Degradation of 3,9-Dimethoxypterocarpan and Medicarpin by *Fusarium proliferatum*

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The degradation of 3,9-dimethoxypterocarpan was investigated in selected strains of *Fusarium*. *Fusarium proliferatum* (i. e. *Gibberella fujikuroi* (SAW)WR) degrades this substrate via 3-methoxy-9-hydroxypterocarpan, 3,9-dihydroxypterocarpan and 2′,4′,7-trihydroxyisoflavan. During degradation by this organism medicarpin is first demethylated to 3,9-dihydroxypterocarpan.

Introduction

Increasing evidence in recent years [1–4] points to the ability of phytopathogenic fungi to degrade fungitoxic phytoalexins to less toxic compounds. Such degradative reactions will contribute to a better understanding of host-parasite relationship in which phytoalexins seem to play an important role as a chemical defense mechanism [5, 6].

Pterocarpans are the main phytoalexins of the Leguminosae [1, 5] and the fungal degradative pathways of such phytoalexins as medicarpin (**I**) or pisatin (**II**) are of great interest. Hydroxilation and O-demethylation reactions as well as conversion of
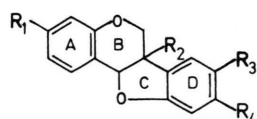
I: $R_1 = OH, R_2 = R_3 = H, R_4 = OCH_3$
II: $R_1 = OCH_3, R_2 = OH, R_3 : R_4 = OCH_2O$
III: $R_1 = R_4 = OCH_3, R_2 = R_3 = H$

the pterocarpan into an isoflavan structure (for instance medicarpin into vestitol, which is 2′,7-dihydroxy, 4′-methoxyisoflavan) have been reported [2, 3, 7–9] for these and related compounds. With respect to O-demethylation of isoflavonoid structures removal of the O-methyl group at position 3 (position 7 in isoflavone numbering) was found to be the opening reaction whenever structurally possible [1, 8–12]. It therefore seemed understandable that in *Fusarium solani*, *F. anguioides* and *F. avenaceum* 3,9-dimethoxypterocarpan (**III**) was found to be one of

the best structural analogs of pisatin for inducing the pisatin 3-O-demethylase [13]. Due to its greater chemical stability in comparison to pisatin, **III** could well serve as a substrate for elucidating pterocarpan catabolism. This is mandatory because complete pathways for isoflavone or pterocarpan degradation are not yet known.

In continuation of our earlier studies [9, 11] the degradation of **III** was measured with our collection of selected strains of *Fusarium*. In this paper we report results with *F. proliferatum* which demonstrate that the expected catabolism sequence from **III** to **I** and finally to vestitol was not followed.

Experimental

Materials

3,9-dimethoxypterocarpan and 2′-hydroxy-7,4-dimethoxyisoflavan were gifts from Dr. D. M. X. Donnelly, Dublin, and medicarpin and vestitol from Dr. P. M. Dewick, Nottingham.

[3-Methyl-^{14}C]-3,9-dimethoxypterocarpan was synthesized from medicarpin with [^{14}C]methyliodide (Radiochemical Center, Amersham, spec. act. 58 mCi/mmol) in dry acetone with anhydrous K_2CO_3 at 70 °C under stirring for 9 h according to [16]. The product (spec. act. 0.42 mCi/mmol) was purified by TLC with S_{19}. For experiments the labelled compound was diluted with inactive material to a spec. activity of 0.12 mCi/mmol. Methylation reactions were carried out with diazomethane (from N-nitrosomethylurea) in 70% methanol for at least 12 h.

Hydrogenation of 3-methoxy-9-hydroxypterocarpan was achieved in abs. ethanol (0.75 ml, 1 mg compound) with Pd on charcoal (0.5 mg) under hy-

drogen for 48 h at normal pressure. The reaction product was purified by TLC (S_2) and sublimation (140 °C).

Spectroscopic methods

UV-spectra were recorded with a Leitz-Unicam SP 8000 spectrophotometer in methanol. Phenolic compounds were analyzed by the addition of diagnostic reagents [17]. Quantitative determinations of medicarpin and 3-methoxy-9-hydroxypterocarpan were carried out at 287 nm (log $\varepsilon = 3.89$).

Mass spectroscopy was carried out with a Hitachi-Perkin-Elmer mass spectrometer RMV-GD.

Growth of fungi

Fungal cultures were kept on a Czapek-Dox-Agarmedium in petri dishes at 4 °C and transferred to new medium every two months. The various strains investigated have been published [11]. Strain XIII previously called *Gibberella fujikuroi* (SAW) WR has been renamed *Fusarium proliferatum* by the Centraalbureau voor Schimmelcultures at Baarn.

For degradative studies fungi were cultivated in a glucose-casein-yeast extract medium [18] for 5 days at 30 °C and 160 rpm on a gyrotory shaker. Prior to incubations the mycelium was washed five times with potassium-phosphate buffer (pH 7.5; 0.05 M).

Degradation experiments

Substrates (app. 1.5 mg) were dissolved in DMSO (0.5 ml) and transferred into 50 ml potassium-phosphate buffer (0.05 M; pH 7.5). After addition of 2.5 g washed mycelium the flasks were incubated at 30 °C/160 rpm on a shaker. Aliquots (5 ml) were periodically taken, freed from mycelium and acidified (pH 2−3). Compounds were extracted with diethylether and the ether extracts used for chromatography.

Chromatography

TLC was conducted on silica gel with the following solvents (v/v):

S_1: benzene:ethylacetate:isopropanol = 45:10:1,
S_2: benzene:ethylacetate:methanol:petrolether = 6:4:1:3,
S_3: benzene:ethylacetate:formic acid = 18:1:1,
S_4: toluene:ethylformate:formic acid = 5:4:1,

S_5: chloroform:isopropanol = 10:1,
S_6: chloroform:methanol = 25:1,
S_7: benzene:ethylacetate:isopropanol = 90:10:1,
S_8: benzene:methanol = 4:1,
S_9: benzene:ethylacetate:methanol:petrolether = 6:4:1:6,
S_{10}: benzene:ethylacetate:methanol = 6:4:1,
S_{11}: benzene:ethylacetate:methanol:petrolether = 6:4:1:10,
S_{12}: methanol,
S_{13}: benzene:methanol = 1:1,
S_{14}: chloroform:methanol = 5:2,
S_{15}: benzene:ethylacetate:isopropanol = 90:1:1,
S_{16}: benzene:ethylacetate = 18:1,
S_{17}: benzene:ethylacetate:methanol:petrolether = 6:1:1:10,
S_{18}: benzene:petrolether:ethylacetate = 5:5:1 and
S_{19}: dichloromethane.

Chromatograms were viewed under UV-light (254 and 365 nm) and fluorescing or absorbing bands were eluted with methanol. Plates were sprayed with diazotized 4-nitro-aniline according to [19].

TLC plates with radioactive compounds were scanned with a Berthold Scanner II, LB 2723.

Results

Degradation of 3,9-dimethoxypterocarpan by strains of Fusarium

3,9-Dimethoxypterocarpan (10^{-4} M) was incubated with suitable amounts of fungal mycelium from our *Fusarium* strains [11]. Pterocarpan metabolism was followed in aliquots by UV-spectroscopy and TLC in solvent S_7. Catabolites were detected by viewing the TLC-plates under the UV-lamp or by spraying with diazotized 4-nitroaniline. 8 out of 16 strains tested [11] were shown to degrade the 3,9-dimethoxypterocarpan. Strains VI (*Fusarium oxysporum* ex Fr. f. *lycopersici* (Sacc.)) and XI (*Fusarium lini*) completely degraded **III** without accumulating any catabolite.

Strains VII (*F. anguioides* Sherbakoff) and VIII (*F. avenaceum* (Fr.) Sacc.) catabolized the substrate within a few hours, but only traces of catabolites could be detected which showed on TLC the same R_f-values as medicarpin ($R_f = 0.35$) and vestitol ($R_f = 0.15$). The strains XIV (*Gibberella fujikuroi* (SAW)) and XVI (*F. aquaeduatium*) showed one in-

termediate only which by TLC appeared to be I. Strain I (*F. sporotrichoides* Sherb.) produced two compounds which again appeared to be medicarpin and vestitol (R_f-value and colour with diazonium reagent). Strain XIII (*Fusarium proliferatum, i. e. Gibberella fujikuroi* (SAW)WR) showed during degradation of the substrate the sequential accumulation of three catabolites.

The time depending formation and transient accumulation of these three catabolites is shown in Fig. 1. As judged from the chromatographic properties it appears that during degradation the compounds become more polar. Fig. 1 further shows that the first and the second compound had the same R_f-values as medicarpin and vestitol. Though *Fusarium proliferatum* had previously been shown not to O-demethylate or degrade pisatin [9] it was decided to isolate the compounds as shown in Fig. 1 and elucidate their structures.
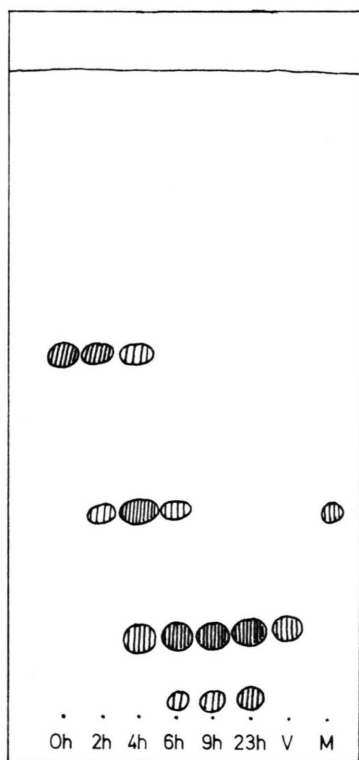

Fig. 1. TLC plate developped with S_7 to demonstrate disappearance of 3,9-dimethoxypterocarpan and sequential formation of 3 catabolites between zero and 23 h of incubation with *F. proliferatum*. Vestitol (V) and medicarpin (M) were applied as references.

Structural elucidation of catabolites

To obtain sufficient amounts of catabolites large scale incubations were worked up after 4 and 9 h. The degradation products were extracted from the nutrient medium with ether and separated by TLC with the solvents S_7 and S_2.

The first degradation product was chromatographically compared with medicarpin in solvents S_1, S_2, S_4, S_5, S_7, S_8, S_9, S_{10} and S_{11}. With respect to R_f-value, absorption under UV-light (254 nm) and yellow-orange colour with diazotized 4-nitroaniline the compound seemed to be identical with medicarpin. After methylation of the first degradation product with diazomethane the starting 3,9-dimethoxyptero-carpan was again obtained as indicated by chromatography with altogether 12 solvents. The UV-spectrum of medicarpin and the first degradation product is shown in Fig. 2. Though the compounds appeared to be identical, addition of NaOH resulted in significant differences in the UV-spectrum. While medicarpin showed maxima at 220, 247 and 286 nm the first degradation product had absorption maxima at 219, 249 sh, 280 sh, 286 and 295 sh (Fig. 3). The UV-spectrum in Fig. 3 shows that the two compounds are obviously not identical. It was therefore assumed that the demethylation had not occured at position 3 but rather at position 9. Attempts to differentiate between the alternative products, namely 3-hydroxy-, 9-methoxypterocarpan and 3-methoxy, 9-hydroxypterocarpan by mass spectrometry failed, because the measured main peaks at m/e 270 (M$^+$; $C_{16}H_{14}O_4$), 269, 255, 161 and 148 could originate from either compound. It is sufficiently substantiated in the literature [14, 15] that due to the fragmentation process of pterocarpans the localization of hydroxy- or methoxy-groups in rings A or D cannot be measured with mass spectrometry. However, the M$^+$-peak of the first degradation product at m/e 270 together with the observation that the remethylated degradation product showed peaks at m/e 284 (M$^+$; $C_{17}H_{16}O_4$), 283, 269, 161 and 148 clearly proved that the first degradation products differs from the 3,9-dimethoxypterocarpan by one methyl group. To convert this first degradation product into a compound amenable to mass spectroscopic analysis it was catalytically reduced to the equivalent isoflavan. During this reaction the benzylphenylether bond at C-11a was cleaved. Hydrogenation after demethylation in position 3 would
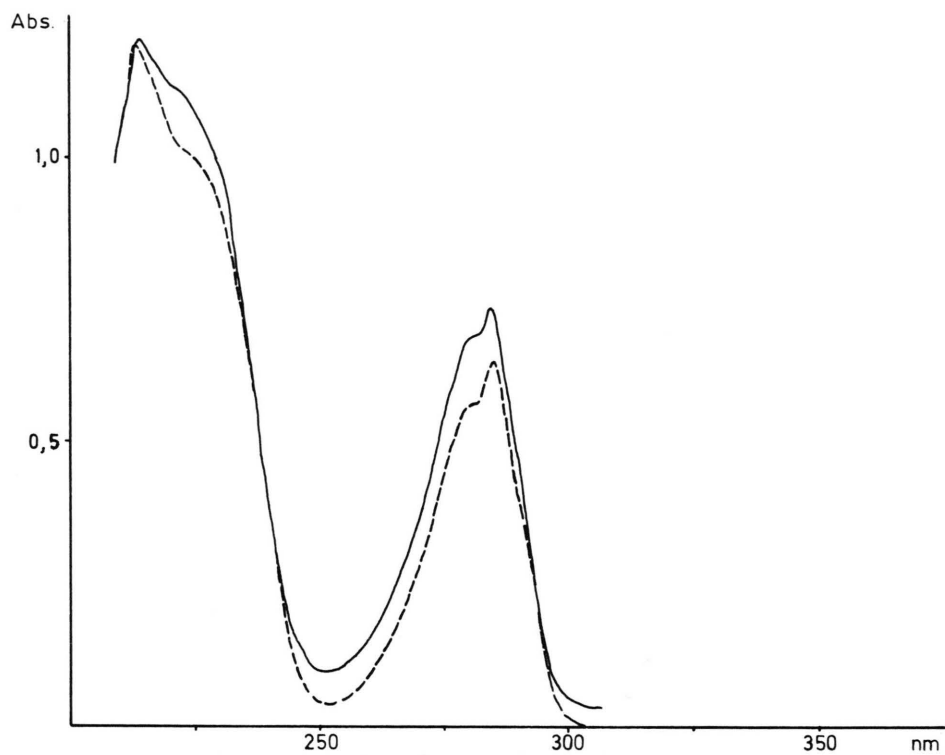
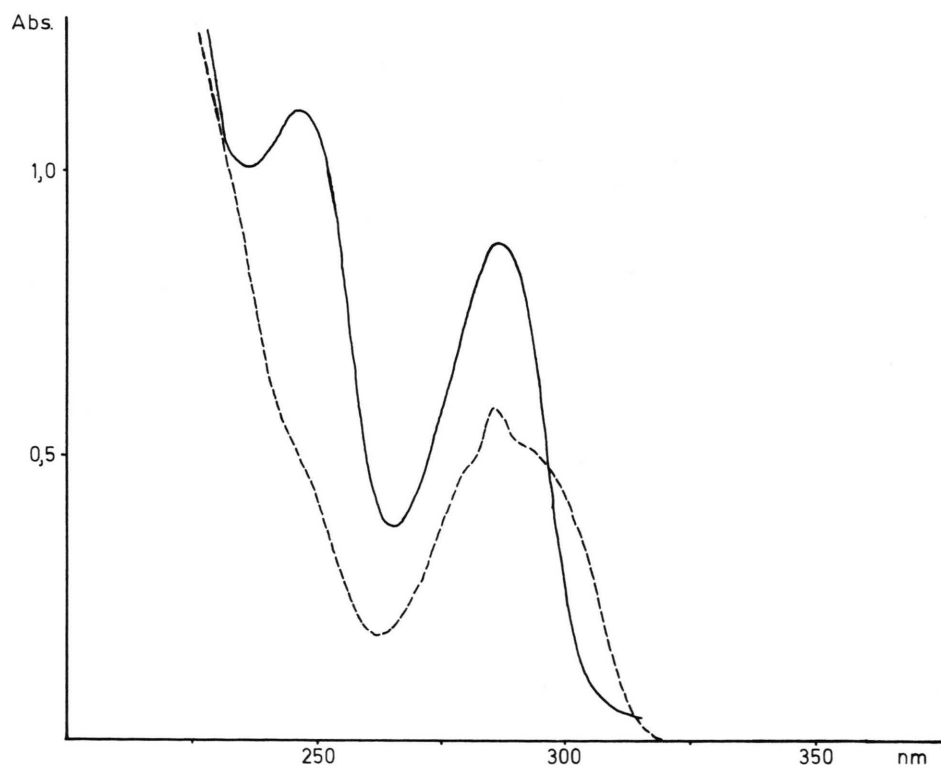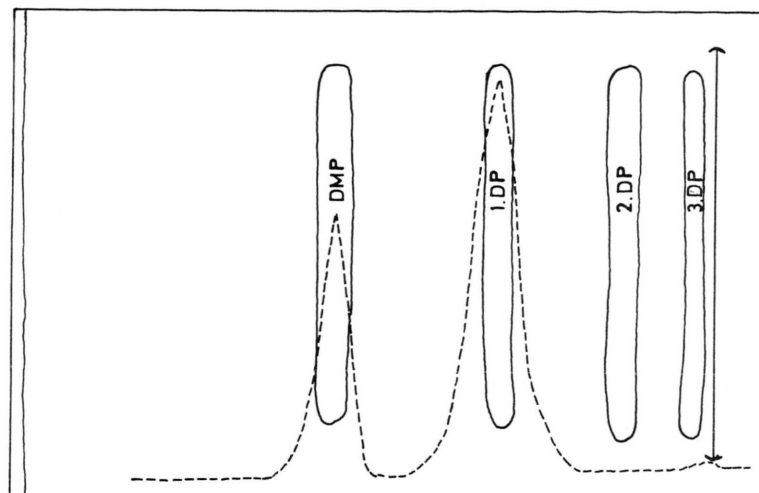Fig. 2. UV-spectrum of medicarpin (———) and the first degradation product (- - - -) of 3,9-dimethoxyptero-carpan.

Fig. 3. UV-spectrum of medicarpin (———) and the first degradation product (- - - -) of 3,9-dimethoxyptero-carpan after addition of NaOH (compare also Fig. 2).

Fig. 4. TLC plate (S$_7$) showing bands of the 3 degradation products (DP) of [3-methyl-^{14}C]-3,9-dimethoxypterocarpan (DMP) together with the corresponding scanner diagram (- - - -).

yield 2',7-dihydroxy-4'-methoxyisoflavan with expected characteristic peaks in the mass spectrum at *m/e* 272 (M$^+$), 150 (B-ring) and 122 (A-ring). Demethylation in position 9 would give 2',4'-dihydroxy, 7-methoxyisoflavan, where the two characteristic fragments of rings A and B would both give a peak in the mass spectrum at *m/e* 136. The isoflavan recovered from the hydrogenation reaction showed in the mass spectrum the expected molecular peak (*m/e* 272) and a very strong signal at *m/e* 136. Peaks at *m/e* 150 and 122 could not be detected in the mass spectrum. These data prove that the first degradation product of 3,9-dimethoxypterocarpan is the 9-hydroxy-, 3-methoxypterocarpan.

This assumption was independently demonstrated by degradation experiments with [3-methyl-^{14}C]-3,9-dimethoxypterocarpan. This compound was obtained by methylation of medicarpin with labelled methyljodide according to previous reports [16]. The labelled substrate was subjected to degradation by strain XIII and the ether extract of the growth medium prepared after 5 h of incubation was separated by TLC. As shown in Fig. 4 the three characteristic catabolites as already shown in Fig. 1 could again be recognized. The scanner diagram in Fig. 4 shows that radioactivity could only be detected in the substrate and in the first degradation product. The substrate was introduced into this experiment with a specific radioactivity of 0.12 mCi/mmol whereas the specific radioactivity of the first degradation product recovered from this experiment was measured to be 0.13 mCi/mmol. These values together with Fig. 4 demonstrate that demethylation

in the first reaction had exclusively taken place at position 9 of the pterocarpan molecule.

The second degradation product (Figs 1 and 4) was shown to possess a pterocarpan and not an isoflavan structure. This was especially indicated by the characteristic UV-spectrum (λ_{max} 214, 225 sh, 281, 285 and 293 sh), because the maxima at 281 and 285 clearly show a pterocarpan skeleton. Addition of NaOH resulted in a bathochromic shift (λ_{max} 220, 246 and 296 nm) where the maximum at 246 nm indicates that the second demethylation reaction had occurred at position 3 because the same maximum can be detected in the UV-spectrum of medicarpin (Fig. 3). The observation that this second degradation product no longer contains radioactivity from the 3-methylgroup of the 3,9-dimethoxypterocarpan (Fig. 4) further proves that this product is the 3,9-dihydroxypterocarpan. This assumption was corroborated by measuring the mass spectrum of the compound (*m/e* 256 (M$^+$, C$_{15}$H$_{12}$O$_4$), 255, 239, 147 and 134). The molecular peak at *m/e* 256 is 28 mass-units lower than the molecular peak of the 3,9-dimethoxypterocarpan, which indicates the loss of two methyl groups. In addition, this degradation product was permethylated and the resulting product was chromatographically indistinguishable (S$_2$, S$_6$, S$_7$, S$_{10}$–S$_{18}$) from 3,9-dimethoxypterocarpan, the UV-spectra of the permethylated product and the original substrate were identical (λ_{max} 280 and 285 nm) and finally the mass spectra of the two compounds were identical. The second compound has therefore been elucidated as 3,9-dihydroxypterocarpan.

The third degradation product with the lowest R_f-value ($R_f = 0.05$ in S_7) could only be isolated in very small amounts. After chromatographic purification (S_7, S_2) the UV-spectrum showed maxima at 220, 280 and 290 sh/nm. The typical double maximum around 280/285 nm of the pterocarpan skeleton could no longer be detected in this spectrum so that cleavage of the benzylphenylether bond at C-11a might have occurred. Addition of NaOH resulted in a bathochromic shift of the UV-spectrum (λ_{max} 280, 245 sh, 295 and 289 sh). Due to the great instability of this compound the small amounts isolated were methylated with diazomethane and after purification (S_7) the mass spectrum of the methylated product was recorded. The spectrum contained the characteristic peaks at m/e 300 (M+, $C_{18}H_{20}O_4$) 164, 149 and 121 and they were identical with the mass spectrum obtained with original 2′,4′,7-trimethoxy-isoflavan. The latter compound was obtained by methylation of 2′-hydroxy-4′,7-dimethoxyisoflavan. The third degradation product was therefore characterized as 2′,4′,7-trihydroxyisoflavan.

When medicarpin was incubated with cells of *Fusarium proliferatum* the substrate rather rapidly disappeared. A first catabolite which accumulated appeared to be chromatographically identical with the second degradation product shown in Figs 1 and 4. This product as derived from medicarpin was isolated and as judged from chromatographic properties, UV-spectrum together with diagnostic reagents and spectroscopic studies was shown to be 3,9-dihydroxypterocarpan.

Discussion

The catabolic sequence of 3,9-dimethoxypterocarpan as elucidated so far with *Fusarium proliferatum* is shown in Fig. 5. Upon demethylation at position 9 medicarpin is funnelled into this sequence at 3,9-dihydroxypterocarpan. The sequence of demethylation steps in Fig. 5 is in so far remarkable, because data published with related compounds and other fungi led to the assumption that demethylation will preferentially begin at position 3 (position 7 of isoflavones) whenever structurally possible. In contrast to experiments described with *Botrytis cinerea* [2] fission of the benzylphenylether bond at C-11a occurs in this case only after both methyl groups have been removed. Vestitol is not an intermediate in the degradation sequence by *Fusarium prolifera-*

tum. The sequence of demethylation steps is therefore inverse to expectation.

The data discussed in connection with Fig. 4 and the comparison of specific radioactivity of [3-14C-methyl]-3,9-dimethoxypterocarpan and the first degradation product reveal that the first demethylation exclusively occurs at position 9. The O-demethylases involved are obviously highly position-specific and can only be induced by specific substrates. Therefore the O-demethylase responsible for removal of the methyl group at position 3 requires as inducing agent a compound with a free hydroxyl group in position 9. This may explain why *Fusarium proliferatum* is not able to demethylate pisatin. Pisatin with its 8,9-methylendioxygroup obviously does not fulfill the structural requirements for acting as an inducer of the 3-O-demethylase.

Formation of 3,9-dihydroxypterocarpan from medicarpin has previously been described for *Botrytis cinerea* [2]. Induction of the demethylase responsible for this conversion seems not to be in line with the above mentioned arguments. One can therefore visualize that different demethylases are
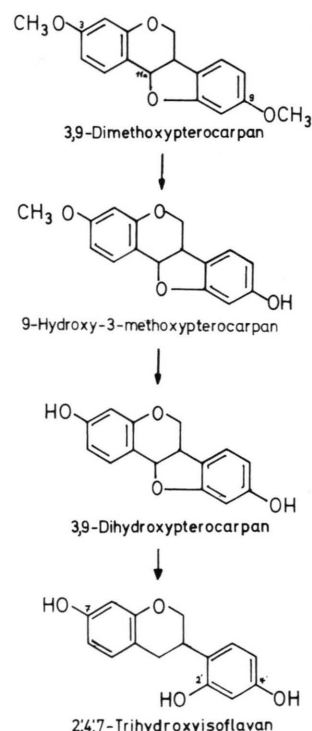

Fig. 5. Catabolic sequence of 3,9-dimethoxypterocarpan in *Fusarium proliferatum.*

involved. Inducing experiments and other studies on the various expected O-demethylases are in progress.

The results with *Fusarium proliferatum* and the other strains capable of degrading 3,9-dimethoxypterocarpan or medicarpin have shown that the 2′,4′,7-trihydroxyisoflavan seems to be an important intermediate in pterocarpan degradation, because in practically all cases this compound seemed to be detectable on chromatograms. Similarly, in case of isoflavone degradation by strains of *Fusarium* the intermediate formation of isoflavan structures has also been observed [20].

We are presently studying degradation of the 3,9-dimethoxypterocarpan in the strains mentioned in the first paragraph. Some of these are known to degrade pisatin such as *Fusarium avenaceum* [9]. In some of these strains the sequence of demethylation steps will not be as strict as shown in Fig. 4 so that alternative demethylation sequences can be found.

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