Degradation of the Isoflavone Biochanin A by *Fusarium javanicum*

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After a lag-phase of 1 h a mycelial suspension of *Fusarium javanicum* completely degraded 5,7-dihydroxy-4′-methoxyisoflavone (biochanin A) to a series of transiently accumulating catabolites. The compounds were elucidated by spectroscopic techniques and are arranged in the first known degradative pathway for an isoflavone: biochanin A → dihydrobiochanin A → 3-(*p*-methoxyphenyl)-4,6-diketo-5,6-dihydro-4H-pyran → *p*-methoxyphenylacetic acid → *p*-hydroxyphenylacetic acid → 3,4-dihydroxyphenylacetic acid → CO_2. The carbon atoms of biochanin A not accounted for by this sequence are discussed in relation to microbial flavonoid pathways.

Introduction

Numerous flavonoids have been investigated for their degradation by fungi and bacteria. In these studies several pathways have been elucidated which demonstrate the conversion of the flavonoid carbon skeleton into aliphatic compounds (review [1]). The isomeric isoflavonoid plant constituents have much less frequently been investigated for their microbial degradation (review [2]). Isoflavonoids are of considerable biological interest due to their fungistatic or fungitoxic properties which qualify several isoflavans, isoflavones and pterocarpans to function as either preinfectional inhibitors [3] or phytoalexins [4].

Despite of these observations no case has so far been reported which shows how an isoflavone type of skeleton is cleaved into smaller compounds suitable for further oxidation by the reactions of primary metabolism.

In continuation of our studies on the degradation of isoflavones and isoflavonoid phytoalexins by fungi of the genus *Fusarium* [5, 6] we now report on the disintegration of the isoflavone biochanin A (5,7-dihydroxy-4′-methoxy-isoflavone (**I**, Fig. 4)) by *F. javanicum*. The main feature of this process is the formation of *p*-hydroxyphenylacetic acid (**V**, Fig. 4)

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derived from the side chain phenylring and carbon atoms 2 and 3 of the isoflavone.

Materials and Methods

Microorganism and culture conditions

Fusarium javanicum Koord (obtained from Centralbureau voor Schimmelcultures at Baarn/Holland; catalogue number CBS 203.32) was maintained on Czapek-Dox agar medium in Petri dishes at 4 °C and transferred to new medium every 5–6 weeks. After transfer the dishes were incubated for 1 week at 25 °C. For degradation experiments the organism was grown in a glucose-caseine-yeast extract medium. For 1 liter of this medium 50 g glucose, 8 g caseinehydrolysate and 500 mg yeast extract were dissolved in 500 ml dest. water. A separate 500 ml batch of potassium phosphate buffer (0.05 M, pH 7.5) contained 10 ml salts A and 3 ml salts B. The two solutions were separately autoclaved (121 °C, 20 min) and combined after cooling to room temperature. The stock solutions of salts A (20 g $MgSO_4 \times 7H_2O$, 2 g $CaCl_2 \times 2H_2O$, 2 g $FeSO_4 \times 7H_2O$ in 1 *l* dest. water) and salts B (250 mg $MnSO_4 \times 4H_2O$, 250 mg $Na_2MoO_4 \times 2H_2O$ in 500 ml dest. water) are kept a 4 °C. Flasks (200 ml Erlenmeyer with 100 ml medium) were shaken at 30 °C and 160 strokes per min for 1 to 5 days depending on the type of experiment.

Degradation experiments

Mycelia were collected by filtration, washed three times with potassium phosphate buffer (0.05 M, pH 7.5) and 3 g were inoculated into 100 ml of the

same buffer (standard assay). Biochanin A and other substrates were investigated at 10^{-4} M; substrates were predissolved in 0.5 ml 2-methoxyethanol prior to transfer into the buffer. These flasks were also incubated at 30 °C and 160 strokes per min.

Isolation of metabolic products

The degradation process was monitored by UV-visible spectroscopy and thin layer chromatography (TLC) of the culture filtrates. Samples (6 ml) were drawn at least every 2 h and mycelium was removed by filtration. For extraction of catabolites the pH of the samples was lowered to pH 1 (5 N H_2SO_4) and products were extracted with diethylether. The organic layer was recovered, evaporated to dryness and the residues were taken for TLC analysis as a methanolic solution. Compounds were detected on TLC plates under a short-wave (366/254 nm) UV light and with spray reagents (fast blue salt B in citrate buffer (0.2 M, pH 4), diazotized *p*-nitro-aniline, 2,4-dinitrophenylhydrazine-HCl, vanillin-H_2SO_4).

For preparative isolation of catabolites incubations (1–6 l of standard medium with 100 ml volume in 200 ml Erlenmeyer flasks) were carried to maximum accumulation of the catabolite under investigation and worked up as described above. Compounds were purified by preparative TLC or by column chromatography. Silica gel (200 mesh, Merck) with the solvent ethylacetate/CHCl$_3$ (2:1) was used for the purification of methylated compounds and silica gel for dry column chromatography (63–200 µm, Woelm) with either ethylacetate/CHCl$_3$ (2:1) or CHCl$_3$/iso-prop-OH (10:1) well separated methylated from non-methylated isoflavones. Phenylacetic acids and other methylated and non-methylated phenols were chromatographed on Sephadex LH 20 (Pharmacia) with methanol as solvent. Neutral Al$_2$O$_3$ in sodium acetate buffer (0.5 M, pH 6.1) when eluted with the same buffer and subsequently with 10% acetic acid was used to separate 3,4- and 2,5-dihydroxyphenylacetic acid.

All substrates were finally crystallized from methanol or sublimated at the lowest temperature possible.

Chromatography

TLC was conducted on silica gel F$_{254}$ plates, silica gel G plates, cellulose F$_{254}$ plates or polyamide F$_{254}$ sheets, all obtained from Merck.

The following solvent systems (V/V) were used:

S$_1$: benzene:ethylacetate:methanol:pet-ether, 6:4:1:3;
S$_2$: benzene:ethylacetate:HCOOH, 18:1:1;
S$_3$: toluene:ethylformate:HCOOH, 5:4:1;
S$_4$: diethylether:petrol-ether, 7:3;
S$_5$: benzene:methanol, 8:2;
S$_6$: methanol;
S$_7$: acetone;
S$_8$: CHCl$_3$:methanol:butanone-2, 12:2:1;
S$_9$: CHCl$_3$:iso-propanol, 10:1;
S$_{10}$: methanol:water, 5:3;
S$_{11}$: methanol:water, 2:3;
S$_{12}$: CHCl$_3$:methanol:17% NH$_3$, 4:4:2;
S$_{13}$: CHCl$_3$:methanol, 10:1;
S$_{14}$: toluene:methanol:butanone-2, 7:3:1;
S$_{15}$: CHCl$_3$:acetic acid, 9:1;
S$_{16}$: CHCl$_3$:acetic acid, 7:3;
S$_{17}$: toluene:butanone-2:HCOOH, 5:4:1;
S$_{18}$: *n*-butanol:acetic acid:water, 40:19:11 and
S$_{19}$: diethylether:CHCl$_3$, 2:1.

Methylation

Product (0.05–0.4 mmol) was dissolved in 60 ml aqueous methanol and excess CH$_2$N$_2$-solution (5 g N-nitrosomethyl urea, 14 g KOH, 20 ml dest. water) was added. After 3 days at 4 °C the yellow colour was destroyed with acetic acid, solvent removed by evaporation and the residue dissolved in acetone for TLC analysis.

Compounds

Biochanin A and all phenylacetic acids were obtained from EGA-Chemie, Germany. Previously published methods were used to synthesize, from biochanin A, genistein [7] 5,7,4′-trimethoxyisoflavone [8] and dihydrobiochanin A [9]. [4-^{14}C]biochanin A (spec. radioact. 6×10^5 dpm/mmol), [2-^{14}C]-biochanin A (spec. radioact. 4.86 µCi/mmol) and [U-^{3}H]biochanin A (spec. radioact. 42.3 mCi/mmol) were all from previous studies [8, 10, 11].

Analytical methods

A Leitz-Unicam SP 8000 spectrophotometer was used to obtain the UV-visible spectra with abs. methanol as solvent. Absorption spectra of phenolic compounds were also assayed by addition of diagnostic compounds [12]. ^{1}H-NMR spectra were taken

with either a WH 90-NMR spectrometer (90 MHz) or a Ha 100-NMR spectrometer (100 MHz) with tetramethylsilane as internal standard. ^{13}C-NMR spectra were recorded with a WH 90-NMR machine. Deuterated solvents (Sigma) were used throughout all recordings. Mass spectra were measured with either a Hitachi-Perkin-Elmer RMV-6 D (70 eV) or a Varian MAT 44 S with MAT 188 S mass spectrometer. IR-spectra (KBr tablets) were recorded with a Perkin-Elmer 457 infrared spectrometer. Radioactive compounds were located on TLC sheets by scanning with a Berthold LB 2723 scanner. For liquid scintillation a betascint GF 5000 counter (Berthold-Frieseke) was used.

In radiorespiratory studies $^{14}CO_2$ was absorbed in ethanolamine/2-methoxyethanol (1:2) and the mixture was assayed for radioactivity with a toluene scintillation cocktail (5 g PPO in 1 l toluene).

Structural elucidation of catabolites

The spectroscopic data which were essential for the determination of structures of isolated catabolites are as follows.

Dihydrobiochanin A (II)

UV: λ_{max} (MeOH) 291, 326 (sh) nm; λ_{max} (NaOAc) 251 (sh) 327 nm; λ_{max} (NaOMe) 245 (sh), 327.5 nm; λ_{max} (AlCl$_3$) 314, 385 (sh) nm; λ_{max} (NaOAc + H$_3$BO$_3$) 292, 325 (sh) nm. MS (m/e): 286 (M$^+$), 153, 152, 134, 124, 119, 91, 77, 69, 65, 51; the ions with m/e 152 and 134 are the isoflavonoid characteristic fragments of a retro-Diels-Alder pattern of fragmentation.

^{1}H-NMR (CD$_3$OD) δ (ppm): 3.79 (s, 3H, 4'-OCH$_3$) 3.92 (t, 1H, H-3, $J_{2,3}$ = 7 Hz), 4.53 (d, 2H, 2x H-2, $J_{2,3}$ = 7 Hz), 5.92 (s, 2H, H-6, H-8), 6.91 (d, 2H, H-3', H-5', $J_{2',3'}$ = 10 Hz), 7.23 (d, 2H, H-2', H-6', $J_{5',6'}$ = 10 Hz). ^{13}C-NMR (acetone-d$_6$) δ(ppm): 50.952 (C-3), 55.534 (4'-OCH$_3$), 72.074 (C-2), 95.633 (C-8), 96.965 (C-6), 114.935 (C-3', C-5'), 128.615 (C-1'), 130.630 (C-2', C-6'), 160.201 (C-4'); the signals of the solvent were at 30.0 (septett) and 206.14 (s).

Dihydrobiochanin A-7-O-methylether

UV: λ_{max} (MeOH) 289, 327 (sh) nm. ^{1}H-NMR (acetone-d$_6$) δ (ppm): 3.76 (s, 3H, 4'-OCH$_3$), 3.83 (s, 3H, 7-OCH$_3$), 3.91 (t, 1H, H-3), 4.62 (d, 2H, 2x H-2, $J_{2,3}$ = 6.5 Hz), 6.02 (s, 2H, H-6, H-8), 6.88 (d,

2H, H-3', H-5', $J_{2',3'}$ = 8.8 Hz), 725 (d, 2H, H-2', H-6', $J_{5',6'}$ = 8.8 Hz), 12.30 (s, 1H, 5-OH).

Dihydrobiochanin A-5,7-dimethylether

UV: λ_{max} (MeOH) 288, 312 (sh) nm; no reaction with diagnostic reagents. IR (KBR) v (cm^{-1}): 2990, 2840, 1678 (C=O), 1605, 1575, 1518, 1470, 1460, 1425, 1385, 1275, 1228, 1212, 1162, 1122, 1025, 970, 947, 857, 827, 811. MS (m/e): 314 (M$^+$), 181, 180, 152 (180-CO), 137 (180-CO, −CH$_3$), 109 (180-CO, −CH$_3$, −CO), 81 (180-CO, −CH$_3$, −CO, −CO), 134, 119 (134-CH$_3$), 91 (134-CH$_3$, −CO), 77, 65, 51. Fragments m/e 180 and 134 represent the retro-Diels-Alder fragmentation typical for isoflavonoids. ^{1}H-NMR (CDCl$_3$) δ (ppm): 3.78 (s, 3H, 4'-OCH$_3$), 3.83 (s, 3H, 5-OCH$_3$), 3.86 (s, 3H, 7-OCH$_3$), 3.91 (t, 1H, H-3), 4.07 (d, 2H, 2x H-2, $J_{2,3}$ = 6.2 Hz), 6.08 (s, 2H, H-6, H-8), 6.84 (d, 2H, H-3', H-5', $J_{2',3'}$ = 8.2 Hz), 7.22 (d, 2H, H-2', H-6', $J_{5',6'}$ = 8.2 Hz).

3-(p-Methoxyphenyl)-6-hydroxy-γ-pyron (III)

UV: λ_{max} (MeOH) 212, 242.5, 273 (sh) nm; λ_{max} (NaOMeOH) 212, 242.5, 275 (sh) nm; λ_{max} (AlCl$_3$) 239, 315, 360 nm; λ_{max} (AlCl$_3$ + HCl) 238, 313, 362 nm. IR (KBr) v (cm^{-1}): 3080, 3010 (arom. CH), 2835 (arom. −OCH$_3$), 1728 and 1645 (>C=O), 1625, 1585, 1528 and 1505 (aryl), 1369, 1325, 1320, 1285, 1265, 1238, 1198, 1145, 1125, 1043, 1003, 890, 845. MS (m/e): 218 (29%) (M$^+$), 176 (86), 148 (14), (176-CO), 120 (100), (176-CO, −CO), 91 (38), 77 (30), 65 (19) (arylether), 42 (11). The fragments m/e 176 and 42 represent characteristic signals of a retro-Diels-Alder fragmentation process. ^{1}H-NMR (MeOH-d$_4$) δ (ppm): 3.71 (s, 3H, 4'-OCH$_3$), 6.83 (d, 2H, H-3' H-5', $J_{2',3'}$ = 9.1 Hz), 7.24 (d, 2H, H-2', H-6', $J_{5',6'}$ = 9.1 Hz), 7.47 (s, 1H, H-2). The hydroxyl group at C-6 did not show up and the proton at C-5 has possibly exchanged with HDO.

3-(p-Methoxyphenyl)-4-methoxy-α-pyrone

UV: λ_{max} (MeOH) 209, 247, 273 (sh); λ_{max} (NaOMe) 223, 247, 273 (sh). No bathochromic shifts were observed with AlCl$_3$, AlCl$_3$ + HCl, NaOAc and NaOAc + H$_3$BO$_3$. IR (KBr) v (cm^{-1}): 3095, 3005, 2840 (arom. −OCH$_3$), 1750 and 1645 (>C=O), 1610, 1515, 1450 and 1405 (aryl), 1285, 1260, 1220, 1185, 1140, 1035, 990, 890, 860, 820.

MS (m/e): 232 (100%) (M$^+$), 233 (16) (M$^+$ + 1), 231 (15.5) (M$^+$ − 1), 217 (10) (232-CH$_3$), 204 (21) (232-CO), 189 (60) (232-CO, −CH$_3$), 175 (29), 161 (24) (189-CO), 145 (16) (189-CO$_2$), 135 (31), 133 (29) (189-CO, −CO), 121 (7), 107 (7), 91 (9), 89 (18) (133-CO$_2$), 77 (16), 69 (16), 65 (12), 44 (16). ^{1}H-NMR (MeOH-d$_4$) δ (ppm): 3.84 (s, 3H, 4′-OCH$_3$), 3.89 (s, 3H, 4-OCH$_3$), 5.76 (s, 1H, H-5), 6.94 (d, 2H, H-3′, H-5′, $J_{2',3'}$ = 9.1 Hz), 7.29 (d, 2H, H-2′, H-6′, $J_{5',6'}$ = 9.1 Hz), 7.57 (s, 1H, H-2). ^{1}H-NMR (CDCl$_3$) δ (ppm): 3.84 (s, 6H, 4′-OCH$_3$, 4-OCH$_3$), 5.64 (s, 1H, H-5), 6.92 (d, 2H, H-3′, $J_{2',3'}$ = 9.1 Hz), 7.25 (d, 2H, H-2′, H-6′, $J_{5',6'}$ = 9.1 Hz), 7.36 (s, 1H, H-2).

p-Methoxyphenylacetic acid (IV)

UV: λ_{max} (MeOH) 268 (sh), 276, 283; MS (m/e): 166 (100%) (M$^+$), 149 (3.9) (166-OH), 122 (35), 121 (92) (166-CO$_2$H), 91 (13), 78 (25), 77 (26), 65 (4.7), 51 (7). ^{1}H-NMR (CDCl$_3$) δ (ppm): 3.57 (s, 2H, CH$_2$), 3.78 (s, 3H, 4-OCH$_3$), 6.86 (d, 2H, H-3, H-5, $J_{3,2}$ = 8.8 Hz), 7.18 (d, 2H, H-2, H-5, $J_{2,3}$ = 8.8 Hz).

p-Methoxyphenylacetic acid methylester

UV: λ_{max} (MeOH) 267 (sh), 275, 282 nm. ^{1}H-NMR (CDCl$_3$) δ (ppm): 3.56 (s, 2H, CH$_2$), 3.68 (s, 3H, −OCH$_3$), 3.79 (s, 3H, 4-OCH$_3$) 6.85 (d, 2H, H-3, H-5, $J_{3,2}$ = 9 Hz), 7.20 (d, 2H, H-2, H-6, $J_{2,3}$ = 9 Hz). MS (m/e): 180 (34%) (M$^+$), 149 (0.5) (180-OCH$_3$), 122 (6.4), 121 (100) (180-CO$_2$CH$_3$), 91 (7.5), 77 (8.6), 65 (2.2), 28 (3.2).

p-Hydroxyphenylacetic acid (V)

UV: λ_{max} (MeOH) 228, 277, 282 (sh) nm. IR (KBr) v (cm^{-1}): 3260 (−OH), 3060 (arom. −CH), 1710 (>C=O), 1610, 1600, 1515 and 1445 (aryl), 1405, 1365, 1350, 1310, 1305, 1230, 1210, 1190, 1170, 920, 830, 820 and 785. ^{1}H-NMR (methanol-d$_4$) δ (ppm): 3.45 (s, 2H, −CH$_2$), 6.71 (d, 2H, H-3, H-5, $J_{5,6}$ = 8.8 Hz), 7.07 (d, 2H, H-2, H-6, $J_{2,3}$ = 8.8 Hz).
^{13}C-NMR (methanol-d$_4$) δ (ppm): 41.057 (−CH$_2$), 116.105 (C-3), 116.235 (C-5), 126.796 (C-1), 131.248 (C-2), 131.345 (C-6), 157.439 (C-4) and 176.188 (−CO$_2$H).

3,4-Dihydroxyphenylacetic acid (VI)

UV: λ_{max} (MeOH) 224, 282 nm. λ_{max} (MeOH + AlCl$_3$) 220, 250 (sh), 291 nm. λ_{max} (MeOH + NaOAc + H$_3$BO$_3$) 211, 234 (sh), 288 nm. IR (KBR) v (cm^{-1}): 3485, 3355, (−OH), 1685 (>C=O), 1615, 1520 and 1470 (aryl), 1420, 1380, 1280, 1260, 1190, 1120, 805, 775 and 625. ^{1}H-NMR (methanol-d$_4$) δ (ppm): 3.40 (s, 2H, −CH$_2$), 6.60 (m, 3H, H-2, H-5, H-6). ^{13}C-NMR (methanol-d$_4$) δ (ppm): 41.431 (−CH$_2$), 116.365 (C-2), 117.582 (C-6), 121.759 (C-5), 127.478 (C-1), 145.415 (C-4), 146.325 (C-3) and 176.256 (−CO$_2$H). MS (m/e): 168 (28%) (M$^+$), 123 (100) (168-CO$_2$H), 105 (7) (168-CO$_2$H, −H$_2$O), 77 (32), 65 (9), 51 (40) and 43 (15).

Results

Choice of organism

Our previous studies on isoflavone degradation by *Fusarium* fungi [5] had demonstrated that several *Fusarium* species readily catabolized biochanin A (I, see Fig. 4). Except for genistein (5,7,4′-trihydroxy-isoflavone) and orobol (5,7,3′-4′-tetrahydroxy-isoflavone) no further catabolites of biochanin A had been isolated in these studies. A more detailed investigation of biochanin A metabolism by our collection of *Fusarium* fungi [13]. revealed that a strain of *F. javanicum* could be used for determination of a degradative pathway of biochanin A catabolism because a sequential accumulation of several metabolites (TLC with SG and solvents S$_1$, S$_4$) was shown to occur. Furthermore, in contrast to several other strains of *Fusarium* this fungis did not produce intensively coloured products which normally hamper the determination and isolation of biochanin A catabolites. The main products have now been characterized, though several minor degradation products await structural elucidation.

Biochanin A degradation products

Dihydrobiochanin A (II)

Incubation of *F. javonicum* mycelium with biochanin A led to the formation of a new compound because the isoflavone absorption (262 nm, Fig. 1) was shifted to 327 nm. Maximum accumulation of the new compound was observed after appr. 3 h with the first hour of incubation being a lagphase. Within an additional 4 to 6 h this new compound had again been consumed by the fungus. Quantitative measurements of the conversion of I into II using [2-^{14}C]- or [4-^{14}C]biochanin A showed that up
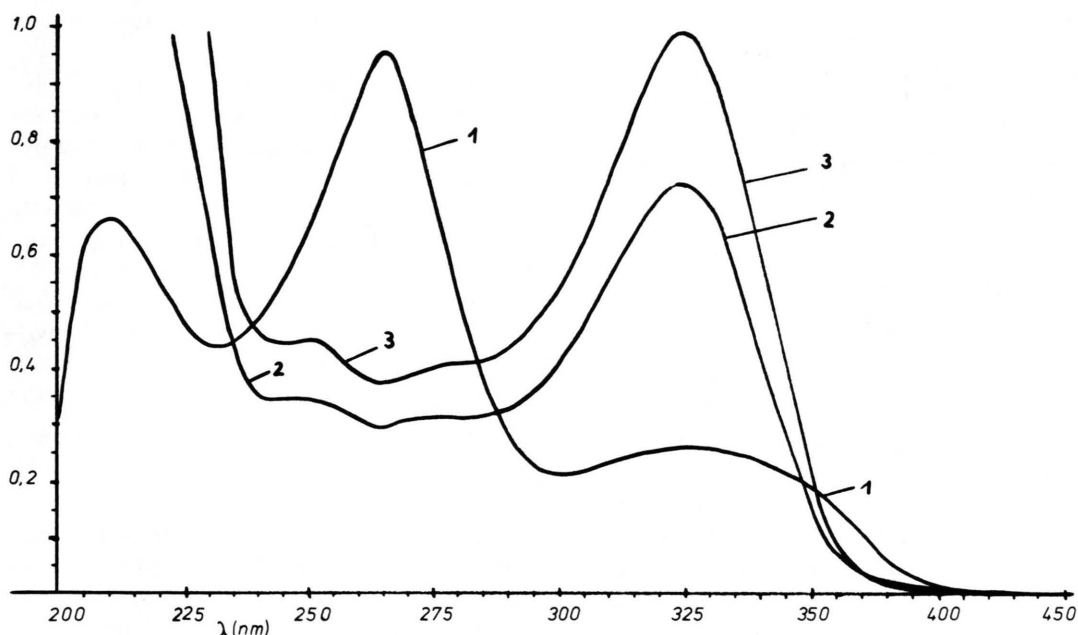
Fig. 1. Changes in the UV-visible absorption of culture filtrates containing biochanin A during incubation with *Fusarium javanicum*. Curve 1 shows the absorption spectrum of biochanin A and curves 2 and 3 the spectrum after 2 and 3 h of incubation. The 327 nm maximum is due to a strong bathochromic shift in the absorption spectrum of dihydrobiochanin A at higher pH values (7.5).
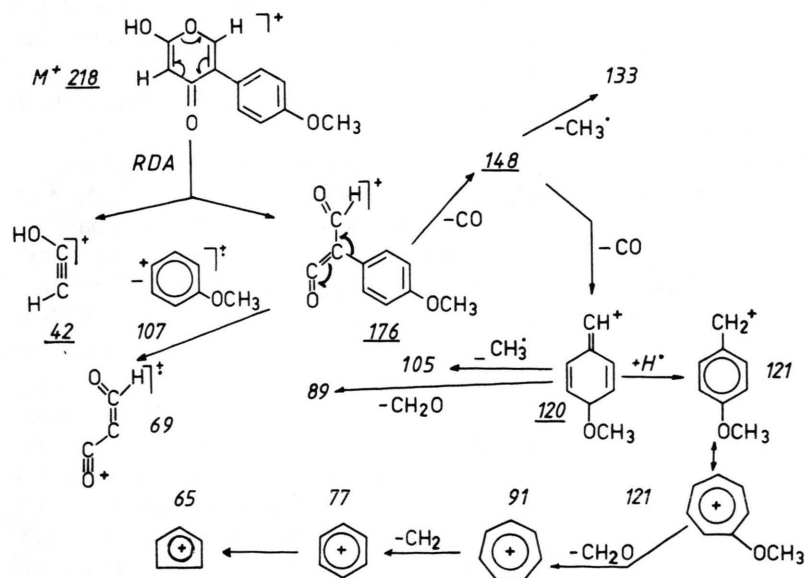

Fig. 2. Mass-spectroscopic fragmentation pattern of the second catabolite (**III**) of biochanin A by *F. javanicum*. The spectrum suggests the structure 3-(*p*-methoxyphenyl)-6-hydroxy-γ-pyrone.
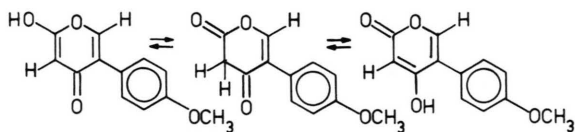
Fig. 3. Proposed keto-enol-tautomerism of catabolite **III** of biochanin A produced by *F. javanicum*.

to 78% of the applied radioactivity could be isolated as dihydrobiochanin A. Preincubation of cells with biochanin A for 6 h led to a mycelium preparation which quantitatively converted **I** to **II** without any lag-phase within 45 min.

Purified **II** (TLC, PA, S_4, S_9; column chromatography) obtained from large scale incubation experiments was shown to possess the same phloroglucinol type of substitution pattern in ring A (purple colour with fast blue salt B, bathochromic shift in the UV spectrum with $AlCl_3$ and sodium acetate (Mabry *et al.* [12], NMR-signals) and the *p*-methoxy-phenyl ring B (NMR) as **I**. The isoflavanone structure became evident from the mass spectrum (M^+ *m/e* 286) and the characteristic ^{1}H-NMR-signals for the protons at C-2 and C-3. Methylation of isolated **II** with CH_2N_2 and separation of the two main products (TLC, SG, SH, R_f 0.39 and 0.63) led to 5,7,4′-trimethoxyisoflavanone and 5-hydroxy-7,4′-dimethoxy-isoflavanone. The fungal dihydrobiochanin A was finally shown to be identical (UV, IR, ^{1}H-NMR and ^{13}C-NMR, methylderivatives, TLC) with synthetic **II**.

3-(p-Methoxyphenyl)-4,6-diketo-5,6-dihydro-4H-pyran (III) (or tautomers)

In standard incubation experiments the formation of **III** (TLC, SG, S_4, R_f 0.23) could first be observed after appr. 6 h with maximum accumulation after 12–15 h. Within altogether 24 h the compound had totally been consumed again. An approximately 25% yield of **III** as based on **I** could be obtained. [^{14}C] **III** was shown to be formed after incubation of *F. javanicum* with both [2-^{14}C]- and [4-^{14}C]biochanin A indicating that the heterocyclic ring of **I** was retained in this compound. The *p*-methoxyphenyl-ring of **I** was also shown to be present in **III** as indicated by the NMR and MS spectra. According to Budzikiewicz *et al.* [14, 15] the MS spectrum of **III** is best interpreted by the fragmentation scheme shown in Fig. 2. Therefore, compound **III** could

be called 3-(*p*-methoxyphenyl)-6-hydroxy-γ-pyrone (Fig. 3, left formula).

However, when **III** was methylated with CH_2N_2 the spectroscopic data (see experimental section) showed that 3-(*p*-methoxyphenyl)-4-methoxy-α-pyrone (methyl ether derivative of the right hand formula in Fig. 3) had been isolated. The catabolite **III** of biochanin A must thus be assumed to undergo a keto-enol-tautomerism as shown in Fig. 3. In the catabolic sequence of biochanin A degradation (see Fig. 4) **III** will be written as 3-(*p*-methoxyphenyl)-4,6-diketo-5,6-dihydro-4H-pyran (middle structure in Fig. 3); this will further be explained in the discussion.

p-Methoxyphenylacetic acid (IV)

This acid was isolated in comparatively small amounts (app. 4% of **I**) (TLC, SGF, S_{13}) from standard incubations after 16–20 h. The isolated acid itself and the methyl ester prepared with CH_2N_2 were identical in all chromatographic and spectroscopic with synthetic material.

p-Hydroxyphenylacetic acid (V)

This acid (TLC, SGF, S_9, R_f: 0.08, yellow colour with fast blue salt B) can first be detected in the incubation medium after 20–24 h of incubation with maximum accumulation (app. 25%) after 30–40 h. While all catabolites of **I** were exclusively isolated from the nutrient medium, some 6–10% of **V** could also be extracted from the fungal mycelium. Structural elucidation was greatly facilitated because the spectroscopic data of the acid and of its permethyl derivative were essentially identical with those of synthetic compounds.

In order to determine the origin of the side chain moiety of **V** from either carbon atoms 2 and 3 or carbon atoms 3 and 4 of biochanin A, comparative tracer studies with labelled **I** were conducted. Parallel incubations of *F. javanicum* with [2-^{14}C]biochanin A (10 μmol; 1.06×10^4 dpm) and [4-^{14}C]biochanin A (10 μmol; 6×10^4 dpm) were carried to the stage of maximum production of *p*-hydroxyphenylacetic acid. While the sample of **V** (0.7 μmol) derived from [4-^{14}C]biochanin A had only negligible radioactivity (40 dpm, 0.06%), the preparation of **V** (0.8 μmol) isolated from the incubation of [2-^{14}C]-biochanin A possessed 9600 dpm (9.06%). TLC (SGF, S_{12}) of the two samples of **V** with subsequent

scanning also demonstrated that only *p*-hydroxy-phenylacetic acid derived from [2-^{14}C]biochanin A contained significant radioactivity. Determination of $^{14}CO_2$ formation in these two experiments further showed that C-4 of **I** is much more rapidly (89%) converted to CO_2 than carbon atom 2 (28%) of biochanin A.

3,4-Dihydroxyphenylacetic acid (VI)

Maximum accumulation (9.8%) of this compound (TLC, SGF, S_{16}, $R_f = 0.35$) was observed after some 50–60 h of incubation and it was mainly isolated from mycelium. During spectroscopic structural elucidation which was again facilitated by reference material, great care was taken to determine the exact hydroxyl group substitution pattern. 2,5-di-hydroxyphenylacetic acid which could also be considered a likely intermediate in *p*-hydroxyphenyl-acetic acid degradation was clearly eliminated in favour of the 3,4-dihydroxy substitution pattern. Experiments with both [2-^{14}C]biochanin A and [U-^{3}H]biochanin A demonstrated (TLC, S_{16}) that **VI** was an intermediate in biochanin A degradation and that it was formed subsequently to the pyrone **III** and to *p*-hydroxyphenylacetic acid.

Longer incubation experiments unequivocally showed that **VI** was further degraded to CO_2 though all attempts to detect any subsequent catabolites have failed so far.

Metabolism of substituted phenylacetic acids

In order to obtain further insight into the catabolism of phenylacetic acids by *F. javanicum*, the oxidation of *p*-methoxyphenylacetic acid (**IV**), *p*-hydroxyphenylacetic acid (**V**), 3,4-dihydroxyphenyl-acetic acid (**VI**) and 2,5-dihydroxyphenylacetic acid (**VII**) as well as *p*-hydroxymandelic acid (**VIII**) was studied. When tested under the conditions of the standard incubation assay described for **I** (at 10^{-5} and 10^{-4} M) only **VI** was slowly oxidized (app. 12%) during an incubation period of up to 60 h; the other acids remained unchanged. However, after preincubation of mycelial preparations with biochanin A (10^{-4} M, 11^h), the cells readily oxidized **V** (48% in 30 h), **VI** and **VII**; **IV** and **VIII** were not attacked by these induced fungal cells. Attempts to detect catabolites of **VI** and **VII** were not successful.

It appears that phenylacetic acid catabolism by *F. javanicum* is only induced as part of isoflavone metabolism and not by the acids when given as sole substrates. Furthermore, though 2,5-dihydroxyphen-ylacetic acid was not detected as an intermediate in the degradation of **I**, preincubation of cells with this isoflavone induced the cells for the oxidation of **VII**. These surprising observations will be further investigated.

Discussion

The catabolities of biochanin A isolated so far in these studies with *F. javanicum* are shown in a proposed sequence in Fig. 4. This pathway appears to be the first example to describe the microbial disintegration of an isoflavonoid skeleton. A great similarity with microbial flavonoid catabolism can be seen [1] in so far that the carbon atoms of ring A
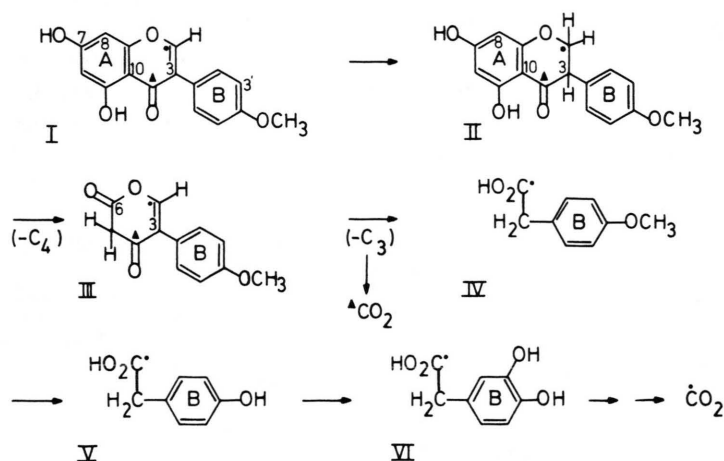

Fig. 4. Proposed degradative pathway of biochanin A as found with *F. javanicum*. Symbols: positionspecific ^{14}C-labelled substrate ● and ⊹. **I**, biochanin A; **II**, dihydro-biochanin A; **III**, 3-(*p*-methoxyphenyl)-4,6-diketo-5,6-dihydro-4H-pyran; **IV**, *p*-methoxyphenylacetic acid; **V**, *p*-hydroxyphenylacetic acid; **VI**, 3,4-dihydroxyphenylacetic acid.

(C-5 to C-8) and of the heterocyclic ring (C-4, C-9, C-10) are removed as aliphatic units with the side chain phenylring being liberated in case of flavonoids as a substituted benzoic acid or, in case of **I**, as a phenylacetic acid. Hydroxyphenylacetic acids as catabolites of flavonoids have only been detected as the result of anaerobic microbial degradative pathways [1].

Though the C_4 and C_3 unit (see Fig. 4) removed during biochanin A degradation are presently unknown, the C_4 moiety of ring A is expected to be oxaloacetate. This assumption is based on the preliminary observation [13] that 8-hydroxydihydrobiochanin A occurs in very small amounts during biochanin A dissimilation by *F. javanicum*. Cleavage of 8-hydroxydihydrobiochanin A by a dioxygenase between C-8 and C-9 with subsequent hydrolytic fission between C-5 and C-10 would lead to oxaloacetate, a keto group at C-9 (C-6 in compound **III**) and a methylene group at C-10 (C-5 in **III**). This assumption also explains why **III** has been written as a 4H-pyranone (Fig. 4). Pyrones of similar structure as **III** are known as natural products [16] and have also been isolated as intermediates in bacterial degradation of flavanonols [17]. It has further been shown that 2,4-pyronones can be methylated at the enol oxygen of either carbonyl [17, 18] so that a keto-enol-tautomerism as shown in Fig. 3 can be justified.

The reduction of biochanin A to dihydrobiochanin A as the introductionary step of an aerobic degradative sequence appears remarkable and highly reminiscent of other reduction reactions catalysed by various fungi such as *Sporotrichum*, *Trametes* and *Polystictus* [19–21]. The high yield of dihydrobiochanin A (up to 78%) also excludes the assumption that the formation of **II** would be the result of a disproportionation reaction of **I** with the oxidized component being rapidly consumed without any significant accumulation. Future enzymatic studies will further characterize this reaction and they will also have to show whether **II** is formed by the fungus as an optically active compound. The comparatively high pH value of the incubation medium (7.5) has undoubtedly led to a racemate due to the rapid isomerisation of isoflavones [22].

Enzymic studies also have to show whether 3,4-dihydroxyphenylacetic acid is catabolized by *ortho*- or *meta*- cleavage of the aromatic ring. Such enzymic experiments should also deal with the question how phenylacetic acid degradation by *F. javanicum* is linked to isoflavone metabolism and why acids such as **IV**, **VI** or **VII** will obviously not act as substrate inducers for suitable oxygenases.

These studies provide another example that fungitoxic defense compound of higher plants such as preinfectional inhibitors (biochanin A or others) [3] or various phytoalexins [6] are degraded by fungi. Phytopathogenic *Fusarium* fungi are especially potent degraders of such inhibitory defense compounds [23] so that this ability might well be related with the infection process of the plant by the microorganism.

The pathway in Fig. 4 as verified in *F. javanicum* represents one metabolic possibility which is also observed in some other *Fusarium* species [13]. Several *Fusarium* fungi have, however, been shown to start biochanin A metabolism by other reactions such as O-demethylation leading to genistein [5, 13], 7-O-methylation or 3'-hydroxilation (Mackenbrock and Barz, unpublished). These data to be published later again point to the great metabolic diversity [5] of this genus of fungi.

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