Biosynthesis of Cladosporone Bisepoxide, A Member of the Spirobisnaphthalene Family

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The biosynthesis of cladosporone 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 [18O]incorporation pattern into palmarumycin C12 (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 cladosporone bisepoxide$^{1,2)}$ (diepoxin $\xi_3$), Sch53514$^5$), palmarumycin C13$^5$) (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 cladosporone 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 cladosporone 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 unambiguously by 2D-NMR measurements in CD$_2$OD. 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]acetate showed complex signals for each carbon from both possible incorporation patterns in each half of the molecule resulting in four isotopomers (Fig. 2).
To establish the biogenesis of the oxygen atoms of 1 we decided to cultivate Sphaeropsidales sp. F-24'707 in an [18O2]-enriched atmosphere. The obtained cladospirone bisepoxide (1) (47% 18O incorporation) showed significant a-isotopic shifts in the 13C 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 C12 (ll)5) (71 % 18O incorporation) was obtained that showed a-isotopic shifts for C-2 (29 ppb), C-3 (33 ppb) and C-8 (11 ppb).

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 monoxygenases 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. 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

Table 1. 13C NMR signals in CD3OD of 1 together with specific incorporations and coupling constants after feeding with sodium [1-13C]acetate (I) and sodium[1,2-13C2]acetate (II).

<table>
<thead>
<tr>
<th>C-atom</th>
<th>δ (ppm)</th>
<th>I</th>
<th>II (J/Hz)</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>96.5</td>
<td>0.0</td>
<td>6.5; 61.0</td>
</tr>
<tr>
<td>2</td>
<td>54.6</td>
<td>9.0</td>
<td>27.0; 61.0</td>
</tr>
<tr>
<td>3</td>
<td>56.7</td>
<td>0.1</td>
<td>27.0; 48.5</td>
</tr>
<tr>
<td>4</td>
<td>62.2</td>
<td>7.9</td>
<td>51.0; 48.5</td>
</tr>
<tr>
<td>4a</td>
<td>72.0</td>
<td>0.1</td>
<td>51.0; 51.0</td>
</tr>
<tr>
<td>5</td>
<td>62.6</td>
<td>9.6</td>
<td>45.0; 51.0</td>
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<tr>
<td>6</td>
<td>144.9</td>
<td>0.2</td>
<td>45.0; 63.0</td>
</tr>
<tr>
<td>7</td>
<td>127.1</td>
<td>8.8</td>
<td>56.0; 63.0</td>
</tr>
<tr>
<td>8</td>
<td>189.9</td>
<td>0.1</td>
<td>56.0; 56.0</td>
</tr>
<tr>
<td>8a</td>
<td>64.2</td>
<td>3.6</td>
<td>66.5; 56.0</td>
</tr>
<tr>
<td>1'</td>
<td>146.9</td>
<td>6.1</td>
<td>75.0; 61.0</td>
</tr>
<tr>
<td>2'</td>
<td>110.2</td>
<td>0.0</td>
<td>75.0; 55.5</td>
</tr>
<tr>
<td>3'</td>
<td>128.8</td>
<td>11.5</td>
<td>61.0; 55.5</td>
</tr>
<tr>
<td>4'</td>
<td>121.90</td>
<td>0.0</td>
<td>61.0; 56.0</td>
</tr>
<tr>
<td>4a'</td>
<td>135.7</td>
<td>12.2</td>
<td>56.0; 56.0</td>
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<tr>
<td>5'</td>
<td>121.86</td>
<td>-0.2</td>
<td>56.0; 61.0</td>
</tr>
<tr>
<td>6'</td>
<td>128.5</td>
<td>9.4</td>
<td>55.5; 61.0</td>
</tr>
<tr>
<td>7'</td>
<td>109.7</td>
<td>0.0</td>
<td>55.5; 75.0</td>
</tr>
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<td>8'</td>
<td>147.2</td>
<td>9.4</td>
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<td>8a'</td>
<td>113.4</td>
<td>-0.3</td>
<td>61.0; 61.0</td>
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Fig. 3. Labeling pattern of 1 and 11 by $^{18}$O$_2$.*

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$_5$ (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$^{21}$ 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 biosynthesis of 1 and further evidence for this hypothesis was the isolation of the first ‘open chain’ palmarumycin from C. palmarum$^{28}$. For the incorporation of 18O$_2$ 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 an apparatus described previously$^{29}$. From 25 to 43 hours pure 18O$_2$ (1260 ml) was fed to the culture followed by addition of 400 ml of 16O$_2$ for the last 5 hours. The rate of oxygen consumption remained steady within the range of 30~40 ml/hour (4X 100ml culture broth) over the production phase (25~48 hour). The cultures were harvested after 48 hours.

Inhibition of the Cladosporine Bisepoxide Biosynthesis by Tricyclazole

Fermentation was carried out as described for biosyn-
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 ([1-13C] acetate), 116 mg/litre ([1,2-13C2] acetate), 95 mg/litre ([18O2]). Isolation of 18O-enriched palmarumycin C12 (11) followed the cladospirone bisepoxide protocol and yielded 150 mg/litre of a white solid.
References


