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Phytotoxic Activity of Bibenzyl Derivatives from the Orchid *Epidendrum rigidum*

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A whole plant chloroform−methanol extract of the orchid *Epidendrum rigidum* inhibited radicle growth of *Amaranthus hypochondriacus* seedlings ($IC_{50} = 300$ μg/mL). Bioassay-guided fractionation furnished four phytotoxins, namely, gigantol (**1**), batatasin III (**2**), 2,3-dimethoxy-9,10-dihydrophenathrene-4,7-diol (**9**), and 3,4,9-trimethoxyphenanthrene-2,5-diol (**11**), along with the known flavonoids apigenin, vitexin, and isovetin and the triterterpenoids 24,24-dimethyl-9,19-cyclolanostane-25-en-3β-ol (**14**) and 24-methyl-9,19-cyclolanostane-25-en-3β-ol (**15**). Stilbenoids **1**, **2**, **9**, and **11** inhibited radicle growth of *A. hypochondriacus* with IC_{50} values of 0.65, 0.1, 0.12, and 5.9 μM, respectively. Foliar application of gigantol (**1**) at 1 μM to 4 week old seedlings of *A. hypochondriacus* reduced shoot elongation by 69% and fresh weight accumulation by 54%. Bibenzyls **1** and **2**, as well as synthetic analogues 4′-hydroxy-3,3′,5-trimethoxybibenzyl (**3**), 3,3′,4′,5-tetramethoxybibenzyl (**4**), 3,4′-dihydroxy-5-methoxybibenzyl (**5**), 3′-*O*-methylbatatasin III (**6**), 3,3′,5-trihydroxybibenzyl (**7**), and 3,4′,5-trihydroxybibenzyl (**8**), were tested for phytotoxicity in axenic cultures of the small aquatic plant *Lemna pausicostata*. All bibenzyls derivatives except **7** and **8** inhibited growth and increased cellular leakage with IC_{50} values of 89.9−180 and 89.9−166 μM, respectively. The natural and synthetic bibenzyls showed marginal cytotoxicity on animal cells. The results suggest that orchid bibenzyls may be good lead compounds for the development of novel herbicidal agents.

KEYWORDS: ***Epidendrum rigidum*; orchidaceae; bibenzyls; phenantrenes; stilbenoids; phytotoxicity; cytotoxicity; *Lemna pausicostata*; duckweed; *Amaranthus hypochondriacus*; gigantol; batatsin III**

INTRODUCTION

Constituents from natural sources may enable the discovery of novel molecular target sites and unknown mechanisms of action that could lead to the discovery of new classes of herbicides. The environmental half-life of many natural compounds is shorter, and they are generally less toxic to the environment than many of the synthetic herbicides (*1*). The stilbenoids so far obtained from Mexican orchids have demonstrated interesting biological properties including phytotoxicity (*2*−*5*). Two phenanthrene derivatives, erianthridin and gymnopusin, isolated from *Maxillaria densa* Lindley, were phytotoxic to *Lemna pausicostata* L. (duckweed), causing electrolyte leakage, chlorophyll loss, and photobleaching (*3*). Gymnopusin induced various ultrastructural changes on duckweed frond and root tissues, which were consistent with membrane destruction (*3*). Other investigators have also demonstrated the herbicidal potential of bibenzyls and phenanthrenes; batatasin III (**2**) (**Figure 1**) inhibited the growth of liverworts, algae, and oat coleoptil (*6*). On the other hand, the phenanthrene batatasin I inhibited the CO_2-dependent O_2 evolution and the flow of electrons from water to methyl viologen in spinach chloroplasts and succinate-dependent O_2 uptake in potato tuber mitochondria (*6*). Continuing with our work on Mexican orchids as a source of phytotoxic agents, we report herein the isolation of the major phytotoxins from *Epidendrum rigidum* Jacq., the most widespread and common *Epidendrum* species in tropical regions of the Western hemisphere. Furthermore, considering that slight structural modifications of active compounds can either increase or decrease their bioactivity, we compared the phytotoxic effects on *L. pausicostata* of several synthetic analogues of gigantol (**1**) and batatasin III (**2**).

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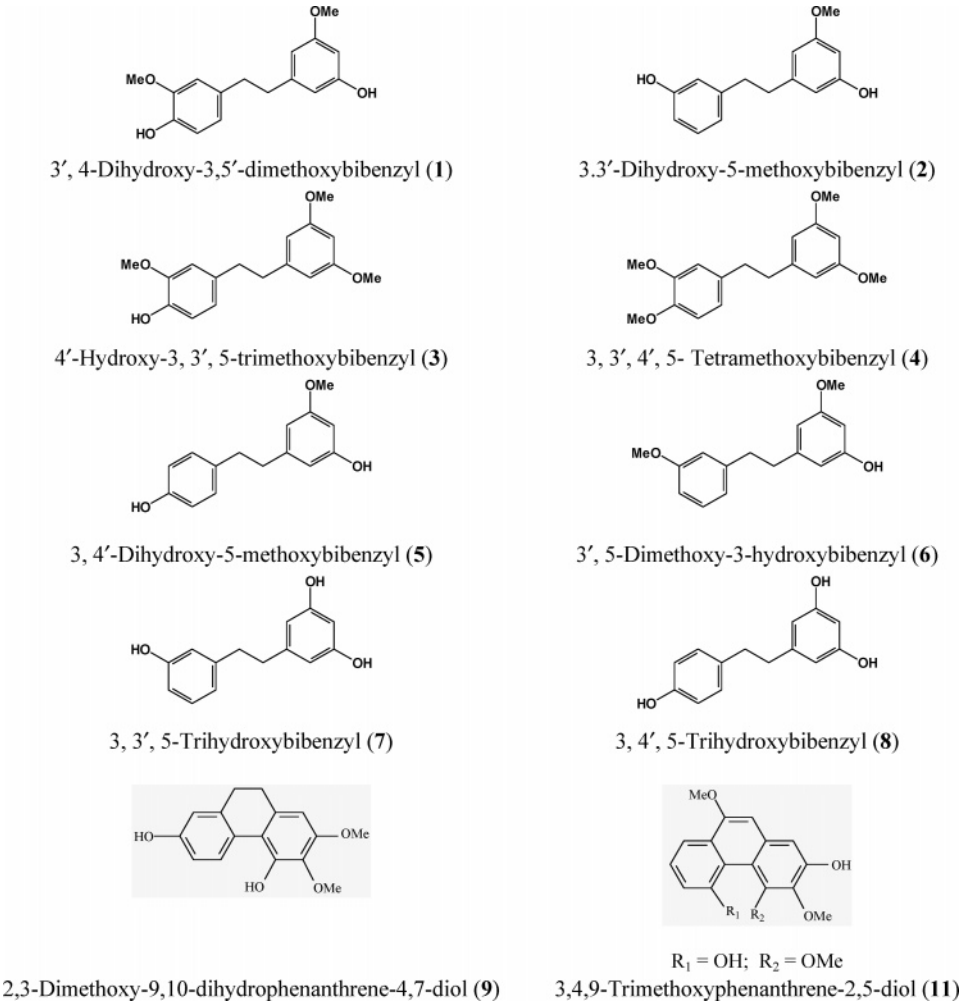

Figure 1. Structures of the bibenzyl derivatives tested for phytotoxicity and in vitro mammalian toxicity.

MATERIALS AND METHODS

General Experimental Procedures. Melting points were determined on a Fisher-Johns apparatus and are uncorrected. IR spectra were obtained using KBr disks on a Perkin-Elmer FT 1605 spectrophotometer. NMR spectra were recorded in $CDCl_3$ on a Varian Unity Plus 500 spectrometer at either 500 (^1H) or 125 (^{13}C) MHz, using tetramethylsilane as an internal standard. Electron impact mass spectra were obtained on a JEOL SX 102 mass spectrometer. Open column chromatography was carried out on silica gel 60 (70−230 mesh, Merck) or ODSA-120-S150 (YMC-Gel). Analytical thin-layer chromatography (TLC) was performed on precoated silica gel 60 F254 plates (Merck). High-performance liquid chromatography (HPLC) was carried out with a Waters HPLC instrument equipped with Waters 996 UV photodiode array detector (900) set at 209−214 nm or a Waters 410 Differential Radiofractometer using a 300 mm × 19 mm i.d. μPorasil column (Waters) at a flow rate of 8.3 mL/min. Control of the equipment, data acquisition, processing, and management of chromatographic information were performed using Millennium 2000 software program (Waters).

Plant Material. *E. rigidum* whole plants were collected in Catemaco, State of Veracruz, Mexico, in October 1996. An authenticated voucher specimen (G. Carmona-Díaz-96-3) was preserved at the Instituto de Ecología Herbarium (XAL), Xalapa, Veracruz.

Extraction and Isolation. Air-dried plant material (1.7 kg) was ground into a powder and extracted exhaustively by maceration at room temperature with $CHCl_3$−MeOH (1:1). The extract was filtered and concentrated in vacuo to yield 340 g of a brown residue, which was subjected to column chromatography over silica gel (1 kg). The column was eluted with a gradient of hexane−EtOAc (1:0 → 0:1) followed by EtOAc−MeOH (1:0 → 1:1) to produce 10 pooled fractions (F-I to F-X). According to the initial phytotoxicity test, only fractions F-VII−F-IX were active, inhibiting the radicle growth of *A. hypochondriacus* by 100% when tested at the IC_{50} of the extract.

The active fraction F-VII (8.0 g, eluted with hexane−EtOAc, 9:1) was chromatographed on a silica gel column (200 g) using hexane−EtOAc (9:1) as a solvent to yield 12 secondary fractions (FVII-1 to FVII-12). The most active fractions according to the bioautographic assay (*15*) were FVII-7 (700 mg) and FVII-9 (1.2 g). From fraction FVII-7 crystallized spontaneously 40 mg of 2,3-dimethoxy-9,10-dihydrophenanthrene-4,7-diol (**9**); the NMR and mass spectra of **9** were identical to those in the literature (*7*). HPLC (hexane-$CHCl_3$-*i*-PrOH-MeOH, 75:23:1:1) separation of active fraction FVII-9 (1 g) afforded gigantol (**1**, 170 mg; R_t = 30 min) and batatasin III (**2**, 34 mg; R_t = 62 min) as the only active compounds. These two bibenzyls were identified by comparison with authentic compounds previously isolated from *Nidema boothi* (*5*). Apigenin (**10**, 18 mg) was crystallized from active fraction VIII (5 g); its spectroscopic properties were identical to those previously described (*8*, *9*). Part of the mother liquor (1 g) of fraction F-VIII was further fractionated by column chromatography on silica gel (190 g), eluting with a gradient of hexane−EtOAc (1:0 →1:1) to yield five secondary fractions (FVIII-1−FVIII-5). From fraction FVIII-1 (680 mg) crystallized 309 mg of a mixture of two cyclolanostane type of triterpenes, which were resolved by HPLC using a gradient of elution with hexane−EtOAc−$CHCl_3$ (1:0:0→2:1:1). The compounds were identified as 24,24-dimethyl-9,19-cyclolanostane-25-en-3β-ol (**14**, 213 mg; R_t = 18.3 min) and 24-methyl-9,19-cyclolanostane-25-en-3β-ol (**15**, 95.8 mg; R_t = 19.3 min) by comparison of the NMR, IR, and MS data with those of authentic samples previously obtained from *Scaphyglottis livida* (*10*). Fraction FVIII-4 (200 mg), eluted with hexane−EtOAc (4:1), was phytotoxic according to the bioautographic assay (*15*). HPLC (hexane-$CHCl_3$-*i*-PrOH-MeOH, 70:28:1:1) purification of

a portion of FVIII-4 (37 mg) yielded 11 mg of 3,4,9-trimethoxyphenanthrene-2,5-diol (**11**, R_t = 27 min) (*11*).

Phytotoxic fraction F-IX (11 g, eluted with hexane−EtOAc 3:7) was chromatographed on a silica gel column (220 g) using hexane−$CHCl_3$ (1:1) as a solvent to yield three secondary fractions (FIX-1 to FIX-3). The most phytotoxic fraction according to the bioautographic assay was FIX-2 (560 mg). Further purification of FIX-2 by HPLC (hexane-$CHCl_3$-*i*-PrOH-MeOH 75:23:1:1) yielded additional amounts of **1** (87 mg) and **2** (140 mg). From inactive fraction IX (18 g) spontaneously precipitated a mixture of isovitexin (**12**) and vitexin (**13**). Column chromatography of part of this precipitate (625 mg) on reverse phase silica gel (19 g), eluting with H_2O−MeOH (1:4→4:1), afforded four fractions (IX-1−IX-4). Fraction IX-2, eluted with H_2O−MeOH (2:3), yielded 125 mg of isovitexin (**12**). Fraction IX-3, eluted with H_2O−MeOH (4:1), yielded 50 mg of vitexin (**13**) (*8, 12*).

Synthesis of Bibenzyls 3−8. 4′-Hydroxy-3,3′,5-trimethoxybibenzyl (**3**), 3,3′,4′,5-tetramethoxybibenzyl (**4**), 3,4′-dihydroxy-5-methoxybibenzyl (**5**), 3′-*O*-methylbatatasin III (**6**), and 3,3′,5-trihydroxybibenzyl (**7**) were prepared using the Wittig reaction as previously described (*5*). 3,4′,5-Trihydroxybibenzyl (**8**) was prepared by catalytic hydrogenation of piceatannol as reported earlier (*5*). In all cases, the spectroscopic properties of the synthetic materials were identical to those previously reported (*5*).

Phytotoxicity Bioassay by Inhibition of Radicle Elongation of *Amaranthus hypochondriacus*. The phytogrowth inhibitory activity of the extract and compounds **1**−**11**, **14,** and **15** was evaluated on seeds of *A. hypochondriacus* by using inhibition of radicle elongation from seeds growing in a Petri dish (*13*). The results were analyzed by analysis of variance (ANOVA) ($p < 0.05$), and IC_{50} values were calculated by Probit analysis based on percent of radicle growth or germination inhibition. The extract was evaluated at 1, 10, 100, and 1000 μg/mL. The pure compounds were tested at 0.1, 1, 10, 100, and 500 μM. 2,4-Dichlorophenoxyacetic acid (2,4-D) was used as the positive control. The bioassays were performed at 28 °C.

Phytotoxicity Bioassay by Direct Bioautography. A direct bioautographic bioassay system was employed to guide secondary fractionation and speed up the isolation of active compounds. The direct bioautographic assay was carried out and interpreted as previously described (*13*). Briefly, TLC was carried out on a 20 cm × 7 cm silica gel glass plate (Merck, Kieselgel 60 F254) 0.25 mm thick using appropriate developing solvents (hexane−$CHCl_3$ 1:1; $CHCl_3$; $CHCl_3$−EtOAc 9:1). The chromatogram was covered with a 0.5% agar layer (2 mm), and the seeds of *A. hypochondriacus* were sown in a row perpendicular to the bands on the chromatogram. The seeds were incubated in a moisture-saturated growth chamber at 28 °C during 1 day, when seed germination and plant growth were examined.

Effect of Gigantol (1) on Fresh Weight and Shoot Elongation of *A. hypochondriacus*. The determination of the effect of **1** on fresh weight and shoot elongation of *A. hypochondriacus* was performed as described by Hoagland (*14*). Three trays of 4 week old seedlings (6−8 seedlings per tray) were treated on the foliage with gigantol (**1**) solutions (0.01, 0.1, and 1 μM) containing Tween 80 [0.01% (vol/vol)] as a wetting agent. Foliar applications were made using a cotton swab. In each case, Tween 80 [0.01% (vol/vol)] and paraquat (0.1 μM) were used as negative and positive controls, respectively. Visual damage in the foliage was observed during 4 weeks after the treatments. All experiments were based on a completely randomized design with a factorial structure. Data on plant elongation and fresh weight were recorded at the end of week four after the application of the testing material. The information was subjected to ANOVA, and mean comparisons were performed using Fischer's protected least significant differences.

Phytotoxicity Bioassay in Duckweed. The duckweed bioassay was carried out in triplicate as described by Tanaka et al. (*15*). Briefly, 20 colonies of three fronds each were incubated for 3 days in 3.5 cm polystyrene Petri dishes containing 3 mL of half-strength Hunter's medium (*16*) with the toxin (compounds **1**−**8**) dissolved in 1% (vol/vol) DMSO at 0, 25, 50,100, 200, and 1000 μM. A conductivity meter was used to determine electrolyte leakage at intervals of 12 h during the incubation period by sampling and returning the bathing medium to each dish. Data are expressed as a percentage increase in conductivity over the control. Chlorophyll bleaching was estimated visually. Growth inhibition was measured by comparing duckweed fresh weight at the beginning and at the end of the experiments. DMSO at the final concentration did not affect any of these three measures of phytotoxicity.

Cytotoxicity Assays. Four permanent mammalian cell lines were used. Untransformed 3T3 Swiss mouse fibroblasts (strain NIH3T3) were obtained from A. Aaronson, National Cancer Institute (Bethesda, MD), and oncogenically transformed 3T3 mouse fibroblasts (strain KA31T, transformed by the Kirsten strain of Moloney sarcoma virus) were obtained from R. Pollack, Columbia University (New York). Rat hepatoma cell line H4TG and dog kidney cell line MDCK were purchased from the American Type Culture Collection (Rockville, MD). The cells were cultured, and the cytotoxicity assays were conducted as described previously (*17, 18*).

Briefly, cytotoxicity bioassays were carried out for each cell line in triplicate 200 μL cultures in 96 well microtiter trays (Nunc MicroWell) with the bibenzyls at 0, 1, 2, 5, 10, 20, 50, 100, and 200 μM in Dulbecco's modified Eagle's medium containing 5% (vol/vol) calf serum. The wells were inoculated with 10000 cells from an actively growing culture and grown for 5 days at 37 °C in a humidified CO_2-containing atmosphere. Growth of the culture was evaluated by fixing washed cultures with 3.7% (wt/vol) formaldehyde in saline, staining with two drops of 0.05% (wt/vol) crystal violet in 20% (vol/vol) aqueous methanol, washing away unbound dye with tap water, drying, extracting bound dye by addition of 200 μL of DMSO, and measuring absorbance of each well at 562 nm using a SPECTRAmax PLUS microplate spectrophotometer (Molecular Devices Corporation, Sunnyvale, CA). The results of percent inhibition at each concentration for each cell line were analyzed by ANOVA. IC_{50} values were estimated from straight lines fitted by the least-squares method from the plots of the mean percent inhibition values against the logarithm of the concentration.

RESULTS AND DISCUSSION

E. rigidum was selected for activity-guided fractionation on the basis of the significant phytotoxicity in a chloroform−methanol (1:1) extract of the whole plant against *A. hypochondriacus* (IC_{50} = 300 μg/mL). Bioassay-guided fractionation of the active crude extract furnished four phytotoxins, namely, gigantol (**1**), batatasin III (**2**), 2,3-dimethoxy-9,10-dihydrophenathrene-4,7-diol (**9**), and 3,4,9-trimethoxyphenanthrene-2,5-diol (**11**). Compounds **1** and **2** were characterized by comparison with authentic samples (*5*), and compounds **9** and **11** were characterized by comparison of their IR, NMR, and MS spectra with those previously reported (*7, 11*). In addition, apigenin, vitexin, and isovetin (*9, 12*) as well as 24,24-dimethyl-9,19-cyclolanostane-25-en-3β-ol (**14**) and 24-methyl-9,19-cyclolanostane-25-en-3β-ol (**15**) were isolated (*10*). Bibenzyls **1**, **2**, **9**, and **11** inhibited radicle growth of *A. hypochondriacus* with IC_{50} values of 0.65, 0.1, 0.12, and 5.9 μM, respectively. The phytotoxic effect was greater (2:9) or lower (1:11) than that of 2,4-dichlorophenoxyacetic acid (IC_{50} = 0.19 μM) used as the positive control. The known flavonoid apigenin was inactive (IC_{50} > 100 μM), to inhibit the growth, although it has been reported that this compound was able to perturb auxin transport (*19*). Thus, apparently, the bibenzyl and phenanthrene derivatives are the main phytotoxic principles of the plant.

Foliar application of gigantol (**1**) at 1 μM to 4 week old seedlings of *A. hypochondriacus* reduced shoot elongation (69%) and fresh weight accumulation (54%). At 0.1 μM, the treatments inhibited only fresh weight accumulation (20%); at these two concentrations, the bibenzyl induced necrosis, desiccation, and leaf abscission. Paraquat (0.1 μM) was used as a standard; the strength of the biological activity of this commercial herbicide was comparable to that of gigantol (**1**).

The studies on phytotoxicity of bibenzyl derivatives were extended to *L. pausicostata* (duckweed) axenic cultures, which

Table 1. Effect of Bibenzyls on *L. pausicostata* Cultures at 72 h[a]

	treatment IC_{50} (μM)			treatment IC_{50} (μM)	
compd	conductivity leakage	growth inhibition	compd	conductivity leakage	growth inhibition
1	166	180	**5**	144	169
2	145	159	**6**	89.9	94.7
3	116	89.9	**7**	725	985
4	148	117	**8**	>1000	>1000

[a] Results are the means of three replicates. All samples exhibited significantly ($P < 0.005$, Student's unpaired *t*-test) higher phytotoxicity than the controls at all concentrations tested.

is one of the best characterized models for assessing phytotoxic activity. The duckweed assay system makes it possible to study toxic effects throughout the plant life cycle, as well as plant specific toxic effects that target photosynthesis. Bibenzyls **1** and **2** as well as the analogues **3**−**8** were tested in triplicate in the small aquatic plant culture. Analogues **3**−**8** were synthesized in order to investigate the effect on phytotoxicity of oxygenated substituents (phenolic vs phenolic methyl ether) and their location on bibenzyl core structure. All synthesized analogues except **8** were obtained using the Wittig reaction, a method widely applied for the synthesis of bibenzyls (*5*, *6*). Analogue **8** was synthesized by catalytic reduction of piceatannol (*5*). In all cases, their spectroscopic data (IR, NMR, and MS) were in agreement with those reported (*5*). Bibenzyl analogues **6**−**8** have also been identified as natural products (*20*−*22*).

The data presented in **Table 1** show that except for compounds **7** and **8**, all of the analogues inhibited growth and caused cellular leakage in duckweed cultures with IC_{50} values ranging between 89.9 and 180 μM and 89.9 and 166 μM, respectively. Compounds **6** and **3** were most active whereas compounds **7** and **8**, with only free hydroxyl groups in both aromatic rings, were the least active. These results are consistent with bibenzyl derivatives requiring at least one methoxyl group at C-3 or C-5 for strong phytotoxicity.

The potential use of natural products as bioherbicides is limited by concerns about safety for animals and humans. Because ideal candidates for commercially viable herbicides should have both strong phytotoxicity to susceptible weeds and low mammalian toxicity, we evaluated compounds **1**−**8** for in vitro toxicity against four cultured mammalian cell lines representing undifferentiated normal and tumor cells and differentiated kidney and liver lines. No obvious structure−activity relationships were observed for mammalian toxicity (**Table 2**). Plant and animal in vitro toxicity (compared as concentrations causing half-maximal inhibition of growth) did not correlate. These results are consistent with toxicity by different mechanisms in plant and animal systems.

In conclusion, we have demonstrated that the orchid bibenzyls gigantol and batasin exhibited good phytotoxicity on both *Amaranthus* and duckweed. A series of synthetic analogues showed similar activity to that of the parent compounds, and it was found that for greatest activity the bibenzyls must have a methoxyl group at C-3 (C-5). These results suggest that these and structurally related bibenzyls are good lead compounds for the development of novel class of herbicidal agents. Because some of these compounds inhibited the ability of calmodulin (CaM) to activate CaM sensitive enzymes such as CaM sensitive phosphodiesterase (*5*), the regulatory protein CaM might be a biological target for the phytotoxic action of orchid bibenzyls gigantol (**1**) and batatasin III (**2**) as previously described for ophiobolin A, a phytotoxin isolated from several species of the genus *Bipolaris* (*23*) and other phytoxins (*13*, *24*).

Table 2. Cytotoxicity of Bibenzyls in Cultured Mammalian Cell Lines[a]

	IC_{50} (μM)			
substance	NIH3T3	MDCK	KA31T	H4TG
1	25.8	35.9	57.3	75.0
2	14.7	81.9	49.4	123.7
3	94.2	97.5	141.6	76.5
4	>200	>200	108.5	195.6
5	29.3	79.6	22.3	83.8
6	60.6	107.1	45.0	126.2
7	32.3	74.2	57.2	118.1
8	65.1	78.3	43.9	165.8

[a] IC_{50}: The concentration of toxin, which causes a 50% reduction in cell-bound dye after 5 days in culture. Cell lines: H4TG, thioguanine resistant rat hepatoma cells; MDCK, Madin-Darb canine kidney cells; NIH3T3, NIH Swiss mouse embryo fibroblasts; and KA31T, Kirsten strain of Moloney sarcoma virus-transformed 3T3 cells.

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