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TUMOR INHIBITORS—XXXIII[1]

CYTOTOXIC FLAVONES FROM *EUPATORIUM* SPECIES

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Abstract—From a cytotoxic extract of *Eupatorium semiserratum* DC., five flavones were isolated. Three of the compounds were characterized as the previously-known pectolinarigenin, and two new flavones, eupatorin and eupatilin. Structural studies are described which have led to assignment of the 5,3'-dihydroxy-6,7,4'-trimethoxyflavone structure (**1**) for eupatorin, and the 5,7-dihydroxy-6,3',4'-trimethoxyflavone structure (**11**) for eupatilin. From a cytotoxic extract of *Eupatorium cuneifolium* (TOURN.) L., two flavones were isolated. The compounds were characterized as the previously-known hispidulin and a new flavone, eupafolin. Structural studies are described which have led to assignment of the 5,7,3',4'-tetrahydroxy-6-methoxyflavone structure (**18**) for eupafolin. The earlier assignment of structure **18** for pedalitin is shown to be incorrect, and the alternative 5,6,3',4'-tetrahydroxy-7-methoxyflavone structure (**24**) is proposed for pedalitin.

IN THE course of our continuing search for tumor inhibitors from plant sources, an alcoholic extract of *Eupatorium semiserratum* DC.* and a chloroform extract of *Eupatorium cuneifolium* (TOURN.) L. were found to have reproducible activity against human carcinoma of the nasopharynx carried in cell culture (KB). We report herein the fractionation of the active extracts and the isolation and characterization of five flavones from *E. semiserratum* and two flavones from *E. cuneifolium*. The flavones from *E. semiserratum* are the previously-known pectolinarigenin and four others, designated flavones K and L, eupatorin and eupafolin. The flavones from *E. cuneifolium* are the previously-known hispidulin and a new flavone, designated as eupafolin. In assays performed by the C.C.N.S.C., the following average ED_{50}'s (i.e. doses inhibiting growth to 50% of control growth) against the KB cell culture were observed: pectolinarigenin, 11·8; flavone K, 9·2; flavone L, 6·9; eupatorin, 4·6; eupatilin, 38; hispidulin, 22; and eupafolin, 18.†

Flavones from Eupatorium semiserratum DC. The preliminary fractionation of the alcoholic extract (A) of *E. semiserratum* DC. is summarized in Fig. 1. The flavonoid-enriched fraction (E) was fractionated by repeated chromatography on silicic acid to give (in order of elution with chloroform) pectolinarigenin, flavone K, eupatilin, flavone L, and eupatorin.

* Stems, leaves, and flowers of *E. semiserratum* were collected in Florida, September, 1963. Stems, leaves, and flowers of *E. cuneifolium* were collected in Florida, August, 1966. The authors acknowledge with thanks receipt of the dried plant material from Dr. Robert E. Perdue, Jr., U.S. Department of Agriculture, Beltsville, Md., in accordance with the program developed with U.S.D.A. by the Cancer Chemotherapy National Service Center (C.C.N.S.C.).

† Cytotoxicity was assayed under the auspices of the C.C.N.S.C. against human carcinoma of the nasopharynx carried in cell culture (KB). The procedures were those described in *Cancer Chemotherapy Rept.* **25**, 1 (1962).

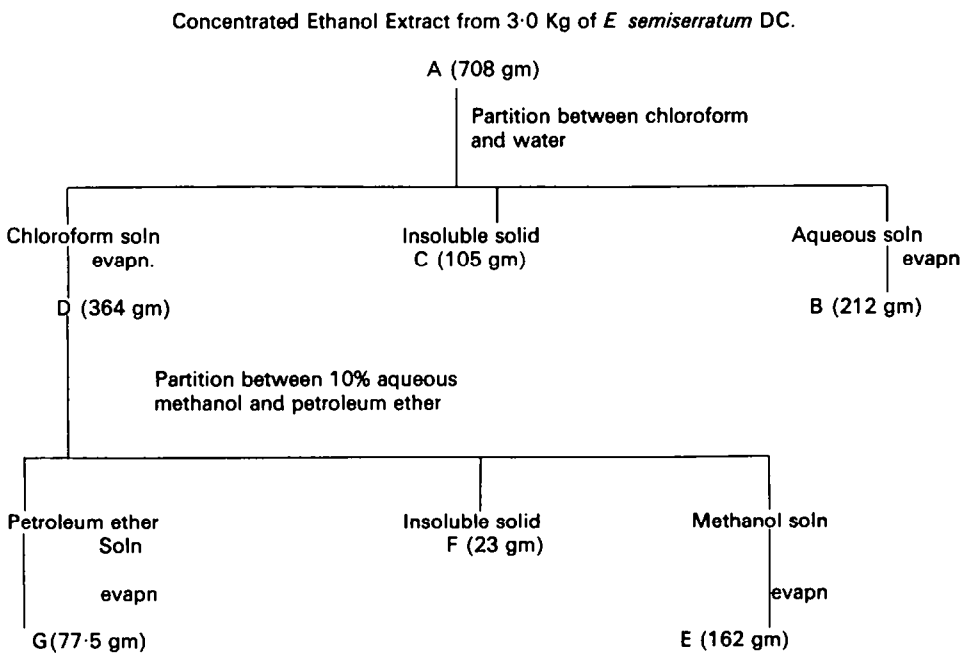


FIG. 1. Flow sheet for fractionation of cytotoxic extract from *E. semiserratum*.

Eupatorin[2] was crystallized as yellow needles from dioxan–water, and elemental analysis supported the molecular formula $C_{18}H_{16}O_7$. The compound gave a purplish brown color with ferric chloride solution and the flavonoid character was shown by the usual reduction test with magnesium-hydrochloric acid (pinkish-red).[3] The UV absorption spectrum showed a sharp band at 342 mμ, indicative that it is a flavone derivative and not a flavonol.[4] The presence of a 5-hydroxy group was indicated by a bathochromic shift of 20 mμ in band I* on the addition of aluminum chloride to an ethanolic solution of eupatorin. Methylation of eupatorin resulted in a hypsochromic shift of 9 mμ of band II and 14 mμ of band I, attributable to the breaking of the hydrogen bond between the 5-OH and the pyrone CO. The UV spectral data for eupatorin and its derivatives are given in Table 1.

The NMR spectral data for eupatorin and its diacetate, **2**, are given in Table 2. These data showed that the flavone was pentasubstituted, with signals for three OMe groups at τ 6·05, 6·12, and 6·23 and signals for two OH groups at τ 0·64 and −2·89. The far-downfield signal indicated the presence of a free 5-OH group which was H-bonded to the pyrone CO.[5] The presence of the 5-OH was also suggested by the spectrum of the acetylated flavone, since a 5-acetate group resonance occurs near τ 7·54, distinct from those of other flavone acetate groups (e.g. τ 7·65 for a 3′-acetate).[5]

The aromatic region of the NMR showed a one-proton doublet centered at τ 2·95 (J = 8 cs) and partly resolved signals at τ 2·46 corresponding to two protons, the pattern being indicative of a 3′,4′-disubstituted B-ring. The remaining two proton

* A band in the 320–380 mμ region is referred to as band I; one in the 240–270 mμ region is called band II.

resonances were singlets at τ 3·26 and 3·18, which were later assigned to the C-3 and C-8 protons, respectively.

Methylation of eupatorin with dimethyl sulfate yielded **3**, which was identified as 5,6,7,3′,4′-pentamethoxyflavone by comparison of its physical properties with those reported previously.[6] The formation of **3** established the oxygenation pattern. Methylation of eupatorin with diazomethane gave **4**, whieh was identified as 5-hydroxy-6,7,3′,4′-tetramethoxyflavone,[7] by direct comparison with an authentic sample.* Acetylation of **4** gave a tetramethoxyflavone 5-acetate, **5**, which was found to have physical properties similar to those described[7] for the known 5-acetoxy-6,7,3′,4′-

TABLE 1. UV SPECTRAL DATA FOR FLAVONOIDS AND THEIR DERIVATIVES

Compound	Normal spectrum (ethanol) λ_{max}^{alc} (mμ)	ε	Spectrum with added reagent: aluminum chloride λ_{max}^{alc} (mμ)	ε	sodium ethoxide λ_{max}^{alc} (mμ)	ε	sodium acetate λ_{max}^{alc} (mμ)	ε
Eupatorin (**1**)	243	17,400	238	16,300	271	13,300	243	20,100
	254	19,300	262	15,300	372	6600	253	19,000
	274	19,800	290	17,900			276	19,200
	342	27,700	362	23,300			342	25,200
Eupatorin diacetate (**2**)	233	27,500						
	265	18,300						
	319	37,200						
5,6,7,3′,4′-pentamethoxy-flavone (**3**)	240	25,900						
	265	16,500						
	328	28,600						
5-hydroxy-6,7,3′,4′-tetramethoxyflavone (**4**)	242	20,400						
	275	19,400						
	339	27,500						
Eupatilin (**11**)	243	17,900	259	15,400	238	29,000	237	23,800
	277	17,000	290	18,400	278	28,400	278	23,800
	340	26,300	363	21,100	312	14,600	322	14,900
					376	17,500	364	15,300
Eupafolin (**18**)	253	10,200	276	13,100			276	14,800
	272	10,100	432	23,500			380	12,200
	342	17,300						
Pedalitin (**22**)	248i	12,000	309	13,400			284	13,000
	284	12,000	436	21,500			359	13,400
	343	19,400						

"i" denotes an inflection in the spectrum.

* The authors thank Professor N. Morita, University of Toyama, Japan, for authentic samples of 5-hydroxy-6,7,3′,4′-tetramethoxyflavone and pedalitin.

TABLE 2. NMR SPECTRAL DATA FOR THE FLAVONES AND THEIR DERIVATIVES

Compound	A-Ring		B-Ring		C-Ring	Methoxyl	Hydroxyl or Acetate
	C-8H	C-2'H	C-5'H	C-6'H	C-3H		
Eupatorin (1)*	3·18 s	2·46 m	2·95 d (J = 8)	2·46 m	3·26 s	6·05 s 6·12 s 6·23 s	C-3'OH, 0·64 C-5OH, −2·89
Eupatorin diacetate (2)†	3·11 s	2·33 m	2·80 d (J = 8)	2·33 m	3·50 s	6·02 s 6·04 s 6·12 s	C-5OAc, 7·54 s C-3'OAc, 7·65 s
Eupatilin (11)*	3·13 s	2·30 m	2·95 d (J = 9)	2·30 m	3·40 s	6·11 s 6·15 s 6·20 s	C-5OH, −3·04 C-7OH, −0·58
Eupafolin (18)*	3·33 s	2·56 m	3·07 d (J = 9)	2·56 m	3·43	6·17 s	C-5OH, −3·26 s C-7OH, C-3'OH, C-4'OH } 0·05 m
Pedalitin (22)*	3·15 s	2·57 m	3·11 d (J = 9)	2·57 m	3·32 s	6·07 s	C-5OH, −2·66 s C-7OH, C-4'OH, C-3'OH } 0·09, 0·61, 1·41

* d_6-DMSO.
† $CDCl_3$.

tetramethoxyflavone. These results confirmed the presence of a suspected 5-OH group. Treatment of eupatorin with 50% alcoholic potassium hydroxide under reflux gave 4,5-dimethoxyresorcinol (7) and isovanillic acid (**8a**). The identification of isovanillic acid located the second OH group of eupatorin at C-3' and the structure of eupatorin was thus defined as 5,3'-dihydroxy-6,7,4'-trimethoxyflavone (**1**). Recently, Wilson *et al.*[8] have reported that NMR solvent shift data can be used to establish the environment and position of OMe groups in flavones. The diethyl ether of eupatorin was prepared in order to utilize and supplement the chemical shift-structural correlations made by these authors. The chemical shifts obtained in $CDCl_3$ and d_6-benzene for eupatorin diethyl ether (**6**) and the ethyl ethers of the other flavones reported in this paper are recorded in Table 3. The solvent shifts observed for the three OMe groups of eupatorin are close to those reported for the C-6, C-7, and C-4' methoxyls of the model compound hexamethylquercetagetin (**10**).[8] This NMR solvent shift data offers further support that eupatorin has structure **1**. Finally, a sample of the diethyl ether **6** was subjected to mild alkaline degradation. The acidic product, **8b**, was identified by its m.p. and NMR spectrum as the expected 3-ethoxy-4-methoxybenzoic acid. The neutral fraction afforded the new acetophenone, **9**, which was characterized as the *p*-nitrobenzoate.

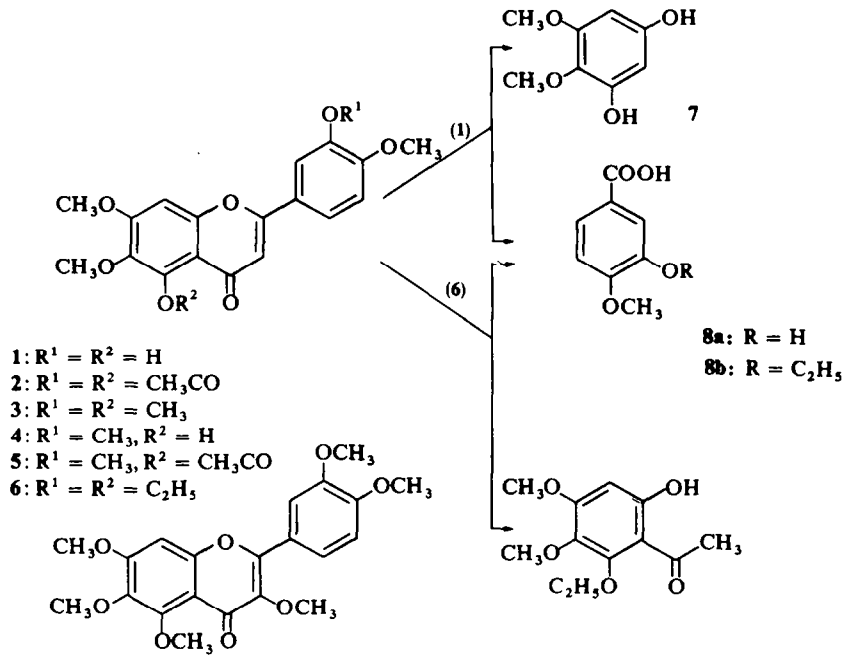
Eupatilin* was assigned the formula $C_{18}H_{16}O_7$ on the basis of elemental analysis. The compound gave a position magnesium hydrochloric acid test and all its spectra

* Eupatilin was also isolated from *Liatris chapmanii* by Dr. P. Bollinger in our laboratory.

TABLE 3. NMR SOLVENT SHIFT DATA FOR ETHYL ETHERS OF FLAVONES

Compound	Chemical shifts (τ) of methoxyl protons				Chemical shifts (τ) of methylene of ethoxy groups			
	d_6-Benzene (TMS)	$CDCl_3$ (TMS)*		Δ (ppm)	d_6-Benzene (TMS)	$CDCl_3$ (TMS)*		Δ (ppm)
Eupatorin diethyl ether (6)	6·15 6·55 6·61	6·00 6·06 6·07	6·03 ±0·03	**0·12** ±0·03 (C-6) **0·52** ±0·03 (C-4′) **0·58** ±0·03 (C-7)	5·60 6·23	5·80 5·80		**−0·20** (C-5) **0·43** (C-3′)
Quercetagetin hexamethyl ether[8] (10)	5·88 6·09 6·20 6·41 6·57 6·75	6·01 6·01 5·96 6·06 6·10 6·01		**−0·13** (C-5) **0·08** (C-6) **0·24** (C-3) **0·35** (C-3′) **0·47** (C-4′) **0·74** (C-7)				
Eupatilin diethyl ether (13)	6·15 6·53 6·60	6·03 6·05 6·09	6·06 ±0·03	**0·19** ±0·03 (C-6) **0·47** ±0·03 (C-3′) and **0·54** ±0·03 (C-4′)	5·57 6·43	5·81 5·81		**−0·24** (C-5) **0·62** (C-7)
Eupafolin tetraethyl ether (20)	6·15	6·09		**0·06** (C-7)	5·62 6·22 6·38 6·38	5·83 5·83 5·83 5·83		**−0·21** (C-5) **0·39** (C-3′) and **0·55** (C-4′) **0·55** (C-7)
Hispidulin triethyl ether (23)	6·16	6·08		**0·08** (C-6)	5·62 6·39 6·39	5·81 5·81 5·81		**−0·19** (C-5) **0·58** (C-4′) **0·58** (C-7)
Pedalitin tetraethyl ether (25)	6·71	6·02		**0·69** (C-7)	5·58 5·87 6·29 6·33	5·81 5·81 5·81 5·86	5·83 ±0·02	**−0·25** (C-5) **0·04** (C-6) **0·46** (C-3′) and **0·50** (C-4′)

* The observed chemical shift for each signal is given. The value used to calculate Δ is the mean of the two extremes.

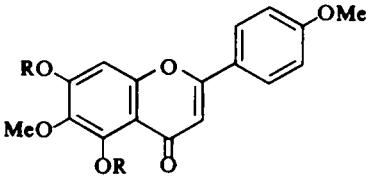


were typical for a flavonoid. The UV spectral data are given in Table 1, the λ_{max} at 340 mμ being indicative of a flavone rather than a flavonol. Further, the bathochromic shift of bands I and II observed on the addition of aluminum chloride indicated the presence of a 5-OH group. The NMR spectrum showed the presence of three OMe's and two protons exchangeable with deuterium oxide, the signal at τ −3·04 being characteristic of a chelated 5-OH. In addition, signals for five aromatic protons were present as two one-proton singlets at τ 3·13 and 3·40, a doublet at τ 2·95 and a two-proton multiplet at τ 2·30, indicative that the oxygenation pattern could well be the same as that of eupatorin. The presence of two OH groups, suggested from the NMR spectrum of eupatilin, was confirmed by acetylation to yield the diacetate, **12**. The NMR spectrum of **12** exhibited two acetate Me signals, the one at τ 7·52 being characteristic of a 5-acetate. Methylation of eupatilin with dimethyl sulfate yielded 5,6,7,3′,4′-pentamethoxyflavone (**3**, previously prepared from eupatorin), which established the oxygenation pattern.

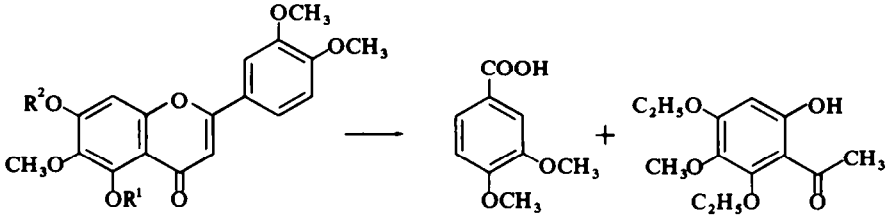
Ethylation of eupatilin with ethyl iodide afforded the diethyl ether, **13**. The NMR spectrum of **13** was measured in d_6-benzene and $CDCl_3$ and the solvent shift data are reported in Table 3. The solvent shifts for the three OMe groups are close to the ones observed for the C-6, C-3′, and C-4′ OMe's in the model compound hexamethylquercetagetin. Alkaline degradation of eupatilin diethyl ether under mild conditions afforded 3,4-dimethyoxybenzoic acid (**14**) and 2,4-diethoxy-6-hydroxy-3-methoxyacetophenone (**15**) which was identified by comparison of its NMR spectrum with that of a sample prepared by the alkaline degradation of hispidulin triethyl ether. The *p*-nitrobenzoates of these two samples were also compared and found to be identical. The results of this alkaline degradation firmly established that C-6,

C-3′, and C-4′ bear methoxy groups in eupatilin and therefore that the two OH groups are located at C-5 and C-7. On this bases, eupatilin has been assigned the structure **11**.

Pectolinarigenin (**16**) was identified by direct comparison with an authentic sample* and by comparison of the physical properties of the acetate ester (**17**) with those previously reported.[9]



16: R = H
17: R = CH_3CO



11: $R^1 = R^2 = H$
12: $R^1 = R^2 = CH_3CO$
13: $R^1 = R^2 = C_2H_5$

14 **15**

Structural studies of the new flavonoids K and L are in progress and will be described in a later publication.

Flavones from Eupatorium cuneifolium (TOURN) L. The chloroform extract of *E. cuneifolium* (TOURN.) L. was solvent-partitioned between 10% aqueous methanol and Skellysolve B. The 10% aqueous methanol solubles were repeatedly chromatographed on silicic acid to give two flavones, eupafolin (**18**) and hispidulin (**22**).

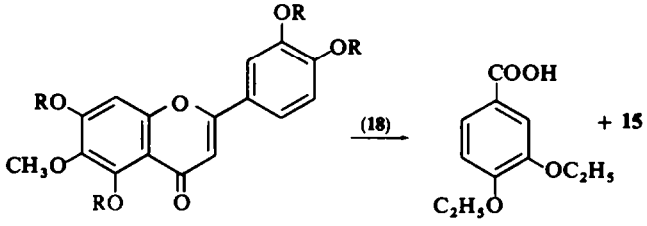
Eupafolin was isolated as pale yellow needles. The elemental analysis and a mass spectrum supported the empirical formula $C_{16}H_{12}O_7$. The UV absorption data are given in Table 1. The λ_{max} of band I at 342 mμ indicated a flavone derivative rather than a flavonol, and the appearance of band II as two peaks at 272 mμ and 253 mμ suggested that the B-ring was 3′,4′-disubstituted. The presence of a 5-OH group was indicated when, upon the addition of aluminum chloride to an ethanolic solution of the flavone, there was a large bathochromic shift of 90 mμ in band I, and band II appeared as a single peak at 276 mμ. The presence of a free 7-OH group was indicated by a bathochromic shift to 276 mμ of the low wavelength band on the addition of sodium acetate. The NMR data for eupafolin are summarized in Table 2. The presence of one OMe group and four OH protons was indicated. A singlet far downfield at τ −3·26 confirmed the presence of the chelated 5-OH group. The signals for the five aromatic protons appeared as a pattern similar to that observed for eupatorin and eupatilin. Two singlets at τ 3·43 and 3·33 could be assigned to the C-3 and C-8

* We thank Professor F. Wesseley for a sample of pectolinarigenin.

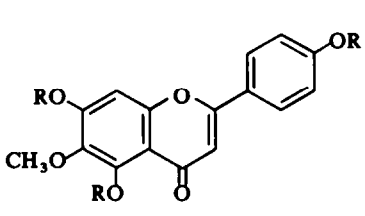
protons. The two-proton multiplet at τ 2·56 could be assigned to the C-2′ and C-6′ protons, and a doublet at τ 3·07 (J = 9 cs) to the C-5′ proton.

Acetylation of eupafolin afforded the tetraacetate **19**, confirming the presence of four OH groups. Methylation with dimethyl sulfate yielded the tetramethyl ether **2**, which was shown to be identical to 5,6,7,3′,4′-pentamethoxyflavone obtained from eupatorin. Treatment of eupafolin with ethyl iodide afforded the tetraethyl ether **20**, the solvent shift data for which are reported in Table 3. The OMe shift of 0·06 ppm is in close agreement with that observed for a C-6 proton in the model compound hexamethylquercetagetin. Alkaline degradation of **20** with 40% potassium hydroxide gave 3,4-diethoxybenzoic acid (**21**) and 2,4-diethoxy-6-hydroxy-3-methoxyacetophenone (**15**) as the major products. A sample of **15** was also prepared by the alkaline degradation of hispidulin triethyl ether (**23**) and the two samples, on comparison of their *p*-nitrobenzoates, were shown to be identical. The properties of the two samples of the *p*-nitrobenzoate of **15** were also similar to those reported earlier for this compound.[10] These experiments established that eupafolin is 5,7,3′,4′-tetrahydroxy-6-methoxyflavone (**18**).*

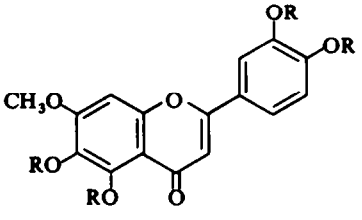
The 5,7,3′,4′-tetrahydroxy-6-methoxyflavone structure (**18**) has been assigned earlier for pedalitin.[7] Since the physical properties of eupafolin differ from those of pedalitin (m.p., mixture m.p., IR, NMR and mass spectra, and paper chromatographic behaviour),



18: R = H
19: R = CH_3CO
20: R = C_2H_5



22: R = H
23: R = C_2H_5



24: R = H
25: R = C_2H_5

the structure of pedalitin warrants further investigation. Upon treatment with diazomethane, pedalitin yields a trimethyl ether identical with the monomethyl ether of eupatorin. Hence, pedalitin must possess the same 5,6,7,3′,4′-oxygenation pattern. The NMR spectrum (Table 2), which shows singlets at τ 3·32 and 3·15 (corresponding to the C-3 proton and the C-8 proton, respectively), a two-proton multiplet at τ 2·57

* After completion of this work, the preparation of 5,7,3′,4′-tetrahydroxy-6-methoxyflavone by hydrolysis of 6-methoxyluteolin glucoside has been reported.[11]

(for the 2′ and 6′-protons), and a doublet at τ 3·11 (J = 9 cs) (for the 5′-proton), confirms the substitution pattern. The presence of one OMe group and four OH groups is also confirmed. The UV spectrum of pedalitin (Table 1) is similar to that of eupafolin. Band I occurs at 343 mμ and band II appears as a sharp peak at 284 mμ and an inflection around 248 mμ. Aluminum chloride causes a large bathochromic shift in both bands, indicating the 5-OH group is not etherified. Sodium acetate, however, does not cause a shift in band II, which suggests that the OMe group is at C-7. The tetraethyl ether of pedalitin (**25**) was prepared in order to utilize chemical shift-structural correlations. The chemical shifts for the ethyl ether of pedalitin obtained in $CDCl_3$ and d_6-benzene are recorded in Table 3. The large solvent shift for the OMe in the pedalitin derivative is consistent with a C-7 OMe group and strongly suggests that the OMe group is not at C-6, C-3′ or C-4′. The latter two positions are also unlikely since Morita reported obtaining 3,4-dihydroxyacetophenone from the alkaline degradation of pedalitin.[7] Thus pedalitin appears to have structure **24**.

Hispidulin* (**22**), obtained as yellow needles, was identified by comparison of its physical properties with those reported by Herz and Sumi.[12] Hispidulin triethyl ether (**23**) was prepared by ethylation with ethyl iodide. The relevant NMR spectral data of this compound are presented in Table 2. Alkaline degradation of hispidulin triethyl ether afforded the acetophenone **15**, identical to the one obtained from eupafolin. The *p*-nitrobenzoates of the acetophenones from both sources were also identical.

The NMR solvent shift technique described by Wilson *et al.*,[8] was found to be useful in assigning provisional methoxyl positions for the flavones reported herein. Wilson *et al.* experienced some difficulty with the solubility of certain flavones in benzene and, to overcome this limitation of the method, our measurements were made on the ethyl ethers of the flavones. It appears from our results that the solvent shifts observed for the OMe groups of the ethyl ethers are in close agreement with those reported for the model compound, hexamethylquercetagetin. In addition the preparation of the ethyl ethers is desirable for degradative studies, as previous workers have shown that alkaline degradations can be performed more efficiently and under milder conditions with fully etherified compounds rather than with the phenols themselves.[13] In an extension of the NMR solvent shift technique, the effect of a change of solvent on the chemical shift of the methylene of the OEt groups was measured. In this manner information concerning the position of the original OH groups could be obtained. The data for the methylene shifts are reported in Table 3. It is apparent from the results that shifts of similar magnitude occur for OMe and methylene groups and assignment of OH's to certain positions, namely C-5 and C-6 can be made on the basis of methylene shifts. For other positions on the nucleus, the assignment of OEt groups to particular positions could be made also, but with less assurance. From the results obtained with a limited range of compounds, the application of the technique appears to warrant further investigation.

EXPERIMENTAL

M.ps were determined on the Fisher-Johns m.p. apparatus. The "Unimelt" (Thomas Hoover capillary m.p. apparatus) was used wherever the m.p. is followed by a subscript $_{(U)}$. UV absorption spectra were determined in EtOH with a Beckman DK 2A recording spectrophotometer. IR spectra were determined

* Hispidulin has also been isolated from *Eupatorium rotundifolium* by Dr. M. Maruyama and Mr. R. Bilous in these laboratories.

on a Beckman IR5A IR spectrophotometer. NMR spectra were determined on a Varian A-60A spectrometer. Paper chromatograms were developed with benzene-petroleum ether–MeOH–water (B.P.M.W.), 50:50:1:50 (upper phase) using Whatman No. 4 paper.

Extraction of flavonoids from E. semiserratum. Coarsely ground *E. semiserratum* (air dried leaves, stems, and flowers, 3 kg) were extracted with 95% EtOH. The plant material was covered with fresh charges of the solvent seven times, over a period of 5 days. Evaporation of the extract under reduced press left a semisolid residue. The residue was treated with $CHCl_3$ and partitioned between $CHCl_3$ and water. Evaporation separately of the two phases yielded $CHCl_3$ solubles (D, 364 g), water solubles (B, 212 g) and a material insoluble in either phase (C, 105 g). The $CHCl_3$ extract (364 g) was partitioned between 10% aqueous MeOH and pet. ether and the two phases evaporated separately to yield 10% aqueous MeOH (E, 162 g), pet. ether solubles (G, 77·5 g) and an insoluble solid (F, 23 g).

Isolation. A sample (1·8 g) of the 90% methanolic extract was chromatographed on a column of silicic acid (120 g), eluting with $CHCl_3$ and finally with 5% MeOH–$CHCl_3$, fractions being combined on the basis of paper chromatography (in B.P.M.W. system which was sprayed with $FeCl_3$–$K_3Fe(CN)_6$, 1:1 soln). The first fraction (0·176 g) was rechromatographed on silicic acid (6 g) to give pectolinarigenin (45 mg), which was crystallized from MeOH as pale yellow needles m.p. 217–218°; R_f in B.P.M.W. system, 0·75; λ_{max} 277 mμ (ε 20,100) and 332 mμ (ε 25,100). (Found: C, 64·62; H, 4·59; OMe, 21·61. $C_{17}H_{14}O_6$ requires: C, 64·96; H, 4·46; 2-OMe, 19·74%). The second fraction (0·164 g) was rechromatographed on a silicic acid column (6 g) to give flavonoid K (78 mg), which was crystallized from MeOH as golden yellow rods, m.p. 243–245°. R_f in B.P.M.W. system is 0·23; λ^{max} 258 mμ (ε 22,100); 273 mμ (ε 15,500) and 366 mμ (ε 23,200). (Found: C, 60·22; H, 4·58. $C_{18}H_{16}O_8$ requires: C, 60·00; H, 4·48%). The third fraction (60 mg) was rechromatographed on a silicic acid (2 g) column to give eupatilin (36 mg), which was crystallized from EtOAc, as yellow rhombohedral plates, m.p. 234–236°; R_f in B.P.M.W. system is 0·55; λ_{max} 243 mμ (ε 17,900); 277 mμ (ε 17,000) and 340 mμ (ε 26,300). (Found: C, 62·93; H, 4·61; OMe, 26·7. $C_{18}H_{16}O_7$ requires: C, 62·79; H, 4·68; 3-OMe, 27·0%). The fourth fraction (90 mg) was crystallized from benzene to give flavonoid L (52 mg), yellow needles, m.p. 146–148°; R_f in B.P.M.W. system 0·12; λ_{max} 255 mμ (ε 20,500) and 356 mμ (ε 23,400). (Found: C, 60·81; H, 4·52; -OMe, 32·09. $C_{19}H_{18}O_8$ requires: C, 60·96; H, 4·85; 4-OMe, 33·15%). The fifth fraction (0·348 g), which was eluted with 5% MeOH–$CHCl_3$, was crystallized from dioxan-water to give eupatorin (200 mg), as yellow needles, m.p. 196–198°$_{(U)}$; R_f in B.P.M.W. system 0·15; λ^{alc}_{max} 243 mμ (ε 17,400); 254 mμ (ε 19,300); 274 mμ (ε 19,800) and 342 mμ (ε 27,700). (Found: C, 62·66; H, 4·84; -OMe, 25·03. $C_{18}H_{16}O_7$ requires: C, 62·79; H, 4·68; 3-OMe, 26·40%).

Extraction of Flavonoids from E. cuneifolium. The dried coarsely ground stems, leaves, and flowers of *Eupatorium cuneifolium* (2·5 kg) were extracted continuously with $CHCl_3$ for 5 hr, and the extraction was repeated with a fresh charge of solvent. The combined extracts were concentrated to dryness under reduced press to yield a thick syrup (382 g).

Isolation. The syrup, dissolved in 10% aqueous MeOH (800 ml), was extracted with pet. ether (4 × 600 ml). The combined pet. ether extracts were washed with 10% aqueous MeOH (400 ml) and the aqueous MeOH solns were combined and evaporated to dryness to yield a semisolid residue (260 g). A portion of this residue (130 g) was rechromatographed on silicic acid (3·0 kg), eluting with $CHCl_3$ and MeOH–$CHCl_3$ mixtures. The 1% MeOH–$CHCl_3$ fraction (7·2 g) was triturated with ether (200 ml) to yield an insoluble residue (1·6 g) which was rechromatographed on silicic acid (90 g). A fraction (170 mg), eluted with 1% MeOH–$CHCl_3$ was crystallized from MeOH to give yellow crystals (68 mg) of hispidulin, m.p. 288–289°$_{(U)}$ (lit. m.p. 291–292°).[12] The 2% MeOH–$CHCl_3$ fraction (29·1 g) was rechromatographed on silicic acid (1·1 kg), eluting with 0·5% MeOH–$CHCl_3$ to afford a hispidulin-rich fraction (795 mg). Trituration of this fraction with ether (10 ml), followed by $CHCl_3$ (5 ml), yielded an insoluble residue (110 mg) which was crystallized from MeOH to give yellow crystals (74 mg) of hispidulin, m.p. 288–289°$_{(U)}$.

The fraction eluted with 5% MeOH–$CHCl_3$ was evaporated to dryness (21·8 g) and triturated with $CHCl_3$ (200 ml). The insolubles were removed by filtration, washed with $CHCl_3$ (50 ml) and dried (6·9 g). Crystallization from MeOH afforded yellow needles of eupafolin (2·7 g), m.p. 271–273°$_{(U)}$; λ^{alc}_{max} 253 mμ (ε 10,200), 272 mμ (ε 10,100) 342 mμ (ε 17,300). (Found: C, 60·57; H, 3·92. $C_{16}H_{12}O_7$ requires: C, 60·76; H, 3·82%).

Acetylation of flavonoids

Eupatorin diacetate (2). Eupatorin (0·10 g) was refluxed with Ac_2O (2 ml) and a few drops pyridine for 2 hr over a direct flame. The reaction mixture was poured into ice water and allowed to stand overnight. The white insoluble material was filtered, dried and crystallized from benzene–pet. ether to give needles

(**2**, 78 mg), m.p. 176–177°; λ_{max} 233 mμ (ε 27,500); 265 mμ (ε 18,300) and 319 mμ (ε 37,200). (Found: C, 61·76; H, 4·79. $C_{22}H_{20}O_9$ requires: C, 61·68; H, 4·71%).

Eupatilin diacetate (**12**). Eupatilin (50 mg) was acetylated by the above method. The product was crystallized from benzene-pet. ether to give **12** (14 mg), m.p. 220–221°.

Pectolinarigenin diacetate (**17**). Pectolinarigenin (39 mg) in Ac_2O (1·0 ml) was treated with 60% perchloric acid (2 drops). After 30 min, the product was poured into ice-water, when a white solid separated. The solid was crystallized twice from $CHCl_3$-pet. ether to give **17**, m.p. 152–158° (lit. m.p. 155·5–156·5°).[9]

Eupafolin tetraacetate (**19**). Eupafolin (75 mg) was refluxed with Ac_2O (2 ml) and pyridine (0·25 ml) in an apparatus protected by a $CaCl_2$ drying tube. After 20 hr, the mixture was poured onto ice (25 g). The aqueous soln was extracted with $CHCl_3$ and the $CHCl_3$ extracts were dried over Na_2SO_4 and concentrated *in vacuo*. The residual white solid (0·108 g) was recrystallized from MeOH to afford white needles (**19**), m.p. 181–183°$_{(U)}$; λ_{max} 301 mμ (ε 18,000), 265 mμ (ε 17,700). (Found: C, 59·43; H, 4·07. $C_{24}H_{20}O_{11}$ requires: C, 59·51; H, 4·16%).

Etherification of flavonoids

5,6,7,3′,4′-*Pentamethoxyflavone* (**3**). (a) Eupatorin (0·10 g) was refluxed on a steam bath with anhydrous (freshly ignited) K_2CO_3 (2 g), distilled Me_2SO_4 (0·5 ml), and dry acetone (20 ml) for 8 hr, with a $CaCl_2$ guard tube. The acetone was evaporated, water was added and the material was filtered. The insoluble material, dissolved in benzene, was chromatographed on a column of neutral alumina (Woelm grade 1), eluting successively with benzene and 5% EtOAc–benzene. The first yellow fraction was concentrated and crystallized from benzene–pet. ether, to give **3**, (45 mg), m.p. 175–176° (lit. m.p. 177°)[6]; λ_{max} 240 mμ (ε 25,900); 265 mμ (ε 16,500) and 328 mμ (ε 28,600).

(b) Eupatilin (0·10 g) was methylated by the procedure used for eupatorin. The product was crystallized from benzene–pet. ether to give pale yellow needles, **3** (62 mg), m.p. 176–177°. The sample was shown to be identical to 5,6.7.3′,4′-pentamethoxyflavone, prepared from eupatorin, by m.p., mixed m.p. and IR spectral comparison.

(c) Eupafolin (0·10 g) was refluxed with Me_2SO_4 (1 ml), dry K_2CO_3 (3·0 g), and dry acetone (20 ml) for 20 hr. The product, obtained as a green oil (0·187 g) was chromatographed on neutral alumina (10 g). Elution with $CHCl_3$ afforded light yellow crystals, **3**, (80 mg) m.p. 174–175°$_{(U)}$. This material was shown to be 5,6,7,3′,4′-pentamethoxyflavone, identical to that prepared from eupatilin, by m.p., mixed m.p. and IR spectral comparison.

5-*Hydroxy*-6,7,3′,4′-*tetramethoxyflavone* (**4**). Eupatorin (0·1 g) in MeOH (40 ml) was treated with excess diazomethane[14] in ether at 0° for 2 hr, and allowed to stand at room temp for 2 hr. The solvent was removed and the product, dissolved in $CHCl_3$, was chromatographed on silicAR CC-7 (12 g) using $CHCl_3$ as solvent. The first fraction eluted (64 mg) was crystallized from benzene-hexane to give pale yellow needles, **4**, (64 mg) m.p. 190–191°; λ_{max} 242 mμ (ε 20,400), 275 mμ (ε 19,400) and 339 mμ (ε 27,500). The m.p., IR spectrum, and R_f in the paper chromatographic system B.P.M.W. were identical to those of an authentic sample of 5-hydroxy-6,7,3′,4′-tetramethoxyflavone generously forwarded by Professor N. Morita. Admixture of the authentic sample with **4** did not depress the m.p. of **4**.

5-*Acetoxy*-6,7,3′,4′-*tetramethoxyflavone* (**5**). Compound **4** (15 mg) was refluxed with Ac_2O (1 ml) and a drop of pyridine for 2 hr over a direct flame, in the same manner as for the preparation of eupatorin diacetate. The product on crystallization from benzene–pet. ether gave **5** (7 mg), as white needles, m.p. 167–168° (lit. m.p. 179–180°).[7]

Eupafolin tetraethyl ether (**20**). Eupafolin (0·5 g) was refluxed with EtI (10 g), dry K_2CO_3 (10 g), and dry acetone (40 ml) for 40 hr. The mixture was filtered and the solvent removed by distillation *in vacuo*. The residue was dissolved in ether (100 ml), washed with distilled water (50 ml), dried over Na_2SO_4 and distilled *in vacuo* to afford a light green solid (0·70 g, m.p. 104–112$_{(U)}$). Chromatography on neutral alumina (15 g), eluting with $CHCl_3$–benzene (1 : 4), gave a yellow solid (0·590 g). Recrystallization from EtOAc-cyclohexane provided white needles, **20**, m.p. 124–126°$_{(U)}$; λ_{max} 238 mμ (ε 17,400) 267 mμ (ε 7900). (Found: C, 67·57; H, 6·44. $C_{24}H_{28}O_7$ requires: C, 67·27; H, 6·59%).

Hispidulin triethyl ether (**23**). Hispidulin (0·320 g) was refluxed with EtI (10 g), anhyd K_2CO_3 (8 g), and dry acetone (25 ml) for 28 hr. The product was obtained as described for eupafolin tetraethyl ether. The triethyl ether gave white needles, **23**, (0·270 g) m.p. 140–142°$_{(U)}$ (lit. m.p. 139–140°)[12] after three recrystallizations from acetone–Skellysolve B.

Eupatorin diethyl ether (**6**). Eupatorin (0·150 g) was refluxed with EtI (3 g), dry K_2CO_3 (3 g), and dry acetone

(20 ml) for 36 hr. The product (0·18 g), obtained as described for eupafolin tetraethyl ether, was crystallized from benzene to yield pale yellow needles, **6**, m.p. 146–147°$_{(U)}$; λ_{max}^{MeOH} 240 mμ (ε 22,100) 265 mμ (ε 12,600) 338 mμ (ε 27,200). (Found: C, 66·07; H, 5·94. $C_{22}H_{24}O_7$ requires: C, 65·99; H, 6·04%).

Eupatilin diethyl ether (**13**). Eupatilin (0·150 g) was refluxed with EtI (3 g), dry K_2CO_3 (3 g), and dry acetone (20 ml) for 36 hr. The product (0·135 g), obtained as described for eupafolin tetraethyl ether, was crystallized without chromatography from EtOAc-cyclohexane to yield pale yellow needles, **13**, m.p. 164–166°$_{(U)}$; λ_{max}^{MeOH} 240 mμ (ε 21,300) 266 mμ (ε 11,800) 338 mμ (ε 25,800). (Found: C, 66·15; H, 6·20. $C_{22}H_{24}O_7$ requires: C, 65·99; H, 6·04%).

Pedalitin tetraethyl ether (**25**). Pedalitin (0·015 g) was refluxed with EtI (0·060 g), anhyd K_2CO_3 (0·10 g), and dry acetone (5 ml) for 36 hr. The product was obtained as described for eupafolin tetraethyl ether. The tetraethyl ether after three recrystallizations from EtOAc–cyclohexane gave white prisms, **25**, (5 mg), m.p. 135–137°$_{(U)}$; (Found: C, 67·21; H, 6·72. $C_{24}H_{28}O_7$ requires: C, 67·27; H, 6·59%).

Alkaline degradation of eupatorin (**1**). Eupatorin (0·50 g) in EtOH (10 ml) was refluxed with 50% KOH aq (40 ml) for 20 hr, over a direct flame, in an atmosphere of N_2. The soln was cooled, acidified and extracted thoroughly with ether. The ethereal extract was treated repeatedly with 8% $NaHCO_3$ aq to remove the acid. The residual ether soln was dried with Na_2SO_4, evaporated, and the residue treated with distilled pet. ether. The pet. ether extract, on concentration, became cloudy, and after standing for 4 days formed colorless needles of **7**, (14 mg), m.p. 76–77° (112–113° after drying *in vacuo*.) (lit. m.p. 76–77°, after drying *in vacuo*, 115–116°).[15]

The $NaHCO_3$ extract was acidified carefully and extracted with ether. The ether extract was dried with Na_2SO_4 and the ether removed. The residue when crystallized from water gave **8a**, (81 mg), m.p. 254–256°.

Alkaline degradation of eupafolin tetraethyl ether (**20**). The tetraethyl ether **20** (0·38 g) was refluxed in a mixture of 50% KOH aq (30 ml) and EtOH (7 ml) under N_2 for 20 hr. The reaction mixture was cooled, acidified with 20% H_2SO_4, and extracted with ether (200 ml). The ether was washed with 5% $NaHCO_3$ (4 × 50 ml), dried over Na_2SO_4, and distilled *in vacuo* to afford the crude acetophenone (0·125 g) as a yellow oil. Chromatography on silicAR CC-7 Mallinckrodt (15 g) afforded (**15**) as a colorless oil (0·085 g) that was spectrally homogeneous (NMR). This material was further characterized by conversion into the *p*-nitrobenzoate, which was crystallized from MeOH as pale yellow needles, m.p. 83–84°$_{(U)}$ (lit., m.p. 74°).[10] The sample was identical (m.p., mixed m.p., IR, TLC) with an authentic sample obtained by preparation of the *p*-nitrobenzoate of the acetophenone derived from the alkaline degradation of **23**.

The bicarbonate extract was acidified with dil HCl and extracted with ether (4 × 50 ml) which was dried over Na_2SO_4 and distilled *in vacuo* to afford a white solid (0·145 g), m.p. 149–156°$_{(U)}$. Two recrystallizations from water gave **21**, as white needles, m.p. 163–164°$_{(U)}$ (lit., m.p. 165).[16]

Alkaline degradation of hispidulin triethyl ether (**23**). The alkaline degradation of **23** (0·135 g), using the same conditions as for eupafolin tetraethyl ether, gave **15**, (0·035 g) after chromatography of the neutral fraction on silicAR CC-7. This material was shown to be identical to the acetophenone obtained from eupafolin tetraethyl by comparison of the TLC behavior, IR and NMR spectra. The *p*-nitrobenzoates were also shown to be identical (m.p. and mixed m.p.).

Alkaline degradation of eupatorin diethyl ether (**6**). The alkaline degradation of **6** (140 mg), using the same conditions as for eupafolin tetraethyl ether, gave an acidic and a neutral material. The acid material (45 mg) was crystallized from EtOH–water to afford colorless microcrystals (35 mg) of **8b**, m.p. 160–162°$_{(U)}$ (lit. m.p. 165–166°).[16] The neutral material (60 mg) after chromatography on silicAR CC-7 (10 g) afforded **9**, as a colorless oil (36 mg) which was spectrally homogeneous (NMR). This material was further characterized by preparation of its *p*-nitrobenzoate which was crystallized from MeOH to afford pale yellow needles, m.p. 106–107°$_{(U)}$. (Found: C, 59·47; H, 5·11; N, 3·59. $C_{18}H_{19}NO_9$ requires: C, 59·83; H, 5·30; N, 3·88%).

Alkaline degradation of eupatilin diethyl ether (**13**). The alkaline degration of **13** (120 mg) using the same conditions as for eupafolin tetraethyl ether gave an acidic and a neutral matrial. The acidic material was crystallized from EtOH–water to afford colorless microcrystals (32 mg) of **14**; m.p. 178–180°$_{(U)}$ (lit., m.p. 180–182°).[16] The neutral material (60 mg) after chromatography on silicAR CC-7 (10 g) afforded **15**, as a pale yellow oil (38 mg) which was spectrally homogeneous (NMR). This material was further characterized by conversion into the *p*-nitrobenzoate which was crystallized from MeOH as pale yellow needles, m.p. 79–81°$_{(U)}$. The sample was identical (m.p., mixed m.p., IR absorption spectrum, and TLC) with an authentic sample obtained by preparation of the *p*-nitrobenzoate of the acetophenone derived from the alkaline degradation of hispidulin.

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