Journal of Natural Products
Vol. 58, No. 1, pp. 128-130, January 1995

ISOLATION OF ANTIFUNGAL AND LARVICIDAL CONSTITUENTS OF *DIPLOLOPHIUM BUCHANANI* BY CENTRIFUGAL PARTITION CHROMATOGRAPHY

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ABSTRACT.—Three phenylpropanoids, myristicin [**1**], elemicin [**2**], and *trans*-isoelemicin [**3**], together with two furanocoumarins, oxypeucedanin [**4**] and oxypeucedanin hydrate [**5**], have been isolated from the leaves of *Diplolophium buchanani* by a separation strategy involving the almost exclusive use of centrifugal partition chromatography. All five compounds were antifungal in a tlc bioautography test using *Cladosporium cucumerinum*. Compounds **1–4** exhibited larvicidal activity against *Aedes aegypti*.

Diplolophium (Umbelliferae) is a small genus of five plant species found in tropical Africa. The representative dealt with in this study, *D. buchanani* (Benth. ex Oliv.) Norman, is endemic to the Zomba and Mlanje Plateaus of Malawi, in central Africa. It is a virgate shrub with large umbels of greenish-white flowers, typical of the family. No previous studies on the phytochemistry and pharmacology of *D. buchanani* have been reported.

The application of centrifugal partition chromatography (cpc) to the isolation of the active constituents of the plant is reported here. The technique of cpc (1) is now being more frequently applied to the separation of natural products because of the advantages it possesses over other chromatographic techniques (2). These include the avoidance of a solid support (and the consequent risks of irreversible adsorption and decomposition) and application to both polar and apolar samples. Not only does cpc allow the separation of purified samples but the method also enables the direct fractionation of crude extracts, as exemplified herein.

Leaves of *D. buchanani* were extracted successively with CH_2Cl_2 and MeOH. During routine screening for biological activities, the CH_2Cl_2 extract was found to possess antifungal activity against the plant pathogenic fungus *Cladosporium cucumerinum* in a tlc bioautography assay (3). The same extract was toxic to larvae of the mosquito *Aedes aegypti*, the vector of yellow fever (4). The MeOH extract, on the other hand, was neither antifungal nor larvicidal.

Cpc of the CH_2Cl_2 extract, employing bioassay-guided fractionation with the *C. cucumerinum* test, led to isolation of the pure furanocoumarin oxypeucedanin [**4**] after crystallization from EtOAc/hexane. A second cpc step using a non-aqueous solvent system gave myristicin [**1**], while another antifungal fraction gave an inseparable mixture of elemicin [**2**] and *trans*-isoelemicin [**3**] after cpc with a different non-aqueous solvent system. Elemicin [**2**] and *trans*-isoelemicin [**3**] were identified by comparison with literature uv, nmr, and ms data (5,6). Operation of the cpc instrument in the reversed-phase mode on the initial CH_2Cl_2 extract gave a fraction containing a second furanocoumarin, oxypeucedanin hydrate [**5**]. Final purification was by a combination of liquid-liquid chromatography and gel filtration. Comparison of physical data of myristicin [**1**] and the furanocoumarins **4** and **5** with literature

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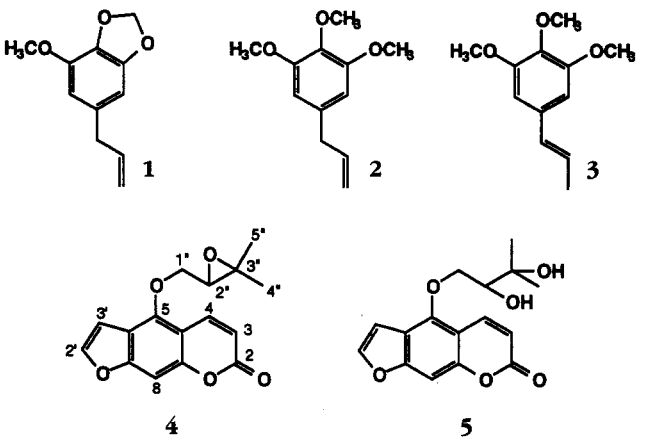

values (7) enabled their identification. However, in the case of oxypeucedanin [**4**], the position of the modified prenyloxy side-chain at C-5 (and not C-8) was confirmed by nOe nmr experiments. Irradiation of the H-4 proton gave an nOe at H-2″, thus confirming the substitution at C-5 and proving that **4** was not the alternative isomer, heraclenin.

The only known report on the phytochemistry of the genus *Diplolophium* concerns the Rwandese medicinal plant *D. africanum*. The coumarin scoparone (2.4% of dry plant material) was isolated from the roots and found to have an inhibitory effect on the growth of wheat rootlets (8). None of the above-mentioned phenylpropanoids or furanocoumarins was identified from *D. africanum*.

Referring to the biological activities, the efficacy of the strongest antifungal compound, oxypeucedanin [**4**], was comparable to the commercially available fungicide miconazole (Table 1). The amount of **4** necessary for inhibition of spore growth in bioautography with *C. cucumerinum* was 1 μg. The other isolated compounds were also active but to a lesser degree. Concerning the larvicidal activity, isolates **1**–**4** were toxic to *Aedes aegypti* but at different concentrations. Myristicin [**1**] and oxypeucedanin [**4**] were larvicidal at concentrations similar to that of the reference compound β-asarone but oxypeucedanin hydrate [**5**] was inactive up to 400 ppm.

TABLE 1. Antifungal and Larvicidal Activities of Compounds Isolated from the Leaves of *Diplolophium buchanani*.

Compound	Antifungal activity[a]	Larvicidal activity[b]
1	20 μg	25 ppm
2+**3**	8 μg	100 ppm
4	1 μg	25 ppm
5	10 μg	inactive
Miconazole .	1 μg	
β-Asarone .		16 ppm

[a]Minimum quantity required to inhibit growth of spores on tlc plate.

[b]LD_{100} after 24 h.

Although furanocoumarins are well-known components of the Umbelliferae (9), little is known about their antifungal activities. Furthermore, this is the first time that their larvicidal activities have been reported.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—Cpc was performed on a CCC-1000 instrument (Pharma-Tech Research Corp., Baltimore, MD). The total volume of the three coils was 660 ml and the rotation speed was 1000 rpm. Detection was at 254 nm. Nmr spectra were measured in $CDCl_3$ at 200 MHz for ^1H and 50.3 MHz for ^{13}C. TMS or the solvent signal were used as internal standards. Eims spectra were recorded on a Finnigan MAT TSQ 700 spectrometer.

PLANT MATERIAL.—Aerial parts of *Diplolophium buchanani* were collected in November 1990 on the Zomba Plateau, Malawi. A voucher specimen has been deposited at the National Herbarium, Zomba, Malawi.

EXTRACTION AND ISOLATION.—Air-dried leaves (360 g) of *D. buchanani* were extracted with CH_2Cl_2 and then subsequently with MeOH, to give 14.4 g of CH_2Cl_2 extract and 41.3 g of MeOH extract. A portion (1.7 g) of the CH_2Cl_2 extract was fractionated by cpc using the solvent system *n*-hexane-EtOAc-MeOH-H_2O (10:5:5:1), with the upper phase as mobile phase. A total of 9 fractions (I–IX) was obtained, together with an additional 3 fractions (X–XII) by elution in the reversed-phase mode (lower phase as mobile phase). Four of the fractions (II, IV, VIII, XI) were active against *C. cucumerinum*. Compound **4** (25 mg) was obtained from the antifungal fraction VIII after crystallization from EtOAc/hexane. A second cpc step on fraction II with the solvent *n*-hexane-*t*-butyl methyl ether-MeCN (5:1:5, upper phase as mobile phase) led to the isolation of **1** (69 mg). A mixture of **2** and **3** (75 mg) was obtained from fraction IV after cpc with the solvent system *n*-heptane-MeCN-MeOH (6:3:1, upper phase as mobile phase). Furanocoumarin **5** (19 mg) was isolated from fraction XI by a combination of cpc ($CHCl_3$-MeOH-EtOAc-H_2O, 5:6:3:4, upper phase as mobile phase) and gel filtration on Sephadex LH-20 ($CHCl_3$-MeOH, 1:1).

BIOASSAYS.—Bioautography with spores of *Cladosporium cucumerinum* for the estimation of antifungal activity was performed by the method of Homans and Fuchs (3). Larvicidal activity, using *Aedes aegypti*, was assessed as previously described (4,10).

Compounds **1–3**.—The uv, ms, ^{1}H-nmr, and ^{13}C-nmr spectra of **1–3** were identical to those previously reported (5,6).

Compound **4**.—Colorless cubes from EtOAc/hexane: mp 139–140° (lit. 142–143° (7)). Uv, ms, ^{1}H-nmr, and ^{13}C-nmr spectra correspond to literature values (7).

Compound **5**.—Colorless crystals from EtOAc/hexane: mp 132–133° [lit. 134° (7)]. Uv, ms, ^{1}H-nmr, and ^{13}C-nmr spectra correspond to literature values (7).

ACKNOWLEDGMENTS

Financial support was provided by the Swiss National Science Foundation, the Directorate for Development Cooperation and Humanitarian Aid (Swiss Federal Department of Foreign Affairs), and the 450th Anniversary Foundation of the University of Lausanne.

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Received 26 May 1994