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PLANT ANTIMUTAGENIC AGENTS, 2.[1] FLAVONOIDS

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ABSTRACT.—A number of known prenylated flavonoids were isolated from *Psoralea corylifolia* using an assay procedure based on inhibition of the mutagenic action of 2-aminoanthracene on *Salmonella typhimurium* (T-98). All of these compounds were toxic rather than antimutagenic or desmutagenic. Bakuchiol [**17**], a known prenylated phenolic terpene, was also isolated; its activity was not due to toxicity. Biochanin A [**4**], a known isoflavone, was similarly isolated from *Cicer arientinum* and was active and nontoxic. Some of the above flavonoids were studied for inhibition of the mutagenicity of several different mutagens with results depending upon the structure of the flavonoid and the mutagen.

Flavonoids are probably the most ubiquitous class of compounds found in nature as secondary plant metabolites. These compounds have been reported to have a broad spectrum of biological activities (1) including frequently reported mutagenic (2) and less frequently reported antimutagenic activities (3,4). During an extensive screening of plant extracts for inhibition of the mutagenic activity of 2-aminoanthracene (2AN) (5), it was found that the crude solvent extract of seeds of *Psoralea corylifolia* L. (Leguminosae) was highly active, inhibition activity (IA) >95% (5). In this paper we wish to report the isolation from this plant of a number of flavonoids and a phenolic terpene, all of which have high IA. The inhibition of the mutagenicity of 2AN and several other mutagens by the above compounds and other flavonoids was determined. Whether the observed inhibition was due to toxicity or antimutagenic activity is discussed.

EXPERIMENTAL

MUTAGENIC INHIBITION.—Inhibition of the mutagenic activity of 2AN toward *Salmonella typhimurium* (T-98) by the various flavonoids was determined by procedures described by us in detail in a previous paper (5). A concentration of 2.5 μg/plate of 2AN was used in all cases; initial concentration of test substance was 600 μg and in some cases (i.e., toxicity or activity determinations) concentrations of 300 and 150 μg were also assayed. In a few cases the inhibitory action of flavonoids was studied on other mutagens; e.g., acetylaminofluorene (AAF), and benzo[*a*]pyrene (B[*a*]P), which require metabolic activation with the Ames S-9 enzyme preparation (6) and 2-nitrofluorene (2NF), which does not require metabolic activation. The concentrations (μg/plate) of various mutagens were 25 (AAF), 1.0 (B[*a*]P), and 2.5 (2NF), respectively. The average numbers of colonies of positive controls (i.e., no test substance present) were 2AN (2500), AAF (743); B[*a*]P (351), and 2NF (533). In most cases the colonies were counted after 72 h incubation at 37.5°. Toxicity determinations (only for 2AN studies) were conducted in the absence of mutagen but presence of histidine, S-9, and test substance (5).

GENERAL ISOLATION AND CHARACTERIZATION PROCEDURES.—Melting points were determined on a Kofler hotstage microscope and are uncorrected. ^{1}H- and ^{13}C-nmr spectra were obtained with a Bruker WM250 spectrometer using TMS as internal standard. High resolution mass spectra (hrms) were obtained with an AEI MS-902 instrument. Uv spectra were obtained in MeOH with a Varian 2290-UV-VIS spectrometer and ir spectra with a Perkin Elmer 467 Grating spectrometer. Standard chromatography was carried out on Si gel E. Merck 230–240 mesh, or Baker Flash chromatography Si gel using in general CH_2Cl_2 as eluent with a gradient of 0.5–10.0% MeOH, collecting 15-ml fractions with an automatic fraction collector. For tlc determinations, precoated Si gel plates were utilized; normal phase, EM precoated Si gel 60, F254, usual solvent 10% MeOH in CH_2Cl_2; reversed phase, Baker precoated Si gel C_{18}-F plates, usual developer 5–10% H_2O in MeOH. Exposure of plates to iodine vapor was used as a general detection agent; alternatively spraying with phosphomolybdate reagent followed by heating was utilized. Preparative hplc was conducted utilizing a Waters Model Prep-3000 instrument. In most cases a Dynamax reversed-phase

[1]For Part 1 of the series, see M.E. Wall *et al.* (5).

TABLE 1. Inhibition of Mutagenicity of 2-Aminoanthracene by Various Flavonoids.

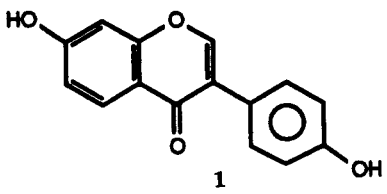
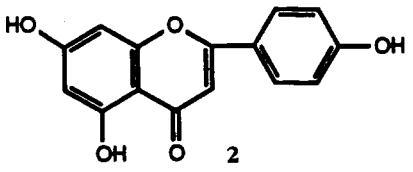
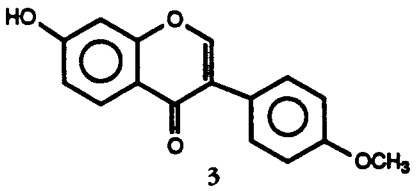
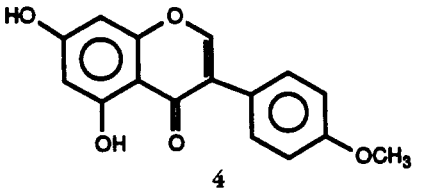
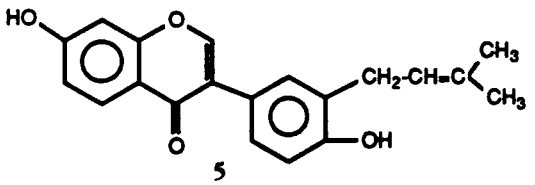
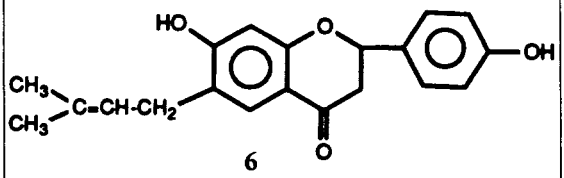
Compound	Structure	% Inhibition of 2 AN Mutagenicity Dose 600 μg/plate
Daidzein[a]	 1	32%
Apigenin	 2	10%
Formononetin[a]	 3	0%
Biochanin A[a]	 4	57%
Neobavaisoflavone[a]	 5	97%
Bavachin[a]	 6	62%

TABLE 1. Continued.

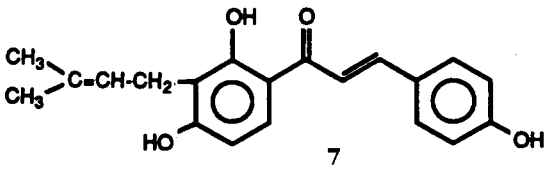
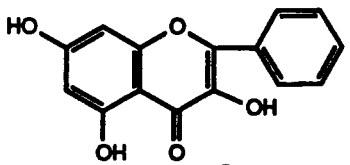
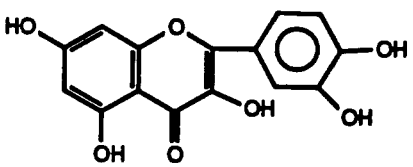
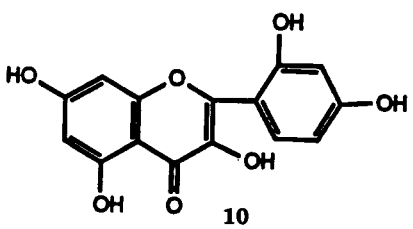
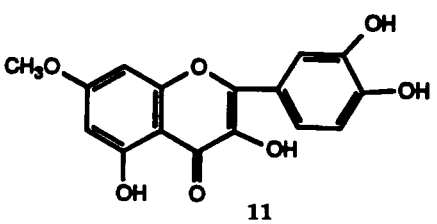
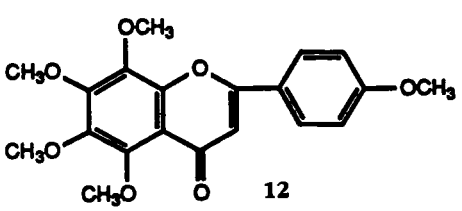
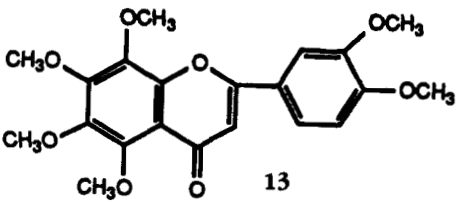
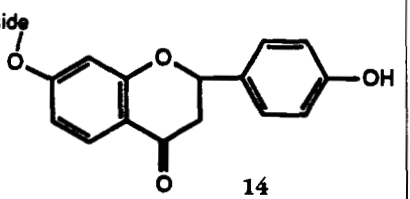
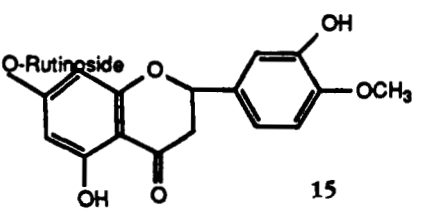
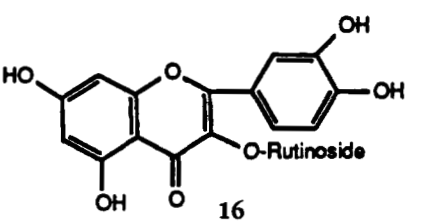
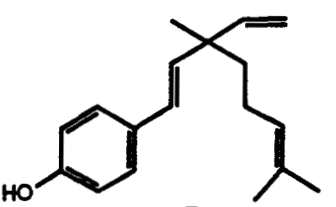
Compound	Structure	% Inhibition of 2 AN Mutagenicity Dose 600 μg/plate
Isobavachalcone[a]	 7	86%
Galangin	 8	97%
Quercetin	 9	20%
Morin	 10	0%
Rhamnetin	 11	78%
Tangeretin	 12	42%

TABLE 1. Continued.

Compound	Structure	% Inhibition of 2 AN Mutagenicity Dose 600 μg/plate
Nobiletin	 13	43%
Naringin	 14	38%
Hesperidin	 15	13%
Rutin	 16	0%
Bakuchiol[a]	 17	81%

[a]Isolated at Research Triangle Institute.

C_{18} column (2.15 × 25 cm) was utilized, with 10–50% H_2O in MeOH as solvent; for highly nonpolar compounds a similar Dynamax 10μ Si column was used.

PLANT MATERIAL.—*P. corylifolia* was supplied through the auspices of the Drug Research and Development Branch, National Cancer Institute by the Medicinal Plant Resources Laboratory, Plant Genetics and Germplasm Institute, Agricultural Research Service, USDA, Beltsville, MD. A herbarium specimen documenting this collection is deposited in the Herbarium of the National Arboretum, Agricultural Research Service, USDA, Washington, D.C. The plant material (seeds) was collected in India in February, 1974. *Cicer arientinum* L. (Leguminosae) seeds were imported from India via a commercial source in North Carolina. A voucher specimen is on deposit in our laboratory.

P. corylifolia seeds (200 g) were extracted with refluxing EtOH, the solvent was concentrated in vacuo, and the residue was partitioned between CH_2Cl_2 and H_2O. The CH_2Cl_2 extract showed 2AN inhibition activity of 75% and 98% in successive 2AN inhibition assays (5). The CH_2Cl_2 extract was concentrated in vacuo and the residue partitioned between MeOH-H_2O (90:10) and petroleum ether. The aqueous MeOH extract (weight 8.9 g) gave IA of 88%. It was chromatographed on 200 g of Si gel (230–400 mesh) with 1% MeOH in CH_2Cl_2 as the packing solvent and initial eluent. Fractions (15-ml) were collected, and fraction combinations were made based on similarity of tlc patterns and retention times.

Fractions 11–27.—These fractions were eluted with 1% MeOH in CH_2Cl_2. They contained one major component and gave high IA values. After further chromatography on Si gel, eluting with C_6H_{14}-CH_2Cl_2 (1:1), 1.3 g of an oil was obtained. The hrms of oil showed $[M]^+$ to be 256.1825 (calcd for $C_{18}H_{24}O$, 256.1827). The ir, uv, and ^{1}H-nmr spectra were all in accord with those of the known phenolic terpene, bakuchiol [**17**] (Table 1), previously isolated from *P. corylifolia* by Mehta *et al.* (7). The 2AN inhibition assay of bakuchiol at a dose of 600 μg/plate gave IA values of 81 and 53% in successive assays.

Fractions 134–142.—These fractions were eluted with 2% MeOH in CH_2Cl_2. After combining they weighed 0.5 g, and showed very high IA, 97%. The combined fraction (100 mg) was subjected to preparative reversed-phase hplc on a Dynamax C_{18} column [2.15 × 25 cm, MeOH-H_2O (80:20), flow rate 12 ml/min]. The major fraction collected (Rt 12–21 min) contained a pure, crystalline yellow solid, mp 158–160°. The hrms of this compound showed $[M]^+$ to be 324.1362 (calcd for $C_{20}H_{20}O_4$, 324.1361). The ms, uv, ir, and nmr spectra of this compound showed that it was the known isobavachalcone [**7**], isolated from *P. corylifolia* seeds by Bhalla *et al.* (8). The 2AN inhibition assay at a dose of 600 μg/plate gave IA values of 79 and 86% in two successive assays.

Fractions 179–186.—Further elution with 2% MeOH in CH_2Cl_2 gave a fraction which weighed 0.2 g. After additional preparative tlc of this fraction on Si gel (20 × 20 × 0.25 cm, 5% MeOH in $CHCl_3$), a crystalline, cream-colored compound was obtained (mp 215°). The hrms of this compound showed $[M]^+$ to be 324.1337 (calcd for $C_{20}H_{20}O_4$, 324.1361). The ms, ir, uv, and ^{1}H-nmr spectra were in accord with that of the known prenylated flavonone, bavachin [**6**] (8–10). Bavachin at 600 μg/plate showed 2AN IA of 62% (cf. Table 3 for additional data at lower concentrations).

Fractions 232–255.—After elution with 4% MeOH in CH_2Cl_2, the entire crude fraction (350 mg) was subjected to preparative hplc on a Dynamax C_{18} column (2.15 × 25 cm, 70% MeOH-30% H_2O, flow rate 10 ml/min). A number of fractions with Rt 24–37 min were collected under a single broad peak. These fractions (170 mg) had identical R_f values and appeared pure by reversed-phase tlc. On concentration of the eluting solvent, crystallization ensued to give a colorless compound, mp 189–191°. The hrms showed $[M]^+$ to be 322.1200 (calcd for $C_{20}H_{18}O_4$, 322.1205). A literature search showed that several prenylated flavones or isoflavones had the above formula: e.g., licoflavone A (6-*C*-prenyl-7,4′-dihydroxyflavone) (11), 6-*C*-prenylchrysin (12), and neobavaisoflavone [**5**] (13). That the isolated compound was indeed **5** was evident from the mp 187–191° and the ir, uv, and ^{1}H-nmr spectra which were in accord with the literature

TABLE 2. Toxicity of Flavonoids to *Salmonella typhimurium* (T-98).[a]

Compound	Dose (μg/plate)		
	600	300	150
Diadzein [**1**]	NT	NT	
Biochanin A [**4**]	NT	NT	
Neobavaisoflavone [**5**]	ST	ST	
Bavachin [**6**]		T	NT
Isobavachalcone [**7**]	ST	ST	
Quercetin [**9**]	T	T	
Morin [**10**]	Slt. T	NT	
Tangeretin [**12**]	T	NT	
Nobiletin [**13**]	NT	NT	
Naringin [**14**]	T	Slt. T	
Hesperidin [**15**]	NT	NT	
Rutin [**16**]	NT	NT	
Bakuchiol [**17**]	Slt. T	NT	NT

[a]Slt. T = slight toxicity, <30% inhibition; T = toxic, 50% inhibition; ST = strongly toxic, >90% inhibition; NT = nontoxic, <20% inhibition.

TABLE 3. Percent Inhibition of Mutagenic Activity by Various Flavonoids.

Compound	Micrograms Mutagen/Plate											
	2AN[a,e]			AFF[b,e]			B[*a*]P[c,e]			2NF[d]		
	600	300	150	600	300	150	600	300	150	600	300	150
Flavonoid												
Apigenin [2]	11	0	0	0	14	0	75	64	67	88	70	60
Neobavaisoflavone [5][f]	97	67	98	96	72	2	97	74	17	66	53	15
Bavachin [6][f]	62	98	98		0	0		0	0		72	52
Quercetin [9]	20	24	16	0	0	0	0	0	0	0	0	0
Rhamnetin [11]	78	0	0		52	11		45	7		41	34
Terpene phenol												
Bakuchiol [17][f]	81	84	91	0	0	0	43	28	0	92	79	75

[a]2-Aminoanthracene.
[b]Acetylaminofluorene.
[c]Benzo[*a*]pyrene.
[d]Nitrofluorene.
[e]Requires metabolic activation by S-9.
[f]Isolated at Research Triangle Institute.

(13). The isoflavone nature of **5** was readily apparent from the ^{1}H-nmr spectra, particularly the C-2 proton which appeared as a singlet at δ 8.26.

Biochanin A [**4**] and formononetin [**3**] were isolated from the seeds of *C. arientum* by literature procedures (14). Hydrolysis of **3** gave daidzein [**1**]. The IAs of these compounds were **1**, 32%; **3**, 0%, and **4** 57% (cf. Table 1). None were toxic.

RESULTS AND DISCUSSION

P. corylifolia seeds have been reported to cause photodermatitis in man; decoctions of the plant have been used as a gargle and for carious teeth (15). The plant has been the subject of chemical investigations for at least 90 years and a number of furanocoumarins have been isolated (16,17). In the studies reported in this investigation the known prenylated phenolic terpene bakuchiol [**17**] was the major compound found based on the 2AN assay procedure. Bakuchiol has been reported to inhibit the growth of *Staphylococcus aureus* at concentrations of 2–4 μg/ml (18–20). Toxicity studies of bakuchiol indicated that it was slightly toxic to *Sa. typhimurium* (T-98) at 600 μg/plate and nontoxic at 300 and 150 μg/plate (Table 3). The prenylated chalcone isobavachalcone [**7**], the prenylated flavanone bavachin [**6**], and the prenylated isoflavone neobavaisoflavone [**5**] have all been reported previously (8). Toxicity tests showed that all of these compounds strongly inhibited *Sa. typhimurium*. The effect in the case of most of these compounds was clearly due to toxicity rather than antimutagenic or desmutagenic action (Table 3).

Seeds of *C. arientinum* (chick pea) are widely used as a food in India. Infusions of chick pea were an ancient remedy to dissolve bladder and kidney stones (20). The isoflavones biochanin A [**4**] and formononetin [**3**] were isolated in low yield; the former had IA of 57%, the latter was inactive. Biochanin A was nontoxic (Table 3). Diadzein [**1**], obtained by the demethylation of formononetin [**3**], was moderately active, IA 32%, and nontoxic.

STRUCTURE-ACTIVITY RELATIONSHIPS.—Small quantities of a number of common, nonprenylated flavonoids were available in our laboratory, and hence it was of interest to compare the various 2AN inhibition activities of these compounds with those isolated during the present investigation. Table 1 shows these compounds and the 2AN IA data at one high dose (600 μg/plate). The compounds are listed more or less in increasing order of degree of hydroxylation and/or polarity. Some relationships were

clear-cut. Thus, flavonoid glycosides such as **14**, **15**, and **16** seem as a class to have weak or no 2AN inhibitory activity. Isoflavones **1** and **5** showed moderate activity and no toxicity; however, **3** was inactive. The presence of a dimethylallyl (prenyl) group seems to convey very strong IA and/or toxicity. For example, compare **1** and **5** which are identical except for presence of a 3′-prenyl moiety in **5**. Why the prenyl group greatly increases the IA and probably the toxicity in the case of 2AN mutagenicity is not clear at this time, but all three flavonoids, **5** (an isoflavone), **6** (a flavonone), and **7** (a chalcone), bearing the prenyl moiety were very active and/or toxic, as was also the phenolic terpene **17**.

Turning next to more hydroxylated compounds, we see large activity differences with relatively minor structural differences when the active compounds **8** and **11** are compared with the inactive compounds **9** and **10**. Table 2 gives toxicity data for some of the compounds shown in Table 1. The toxicity tests were conducted in the absence of a mutagen but with histidine and S-9 enzyme added. It is apparent that the inhibitory activity of the prenylated flavonoids is due to toxicity, with some evidence that it is due to the dimethylallyl moiety.

COMPARISON OF INHIBITION OF SELECTED FLAVONOIDS BY SEVERAL MUTAGENS.—Table 3 compares the effects of a few flavonoids and a phenolic terpene bakuchiol on four mutagens, at three dose levels. Three of the mutagens, 2AN, AAF, and B[*a*]P, require metabolic activation; the fourth, 2NF, does not. Inspection of Table 3 indicates that in some cases the inhibitory activity of the compound can be strongly influenced by the nature of the mutagen. Thus, apigenin [**2**] is essentially inactive in 2AN and AAF but is very active in inhibition of B[*a*]P. Neobavaisoflavone [**5**] is active in inhibition of every mutagen (possibly due to toxicity), and quercetin [**9**] is inactive in all systems. As discussed above, bakuchiol [**17**], a prenylated phenolic terpene, shows strong inhibition of 2AN. In addition, it also shows very strong inhibition of 2NF and moderate to weak activity in B[*a*]P and is inactive in AAF. A number of questions are raised. One of the most perplexing is the following: All of the assays were conducted with *Sa. typhimurium* (T-98). All the prenylated flavonoids including bavachin [**6**] were shown to be toxic to this organism in the absence of mutagen. Yet, bavachin caused no inhibition of the mutagenic activity of AAF or B[*a*]P (Table 3). In this case, are the mutagen and flavonoid interacting or reacting so that toxicity is avoided?

It is apparent that depending on the structure of the flavonoid and mutagen, the flavonoids can exhibit a variety of activities, ranging from extremely toxic to desmutagenic or possibly even antimutagenic activity. The literature has few examples of flavonoid antimutagenic activity, and some are contradictory. Birt *et al.* (4) found that apigenin [**2**] and robinetin had strong inhibition in 2AN and were not toxic and less active in B[*a*]P. In our hands apigenin [**2**] was virtually inactive in 2AN and showed high activity in B[*a*]P. Mitscher *et al.* (3) showed that glabrene, an isoflavone, was highly antimutagenic and nontoxic against ethyl methanesulfonate induced mutations. This mutagen does not require metabolic activation. It is evident that the mechanism of inhibitory mutagenic activity exhibited by flavonoids is complex and will require much more mechanistic study.

ACKNOWLEDGMENTS

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