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Biosynthesis of Cladospirone Bisepoxide, A Member of the Spirobisnaphthalene Family

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The biosynthesis of cladospirone bisepoxide (**1**) was investigated by feeding ^{13}C-labeled acetate to growing cultures of the fungus *Sphaeropsidales* sp. (strain F-24′707). ^{13}C NMR spectral analysis demonstrated the polyketide origin of both naphthalene units. The origin of two epoxide oxygens was confirmed as from air by cultivation of the strain in an $^{18}O_2$-enriched atmosphere. The [^{18}O]incorporation pattern into palmarumycin C_{12} (**11**), the putative precursor of **1** led to the hypothesis that the carbonyl oxygen of **1** is derived from water by exchange of an oxygen atom. Inhibition of the biosynthesis of **1** with tricyclazole, an inhibitor of the 1,8-dihydroxynaphthalene (DHN) melanin biosynthesis, confirmed the connection of both biosynthetic pathways.

The fungal metabolite cladospirone bisepoxide[1,2] (diepoxin ζ[3], Sch53514[4], palmarumycin C_{13}[5]) (**1**) is a member of the rapidly growing group of the so-called spirobisnaphthalenes showing antitumor[4,6], antifungal[1,3] and antibacterial[1,3] activity. The absolute structure of **1** as a result of spectroscopical analysis, X-ray analysis[7] and ECCD spectroscopy[8] is shown in Fig. 1. This paper deals with the biogenetic origin of all carbon atoms and oxygens of cladospirone bisepoxide (**1**) from *Sphaeropsidales* sp. (strain F-24′707).

Fermentation and Isolation

Fermentations of strain F-24′707 were carried out as described previously[1,9]. Production of cladospirone bisepoxide (**1**) started at about 31 hours and reached a maximum after 47 hours. For feeding experiments the strain was cultivated in 350 ml Erlenmeyer flasks for 60 hours yielding 40~50 mg/litre of **1**. **1** was isolated from the culture broth after homogenisation in the presence of ethyl acetate and centrifugation to remove unsoluble material. The aqueous phase was separated and extracted twice with ethyl acetate. The combined organic phases were dried over anhydrous sodium sulfate and evaporated to dryness. Repeated silica gel chromatography followed by a chromatography step using Sephadex LH-20 yielded pure **1** that crystallized from methanol.

Feeding Experiments

The NMR signals of **1** were assigned unambigously by 2D-NMR measurements in CD_3OD. Feeding of sodium [1-^{13}C]acetate resulted in signal enhancement of C-2, C-4, C-5, C-7, C-8a, C-1′, C-3′, C-4a′, C-6 and C-8′ as depicted in Table 1. The ^{13}C NMR spectrum of **1** after feeding of sodium [1,2-$^{13}C_2$]acetate showed complex signals for each carbon from both possible incorporation patterns in each half of the molecule resulting in four isotopomers (Fig. 2).

Fig. 1. Structural formula of cladospirone bisepoxide (**1**).

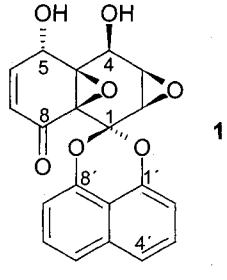
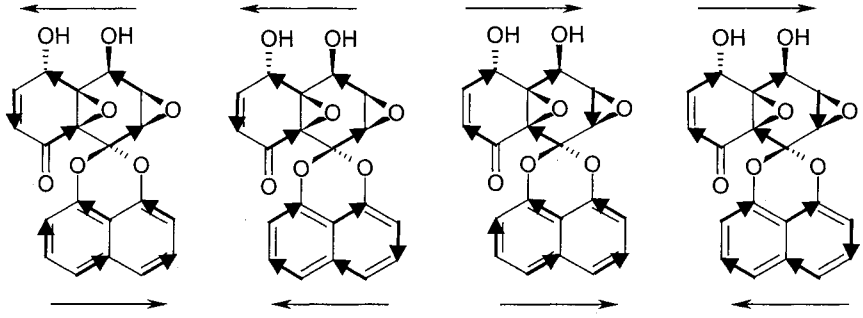


Fig. 2. Incorporation pattern of the four different isotopomers of **1** after feeding with sodium[1,2-$^{13}C_2$]acetate. The long arrows indicate the overall direction of the biosynthesis for each naphthalene subunit.



To establish the biogenesis of the oxygen atoms of **1** we decided to cultivate *Sphaeropsidales* sp. F-24'707 in an [$^{18}O_2$]-enriched atmosphere. The obtained cladospirone bisepoxide (**1**) (47% ^{18}O incorporation) showed significant α-isotopic shifts in the ^{13}C NMR spectrum for C-2 (28 ppb), C-3 (33 ppb), C-4a (34 ppb) and C-8a (25 ppb). During the same experiment palmarumycin C_{12} (**11**)[5] (71 % ^{18}O incorporation) was obtained that showed α-isotopic shifts for C-2 (29 ppb), C-3 (33 ppb) and C-8 (11 ppb).

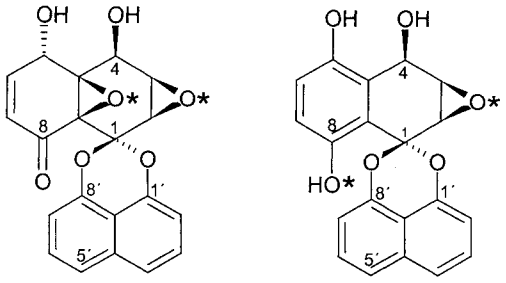
Table 1. ^{13}C NMR signals in CD_3OD of **1** together with specific incorporations[10] and coupling constants after feeding with sodium [1-^{13}C]acetate (I) and sodium[1,2-$^{13}C_2$]acetate (II).

C-atom	δ_c (ppm)	I	II ($^1J_{CC}$/Hz)
1	96.5	0.0	66.5; 61.0
2	54.6	**9.0**	27.0; 61.0
3	56.7	0.1	27.0; 48.5
4	62.2	**7.9**	51.0; 48.5
4a	72.0	0.1	51.0; 51.0
5	62.6	**9.6**	45.0; 51.0
6	144.9	0.2	45.0; 63.0
7	127.1	**8.8**	56.0; 63.0
8	189.9	0.1	56.0; 56.0
8a	64.2	**3.6**	66.5; 56.0
1'	146.9	**6.1**	75.0; 61.0
2'	110.2	0.0	75.0; 55.5
3'	128.8	**11.5**	61.0; 55.5
4'	121.90	0.0	61.0; 56.0
4a'	135.7	**12.2**	56.0; 56.0
5'	121.86	-0.2	56.0; 61.0
6'	128.5	**9.4**	55.5; 61.0
7'	109.7	0.0	55.5; 75.0
8'	147.2	**9.4**	61.0; 75.0
8a'	113.4	-0.3	61.0; 61.0

Discussion

Our experiments have provided a nearly complete picture of the biosynthesis of cladospirone bisepoxide (**1**). There is evidence that both naphthalene moieties derive from the same pentaketide precursor *via* a fungal polyketide synthase (PKS). Condensation, further modifications and incorporation of oxygen from air *via* postulated monooxygenases led to **1**. The results indicated that the biosynthesis of both naphthalene subunits of **1** proceeds *via* a symmetric intermediate. The observed inhibition of the biosynthesis after addition of tricyclazole confirmed that possible precursors could derive from the 1,8-dihydroxynaphthalene (DHN) biosynthesis as proposed by KROHN *et al.*[5]. The biosynthetic pathway of DHN melanin has been elucidated using melanin-deficient mutants of the fungi *Wangiella dermatidis*, *Colletotrichium lagenarium*, *Magnaporthe grisea* and *Verticillium dahliae* which accumulate shunt products and exhibit pigmentation phenotypes[11~18]. The biosynthesis in *C. lagenarium* starts from pentaketide synthesis and proceeds to form scytalone (**3**) *via* reduction of 1,3,6,8-tetrahydroxynaphthalene (**2**), the first free product of the polyketide synthase. Dehydration of **3** to 1,3,8-trihydroxynaphthalene (**4**), reduction of **4** to vermelone (**5**) and dehydration of the latter yielded 1,8-dihydroxynaphthalene (DHN, **6**) that is oxidized and polymerized to DHN melanin. Although tricyclazole inhibits the reduction

Fig. 3. Labeling pattern of **1** and **11** by [$^{18}O_2$*].



1 **11**

of **4** to **5**[19,20] no accumulation of **4** could be detected. The dark colouring of the cultures differs slightly from control cultures where no tricyclazole was added. This is consistent with the formation of melanin-like dyes from accumulated intermediates.

In contrast to the fungal PKS recent findings suggest the involvement of a chalcone synthase (CHS) in the biosynthesis of similar compounds from bacteria[21~24]. FUNA *et al.*[25] isolated the first bacterial chalcone synthase (CHS) and the corresponding gene from *Streptomyces griseus* that produces a red-brown pigment and they were able to show the synthesis of **2** *in vitro* using the purified enzyme.

Besides new spirobisnaphthalenes and the new macrolide mutolide[6] we were able to isolate palmarumycin C_2 (**9**), palmarumycin C_3 (**10**) and palmarumycin C_{12} (**11**)[5] from the fungus F-24'707 by variation of the culture conditions[9]. In addition with palmarumycin CP_1 (**8**) which was isolated from the fungus *Coniothyrium palmarum*[27] these compounds are putative intermediates in the biosynthesis of **1** and further evidence for this hypothesis was the isolation of the first 'open chain' palmarumycin from *C. palmarum*[28]. Following this hypothesis cladospirone bisepoxide is the last product of a stepwise oxygenation that starts with the oxidative coupling of two DHN molecules. Interestingly, the similar 'open chain' compound **7** and the first cyclisation product **8** could not yet be detected in the fermentation broth of the fungus F-24'707. However, cladospirone bisepoxide (**1**) is a highly oxygenated compound and **7** and **8** as putative intermediates may be rapidly oxidized to the isolated members of this interesting group of compounds during the fermentation process. The last step of this pathway is the epoxidation of palmarumycin C_{12} (**11**) followed by proton rearrangement which is accompanied by oxygen exchange at C-8 leading to **1**. This reaction may start with the attack of water at C-8 of **11** resulting in the hydrate of **1**, which looses water in an equilibrium. Although addition of **9** and **10** to growing cultures of the strain and simultaneously addition of tricyclazole did not lead to production of **1** in a detectable amount, further experiments feeding labeled palmarumycin C_{12} (**11**) are planned. To exclude a failure of these experiments due to cell permeability problems of the highly lipophylic precursors, experiments with a cell-free system are in progress.

Experimental

General

^{13}C NMR spectra of pure cladospirone bisepoxide (**1**) were recorded with a Varian VRX 500 and a Varian Inova 500 using standard Varian software. Chemical shifts are expressed in δ values (ppm) using the solvent as internal reference (CD_3OD: δ_c=49.0).

Labeled Compounds

^{13}C-labeled compounds were 99% ^{13}C atom purity. 10.1 mmol/litre of sodium [1-^{13}C]acetate (Campro Scientific) and 8 mmol/litre of sodium [1,2-$^{13}C_2$]acetate (Campro Scientific) were fed. $^{18}O_2$ (99% purity, 1260 ml for 400 ml culture broth) was purchased from Campro Scientific.

Incorporation of Isotope-labeled Compounds to 1

Spaeropsidales sp. F-24'707 was grown as shaking cultures in 8×350 ml-Erlenmeyer flasks with three intrusions and 100 ml medium consisting of: glucose 2%, degreased soy bean meal 2% and oat meal 2% in deionized water without pH adjustment. The cultures were inoculated with 2.5 vol-% of a pre-culture grown for 72 hours on a rotary shaker at 250 rpm and 28°C. The precursors were added to each culture following the pulse feeding method in 1 ml portions at 32, 35, 38, 41 and 44 hours after incubation.

For the incorporation of $^{18}O_2$ the following experiment was conducted: The cultures were grown until the start of the production phase (25 hours). After flushing the flasks with nitrogen for 3 minutes they were connected to a apparatus described previously[29]. From 25 to 43 hours pure $^{18}O_2$ (1260 ml) was fed to the culture followed by addition of 400 ml of $^{16}O_2$ for the last 5 hours. The rate of oxygen consumption remained steady within the range of 30~40 ml/hour (4×100 ml culture in 350 ml-Erlenmeyer flasks with three intrusions) over the production phase (25~48 hour). The cultures were harvested after 48 hours.

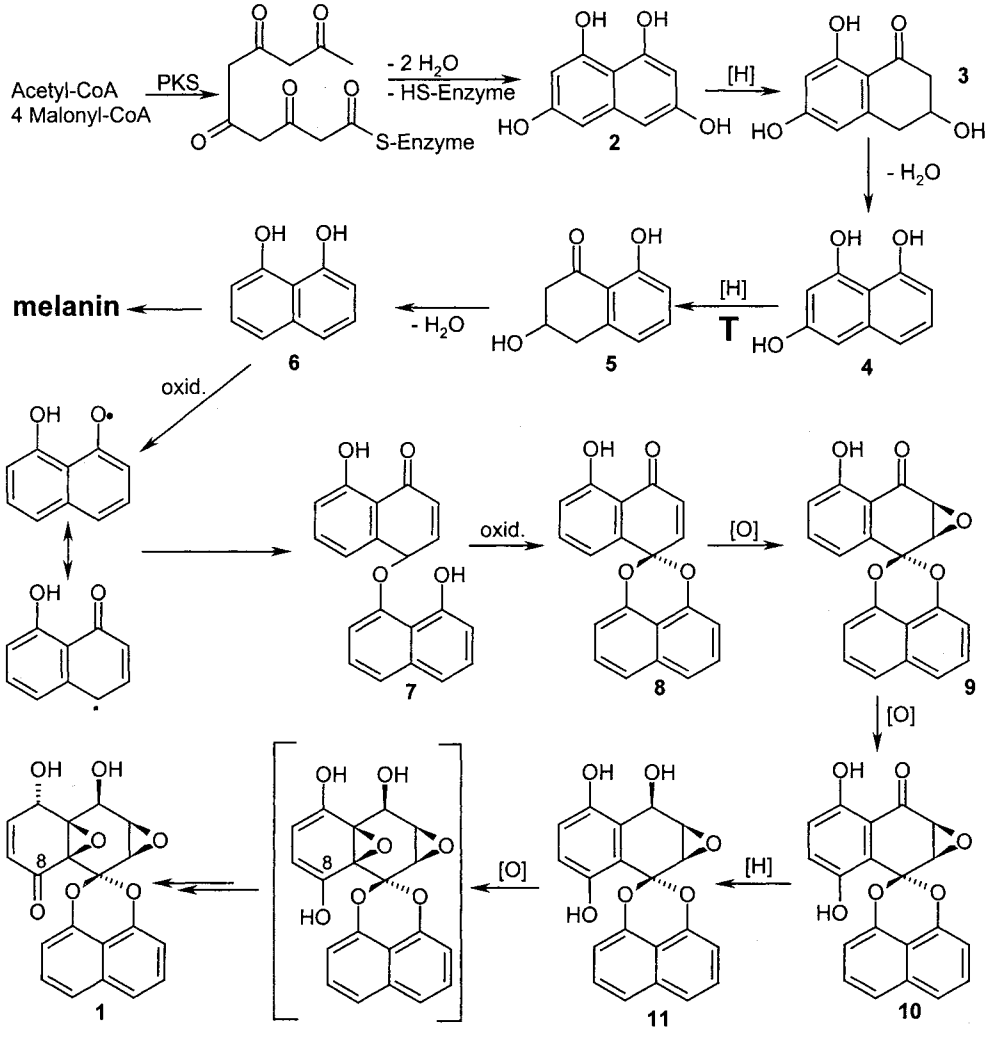
Inhibition of the Cladospirone Bisepoxide Biosynthesis by Tricyclazole

Fermentation was carried out as described for biosyn-

Scheme 1. Proposed intermediates of the cladospirone bisepoxide biosynthesis.

1,3,6,8-Tetrahydroxynaphthalene (**2**), scytalone (**3**), 1,3,6-trihydroxynaphthalene (**4**), vermelone (**5**), 1,8-dihydroxynaphthalene (**6**), palmarumycin CP_1 (**8**), palmarumycin C_2 (**9**), palmarumycin C_3 (**10**), palmarumycin C_{12} (**11**), cladospirone bisepoxide (**1**).

T: Inhibition site of tricyclazole.



thetic studies but with addition of tricyclazole (BASF AG, Ludwigshafen; 0.1, 1 and 10 mg/litre) from the beginning of the fermentation. After 72 hours the cultures were harvested and worked-up as described before. TLC analysis of the ethyl acetate extract showed no detectable amount of **1** for the different concentrations of tricyclazole.

Isolation of **1**

An equal amount of ethyl acetate was added to the culture broth and the mixture was homogenized for 5 minutes with a blender (Ultra-Turrax, Janke & Kunkel KG). After centrifugation to remove unsoluble material the phases were separated and the aqueous phase was extracted twice with ethyl acetate. The combined organic phases were dried over anhydrous sodium sulfate and concentrated *in vacuo* to an oily residue that was subjected to silica gel chromatography (30×2.5 cm, ICN SiliTech 32-63, 60 Å) with ethyl acetate/petrol ether (1 : 1) as eluent. Gel permeation chromatography (Sephadex LH-20, 100×2.5 cm, acetone) of fractions containing **1** yielded the labeled metabolite in the following amounts: 69 mg/litre ([1-^{13}C] acetate), 116 mg/litre ([1,2-$^{13}C_2$]acetate), 95 mg/litre ([$^{18}O_2$]). Isolation of ^{18}O-enriched palmarumycin C_{12} (**11**) followed the cladospirone bisepoxide protocol and yielded 150 mg/litre of a white solid.

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