POTENTIAL ANTICANCER AGENTS. XIV. ISOLATION OF SPRUCEANOL AND MONTANIN FROM *CUNURIA SPRUCEANA* (EUPHORBIACEAE)[1]

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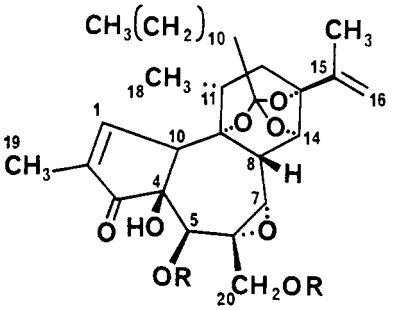
ABSTRACT.—Montanin (**1**) and spruceanol (**2**), two quite different diterpenes, were found to be responsible for the cytotoxic and antitumor activity of the root and root bark of *Cunuria spruceana* (Euphorbiaceae). The structure of spruceanol (**2**) was deduced from spectral interpretation and chemical correlation with 12-methoxycleistanth-8,11,13-trien-3-one (**4**).

An observation that alcohol extracts of *Cunuria spruceana* Baill. (Euphorbiaceae) displayed cytotoxic and antitumor activity[2] prompted us to investigate this plant for its active constituents. There have apparently been no previous biological or phytochemical investigations of this genus. Fractionation, monitored throughout by concomitant bioassay, established that two quite different diterpene derivatives were responsible for the anticancer activity of the below-ground parts of this plant.

One of the diterpenes was the ortho ester derivative montanin (**1**), previously obtained by us (3) from the roots of *Baliospermum montanum* (Wild.) Muell.-Arg. (Euphorbiaceae). A second diterpene, spruceanol (**2**), was found to be a new compound in the rare cleistanthol series. Cleistanthol (**3**), the only other member of this series, was previously isolated from the heartwood of *Cleistanthus schlechteri* var. *schlecteri* (Euphorbiaceae) (4).



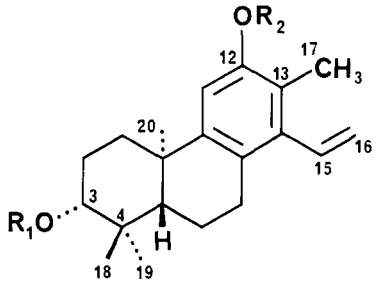
1 montanin, R = H

5 R = $COCH_3$

The structure of spruceanol (**2**) was determined by interpretation of its spectroscopic parameters and by its chemical correlation with 12-methoxycleistanth-8,11,13-trien-3-one (**4**).

[1]For the previous paper in this series see reference 1.

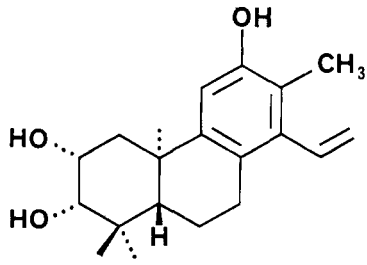
[2]Extracts, fractions and pure compounds were tested under the auspices of the Developmental Therapeutics Program of the National Cancer Institute (2). An isolate is considered active if it shows an $ED_{50} \leq 4$ μg/ml in the Eagles carcinoma of the nasopharynx (KB) or P-388 lymphocytic leukemia (PS) test systems in cell culture, or a prolongation of life of 30% or more in the P-388 test system *in vivo*.



2 spruceanol, $R_1, R_2 = H$

6 $R_1, R_2 = COCH_3$

7 $R_1 = H$, $R_2 = CH_3$



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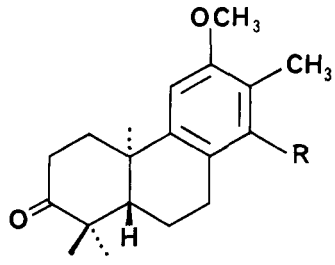


4 $R = C_2H_5$

8 $R = CH=CH_2$

EXPERIMENTAL[3]

PLANT MATERIAL.—The plant material was collected in Peru in April, 1976, and identified as *Cunuria spruceana* Baill. (Euphorbiaceae)[4].

EXTRACTION AND FRACTIONATION.—Air-dried and milled (⅛″ mesh) whole root and root bark of *Cunuria spruceana* (15.5 kg) was successively extracted with petroleum ether (b.p. 60–80°) (A), chloroform (B) and methanol (C). Concentration of the extracts *in vacuo* afforded residues weighing A 52.5 g, B 42.1 g, and C 505 g, respectively. Partition of the methanol extract between chloroform and water afforded, after the usual work-up, a chloroform soluble fraction (D) weighing 20.1 g.

[3]Melting points were determined with a Kofler hot plate and are uncorrected. The uv spectra were obtained with a Beckman, model DB-G grating spectrophotometer. The ir spectra were determined with a Beckman, model 18-A spectrophotometer with polystyrene calibration at 1601 cm^{-1}; absorption bands were recorded in wave numbers (cm^{-1}). Pmr spectra were recorded in $CDCl_3$ solution with a Varian model T-60A instrument operating at 60 MHz with a Nicolet, model TT-7, Fourier Transform attachment. Tetramethylsilane was used as an internal standard, and chemical shifts are reported in δ (ppm) units. Low resolution mass spectra were obtained with a Hitachi Perkin-Elmer, model RMU-6D, single-focusing spectrometer operating at 70 ev. High resolution mass spectra were obtained with a Varian MAT 112S double-focusing spectrometer operating at 70 ev.

[4]The plant material was supplied and identified through the auspices of Drug Research and Development Program of the National Cancer Institute by the Medicinal Plant Resources Laboratory, Agricultural Research Service, U.S.D.A., Beltsville, MD. An herbarium specimen documenting this collection is deposited in the Herbarium of the National Arboretum, Agricultural Research Service, U.S. Department of Agriculture, Washington D.C.

BIOASSAY OF THE CRUDE EXTRACTS.—The petroleum ether and chloroform fractions displayed ED_{50} 2.8 and 0.18 μg/ml, respectively, in the P-388 lymphocytic leukemia system in cell culture. The aqueous phase was devoid of activity.

CHROMATOGRAPHIC SEPARATION OF THE PETROLEUM ETHER EXTRACT.—A portion of the petroleum ether extract (fraction A) (2 g) was chromatographed on a column of silica gel[5] (90 g) packed in benzene. A total of 23 fractions (500 ml each) were collected as the solvent was progressively changed to increasingly polar mixtures of benzene-chloroform, chloroform, and chloroform-methanol.

ISOLATION AND IDENTIFICATION OF MONTANIN (**1**).—Fraction 23 (420 mg), obtained by elution with chloroform—2% methanol, was further separated by preparative tlc on silica gel PF-254[5] (20 x 20 x 0.2 cm) eluted with 8% methanol-chloroform. A short wave uv visible band R_f 0.23) was removed and extracted with chloroform—2% methanol to yield montanin (**1**) (165 mg, 0.027%) as a pale yellow oil. The isolate was identical (uv, ir, nmr and tlc) with an authentic sample obtained previously from roots of *Baliospermum montanum* (3).

ACETYLATION OF MONTANIN (**1**).—Montanin (**1**, 30 mg) was treated with acetic anhydride-pyridine (1:10, 2 ml) at room temperature overnight. Work-up in the usual way and crystallization from hexane afforded montanin diacetate (**5**, 25 mg) as white crystals, mp 54.5° [lit. (3) 54°]: The sample was identical (mmp, ir, nmr and tlc) with an authentic sample (3).

CHROMATOGRAPHIC SEPARATION OF THE CHLOROFORM EXTRACT.—A portion (55 g) of the combined extracts B and D was chromatographed on a column of silica gel PF-254 (1.2 kg) packed in chloroform. A total of 16 fractions (2 liters each) were collected as the solvent was progressively changed to increasingly polar mixtures of chloroform-methanol.

ISOLATION OF SPRUCEANOL (**2**).—Fraction 13 (470 mg) obtained from the above column by elution with chloroform: 1%methanol was rechromatographed on a column of silica gel PF-254 (30 g); elution with chloroform yielded crude spruceanol. Preparative tlc on silica gel (20 x 20 x 0.2 cm) plates eluted with chloroform: 8% methanol afforded spruceanol (**2**) (22 mg, 0.0015%) as a pale yellow gum, R_f 0.25; $[\alpha]^{25}D-3.0$ (c 0.52, $CHCl_3$); uv, λ max (MeOH) 218 (log ε 4.48) 278 nm (3.58), (MeOH/KOH) 221 (4.81), 289 (3.49); ir ν max (thin film, NaCl plates) 3375, 2940, 1595, 1465, 1430, 1400, 1380, 1040, 1015, 1000, 979, 927, 862 and 760 cm^{-1}; pmr ($CDCl_3$) δ 0.87 (3H, s, 18-H_3), 1.05 (3H, s, 19-H_3), 1.19 (3H, s, 20-H_3), 2.17 (3H, s, 17-H_3), 3.14 (2H, m, 7-H), 3.29 (1H, dd, J=7.5, 5.7 Hz, 3-H), 3.45 (1H, s, disappeared with D_2O, 12-OH), 4.97–5.62 (2H, m, 16-H_2), 6.59 (1H, dd, J=17.7, 11.3 Hz, 15-H), 6.65 (1H, s, 11-H); ms, *m/e*, 300 (M^-, 100%), 285 (12, M^+-15), 274 (11), 267 (47), 241 (13), 225 (11), 213 (24), 211 (15), 199 (35), 197 (42), 185 (23), 173 (37) and 147 (19). Mass measurement calculated for $C_{20}H_{28}O_2$, 300.2089; found, 300.20102.

ACETYLATION OF SPRUCEANOL (**2**).—Spruceanol (**2**, 8 mg) was treated with acetic anhydride-pyridine (1:10, 2 ml) at room temperature overnight. Work-up in the usual way and crystallization from petroleum ether afforded spruceanol diacetate (**6**, 8 mg) as white crystals, mp 97–8°; uv, λ max (MeOH) 220 (log ε 4.15) and 278 nm (3.11); ir, ν max (KBr) 2920, 2870, 1775, 1745, 1601, 1470, 1375, 1250, 1215, 1040, 950, 930, 910, and 710 cm^{-1}; pmr ($CDCl_3$) δ 0.94 (6H, s, 18-H_3 and 19-H_3), 1.22 (3H, s, 20-H_3), 2.06 (6H, s, 2 x -$COCH_3$), 2.29 (3H, s, 17-H), 2.54 (2H, m, 7-H_2), 4.50 (1H, m, 3-H), 5.01–6.64 (2H, m, 16-H_2), 6.58 (1H, dd, J=18.7, 12.1 Hz, 15-H), 6.81 (1H, s, 11-H); ms, *m/e* 384 (M^+, 24%), 369 (1), 342 (100), 309 (5), 299 (4), 281 (6), 267 (67), 213 (21), 197 (34), 185 (31), 173 (35) and 147 (22).

METHYLATION OF SPRUCEANOL (**2**).—Spruceanol (**2**, 6 mg) was treated with excess ethereal diazomethane at 0° for 3 days. Work-up in the usual way afforded 12-O-methylspruceanol (**7**, 6 mg) as a gum; ms, *m/e* 314 (M^+, 100%), 299 (9), 281 (30), 213 (13), 211 (15), 187 (18), 141 (17) and 113 (21).

OXIDATION OF 12-*O*-METHYLSPRUCEANOL (**7**).—12-*O*-Methylspruceanol (**7**, 4 mg) was oxidized with chromium trioxide (4 mg) in pyridine (1 ml) at 5° overnight. Work-up in the usual way followed by purification on a silica gel plate (10 x 20 x 0.025 cm) and crystallization from hexane afforded 12-*O*-methyl-spruceanone (**8**, 2.6 mg) as white crystals, mp 106–107°, $[\alpha]^{25}D-90.1$ (c 0.4, $CHCl_3$); ir, ν max (KBr) 2930, 2860, 1715, 1600, 1468, 1387 and 1272 cm^{-1}.

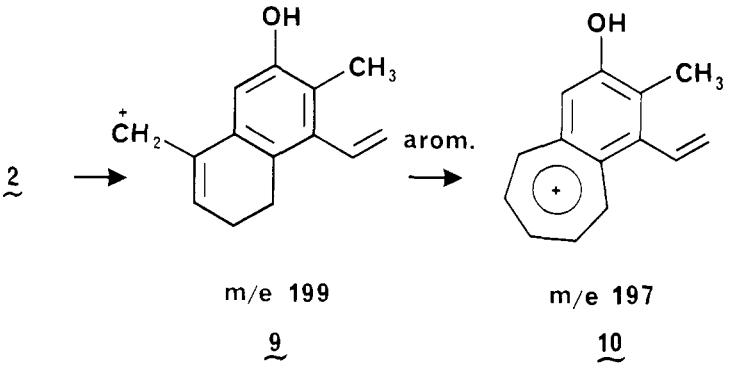
HYDROGENATION OF 12-*O*-METHYLSPRUCEANONE (**8**).—12-*O*-Methylspruceanone (**8**, 2.4 mg) in ethanol (0.8 ml) was hydrogenated over prereduced platinum oxide (2.9 mg) at room temperature and at atmospheric pressure for 12 hrs. Work-up in the usual way and crystallization from petroleum ether afforded 12-methoxycleistanth-8,11,13-trien-3-one (**4**, 2.1 mg) (4), mp 111–112°, $[\alpha]^{26}D-94.5$ (c 0.3, $CHCl_3$), lit (4), mp 112°, $[\alpha]D-96$ ($CHCl_3$); ir, ν max (KBr) 2940, 2870, 1705, 1600, 1585, 1465, 1388, 1272, 1120, and 845 cm^{-1}; ms, *m/e* 314 (M^+, 100%), 299 (30), 285 (3), 271 (2), 257 (18), 243 (5), 229 (8), 213 (18), 201 (8), 189 (9), 163 (16) and 125 (12). The product was identical (mmp, ir and tlc) with an authentic sample of **4**.

[5]E. Merck, Darmstadt, W. Germany.

STRUCTURE ELUCIDATION OF SPRUCEANOL (**2**).—Spruceanol was obtained as a homogeneous, pale yellow gum which failed to crystallize from many solvents and solvent combinations, but which did give a characteristic green coloration with ferric chloride solution for the presence of a phenolic group. The mass spectrum showed a molecular ion at *m/e* 300 analyzing for $C_{20}H_{28}O_2$ and thereby indicated the compound belonged to the diterpenoid series. Strong absorptions in the ir spectrum at 3375 and 1595 cm^{-1} and a uv absorption maxima at 289 nm, shifting bathochromically in base, confirmed the presence of a phenolic group.

Spruceanol showed signals in the proton nmr spectrum for an aromatic methyl group (2.17 ppm), three tertiary methyl groups (0.87, 1.05 and 1.19 ppm), a secondary non-benzylic alcohol group (methine proton at 3.29 ppm), a single aromatic proton (6.65 ppm), and a vinyl group attached to an aromatic nucleus. The combination of these groups in a single nucleus suggested the compound was a member of the cleistanthane series, of which cleistanthol (**3**) (4) is the only previously isolated example.

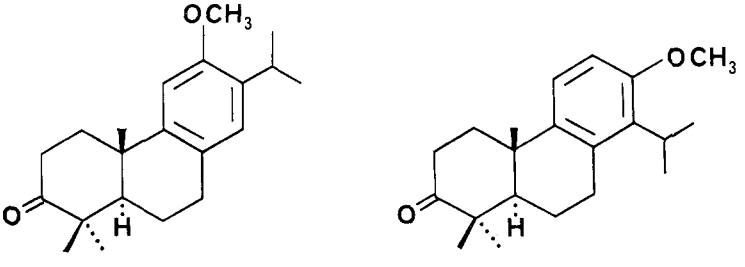
Acetylation of spruceanol afforded a diacetate derivative **6** in which a secondary methine proton was now shifted downfield to 4.50 ppm. In the mass spectrum of spruceanol, the presence of a relatively weak M^+-15 ion suggested (5) that the phenolic group might be located at C-12. In support of this assignment, both spruceanol and cleistanthol display the lone aromatic proton at 6.65 ppm. Fragment ions at *m/e* 199 and 197 were interpreted as having the structures **9** and **10**, respectively, implying that the secondary hydroxy was located in ring A of spruceanol.



2 **9** **10**

Of the three possible positions (C-1, C-2 and C-3) for this group, C-2 could be eliminated from the observation of the methine proton as a doublet of doublets (J=7.5 and 5.7 Hz); distinction between C-1 and C-3 was made from the nmr spectrum of the diacetate derivative. One of the singlet methyl groups at C-4 showed a downfield shift of 6.4 Hz in the diacetate derivative, whereas the other showed an upfield shift of 4.1 Hz. These shifts are characteristic (6) of a secondary hydroxy group located at C-3 and having the α-stereochemistry. Such a stereochemical assignment is supported by the coupling constants of this proton, which indicated a combination of axial-axial and axial-equatorial coupling constants.

Spruceanol (**2**) formed a monomethyl ether with diazomethane which could be oxidized to 12-*O*-methylspruceanone (**8**) with Sarrett's reagent at low temperature. The only asymmetric centers in the molecule are now C-5 and C-10, the A/B ring junction. Comparison of the specific rotation of 12-*O*-methylspruceanone (**8**), $[\alpha]D-90.5°$, with those of 12-methoxycleistanth-8,11,13-trien-3-one (**4**) (4), $[\alpha]D-96°$, hinokione methyl ether (**11**) (7), $[\alpha]D+119°$, and totarolone (**12**) (7), $[\alpha]D+99°$ indicated spruceanol had the A/B ring junction configuration shown in **2**.



11 **12**

Final proof of the structure was obtained when 12-*O*-methylspruceanone (**8**) was reduced catalytically to 12-methoxycleistanth-8,11,13-trien-3-one (**4**), identical (mmp, ir and tlc) with an authentic sample. Spruceanol is, therefore, represented by the structure 3α,12-dihydroxycleistanth-8,11,13,15-tetraene (**2**).

BIOLOGICAL ACTIVITY OF THE ISOLATES.—Montanin (**1**, NSC-282158) has previously (3) shown marginal activity in the P-388 lymphocytic leukemia test system *in vivo* and cytotoxicity (ED_{50} 0.06 μg/ml) against the same system *in vitro*. Spruceanol (**2**, NSC-312885) displayed only marginal activity (ED_{50} 3.2 μg/ml) against the P-388 test sytem *in vitro*.

ACKNOWLEDGMENTS

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