Degradation of the Pterocarpan Phytoalexin (−)-Maackiain by *Ascochyta rabiei*

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Ten strains of *Ascochyta rabiei* pathogenic to chickpea (*Cicer arietinum* L.) were shown to be potent degraders of the chickpea pterocarpan phytoalexin (−)-maackiain (**1**) ([6aR:11aR]-3-hydroxy-8,9-methylenedioxypterocarpan). In degradative studies with mycelial preparations and crude protein extracts seven catabolites could be isolated and structurally elucidated by spectroscopic techniques. The main routes of maackiain degradation are reduction to a 2′-hydroxyisoflavan (**2**) and oxidation to an 1a-hydroxy-pterocarp-1,4-diene-3-one (**3**) with subsequent reductions of the early catabolites in ring A. A catabolic sequence for maackiain is compared with the degradation pattern of pterocarpan phytoalexins observed with other fungi.

Introduction

Ascochyta rabiei, a pathogenic deuteromycete parasite of chickpea (*Cicer arietinum* L.) is the causal agent of the economically important blight of this grain legume crop [1]. This fungus (the sexual stage is named *Mycosphaerella rabiei*) has previously been shown to be a potent degrader of the isoflavone biochanin A [2] and the pterocarpan medicarpin [3] which are important defense compounds of chickpea plants [4]. The ability of phytopathogenic fungi to degrade the preinfectional and postinfectional fungal inhibitors of higher plants is considered to be a major feature of pathogenicity [5–7].

In continuation of our studies on the fungal degradation of pterocarpan phytoalexins [3, 8, 9] we now report on the catabolism of the chickpea phytoalexin (−)-maackiain ([6 aR:11 aR]-3-hydroxy-8,9-methylenedioxypterocarpan; (**1**) in Fig. 1) by the chickpea pathogenic strains of *A. rabiei*. All strains were shown to be potent degraders of this phytoalexin and by structural elucidation of various aromatic catabolites two predominant types of conversion reactions of **1** could be clarified.

Materials and Methods

Fungal strains

A. rabiei strains I to IV have previously been used in degradative studies on chickpea phenolic constituents [2, 3]. The other strains, obtained from M. C. Saxena, ICARDA, Aleppo, Syria, were named as follows: V, Jisir Elchoutan; VI, Sarmin; VII, TH old; VIII, Isra; IX, Maret Musrin; X, Atareb-edleb.

The growth of strains on chickpea-seed-meal-glucose or potato-glucose media, as well as cultivation and harvest of mycelia for incubation experiments were as described [2, 3].

Incubation conditions

The standard incubation assays of mycelium with substrates (10^{-4} M) and the isolation of catabolites followed earlier reports [2, 3].

Protein preparations

The preparation of protein extracts and the incubation of such cell free enzyme mixtures with substrates (10^{-4} M), NADPH (0.4–2 mM) and dithioery-

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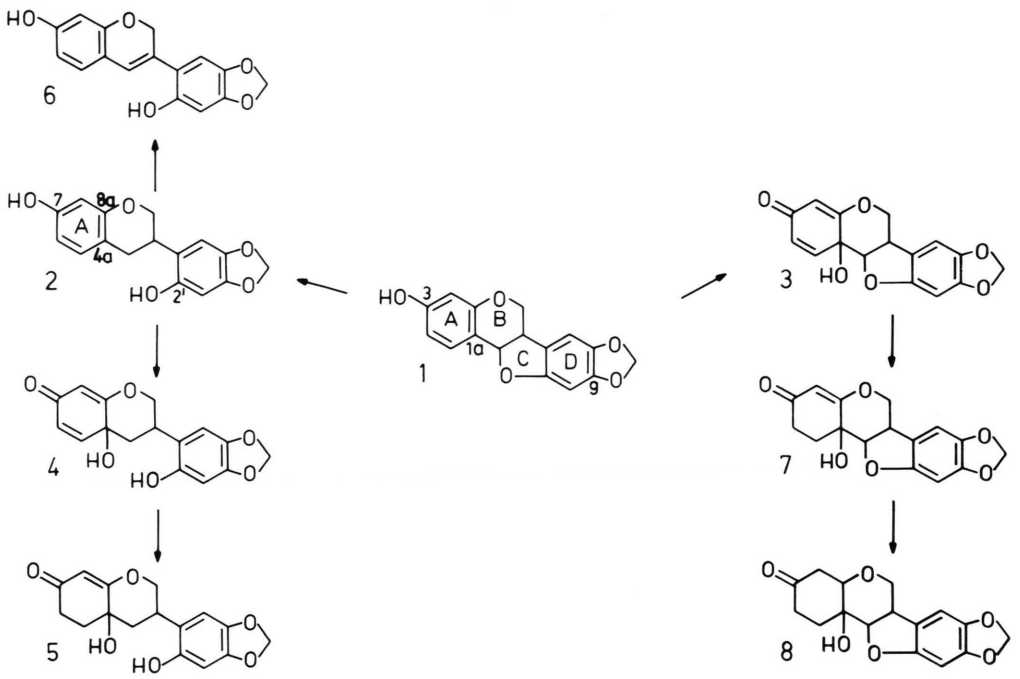

Fig. 1. Postulated scheme of initial reactions involved in maackiain (**1**) degradation by *Ascochyta rabiei*.

thritol (1 mM) have been described [3]. Control assays were incubated, respectively, with boiled protein extracts, NADH as coenzyme or with oxygen-free nitrogen instead of normal air.

Product analyses

Thin-layer chromatography (TLC) was performed with silica gel plates and the solvents

S1: benzene : ethyl acetate : petrol ether (b.p. 60–80 °C) : methanol = 6:4:3:2 (v/v), and
S2: benzene : ethyl acetate : petrol ether : methanol = 6:4:3:1.

Phenolic catabolites were detected by spraying with diazotized *p*-nitroaniline. The procedures for separation and quantitation of compounds by HPLC have been reported [2, 3, 16].

UV-visible absorption spectra and gaschromatography-mass-spectroscopy (GC-MS) were as previously described [3]. For GC-MS phenolics were either measured in form of their trimethylsilyl (TMSi) or acetyl derivatives [19] or in underivatized form. ^{1}H-NMR-spectroscopy was performed with a Bruker WM 300 MHz-NMR-spectrometer with tetramethylsilan as internal standard.

Compounds

Dihydromaackiain (**2**) was synthesized from maackiain by reduction with $NaBH_4$ according to R. M. Cooper (pers. communication). 1a-Hydroxy-8,9-methylenedioxy-pterocarp-1,4-diene-3-one (**3**) had been isolated from degradative studies of **1** with *Nectria haematococca* [12]. All other compounds were from the collection of the institute.

Structural elucidation of catabolites from mycelial incubations

The phenolic catabolites of **1** as isolated from the growth medium were separated and purified by TLC (S_1, S_2). All fractions were also assayed by HPLC and individually subjected to UV-, ^{1}H NMR and GC-MS-spectroscopy. The interpretation of MS-spectra of isoflavonoids and pterocarpans as well as the nomenclature of molecular fragments follows earlier reports [3, 8–11]. Reference compounds were included in the identification process whenever possible.

The spectral data of all identified catabolites are subsequently given with their R_f-values on TLC (S_1), the retention time R_T during HPLC [3] and their number according to Fig. 1.

*7,2'-Dihydroxy-4',5'-methylenedioxy-isoflav-3-ene (**6**)*

R_f 0.57; R_T 27.6 min; UV: λ_{max} 240 (sh), 325 and 350 (sh) nm. MS (TMSi) *m/e* 428 (M^+), 413 (M^+-15), 355 (M^+−TMSi), 339 (M^+−OTMSi), 248 (fragment B), 220, 219 (fragment G), 209 (fragment H), and 180 (fragment A).

*1a-Hydroxy-8,9-methylenedioxy-pterocarp-1,4-diene-3-one (**3**)*

R_f 0.50; R_T 19.2 min. UV: λ_{max} 288 (sh), 308 nm. MS *m/e* 300 (M^+), 176 (fragment B), 175, 163 (fragment D), 162, 138, 137 (fragment C) and 133. MS (TMSi) *m/e* 372 (M^+), 357 (M^+-15), 327, 299 (M^+−TMSi), 236 (fragment G), 235, 234, 176 (fragment B), 175, 163 (fragment D), 162 and 133. These spectra were confirmed with reference compound [12].

*7,2'-Dihydroxy-4',5'-methylenedioxyisoflavan (**2**)*

R_f 0.48; R_T 27.6 min; λ_{max} 225, 287 (sh), 291 and 300 (sh) nm; λ_{max} ($NaOCH_3$) 230 and 302 nm. MS (TMSi) *m/e* 430 (M^+), 415 (M^+-15), 398, 355 (M^+−TMSi), 341 (M^+−OTMSi), 281, 269 (M^+−TMSi−OTMSi), 236 (fragment B), 223 (fragment D), 221, 207 and 194 (fragment A).

^{1}H NMR (acetone-d_6) δ (ppm): 2.81 (ddd, 1H, $J_{2\,Heq,\,2\,Hax}$ = 15.7 Hz, $J_{2\,Heq,\,3\,Hax}$ = 5.5 Hz, $J_{2\,Heq,\,4\,Heq}$ = 1.7 Hz; Heq-2), 2.94 (ddd, 1H, $J_{2\,Hax,\,2\,Heq}$ = 15.7 Hz, $J_{2\,Hax,\,3\,Hax}$ = 10.6 Hz, $J_{2\,Hax,\,eq}$ = 0.5 Hz; Hax-2), 3.52 (dddd, 1H, $J_{3\,Hax,\,2\,Heq}$ = 5.5 Hz, $J_{3\,Hax,\,2\,Hax}$ = 10.6 Hz, $J_{3\,Hax,\,4\,Heq}$ = 3.5 Hz, $J_{3\,Hax,\,4\,Hax}$ = 10.0 Hz; Hax-3), 3.99 (dd, 1H, $J_{4\,Hax,\,4\,Heg}$ = $J_{4\,Hax,\,3\,Hax}$ = 10.0 Hz; Hax-4), 4.21 (dddd, 1H, $J_{4\,Heq,\,4\,Hax}$ = 10.0 Hz, $J_{4\,Heq,\,3\,Hax}$ = 3.5 Hz, $J_{4\,Heq,\,2\,Heq}$ = 1.7 Hz, $J_{4\,Heq,\,2\,Hax}$ = 0.4 Hz, Heq-4), 5.88 (m; 2H, methylenedioxy), 6.28 (d, 1H, J = 2.5 Hz; H-8), 6.36 (dd, 1H, $J_{6,\,5}$ = 8.2 Hz, $J_{6,\,8}$ = 2.5 Hz; H-6), 6.52 (s; 1H, H3'), 6.70 (s; 1H, H-6'), 6.89 (d, 1H, J = 8.2 Hz; H-5), 8.15, 8.34 (2 br. s; 1 H each, Ar-OH).

These spectra were confirmed with reference material.

*1a-Hydroxy-8,9-methylenedioxy-pterocarp-3-one (**8**)*

R_f 0.34, R_T 20.3 min; λ_{max} 300 nm; MS (TMSi) *m/e* 376 (M^+), 361 (M^+-15), 333, 317, 200 (fragment A), 176 (fragment B), 163 (fragment D) and 133.

*10,2'-Dihydroxy-4',5'-methylenedioxy-isoflav-8-ene-7-one (**5**)*

R_f 0.29; R_T 18.4 min. UV: λ_{max} 256 and 300 nm. MS (di-TMSi) *m/e* 448 (M^+), 433 (M^+-15), 358 (M^+-90), 343 (M^+-90-15), 332, 330 (M^+−OTMSi−28) 256, 249, 239 (fragment E), 236 (fragment B), 223 (fragment D), 210, 209 (fragment F). MS (tri-TMSi) *m/e* 520 (M^+), 505 (M^+-15), 448, 430, 415, (430−15), 364, 297, 236 (fragment B), 223 (fragment D) and 221. The formation of a tri-trimethylsilyl derivative can be explained by derivatization of a hydroxyl group at C-7 generated by enolization [11, 19]. MS *m/e* 304 (M^+), 286, 284, 245, 215, 202, 177, 176, 164, 163, 151 (fragment D), 147, 138 (fragment F), 133, 128 ($C_5H_8O_3$), 119, 105, 91 and 77.

^{1}H NMR ($CDCl_3$:C_6D_6; 1:1): δ (ppm): 1.55 (mc; 1H; H-4), 1.69 (mc; 2H, H-5), 1.81 (mc; 1H; H-4), 2.24, 2.46 (2 mc; je 1H; H-6), 3.47 (dd, J = 10.9 Hz, J = 10.9 Hz; 1H; Hax-2), 3.75 (mc; 1H; H-3), 4.23 (ddd; $J_{2\,Heq,\,2\,Hax}$ = 10.5 Hz, $J_{2\,Heq,\,3\,H}$ = 4.8 Hz, $J_{2\,Heq,\,4\,Heq}$ = 2.0 Hz; 1H; Heq-2), 5.54 (s; 1H; H-8), 5.58 (m; 1H; methylenedioxy), 5.94 (s; 1H; H-3'), 6.36 (s; 1H; H-6').

The signals were assigned by decoupling experiments shown in the Table.

Table. Decoupling experiments used to assign the NMR signals obtained with compound **5**.

Entry	Signal irradiated [ppm]	Signal multiplicity collapsed [ppm]
1	3.75	3.47; 4.23; 1.81; 1.55
2	2.46	1.69; 2.24
3	2.24	2.46; 1.69
4	1.81	3.47; 3.75; 4.23
5	1.69	2.24; 2.46
6	1.51	1.81; 3.75

The interpretation of the spectrum is based on the assumption that the 2 protons H-6 ortho to the carbonyl group absorb at lower field in comparison to the H-5 protons.

*1a-Hydroxy-8,9-methylenedioxy-pterocarp-4-ene-3-one (**7**)*

R_f 0.29, R_T 19.2 min. MS (TMSi) *m/e* 374 (M^+), 346, 329, 317, 238 (fragment G), 237, 236, 176 (fragment B), 175 and 163 (fragment D).

*10,2'-Dihydroxy-4',5'-methylenedioxyisoflav-5,8-diene-7-one (**4**)*

R_f 0.24; R_T 18.4 min. MS (TMSi) *m/e* 446 (M^+), 431 (M^+-15), 356 (M^+-90), 341 (M^+-90-15), 287, 237 (fragment E), 236 (fragment B), 223 (fragment C and D) and 175.

Catabolites from protein incubation

Compounds formed in incubation assays with NADPH (1.2 mM) and *A. rabiei* protein preparations and **1**, **2** or **3** as substrates (10^{-4} M) were extracted with ethylacetate. Separation of compounds was performed by TLC (S_1) and finally by HPLC [2, 3]. Purified catabolites were subjected to UV- and NMR-spectroscopy as well as GC-MS analyses. The spectral data obtained are summarized under "mycelial catabolites".

Results

Catabolites of Maackiain

In a comparative study on the degradation of maackiain (**1**) by the strains of *A. rabiei* (see below) all isolates were shown (TLC, HPLC) to be potent degraders of the phytoalexin. Conversion of **1** commenced without any detectable lag-phase with the transient accumulation of numerous catabolites. Many of these compounds occurred in only low amounts insufficient for structural elucidation. Maackiain was totally consumed within 6–10 h and finally all aromatic intermediates had completely disappeared from the incubation medium within altogether 24 h. Extraction of mycelia with ether after this period of incubation did not lead to any residual UV-absorbing catabolites indicating that complete degradation of **1** and its disintegration products had occurred. Since only quantitative differences in the accumulation of catabolites could be observed among the various fungal isolates, strain III was chosen for a thorough analysis of the intermediates in the degradation sequence of maackiain.

Mycelial preparations of *A. rabiei* strain III were incubated with **1** (10^{-4} M; total volume up to 2000 ml) for various periods of time (2, 5–4 h) and the catabolites extracted from the growth medium were separated by TLC (S_1). Six bands were observed and the compounds extracted from them were all rechromatographed (TLC; S_1, S_2). They were individually subjected to GC-MS-analyses and UV-spectroscopy as well as to ^{1}H-NMR-spectroscopy whenever sufficient material was available. The spectral data (see Materials and Methods) are interpreted on the basis of our earlier detailed report [3] on MS fragmentation pattern of pterocarpans and isoflavans and of other published data [9–12]. The structures of the elucidated compounds are arranged in Fig. 1 according to the most likely degradative sequence as based on time course studies (see below) and chemical structure. It is evident that both reductive and oxidative reactions are involved in maackiain degradation by *A. rabiei*.

Maackiain (**1**) degradation starts with the formation of compounds **2** and **3** which were already known as conversion products of **1** from other fungi [13–15]. The oxidation product **3** accumulated in only small amounts because it was readily reduced to compounds **7** and **8**. The MS-data of **7** and **8** clearly indicated that the reduction reactions had occurred in ring A of **3** because the two characteristic mass fragments of pterocarpans representing rings C and D (*m/e* 176 and 163) were both found in the spectra of compounds **3**, **7** and **8**.

Among the conversion products of **2** compound **5** is one of the most prominant intermediates in the catabolism of maackiain. The homology in the oxidation pattern of ring A between compounds **5** and **7** was clearly supported by the MS-data and the NMR-spectrum. The chemically unexpected stability of **5** and **7** towards aromatization by dehydration (which would lead to **2** and **1**, respectively) deserves further investigations.

Compound **4** accumulating in low amounts only, was structurally elucidated by the fragmentation pattern visible in the MS-spectrum and by the analogy with the MS-spectrum of compound **3**.

Compound **6**, a dehydrogenation derivative of **2**, showed a fragmentation pattern in the MS-spectrum which had been found earlier [3, 9] in studies on an analogous product formed during medicarpin degradation by *A. rabiei* and *Fusarium oxysporum*.

Enzymatic investigations

Further insight into maackiain catabolism by *A. rabiei* strain III could be obtained by incubation experiments with cell free crude protein preparations carried out according to our previous report [3]. In the presence of NADPH (NADH was not effective) the rapid formation of three products was observed

(HPLC) which were identified as **2** and **5** (GC-MS, NMR) and **3** (GC-MS). Exclusion of oxygen from these incubation assays prevented the accumulation of compounds **3** and **5** and solely led to **2**. When dihydromaackiain (**2**) was used as substrate in the presence of NADPH and oxygen the polar catabolite **5** was formed; compound **4** was not observed as an intermediate. The enzymatic formation of **5** from **2** but not from **3** was further demonstrated by the observation that compound **3** was not converted to products **4** or **5** by such cell free protein preparations either with or without NADPH.

Furthermore, these enzymatic investigations have substantiated our assumption that the enzymatic activities for the reductive (*i.e.* formation of **2**) and the oxidative (*i.e.* formation of **3**) conversion of **1** are constitutively expressed in *A. rabiei* [3]. Preincubation of mycelial preparations of strain III with maackiain (**1**) (10^{-4} M; 6 h) did not lead to any higher enzyme activities than measured in uninduced cells.

Comparison of A. rabiei strains for maackiain degradation

As indicated before the 10 strains of *A. rabiei* all possess the ability for the degradation of **1** regardless of whether chickpea-seedmeal or potato-homogenate were used as growth medium. Preincubation of various strains with **1** for up to 6 h did not lead to any accelerated degradation of the phytoalexin so that constitutive expression of degradative enzymes by *A. rabiei* must be assumed. The strains greatly differ in their ability to accumulate the various intermediates shown in Fig. 1. The analyses (TLC, HPLC), however, support the conclusion that all strains convert maackiain both oxidatively (formation of **3**) and reductively (formation of the isoflavan **2**). Further investigations must show to which extent these alternative enzyme reactions are expressed in the *A. rabiei* strains employed in these investigations.

Discussion

Chickpea pathogenic isolates of *A. rabiei* readily degrade the two pterocarpan phytoalexins medicarpin [3] and maackiain (Fig. 1) of this crop plant. These results again show that the potential for phytoalexin disintegration must be assumed to be an important trait of phytopathogenic fungi [5–7].

As previously demonstrated for medicarpin [3] *A. rabiei* also degrades maackiain along two major routes as indicated by the initial catabolites **2** and **3**. It is important for our future investigations on the virulence mechanisms of the *A. rabiei* isolates to determine to which extent these two alternative enzyme reactions are expressed in *A. rabiei* under *in vivo* conditions and how they contribute to the degradation of the pterocarpan phytoalexins during the infection process of chickpea plants. Such investigations will be facilitated by our present observations that these two enzyme reactions can be measured with cell-free protein preparations; a thorough characterization of the two enzyme reactions is presently carried out.

Reductive cleavage of the benzylphenylether bond of **1** yielding the 2′-hydroxyisoflavan **2** has repeatedly been observed with several pterocarpans in various different fungi [3, 5, 8, 9–11, 13–16]. Recent investigations [20] on the enzyme partially purified from *A. rabiei* strain III have shown that the reaction strictly depends on NADPH and that the enzyme is specific for medicarpin and maackiain. This fact readily explains our observation that compounds **4** and **5** are formed from **2** and not from **3** or **7**, respectively.

Formation of the 1a-hydroxy-pterocarp-1,4-diene-3-one (**3**) from **1** has so far only been reported for maackiain metabolism by *Nectria haematococca* [12, 17] where oxidative attack represents the sole route of pterocarpan conversion. *A. rabiei* is at present the only example in pterocarpan fungal metabolism where both reductive cleavage to **2** and oxidative conversion to **3** occur in one fungus. The pronounced ability of *A. rabiei* for such oxidation reactions at ring A is demonstrated by the formation of the analogous compounds **3** and **4**. Future investigations must show whether these reactions are catalyzed by one or two enzymes.

The reduction reactions as indicated by the structures of compounds **5**, **7** and **8** have hitherto not been observed in pterocarpan degradation with any fungus other than *A. rabiei* [3]. Reductases for the NADPH-hydrogenation of α,β-unsaturated ketones are, however, well known from various fields such as steroid metabolism in numerous microorganisms [18]. Future investigations will have to elucidate the degradative pathways of the keto compounds **5** and **8** because they are only transiently accumulated by *A. rabiei.*

2,4-Dihydroxybenzoic acid has recently been found as a late aromatic catabolite of medicarpin by *A. rabiei* [3]. In case of this phytoalexin it could not

be decided whether the benzoic acid had been formed from either ring A or ring D of the pterocarpan molecule. In view of the pronounced accumulation of compounds **3**–**8** with a modified structure in ring A and the fact that 2,4-dihydroxybenzoic acid could not be isolated during maackiain catabolism by *A. rabiei* this benzoic acid might have originated from ring D.

Future investigations with *A. rabiei* and maackiain will be devoted to a more complete analysis of the later stages of the degradation sequence of **1** by this fungus and characterization of the enzymes involved. Furthermore, detailed analyses are required concerning the stereochemistry of the isolated catabolites both with respect to the astonishing stability of compounds **5** and **7** towards aromatization and to the steric course of the enzymatic transformations.

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