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PLANT ANTIMUTAGENIC AGENTS, 1. GENERAL BIOASSAY AND ISOLATION PROCEDURES

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ABSTRACT.—An antimutagenic assay in *Salmonella typhimurium* has been utilized for a study of the inhibition of the mutagenic activity of 2-aminoanthracene in the presence of the Ames S-9 metabolic activation preparation by crude organic solvent extracts of plant materials. More than 2000 extracts representing 39 families have been tested to date. Confirmed inhibitory activity has been found in 80 samples. More than 60% were nontoxic. Methods for isolation and characterization of pure compounds are presented. Of particular interest is the utilization of large scale preparative hplc for rapid purification of inhibitory chromatographic fractions that were still highly impure.

In recent years there has been increasing interest in anticarcinogens of plant origin, particularly in edible plants (1,2). Relatively less work has been carried out on wild plants; the literature shows a few studies, largely from Kada's laboratory 3–5, and some work from Mitscher's laboratory (6). It is now becoming clear that higher plants contain a variety of preformed secondary metabolites that represent a structurally diverse array of mutagenic, antimutagenic, and desmutagenic substances. Study of such substances can lead to much interesting detail about the processes of mutagenicity and antimutagenicity. In addition, it is possible that some of the plant antimutagens could have therapeutic use, such as prophylaxis for such high risk groups as petrochemical workers, etc. According to Kada *et al.* (3), antimutagens can be classified into two categories, desmutagens and bioantimutagens. Desmutagens are agents that cause chemical or biochemical modifications of mutagens outside cells; bioantimutagens increase the fidelity of DNA replication, promote the repair of DNA damage, and/or are involved in inhibition of error-prone repair.

A systematic screening of a large number of plants for antimutagenic agents has not been conducted to date. In this report we present rapid methods for screening, isolation, and characterization of antimutagenic or inhibitory agents; these methods utilize relatively small quantities of plant samples or extracts. By these methods 2060 plant samples representing 39 families have been screened in one year. Eighty samples have shown confirmed inhibitory "activity" in a *Salmonella* bacterial test system.

EXPERIMENTAL

ANTIMUTAGENIC SCREENING.—The basic procedures followed were the general mutagenicity procedures of Ames and co-workers (7,8), combined with the specific antimutagenicity procedure of Birt *et al.* (9). Plates were prepared the day before assay (8). Top agar was prepared in 500-ml bottles and stored at 4°. Agar was melted in an autoclave the morning of the experiment (5 min autoclave time) and kept in 45° waterbath until used. The bacterial strain used was *Salmonella typhimurium* (TA 98) obtained from Dr. Bruce Ames, University of California, Berkeley. TA 98 frozen stock (10 μl) was placed in 50 ml of Oxoid nutrient broth. The flask was shaken in a warm room at 100 rpm at 37° for 16 h. Broth (100 μl) containing $1.0–2.0 \times 10^7$ bacteria per plate was used. For metabolic activation a standard S-9 preparation was prepared by the method of Maron and Ames (8). The S-9 preparation was made from the livers of Aroclor-1254-induced Sprague-Dawley male rats. A 5% S-9 mix was made the morning of the experiment and S-9 mix, 500 μl, per plate was used. Bacteria were added to the S-9 mix in a 1:5 ratio. Crude plant samples prepared from CH_2Cl_2- or $CHCl_3$-soluble fractions as described below were added to one-dram vials and diluted with DMSO (Mallinkrodt) to 6 mg/ml on the morning of the experiment. DMSO crude sample solution (100 μl) was added to the bacteria-plus-S-9 mix for a dose of 600 μg/plate. For lower doses, 50, 25, or 10 μl of DMSO solution/plate was utilized. Each dose was tested in triplicate. Pure samples were also tested at this level, but frequently lower concentrations were also tested to determine if there was a dose response.

The mutagen was 2-aminoanthracene (2AN) (9). The sample was weighed and diluted on the morning of the experiment. The 2AN was weighed in a sterile scintillation vial and diluted to 2.5 μg/10 μl in DMSO. This dose produced between 2500 and 3000 revertant colonies/plate. As a positive control, the 2AN without added antimutagen was employed. After every set of five samples, a 2AN positive control (triplicate plates) was performed.

ASSAY PROCEDURE.—Into sterile 13 × 100 mm culture tubes the following components were added in order: (a) 100 μl of bacteria plus 500 μl of S-9 mix, (b) either 50 μl or 100 μl of antimutagen sample (to give 300 or 600 μg/plate, respectively), (c) 10 μl of 2AN solution at 2.5 μg/10 μl concentration.

The S-9-plus-bacteria mix and the 2AN were added with an Eppendorf Repeater Pipettor. The antimutagen samples were added with a Pipetman P100 and P200. A rack of 72 culture tubes at a time received aliquots.

The rack was covered with foil and placed in a rack holder in the shaker H_2O bath and shaken at 100 rpm at 37° for 20 min. Racks were immediately removed from the shaker and allowed to sit at room temperature until plating occurred (usually not longer than 20 min). A Cornwall syringe was used to make aliquots of the top agar (2.0 ml) into the 13 × 100 mm culture tubes, 36 tubes at a time. Each tube was vortexed for about 2 sec and poured onto base layer plates. Plates were allowed to harden, then inverted and incubated for 48–72 h at 37° in a vented and timed bacterial incubator. The longer incubation periods were particularly useful when dealing with toxic samples. The number of colonies per plate was counted using an ARTEX 880 Cell Counter.

CALCULATIONS.—The % inhibition of mutagenicity was calculated for all samples as follows:

$$\% \text{ Inhibition} = [1 - (N_T/N_C)] \times 100$$

where N_T = the number of revertant colonies observed on test plates (plant extract with 2AN) and N_C = the average number of colonies on control plates (2AN with no plant extract).

INTERPRETATION OF DATA.—Compounds were classified as positive antimutagens based on the % inhibition of the mutagenicity of 2AN. Data were qualitatively ranked according to the following scheme:

% Inhibition	Ranking for Antimutagenicity
0–20%	Negative (−)
20–40%	Weak (W +)
40–60%	Positive (+)
60–90%	Strong (S +)
>90%	Toxicity suspected (T)

Positive antimutagens should be dose-responsive (more antimutagenic at the 600 μg/plate dose than at the 300 μg/plate dose). Inhibition values greater than 90% were suggestive of a toxic effect, but quantitative toxicity data was obtained on all samples from W + to T as described below. Samples with positive inhibitory responses were reassayed (new sample, new assay). A sample was considered confirmed if two consecutive or two out of three consecutive assays showed 30% or better 2AN inhibition. Toxicity was evaluated in a separate assay by plating 2000–3000 *Salmonella* colonies on complete medium (containing histidine) in the presence of the test substance and absence of mutagen. Inhibition values >50% indicated that the test substance was toxic rather than desmutagenic or antimutagenic.

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 were obtained with an AEI MS-902 instrument. Uv spectra were obtained in MeOH with a Varian 2290-UV-VIS spectrometer, ir spectra with a Perkin-Elmer 467 Grating spectrometer. Standard chromatography was carried out on Si gel E. Merck 230–40 mesh or Baker Flash chromatography Si gel using in general $CHCl_2$ as eluent with a gradient of 0.5–10% 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 centrifugal tlc was performed on a chromatotron, Model 7924, Harrison Research. Rotors were poured using Si gel PF_{254} (E. Merck). Preparative hplc was conducted utilizing a Waters Model Prep-3000 instrument. In most cases a Dynamax reversed-phase C_{18} column, 2.15 × 25 cm, was utilized, with 10–50% H_2O in MeOH as solvent phase; for highly nonpolar compounds a similar Dynamax 10μ Si column was used.

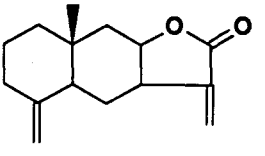
EXTRACTION AND ISOLATION PROCEDURES.—EtOH extracts were prepared by percolation at room temperature or hot continuous extraction. After concentration of the EtOH extract to a syrup at 35–

40°, the residual material was partitioned between equal volumes of H_2O and CH_2Cl_2 or $CHCl_3$ in our usual manner (10). The organic-solvent-soluble fraction was then concentrated, dissolved in a minimal volume of CH_2Cl_2 or $CHCl_3$, and subjected to flash chromatography. For the relatively small quantity of extract, usually from 100–200 g of plant material, Si gel columns (4.5 × 45 cm) containing 100 g of the adsorbent were utilized. Using gradients of 0.5–10% MeOH in CH_2Cl_2, 15-ml fractions were collected and monitored by normal phase tlc. Fractions with similar patterns were combined and, after removal of solvent, weighed and tested for 2AN inhibition. Active fractions with sufficient weights (at least 50–100 mg) were subjected to additional purification by chromatography on a finer grade Si gel such as EM 230–240 mesh, or occasionally on Sephadex LH-20. Frequently, after initial purification by flash chromatography, the active fractions could be purified by one pass through preparative hplc, usually reversed-phase. Fractions (20 ml) were collected and monitored by reversed-phase tlc, and fractions with similar patterns were combined. In most cases, crystalline products were obtained without further treatment. Spectra routinely obtained were hrms, ^{1}H nmr, ir, and uv. Where required, various ^{13}C-nmr and/or 2D-nmr experiments were carried out.

PLANT MATERIAL.—All plant materials were supplied through the auspices of the Natural Products Branch, DCT, National Cancer Institute, by the Medical Plant Resources Laboratory, ARS, USDA, Herbarium specimens documenting these collections are deposited in the Herbarium of the National Arboretum, ARS, USDA.

The following examples illustrate our procedures: *Example 1.—Aristolochia debilis* Sieb et Zucc. (Aristolocheaceae) was collected in China. A CH_2Cl_2 extract of root, stem, and stem bark was shown to have very strong inhibition (>95%) to 2AN mutagenesis. After partitioning between 90% MeOH/10% H_2O and petroleum ether, the MeOH phase was concentrated and chromatographed on Si gel. On elution with 7% Et_2O in C_6H_{14}, two crystalline fractions of 46 and 10 mg were obtained. The major fraction was highly active and/or toxic (inhibition: 99% at 300 μg, 97% at 150 μg, 33% at 75 μg). The minor fraction, which was somewhat more polar, was much less active and/or toxic (inhibition: 35% at 300 μg, 27% at 150 μg). After crystallization from hexane, the mp of the major fraction was 108–110°, and that of the minor fraction was 163–168°. The hrms of the major fraction showed $[M]^+$ to be 232.1440 (calcd for $C_{15}H_{20}O_2$, 232.1463) and that of the minor fraction showed $[M]^+$ to be 234.1624, (calcd for $C_{15}H_{22}O_2$, 234.1620). A computer search of *Chemical Abstracts* indicated that the major compound was the known isoalantolactone [**1**] (11), and the minor constituent was dihydroisoalantolactone [**2**] (11). The mp and ^{1}H nmr of **1** and **2** were in accord with literature values. Toxicity tests on **1** showed it was toxic at doses of 300 and 150 μg per plate, whereas **2** was nontoxic at these doses.

Example 2.—Use of preparative hplc: *Psorolea corylifolia* L. (Fabaceae), seeds, India. A sample of seeds (200 g) was extracted with EtOH. The CH_2Cl_2-soluble fraction obtained as described above gave high inhibition activity (IA), average 85%. The 90% MeOH fraction (8.9 g) (10) also showed IA 85%. After flash chromatography on 200 g Si gel, a number of crude fractions with strong IA activity were obtained as shown in Table 1. Many of these fractions have been further purified and their structures elucidated. The use of preparative hplc for the purification of fractions 219–231 is exemplified in this paper. Silica gel column fraction 219–231 weighing 325 mg was triturated in MeOH-H_2O (7:3) with some heat. The soluble portion was chromatographed using reversed-phase preparative hplc and the following conditions: Dynamax C_{18} 2.15 × 25 cm column, 70% MeOH + H_2O solvent, 210 nm uv detection, 10 ml/min flow



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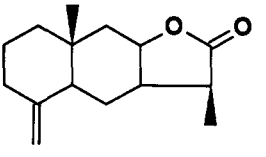

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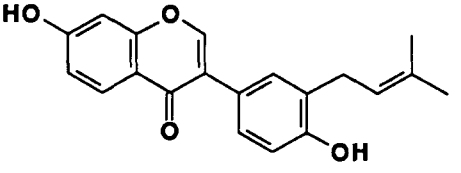

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TABLE 1. Inhibition of Mutagenic Activity of 2-Aminoanthracene (2AN) by Fractions Obtained from Flash Chromatography of the EtOH Extract of Seeds of *Psorolea corylifolia*.

15-ml Fraction	Wt (mg)	Eluting Solvent	Inhibition of 2AN Activity
1–10	35	1% MeOH + CH_2Cl_2	—
11–27	1521	" "	96%
28–46	279	" "	—
47–63	49	" "	—
64–77	62	" "	85%
78–87	67	" "	neg[a]
88–110	107	" "	—
111–125	203	" "	neg
126–133	328	2% MeOH + CH_2Cl_2	—
134–142	577	" "	97%
143–166	568	" "	—
167–173	92	" "	91%
174–178	94	" "	—
179–186	216	" "	98%
187–206	494	" "	56%
207–218	381	" "	—
219–231	325	" "	98%
232–255	345	4% MeOH + CH_2Cl_2	—
256–282	200	" "	neg
283–298	94	" "	—
299–330	129	" "	neg
331–355	106	6% MeOH + CH_2Cl_2	—
356–393	91	" "	—
394–415	55	" "	—
416–445	64	8% MeOH + CH_2Cl_2	—
446–475	82	" "	neg
S.M.[b]	—	" "	95%

[a]neg = negligible.
[b]S.M. = starting material.

rate. The preparative hplc results are shown in Figure 1. The fractions (20 ml) were collected every 2 min and monitored by reversed-phase tlc. Fractions with similar tlc patterns were combined. The fractions collected at 24–36 minutes had similar Rt values, appeared pure (one spot on tlc), and weighed 170 mg. All of the earlier fractions had low weight. As the preparative hplc solvent was concentrated, crystallization ensued. The mol wt of the compound was found to be 322.1200 by hrms (calcd for $C_{20}H_{18}O_4$, 322.1205). Literature search showed that several prenylated flavonoids could have this formula. However, the mp and ^1H nmr of only neobavaisoflavone [**3**] (12,13) were identical to those of the isolated compound.

RESULTS AND DISCUSSION

To date we have obtained about 80 active samples representing 39 families. These are listed in Table 2. It will be noted that only two samples are H_2O-soluble extracts. Activity in this case was found only after extraction of aqueous samples with EtOAc or *n*-BuOH. Pure compounds from six samples have been isolated and characterized to date; we are working on a number of the others. Although we are entitling our new series "Plant Antimutagenic Agents," it should be recognized that, strictly speaking, antimutagenesis takes place only when the potential agent interacts with DNA; prior to this the inactivation of mutagens and carcinogens by whatever means [antioxidants, action on P-450 enzymes, or actual reaction with toxic substances, such as the reaction of ellagic acid with the active metabolite of benzo[*a*]pyrene (14)] is termed desmutagenesis (15). It should also be realized that with the very high dose used for initial

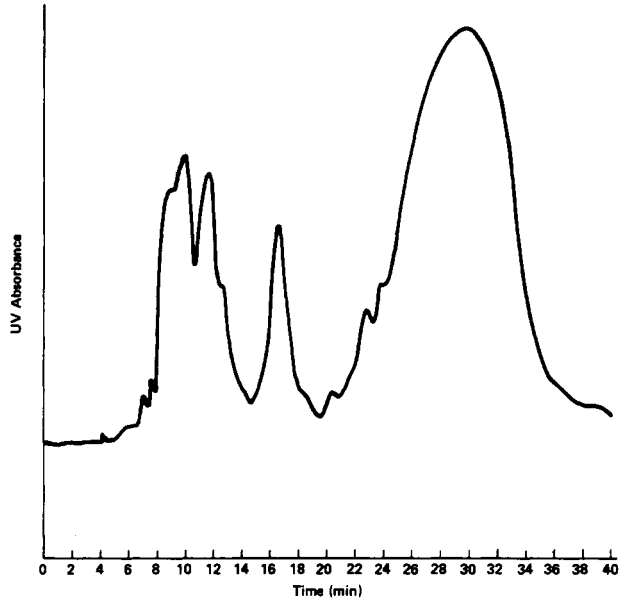

FIGURE 1. Preparative hplc of a crude flavonoid fraction obtained from seeds of *Psorolea corylifolia*.

screening, 600 μg of crude sample, we may be dealing with toxic responses in some cases. The high dose is necessary because the "active" plant constituent may be present in ≤1%. The method, nevertheless, shows considerable selectivity, and the active plants constitute only about 4% of the total screened. As our investigations proceed we will look further into the mechanism of action of the compounds we isolate; at this time we have done only toxicity determinations. All of the active extracts have been tested for toxicity by the standard Ames procedure (8). Of the 80 "active" samples tested to date, approximately 60% are nontoxic, and these will receive highest priority.

The current study is the first in which large-scale screening for antimutagenicity has been carried out using 2AN as the mutagenic agent. 2AN must be metabolically activated. It is believed that like most aromatic amines, hydroxylation on nitrogen occurs first (16), leading to a final metabolite, possibly an ester, which is the active agent. The literature on previous plant antimutagenic studies has been reviewed by Mitscher *et al.* (6). A variety of antimutagenic agents from plants have been screened with various mutagens such as uv irradiation, acriflavine, 9-aminoacridine, and ethyl methane sulfonate. We have recently compared other mutagens, benzo[*a*]pyrene, 2-aminoacetylfluorene, and 2-nitrofluorene with 2AN. The advantage of 2AN was that it is utilized in a published procedure (9), and in our hands it gave reproducible data and about five times more colonies per plate than the others, which leads to greater sensitivity.

Although we have conducted too few assays on H_2O-soluble fractions to make final conclusions, it would seem that organic-solvent-soluble fractions yield a higher percentage of samples active in 2AN inhibition because aqueous samples may contain sufficient histidine to counteract the antimutagenic effect. Hence, in this case it may be necessary to evaluate the test material in the absence of mutagen.

To date using the 2AN assays we have isolated as active pure compounds a series of prenylated flavonoids from *Psoralea corylifolia*; several prenylated coumarins from *Cnidium monnniere*, a new dimeric phenolic compound from *Maesa montana*, and a toxic sesquiterpene from *Aristolochia debilis*. Although only the dimeric compound is new, none of the known compounds have been tested for antimutagenicity previously. Isola-

TABLE 2. Plants Active in 2-Aminoanthracene (2AN) Antimutagenesis Screening.

Plant	Plant Part[a]	Source	Fraction	% Inhibition	Toxicity[b]
Anacardiaceae					
Rhus undulata	tw,lf	South Africa	CH_2Cl_2	45,83	No tox
Annonaceae					
Artobotrys velutimus	tw,lf	Ghana	CH_2Cl_2	99,97	Toxic 300,600
Guatteria acutissima	tw	India	CH_2Cl_2	83,68	Toxic 600
Apiaceae					
Ferula sp.	st,sd,lf,fl	Turkey	EtOH	81,83	Sl tox 600 No tox 300
Cnidium monniere	sd	China	CH_2Cl_2	93,97,90	No tox
Araceae					
Anthuricum lancea	lf	Ecuador	$CHCl_3$	98,94,99	Toxic 300,600
Wettenia angusta	st	Peru	CH_2Cl_2	44,42	No tox
Aristolocheaceae					
Aristolochia debilis	ws,sb,rt	China	CH_2Cl_2	69,89,99	No tox
Asteraceae					
Lagascea mollis	rt,st,lf	India	CH_2Cl_2	96,60,59	No tox
Carpesium abrolanoides	st,lf	China	CH_2Cl_2	42,42	
Mikania cordata	rt,st,lf,fr	India	CH_2Cl_2	62,44,89,47	No tox
Bignoniaceae					
Markhamia obtusifolia	tw,lf	Tanzania	CH_2Cl_2	80,78	No tox
Oroxylum indicum	tw,lf	Thailand	CH_2Cl_2	78,99,89	No tox
Boraginaceae					
Lithospermum erythrorhizin	st	China	CH_2Cl_2	50,53	No tox
Clusiaceae					
Vismia amazonia	tw	Peru	CH_2Cl_2	60,36	No tox
Combretaceae					
Terminalia boivinii	lf	Tanzania	$CHCl_3$	85,87,88	No tox
Euphorbiaceae					
Cleistanthus collinus	fr	India	$CHCl_3$	82,36,63	No tox
Euphorbia corallata	pl	North Carolina	H_2O, EtOAc	24,44	No tox
Fabaceae					
Cassia sp.	rt	Thailand	CH_2Cl_2	50,58	No tox
Indiofera elliptica	sd	Thailand	EtOH	95	Toxic 600
Psoralea corylifolia	sd	India	CH_2Cl_2	75,98,88	No tox
Fagaceae					
Castanopsis hystrix	st,fr	India	EtOH	94	Toxic 600
Flacourtiaceae					
Casearia arborea	st	Costa Rica	CH_2Cl_2	43,46,87	No tox
Lacistema aggregatum	ws,sb	Peru	CH_2Cl_2	44,53	No tox
Guttiferae					
Garcinia mangostana	sd	India	CH_2Cl_2	95,40	No tox
Juglandaceae					
Juglans nigra	fr	North Carolina	H_2O, *n*-BuOH	46,88	No tox
Lamiaceae					
Mentha arvensis	lf	China	CH_2Cl_2	70,80	Toxic 600
Phlomis sp.	st,lf,fl	Turkey	CH_2Cl_2	54,36	No tox
Salvia miltiorrhiza	rt	China	CH_2Cl_2	81,84,78	Toxic 600
Thymus sp.	st,lf,fl,rt	Turkey	CH_2Cl_2	30,44	No tox
Loganiaceae					
Fagraea racemosa	sd	Thailand	EtOH	92	No tox
Marcgraveaceae					
Marcgravia trinititis	lf	Peru	CH_2Cl_2	72,47,79	No tox
Melastomalaceae					
Bellucia pentamera	ws,sb	Peru	CH_2Cl_2	44,54	No tox
Oxyspora paniculata	rt,st,lf	India	EtOH	95	No tox
Monimiaceae					
Siparuna decipiens	tw	Peru	EtOH	64,37,31,42	
Myrsinaceae					
Maesa indica	sd	India	CH_2Cl_2	52,44	No tox
Maesa montana	sd	India	CH_2Cl_2	75,54	No tox
Maesa tabacifolia	ws,sb	W. Samoa	CH_2Cl_2	42,41	
Rapanea allenii	tw,lf	Panama	CH_2Cl_2	86,52	No tox
Virola elongata	ws,sb	India	CH_2Cl_2	93,94	Toxic 600
Myrtaceae					
Baeckea latragina	rt,st,lf,fl	W. Australia	CH_2Cl_2	45,73,66	No tox
Eugenia egensis	tw	Peru	$CHCl_3$	90,66	No tox
Syzygium gratum	sb	Thailand	CH_2Cl_2	27,75	No tox
Olaceae					
Heisteria spruceana	tw	Peru	CH_2Cl_2	47,51	No tox
Papaveraceae					
Papaver sp.	rt,st,lf,fl	Turkey	CH_2Cl_2	89,91,81	Toxic 600

TABLE 2. Continued.

Plant	Plant Part[a]	Source	Fraction	% Inhibition	Toxicity[b]
Polygonaceae					
Coccaloba densifrons	tw	Peru	EtOH	66,20,75,77	Toxic 600
Polytrichaceae					
Polytrichia commune	moss	Maine	CH_2Cl_2	62,71	No tox
Polytrichia juniperus	moss	Pennsylvania	$CHCl_3$	61,65	No tox
Proteaceae					
Grevillia baxteri	rt	W. Australia	CH_2Cl_2	64,98	No tox
Grevillia leucopterus	rt	W. Australia	CH_2Cl_2	92,87,72	Toxic 600
Hakea costata	rt	W. Australia	CH_2Cl_2	70,95	No tox
Rhamnaceae					
Rhamnus prinoides	st,lf,fr	South Africa	CH_2Cl_2	89,97,74	No tox
Rhanunculaceae					
Clematis buchaniana	st,lf,fr	India	EtOH	84,28,91	No tox
Rubiaceae					
Ixora ulei	rt,st,tw	Peru	CH_2Cl_2	78,33,45,65	No tox
Rubia cordifolia	st,ws,sb	China	CH_2Cl_2	78,83,88	No tox
Rutaceae					
Boronia inornata	rt,st,lf,fl	W. Australia	CH_2Cl_2	52,92	No tox
Euodia ruticarpa	fr	China	CH_2Cl_2	45,49	No tox
Melicope sissiliflora	lf	Australia	$CHCl_3$	91,99	Toxic 600
Zanthoxylum leibmannianum	tw,lf,inf	Mexico	$CHCl_3$	40,41	No tox
Sapindaceae					
Dodonea viscosa	tw,lf	South Africa	CH_2Cl_2	36,57	No tox
Solanaceae					
Brunfelsia grandiflora	tw	Peru	CH_2Cl_2	53,85	No tox
Thymelacaceae					
Daphne sp.	lf,fl	Turkey	CH_2Cl_2	59,89,52,43,48	No tox
Daphne genkwa	fl	China	CH_2Cl_2	63,53	No tox
Turneraceae					
Turnea acuta	lf	Peru	CH_2Cl_2	98,87	Toxic 600
Verbenaceae					
Vitex quinata	tw,lf	Thailand	CH_2Cl_2	49,59,47,56	No tox
Vitex negundo	lf	Thailand	CH_2Cl_2	46,34	
Vitaceae					
Cayratia japonica	st,lf	China	CH_2Cl_2	78,33,45,65	No tox
Xanthorrhoeaceae					
Xanthorrhoea preissii	st,lf	W. Australia	CH_2Cl_2	81,84,88	Toxic 300,600

[a]fl, flower; fr, fruit; inf, infloresence; lf, leaf pl, whole plant; rt, root; sb, stem bark; sd, seed; st, stem; tw, twig; ws, wood stem.
[b]No tox, nontoxic; Toxic 300, toxic at 300 μg/plate; Toxic 600, toxic at 600 μg/plate; Sl tox 600, slightly toxic at 600 μg/plate.

tion, structural elucidation, and SAR studies will be presented in subsequent papers. Other workers using inhibition of various mutagens, but not 2AN, have found that compounds such as cinnamaldehyde, coumarin, umbelliferone, protoanemonin, the diterpenoid enmein, tannic acid [see Kada *et al.* (15) for a review], and a novel flavonoid, glabrene (6), were inhibitors. We have initiated studies which compare the inhibition by some of the above compounds with those we have isolated using inhibition of 2AN and several other common mutagens.

The initial isolation procedure (extraction with EtOH and transfer to CH_2Cl_2 or $CHCl_3$, followed by Si gel chromatography) is a fairly standard procedure used particularly in the isolation of plant antitumor agents (10). The novel feature is preparative hplc of active fractions after only one Si gel chromatography. The apparatus we used was a Waters Prep 3000, but other similar commercial instruments could be used. It utilizes large pumps and large columns (2.1 × 25 cm) with flow rates as high as 10–20 ml/min. As shown in Figure 1, very pure material can be obtained in one pass within 1–2 h, thus greatly simplifying and shortening the procedure.

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