Phytoalexin-like Activity of Abietic Acid and Its Derivatives

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Abietic acid and some of its derivatives, not known to be naturally occurring phytoalexins, were examined for their ability to promote H^+ conductance across membranes and to inhibit growth of two fungal species—*Aphanomyces euteiches* and *Fusarium moniliforme*. These results were compared with those of phaseollin, a phytoalexin isolated from kidney beans. Ion leakage and fungal growth inhibition were shown to be a function of structure, results that suggest that pterocarpan phytoalexin activity requires the presence of a polar, protic functional group situated at the end of a large, rigid hydrophobic moiety. Log *P* values for phaseollin, abietic acid, and some derivatives of abietic acid were also determined.

Keywords: *Abietic acid; H^+ leakage; antifungal activity; log P; phaseollin*

INTRODUCTION

Phytoalexins are relatively low molecular weight compounds produced when some plants are challenged by pathogenic organisms. These molecules appear to play a role in conferring disease resistance to the host plant (Chamberlin and Paxton, 1968). Over 100 phytoalexins have been isolated, and the majority of these are found in the dicotyledons, the best studied of which are species in the nightshade and legume families (Ersek and Kiraly, 1986).

While the mechanism of phytoalexin action is still not well understood, the ability of many phytoalexins to cause membrane damage is well documented (Smith, 1982). Glyceollins I and III and phaseollin—pterocarpan phytoalexins—cause membrane damage to both plant and fungal systems (Hargreaves, 1980; Giannini et al., 1990). We have shown specifically that glyceollins I and III (Giannini et al., 1991) and phaseollin (Spessard et al., 1994) cause proton leakage across isolated red beet tonoplast vesicles and vacuoles without significant inhibition of ATPase. These data suggest that the cause of proton leakage in the presence of pterocarpans may be due to a direct interaction of the phytoalexin with the lipid bilayer of the membrane.

We have directed much of our research effort toward the determination of a structure–activity relationship for the pterocarpan phytoalexins. Several other groups have investigated the effect of lipophilicity (log *P*) on fungitoxicity and the nature of substituents required for activity (Stössel, 1985; Adesanya et al., 1986; Laks and Pruner, 1989; Arnoldi et al., 1986, 1989; Arnoldi and Merlini, 1990). In all cases, the compounds chosen for study either were known pterocarpan or isoflavanoid phytoalexins or were compounds closely related in structure to these molecules.

It occurred to us that compounds might exist, which were not known to be phytoalexins, that would behave as the glyceollins and phaseollin in their ability to cause ion leakage or cell death. We considered a number of candidates that shared some of the characteristics of the pterocarpan phytoalexins, i.e., a rigid polycyclic ring structure, a large hydrophobic moiety, and the presence of a protic functional group such as OH or CO_2H. The diterpene abietic acid, which possesses these characteristics, was shown recently to inhibit animal-derived

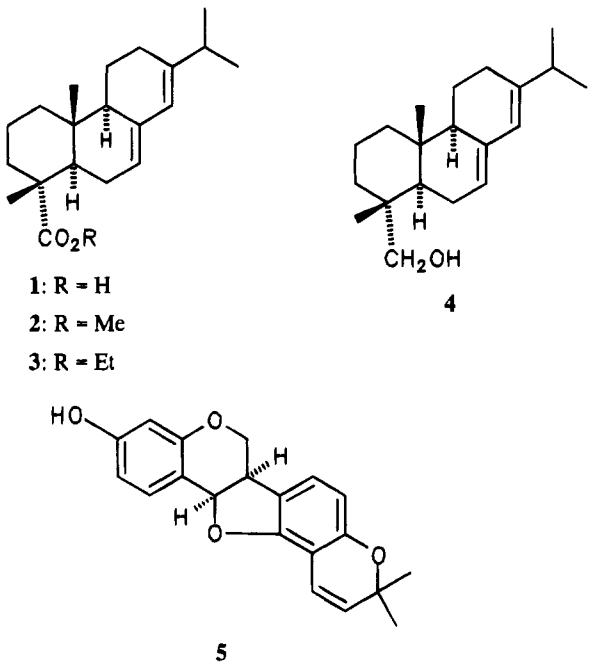

Figure 1. Abietic derivatives (**1**–**4**) and phaseolin (**5**).

ATPases (Sediko et al., 1990). These workers hypothesized that abietic acid acted as a nonspecific inhibitor capable of inducing disorganization of cell membrane systems.

Abietic acid and its derivatives (Figure 1) were of particular interest to us because *(1) they resemble structurally the pterocarpans, (2) they are easy to modify chemically, and (3) they are not known to be present in the tissue of soybeans or red beans, the sources of the glyceollins and phaseollin, respectively.* We report herein that abietic acid and its derivatives display variable ability to cause proton leakage across red beet membrane systems and to exhibit fungitoxicity toward two different fungal species. The activity of these compounds is a function of (1) the nature of the polar group located at one end of the molecule and (2) the compound's log *P* value (octanol/H_2O partition coefficient), an indication of lipophilicity.

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Table 1. Log *P* Values for Standard Compounds

compd	log P^a	log P^b	log *P* (calc)^c
phenol	1.49	1.53	1.42
2-methylphenol	1.95	1.98	2.03
4-chlorophenol	2.39	2.19	2.28
bromobenzene	2.99	3.20	3.18
naphthalene	3.37	3.45	3.46
biphenyl	4.04	3.87	3.84

[a] Nys and Rekker (1974). [b] Arnoldi et al. (1990). [c] This work.

MATERIALS AND METHODS

General. Red beet membrane vesicles were isolated according to a previously reported procedure (Giannini et al., 1990). Phaseollin was obtained from kidney beans (*Phaseolus vulgaris*) according to a method previously reported (Spessard et al., 1994). Abietic acid was purchased commercially and recrystallized twice from EtOH before use. Melting points were checked in capillary tubes using a Thomas-Hoover melting point apparatus and are uncorrected. FT-IR spectra were recorded on an IBM-Nicolet IR-32 spectrophotometer on KBr disks or as neat liquids on salt plates. Proton NMR spectra were taken with a Varian VXR-300 spectrometer in $CDCl_3$ solution. A Hewlett-Packard 5890 gas chromatograph with an HP 5970 mass selective detector attached was used to obtain mass spectra. Flash chromatography over silica gel 60 (200–400 mesh) was accomplished according to a standard procedure (Still et al., 1978). Organic extracts were dried over $MgSO_4$ and solvents removed under reduced pressure by rotary evaporator. THF was refluxed over and distilled from sodium benzophenone ketyl. All other anhydrous solvents were purchased commercially. HPLC grade solvents were used without further purification for both HPLC and flash chromatography.

Optical Measurement of Proton Transport. Proton transport in membrane vesicles was measured by the decrease in *A* of acridine orange dye (Giannini et al., 1988). Leakage was determined by allowing the vesicles to transport protons for 6 min, followed by the addition of 10 mM EDTA along with 60 μM of **1**, **2**, **3**, **4**, or **5** (Figure 1). A control using EDTA and EtOH was also measured. After 5 min of H^+ transport, 3 μM gramicidin D was added to collapse any of the H^+ gradient still remaining. The assay buffer contained 100 mM KCl, 25 mM bis-TRIS propane–MES (pH 7.75), 250 mM sorbitol, 3.75 mM $MgSO_4$, and 3.75 mM ATP. The assays were conducted at 22 °C, and the absorbance was measured at 490 nm.

Stock Cultures. Cultures of *Aphanomyces euteiches* and *Fusarium moniliforme* were maintained on cornmeal agar (17 g of cornmeal agar Difco in 1 L of H_2O) at 23 °C in the dark. Fungal growth plugs were cut using a 4.5 cm cork borer and transferred from stock plates to fresh agar biweekly to maintain actively growing fungus.

Radial Fungal Growth Assays. Assays were conducted on cornmeal agar at 23 °C in the dark. Prior to the agar being poured into the plates, 10 mM stock solutions of phytoalexins in EtOH were added to the molten agar, giving a final concentration of 159 μM. Agar (ca. 5 mL) was poured into three control (EtOH was the control) and three test plates for each phytoalexin. Fungal plugs (4.5 cm in diameter) of either *A. euteiches* or *F. moniliforme* were transferred to the middle of each of the six plates. Measurements were taken three times daily over a period of 4–7 days, noting the distance from the edge of the fungal plug to the edge of the actively growing fungus. Measurement ceased when fungal growth reached the edge of the ethanol control plates.

Log *P* Measurements. The octanol/water partition coefficients, log *P*, were measured using HPLC according to a previously described method (Arnoldi and Merlini, 1990). Six reference standards (Table 1), whose values were known from previous work (Nys and Rekker, 1974), were chosen to cover the range of log *P* from ca. 1 to 4 at intervals approximating 0.5 log *P* unit. Table 1 compares our calculated log *P* values for the six standards with previous work. Our standard log *P* values were then used to determine log *P* values for compounds **1**–**5** (Table 2).

Methyl Abietate (2). A mixture of 0.6 mL of H_2SO_4 and 3.00 g (9.9 mmol) of **1** in 16 mL of dry MeOH was heated at reflux temperature for 36 h. Upon cooling, the reaction mixture was extracted and the extract washed with saturated aqueous $NaHCO_3$, dried, and concentrated to give 2.89 g of yellow oil. Purification by flash chromatography (2% EtOAc in hexane) provided 1.41 g (45%) of **2** as a clear, colorless oil: ^1H NMR δ 0.80 (s, Me), 1.00 (d, *gem*-dimethyl), 1.25 (s, Me), 1.05–2.05 (m, 14 H), 2.11 (septet, 1 H), 3.62 (s, OMe), 5.17 (m, 1 H, vinylic), 5.88 (broad s, 1 H, vinylic); IR ν_{max} = 2945 (s), 1728 (s, C=O), and 1245 cm^{-1} (m, acyl CO).

Table 2. Proton Leakage, Fungal Growth, Log *P* Values, and Molecular Properties

		% fungal growth^b				
compd	H^+ leakage^a	*A. euteiches*^{c}	*F. moniliforme*^{d}	log *P*	surface area (Å^2)	vol (Å^3)
1	+++++	28	57	2.88	357	310
2	+	83	95	3.37	379	327
3	+	90	96	3.34	400	344
4	+++++	18	41	2.90	354	308
5	+++	28	38	3.59	338	286

[a] Relative activity (Figure 1). [b] Expressed as a percent of EtOH control (EtOH = 100%); concentration of compound **1**, **2**, **3**, **4**, or **5** = 159 μM. [c] After 4 days. [d] After 7 days

Ethyl Abietate (3) via the Acid Chloride. A solution of 3.00 g (9.9 mmol) of **1** in 40 mL of anhydrous ether was cooled to 0 °C before 0.85 mL (9.9 mmol) of oxalyl chloride (Spessard et al., 1983) was added dropwise with stirring. The solution was stirred at 25 °C for 40 h, at which time IR analysis of the reaction mixture showed the presence of a strong peak at 1782 cm^{-1} (acid chloride). Next, 2.6 mL of absolute ethanol and 1.5 mL of Et_3N were added to the reaction mixture and the solution was heated to reflux for an additional 72 h. Extraction with ether, $NaHCO_3$ washing, drying, and concentration gave 2.52 g of crude ester. Purification by flash chromatography (1.5% EtOAc in hexane) provided 1.59 g (48%) of **3** as a colorless, opaque oil: ^1H NMR δ 0.80 (s, Me), 1.00 (d, *gem*-dimethyl), 1.22 (t, Me, from the Et ester group), 1.24 (s, Me), 1.05–2.05 (m, 14 H), 2.22 (septet, 1 H), 4.08 (q, 2H, from the Et ester group), 5.38 (m, 1H, vinylic), and 5.78 (broad s, 1 H, vinylic); IR ν_{max} = 2365 (s), 1725 (s, C=O), and 1245 cm^{-1} (m, acyl CO); mass spectrum, mass (% of base peak, type) = 121 (56, bicyclic ion characteristic of tricyclic diterpenes), 256 (100, M – HCO_2Et), 330 (35, $M^{\bullet+}$).

7,13-Abietadien-18-ol (Abietinol) (4). A solution of 5.01 g (16.0 mmol) of **1** in 15 mL of dry THF was added dropwise to a mixture of 0.63 g (16 mmol) of $LiAlH_4$ suspended in 10 mL of THF. The reaction mixture was heated first at reflux for 1.5 h and then at 25 °C for 16 h. Quenching by sequential addition of 1 mL of water, 1.5 mL of 15% NaOH solution, and then 3 mL of H_2O gave a slurry that was diluted with 50 mL of ether. The mixture was washed with saturated $NaHCO_3$ and H_2O, dried, and concentrated to give 3.66 g (79%) of viscous oil that eventually solidified. Recrystallization from 1:1 pentane–CH_2Cl_2 provided **4** as white crystals: mp 82–86 °C [lit. mp 84–85 °C (Lisina and Pentegova, 1965)]; ^1H NMR δ 0.80 (s, Me), 0.90 (s, Me), 1.00 (d, *gem*-dimethyl), 1.20–2.10 (m, 14 H), 2.22 (septet, 1H), 3.15 (d, 1 H, J = 12 Hz, CH_2OH), 3.35 (d, 1H, J = 12 Hz, CH_2OH), 5.40 (broad s, 1 H, vinylic), and 5.78 (s, 1 H, vinylic); IR ν_{max} = 3350 (s, broad, OH), 2926 (s), and 1050 cm^{-1} (m, CH_2OH).

Molecular Modeling. Energy-minimized structures were produced using a CAChe Scientific stereo worksystem. Energy minimization was accomplished using CAChe Scientific versions of MM2 and MOPAC. Molecular surface and volume calculations were obtained using Bodor's (Bodor et al., 1989) log *P* program, also sold by CAChe Scientific.

RESULTS

Abietic acid (**1**) was converted to methyl abietate (**2**), ethyl abietate (**3**), and abietinol (**4**) by standard procedures. The effect of these compounds and phaseollin (**5**) on proton conductance was measured. Tonoplast membrane vesicles were allowed to transport protons for 6 min; at the end of this time, 10 mM EDTA was

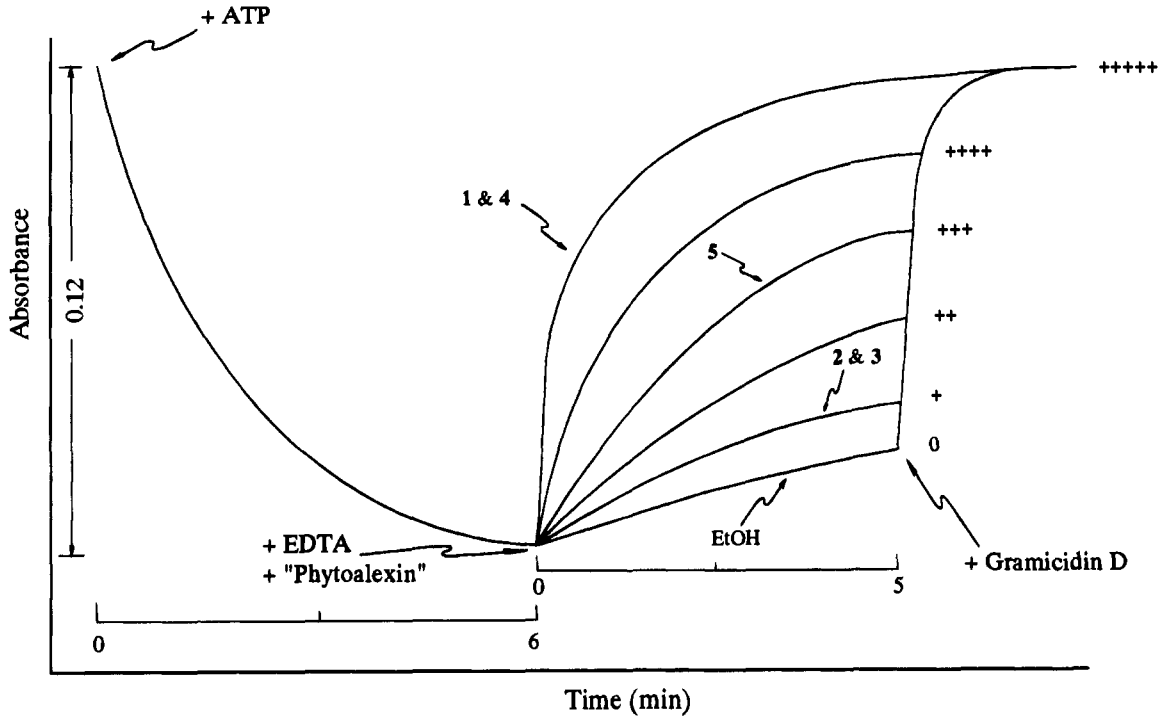
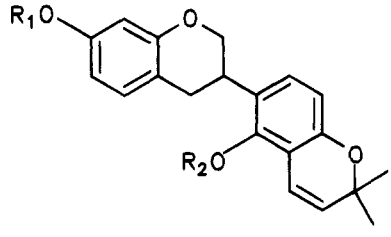
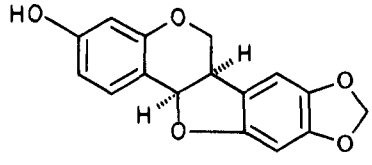


Figure 2. Effect of compounds **1**–**5** on H^+ leakage using red beet tonoplast vesicles. Assay conditions are described under Materials and Methods; 16.24 μg of membrane protein was used. The relative leakage activity scale (+ to +++++) is based upon assays described previously (Giannini et al., 1991; Spessard et al., 1994). The curve labeled 0 represents the control when only ethanol is present. All six curves are shown for relative comparison.



6: R_1, R_2 = H
7: R_1 = Me, R_2 = H
8: R_1 = H, R_2 = Me
9: R_1, R_2 = Me



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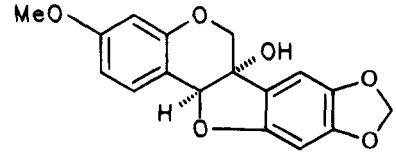

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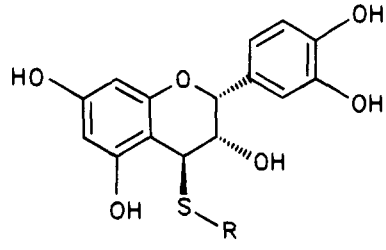
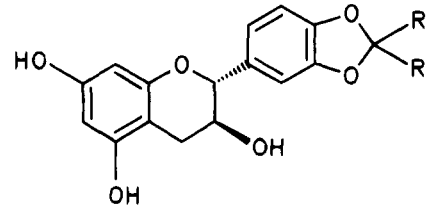


12 R = C_6 to C_{16} *n*-alkyl



13 R = C_2 to C_7 *n*-alkyl

Figure 3. Isoflavanoid phytoalexins.

added to inhibit the ATPase. The passive proton efflux was then monitored in the presence (60 μM) and absence of **1**, **2**, **3**, **4**, or **5**. Figure 2 shows the relative rates of leakage for the five compounds. Whereas the methyl and ethyl esters of abietic acid are relatively inactive (relative activity = +), abietic acid and the corresponding alcohol are indeed active (relative activity = +++++). This is true especially with respect to phaseollin (relative activity = +++), whose activity has already been shown to be comparable to that of the glyceollins (Spessard et al., 1994). Abietic acid and **4**, in fact, show an ability to promote proton leakage that closely resembles that of the ionophore gramicidin D.

Phaseollin and the glyceollins inhibit growth of a number of fungal species. The propensity of abietic acid derivatives to inhibit growth in two species of fungi—*A. euteiches* and *F. moniliforme*—was measured. Inhibition due to **1**, **2**, **3**, or **4** was compared with that of phaseollin. All inhibitions were normalized as percent inhibition compared to the control ethanol. Table 2 shows the

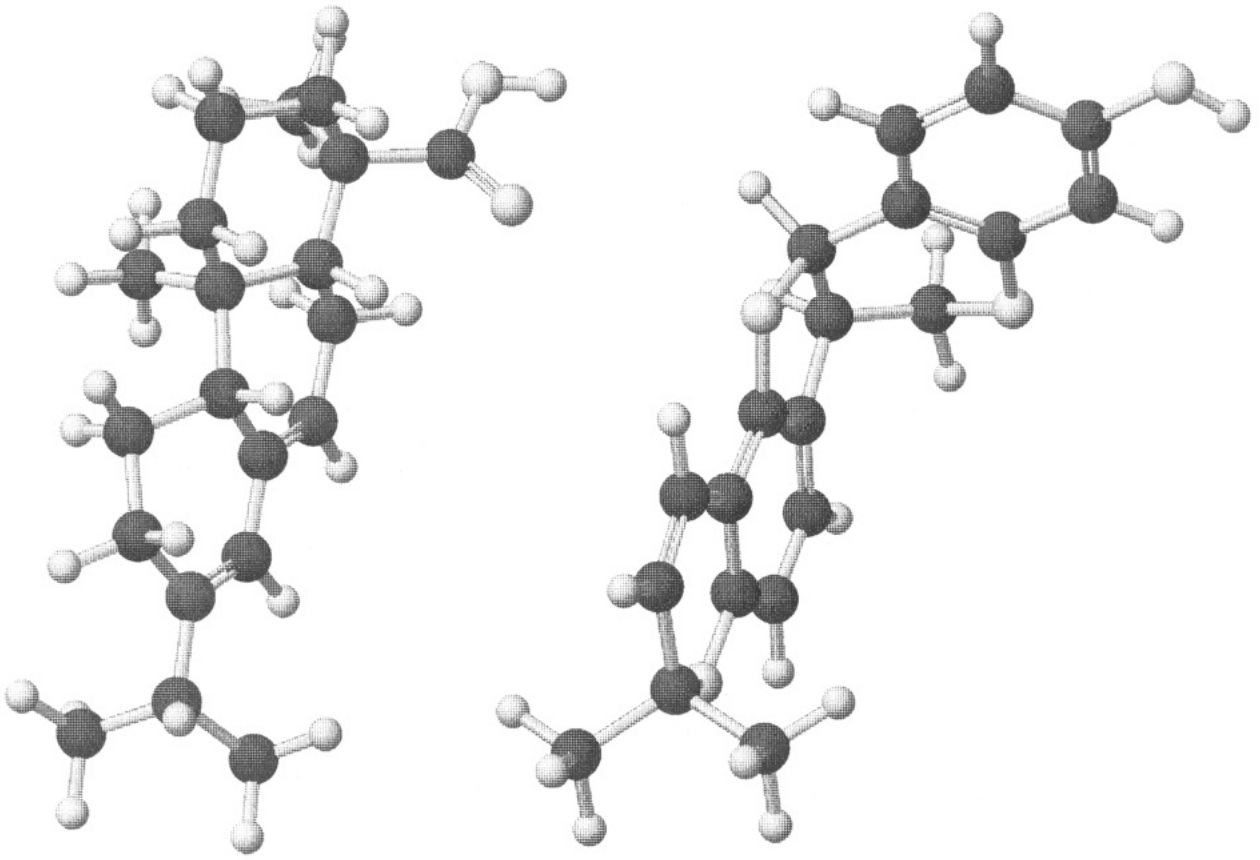


Figure 4. Energy-minimized structural representations of abietinol (left) and phaseollin (right).

relative percent inhibition for abietic acid and its derivatives compared to phaseollin. The two esters, **2** and **3**, are clearly noninhibitory agents, whereas **1** and **4** show about the same growth inhibition as phaseollin.

Several groups have investigated the relationship between lipophilicity and fungal growth inhibition of phytoalexins or closely related analogs (Stössel, 1985; Adesanya et al., 1986; Laks and Pruner, 1989; Arnoldi et al., 1986, 1989; Arnoldi and Merlini, 1990). Using the method reported by Arnoldi et al. (1986), in which log P values were determined using reversed phase HPLC, we measured log P values for phaseollin, abietic acid, and compounds **2**–**4**. These values appear in Table 2. The esters of abietic acid are significantly more lipophilic than abietic acid and abietinol.

DISCUSSION

There appears to be a definite correlation between structure and activity for abietic acid and its derivatives. The presence of a polar, protic functional group is clearly requisite for both promotion of ion leakage through membranes and fungitoxicity. When the molecule becomes more hydrophobic by the conversion of the acid to the ester, activity decreases drastically. Stössel has already demonstrated the effect of converting a phenolic OH to the corresponding methyl ether on inhibition of fungal growth (Stössel, 1985). By comparing growth of several fungal species in the presence of phaseollin-isoflavan (**6**) and methylated derivatives **7**–**9** (Figure 3), he found that the monomethylated derivatives were less inhibitory than **6** and that **9**, the dimethylated and most hydrophobic derivative, was the least fungitoxic.

It has been suggested (Laks and Pruner, 1989) that acidity of a polar, protic functional group is somehow related to biological activity in pterocarpan phytoalexins. Maackiain (**10**) is a significantly better growth inhibitor toward both *A. euteiches* and *F. solani* than is pisatin (**11**). The phenolic OH of **10** is more acidic than the alcohol function of **11** by ca. 5 pK_a units. Our data, on the other hand, indicate that acidity does not influence biological activity. Our two most active phytoalexin analogs, **1** and **4**, differ in acidity by at least 10 orders of magnitude, and yet they have similar biological activity! It is possible that **10** is more active than **11** due to the *position* of the polar group on the molecule and not because of the group's proton donating ability. Maackiain, the glyceollins, phaseollin, and our most active molecules—**1** and **4**—all possess one common structural characteristic: a polar, protic functional group at one *end* of a large, hydrophobic molecular fragment.

Lipophilicity seems to correlate with biological activity but in an apparently inverse manner in our case. Arnoldi (Arnoldi et al., 1986) has shown that increased lipophilicity correlates with higher antifungal activity within families of molecules with similar structure (such as the isoflavanoids and pterocarpans). On the other hand, two series of flavanoids (**12** and **13**) that are not naturally occurring were synthesized by Laks and analyzed for fungitoxicity (Laks and Pruner, 1989). The relationship between fungal growth inhibition and lipophilicity was bell-shaped, indicating an optimal log P for biological activity; the compounds with high lipophilicities were relatively poor inhibitors.

In general, compounds with high log P values tend to be retained in membranes and lack mobility for effective systemic activity. The log P values of abietic esters **2** and **3**, the least active compounds in the series, are close to 3.4. Compounds **1** and **4**, on the other hand, have log P values of ca. 2.9. Our data and those of Laks suggest that for phytoalexin activity of nonnaturally occurring analogs to be optimal, lipophilicity must be

sufficiently high for facile interaction with membranes, yet sufficiently low to promote mobility for ready distribution within the living system. Phaseollin, interestingly, has a relatively large log P value that we measured to be 3.59. This suggests a high membrane affinity and low systemic mobility. Since phytoalexins are toxic to both microorganism and plant cells, it is important that phytoalexins remain localized at the site of infection. The relatively high log P value for phaseollin compared to synthetic agents, therefore, may not be surprising. In any event, the use of log P values as a predictor of phytoalexin activity seems clearly limited to correlation among compounds that are closely related structurally.

Figure 4 shows computer-generated, energy-minimized structures for abietinol (**4**) and phaseollin (**5**). The two molecules along with abietic acid (**1**) are similar not only in shape but also in surface area and volume (Table 2). The surface area and volume of biologically inactive ethyl ester **3** are significantly larger than those of either phaseollin or abietinol. Our work and that of other groups hint that no one factor is responsible for the activity of the pterocarpan and isoflavanoid phytoalexins. One paper (Stipanovic et al., 1991) suggests that pterocarpans are active as phytoalexins because the potential exists for their conversion into benzylic radicals. Stipanovic's work strongly suggests that such radicals, derived from the cotton phytoalexin desoxyhemigossypol, are the species responsible for activity against the fungus *Verticillium dahliae*. Abietic acid and its derivatives, lacking benzylic positions, would not be expected to form free radicals and thus may have a mode of action different from the pterocarpans. Assuming, however, that pterocarpans and abietic acid derivatives act in the same manner—perhaps by disrupting cell membrane systems—minimum requirements for activity seem to be the presence of at least one polar, protic group at the end of the molecule and lipophilicity sufficiently high for ready molecular attraction to membranes. It may also be true that rigid, hydrophobic, polycyclic ring systems, all with approximately the same volume and surface area, are also required. Work is ongoing in our laboratories to try to determine if molecular size and shape parameters along with lipophilicity correlate with phytoalexin activity.

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