Phytochemistry, Vol. 28, No. 1, pp. 87–91, 1989.
Printed in Great Britain.

0031-9422/89 $3.00+0.00

FLAVONOID BIOCIDES: STRUCTURE/ACTIVITY RELATIONS OF FLAVONOID PHYTOALEXIN ANALOGUES

PETER E. LAKS and MARCIA S. PRUNER

Institute of Wood Research, Michigan Technological University, Houghton, MI 49931, U.S.A.

(*Received* 25 *April* 1988)

Key Word Index—Phytoalexin; flavonoid; antibacterial; antifungal; condensed tannin; structure/activity relationship.

Abstract—Two sets of semisynthetic flavonoid phytoalexin analogues were synthesized and their lipophilicity (R_M) correlated with their antifungal and antibacterial activity. Two natural phytoalexins, pisatin and maackiain, were also tested under these same conditions, and their R_M's determined. The observed structure/activity relationships suggest that these analogues, and the phytoalexins, function primarily as uncouplers of oxidative phosphorylation. The relative acidity and number of hydroxyl groups per molecule appear to be the main factors affecting the antifungal activity of flavonoids. With the bacterium, *Streptococcus faecium*, there is not the same simple correlation between lipophilicity and antibacterial activity for the two sets of analogues, implying they have different modes of action.

INTRODUCTION

Phytoalexins are biocides produced by plants in response to attack by pathogens such as fungi. Their synthesis can also be stimulated by a variety of abiotic elicitors, including metal salts and irradiation. Many classes of phytochemicals are represented among the phytoalexins, but probably the most commonly described are flavonoids. It has been suggested that the phytoalexin response can be used for crop protection. Application of elicitors to plants causes them to produce phytoalexins prior to infection and make them more resistant to fungal attack [1, 2]. This concept, however, is not practical due to the phytotoxicity and possible mammalian toxicity of the induced phytoalexins. The latter effect would be particularly undesirable in a crop plant. Another approach is to tailor the structure of the phytoalexin to be effective against specific pathogens. Plant pathogenic fungi have evolved mechanisms to detoxify phytoalexins. It might be possible to biologically engineer the phytoalexin biosynthetic mechanism of the plant to produce a different phytoalexin which the fungal pathogens cannot detoxify [3]. Information on the structure/activity relationships and mode of action of the phytoalexins would be valuable in developing this type of approach.

For a biologically active compound like a fungicide to have activity it must first diffuse from its site of application, usually the exterior of the cell, to its site of action, often within the cell, then partition itself onto the active site [4]. The rate of both these events will depend on the lipophilicity of the compound. Once at the active site, the compound has some chemical or physical effect that accounts for its activity. It is well known that for a given set of related fungicides or other biologically active compounds, the most active chemicals will often have a similar lipophilicity [4]. This might be expected since a given lipophilicity will allow the most efficient transportation of a chemical to an active site within the target cell. Natural products that are biologically active, such as the phytoalexins, would have similar polarity requirements.

In this paper, correlations between structural features, lipophilicity, and antifungal and antibacterial activities for some analogues of flavonoid phytoalexins, as well as some natural phytoalexins, are presented.

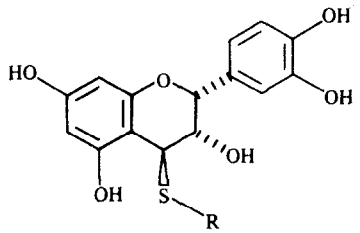
RESULTS AND DISCUSSION

A standard measure of lipophilicity is P, the octanol/water partition coefficient. However, P is often difficult to measure directly. In order to compare the lipophilicity of different compounds, a relative parameter, R_M, is often used [5]. An R_M can be calculated using a number of techniques, but most commonly by TLC using the R_f of the compound for a given set of TLC conditions (eqn 1) [5–7].

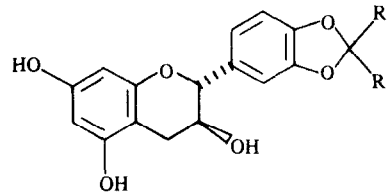
$$R_M = \log(1/R_f - 1) \qquad \text{(eqn 1)}$$

In a previous paper [8], a series of flavonoid derivatives were described (epicatechin-4-alkylsulphides, **1**, **2** and **4**–**6**) that differed in the overall lipophilicity of the molecule. The antifungal and antibacterial properties of the series also varied, with the maximum activity occuring at the decylsulphide derivative (**4**). In order to obtain further information on the relationship between lipophilicity and biocidal activity for flavonoids, a different set of derivatives, catechin dialkyl ketals (**7**–**12**), were prepared and tested for antifungal and antibacterial activity. An additional epicatechin derivative, epicatechin-4-nonylsulphide (**3**) was also prepared. The R_M's of both sets of flavonoid derivatives were determined.

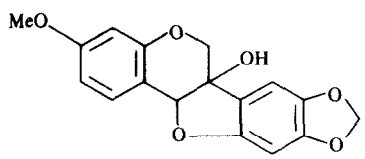
The relationships between R_M and activity for the phytoalexin analogues against *Aphanomyces euteiches*, *Fusarium solani*, and *Streptococcus faecium* are shown in Figs 1–3. Radial growth inhibition was determined for the first two organisms on agar plates doped with the test compound at 0.1 or 0.05 mM. For the bacterium, how-



1 R = —$(CH_2)_5Me$
2 R = —$(CH_2)_7Me$
3 R = —$(CH_2)_8Me$
4 R = —$(CH_2)_9Me$
5 R = —$(CH_2)_{11}Me$
6 R = —$(CH_2)_{15}Me$



7 R = —CH_2Me
8 R = —$(CH_2)_2Me$
9 R = —$(CH_2)_3Me$
10 R = —$(CH_2)_4Me$
11 R = —$(CH_2)_5Me$
12 R = —$(CH_2)_6Me$



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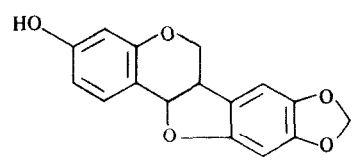

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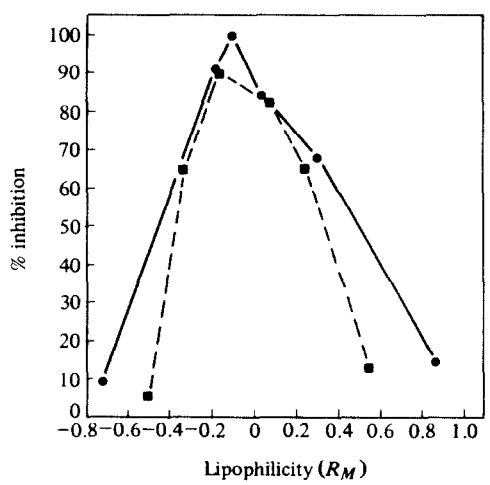

Fig. 1. Sensitivity of *Aphanomyces euteiches* to epicatechinalkylsulphides (●, **1** [$R_M = -0.72$], **2** [−0.18], **3** [−0.10], **4** [0.035], **5** [0.31], **6** [0.87]) and catechin dialkyl ketals (■, **7** [$R_M = -0.50$], **8** [−0.33], **9** [−0.16], **10** [0.07], **11** [0.25], **12** [0.55] of differing lipophilicities at a concentration of 0.05 mM.

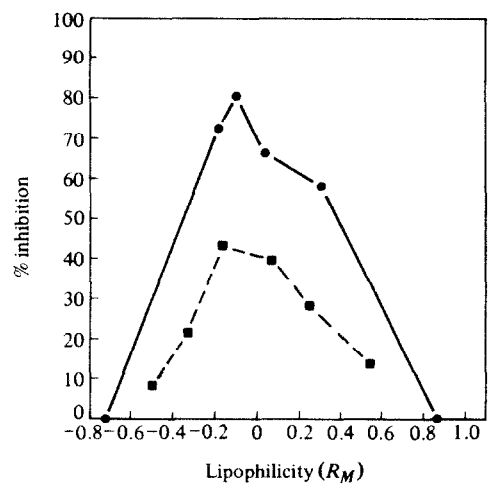

Fig. 2. Sensitivity of *Fusarium solani* to epicatechinalkylsulphides (●, **1** [$R_M = -0.72$], **2** [−0.18], **3** [−0.10], **4** [0.035], **5** [0.31], **6** [0.87]) and catechin dialkyl ketals (■, **7** [$R_M = -0.50$], **8** [−0.33], **9** [−0.16], **10** [0.07], **11** [0.25], **112** [0.55]) of differing lipophilicities at a concentration of 0.1 mM.

ever, the minimum inhibitory concentrations (MIC) of the flavonoids were determined.

Flavonoid phytoalexins have a wide variety of structures [e.g. 9]. The two phytoalexin analogues used here are also different in functional groups present, substitution pattern, and 3-D shape. It is unlikely that both could have the same mechanism of action if a specific molecular shape is required for activity. Against both fungi, the most active members of the two sets of flavonoid derivatives have a very similar R_M, *ca* −0.1, and a level of activity within a factor of two. Against the bacterium *Streptococcus faecium*, however, the most active epicatechin-4-alkylsulphide and catechin dialkylketal differed in R_M's by more than 0.2 units and have a difference in activity greater than an order of magnitude. These observations are consistent with the two types of derivatives having similar modes of action against fungi, but a different mechanism on the bacterium.

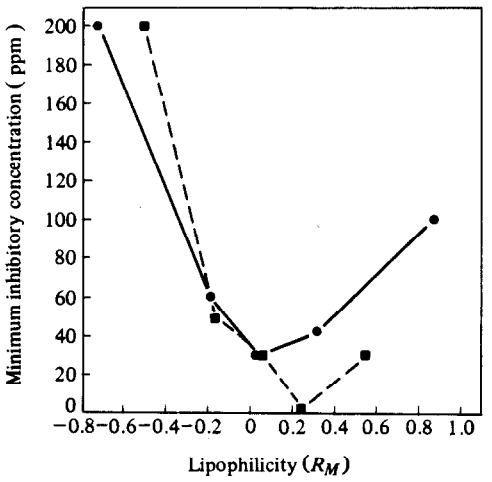

Fig. 3. Minimum inhibitory concentrations of epicatechinalkylsulphides (●, **1** [$R_M = -0.72$], **2** [−0.18], **3** [−0.10], **4** [0.035], **5** [0.31], **6** [0.87]) and catechin dialkyl ketals (■, **7** [$R_M = -0.50$], **9** [−0.16], **10** [0.07], **11** [0.25], **12** [0.55]) of differing lipophilicities toward *Streptococcus faecium*.

There is a growing consensus that, in most systems, flavonoid phytoalexins exert their toxicity by some membrane-associated phenomenon [10–17], again indicating the possible importance of lipophilicity for their activity. The relative lipophilicities of flavonoid phytoalexins have been qualitatively compared [10, 18], but no clear trends have been reported. Similarly, the structural requirements for activity have not been well defined [10, 18–20]. It is generally agreed, however, that at least one hydroxyl group and a certain degree of lipophilicity are required for biocidal activity.

These same requirements for a hydroxyl group and a degree of lipophilicity are also found in the simpler commercial phenolic fungicides such as *p*-pentyl phenol, dinitrophenol and pentachlorophenol. These compounds exert their toxicity through the acidity of the hydroxyl group by uncoupling oxidative phosphorylation. Protons are conducted across the lipid-containing mitochondrial membrane, thereby destroying the proton differential produced by electron transport that is required for the formation of ATP [21, 22]. It is possible that the flavonoid phytoalexins exert their fungitoxicity through a similar mechanism.

To obtain more information on the structural requirements for the antifungal activity of flavonoid phytoalexins, the lipophilicity and activity of pisatin (**13**) and maackiain (**14**) were compared to the analogues described above (Table 1). Pisatin and maackiain have a similar structure, both being based on the pterocarpan skeleton with a dioxole ring fused to the flavonoid B-ring and contain a single hydroxyl group, aliphatic in the former and phenolic in the latter. With these structural similarities, it is not surprising that they have similar R_M's, −0.14 for pisatin and −0.18 for maackiain (Table 1).

During the evolution of the phytoalexin system in the plant, it would be expected that the more fungitoxic phytoalexins will be selected for, as this will help maximize a plant's reproductive potential. Overall lipophilicity of the phytoalexin can be readily changed by the plant during the biosynthetic process through hydroxylase and dehydroxylase enzymes, and by methylation and addition of isoprenyl substituents. If the analogues described here have the same fungitoxic mechanism as the phytoalexins, one might expect the R_M's of the most active derivatives to be similar to that of the evolution-optimized flavonoid phytoalexins. This is indeed what is observed. The R_M's for the most active flavonoid analogues, −0.10 for epicatechin-4-nonylsulphide (**3**) and −0.16 for catechin-nonanone ketal (**9**), are very close to values for the phytoalexins (Table 1).

The effects of pisatin and maackiain on the growth of the two fungi tested are quite different. Maackiain is a significantly better inhibitor of radial fungal growth than pisatin. If these compounds act as uncouplers, this difference could be due to the relative acidity of the hydroxyl groups of the two compounds. Within certain limits, stronger acids are better uncouplers than weaker acids [23]. Since maackiain has a phenolic hydroxyl, it will be more acidic than the aliphatic hydroxyl of pisatin and so be expected to be the more fungitoxic of the two compounds.

Even with having essentially the same R_M's, the phytoalexins have lower antifungal activity than epicatechin-4-nonylsulphide (**3**) and catechin 5-nonanone ketal (**9**) (see Table 1). This can be explained in terms of the number of hydroxyl groups per molecule. It might be expected that for phenolics of a given lipophilicity, compounds with a greater number of hydroxyl groups will be more efficient uncouplers on a molar basis since they will be able to transfer more protons per molecule. The most active analogue, **3**, has five hydroxyl groups, while the less

Table 1. R_M, fungal radial growth inhibition and bacterial MIC of pisatin (**13**) and maackiain (**14**) compared to epicatechin-4-nonylsulphide (**3**), catechin 5-nonanone ketal (**9**), and catechin 7-tridecanone ketal (**11**) (The MIC for the latter is estimated from Fig. 3)

		Per cent inhibition		MIC (ppm)
	R_M	*A. euteiches**	*F. solani*†	*S. faecium*
Epicatechin-4-nonylsulphide	−0.10	100	80.6	45 (est.)
Catechin 5-nonanone ketal	−0.16	89.9	43.3	50
Catechin 7-tridecanone ketal	0.25	65.0	28.2	2.5
Pisatin	−0.14	6.2	9.4	>200
Maackiain	−0.18	34.5	39.2	>200

*Concentration of test compound: 0.05 mM.
†Concentration of test compound: 0.1 mM.

active **9** has three. Both pisatin and maackiain are less active still, and have only one hydroxyl group.

The modes of action of some flavonoid phytoalexins against organisms other than fungi have been reported. Glyceollin is an electron transport inhibitor when tested against the root knot nematode, *Meloidogyne incognita* [24], and soybean mitochondria [25]. Glycinol, glyceollin, and coumestrol were found to have antibacterial activity due to a general interaction with the bacterial membrane [26].

The observed structure/activity relationships suggest that the phytoalexin analogues, as well as pisatin and maackiain, function primarily against fungi as uncouplers of oxidative phosphorylation. The relative acidity and number of hydroxyl groups per molecule appear to be the main factors affecting the antifungal activity of flavonoids. The situation with the bacteria is more complex. In this case, there is not the same, simple correlation between lipophilicity and antibacterial activity for both analogues, indicating they have different modes of action. This is probably also true for the natural phytoalexins.

EXPERIMENTAL

General. Epicatechin-4-alkylsulphides were prepared and MIC's determined as described previously [8]. ^{13}C NMR were run at 50 MHz. High resolution mass spectra were obtained from the Midwest Center for Mass Spectrometry, a National Science Foundation Regional Instrumentation Facility (Grant No. CHE 8211164). Samples of pisatin and maackiain were supplied by Dr H. D. VanEtten (Cornell University).

Determination of R_M. Merck RP-18$_{254}$TLC plates were activated by heating at 120° for 15 min. An EtOH solution of the test compound (vol. 0.5 μl, concn 0.17 M) was then spotted on the plate and allowed to evap. The solvent system (13:7, acetonitrile–H_2O) was equilibrated in the tank for at least 15 min before putting in the plate. After development, the plate was visualised using UV light and/or I_2 vapour. The R_M was then calcd from the measured R_f using eqn 1, above.

Typical preparation of a catechin ketal. The procedure given here is for preparation of the catechin 5-nonanone ketal. The 3-pentanone, 4-heptanone, 6-undecanone, 7-tridecanone, and 8-pentadecanone ketals are synthesized in a similar manner except with different proportions of THF cosolvent (the larger the ketone, the more cosolvent is required) and a variation in yield (the larger the ketone, the lower the molar yield).

Catechin (5.0 g) was combined with nonanone (35.0 g) in 35 ml of THF. The solution was cooled to 0°, then HCl gas bubbled through until the solution was satd. After allowing it to sit for 10 min, a smaller amount of HCl gas was again added to saturate the solution. After an additional 10 min, the reaction solution was poured slowly into 400 ml of ice-cold 6% $NaHCO_3$. When the neutralization reaction had subsided, the resulting suspension was extracted with EtOAc (3 × 100 ml). Combination and evapn of the organic extracts gave a solid that was purified by repeated CC over Sephadex LH-20 with 95% EtOH. The separation was monitored by TLC (silica gel and 4:1 $CHCl_3$–MeOH), visualized by spraying with vanillin–HCl. A yield of 0.50 g was obtained.

Numbering of carbon atoms. Conventional numbering is used for the flavonoid ring system with primed numbers indicating the B-ring. Double primes denote the symmetrical alkyl substituents.

Catechin 3-pentanone ketal (**7**). ^{13}C NMR (50 MHz, Me_2CO-d_6): δ8.12, 8.42 (C-3″), 27.64 (C-4), 33.00, 33.44 (C-2″), 67.07 (C-3), 73.90 (C-2), 80.58 (C-1″), 95.73, 96.43 (C-6, 8), 100.82 (C-10), 112.15, 112.37 (C-2′, 5′), 126.93 (C-6′), 133.27 (C-1′), 144.26, 145.50 (C-3′, 4′), 156.48, 157.24, 157.82 (C-5, 7, 9). HREIMS, *m/z* (rel. int.): 358.1418 ([M]$^+$, calcd for $C_{20}H_{17}O_6$: 358.1410) (5.08), 329 [M − Et]$^+$ (95.44).

Catechin 4-heptanone ketal (**8**). ^{13}C NMR (50 MHz, Me_2CO-d_6): δ13.12, 13.18 (C-4″), 23.04, 23.10 (C-3″), 27.64 (C-4), 33.05, 33.44 (C-2″), 67.11 (C-3), 73.91 (C-2), 80.58 (C-1″), 95.68, 96.40 (C-6, 8), 100.82 (C-10), 112.13, 112.38 (C-2′, 5′), 126.85 (C-6′), 133.37 (C-1′), 144.26, 145.49 (C-3′, 4′), 156.51, 157.23, 157.82 (C-5, 7, 9). HREIMS, *m/z* (rel. int.): 386.1728 ([M]$^+$, calcd for $C_{22}H_{26}O_6$: 386.1722) (15.61), 343 [M − Pr]$^+$ (70.44).

Catechin 5-nonanone ketal (**9**). ^{13}C NMR (50 MHz, Me_2CO-d_6): δ14.21 (C-5″), 23.24, 23.50 (C-4″), 26.15, 26.40 (C-3″), 27.39 (C-4), 40.67, 40.85 (C-2″), 67.00 (C-3), 73.60 (C-2), 80.40 (C-1″), 95.55, 96.38 (C-6, 8), 100.57 (C-10), 112.04, 112.20 (C-2′, 5′), 126.00 (C-6′), 133.43 (C-1′), 143.97, 145.25 (C-3′, 4′), 156.03, 156.93, 157.06 (C-5, 7, 9). HREIMS, *m/z* (rel. int.): 414.2037 ([M]$^+$, calcd for $C_{24}H_{30}O_6$: 414.2034) (0.37), 357 [M − Bu]$^+$ (100).

Catechin 6-undecanone ketal (**10**). ^{13}C NMR (50 MHz, Me_2CO-d_6): δ14.23, 14.26 (C-6″), 23.10, 23.17 (C-5″), 23.80, 24.10 (C-4″), 27.69 (C-4), 32.74, 32.96 (C-3″), 41.12, 41.30 (C-2″), 67.12 (C-3), 73.90 (C-2), 80.38 (C-1″), 95.72, 96.41 (C-6, 8), 100.78 (C-10), 112.10, 112.35 (C-2′, 5′), 126.50 (C-6′), 133.86 (C-1′), 144.18, 145.17 (C-3′, 4′), 156.49, 157.22, 157.60 (C-5, 7, 9). HREIMS, *m/z* (rel. int.): 442.2350 ([M]$^+$, calcd for $C_{26}H_{34}O_6$: 442.2346) (2.10), 371 [M − pentyl]$^+$ (100).

Catechin 7-tridecanone ketal (**11**). ^{13}C NMR (50 MHz, Me_2CO-d_6): δ14.16 (C-7″), 23.02, 23.12 (C-6″), 24.11, 24.82 (C-5″), 27.69 (C-4), 30.19, 30.46 (C-4″), 32.23, 32.43 (C-3″), 41.19, 41.35 (C-2″), 67.09 (C-3), 73.90 (C-2), 80.33 (C-1″), 95.68, 96.36 (C-6, 8), 100.73 (C-10), 112.08, 112.33 (C-2′, 5′), 126.43 (C-6′), 133.85 (C-1′), 144.21, 145.17 (C-3′, 4′), 156.38, 157.25, 157.68 (C-5, 7, 9). HREIMS, *m/z* (rel. int.): 470.2675 ([M]$^+$, calcd for $C_{28}H_{38}O_6$: 470.2658) (0.71), 385 [M − hexyl]$^+$ (97.71).

Catechin 8-pentadecanone ketal (**12**). ^{13}C NMR (50 MHz, Me_2CO-d_6): δ14.24 (C-8″), 23.15, 23.12 (C-7″), 24.15, 24.38 (C-6″), 27.69 (C-4), 29.36 (C-5″), 30.51 (C-4″), 32.44 (C-3″), 41.19, 41.34 (C-2″), 67.12 (C-3), 73.90 (C-2), 80.37 (C-1″), 95.68, 96.38 (C-6, 8), 100.73 (C-10), 112.09, 112.33 (C-2′, 5′), 126.49 (C-6′), 133.85 (C-1′), 144.18, 145.50 (C-3′, 4′), 156.48, 157.24, 157.63 (C-5, 7, 9). HREIMS, *m/z* (rel. int.): 498.2985 ([M]$^+$, calc. for $C_{30}H_{42}O_6$: 498.2970) (1.39), 399 [M − heptyl]$^+$ (100).

Radial growth bioassay. The procedure of VanEtten [20, 27] was used. Briefly, plates (15 × 60 mm) of Martin's peptone–glucose medium (PGA) were innoculated with 4 mm plugs obtained from the actively growing margin of a *Aphanomyces euteiches* (ATCC # 46690) or *Fusarium solani* (ATCC # 16372) culture grown on PDA. The PGA had been doped at a concn of 0.1 mM by mixing DMSO solutions containing the test compound with the molten agar. The final concentration of DMSO in the agar was 2%. Controls were also prepared containing DMSO at this concentration.

Plates were grown in the dark at 23–25° until the mycelium of the control plates had almost reached the sides of the petri dish (*ca* 2 days). Cultures were run in triplicate. Per cent inhibition was determined by averaging the radial growth of the mycelial mass in three directions on the cultures grown on doped media, subtracting the diameter of the inoculum plug, and expressing it as 100 minus the percentage of the averaged diameters of test plates over the same average of the control plates.

Acknowledgements—The authors are grateful to the USDA for support in this project through grant 85-FSTY-9-0125, the Michigan Research Excellence Fund for additional support, and Dr H. D. VanEtten for supplying authentic samples of pisatin

and maackiain. The laboratory assistance of Mr G. Belkola is also gratefully acknowledged.

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