 mg/kg (0.02–0.03%). Although no experimental details were given, a more recent report (5) by the same group cites an improved yield of 1 g/kg (0.1%) of compound **5** from fresh *T. baccata* needles.

Taxol was isolated from the CH_2Cl_2 solubles of *T.* × *media* cv. Hicksii needles. First, the CH_2Cl_2 solubles were filtered through celite using batch solvent elution starting with hexane, then CH_2Cl_2, EtOAc, and finally MeOH. Hplc analysis of the resulting fractions showed that taxol was located primarily in the fraction that eluted with CH_2Cl_2. This step facilitated the removal of a large quantity of hexane-soluble

TABLE 2. Summary of Percent 10-Deacetylbaccatin III in Six *Taxus* spp.

Taxus spp.	10-Deacetylbaccatin III content			
	Plant part[a]	Dry wt (%)	Crude residue (%)	CH_2Cl_2 solubles (%)
baccata cv. Repandens	N	0.02	0.1	0.4
	S	[b]	[b]	[b]
brevifolia	N	0.01	0.09	0.3
canadensis	N	0.002	0.01	0.09
	S	0.005	0.08	0.6
cuspidata cv. Capitata	N	0.002	0.01	0.05
	S	0.002	0.04	0.1
× *media* cv. Densiformis	N	0.007	0.03	0.1
	S	0.002	0.03	0.1
× *media* cv. Hicksii	N	0.009	0.03	0.2
	S	0.002	0.03	0.1

[a]N = needles, S = stems.
[b]Not quantifiable.

compounds (approximately 72% by wt) that are present in the CH_2Cl_2 solubles derived from the needles.

Next, the taxol-containing fraction was vacuum chromatographed over Si gel using a 5% step gradient of hexane/Me_2CO starting with 75% hexane. From this chromatography, more than 95% of the taxol was concentrated into the fraction eluting with 55% hexane.

In a final step, taxol (>98% pure) was isolated by reversed-phase preparative hplc using an isocratic elution profile. The isolated product was identical to taxol, as determined by hplc analysis and spectral data. The overall yield of taxol was 0.006% dry wt of the needles.

In summary, our findings show that the needles of four species of *Taxus* contain amounts of taxol comparable to quantities reported from the bark of *T. brevifolia*. The four species, *T. brevifolia, T. canadensis, T. cuspidata* cv. Capitata, and *T.* × *media* cv. Hicksii, may offer a practical solution to the problem of taxol supply because the needles represent a potentially renewable source of raw material. In addition, quantities of 10-deacetylbaccatin III roughly equivalent to the amount in the needles of *T. baccata* were found in the needles of three other species of *Taxus*. In fact, the needles of *T.* × *media* cv. Hicksii contain as much 10-deacetylbaccatin III as taxol and could be used to obtain both compounds. Finally, from the needles of *T.* × *media* cv. Hicksii, we purified taxol in three steps from the CH_2Cl_2 partition fraction.

While our results do not account for temporal and infraspecific variation in secondary metabolite production (because only one plant of each species was investigated), these data show that *Taxus* needles could provide a renewable source of taxol and compound **5**. Further research is necessary to optimize the conditions for isolation and extraction of taxol from *Taxus* needles (e.g., addition of an initial hexane defatting step). Other studies will be required to determine the highest yielding species and/or varieties of *Taxus* and the optimum harvesting conditions.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—Instrumentation consisted of a Perkin-Elmer 410 LC Pump, Perkin-Elmer ISS-100 Autoinjector, and Waters 990 Photodiode Array (PDA) Detector with an NEC APC IV computer (NEC Information Systems) for data processing. A Dynamax-60 A 8 μm phenyl column (4.6 mm × 250 mm) with guard module (Rainin Instrument Company) was used for analytical analyses. Preparative instrumentation consisted of a Milton Roy CM 4000 LC Pump and an SM 4000 variable wavelength detector with a CI 4000 integrator. A Dynamax 60 A 8 μm phenyl column (10 mm × 250 mm) with guard module (Rainin Instrument Company) was used for the preparative hplc studies. Hplc grade MeCN, MeOH, CH_2Cl_2, and H_2O were purchased from Burdick and Jackson. All solvents were filtered through 0.2 μm Nylon 66 filters (Rainin). Samples were filtered (0.2 μm) prior to hplc analysis. Standards **1** and **5** were weighed on a Cahn 21 automatic electrobalance. ^{1}H-nmr spectra were recorded in $CDCl_3$ solution on a Varian 500 MHz spectrometer.

PLANT MATERIALS.—Four of the *Taxus* species investigated were purchased from the Dutch Plant Farm of Frederick, Maryland, in January 1989. The young plants, approximately 6–7 years old[1] were purchased with burlap-wrapped rootballs for outdoor planting. These species are: *T. baccata* cv. Repandens, *T. cuspidata* cv. Capitata, *T.* × *media* cv. Densiformis, and *T.* × *media* cv. Hicksii. Cuttings of *T. canadensis* were collected near McConnell's Mill State Park in west-central Pennsylvania by KMW. The needles of *T. brevifolia* were collected in southern Oregon and supplied for this study by Mr. Charles Edson, Research Resources, Jacksonville, Oregon. Stem material from *T. brevifolia* was not requested. Voucher samples were deposited with the University of Mississippi Herbarium, University, Mississippi; voucher numbers are available upon request. The stems and needles were segregated from the fresh plant material and dried under vacuum at room temperature for 48 h. Dried stems and dried needles were ground in a Wiley mill prior to extraction.

[1]Personal communication from Dr. Ed Croom.

EXTRACTION.—Ground stems and ground needles were extracted in a glass percolator with CH_2Cl_2-MeOH (1:1) for 16 h at room temperature. The resulting organic extracts were evaporated in vacuo to give crude residues. A portion (approximately 500 mg) of each crude residue was subsequently partitioned between CH_2Cl_2 and H_2O (1:1) to yield, after evaporation of solvent and drying, a CH_2Cl_2 partition fraction (CH_2Cl_2 solubles). The amount of dried plant material, crude residue obtained, the portion of crude residue partitioned, and quantity of CH_2Cl_2 solubles that resulted from each extraction are summarized below.

T. baccata cv. Repandens.—From 83.5 g dried stems, 7.8 g crude residue was obtained; of the crude residue, 494.4 mg was partitioned to yield 87.2 mg CH_2Cl_2 solubles. The dried needles (247.1 g) yielded 50.7 g crude residue, of which 489.7 mg was partitioned to give 131.0 mg CH_2Cl_2 solubles.

T. brevifolia.—From 332.0 g dried needles, 53.1 g crude residue was obtained; of the crude residue, 513.4 mg was partitioned to yield 91.8 mg CH_2Cl_2 solubles.

T. canadensis.—The dried stems (941.0 g) yielded 56.4 g crude residue, of which 478.2 mg was partitioned to give 68.1 mg CH_2Cl_2 solubles. From 786.5 g dried needles, 150.6 g crude residue was obtained; 468.1 mg of the crude residue was partitioned to yield 65.7 mg CH_2Cl_2 solubles.

T. cuspidata cv. Capitata.—From 557.9 g dried stems, 31.0 g crude residue was obtained; of the crude residue, 475.9 mg was partitioned to yield 126.1 mg CH_2Cl_2 solubles. The dried needles (514.2 g) yielded 84.7 g crude residue, of which 494.4 mg was partitioned to give 142.2 mg CH_2Cl_2 solubles.

T. × *media* cv. Densiformis.—From 136.0 g dried stems, 10.8 g crude residue was obtained; of the crude residue, 490.5 mg was partitioned to yield 89.0 mg CH_2Cl_2 solubles. The dried needles (242.9 g) yielded 59.1 g crude residue, of which 486.8 mg was partitioned to give 138.1 mg CH_2Cl_2 solubles.

T. × *media* cv. Hicksii.—The dried stems (116.0 g) yielded 9.7 g crude residue, of which 493.7 mg was partitioned to give 106.0 mg CH_2Cl_2 solubles. From 387.3 g dried needles, 100.2 g crude residue was obtained; 486.3 mg of the crude residue was partitioned to yield 102.9 mg CH_2Cl_2 solubles.

STANDARDS.—Baccatin III and 10-deacetylcephalomannine were provided by Drs. David G.I. Kingston, Virginia Polytechnic Institute, Blacksburg, Virginia, and Richard G. Powell, United States Department of Agriculture, Peoria, Illinois, respectively. A sample of 10-deacetylbaccatin III was kindly provided by Dr. Pierre Potier, Institut de Chimie des Substances Naturelles du CNRS, 91190 Gif-sur-Yvette, France. Taxol and cephalomannine were isolated in our laboratory from the bark of *T. brevifolia* and were identified based on their spectral properties. Samples of **1** and **5** for the standard addition experiments were diluted in MeOH. The mixture of standards **1–5** was prepared in MeOH such that each compound was present at a concentration of approximately 0.25 mg/ml.

ANALYTICAL HPLC METHOD.—Analyses were performed on a column packed with a phenyl-bonded Si gel phase operated in the reversed-phase mode using an MeOH/H_2O/MeCN mobile phase. The linear gradient elution profile started with MeOH-H_2O-MeCN (20:67:13) and ended with MeOH-H_2O-MeCN (20:27:53) within 50 min. The flow rate was 1 ml/min, and all chromatograms were plotted at the absorption maximum of taxol, 227 nm.

Throughout the study, sample injection volumes were 5 μl. Samples for injection were prepared by dissolving the dried CH_2Cl_2 partition fraction in MeOH at a concentration of 25 mg/ml (125 μg/injection). Spiking of the CH_2Cl_2 partition fraction was accomplished by adding the mixture of standards such that approximately 0.5 μg of each compound was present in the spiked injection.

Analysis of fractions generated during the isolation of taxol from the needles of *T.* × *media* cv. Hicksii was performed by isocratic elution using a MeOH-H_2O-MeCN (20:43:37) mobile phase, which eluted taxol within 20 min (2).

ISOLATION OF TAXOL: CH_2Cl_2 PARTITION.—From the 100.2 g of crude residue obtained from extraction of the *T.* × *media* cv. Hicksii needles, a 99.0 g portion was partitioned between CH_2Cl_2 and H_2O (1:1) to give 29.0 g of CH_2Cl_2 solubles after solvent evaporation and drying.

ISOLATION OF TAXOL: BATCH FILTRATION.—The CH_2Cl_2 soluble fraction derived from *T.* × *media* cv. Hicksii (29.0 g) was dissolved in EtOAc (approximately 500 ml) and coated onto 580 g of Celite-545. The EtOAc was removed under reduced pressure facilitated by the addition of several portions of hexane (5 × 100 ml). The resulting free-flowing powder was placed into a 4-liter glass column (equipped with a vacuum adapter) in which a 2.5-cm layer of fresh Celite-545 was present. Fractions were eluted by suction from this column with 2-liter volumes each of hexane, CH_2Cl_2, EtOAc, and MeOH. Analytical hplc showed that taxol was concentrated in the fraction eluting with CH_2Cl_2 (4.6 g after vacuum drying).

ISOLATION OF TAXOL: VACUUM CHROMATOGRAPHY.—The vacuum-dried CH_2Cl_2 fraction (4.6

g) was coated onto 30 g Celite-545 and loaded onto a 5 cm × 60 cm column dry-packed with a 12 cm bed of Fluka Si gel 60 (230–400 mesh). Fractions were eluted with a 5% step gradient starting with hexane-Me_2CO (75:25) and ending with 100% Me_2CO. Taxol was found to be concentrated (>95%) in the fraction (530 mg) that eluted with 55% hexane.

ISOLATION OF TAXOL: PREPARATIVE HPLC.—The fraction was dissolved in Me_2CO at a concentration of 0.5 g/ml. Five injections were needed to separate the sample (approximately 100 mg/injection). For each injection, purification of taxol was accomplished within 30 min operating isocratically in the reversed-phase mode on a column packed with phenyl phase-bonded Si gel using an MeOH-H_2O-MeCN (10:46:44) mobile phase. The flow rate was 5 ml/min, and the elution of taxol was monitored at 227 nm. The peak corresponding to taxol was collected. Organic solvents were removed by evaporation in vacuo, and the remaining aqueous solution was freeze-dried to yield 21.2 mg taxol (0.006% overall yield from dry needles).

TAXOL.—A taxol reference standard was provided by the Developmental Therapeutics Program, National Cancer Institute. The sample of taxol isolated in this study was evaluated for purity using analytical hplc and found to be >98% pure. This sample was shown to be identical to taxol by its characteristic ^{1}H-nmr spectrum.

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