Demethylation, Methylation and 3′-Hydroxylation of Isoflavones by *Fusarium* Fungi

K.-M. Weltring, K. Mackenbrock, and W. Barz

Lehrstuhl für Biochemie der Pflanzen, Westfälische Wilhelms-Universität, Hindenburgplatz 55, D-4400 Münster

Z. Naturforsch. **37 c**, 570 – 574 (1982); received March 29, 1982

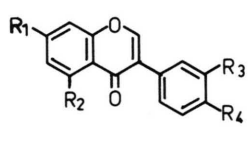
Isoflavones, *Fusarium*, Methylation, Demethylation, 3′-Hydroxylation, Metabolism

Several introductionary reactions of isoflavone metabolism by *Fusarium* fungi are reported. *Fusarium avenaceum* hydroxylates formononetin in the 3′-position yielding 3′,7-dihydroxy-4′-methoxyisoflavone (calycosin). 3′-Hydroxylation of biochanin A to 3′,5,7-trihydroxy-4′-methoxyisoflavone (pratensein) has been observed with *Fusarium oxysporum* f. sp. *lycopersici* and *Fusarium oxysporum* f. sp. *lini*. Depending on the growth medium *Fusarium proliferatum* metabolises formononetin either by 7-O-methylation or by 4′-O-demethylation.

Introduction

Isoflavonoid plant constituents are of considerable biological interest because numerous isoflavones, isoflavanones and pterocarpans are plant defense compounds due to their fungitoxic properties [1, 2]. The ability of various fungal pathogens to degrade such isoflavonoid plant products (*i. e.* preinfectional inhibitors and phytoalexins) is of great interest because this ability could well contribute to fungal pathogenicity [3].

During our studies on the degradation of a variety of plant polyphenols [4] and phytoalexins [5] by fungi of the genus *Fusarium* we have recently reported on the catabolism of the 5-hydroxyisoflavones biochanin A (**V**, in the figure) and genistein (5,7,4′-trihydroxyisoflavone) [6, 7]. Reduction one to an isoflavanone and alternatively, O-demethylation at the 4′-position were found to be introductionary reactions of isoflavone metabolism by *Fusarium* fungi.



I : R_1=OH, R_2=R_3=H, R_4=OCH_3
II : R_1=R_4=OCH_3, R_2=R_3=H
III: R_1=R_4=OH, R_2=R_3=H
IV: R_1=R_3=OH, R_2=H, R_4=OCH_3
V : R_1=R_2=OH, R_3=H, R_4=OCH_3
VI: R_1=R_2=R_3=OH, R_4=OCH_3

Figure: Structures of isoflavones mentioned in the text.

Reprint requests to Prof. Dr. W. Barz.

0341-0382/82/0700-0570 $ 01.30/0

We now report our observations on hydroxylation reactions in the 3′-position of **V** and furthermore describe studies on the metabolism of the 5-deoxyisoflavone formononetin (**I**) by *Fusarium* fungi. **I** is also considered to be a preinfectional inhibitor [8] of widespread occurrence in the *Leguminosae* [9].

Experimental

Materials

Formononetin, daidzein, biochanin A, pratensein, [U-^{14}C]formononetin and [methyl-^{14}C]formononetin were from previous studies [12, 26]. Calycosin was obtained from Dr. P. M. Dewick, Nottingham.

[Ring A-^{14}C]biochanin A and [ring A-^{14}C]formononetin were obtained by feeding [U-^{14}C]acetate (1 mCi; spec. radioact. 57.3 mCi/mmol) to the roots of 200 *Cicer arietinum* L. plants for 5 days beginning at day 8 after germination. The roots were homogenized in water with a Waring Blender and isoflavone-glucosides were allowed to be hydrolyzed by endogenous glucosidases. The isolation of the aglycones was by previous methods [18, 26]. The isoflavones were purified by repeated thinlayer chromatography on silica gel plates with solvents S_1 and S_2 and by Lobar column chromatography with 45% acetonitrile in water. Yield: 180 mg [ring A-^{14}C]biochanin A (spec. radioactivity 13.4 μCi/mmol and 40 mg [ring A-^{14}C]formononetin (spec. radioact. 4,4 μCi/mmol). 7,4′-dimethoxyisoflavone was synthesized from formononetin by methylation [18].

Fungi

The origin and the method of storage of the various *Fusarium* fungi has been published [4].

Growth of fungi

The fungi were either grown on a glucose-casein-yeast extract medium (SM) [7] or on a special *Fusarium* medium (FM) according to Brewer *et al.* [17]. Mycelium was grown in Erlenmeyer flasks for 5 days at 30 °C on a gyrotory shaker at 160 strokes per min.

Standard incubation assays

All degradative studies were carried out with 3 g wet weight of cells in 100 ml of potassium phosphate buffer (pH 7.5; 0.05 M) with 10^{-4} M substrate. These flasks were incubated at 30 °C/160 rpm. For isolation of catabolites cells were removed by filtration, the medium was acidified (5 N H_2SO_4) to pH 2–3 and the compounds wer extracted with diethylether. For chromatographic separation the extracts were applied as a methanolic solution.

Chromatography

Thin layer chromatography (TLC) was performed on silica gel plates with the following solvents (V/V):

S_1 dichlormethane: methanol = 30:1,
S_2 diethylether: petroleum ether (60–80 °C) = 7:3,
S_3 dichlormethane: methanol = 15:1 and
S_4 chloroform: methanol = 10:2.

Chromatograms were viewed under UV-light (254/366 nm) and sprayed with diazotized 4-nitroaniline or fast blue salt B. Compounds were eluted from the gel with methanol.

High-performance-liquid-chromatography (HPLC) was carried out with a KONTRON chromatograph with a Li Chrosorb RP 8 column (5 µ, 125×4 mm) with a flow of 1.2 ml/min (formononetin) and a Li Chrosorb RP 8 column (5 µ, 250×4 mm) with a flow of 0.8 ml/min (biochanin A). Various gradients of acetonitril in water were applied.

LOBAR column (Merck) chromatography was performed with size A columns (40–63 µ) with a Duramat pump (Watek, Heidelberg) with various acetonitril/water mixtures.

Analytical methods

The detection and measurement of ^{14}C-labelled compounds and the various spectroscopic methods together with the machines used have previously been described [6].

Results

Experiments with biochanin A

Our previous reports on biochanin A (V) metabolism by *Fusarium* fungi [6, 7] had presented the first known, rather detailed catabolic sequence of an isoflavonoid carbon skeleton. In these studies evidence had been obtained that an isoflavanone with a hydroxyl group in position 8 might occur as an intermediate of biochanin A catabolism.

A systematic search for such an intermediate [10] had shown that strains of *Fusarium oxysporum* f. sp. *lycopersici* and *Fusarium oxysporum* f. sp. *lini* when incubated with **V** (10^{-4} M) readily accumulated a hydroxylated derivative with an intact isoflavonoid skeleton (UV-spectrum and red-blue colour with fast blue salt B).

Our present studies with these both *Fusarium* strains now revealed that neither dihydrobiochanin A nor genistein were formed during biochanin A metabolism (TLC analysis of aliquots from standard incubation assays with S_3) whereas the aforementioned higher hydroxylated compound (TLC, S_3, $R_f = 0.5$) was formed by both strains. For structural elucidation of the new metabolite incubation assays of both strains were carried to maximum production, the compound was extracted from the nutrient medium and purified by preparative TLC (S_3). The UV-spectrum in methanol (λ_{max} 261, 290 (sh), 328 (sh) nm and the UV-data obtained by the addition of diagnostic reagents (11) (λ_{max} (NaOH) 270, 325 nm; ($AlCl_3$) 272, 310 (sh), 366 nm, this spectrum was not reversed by the addition of HCl; (Na-acetate) 268, 325 (sh/nm) were identical from both samples and reminiscent of the values obtained for the isoflavone pratensein [5,7,3′-trihydroxy-4′-methoxyisoflavone (**VI**) [11, 12]. This assumption was corroborated by the mass spectrum [*m/e* 300 (M^+)], 285, 257, 229, 201, 153, 148, 133, 120, 105, 72 and 69) which showed a molecular weight of 16 mass units higher than that of **V** (MW 284) and the isoflavone-characteristic *retro* Diels-Alder fragments of *m/e* 153 (152 + H) and *m/e* 148. These values prove that the additional oxygenation is located in the side chain phenyl ring of **VI.** HPLC-cochromatography of the samples isolated from the fungal cultures with a sample of previously isolated pratensein [12] further showed identity of the structures.

The two strains of *F. oxysporum* f. sp. *lycopersici* and *F. oxysporum* f. sp. *lini* greatly differed in their

ability to convert **V** into **VI**. The latter strain produced **VI** only very slowly, with pratensein being the sole metabolite even after longer incubation periods. *F. oxysporum* f. sp. *lycopersici*, however, converted biochanin A almost quantitatively into **VI** within approximately 12 h. Using [ring A-^{14}C]biochanin A as substrate it could be shown that 97% of **V** had been dissimilated with 89% of the applied radioactivity being found in pratensein. Soon after maximum accumulation of **VI** has occurred some 60% of this product are subsequently degraded as measured by disappearance of product and a high rate of $^{14}CO_2$ formation. Various catabolites of **VI** observed in these studies remain to be elucidated.

Experiments with formononetin

Numerous strains of *Fusarium* fungi have recently been shown to be potent degraders of 5-hydroxyisoflavones such as **V** [6, 7, 10]. Much less is known about the fungal metabolism of 5-deoxyisoflavones (review 13) which, in general, appear to be metabolized by *Fusarium* fungi much less rapidly than the 5-hydroxyderivatives [14].

Studies with Fusarium avenaceum

In standard incubation experiments with *Fusarium avenaceum* formononetin (**I**) was shown to be quantitatively converted to a new compound (TLC, S_4, $R_f = 0.42$, orange colour with diazotized 4-nitroaniline) within some 22 h. [Methyl-^{14}C]formononetin was transformed into this compound without any loss of radioactivity indicating that the new compound did not originate from an O-demethylation reaction of **I**. Preparative isolation of this compound from an ether extract of the incubation medium was carried out by two successive chromatographic steps on a LOBAR-RP 8 column with 40% acetonitril and 38% acetonitril, respectively, as solvents. The mass spectrum *m/e* 284 (M^+), 269, 241, 213, 184, 148, 137, 133, 108, 103, 80 and 77 showed that a hydroxyl group had been introduced into ring B of **I**. Thus, both the molecular weight and the isoflavone-characteristic *retro*-Diels-Alder fragment of ring B (*m/e* 148) of the new compound were 16 mass units higher than the values obtained with **I** (MW 268; *m/e* 132). The UV-spectra (λ_{max} (methanol) 248, 258 (sh), 290, 308 (sh) nm; λ_{max} (methanol with Na-acetate) 256, 290, (sh), 305 (sh), 330 (sh) nm; λ_{max} (methanol with Na-methylate) 255, 330 nm did not show a maximum at 240 nm which has been reported for 2′-hydroxyformononetin [15]. The new compound was finally characterized as 7,3′-dihydroxy-4′-methoxyisoflavone (calycosin, **IV**) by its NMR-spectrum ((CD$_3$OD) δ (ppm): 8.15 (s, 1H, H-2), 8.05 (d, 1H, H-5), 6.9 – 7.08 (m, 5H, H-2, H-5′, H-6′, H-6, H-8) and 3.9 (s, 3H, 4′-OCH$_3$)) and HPLC-chromatographic comparison with an authentic sample of calycosin [16].

Other metabolites of **I** or degradation products of **IV** in incubations with mycelial preparations of *F. avenaceum* have not been observed.

Studies with Fusarium proliferatum

Metabolism of formononetin by mycelial preparations of *Fusarium proliferatum* greatly depended on the applied growth medium.

When grown on SM-medium [7] and incubated with **I** (10^{-4} M) this fungus converted the substrate rather slowly. When incubated with [U-^{14}C]formononetin small amounts of a metabolite (TLC, S_4, $R_f = 0.34$) could be observed after an induction period of 2 – 5 h. When isolated on a preparative scale (LOBAR-RP 8 column chromatography with 43% acetonitril) this material was unequivocally identified as 7,4′-dihydroxyisoflavone (daidzein (**III**)) by its UV spectrum (λ_{max} (MeOH) 237 (sh), 248, 255 (sh), 303 (sh) nm), its mass spectrum (*m/e* 254 (M^+), 225, 197, 137, 118, 108, 95, 90, 89 and 80) and by HPLC cochromatography with an authentic sample. Similar studies with mycelial preparations of *F. proliferatum* after growth on FM-medium (17) showed (TLC, S_4) that the O-demethylation of **I** yielding **III** occurred to a very low extent only. As main reaction the formation of a product (TLC, S_4, $R_f = 0.7$) was now observed which seemed to be formed without any lag-phase. Using chromatography (HPLC, TLC) and spectroscopic techniques (UV, mass spectrum) this compound was identified as 7-O-methylformononetin (**II**) which was available from various previous studies [18]. Subsequent degradation of **II** by *F. proliferatum* to formononetin and daidzein occurred only slowly when mycelial preparations after growth on FM-medium were used. This sequence was investigated by HPLC chromatography of aliquots of the nutrient medium of standard assays. Other, still unidentified catabolites of **II** could also be detected in small amounts in these studies.

The degradative sequence 7-O-methylformononetin → formononetin → daidzein could, how-

ever, readily be measured in mycelial preparations of *F. proliferatum* after growth on SM medium. After short incubation periods **II** (10^{-4} M) was readily O-demethylated first in position 7 yielding **I** and then in position 4′ yielding **III**. The intermediate formation of 7-O-methyl-daidzein (**III** with $R_1 = OCH_3$) could not be observed in these studies.

Discussion

When compared to 5-hydroxyisoflavones such as biochanin A the 5-deoxyisoflavones formononetin (**I**) and daidzein (**III**) are much less rapidly and efficiently metabolized by *Fusarium* fungi. Accumulation of catabolites of **III** or other fragments of such an isoflavone skeleton have so far not been observed though numerous *Fusarium* strains have now been investigated [4, 14]. The actual reason for this decisive difference remains to be elucidated. It can, however, be postulated that microorganisms such as several *Fusarium* strains [6], *Aspergillus niger* [19] *Streptomyces* [20] and *Pseudomonas* species [21] readily hydroxilate isoflavones and flavonoids in position 8 but not in position 5. Thus, conversion of **I** into **V** appears to be impossible. Similarly, hydroxylation in position 3′ seems to be possible as shown by our results with both formononetin and biochanin A as well as by previous reports from Japanese laboratories [20, 21]. Hydroxylation adjacent to an existing methoxyl group such as observed during formation of **IV** and **VI** has only rarely been found because in most cases the introduction of a second oxygen function occurs next to a hydroxyl group. The conversion of formononetin into calycosin has been postulated to occur in various plants as part of the biosynthesis of the phytoalexins pisatin and maackiain [16, 22].

In case of *F. oxysporum* f. sp. *lycopersici* (producing **VI**) and *F. avenaceum* (forming **IV**) hydroxylation in position 3′ proceeded so efficiently that this reaction could well be used for the preparative production of pratensein and calycosin, respectively, from labelled **V** and **I**. The extreme accumulation of these 3′-hydroxyisoflavones indicates that this type of hydroxylation is not part of a normal catabolic sequence but rather a side reaction which adds to the metabolic diversity of the *Fusarium* fungi.

Of considerable interest is the observed change in the pattern of formononetin metabolism upon changes of the growth medium of *Fusarium proliferatum*. Growth on a glucose-caseine-yeast extract medium favoured expression of O-demethylating activity in position 4′ of **I** whereas growth on a special "Fusarium-medium" [17] led to a constitutive O-methyltransferase. This result is very similar to studies by Thomas and associates [23] who have determined that species of the yeast *Candida* contain O-methyltransferases for phenolic substrates. Other reports on microorganisms with the ability to methylate phenolic and isoflavone substrates have appeared [19, 20].

The sequence of O-demethylation reactions observed with **II** and *Fusarium proliferatum* (**II** → **I** → **III**) again documents the pronounced ability of this fungus for the removal of O-methylgroups such as previously shown to occur with other related phenolic substrates [24] and other strains [4, 25].

In summary, the reactions observed with isoflavones (3′-hydroxylation, O-methylation, O-demethylation) in these studies further document the great metabolic diversity of *Fusarium* fungi during the metabolism of phenolic plant constituents.

Acknowledgements

Dr. P. M. Dewick, Nottingham, has kindly provided a sample of authentic calycosin. Financial support by Deutsche Forschungsgemeinschaft and Fonds der Chemischen Industrie is gratefully acknowledged.

[1] D. Gross, Fortsch. Chem. Org. Naturstoffe **34,** 187 (1977).
[2] H. D. VanEtten and S. G. Pueppke, Biochemical Aspects of Plant-Parasite Relationships (J. Friend and D. R. Threefall, eds.), chapter 13, Academic Press, London, New York, San Francisco
[3] H. D. VanEtten, P. S. Matthews, K. J. Tegtmeier, M. F. Dietert, and J. I. Stein, Physiol. Plant Pathol. **16,** 257 (1980).
[4] W. Barz, R. Schlepphorst, and J. Laimer, Phytochemistry **15,** 87 (1976).
[5] U. Lappe and W. Barz, Z. Naturforsch. **33 c,** 301 (1978).
[6] U. Willeke and W. Barz, Z. Naturforsch. 1982, in press.
[7] U. Willeke and W. Barz, Arch. Microbiol., 1982, submitted.
[8] M. Naim, B. Gestetner, S. Zilkah, Y. Birk, and A. Bondi, J. Agric. Food Chem. **22,** 806 (1974).
[9] J. L. Ingham, Z. Naturforsch. **32 c,** 449 (1977).
[10] U. Willeke, Doctoral thesis, University of Münster (1981).
[11] T. J. Mabry, K. R. Markham, and M. B. Thomas, The Systematic Identification of Flavonoids, Springer-Verlag, Berlin 1970.
[12] W. Hösel and W. Barz, Phytochemistry **9,** 2053 (1970).
[13] W. Barz, U. Willeke, and K.-M. Weltring, Ann. Phytopathol. **12,** 435 (1980).
[14] K.-M. Weltring, Diplomarbeit, University of Münster (1980).
[15] P. M. Dewick, Phytochemistry **16,** 93 (1977).
[16] P. M. Dewick and D. Ward, Phytochemistry **17,** 1751 (1978).
[17] D. Brewer, G. P. Arsenault, J. L. C. Wright, and L. C. Vining, J. Antibiotics **26,** 778 (1973).
[18] W. Barz and H. Grisebach, Z. Naturforsch. **22 b,** 627 (1967).
[19] H. Umezawa, H. Tobe, N. Shibamoto, F. Nakamura, K. Nakamura, M. Matsuzaki, and T. Takeuchi, J. Antibiotics **XXVIII,** 947 (1975).
[20] H. Chimura, T. Sawa, Y. Kumada, H. Naganawa, M. Matsuzaki, T. Takita, M. Hamada, T. Takeuchi, and H. Umezawa, J. Antibiotics **XXVIII,** 620 (1975).
[21] E. Schultz, I. E. Engle, and J. M. Wood, Biochemistry **13,** 1768 (1974).
[22] P. M. Dewick and S. W. Banks, Planta medica **39,** 287 (1980).
[23] J. Veser, P. Geywitz, and H. Thomas, Z. Naturforsch. **34 c,** 709 (1979).
[24] K.-M. Weltring and W. Barz, Z. Naturforsch. **35 c,** 399 (1980).
[25] K.-M. Weltring, W. Barz, and P. M. Dewick, Arch. Microbiol. **130,** 381 (1981).
[26] W. Barz, Ch. Adamek, and J. Berlin, Phytochemistry **9,** 1735 (1970).