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NATURAL PRODUCTS AS A SOURCE OF POTENTIAL CANCER CHEMOTHERAPEUTIC AND CHEMOPREVENTIVE AGENTS[1]

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ABSTRACT.—Recent advances in the chemistry of novel bioactive natural products are reported. This research is directed to the exploration of plants with confirmed activity in bioassays designed to detect potential cancer chemotherapeutic and chemopreventive agents. Structural work and chemical studies are reported for several cytotoxic agents from the plants *Annona densicoma, Annona reticulata, Claopodium crispifolium, Polytrichum ohioense,* and *Psorospermum febrifugum.* Studies are also reported based on development of a mammalian cell culture benzo[*a*]pyrene metabolism assay for the detection of potential anticarcinogenic agents from natural products. In this study a number of isoflavonoids and flavonoids with antimutagenic activity have been discovered.

Advances in the prevention and treatment of cancer will require the continued development of novel and improved chemotherapeutic and chemopreventive agents. In the short history of the cancer drug development effort a number of clinically useful agents have been developed by screening programs coordinated by the pharmaceutical industry, research institutes, and the National Cancer Institute (1–11). Although chemotherapy for cancers with a high growth fraction has achieved important advances and has resulted in improved cure rates for these diseases, little impact has been made on the solid human cancers such as lung, colon, breast, ovarian, prostate, pancreas, and brain. This situation suggests that new directions must be taken in the approach to discovery of drugs for these diseases. In response to this need, new screens designed to uncover agents specific for these forms of human disease have been initiated. In addition, an expanded search is underway to identify novel therapeutic targets for these diseases and to uncover nontoxic agents which might prevent the development of cancer (10–14). In this review we will report on recent research in our laboratory using bioassays which fall into these categories.

CYTOTOXIC AGENTS FROM HIGHER PLANTS.—*Bioassays for antitumor activity.*—The efficiency of uncovering effective antitumor agents from higher plants strongly depends on the prescreening/screening methodology utilized for guiding the fractionation and separation of the crude extracts. There are numerous factors to be considered in the selection of bioassay methods for screening crude plant extracts/fractions. The crude extracts/fractions are not always H_2O-soluble. H_2O-soluble organic solvents, such as DMSO or MeOH, are often added to enhance solubility. The crude extracts/fractions usually contain various pigments, that may impose serious interference in spectrophotometric analysis. Furthermore, the active constituents frequently exist in a chemically complex matrix and in low concentration. Therefore, the assay systems must have sufficient specificity to exclude a great variety of inactive constituents and sensitivity to detect a minute amount of active compound in the crude extract.

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Cytotoxicity-Based Bioassays.—Various bioassay systems have been used to screen for antitumor agents since the establishment of the National Cancer Institute (NCI) natural product program in 1956 (1,10,15). The initial assay system was based on an increase in the life-span of tumor-bearing mice. The early Walker carcinosarcoma 256 was abandoned because of its excessive sensitivity, which gave a large number of false positive leads. This tumor system was also sensitive to the ubiquitously occurring tannins. The screening for antitumor agents from natural sources was then guided by the in vivo activity in the mouse L-1210 leukemia. During the past decade, the P-388 murine leukemia system was found to be more sensitive and predictive than the L-1210 system and has been used as a primary screening system for selecting active plant materials and directing further fractionation and separation. The turn-around time and the cost for the in vivo testing are high. In many cases, in vitro prescreens, such as cytotoxicity against human nasopharynx carcinoma (9KB) or murine P-388 leukemia (9PS), have been employed to facilitate the activity-directed separation process if the in vivo activity is coincidental with the in vitro cytotoxicity. However, this approach may miss antitumor compounds which are not cytotoxic.

A large number of active natural products has been isolated using the above screening methods. However, the anticancer activities of these compounds were predominantly noted in leukemia, lymphoma, and a few rare tumors. The low efficacy or, for most drugs (natural and synthetic ones), the total lack of clinical efficacy against slower growing solid tumors, is a serious concern. It has been recognized that the use of a single antileukemia screening system could bias the end results and lead to the isolation of compounds active only in the treatment of fast growing tumors. It is also likely that many novel compounds with possible antitumor activity could have remained undetected by the less sensitive in vivo models due to the low concentrations at which many active natural products occur.

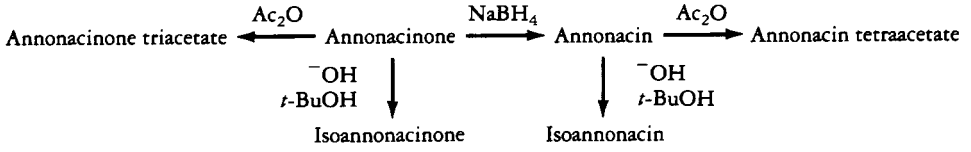
Considering the diversity and specificity of tumors, NCI has been developing a "disease-oriented" approach to antitumor activity screening (11, 16–18). Each extract or compound may be tested directly against a spectrum of human tumor types. The logistics of screening large numbers of plant extracts and fractions using multiple in vivo models is prohibitively expensive and time-consuming. Thus, NCI has begun the development of an in vitro prescreening system to measure antitumor cytotoxicity against human tumor cell line panels consisting of 60–100 cell lines of major human tumors (lung, colon, breast, skin, kidney, etc.). Compounds showing differential cytotoxicity for particular tumor types will be followed up with in vivo testing using the same sensitive cell lines. The most important advantage of the new in vitro screening panel is the potential to identify tumor-type-selective compounds. The enhanced sensitivity of the in vitro screen will also enable the discovery of active constituents present in low concentration in the plant extracts. The success of this strategy will be assessed by (a) the effectiveness for isolation of pure and selective cytotoxins from crude plant extracts and (b) the confirmation of their selective antitumor activity in the animal tumor models. In order to validate this strategy, NCI has awarded three contracts to collect about 20,000 plants from Africa and Madagascar, Central and South America, and Southeast Asia. An in-house natural product research group has also been established to isolate the selectively active constituents. Ultimately, the effectiveness of any in vitro prescreening or in vivo screening model must be assessed by its impact on clinical advances and overall cost.

Mechanism-based bioassays.—Recent studies of the molecular pharmacology of new antitumor agents and tumor cell biology have disclosed numerous molecular targets for discovery of novel anticancer agents (19,20): specific inhibition of the expression of oncogenes or oncogene products; modulation of anticancer drug resistance; interference of

growth factors, angiogenesis, mitosis, intercellular signal transduction, and cellular proliferation; inhibition of specific processes in tumor cell invasion and metastasis; and inhibition of specific enzymes involved in the synthesis and processing of essential biomolecules such as DNA topoisomerases, polymerases, nucleoside reductase, and aromatase.

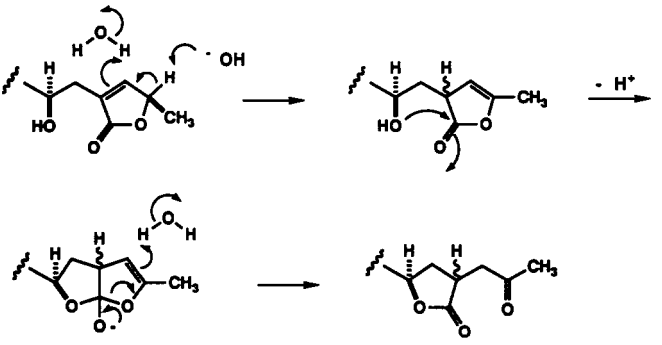
RECENT STUDIES ON TUMOR CELL CYTOTOXIC AGENTS FROM PLANTS.—*The family Annonaceae.*—Studies on the extract of the stem bark of *Annona densicoma* have led to the isolation of several novel cytotoxic polyketides (21,22), including the parent annonacin, which was the first member of the C_{35} polyketide (acetogenin) series to be discovered. This series was found to possess a single tetrahydrofuran ring in contrast to the more common C_{37} polyketide (acetogenin) series, which has two adjacent tetrahydrofuran groups (23–28). Rupprecht *et al.* (28) have recently reported another relative of the annonacin series named goniothalamicin. In addition to annonacin, the related compounds annonacinone, isoannonacin, and isoannonacinone have been interrelated and elucidated. These structures were elucidated based on detailed analysis of their ms, ir, uv, and high field nmr spectra (21,22).

The isomeric relationship of annonacin to isoannonacin and annonacinone to isoannonacinone was confirmed by a base-catalyzed isomerization reaction. The major product from treatment of annonacin with KOH in *t*-BuOH was identical to isoannonacin by ir, ms, and nmr data. A similar relationship was established between annonacinone and isoannonacinone (Scheme 1). A minor amount of an epimer was detected by nmr, which would be expected based on the proposed mechanism for the rearrangement reaction (Scheme 2).



SCHEME 1. Chemical Interrelationships Among Annonacins.

Isoannonacin and isoannonacinone are the first reported members of the iso series of C_{35} polyketides. These compounds have demonstrated very interesting selective cytotoxicity in human tumor cell lines. The iso series is 10,000× less active against leukemia cells and 1000× more active against the HT-29 colon tumor cells than the parent butenolide (Table 1). Based on this, further research is under way to establish the complete stereochemistry, mechanism of action, and in vivo antitumor activity of this series.



SCHEME 2

TABLE 1. Selective Cytotoxicity in the Annonacin Series.[a]

Compound	Cell Line[b]		
	9PS	A-549	HT-29
Annonacin	1×10^{-5}	1×10^{-3}	3×10^{0}
Annonacinone	1×10^{-6}	1×10^{-1}	1×10^{0}
Isoannonacin	3×10^{0}	2×10^{-2}	2×10^{-3}
Isoannonacinone	5×10^{-1}	7×10^{-2}	9×10^{-3}

[a]ED_{50} (μg/ml)

[b]9PS = mouse leukemia, H-549 = human non-small lung carcinoma, and HT-29 = human colon adenocarcinoma.

Bioassay-directed fractionation of *Annona reticulata* has resulted in isolation of active members of both the mono- and bis-tetrahydrofuran series. The mono-series is closely related to the annonacin series, and structural work has been initiated on two active compounds in this group. In addition, we have isolated a member of the bis-tetrahydrofuran group called annonareticin ($C_{37}H_{66}O_7$) which is isomeric with asimicin and bullatacin, which are cytotoxic, pesticidal acetogenins isolated and elucidated by McLaughlin and co-workers (28–30).

Analysis of uv, ir, nmr, and ms established the close similarity of annonareticin, asimicin, and bullatacin and suggested that these compounds were stereoisomers. The only member of the series which has been subjected to X-ray analysis is rolliniastatin, which was reported by Pettit *et al.* (31). Hoye and co-workers have reported a very valuable new method for determining the stereochemistry of the bis-(tetrahydrofuranyl) moiety in compounds of this type (32). This method, which utilizes ^{1}H-nmr chemical shift data correlations, has been applied to assignment of the configuration of uvaricin and asimicin by Hoye's group (33). McLaughlin *et al.* (34) have independently applied this method to the assignment of the configuration of asimicin and the related bullatacin. Conversion of annonareticin to its triacetate and ^{1}H-nmr analysis provided evidence that this compound possessed the same relative stereochemistry around the bis-(tetrahydrofuranyl) moiety as uvaricin and bullatacin (Figure 1).

Further research is under way to establish the stereochemistry around the butenolide ring and at the 4-hydroxy position. This series has exhibited very potent cytotoxicity, and correlation of relative cytotoxicity and stereochemistry should be of interest in further development of these potential antineoplastic agents.

Mosses from the Thuidiaceae.—Antitumor activity was discovered in bryophytes in 1980, and the NCI has placed some emphasis on collecting and screening members of this group since that time. In their initial survey forty-three species showed activity (35). One of these, *Claopodium crispifolium*, showed significant antitumor activity, and an investigation was initiated on this species and *Anomodon attenuatus*, both in the Thuidiaceae (36).

The fractionation of both plants proceeded along parallel schemes, and in each case a minute quantity of an intensely cytotoxic component was isolated after intensive fractionation. In each case, the minor, cytotoxic constituent was identified as the known maytansinoid, ansamitocin P-3 [**1**] (37). Ansamitocin P-3 is a member of the group that was previously isolated from the culture broth of a *Nocardia* sp. (38). Recently Sakai *et al.* (39) reported the occurrence of maytansinoids, including 15-methoxyansamitocin P-3, in the Japanese mosses *Isthecium subdiversiforme* (Lembophyllaceae) and *Thamnobryum sandei* (Neckeraceae). The co-occurrence of ansamitocin P-3 in these mosses in very low concentrations suggests a possible association between this com-

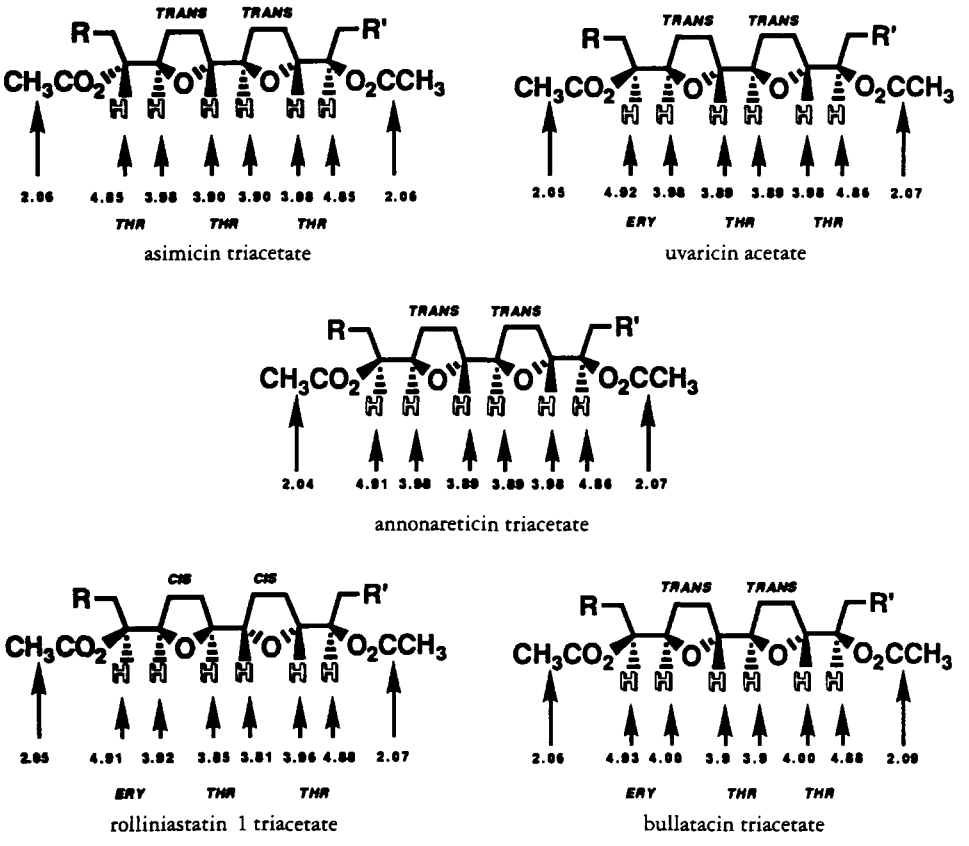
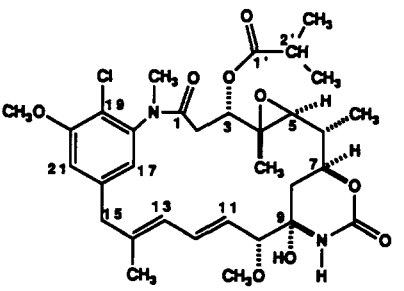


FIGURE 1. ^{1}H-nmr comparison of polyketides (acetogenins).

pound in mosses and closely associated actinomycetes which may produce these metabolites.

Studies on Polytrichum ohioense *(Polytrichaceae).*—Another family that produced active species in the Spjut study was the Polytrichaceae. One member of this family, *Polytrichum ohioense*, showed cytotoxicity in the 9KB system. A re-collection of this moss produced an extract which was active in both 9PS and 9KB. Activity-directed fractionation has yielded a series of novel natural products named ohioensins A [**2**], B [**3**], C [**4**], D [**5**], and E [**6**]. The structure of the isomer ohioensin A was established unequivocally by X-ray analysis to be a novel polycyclic benzo[*c*]naphthoxanthenone system that apparently results from coupling of *o*-hydroxycinnamate and hydroxylated



1 ansamitocin P-3

TABLE 2. Bioactivity Data of Ohioensins.[a]

Compound	Cell Line[b]				
	9KB	9PS	A-549	MCF-7	HT-29
Ohioensin A [2]	>10	1	>10	9	>10
Ohioensin B [3]	>10	1	8	7	>10
Ohioensin C [4]	10	>10	>10	3	4
Ohioensin D [5]	>10	1	>10	>10	>10
Ohioensin E [6]	>10	1	6	>10	>10

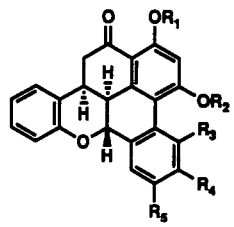
[a]ED_{50} (μg/ml)

[b]9KB = human epidermoid carcinoma, 9PS = mouse leukemia, A-549 = human lung carcinoma, MCF-7 = human colon adenocarcinoma, and HT-29 = human breast adenocarcinoma.

bibenzyl precursors (40). This series has borderline cytotoxicity versus 9PS, and ohioensin C is borderline cytotoxic versus MCF-7 and HT-29.

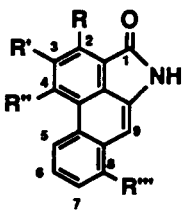
Studies on Pararistolochia flos-avis.—Chemical studies of *Pararistolochia flos-avis* were prompted by the initial antileukemic activity (T/C, 160% at 200 mg/kg) of the crude EtOH extract. Two new aristolactams, FI [**7**] and FII [**8**], were isolated from the root and stem (41). Their structures were elucidated on the basis of nmr, ms, uv, and ir spectral analyses. It was noted that the 2-methoxy protons were significantly shifted to downfield (δ 4.61) by the peri carbonyl group. Aristolactam I [**9**] and Aristolactam AII [**10**] were also isolated from this plant. Aristolactam AII showed approximately tenfold selective cytotoxicity against human small cell lung and colon tumors in the NCI human tumor cell line evaluation. Further development of this active lead appears worthwhile. Further cytotoxicity-directed fractionation led to the isolation of two other new compounds, flossonol [**11**] and (−)-phillygenin [**12**] (42). The relative configuration of the new tetralone was established by the ^{1}H-nmr coupling analysis and nOe experiments. It was cytotoxic to murine leukemia cells in culture.

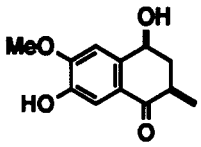
2 $R_1=R_2=R_3=R_4=H$, $R_5=OH$ Ohioensin A
3 $R_1=R_2=R_4=R_5=H$, $R_3=OH$ Ohioensin B
4 $R_1=R_4=R_5=H$, $R_2=Me$, $R_3=OH$ Ohioensin C
5 $R_1=R_5=H$, $R_2=Me$, $R_3=R_4=OH$ Ohioensin D
6 $R_1=R_5=H$, $R_2=Me$, $R_3=OH$, $R_4=OMe$ Ohioensin E



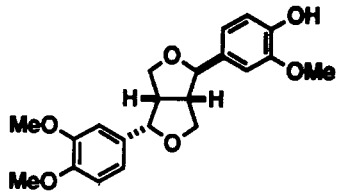
Studies on Podocarpus.—The genus *Podocarpus* (Podocarpaceae) is widely distributed in tropical and subtropical areas of eastern Asia and the southern hemisphere (43). Various terpenoids have been isolated from a number of species (44,45). One of the biologically active terpenoid groups is the norditerpene dilactone. This group of com-

7 $R=R'''=H$, $R'=OMe$, $R''=OH$ aristolactam FI
8 $R=R''=OMe$, $R'=OH$, $R'''=H$ aristolactam FII
9 $R=H$; $R',R''=O\text{-}CH_2\text{-}O$; $R'''=OMe$ aristolactam I
10 $R=R'''=H$, $R'=OH$, $R''=OMe$ aristolactam AII





11 flossonol

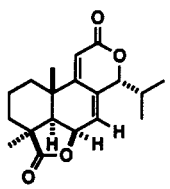


12 (−)-phillygenin

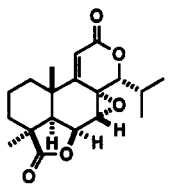
pounds shows antitumor activity (45–50), plant growth regulatory activity (51,52), termiticidal activity (53), and toxicity toward insect larvae (54,55). In the search for antitumor constituents, we isolated the cytotoxic norditerpene dilactones, nagilactone F [**13**] and its new congener nagilactone G [**14**], from the stem bark of *Podocarpus sellowii* and *Podocarpus milanjianus* (49). Two new cytotoxic dilactones, milanjilactones A [**15**] and B [**16**], were also isolated from *P. milanjianus* and characterized on the basis of spectroscopic evidence as 1,2-dehydro derivatives of nagilactones F and G, respectively (50). This series was shown to be inactive in P-388 in vivo.

From the P-388 in vivo active fraction of *P. milanjianus*, three known dilactones, podolactone C [**17**], nagilactone C [**18**] and sellowin A [**19**], were isolated. Podolactone C was previously determined by nmr to be a 1,2-α-epoxide in ring A. However, our single-crystal X-ray analysis indicated that it is a 2,3-β-epoxide (45). Podolactone C (T/C, 151% at 20 mg/kg) (45) and nagilactone C (T/C, 145% at 20 mg/kg) (47) showed moderate antitumor activity against P-388 in vivo (45). Besides podolactone C and nagilactone C, podolide (T/C, 127% at 50 mg/kg) (48) is the only other in vivo active norditerpene dilactone. Insufficient numbers of compounds of this type have been evaluated for antitumor activity for any firm in vivo structure-activity correlation to be derived. Most of the diterpene dilactones are active in the human non-small cell lung tumor (A-549) and colon tumor (HT-29) cells in culture. Our preliminary data suggest that the $\Delta^{7,8}$ double bond or its epoxide is essential in addition to the dilactone groups.

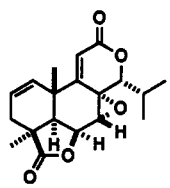
Our previous study indicated the best source for nagilactone C is *Podocarpus purdieanus* collected in Jamaica (56). A sufficient quantity of nagilactone C has been reisolated from this plant for further antitumor evaluation by NCI. In order to facilitate the reisolation process, we utilized a newly developed method employing tandem mass spectrometry (ms/ms) to selectively identify the plant parts and fractions that contain



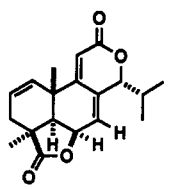
13 nagilactone F



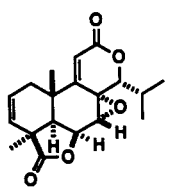
14 nagilactone G



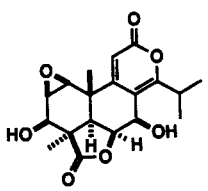
15 milanjilactone A



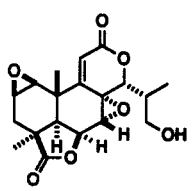
16 milanjilactone B



17 podolactone C



18 nagilactone C



19 sellowin A

the target compounds (57). This approach is based on the selection of ions of a specific mass-to-charge ratio from the ion source, and analysis of the dissociation products obtained upon inelastic collisions of these ions with an inert target gas allows individual components of complex mixtures to be determined. This technique can reduce many laborious separation procedures. Our studies have also revealed that the conventional ms/ms analysis using an inert collision reagent often could not differentiate structural isomers. Recently we have extended the ms/ms studies of the *Podocarpus* norditerpenoid dilactones to include spectral analysis utilizing reactive collision reagents, such as ethyl vinyl ether (58). Minor differences in the structures of the compounds led to different reactivities toward the reagent, as indicated by the relative abundance of collision products, such as the ethylated and vinylated adducts. Additional information can be obtained from the fragmentation products formed from nonreactive, inelastic collisions with neutral reagent. The results demonstrate that the use of reactive collisions can supplement collision-activated fragmentation in chemical analysis of natural products to probe specific functionality and stereochemistry.

Constituents of Psorospermum febrifugum.—Our laboratory has carried out extensive studies on the cytotoxic constituents of the African tropical shrub *Psorospermum febrifugum*. These studies and reports from other laboratories have elucidated numerous cytotoxic anthrones (59–62), vismiones (60, 63–65), anthraquinones (65,66), and xanthones (67–70) from this species.

The furanoxanthone psorospermin [**20**] has been studied in detail because of its significant cytotoxicity and antitumor activity. This research has established the stereochemistry of [**20**] and synthetic routes to [**20**] and analogues (71–73). Recently a new series of cyclo derivatives of the psorospermin group have been discovered. These compounds, named psorofebrin [**23**] and 5′-hydroxyisopsorofebrin [**24**] possess the novel tetrahydrofurobenzofuranoxanthone skeleton. After examination of numerous analogues of psorospermin, e.g., psorospermin-diol [**21**] and 3′-*O*-acetylpsorospermin-diol [**22**], it is clear that the most potent cytotoxic agents in the series possess an alkylating group at the 3′,4′ position (Table 3). Compounds lacking this group show only borderline cytotoxicity. In addition, at the present time none of the psorospermin analogues has shown selective activity versus the human tumor panel. Further investigations are underway on heterocyclic analogues of **20**.

Another group of cytotoxic constituents of *P. febrifugum* that have been uncovered in our studies include vismiones D and F. These compounds appear to be less cytotoxic than vismione A (63). It is interesting to note that psorospermin [**20**] and vismione F have shown preliminary activity in a screen for antimalarial activity.[2]

TABLE 3. Cytotoxicity of Xanthones from *Psorospermum febrifugum*.[a]

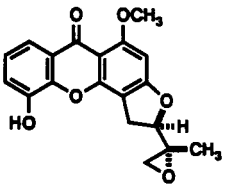
Compound	Cell Line[b]		
	HT-29	A-549	MCF-7
Psorospermin [**20**]	$>10^{-5}$	1.6×10^{-3}	1.2×10^{-2}
Psorospermin-diol [**21**]	2.3×10^{0}	$>10^{1}$	$>10^{1}$
3′-*O*-Acetylpsorospermin-diol [**22**]	1.6×10^{-2}	2.0×10^{0}	2.5×10^{0}
Psorofebrin [**23**]	8.5×10^{0}	$>10^{1}$	$>10^{1}$

[a]ED_{50}

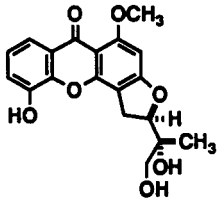
[b]HT-29 = human breast adenocarcinoma, A-549 = human lung carcinoma, MCF-7 = human colon adenocarcinoma.

[2]Private communication from M.J. O'Neill and J.D. Phillipson.

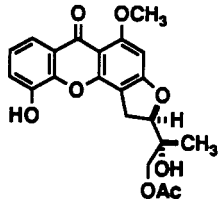
Recent investigations have led to the elucidation of a group of xanthonolignoids **25–31** from *P. febrifugum*. Five of these compounds are novel; two were previously reported (74–77). Bioactivity data are summarized in Table 4.



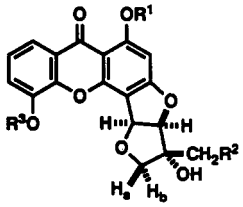
20 psorospermin



21 psorospermin-diol

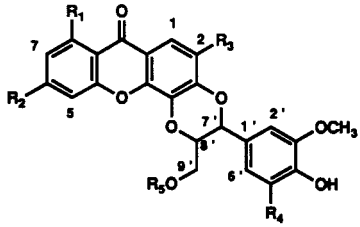


22 3′-*O*-acetylpsorospermin-diol



23 R^1=Me, R^2=R^3=H psorofebrin

24 R^1=H, R^2=OH, R^3=Me 5′-hydroxyisopsorofebrin



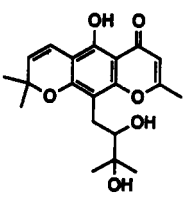
25 R_1=R_2=R_4=R_5=H, R_3=OMe
26 R_1=R_2=R_5=H, R_3=R_4=OMe
27 R_1=R_3=OMe, R_2=R_4=R_5=H
28 R_1=R_3=OMe, R_2=R_4=H, R_5=Ac
29 R_1=R_3=R_4=OMe, R_2=R_5=H
30 R_1=R_3=R_4=OMe, R_2=OH, R_5=H
31 R_1=R_2=OH, R_3=R_5=H, R_4=OMe

Studies on Spathelia sorbifolia.—Cytotoxicity-directed fractionation and separation led us to the isolation of three new chromones **32–34** and five known chromones from the 10% aqueous MeOH extract from the twigs and leaves of *Spathelia sorbifolia* (78). Compound **33** and O^5-methylsorbifolin [**35**] showed in vitro cytotoxicity against P-388 leukemia cells.

TABLE 4. Bioactivity of Xantholignoids **25–31** from *Psorospermum febrifugum*.

Compound	Cell line[b]		
	A-549	MCF-7	HT-29
25	3	>10	3
26	>10	>10	>10
27	>10	>10	>10
28	4	>10	4
29	>10	>10	>10
30	N.T.[c]	N.T.	N.T.
31	3	>10	4×10^{-1}

[a]ED_{50} (μg/ml).
[b]A-549 = human lung carcinoma, MCF-7 = human colon adenocarcinoma, HT-29 = human breast adenocarcinoma.
[c]N.T. = not tested.



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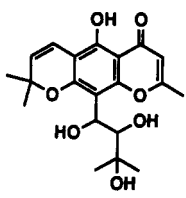

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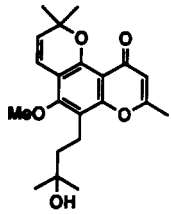

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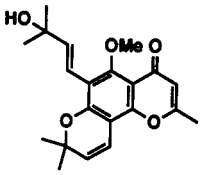


35

DISCOVERY OF NATURAL CHEMOPREVENTIVE AGENTS.—Dietary factors are now recognized as one of the major determinants in the incidence of human cancer (79–81). These dietary influences on cancer incidence in humans involve a complex interaction of a number of factors; however, a number of dietary components have been clearly related to a decreased incidence of tumors in humans. Consumption of cruciferous vegetables is linked to a decreased incidence of colo-rectal cancer in humans (80). Wattenberg and his co-workers have succeeded in isolating two major classes of chemopreventive agents from cruciferous vegetables, a series of indoles including indole 3-acetonitrile, 3,3′-diindolylmethane, and indole 3-carbinol (82,83) and a series of aromatic isothiocyanates such as benzyl or phenethyl isothiocyanate (82,84). Onion and garlic oils have also demonstrated anticarcinogenic activity (85,86): allylsulfide compounds from garlic and onions have been shown to inhibit tumorigenesis (87,88).

The biochemical effects of the above compounds and other naturally occurring inhibitors of carcinogenesis such as certain terpenoids (89,90) and coumarins (91) are complex and may result from various biochemical alterations in the target organs. Some other compounds with potential to be chemopreventive agents include reserpine (92), protoanemonin (93), calcium glucarate (94,95), curcumin (96), glabrene (97), hydroxychavicol (98), 9-hydroxyellipticine (99), and certain fatty acids (100). The various types of inhibitors of carcinogenesis and mutagenesis and their mechanisms have been reviewed by others (82,101,102). Wattenberg (82) described three categories of inhibitors: (a) agents that prevent the formation of carcinogens, (b) "blocking" agents that prevent carcinogens from binding to critical targets, (c) "suppressive agents" that prevent the development of tumors after exposure to a carcinogen. Many chemicals act by more than one of these mechanisms. The blocking agents that may act by several mechanisms to inhibit various classes of chemical carcinogens offer excellent potential for finding widely useful inhibitors of carcinogenesis.

The blocking agents themselves can be divided into several subcategories (82). One type inhibits the metabolic activation of carcinogens to their ultimate carcinogenic forms. Another increases the activity of the detoxification system, and a third scavenges the reactive forms of carcinogens (103). This diversity of action has resulted in the development of a number of types of assays for detection of the blocking agent group of chemopreventive agents. One of the best characterized assays involves examining the effect of potential chemopreventive agents on the detoxifying enzyme glutathione transferase (82). This assay has been used by Wattenberg and co-workers to isolate active compounds from a number of classes of plants including cruciferous vegetables, green coffee beans, onions, and garlic (82,88,89). Another assay which has been widely used for the detection of potential chemopreventive agents is the mutation assay in *Salmonella typhimurium* (104). This assay takes advantage of the fact that many classes of chemical carcinogens are also mutagens and may be rapidly detected by a reversion assay in *S. typhimurium* (102,104). Examples of assays using this technique will be discussed further in the section dealing with flavonoids. A third major approach has been the use of assays that measure either the metabolism of the carcinogen itself or the interaction of

carcinogens with DNA. Carcinogen DNA interactions are also known to correlate with carcinogenic activity for a number of classes of chemical carcinogens. Such assays can be carried out either in tissue culture (105) using mammalian cells or in animals (106,107). A major requirement for such assays is the ability to carry them out in a short time period to permit efficient activity-directed fractionation of plants. This has resulted in the majority of activity-directed fractionation studies to date being carried out with either the Ames mutation assay or the carcinogen metabolism assays.

The assay which we have developed for use in isolating potential chemopreventive agents from plants is based upon the effect of these plant extracts on metabolism of the carcinogen benzo[*a*]pyrene B[*a*]P in hamster embryo cells in tissue culture (105). B[*a*]P is a common environmental contaminant that is metabolized in cells by a number of enzyme systems (108). It is activated to an ultimate carcinogenic form through two oxidations by the mixed function oxidase (cytochrome P_{450}) system and an intermediate conversion of the initial arene oxide to a dihydrodiol by epoxide hydrase (108). It is subject to multiple pathways of oxidation as well as conjugation with glucuronic acid, sulfate, and glutathione. The interrelationship of all of these activities determines the relative proportion of hydrocarbon detoxified compared with that activated to ultimate carcinogenic diol-epoxides. These reactive metabolites may be measured by analyzing the binding of B[*a*]P to DNA (109). Hamster embryo cells were chosen for these studies because they are known to have very high metabolizing and metabolic activation capacity for B[*a*]P. The assay involves plating hamster embryo cells in culture vessels and then adding the test compound and tritiated B[*a*]P (105). After an incubation of approximately 18–24 h, the culture medium is removed and aliquots are extracted with a mixture of $CHCl_3$, MeOH, and H_2O. The $CHCl_3$ phase contains the primary hydrocarbon oxidation products as well as unmetabolized B[*a*]P. Multiple oxidation products as well as conjugates remain in the aqueous phase. The amount of radioactivity in each phase is determined by liquid scintillation counting, and the ratio provides a rapid measure of the relative effect of the test compound on the metabolism of B[*a*]P (105). The advantage in such studies is that further analysis may be carried out through the use of hplc or ion-pair chromatography to determine which specific pathways of hydrocarbon metabolism are altered by the test compound (105).

When a test material, usually the initial plant extract, shows an effect on B[*a*]P metabolism, it is then further investigated by analysis of the effect of the test material on the binding of B[*a*]P to DNA (105). This is carried out by treating hamster embryo cells in larger culture vessels (175 cm^2 flask) with the test material and tritiated B[*a*]P for 24 h. The cells are then harvested, and DNA is isolated. The total level of binding of B[*a*]P to DNA is calculated based upon the radioactivity bound to the DNA, and the specific B[*a*]P DNA adducts are then analyzed through enzymatic degradation and reversed-phase hplc (105). If a test fraction has a significant inhibitory effect on B[*a*]P-DNA binding it is considered a good candidate for fractionation to isolate the pure active compound and examine it for potential chemopreventive activity.

Recently, we have incorporated two improvements in the B[*a*]P metabolism assay described by Cassady *et al.* (105). One is an initial assay for cytotoxicity of fractions using a 96-well titer plate and staining for total protein using the BCA assay. (BCA is a trademark of Pierce for protein assays using bicinchoninic acid.) This is essential, for a compound which causes toxicity to the cells would alter B[*a*]P metabolism nonspecifically. Thus, nontoxic doses are selected for our assays. The second improvement involves treating the initial cultures in 12-well culture dishes. This allows us to use only 3.5 ml of media per culture as opposed to the previous 10 ml permitting a rapid assay with a very small amount of test material.

Using this approach, we have screened a number of plants for potential chemo-

preventive agents (105). One example of this is *Trifolium pratense* L. An initial extract of this plant showed high inhibition of B[*a*]P metabolism in the cell culture assay (105). This extract also inhibited the binding of B[*a*]P to DNA in hamster embryo cell cultures (105). The *T. pratense* extract was then fractionated using an activity-directed fractionation based upon effects on B[*a*]P metabolism. From this procedure, a major B[*a*]P metabolism inhibitory component was found to be biochanin A [**36**] (105). Biochanin A produced inhibitions of B[*a*]P metabolism of 30–50% in the range of 10–25 μg/ml of culture medium.

Various flavonoids have previously been demonstrated to have anticarcinogenic activity (82). The synthetic compound 7,8-benzoflavone is known to inhibit tumorigenesis by the polycyclic hydrocarbon 7,12-dimethylbenz[*a*]anthracene in mouse skin (110). Van Duuran and co-workers (111,112) demonstrated that the flavonoids, rutin, morin, and quercetin inhibited tumorigenesis by B[*a*]P in mouse skin. Quercetin was also shown to be an inhibitor of tumor-initiating activity in mouse skin (113). Recently, Mukhtar and co-workers (106,107,114) have investigated the effect of a number of plant phenols on carcinogen metabolism and tumorigenesis in mouse skin. The two flavonoids tested, quercetin and myricetin, had similar activity in inhibiting B[*a*]P metabolism and B[*a*]P DNA adduct formation in Sencar mouse epidermis (106,107). Both flavones also demonstrated activity in inhibiting complete carcinogenesis by hydrocarbons such as B[*a*]P and 3-methylcholanthrene and also by the direct-acting carcinogen *N*-methyl-*N*-nitrosourea (114). This suggests that the inhibitory activity of these compounds may be related to inhibition of carcinogen metabolism and to other effects, for they inhibited both carcinogens requiring metabolic activation and a direct-acting carcinogen.

One mechanism by which plant flavonoids can inhibit the activity of ultimate carcinogenic metabolites is direct reaction with these compounds resulting in their detoxification. The ultimate carcinogenic metabolite of B[*a*]P, B[*a*]P-7,8-diol-9,10 epoxide, was shown to undergo rapid reaction with the plant phenol ellagic acid (115). In a study of the effect of a series of phenolic plant flavonoids on mutagenic activity of B[*a*]P diol-epoxide, Huang *et al.* (116) found that a large number of plant flavonoids were highly effective inhibitors of mutation induction in *S. typhimurium* by B[*a*]P diol-epoxide. Flavonoids that lacked a free hydroxyl were inactive in this assay (116). Thus, one of the major mechanisms of action of these flavonoids in inhibiting mutagenic activity by B[*a*]P diol-epoxide appears to be their interaction with the diol-epoxide rather than with the target cells. Similar conclusions were reached in a study of the effects of plant flavonoids on microsome-mediated binding of B[*a*]P to DNA (117). Although a number of the flavonoids inhibited metabolism of B[*a*]P to B[*a*]P-7,8-dihydrodiol, the extent of inhibition of metabolism did not correlate with their ability to inhibit adduct formation (117). Shah and Bhattacharya (117) conclude that interaction of these compounds with B[*a*]P diol-epoxide may therefore be a major mechanism by which adduct formation is inhibited.

Flavonoids have also been demonstrated to have antimutagenic properties against other types of mutagens and carcinogens. Studies of a series of plant phenolic flavonoids on the metabolic activation of aflatoxin B_1 to metabolites that react with DNA and to mutagenic metabolites in *S. typhimurium* have indicated that a number of these compounds are very effective inhibitors of the activation of aflatoxin B_1 (118,119). Among the most effective inhibitors of S-9-mediated mutagenic activity were a series of flavonols and the isoflavone biochanin A (118,119). (+)-Catechin has been shown to inhibit mutagenesis by 2-aminofluorene and 4-aminobiphenyl in *S. typhimurium* (120). Wall *et al.* (121) recently investigated the effect of a number of flavonoids on mutagenesis in *S. typhimurium* by various types of mutagens. Biochanin A was a very ef-

fective inhibitor of 2-aminoanthracene without toxicity whereas a number of the other compounds tested showed toxicity in this assay. Apigenin was found to have high inhibitory activity against both 2-aminoanthracene and acetylaminofluorene, but much less activity against B[*a*]P (121). In contrast, Birt *et al.* (122) found apigenin to have inhibitory activity against both B[*a*]P and 2-aminoanthracene; however, a lower dose was tested.

Some flavonoids have also been shown to inhibit tumor promotion. Quercetin inhibited tumor promotion by 12-*O*-tetradecanoylphorbol-13-acetate in a mouse skin initiation-promotion assay (123). Apigenin and robinetin both inhibited the TPA-induced elevation of epidermal ornithine decarboxylase activity that is associated with tumor promotion (122). In an in vitro assay, various isoflavonoids from *Wysteria brachybotrys* have been shown to inhibit Epstein-Barr virus early antigen activation in Raji cells (124). The authors had previously shown a relationship between compounds which inhibit this activation and tumor promoters, although a mechanistic rationale for this relationship has yet to be established (124).

To determine the mechanism of inhibition of B[*a*]P activation by biochanin A [**36**], studies were carried out in the hamster embryo cell culture system. Initial studies of the effect of **36** on [^{3}H]B[*a*]P metabolism demonstrated that **36** inhibited formation of both the 9,10- and 7,8-diol as well as the H_2O-soluble metabolites. However, both the 9-hydroxy and 3-hydroxy B[*a*]P present in the media increased in biochanin-A-treated cultures. Further studies, using β-glucuronidase to cleave glucuronide conjugates in the media, demonstrated that in fact, the control group had higher amounts of 9-hydroxy and 3-hydroxy B[*a*]P than the biochanin-A-treated group. Thus, the elevated levels of the 3- and 9-hydroxy B[*a*]P in the biochanin-treated samples were due to inhibition of the formation of the glucuronides of these hydrocarbon phenols. This decrease in glucuronide formation also accounted for most of the decrease in H_2O soluble metabolites caused by biochanin A treatment.

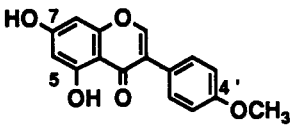
To establish whether biochanin A [**36**] decreases the formation of phenol-glucuronides through inhibition of formation of the glucuronide conjugates, cell cultures were treated with tritiated 3-hydroxyB[*a*]P or tritiated 9-hydroxyB[*a*]P. In both cases, almost three times as much unmetabolized hydroxyB[*a*]P was recovered in the biochanin-A-treated cultures as in the controls. However, subsequent treatment of the media with β-glucuronidase resulted in recovery of similar amounts of these phenols in both cases. Thus, the major effect of **36** on metabolism of hydrocarbon phenols is to greatly reduce the formation of phenol glucuronide conjugates. These results, taken in conjunction with those obtained for B[*a*]P diols, indicate that **36** is affecting at least two metabolic pathways in hamster embryo cells: the oxidation of B[*a*]P by cytochrome P_{450} to form compounds such as the dihydrodiols and the conjugation of B[*a*]P phenols to glucuronides.

In studies of the effect of biochanin A [**36**] on the binding of B[*a*]P to DNA, it was demonstrated that biochanin A treatment resulted in a decrease in the total level of binding at all time points tested between 24 and 120 h. This decrease resulted from a decrease in formation of both the (+)-*anti*-B[*a*]P diol-epoxide and the *syn*-B[*a*]P diol-epoxide. Although the proportional reduction in each was not greatly different, at later time points there was almost 10 times as much (+)-*anti*-diol-epoxide formed in control cultures as *syn*-diol-epoxide. Thus, the major effect of **36** in this system is to reduce the formation of (+)-*anti*-B[*a*]P diol-epoxide. This isomer of the diol-epoxide is the one with the highest carcinogenic activity in rodent bioassays.

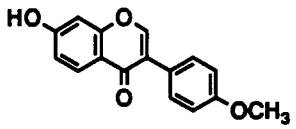
Biochanin A [**36**] was also tested for its ability to inhibit B[*a*]P-induced mutagenesis in Chinese hamster V79 cells. The assay was a cell-mediated mutation assay using hamster embryo cells to activate the B[*a*]P and V79 cells to detect 6-

thioguanine-resistant mutants. After 24 h of exposure to B[*a*]P in the presence or absence of **36**, biochanin treatment reduced the number of mutants per 10^5 cells from 19 in the B[*a*]P-alone group to 1 in the group treated with B[*a*]P plus biochanin A. Biochanin A itself had no mutagenic activity in this assay. Thus, **36** is a very effective inhibitor of mutation induction by the carcinogen B[*a*]P.

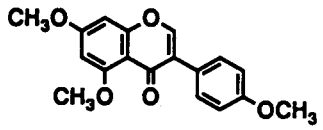
To determine the structural features of the biochanin A molecule responsible for inhibition of B[*a*]P activation, a series of isoflavones and flavones were tested for their ability to inhibit B[*a*]P metabolism to H_2O-soluble derivatives in the hamster embryo cell assay. The results are shown in Figures 2 and 3 which show the structures of the



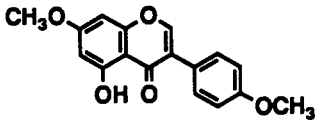
36 biochanin A (25 μg/ml)



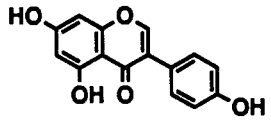
37 formononetin (no effect)



38 4′,5,7-trimethoxyisoflavone (50 μg/ml)



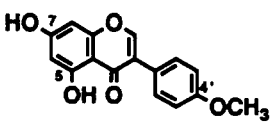
39 5-hydroxy-4′,7-dimethoxyisoflavone (>50 μg/ml)



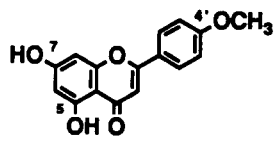
40 genistein (30 μg/ml)

FIGURE 2. Effect of isoflavonoid analogues on B[*a*]P metabolism showing that the 5,7-hydroxyl groups are critical.

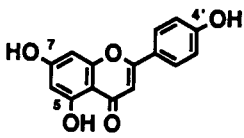
compounds tested and their ID_{50}'s in μg/ml. Neither formononetin [**37**] nor 4′,7-dimethoxyisoflavone (structure not shown) exhibited any significant effect on B[*a*]P metabolism. This would suggest that functionality in the 5 position may be necessary for activity. 4′,5,7-Trimethoxyisoflavone [**38**] had some activity, but it was much weaker than that of biochanin A [**36**]. 5-Hydroxy-4′,7-dimethoxyisoflavone [**39**] was not very soluble and showed even less inhibitory activity, with inhibition measured only at the highest dose tested. Genistein [**40**], the analogue of biochanin A with the 4′ as a free hydroxyl, had activity very similar to biochanin A. It was, however, more



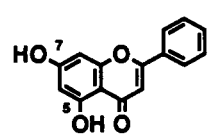
36 biochanin A (25 μg/ml)



41 5,7-dihydroxy-4′-methoxyflavone (15 μg/ml)



42 apigenin (17 μg/ml)



43 chrysin (12 μg/ml)

FIGURE 3. Effect of flavonoid analogues on B[*a*]P metabolism showing that the 4′-hydroxyl group is not critical.

cytotoxic to the cells. 5,7-Dihydroxy-4′-methoxyflavone [**41**], the flavone analogue of biochanin A, exhibited slightly greater inhibitory activity than biochanin A [**36**] itself. Apigenin [**42**], the trihydroxyflavone, also had similar activity. In addition, preliminary data with chrysin [**43**] (5,7-dihydroxyflavone) suggests that it also has a very high inhibitory activity. Thus, present structural information suggests that the presence of both free 5 and 7 hydroxyls is required for high inhibitory activity. However, the role of other portions of the molecule in this activity remains to be established.

One consideration in the use of flavonoids as chemopreventive agents is that there have been several reports of weak mutagenic activity by certain flavonoids themselves. These are reviewed by MacGregor (125) and Natori and Ueno (126). A number of investigators have demonstrated mutagenic activity by certain flavonoids in *S. typhimurium* assays (125–131), and some flavonoids are co-mutagenic with 2-acetylaminofluorene (132). The structural features required for mutagenic activity in this assay, as defined by MacGregor and Jurd (127), include the presence of a free hydroxyl group at the 3 position and a 2,3-double bond. This process requires in addition an intact 3′,4′-catechol group. Because the flavones that inhibit B[*a*]P metabolizing activity in our assay lacked this, as did the isoflavones in the analogous 2 position, it seems likely that the structural features responsible for mutagenic activity will not be important for anticarcinogenic activity and should provide a method of differentiating these two activities in the synthesis of potential chemopreventive agents. Carver *et al.* (133) tested a series of flavonols in Chinese hamster ovary cells and found that they failed to induce significant mutations at the HPRT, APRT, or Na^+/K^+ ATPase loci. This would agree with our finding that biochanin A did not induce significant mutations at the HPRT locus. They did find that certain flavonoids increased the incidence of chromosomal aberrations in Chinese hamster ovary cells, but the small number of compounds tested precluded determination of the structural features responsible for this. Both MacGregor (125) and Natori and Ueno (126) review the studies on carcinogenic activity of flavones. Both point out that early studies may have been subject to problems from contaminants and that the majority of the studies fail to demonstrate carcinogenic activity of flavonoids. Thus, the flavones appear to be excellent candidates for potential chemopreventive agents. After completion of our structure-activity relationship studies to determine the most effective compounds, we plan to test these in animal bioassays as chemopreventive agents.

Another class of common components of cruciferous vegetables that were investigated in the B[*a*]P metabolism assay were the glucosinolates. Wattenberg has demonstrated that benzylisothiocyanate and phenethyl isothiocyanate inhibit tumor induction by 7,12-dimethylbenz[*a*]anthracene (6) and by B[*a*]P (134). Isothiocyanates also inhibit tumor induction by several nitrosomines in rat tissues (135,136). The isothiocyanates in plants are often found as glucosinolates. These are subjected to enzymatic hydrolysis by myrosinase during the processing and digestion of foods (137). Chung *et al.* (135) reported that sinigrin, the glucosinolate of allyl isothiocyanate, decreased nitrosamine demethylation by liver microsomes and decreased DNA binding of two nitrosamines. In our B[*a*]P metabolism assay, neither benzyl glucosinolate nor allyl glucosinolate (sinigrin) had any effect on B[*a*]P metabolism (138). In addition, glucosinolate-containing plant fractions from two other species failed to alter B[*a*]P metabolism. However, a glucosinolate-containing fraction from *Tropaeolum majus* did increase B[*a*]P metabolism by 35%. Glucolimnanthin (*m*-methoxybenzyl glucosinolate) isolated from *Limnanthes douglasii* increased B[*a*]P metabolism by 71% (138). Analysis of the metabolites formed in glucolimnanthin-treated cultures demonstrated that this treatment increased the formation of a number of metabolites, especially the B[*a*]P phenol glucuronides (138). Glucolimnanthin treatment also caused an increase

of greater than 100% in the amount of B[*a*]P bound to DNA. In addition, it caused a substantial increase in the proportion of B[*a*]P activated to the ultimate carcinogenic metabolite (+)-*anti*-B[*a*]P diol-epoxide. Studies with one of the potential metabolites of glucolimnanthin, *m*-methoxybenzyl isothiocyanate, demonstrated that at high doses, this compound was very toxic to the cells; however, at low doses it caused an increase in B[*a*]P metabolism. Thus, if it were formed from glucolimnanthin, it could be responsible for the increase observed in B[*a*]P metabolism observed in glucolimnanthin-treated cultures. These results demonstrate that certain glucosinolates can increase the metabolic activation of the carcinogen B[*a*]P (138). This is in contrast to other studies where certain glucosinolates have been shown to decrease the activation of nitrosamines (135). This suggests that the use of glucosinolates as chemopreventive agents will require analysis of their effects upon a broad range of carcinogens to ensure that they do not have the effect of increasing activation of one class of carcinogen and decreasing that of another. The fact that sinigrin showed no activity in the B[*a*]P metabolism assay while it does inhibit carcinogenesis by nitrosamines suggests that it should be possible to find glucosinolates which would have a chemopreventive action without increasing activation of other forms of carcinogens.

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