Phytochemistry, 1980, Vol. 19, pp. 917–919. © Pergamon Press Ltd. Printed in England. 0031-9422/80/0501-0917 $02.00/0

3-HYDROXYMAACKIAINISOFLAVAN, A PISATIN METABOLITE PRODUCED BY *FUSARIUM OXYSPORUM F. SP. PISI*

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(*Revised received* 15 *August* 1979)

Key Word Index—*Pisum sativum*; Leguminosae; *Ascochyta pisi*; *Fusarium oxysporum f. sp. pisi*; pterocarpans; isoflavans; pisatin; 6a-hydroxymaackiain; 3-hydroxymaackiainisoflavan; phytoalexins; fungal metabolism.

Abstract—3,7,2′-Trihydroxy-4′,5′-methylenedioxyisoflavan, a novel fungal metabolite of pisatin, was identified on the basis of NMR and MS data. The isoflavan arises by reductive opening of the dihydrofuran ring in the pterocarpan 6a-hydroxymaackiain, the first breakdown product of pisatin. The trivial name 3-hydroxymaackiainisoflavan is proposed for this substance.

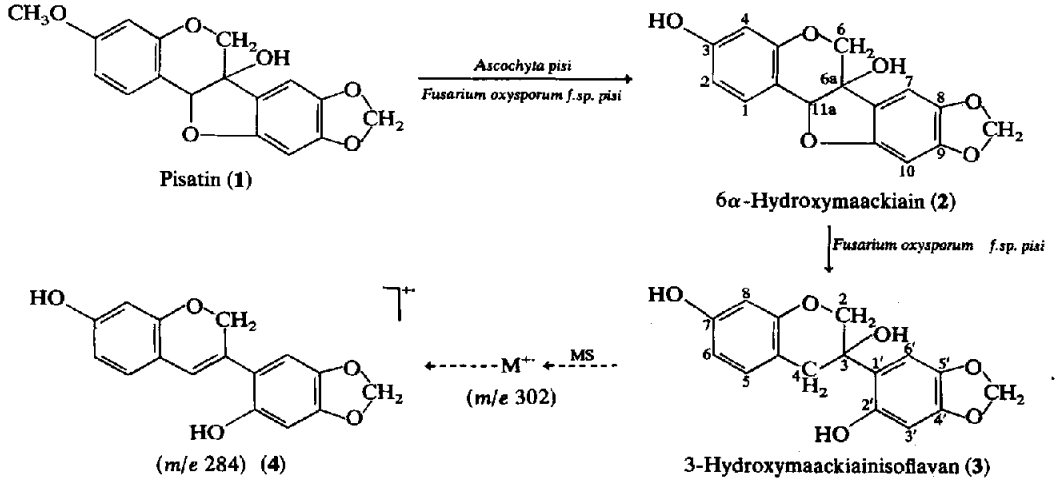
INTRODUCTION

Previous studies [1, 2] have shown *Fusarium oxysporum f. sp. pisi* to be capable of degrading pisatin (**1**), the phytoalexin of pea, *Pisum sativum*, with CO_2 as one of the end-products. As early as 1971 [2] an intermediate of pisatin breakdown produced by *Ascochyta pisi* was partially characterized. On the basis of UV and IR spectra, and NMR and MS measurements, it was later [3] identified as 3,6a-dihydroxy-8,9-methylenedioxypterocarpan (**2**). It arises by *O*-demethylation of pisatin and can thus be considered to be 6a-hydroxylated maackiain (= inermin), although it is probably enantiomeric with the 6a-hydroxymaackiain as reported by Bilton *et al.* [4]. The same metabolite is produced by a number of other fungi, such as *Fusarium solani f. sp. pisi* [5], *Stemphylium botryosum* ([6] cf. [5, 7]), *Fusarium anguioides* and *F. avenaceum* [8] and *Mycosphaerella pinodes* [9].

Time-course studies on the degradation of pisatin by *Fusarium oxysporum f. sp. lycopersici* and *F. oxysporum f. sp. pisi* demonstrated that only the pea-pathogenic *forma specialis pisi* was able to metabolize pisatin [10, 11]. The first metabolite formed was identified once again as 6a-hydroxymaackiain; the second one proved to be an isoflavan [10, 11]. This paper presents evidence for its structure (**3**).

RESULTS AND DISCUSSION

TLC, UV and MS data for 6a-hydroxymaackiain were comparable with those given in the literature [3, 5, 8]. The NMR spectrum of 6a-hydroxymaackiain (see Experimental) was only slightly different from that of pisatin [12]. Only the aromatic protons H-1, H-2 and H-4 showed small upfield shifts (0.05, 0.12 and 0.05 ppm, respectively) relative to those of



Pisatin (**1**)

6α-Hydroxymaackiain (**2**)

(*m/e* 284) (**4**)

3-Hydroxymaackiainisoflavan (**3**)

pisatin, which is expectable in view of the replacement of methoxyl by hydroxyl at position 3.

Upon prolonged incubation of *Fusarium oxysporum f. sp. pisi* [10, 11], a compound was produced which showed TLC mobilities in chloroform–methanol, 97:3 (R_f 0.20) and ethyl acetate–diethyl ether, 1:1 (R_f 0.74) not significantly different from those of 6a-hydroxymaackiain in the same solvent systems (R_f 0.24 and 0.80, respectively) [11]. However, its UV spectrum (λ_{max}^{EtOH} nm: 291, 300 (sh)) distinctly differed from that of pisatin (λ_{max}^{EtOH} nm: 281, 286 and 309) and from that of 6a-hydroxymaackiain (λ_{max}^{EtOH} nm: 282, 287, 309 (for full spectral data, see [11]). Acid dehydration in the presence of formic acid was accompanied by marked shifts in the UV spectrum of pisatin and 6a-hydroxymaackiain, with new absorption maxima produced at 339 and 357 nm; preliminary experiments with the novel metabolite showed no such peaks at similar wavelengths; however, prolonged incubation and partial purification of the product obtained led to the isolation of an unidentified compound with λ_{max}^{EtOH} at 317 and 276 nm. These spectral characteristics suggest some chemical relationship with 2-(2-hydroxy-4-methoxyphenyl)-3-methyl-6,7-methylenedioxybenzofuran [13] or more probably with an ethanolysis product comparable to that derived from anhydropisatin [15].

A sufficiently large quantity of the compound could be isolated to carry out NMR and MS measurements. The novel metabolite proved to be rather unstable in solution as was shown by its decomposition within 3–4 days when stored in chloroform in the dark at 4°. The similarity of the NMR spectrum of 6a-hydroxymaackiain (**2**) to that of the newly isolated compound suggested a close chemical relationship between the two metabolites. Only in one respect was there a distinct difference: in contrast to the NMR spectrum of **2**, in which a singlet was observed at δ 5.28, that of the novel metabolite showed two doublets at δ 3.34 and 3.05 ($J = 17$ Hz) of which the one at high-field (δ 3.05) showed a long-range coupling ($J =$ 2.3 Hz). These signals can be most readily ascribed to a third CH_2 group (6a-hydroxymaackiain has a methylenedioxy group and a CH_2 group at position 6). One could explain the presence of three CH_2 groups in the NMR spectrum of the new compound by postulating an isoflavan structure.

In accordance with the molecular structure (**3**), the NMR spectrum of the postulated isoflavan exhibited signals from five aromatic protons; four were found in the range of δ 6.60–6.34 (H-6, H-8, H-3′, H-6′) and one at 7.00 ($J = 8$ Hz). The latter absorption can be ascribed to H-5 because of the lack of (long-range) *meta*-coupling. The protons of the methylenedioxy group showed one sharp absorption at δ 5.95. Two doublets were observed at lower field (δ 4.28 and 4.07) of which one (4.28) showed long-range coupling ($J = 2.3$ Hz); it can be ascribed to the CH_2 group at position 2. From these observations, the conclusion can be drawn that one proton of a CH_2 group at position 4 is coupled with one of the two protons of the CH_2 group at position 2.

At all three probe temperatures chosen (see Experimental), major peaks in the MS were found at *m/e* 137 (80%), 138 (100%) and 164 (30–40%). At *m/e* 284 a peak was found which, relative to the base peak (*m/e* 138), increased with temperature from 5% at 120° to 30% at 150° and 40% at 200°. Smaller peaks (*ca* 5–8%) were present at *m/e* 162 and 283. Exact mass measurements of the ions at *m/e* 138, 164 and 284 showed their elemental compositions to be $C_7H_6O_3$, $C_9H_8O_3$ and $C_{16}H_{12}O_5$, respectively. Only a very small peak (± 0.5%) was found at *m/e* 302, the mass of the supposed molecular ion. However, this is not surprising since tertiary alcohols are known to lose water quite readily, especially in this case where an extensive conjugated system can be formed. The fact that the peak at *m/e* 284 increased with temperature is undoubtedly due to thermal dehydration. The elemental composition of the ion concerned is in agreement with the proposed dehydration product **4**. In 6a-hydroxypterocarpans the dehydration during MS measurement takes place across the C6a–C11a bond. Analogously, our isoflavan can be assumed to give rise to the major ion at *m/e* 284 by a dehydration across the comparable C3–C4 bond. The presence of an ion at *m/e* 162 might be due to a retro-Diels–Alder fragmentation of the 284 ion, as reported for other isoflavans [15–17].

Both the NMR and MS data are consistent with the assumption that the isolated pisatin metabolite is an isoflavan with structure **3**. Since the numbering of the isoflavan ring system is different from that of the pterocarpan ring system (cf. [7]), its semisystematic name, 3,7,2′-trihydroxy-4′,5′-methylenedioxyisoflavan, has little relationship with that of the parent compound(s); therefore, in accordance with the habit of naming isoflavans after their parent compound (cf. phaseollinisoflavan [15], 2′-*O*-methylphaseollidinisoflavan [17]), we propose as a trivial name 3-hydroxymaackiainisoflavan (elsewhere [11] indicated as 3-hydroxyinerminisoflavan).

Several fungal species are known to be able to degrade pisatin, with CO_2 being an end-product [1, 2]. *Fusarium oxysporum f. sp. pisi* is the first species of which the first two intermediates in the degradative pathway are now known: the first step involves *O*-demethylation of the methoxyl at C-3, the second reductive opening of the dihydrofuran ring of the pterocarpan skeleton. The isoflavan resulting from the latter step is rapidly broken down, without intermediates with distinctive UV-spectral characteristics being isolated [11]. *Mycosphaerella pinodes* is another fungal species which most probably degrades pisatin in a similar way (Fuchs and De Vries, unpublished data). It will be of interest to investigate whether *O*-demethylation of pisatin is a prerequisite for further degradation; the evidence so far indicates that it is an introductory step to further microbial breakdown, 6a-hydroxymaackiain being found as its first metabolite.

EXPERIMENTAL

Fusarium oxysporum f. sp. pisi was grown in shake cultures [11] in 200 ml Lilly and Barnett's medium [18] with 1% glucose. After 1 week, the whole cultures, together with 10 ml fresh medium with 0.1% glucose, were transferred to Erlenmeyer flasks in which pisatin had been dried down; incubation took place at *ca* 22°. At intervals, samples were taken which were fractionated as described elsewhere [11]. Some EtOAc fractions contained either 6a-hydroxymaackiain or its isoflavan breakdown product, or both. Both

compounds were isolated and purified by TLC and subjected to UV, NMR and MS analysis. ^{1}H NMR spectra were recorded on a Varian XL-100-15 spectrometer in FT mode, equipped with a pulse unit and a 620 L-16K on-line computer system using TMS as an internal standard. (Typical spectral parameters were as follows: spectral width 1000 Hz (0.25 Hz/point), acquisition time 4 sec, pulse width 25 μs and number of transients 150–400.) MS were obtained using a heated direct insertion probe at three different probe temp. (120, 150 and 200°). Peak intensities at 200° of the main peaks were: *m/e* 137: 80%, 138: 100%, 139: 10%, 162: 5%, 164: 30%, 283: 7%, 284: 40%, 285: 7%, 302: <0.5%. Exact mass measurements were *m/e* 138, experimental value 138.0316 (theor. 138.0317), *m/e* 164, exper. 164.0479 (theor. 164.0473), *m/e* 284, exper. 284.0675 (theor. 284.0685). Elemental compositions were $C_7H_6O_3$, $C_9H_8O_5$ and $C_{16}H_{12}O_5$, respectively. No exact mass measurement could be made of the molecular ion, since the peak intensity was too low to enable an accurate estimation.

6a-Hydroxymaackiain. ^{1}H NMR ($CDCl_3$) δ ppm: 7.37 (*d*, $J = 8$ Hz, H-1), 6.82 (s, H-10), 6.56 (*dd*, $J = 8$ and 2 Hz, H-2), 6.43 (*d*, $J = 2$ Hz, H-4), 6.42 (s, H-7), 5.96 and 5.93 (*d's*, $J \sim 1$ Hz, 2H, O-CH_2-O), 5.28 (s, H-11a), 4.18 and 4.00 (*d's*, $J = 11$ Hz, 2H, CH_2 position 6).

3,7,2'-Trihydroxy-4',5'-methylenedioxyisoflavan (= *3-hydroxymaackiainisoflavan*). ^{1}H NMR ($CDCl_3$) δ ppm: 7.00 (*d*, $J = 8$ Hz, H-5), 6.60–6.34 (*m*, 4H: H-6, H-8, H-3', H-6') 5.95 (s, 2H, O-CH_2-O), 4.28 (*dd*, $J = 11$, 2.3 Hz) and 4.07 (*d*, $J = 11$ Hz) (2H, CH_2 position 2), 3.34 (*d*, $J = 17$ Hz) and 3.05 (*dd*, $J = 17$, 2.3 Hz) (2H, CH_2 position 4).

Acknowledgement—The authors are indebted to Dr. P. Smit, Laboratory of Organic Chemistry, for advice in preparing the manuscript.

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