PLANT ANTICANCER AGENTS. XIII. CONSTITUENTS OF *AUSTROCEDRUS CHILENSIS*[1]

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ABSTRACT.—As a result of the continuing search for plants having cytotoxic or tumor-inhibiting constituents, the chloroform-soluble fraction of *Austrocedrus chilensis* was found to be strongly cytotoxic. Fractionation of this extract guided by bioassay yielded desoxypodophyllotoxin (**1**) as the major cytotoxic constituent, together with the new diterpene **2** and the known diterpene pisiferol (**3**).

In continuation of our search for tumor inhibitors of plant origin, an alcoholic extract of *Austrocedrus chilensis* (D. Don) Florin and Boutelje (Cupressaceae) was found to show significant cytotoxicity in the KB cell culture assay. Partitioning of this extract between water and chloroform yielded an active chloroform fraction, and partitioning of this fraction between 90% aqueous methanol and hexane yielded an active aqueous methanol fraction.

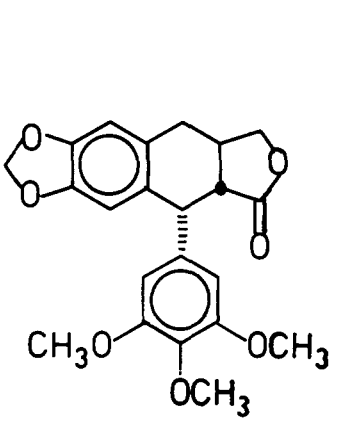
Fractionation of the aqueous methanol fraction was effected by a combination of normal-phase and reversed-phase liquid chromatography, monitored by bioassay of the resulting fractions. The major cytotoxic component, **1**, was isolated both by normal phase chromatography followed by crystallization, and also by preparative reversed-phase high performance liquid chromatography, as previously described (2). Identification of **1** as desoxypodophyllotoxin was accomplished by spectroscopic means and by comparison with an authentic sample.

A second component of the active fraction yielding desoxypodophyllotoxin was isolated by preparative reversed-phase liquid chromatography and obtained as colorless needles. The compound was identified as the unusual diterpene dienone **2** on the basis of the following evidence.

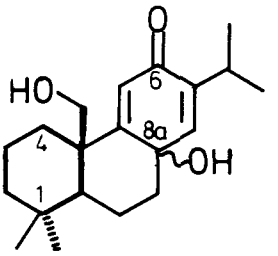
Mass spectrometry of the new compound indicated a molecular weight of 318, and both exact mass measurement of the parent ion and elemental analysis indicated a composition of $C_{20}H_{30}O_3$. Its ^1H nmr spectrum in $CDCl_3$ indicated the presence of two methyl groups on quaternary carbons and an isopropyl group, together with a group $-CH_2-X$ giving rise to an AB quartet at 4.2 ppm and two vinyl protons giving rise to one-proton singlets at 6.07 and 6.45 ppm. The compound's uv spectrum showed absorption at 242 nm, corresponding to an $\alpha\beta$-unsaturated ketone with two β-substituents (or one α and one β substituent) (3), and its ir spectrum showed the presence of a carbonyl group and at least one hydroxyl group. The carbonyl stretching frequency of 1670 cm^{-1} was accompanied by two vibrations at 1625 and 1610 cm^{-1}, suggestive of an unsymmetrical system.

These data, taken into consideration with the known diterpene ring systems, suggested a diterpene of the abietane class, most probably the dienone **2** or **3**. The mass spectrum of the new compound lends support to the structural assignment **2** (scheme). The prominent ion at m/z 271 is presumably formed as indicated by the loss of the fragments CH_2O and OH, followed by tautomerization, and it would have the same structure as that postulated for the corresponding ion derived from ferruginol (4). The ions at m/z 201, 189, 175, and 69 can then be seen as derived from this ion, as is the case with ferruginol. The important ion at m/z 202 can also be derived from the ion at m/z 271; the fact that this ion is more abundant than the corresponding ion in the ferruginol spectrum could be due to differing energy contents or differing structures for these ions. The formation of the ion at m/z 69 is particularly significant for the structural assignment since

[1]For Part XII, see ref. 1.



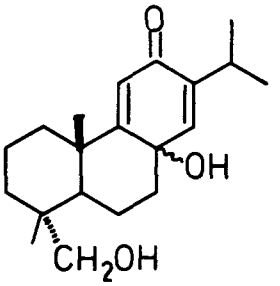
1



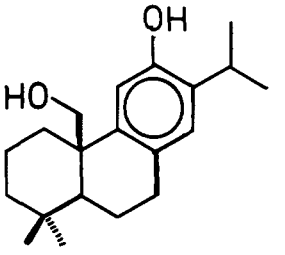
2

1. $NaBH_4$

2. HCl



3



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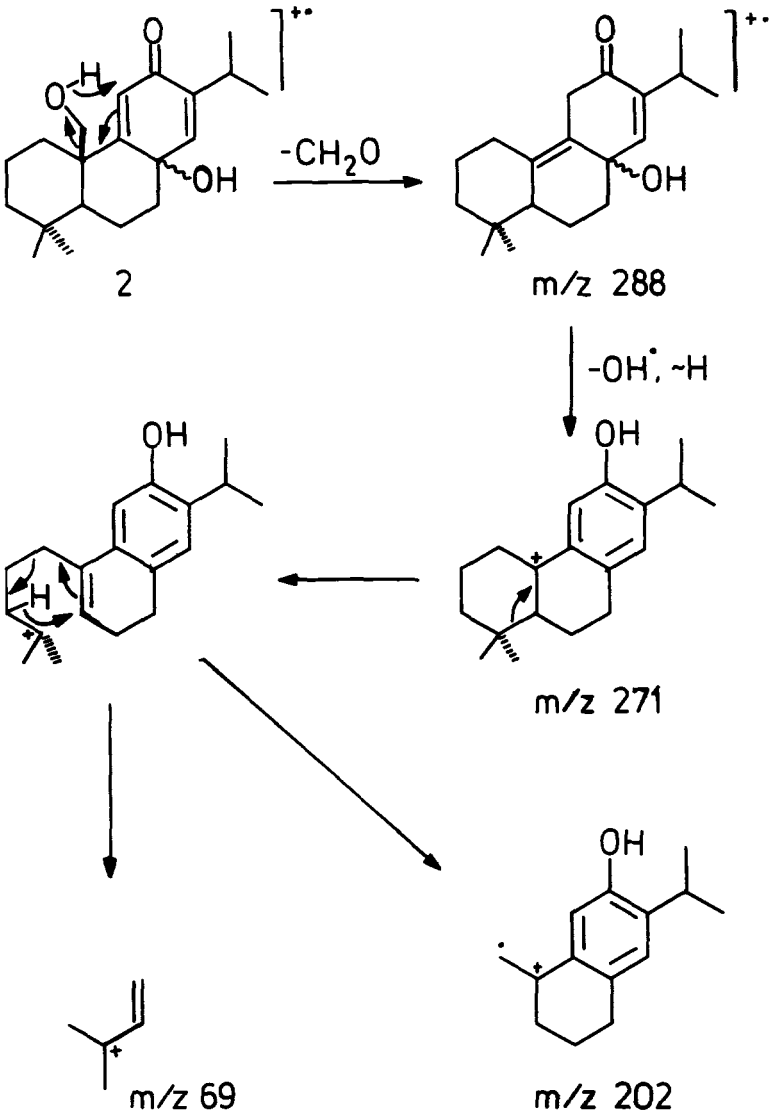
a compound with the alternate possible structure **3** would yield an ion at *m/z* 85 if it underwent fragmentation by comparable pathways.

Final proof of the structural assignment for compound **2** was obtained when pisiferol (**4**) became available. Reduction of **2** with sodium borohydride followed by acid work-up yielded a compound identical (hplc and ^{1}H nmr) to an authentic sample of pisiferol. This reaction thus confirms the structure and stereochemistry of **2**, except for the stereochemistry of the tertiary hydroxyl group at position 8a.

The ^{1}H nmr spectrum of **2** in deuterochloroform showed a single doublet for the methyl protons of the isopropyl group at position 7. However, when a spectrum was obtained in deuterobenzene, the signal for these methyl protons was separated into two overlapping doublets. This finding suggests that the diterpene **2** exists as a mixture of diastereomers epimeric at carbon 8a, in spite of the fact that it gave one symmetrical peak on hplc, and is a reasonably sharp-melting solid. If this interpretation of the evidence is correct, it would further suggest that **2** may not be a true natural product but may, instead, have been formed by aerial oxidation of pisiferol **4**. Attempts to carry out this conversion in the laboratory under a variety of conditions yielded traces of material with the same retention time on hplc as **2**, but conclusive evidence for the formation of **2** was not obtained.

A third product derived from the same fraction that yielded **2** was identified as the diterpene pisiferol (**4**) by comparison with an authentic sample obtained from the leaves of *Chamaecyparis pisifera* (5,6).



SCHEME. Mass Spectrometric Fragmentations of Diterpene **2**.

Although both diterpenes **2** and **4** were obtained from a fraction that showed modest cytotoxicity in the KB cell culture bioassay, neither pure compound showed significant activity in this system. This result is not entirely surprising since, although compound **2** is a Michael acceptor which might be expected to show activity in the KB system (7), it is structurally similar to taxodone which is also inactive in this system (8). The activity of the fraction containing the diterpenes was almost certainly due to traces of desoxypodophyllotoxin since this strongly cytotoxic compound was found in adjacent fractions. We conclude, therefore, that desoxypodophyllotoxin is the major cytotoxic constituent of *Austrocedrus chilensis*.

EXPERIMENTAL[2]

[2]Carbon and hydrogen analysis was performed by Galbraith Laboratories. Mass, ^{1}H nmr, uv, and ir spectra were recorded on a Kratos MS-9 mass spectrometer, Bruker WP-200 or JEOL FX-200 Spectrometers, and a Cary 14 spectrophotometer, respectively. The optical rotations were obtained on a Rudolph model 70 polarimeter, and melting points were determined on a Kofler hot stage apparatus and are uncorrected. Hplc was carried out on a home-assembled apparatus consisting of a Waters M6000A pump, a Valco injection valve, and a Pharmacia uv detector, with E. Merck RP-8 columns, 4.6 x 250 mm.

PLANT MATERIAL.—Dried stems, twigs, and leaves of *Austrocedrus chilensis* (B642943, PR-49530) were collected in Chile in 1977 and authenticated by the Economic Botany Laboratory, USDA, Beltsville, Maryland. A reference spectrum documenting the collection is deposited in the Herbarium of the National Aboretum, Agricultural Research Service, USDA, Washington, DC. The material was ground in a hammermill.

EXTRACTION PROCEDURE.—Ground stems, twigs, and leaves of *A. chilensis* (2 kg) were extracted exhaustively with ethanol, the extract concentrated, and the gummy residue partitioned between water and chloroform. The chloroform fraction was evaporated to yield a gummy residue which was partitioned between methanol-water (90:10) and hexane. Evaporation of the aqueous methanol fraction yielded a gummy residue (50 g), ED_{50} 5.5 x 10^{-2} μg/ml in KB cell culture.

ISOLATION AND CHARACTERIZATION OF DESOXYPODOPHYLLOTOXIN.—The residue from the methanol fraction (above, 50 g) was subjected to chromatography over silica gel 60 (E. Merck, 1 kg). Elution with chloroform with increasing amounts of methanol yielded two active fractions. Fraction A, 17.1 g, eluted with chloroform-methanol (95:5), had ED_{50} 4.9 x 10^{-2} μg/ml cell culture, and fraction B, 3.1 g, eluted with chloroform-methanol (80:20) had ED_{50} 5.4 x 10^{-2} μg/ml in KB cell culture. Further chromatography of fraction B on silica gel 60 (E. Merck, 230–400 mesh, 25 x 1000 mm) in the apparatus previously described (9) and elution with a gradient from ethyl acetate: hexane (50:50) to pure ethyl acetate and collection of 55 x 42 ml fractions yielded a strongly cytotoxic material in fractions 13–22. Crystallization from methanol yielded desoxypodophyllotoxin (17 mg, ED_{50} less than 1 x 10^{-4} μg/ml in KB cell culture) as colorless crystals, mp 164.5–166°, undepressed in admixture with authentic material (10) and showing chromatographic (hplc, tlc) and spectroscopic (ir, uv, ^{1}H nmr) properties identical to those of authentic material.

ISOLATION OF DITERPENE **2** AND PISIFEROL (**4**).—Fraction A from the separation described above was subjected to chromatography on silica gel 60 (E. Merck, 230–400 mesh, 25 x 1000 mm) (9) and elution with a gradient from hexane-dichloromethane (75:25) to hexane-dichloromethane-methanol (49:49:1). Collection of 47 fractions yielded a weakly cytotoxic fraction 43 (ED_{50} 0.16 μg/ml in KB cell culture) together with the strongly cytotoxic fractions 13–42. Analysis of these earlier fractions by hplc (2) indicated that they contained desoxypodophyllotoxin and other lignans, and their cytotoxicity could be accounted for on the basis of their desoxypodophyllotoxin content. Fraction 43 was subjected to chromatography on Lichroprep RP-8 (E. Merck Lobar column size B) with elution with a gradient from acetonitrile-water, 40:60 to 80:20, and collection of 20 ml fractions. Fractions 16 and 17 (179 mg) yielded crude diterpene **2**, and fractions 24–31 yielded crude pisiferol (870 mg).

DITERPENE **2**.—Crystallization of fractions 16 and 17 from aqueous methanol yielded compound **2**: mp 149–153°C; $[\alpha]^{25}D$+19° (c 2.6, $CHCl_3$); ir, (KBr) 3530, 3300, 1670, 1625, 1610 cm^{-1}; uv (EtOH) λmax (ε) 242 nm (12,600); ^{1}H nmr (C_6D_6) δ 0.68 (3H, s), 0.72 (3H, s), 1.063, 1.077 (3H each, d, J=7Hz), 3.82 (1H, d, J=11 Hz) 4.40 (1H, d, J=11 Hz), 4.46 (1H, bd s), 6.25 (1H, s), 6.47 (1H, s); mass spectrum (electron impact), m/z 318.2198 (M^+; calcd for $C_{20}H_{30}O_3$, 318.2194), 300, 288, 287, 271, 270, 255, 227, 202 (base), 201, 200, 199, 189, 188, 187, 185, 179, 178, 175, 171, 165, 164, 157, 149, 147, 145, 135, 69. ED_{50} 16 μg/ml in KB cell culture.

Anal. Calcd for $C_{20}H_{30}O_3$: C, 75.43; H, 9.50. Found: C, 75.45; H, 9.79.

PISIFEROL (**4**).—Crystallization of fractions 24–31 from aqueous methanol yielded pisiferol (**4**), mp 139–140°. Crystallization from benzene-ether yielded material with mp 109–11°, undepressed in admixture with authentic material, mp 108–110° (5,6). Pisiferol showed the following properties: $[\alpha]^{25}D$+29° (*c* 2.7, $CHCl_3$); uv (EtOH) λmax (ε) 232 (3,900), 284 (3,000); ir and ^{1}H nmr; identical to the published spectra (5,6); mass spectrum (electron impact), m/z 302.2248 (M^+; calcd for $C_{20}H_{30}O_2$ 302.2246), 271 (base), 229, 215, 201, 189, 175, 69; ED_{50}, greater than 100 μg/ml in KB cell culture.

CONVERSION OF DITERPENE **2** TO PISIFEROL (**4**).—Diterpene **2** (3.2 mg) was dissolved in ethanol (0.5 ml) and treated with sodium borohydride (6 mg) with stirring overnight at room temperature. The reaction mixture was then treated with dilute hydrochloric acid and worked up in the usual way to yield a product which showed retention time on hplc (acetonitrile-water, 50:50) identical to that of an authentic sample of pisiferol. A ^{1}H nmr spectrum was obtained on a sample (0.6 mg) purified by preparative hplc, and the major resonances corresponding to pisiferol could clearly be distinguished.

ACKNOWLEDGMENTS

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