Available online at www.sciencedirect.com
SCIENCE DIRECT
Bioorganic & Medicinal Chemistry Letters
Bioorganic & Medicinal Chemistry Letters 16 (2006) 2446–2449

Taxoid from the needles of the Himalayan yew *Taxus wallichiana* with cytotoxic and immunomodulatory activities☆

Sunil K. Chattopadhyay,[a,*] Anirban Pal,[a] Prakas R. Maulik,[b] Tanpreet Kaur,[a] Ankur Garg[a] and Suman Preet S. Khanuja[a]

[a]*Central Institute of Medicinal and Aromatic Plants, PO CIMAP, Lucknow 221005, India*
[b]*Central Drug Research Institute, Lucknow 226001, India*

Received 24 October 2005; revised 9 January 2006; accepted 24 January 2006
Available online 9 February 2006

Abstract—The needles of *Taxus wallichiana* gave a taxoid 1-hydroxy- 2-deacetoxy-5-decinnamoyl-taxinine j, whose structure has been established by spectroscopic data and confirmed by X-ray crystallography. The taxoid possesses significant cytotoxic and immunomodulatory activity.

The Himalayan yew (*Taxus wallichiana*) is a small medium-sized evergreen tree growing in the temperate Himalayas at an altitude of 1800–3300 m and in the Khasia hills at altitudes of 1500 m.[1]

Genus *Taxus* is the most extensively investigated genus for its taxoids content. Almost all its species have been chemically investigated in the search for paclitaxel, its important precursor 10-deacetyl baccatin-III, and other taxoids with the hope of finding additional taxoids with better activity profile than paclitaxel. Over 300 taxoids have been isolated from the genus *Taxus* and excellent reviews have appeared on this important class of molecules.[2,3] The needles,[4,3,5,6] stem bark,[7–9] heartwood,[10] roots,[11] and seeds[12] of *Taxus wallichiana* were separately investigated yielding several structurally unique taxoids.

As part of an ongoing research program on the isolation of paclitaxel analogues/precursors from the Himalayan yew *T. wallichiana*, we have isolated a taxoid from the needles of this plant. This paper describes the isolation and characterization of the taxoid **1** which has been identified as 1-hydroxy-2-deacetoxy-5-decinnamoyl-taxinine j with the help of detailed spectroscopic analysis and confirmed by its X-ray analysis.

The anticancer and immunomodulatory properties of the taxoid are also reported in this paper.

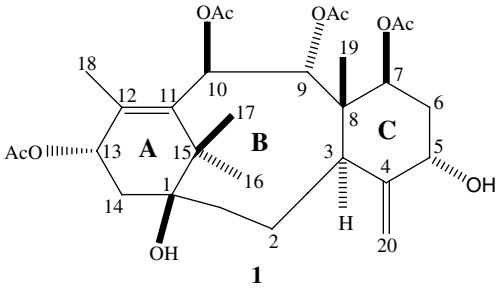
Taxoid **1**, $[\alpha]_D^{23}$ +170° (*c* 0.1, MeOH), was isolated as a crystalline material in 0.00315% yield (dry plant material) from the chloroform-soluble fraction of the methanol extract of the needles of *T. wallichiana*. Elemental analysis, ESI-MS *m/z*: 559 $(M+Na)^+$, and ^{13}C NMR of the compound established its molecular formula as $C_{28}H_{40}O_{10}$.

The ^1H NMR of the compound showed characteristic taxoid signals for four methyl groups, four acetoxy groups, and C-4(20) exo-cyclic methylene group.[4] ^1H and ^{13}C NMR of the compound **1** was very much similar to 2-deacetoxy-5-decinnamoyl-taxinine j,[5] the main difference being the disappearance of the H-1 multiplet at δ1.78 and the downfield shift of C-1 $\Delta\delta$ (36 ppm) in **1** as compared to 2-deacetoxy-5-decinnamoyl-taxinine j. Full assignment of ^1H and ^{13}C NMR spectrum of **1**[14] was made by a combination of ^1H–^1H COSY, DEPT, HMQC, and HMBC techniques. The results show that the taxoid **1** is 1-hydroxy-2-deacetoxy-5-decinnamoyl-taxinine j, related to 2-deacetoxy-5-decinnamoyl-taxinine j having one extra hydroxyl group at C-1.[5] Search in the literature revealed that a taxoid of molecular weight of 536 was previously isolated from the leaves of the Himalayan yew *T. wallichiana* and it was wrongly characterized as 7,13-diacetyl-7-debenzoyl brevifoliol.[4] In a later publication, the structures of brevifoliol and its four other derivatives were revised.[13]

Keywords: *Taxus wallichiana*; Taxaceae; Taxoid; Structure determination; X-ray analysis; Cytotoxic; Immunomodulatory.
☆ CIMAP communication No. 2005-59J.
* Corresponding author. Tel./fax: +91 0522 2342666; e-mail: chattsk@yahoo.com

0960-894X/$ - see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmcl.2006.01.077

However, the structure of the taxoid of molecular weight 536 was never characterized unambiguously. Therefore, we have grown crystals of the above taxoid **1** suitable for X-ray crystallography. Final confirmation of the structure **1** was achieved by single crystal X-ray analysis[15] of the taxoid **1** and it was characterized as 1-hydroxy-2-deacetoxy-5-decinnamoyl-taxinine j unambiguously.



1

Figure 1 shows the molecular structure and conformation of **1** with the atomic numbering scheme. The molecule contains a fused three-ring system A/B/C with transfused B/C junction due to the trans-axial dispositions of C9 at C8 and H3 at C3, the ring A being in a syn conformation with respect to ring C. Thus, the molecule as a whole adopts folded cage-type conformation. The eight-membered B ring is puckered to form sofa like conformation [the deviations of atoms C1, C8, C9, and C10 are −0.756(6), 1.256(6), 1.402(6) and 2.041(5) Å, respectively, from the least-squares plane through atoms C2, C3, C11, and C15]. The six-membered ring A is in a boat conformation, while ring B adopts a chair conformation.

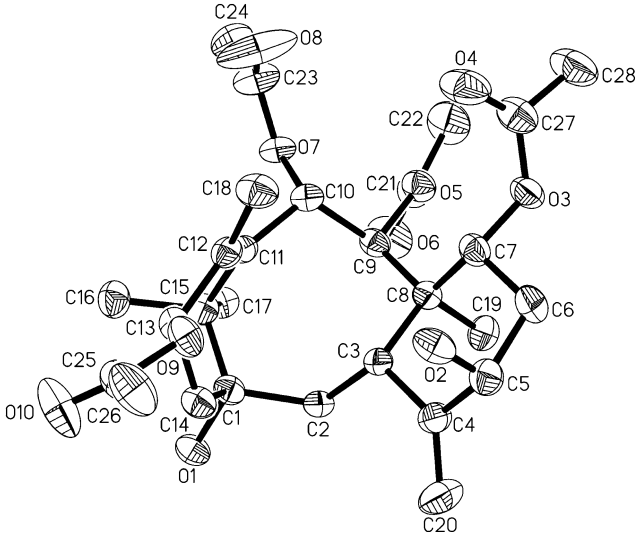


Figure 1. ORTEP diagram showing a view of the molecule **1**. Displacement ellipsoids are drawn at the 30% probability. Hydrogen and solvent water atoms are omitted for clarity.

The molecular packing in the crystal shows that both the hydroxyl groups are involved in intermolecular hydrogen bonding of the type O–H···O [O1–H1···O4: 2.821 (5) Å, H1···O4: 2.01 Å, O1–H1–O4: 170°; O2–H2···O11: 2.800(5) Å, H2–O11: 2.00 Å, O2–H2–O11: 165.5°. In addition, the crystal packing further reveals the O–H···O hydrogen bonding by the solvent water molecule [O11–H11···O1: 2.915(6) Å and O11–H11B···O2: 2.859(5) Å, respectively].

Taxoid **1** was found to possess dose-dependent cytotoxic activity against five human cancer cell lines as determined by MTT[16] and clonogenic assays (Table 1). However, it was found to be nontoxic on normal hepatocytes of mice even at 50 μg/ml. The compound displayed nearly similar cytotoxic potency with IC_{50} values equal to or even better than those of the controls which are clinically used drugs vincristine and vinblastine sulfates.

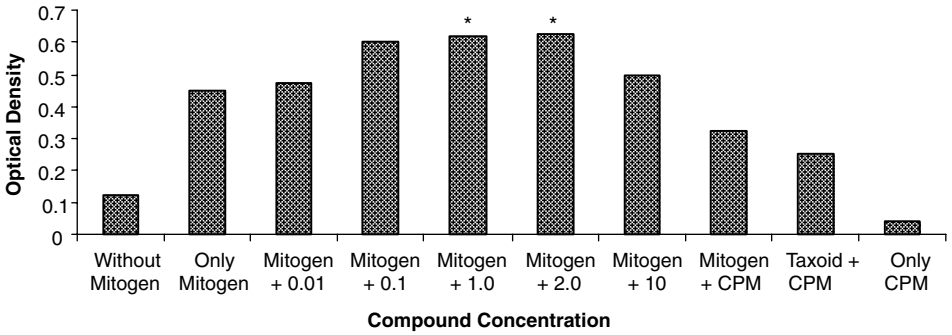
The immunomodulating activity of the cytotoxic compound **1** was evaluated using human lymphocytes. It was observed that the taxoid **1** exhibited negligible proliferative response in the absence of mitogen (concanavaline A). However, in the presence of optimal dose of ConA (5μg/ml), taxoid **1** showed maximum proliferation at a concentration of 1.0 μg/ml. Cyclophosphamide represented suppression of lymphocytes to 0.04 which was significantly enhanced many fold when cotreated with 1μg/ml of ConA and taxoid **1** independently. Hence it can be said that taxoid **1** represents similar immunomodulant properties as shown by ConA (Fig. 2).

At present, the mechanism of tumor reduction and the process responsible for immunomodulation are not known. Although taxoid **1** was cytotoxic on cancer

Table 1. Cytotoxicity of taxoid **1** against human tumor cell lines by MTT and clonogenic assays

Compound	IC_{50} values (μg/ml)				
	MCF-7	WRL-68	KB	PA-1	Colo-320DM
Taxoid **1**	0.18 ± 0.020 (8.42) ± 1.150	1.65 ± 0.050 (12.8) ± 0.100	0.04 ± 0.002 (1.23) ± 0.050	0.01 ± 0.0025 (0.85) ± 0.0062	0.23 ± 0.0035 (1.82) ± 0.050
Vincristine	0.05 ± 0.002 (0.15) ± 0.005	1.42 ± 0.040 (8.5) ± 0.540	0.02 ± 0.004 (0.25) ± 0.005	0.01 ± 0.004 (1.0) ± 0.06	0.46 ± 0.002 (1.5) ± 0.040
Vinblastine	0.02 ± 0.002 (0.22) ± 0.050	1.45 ± 0.080 (2.6) ± 0.050	0.046 ± 0.004 (0.46) ± 0.068	0.025 ± 0.0028 (1.15) ± 0.240	0.52 ± 0.010 (1.60) ± 0.080

Values in parentheses indicate IC_{50} values (μg/ml) of respective compounds as determined by clonogenic assay. Each result is the mean ± SD (Standard Deviation).



CPM = Cyclophosphamide; Mitogen = Concavaline A;

S.No	Optical Density	Compounds/combinations
1	0.12	Without Mitogen
2	0.45	Only Mitogen (Concavaline)
3	0.47	Mitogen + 0.01 (Taxoid)
4	0.6	Mitogen + 0.1 (Taxoid)
5	0.62	Mitogen + 1.0 (Taxoid)
6	0.625	Mitogen + 2.0 (Taxoid)
7	0.5	Mitogen + 10 (Taxoid)
8	0.325	Mitogen + CPM (Cyclophosphamide)
9	0.25	Taxoid (1ug/ml)+ CPM (Cyclophosphamide)
10	0.04	Only (Cyclophosphamide)

Figure 2. Estimation of lymphocytes proliferation by taxiod **1** in presence of mitogen (ConA 5 μg/ml). * Indicates maximum proliferation found as compared to control (only mitogen).

cells, it was not toxic on normal primary cultured hepatocyte cells used. Therefore, the probable mechanism of action of taxoid **1** may be through the stimulation of effector cells that retard/destroy the tumor cells.

References and notes

1. Kapoor, I. D. *Handbook of Ayurvedic Medicinal Plants*; CRC: Boca Raton, FL, 1990, p 317.
2. Baloglu, E.; Kingston, D. G. I. *J. Nat. Prod.* **1999**, *62*, 1448.
3. Appendino, G. *Nat. Prod. Rep.* **1995**, *12*, 349.
4. Barboni, L.; Gariboldi, P.; Torregiani, E.; Appendino, G.; Gabetta, B.; Zini, G.; Bombardelli, E. *Phytochemistry* **1993**, *33*, 145.
5. Chattopadhyay, S. K.; Sharma, R. P.; Appendino, G.; Gariboldi, P. *Phytochemistry* **1995**, *39*, 869.
6. Chattopadhyay, S. K.; Tripathi, V.; Sharma, R. P.; Shawl, A. S.; Joshi, B. S.; Roy, R. *Phytochemistry* **1999**, *50*, 131.
7. Chattopadhyay, S. K.; Tripathi, V. K.; Thakur, R. S.; Sharma, R. P.; Jain, S. P. *Indian J. Chem.* **1994**, *33B*, 409.
8. Chattopadhyay, S. K.; Sharma, R. P. *Phytochemistry* **1995**, *39*, 935.
9. Chattopadhyay, S. K.; Saha, G. C.; Sharma, R. P.; Kumar, S.; Roy, S. *Phytochemistry* **1996**, *42*, 787.
10. Chattopadhyay, S. K.; Kulshrestha, M.; Saha, G. C.; Sharma, R. P.; Jain, S. P.; Kumar, S. *Planta Med.* **1996**, *62*, 482.
11. Chattopadhyay, S. K.; Saha, G. C.; Kulshrestha, M.; Tripathi, V.; Sharma, R. P.; Mehta, V. *Planta Med.* **1998**, *64*, 287.
12. Chattopadhyay, S. K.; Tripathi, V.; Sashidhara, K. V.; Mehta, V. K. *Indian J. Chem.* **2002**, *41B*, 225.
13. Appendino, G.; Barboni, L.; Gariboldi, P.; Bombardelli, E.; Gabetta, B.; Viterbo, D. *J. Chem. Soc. Chem. Commun.* **1993**, 1587.
14. *Selected NMR data*: **1:** ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.70 (m, 1H, 2α-H), 1.85 (m, 1H, 2β-H), 3.16 (d, J = 4.89 Hz, 1H, 3-H), 4.32 (br s, 1H, 5-H), 2.13 (m, 1H, 6α-H), 2.0 (m, 1H, 6β-H), 5.66 (dd, J = 11.3 and 5.0 Hz, 1H, 7-H), 5.83 (d, J = 11 Hz, 1H, 9-H), 6.27 (d, J = 11 Hz, 1H, 10-H), 5.90 (distorted dd, 1H, 13-H), 1.56 (m, 1H, 14α-H), 2.70 (dd, J = 4.7 and 10.4 Hz, 1H, 14β-H), 1.55 (s, 3H, 16-CH_3), 1.07 (s, 3H, 17-CH_3), 2.17 (s, 3H, 18-CH_3), 0.82 (s, 3H, 19-CH_3), 5.20 (br s, 1H, 20α-H), 4.90 (br s, 1H, 20β-H), 1.99, 2.03, 2.05, 2.07(4s, 4× 3H, $OCOCH_3$); ^{13}C NMR (75 MHz, $CDCl_3$) δ 75.5 (C-1), 36.4 (C-2), 39.8 (C-3), 151.4 (C-4), 73.6 (C-5), 37.1 (C-6), 70.0 (C-7), 47.4 (C-8), 75.7 (C-9), 72.2 (C-10), 136.2 (C-11), 139.7 (C-12), 71.4 (C-13), 42.1 (C-14), 43.6 (C-15), 21.2 (C-16), 28.5 (C-17), 16.0 (C-18), 12.9 (C-19), 112.0 (C-20), 20.8, 20.9, 21.1, 21.6, 169.3, 169.8, 170.1, 170.4 (4× $OCOCH_3$).
15. *X-Ray crystallography data of* (***I***):$C_{28}H_{40}O_{10}$. H_2O, Mol. Wt. = 554.62, orthorhombic, space group $P2_12_12_1$, a = 9.030 (1), b = 18.051(2), c = 18.374(2) Å, V = 2995.0(6) Å^3, Z = 4, D_c = 1.283 g cm^{-3}, $F(000)$ = 1240, μ(Mo-K$_\alpha$) = 0.098 mm^{-1}, rectangular transparent block. A single crystal of approximate dimensions 0.325 × 0.25 × 0.25 mm was used for all X-ray measurements. Total reflections read = 3813 (R_{int} = 0.022) of which 3605 Unique. Conventional R = 0.0520 [$(\Delta/\sigma)_{max}$ = 000)] on F values of 2114 reflections with $I > 2\sigma(I)$, S = 1.028 for all data and 371 parameters. The unit cell determination and intensity data collection (2θ = 49.98°) of all unique reflections were performed on a Bruker P4 diffractometer at 273 K. There was no significant intensity decay. Structure

solutions were performed by direct methods and refinements by full-matrix least-squares method on F^2. All the non-hydrogen atoms were generated in the ideal positions and the parameters were constrained during the refinements. Programs: XSCANS [(Siemens Analytical X-ray Instrument Inc.: Madison, Wisconsin, USA, 1997) used for data collection and data processing] and SHELXTL-NT [(Bruker AXS: Madison, Wisconsin, USA, 1997) used for structure determination, refinements, and molecular graphics]. CCDC (Deposit No: 294564) contains the supplementary crystallographic data. These data can be obtained free of charge from www.ccdc.cam.uk/conts/retrieving.html or from the Cambridge crystallographic data Centre, 12, Union road, Cambridge, CB2 1EZ, U K; Fax (internat.): +44 1223 336 033; E-mail: deposit@ccdc.cam.ac.uk.

16. Mosmann, T. *J. Immunol. Methods* **1983**, *65*, 55.
17. Beekman, A. C.; Barentsen, A. R. W.; Woerdenbag, H. J.; Uden, W. V.; Pras, N. *J. Nat. Prod.* **1997**, *60*, 325*Cytotoxicity Bioassay: MTT Assay:* IC_{50} values were determined and the cell survival was measured by using the MTT-microculture tetrazolium assay described by Mosmann.[16] Briefly, 0.5 or 1×10^5 cells/ml cells at the exponential growth phase were taken in a flat-bottomed 96-well polystyrene-coated plate and were incubated for 24 h in CO_2 incubator at 5% CO_2 and 37 °C. Compound **1** was added in concentrations of 100, 10, 1 0.1, 0.01, and 0.001 μg/ml medium. After 48 h incubation, 10 μl/well MTT (stock solution 5 mg/ml PBS) was added for 4 h and formazan crystals so formed were dissolved in 100 μl DMSO. The plates were read immediately in a microplate reader (Spectramex, 190 Molecular Devices Inc., USA) operating at 570 nm. Wells with complete medium, compound, and MTT, but without cells were used as blanks. IC_{50} values were expressed as micrograms of compound concentration per milliliter that caused a 50% inhibition of growth compared with controls. Vincristine sulfate (Sigma) and Vinblastine (Sigma) were used as positive controls in every experiment; *Clonogenic Assay:* Taxoid **1** along with the positive controls representing potential activity in MTT was confirmed by Soft Agar Colony-Forming Assay as described by Beekman et al.[17] for their cytotoxicity. Briefly, cells embedded in soft agar were mixed with test compound in different doses and were incubated at 37 °C, 5% CO_2, 95% atmosphere for 15 days. The colonies consisting of more than 40 cells were scored on day 14 using dissecting microscope and were stained with methylene blue. Taking untreated cells as control the inhibition was calculated as IC_{50} values of the compound, that is, 50% inhibition concentration and was calculated as results of three assays in triplicate with at least five concentrations.
18. Nakamura, A.; Nagai, K.; Suzuki, S.; Ando, K.; Tamura, G. *J. Antibiot.* **1986**, 39 *Lymphocyte proliferation assay for immunomodulation:* Peripheral blood lymphocytes were isolated using the protocol of Nakamura et al.[18] and were suspended in RPMI 1640 medium with 10% fetal bovine serum. The cell titer was adjusted to 1×10^6 cells/ml in growth medium. Studies were carried out to evaluate the effects of taxoid **1** on the prolife ration of human lymphocytes under various conditions, in the presence of sub-optimal doses of ConA (0.3 μg/ml, optimal doses of ConA (5 μg/ml) and 1 μg/ml of cyclophosphamide (immunosuppressant drug) or without mitogens. The lymphocytes were incubated for 72 h at 37 °C in 5% CO_2 incubator in 95% humidified CO_2 incubator with serially diluted compounds starting from 100μg/ml up to 10^{-5} μg/ml in triplicate wells. Cell activation and proliferation were carried out using the MTT tetrazolium assay as described above; *Statistical analysis:* Student's *t* test was used to evaluate the presence of significant difference between control and treated test. Differences with P values <0.05 were considered to be statistically significant.