Biosynthetic Studies on the Decarestrictine Family

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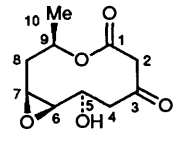
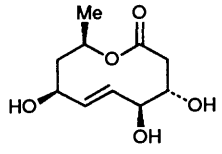
Feeding experiments with sodium [^{13}C]-labelled acetates and [2-^{13}C]-malonic acid to *Penicillium simplicissimum* (strain FH-A 6090) proved the polyketide origin of the ten-membered lactones of the decarestrictines. The oxygenation pattern was investigated by incorporation studies with sodium [1-^{13}C, $^{18}O_2$]-acetate and [$^{18}O_2$]-gas, resulting in a detailed biosynthetic analysis of the main products decarestrictine B, **1**, and D, **2**. The various decarestrictines seem to arise from a common pentaketide precursor, which undergoes subsequent post-polyketide synthase modifications. In combination with further experiments (*e.g.* product profiles during fermentation, pH-static fermentations) their biosynthetic relationships are described. These results were used to manipulate the metabolite pattern of strain FH-A 6090 directing the fermentation process to produce the desired decarestrictines.

The decarestrictine family of secondary metabolites[1,2] was discovered by chemical screening,[3] as products of different *Penicillium* species. These compounds were found to be specific inhibitors of the *de novo* cholesterol biosynthesis, demonstrated in cell line tests with HEP-G2 liver cells and *in vivo* studies with normolipidemic rats. Beside the main products, decarestrictine B, **1**, and the biologically most active decarestrictine D, **2**, a



1 Decarestrictine B



2 Decarestrictine D

number of minor components have been isolated from the culture broth of *Penicillium simplicissimum* (strain FH-A 6090).[4] Most of these natural products exhibit a ten-membered lactone ring with an exocyclic methyl group as the typical structural element. The different decarestrictines bear a unique variation in the oxygenation pattern ranging from C-3 to C-7, involving the creation of a double bond located either at C-4, C-5 or C-6. By the use of single crystal diffraction analysis the absolute configuration of **1** and the relative configuration of **2** has been determined.[2] In addition to the ten-membered lactones, the related 8-membered bicyclic lactone decarestrictine M, **7**, as well as the tetrahydropyran derivative decarestrictine L were discovered as minor components in the culture broth of the producing organism.[4] Interestingly, one member of the decarestrictine family, decarestrictine D, **2**, has recently been isolated from the sclerotium of *Polyphorus tuberaster*.[5]

Substituted decan-9-olides were also found in other fungal sources. Examples are the diplodialides, steroid hydroxylase inhibitors from *Diplodia pinea*,[6] which exhibit close structural relationships to the decarestrictines (*e.g.* a keto or hydroxy group at C-3 and a double bond at C-4). The pyrenolides from *Pyrenophora teres*[7] were described as fungal morphogenic substances, bearing an epoxide ring at C-7/C-8 and a keto group at C-4. A related group of lactones, named cephalosporolides, were isolated from *Cephalosporium aphidicola*.[8] In addition, a mutant of this strain produced thiobiscephalosporolide A, forming a dimeric thiomacrolide of two ten-membered lactone moieties. The peculiarity of another unsaturated ten-membered hydroxylated lactone, achaetolide A from *Achaetomium crystalliferum*,[9] lies in its substitution with an exocyclic apolar heptane-side chain at C-9. The first biogenetic studies by feedings with [^{13}C]-labelled acetates showed that the carbon skeleton of achaetolide A was built up *via* the polyketide pathway.[9] In contrast to these metabolites of fungal origin, ten-membered lactones were discovered in the case of the phoracantholides, metasternal secretions of the eucarypt longicorn *Phoracantha synonyma*.[10]

In agreement with the polyketide origin of achaetolide A, we assume an analogous biosynthetic pathway for the carbon skeleton of the decarestrictines.[11] Our attention was mainly attracted by their unusual oxygenation pattern. Because of the variety of metabolites produced, we anticipated interesting enzyme-catalysed reactions, which seem to be involved in the biosynthetic sequence leading to the decarestrictine family. The decarestrictines may lead to an additional fungal model for the process of growing polyketide chains as recently described for the procaryotic streptomycete metabolite erythromycin[12] at the level of polyketide synthase reactions.

Results and Discussion

Our working hypothesis for the biosynthesis of the ten-membered lactone skeleton of the decarestrictines envisioned a similar polyketide-type pathway as in the case of achaetolide A.[9] Considering the variety of the metabolite pattern of the producing strain, biosynthetic studies were carried out in small (600 cm^3) fermentation scales in 300 cm^3 Erlenmeyer flasks each with 100 cm^3 of culture broth, which allowed us to obtain sufficient yields for detailed NMR spectroscopic analysis of the main products decarestrictine B, **1**, and D, **2**. Therefore, incorporation experiments with small amounts of selected precursors, especially exhibiting the [^{18}O]-label, could be realized. In combination with the fermentation curves, which have already been described in parts for the main products,[1] we used pulse feeding experiments (mostly between 112 and 144 h) to incorporate the precursors successfully within the time of optimum production of both, **1** and **2**.

Feeding experiments with the typical polyketide precursors, sodium [1-^{13}C]- and [2-^{13}C]-acetate, labelled the positions as depicted in Fig. 1, resulting in the anticipated alternate labelling pattern in both, decarestrictine B, **1**, and D, **2**. Generally, in each feeding experiment the incorporation rates (Table 1) were found to be higher for **1** (*e.g.* sodium [1-^{13}C]-acetate feeding: spec. inc. ranging from 1.6–11%), than for **2** (1.1–1.6%). The fact that feeding with [2-^{13}C]-acetate gave less enrichment compared with [1-^{13}C]-acetate, led to a minimized (spec. inc. 2.4–3.6% into **1**) or failed (into **2**) incorporation of this precursor. The deviations in the incorporation rates of **1** and **2**

Table 1 Chemical shifts, specific incorporation of different [^{13}C]-labelled precursors and $J_{C,C}$ coupling constants of **1** to **5**

Carbon no.	δ_C					Specific incorporation						$^1J_{C,C}$/Hz [1,2-$^{13}C_2$]-acetate		
						[1-^{13}C]-acetate			[2-^{13}C]-acetate	[2-^{13}C]-malonate				
	1	**2**[a]	**3**	**4**	**5**	**1**	**2**[a]	**5**[f]	**1**	**1**	**2**	**1**	**2**[a,b]	**3,4**[b]
1	165.2	174.7	170.7	171.1	165.6	1.6	1.1	5.9	−0.1	−0.3	0.1	55.5	57.5	58.5
2	52.0	35.6	45.7	44.4	51.4	−0.2	0	0.3	3.6	41.9	18.0	55.5/38.0[e]	57.5/35.0[e]	58.5/32.5[e]
3	200.1	75.4	71.6[c]	68.2	198.4	3.6	1.6	7.6	−0.3	−0.6	0	41.0/38.0[e]	41.5/35.0[e]	45.0/32.5[e]
4	48.4	73.1	133.9	136.7	136.4	−0.2	−0.1	0.1	3.2	44.9	12.4	41.0/37.0[e]	41.5/45.0[e]	45.0/75.0[e]
5	67.8	129.4	129.7	120.1	124.7	5.5	1.3	19.6	0	−0.3	−0.2	48.5/37.0[e]	72.5/45.0[e]	57.5/75.0[e]
6	60.5	135.9	55.2[d]	54.1	53.5	−0.1	0	0.4	2.4	25.7	12.6	48.5/28.0[e]	72.5/47.5[e]	57.5/27.5[e]
7	56.3	73.6	55.0[d]	53.5	63.7	11.0	1.2	37.5	0	−0.2	−0.1	45.0/28.0[e]	37.5/47.5[e]	45.0/27.5[e]
8	36.7	44.2	34.6	29.0	30.5	0.1	0.1	0.2	2.7	31.0	16.1	45.0/37.5[e]	37.5/40.0[e]	45.0/36.5[e]
9	69.0	69.4	71.7[c]	69.3	69.7	7.9	1.2	18.3	0	0	0	39.5/37.5[e]	39.5/40.0[e]	39.0/36.5[e]
10	21.6	21.6	21.5	19.0	17.8	0	0	0	2.7	15.5	8.3	39.5	39.5	39.0

[a] Recorded in CD_3OD. [b] 125 MHz. [c,d] The assignments may be exchangeable. [e] Statistical $^1J_{C,C}$ coupling. [f] Internally referenced to C-10.

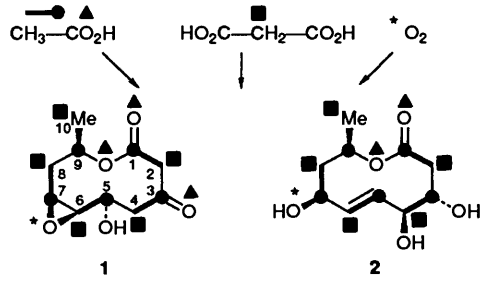


Fig. 1 Biosynthesis of decarestrictine B, **1**, and D, **2**

from the acetate feedings reflected, that both main products were formed at different times during fermentation. In combination with the fermentation curve (Fig. 4) we concluded, that **2** seemed to be an early, stable biosynthetic product, whereas **1** represents an advanced metabolite in the fermentation process. Single labelled [^{13}C]-acetate gave information about the direction of the acetate incorporation, whereas intact incorporation of the C_2-units was examined with sodium [1,2-$^{13}C_2$]-acetate. We found intact acetate building blocks in C-1/C-2, C-3/C-4, C-5/C-6, C-7/C-8 and C-9/C-10 in **1** and **2** as well as in the minor components decarestrictine A_1,A_2 (**3**, **4**). In this feeding experiment the latter metabolites could be isolated in yields sufficient for NMR spectroscopic analysis. Beside the biosynthetic information, this experiment allows proof of the ^{13}C-assignments of these decarestrictines by the use of the $^1J_{C,C}$ coupling constants. In the case of **3** and **4** some ^{13}C-signal assignments have to be corrected (Table 1).[2] The results of the acetate experiments led us to investigate the initiator of the polyketide chain of the decarestrictines. A feeding experiment with [2-^{13}C]-malonic acid resulted in highly enriched samples of **1** and **2**, labelled in positions C-2, C-4, C-6 and C-8, whereas about half the amount of signal enhancement was found for C-10 (Table 1). This contrasts to the more deviated incorporation rates obtained from the acetate feeding experiments and clearly indicated acetate to be the polyketide starter.

In combination, the results have proved a complete picture of the biosynthetic origin of the carbon skeleton of the decarestrictines. Their common ten-membered lactone ring derives from five C_2-building blocks *via* the polyketide pathway. Acetyl-CoA, represented in C-10/C-9, is used as the polyketide starter, which is subsequently elongated by four malonyl-CoA units. Consequently, this type of polyketide biosynthesis using acetate and malonate building blocks to form a ten-membered lactone skeleton seems to be transferable to achaetolide A,[9] and the fungal metabolites described above, namely the diplodialides,[6] the pyrenolides [7] and the cephalosporolides.[8]

To investigate the origin of the hydrogen atoms in decarestrictine B, **1**, and D, **2**, a feeding experiment with [2,2,2-$^2H_3$]-acetate was performed. Because of possible exchanges of the deuterium label in the fermentation broth (pH 2.5) at the step of the malonyl-CoA precursor as well as in the growing polyketide chain, the deuterated precursor was fed in nearly double amounts (48 mmol) than in previous acetate experiments. Unfortunately, ^{1}H- as well as ^{2}H-NMR spectral analysis of the isolated samples of **1** and **2** gave no evidence for a successful incorporation of the deuterium label.

Our main interest focused in studies about the source of the oxygen atoms, which constitute the unique characteristic in the decarestrictine family. Therefore sodium [1-^{13}C, $^{18}O_2$]-acetate was selected as a useful precursor, but in consideration to the acidic pH-value during the fermentation process, we assume a possible loss of the [^{18}O]-label of the acetate precursor.[13] Actually, in a first feeding experiment, carried out under analogous fermentation conditions, significant [^{13}C]-, but no [^{18}O]-enriched samples of **1** and **2** were obtained. However, the addition of undissolved sodium acetate to the fermentation broth resulted in a drastic pH-shift from 2.5 to 6.5–7.0, accompanied by reduced growth of the producing strain and, consequently, in weak production of the decarestrictines. These general feeding problems into acidic fermentation broths, which often occur in fungal metabolism studies, were successfully overcome by the use of a 1 dm^3 fermenter with continuous feeding using a tubing pump as well as pH-measuring and -regulating equipment. The experiment was carried out by continuous feeding of 13 mmol of sodium [1-^{13}C, $^{18}O_2$]-acetate (dissolved in 100 cm^3 of sterile water; pH = 8.9) to minimize oxygen label exchange,[13] while maintaining pH = 2.5 in the fermentation vessel. In the 125 MHz ^{13}C NMR spectra of **1** and **2**, isolated from this feeding experiment, the positions of incorporation were determined by observation of [^{18}O] isotope-induced shifts. The results are summarized in Table 2. Beside an identical [^{13}C]-labelling pattern as in the case of the sodium [1-^{13}C]-acetate experiment, [^{18}O]-label was observed at both oxygen atoms of the lactone moiety (C-1) in **1** and **2**. Furthermore an [^{18}O]-enrichment at the keto group at C-3 in **1** was observed, indicating the intact incorporation of [^{13}C–^{18}O]-assemblies from the carboxy group of acetate into these positions. The biosynthetic origin of the remaining oxygen atoms was studied by a fermentation, conducted in a closed system under an atmosphere of 20% [$^{18}O_2$] and 80% N_2.[14] In an isolated sample of decarestrictine B, **1**, a significant [$^{18}O_2$]

Table 2 [$^{18}O_2$] Isotope-induced shifts in the ^{13}C NMR spectra of **1** and **2** enriched with [1-^{13}C, $^{18}O_2$]-acetate and with [$^{18}O_2$]-gas

Decarestrictine B, **1**				Decarestrictine D, **2**			
Carbon no.	δ_C	$\Delta\delta_C$	$^{16}O:^{18}O$	Carbon no.	δ_C	$\Delta\delta_C$	$^{16}O:^{18}O$
1	165.2	0.040[a,c]	n.d.	1	174.7	0.040[a,c]	n.d.
3	200.1	0.020[a]	61:39	3	75.4	—	—
5	67.8	—	—	4	73.1	—	—
6	60.5	0.032[b]	54:46	7	73.6	0.018[b]	57:43
7	56.3	0.034[b]	54:46	9	69.9	0.020[a,c]	63:37
9	69.3	0.025[a,c]	64:37				

n.d. = not determined. [a] Feeding experiment with sodium [1-^{13}C, $^{18}O_2$]-acetate. [b] Fermentation in an [$^{18}O_2$]-enriched atmosphere. [c] Additionally $^2J_{C,C}$ coupling is observable.

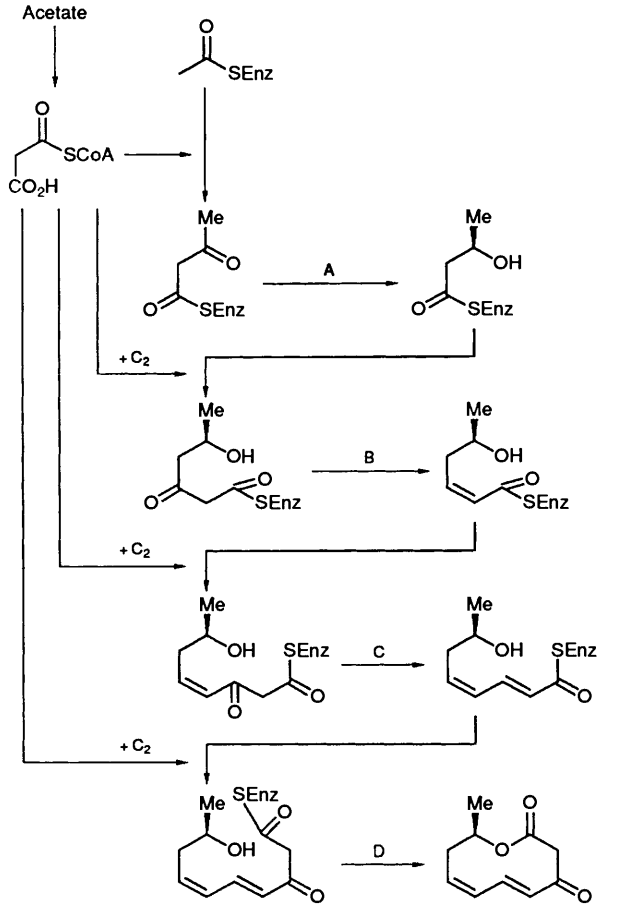


Fig. 2 Formation of the hypothetical, common decarestrictine precursor by the polyketide synthase

isotope-induced shift (δ_C 0.032 and 0.034, $^{16}O:^{18}O$ ratio = 54:46) was only found for the carbon signals at C-6 and C-7, indicating that molecular oxygen was introduced into the epoxide ring. Decarestrictine D, **2**, showed one isotopically shifted [^{13}C]-signal assigned to C-7 (δ_C 0.018) with an analogous enrichment as in **1** ($^{16}O:^{18}O$ = 57:43). The origin of the oxygen atoms in the hydroxy group at C-5 in **1**, C-3 and C-4 in **2** remain open.

The results of incorporation experiments with [^{18}O]-labelled precursors illuminated the biosynthetic origin of the oxygen atoms in decarestrictine B, **1**, and D, **2**. The feeding experiment with sodium [1-^{13}C, $^{18}O_2$]-acetate indicated, that in **1** the oxygen atoms of the lactone moiety as well as the keto group at C-3 derives from this acetate precursor, whereas the epoxide ring at C-6/C-7 originates from molecular oxygen. In **2**, we detected the analogous oxygenation label to **1** from acetate in the lactone ring, but the anticipated [^{18}O] enrichment of the hydroxy group at C-3 could not be determined. This may be due to signal broadening in the NMR experiment, resulting in a non-detectable isotopic shift (expected δ_C 0.015–0.02). The fermentation performed in an [$^{18}O_2$]-enriched atmosphere contributes the origin of one remaining oxygen in **2**, as the hydroxy group at C-7 derived from [$^{18}O_2$]-gas. Knowledge about the biosynthesis of decarestrictine B, **1**, and D, **2**, allows further biosynthetic details for the decarestrictine family to be discussed. In a common biosynthetic process the typical ten-membered lactone ring, whose carbon skeleton derives from an acetate- and four malonate-building blocks, is formed *via* attack of the C-9 hydroxy group to the probably CoA-activated carboxy group (C-1) after the termination step of the already formed polyketide intermediate. In addition, the keto/hydroxy group at C-3 in the decarestrictine family suggests a similar biosynthetic origin. As the keto group in **1** derives from acetate it seems likely that the origin of the oxygen atom in the C-3 position of the decarestrictines generally derives from this precursor. Furthermore, the results of the [$^{18}O_2$]-experiment reflected an unexpected structure–biosynthesis relationship. The origin of the epoxide ring in **1** from molecular oxygen suggested an oxygenase catalysed reaction to a previous existing double bond located at C-6/C-7. Because of an incorporation of [$^{18}O_2$] into the hydroxy group at C-7 in **2**, this oxygen atom seems to derive from an analogous or identical reaction to that in **1** by an oxygenase attack forming an epoxide ring. Resulting in the stable product **2**, further reactions (*e.g.* opening of the epoxide ring) seem to be involved in the biosynthesis of **2**.

As a consequence, the decarestrictines seem to arise from a common pentaketide precursor, which is formed by a polyketide synthase. The information from our feeding experiments allowed us to formulate a hypothetical biosynthetic pathway at the level of the polyketide synthase reactions. We assume, that the organization of this fungal polyketide synthase involves four repeated units, which each designated a module as in the erythromycin case[12] (Fig. 2). After the first condensation step of the acetate starter molecule with a malonate building block, a β-ketoreductase reaction led to the hydroxy group at C-9 in the decarestrictines. The next two condensation steps exhibit additional dehydratase activities to create a (*Z*)- (at C-6/C-7) and an (*E*)-configurated double bond (C-4/C-5) in the decarestrictines. The final condensation and cyclization step to the ten-membered lactone is catalysed by a thioesterase, forming a common, hypothetical precursor. We assume, that the reduction step of the keto functionality at C-3 as well as further enzymatic reactions leading to the different decarestrictines seem to be post-polyketide modifications. However, an alternative hypothetical precursor with a hydroxy group at C-3 is not probable, as we have observed decarestrictines with both

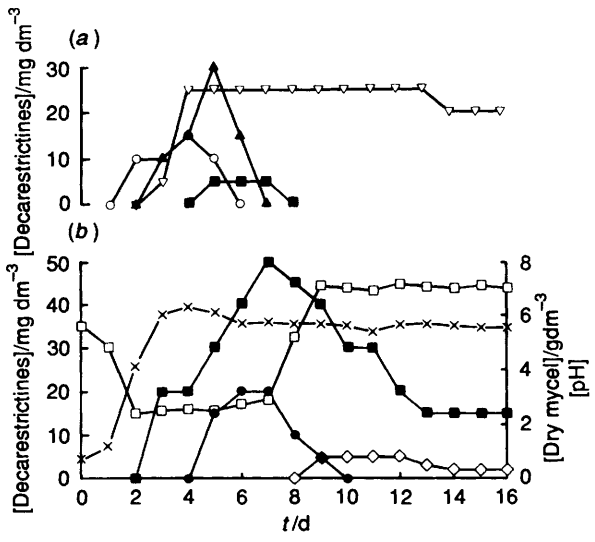


Fig. 3 Typical fermentation time course of *Penicillium simplicissimum* (FH-A 6090) and production of the decarestrictines (concentrations estimated by TLC analysis) (*a*) 'Decarestrictine D related compounds' ▽, Decarestrictine D (**2**); ○, Decarestrictine F (**5**); ▲, Decarestrictine A_1,A_2 (**3**, **4**); ■, Decarestrictine N; (*b*) 'Decarestrictine B related compounds', □, pH; ×, Dry weight; ■, Decarestrictine B (**1**); ●, Decarestrictine E (**6**); ◇, Decarestrictine M (**7**)

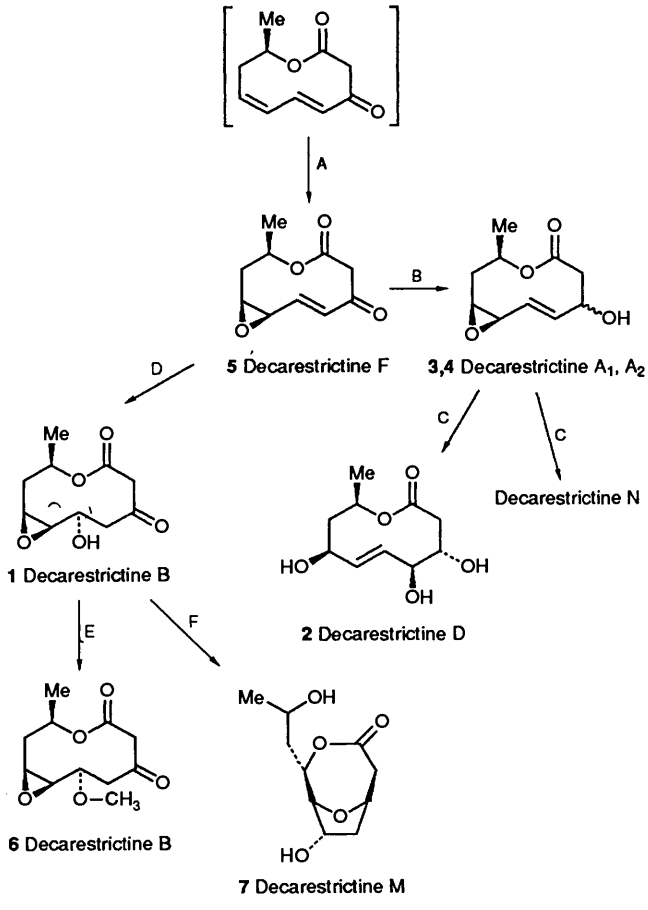


Fig. 4 Biosynthetic relationships of the decarestrictine family

configurations at C-3. Experiments to isolate the postulated decarestrictine precursor from the culture broth of strain FH A-6090 as well as force field calculations in order to illustrate the ring cyclization reaction of the ten-membered lactone are in progress.

Our biosynthetic studies with feeding experiments using labelled precursors were supplemented by the results from the fermentation curves. In addition to the work already presented,[1] we focused our interest on a selection of minor components[4] to get a more complete picture of the biosynthetic relationship of the decarestrictines during the fermentation process. Regarding dry weight, pH-course as well as the producing time course of the decarestrictines, we intersected the fermentation procedure of *Penicillium simplicissimum* (FH-A 6090) in different parts. The first section (between 2 and 4 days of growth at pH 2.5) was characterized by the appearance and maximum production of 'decarestrictine D-related compounds', depicted in Fig. 3a. Whereas **2** emerged a stable product during the whole fermentation, decarestrictine F, **5**, decarestrictine A_1,A_2, **3**, **4**, and a new metabolite, decarestrictine N disappeared after 6 to 7 days of inoculation. In the second section of the fermentation procedure a drastic pH shift to pH 6.5–7 is observable, accompanied by a diversion of the decarestrictine production to 'decarestrictine B-related compounds' (Fig. 3b). The main metabolite decarestrictine B, **1**, and the minor compound decarestrictine E, **6**, reached their maximum amounts at 7–8 days, followed by a rapid decrease during further fermentation. Decarestrictine M, **7**, appeared after 8 days and remained constant until the end of the fermentation.

As a consequence, the production of the different decarestrictines seems to be mainly influenced by duration and pH of the fermentation. Therefore, strain FH-A 6090 was cultured at constant pH while analysing the metabolite pattern. A fermentation at pH 3.5 (duration 6 days) led to the anticipated optimum of both main products **1** (40 mg dm^{-3}) and **2** (25 mg dm^{-3}) as well as the typical palette of minor components. Below pH 2.5 we detected exclusively **2** (20 mg dm^{-3}) and small amounts of decarestrictine N (5 mg dm^{-3}). Whereas **2** reflected a particularly stable product, decarestrictine N disappeared during further fermentation. In conclusion, the production or the conversion into **2** is favoured under these more extreme acidic conditions. To detect possible acid-dependent reactions, which may be involved in the biosynthetic sequence of the decarestrictines, a fermentation at pH 5–7 provided useful information. Considering the experience of insufficient growth at pH 6.5–7 from the feeding experiment with [1-^{13}C, $^{18}O_2$]-acetate, the fermentation was first started without pH-control. At the beginning of decarestrictine production (48 h after inoculation), the pH was changed from 2.5 to 5.5. Further fermentation until 72 h led to growth and production of **1** (10 mg dm^{-3}). Remarkably, the formation of **2** could not be detected. On the other hand, **3** and **4** were produced in twice the amounts (20 mg dm^{-3}) than in fermentations without pH-control, indicating a hypothetical biosynthetic relationship between decarestrictine A_1,A_2, **3**, **4** decarestrictine D, **2**, and decarestrictine N. This result led to the assumption, that we had intercepted the acid-dependent conversion of **3** and **4** into **2** and decarestrictine N at pH 5.5.

In combination, the results from the feeding experiments with labelled precursors, the fermentation curves and the pH-static fermentations illustrate the biosynthetic relationships of the decarestrictine family, depicted in Fig. 4. The different decarestrictines seem to arise from a common pentaketide precursor, which is attacked *via* an oxygenase catalysed step (A), leading to decarestrictine F, **5**, the first biosynthetic product detectable in the fermentation broth. The highly specific incorporation rates of **5** from feeding with [^{13}C]-labelled acetates (Table 1) can be explained by feeding the precursors within the time of optimum production of decarestrictine F, **5**. The following transformations into the main products decarestrictine B, **1** or D, **2**, depend on the fermentation

Table 3 Labelled precursors used for biosynthetic studies of the decarestrictines

Precursor	Enrichment (%)	Amount (mmol dm^{-3})	Yield (mg dm^{-3}) 1	2	3, 4	5
Sodium [1-^{13}C]-acetate	99.0	27	61	35	—	7
Sodium [2-^{13}C]-acetate	99.5	27	56	43	—	—
Sodium [1,2-$^{13}C_2$]-acetate	99.0	16	24	16	12	—
Sodium [2-^{13}C]-malonate	99.0	16	12	14	—	—
Sodium [1-^{13}C, $^{18}O_2$]-acetate	99.0 ^{13}C, 96.0 ^{18}O	13	41	28	—	—
Sodium [2,2,2-$^{2}H_3$]-acetate	99.0	48	23	17	—	—
[$^{18}O_2$]-gas	50.0	67	10	16	—	—

conditions (see above). In the early fermentation process, the formation of the 'decarestrictine D-related compounds' is preferred. The reduction of the keto group of **5** (B) leads to a diastereoisomeric mixture of decarestrictine A_1,A_2, **3**, **4**, in the ratio of 3:1. We presume that these metabolites were immediately transformed *via* an acid-dependent step (C) to end in the main product decarestrictine D, **2**, and decarestrictine N with the same ratio (3:1). The chemical structure of the latter compound, decarestrictine N, as well as the studies of this transformation is still under investigation. The pH-shift to 6.5–7 after 6–7 days of fermentation showed an interesting reverse of the observed metabolite pattern, leading to the production of 'decarestrictine B-related compounds'. A hydration (D) of the double bond at C-4/C-5 in **5** results in the main product, decarestrictine B, **1**. As deduced from the fermentation curve (Fig. 3b), **1** obviously undergoes further modifications, *e.g.* a methylation step (E) to decarestrictine E, **6**. In addition, a reduction of the keto group at C-3 and an opening of the epoxide ring at C-6/C-7 (F) leading to the stable fermentation product decarestrictine M, **7**, is plausible.

Experimental

General.—Stable isotopes were purchased from Cambridge Isotopes (Table 3), malt and yeast extracts from Difco Ltd., and all chemicals from Riedel de Haen. 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). Fermentation was carried out in 1 and 10 dm^3 fermenters (Biostat M and E) from Braun Dissel (Melsungen, Germany). NMR spectra were measured with Bruker AM 360, Varian VXR-200 and Varian VXR-500S. Chemical shifts are expressed in δ values, and J values in Hz, with tetramethylsilane (TMS), $CDCl_3$ or CD_3OD as internal standards.

Culture.—*Penicillium simplicissimum* (strain FH-A 6090, deposited in the German Culture Collection: DSM 4209) 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 sterilization (medium A).

Fermentation.—A piece of agar from the storage culture described above (1 cm^2) was used to inoculate a 300 cm^3 Erlenmeyer flask containing the same medium omitting agar (medium B, 100 cm^3). These flasks were cultivated for 7 days at 25 °C on a rotary shaker (140 rpm). Each culture (300 cm^3) was used to inoculate a fermenter (10 dm^3 working volume) containing medium B (200 rpm, 7 days, aeration 5.0 dm^3 min^{-1}). The total fermentation process was analysed using a cultivation time of 16 days. The pH value was determined by using a pH-electrode. Mycelium dry weight was determined after filtration of culture broth (10 cm^3) and drying the filter cake at 105 °C. The culture broth (10 cm^3) was extracted twice with ethyl acetate (5 cm^3). The combined organic layers were evaporated to dryness and the oily crude extract was dissolved in chloroform–methanol 9:1 (200 mm^3). The production of secondary metabolites was visualized by TLC analysis on silica gel plates (solvent system: chloroform–methanol 9:1, staining reagent: anisaldehyde–H_2SO_4).

Feeding Experiments.—A piece of agar from the storage culture described above (1 cm^2) was used to inoculate 300 cm^3 Erlenmeyer flasks containing medium B (100 cm^3). These cultures were incubated at 25 °C on a rotary shaker (140 rpm). Labelled precursors (Table 3) were added to the fermentation in pulse feeding experiments at 112, 120, 136 and 144 h. Precursors were dissolved in sterile water (20 cm^3) and adjusted to pH 3.0. The cultures were harvested at 168 h.

Feeding Experiments with Sodium [1-^{13}C, $^{18}O_2$]-*Acetate.*—A 4-day-old culture (80 cm^3), which was grown using standard conditions, was used to inoculate a 1 dm^3 fermenter containing cultivation medium B (800 cm^3) (200 rpm, aeration 5 dm^3 min^{-1}). Between 94 and 128 h, 13 mmol of sodium [1-^{13}C, $^{18}O_2$]-acetate, dissolved in sterile water (100 cm^3) (pH 8.9, immediately prepared before use), was continuously added to the fermentation vessel using a tubing pump (3 cm^3 h^{-1}). During this feeding time the pH-value of the culture broth was controlled by a pH-electrode and maintained at pH 2.5 by addition of 0.667 mol dm^{-3} citric acid.

Fermentation Under [$^{18}O_2$]-*Enriched Atmosphere.*—The fermentation under [$^{18}O_2$]-enriched atmosphere was carried out in a closed vessel as previously described.[14] Cultures were grown under standard conditions in 250 cm^3 Erlenmeyer flasks containing medium B (100 cm^3). After 60 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 (3 dm^3) was pumped continuously from a reservoir into the fermentation vessel. The output from the flasks was directed into a KOH solution (5 mol dm^{-3} in water) to trap the CO_2 produced. During fermentation under the [$^{18}O_2$]-enriched atmosphere (84 h) the O_2 consumption was found to be 10–25 cm^3 h^{-1}. The cultures were harvested after 144 h.

Isolation and Purification of the Decarestrictines **1**–**5**.—The fermentation broth (600 cm^3) of each labelling experiment was filtered, the filtrate was extracted twice with ethyl acetate (300 cm^3) and concentrated under reduced pressure to dryness. The oily crude product obtained was chromatographed on silica gel (column: 40 × 3.5 cm) using a gradient of chloroform–methanol (30:1 to 10:1). Two fractions were collected, containing **1** and **3**–**5** (fraction 1), and **2** (fraction 2).[1] Fraction 1 was chromatographed on silica gel (column: 25 × 2 cm, ethyl acetate–hexane, 1:1) to give pure **1**, **3**, **4** and **5** (Table 3). Gel

filtration of fraction 2 on Sephadex LH-20 (column: 100 × 3 cm, MeOH) resulted in pure **2**.

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