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Monroe E. Wall

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ANTIMUTAGENIC AGENTS FROM NATURAL PRODUCTS[1]

MONROE E. WALL

Research Triangle Institute, Box 12194, Research Triangle Park, North Carolina 27709

Certain secondary metabolites found in terrestrial and marine plants and organisms have evinced the capability for inhibiting the mutagenicity toward *Salmonella typhimurium* of a number of mutagens. These include 2-aminoanthracene (2AN), ethylmethanesulfonate (EMS), and benzo-[*a*] pyrene (B[α]P). The sensitivity of the antimutagenicity assay is such that crude extracts can be evaluated and purification of extracts readily followed. Major classes of antimutagenic compounds that have been isolated include flavonoids, coumarins, and cymopols.

Both chemists and biologists have been intrigued for many years concerning the role of secondary metabolites in plants of terrestrial and marine origins. Of equal interest is whether such compounds have antitumor, antiviral, or antimutagenic properties. For example, two highly active antitumor agents, camptothecin and taxol, have been isolated from the bark and wood of trees (1,2) and another active agent, bryostatin I, from a marine animal (3). Dudley Williams has proposed that "secondary metabolites are a measure of the fitness of the organism to survive by repelling or entrapping other organisms" (4). Mitscher has stated that "as some constituents of higher plants are mutagens, it seems reasonable that substances capable of antimutagenicity also be produced by such plants" (5).

Initially influenced by the first Symposium on Antimutagensis and Anticarcinogenesis Mechanisms (6), we have been working for several years on isolation and structure determination of potential antimutagens from terrestrial and marine sources.

GENERAL METHODS

ANTIMUTAGENIC SCREENING.—Our procedure has been described in detail (7–9). It is based on the inhibition of the mutagenicity of 2-aminoanthracene (2AN) by crude or semi-purified plant extracts (7). The procedure incorporates methodology first described by Birt *et al.* (10) and utilizes an enzyme metabolic activator for the mutagen (11). In brief, 300–600 μg aliquots of crude extract in DMSO solution are added to the nutrient broth containing *Salmonella typhimurium* TA-98, the Ames S-9 metabolic activation preparation, and the mutagen, 2AN. The procedure is outlined in Scheme 1.

EXTRACTION AND ISOLATION OF ANTIMUTAGENIC COMPOUNDS.—The extraction and isolation of antimutagenic agents which, as defined by Kada *et al.*, may include both desmutagenic and bioantimutagenic agents (12), is guided by bioassay at every step. The general extraction and isolation procedure has been given in detail in previous papers from our laboratory (7–9,13,14). In brief, antimutagenic EtOH extracts are concentrated and partitioned between CH_2Cl_2 or $CHCl_3/H_2O$; the organic solvent fraction is concentrated; and the residue is partitioned between 90% MeOH/10% H_2O and hexane. The MeOH fraction is concentrated and chromatographed on Si gel or Sephadex LH-20. Chromatographic fractions are tested for inhibition of 2AN and toxicity. Active, nontoxic fractions are usually rechromatographed and then subjected to preparative hplc for final purification.

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INHIBITION PROCEDURE

1. *Salmonella typhimurium* (TA-98)
+
2. Ames S-9 preparation
+
3. 600 μg/plate of test substance
+
4. 2-Aminoanthracene (2AN) (2.5 μg/plate)
5. Mix with top agar and incubate 48–72 h

TOXICITY PROCEDURE

1. Steps 1–3 as above
2. Omit 2AN in step 4 and replace by histidine
3. Step 5 as above

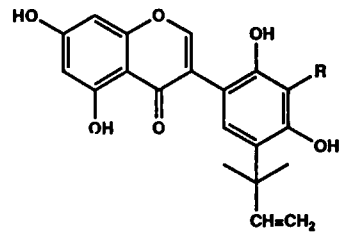
SCHEME 1

DETERMINATION OF PHYSICAL PROPERTIES.—Isolated compounds are initially crystallized, and melting points and optical rotations are determined. If the compounds have uv absorption, purity can then be carefully checked by a technique involving hplc and diode array uv. Ir, ^{1}H and ^{13}C nmr, and hrms, which gives the molecular formula, are obtained. By means of the molecular formula, the compounds can be rapidly checked via *Chemical Abstracts* and other computer search compendiums to determine if the compounds are novel, or, if previously known, have ever received antimutagenic testing. In certain cases, when it is evident we are dealing with a complex structure with several asymmetric centers, the precise structure is determined by X-ray crystallography which, in spite of all the improvements in nmr techniques, is probably still the best single structural technique. It is limited, however, to compounds or their derivatives which are available in appropriate crystal form.

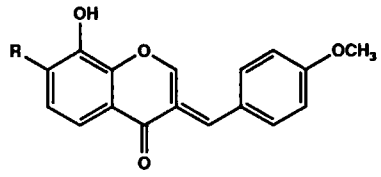
ACTIVE PLANTS.—*Edible plants.*—As described previously, we have had little or no success in finding antimutagenic agents by our techniques in edible plants (15). A number of edible vegetables in the Brassicaceae found to have anticarcinogenic constituents (16) were inactive when screened for antimutagenicity toward 2AN (15).

Wild plants.—We have had considerably more success in studying wild plants of both terrestrial and marine origin. Of approximately 2750 plants, 3.5% were found to be active and nontoxic (15). In the case of marine samples, 50 algae samples gave 20% active, nontoxic extracts; from almost 100 samples of corals and sponges, only 3.1% were active; and from various marine animals, only one out of approximately 185 samples showed activity. Recently, plants with reputed therapeutic activity were examined, which gave a much higher success rate. From 100 plants obtained from Sri Lanka, accurately botanically identified and reported to have various therapeutic activities, the success rate was almost 20%. We plan to investigate this specialized area and obtain additional plants utilized in other cultures and areas for medicinal activity.

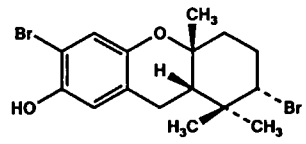
ANTIMUTAGENIC COMPOUNDS ISOLATED.—*Flavonoids.*—Figure 1 shows a number of putative antimutagenic agents, all of which were isolated in our laboratory and received antimutagenicity testing for the first time. In addition, some of these compounds also have completely novel structures. Flavonoids have received considerable attention, both



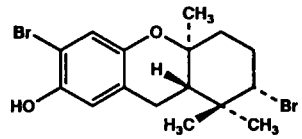
Fremontin,* R=H
Fremontone,* R=H_2C-CH=$C(CH_3)_2$



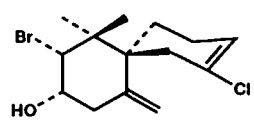
Intricatol,* R=OH
Intricatinol,* R=OMe



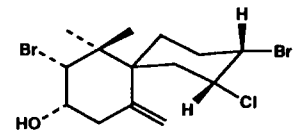
Cymobarbatol*



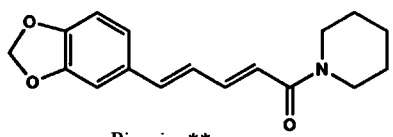
4-Isocymobarbatol*



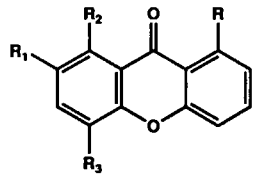
Elatol**



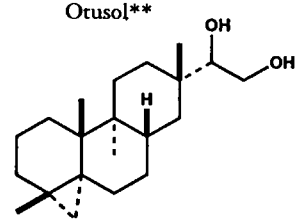
Otusol**



Piperine**



1,7-Dihydroxyxanthone
(Euxanthone)**
R=R_1=OH, R_2=R_3=H
1,5-Dihydroxy-8-methoxyxanthone
R=R_3=OH, $R_2$5OMe, R_1=H



Erythoxydiol X**

FIGURE 1. New* and known** putative antimutagens

in our laboratory and by other groups. Previously, we have described the antimutagenic activity of a number of known flavonoids, including, in some cases, flavonoid glycosides (8). The latter category has never shown any activity. Recently, we have isolated from the roots of *Psorothamnus fremontii* (Fabiaceae) two new isoflavonoids, fremontin and fremontone (13). The latter is considerably more potent as an antimutagen than the former compound. Two homoisoflavonoids which have novel structures are the compounds intricatol and intricatinol, isolated from *Hoffmanosseggia intricata* (14). Of these, we have found that intricatinol is much more potent against a number of mutagens, including 2AN, acetylaminofluorine (AAF), and ethyl methanesulfonate (EMS) (14). We have developed synthetic schemes for the synthesis both of the fremontin and the intracatinol systems, and these compounds can be produced in quantity if further study shows good therapeutic activity.

Flavonoids isolated by other groups include the compound glabrene, a known isoflavone isolated from *Glycyrrhiza glabra* (5). Glabrene was highly active against EMS-induced mutations of *S. typhimurium* (TA-100). Recently, the well known flavonoid biochanin A has been reported to inhibit B[*a*]P-induced mutations by hamster embryo cells in tissue culture as a test system (17). In general, the flavonoid group will require careful study in a variety of antimutagenic assays and should also be tested for mutagenic activity. Flavonoids have been reported to have a wide variety of biological activities (18). Certain flavonoids have been considered to display mutagenic and carcinogenic effects (18) whereas other and sometimes the same flavonoids have been reported to exert inhibition of tumor promotion (16). The multitudinous biological activities attributed to flavonoids have been well summarized in an excellent symposium report (18).

Coumarins.—Coumarins are a group of well known compounds which have received attention both as anticarcinogens (16) and antimutagens (12). Coumarin, umbelliferone, and 8-methoxysoralin have been previously described by Kada *et al.* (12). Recently we have made a thorough investigation of antimutagenic coumarins isolated from various sources (9). We found, in particular, that two known coumarins, imperatorin and osthol, isolated from *Selinum monniere*, have particularly high activity in the inhibition of 2AN and B[*a*]P. Because of the well known photochemical activity of these compounds, we have not investigated this group further.

Xanthones.—The two xanthones shown in Figure 1 were isolated from *Vismia amazonica* (Clusiaceae). Both xanthones are known (19,20) but had never received antimutagenicity testing. Both compounds display considerable antimutagenicity at a number of dose levels against 2AN and EMS (21).

Acetophenone analogue.—A simple new acetophenone analogue, 2,3-dihydroxy-5-ethoxyacetophenone, was isolated from a rather unusual source, a lichen, *Lasallia papulosa* (Umbellicariaceae). This acetophenone analogue was active and nontoxic in the 2AN assay (21).

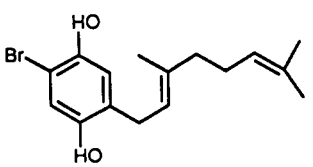
Piperine.—The structure of piperine is shown in Figure 1. This is a known compound (22), but it had never received antimutagenicity testing. Piperine was isolated from *Scindapsus officianallis* (Araceae) (21), a plant used as a medicinal herb in Sri Lanka. The compound showed considerable activity in the 2AN antimutagenicity assay and was nontoxic. A number of synthetic analogues of piperine were prepared and have also been active. Further studies of the activity in this series in other in vitro test systems are in progress.

Erythoxydiol, a known diterpene (23) was isolated from *Aquillaria agalloche* (21), a medicinal herb from Sri Lanka. In addition to antimutagenic activity toward 2AN, it recently has been shown to have considerable activity in the inhibition of DMBA-

induced mammary tumor (private communication from Dr. John Pezzuto, School of Pharmacy, University of Illinois at Chicago.).

MARINE ANIMALS AND PLANTS.—*Elatol and Obtusol.*—The structures of these known compounds (24,25) are shown in Figure 1. Of interest is the fact that they are halogenated, both containing bromine and chlorine. These compounds were isolated from extracts from a marine animal known as the "sea hare," *Aplysia dactylomela* (21). It is almost certain that these and a large number of similar compounds are derived originally from marine algae belonging to the *Laurencia* (24,25). Although both elatol and obtusol show significant activity in the inhibition of 2AN mutagenicity, the latter compound is far more potent (21).

ISOLATION AND STRUCTURE OF CYMOBARBATOL AND RELATED COMPOUNDS FROM GREEN ALGAE.—*Background.*—The following sections, which exemplify our methodology, describe the isolation, structure, and antimutagenic activity of several new secondary metabolites from a green alga, *Cymopolia barbata*. Marine algae have been the subject of many investigations dealing with the structure of various secondary metabolites. Numerous halogenated metabolites have been found in red and brown algae. Green algae received lesser attention until a group of prenylated bromohydroquinones, collectively termed cymopols, were isolated from *Cymopolia barbata* (L.) Lamouroux (Dasycladaceae) by Hogberg *et al.* (26). Of particular interest were two compounds, cymopol [**1**] and cyclocymopol [**2**], the structures of which are shown below. The structure of one of the diastereomers of **2** as the monomethyl ether was confirmed in an X-ray crystallographic study (27). We have found two new cymopols in *C. barbata* extracts guided by antimutagenicity assay (28).



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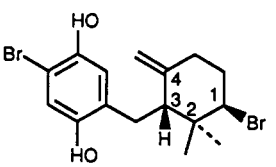
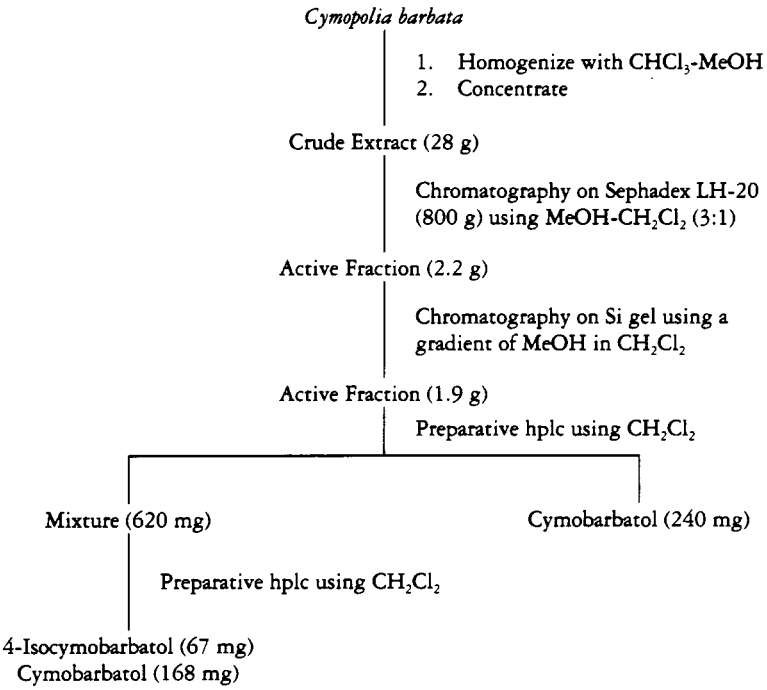


2

Isolation of new cymopols from C. barbata.—Crude methanolic $CHCl_3$ extracts of *C. barbata*, collected by Professor William Gerwick, Oregon State University, Corvallis, Oregon, were tested for both antimutagenicity and toxicity and were found to be active in the inhibition of 2AN mutagenesis and relatively nontoxic. Guided by antimutagenic bioassay, two new compounds were obtained, cymobarbatol [**3**] and 4-isocymobarbatol [**4**], isolated by the procedure presented in Scheme 2 (28). In addition, a somewhat more polar compound was isolated from the same preparation in a fraction obtained from a more polar gradient. A pure known compound, cymopol [**1**] (26), was isolated utilizing techniques involving preparative hplc similar to those shown in Scheme 2.

Physical properties of compounds **3** *and* **4**.—Some of the important physical properties of compounds **3** and **4** are as follows. Cymobarbatol [**3**]: mp 166° (aqueous MeOH); $[\alpha]^{23}$ −15.4°: uv (MeOH) nm (log ε) 306 (3.72), 225 sh (4.00), (MeOH+NaOH) 327 (3.84); hrms *m/z* 401.9831 ($C_{16}H_{22}Br_2O_2$=401.9831). 4-Isocymobarbatol [**4**]: mp 147° (aqueous MeOH); $[\alpha]^{23}$ −51.4°; uv (MeOH) nm (log ε) 305 (3.76), 225 sh (3.76, (MeOH+NaOH) 326 (3.79); hrms *m/z* 401.9835 ($C_{16}H_{22}Br_2O_2$=401.9831). Both compounds show similar uv spectra and high resolution mass spectra. However, the optical rotations of **3** and **4** differ considerably.

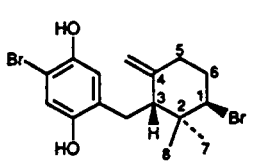
ISOLATION OF CYMOBARBATOL AND 4-ISOCYMOBARBATOL FROM *CYMOPOLIA BARBATA*



SCHEME 2

Structure of cymobarbatols.—The structure of the cymobarbatols was deduced in part from nmr data using both ^1H and ^{13}C analyses. From the ^1H spectra of **3** and **4** (Figure 2), it was immediately evident that **3** and **4** had identical structures in the aromatic ring, but there was an obvious differentiation in the ring fusion including carbons 3 and 4. X-ray crystallography gave complete structural proof as follows (28). Hrms shows that cymobarbatol and cyclocymopol are isomeric with a molecular formula of $C_{16}H_{20}Br_2O_2$. Acetylation of cymobarbatol gives a monoacetate. Cyclocymopol gives a diacetate. ^1H-nmr of cymobarbatol shows the presence of three Me groups whereas that of cyclocymopol shows two Me groups and an olefinic CH_2 group. Therefore, cymobarbitol appears to be formed by the cyclization of cyclocymopol. The stereochemistry of ring fusion at C-3 and C-4 and the configuration of Br at C-1 in both cymobarbatol and 4-isocymobarbatol were determined by single crystal X-ray crystallography.

Antimutagenic activity of cymopols.—Cymobarbatol and 4-isocymobarbatol were nontoxic at doses of 75–300 μg/plate in both *S. typhimurium* TA-98 and TA-100 strains, whereas cymopol was very toxic in both these strains at doses of 75–600 μg/plate. Cymobarbatol and 4-isocymobarbatol were highly active in the inhibition of 2AN mutagenicity toward *S. typhimurium* TA-98 strain at doses of 75, 150, 300 μg/plate.



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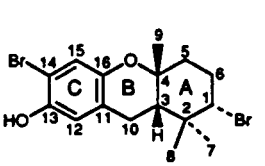

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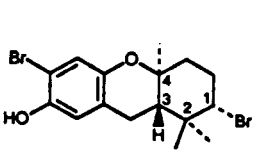

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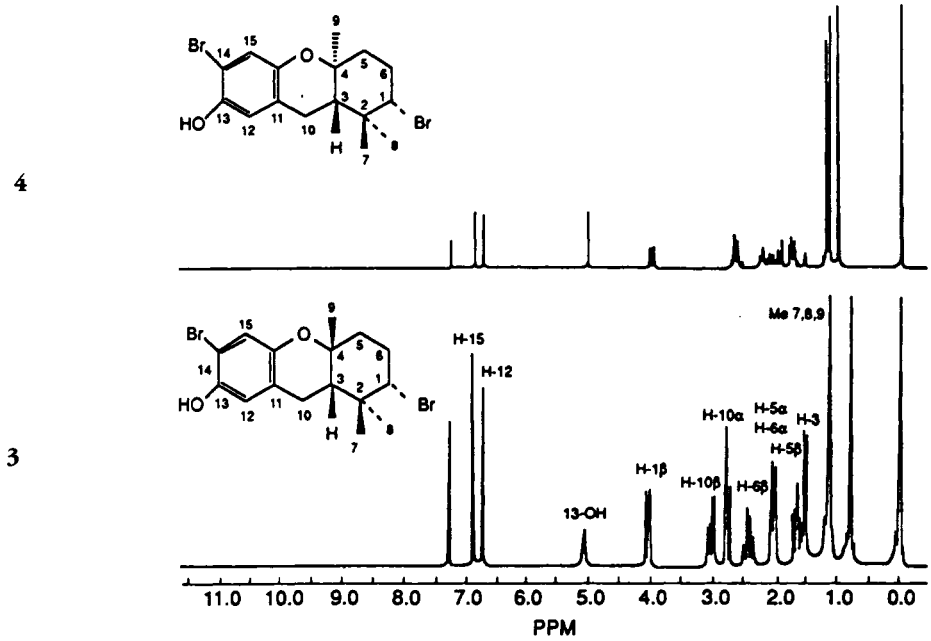

FIGURE 2. (Top) ^{1}H-nmr (250 MHz) spectrum of 4-isocymobarbatol [**4**] in $CDCl_3$. (Bottom) ^{1}H-nmr (250 MHz) spectrum of cymobarbatol [**3**] in $CDCl_3$.

These two compounds showed even greater (80–95%) activity in the inhibition of EMS mutagenicity toward *S. typhimurium* (TA-100) at doses of 32–300 μg/plate. On the other hand, cymopol was toxic even at low concentrations.

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