CYTOTOXIC PRINCIPLES FROM THE SAP OF *KALMIA LATIFOLIA*

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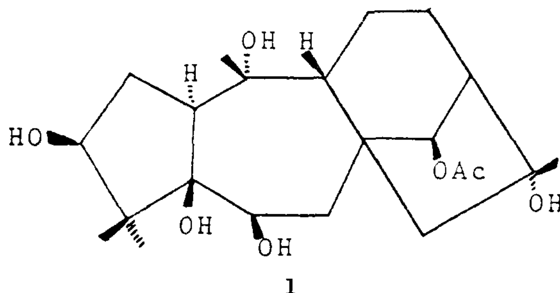
ABSTRACT.—Examination of the sap of *Kalmia latifolia* has revealed Grayanotoxin I (**1**), phloretin (**3**), and 2',6'-dihydroxy-4-methoxyacetophenone (**5**) as cytotoxic components. Twenty-one dihydrochalcones have been synthesized and tested for cytotoxic activity. 2',3,3',4,4'-Pentahydroxydihydrochalcone (**8**) has been found to be cytotoxic and to have marginal activity *in vivo*. Seven new dihydrochalcones are described.

Kalmia latifolia L. (Mountain laurel) is a well-known perennial herb of the family Ericaceae, commonly found in the woods of Connecticut. Previous chemical investigation of the species has demonstrated the presence of the toxic diterpenoid grayanotoxin I (andromedotoxin, acetylandromedol) [**1**] (1), the dihydrochalcones phlorizin [**2**] (2), the glycoside of phloretin [**3**], and asebotin [**4**] (3).

The toxicity of the grayanotoxins is well established *in vivo*. For example, **1** has an LD_{50} (I.P. in mice) of about 1.3 mg/kg (4), but no *in vitro* cytotoxicity data have been published.



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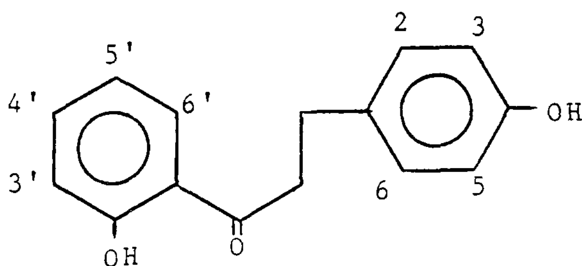


2 4'OH, 6'OGlu.
3 4'OH, 6'OH.
4 4'OMe, 6'OGlu.

As a class, the dihydrochalcones seem to be remarkably free of toxicity in laboratory animals (5) and *in vitro* testing against the 9KB cell line indicates ED_{50} values generally >20 $\mu g/ml$ (6).

Recently however, uvaretin, a 3'-benzyldihydrochalcone isolated from *Uvaria*

acuminata, and some of its derivatives have shown activity (T/C 132–144) in the P388 lymphocytic leukemia screen (7).

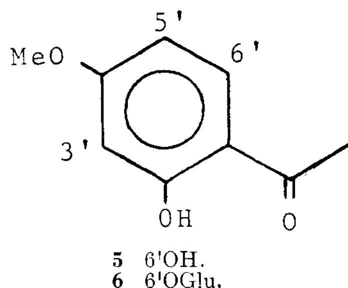
DISCUSSION

Normal solvent extraction of oven-dried *K. latifolia* gave fractions which were inactive in the P388 system, whereas the expressed sap (leaves and stems) had reproducible activity (T/C = 150). Further solvent fractionation of the concentrated sap with chloroform, ethyl acetate, and butanol gave active extracts (see experimental).

The butanol extract was subjected to chromatography over silica gel with chloroform, ethyl acetate, ethyl acetate-ethanol, and ethanol used sequentially as eluting solvents. The fractionation was followed by a quantitative, *in vitro*, cytotoxicity screen involving the single cell plating of 9KB cells. Briefly, inocula of *ca* 200, 2000, and 20,000 9KB cells were made; the test compound at 1, 10 and 100μg/ml was added. After incubation and staining, the cell colonies were counted, thus giving a direct determination of surviving cells. This screening procedure is different from that used by the National Cancer Institute. The modification was developed so that test results could be obtained quickly (in about two weeks); the single plating technique has the additional advantage that it shows changes in cell morphology in response to the drug under test, and further that it can differentiate between, for example, the death of half the cells and a decrease in growth rate of 50%, and between cell multiplication and cell growth without division. Neither of these is possible if the NCI protocol is followed.

The chloroform fractions from the column were uniformly inactive and were not further investigated. Elution with ethyl acetate yielded a white crystalline compound, mp 262–4°, which was identified as phloretin [**3**]. The dihydrochalcone had an ED_{50} of 28 μg/ml but proved to be inactive in extensive *in vivo* testing. The initial tests had used a saline vehicle, but since it was felt that [**3**] might still be the active component of the sap and that there might be a transport or solubility problem, additional vehicles were investigated. No increased activity was observed in tween-80, saline and alcohol, or Klucel. A careful tlc-examination of the ethyl acetate fractions, which contained [**3**] and which were more active *in vitro* than the purified phloretin, indicated the presence of an additional component having the same R_f as [**3**] in our analytical system. The compound was identified as 2',6'-dihydroxy-4'methoxyacetophenone [**5**]. The acetophenone had an ED_{50} of 4.7 μg/ml, but had no activity *in vivo*.

Further elution with ethyl acetate gave asebotin [**4**], which was inactive *in vitro*, and grayanotoxin I [**1**], which was mildly active *in vitro* (ED_{50} 60 μg/ml) but was toxic *in vivo* (no survivors at >1.5 mg/kg). Further chromatography gave phlorizin [**2**], which was inactive *in vitro*.



5 6'OH.
6 6'OGlu.

Although some of the compounds isolated from the sap were cytotoxic to a degree, none was active *in vivo*. We were unable to identify any crude fractions having greater *in vitro* activity than the original butanol extract; thus, further investigation must rely on *in vivo* testing to guide the fractionation.

In view of the above findings, we undertook the synthesis of several dihydrochalcones and acetophenones in order to explore their potential as cytotoxic agents. The compounds and their activities are listed in tables 1 and 2. Eighteen

TABLE 1. Cell survival data for dihydrochalcones.

Compound Number	2'	3'	4'	5'	6'	3	4	% Survivors at 100 μg/ml	% Survivors at 10 μg/ml	ED_{50} μg/ml
	H	H	H	H	H	H	H	0	100	
	OH	OH	OH	H	H	H	OH	0	100	
	H	H	H	H	OH	H	OH	0	100	
	OH	H	OMe	H	OH	H	OH	0	100	
	OH	H	OMe	H	H	H	OH	0	100	
10	OH	H	H	H	OH	H	OH	20	100	
16	OH	H	OH	H	OH	OH	OMe	46	100	
	OH	H	OH	H	OH	H	H	0	100	
9	H	H	H	H	OH	OH	OH	0	100	
15	OH	H	OH	H	OH	OH	OH	0	95	
7	H	H	OH	H	H	H	OH	0	90	
	H	H	H	H	H	H	OH	0	89	
	H	H	H	H	H	OMe	OH	0	82	
	H	H	H	H	OH	OMe	OH	0	76	
3	OH	H	OH	H	OH	H	OH	0	76	28
12	H	OH	H	H	H	H	OH	0	71	
13	OMe	H	OMe	H	OH	H	H	5	59	
11	H	OMe	OH	H	H	H	OH	0	57	
	H	H	H	H	OH	H	H	0	52	30
	OH	H	OH	H	H	H	OH	0	50	
8	OH	OH	OH	H	H	OH	OH	0	0	2.5
2	OH	H	OH	H	OGlu	H	OH	47	100	100
4	OH	H	OMe	H	OGlu	H	OH	100	100	

TABLE 2. Cell survival data for acetophenones.

Compound Number	2'	3'	4'	5'	6'	% Survivors at 100 μg/ml	% Survivors at 10 μg/ml	ED_{50} μg/ml
	OH	H	OH	H	OH	29	100	
5	OH	H	OMe	H	OH	0	0	4.7
14	OH	H	OMe	H	OMe	0	100	
6	OH	H	OMe	H	OGlu	39	100	
	OH	OH	OH	H	H	0	81	

dihydrochalcones were synthesized by condensation of an acetophenone with a suitable aromatic aldehyde in the presence of 60% KOH (8) to give the chalcone, followed by reduction; the remaining three required special procedures (see experimental). The synthetic dihydrochalcone showing the most promise in *in vitro* activity was 2',3,3',4,4'-pentahydroxychalcone [**8**], which had an ED_{50} of 2.5 μg/ml; the compound was also marginally active *in vivo* (T/C=126 at 65

mg/Kg). The synthesis of asebotin [4] was through the glucoside pleoside [**6**], a natural product of unknown stereochemistry, isolated from *Pleopoltis thunbergiana* and not previously synthesized (9). A Knoenigs-Knorr synthesis using **5** and tetra *O*-acetyl-α-D-bromoglucose in toluene, in the presence of $CdCO_3$ (10), gave the required tetraacetate, which was deacetylated to **6** under Zemplen conditions (11). The synthetic and natural pleosides were identical (tlc, ir, optical rotation). The natural product is, thus, a β-glucoside.

EXPERIMENTAL[1]

PRELIMINARY FRACTIONATION.—Fresh leaves and stems of *Kalmia latifolia* L. (Ericaceae) were chopped in a silage mill, and a hydraulic press was used to express the sap (5 liters). The sap was concentrated at 40° to about ⅕ of its original volume and extracted to give the following fractions [wt; T/C (dose mg/kg); ED_{50}]: chloroform [10g; 138 (100), 157 (12.5); 65 μg/ml]; ethyl acetate [8g; 120 (100), 113 (50); 98 μg/ml]; butanol [21g; 166 (200), 120 (400); 14 μg/ml); aqueous residue (T/C:104).

CHROMATOGRAPHY OF THE BUTANOL EXTRACT.—The butanol extract was subjected to chromatography over silica gel (1 kg); 500 ml fractions were collected. No active compounds were eluted by chloroform (fraction 1-24). Fractions 29+30 (ethyl acetate) contained 2.75 gm of **3**: white needles mp 262–4° from ethanol water [lit (13) 262–4°], ir and tlc (systems a-g) identical with an authentic sample. Fractions 34–44 (ethyl acetate) were combined on the basis of tlc analysis (system g) and rechromatographed over silica (80 gm) with ethyl acetate-ethanol, 20/1 as the eluting solvent. Asebotin [4] was eluted in the early fractions: the glycoside was identified by co-chromatography (systems h, i) with an authentic sample, and by hydrolysis to asebogenin and correlation of the aglycone with authentic material. Later fractions contained phlorizin [**2**]; the glycoside was identical with authentic material (ir, mp, tlc) and gave **3** on acid hydrolysis. Tlc (system g) of those fractions containing **2** and **4** indicated the probable presence of grayanotoxin I [**1**] (blue color with Godin's reagent (14)); confirmation was provided by co-chromatography of the compound present in the extracts, with authentic grayanotoxin I in several solvent systems (a, c, and g).

ISOLATION OF 2',6'-DIHYDROXY-4'-METHOXYACETOPHENONE [5].—Sap (150 ml) was diluted with water (150 ml) and extracted with ether (5 x 100 ml). After drying, evaporation, preparative layer chromatography (silica gel; chloroform-methanol, 8/1), and further tlc (silica gel; chloroform), pure **5** was obtained. Mp, ir, and tlc behavior were identical with synthetic material (see below).

2',6'-DIHYDROXY-4'-METHOXYACETOPHENONE [5] AND 4',6'-DIMETHOXY-2'-HYDROXYACETOPHENONE [**14**].—The method of Cavell and MacMillan (15) gave **5**, mp 138°, and **14**, mp 85–86°, in 27 and 14% yields, respectively.

PLEOSIDE [**6**].—Compound **5** (0.182 gm, 1 mM) and $CdCO_3$ (0.344 gm, 2mM) were added to dry toluene (20 ml) in a flame-dried round-bottom flask (100 ml). Tetra-*O*-acetyl-α-D-bromoglucose (0.822 gm, 2mM) was dissolved in dry toluene (20 ml) and added to the stirred, refluxing mixture over 1 hr. The mixture was heated at reflux for an additional 30 min and filtered hot through a pad of Celite. The filtrate was evaporated to an oil which crystallized overnight. Crystallization from methanol gave the tetraacetate as white crystals: 0.195 gm (38%), mp 182–3° (lit mp 119–20° (9))[2]; δ: 2.02 (s;12H), 2.50 (s;3H),2.74 (s;3H), 4.14 (bs;2H), 5.25 (s;3H), 6.02 (d, J=2Hz; 1H), 6.10 (d, J=2Hz; 1H), and 13.20 (s;1H); ms: 512 (M^+), 453.138 (M–OAc); $C_{23}H_{25}O_{13}$–OAc requires 453.139.

This tetraacetate (40 mg, 0.078mM) was suspended in dry methanol (5 ml). The suspension was treated with NaOMe [2 ml of a solution prepared by adding Na (2.3 mg) to methanol (5 ml)] and stirred at room temperature for 2 hr. The mixture was neutralized with Dowex 50 (H^+ form), filtered, and evaporated. Crystallization from ethyl acetate-hexane gave **6**, 22

[1]Mps were determined on a Kofler hot stage apparatus and are uncorrected. Nmr spectra were obtained on a Hitachi Perkin-Elmer R-24 spectrometer in D_6-acetone unless otherwise noted. Mass spectra were recorded on a CEC 21–110B spectrometer. Ir spectra were recorded on a Beckman Acculab 3 spectrophotometer. Tlc analysis was on silica gel using the following systems: a. chloroform-methanol, 15/1; b. ethyl acetate-hexane-acetic acid, 50/50/0.5; c. ethyl acetate-ethanol, 20/1; d. ethyl acetate-isopropanol-water, 6/2/1; e. chloroform-benzene-ethanol, 75/25/3; f. benzene-methanol-acetic acid, 75/25/3; g. ethyl acetate-ethanol, 20/1; toluene-acetic acid-formic acid, 5/4/1; i. chloroform-acetic acid-formic acid, 5/4/1. Visualization was with Godin's reagent (12) or diazotized *p*-nitroaniline/10% Na_2CO_3. *In vivo* testing was carried out by the National Cancer Institute, NIH, Bethesda, MD.

mg (80%), mp 180–5° (lit mp 200–3° (9)); νmax 3400, 2895, 1610 and 1565 cm^{-1}; $[\alpha]D = -40.3°$. (c=0.195, pyridine) (lit $[\alpha]D = -41.6°$ (9)).

PREPARATION OF DIHYDROCHALCONES.— Except in the cases of compounds **15** and **16**, the acetophenone (0.02 ml) and the benzaldehyde (0.02 mole) were dissolved in a minimum volume of ethanol. The solution was cooled in ice. Cold aqueous KOH (60%, 18.2 gm, 0.445 mole) was added slowly. The reaction mixture was kept at room temperature for 3 days and acidified by pouring into ice-cold 5% HCl. The precipitate was rapidly filtered and washed with cold water. The crude chalcones were crystallized from ethanol, and subsequently reduced in a Parr hydrogenator at 3 atmosphere pressure over 5% Pd/C. The dihydrochalcones were crystallized from hexane or benzene-hexane.

The method of Swaleh *et al.* (16) was followed in the preparation of **15**. Hesperitin was isomerized with 25% KOH to give **16**.

Seven of the dihydrochalcones had not been previously described. Each one had spectral (ir, nmr, and ms) characteristics compatible with the assigned structure; the observed and calculated values for the molecular ions, the mps and νco, are recorded below.

Compound No.	mp°	νco cm^{-1}	Formula	Calculated MW	Observed MW
7	98–101	1650	$C_{15}H_{14}O_3$	242.0943	242.0941
8	155–6	1620	$C_{15}H_{14}O_5$	290.0786	290.0786
9	142–4	1620	$C_{15}H_{14}O_4$	258.0891	258.0896
10	147–9	1615	$C_{15}H_{14}O_4$	258.0891	258.0894
11	137–9	1655	$C_{16}H_{16}O_4$	272.1047	272.1045
12	90–2	1650	$C_{15}H_{14}O_3$	242.0943	242.0944
13	106–7	1610	$C_{17}H_{18}O_4$	286.1204	286.1204

SINGLE CELL PLATING (CLONING) PROCEDURE. CELLS: GROWTH CONDITIONS.—The human 9KB cells were maintained in culture as monolayers. The cells were grown in DME (Dulbecco's Modified Minimal Essential Medium of Eagle) and 5% calf serum. Plastic dishes (60 x 15 mm) were seeded with 1 x 10^6 cells and used after incubating 24 hrs at 37°. For testing, cells from the monolayers were dispersed into suspensions of single cells with a trypsin-EDTA solution (0.05% trypsin, Nutritional Biochemical Corporation; 1:300 and 5 x 10^{-4} M EDTA, ethylenediaminetetraacetic acid in Saline D), counted in a hemocytometer, and plated in DME supplemented with 10% calf serum plus the test compound (see below). The progeny from individual cells which survive exposure to the test compound form macroscopically visible colonies in 14 days at 37°. These colonies were washed with non-sterile PBS (phosphate buffered saline), fixed with 10% formalin, stained with Giesma, and counted over a photographic light box.

SAMPLE PREPARATION.—The compound to be tested was dissolved in either ethanol or water and added to the DME and 10% calf serum solution to a final concentration of 100 μg/ml. This solution was sterilized by filtration through a millipore filter, and further dilutions were made (10 and 1 μg/ml).

COMPOUND TESTING PROCEDURE: EFFECT ON PLATING EFFICIENCY OF SINGLE CELLS.—The general testing included three dose levels of the test compound which were measured by weight at 100, 10, and 1 μg/ml per sample, with duplicates for each sample. The cells were plated at three levels: 200, 2000, and 20,000 cells per dish with duplicates for each dish. Controls (DME and 10% calf serum and ethanol or water) were plated in duplicate at 200, 2000, and 20,000 cells per plate.

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[2]Hikino (9) made the tetraacetate from **6** with acetic anhydride and pyridine. His analytical figures (C,54.37; H,5.31) correspond better to a pentacetate (C,54.16; H,5.47) than to the tetraacetate (C,53.9; H,5.47).

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