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Isolation and Structure Elucidation of Aryltetralin Lignans from *Linum tauricum* ssp. *bulgaricum*

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ABSTRACT - A phytochemical investigation of the aerial parts of *Linum tauricum* ssp. *bulgaricum* (Linaceae) led to the isolation of five lignans using semi-preparative HPLC. LC-MS and NMR studies were used to characterize the structures of the isolated compounds. This is the first report of podophyllotoxin, 4′-demethylpodophyllotoxin, 4′-demethyl-6-methoxypodophyllotoxin and isolariciresinol in *L. tauricum*. Thus, the combination of semi-preparative HPLC, LC-MS and NMR techniques represents a powerful tool for unraveling the structure of naturally occurring lignans.

KEY WORDS - *Linum tauricum* ssp. *bulgaricum;* lignans; NMR; LC-MS.

INTRODUCTION

Lignans exert various pharmacological activities of clinical interest (1) The aryltetralin lactone podophyllotoxin is the most important lignan used as an antiviral agent and a starting material for the semi-synthetic production of the anticancer drugs Etoposide, Teniposide and Etopophos (2, 3). However, continued supply of podophyllotoxin is not compatible with the conservation of the wild *Podophyllum* plants. Therefore the identification of other sources of this rare natural lignan is required (4). Screening for rapid growth and high lignan yield showed that *Linum* species belonging to the *Syllinum* section are promising for exploitation *in vitro* (5). *Linum tauricum* Willd. (section *Syllinum*) is well distributed in South Eastern Europe (Balkan Peninsula and Krym) (6).

In continuation of our research on lignans in *Linum* species, we have investigated *Linum tauricum* Willd. ssp. *bulgaricum* (Podp.) Petrova, is a endemic subspecies in Bulgaria (7, 8). We utilized a combination of HPLC, LC-MS and NMR techniques to isolate and elucidate the structures of naturally occurring aryltetralin lignans.

MATERIALS AND METHODS

Instrumentation and chromatography

A Brüker (Karlsruhe, Germany) model AVANCE 500 spectrometer, operating at 500.13 MHz for ^{1}H NMR and 125.77 MHz for ^{13}C NMR, was used and all spectra were measured in *deutero*-chloroform and referenced to a residual solvent peak.

Semi-preparative HPLC separations were carried out with a Hewlett Packard 1050 Gradient HPLC system equipped with an Agilent UV photodiode array detector and a Chrom Jet chromatointegrator. The wavelength of the UV detector was set to 290 nm. The semi-preparative reverse-phase HPLC column was a Phenomenex Luna C-18 column with a guard column (250 x 10 mm and 50 x 10 mm, respectively; 10 µm particles). The gradient system consisted of acetonitrile-water-phosphoric acid (75%:24.9%:0.1% v/v) (A) and acetonitrile-water-phosphoric acid (10%:89.9%:0.1% v/v) (B). The flow rate was 3.0 ml/min. A gradient method of, 0 min: 60% B; 5 min: 55% B; 30 min: 15% B; 33 min: 5% B; 34 min: 60% B; 35 min: 5% B. The method was stopped at 40 min. Ten min equilibration time was allowed between all runs. The injection volume was 100 µl.

A Perkin-Elmer SCIEX API-3000 apparatus (Toronto, Canada) with atmospheric pressure electrospray ionisation and triple-quadruple mass spectrometer was used for the LC-MS analysis. The machine was equipped with a Perkin-Elmer series 200 LC micro-gradient pump and autosampler. The mass spectrometer was interfaced to a Lichrosphere 100 RP-18 column (250 mm x 4 mm, 5 µm particle size) equipped with a guard column. The mobile phase was 95% H_2O / 4.9% MeCN (solvent A) and 95% MeCN / 4.9% H_2O (solvent B), both containing 0.1% HCOOH and 2 mM ammonium formate. The sample was commenced at 70:30 (v/v) of A:B for 5 min, followed by a gradient method of 5 min: 30% B; 30 min: 90% B; 35 min: 90% B;

37 min: 30% B; 40 min: 30% B. The method was stopped at 40 min. The flow rate was 1 ml/min at room temperature. The injection volume was 25 µl. The positive ion mode was employed and spectra were obtained with a spray voltage of 5.2 kV. The source temperature was 400°C. The scan rate was 2 sec/scan, a full ion scan was applied in the range 100–1200 amu with a step size of 1 amu and 10 V entrance potential. Nitrogen was both used as the nebulizer and curtain gas at a pressure of 0.8 Torr and a flow rate of 20 mL/min. Data processing was performed using Analyst 1.4 Software (Sciex).

Plant material

The plant material was collected and identified by Prof. Dr. Ana Petrova (Institute of Botany, Bulgarian Academy of Sciences) near the town of Sozopol, South Black Sea Coast. A voucher specimen is deposited in the herbarium of the Institute of Botany, Bulgarian Academy of Sciences (SOM 126 808).

Extraction and isolation

Air-dried plant material (20 g) was extracted for 30 min once with 100 ml of simmering ethanol (96%) and twice with ethanol (70%). The resultant extracts were combined, concentrated, treated with water to 100 ml final volume, then extracted with EtOAc (3 x 100 ml). The combined EtOAc layers were evaporated, freeze-dried (1.08 g) and underwent semi-preparative HPLC.

Multiple fractions eluting between 11.8 and 13.0 min (fr. I), 15.4 and 16.2 min (fr.II), 16.8 and 17.8 min (fr.III) and between 21.4 and 22.8 min (fr. IV) were collected from the semi-preparative HPLC, as individual well-defined peaks and then pooled. These pooled aqueous fractions were extracted with dichloromethane to remove the lignans and dried. Fractions I, II, III and IV were purified by repeated chromatography as described above to yield compounds **3** (12.06 mg) and **5** (0.59 mg), **4** (1.38 mg), **1** (5.46 mg) and **2** (6.33 mg) respectively.

RESULTS AND DISCUSSION

Compounds were identified by mass spectra obtained from LC-MS and NMR data in comparison with the literature.The collected and purified fractions were subjected to LC-MS in order to obtain information on molecular weight of the isolated compounds. LC-MS analysis led to good responses and produced protonated ($[M+H]^+$), ammoniated ($[M+NH_4^+]$) and sodiated ($[M+Na^+]$) species, typical for electrospray ionisation (9). Specifically, the LC/(+)ESI-MS spectrum of **1** (molecular weight 414) showed abundant ion peaks at m/z 415 ($[M+H]^+$), 432 ($[M+NH_4^+]$) and 437 ($[M+Na^+]$). Ions of $[M+81]^+$ and $[M+91]^+$ were also observed in the spectrum of **1**, corresponding to the following clusters of the formate ion: $[M+((NH_4)_2HCOO)^+]$ and $[M+(Na_2HCOO)^+]$ (10). The product ion scans in the fragmentation pattern of **1** led to detection of ($[M+H]^+$-18) ions, primarily derived from successive loss of water $[M+H-H_2O]^+$. Our literature search gave a suggestion for two potential lignans with a molecular mass of 414, characteristic for Section *Syllinum* of the genus *Linum*: podophyllotoxin and β-peltatin.

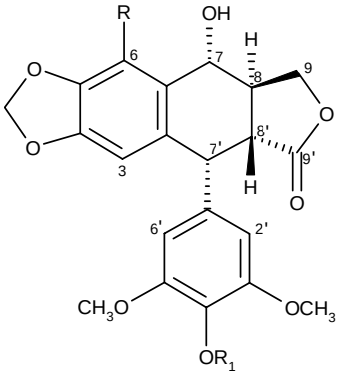
In a follow-up step, the structure of compound **1** was assigned by one- and two-dimensional NMR spectra. ^1H NMR spectrum of **1** indicated an aryltetralin structure. The signal at δ 2.79 (1H, *m*) and δ 2.85 (1H, *dd*, *J*=4.5, 14.0 Hz) were easily assigned to H-8 and H-8′ respectively, based on their chemical shift and multiplicity. ^1H-^1H-COSY spectra showed that a doublet at δ 4.78 was coupled to H-8 which allowed this signal to be unambiguously assigned to H-7. COSY spectra also assisted in the assignment of the other three alicyclic hydrogens: the diastereotopic protons at C-9 and H-7′. COSY connectivities revealed that the doublet of doublets at δ 4.10 was coupled to the multiplet at δ 4.61-4.64 and to H-8. Therefore the signal at δ 4.10 could be ascribed to one of the two H-9a or H-9b protons. The multiplicity and coupling-constant (*J*= 9.5 Hz) were consistent with the H-9b proton, which showed diaxial coupling with H-8. The integration of the signal intensity in the range of δ 4.61-4.64 showed the presence of two protons: the second diastereotopic proton and H-7′. Despite the complexity of the signal it was possible to assign the doublet at δ 4.61 to H-7′ based on the coupling with H-8′ (*J*=4.5 Hz). Finally, the residual aliphatic proton at δ 4.63 was conclusively assigned to H-9a. The doublet at δ 5.99 was typical for a methylenedioxy group commonly found in aryltetralin lignans. Another characteristic feature of podophyllotoxin and all of its derivatives is that the 2′,6′-protons and the 3′,5′-methoxy protons are observed as single resonance peaks. The equivalence of the 2′- and 6′- protons and the equivalence of the 3′- and 5′- methoxy protons result from a rapid 180˚ rotation of the E ring about the C-1′,7′ bond (11). On this ground the singlets at δ 6.38 and δ 3.79 were attributed to the 2′,6′ aromatic and 3′,5′ methoxy protons. The signal at δ 3.85 (3H, *s*) was ascribed to the 4′-methoxy protons. The two aromatic protons at H-3 and H-6 gave rise to single peaks at δ 6.52 and 7.12, respectively. In conclusion compound **1** possesses four aromatic protons which were consistent with the structure of podophyllotoxin,

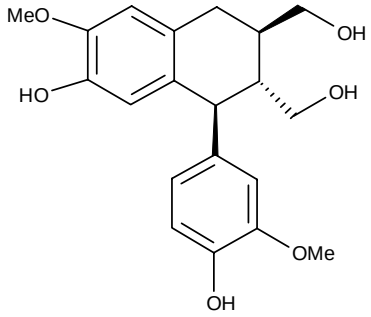
not β-peltatin. All ^{1}H NMR data are in accordance with the previous reports for podophyllotoxin (11, 12).

The structure of podophyllotoxin was confirmed by ^{13}C-NMR spectrum which showed characteristic signals for alicyclic, methoxy, aromatic carbons and a C=O moiety. The exact assignment of the carbon nuclei of **1** was conducted by heteronuclear correlation techniques. HMQC enabled association of all alicyclic carbons with their directly bonded protons. Consequently, signals at 41.1, 44.4 and 45.6 ppm belong to C-8, C-7′ and C-8′ respectively. The alicyclic signals of C-9 and C-7 were downfield at 71.5 and 73.2 ppm due to the oxygen atoms directly bonded to them. The negative amplitude at 71.5 ppm in the DEPT spectrum allowed the assignment of this signal as C-9 and the peak at 73.2 ppm was attributed to C-7. DEPT spectra also helped to identify the carbon atom from the methylenedioxy bridge at 101.7 ppm. Two signals (56.6 and 61.0 ppm) were observed in the area of the methoxy carbons and the distinction between them was made on the basis of the integration intensity: OCH_3 at C-3′,5′ (56.6 ppm) and OCH_3 at C-4′ (61.0 ppm). The identified CH connectivities between 106.5 ppm and δ 7.12 (H-6), 108.9 ppm and δ 6.38 (H-2′,6′), 110.1 ppm and δ 6.52 (H-3), allowed assignment of the protonated aromatic carbons as follows: C-6 (106.5 ppm), C-2′,6′ (108.9 ppm) and C-3 (110.1 ppm). The assignments of the non-protonated non-oxygenated carbons were obtained using HMBC data. HMBC correlations were identified between 131.5 ppm and H-6, 133.4 ppm and H-3, 135.6 ppm and 2′,6′-H, which permitted assignment of the above signals to C-1, C-2 and C-1′, respectively. Non-protonated oxygenated carbons C-4′, C-4, C-5 and C-3′,5′ were also assigned on the basis of their HMBC correlations and signal intensities. Finally, the signal at 174.5 ppm was readily attributed to the C=O of the lactone moiety. ^{13}C NMR of podophyllotoxin is shown in Table 1. The ^{13}C NMR data is in agreement with that previously reported (13).

Ion peaks at m/z 445 ($[M+H]^+$), 462 ($[M+NH_4^+]$), 467 ($[M+Na^+]$), 535 $[M+(Na_2HCOO)^+]$ and 427 $[M+H-H_2O]^+$ were observed in LC-MS spectrum of compound **2.** Therefore molecular weight of 444 was implied for compound **2**, which is consistent with 6-methoxypodophyllotoxin. This assertion was proved by inspection of its ^{1}H and ^{13}C NMR spectra which were similar to **1**. Comparison of the proton NMR spectrum of **2** with that of podophyllotoxin showed it to have an extra methoxy signal at δ 4.17 and one of the signals for the aromatic protons (H-6) was absent. The assignment of the additional OCH_3 at C-6 was supported by the fact that (i) the pendent aryl ring had the same trimethoxy substitution pattern as podophyllotoxin and (ii) the NOESY spectrum revealed a correlation between the additional methoxy group and H-7. Another specific feature of the ^{1}H NMR of compound **2** compared to podophyllotoxin is that the signals of H-8 and H-8′ as well as H-7′ and H-9a are well-resolved. The ^{1}H NMR data is in agreement with those previously reported in literature for 6-methoxypodophyllotoxin (12). The ^{1}H NMR data enabled the ^{13}C NMR spectrum to be assigned completely. The ^{13}C NMR spectral data of 6-methoxypodophyllotoxin is similar to that of podophyllotoxin and one extra carbon from a methoxyl group was evident. HMQC spectra clearly located the signal at 60.1 ppm to the carbon of the methoxy group bonded to C-6. A characteristic feature is that the signal of C-6 (106.5 ppm for podophyllotoxin) was downfield at 125.2 ppm for 6-methoxypodophyllotoxin due to the deshielding effect of the attached methoxy group. The OCH_3 at C-6 also influenced C-4 and C-5 signals which appeared as well-resolved peaks at 149.7 and 141.8 ppm respectively. The complete ^{13}C NMR of 6-methoxypodophyllotoxin is given in Table 1.

The LC/(+)ESI-MS spectrum of **3** produced ion peaks at m/z 401 ($[M+H]^+$), 418 ($[M+NH_4^+]$), 423 ($[M+Na^+]$) and 491 $[M+(Na_2HCOO)^+]$, indicating a molecular weight of 400. To our knowledge this might be one of the following lignans found in the *Linum* genus: α-peltatin, yatein or 4′-demethlypodophyllotoxin. ^{1}H NMR of compound **3** was very similar to those of podophyllotoxin with respect to the chemical-shift positions, multiplicity and coupling constants of protons that are common to both structures. The ^{1}H NMR spectrum of **3** showed the presence of only one methoxy signal (δ 3.78, 6H) which was relevant for the two methoxyl groups at 3′- and 5′-positions. All proton NMR data match with those reported in the literature (14). The ^{13}C NMR spectrum (Table 1) also confirmed the presence of one methoxyl signal which was related to a 3′,5′-methoxy-phenyl group in the pendent ring according to the HMBC spectrum. These findings led to identification of compound **3** as 4′-demethlylpodophyllotoxin. Demethylation was also consistent with data from LC-MS which showed loss of 14 amu compared to podophyllotoxin. This is the first report on the assignments of ^{13}C NMR signals of compounds **2** and **3**. In an analogous manner the structure of compound **4** was assigned and revealed a molecular weight of 430. The ion peaks identified by LC-MS were m/z 431 ($[M+H]^+$), 448 ($[M+NH_4^+]$), 453 ($[M+Na^+]$), 511 $[M+((NH_4)_2HCOO)^+]$, 521 $[M+(Na_2HCOO)^+]$ and 413 $[M+H-H2O]^+$. The ^{1}H NMR spectrum showed close similarity to 6-methoxypodophyllotoxin





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1 R=H, R_1=CH_3 ; **2** R=OCH_3, R_1=CH_3 ; **3** R=H, R_1=H ; **4** R=OCH_3, R_1=H

*Fig.1. Structures of the lignans isolated from Linum tauricum Willd. ssp. bulgaricum (Podp.) Petrova: podophyllotoxin (**1**), 6-methoxypodophyllotoxin (**2**), 4'-demethylpodophyllotoxin (**3**), 4'-demethyl-6-methoxypodophyllotoxin (**4**) and isolariciresinol (**5**).*

Table 1. ^{13}C spectra of podophyllotoxin (1) 6-methoxypodophyllotoxin (2) and 4'-demethylpodophyllotoxin (3)

C-	**1** δ (ppm)	**2** δ (ppm)	**3** δ (ppm)
1	133.4	134.9	131.7
2	131.5	133.1	131.2
3	110.1	104.6	110.1
4	148.0	149.7	147.9
5	148.1	141.8	148.1
6	106.5	125.2	106.5
7	73.2	72.1	73.1
8	41.1	39.2	41.0
9	71.5	70.7	71.5
1′	135.4	135.1	133.4
2′	108.9	108.3	108.5
3′	152.9	152.8	146.8
4′	137.8	137.3	134.5
5′	152.9	152.8	146.8
6′	108.9	108.3	108.9
7′	44.4	44.8	44.2
8′	45.6	45.3	45.7
9′	174.5	174.6	174.6
-OCH_2O-	101.7	101.6	101.7
-OCH_3 at 3′ + 5′	56.6	56.4	56.8
-OCH_3 at 4′	61.0	61.0	61.0
OCH_3 at 6	n.a.	60.1	n.a.

**n.a.: not applicable*
*** ^{13}C-NMR for compounds **4** and **5** are not available due to sample paucity*

except for the methoxy signal at C-4′ which was absent. Therefore the structure of 4′-demethyl-6-methoxy-podophyllotoxin could be assigned to compound **4** which corresponded to a molecular mass 430. The ^{1}H NMR is in complete accordance with the literature (15).

Despite the paucity of compound **5** (0.59 mg), it was possible to assign its structure. The open lactone ring (^{1}H NMR data) and the molecular weight of 360 (LC-MS data), indicated that **5** is isolariciresinol, which is a common precursor in the biosynthesis of lignans. In our assignment we used isolariciresinol dimethyl ether as a model (16). H-5′ (δ 6.85, d), H-6′ (δ 6.66, dd) and H-2′

(δ 6.60, d) aromatic protons were identified due to the *AMX* system they formed. The multiplets at δ 1.85 and δ 2.05 were attributed to H-8′ and H-8 respectively according to the literature data (16). The doublet of doublets at δ 2.75 and 2.81 were assigned to the diastereotopic protons H-7a,b. The multiplet signal at δ 3.53 was assigned to one of the CH_2 protons at C-9′. The multiplet δ 3.72-3.91 (10H) corresponded to the second proton from C-9′, H-9a,b, H-7′ and the protons from the two methoxy groups at C-3′ and C-5. The other two aromatic protons at δ 6.58 and 6.30 were ascribed to H-6 and H-3 respectively, based on the NOESY correlation of the δ 6.58 signal and δ 3.72-3.91 (H-7 and 5-OCH_3). Moreover, it has been reported that a conversion of lariciresinol into isolariciresinol can occur during HPLC fractionation in studies on the *in vitro* metabolism of secoisolariciresinol with liver microsomes (17). Therefore, it is not clear at this time whether isolariciresinol in fraction I is a natural product extracted from the plant material or an artifact due to the instability of lariciresinol. Further studies are needed in order to answer this question.

6-Methoxypodophyllotoxin is reported as a common component of many *Linum* species (18) while podophyllotoxin, 4′-demethyl-6-methoxypodophyllo toxin and 4′-demethyl-podophyllotoxin are not previously isolated from *L. tauricum* (19). This finding reconfirms the supposal that aryltetralin lignans are characteristic for the section *Syllinum* of the genus *Linum* (12).

It has been reported that *Podophyllum* lignans with a 3,4,5-trimethoxy substitution in the pendant aryl ring and those with a 4-hydroxy-3,5-dimethoxy-substituted pendent ring undergo analogous reactions of oxygenation at C-6 and C-7 (20). Our finding of 6-methoxypodophyllotoxin and podophyllotoxin and their 4′-demethyl analogues 4′-demethyl-6-methoxy-podophyllotoxin and 4′-demethyl-podophyllotoxin in *L. tauricum* ssp. *bulgaricum* suggests that lignan biosynthesis in the latter may occur via a similar mechanism.

CONCLUSION

The combination of semi-preparative HPLC, LC-MS and one- and two- dimensional NMR techniques enable structure elucidation of aryltetralin lignans in a convenient and unambiguous manner from small amounts of plant material. In addition, phytochemical results can cast light on the systematic classification of *L. tauricum* species.

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