THE LIGNANS OF *POLYGALA POLYGAMA* (POLYGALACEAE): DEOXYPODOPHYLLOTOXIN AND THREE NEW LIGNAN LACTONES

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ABSTRACT.—In a continuing phytochemical investigation of *Polygala polygama* Walt. (Polygalaceae), four major constituents were obtained when a complex mixture of lignan lactones was separated by high performance liquid chromatography. The structural characterization of these materials as the known lignan deoxypodophyllotoxin (**3**) and the new compounds polygamain (**4**), polygamatin (**5**) and β-apopolygamatin (**6**) is described. Structural assignments were based upon analysis of spectral data and comparison with values reported in the literature.

In a previous report (1) fractionation of a tumor-inhibiting extract of *Polygala polygama* Walt. (Polygalaceae) leading to the isolation of the known lignans podophyllotoxin (**1**) and 4'-demethylpodophyllotoxin (**2**) was described. In a continuing phytochemical investigation of the plant, a separate and complex mixture of lignans, derived from the same parent fraction as **1** and **2**, when separated by means of high performance liquid chromatography (hplc), yielded four major and two minor constituents. This report details the separation of the lignan mixture and describes the identification of the major components as the known compound deoxypodophyllotoxin (**3**) and the new lignans polygamain, polygamatin, and β-apopolygamatin, assigned structures **4**, **5** and **6**, respectively, on the basis of spectral evidence.

The concentrated ethanolic extract of *P. polygala* was partitioned between chloroform and water, with the chloroform-soluble fraction being further partitioned between hexane and aqueous methanol. The concentrated methanolic fraction was separated into two lignan-containing fractions by conventional gradient elution column chromatography on silica with benzene-chloroform and chloroform as the eluents. The identification of compounds **1** and **2** from the chloroform eluates has previously been described (1). Concentration of the benzene-chloroform fraction yielded a dark green tar from which green pigments were removed by filtration through Florisil. The resulting yellow film, exhibiting one large spot on tlc in several solvent systems, resisted crystallization and, when subjected to ir and pmr analysis, gave characteristics of a complex mixture of aryl-tetrahydronaphthalide lignans. Following several unsuccessful attempts to resolve the mixture by means of conventional absorption chromatography, the material was subjected to hplc analysis.

Analytical-scale hplc on a μ-Porasil prepacked column (Waters Assoc.) with chloroform-hexane mixtures as the mobile phase resulted in the resolution of the lignan mixture into four major and two minor components. In an attempt to prepare sufficient material for structural analysis, semi-preparative hplc was performed on a Porasil Type A prepacked column (Waters Assoc.) eluted with hexane-chloroform (55:45). Separation of components was monitored with a variable wavelength UV detector (Schoeffel) operating at 288 nm, allowing the collection of six primary fractions labelled A–F according to their order of elution. After several chromatographic runs, identical fractions were combined, concentrated, and analyzed by analytical hplc, to confirm that each major fraction contained a

single component. Concentration of fractions A, B, D and F resulted in the recovery of four white solids, subsequently identified as compounds A, B, D and F. Fractions C and E contained only trace amounts of material and were not examined further.

Ir, pmr and uv analysis of the isolates suggested tentative assignment to the podophyllotoxin lignan series. Compound F was readily identified as deoxypodophyllotoxin (**3**) by comparison of mp, uv, mass spectral and optical rotation values with those reported in the literature (2–5), and by comparison of pmr and ir spectra with those reproduced in the literature (3).

Compound A, a new compound for which the name polygamain is suggested, was assigned the molecular formula $C_{20}H_{16}O_6$ on the basis of high resolution mass spectrometry. The uv [λmax 288 (log ε 3.94)] and ir spectra (1770^{-1}, γ-lactone), as well as the general appearance of the pmr spectrum all were in close agreement with the assignment of the isolate to the podophyllotoxin series. Noteworthy in the proton spectrum, however, was the absence of peaks corresponding to OCH_3 groups and the appearance of a pair of singlets (δ 5.93 and 5.95), each integrating for two protons, in the region characteristic of aromatic methylenedioxy groups (OCH_2O). On the basis of the stated evidence, polygamain was tentatively formulated as **4**, a 1-aryl-1, 2, 3, 4-tetrahydronaphthalene lignan possessing methylenedioxy substituents on rings A and C. Several examples of similarly substituted aryl-naphthalide lignans have been reported in the literature, including helioxanthin (**7**), taiwanin-C (**8**) and otobain (**9**) (references 6–8, respectively). Of special interest is the occurrence of **7** in *Polygala chinensis*, suggesting that polygamain may have the alternate arrangement of the ring-A methylenedioxy at the 7,8 position. Fine analysis of the proton spectrum, however, appears to eliminate this possibility. In previous studies of the pmr spectra for similar compounds (8, 9), it has been suggested that methylenedioxy group resonances will appear as singlets if located in the 6, 7 or 3', 4' positions;[1] whereas a pair of doublets will be observed with substitution in the 7, 8 or 2', 3' positions. For example, in the spectrum of otobain (8), the ring-A OCH_2O protons appear as a pair of doublets (J=1.5 Hz) centered at δ 5.67 and 5.58. In the spectrum of polygamain, the resonance signals for both methylenedioxy groups appear as singlets, thus ruling out 7, 8 and 2', 3' substitution. The final possibility for the location of the OCH_2O grouping in ring-A of the unknown would be at the 5, 6 position, although this appears unlikely on biogenetic grounds. Such substitution, however, would be expected to result in ortho-coupling of H–7 and H–8, while in the spectrum of polygamain the peak assigned to H–8 (δ 6.52) by analogy to other members of the podophyllotoxin series occurs as a singlet.

From the above evidence and the optical rotation value ($[\alpha]^{24}D-127°$), which is consistent with the 1, 2-*cis*, 2, 3-*trans* configuration as in compounds **1**, **2** and **3**, it would appear likely that the isolate has structure **4**. The alternate orientation of the γ-lactone ring, however, as observed with conidendrin (**10**) (ref. 10), cannot be definitively ruled out on the basis of present evidence.

Analysis of the mass spectral fragmentation for polygamain lends further support to its formulation as **4**. All data is consistent with fragmentation schemes which have been described for similar aryl-tetrahydronaphthalene lignans (4, 11). As for other members of the podophyllotoxin series, the base beak for **4** is the molecular ion (M^+ 352). The second most abundant peak is located at m/e 135 (39.1%)

[1]For certain lignans the signal for these protons in high resolution spectra appears as an AB quartet with small coupling constant ($J \ll 2$ Hz).

consistent with benzylic cleavage to yield a methylenedioxybenzyl ion. Other ions of significance may be viewed as being the result of a retro-Diels-Alder cleavage accompanied by loss of hydrogen (M–85, *m/e* 267, 16.3%), the loss of methylenedioxyphenyl and hydrogen from the parent ion (M–122, *m/e* 230, 12.1%) followed by loss of CO_2 and hydrogen from the γ-lactone ring [(M–122)–45, *m/e* 185, 18.4%] and the direct loss of the latter from the parent ion (M–45, *m/e* 307, 14.3%). (For structural representations of similar fragments see ref. 4).

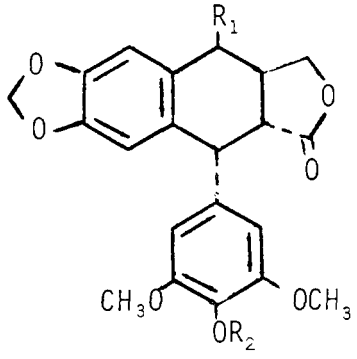

FIG. 1. **1** R_1=OH; R_2=CH_3
2 R_1=OH; R_2=H
3 R_1=H; R_2=CH_3

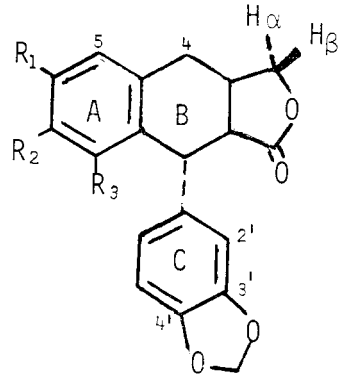

FIG. 2. **4** R_1, R_2=OCH_2O; R_3=H
5 R_1=H; R_2=R_3=OCH_3

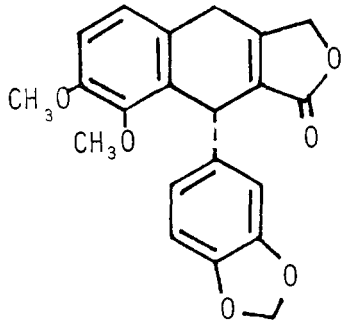

FIG. 3. **6**

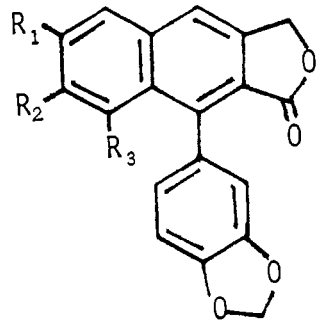

FIG. 4. **7** $R_1=H$; R_2, $R_3=OCH_2O$
8 R_1, $R_2=OCH_2O$; $R_3=H$

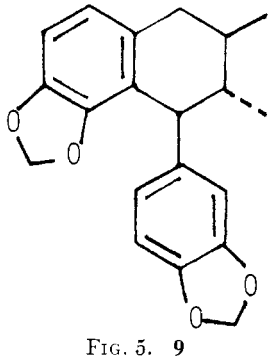

FIG. 5. **9**

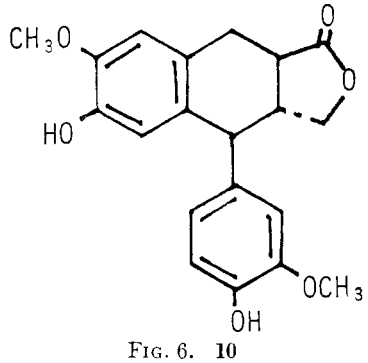

FIG. 6. **10**

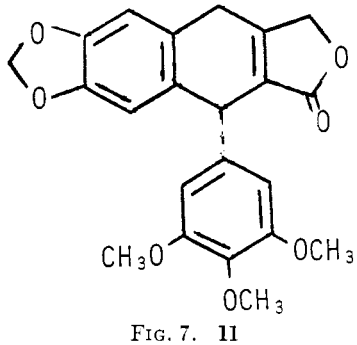

FIG. 7. **11**

Compound B, for which the name polygamatin is suggested, crystallized from methanol as colorless prisms, mp 184–186°, $[\alpha]^{24}D-115°$ (C=0.1 $CHCl_3$), and was assigned the formula $C_{21}H_{30}O_6$ on the basis of the high resolution mass spectrum (M^+ 368.126; $C_{21}H_{20}O_6$ requires 368.126). In the mass spectrum of polygamatin the base peak again coincided with the molecular ion. The only additional ion of significant abundance was observed at *m/e* 135 (43.3%). The ir spectrum exhibited carbonyl absorption at 1780 (γ-lactone) and 945 cm^{-1} (methylenedioxy), while the uv spectrum showed λ max (EtOH) 286 (log ε 3.84). The pmr spectrum confirmed the presence of a methylenedioxy group (δ5.92, s, 2H) and indicated the presence of two aromatic methoxy groups (δ3.85 and 3.40, singlets, 3H each) as well as five aromatic protons (δ6.61 and 6.95, multiplets, 3H and 2H respectively). These data suggested that polygamatin could be assigned structure **5**, a new aryltetrahydronaphthalide lignan lactone. Based upon this structure, the mass spectral fragment at *m/e* 135 could now be envisioned as being the result of benzylic cleavage of the parent ion to yield a methylenedioxybenzyl ion representing ring-C, with the substitution at C_3', $_4'$ consistent with the appearance of the OCH_2O protons in the pmr spectrum as a singlet. Additional analysis of the proton spectrum confirms the ring-A substitution pattern of **5**, with the assignment of methoxy groups to C_7 and C_8 on the basis of the singlets observed at δ3.85 and 3.40, the latter representing the C_8-OCH_3 shielded by the influence of ring-C (9, 12). With the assignment of aromatic proton resonances at δ6.61 and 6.95to C_2',$_5'$,$_6'$-H and $C_{5,6}$-H respectively, consistent with location of signals for these protons in similarly substituted lignans (8), the identity of polygamatin was confirmed.

Compound D, crystallized from methanol as colorless needles, mp 170–172°, $[\alpha]^{24}D-75°$ (c=0.2 $CHCl_3$), was assigned the formula $C_{21}H_{18}O_6$ from ms data (M^+ 366.111; $C_{21}H_{18}O_6$ requires 366.110). The mass spectrum of D again showed the base peak to be the molecular ion; however, little additional fragmentation was observed with the next abundant peak again located at *m/e* 135 (10.7%). The ir spectrum showed carbonyl absorption at 1755 (α,β-unsaturated γ-lactone) and 930 cm^{-1} (methylenedioxy), while the uv spectrum exhibited λ max (EtOH) 293 (log ε3.98). As observed with polygamatin the pmr spectrum of compound D again showed methylenedioxy (δ5.98, s, 2H), two aromatic methoxy groups (δ3.84 and 3.25, singlets, 3H each) and five aromatic protons (δ6.74 and 6.95, multiplets, 3H and 2H respectively). The data presented are in accordance with the formulation of the isolate as a lignan lactone similar to **5**, but possessing unsaturation in ring-B as suggested by the molecular formula and ir data. A review of spectral data for similarly unsaturated compounds in the apopodophyllotoxin series (2) suggests that compound D resembles β-apopodophyllotoxin (**11**). Only the location of ring-B unsaturation in the 2,3-position is consistent with the uv data and the lack of signals in the pmr spectrum corresponding to vinyl protons. The unknown lignan is thus assigned structure **6** and, consistent with the nomenclature in the podophyllotoxin series, the name β-apopolygamatin is suggested.

Compounds **1–6** have been isolated from the methanolic partition fraction of *P. polygama* which exhibited marginal activity in the P 388 lymphocytic leukemia *in vivo* assay (% T/C 144, 40 mg/Kg)[2]. While the tumor inhibiting and cytotoxic potential of **1**, **2** and **3** have been documented (3, 5, 14, 15), the new compounds

[2]Extracts and fractions were tested under the auspices of the Drug Research and Development Branch of the National Cancer Institute (13). Due to the marginal nature of the activity, fractions subsequent to the aq. methanolic fraction were not accepted for bioassay.

4, **5** and **6**, whose structures suggest a high likelihood of activity, have not been tested; and insufficient ioslate is available for bioassay. Studies concerning their synthetic preparation are currently in progress.

EXPERIMENTAL[3]

EXTRACTION AND FRACTIONATION.—The extraction and preliminary fractionation of all plant parts of *Polygala polygama* Walt. (Wayne State Univ., Dept. of Biology herbarium, voucher accession No. 16235) have been previously described (1). An ethanolic extract was partitioned against chloroform and water, and the chloroform-soluble material was subsequently partitioned between hexane and aqueous methanol. A portion of the methanolic fraction (1.4 g) was chromatographed on a column of silica gel 60[4] (70 g), prepared in benzene and eluted with benzene, benzene-chloroform, chloroform and chloroform-methanol. The fraction which eluted with benzene-chloroform (1:1) was concentrated to dryness and yielded 329 mg of green tar. Filtration of the tar in chloroform through a column of Florisil (60–100 mesh)[5] (10 g) and collection of filtrate until green pigments eluted afforded 130 mg of yellow film following concentration *in vacuo*.

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY.[6]—The yellow film resulting from Florisil filtration was characterized as a mixture of aryl-tetrahydronaphthalide lignan lactones on the basis of uv (λmax 288) and ir (multiplet of peaks centered at 1770 cm^{-1}) spectra as well as the general appearance of the pmr spectrum. Analytical-scale hplc analysis of the mixture, performed by injecting 2 μl of the mixture in chloroform (approximately 10 mg/ml) and eluting with a solvent mixture composed of hexane-chloroform (55:45), resulted in the resolution of six distinct peaks labelled A–F in order of elution. Intense peaks were observed for component A (retention time 7.6 minutes), B (9.5), D (13.25) and F (22). Significantly less intense peaks were observed for components C (11 minutes) and E (18 minutes).

Semi-preparative separations of the lignan mixture were performed in ten separate runs injecting each time approximately 10 mg of sample dissolved in 200 μl of chloroform. Separation was monitored by observation of the uv absorption as recorded throughout the procedure. Six major and five intermediate fractions, corresponding to the separation of components as described above, were collected and labelled Fraction A, A–B, B, etc. The average time required for separation and collection of all fractions was approximately 90 minutes.

Following the combination of similar eluates from all chromatographic trials the eleven resultant fractions were concentrated *in vacuo* and analyzed by analytical hplc. As expected, intermediate fractions (A–B, B–C, etc.) consisted of mixtures of materials. Fractions A through F, however, were shown to be homogeneous. Concentration of these fractions to dryness *in vacuo* afforded the white amorphous solids compounds A (from Fraction A, 14 mg), B (21 mg), D (15 mg) and F (26 mg). The residues from Fractions C (2 mg) and E (1 mg) provided insufficient material for comprehensive analysis and were not investigated further.

DEOXYPODOPHYLLOTOXIN (**3**).—Compound F crystallized from methanol as colorless needles, mp 167–168°, $[\alpha]^{24}D-115.6°$ (C=0.16 $CHCl_3$). The material exhibited the following spectra properties: ms, *m/e*, M^+ 398 (base peak), 283, 230, 185, 181, 173; ir: ν max (KBr), cm^{-1}, 1780 (C=O), 1490, 1230, 1125, 1040, 1000, 930; uv, λ max (log ϵ) (EtOH), 291nm (3.79); pmr ($CDCl_3$), δ2.58–2.98 (m, 4H, H-2, 3, 4), 3.7–4.0 (m, 1H, Hα), 3.80 (s, 6H, $C_{3',5'}$-OCH_3), 3.85 (s, 3H, $C_{4'}$-OCH_3), 4.3–4.7 (m, 2H, H-1, Hα), 5.98 (s, 2H, OCH_2O), 6.40 (s, 2H, H-2', 6'), 6.57 (s, 1H, H-8), 6.73 (s, 1H, H-5).

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[3]Melting points were taken on an Electrothermal capillary melting-point apparatus and are uncorrected. Uv absorption spectra were determined in 95% ethanol on a Beckman model DK2A recording spectrophotometer. Ir absorption spectra were recorded on a Beckman model 33 recording spectrophotometer. Pmr spectra were determined in deuteriochloroform, with tetramethylsilane as the reference standard, on a Varian EM 360 instrument. Ms data were recorded by the Wayne State University Department of Chemistry on an AEI-MS9 mass spectrometer.

[4]E. Merck, Darmstadt, Germany.

[5]Fisher Scientific Company.

[6]Hplc analysis was performed on a Waters Assoc. model ALC 201 Liquid Chromatograph equipped with a Schoeffel model SF 770 variable wavelength detector operating at 288 nm. Analytical separations were achieved with a Waters 4 mm x 30 cm μ Porasil column. Preparative separations were achieved on a Waters 4 mm x 61 cm Porasil Type A column. Filtered and degassed Burdick and Jackson solvents were employed throughout as the mobile phase. A flow rate of 1 ml/min was maintained during analytical-scale runs, increased to 2.5 ml/min for semi-preparative separations. Detector sensitivity(uv) was set at 0.1 AUFS during analytical analysis and 1.0 AUFS for preparative work. All samples in solution were filtered prior to injection.

POLYGAMAIN (**4**).—Compound A, which resisted repeated attempts at crystallization, yielded a white amorphous solid from methanol, mp 148–150°, $[\alpha]^{24}D-127°$ (C=0.3, $CHCl_3$). The material exhibited the following spectral properties: ms, *m/e* (% of base), M^+ 352 (100) (high resolution 352.096; $C_{20}H_{16}O_6$ requires 352.095), 307 (14.3), 267 (16.3), 230 (12.1), 185 (18.4), 135 (39.1); ir, υ max (KBr), cm^{-1}, 1780 (C=O, γ-lactone), 1490, 1445, 1225, 1030, 980, 925; uv, λ max (log ϵ), (EtOH), 288nm (3.49); pmr ($CDCl_3$), δ2.53–3.0 (m, 4H, H–2, 3, 4), 3.76–3.98 (m, 1H, Hβ), 4.35–4.67 (m, 2H, H–1, Hα), 5.93 (s, 2H, OCH_2O), 5.95 (s, 2H, OCH_2O), 6.52 (s, 1H, H–8), 6.70 (m, 4H, H–5, 2', 5', 6').

POLYGAMATIN (**5**).—Compound B crystallized from methanol as colorless prisms, mp 184–186°, $[\alpha]^{24}D-115°$ (C=0.1, $CHCl_3$). The material exhibited the following spectral properties: ms, *m/e* (% of base), M^+ 368 (100) (high resolution 368.126; $C_{21}H_{20}O_6$ requires 368.126), 135 (43.3%); ir, υ max (KBr), cm^{-1}, 1780 (C=O, γ-lactone), 1495, 1450, 1290, 1240, 1045, 1010, 945; uv, λ max (log ϵ) (EtOH), 286nm (3.84); pmr ($CDCl_3$), δ2.5–3.1 (m, 4H, H–2, 3, 4), 3.40 (s, 3H, C_8–OCH_3), 3.7–3.95 (m, 1H, Hβ), 3.85 (s, 3H, C_7–OCH_3), 4.3–4.55 (m, 1H, Hα), 4.8–4.98 (m, 1H, H–1), 5.92 (s, 2H, OCH_2O), 6.55–6.66 (m, 3H, H–2', 5', 6'), 6.95 (bs, 2H, H–5, 6).

β-APOPOLYGAMATIN (**6**).—Compound D crystallized from methanol as colorless needles, mp 170–172°, $[\alpha]^{23}D-75°$ (C=0.2, $CHCl_3$). The material exhibited the following spectral properties: ms, *m/e* (% of base), M^+ 366 (100) (high resolution 366.111; $C_{21}H_{18}O_6$ requires 366.110), 135 (10.7); ir, υ max (KBr), cm^{-1}, 1755 (C=O, α β-unsaturated γ-lactone), 1495, 1445, 1245, 1120, 1045, 1010, 930; uv, λ max (log ϵ) (EtOH), 293nm (3.98); pmr ($CDCl_3$), δ2.90 (bs, 2H, H–4), 3.25 (s, 3H, C_8–OCH_3), 3.84 (s, 3H, C_7–OCH_3), 4.0 (bs, 1H, Hβ), 4.53 (bs, 1H, Hα), 4.71 (bs, 1H, H–1), 5.98 (s, 2H, OCH_2O), 6.65–6.84 (m, 3H, H–2', 5', 6'), 6.95 (bs, 2H, H–5, 6).

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