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Absolute Configuration and Anticancer Activity of Taxiresinol and Related Lignans of *Taxus wallichiana*

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Abstract—Absolute configuration of taxiresinol **1**, a lignan from the heartwood of *Taxus wallichiana* has been determined as 8*R*, 8′*R*, and 7′*R* with the help of chemical correlation method and X-ray crystallography. The anticancer activity of taxiresinol **1** and other two lignans **2**, **3** were also studied. Taxiresinol **1** showed notable anticancer activity in the in vitro bioassays against colon, liver, ovarian and breast cancer cell lines.

Introduction

The Himalayan yew (*Taxus wallichiana* Zucc.) is a high value tree species that naturally grows on either side of Himalayas. In contrast to the European yew (*Taxus baccata* L), the Himalayan yew, *T. wallichiana* has a remarkable history of medicinal use and is also used as a coloring matter and as incense.[1]

As a part of our systematic studies on the chemical constituents of the different parts of *T. wallichiana*, we have isolated and identified several taxoids of different structural types and five of them were new molecules.[2–6] Though the main thrust of phytochemical work on *T. wallichiana* has been concentrated mainly on the isolation and identification of various taxoids, there has not been much work reported on the non-taxoids constituent of the plant in comparison to its taxoid constituents.

In continuation of our studies on the isolation of anticancer compounds from the plant *T. wallichiana*, anticancer activity has been detected for three lignans, which have been isolated from the heartwood of the plant. The three lignans have been characterized as taxiresinol **1**, isotaxiresinol **2** and (−)-secoisolariciresinol **3** on the basis of their spectral characteristics.[7] Recently, we have done a crystal structure of (−)-secoisolariciresinol and its absolute stereochemistry has been confirmed as 8*R*, 8′*R* as previously determined by chemical correlation method.[8]

Taxiresinol **1** was previously isolated from the heartwood of *T. wallichiana* and its structure was established by Mujumdar et al. in 1972 with the help of low-resolution NMR.[7] However, the orientation of the vanillyl and the catechol moiety with respect to the tetrahydrofuran ring has not been established conclusively, and no 2D NMR data is available for the compound. Most importantly, the absolute stereochemistry of taxiresinol **1** has not yet been established.

In this paper, we would like to report the absolute configuration of taxiresinol **1**, which has been established with the help of chemical correlation methods and X-ray crystallography. Furthermore, the anticancer properties of taxiresinol **1** and two other lignans, (−)-secoisolariciresinol **3** and isotaxiresinol **2** are also reported for the first time for the above molecules.

Results and Discussion

Taxiresinol **1** was isolated from the ethyl acetate soluble fraction of the methanol extract of the heartwood of *T. wallichiana*. The molecular formula of $C_{19}H_{22}O_6$ for the

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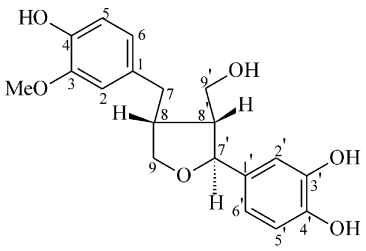

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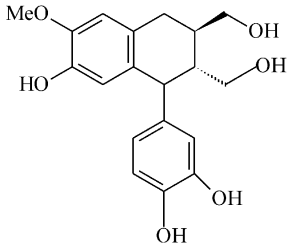

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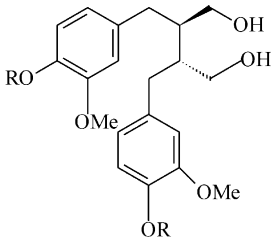

3 R = H
4 R = Me

molecule was established from elemental analysis, ^{1}H and ^{13}C NMR data. Detailed analysis of the ^{1}H and ^{13}C NMR spectra including 2D NMR studies (HMQC) revealed that it belongs to a substituted furan type lignan like lariciresinol.[9] Furthermore, examination of the HMBC spectrum of taxiresinol **1** confirmed a three bond correlation between 7′-H (δ 4.53) and the carbon (δ 58.5) of the primary alcohol. Also, the aromatic proton at δ_H 6.63 (1H, d, $J=1.8$ Hz) and δ_H 6.46 (1H, dd, $J=1.8$, 7.8 Hz) correlated with the same carbon resonance at δ_C 81.6. Thus, the catechol and the primary alcohol constitute the C_6C_3 unit of one half of the tetrahydrofuran ring. A single crystal X-ray crystallography (Fig. 1) of the molecule confirmed the structure previously established by Mujumdar et al.[7] However, the absolute configuration of the molecule **1** could not be determined from the X-ray analysis. In order to establish the absolute configuration of the molecule, it was subjected to catalytic hydrogenation and it formed a triphenolic compound, which upon methylation with dimethylsulphate formed a compound, which was identified as (−)-secoisolariciresinol dimethylether **4** mp 123–125 °C, $[\alpha]_D^{25}$ −35° (*c* 1.0, $CHCl_3$) on the basis of spectral characteristics[10] and by direct comparison with an authentic sample prepared from the methylation of (−)-secoisolariciresinol **3** with dimethylsulphate. Since the absolute configuration of (−)-secoisolariciresinol **3** has been established as (8*R*, 8′*R*) by chemical correlation as well as by X-ray analysis, the absolute configurations of the asymmetric carbons of taxiresinol **1** was established as (8*R*, 8′*R*).

A single crystal X- ray analysis[11] of taxiresinol **1** was carried out and the conformation of the molecule is shown in Figure 1. The present crystallographic study alone could not establish the absolute configuration of the molecule. After establishing the absolute configuration of the asymmetric centers of taxiresinol as 8*R* and 8′*R*, the X-ray crystal structure of the molecule helped in assigning the absolute configuration of the remaining center at 7′ as 7′*R* also. Thus, with the help of chemical correlation and X-ray analysis, the absolute configuration of taxiresinol **1** has been assigned as (8*R*, 8′*R*, 7′*R*) surrounding its three asymmetric centers at 8, 8′, 7′. Accordingly, the structure was inverted to get its chiral centers as *R* configuration during final cycles of refinement. Two of the H-atoms of the chiral centers, 3-H and 4-H have a β orientation while the third 2-H, has a α-orientation. The tetrahydrofuran ring is puckered to adopt an envelope conformation [deviation of atom C-5 from the least square plane through O-1, C-2, C-3, and C-4 is −0.565 (4) Å]. The molecular packing in the crystal shows that the hydroxyl groups are involved in both intra- and intermolecular hydrogen bonding of the type O–H...O (Table 1).

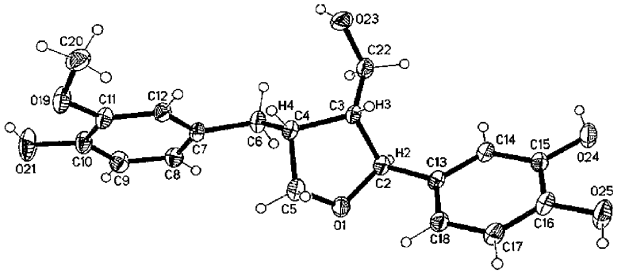

Figure 1. An ORTEP diagram showing the molecular structure of taxiresinol **1** with atomic numbering scheme. Only H atoms attached to chiral centers are numbered.

Table 2 indicates the IC_{90} values obtained for the three lignans **1**–**3** against six different tumor cell lines. All the three lignans were active against colon adenocarcinoma

Table 1. Hydrogen bonding geometry (Å)

D–H	H–A	D–A	<(DHA)	D–H–A
0.82	2.20	2.964(5)	155.4	O21–H21...O25[a]
0.82	2.20	2.641(4)	114.1	O21–H21...O19
0.82	2.10	2.903(4)	165.2	O23–H23...O21[b]
0.82	1.89	2.698(4)	169.6	O24–H24...O1[c]
0.82	1.91	2.715(4)	167.9	O25–H25...O23[d]

[a] $X+1, Y, Z+1$.
[b] $2-X, Y-0.5, 2-Z$.
[c] $1-X, 4-0.5, 1-Z$.
[d] $-X, 4+0.5, 1-Z$.

Table 2. Cytotoxicity of lignans (**1**–**3**) against human tumor cell lines

Compd	IC_{90} (μg/mL)					
	PA-1	MCF-7	COLO-320 DM	CaCo2	KB-403	WRL
3	—	—	1 (1.99)*	0.08 (0.251)	—	—
2	—	—	0.75 (7.08)	0.08 (0.056)	—	—
1	50 (>50)	4 (10)	1 (31.62)	3.5 (15.84)	—	>55.0 (55)
Paclitaxel	0.9 (2.23)	0.85 (1.82)	0.01 (0.79)	0.065 (1.25)	0.047 (7.94)	2.5 5.62
Doxorubicin	0.5 (10)	0.01 (0.02)	0.08 (0.08)	0.052 (0.063)	0.06 (0.79)	1 (3.98)

*Values in parenthesis indicates IC_{90} (μg/mL) of respective compounds as determined by clonogenic assay.

cell lines in MTT assay. However, lignans **2** and **3** were most active against Caco-2 cell line with an IC_{90} value of 0.08 μg/mL in MTT assay and 0.056 and 0.251 μg/mL, respectively in clonogenic assay. Lignan **2** was equal to or even better than standard refernce compounds such as taxol and doxorubicin against colon adenocarcinoma (Caco-2) in both the assay systems. Lignans **2** and **3** were however inactive against other cell lines. Taxiresinol **1** was active against ovary teratocarcinoma and breast adenocarcinoma cell lines albeit at higher concentration.

Conclusion

Thus the absolute configuration of taxiresinol **1**, having anticancer activity against colon, ovary, breast and liver cancer cell lines, was determined as 8*R*, 8′*R*, *and* 7′*R* with the help of chemical correlation method and X-ray crystallographic studies.

Experimental

General

Melting points were determined in open capillaries and are uncorrected. The NMR spectra were recorded on a Brucker DRX-300 (300 MHz) with TMS as internal standard. Chemical shifts were expressed as δ in ppm. Optical rotations were recorded on JASCO DIP-180 digital polarimeter. Elemental analysis was carried out on HERAEUS CHN-*O*-RAPID elemental analyzer.

Crystal data for 1. $C_{19}H_{22}O_6$, MW = 346.4; monoclinic; colourless rectangular crystal, size 0.35×0.25×0.125 mm; $P2_1$, $a = 5.446(0)$, $b = 9.983(1)$, $c = 15.355(1)$ Å; $\beta = 98.96(0)$ Å, $V = 824.62(10)$ Å^3; $Z = 2$; $D_c = 1.395$ g cm^{-1}; μ(Mo-K$_\alpha$) = 0.71073 mm^{-1}; $F(000) = 368.0$ mm, 2857 reflections measured (2197unique), Rw = 0.11 for all the data, conventional $R = 0.0412$ on F values of 1853 reflections with $I > 2\sigma$ (I), S = 1.074 for all data. Unit cell determination and intensity data collection ($2\theta = 50°$) was performed on a Bruker P4 diffractometer at 293(2) K.c. Structure solutions[11,12,15] by direct methods and refinement by full-matrix least squares methods on F^2.

Isolation of the lignans (1–3)

The heartwood of *T. wallichiana* was collected in Kashmir, India, in October 1998, and was identified by V. K. Mehta, CIMAP, Lucknow. A voucher specimen is deposited in the herbarium of CIMAP. The dried and ground heartwood (1.0 kg) was extracted with MeOH exhaustively at room temperature by percolation. After removal of the solvent, the extract was diluted with water (1 L) and extracted with $CHCl_3$ (3×1 L) and EtOAc (3×1 L) to give crude $CHCl_3$ extract (22 g) and EtOAc extract (35 g), respectively. $CHCl_3$ extract was chromatographed over SiO_2 (60–120 mesh, 600 g) with $CHCl_3$ followed by $CHCl_3$–MeOH (98:2). (−)-Secoisolariciresinol **3** (2 g) was isolated from the later fraction. Taxiresinol **1** (4 g) and isotaxiresinol **2** (4 g) were isolated from the EtOAc extract upon chromatography with SiO_2 and eluted with EtOAc–petrol ether (3:1) and EtOAc, respectively. Lignan **3** was identified by comparison with its physical and spectral data with the reported values.[7] Isotaxiresinol **2** was easily formed from taxiresinol **1** by acid treatment and thus it was identified as isotaxiresinol.[7]

Taxiresinol 1. Crystal, mp 157–158 °C (MeOH); [Found: C, 65.82; H, 6.66. $C_{19}H_{22}O_6$ (346), requires C, 65.9; H, 6.4%]; $[\alpha]_D^{25}$ +68° (*c* 1.0, MeOH); δ_H (300 MHz, DMSO-d_6), 6.68 (1H, d, $J = 1.8$ Hz, 2-H), 6.62 (1H, d, $J = 7.8$ Hz, 5-H), 6.51 (1H, dd, $J = 1.8$ and 7.8 Hz, 6-H), 2.34 (1H, dd, $J = 10.8$ and 13.2 Hz, 7-H), 2.75 (1H, dd, $J = 4.2$ and 13.2 Hz, 7-H), 2.48 (1H, m, 8-H), 3.49 (1H, dd, $J = 6.6$ and 8.1 Hz, 9-H), 3.77 (1H, dd, $J = 6.6$ and 8.1 Hz, 9-H), 6.63 (1H, d, $J = 1.8$ Hz, 2′-H), 6.59 (1H, d, $J = 7.8$ Hz, 5′-H), 6.46 (1H, dd, $J = 1.8$ and 7.8 Hz, 6′-H), 4.53 (1H, d, $J = 6.3$ Hz, 7′-H), 2.08 (1H, m, 8′-H), 3.58 (1H, m, 9′-H), 3.39 (1H, 9′-H), 3.68 (3H, s, OMe), 8.62, 8.74, 8.66, (s each, ph-OH), 4.61 (1H, t, $J = 4.5$ Hz, 9′-OH); δc (75.5 Hz, DMSO-d_6, assignment by DEPT, HMQC and HMBC NMR experiments) 131.7 (C-1), 112.6 (C-2), 147.4 (C-3), 144.5 (C-4), 115.3 (C-5), 120.5 (C-6), 32.1 (C-7), 41.9 (C-8), 71.7 (C-9), 134.8 (C-1′), 113.1 (C-2′), 144.1 (C-3′), 144.9 (C-4′), 115.1 (C-5′), 116.5 (C-6′), 81.6 (C-7′), 52.5 (C-8′), 58.5 (C-9′), 55.5 (OMe).

Conversion of taxiresinol into (−)-secoisolariciresinol dimethyl ether 4

Taxiresinol (100 mg) was dissolved in MeOH (3 mL) and added with Pd/C (100 mg) and was subjected to catalytic hydrogenation for 24 h. Usual workup of reaction mixture gave a semi-solid residue, which was dissolved in anhydrous acetone and treated with anhydrous K_2CO_3 (2 g) and dimethyl sulphate (0.3 mL). The reaction mixture was refluxed for 4 h; usual work up of the reaction mixture gave **4** (60 mg), mp 123–125 °C, $[\alpha]_D^{25}$ −40° (*c* 1.0, $CHCl_3$); ^{1}H and ^{13}C NMR data of **4** were identical with those reported in the literature.[10] Compound **4** was found to be identical with an authentic sample of (−)-secoisolariciresinol dimethyl ether prepared by the methylation of an authentic sample of (−)-secoisolariciresinol **3**.

Cytotoxicity testing of the lignans 1–3

Cytotoxicity testing in vitro was done by the method of Woerdenbag et al.[13] 2×10^3 cells/well were incubated in the 5% CO_2 incubator for 24 h to enable them to adhere properly to the 96-well polysterene microplate (Grenier, Germany). Test compounds dissolved in 100% DMSO (Merck, Germany) in at least five doses were added and left for 6 h after which the compound plus media was replaced with fresh media and the cells were incubated for another 48 h in the CO_2 incubator at 37 °C. The concentration of DMSO used in our experiments never exceeded 1.25%, which was found to be non-toxic to cells. Then, 10 μL MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma M 2128] was added, and plates were incubated at 37 °C for 4 h. 100

μL dimethyl sulfoxide (DMSO, Merck, Germany) were added to all wells and mixed thoroughly to dissolve the dark blue crystals. After a few min at room temperature to ensure that all crystals were dissolved, the plates were read on a SpectraMax 190 Microplate Elisa reader (Molecular Devices Inc., USA), at 570 nm. Plates were normally read within 1 h of adding the DMSO. The experiment was done in triplicate and the inhibitory concentration (IC) values were calculated as follows:% inhibition = [1 − OD (570 nm) of sample well/OD (570 nm) of control well] × 100. IC_{90} is the concentration μg/mL required for 90% inhibition of cell growth as compared to that of untreated control.

The clonogenic assay for tumor cells which determines the actual cell death was performed to determine the cytotoxic potential of test compounds. The principle of clonogenic assay is to investigate the ability of an individual cell to form a colony on a soft agar plate containing various concentrations of test compounds. Cells not able to form colonies are considered clonogenically dead.[14] The concentration of test compound resulting in 90% of the control (untreated) colonies was denoted as IC_{90} and was used as a parameter for cytotoxicity. The assay was performed as described previously except that the test compounds were added into the top soft agar and the cells were plated out to form colonies.

The anthracycline derivative doxorubicin and microtubule depolymerization inhibitor paclitaxel (Sigma Chem. Co., St. Louis, USA) both established anticancer agents were included as standard reference drugs.

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15. Crystallographic data (excluding structure factors) for the structure reported in this paper have been deposited with the Cambridge Crystallographic Data Centre as supplementary data. Copies of the data can be obtained free of charge on application to from the CCDC, 12 Union Road, Cambridge, CB2 1 EZ, UK [Fax: (internat.) +44-1223-/336-033; e-mail: deposit@ccdc.cam.ac.uk].