NEW TAXANES FROM *TAXUS BREVIFOLIA*

DAVID G. I. KINGSTON,* DOUGLAS R. HAWKINS, and LIZA OVINGTON

Department of Chemistry, Virginia Polytechnic Institute and State University, Blacksburg, Virginia 24061

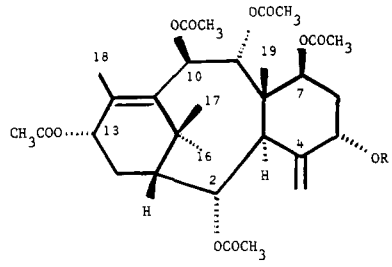
ABSTRACT.—The isolation of two taxanes from *Taxus brevifolia* Nutt. is described, together with the isolation of two acetylated taxol derivatives from an acetylated fraction from the same plant. The compounds were identified on the basis of their spectroscopic properties as taxa-4(20),11-diene-2α,5α,7β,9α,10β,13α-hexaol 2,7,9,10,13-pentaacetate (**1**) (decinnamoyltaxinine J), 10-deacetylbaccatin III (**2**), taxol 2',7-diacetate (**3**), and 10-deacetyltaxol 2',7-diacetate (**4**).

The unusual diterpene taxol (**5**) was reported in 1971 as the major cytotoxic and antileukemic constituent of *Taxus brevifolia* Nutt. (1). It possesses confirmed activity in the L-1210, P-388, and P-1534 leukemia, the B-16 melanoma, and the Walker 256 carcinosarcoma *in vivo* assays, and it also shows strong cytotoxicity in KB cell culture (1, 2). Taxol is an interesting drug in that it appears to act by a mechanism that is different from other known anticancer drugs; it is an antimitotic agent, but it acts by promoting microtubule formation by decreasing the lag time for microtubule assembly and shifting the equilibrium in favor of the microtubule (3). Taxol is currently undergoing preclinical toxicology testing and is scheduled to go into clinical trial at the National Cancer Institute in the summer of 1982.

Although taxol is an interesting and effective anticancer agent, it suffers from some disadvantages which might limit its usefulness. One major problem with the drug is its insolubility in water, which makes its formulation for intravenous infusion difficult. Other problems are associated with its limited availability by isolation from natural sources and with its potential instability. In an attempt to address these problems, we have begun a systematic investigation of structure-activity relationships in the field of the taxane diterpenes related to taxol. Although over thirty taxane diterpenes have been described in the literature (4), very few of them have undergone any sort of biological evaluation, and most of them lack the α-hydroxyester side-chain characteristic of taxol. The isolation and biological testing of new or of previously isolated taxanes thus represents one approach to the determination of structure-activity relationships, while a second approach is *via* the structural modification of known compounds. This paper presents the results of our initial studies in this area, and reports the isolation of two taxane diterpenes (one of which is new) and of taxol and two modified taxols. Biological activity in KB cell culture and the astrocyte reversal assay is given for the five compounds. A study of the tubulin binding of the two modified taxols is reported fully elsewhere (5).

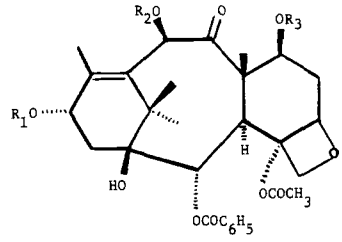
The starting material for our study was a "post-taxol" fraction produced in the course of the pilot-plant preparation of taxol. This fraction consisted of materials eluting from a Florisil column with acetone-hexane after taxol and was obtained as a dark brown viscous oil. Chromatography of this material on silica gel yielded nine major fractions. Subsequent chromatography of the third fraction, first on silica gel and then on a reversed-phase column, yielded two homogeneous products identified as taxol (**5**) and the new compound taxa-4(20),-11-diene-2α,5α,7β,9α,10β,13α-hexaol 2,7,9,10,13-pentaacetate (decinnamoyltaxinine J, **1**). A third compound was isolated by further chromatography of fraction six from the original column and was identified as 10-deacetylbaccatin III (**2**).

Structural assignment of decinnamoyltaxinine J (**1**) was accomplished primarily by comparison of its ms and nmr spectra with the spectra of related compounds (4). Its cims indicated a molecular weight of 578, and exact mass measurement was consistent with the composition $C_{30}H_{42}O_{11}$.



1 R=H

6 R=$COCH_3$



2 $R_1 = R_2 = R_3 = H$

3 $R_1 = COCH(OCOCH_3)CH(C_6H_5)NHCOC_6H_5$, $R_2 = R_3 = COCH_3$

4 $R_1 = COCH(OCOCH_3)CH(C_6H_5)NHCOC_6H_5$, $R_2 = H$, $R_3 = COCH_3$

5 $R_1 = COCH(OH)CH(C_6H_5)NHCOC_6H_5$, $R_2 = COCH_3$, $R_3 = H$

The ^1H nmr spectrum of **1** (table 1) showed the characteristic signals for a taxa-4(20),11-diene, with a methyl doublet at 2.23 ppm and signals for the exocyclic methylene protons at 4.84 and 5.28 ppm. The presence of a proton at C–1 was shown by the fact that the C–2 proton appeared as a doublet of doublets at 5.46 ppm; this signal collapsed to a doublet on irradiation of the C–3 proton at 3.49 ppm, and also collapsed to a doublet on irradiation of the C–1 proton at 1.94 ppm. Assignment of the signal at 4.24 ppm to the C–5 proton was made on the basis of its multiplicity, since C–7 protons on oxygenated carbons usually appear as clearly resolved doublets of doublets; this assignment indicates that the free hydroxyl group must be at C–5. The C–7 proton appeared as a well-resolved doublet of doublets at 5.6 ppm; this signal collapsed to a doublet on irradiation at 2.0 ppm, indicating that one of the C–6 proton resonances occur at this chemical shift.

The triplet of quartets at 5.71 ppm was assigned to the C–13 proton by analogy with other taxane acetates, with the smaller coupling constant assigned to the long-range coupling to the C–18 methyl group. Irradiation at 2.6 ppm collapsed the signal to a very broad singlet, indicating that one of the C–14 protons absorbs at 2.6 ppm.

The stereochemistry of compound **1** was assigned by comparison of its ^1H nmr spectrum, and particularly the coupling constants, with the spectra of related compounds. The general similarity of the spectrum to the spectra of similar compounds such as the hexaacetate **6** (4) confirms the stereochemistry given.

The second compound isolated had an essentially identical ^1H nmr spectrum with that published for 10-deacetylbaccatin III (**2**). This compound was previously obtained as one component of a mixture of products formed by mild methanolysis of cephalomannine (6). The mass spectrum of our compound was

TABLE 1. ^{1}H nmr chemical shifts for taxanes from *T. brevifolia*.[a]

Protons on	1[b]	2[b]	3[c]	4[c]	5[b]
C-1	1.94	—	—	—	—
C-2	5.46 dd J=6,2	5.63 d J=7	5.60 d J=7	5.62 d J=7	5.62 d J=7
C-3	3.49 d J=6	4.00 d J=7	3.87 d J=7	3.94 d J=7	3.80 d J=7
C-5	4.24 br t	4.96 d J=9	4.90 d J=9	4.88 dd J=1,7	4.92 dd J=2,8
C-6	2.0 m	2.26 m	2.2 m	—	—
C-7	5.6 dd J=4,10	4.2 m	5.53 m	5.40 m	4.33 m
C-9	5.84 d J=9	—	—	—	—
C-10	6.21 d J=9	5.24 s	6.18 s	5.26 br s	6.26 s
C-13	5.71 tq J=4,1	4.8 br t	6.15 t J=8	6.15 t J=7	6.15 t
C-14	2.6 m	2.6 m	2.55 m	2.45 m	2.5 m
C-16	1.72 s	1.10 s	1.14 s	1.12 s	1.25 s
C-17	1.03 s	1.10 s	1.09 s	1.00 s	1.14 s
C-18	2.23 d J=1	2.06 br s	1.74 br s	1.77 s	1.78 s
C-19	0.96 s	1.74	1.91 s	1.92 s	1.67 s
C-20	4.84 br t	4.17 d J=9	4.11 d J=8	4.17 d J=8	4.17 d J=8
	5.28 br s	4.32 d J=9	4.25 d J=8	4.27 d J=8	4.27 d J=8
C-2 OBz	—	7.48 d J=7	7.45 t J=7	7.46 t J=7	7.4 m
		7.58 d J=7	7.52 t J=7	7.52 t J=7	8.11 dd
		8.09 d J=7	8.06 dd J=1,7	8.06 dd J=1,7	
OAc	2.09 s	—	—	—	—
	2.05 s, 6H	2.28 s	1.96 s	1.92 s	2.23 s
	2.03 s		2.08 s, 6H	2.09 s	2.38 s
	1.98 s		2.36 s	2.37 s	
C-2'	—	—	5.50 d J=3	5.48 d J=3	4.71 d J=3
C-3'	—	—	5.89 dd J=3,9	5.87 dd J=3,8	5.72 dd J=3,9
C-3' Ph	—	—	7.3 m	7.3 m	7.4 m
C-3' NH	—	—	6.83 d J=9	6.84 d J=8	7.00 d J=9
C-3' NBz	—	—	7.33 m	7.3 m	
	—	—	7.45 t J=7	7.46 t J=7	7.4 m
	—	—	7.67 dd J=1,7	7.67 dd J=1,7	7.7 dd

[a]Chemical shifts (δ) are expressed in ppm from TMS and coupling constants (J) in Hz.
[b]Measured at 200 MHz. [c]Measured at 400 MHz.

also in agreement with the published spectrum of compound **2**, and its other physical constants were consistent with the structural assignment as 10-deacetylbaccatin III (**2**). This is the first reported isolation of this compound from a natural source, although the possibility of hydrolysis of an ester group at the 13 position during isolation cannot be completely excluded (7).

A second study was conducted on the isolation of materials from acetylated crude material. Acetylation of the "post-taxol" fraction followed by chromatographic separation of the resulting crude acetate mixture led to the isolation of two acetylated derivatives.

The first acetate was identified as taxol 2',7-diacetate (**3**) on the basis of its spectroscopic properties. Its cims showed an (MH^+) ion at m/z 938, consistent with the formulation of the compound as a diacetate of taxol. Its ^{1}H nmr spectrum (table 1) was definitive for this structural assignment. The spectrum was very similar to that of taxol (4), except that the resonances of the C–1' and C–7 protons were shifted from 4.71 and 4.33 ppm to 5.50 and 5.53 ppm respectively, indicating acetylation at both these positions.

The second acetate was similarly identified as 10-deacetyltaxol 2',7-diacetate (**4**). Its cims showed an (MH^+) ion at m/z 896, and its ^{1}H nmr spectrum (table 1) was very similar to that of compound **3** except that the resonance of the C–10 proton was shifted from 6.18 ppm in **3** to 5.26 ppm in **4**, comparable to the 5.18 ppm observed for 10-deacetyltaxol (7).

The activities of the four compounds in the astrocyte reversal assay (8) and in KB cell culture are indicated in table 2. None of the compounds showed significant astrocyte reversal activity, in agreement with the proposed mechanism of action of taxol (5). In contrast, however, the two acetylated taxol derivatives showed significant activity in the KB cell culture assay, comparable to the activity

shown by taxol itself. Although *in vitro* cytotoxicity is not necessarily a reliable predictor of *in vivo* activity, this result does support earlier results that indicate that the acetylated taxols may be converted intracellularly to taxol or other compounds with taxol-like activity (5).

TABLE 2. Biological activity of isolated compounds.

Compound	Dose, μg/ml	% Astrocyte reversal (duplicate assay)	ED_{50} (μg/ml) in KB cell culture
1	100	6–13, 16–30	28
2	100	16–30, 16–30	1.0
3	100	16–30, 16–30	0.03
4	100	16–30, 16–30	<0.01
5 (taxol)	100	31–50, 31–50	5.5×10^{-5}[a], <0.01

[a]Ref. 1.

EXPERIMENTAL[1]

STARTING FRACTION.—The starting fraction was a "post-taxol" fraction obtained by Polysciences, Inc., during the large-scale preparation of taxol. *Taxus brevifolia* Nutt. (3863 kg) was extracted with methanol, and the concentrated extract was partitioned between chloroform and water. The chloroform-soluble fraction (56.8 kg) was treated to remove material soluble in hexane and material insoluble in acetone-hexane (1:1), and the resulting fraction was subjected to chromatography on Florisil with elution by acetone-hexane (1:1). The fractions eluting immediately after taxol, when combined and evaporated, yielded 1800 g of "post-taxol" fraction (10).

FRACTIONATION OF THE POST-TAXOL FRACTION.—A portion of the post-taxol fraction (12.9 g) was subjected to chromatography on a column of silica gel, 4 x 70 cm. Elution with 1000 ml. portions of 0%, 2%, 5%, 10% and 20% methanol in chloroform, followed by 500 ml of 50% methanol in chloroform and 500 ml of methanol yielded 48 fractions (125 ml each). Those fractions, when combined on the basis of their weight and tlc characteristics, gave nine major fractions as follows: 1(1–10), 2(11–18), 3(19–22), 4(23–24), 5(25–26), 6(27–32), 7(33–42), 8(43–46), and 9(47–48). Fraction 3 (5.0 g) was then subjected to chromatography on a prepacked column of silica gel 60 (E. Merck "Lobar" size C), with elution by methylene chloride:ethyl acetate (70:30). Fractions of 50 ml were collected and combined on the basis of tlc to give 18 fractions.

Fraction 8 from the "Lobar" column was subjected to reversed-phase chromatography on Lichroprep RP-8, 4 x 30 cm, with elution by water:acetonitrile (70:30) and collection of 80 ml fractions. Crystallization of fraction 9 from methanol yielded the pentaacetate (**1**) as a single pure compound (75 mg).

Fraction 10 from the "Lobar" column was subjected to preparative tlc with development by chloroform:ethyl acetate:methanol (82:10:8). The major uv absorbing band was scraped and eluted with chloroform:methanol (1:1), and the substance recrystallized from aqueous methanol; taxol (**5**) was obtained as white needles (80 mg).

Fraction 6 (8.5 g) from two runs of the original large silica gel column was subjected to chromatography on a prepacked silica gel column (L. Merck "Lobar" size C) with elution by ethyl acetate:chloroform:acetone:methanol (50:50:0:0, 500 ml), (55:35:10:0, 1000 ml), (54:34:10:2, 3000 ml), (60:20:20:10, 500 ml), (50:10:10:20, 500 ml), and (50:0:0:50, 5000 ml). Fifty fractions of 125 ml were collected and combined to give 10 combined fractions. Crystallization of fraction 3 from aqueous acetonitrile yielded 10-deacetylbaccatin III (**2**) as white needles (28 mg), together with approximately 100 mg of partially pure material.

[1]Melting points were determined on a Kofler hot-stage, and are uncorrected. Analytical tlc was performed on silica gel 60 F_{254} plates, 0.25 mm (E. Merck), and preparative tlc was performed on 0.5 mm layers, with development in chloroform-methanol (9:1), except as noted. Analytical plates were charred after spraying with sulfuric acid in ethanol. Normal phase column chromatography was carried out on silica gel 60, 70–230 mesh or 230–400 mesh (E. Merck), and reversed-phase chromatography was carried out on LiChroprep RP-8, 25–40 μM (E. Merck). Analytical hplc was carried out on the apparatus previously described (9); a LiChrosorb RP-8 column, 25 cm x ¼″ (E. Merck) was used, and the solvent was acetonitrile-water (60:40 or 50:50). ^{1}H nmr spectra were recorded in deuterochloroform at 200 MHz on a JEOL FX-200 spectrometer or at 400 MHz on a Bruker WH-400 spectrometer. The low resolution electron impact mass spectrum of compound 1 was obtained on a Varian MAT-112 mass spectrometer, and high resolution electron impact and chemical ionization mass spectra were obtained on a Kratos MS-50 mass spectrometer. Infrared spectra were obtained on a Beckman IR-20 spectrometer, ultraviolet spectra on a Hitachi model 100-60 spectrophotometer, and optical rotations on a Rudolph model 70 polarimeter. The astrocytoma and KB cell culture assays were conducted at the University of Miami School of Medicine according to established protocols (8,11).

ISOLATION OF ACETATES **3** AND **4**.—The post-taxol fraction previously described (10 g, containing approximately 7 g solid material) was treated at room temperature for 24 hr with acetic anhydride (20 ml) and pyridine (100 ml). The crude mixture was then treated with water, extracted with chloroform, and the chloroform extract washed (dilute hydrochloric acid, water), dried ($MgSO_4$), filtered, and evaporated; a crude acetate mixture (6.9 g) was obtained. This mixture was then subjected to chromatography on silica gel, 50 x 2.5 cm, with elution by methylene chloride:hexane:methanol, 99:99:2, and collection of 20 ml fractions.

Fractions 180–185 were combined and recrystallized from methylene chloride-hexane to yield the taxol diacetate **3** (75 mg). Fractions 248–254, when combined and crystallized from methylene chloride-hexane, yielded the deacetyltaxol diacetate **4** (44 mg).

CHARACTERIZATION OF TAXA-4(20),11-DIENE-2α,5α,7β,9α,10β,13α-HEXAOL 2,7,9,10,13-PENTAACETATE (DECINNAMOYLTAXININE J) (**1**).—Compound **1** crystallized from methanol, mp 242–244° (dec); $[\alpha]^{23}D+35°$ (*c* 0.026, $CHCl_3$). Mass spectrum (electron impact), *m/z* 578.2719 M^+; $C_{30}H_{42}O_{11}$ requires 578.2728), *m/z* (relative intensity) 578 (M^+, 13), 560 (M^+-H_2O, 14), 519 M^+–OAc, 2), 518 (M^+–HOAc, 14), 501 (M^+–OAc-H_2O, 3), 458 (M^+–2 HOAc, 24), 416 (27), 399 (13), 398 (37), 374 (15), 356 (42), 338 (54), 296 (78), 278 (87), 263 (70), 235 (55), 147 (65), 145 (78), 135 (80), 133 (100), 121 (82), 119 (75), 107 (57), 105 (74); νmax ($CHCl_3$) 3600, 1740 cm^{-1}; ^1H nmr spectrum, see table 1.

CHARACTERIZATION OF 10-DEACETYLBACCATIN-III (**2**).—Compound **2** crystallized from aqueous acetonitrile, mp 223–225° (lit. 234–236° (6)); $[\alpha]^{23}D-249°$ (*c* 0.009, EtOAc). Mass spectrum (electron impact), *m/z* 543.2207 ($M-H^+$; $C_{29}H_{35}O_{10}$ requires 543.2232); νmax ($CHCl_3$) 3540, 1730, 1710 cm^{-1}; λmax (EtOH) 228 (log ϵ 4.11), 254 (3.03); ^1H nmr spectrum, see table 1.

CHARACTERIZATION OF TAXOL (**5**).—Taxol (**5**) crystallized as white needles, mp. 194–197° (dec) (lit. 198–203° (6), 213–216 (1)); $[\alpha]^{23}D-54°$ (c 0.026, MeOH) (lit. $[\alpha]^{23}D-49°$ (1)). The ^1H nmr spectrum of **5** was identical with that recorded for taxol (1,6), and the substance had an identical retention time on hplc (RP-8 column; methanol:water, 65:35) as an authentic sample of taxol.

CHARACTERIZATION OF TAXOL 2'7-DIACETATE (**3**).—Compound **3** recrystallized as white needles, m.p. 227–228°. Spectroscopic data: νmax ($CHCl_3$) 3450, 1750, 1730 cm^{-1}; ^1H nmr spectrum, see table 1; mass spectrum (isobutane chemical ionization), *m/z* 938 (MH^+, 20), 878 (15), 860 (42), 816 (7), 756 (37), 611 (28), 551 (73), 535 (35), 489 (15), 429 (22), 369 (12), 328 (73), 310 (35), 284 (10), 224 (12), 149 (8), 123 (100), 105 (10).

CHARACTERIZATION OF 10-DEACETYLTAXOL 2',7-DIACETATE (**4**).—Compound **4** crystallized as white needles, mp 173°. Spectroscopic data: νmax ($CHCl_3$) 3450, 1750, 1730 cm^{-1}; ^1H nmr spectrum, see table 1; mass spectrum (isobutane chemical ionization), *m/z* 896 (MH^+, 45) 878 (50), 860 (55), 856 (18), 838 (20), 774 (18), 756 (25), 569 (20), 551 (50), 447 (70), 387 (28), 328 (100), 306 (35), 123 (78).

ACKNOWLEDGMENTS

DRH and LO acknowledge support as undergraduate research participants on National Science Foundation grants SPI-8926151 and SPI-7926873, respectively. The authors gratefully acknowledge the National Science Foundation regional nuclear magnetic resonance facilities at the University of South Carolina (CH78-18723) and mass spectrometric facilities at Johns Hopkins University (CHE78-18386), and financial support from the Research Division, Virginia Polytechnic Institute and State University. The assistance of Dr. M. Suffness and Dr. F. Boettner in providing a sample of the post-taxol fraction is also acknowledged with thanks.

Received 28 December 1981

LITERATURE CITED

1. M. C. Wani, H. L. Taylor, M. E. Wall, P. Coggon, and A. I. McPhail, *J. Am. Chem. Soc.*, **93**, 2325 (1971).
2. J. M. Cassady and M. Suffness, "Terpenoid Antitumor Agents" in J. M. Cassady and J. D. Douros, eds, "Anticancer Agents Based on Natural Product Models" Academic Press, New York, 1980, pp 201–269.
3. P. B. Schiff, J. Fant, and S. B. Horwitz, *Nature*, **277**, 665 (1979).
4. R. W. Miller, *J. Nat. Prod.*, **43**, 425 (1980).
5. J. Parness, D. G. I. Kingston, R. G. Powell, C. Harracksingh, and S. B. Horwitz, *Biochem. Biophys. Res. Comm.*, **105**, 1082 (1982).
6. R. W. Miller, R. G. Powell, C. R. Smith, Jr., E. Arnold, and J. Clardy, *J. Org. Chem.*, **46**, 1469 (1981).
7. J. L. McLaughlin, R. W. Miller, R. G. Powell, and C. R. Smith, Jr., *J. Nat. Prod.*, **44**, 312 (1981).
8. K. Igarashi, S. Ikeyama, M. Takeuchi, and Y. Sugino, *Cell Struct. Funct.*, **3**, 103 (1978).
9. D. G. I. Kingston and B. T. Li, *J. Chromatogr.*, **104**, 431 (1975).
10. F. E. Boettner, T. M. Williams, R. Boyd, and B. D. Halpern, Preparation Report 9. Polysciences, Inc., Paul Valley Industrial Park, Warrington, PA 18976, November 27, 1979.
11. R. I. Geran, N. H. Greenberg, M. N. Macdonald, A. M. Schumacher, and B. J. Abbott, *Cancer Chemother. Rep. Part 5*, **3**, 1 (1972).