Articles

Biosynthesis of Streptazolin

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The biosynthetic origin of streptazolin (1) was studied by feedings of sodium [^{13}C]acetates, sodium [^{13}C]formate, [^{13}C]urea, L-[*methyl*-^{13}C]methionine, [$^{15}N_2$]ammonium sulfate, and L-[^{15}N]glutamic acid to the producing microorganism *Streptomyces* sp. (strain FH-S 2184). NMR spectra analysis of the produced streptazolin (1) provided information about the biogenetic construction of its carbon skeleton. The unique tricyclic streptazolin (1) is assembled by a mixed biosynthesis *via* the polyketide pathway, the one-carbon and nitrogen pool, as well as oxygen from air. Acetyl-CoA seems to act as the pentaketide starter (C-12/C-13), which is subsequently elongated by four malonate units and the linkage of a building block deriving from the one-carbon pool. Urea, carbamoyl phosphate, or even carbon dioxide are discussed as ultimative precursors of the C_1-pool moiety in 1. A fermentation in an [$^{18}O_2$]-enriched atmosphere established the origin of the oxygen atom at 5-OH from molecular oxygen. The origin of the nitrogen atom was investigated by feedings of [^{15}N]ammonium sulfate and L-[^{15}N]glutamic acid indicating its introduction into 1 from the common nitrogen pool *via* a C_1-pool metabolite (C_1N-unit). A biosynthetic scheme for 1 including mechanistic aspects of the ring cyclization reactions leading to 1 is discussed.

Introduction

Streptazolin (1) was originally discovered by a chemical screening as a secondary metabolite of *Streptomyces viridochromogenes* (strain Tü 1678).[1] Meanwhile, further producers like *Streptomyces luteogriseus* (strain FH-S 1307)[2] or the high-producing strain *Streptomyces* sp. (strain FH-S 2184) have been found during a routine chemical screening on a large number of streptomyces isolates.[3-5] The constitution of streptazolin (1) was established by both spectroscopic and classical chemical degradation studies.[1] Stereochemical information leading to the absolute configuration of all centers of chirality was obtained by the application of the dibenzoate chirality method[6] as well as by crystallographic analysis of *O*-acetyldihydrostreptazolin.[7] While streptazolin exhibits limited antimicrobial activities,[5] structural variations led to potent antimicrobial and cytotoxic derivatives.[8,9] In addition, streptazolin was a target of total synthesis efforts.[10-13] The striking structural feature of streptazolin (1) is a urethane moiety of natural origin in the tricyclic skeleton. Urethane units are rarely distributed in nature. Examples were found in the 3-*O*-carbamoyl-2-deoxy-α-D-rhamnoside moiety of the macrolactone irumamycin[14,15] or in the aglycon of the enediyne-type antibiotics calicheamicin and esperamicin.[16,17]

The unique structure of streptazolin (1) attracted our interest to study its biosynthetic origin. On the basis of feeding experiments with selected labeled precursors we present a proposal for the biogenetic origin of 1. It is formed *via* polyketide, C_1-, and N-pool building blocks as well as oxygen from air. On the basis of the results of the incorporation experiments we suggest possible cyclization reactions of the proposed intermediate leading to 1.

Experimental Section

Fermentation. *Streptomyces* sp. (strain FH-S 2184) was grown on agar slants containing medium A (2% soybean meal, degreased; 2% mannitol, 1.5% agar, pH = 7.2 prior to sterilization; incubation time 1–3 weeks at 30 °C). A loopful of agar from the storage culture was used to inoculate a 300-mL Erlenmeyer flask containing 100 mL of medium A omitting agar (medium B) shaken at 180 rpm on a rotary shaker at 30 °C for 3 days. An aliquot of this seed culture (3%) was used to inoculate medium B for production (harvest after 72 h). The production time course was examined during a 10-L fermentation. Ten mL samples of the fermentation broth were taken every 4 h and centrifuged (15 min, 3000 rpm). The supernatant was adsorbed on Amberlite XAD-16. After the resin was washed with deionized water, the metabolites were eluted with a 4:1 mixture of methanol/water

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Table I. Labeled Precursors Used for the Biosynthetic Studies (Amounts Added to the Culture Broth and Yields of Streptazolin (1))

precursor	enrichment (%)	amt (mmol/L)	yield of 1 (mg/L)
sodium [1-^{13}C]acetate	99.0	24	146.6
sodium [2-^{13}C]acetate	99.5	36	66.0
sodium [1,2-$^{13}C_2$]acetate	99.0	12	79.4
sodium [1-^{13}C,$^{18}O_2$]acetate	99.0 ^{13}C, 96.0 ^{18}O	13	96.2
sodium [^{13}C]formate	99.3	18	156.8
[^{13}C]urea	99.0	11	40.8
L-[*methyl*-^{13}C]methionine	98.0	7	6.8
[^{15}N]ammonium sulfate	99.0	0.8	29.4
L-[^{15}N]glutamic acid	99.0	0.8	42.2
[$^{18}O_2$] gas	50.0	156.2	26.7

and concentrated to dryness. The production of streptazolin (1) was analyzed by TLC chromatography (HPTLC-silica gel 60F_{254} on glass; solvent, chloroform/methanol (9:1); detection, UV-extinction at 256 nm and staining with Ehrlich's reagent).[1] In a typical fermentation, the production of streptazolin (1) started 16 h after inoculation and increased by further cultivation; after ca. 64 h the concentration of 1 remained constant (maximum yield, about 300 mg/L) and decreased slightly after 144 h.

Feeding Experiments. Pulse feeding experiments were carried out by addition of labeled precursors in equal portions after 16, 24, and 40 h of inoculation (total amounts, see Table I). Precursors were dissolved in 10 mL of sterile water and adjusted to pH = 7.0. Each feeding experiment with labeled precursors was performed with a total of 500 mL of culture broth except with the [^{15}N]-labeled precursors (1 L of culture broth). The fermentation under [$^{18}O_2$]-enriched atmosphere was carried out in a closed vessel as previously described.[18,19] Cultures were grown under standard conditions in 250-mL Erlenmeyer flasks containing 100 mL of the production medium. The fermentation was started with [$^{16}O_2$]; 16 h after inoculation the atmosphere in the closed vessel was replaced by an [$^{18}O_2$]-containing gas mixture (20% O_2, which was 50% enriched with [$^{18}O_2$], and 80% N_2). During fermentation (42 h) under the [$^{18}O_2$]-enriched atmosphere the O_2 consumption was found to be 40–55 mL/h for a total of 300 mL of culture medium.

Isolation Procedure. Each fermentation broth was centrifuged, and the supernatant was adsorbed on Mitsubishi HP-20 (column, 25 × 2.5 cm). After the resin was washed with deionized water (double bed volume) the bound compounds were eluted with a 4:1 mixture of methanol/water. *In vacuo* the eluate was concentrated to dryness. The crude product was chromatographed on silica gel (column, 20 × 2 cm, chloroform/methanol (9:1)). The streptazolin containing fractions were evaporated to dryness and further purified by gel permeation chromatography on Sephadex LH-20 (column, 45 × 1.8 cm, methanol) to yield pure oily 1 (yields, see Table I). The isolation and purification of 1 is difficult because of its propensity to polymerize upon concentration from organic solvents,[1,10] but usually enough material could be redissolved after the final purification step for NMR analysis.

NMR Experiments. ^{13}C-NMR spectra of the labeled streptazolin samples were recorded at 50.3 MHz in acetone-d_6. Chemical shifts are expressed in δ values (ppm) with tetramethylsilane (TMS) or $CDCl_3$ as internal standard. The signals of 1 were unequivocally assigned in detailed 1D- and 2D-NMR studies (δ_H = 1.90 (d, $J_{13,12}$ = 7.5 Hz, 13-H_3), 2.09–2.60 (m, 9-H_2), 3.35–3.49 (m, 10-H_2), 3.62 (s, broad, exchangeable with D_2O, 5-OH), 4.28 (d, $J_{11,4}$ = 6.8 Hz, 11-H), 4.83 (d, 4-H), 4.89 (s, broad, 5-H), 6.03 (m, 8-H), 6.15 (q, 12-H); δ_C see Table II).

[^{15}N]-Determination. An emission-spectrometric [^{15}N] analysis system, which makes use of the isotopic shifts in the emission spectrum of nitrogen, was employed.

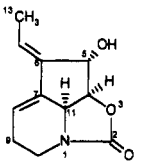
Results and Discussion

A working hypothesis for the biosynthesis of streptazolin (1) followed from the observation that 1 was found to be a cometabolite of a streptenol- (e.g., streptenol D (2)) and piperidine-producing organism (e.g., 3).[2] As these secondary metabolites bear analogous structural elements biosynthetical relationships are assumed. Anticipating a polyketide origin of the streptenols and piperidines, we started incorporation experiments with [^{13}C]-labeled acetates as typical polyketide precursors. The isolated samples of streptazolin (1) yielded from the sodium [1-^{13}C]- and [2-^{13}C]acetate feedings were analyzed by ^{13}C-NMR spectroscopy resulting in the labeling pattern depicted in Figure 2 (for enhancements of the signals see Table II). The feeding of sodium [1-^{13}C]acetate led to significant ^{13}C-signal enhancements of C-5, C-8, C-10, C-11, and C-12 (spec inc:[20] 3.3–4.8%), while sodium [2-^{13}C]acetate resulted in the alternative labeling pattern. Because of the high incorporation rates in the latter experiment the neighboring carbons C-6 and C-7 show the typical signal splittings of $^1J_{C-C}$ couplings. In addition to the direction of the acetate incorporation obtained by the feeding experiments with single labeled acetates incorporation of sodium [1,2-$^{13}C_2$]acetate provided information about the integrity of the C–C bond of these C_2-building blocks. In 1, we found intact acetate incorporation at C-12/C-13, C-5/C-6, C-4/C-11, C-7/C-8, and C-9/C-10. The obtained $^1J_{C-C}$ coupling constants are summarized in Table II. These

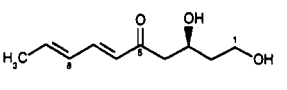
Table II. Chemical Shifts, Specific Incorporations (Standardized on the C-13 Signal Intensity), and J_{C-C} Coupling Constants of the Proton Noise Decoupled ^{13}C NMR Resonances of Streptazolin (1) in Acetone-d_6 after Feeding with Different [^{13}C]-Labeled Precursors

C-atom	δ (ppm)	A[a]	B[a,c]	C[b]	D[a]	E[a]
2	159.8	2.4	0.8		2.1	3.5
4	82.6	0.2	9.2	32.1	0.0	0.2
5	74.7	3.3	–0.1	46.3	–0.2	0.0
6	140.2	0.3	4.2[d]	46.3	–0.1	0.3
7	144.5	0.1	3.5[d]	72.9	–0.3	0.0
8	118.7	3.5	0.0	72.9	0.1	0.1
9	23.1	0.2	9.0	32.8	0.1	0.2
10	40.6	4.8	0.2	32.8	0.0	0.1
11	59.7	4.1	–0.1	32.1	0.1	0.1
12	123.2	3.7	0.0	43.5	0.1	0.1
13	14.9	0.0	9.2	43.5	0.0	0.0

[a] Specific incorporations. [b] $^1J_{C-C}$ coupling constants in Hz. [c] Standardized on the C-12 signal intensity. [d] Both signals exhibit strong $^1J_{C-C}$ coupling satellites ($J_{C-6,C-7}$ = 56.6 Hz), spec inc C-6, 9.6%; C-7, 7.3%; A, sodium [1-^{13}C]acetate; B, sodium [2-^{13}C]acetate; C, sodium [1,2-$^{13}C_2$]acetate; D, sodium [^{13}C]formate; E, [^{13}C]urea.



Streptazolin (1)



Streptenol D (2)

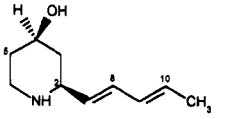


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Figure 1. Streptazolin (1), streptenol D (2), and the piperidine 3.

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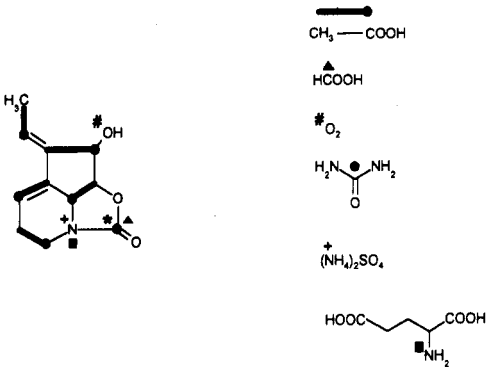

Figure 2. Biogenesis of the carbon, oxygen, and nitrogen atoms of streptazolin (1).

experiments proved the polyketide origin of 1. It seem likely that the formation of a pentaketide precursor starts with an acetyl-CoA unit (C-12/C-13) acting as the starter, which is then elongated by the subsequent condensation with four malonyl-CoA building blocks (C-6/C-5, C-4/C-11, C-7/C-8, C-9/C-10) in the typical fashion of polyketide assembly.

In analogy to the findings for the carbamoyl groups of mitomycin,[21] geldanamycin,[22] and streptothricin[23] the solitary carbon atom of the carbamoyl ester moiety (C-2) in 1 is expected to arise from the one-carbon pool. A possible precursor for labeling of C_1-pool carbon atoms is L-methionine. However, the addition of about 7 mmol/L of L-[*methyl*-^{13}C]methionine to the culture broth resulted in a 10-fold decrease of the streptazolin production. A close examination of the isolated sample of 1 with ^{13}C NMR spectroscopy gave no evidence for any label incorporation ruling out the participation of L-methionine in its biosynthesis. In the feeding experiment with sodium [1-^{13}C]acetate a distinct enrichment of the carbon signal C-2 was observed. The specific incorporation obtained was significantly lower (spec inc = 2.4%) than for the direct acetate incorporations into the pentaketide chain (spec inc = 3.3–4.8%). This argues for biosynthetic relationships between acetate and the C_1-pool in the streptazolin-producing organism. Two hypothelical pathways can be discussed. On the one hand complete oxydation of acetate in the TCA-cycle can result in CO_2, which may be used in the biosynthetic sequence. On the other hand, however, formic acid can derive from decarboxylation reaction of oxalic acid arising from glyoxylic acid oxidation. The latter pathway points to formate as a possible precursor. As expected a feeding experiment with sodium [^{13}C]formate resulted in specific incorporation into C-2. Furthermore, the incorporation of [^{13}C]urea into 1 reflects the fact that this C_1-pool metabolite, which is closely related to formate, may serve as a source of both the nitrogen atom and C-2.

In order to determine the origin of the nitrogen atom in 1, feeding experiments were carried out with [^{15}N]-labeled precursors. In a parallel attempt [$^{15}N_2$]ammonium sulfate and L-[^{15}N]glutamic acid were fed to the streptazolin-producing organism. Analyzing the purified streptazolin samples of each experiment by the isotope shifts in the emission spectrum of nitrogen, [$^{15}N_2$]ammonium sulfate resulted in a 2.37% and L-[^{15}N]glutamic acid in a 2.52% incorporation with respect to an unfed control exhibiting 0.38% [^{15}N]-label. Obviously, both precursors represent substrates for 1. Because no preference of incorporation was found, the fed [^{15}N]-label from these two precursors seems to be incorporated *via* the common nitrogen pool of the cells. At present we discuss urea, carbamoyl phosphate, or even CO_2 to be the ultimate precursor of the carbamoyl ester moiety in streptazolin (1). Feeding experiments, especially in order to prove the hypothesis of an intact C_1N-unit incorporation (e.g., [^{13}C,^{15}N]urea and L-[*guanidino*-^{13}C,^{15}N]arginine), are currently underway.

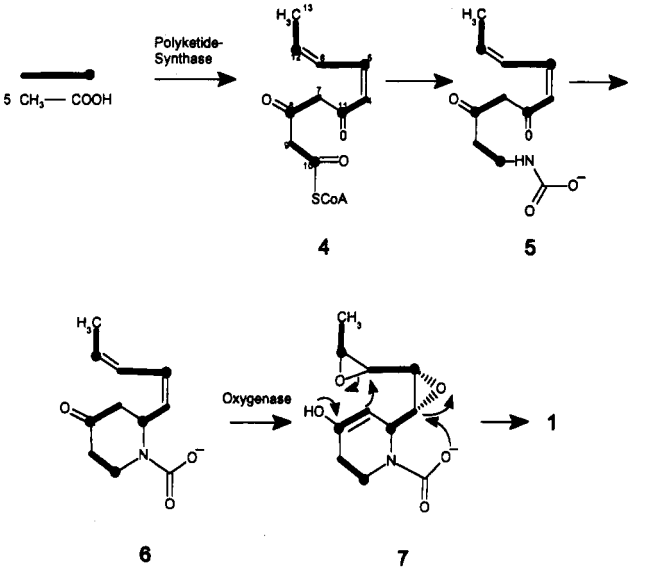

Figure 3. Proposed biosynthetic pathway of streptazolin (1).

Considering the label pattern of the acetate feedings the oxygen atom of 5-OH could derive from the acetate precursor *via* polyketide synthase reactions. From an experiment with sodium [1-^{13}C,$^{18}O_2$]acetate an identical [^{13}C]-labeling pattern as in the case of the sodium [1-^{13}C]-acetate feeding was observed, but the anticipated [^{18}O] isotope-induced signal shift was not observable in the ^{13}C NMR spectrum. Therefore, the introduction of oxygen atoms into 1 *via* oxygenases was studied by cultivation under an [$^{18}O_2$]-enriched atmosphere using a closed vessel fermentation system.[18,19] In the isolated sample of streptazolin (1) a significant [$^{18}O_2$] isotope-induced shift ($\Delta\delta_{C\text{-}5}$ = 0.02 ppm) was found for the signal of C-5, indicating that the oxygen atom of the 5-OH group was introduced *via* molecular oxygen. EI-MS spectroscopy independently supported this result (70 eV, *m/e* = 207 (100); 209 (58). It seems likely that the oxygenase attack occurs at a (*Z*)-configurated double bond (C-4/C-5) of a hypothetic pentaketide precursor leading to an oxirane intermediate like 7. We discuss the epoxide opening by the attack of the hydroxy group of a carbamoyl moiety as depicted in Figure 3. The opposite facial positions of 5-OH and 3-O support this assumption.

A necessary activation for carbon–carbon bond formation between C-6 and C-7, which both originate from a CH_2-group of malonyl-CoA, predict appropriate functionalization of the growing pentaketide chain. Mechanistic aspects of this ring closure reaction can be discussed in a plausible manner by a keto group at C-8 and a second oxyrane ring located at C-6/C-12 of a postulated pentaketide precursor. However, the hypothetic intermediate

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7 illustrates only one possible mechanism. The linkage of nitrogen at the terminating carboxyl group of a pentaketide chain followed by ring closure reaction to a piperidine has already been reported in the case of coniine.[24] We assume an analogous mechanism (4 and 5), in which a C_1N-unit like urea or carbamoyl phosphate is involved. This sequence predicts a keto functionality of the pentaketide chain at C-11 (4). Regarding the biosynthetic relationships of 1, streptenol D (2), and the piperidines of type 3, some analogies can be seen. In streptenol D (2) the terminating carboxy functionality is reduced to a hydroxy group, whereas an identical piperidine ring formation as in the case of streptazolin (1) by introduction of a nitrogen atom seems plausible for the biosynthesis of 3. This is in accordance with the location of a keto group at C-5 in 2 and hypothetically at C-2 in 3. It seems likely that the availability of nitrogen during the fermentation process either from the nitrogen pool or in combination with a one-carbon metabolite is able to direct the biosynthesis to defined end products. Further studies are in progress.

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