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ANTIFUNGAL ACTIVITY OF PTEROCARPANS AND OTHER SELECTED ISOFLAVONOIDS

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Key Word Index—*Fusarium solani* f. sp. *cucurbitae*; *Aphanomyces euteiches*; fungi; phytoalexin; isoflavonoid; pterocarpan; 6a,11a-dehydropterocarpan; isoflavan; isoflavone; coumestan.

Abstract—Six pterocarpans and 11 structurally related isoflavonoids were tested for antifungal activity against *Fusarium solani* f. sp. *cucurbitae* and *Aphanomyces euteiches*. Representatives from the pterocarpan, isoflavan, and 6a,11a-dehydropterocarpan classes of isoflavonoids were found that were antifungal. The activity of the antifungal isoflavonoids does not appear to be dependent on a common 3-dimensional shape.

INTRODUCTION

Pterocarpans are isoflavonoids that have the basic ring structure illustrated in Fig. 1. Several optical isomers are possible because of asymmetric centers at C-6a and 11a. However, since the 6a-11a junction has been established as a *cis*-fusion of the two heterocyclic rings, only a pair of enantiomers can occur [1, 2]. Indeed such enantiomeric forms of several pterocarpans have been isolated [3, 4]. On the basis of NMR studies, Pachler and Underwood [5] have concluded that the dihydrofuran ring is planar, while the dihydropyran ring exists in a staggered half-chair form. The overall shape of the molecule is thus one in which the two aromatic rings are almost perpendicular to each other [5, 6] (Fig. 2).

The results of a study of eight pterocarpans and related compounds led Perrin and Cruickshank [6] to propose that the characteristic antifungal activity of the pterocarpans may depend on the above described molecular shape. In addition, they suggested that small oxygen containing substituents, especially at C-3 and C-9, were required for activity. These apparent requirements led them to speculate that there is a structure-specific, bio-receptor site for the pterocarpans in sensitive fungi. Our own studies [7, 8] on the mode of action of the pterocarpan phaseollin (Fig. 3, **11**) led us to believe that this pterocarpan interferes with some process needed for membrane function. Thus the intriguing possibility exists that there is a specific receptor site for the pterocarpans in the membranes of sensitive fungi.

Since Perrin and Cruickshank completed their study several new compounds which can provide information pertinent to the structure–activity model have been described. The purpose of the present study was to reevaluate the model utilizing these and additional compounds.

RESULTS AND DISCUSSION

The 6 pterocarpans (**1**, **2**, **5**, **11**, **14**, **16**), four 6a,11a-dehydropterocarpans (**3**, **4**, **15**, **17**), 4 isoflavans (**6**, **7**, **12**, **13**), 2 coumestans (**9**, **10**) and one isoflavone (**8**) that were tested for antifungal activity are illustrated in Fig. 3. Non-pterocarpanoid isoflavonoids with the same substituent patterns as the pterocarpans were purposely selected. Thus a more direct indication of the influence of molecular shape on antifungal activity is possible because the possible confounding effects of diverse substituents is minimized.

Fusarium solani (Mart.) Sacc. f. sp. *cucurbitae* (F. R. Jones) Snyd. & Hans. (Class Ascomycetes) and *Aphanomyces euteiches* Drechs. (class Oomycetes) were used as test fungi. It was demonstrated previously that these fungi are sensitive to the pterocarpans pisatin (**1**) and phaseollin (**11**) [9]. To permit a semiquantitative comparison of their relative antifungal activity, all compounds were tested at the same molarity (0·1 mM) [10]. The effect of each isoflavonoid on the radial growth of the fungi on solid medium was the major criterion of antifungal activity. Since some of the compounds possibly could be altered during the incubation period of the radial growth bioassays, a rapid indication of antifungal activity was desired. Therefore the immediate effects of the compounds on both motile zoospores and non-motile encysted spores of *A. euteiches* were observed

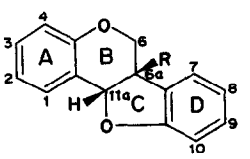

Fig. 1. (6aS,11aS) Pterocarpan ring structure.

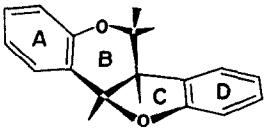

Fig. 2. (6a,R,11aR) Pterocarpan ring structure.

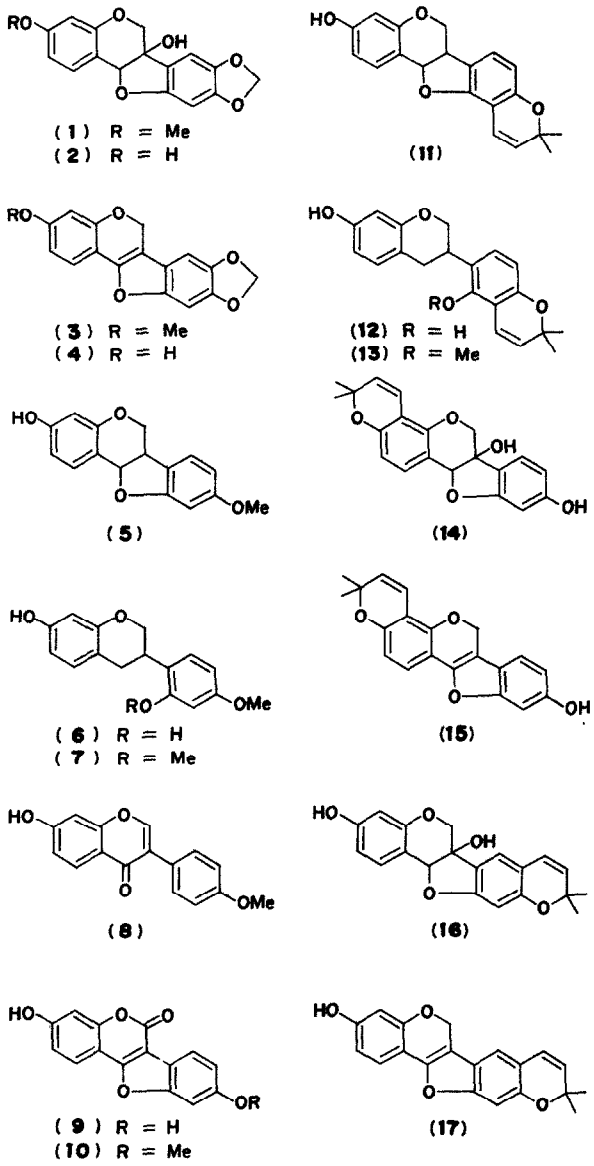

Fig. 3.

visually. In addition, the effect of a 5 min exposure to the compounds on the viability of these spores was determined.

In Table 1 (and Table 2) the isoflavonoids are ranked according to their increasing ability to restrict the radial growth of *A. euteiches*. Although the sequence was not identical, the same general pattern of radial growth restriction is evident for *F. solani* f. sp. *cucurbitae* ($r = 0{\cdot}77$, $p = 0{\cdot}01$). Except for glyceollin (**14**), the visual rating of "death" (a rating of 2 and 3) of *A. euteiches* spores was consistent with the subsequent growth response of the treated spores (Table 2). However, several discrepancies exist between the effects on radial growth and those on spore viability (Tables 1 and 2). Both tuberosin (**16**) and glyceollin (**14**) markedly inhibited the linear growth of *A. euteiches* but appeared to have little effect on spore viability. In contrast 6a,11a-dehydrotuberosin (**17**) and 6a,11a-dehydroglyceollin (**15**) had a greater effect on spore viability than might be expected from their effect on the radial growth of *A. euteiches*.

Central to Perrin and Cruickshank's structure–activity hypothesis is their finding that 6a,11a-dehydropisatin (**3**) and coumestans, such as coumestrol (**9**) and 4′-*O*-methylcoumestrol (**10**), lack antifungal activity. Although the carbon skeleton of these compounds is analogous to that of pterocarpans, they have a markedly different molecular shape. Because of the double bond at 6a-11a, such compounds are almost planar. Our tests confirm that 6a,11a-dehydropisatin, coumestrol and 4′-*O*-methylcoumestrol lack antifungal activity (Tables 1 and 2). However, in aqueous environments 6a,11a-dehydropisatin and 4′-*O*-methylcoumestrol, may not remain in a soluble form (see Experimental). Thus lack of activity may simply result from insolubility. The other 6a,11a-dehydropterocarpans that were tested, 3-hydroxy-8,9-methy-

Table 1. Effect of 0·1 mM isoflavonoids on the radial growth of *Aphanomyces euteiches* and *Fusarium solani* f. sp. *cucurbitae*

	% Inhibition*	
Isoflavonoid†	*Aphanomyces euteiches*	*Fusarium solani* f. sp. *cucurbitae*
6a,11a-Dehydropisatin (**3**)	$-2{\cdot}3^{a}$	$0{\cdot}5^{a}$
4′-*O*-Methylcoumestrol (**10**)	$0{\cdot}5^{a}$	$4{\cdot}3^{b}$
Coumestrol (**9**)	$3{\cdot}8^{a}$	$4{\cdot}2^{ab}$
(+)-3,6a-Dihydroxy-8,9-methylenedioxy-pterocarpan (**2**)	$5{\cdot}5^{a}$	$2{\cdot}8^{ab}$
Formononetin (**8**)	$16{\cdot}7^{b}$	$18{\cdot}1^{c}$
(−)-Vestitol (**6**)‡		25·0
6a,11a-Dehydroglyceollin (**15**) [10]	$18{\cdot}4^{bc}$	$35{\cdot}6^{d}$
(+)-Pisatin (**1**)	$19{\cdot}7^{bc}$	$52{\cdot}0^{e}$
(±)-3-Hydroxy-9-methoxypterocarpan (**5**)	$25{\cdot}5^{bcd}$	$78{\cdot}1^{ghi}$
(+)-3-Hydroxy-9-methoxypterocarpan (**5**)	$30{\cdot}3^{bcd}$	$64{\cdot}6^{ef}$
(−)-3-Hydroxy-9-methoxypterocarpan (**5**)	$30{\cdot}9^{bcd}$	$68{\cdot}7^{fg}$
(−)-Sativan (**7**)	$39{\cdot}8^{cd}$	$54{\cdot}3^{e}$
6a,11a-Dehydrotuberosin (**17**)	$46{\cdot}0^{d}$	$37{\cdot}4^{d}$
3-Hydroxy-8,9-methylenedioxy-6a-11a-dehydropterocarpan (**4**)	$46{\cdot}1^{d}$	$38{\cdot}0^{d}$
(−)-Glyceollin (**14**)‖	$77{\cdot}3^{e}$	$75{\cdot}4^{fgh}$
(−)-Phaseollin (**11**)	$85{\cdot}8^{ef}$	$85{\cdot}6^{hi}$
(+)-2′-Methoxyphaseollinisoflavan (**13**)	$88{\cdot}5^{efg}$	$79{\cdot}9^{ghi}$
(+)-Tuberosin (**16**)	$96{\cdot}9^{fg}$	$55{\cdot}0^{e}$
(−)-Phaseolliniso-flavan (**12**)	$96{\cdot}9^{g}$	$86{\cdot}7^{i}$

* Radial growth measurements were recorded when net growth (diam.) in controls was 24 ± 3 mm. Values not followed by the same letter are significantly different at the 5% level. Values are the mean of 3 replicate experiments. † The bold Arabic numeral in parenthesis following each isoflavonoid referred to the structures given in Fig. 3. ‡ This isoflavonoid was bioassayed only once and the datum obtained was not included in the statistical analysis. ‖ This compound, originally incorrectly identified as 6a-hydroxyphaseollin [24], has recently been assigned structure (**14**) [29]. It has been assigned the trivial name glyceollin (Burden, Bailey and Keen, personal communications).

Table 2. Effect of 0·1 mM isoflavonoids on spores of *Amphanomyces euteiches*

Treatment	Immediate effects on spores (Visual observation)*	Effect on viability (Maximum dilution of spore suspension at which growth occurred)†
2% DMSO	0	10^5
6a,11a-Dehydropisatin (3)	1	10^5
4′-*O*-Methylcoumestrol (10)	1	10^4
Coumestrol (9)	1	10^5
(+)-3,6a-Dihydroxy-8,9-methylenedi-oxypterocarpan (2)	0	10^6
Formononetin (8)	0	10^4
(−)-Vestitol (6)	1	
6a,11a-Dehydroglyceollin (15) [10]	3	10^2
(+)-Pisatin (1)	1	10^5
(±)-3-Hydroxy-9-methoxypterocarpan (5)	1	10^5
(+)-3-Hydroxy-9-methoxypterocarpan (5)	1	10^5
(−)-3-Hydroxy-9-methoxypterocarpan (5)	1	10^5
(−)-Sativan (7)	1	10^4
6a,11a-Dehydrotuberosin (17)	3	no growth
3-Hydroxy-8,9-methyl-enedioxy-6a,11a-dehydropterocarpan (4)	2	no growth
(−)-Glyceollin (14)	2	10^4
(−)-Phaseollin (11)	3	10^2
(+)-2′-Methoxyphaseol-linisoflavan (13)	3	10^3
(+)-Tuberosin (16)	1	10^5
(−)-Phaseollinisoflavan (12)	3	10^2

* The non-treated control spore suspension consisted of 30–50% swimming zoospores; the rest were non-motile primary spores or encysted zoospores. Data in the table represents observations at 5 min after treatment. 0, no obvious difference from control; 1, zoospores responded to the treatments by ceasing to swim and/or erratic movement, and/or encystment. Few if any spores assumed an irregular shape and contained granulated protoplasm; 2, some spores irregularly shaped with protoplasm appearing granular; 3, most spores irregularly shaped with protoplasm appearing granular. Spores with an irregular shape and granulated protoplasm were judged to be dead. † Effect of the compounds on viability of *A. euteiches* spores was estimated by exposing the spores to the compounds for 5 min and then serially diluting with liquid growth medium to a 10^6 dilution. Presence or absence of growth in each dilution was recorded after 3 weeks. This table represents a composite of many individual experiments. The initial spore concentration was adjusted to 1–2 × 10^5/ml. The data in the table was selected only from experiments in which the controls showed growth at 10^5 and no growth at 10^6.

lenedioxy-6a,11a-dehydropterocarpan (4), 6a,11a-dehydroglyceollin (15), and 6a,11a-dehydrotuberosin (17), remained soluble in the media and all exhibited antifungal activity by all criteria used (Tables 1 and 2).

The comparison between 3-hydroxy-8,9-methylenedioxy-6a,11a-dehydropterocarpan (4) and the analogous pterocarpan, 3,6a-dihydroxy-8,9-methylenedioxypterocarpan (2) is particularly interesting. The aplanar pterocarpan appeared to lack antifungal activity, yet the planar 6a,11a-dehydropterocarpan was inhibitory. The proposed structure–activity hypothesis predicts exactly the opposite.

The previous structure-activity study reported that both enantiomers of 3-hydroxy-8,9-methylendioxypterocarpan were equally inhibitory. This original observation appears to contradict the hypothesis of a specific sterochemical requirement of the pterocarpans for antifungal activity. A specific structural interaction where both stereochemical isomers are equally active seems unlikely. Nevertheless Perrin and Cruickshank recognized that the shape of the pterocarpan molecule is such that it is possible to superimpose rings A, C and D (but not ring B) of enantiomer pairs (Fig. 4). They interpreted this to mean that binding to bio-receptors through the heterocylic rings is not important. When enantiomer models are superimposed on each other as outlined above, not every aromatic carbon superimposes on the same carbon of the enantiomer. Thus C-3 of one member is superimposed on C-3 of its opposite enantiomer, but the C-9's are superimposed on C-8's (Fig. 4). Perrin and Cruickshank have suggested that there is specific binding of the bioreceptor site to oxygen substituents at C-3 and C-9. The enantiomeric pterocarpans that Perrin and Cruickshank tested have oxygen substitution at both C-8 and C-9. Thus in the context of their model it makes little difference that C-8 of one enantiomer superimposes with C-9 of the other. Substitution at position 8 could mimic the group at C-9 of its enantiomer. However, the enantiomeric pair of pterocarpans, (−),(+) 3-hydroxy-9-methoxypterocarpan (5), that were found to be equally antifungal (Tables 1 and 2) did not have oxygen substitution at C-8. That both enantiomorphs have equal activity is inconsistent with the necessity for specific binding to substituents at C-9 (C-8 of the active enantiomer).

Perrin and Cruickshank tested one representative of the isoflavan class of isoflavonoids and observed that the compound lacked significant antifungal activity. We tested 4 other isoflavans: sativan (6), vestitol (7), phaseollinisoflavan (12), and 2′-methoxyphaseollinisoflavan (13). All possessed antifungal activity (Tables 1 and 2), as has been reported previously [11–14]. Except for vestitol, the activity was of the same magnitude as the analogous pterocarpans, 3-hydroxy-9-methoxypterocarpan (5) and phaseollin (11). If the conformation for antifungal activity suggested by Perrin and Cruickshank is required, it might be expected that the isoflavans would have antifungal activity. Although the aromatic rings of the isoflavans would not be maintained in the rigid conformation relationship of the pterocarpans, the isoflavans and pterocarpans share a heterocyclic asymmetric carbon atom.

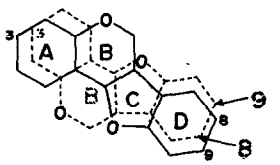

Fig. 4. Drawing based on Dreiding stereomodels of a superimposed enantiomeric pair of pterocarpans.

In isoflavans there is free rotation about the carbon–carbon bond connecting the aromatic ring to this asymmetric carbon. Therefore the isoflavans can assume a conformation similar to the 3-dimensional shape of the pterocarpans, and thus they may interact with the same hypothetical fungal bioreceptor site as the pterocarpans. The antifungal activities of the series of isoflavonoids (**6**, **7**, **8**, **9**) that are closely related to 3-hydroxy-9-methoxypterocarpan (**5**) supports this contention. The pterocarpan (**5**) and the isoflavans (**6**, **7**) are most active, while the isoflavone (**8**) and coumestan (**9**) are less active (Table 1). Neither the isoflavones nor the planar coumestans can assume a conformation similar to the 3-dimensional shape of the pterocarpans.

Nevertheless, based on the response of the 17 tested isoflavonoids it appears unlikely that all antifungal isoflavonoids must assume a common 3-dimensional shape before they can express activity. Antifungal activity was associated with both aplanar pterocarpans and isoflavans, and planar 6a,11a-dehydropterocarpans. The fact that 6a,11a-dehydropterocarpans possess antifungal activity also negates one of the major lines of evidence that was used by Perrin and Cruickshank to propose their structure-activity model for the pterocarpans. Perhaps the critical factor for antifungal activity of isoflavonoids is some physiochemical property other than three-dimensional shape. Alternatively, it may be that different classes of isoflavonoids or individual members within a class are antifungal, because they have different modes of action. Therefore, different receptor sites may exist in the fungal cell, and a common physiochemical bases for activity of antifungal isoflavonoids may not exist.

EXPERIMENTAL

Bioassays. Radial growth. These were performed as described previously [9]. The bioassay medium was Martin's [15] peptone–glucose agar (PGA) medium (10 g glucose, 5 g peptone, 1 g KH_2PO_4, 0·5 g $MgSO_4 \cdot 7H_2O$, 22 g agar and 1 l. H_2O). Each isoflavonoid was bioassayed 3 times in duplicate against each organism.

A. euteiches *spores.* The fungus was grown in Yang and Schulties [16] (Y & S) medium (5·5 g glucose, 4·0 g asparagine, 0·1 g glutathione, 0·02 g $CaCl_2 \cdot 2H_2O$, 0·4 g $MgCl_2 \cdot 6H_2O$, 0·7 g KH_2PO_4, 0·4 g K_2HPO_4, 1 l. H_2O) and was induced to form zoospores by techniques similar to those used by Mitchell and Yang [17]. To observe the visual effects of the isoflavonoids on the spores, a 0·5 ml aliquot of the spore suspension ($1–2 \times 10^5$ spores/ml) was treated with 10 μl of DMSO containing the isoflavonoid. Immediately, *ca* 50 μl of the treated suspension was withdrawn, placed on a glass slide and observed at $\times 100$ and $\times 400$. Observations were recorded at *ca* 1–5 min. Care was taken at all times to avoid any sudden vibration of the spore suspension as this would cause the motile zoospores to encyst. All treatments were coded so that the observer was unaware of the compound being tested. To test the effects on spore viability of a short exposure to the isoflavonoids, 0·5 ml of spore suspensions, in duplicate, were treated with the isoflavonoid as above. After 5 min, 4·5 ml of Y & S medium was added. After mixing, 0·5 ml was withdrawn and added to another 4·5 ml of Y & S medium. Serial dilution was repeated to a final dilution of 10^6. All dilutions were incubated at 24° ± 2° for 3 weeks and then the presence or absence of fungal growth was recorded. Each isoflavonoid was bioassayed in this manner at least twice.

Isoflavonoids. Published procedures (some with minor modification) were used to obtain (+)-pisatin [18], (+)-3,6a-dihydroxy-8,9-methylenedioxypterocarpan [18], 6a,11a-dehydropisatin [19], 3-hydroxy-8,9-methylenedioxy-6a,11a-dehydropterocarpan [18], (−)-3-hydroxy-9-methoxypterocarpan [14, 20], (−)-sativan [13, 14], (−)-vestitol [13], (−)-phaseollin [21], (−)-glyceollin [22], (−)-phaseollinisoflavan [23], (+)-2′-methoxyphaseollinisoflavan [11], 6a-11a-dehydroglyceollin [24], and 6a,11a-dehydrotuberosin [25]. Additional samples of (−)-3-hydroxy-9-methoxypterocarpan, (−)-vestitol, and (−)-sativan were supplied by J. L. Ingham, M. R. Bonde, and P. W. Steiner. A sample of (−)-glyceollin was supplied by N. T. Keen. All the (+)- and (±)-3-hydroxy-9-methoxypterocarpan was supplied by W. D. Ollis. Formononetin was supplied by A. B. Beck, and S. Shibata. Coumestrol and 4′-*O*-methylcoumestrol was supplied by E. M. Bickoff, and the (+)-tuberosin was supplied by B. S. Joshi. The identity of all compounds was verified by comparing the UV absorption spectrum, MS, optical rotation, and mp (when available) with literature values. All compounds were purified to at least TLC homogeneity. All isoflavonoids (except 6a,11a-dehydroglyceollin [10]) were bioassayed at a final concentration of 0·1 mM. Molar extinction coefficients from the cited references were used to quantify pisatin [19], 3,6a-dihydroxy-8,9-methylenedioxypterocarpan [18], 6a,11a-dehydropisatin [19], 3-hydroxy-8,9-methylenedioxy-6a,11a-dehydropterocarpan [18], 3-hydroxy-9-methoxypterocarpan [26], vestitol [13], sativan [14], phaseollin [27], glyceollin [24], phaseollinisoflavan [12], 2′-methoxyphaseollinisoflavan [11], tuberosin [25], 6a,11a-dehydrotuberosin [25], and formononetin [28]. Coumestrol and 4′-*O*-methylcoumestrol were quantified by wt. The isoflavonoids were added to the bioassay media in DMSO to facilitate maintainance of the compounds in soln. However, even in the presence of DMSO, 6a-11a-dehydropisatin and 4′-*O*-methylcoumestrol formed a ppt. in the media. DMSO had no noticeable effect on the linear growth of *F. solani* f. sp. *cucurbitae* or on spores of *A. euteiches*. DMSO inhibited radial growth of *A. euteiches* 30–40% in comparison to treatments not containing DMSO. The reported values of the effects of the isoflavonoids on the fungi are in comparison to a DMSO control.

Statistical analysis. Relative growth rate (RGR) for the test organism in the presence of each isoflavonoid was expressed as a proportion of the growth rate of the comparable control. Thus [% inhibition = 100 (1-RGR)]. These RGR's were converted using the arcsine square root transformation, and an analysis of variance was performed on the transformed values. Significant differences between individual RGR's were determined by Duncan's New Multiple Range Test.

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10. The molar extinction coefficient for 6a,11a-dehydroglyceollin has not been reported and a sufficient amount was not isolated to do the calculation. The concentration of

6a,11a-dehydroglyceollin used in this study was one A unit at λ_{max} (344 nm)/ml of medium. Based on comparisons with the molar extinction coefficient of the other 6a,11a-dehydropterocarpans [18, 19, 25], the soln was less than 0·1 mM.

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