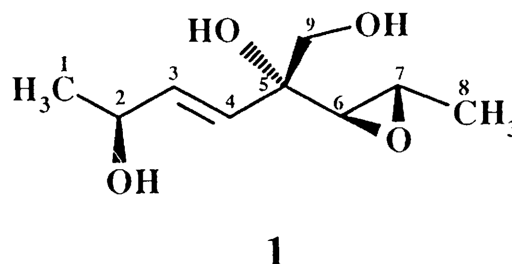
Biosynthesis of aspinonene, a branched pentaketide produced by *Aspergillus ochraceus*, related to aspyrone

Jens Fuchser,[a] **Ralf Thiericke**[b] **and Axel Zeeck***[,a]
[a] *Institut für Organische Chemie, Universität Göttingen, Tammannstrasse 2, D-37077 Göttingen, Germany*
[b] *Hans-Knöll-Institut für Naturstofforschung e. V. Beutenbergstrasse 11, D-07745-Jena, Germany*

Feeding experiments with [^{13}C]-labelled acetates and [$^{18}O_2$] to *Aspergillus ochraceus* (strain DSM-7428) have been carried out to evaluate the biosynthesis of aspinonene **1**. The results reveal a close relationship to aspyrone **2**. After a rearrangement reaction of a pentaketide intermediate the hypothetical bisepoxide **5** is either oxidised leading to **2**, or reduced leading to **1**. Both pathways were found to be active in *A. ochraceus*, and could be directed towards **2** by using increased dissolved oxygen concentrations during fermentation.

In the course of our chemical screening routine we discovered a new fungal metabolite in the culture broth of *Aspergillus ochraceus* (DSM-7428), named aspinonene **1**.[1] The branched chain as well as the unusual oxygenation pattern of aspinonene **1** stimulated us to investigate its biosynthesis. Evaluating the structure of **1** we presumed a polyketide-like pathway. In order to examine the biogenesis of the carbon skeleton and the origin of the oxygen atoms we carried out feeding experiments with sodium [1-^{13}C]-, [2-^{13}C]- and [1,2-$^{13}C_2$]-acetate as well as a fermentation in an [$^{18}O_2$]-enriched atmosphere.



Results and discussion

Due to the acidity of the culture medium, which appeared to be crucial for aspinonene production, typical polyketide precursors like sodium [1-^{13}C]-, [2-^{13}C]- and [1,2-$^{13}C_2$]-acetate were added to cultures (stirred vessels, 1.5 dm^3) of strain DSM-7428 by pulse-feeding in pH-static fermentations ($4.5 > pH > 3.5$). Using these conditions the logarithmic growing phase of the strain ends after 100 h of cultivation, thus, the addition of the precursors was started after 120 h. The feeding experiment with sodium [1-^{13}C]acetate led to the labelling of C-2, C-4, C-7 and C-9 of **1**, which indicates irregularities in the proposed polyketide assembly. The remaining carbon atoms (C-1, C-3, C-5, C-6, C-8) were enriched by sodium [2-^{13}C]acetate. The analysis of the ^{13}C-coupling constants of [1,2-$^{13}C_2$]acetate enriched aspinonene **1** revealed three intact acetate building blocks, which are positioned at C-2/C-3, C-4/C-5 and C-7/C-8. These results suggest a rearrangement of a hypothetical pentaketide intermediate, which is generated by a subsequent elongation of an acetyl-CoA starter by four malonyl-CoA building blocks. This well established pentaketide formation is described for a number of fungal secondary metabolites such as the decarestrictines,[2] the diplodialides[3] or aspyrone **2**.[4] However, in the case of aspyrone **2** a carbon skeleton rearrangement during the biosynthetic cascade has been proved recently.[5]

Due to the labelling pattern arising from the [1,2-$^{13}C_2$]acetate feeding experiment only the oxygen atom at C-2 can be of acetate origin. Based on this fact and considering the acidic pH-value of the fermentation broth we decided to cultivate DSM-7428 in an [$^{18}O_2$]-enriched atmosphere rather than feeding [1-^{13}C,$^{18}O_2$]acetate to enlighten the biogenesis of the unusual oxygenation pattern of **1**.

In contrast to the fermentations in stirred vessels the cultivation in shaken flasks in an [$^{18}O_2$]-enriched atmosphere was much faster, which obviously is due to better aeration. To enable good incorporation rates, the [$^{18}O_2$]-enriched atmosphere was established 54 h after inoculation. The obtained aspinonene **1** shows significant α-isotopic shifts in the ^{13}C-NMR spectrum for C-2, C-6 and C-7 (Table 1). The ^{13}C NMR signal of C-9 is broad, thus indicating an incorporation of [$^{18}O_2$] into the primary hydroxy group. An analysis of the DCI-MS of [$^{18}O_2$]-enriched aspinonene **1** supports the incorporation of three oxygen atoms demonstrated by a peak at *m/z* 212 (12%), besides ions at *m/z* 206 (56%), 208 (100) and 210 (55). The tertiary hydroxy group located at C-5 is not labelled by [$^{18}O_2$], therefore the ^{13}C NMR signal of C-5 appears as a singlet. The complete labelling pattern of **1** is given in Fig. 1.

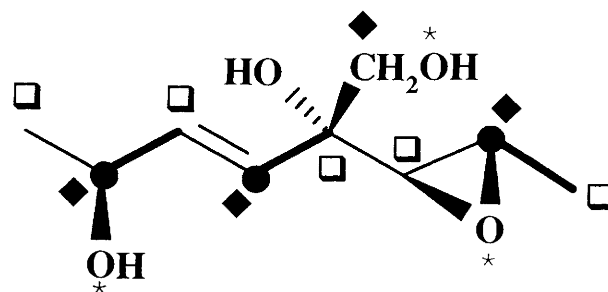


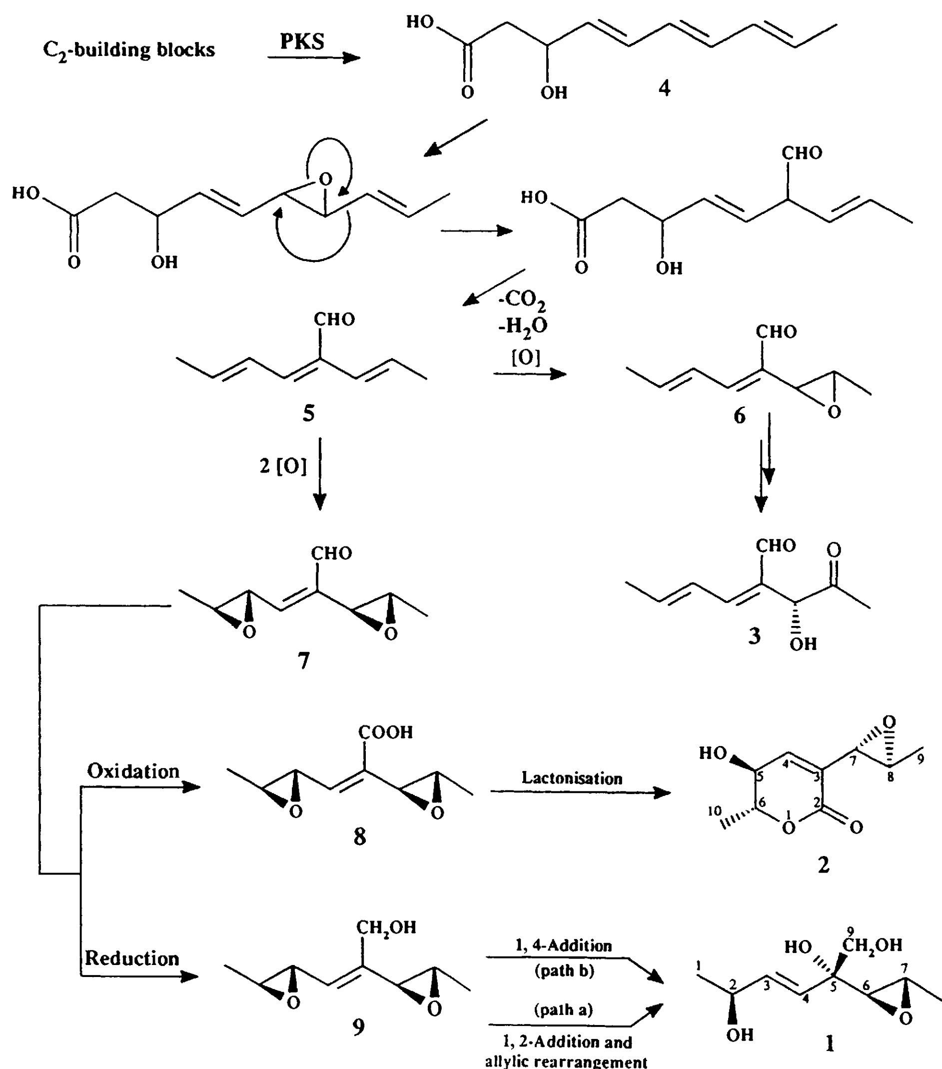
Fig. 1 Labelling pattern of aspinonene **1**; * = ^{18}O; ◆ = [1-^{13}C]-acetate; ☐ = [2-^{13}C]acetate

The similarity of the labelling pattern with the ones found for **2**[6] and avellaneol[7] **3**, which is produced by the Pyrenomycete *Hypocrea avellanea*, led us to re-examine the metabolite composition of *A. ochraceus* (strain DSM-7428). Cultivation for 21 d in static (not shaken) cultures enabled us to detect 8 mg dm^{-3} of aspyrone **2**, accompanied by decreased amounts (9 mg dm^{-3}) of aspinonene **1**. Since the strain is able to produce both **1** and **2** we suggest analogous biosynthetic pathways leading to these metabolites (Scheme 1). The enzyme bound intermediates of aspyrone biosynthesis have already been determined by

Table 1 Incorporation rates observed in aspinonene **1** by feeding experiments with [1-^{13}C]-, [2-^{13}C]- and [1,2-^{13}C]-acetate as well as fermentations in an [$^{18}O_2$]-enriched atmosphere

Carbon	δ_C	Specific incorporation [1-^{13}C]acetate	Specific incorporation [2-^{13}C]acetate	$^1J_{C\text{-}C}$/Hz [1,2-$^{13}C_2$]acetate	^{18}[O]-α-Isotopic shift $\Delta\delta_C$(ppm)	^{18}[O]-α-Isotopic shift ^{18}O:^{16}O (%)
1	23.1	0	3.44	38.0[b]	—	—
2	68.2	6.89	0	46.0[a]/38.0[b]	0.03	36:64
3	136.2	0.24	2.39	46.0[a]/72.5[b]	—	—
4	128.2	6.88	−0.08	48.0[a]/72.5[b]	—	—
5	72.2	−0.10	2.28	48.0[a]/48.0[b]/40.0[b]	—	—
6	61.9	0.20	2.25	31.5[b]/48.0[b]	0.04	34:66
7	50.6	6.27	−0.15	39.8[a]/31.5[b]	0.04	39:61
8	17.1	0	3.07	39.8[a]	—	—
9	67.6	9.94	0.27	40.0[b]	N.d.	N.d.

N.d. = not determinable. [a] Intact coupling. [b] Statistical coupling.



Scheme 1 Biosynthesis of aspinonene **1**, aspyrone **2** and avellaneol **3**

feeding experiments with ^{2}H-labelled *N*-acetylcysteamine thioesters to *Aspergillus melleus*.[5] It has been demonstrated that the linear β-hydroxy-acid **4** is the ultimate product of the polyketide synthesis (PKS) and that **4** is further modified by post-polyketide enzymes. The key step in aspyrone biosynthesis is a Favorsky-type rearrangement,[8] which we also presume to occur in aspinonene biosynthesis. This is a good explanation of the unexpected enrichment of C-9 by [1-^{13}C]acetate. A triene intermediate **5** is further epoxidised by monooxygenases resulting in the bisepoxide **7**. The formyl group of **7** is either oxidised to the related carboxylic acid, which forms the δ-lactone aspyrone **2** or reduced to the primary alcohol **9** which is further modified to furnish aspinonene **1**. This sequence of modification reactions is plausible because the intermediate **5** serves as the substrate for the epoxidation steps, which, as a consequence, establishes the corresponding stereochemistry in both metabolites **1** and **2**. The inversion of C-6 in **2** occurs by the nucleophilic attack of the carboxylic oxygen on the oxirane ring at C-2 in **8** forming the six-membered lactone. In contrast to this the analogous oxirane **9** is opened by a nucleophilic attack of

Table 2

P/bar	$P[O_2]$/mbar	Yield (mg dm^{-3})	
		1	2
1	210	14	7
2.5	525	12	64
5	1050	3	94

water at C-3 followed by an allylic rearrangement leading to **1** (path a). Alternatively, the oxirane **9** could undergo an 1,4-addition of water directly creating aspinonene **1** (path b). Both ways establish the (*S*)-configuration at C-2 as observed in aspinonene **1**.

The results of the feeding experiments and the metabolite composition clearly underline the presence of the common precursor **5** in our strain and therefore indicate a relationship between the PKS of *A. melleus* and *A. ochraceus* (DSM-7428). Furthermore **5** could be assumed to be an intermediate in the avellaneol biosynthesis in *Hypocrea avellanea*. To derive avellaneol **3** from **5** a single epoxidation leading to **6** followed by hydrolysis and oxidation must occur. Thus, **5** seems to be a more general intermediate in fungal pentaketide biosynthesis.

In the next step we focused our interest on the regulation of the oxidation/reduction process of the hypothetical intermediate **7**. This post-polyketide modification appears to be the key step directing the biosynthesis either to aspinonene **1** or to aspyrone **2**. Therefore, we cultivated *A. ochraceus* (DSM-7428) in a pressurisable air-lift loop fermentor[9] at different pressures (1, 2.5 and 5 bar†), thus enabling increased dissolved oxygen concentrations (pO_2 = 210, 525 and 1050 mbar, respectively) in the culture broth. Higher dissolved oxygen concentrations led to a reduced production of aspinonene **1** contrasted by remarkably increased yields of aspyrone **2** (Table 2). Thus, the regulation phenomenon *via* O_2-concentrations in the aspinonene/aspyrone biosynthesis is a further example showing that the secondary metabolite pattern in streptomyces and fungi can be effected easily.[10,11] This seems to be a more general method for directing biosynthetic pathways and, probably, enhancing the product diversity of selected strains.

Experimental

General

Stable isotopes were purchased from Cambridge Isotopes, malt and yeast extracts from Difco Ltd., and all chemicals from Riedel–de Haën. Sodium [1-^{13}C]- and sodium [1,2-$^{13}C_2$]-acetate were 99% enriched, sodium [2-^{13}C]acetate 99.5%. The [$^{18}O_2$] enrichment was 50%. TLC was performed on silica gel plates (Merck, HPTLC ready-to-use plates, silica gel 60F$_{254}$ on glass), and column chromatography on silica gel 60 (0.040 × 0.063 mm, Merck) or Sephadex LH-20 (Pharmacia). CD spectra were recorded on a JASCO J 500 spectrometer. DCI-MS were recorded on a Finnigan MAT 95, 200 eV, reaction gas: NH_3. Fermentation was carried out in 1 dm^3 and 10 dm^3 fermentors (Biostat M and E) from Braun Dissel (Melsungen, Germany). NMR spectra were measured with Bruker AM 360, Varian VXR-200 and Varian VXR-500S spectrometers. Chemical shifts are expressed in δ values (ppm) with tetramethylsilane (TMS), $CDCl_3$ or CD_3OD as internal standards.

Culture

Aspergillus ochraceus, deposited as DSM-7428 in the German Culture Collection, was grown on agar slates containing malt extract 2%, yeast extract 0.2%, glucose 1%, $(NH_4)_2HPO_4$ 0.05%, agar 1.5%, pH = 6.0 prior to sterilization (medium A).

Feeding experiments

A 1 cm^2 piece of agar from 7 d old cultures grown on medium A was used to inoculate 100 cm^3 of medium B (medium A omitting agar) in 300 cm^3 Erlenmeyer flasks. These cultures were cultivated on a rotary shaker (180 rpm) at 25 °C for 4 d. A 100 cm^3 portion of this culture was used to inoculate a 1.5 dm^3 fermentor containing 1.2 dm^3 of the cultivation medium B (500 rpm, aeration 3.5 dm^3 min^{-1}).

Labelled precursors were added to the fermentation in pulse feeding experiments after 120, 132, 144, 156, 168 and 180 h. During this feeding time the pH-value of the culture broth was controlled by a pH-electrode and maintained at pH 3.5–4.0 by addition of 0.667 mol dm^{-3} citric acid. Precursors were dissolved in 20 cm^3 sterile H_2O and adjusted to pH 3.5. The cultures were harvested after 192 h.

Fermentation under an [$^{18}O_2$]-enriched atmosphere

The fermentation under an [$^{18}O_2$]-enriched atmosphere was carried out in a closed vessel as previously described.[12] Cultures were grown under standard conditions in 250 cm^3 Erlenmeyer flasks containing 100 cm^3 of medium B. After 54 h of cultivation N_2 was pumped into the fermentation flasks to remove oxygen. The following cultivation was carried out in an atmosphere of [$^{18}O_2$] and N_2 (4:1). [$^{18}O_2$] Gas (2.5 dm^3) was pumped continuously from a reservoir into the fermentation vessel. The output from the flask was directed into aqueous KOH (5 mol dm^{-3}) to trap the CO_2 produced. During fermentation under the [$^{18}O_2$]-enriched atmosphere (90 h) the O_2 consumption was determined between 20–45 cm^3 h^{-1}. The cultures were harvested after 140 h.

Fermentation in static (not shaken) cultures

A 4 cm^2 piece of agar from 7 d old cultures grown on medium A was used to inoculate 1000 cm^3 of medium B in 1.5 dm^3 Penicillium-flasks. Six P-flasks were cultivated at 26 °C for 21 d.

Fermentation in 10 dm^3 air-lift loop fermentor

A portion of 10.3 dm^3 of medium B was inoculated with 700 cm^3 of a 4 d old culture of *A. ochraceus* grown in Erlenmeyer flasks (medium B). Fermentations were carried out at 1 bar, 2.5 bar and 5 bar, respectively,† with an aeration of 37 dm^3 min^{-1} (tantamount to a volumetric gas flow of 1.95 VVM) at 25 °C. The cultures were harvested after 168 h.

Isolation procedure for aspinonene 1

The culture broths of *A. ochraceus* were separated from the mycelium by filtration and the culture filtrates were extracted three times successively with equal volumes of chloroform and ethyl acetate. The combined ethyl acetate layers were washed with saturated aqueous Na_2CO_3 and concentrated under reduced pressure. The resulting brown oils were chromatographed on silica gel (column: 40 × 3.5 cm, chloroform–methanol, 9:1) and further purified by gel-permeation chromatography (Sephadex LH-20, column: 100 × 2.5 cm, methanol) to yield 68 mg dm^{-3} (10 dm^3 fermentor) **1**.

In contrast to the usual work-up procedure described above the culture broths of each labelling experiment were separated from the mycelium by filtration and the culture filtrates (about 1000 cm^3) were lyophilised. The dark brown residues (about 6 g) obtained were extracted three times with 500 cm^3 of ethyl acetate each. The combined organic layers were washed with a saturated aqueous Na_2CO_3 and concentrated under reduced

† 1 bar = 10^5 Pa.

pressure. Further purification was carried out by chromatography (see above) to yield 53.3 mg dm^{-3} ([1-^{13}C]-), 37.0 mg dm^{-3} ([2-^{13}C]-) and 24.8 mg dm^{-3} ([1,2-$^{13}C_2$]-acetate feeding experiment) of **1**. The spectroscopical data appeared to be identical to formerly isolated aspinonene **1**.[1]

Isolation of aspyrone 2

The culture broth of static (not shaken) cultures was separated from the mycelium by filtration and the culture filtrate (about 6.0 dm^3) was extracted twice with 3.0 dm^3 of chloroform. Further extraction with ethyl acetate (4 dm^3) led to the aspinonene-enriched crude product, which was purified as described above. The chloroform layer was washed with saturated aqueous Na_2CO_3 and concentrated under reduced pressure. The remaining residue (400 mg) was chromatographed on silica gel (column: 40 × 3 cm, chloroform–methanol, 9:1) and further purified by gel-permeation chromatography (Sephadex LH-20, column: 100 × 2.5 cm, methanol) to yield 48 mg of aspyrone **2**, as colourless needles. The spectroscopical data were identical with those already published,[13] CD λ_{max}/nm 231 (8046) and 261 (−8095).

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