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UV mutagenesis and enzyme inhibitors as tools to elucidate the late biosynthesis of the spirobisnaphthalenes

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Abstract

The metabolite pattern of UV mutants of the spirobisnaphthalene producing fungus F-24′707 by TLC and HPLC analysis has been investigated. Mutants with differences in colony morphology or colour compared to the parent strain were isolated. Cultivation in shaking flasks and P flasks showed differences in the metabolite pattern of some of the strains. Furthermore, enzyme inhibitors were used to block the spirobisnaphthalene biosynthesis of the parent strain at different steps. Feeding of precursors and intermediates of cladospirone bisepoxide (**15**) led to a two-fold increase of the production of **15**. From these data and preceding biosynthetic studies we deduced a general pathway for the biosynthesis of all spirobisnaphthalenes of the fungus F-24′707. This enables us to present the hypothesis that all bisnaphthalenes described so far are produced using a common pathway with only a few intermediates as central branching points.

Keywords: Fungal metabolites; DHN biosynthesis; *Sphaeropsidales* sp.; Bisnaphthalenes

1. Introduction

Spirobisnaphthalenes represent a growing class of fungal secondary metabolites with different biological activities. A previous paper has reported on the isolation and structure elucidation of 15 spirobisnaphthalenes from the fungus *Sphaeropsidales* sp. F-24-707, eight of them proved to be new (Bode et al., 2000a). UV mutagenesis of the wild type strain led to the isolation of 25 different mutants (named mutant A to Y) differing from the parent strain in colony morphology and colour (Bode et al., 2000b). The parent strain has a dark black substrate and a dark grey aerial mycelium indicating the presence of 1,8-dihydroxynaphthalene (DHN) melanin in the cell walls. Most of the mutants have a brown or grey substrate and a grey to white aerial mycelium. The production of a new macrolide named mutolide with a totally different carbon backbone by one of the mutants indicates the potential of this method, activating or silencing of biosynthetically interesting genes (Bode et al., 2000b). We have analyzed all mutants by TLC and HPLC for their production of secondary metabolites and observed the accumulation of different spirobisnaphthalenes in some cases. Furthermore, we demonstrated the influence of different enzyme inhibitors on the metabolite pattern of the wild type strain. In this paper we present the results of these experiments and give further evidence for a common biosynthetic origin of all bisnaphthalenes described so far. Previously described results, e.g. biosynthetic studies of cladospirone bisepoxide (**15**) and its putative precursor palmarumycin C_{12} (**12**) (Bode et al., 2000c) and the isolation of sphaerolone (**4**) and dihydrosphaerolone (**5**) (Bode and Zeeck, 2000d), two shunt products of the 1,8-dihydroxynaphthalene biosynthesis, confirm this hypothesis.

2. Analysis of the mutants

The picture we obtained from the different experiments was rather complex and consisted of three main groups of mutants. The first group has a difference in colony morphology or colony colour but no difference in the metabolite pattern compared to the wild type; these mutants are not discussed any further. The second group shows a general reduction of the metabolite production. As an example, mutant W becomes only grey after 3 days in shaking flasks (250 rpm, 28°C) compared to the

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dark black colour of the wild type and does not produce any bisnaphthalenes during this time (data not shown). Furthermore, mutant W is the only strain with white aerial mycelium on agar plates (10 days, 28°C). This is a good evidence for a mutation in the polyketide synthase (PKS) gene or in a gene involved in its regulation resulting in the loss of the DHN melanin production. The loss of DHN melanin and bisnaphthalene production indicates the common biosynthetic pathway of both compounds.

Mutants of the third group produce additional spirobisnaphthalenes or accumulate some known intermediates. Mutant T seems to be a cladospirone bisepoxide (**15**) high-producing strain with a more than a two-fold increase of **15** in P flasks (Fig. 1, II). Almost all mutants that accumulate **10** in high amounts (e.g. mutant O, see Fig. 1, III) in the oxygen limited static surface culture, show comparable production of all metabolites in shaking culture compared to the parent strain.

3. Influence of enzyme inhibitors to the metabolite pattern

Addition of ancymidol, an inhibitor of P_{450} dependent mono-oxygenases (Oikawa et al., 1988), resulted in a different metabolite pattern compared to the control experiment. Despite the high concentrations of inhibitor used (100 mg/l) there was no loss but an increase of the cladospirone bisepoxide (**15**) production and additionally **9** and **10** were detected (Fig. 1, I_2). After addition of tetcyclacis, another inhibitor of monooxygenases, only traces of **15** and small amounts of **9** were detected (data not shown).

As described previously, addition of tricyclazole (5 mg/l) suppressed any spirobisnaphthalene biosynthesis due to its inhibition of the reduction of 1,3,8-trihydroxynaphthalene (**2**) to vermelone (**6**), the direct precursor of 1,8-dihydroxynaphthalene (**7**). Instead of different cladospirones, the macrolide mutolide (Bode et al., 2000b) and the bisnaphthalenes sphaerolone (**4**) and dihydrosphaerolone (**5**) and 2-hydroxyjuglone (**3**) (Bode and Zeeck, 2000d) have been isolated from the culture broth. Furthermore, ancymidol causes a reduction of the mutolide biosynthesis by simultaneous addition of tricyclazole (data not shown).

4. Feeding of precursors of the cladospirone bisepoxide biosynthesis

1,8-Dihydroxynaphthalene (**7**), palmarumycin C_2 (**9**) and palmarumycin C_3 (**10**) were fed to growing cultures

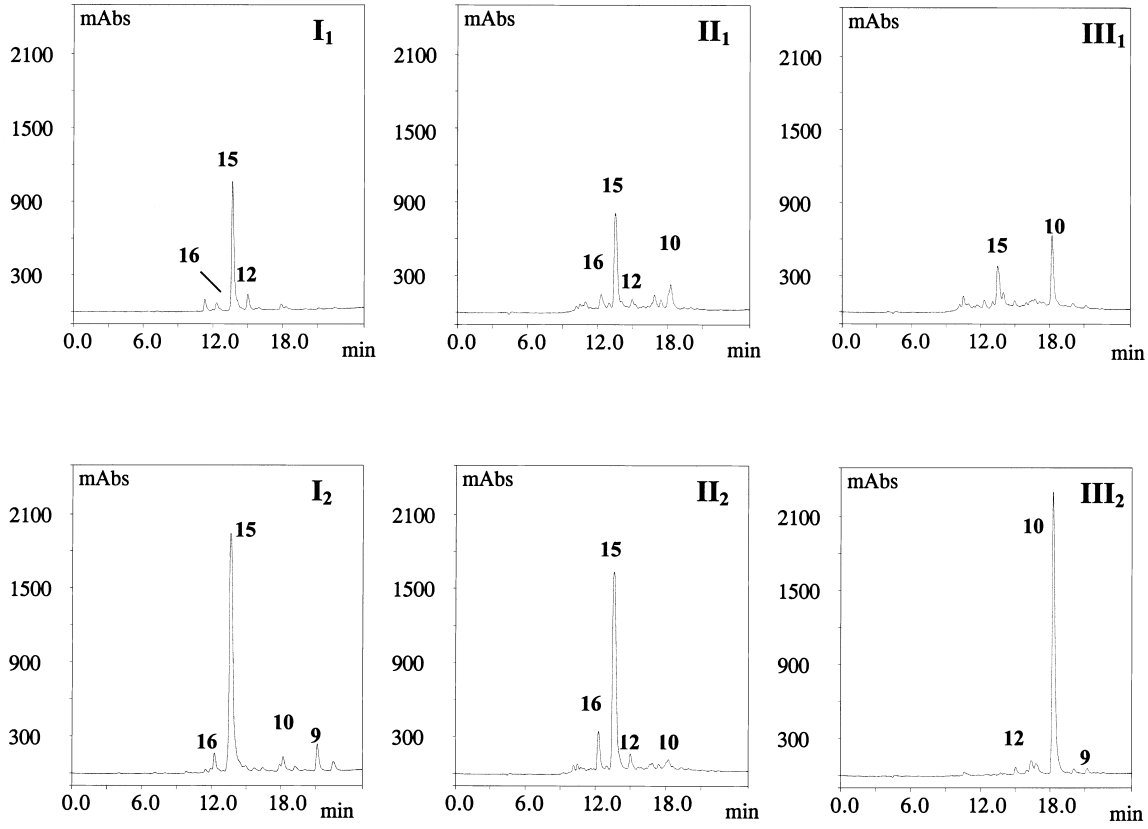
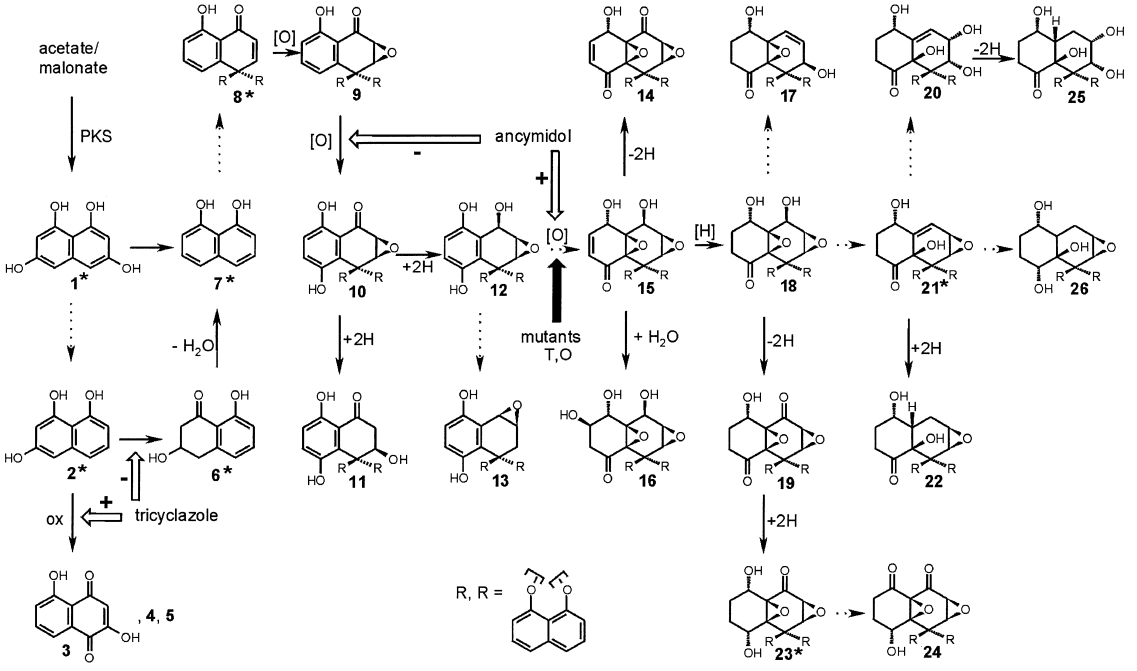


Fig. 1. HPLC chromatograms of crude extracts of the wild type (top) compared to the wild type with ancymidol (I_2), mutant T (II_2) and mutant O (III_2). I: Shaking flasks, medium 1; II: *Penicillium* flasks (P flasks), medium 1; III: P flasks, medium 2.

of the strain in one portion (0.5 g/l) after 20 h in order to investigate the role of these putative intermediates in the biosynthesis of cladospirone bisepoxide (**15**). Almost a doubling of the production of **15** was observed in all three experiments compared to the control experiment by HPTLC analysis. HPLC analysis confirmed these results (data not shown). Feeding of **7**, **9** or **10** and inhibition of the DHN biosynthesis by tricyclazole at the same time did not restore the production of **15** and no new metabolites were observed by HPTLC or HPLC analysis.

5. Discussion

Scheme 1 shows the proposed biosynthetic pathway of the isolated bisnaphthalenes of strain F-24′707. The marked compounds (*) represent intermediates that have not been isolated so far from our strain. The first steps are common to the biosynthesis of DHN (Wheeler and Bell, 1988), spirobisnaphthalenes (Krohn et al., 1994b; Bode et al., 2000a) and of sphaerolone (**4**)/dihydrosphaerolone (**5**) (Bode and Zeeck, 2000d) and have been presented previously. Besides the unexpected isolation of mutolide, the isolation of **4** and **5** and the loss of the spirobisnaphthalene production after inhibition of the DHN biosynthesis with tricyclazole clearly demonstrates the DHN origin of all isolated bisnaphthalenes. The results obtained from the analysis of the different mutants and the biosynthetic studies led us to propose a main biosynthetic route starting from palmarumycin CP_1 (**8**) (Krohn et al., 1994a), the first postulated spirobisnaphthalene that was isolated from the fungus *Coniothyrium palmarum*. In our strain, **8** is oxidized to palmarumycin C_2 (**9**) (Krohn et al., 1994b) by an oxygenase that is not inhibited by ancymidol or tetcyclacis. **9** is oxidized by a P_{450}-dependent monooxygenase to **10** as deduced from its accumulation after addition of ancymidol. After reduction of **10** to palmarumycin C_{12} (**12**), **12** is further oxidized leading to cladospirone bisepoxide (**15**) (Petersen et al., 1994; Thiergardt et al., 1994, 1995). **10** is the main metabolite at oxygen limited conditions (static surface culture) together with cladospirone B (**11**) and F (**13**) (Bode et al., 2000a) as reduced shunt products. **15** is the main product in shaking flasks and high oxygen supply (e.g. airlift fermenter, data not shown). Oat grains with their large solid surface and long incubation times promote the production of **15**. As a result of this accumulation, several shunt products of **15** can be found [cladospirones C (**22**), D (**24**), E (**17**), G (**26**), H (**25**), I (**20**) (Bode et al., 2000a), diepoxins α (**19**), δ (**16**), σ (**14**) [(Schlingmann et al., 1993, 1996) and SCH49210 (**23**) (Chu et al., 1994)] derived by simple reductions/oxidations of the keto/hydroxy groups or by more complex reaction sequences. Reductive opening of one of both epoxides followed by dehydratation of the resulting hydroxy group would result in the formation of cladospirone E



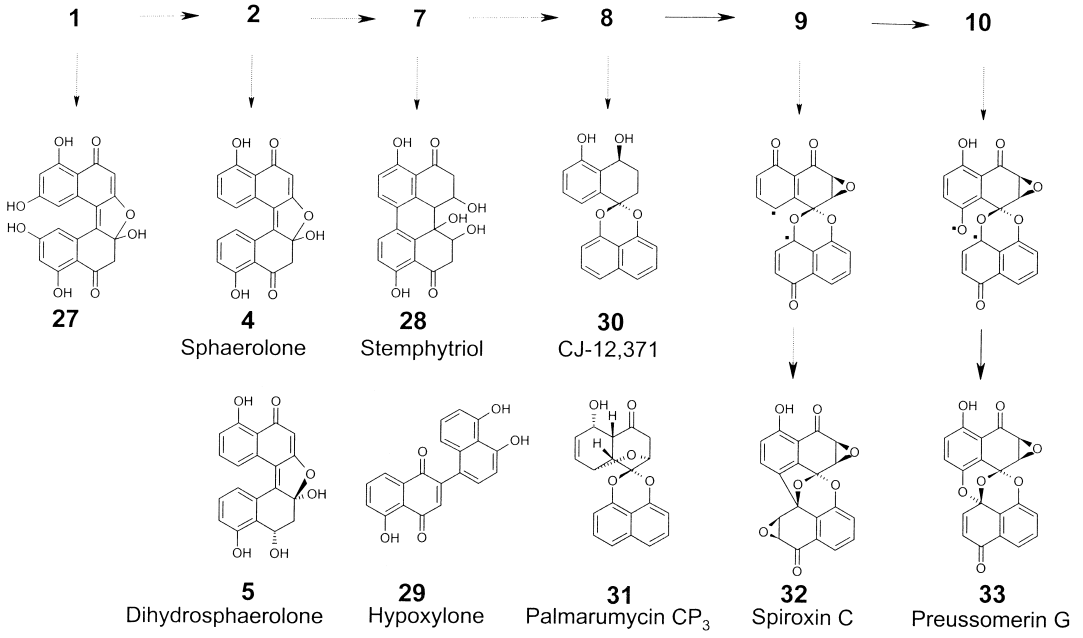
Scheme 1. Biosynthetic origin of the bisnaphthalenes isolated from *Sphaeropsidales* sp. F-24′707. -2H = reduction reaction, [O] = oxygenation reaction, $-H_2O$ = dehydratase reaction, + = site of stimulation, – = site of inhibition, PKS = polyketide synthase. Broken arrows indicate multistep reactions. Marked compounds (*) have not been isolated from strain F-24′707 so far but have been isolated either from other fungi (**1**, **2**, **6–8**, **23**) or represent postulated intermediates (**21**).

(**17**) and the postulated intermediate **21** that is modified in further reactions to give **20**, **22**, **25** and **26** (Bode et al., 2000a). The increase of the cladospirone bisepoxide production after feeding palmarumycin C_2 (**9**) and C_3 (**10**) to the strain further supports the biosynthesis of hydroxylated and epoxidized compounds via stepwise oxygenation of reduced precursors.

One can speculate about the conversion of palmarumycin C_{12} (**12**) to cladospirone bisepoxide (**15**), a formal epoxidation of an aromatic compound and an oxygen exchange at C-8 (Bode et al., 2000c). Eight of 25 mutants show differences in this transformation and this might support a multi-enzyme sequence with many potential targets for UV mutagenesis. Nevertheless, no mutant could be isolated that show the complete loss of the bisnaphthalenes production, probably due to the small number of mutants isolated. Although such mutants should not be lethal, the tricyclazole experiments show that only a defect in the PKS gene would result in a DHN and bisnaphthalene non-producing 'white' strain.

All bisnaphthalenes described so far can be explained as branches of the postulated biosynthetic main route as depicted in Scheme 2. Compound **27** (Yoshida et al., 1993), patented as aldose reductase inhibitor, and **4** and **5** are dimerisation products of the early DHN intermediates 1,3,6,8-tetrahydroxynaphthalene and 1,3,8-trihydroxynaphthalene, respectively. Dimerisation of DHN (**7**) would result in the production of the perylenes [e.g. stemphytriol (**28**) (Krohn et al., 1999)] bearing two C–C bridges, simple dimers as hypoxylone (**29**) (Bodo et al., 1983) with one C–C bridge or in the formation of palmarumycin C_P1 (**8**) bearing the typical spiro-ketal. **8** is the putative precursor of some reduced derivatives [e.g. CJ-12,371 (**30**) (Sakemi et al., 1995)] and of the oxygen-bridged palmarumycins CP_3 (**31**) (Krohn et al., 1994a; Bringmann et al., 1997) or CP_{4a} (Krohn et al., 1997). Palmarumycin C_2 (**9**) is the precursor of palmarumycin C_3 (**10**) and of the latest subtype of bisnaphthalenes, the spiroxins (McDonald et al., 1999). Oxygenation at C-5′ and oxidation to a diradical similar to the formation of palmarumycin CP_1 (**8**), intramolecular dimerisation and epoxidation of these so far unknown intermediates would give spiroxin C (**32**) that can be modified further by oxidation, reduction or chlorination leading to the spiroxins A, B, D and E. Palmarumycin C_3 (**10**) is the direct precursor of a number of palmarumycins [e.g. palmarumycin C_{12} **12**)] and other spirobisnaphthalenes bearing the typical hydroquinone chromophore (Chu et al., 1996). After oxygenation at C-5′, diradical formation and intramolecular dimerisation similar to the spiroxin biosynthesis, Preussomerin G (**33**) (Singh et al., 1994) is formed, which can be reduced in additional steps leading to the preussomerins A–F and H, potent RAS-farnesyl transferase inhibitors (Weber et al., 1990; Weber and Gloer, 1991).

More than 70 bisnaphthalenes are described in the literature. Most of them show interesting biological activities (see references cited) and all of them are interconnected by only a small set of similar biosynthetic modifications (e.g. oxidation, reduction, epoxidation, chlorination) starting from precursors that are derived from the fungal DHN biosynthesis. Therefore, one can postulate a much greater variety of bisnaphthalenes which need to be identified either by chance (e.g. UV mutagenesis) or by directed



Scheme 2. Proposed biosynthetic relationship between the known bisnaphthalenes. Broken arrows indicate multistep reactions.

genetic engineering. Using an advanced combinatorial biosynthetic approach, it should be possible to express selected genes of these interesting pathways in other fungi in order to generate predicted bisnaphthalenes that can be tested in various bioassays.

6. Experimental section

6.1. General

The strain was described previously (Petersen et al., 1994). For UV mutagenesis, cultivation of the mutants and the wild type, and HPTLC analysis of the crude extracts (see Bode et al., 2000a,b). Medium 1 (2% defatted soybean meal, 2% oatmeal, 2%, glucose, deionised water), medium 2 (2% glycerol, 1% malt extract, 0.4% yeast extract, deionised water). Palmarumycin CP_1 (**8**) was prepared as described previously (Ragot et al., 1998, 1999; Barrett et al., 1998). The influence of enzyme inhibitors on the wild type was investigated by addition of the inhibitor from the beginning of the fermentation (tricyclazole) (Bode et al., 2000b) or during the production phase (ancymidol) in three portions after 44, 50 and 54 h. Feeding experiments were performed as described previously (Bode et al., 2000c). 50 mg of palmarumycin C_2 (**9**) or palmarumycin C_3 (**10**) were fed in one portion to two 100 ml cultures in 350 ml Erlenmeyer flasks to *Sphaeropsidales* sp. F-24′707 after 20 h and the cultures were harvested after 36 h. After extraction of the culture broths with ethyl acetate, the obtained crude extracts were analyzed by HPTLC (prescreening) and HPLC using palmarumycin CP_1 (**8**) [$R_t = 23.1$], palmarumycin C_2 (**9**) [$R_t = 21.1$], palmarumycin C_3 (**10**) [$R_t = 18.2$], palmarumycin C_{12} (**12**) [$R_t = 15.0$], cladospirone bisepoxide (**15**) [$R_t = 13.7$] and diepoxin δ (**16**) [$R_t = 12.3$] as standards.

6.2. HPLC analysis

The following equipment was used: Kontron P 322 (0.05–10 ml/min), Kontron Sa 360, Kontron DAD 440, Kontron Multiport RS 232, Kontron KromaSystem 2000, analytical 20 μl-sample volume. Pre-column Beckmann Ultrasphere ODS (5 μm, 4.6×45 mm), column Knauer Nucleosil 100 C18 (analytic, 5 μm, 3×250 mm). Solvents: A 0.1% H_3PO_4; B: Acetonitrile; Gradient: 100% B to 20% B in 5 min, 20% B to 100% B in 20 min, 100% B for 5 min. Flowrate 0.5 ml/min. Detection 220, 254, 270 and 366 nm.

All crude extracts were dissolved in equal amounts of acetone (P flasks 10 ml, shaking flasks 5 ml) and aliquots were evaporated in 0.5 ml-Eppendorf reaction cups. After evaporation the brown residues were dissolved in 0.5 ml of acetonitrile/water 1:1, centrifuged and subjected to HPLC analysis.

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