Phytochemistry, 1975, Vol. 14, pp. 1103–1105. Pergamon Press. Printed in England.

3,6a-DIHYDROXY-8,9-METHYLENEDIOXYPTEROCARPAN AS A METABOLITE OF PISATIN PRODUCED BY *FUSARIUM SOLANI* f. sp. *PISI*

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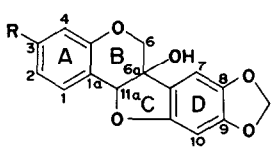
(*Revised Received* 4 *October* 1974)

Key Word Index—*Pisum sativum*; Leguminosae; *Fusarium solani* f. sp. *pisi*; isoflavonoids; pterocarpans; pisatin; 3,6a-dihydroxy-8,9-methylenedioxypterocarpan; fungal metabolism; detoxification; phytoalexins.

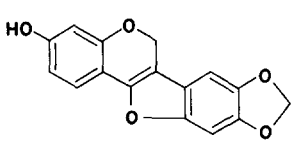
Abstract—One of the fungal metabolites of pisatin has been identified as resulting from the demethylation of pisatin at position 3.

INTRODUCTION

Previous studies [1–4] have demonstrated that fungi are able to metabolize pisatin (**1**), a pterocarpanoid phytoalexin produced by pea, *Pisum sativum* L. [5]. In Several of these studies [1,2] an unidentified metabolite that had a UV spectrum similar to that of pisatin was observed. We have found that *Fusarium solani* (Mart.) Sacc. f. sp. *pisi* (F. R. Jones) Snyd. and Hans., a pathogen of pea, is able to metabolize pisatin to what appears to be the same product [6]. This report presents evidence that the fungal metabolite of pisatin produced by *F. solani* f. sp. *pisi* is 3,6a-dihydroxy-8,9-methylenedioxypterocarpan (**2**), a previously undescribed pterocarpan.



(**1**) R = OMe
(**2**) R = OH



(**3**)

RESULTS AND DISCUSSION

The needle-shaped crystals of the metabolite m.p. 178–181°, dec. (uncor.) have absorption at [λ_{max}^{EtOH} (log ϵ)] 281 (3·64), 286·5 (3·71) and 309 (3·88) nm, and they exhibit an optical rotation of $[\alpha]_D^{21}$ +337° (EtOH, c = 0·944). Low and high resolution MS (obtained using a heated direct insertion probe) revealed a parent ion with an elemental composition of $C_{16}H_{12}O_6$ at *m/e* 300 (30%), 14 m.u. less than pisatin. Other major ions in the spectrum are at *m/e* 282 (100%, $C_{16}H_{10}O_5$) and 281 (86%, $C_{16}H_9O_5$). A major ion at M-18 has been observed for other 6a-hydroxypterocarpans [7–9] and is consistent with a dehydration across the 6a—11a bond resulting in an ion with the structure of **3**. The *m/e* 281 ion is consistent with a further loss of a proton at C_6, as observed previously for anhydrohomopisatin [10].

Unlike pisatin, the metabolite reacts strongly with diazotised *p*-nitroaniline to give an orange product. Upon the addition of NaOH the UV spectrum in EtOH shifts to 252 and 301 nm. The MS of the acetate, formed on reaction with acetic anhydride–pyridine, reveals a parent ion of *m/e* 342 (base peak) and major ions at *m/e* 324 (M-18), *m/e* 300 (M-42), *m/e* 282 (M-18-42) and *m/e* 281. These properties suggest the presence of an aromatic hydroxyl group, which pisatin lacks.

Like pisatin, the metabolite gives a positive Labat test [11] for methylenedioxy groups and a negative Gibbs [12] test. The IR spectrum (film) of the metabolite is very similar to that of pisatin, except for an intensification of the OH band centered at 3370 cm^{-1} and the lack of absorption at

1322 and 1192 cm^{-1}. The latter two bands most likely are due to the methoxyl group of pisatin [13].

Methylation of the metabolite with diazomethane afforded a compound with the same UV properties, TLC mobility and MS as pisatin. Acidic dehydration [5] of the metabolite yielded a product with the same m.p. and UV spectrum as anhydrosophorol (**3**) [14] with [λ_{max}^{EtOH} log ϵ)] at 340(4·53) and 357(4·53) nm. The presence of a parent ion at *m/e* 282 (78%) and a base peak at *m/e* 281 in the MS of this product indicates that it is anhydrosophorol and confirms that the metabolite is 3,6a-dihydroxy-8,9-methylenedioxypterocarpan.

The identification of 3,6a-dihydroxy-8,9-methylenedioxypterocarpan as a fungal metabolite of pisatin exemplifies yet another way in which fungi initiate metabolism of pterocarpans. Previous studies on the metabolism of the pterocarpans phaseollin [15], 3-hydroxy-9-methoxypterocarpan [16], and 3-hydroxy-8,9-methylenedioxypterocarpan [17] by *Stemphylium botryosum* have shown that the initial fungal metabolites are the analogous isoflavans formed by the cleavage of the benzyl ether linkage of the dihydrofuran ring. Other studies have shown that one of the first metabolites of phaseollin produced by *F. solani* f. sp. *phaseoli* [18,19] is the result of hydroxylation at position 1a with concommitant dienone formation in ring A. However, the metabolism by *Colletotrichum lindemuthianum* [20] of phaseollin results in hydroxylation at 6a and 7.

Whether the metabolism of these pterocarpans is always a detoxification mechanism is still unclear. In some cases [18,21] the metabolites are less antifungal than the parent pterocarpans, while in others (i.e. the isoflavans) the same order of antifungal activity exists. In this study the radial mycelial growth of *F. solani* f. sp. *pisi* was not significantly inhibited by 100 μg of pisatin (10%) or 100 μg of 3,6a-dihydroxy-8,9-methylenedioxypterocarpan (12%) per ml of medium. However, at the same conc., *Fusarium solani* f. sp. *cucurbitae*, a nonpathogen of pea, is inhibited 100% by pisatin [22] but only 12% by 3,6a-dihydroxy-8,9-methylenedioxypterocarpan. Even in this case, our preliminary results on the rate of metabolism of pisatin vs rate of growth by *F. solani* f. sp. *pisi* suggest that the tolerance of the organism to pisatin is due to some mechanism other than metabolic alteration of pisatin to a non-inhibitory metabolite.

EXPERIMENTAL

Isolation of pisatin (**1**). Pea (*Pisum sativum* L. 'Progress No. 9') seeds were germinated in dark in wet paper towels at *ca* 25° in conditions of high humidity. After extensive fungal infestation and growth (1–2 weeks) on germinating seeds, they were dried at 75° (2 days) and ground to powder (60 mesh). The powder was exhaustively extracted with Et_2O (4–5 days) in a Soxhlet. The Et_2O was evaporated, and oily residue was suspended in 0·1 M phosphate buffer, pH 7·0 (*ca* 50 ml added to the extract from 50 g of powder, dry wt). The buffered suspension was partitioned 2× against 2 vol of $CHCl_3$ and the $CHCl_3$ fractions collected. After removal of $CHCl_3$, Me_2CO (*ca* 25 ml) was added to the oily residue and the Me_2CO-soluble substances collected. After solvent removal, this material was chromatographed on a column (20 cm) of Si gel (75 g, grade 950) with hexane–Et_2O, 1:1 as the eluting solvent. The pisatin obtained from the column was further purified by PLC (Si gel-coated glass plates, Brinkmann) in $CHCl_3$–MeOH, 25:1 (R_f 0·57), C_6H_6–EtOAc–*iso*-PrOH, 90:10:1 (R_f 0·44), and toluene–EtOAc, 7:1 (R_f 0·28).

Production and isolation of 3,6a-dihydroxy-8,9-methylenedioxypterocarpan (**2**). Actively growing mycelium of *F. solani* f. sp. *pisi* was obtained by the procedure used previously for *F. solani* f. sp. *phaseoli* [18]. Pisatin, dissolved in EtOH, was added to cultures (final pisatin conc. was 50–100 μg/ml and final EtOH conc. was 0·5%) containing 1·5 mg (dry wt.) of fungus/ml of medium. The cultures (100 ml/500 ml flask) were incubated at 24° on a reciprocal shaker (125 strokes/min). The production of **2** was monitored by assaying small aliquots of the culture. When approx. 50% of pisatin had been converted to **2** (20–30 hr) the cultures were partitioned against 4 vol of $CHCl_3$, then against 1 vol of $CHCl_3$. After evaporation of $CHCl_3$ **2** was separated from the residue by preparative TLC in $CHCl_3$–MeOH, 25:1 (R_f 0·24) and Et_2O–hexane, 5:1 (R_f 0·27). Crystallization was from aq. EtOH.

Bioassays. Bioassays were run as previously described [22].

Acknowledgements—This research was supported by Hatch project 423. The authors would like to thank J. D. Henion for MS service.

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