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FAD-DEPENDENT EPOXIDASE AS A KEY ENZYME IN FUNGAL METABOLISM OF PRENYLATED FLAVONOIDS

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Key Word Index—*Botrytis cinerea*; Hyphomycetes; epoxidase; prenylflavonoids; FAD-dependent monooxygenase; prenyl cyclization; fungal metabolism.

Abstract—Crude protein extracts from *Botrytis cinerea* preincubated with 6-prenylnaringenin (6-PN) for 20 hr catalysed the prenyl epoxidation of 7-*O*-methyl-luteone. The resulting epoxide was non-enzymatically and slowly converted into the corresponding dihydrofurano derivative in a buffer solution at pH 7.5. Preparation of cell-free extracts in the presence of 6-PN from the mycelia without preincubation with 6-PN hardly showed the epoxidizing activity. These facts revealed that the substrate analogue 6-PN has a role as an enzyme inducer rather than stabilizer. The enzyme reaction depends on molecular oxygen and NADPH. Low amounts of FAD were necessary for maximal enzyme activity. The enzymatic activity was not inhibited by various inhibitors of cytochrome P-450 tested, in addition to carbon monoxide and cytochrome c. The results indicated that this enzyme does not belong to the monooxygenases dependent on cytochrome P-450, but to those dependent on FAD. About half of the total enzyme activity was found in the 125 000 *g* supernatant, but the specific activity for the epoxidation reaction in the 125 000 *g* pellet was 3.7-fold higher than in the soluble fraction. The enzyme showed high specificity to monoprenyl isoflavones. Finally, a preliminary experiment using a cell-free system from white lupin hypocotyls resulted in formation of small amounts of an epoxide corresponding to 7-*O*-methyl-luteone used as the substrate. © 1997 Elsevier Science Ltd

INTRODUCTION

ortho-Prenylated phenols (**a**) are believed to be the precursors of cyclic ether derivatives possessing pyrano (**b**), dihydropyrano (**f**) and dihydrofurano (**c**, **d**) ring systems widely found in secondary metabolites such as flavonoids [1], coumarins [2], alkaloids [3], chromones [4], and xanthones [5] (Fig. 1). A corresponding epoxy derivative has long been postulated as a reaction intermediate to the cyclic ether products [2, 6]. Enzymatic cyclization of *ortho*-prenylated phenols has been studied for rotenoid [7], pterocarpanoid [8], and coumarin metabolism [9]. It has been found that oxygen-dependent cyclization of rot-2-enoic acid to deguelin is catalysed by a non-heme iron protein, deguelin cyclase, from *Tephrosia vogelii* [7]. On the other hand the prenylated pterocarpan, 2-prenylglycinol, was transformed to glyceollin II (pyrano-type) and glyceollin III (dihydrofurano-type) by cytochrome P-450 dependent monooxygenase(s) from elicitor-treated soybean cells [8]. Cyclization of a prenylated coumarin, demethylsuberosin, was also catalysed by a microsomal cytochrome P-450 dependent monooxygenase extracted, from elicitor-stimulated suspension cultures of *Ammi majus*, to give (+)-marmesin, possessing a 2-(1-hydroxy-1-methylethyl)furano side attachment [9]. The presence of an epoxy-intermediate was unambiguously ruled out in the route from rot-2-enoic acid to deguelin [7], and could neither be observed with the enzymatic formation of glyceollins [8] and marmesin [9]. Fungitoxic prenylated isoflavones from lupins, luteone (**1**) [10], wighteone (**2**) [11], licoisoflavone A (**3**) [12] and 2,3-dehydrokievitone (**4**) [13], the prenylflavone topazolin (**5**) [14], and the prenylflavanone 6-prenylnaringenin (**6**) [15] have been reported to be metabolized by *Botrytis cinerea* into the corresponding non-toxic dihydrofurano (**d**), dihydropyrano (**f**) and glycol-type (**g**) derivatives. These structural types (**a**–**d** and **f**–**g** in Fig. 1) were also found in the roots of lupins [16], and presumed to be formed via epoxidation of prenylated isoflavones according to the proposed pathway for the fungal metabolism [16, 17].

As described in our previous paper, an epoxy-intermediate in the metabolism of the prenylated isoflavone 7-*O*-methyl-2,3-dehydrokievitone (**7**) has been isolated and characterized from a metabolic culture of *B. cinerea* [18]. We now report the formation of epoxy-intermediates from prenylisoflavones in a cell-free

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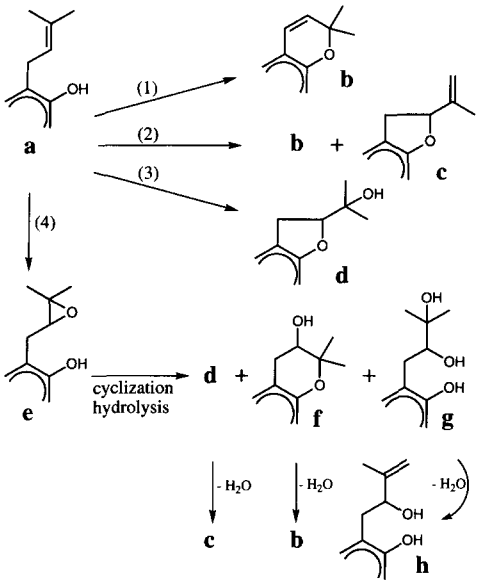
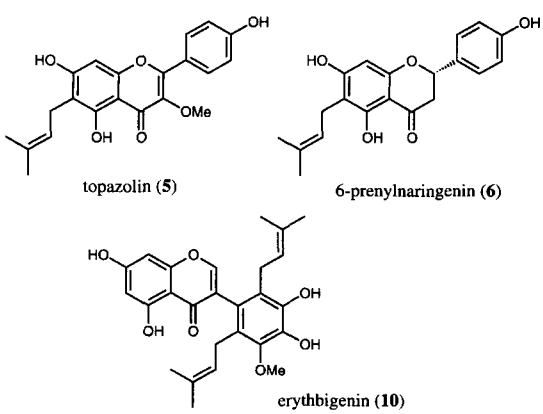


Fig. 1. Structural classes formed from *ortho*-prenylphenol by oxidative enzyme reactions. Reactions catalysed by (1) deguelin cyclase to yield deguelin [7], (2) soybean monooxygenase(s) to yield glyceollins I-III [8], (3) marmesin synthase to yield (+)-marmesin and (4) *Botrytis* epoxidase to yield prenylflavonoid epoxides. Cyclization of hydrolysis of the epoxide to yield **d**, **f** and **g** occurs spontaneously in neutral or weakly acidic aqueous solution.

extract from *B. cinerea* and describe some properties of the enzymatic reaction. A preliminary account of the prenylflavonoid epoxidase reaction has been presented elsewhere [19]. This is the first evidence for enzymatic prenyl cyclization of *ortho*-prenylphenols via prenyl epoxidation.

RESULTS AND DISCUSSION

In a previous paper [19], we have shown a cell-free system consisting of 7-*O*-methyl-luteone, NADPH and a crude protein extract from *Botrytis cinerea* preincubated with 6-prenylnaringenin (**6**) catalysed prenyl epoxidation, a key step in the fungal metabolism of fungitoxic prenylated flavonoids, giving less toxic dihydrofurano (**d**), dihydropyrano (**f**) and glycol-type (**g**) derivatives (Fig. 1). The reaction depended on molecular oxygen and NADPH, and the reaction product (or chemically formed products) possessed an oxygen atom derived from $^{18}O_2$, but not from $H_2^{18}O$. Some properties of the epoxidase were further examined and results are described in the present paper. A linear relationship was confirmed for the amount of consumed substrate **8** (24–70%) and concentration of the crude protein in the reaction mixture (2.3–9.2 mg ml^{-1}).



Inducibility of epoxidase activity in Botrytis cinerea

Preincubation of *Botrytis cinerea* with a substrate analogue 6-prenylnaringenin (**6**) enhanced markedly the epoxidase activity of the cell-free system ([19] and Fig. 2). Effects of the preincubation of the mycelia with **6**, and the presence of **6** throughout the extraction of the crude protein from *B. cinerea* on the epoxidizing activity were compared. The chromatograms in Fig. 2 clearly indicated that the appearance of epoxidase activity depended on the preincubation with a substrate analogue **6** and not on the presence of **6** during the crude protein extraction. Obviously, compound **6** functioned as an epoxidase inducer of the fungal culture and was not a stabilizer for a constitutively present enzyme activity. Metabolism of **6** in the cultures of *B. cinerea* or *A. flavus* has been reported elsewhere [15].

Subcellular distribution of Botrytis *epoxidase*

Crude extracts of *B. cinerea* mycelia were fractionated by subsequent centrifugation steps giving (1) 3000 *g* debris, (2) 15000 *g* precipitate, (3) 125000 *g* supernatant, and (4) 125000 *g* pellet. The latter three fractions were used for quantification of protein and epoxidase activity using 7-*O*-methyl-luteone (**8**) as a substrate. Results are summarized in Table 1. The highest specific activity was observed in the 125000 *g* pellet. However, about half of the total activity each was found in the 125000 *g* supernatant and the pellet, respectively. Therefore, it is not clear at present if the epoxidase is a soluble or a membrane bound enzyme.

Substrate specificity of Botrytis *epoxidase*

The substrate specificity of *Botrytis* epoxidase was determined using several prenylated isoflavones differing in position and number of prenyl substitution and other substituents, e.g. hydroxyl and *O*-methyl groups. Results are summarized in Table 2. The structures of the reaction products were confirmed by direct comparison with authentic compounds (see Fig. 3).

Isoflavones monoprenylated at C-3′ and C-6 revealed to be good substrates under the reaction conditions used, while monoprenylation at C-8 and diprenylation at C-6 and C-3′ decreased the reaction rate. In our earlier studies, 7-*O*-methyl derivatives **8** and **7**

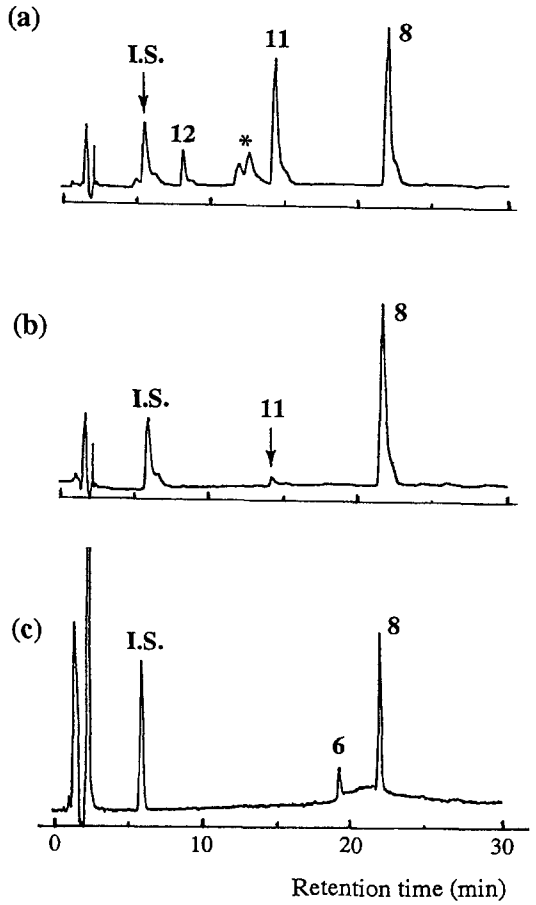


Fig. 2. HPLC analysis of epoxidase activity induced by preincubation of mycelia with 6-prenylnaringenin (**6**). The reaction products from 7-*O*-methyl-luteone (**8**) were analysed by HPLC. The substrate **6** was incubated at 20° for 60 min with crude protein extracts (15 000 *g* supernatant) prepared: (a) from the mycelia preincubated with 6-prenylnaringenin (**6**) (protein 4.5 mg/ml^{-1} reaction mix.) and (b) from the mycelia preincubated without **6** (protein 6.7 mg/ml^{-1}). In (c), preparation of the crude protein extract was conducted in the presence of **6** (as a stabilizer, *ca* 75 ppm) from the mycelia preincubated without **6** (protein 6.2 mg/ml^{-1}). **11**: 7-*O*-methyl-luteone epoxide (see Fig. 3), **12**: dihydrofurano derivative (see Fig. 3), and **I.S.**: methyl *p*-hydroxybenzoate as an internal standard. *Metabolites of **6** used as an enzyme inducer.

of luteone (**1**) and 2,3-dehydrokievitone (**4**), respectively, were metabolized more rapidly than the non-methylated parent isoflavones by the metabolic culture of *B. cinerea* [18, 20]. This was presumably the result of differences in cell permeation.

Lonchocarpol A (6,8-diprenylflavanone) or the 2′,6′-diprenylated isoflavone erythbigenine (**10**) was hardly metabolized by the culture of *B. cinerea*, and the latter compound was not transformed by the cell-free system at all. 6-Prenylnaringenin (**6**) has been shown to be metabolized to the corresponding dihydrofurano, dihydropyrano and glycol-type derivatives. It seemed that the reaction proceeded via an epoxy-intermediate in the cultures of *B. cinerea* and *A. flavus*. The speed of metabolism was lower than that of luteone (**1**) [15]. 6-Prenylnaringenin (**6**) or its metabolites distinguishable from complex isoflavones used in the present experiment by HPLC, remained in the crude protein extract or the microsomal fraction, when **6** had been used as an epoxidase inducer. Therefore, **6** could not be used in the present substrate specificity test.

Effect of cytochrome P-450 inhibitors and other factors on the epoxidase activity

The major part of enzymatic monooxygenation is mediated by P-450 dependent oxygenases coacting with a cytochrome P-450 reductase using NAD(P)H as a reducing cofactor. However, the present epoxidase showed no practical sensitivity to the following cytochrome P-450 inhibitors; SKF-525A (100 μM, 1 mM and 2 mM), ketoconazole (50 μM), ancymidole (100 μM), juglone (10 μM), xanthotoxin (10 μM), and cytochrome c (10 μM) inhibited less than 10% of the epoxidase activity with 7-*O*-methyl-luteone (**8**) or luteone (**1**) under standard reaction conditions. Furthermore, neither bubbling CO gas through the reaction mixture for 2 min and incubation in the dark, nor the addition of terbinafine (1 mM), a known squalene epoxidase inhibitor, resulted in significant inhibition of the enzyme reaction. Squalene epoxidases that were insensitive to cytochrome P-450 inhibitors, have been reported from rat liver [21]. *Candida albicans* [22] and *Saccharomyces cerevisiae* [23], and were identified as flavoprotein monooxygenases. The *Botrytis* epoxidase was stimulated a little in the presence of 1 mM EDTA or $CuSO_4$ (*ca* 10%), whilst 1 mM $FeSO_4$ decreased the reaction to 67% of the control level.

Table 1. Subcellular distribution of 7-*O*-methyl-luteone (**8**) epoxidizing activity

Fraction	Total protein (mg)*	Relative specific activity†	Distribution of enzyme activity (%)
15 000 *g* precipitate	4	21	3
125 000 *g* supernatant	48	27	50
125 000 *g* pellet	12	100	47

* From *ca* 7.5 g of wet mycelia.

† Enzyme reactions were carried out at 20° for 30 min and at the protein concentration of 3 mg ml^{-1}. About 80% of the substrate (**8**) was consumed in the reaction mixture catalysed by the 125 000 *g* pellet.

Table 2. Substrate specificity of *Botrytis* epoxidase

Substrate	Conversion ratio (%)*	Ratio to luteone
Luteone (**1**)	87	1.00
Wighteone (**2**)	94	1.08
Licoisoflavone A (**3**)	92	1.06
2,3-Dehydrokievitone (**4**)	56	0.64
7-*O*-Methyl-2,3-dehydrokievitone (**7**)	46	0.53
7-*O*-Methyl-luteone (**8**)	73	0.84
2′-Hydroxylupalbigenin (**9**)	21	0.24
Erythbigenin (**10**)	0	0

* The conversion ratio was calculated from the remaining substrate in the reaction mixture catalysed by the microsomal fraction (protein 2.2 mg ml^{-1}) at 20° for 20 min.

Conversion ratio = (1-S/S′) × 100, average of duplicated experiments.

S: HPLC peak area of the remaining substrate in the reaction mixture after incubation/that of the internal standard.

S′: the corresponding ratio before incubation

Internal standard: methyl *p*-hydroxybenzoate.

Conversion ratios for **1**–**3** do not reflect their reaction rate well, because the reaction proceeded into the region out of reaction linearity.

Structures for the reaction products, see Fig. 3.

Cofactor requirements for the Botrytis *epoxidase*

NADPH was used in our previous paper as an effective cofactor for prenyl epoxidation of 7-*O*-methyl-luteone (**8**). The efficiency of NADH as a cofactor was confirmed to be one-tenth of that of NADPH. In the present study, we concentrated on the effect of flavin cofactors. Addition of NAD^+, $NADP^+$, FAD and FMN alone to the reaction mixture, no substrate consumption was observed. The contribution of a flavin cofactor in the presence of NADPH to the reaction was demonstrated by the addition of small amounts of FAD (1–2 μM) to the reaction mixture to give maximal enzyme activity which was about twofold compared to NADPH alone (Table 3). FAD also enhanced the efficiency of NADH about 10 times to the level of that of NADPH alone in the reaction mixture.

The notable feature of the present *Botrytis* epoxidase consists in the reaction catalysing the transformation of *ortho*-prenylphenol part structures into dihydrodihydroxyprenylphenol and/or cyclic ether derivatives via the corresponding epoxy-intermediates (Fig. 1). It is not clear at present if hydrolysis of the epoxy-intermediate into a glycol-type product (**g**) is catalysed by any hydrolase [24]. In addition to the fact that the epoxidase was insensitive to cytochrome P-450 inhibitors, the positive effect of low amounts of FAD on the enzyme activity is indicative of the character of flavoprotein monooxygenase for the epoxidase from *B. cinerea*. As a matter of fact, the epoxidase has not been strictly confirmed if the enzyme is one of the membrane bound and flavoprotein monooxgenases. However, this kind of cyclic ether or glycol forming enzyme has not been reported so far in complex phenol biosynthesis or biodegradation of prenylphenols.

Table 3. Effects of cofactors on the epoxidation of luteone (**1**) to yield a dihydrofurano derivative (**13**, R = OH) via an epoxy-intermediate

Cofactor (μM)*	Conversion ratio (%)‡	Relative efficiency
NADPH (400)	28	1.00
NADPH (400) + FAD (1)	59	2.11
NADPH (400) + FAD (5)	64	2.29
NADPH (400) + FAD (10)	63	2.25
NADPH (400) + FAD (20)	62	2.21
NADPH (400) + FMN (20)	28	1.00
NADH (400) + FAD (20)	29	1.04
None†	0	0

* It was previously confirmed that relative amounts of **13** (R = OH) produced by the microsomal epoxidase from **1** in the reaction mixture with 400 μM NADPH, 400 μM NADH and no cofactor (control) were 1.00, 0.13 and 0.03, respectively, and the latest amount could not be enhanced by the addition of 50 μM of FAD or FMN alone.

† The reaction mixture consisting of **1** as a substrate, buffer and the boiled microsomal fraction.

‡ The conversion ratio was calculated from the remaining substrate in the reaction mixture catalysed by the microsomal fraction (protein 1.0 mg ml^{-1}) at 20°C for 30 min.

Conversion ratio = (1-S/S″) × 100

S: HPLC peak area of the remaining substrate in the reaction mixture after incubation/that of the internal standard.

S″: the corresponding ratio using the denatured microsomal protein

See also the footnotes to Table 2.

There are three examples for enzymatic cyclic ether formation from *ortho*-prenylphenols [7–9], which seem remarkably different from that of *B. cinerea* in reaction mechanism. Squalene epoxidase catalysing

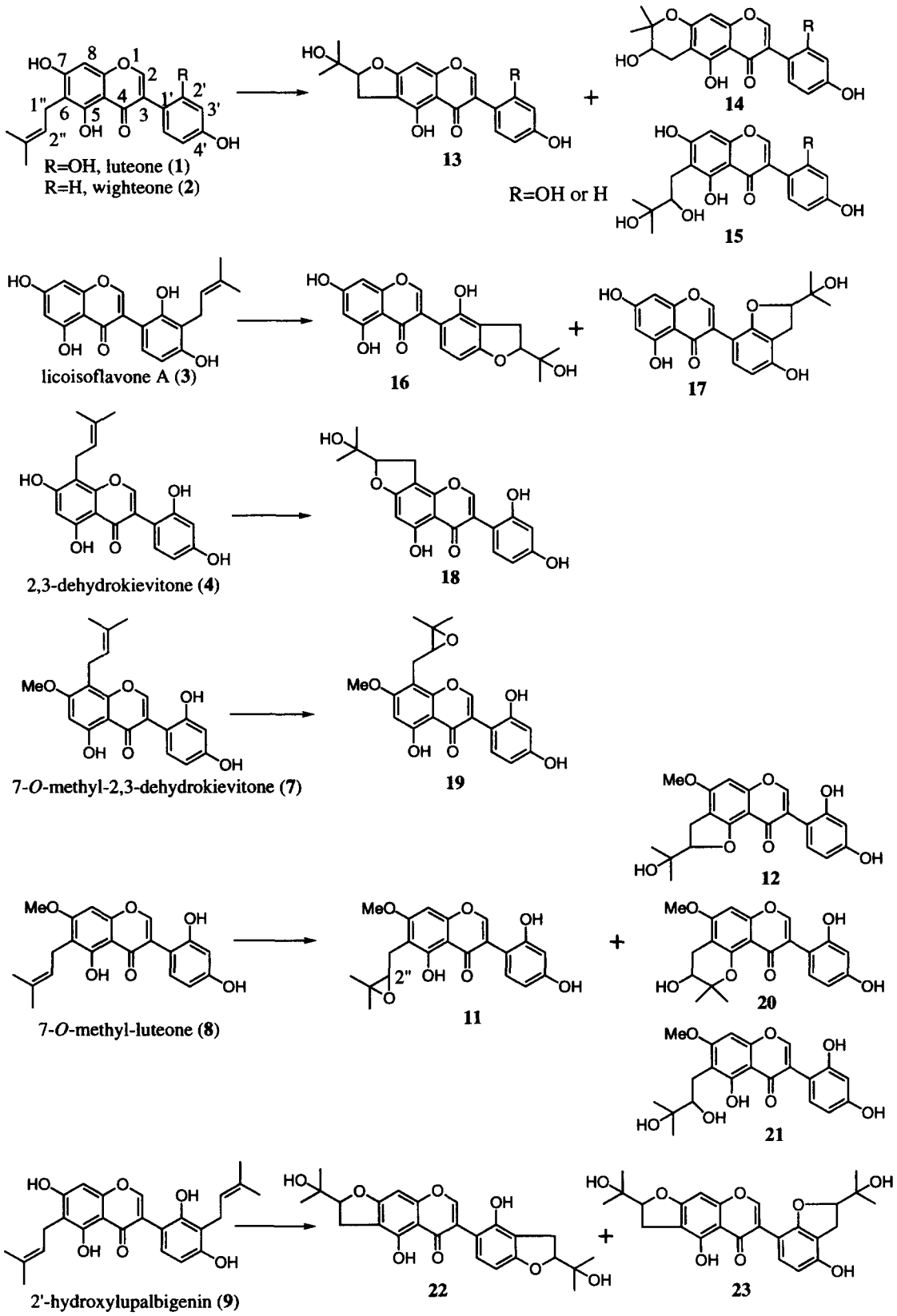


Fig. 3. Structures of epoxidase substrates and reaction products. **14** and **15**: metabolites found in the *Botrytis cinerea* culture administered **1** or **2**. **12**: minor metabolite found in the *B. cinerea* culture administered **8**. **20**: one of major metabolites of **8** found in the *B. cinerea* culture, cyclization product of **11**. **21**: one of major metabolites of **8** found in the *B. cinerea* culture, hydrolysis product of **11**. **22** and **23**: Reaction products have not been identified, but possible structures are shown (see ref. 27 for details).

epoxidation at the terminal dimethyallyl groups has long been found in rat liver [21 and references cited therein], *Candida albicans* [22], and *Saccharomyces cerevisiae* [23]. These enzymes were insensitive to cytochrome P-450 inhibitors, and small amounts of FAD was essential or effective to enhance the reaction. The *Saccharomyces* enzyme was inhibited by terbinafine in a non-competitive manner [23], unlike *Botrytis* epoxidase. Methyl farnesoate 10,11-epoxidase from African migratory locusts was supposed to be a cytochrome P-450 isozyme [25], whilst simple alkene epoxidase from *Nocardia corallina* [26] consists of a non-heme iron containing epoxidizing protein, an FAD and an Fe_2S_2 cluster.

Some aromatic ring hydroxylases, all soluble enzymes, were also known as flavoprotein monooxygenases, e.g. 4-aminobenzoate hydroxylase from mushroom (*Agaricus bisporus*) [27], 3-hydroxyphenylacetate 6-hydroxylase from a *Flavobacterium* species [28] and pterocarpan hydroxylase from *Ascochyta rabiei* [29]. The latter enzyme from *A. rabiei* is a constitutive one which catalyses the conversion of medicarpin and maackiain to their 1a-hydroxy-1,4-dien-3-one derivatives. Although the reaction centres in their substrate and the class of reaction products are apparently different, the *Ascochyta* hydroxylase and *Botrytis* epoxidase showed common properties; (a) low amounts of FAD are necessary for maximal enzyme activity, (b) NADPH is far more effective (3–10 times) than NADH in the reaction mixture without additional FAD, and (c) in the presence of enough amounts of FAD, the efficiency of NADH as a hydride donor increases comparably to that of NADPH (see ref. [29] and Table 3). Interestingly, *A. rabiei* a causative fungus of blight in chick pea which can produce pterocarpan phytoalexins has shown to have small metabolic activity to prenylflavonoids, e.g. luteone (**1**) and 6-prenylnaringenin (**6**) [15].

Although a prenylisoflavone epoxidizing enzyme has not been confirmed in plants, our earlier studies on lupin isoflavonoids revealed the presence of complex isoflavones possessing variously modified side attachments (**b–d**, **f–h** in Fig. 1) [16] possibly being transformed from the corresponding prenylisoflavones via epoxide intermediates. When incubated together with NADPH in the crude protein extract of lupin hypocotyls from seedlings stressed with $CuCl_2$, 7-*O*-methylluteone (**8**) yielded HPLC detectable amounts of the corresponding epoxy-intermediate (**11**), which was hardly found in the reaction mixture using the heat denatured extract. These facts suggest that prenylisoflavone epoxidizing enzyme(s) is presumably present in lupin and taking some roles in the formation of its complex isoflavones.

EXPERIMENTAL

Chemicals. Substrates, 7-*O*-methyl-luteone (**8**), 7-*O*-methyl-2,3-dehydrokievitone (**7**) and 6-prenylnaringenin (**6**), were prepd in our laboratory from luteone (**1**), 2,3-dehydrokievitone (**4**) and commercially available naringenin [18, 30, 31]. Other substrates were isolated from suitable Luguminosae species, e.g. **1**, wighteone (**2**), licoisoflavone A (**3**), and 2′-hydroxylupalbigenin (**9**) from *Lupinus albus* [17], **4** from *L. luteus* [32], and erythbigenin (**10**) from *Piscidia erythrina* [33, 34]. Coenzymes (FAD, FMN, NAD, NADP, NADH and NADPH), glutathione, inhibitors (CO, SKF-525A, ketoconazole, ancymidole, juglone, xanthotoxin and cytochrome c) and chemicals for buffer solns and fungal media were purchased. Terbinafine [(*E*)-*N*-(6,6-dimethyl-2-hepten-4-ynyl)-*N*-methyl-1-naphthalene methanamine, Sandoz Lamisil®] was a gift from SDS Biotech Co. Ltd.

Fungus and culture conditions. Botrytis cinerea AHU 9424 kindly supplied from the Laboratory of Applied Microbiology in our Faculty was maintained on potato dextrose agar slants. Flasks (300 ml) containing 100 ml of a liquid medium prepd and sterilized according to Fritz *et al.* [35] were inoculated with 1 ml each of a conidia suspension from a 10- to 14-day old culture of *B. cinerea*, and incubated at 20° on a rotary shaker (130 rpm) for 70 hr. After this period, 5 mg of 6-prenylnaringenin (**6**) in 1 ml of EtOH was added to each flask, and the incubation was continued for another 20 hr (induction period) before harvesting the mycelia.

Preparation of crude protein extracts and microsomes

All preps described were carried out between 0 and 4° unless otherwise specified. After the induction period, the mycelia (7.5 g, wet wt.) were harvested by suction filtration and washed with 80 mM K-Pi buffer (pH 7.5). To the mycelia were added silica sand (15 g) and 5.0 ml of the buffer soln containing 1 mM of glutathione and glycerol (20%) and the mixt. was ground thoroughly for 15 min using a pre-cooled mortar and pestle. To the resulting suspensions, the buffer (15 ml) was added, mixed well and filtered through fouth-layered gauze. After adding toluene sulphonyl fluoride (1 mM), the filtrate was centrifuged at 3000 *g* for 10 min and the supernatant was subsequently centrifuged at 15 000 *g* for 20 min. The supernatant of the second centrifugation was finally centrifuged at 125 000 *g* for 90 min to give a pellet designated as the microsomal fr. The pellet was resuspended in the buffer (2 ml) and gently homogenized with a Potter–Elvehjen homogenizer. Microsomal prepn thus obtained from *B. cinerea* was stored at −80° for 15 days without remarkable loss of the epoxidase activity and was used as a crude enzyme in the present study if not otherwise mentioned.

Enzyme assay. Substrate (40 μM), NADPH (400 μM) and microsomal protein (250–750 μg) or the crude protein extract (protein, 550–2300 μg) and buffer, then in a total volume of 250 μl was incubated at 20° for 20–60 min. The reaction mixt. was then shaken with 500 μl EtOAc and 10 μl methyl *p*-hydroxybenzoate soln (4 mg/100 ml MeOH) as the

int. standard. An aliquot (400 μl) from the EtOAc layer sepd by centrifugation at 12 000 *g* for 5 min was concd to dryness. The residue solved in 100 μl MeOH was charged on Sep-Pak Plus C_{18} Cartridge (Waters), and the eluate from the cartridge using 500 μl of MeOH was reduced *in vacuo* to adjust the vol. to 50 μl. The final soln (*ca* 30 μl) was analysed by HPLC in an Inertsil ODS-2 column eluting with an aq. MeCN system. The isoflavonoids were sepd by an MeCN gradient from 30 to 70% in H_2O over 20 min, followed by isocratic elution with 70% MeCN for further 30 min, monitoring the eluting compounds with a UV detector at 265 nm. The reaction products were identified by cochromatography with authentic compounds [10–15, 18, 20, 36]. Microsomal and cytosolic proteins were determined using a Bio-Rad protein assay based on Lowry's method [37] and bovine serum albumin as a standard.

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