Umbelliferone Analogues and Their Potential to Inhibit Benzo(*a*)pyrene- and Hydrogen Peroxide-Induced Mutations

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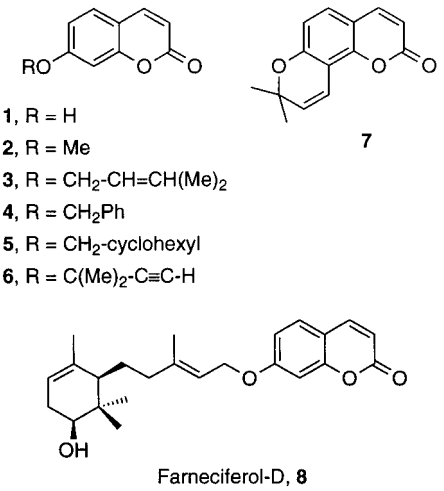
Following the natural product lead, farneciferol-D (kopetdaghin, **8**), some ether analogues of umbelliferone were synthesized and assayed for their potential to be antimutagenic/anticarcinogenic against mutations induced by benzo(*a*)pyrene, a potent mutagen/carcinogen, and hydrogen peroxide, and for their ability to function as free radical scavengers. The "true" antimutagenic effect of these compounds was determined at half the nontoxic concentration in *Salmonella typhimurium* strains utilizing a modified Ames test protocol, and their free radical-scavenging ability was assayed utilizing a nonenzymatic phenazine methosulfate (PMS)−NADH system. Umbelliferone analogues **4** and **5** demonstrated good potential in preventing mutations induced by benzo(*a*)pyrene and hydrogen peroxide and also exhibited good superoxide scavenging ability in the PMS−NADH assay, suggesting that the antimutagenic activity of these analogues may be linked to their antioxidative properties.

One can assume that health hazards from mutational events involve somatic as well as germinal tissue and can lead to various somatic diseases, to teratogenic effects, and to heritable disorders. This is particularly true for the relationship between somatic mutations and cancer, where analyses of oncogene activation during recent years have indicated strongly that specific alterations of DNA and of chromosomes are intimately involved with the carcinogenic process. Evidence for the involvement of somatic mutations in other diseases is not as well founded, but they are believed to be of importance in arteriosclerosis, in some neurological disorders, and in aging.[1]

According to estimates from epidemiological data, at least 75−80% of cancer cases in the United States are caused by environmental factors and could be considered, in principle, as avoidable.[2] Considering the fact that tumor formation is a complex process involving a series of steps, some of which are probably epigenetic, we do not know at present to what extent such environmental factors operate at the level of mutations. However, experimental data on chemical mutagenesis and carcinogenesis lead to the view that mutagenic factors are important in carcinogenesis. These circumstances suggest that certain human cancers might be prevented by identification of antimutagenic agents present in our environment which may be capable of partially protecting humans from exposures to the mutagenic agents deemed hazardous.

In recent years there has been an increasing interest in anticarcinogens of plant origin, particularly those from edible plants.[3−10] It is now becoming clear that higher plants contain a variety of preformed secondary metabolities that represent a structurally diverse array of mutagenic and antimutagenic substances. Study of such substances can lead to much interesting detail about the processes of mutagenicity and antimutagenicity. In only a very few instances are the molecular details presently understood.

Coumarins are one of the most common families of plant secondary metabolites, several of them being reported in a comprehensive survey.[11] Recently there has been considerable interest in the anticarcinogenic and/or antimutagenic activity of some of these compounds. Thus, coumarin itself has been reported to be an inhibitor of dimethylbenz(*a*)anthracene-induced mammary neoplasia, where umbelliferone (7-hydroxycoumarin, **1**) was inactive.[12] Further, Ohta et al. demonstrated that both of the above exhibited antimutagenic activity against 4-nitroquinoline-1-oxide and UV irradiation in *Escherichia coli* (WP2s).[13]



Farneciferol-D, **8**

During the course of an investigation aimed at identifying medicinal agents/leads from plants of Indian and Russian origin, farneciferol-D (kopetdaghin, **8**), a complex ether derivative of umbelliferone was isolated in our laboratory from *Ferula asafoetida* (Indian) and was found to exhibit antimutagenic activity (at a concentration of 32 μg/plate, 45% protection against benzo(*a*)pyrene-induced mutations) in *Salmonella typhimurium* strain TA 100 utilizing the modified Ames test.[14] Farneciferol-D (**8**) is a comparatively rare and complex natural product not serving easily as the starting material for a systematic study of structure−activity relationships (SAR). In a related study, Wall et al. have previously shown that some naturally occurring coumarin analogues exhibit antimutagenic activity against 2-aminoanthracene, acetylamino-

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Table 1. Percent Protection by Umbelliferone Ethers against Benzo(*a*)pyrene-Induced Mutations in *S. typhimurium* TA 100 Strain

compound	concentration per plate (μg)	no. of His^+ revertants (3 plates)	av no. of His^+ revertants	% protection[a]
DMSO[b]	–	129; 150; 139	139	–
benzo(*a*)pyrene	5	869; 882; 865	872	–
1	4	810; 795; 790	798	10
	8	714; 708; 718	713	22
	16	551; 581; 630	587	39
2	2	736; 696; 725	719	21
	4	642; 671; 699	671	28
	8	588; 590; 659	612	36
3	2	747; 733; 721	734	19
	4	668; 665; 638	657	29
	8	633; 583; 601	606	36
4	8	592; 599; 615	602	37
	16	553; 518; 540	537	46
	32	403; 422; 448	424	61
5	4	631; 607; 652	630	33
	8	555; 538; 555	549	44
	16	454; 480; 494	476	54
6	2	794; 811; 801	802	10
	4	726; 780; 771	759	15
	8	703; 663; 694	687	25
7	4	862; 869; 869	867	1
	8	800; 837; 805	815	8
	16	675; 755; 754	728	20

[a] See Experimental Section for description. [b]Negative control.

fluorene, benzo(*a*)pyrene, and nitrofluorene, though at rather high concentrations (150−600 μg/plate).[6] It seems plausible that at the high test concentrations employed, some of the observed end results may be due to an "apparent" antimutagenic effect caused by cellular toxicity rather than resulting from a "true" antimutagenic effect. In our hands such high concentrations have usually been observed to be toxic to the tester strain. As part of a further exploration of the SAR for farneciferol-D (**8**), we decided to synthesize and examine the antimutagenic potential of some structurally simple umbelliferone ethers against mutations induced by benzo(*a*)pyrene in *Salmonella typhimurium* strain TA 100. We wish to report here our findings on the "true" antimutagenic potential of these umbelliferone ethers against benzo(*a*)pyrene- and hydrogen peroxide-induced mutations, determined at half the maximum nontoxic dose using a modified Ames test protocol, and their scavenging potential for free radicals in a nonenzymatic phenazine methosulfate (PMS)−NADH assay.

Results and Discussion

We wish to define clearly what we mean by "true" antimutagenesis. "True" antimutagenesis will involve any agent or effect that specifically or preferentially reduces the *frequency* of mutants, i.e., "true" antimutagenesis is defined as an event wherein an increase in the dose of an agent results in a decrease in mutations in the tester strain without affecting cell survival, or results in a greater decrease in mutations than the decrease in survival. Any effects arising only from altered survival (e.g., cell death) would therefore only constitute "apparent" antimutagenesis.[15] To evaluate the "true" antimutagenic potential of our umbelliferone analogues against benzo(*a*)pyrene-induced mutations, we have utilized half the maximum nontoxic dose of each test compound, determined from a preliminary cytotoxicity assay, in modified Ames tests.

Utilizing this test protocol, the percent inhibition of benzo(*a*)pyrene-induced mutagenesis determined for the umbelliferone analogues **1**−**7** is listed in Table 1. Examination of the percent inhibition of benzo(*a*)pyrene-induced mutagenesis by these umbelliferone ethers at a single, comparable test concentration (8 μg/mL) indicates that alkylation of umbelliferone (with the exception of analogue **7**) improves its antimutagenic activity. Analogues **4** and **5** are observed to be significantly more active than umbelliferone itself at their maximum nontoxic test concentrations (32 and 16 μg/mL, respectively, for **4** and **5**) in this assay.

It is well established that benzo(*a*)pyrene is a promutagen/carcinogen, i.e., it requires metabolic conversion by the cytochrome P-450 enzyme system to its 7,8-diol-9,-10-epoxide form to become a potent mutagen/carcinogen.[16,17] The cytochrome P-450 enzyme system is an extremely important metabolic pathway for the oxidation, and subsequent solubilization and detoxification of lipophilic molecules such as xenobiotics. The enhanced antimutagenic activities for analogues **4** and **5** over umbelliferone suggests that the increased lipophilicity of these analogues, attributable to their greasy side chains, may be a factor contributing to this effect.[18]

The process of metabolic activation of benzo(*a*)pyrene by the cytochrome P-450 enzyme system is postulated to involve an oxenoid complex which serves as an oxygen atom donor.[19] This oxygen-atom transfer process, mediated by the cytochrome P-450 system, may not involve isolatable radical intermediates; however, for some substrates, free radicals are clearly involved.[20−23] To help elucidate the mechanism of inhibition of benzo(*a*)pyrene-induced mutagenesis by these analogues, we next looked at their potential to inhibit mutations induced by hydrogen peroxide in *S. typhimurium* TA 102 strains in which case the cellular insult is caused by hydroxy radicals. Inspection of Table 2 reveals that analogues **4** and **5**, at their maximum nontoxic doses, are the most active of this series in inhibiting hydrogen peroxide-induced mutations in the tester strain, suggesting that they may be functioning as free radical scavengers in these assays.[24,25] However, it must be noted that at comparable nontoxic doses (4 μg/mL), the relative activities of these umbelliferone ethers do not conform to this order possibly due to the nonselective, random nature of the scavenging processes involved in this assay.

To further verify this point, we utilized a scavenger assay to determine the potential of these umbelliferone ethers to scavenge for free radicals generated by a nonenzymatic

Table 2. Percent Protection by Umbelliferone Ethers against Hydrogen Peroxide-Induced Mutations in *S. typhimurium* TA 102 Strain

compound	concentration per plate (μg)	no. of His^+ revertants (3 plates)	av no. of His^+ revertants	% protection[a]
DMSO[b]	–	203; 208; 202	204	–
hydrogen peroxide	100	463; 454; 461	459	–
1	2	434; 443; 445	441	7
	4	421; 428; 429	426	13
	8	381; 399; 387	389	28
2	1	456; 471; 462	463	0
	2	441; 429; 433	434	10
	4	443; 418; 430	430	11
3	1	429; 434; 446	436	9
	2	422; 427; 419	422	14
	4	403; 419; 430	417	17
4	4	436; 435; 429	433	10
	8	420; 422; 418	420	15
	16	389; 346; 380	371	34
5	2	477; 461; 472	470	0
	4	444; 441; 453	446	5
	8	372; 343; 362	359	39
6	1	463; 470; 468	467	0
	2	459; 462; 456	459	0
	4	431; 422; 442	431	11
7	2	470; 467; 471	469	0
	4	439; 441; 435	438	8
	8	386; 419; 402	402	22

[a] See Experimental Section for description. [b] Negative control.

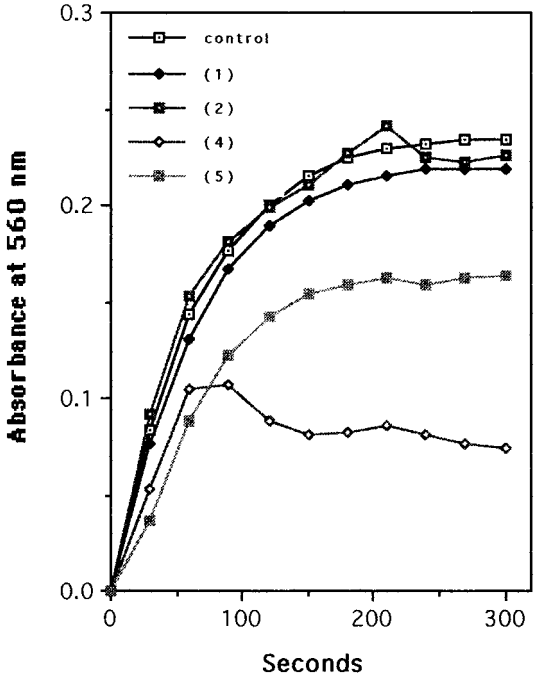

Figure 1. Free radical scavenging potential of umbelliferone and its analogues in the PMS–NADH assay.

system (PMS–NADH). The results of the scavenger assays are shown in Figure 1. We found that analogues **4** and **5** demonstrated the best free radical scavenging potential in this series, an effect that mirrors their efficacy as antimutagenic agents in the benzo(*a*)pyrene- and hydrogen peroxide-induced mutagenesis assays. These results suggest strongly that the antimutagenic activities of analogues **4** and **5** may be related to their antioxidative properties. Thus, it appears from these assays that analogues **4** and **5** may be functioning as antimutagens in the benzo(*a*)pyrene assay by preventing the cytochrome P-450 enzyme system mediated metabolic activation of the pro-mutagen benzo(*a*)pyrene to its mutagenic/carcinogenic 7,8-diol-9,10-epoxide form. However, it must be emphasized that other modes of action cannot be ruled out at present, especially since most antimutagenic agents are well-known to possess multiple mechanisms of action.

Kada et al. have emphasized the necessity for distinguishing "desmutagenic" events occurring outside the cell from "bioantimutagenic" events taking place inside the cell.[26] These desmutagenic events correspond to stage 1 inhibitors acting by preventing the formation of genotoxic precursor compounds. Desmutagens can be further classified into three categories: (1) those which directly (chemically and/or enzymatically) inactivate mutagens; (2) those which inhibit formation of active forms of mutagens, by inhibition of (a) metabolic activation or (b) of formation of mutagens from precursors (such as *N*,*N*-dimethylnitrosamine [DMN] formation from nitrite and amines); and (3) those such as fibers or other high molecular weight substances which adsorb mutagens. On the other hand, the factors or agents which modulate mutagenesis at the cellular levels (inside the cell) are classified as bioantimutagens. Using the classification of Kada et al. described above, we can group analogues **4** and **5** under the category "desmutagens 2a" since they serve to inhibit the metabolic activation of the pro-mutagen, benzo(*a*)pyrene by their ability to scavenge for free radicals.

As a preliminary conclusion, it would appear that the comparatively complex terpenoid side chain of farneciferol-D (**8**) is not needed for antimutagenic activity. Indeed, rather more easily accessible analogues **4** and **5** are significantly more potent and efficacious in the assay systems employed. Of the umbelliferone ethers assayed in this study, **4** and **5** exhibit the least toxicity on account of which higher safe concentrations of these compounds are attainable with the net result that they are far superior in efficacy to the rest. On the basis of our findings here, in particular, the fairly impressive antimutagenic/anticarcinogenic potential demonstrated by analogues **4** and **5** in this series, we conclude that a more extensive investigation into the "true" antimutagenic potential and mechanisms of action of umbelliferone ether derivatives using other test models, e.g., tissue culture systems using murine FM3A cell lines,[27] or animal models using the big blue mouse system,[28] is of considerable practical interest.

Experimental Section

General Experimental Procedures. Proton nuclear magnetic resonance spectra (^{1}H NMR) were recorded on a GE QE-300 spectrometer and chemical shifts (δ) are reported in parts per million (ppm) downfield from tetramethylsilane (Me_4Si; 0.0 ppm). Electron-impact mass spectra (EIMS) were obtained on a Nermag R 10-10 mass spectrometer. The intensity of each peak in the mass spectrum relative to the base peak is reported in parentheses. Microanalyses were performed on a Perkin-Elmer 2400 CHN elemental analyzer at the University of Kansas. Melting points were determined in open capillaries on a Thomas-Hoover capillary melting point apparatus and are uncorrected.

Umbelliferone (**1**) was purchased from Aldrich Chemical Co.

Coumarins (**2**−**4**)[29,30] were prepared from umbelliferone using standard alkylation procedures (alkyl halide, K_2CO_3, acetone, or DMF; see experimental procedure for **5** below). Coumarin (**6**) and seselin (**7**, from **6**) were prepared by the general procedure of Hlubucek et al.[31] The identity of these known coumarin derivatives was confirmed based on their physical properties and spectral data, and their purity was established by elemental analysis. Benzo(*a*)pyrene and S9 were purchased from Moltox, Annapolis, MD; hydrogen peroxide from Fisher Scientific; and Oxoid Nutrient Broth No. 2 from Unipath, Ogdensburg, NY. The remaining biochemicals used in this study were obtained from Sigma.

Umbelliferone Cyclohexylmethyl Ether (5). (Bromomethyl)cyclohexane (1.10 mL, 7.89 mmol) was added to a mixture of umbelliferone (1.00 g, 6.17 mmol) and K_2CO_3 (1.02 g, 7.39 mmol) in dry DMF (10 mL) and the reaction mixture was heated at 70 °C for 24 h. The undissolved material was filtered off with suction and the filter bed was washed with $CHCl_3$. After concentration of the filtrate under reduced pressure, the residual DMF was distilled off on the kugelrohr. The solid residue was taken up into $CHCl_3$ (125 mL) and subsequently extracted with 10% aqueous Na_2CO_3 (2 × 50 mL). The combined aqueous phase was re-extracted once with $CHCl_3$ (50 mL). The organic extracts were pooled, washed with water (50 mL) followed by brine, and finally dried over anhydrous Na_2SO_4. Evaporation of solvent under reduced pressure yielded 1.47 g (92%) of **5**, an analytical sample of which was obtained by recrystallization from EtOAc: mp 121−122 °C; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 7.63 (1H, d, J = 9.5 Hz, H-4), 7.36 (1H, d, J = 8.5 Hz, H-5), 6.86−6.79 (2H, m, H-6, H-8), 6.22 (1H, d, = 9.5 Hz, H-3), 3.81 (2H, d, J = 6.1 Hz, OCH_2), 1.92−1.00 (11H, m, cyclohexyl); EIMS *m*/*z* 259 (M + 1, 42), 258 (M^+, 30), 163 (32), 162 (60), 134 (43), 55 (100); *anal.* C 74.21%, H 6.68%, calcd for $C_{16}H_{18}O_3$, C 74.40%, H 7.02%.

Cytotoxicity Assays. Cytotoxicity assays were performed using *S. typhimurium* strain TA 100 or TA 102 grown in Oxoid Nutrient Broth No. 2 using a 96-well ELISA plate containing varying concentrations of test compounds incubated for 15 h at 37 °C. After incubation, the plates were subjected to readings at absorbance 570 nm using a Cambridge Technology, Inc. Plate Solver, Version 4.00.

Modified Ames Tests. The modified Ames tests were performed based on the initial protocol described by Maron and Ames[32] using *S. typhimurium* TA 100 or TA 102, with the incorporation of a mutagenic agent, e.g., benzo(*a*) pyrene or hydrogen peroxide. The cells were grown for 15 h in Oxoid Nutrient Broth No. 2 at 37 °C with shaking to an approximate density of (1−2) × 10^8 cells/mL prior to each assay.

To initiate the experiment, 0.1 mL of the culture was added to test tubes containing 2 mL of Ames top agar supplemented with 50 μg/mL of L-histidine and 0.74 μg/mL of D-biotin. To induce His^+ revertants, 100 μg of hydrogen peroxide (TA 102) or 5 μg of benzo(*a*)pyrene with 50 μL of S9 (TA 100) was used. To determine the antimutagenic potential of the umbelliferone derivatives in this investigation, varying concentrations of test compounds were added to the above and the mixture was then plated on VogelBonner E Medium using the top agar layer method as described by Maron and Ames.[32] After incubation at 37 °C for 2 days the plates were scored for His^+ revertant colonies using a Dynatech Autocount colony counter. From these data, the percent inhibition values for the test compounds in this study were calculated using the following equation:

% inhibition = 100% − [(induced revertants in the presence of test compounds, 5 μg of benzo(*a*)pyrene and 50 μL of S9) − (spontaneous revertants)/(induced revertants in the presence of 5 μg of benzo(*a*)pyrene and 50 μL of S9) − (spontaneous revertants) × 100].

Superoxide Scavenging Assays (Nonenzymatic PMS−NADH Assays). The nonenzymic generation of superoxide anions was measured utilizing samples containing 10 μmol of phenazine methosulfate, 78 μmol of NADH, and 250 μmol of nitro blue tetrazolium in 100 mmol of phosphate buffer (pH 7.4) in the presence or absence of test compounds. Superoxide anions were estimated by the spectrophotometric measurement of the product of the reduction of nitro blue tetrazolium, recorded every 30 s for 5 min at 560 nm against blank samples which contained no phenazine methosulfate.[33]

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