Pergamon 0031-9422(95)00195-6 Phytochemistry, Vol. 40, No. 2, pp. 427 431, 1995
Elsevier Science Ltd
Printed in Great Britain
0031 9422/95 $9.50 + 0.00

ANTIFUNGAL ARYLTETRALIN LIGNANS FROM LEAVES OF *PODOPHYLLUM HEXANDRUM*

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(*Received in revised form* 14 *February* 1995)

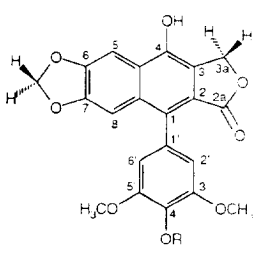
Key Word Index—*Podophyllum hexandrum*; Podophyllaceae; leaves; aryltetralin lignans; 4′-*O*-demethyldehydropodophyllotoxin; picropodophyllone; isopicropodophyllone; dehydropodophyllotoxin; antifungal activity.

Abstract—Two aryltetralin lignans, 4′-*O*-demethyldehydropodophyllotoxin and picropodophyllone, which were earlier reported as semi-synthetic products, were isolated from the leaves of *Podophyllum hexandrum* of Pakistani origin. Two known aryltetralin lignans, isopicropodophyllone and dehydropodophyllotoxin, were also isolated. Structures were identified by spectroscopic methods. The two new lignans showed strong antifungal activity against *Epidermophyton floccosum*, *Curvularia lunata*, *Nigrospora oryzae*, *Microsporum canis*, *Allescheria boydii* and *Pleurotus ostreatus*, while picropodophyllone also showed activity against *Drechslera rostrata*.

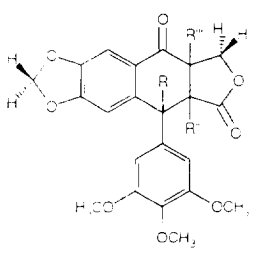
INTRODUCTION

The genus *Podophyllum* (Podophyllacea) has four well known species, one Himalayan, one American and two of Chinese origin [1]. Several other less-known Chinese species have also been identified and chemically investigated. The genus was the subject of vigorous phytochemical investigations in the early 60's after the isolation of several antitumour lignans, such as podophyllotoxin, etc. A number of lignans isolated from *Podophyllum* species have shown a wide range of biological activities, such as antitumour, antimitotic and antiviral activities. Some of them have also shown toxicity to fungi, insects and vertebrates [2]. The successful chemical conversion of the major constituent podophyllotoxin into the clinically useful anticancer drug Etoposide® and Teniposide® has also triggered further researches in this area [3].

Our investigation on *P. hexandrum* has resulted in the isolation of two aryltetralin lignans, 4′-*O*-demethyldehydropodophyllotoxin (**1**) and picropodophyllone (**2**), earlier reported as semisynthetic products. Isopicropodophyllone (**3**) and dehydropodophyllotoxin (**4**) were also isolated which have been reported previously as constituents of *P. pleianthum* and *P. peltatum*, respectively [4, 5].



1 R = H
4 R = CH_3



2 R′ = Hβ, R″ = Hα, R‴ = Hα
3 R′ = Hβ, R″ = Hβ, R‴ = Hβ

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RESULTS AND DISCUSSION

Leaves of *P. hexandrum* were collected from Muzaffarabad, Pakistan. The concentrated ethanolic extract of the leaves was partitioned between water and chloroform. The concentrated chloroform extract was subjected to VLC and then to column chromatography. The fractions thus obtained were purified by repeated TLC to yield compounds **1**–**4**.

Compound **1** was isolated as an amorphous powder. The molecular formula $C_{21}H_{16}O_8$ was obtained from high resolution EI mass measurements of the $[M]^+$ (*m/z* 396.0799) indicating the presence of 14 double bond equivalents in the molecule. The $[M]^+$ was further confirmed by positive FAB mass spectrometry. The overall spectral behaviour of **1** was identical to that of the semisynthetic product 4′-*O*-demethyldehydropodophyllotoxin [6]. The UV spectrum exhibited absorption bands at 205, 229, 264, 314 and 357 nm, characteristic of a tetradehydropodophyllotoxin lignan nucleus [7]. The absorption bands in the IR spectrum at 3437, 2900, 1742, 1609 and 1448 and 1029 cm^{-1} indicated the presence of phenolic OH, C–H, lactone carbonyl, aromatic C=C and C–O functionalities, respectively.

The ^{13}C NMR spectra (broadband decoupled and DEPT) [8] showed 16 signals representing 19 carbons.

An examination of the structure shows that the aromatic ring substituted at C-1 contains three pairs of identical carbons (the two $-OCH_3$ carbons appearing at δ56.3, the two carbons at which the $-OCH_3$ groups are attached resonating at δ148.7 and the two carbon *ortho* to the methoxy group resonating at δ109.3). The signals for the C-1′ and C-4′ quaternary carbons were too weak to be detected in the broadband decoupled ^{13}C NMR spectrum. An ester carbonyl carbon resonated at δ170.0, a methylenedioxy carbon appeared at δ102.1 and a downfield methylene carbon resonated at δ66.9, while the remaining 12 signals appeared between δ100.0 and 150.0, again confirming a functionalized aromatic system.

In the ^1H NMR spectrum of **1**, a 6H singlet resonating at δ3.81 represented two $-OCH_3$ groups in an identical magnetic environment. A downfield methylene singlet at δ 6.11 was characteristic of a methylenedioxy group and its appearance as a singlet was indicative of the lack of chirality in the molecule. Another 2H downfield singlet at δ5.50 was due to the methylene protons (C–3H) sandwiched between an oxygen function and the quaternary carbon. A 2H singlet in the aromatic region of the spectrum at δ6.97 was assigned to the two aromatic protons in an identical magnetic environment, i.e. the C-2′ and C-6′ protons. Two more downfield signals appearing as broad singlets at δ8.14 and 7.46 were ascribed to the two remaining aromatic protons of the skeleton. The absence of coupling interactions between these aromatic protons indicated their *para*-disposition and they were therefore assigned to the C-5 and C-8 protons, respectively.

An heteronuclear multiple quantum coherence (HMQC) experiment [9] was performed to establish connectivities between the protons and their respective carbons (Table 1). Hence, the C-5 and C-8 carbons (δ99.0 and 104.0) in the aromatic moiety displayed one-bond interactions with the protons resonatng at δ8.14 and 7.46, respectively, while another set of carbons resonating at δ109.3 (C-2′ and C-6′) in ring D showed correlation with the C-2′ and C-6′ protons (δ6.97). The C-3a methylenic protons exhibited heteronuclear couplings with the C-3a carbon (δ66.9). The various proton–carbon connectivities based on the HMQC data are summarized in Table 1.

A heteronuclear multiple bond connectivity (HMBC) experiment [10] was used for unambiguous chemical shift assignments. Thus, the long-range interactions between the protons at δ6.97 (C-2′H and C-6′H) with the carbons resonating at δ148.7 (C-5′) and δ137.4 (C-4′) suggested

Table 1. ^{13}C NMR data and ^1H ^{13}C connectivities of compounds **1** and **2**

	1			**2**		
Carbon	Chemical shift (δ)	Multiplicity†	^1H–^{13}C connectivity‡ (J = in Hz)	Chemical shift (δ)	Multiplicity	^1H–^{13}C connectivity‡ (J = in Hz)
C-1	126.2	C	—	43.4	CH	4.67 *br s*
C-2	125.9	C	—	46.7	CH	3.28 *m*
C-2a	170.0	C	—	175.5	C	—
C-3	120.0	C	—	43.5	CH	3.28 *m*
C-3a	66.9	CH_2	5.50*s*	70.5	CH_2	4.32 *m* Hα 4.73 *d* Hβ (J = 9.5)
C-4	*	C	—	193.4	C	—
C-4a	132.5	C	—	127.3	C	—
C-5	99.0	CH	8.14*s*	106.0	CH	7.47 *s*
C-6	146.6	C	—	148.4	C	—
C-7	148.9	C	—	153.8	C	—
C-8	104.0	CH	7.46*s*	109.4	CH	6.66 *s*
C-8a	132.3	C	—	139.5	C	—
C-1′	*	C	—	137.0	C	—
C-2′	109.3	CH	6.97*s*	104.9	CH	6.21 *s*
C-3′	148.7	C	—	153.8	C	—
C-4′	137.4	C	—	137.9	C	—
C-5′	148.7	C	—	153.8	C	—
C-6′	109.3	CH	6.97*s*	104.9	CH	6.21 *s*
OCH_2O	102.1	CH_2	6.11*s*	102.2	CH_2	6.02 *d* (J = 1.0) 6.01 *d* (J = 1.0)
C-3′/C-5′–OCH_3	56.3	CH_3	3.81*s*	56.3	CH_3	3.73 *s*
C-4–OCH_3	—	—	—	60.8	CH_3	3.73 *s*

*Signals too weak to be detected.

†Multiplicity determined by DEPT.

‡One-bond heteronuclear connectivities determined by HMQC.

that they are part of ring D. The aromatic C-5 proton resonating at δ8.14 exhibited long-range interactions with the carbons at δ148.9 (C-7) and δ132.5 (C-4a). The C-3a methylene protons displayed HMBC interaction with C-3 (δ120.0), while C-8H showed interactions with C-7, C-8a and C-1.

The mass spectrum included peaks at *m/z* 396 $[M]^+$ 353, 334, 281 and 139. This spectroscopic data showed unambiguously that **1** is the naturally occurring 4′-demethyl derivative of dehydropodophyllotoxin (**4**). Compound **1** has exhibited some cytotoxic and antitumour activity [6].

Picropodophyllone (**2**), a 1*R*, 2*S*, 3*R* isomer of podophyllotoxone, has been known synthetically for several years, but has not previously been reported as a natural product [11]. The possibility of heat and base-catalysed isomerization did not exit since no base was employed during the isolation process. The molecular formula $C_{22}H_{20}O_8$ of **2** was again determined by HR EI mass spectrometry (*m/z* 412.1138). The UV spectrum of **2** displayed absorption bands at 206, 240, 280 and 324 nm characteristic of a podophyllotoxone skeleton [12]. The IR spectrum of **2** contained bands at 2839 (C–H), 1772 (lactone carbonyl), 1661 (ketone carbonyl), 1584 (C=C) and 1125 (C–O) cm^{-1}.

The ^{13}C NMR spectra (broadband decoupled and DEPT) of **2** contained a lactone carbonyl signal at δ175.5 and an α,β-unsaturated ketonic resonance at δ193.4. The three methoxy carbons appeared at δ56.3 ($2\times OCH_3$) and δ60.8 ($-OCH_3$). The methylenedioxy carbon yielded a characteristic signal at δ102.2. A downfield methylene signal at δ70.5 was assigned to the methylene carbon containing an oxygen function. The methine carbons resonated at δ43.5, 46.7 and 43.4 representing the carbon atoms of ring B. The aromatic carbons appeared in two groups. The signals between δ104 and 107 were due to unsubstituted aromatic carbons, while the signals resonating between δ127.0 and 154.0 represented either oxygen-bearing or quaternary aromatic carbons. Only 17 signals were visible in the ^{13}C NMR spectrum representing 22 carbons.

The ^1H NMR spectrum of **2** contained one 6H and one 3H singlets at δ3.73 and 3.77, which could be assigned to three methoxy groups, two of which have identical magnetic environments. The methylenedioxy protons appeared as two AB doublets at δ6.02 and 6.01 indicating the presence of chirality in the molecule. Similarly, a set of geminally-coupled protons resonating at δ4.73 as a doublet and δ4.32 as multiplet represented a methylene, i.e. C-3a protons sandwiched between an oxygen and methine. The COSY 45° [13] spectrum displayed cross-peaks between signals at δ4.73 and 4.32 due to their geminal disposition, while the cross-peak between δ4.32 and 3.28 represented vicinal coupling between one of the methylenic protons with the neighbouring methine proton (C-3H).

The spin system comprising C-3a, C-3, C-2 and C-1 in picropodophyllone (**2**) was further investigated by HOHAHA experiments [14] recorded with mixing intervals of 20, 60 and 100 ms. Thus, the C-3a α and β protons resonating at δ4.73 and 4.32 showed geminal couplings and also HOHAHA interactions with the C-3 and C-2 protons (δ 3.28).

The HMQC spectrum of **2** showed that the most downfield proton resonating at δ7.47 had a heteronuclear interaction with the C-5 carbon (δ106.0). The C-8 proton (δ6.66) showed correlation with its respective carbon resonating at δ109.4. The methylenic C-3a α and β protons (δ4.32 and 4.73) displayed connectivities with the C-3a carbon (δ70.5). The HMQC data is summarized in Table 1. The HMBC spectrum indicated that the proton resonating at δ4.67 (C-1H) had long-range interactions with C-8a, C-4a, C-3, C-2 and also with the C-2′ and C-6′ carbons in ring D. Similarly, the C-5 proton (δ7.47) in the aromatic moiety exhibited interactions with C-6, C-7 and C-8a, while the aromatic proton resonating at δ6.66 (C-8H) showed interaction with C-4a, C-6 and C-7.

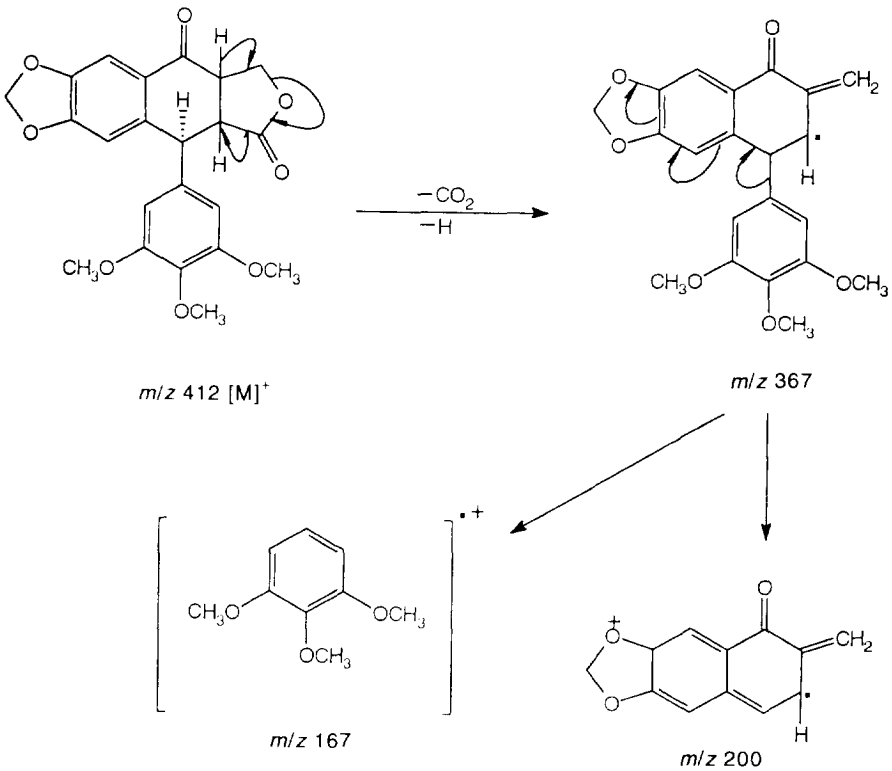
The EI mass spectrum of **2** displayed a $[M]^+$ at *m/z* 412.1138 ($C_{22}H_{20}O_8$). The ion at *m/z* 367 $[M-HCO_2]^+$ represented the fragment $C_{21}H_{19}O_6$. The peaks at *m/z* 167 $[M-245]^+$ and 200 $[M-212]^+$ were due to the fragments $C_9H_{12}O_3$ and $C_{12}H_8O_3$, respectively (Scheme 1). Other peaks at *m/z* 353, 337, 297, 227, 139, etc., were also in complete agreement with the structure **2**. Complete analysis of the ^1H and ^{13}C NMR spectra with the aid of COSY, HMQC and HMBC experiments led to the identification of the compound as shown in structure **2**.

Isopicropodophyllone (**3**) and dehydropodophyllotoxin (**4**) have been reported earlier as naturally occurring constituents of *P. pleianthum* and *P. peltatum*, respectively [4, 5]. Dehydropodophyllotoxin (**4**) is also known synthetically [11]. Our compounds had identical spectral characteristics (UV, IR, mass spectra, ^1H NMR) to those reported in the literature [4, 12, 7]. However, the ^{13}C NMR spectral data of these compounds are reported here for the first time.

EXPERIMENTAL

General. UV spectra were taken in EtOH or MeOH and optical rotations in $CHCl_3$. ^1H NMR spectra (δJ in Hz) were obtained in deuterated solvents (pyridine and chloroform) on 400 or 500 MHz spectrometers. ^{13}C NMR were also recorded in d_5-pyridine and $CDCl_3$ at 100 MHz. MS were recorded on double focusing spectrometers connected to computer systems. IR spectra were taken in KBr or $CHCl_3$. Purity of samples was checked by TLC (silicia gel, precoated plates, Merck).

Plant material. Leaves (32 kg) of *P. hexandrum* Royle were collected from Jagran village, Neelum valley, District Muzaffarabad, Azad Kashmir, during the month of September, 1992. Plant material was identified by the plant taxonomist, Prof. Shafiq-ur-Rahman, and voucher specimen is deposited in the Botany Department, University of Azad Jammu and Kashmir, Muzaffarabad, Pakistan.



Scheme 1. Mass fragmentation of **2**.

Extraction and isolation. An EtOH extract of air-dried leaves was partitioned between H_2O and $CHCl_3$. The $CHCl_3$ extract (57 g) was subjected to VLC on a column packed with silica gel (plate silica) and eluted with petrol (40–60°), $CHCl_3$–petrol and MeOH–$CHCl_3$. As a result, seven frs of different polarities were collected. The fr. (3.35 g) collected on elution with $CHCl_3$–petrol (1:1) was further loaded onto a silica gel column (70-230 mesh, 105 g) which resulted in several frs. The fr. eluted with $CHCl_3$–petrol (1:1) contained two major bands which were purified by TLC (silica gel) using MeOH–CH_2Cl_2 (1.5:98.5) to yield *iso*picropodophyllone (**3**) and picropodophyllone (**2**). Another fr. eluted from the same column with MeOH–$CHCl_3$ (3:97) also contained two major bands which were purified by CC MeOH–$CHCl_3$ (1:19). The upper band was identified as dehydropodophyllotoxin (**4**), while the lower one was 4′-demethyl dehydropodophyllotoxin (**1**).

Antifungal activity. Antifungal activity of compounds **1** and **2** were determined using the agar diffusion method [15]. Grisiofulvin was used as the standard. Compounds were dissolved in DMSO (2.5 mg ml^{-1}). Sterile SDA (Sabouraud's dextrose agar) medium (5 ml) was placed into test tubes and inoculated with sample solns (200 $\mu g\ ml^{-1}$) and kept in a slanting position at room temp. overnight. Fungal culture was then inoculated onto the slants. Samples were incubated for 7 days at 29° and growth inhibition measured.

4′-Demethyldehydropodophyllotoxin (**1**). Amorphous (21 mg, 6.56×10^{-5}% yield). UV λ_{max} (EtOH) nm (logε) 205 (4.54), 229 (4.35), 264 (4.46), 314 (3.83), 357 (3.60). IR(KBr) cm^{-1} ν_{max} 3437 (phenolic OH), 2900 (C–H), 1742 (lactone carbonyl), 1609, 1448 (aromatic C=C), 1240, 1029 (C–O). EIMS *m/z* (rel. int.): 396 (100) $[M]^+$, 352 (13), 334 (11), 281 (9), 139 (7). ^1H NMR (C_5D_5N, 400 MHz) in Table 1. ^{13}C NMR (C_5D_5N, 100 MHz) in Table 1.

Picropodophyllone (**2**). Amorphous (33 mg, 1.03×10^{-4}% yield). $[\alpha]_D^{25} = -109°$ ($c = 0.073$, $CHCl_3$). UV λ_{max} (EtOH) nm (logε) 206 (4.76), 240 (4.44), 280 (3.97), 324 (3.92). IR ($CDCl_3$) cm^{-1} ν_{max} 2839 (C–H), 1772 (lactone carbonyl), 1661 (ketone carbonyl), 1584 (C=C), 1125 (C–O). EIMS *m/z* (rel. int.): 412 (100) $[M]^+$, 367 (67), 353 (10), 337 (14), 297 (12), 227 (4), 200 (2), 167 (11), 139 (7). ^1H NMR ($CDCl_3$, 500 MHz) in Table 1. ^{13}C NMR ($CDCl_3$, 100 MHz) in Table 1.

Isopicropodophyllone (**3**). Amorphous (23 mg, 7.18×10^{-5}% yield). UV λ_{max} (MeOH) nm (logε), 202 (4.7), 237 (4.4), 275 (4.0), 323 (3.9). IR ($CHCl_3$) cm^{-1} ν_{max} 2840 (C–H), 1762 (lactone carbonyl), 1668 (ketone carbonyl), 1587 (C=C), 1125 and 1025 (C–O). EIMS *m/z* (rel. int.): 412 (53) $[M]^+$, 367 (11), 297 (12), 227 (1), 188 (20), 83 (100). ^1H NMR ($CDCl_3$, 500 MHz): δ4.55 (1H, *d*, $J = 6.0$ Hz, C-1H), 3.51–3.60 (2H, *m*, C-2H and C-3H), 3.82 (1H, *t*, $J = 9$ Hz, C-3aHα), 4.50 (1H, *t*, $J = 9$ Hz, C-3aHβ), 7.24 (1H, *s*, C-5H), 6.66 (1H, *s*, C-8H), 6.26 (2H, *s*, C-2′H and C-6′H), 6.04 (1H, *d*, $J = 1.0$ Hz, O–CH_2–O), 6.06 (1H, *d*, 1.0 Hz, O–CH_2–O), 3.70 (6H, *s*, C-3′ and 5′-OCH_3), 3.78 (3H, *s*, C-4′-OCH_3). ^{13}C NMR ($CDCl_3$, 100 MHz): δ44.2 (C-1), 45.0 (C-2), 175.2 (C-2a), 44.7

(C-3), 69.4 (C-3a), 193.0 (C-4), 128.8 (C-4a), 108.5 (C-5), 148.3 (C-6), 153.4 (C-7), 106.09 (C-8), 133.8 (C-1'), 106.7 (C-2'), 153.3 (C-3'), 139.0 (C-4'), 153.3 (C-5'), 106.7 (C-6'), 102.2 (O–CH_2–O), 56.2 (C-3' and C-5' OCH_3), 60.8 (C-4'–OCH_3).

Dehydropodophyllotoxin (**4**). Amorphous (16 mg, 5×10^{-5}% yield). UV λ_{max}(MeOH) nm (logε), 206 (4.4), 225 (4.2), 263 (4.4), 322 (3.8), 356 (3.4). IR (KBr) cm^{-1} ν_{max} 3400 (phenolic OH), 2903 (C–H), 1725 (lactone carbonyl), 1589, 1427 (aromatic C=C), 1216, 1109, 1021 (C–O). EIMS *m/z* (rel. int.): 410 (100) $[M]^+$, 395 (50), 367 (12), 350 (4), 337 (5), 239 (3), 235 (2), 167 (5), 139 (5). ^{1}H NMR (C_5D_5N, 500 MHz): δ5.49 (2H, *s*, C-3a Hs), 8.12 (1H, *s*, C-5H), 7.36 (1H, *s*, C-8H), 6.92 (2H, *s*, C-2'H and C-6'H), 6.09 (2H, *s*, O–CH_2–O), 3.80 (6H, *s*, C-3' and 5' –OCH_3), 3.97 (3H, *s*, C-4' –OCH_3). ^{13}C NMR ($CDCl_3$, 125 MHz): δ126.5 (C-1), 123.0 (C-2), 170.2 (C-2a), 120.3 (C-3), 67.2 (C-3a),* (C-4), 132.5 (C-4a), 99.4 (C-5), 147.1 (C-6), 149.3 (C-7), 104.0 (C-8), 132.3 (C-8a), 131.9 (C-1'), 109.5 (C-2'), 153.9 (C-3'), 139.0 (C-4'), 153.9 (C-5'), 109.5 (C-6'), 102.4 (O–CH_2–O), 56.4 (C-3' and C-5' –OCH_3), 60.8 (C-4' –OCH_3). *Signal too weak to be detected.

Acknowledgement—This work was supported by the Third World Academy of Sciences (BC-91-016).

REFERENCES

1. Dymock, W. (1972) *Pharmacographia Indica* **1**, 69, republished by the Institute of Health and Tibbi Research under the auspices of Hamdard National Foundation, Pakistan.
2. Macrae, W. D. and Towers, G. H. N. (1984) *Phytochemistry* **23**, 1207.
3. Issel, B. F., Muggia, F. M. and Carter, S. K. (1984) *Etoposide (VP–16) Current Status and New Development*. Academic Press, Orlando.
4. Chang, F. C., Chiang, C. K. and Aiyar, V. N. (1975) *Phytochemistry* **14**, 1440.
5. Kofod, H. and Jørgensen, C. (1954) *Acta Chem. Scand.* **8**, 1296.
6. Beers, S. A., Imakura, Y., Dai, H. J., Li, D. H., Cheng, Y. C. and Lee, K. H. (1988) *J. Nat. Prod.* **51**, 901.
7. Tanoguchi, M., Arimoto, M., Saiki, H. and Yamaguchi, H. (1987) *Chem. Pharm. Bull.* **35**, 4162.
8. Doddrell, D. M., Pegg, D. T. and Bendall, M. R. (1982) *J. Magn. Res.* **48**, 323.
9. Bax, A. and Subramanian, S. (1986) *J. Magn. Res.* **67**, 565.
10. Bax, A. and Summers, M. F. (1986) *J. Am. Chem. Soc.* **108**, 2093.
11. Gensler, W. J., Johnson, F. and Sloan, A. D. B. (1960) *J. Am. Chem. Soc.* **82**, 6074.
12. Dewick, P. M. and Jackson, D. E. (1981) *Phytochemistry* **20**, 2277.
13. Atta-ur-Rahman (1989) *One- and Two-Dimensional NMR Spectroscopy*, p. 269. Elsevier, Amsterdam.
14. Braunschweiler, L. and Ernst, R. R. (1983) *J. Magn. Res.* **53**, 521.
15. Paxton, J. D. (1991) *Meth. Plant Biochem.* **6**, 33.