PLANT ANTICANCER AGENTS. IX.[1] CONSTITUENTS OF *HYPTIS TOMENTOSA*

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ABSTRACT.—The twigs, leaves, and flowers of *Hyptis tomentosa* were found to owe their major cytotoxic activity to the presence of desoxypodophyllotoxin (**4**), but the two weakly cytotoxic flavones 5-hydroxy-4',6,7,8-tetramethoxy flavone (**3**) and 5-hydroxy-4',3,6,7,8-pentamethoxy flavone (**2**) also contributed to the total cytotoxicity of the crude extracts. The flavones eupatorin (**6**) and 5-hydroxy-3',4',6,7-tetramethoxy flavone (**5**) were isolated as inactive constituents, together with the lignan sesamin (**1**).

Plants from the genus *Hyptis* (Lamiaceae) have been studied fairly extensively from a phytochemical point of view, and the anticancer agents 4'-demethyldesoxypodophyllotoxin (1) and betulinic acid (2) have been isolated from plants of this genus. It is also likely that 14-methoxytaxodione, obtained from *H. fructicosa* (3), is responsible for the reported (3, 4) anticancer activity of this species. Other compounds isolated from *Hyptis* species include hyptol (5), suaveolic acid (6) and hyptolide (7).

In a continuation of our search for anticancer agents of plant origin, we examined an ethanolic extract of *Hyptis tomentosa* Poit. which showed activity in the KB cell culture system and the P–388 lymphocytic leukemia system. Fractionation of this extract by liquid-liquid partition followed by extensive chromatography over silica gel monitored by the KB assay system yielded three compounds which showed cytotoxicity in the KB cell culture system, together with three inactive compounds.

The primary cytotoxic component was identified as desoxypodophyllotoxin (**4**) on the basis of its spectroscopic properties, its melting point, and comparison with an authentic sample. The two flavones 5-hydroxy-4',3,6,7,8-pentamethoxy flavone (**2**) and 5-hydroxy-4',6,7,8-tetramethoxy flavone (**3**) were also isolated and found to show mild cytotoxicity. Three inactive compounds were obtained in the course of the isolation procedures and were shown to be sesamin (**1**), 5-hydroxy-3',4',6,7-tetramethoxy flavone (**5**), and 3',5-dihydroxy-4',6,7-trimethoxy flavone (eupatorin) (**6**). Eupatorin was originally reported to be mildly cytotoxic (8, 9), but a recent paper records that it was found not to be cytotoxic (10), and our results confirm this finding. The weak cytotoxicity of compound **3** is recorded in a recent review of the antineoplastic activity and cytotoxicity of flavones and related compounds (11), but the cytotoxicity of compound **2** and the lack of activity of compound **5** are reported for the first time.

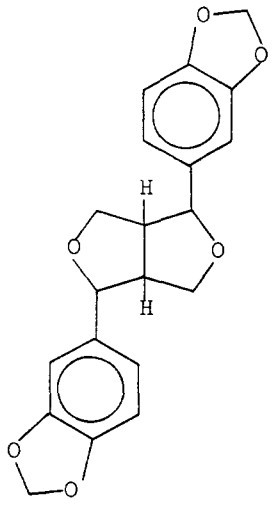
We have been unable to detect any highly active compounds other than desoxypodophyllotoxin in the active fractions obtained in this work, and we thus conclude that this compound is responsible for the cytotoxicity of the crude extract. It is probable that it is also responsible for the observed P–388 *in vivo* activity of the crude extract since it is known to be active in this system (12).

EXPERIMENTAL[2]

BIOLOGICAL ACTIVITY.—A 50% aqueous ethanol extract of *H. tomentosa* was evaluated for

[1]For Part VIII, see D. G. I. Kingston and R. C. Munjal, *Lloydia* **41**, 499 (1978).

[2]General experimental details are given in Part III of this series; D. G. I. Kingston, B. T. Li and F. Ionescu, *J. Pharm. Sci.* **66**, 1135 (1977).



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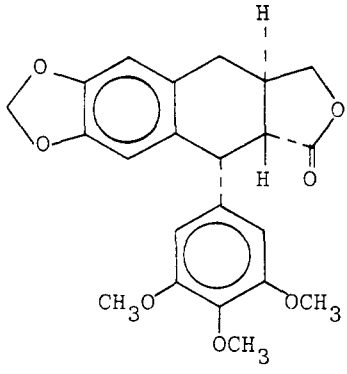

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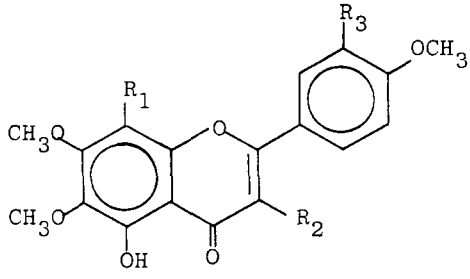

2	R_1, R_2 = OCH_3	R_3 = H
3	R_1 = OCH_3	R_2, R_3 = H
5	R_1, R_2 = H	R_3 = OCH_3
6	R_1, R_2 = H	R_3 = OH

cytotoxic and antitumor activity;[3] it was active against the P-388 lymphocytic leukemia in mice (T/C 140 at 200 mg/kg) and in the KB cell culture system (ED_{50} 11, 2.6 μg/ml).

EXTRACTION AND FRACTIONATION.—A sample of ground twigs, leaves, and flowers of *H. tomentosa*[4] (4 kg) was extracted three times with 95% ethanol. The combined ethanol extracts were evaporated and partitioned between water and chloroform, and the chloroform fraction was evaporated and partitioned between 10% aqueous methanol and hexane. The methanol fraction was evaporated to yield 120 g of a thick dark syrup (Fraction A). This fraction had an ED_{50} of 0.16 μg/ml in the KB cell culture.

Fraction A was subjected to chromatography on a column of silica gel (900 g) with elution by a chloroform to methanol gradient. Fractions of 1000 ml were collected and combined on the basis of their similarity following tlc to yield thirteen major fractions B-N. Fractions C-E (18.5 g total weight) were all highly cytotoxic (ED_{50}<0.01 μg/ml in KB), and fraction I (25 g) also showed significant activity (ED_{50}=0.18 μg/ml in KB).

[3]The test methods employed were those of the Drug Research and Development Program of the National Cancer Institute (13). Active fractions are defined as those that show a T/C value $\geq$130% in the P-388 system, or ED_{50} values $\leq$20 μg/ml in the KB cell culture system.

[4]The plant was collected in Mexico in November 1976, and was supplied through the auspices of the 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, DC.

CHROMATOGRAPHY OF FRACTION D.—Fraction D (8.0 g) was subjected to chromtogaraphy on silica gel with elution by chloroform-methanol, 95:5, to yield two major active fractions O and P. Fraction 0 (3.5 g) was crystallized from hexane-ether-ethyl acetate to give a crude crystalline fraction consisting largely of sesamin and a mother liquor, which was subjected to chromatography on silica gel with elution by hexane:ether:ethyl acetate, 35:45:20. Five major fractions Q–U were obtained; fractions T and U both showed significant cytotoxicity ($ED_{50}<0.01$ μg/ml).

ISOLATION AND CHARACTERIZATION OF SESAMIN (1).—Fraction Q (0.76 g) was crystallized from methanol to give colorless needles, mp 123°, $[\alpha]_D+68.8°$ (c=0.035 in chloroform); lit. (14) mp 122.5°, $[\alpha]_D+68.2°$.

The spectroscopic properties of the isolated material were entirely in accord with the assigned structure. In particular, its mass spectrum showed a molecular ion at *m/z* 354.1107; $C_{20}H_{18}O_6$ requires 354.1103.[5] Its pmr spectrum was fully in agreement with that previously published for sesamin (15), and its ir and uv spectra were also consistent with the assigned structure.

Sesamin showed no activity ($ED_{50}>100$ μg/ml) in the KB cell culture system.

ISOLATION AND CHARACTERIZATION OF 5-HYDROXY-4',3,6,7,8-PENTAMETHOXY FLAVONE (**2**).—Fraction S (0.32 g) was separated into four fractions by preparative tlc with elution by hexane:chloroform:methanol (53:45:2). The first fraction ($R_f=0.66$, 0.13 g) was crystallized from methanol to yield the pentamethoxy flavone **2** as yellow needles, mp 122–3°; lit. (16) mp 122–3°.

The uv spectrum of **2** in ethanol exhibited absorptions with λ_{max} 336 nm (log ε 4.24), 282 (4.23), and λ_{inf} 225 (4.21). Its mass spectrum showed a molecular ion at *m/z* 388, consistent with the composition $C_{20}H_{20}O_8$, and its pmr spectrum showed signals for the protons of five methoxyl groups, together with signals at δ=8.09 (2H, d, *J*=9 Hz) and 6.99 ppm (2H, d, *J*=9 Hz) which could be assigned to the protons of a ring B substituted in the 4' position. The ir spectrum showed a peak at 1645 cm^{-1} (chelated CO) and no peak attributable to a hydroxyl group.

These data demand that the free hydroxyl group required by the composition of **2** be located in the 5 position, and also that the A ring be fully substituted and the B ring be substitued only at the 4'-position. The structure 5-hydroxy-4',3,6,7,8-pentamethoxy flavone (4'-O-methylcalycopterin) may thus be assigned to this compound.

Compound **2** had an ED_{50} of 1.8 μg/ml in the KB cell culture system.

ISOLATION AND CHARACTERIZATION OF 5-HYDROXY-4',6,7,8-TETRAMETHOXY FLAVONE (**3**).—Fraction T (0.16 g) was crystallized from methanol to yield the tetramethoxyflavone **3** as yellow needles, mp 178.5–179.5°; lit. (17) 176–177°, (18) 179–180°.

The uv spectrum of **3** in ethanol exhibited absorptions with λ_{max} 328 nm (log ε 4.39), 289 (4.33), and λ_{inf} 217 (4.43), and its mass spectrum showed a molecular ion at *m/z* 358.1054 ($C_{19}H_{18}O_7$ requires 358.1051). Its pmr spectrum in $CDCl_3$ showed signals for the protons of four methoxyl groups, together with signals at δ=7.82 (2H, d, *J*=9 Hz) and 6.96 ppm (2H, d, *J*=9 Hz) which could be assigned to the protons of a ring B substituted in the 4' position, and a signal at 6.54 ppm (1H, s), which could be assigned to the proton in the 3 position.

These data indicate that compound **3** can be assigned the structure 5-hydroxy-4',6,7,8-tetramethoxy flavone (5-*O*-desmethyltangeretin). The material had an ED_{50} of 6.0 μg/ml in the KB cell culture system.

ISOLATION AND CHARACTERIZATION OF DESOXYPODOPHYLLOTOXIN (**4**).—Fraction U (0.17 g) was subjected to ptlc with development by chloroform-hexane-methanol, 50:50:3. The major active band (80 mg) was crystallized from ether to yield desoxypodophyllotoxin (**4**) as colorless platelets, mp 166–167°, undepressed in admixture with an authentic sample mp 166–169°. The isolated material had spectroscopic properties (uv, ir, ms, nmr) identical with those of authentic material. Desoxypodophyllotoxin had an ED_{50} of 0.032 μg/ml in the KB cell culture system.

ISOLATION AND CHARACTERIZATION OF 5-HYDROXY-3',4',6,7-TETRAMETHOXYFLAVONE (**5**).—Fraction P from the second chromatographic separation described earlier was treated with cold methanol, and the insoluble portion recrystallized from chloroform-hexane to yield the tetramethoxy flavone **5** as yellow needles, mp 192°; lit. (9) 190–191°, (19) 192–194°.

The uv spectrum of compound **5** in ethanol exhibited absorptions with λ_{max} 338 nm (log ε 4.44), 275 (4.28), and 242 (4.27), and its mass spectrum showed a molecular ion at *m/z* 358.1058 ($C_{19}H_{18}O_7$ requires 358.1051). Its pmr spectrum in dimethyl sulfoxide showed signals for the protons of four methoxyl groups, together with signals at δ=8.24 (1H, s, 5-OH), 7.63 (1H, dd, *J*=8 Hz, 2 Hz, H-6'), 7.51 (1H, d, *J*=2 Hz, H-2'), 7.05 (1H, d, *J*=8 Hz, H=5'), 6.96 (1H, s) and 6.88 (1H, s). These data indicate that compound **5** has the structure 5-hydroxy-6,7,3',4'-tetramethoxyflavone. The material showed no activity ($ED_{50}>100$ μg/ml) in the KB cell culture system.

[5]High resolution mass spectra were obtained on an AEI MS-902 mass spectrometer at the Research Triangle Institute, N.C.

ISOLATION AND CHARACTERIZATION OF 3',5-DIHYDROXY-4',6,7-TRIMETHOXY FLAVONE (**6**).—Fraction G from the original large chromatographic separation (2.7 g) was triturated with cold methanol, and the insoluble portion was dissolved in chloroform and filtered to remove a small amount of suspended material. Concentration of the chloroform solution yielded compound **6** as yellow needles, which had mp 197–198° after recrystallization from methanol, undepressed in admixture with authentic material. The isolated material had spectroscopic properties (ir, uv, nmr, ms) identical with those reported for eupatorin (9, 10) and showed identical behavior on tlc. The material showed no activity (ED_{50}>50 μg/ml) in the KB cell culture system.

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