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BIOSYNTHESIS OF AXENOMYCIN D: INCORPORATION OF ^{13}C-LABELLED PRECURSORS INTO THE MENADIONE CHROMOPHORE, DESOXYSUGARS AND THE AXENOLIDE

V. FRIESE, A. BOOS, H.-J. BAUCH* and E. LEISTNER†

Institut für Pharmazeutische Biologie der Universität Bonn, 5300 Bonn, Germany; *Zentrallaboratorium, St Johannes Hospital, 4600 Dortmund, Germany

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IN HONOUR OF PROFESSOR MEINHART ZENK'S SIXTIETH BIRTHDAY

Key Word Index—*Streptomyces lisandri*; axenomycin; menadione; biosynthesis; ^{13}C NMR spectroscopy.

Abstract—Experiments on the biosynthesis of the menadione chromophore of axenomycin D in *Streptomyces lisandri* resulted in the specific incorporation of label from L-[^{13}C-Me]methionine, [1-^{13}C]propionate, [2,3-$^{13}C_2$]propionate, [3-^{13}C]propionate, [U-$^{13}C_3$]glycerol and [U-$^{13}C_6$]glucose. The data indicate that the menadione chromophore is derived from a metabolite of the shikimate pathway. The mode of incorporation shows, however, that the biosynthesis of the menadione chromophore of axenomycins differs from the biosynthesis of the menadione chromophore of menaquinones.

INTRODUCTION

Antibiotics are a group of biologically active compounds of high structural diversity. This diversity is also seen in individual molecular structures of antibiotics like axenomycins. They consist of three main components, namely a menadione chromophore, two desoxysugars (amicetose and axenose) and a macrocyclic lactone called axenolide (Fig. 1) [1–4]. Axenomycins A, B and D are assumed to differ in the structure of the axenolide [1, 2]. Axenomycins exhibit antifungal, antiprotozoal and anthelminthic activity by inhibiting protein synthesis [3, 4].

Factors that influence the formation of axenomycins in batch cultures of *Streptomyces lisandri* have been studied [5]: axenomycin formation can be influenced by various media components. Selection of a high yielding strain and improvement of the medium led to a culture producing 1.5 g axenomycins per litre.

We focused our interest on the biosynthesis of the menadione (2-methyl-1,4-naphthoquinone) chromophore of axenomycins. Menadione is also a structural unit of menaquinones (vitamin K_2) and phylloquinone (vitamin K_1). The biosynthesis of the latter compounds has been extensively studied during recent years [6, 7]. Attempts to specifically incorporate isotopically labelled precursors of the K-vitamins like *o*-succinylbenzoic acid into the menadione chromophore of axenomycins were unsuccessful, however. Experiments which are suitable to demonstrate the presence of enzymes involved in the formation of the menadione chromophore of K-vitamins gave negative results when protein extracts of the axenomycin-producing *Streptomyces lisandri* strain were employed. This seemed to indicate that the menadione chromophore of axenomycins is derived by a sequence of reactions different from the pathway known to lead to K-vitamins.

In the present manuscript we describe experiments on the biosynthesis of the menadione chromophore of axenomycins and its isobutyryl side chain with some observations on the incorporation of precursors into desoxysugars and the axenolide.

RESULTS

Structure and ^{13}C NMR signal assignment of axenomycin D

When a mycelial extract of our *Streptomyces lisandri* strain 3935-1-18 was submitted to bioautography [5] not only axenomycins B (R_f 0.62), A (R_f 0.42) and D (R_f 0.15) became visible but also another antibiotic (R_f 0.25) which we called axenomycin X. Axenomycin D was most abundant followed by axenomycin A, B and X. When *Streptomyces lisandri* was grown in the presence of

†Author to whom correspondence should be addressed.

$BaCO_3$ (instead of $CaCO_3$ [5]) another antibiotic appeared which was called axenomycin F. In this case axenomycin X occurred in minute amounts only.

The structure of the chromophore of the axenomycins is common to all known members of this group of antibiotics. They differ, however, in the structure of the axenolide [1, 2]. A structural formula has only been published for axenomycin B (Fig. 1) [1–4]. This formula, however, is neither consistent with the stated calculated nor the experimentally determined molecular weight [1–4]. Moreover, the structure of the small lactone ring of the axenolide was only supposed [2] to be six-membered. Reduction of the keto function at C-17'''' of axenomycin B (Fig. 1) was assumed to give axenomycin A whereas esterification with sulphuric acid at C-23'''' of the axenolide of axenomycin B would give axenomycin D [F. Arcamone, personal communication]. The presence of a sulphate ester would be consistent with the observation that axenomycin D can be precipitated from a butanol extract with ether [3]. No evidence is available for the position of the ester group in axenomycin D or the keto function in axenomycin A.

Since the structure elucidation of axenomycins was first published [1, 2] FAB mass spectrometry became available. We used this technique for the investigation of the structure of axenomycin D, the most abundant antibiotic produced by *Streptomyces lisandri*. It was useful to compare the data obtained for axenomycin D with those of axenomycin F and X. The molecular weights are given in Table 1. The differences in molecular weight between axenomycins can best be explained by the assumption that axenomycin X lacks an oxygen function and that axenomycin F lacks a SO_3 group when compared to axenomycin D. Some of the more prominent fragments are also listed in Table 1.

The ^{1}H NMR spectrum of axenomycin D was recorded in MeOD. It showed two methyl groups which

Table 1. FAB mass spectroscopic data of axenomycins D, X and F

Axenomycin	M_r	Functional group O	Functional group SO_3	Main fragments (negative ions)
D	1574	+	+	97, 513, 1059
X	1558	−	+	97, —, 1043
F	1494	+	−	—, 513, 979

For explanations see text. [Possible absence (−) or presence (+) of functional groups is indicated].

appeared as singlets (C-2' at 2.175 ppm and C-3''' of axenose at 1.895 ppm) and a total of 20 signals (0.7–1.3 ppm) which are part of 10 doublets corresponding to 10 methyl groups each of which is attached to CH. Since axenomycins differ in the axenolide [1, 2] it follows that axenomycin D contains an additional methyl group in this portion when compared to axenomycin B [2 Me (*s*) and 9 Me (*d*), compare Fig. 1 and Table 2]. The same conclusion was drawn from a broad band decoupled ^{13}C NMR and a DEPT experiment (Table 2). These experiments also showed that axenomycin D contained a total of 75 carbons only (instead of 78 in axenomycin B) (Table 2).

The numbers of quaternary carbons (Cq), methine and methylene groups differ in axenomycin D and B as shown in Table 2. The data also revealed that axenomycin D contains only three quaternary carbons in the axenolide when compared to axenomycin B (four quaternary carbons). The ^{13}C NMR signals of these quaternary carbons were at 209.349 ppm, 168.666 ppm and 99.901 ppm. Since these chemical shifts differ significantly, it is evident that two of these signals cannot be assigned to carbons which are part of two identical structural units such as lactone groups. [Two lactone groups were proposed for axenom-

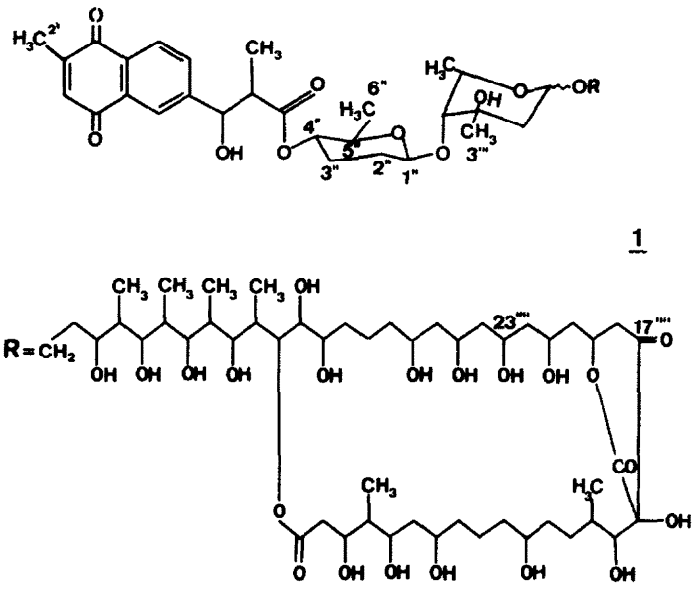

Fig. 1. Structure of axenomycin B (**1**) [1, 2].

Table 2. Comparison of numbers of different carbons in axenomycin B as deduced from degradation, derivatization, ^{1}H NMR and EIMS [1, 2] and of axenomycin D as deduced from a broad band decoupled ^{13}C NMR spectrum and a DEPT experiment (this manuscript) (Cq = quaternary carbon)

	Axenomycin B (Fig. 1 [1–4])	D
Cq	12	11
CH	35	38
CH_2	20	14
Me	11	12
Sum (carbons)	78	75

ycin B (Fig. 1).] Hence it was concluded that axenomycin D may have one lactone ring but is unlikely to have a second one as seems to be the case with axenomycin B (compare Fig. 1).

For the experiments described herein ^{13}C NMR signal assignments of the menadione chromophore and its isobutyryl side chain were most important. The assignments were obtained by spectroscopic techniques, by comparison with published data of menadione (**2**) [8] and by comparison with ^{13}C NMR signals of authentic samples of compounds **3**–**6** (Fig. 2) (*vide supra* or compare Experimental). The authentic samples were obtained in two ways, namely by synthesis of **3**, **4** and **5** as described in the Experimental and by degradation of axenomycin D by acid hydrolysis (which gave **6**) or by alkaline hydrolysis (which gave **5**). Compounds **3** and **4** were either obtained by synthesis or by mild oxidation of **5** or **6**. The ^{13}C NMR signals of **6** (amicetoside of **5**) which was obtained by acid hydrolysis of axenomycin D are given in Table 3. The signals of the synthetic reference compounds **3**–**5** are presented in the Experimental.

Incorporation of precursors into axenomycins

A time course study of growth and axenomycin production of *Streptomyces lisandri* is shown in Fig. 3. While growth of the strain levelled off 22 hours after inoculation, production of axenomycins continued for up to 96 hours.

The time of application of precursors to the culture was crucial. Incorporation of precursors into the axenolide was highest when precursors were fed at a late stage of growth (72–96 hours after inoculation) but incorporation into the quinonoid carbon skeleton was most efficient when precursors were added 24–48 hours after inoculation. This observation was made with different precursors such as acetate, propionate or glucose, labelled with either ^{14}C or ^{13}C in different positions. Incorporation of labelled methionine, however, was observed into the 2'-methyl group of the menadione chromophore after a late feeding experiment (Exp. 1, Table 4). Degradation

Table 3. ^{13}C NMR spectral data (300 MHz) for the α- and β-anomer of **6** (i.e. amicetoside of **5**) in $CDCl_3$

C		δ*	C		δ*
1	Cq	185.2	α-4″	CH	73.9
4	Cq	184.8	β-5″	CH	73.4
14	Cq	174.6	β-4″	CH	73.0
2	Cq	148.4	α-5″	CH	66.5
6	Cq	147.9	12	CH	46.9
3	CH	135.7	β-2″	CH_2	31.6
10	Cq	132.3	α-2″	CH_2	29.0
9	Cq	131.7	β-3″	CH_2	27.2
7	CH	131.6	α-3″	CH_2	23.2
8	CH	127.0	β-6″	Me	17.8
5	CH	124.2	α-6″	Me	17.6
β-1″	CH	95.7	2′	Me	16.5
α-1″	CH	90.7	13	Me	14.5
11	CH	75.5			

*ppm downfield of TMS.

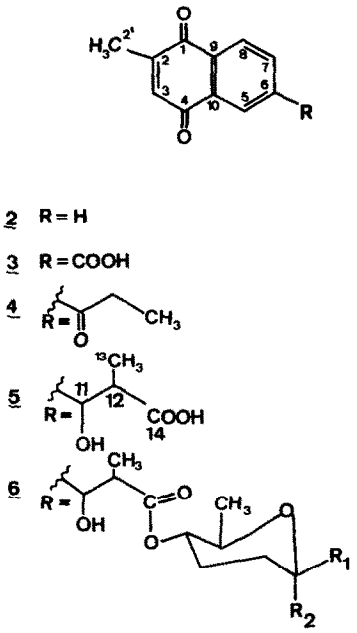

Fig. 2. Structure of menadione derivatives obtained by synthesis (**3**–**5**) or by degradation (**3**–**6**) of axenomycins.

of axenomycin D to **5** (alkaline hydrolysis) and ^{13}C NMR spectroscopy revealed that the 2'-methyl group of the menadione chromophore was specifically labelled with a 6.9-fold enrichment. No label was detected in C-13 which is also part of a methyl group (Fig. 2).

Table 4. Incorporation of ^{13}C-labelled precursors into **5** or **6**

Experiment number	Precursor fed: gram of precursor per litre of medium	labelled compound	Atom % excess	Addition of precursor—harvest of mycelium (hr after inoculation)	Positions labelled (carbons)	δ (ppm)	J(Hz)	^{13}C enrichment observed (fold)	Reference carbon (1.1 atom% ^{13}C) δ
1	0.3/1	L-[Me-^{13}C]methionine	90	72–96	2′	16.4	-	6.9	8 (126.1)
2	1/1	Na-[1-^{13}C]propionate	91.5	72–96	14	175.5	-	4.9	2′ (16.4)
3	0.5/1	Na-[3-^{13}C]propionate	90	72–96	13	14.5	-	6.5	2′ (16.4)
4	0.25/0.75	Na-[2,3-$^{13}C_2$]propionate	80	24–96	12 13	46.9 14.3	33.6 33.4	13 13	2′ (16.4) 2′ (16.4)

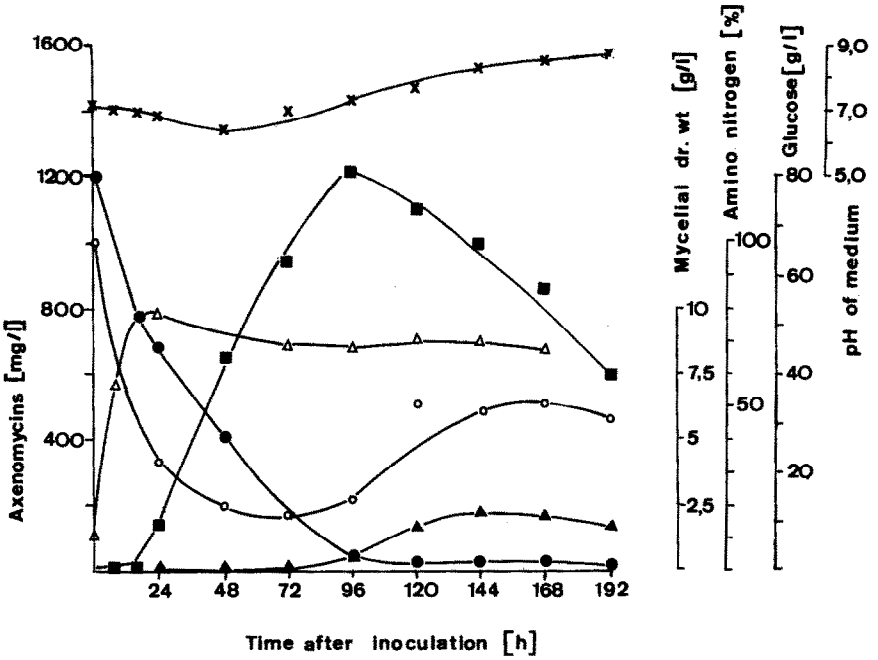

Fig. 3. Time course study of growth (mycelial dry weight (-△-△-) of *Streptomyces lisandri*, amino nitrogen concentration (-○-○-) (% of initial amount present in the medium), glucose concentration (-●-●-), pH of the medium (-×-×-) and occurrence of axenomycins in the mycelium (-■-■-) and in the medium (-▲-▲-).

Indeed, label in this carbon was observed after a Na [3-^{13}C]propionate feeding experiment (Exp. 3, Table 4) suggesting that carbons 13, 12 and 14 of **5** are derived from an intact propionate unit. This assumption was confirmed by experiments 2 and 4 (Table 4) which showed that C-14 of the isobutyryl side chain is specifically labelled from Na [1-^{13}C]propionate whereas both carbons 12 and 13 are labelled during an experiment with Na [2, 3-^{13}C]propionate. The labelled carbons 12 and 13 are coupled, indicating that they are derived from an intact C-2 unit comprising carbons 2 and 3 of propionate.

The origin of the carbon skeleton of the menadione chromophore was investigated using [U-^{13}C]glucose and [U-^{13}C]glycerol as labelled precursors (Exp. 5 and 6, Table 5).

The ^{13}C NMR spectrum of axenomycin D revealed that most of the signals showed satellites. The satellites of carbons 2 and 6 differed. While C-2 of the chromophore had one pair of satellites with a coupling constant of 48.0 or 49.3 Hz, C-6 showed two pairs of satellites. Thus, the nucleus of C-2 was coupled with an adjacent ^{13}C-nucleus whereas C-6 was the centre atom of a biogenetic unit also comprising carbons 5 and 7 or 5 and 11. Axenomycin D from this experiment was hydrolysed and the amicetoside (**6**) subjected to NMR spectroscopy. The interpretation of the spectral data is identical to the interpretation obtained after a [U-$^{13}C_3$]glycerol experiment (Table 6). A coupling was observed between carbons 12 and 13 consistent with the assumption that glucose was channelled into the TCA cycle via acetyl CoA and incorporation into the isobutyryl side chain via succinyl CoA and methylmalonyl CoA. This observation confirmed the conclusions drawn from experiments 2–4.

C-11 showed no coupling with C-12 but with C-6. C-5 had two satellites only and coupled with C-6 rather than C-10. In both experiments 5 and 6 the satellites of C-5 and C-11 were split (3 Hz) due to a long range coupling between the nucleus of C-5 and C-11. This again was a strong indication for the incorporation of carbons 5, 6 and 11 as an intact unit. Moreover, C-10 did not couple with C-4 which had two satellites due to labelling at C-3. Thus, C-3 and 4 represent an intact two-carbon unit. The situation is similar with C-1 and 2. C-7–9 represent another three-carbon unit: while C-7 and C-9 couple with

Table 5. Application of ^{13}C labelled precursors

Experiment number	Precursor fed: gram of precursor per litre of medium	Precursor fed: labelled compound	Atom % excess	Addition of precursor harvest of mycelium (hr after inoculation)
5	0.5/1	[U-$^{13}C_6$]glucose	99	24–96
6	0.5/0.5	[U-$^{13}C_3$]glycerol	99.5	48–96

Table 6. Coupling constants of **6** after incubation of *Streptomyces lisandri* with [U-$^{13}C_6$]glucose (Exp. 5) or [U-$^{13}C_3$]glycerol (Exp. 6). Data for the amicetose are not listed

C	Chemical shifts ($CDCl_3$) [ppm]	Coupling constants [U-$^{13}C_6$]glucose (Exp. 5)	[U-$^{13}C_3$]glycerol (Exp. 6)
1	185.158	49.79	48.68
4	184.810	52.58	53.94
14	174.600	—	—
2	148.356	49.30	48.00
6	147.926	59.08	58.35
		48.47	48.00
3	135.651	53.70	53.61
10	132.286	—	—
9	131.716	58.77	59.81
7	131.645	56.74	56.57
8	126.985	58.25	59.80
		56.75	56.70
5	124.184	58.70	58.81
		3.0	3.30
11	75.437	47.90	47.87
		3.0	3.20
12	46.888	33.64	33.69
2′	16.460	—	—
13	14.517	33.57	33.63

one nucleus only (one pair of satellites in each case), C-8 had two pairs of satellites. The data from both experiments 5 and 6 suggest that carbons 7–10 are derived from erythrose 4-P and carbons 5, 6 and 11 from phosphoenol-pyruvate via a metabolite of the shikimate pathway. The incorporation pattern observed in experiments 1–6 is summarized in Fig. 4. Incorporation of the labelled precursors (Exp. 1–6) also took place into the desoxysugars and the axenolide. Incorporation of label into the amicetose was uneven after [U-$^{13}C_6$]glucose and [U-$^{13}C_3$]glycerol feeding. [U-$^{13}C_3$]Glycerol was incorporated into carbons 4″,5″ and 6″ of the desoxysugar, as indicated by satellites. Carbons 1″, 2″ and 3″, however, showed no satellites. The incorporation of label from [U-$^{13}C_6$] glucose into axenose was also observed, again, with an uneven distribution of enrichments in the sugar molecule. The mode of incorporation into the amicetose suggests that both glucose and glycerol may equilibrate with two different triose pools. Studies on the biosynthesis of acarbose led to a similar conclusion [9].

Observations on the incorporation of precursors into the axenolide also seemed to be noteworthy. After 2,3-^{13}C propionate feeding, eight signals derived from labelled methyl groups were visible. One of these signals was assigned to C-13 whereas seven signals were due to labels in the axenolide. This observation again confirms our previous finding that axenomycin D contains one additional methyl group when compared to axenomycin B which has six methyl groups in the axenolide (compare Fig. 1 and Table 2).

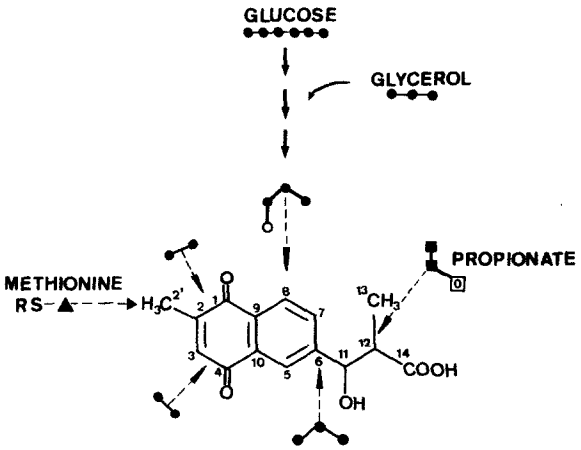

Fig. 4. Origin of carbons and coupling pattern observed after incubation of *Streptomyces lisandri* with ^{13}C-labelled precursors.

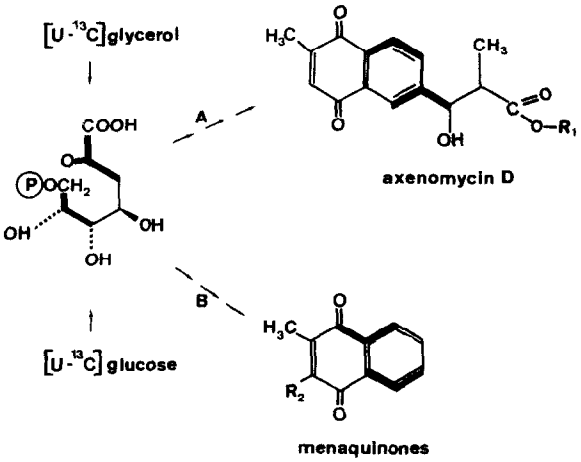

Fig. 5. 'Bond labelling' of the menadione chromophore observed in axenomycin D (A) or expected for menaquinones (B) after incorporation of label from [U-$^{13}C_3$] glycerol or [U-$^{13}C_6$] glucose via 2-deoxy-d-arabinoheptulosonic acid 7-phosphate (DAHP) and the shikimate pathway. The 'bond labelling' pattern in axenomycins is based on the experiments described here whereas the pattern in menaquinones is based on its known [6, 7] biosynthesis.

DISCUSSION

Axenomycins and menaquinones have a menadione chromophore in common. Experiments reported in this paper show that not only the menaquinone chromophore but also the axenomycin chromophore is derived from a metabolite of the shikimate pathway.

While menaquinones originate from isochorismate via *o*-succinylbenzoate [6, 7], the point at which the reactions leading to axenomycins branch off from the shikimate pathway is not known. The incorporation of metabolites of the shikimate pathway into menaquinones (B) and axenomycins (A) differs as shown in Fig. 5. The labelling pattern observed for the axenomycin chromophore after

glycerol and glucose feeding is typical of natural products derived from the shikimate pathway [10]. The pattern of 'labelled bonds' is identical after both [U-$^{13}C_3$]glycerol and [U-$^{13}C_6$]glucose feeding. Glycerol is metabolized to dihydroxyacetone phosphate. The pathway of both labelled glycerol and glucose merge at this metabolite resulting in an identical labelling pattern in the product (axenomycin D).

In an attempt to get some information about the immediate precursor of the menadione chromophore of axenomycins, we fed [G-^{14}C]shikimate 48 hours after inoculation (data not shown). Seventy three per cent of the radioactivity, however, was recovered from the medium, indicating that uptake of the precursor was low and did not allow unequivocal conclusions to be drawn. A repeated feeding of labelled shikimate in the presence of aromatic amino acids and glyphosate, an inhibitor of the EPSP synthase [11], was also carried out. It was hoped that the labelled shikimate would not be metabolized to aromatic amino acids but rather incorporated into axenomycins. The incorporation observed, however, did not exceed the data obtained in the previous experiment. Aromatic acids like phenylalanine, tyrosine and cinnamic acid also were not specifically incorporated during an early feeding experiment (addition of precursor 48 hours after inoculation).

Cell-free protein extracts prepared from mycelium of *Streptomyces lisandri* and incubation of the protein with radiolabelled chorismate and isochorismate did not give a hint as to the identity of the possible shikimate pathway precursor of axenomycins. While isochorismic acid was not converted to any detectable metabolite, chorismate was converted to phenylpyruvate and phenylalanine. Thus the nature of the immediate precursor of the menadione chromophore of axenomycins remains obscure. Carbons 2–4 of α-ketoglutarate supply carbons 2–4 of the quinonoid ring of menaquinones [6, 7]. The pattern of incorporation of [U-$^{13}C_{13}$]glycerol and [U-$^{13}C_6$]glucose into carbons 1–4 of axenomycins with 'bond labelling' between carbons 1 and 2 as well as 3 and 4 suggests, however, that this unit is not derived from a metabolite of the tricarboxylic acid cycle.

Finally it should be mentioned that the parts of the complete axenomycin molecule seem to be synthesized at different times in the growth cycle. As pointed out earlier, the carbon skeleton of the quinonoid chromophore may be completed at a rather early stage of the biosynthesis, whereas the axenolide is hooked up to the chromophore at a rather late stage of growth.

EXPERIMENTAL

Organisms. The origins of *Streptomyces lisandri* and *Paecilomyces varioti* (test organism) were previously described [5].

Media. Paecilomyces varioti was kept on malt agar which was also used for quantitative determination of axenomycins and bioautography [5]. *Streptomyces lisandri* was maintained on a yeast/malt/mannitol agar [5]. Before incubations the organism was raised in an inoculum medium [5]. However, caseine was replaced by NZ-Amine Type A. Cell suspension (7.5 ml) was withdrawn from the inoculum culture and added to a fermentation medium [5] which contained the following components per litre medium: glucose (70 g), L-threonine (7.0 g), cornsteep liquor (20 g), $CaCO_3$ (20 g), $MgSO_4 \times 7H_2O$ (0.5 g), KCl (0.5 g), $FeSO_4 \times 7H_2O$ (0.02 g), $ZnSO_4 \times 7H_2O$ (0.02 g), $MnSO_4 \times H_2O$ (0.002 g), pH 7.2 (NaOH, 1 M). Fermentations were carried out as described [5], however, in 500-ml flasks containing 50 ml medium. For the time course study (Fig. 3) amino nitrogen was determined using the ninhydrin method of Moore and Stein whereas glucose was determined with glucoseoxidase according to Bergmeyer. The methods are quoted in ref. [5].

Extraction and purification of axenomycins. The mycelium was harvested by centrifugation (15 min; 4000 rpm; 'Macrofuge', Heraeus). The supernatant was discarded and the pellet resuspended in H_2O (500 ml) and again centrifuged. The pellet was frozen to −15° and freeze dried. The powdered mycelium was stirred in toluene (10 g mycelium in 250 ml) for 2–3 hr at room temp. The mycelium was collected on a Büchner funnel and toluene removed by suction and the mycelium air dried. The mycelium was extracted by two different methods. Method (a) gave mainly axenomycin D whereas method (b) was more efficient but gave a mixture of axenomycins.

Method (a). The mycelium was stirred in BuOH for 6 hr at room temperature. Extraction was repeated twice. The combined extracts were concd at 40° on a rotavapor until a vol of 100–150 ml was obtained. The soln was mixed with a 6-fold excess of Et_2O. The mixt. was kept overnight at 4°. The ppt. was collected by centrifugation and dried in a desiccator. The ppt. contained mainly axenomycin D.

Method (b). The mycelium was stirred in 500 ml MeOH for 3–5 hr at room temp. Extraction was repeated twice. The filtered and combined extracts were kept at 4° for several hours and again filtered. The solution was evapd at 40° and the residue dissolved in a small amount of MeOH. The soln was mixed with a small amount of $CHCl_3$ and silica gel. Silica gel (Kieselgel 60, Merck, Darmstadt, 300 g) was also suspended in ETOAc–MeOH–H_2O = 125:25:17 and poured into a column (3.8 × 60 cm). The dried mixture of silica gel and axenomycins (*vide supra*) was also suspended in a small amount of the same solvent and placed on top of the column. Axenomycins were eluted from the column using the solvent system given above. The method was suitable to separate the mixture of axenomycins A and B from the mixture of D and X.

The fractions containing axenomycins were now evapd and sepd on a Lobar RP8 Lichroprep column, size B (40–63 μm, Merck, Darmstadt). The solvent was passed through the column with a pressure of 30 bar (Duramat pump, CFG Verfahrenstechnik, Heidelberg, Germany). Impurities were washed down from the column with MeOH–H_2O = 3:2. Axenomycins were eluted from the column with a solvent mixt. of MeOH–H_2O = 7:3. Axenomycin D was also obtained after preparative TLC on

silica gel using EtOH–iso-PrOH–H_2O (20:7:3) (Axenomycin B R_f = 0.7, A = 0.5, X = 0.34, D = 0.22.)

Axenomycins prepared for mass spectrometry were also purified by TLC (silica gel) in the following solvent systems: 1. EtOAc–EtMeCO–MeOH–H_2O = 12:8:2:1. Axenomycin B R_f = 0.5, A = 0.37, X = 0.20, D = 1.0. 2. EtOAc–EtMeCO–MeOH–H_2O = 12:2:2:1. Axenomycin B R_f = 0.6, A = 0.5, F = 0.33, X = 0.27, D = 0.2.

Prior to spectroscopy, axenomycins were purified on a Sephadex LH-20 column with MeOH as solvent. (The silica gel was washed with acid (2 M HCl) and H_2O until the effluent was neutral. Eventually the silica gel was dried at 100° and then employed in either CC or TLC.)

Isolation of axenomycin F. The medium used for production of axenomycins contained $CaCO_3$ (20 g l^{-1}). When $CaCO_3$ was replaced by increasing amounts of $BaCO_3$ (1. 20 g l^{-1} $CaCO_3$; 2. 10 g l^{-1} $CaCO_3$ + 20 g l^{-1} $BaCO_3$; 3. 10 g l^{-1} $BaCO_3$; 4. 40 g l^{-1} $BaCO_3$), dry weight (1. 35 g l^{-1}; 2. 38.7 g l^{-1}; 3. 38.0 g l^{-1} and 43.8 g l^{-1}) and amount of axenomycin (1. 1079 mg l^{-1}; 2. 1209 mg l^{-1}; 3. 1195 mg l^{-1} and 4. 1375 mg l^{-1}) isolated from the mycelium increased. Moreover the spectrum of axenomycins changed. TLC [silica gel; EtOAc–EtMeCO–MeOH–H_2O (12:2:2:1)] showed that only trace amounts of axenomycin X (R_f 0.27) were present in medium containing $BaCO_3$. But increasing amounts of a new axenomycin were formed which we called F (R_f 0.33). The UV spectrum of this new axenomycin was superimposable with that of axenomycin D indicating that axenomycin F contained also a menadione chromophore. The ^1H NMR spectrum of axenomycin F could not be distinguished from a spectrum of axenomycin D.

Compound **6** *by acid hydrolysis of axenomycins.* Acid hydrolysis of an axenomycin mixt. or axenomycin D gave **6** (i.e. amicetoside of **5**). The reaction was carried out in 50–200 ml H_2O in which the axenomycin (*ca* 1 g) was suspended with the aid of an ultrasonic bath. The mixt. was heated until reflux commenced and then acidified (conc. HCl) to pH 1. Heating was continued until the soln became clear and then filtered into a sepn funnel. The cold filtrate was extracted several times with $CHCl_3$ (10–15 ml each) and the combined organic layers dried (Na_2SO_4). The solution was evapd and either sepd by TLC (silica gel) ($CHCl_3$–HOAc, 9:1, R_f 0.65) (toluene–dioxane–HOAc, 95:25:4, R_f 0.36) or by chromatography on a silica gel column. The residue was dissolved in a $CHCl_3$–MeOH mixt. and mixed with a small amount of acid-washed silica gel 60 (Merck, Darmstadt). The silica gel was air-dried at room temp. and placed on top of a column which was prepared as follows: 90 g acid-washed silica gel 60 was suspended in $CHCl_3$–HOAc, 9:1 and the suspension poured into a glass column (2.5 cm diameter). The product of the acid hydrolysis was eluted with the same solvent mixture (yield 10 mg).

Characterization of **6** *(i.e. amicetoside of* **5***).* When **6** was submitted to alkaline hydrolysis (*vide supra*), **5** was obtained. The UV spectrum (MeOH) of **5** showed two maxima at 225 and 335 nm with two shoulders at 253 and 270 nm. The spectrum was superimposable with that of **5**. The amicetoside (**6**) was submitted to EIMS which revealed a molecular mass of 388.1543 corresponding to $C_{21}H_{24}O_7$. EIMS m/z = 388 ($[M]^+$, 1.1%); (372, 2.8%); (370, 2.7%); (272, 28.4%); (258, 57.4%); (202, 100%); (201, 95.7%). The ^{13}C NMR-APT spectrum showed in addition to the ^{13}C signals of **5** six CH-, four CH_2- and two Me groups, indicating that hydrolysis had taken place between the two desoxysugars (Fig. 1) and that two anomers (α and β) of the amicetoside were formed. The relative amount of both anomers was equal as judged from the size of the ^{13}C NMR signals. Assignments for both ^{13}C and ^1H signals were carried out by a HETCOR (heteronuclear correlation spectroscopy) and H, H-COSY (correlated spectroscopy) experiment. The data for the amicetoside are given in Table 3. Signals of C-9 and C-10 were distinguished by selective irradiation of H-3 which gave a doublet for C-10 at 13.343 ppm. Assignment of signals for C-1/C-4 and C-2/C-6 were possible by comparison of signals of synthetic menadiones (Table 7) and after a ^{13}C-glucose feeding experiment (*vide supra*).

Compound **5** *by alkaline hydrolysis of axenomycins.* A crude mixture of axenomycins (3 g) was suspended in H_2O (50 ml). N_2 was flushed through the mixt. for 60 min at room temp. and NaOH (50 ml, 2 M) was added. The mixt. was kept at room temp. for 10 min and acidified (2 M HCl) until a pH of 2–3 was reached. The aq. phase was extracted ×5 with EtOAc (150 ml) and the combined organic layer dried (Na_2SO_4). The 2-methyl-6-(2-carboxy-1-hydroxypropyl)-1,4-naphthoquinone (**5**) was purified by TLC (toluene–dioxane–HOAc, 92:25:4; R_f 0.31) or by DCCC. The compound was dissolved in a small amount of $CHCl_3$ and sepd by the descending mode with a solvent system containing $CHCl_3$–MeOH–H_2O = 13:7:4.

Compounds **3** *and* **4** *by oxidation of* **5** *or* **6**. Compound **5** or **6** (4 mg) was dissolved in HOAc (1 ml) and a soln of K_2CrO_4 (50 mg) in H_2O (1 ml) added. The reaction mixt.

Table 7. ^{13}C NMR spectral data (300 MHz) in DMSO of **3**–**5** as compared to data published for menadione (**2**) [8]

C	5	4	3	2
1	184.9	184.5	184.4	184.3
4	184.7	184.2	183.7	184.9
14	175.5	—	—	—
6	149.7	140.4	134.2	133.3
2	148.2	148.7	148.3	147.8
3	135.3	135 7	135.4	135.4
7	132.3	132.8	133.9	133.3
10	131.7	132.1	131.7	131.9
9	131.0	134.4	135.2	131.9
8	126.1	126.9	126.1	126.2[a]
5	123.8	124.8	126.5	125.8[a]
11	74.1	202.0	165.5	—
12	47.1	32.0	—	—
2'	16.0	16.1	15.9	16.3
13	13.2	8.0	—	—

[a]These assignments may be reversed.

was cooled (0°) during the oxidation. Then the soln was heated on a boiling water bath for 90 min. Fifteen minutes after the start of the reaction TLC (silica gel) (toluene–dioxane–HOAc, 95:25:4) revealed the presence of **4** (R_f 0.80). Compound **3** (R_f 0.64) was detectable after 25 min. Oxidation of **5** or **6** was completed after 25 min. No **4** but only **3** was detectable after 90 min. Yield of **3** and **4** was 12%. The reaction products were isolated preparatively and identified by comparison with authentic samples (*vide infra*).

Synthesis of **3** *from* 2-*methylnaphthalene* (*modified after ref.* [12]). 2-Methylnaphthalene (46 g) and $AlCl_3$ (88 g) were dissolved in nitrobenzene and cooled. Acetylchloride (23 g) was added dropwise and the mixt. kept at room temp. for 24 hr. HCl (1 M) and ice were added and the soln extracted with Et_2O. The Et_2O was washed with NaOH and H_2O, dried (Na_2SO_4), filtered and evapd. The residue was distilled (1.0 Torr, 56°) and the nitrobenzene discarded. A mixt. of 6-acetyl and 8-acetyl-2-methylnaphthalene distilled at 139.5° (0.1 Torr). Yield 77%. The mixt. of naphthalenes was sepd by crystallization of their semicarbazones. The 8-acetyl derivative was very soluble in EtOH, whereas the desired 6-acetyl derivative crystallized from hot EtOH, mp 236°, yield 36%.

The semicarbazone of 6-acetyl-2-methylnaphthalene (10 g) was stirred in conc. HCl (200 ml) for 15 min at 100°. After cooling, the soln was diluted 3-fold (H_2O) and extracted with Et_2O. The organic layer was washed, dried (Na_2SO_4) and evapd. 6-Acetyl-2-methyl-naphthalene crystallized from the oily residue. Yield 77%.

The 6-acetyl-2-methylnaphthalene was submitted to hypobromite oxidation (10 g NaOH and 2.5 ml Br in H_2O (50 ml) per 1 g of naphthalene derivative). Hypobromite oxidation was carried out at room temp. After 2 hr the mixt. was heated to 100°. The crystals formed were collected, dissolved in NaOH (5.0 M), the solution filtered and the naphthoic acid pptd by addition of H_2SO_4 (2 M). The crystals were collected, washed with H_2O and dried, mp 229°. Yield 78%.

2-Methyl-6-carboxynaphthoic acid (4.65 g) was dissolved in HOAc (49 ml) and a solution of CrO_3 (16.4 g) in H_2O (11.5 ml) and HOAc (11.5 ml) was added dropwise at 60°. After addition the mixt. was heated to 85–90° for 1 hr. The mixt. was diluted to 200 ml (H_2O) and cooled on ice. The 2-methyl-6-carboxy-1,4-naphthoquinone was collected, washed (H_2O) and dried. The product (**3**) was recrystallized from EtOH. Yield 45%. UV λ_{max}^{MeOH} (ε): 251 (31 272), 256 (31 887), 335 (3884). IR ν^{KBr} cm^{-1}: 1685, 1660, 1622, 1600, 1570 (C=C aro), 1480 (C=CH aro); 940, 910, 878, 760, 755, 690. MS *m/z* 216 ($[M]^+$, 100%); 199 ($[M]^+ - OH$; 3.2%); 160 (188 − CO; 6.6%). ^1H NMR (90 MHz) Me_2CO δ (ppm vs. TMS): 8.75 (H, *d*), 8.47 (1H, *dd*), 8.22 (1H, *d*), 7.02 (1H, *q*), 2.22 (3H, *d*).

Synthesis of **4** *from* 2-*methylnaphthalene* (*modified after refs* [12] *and* [13]). Propionylchloride (15 g) was added to a soln of 2-methylnaphthalene (23 g) and $AlCl_3$ (44 g) in nitrobenzene (100 ml). The mixt. was stirred (48 hr) and HCl (0.1 M, 70 ml) added. The soln was cooled and extracted with ether (150 ml × 6). The Et_2O was dried (Na_2SO_4), filtered and concd. The nitrobenzene was removed by vacuum distillation (46 Torr, 110–120°). 2-Methyl-6-propionylnaphthalene was subsequently distilled at 210–224°. 2-Methyl-6-propionylnaphthalene crystallized at room temp. The product was recrystallized from petrol (40–60°). Needles, mp 53°. Yield 14%. 2-Methyl-6-propionylnaphthalene (500 mg) was dissolved in HOAc (5 ml). The soln was cooled and a soln of CrO_3 (500 mg) in HOAc (5 ml) added for 5 min. The mixt. was kept at 75° for 10 min and diluted with H_2O. The mixt. was extracted with EtOAc (30 ml × 5). TLC revealed the presence of 2-methyl-6-carboxy-1,4-naphthoquinone (**3**) and 2-methyl-6-propionyl-1,4-naphthoquinone (**4**).

Compound **3** was removed from the EtOAc by extractions with $NaHCO_3$ (1% w/w). The EtOAc containing **4** was washed with HCl (0.01 M) and H_2O and EtOAc dried and concd.

The yellow product was recrystallized from HOAc, mp 147°, yield 43.3%. UV λ_{max}^{MeOH} (ε): 334 (27 19), 254 (24 474). IR ν^{KBr} cm^{-1}: 1708 (C=O), 1660 (C=O), 1620 (C=C aro), 1598 (C=C aro), 1480 (CH aro) 940, 910, 890, 795, 690. ^1H NMR (90 MHZ), δ (ppm vs TMS): 8.58 (H, *d*), 8.3 (H, *dd*), 8.2 (H, *d*), 6.92 (H, *q*), 3.1 (2H, *q*), 2.22 (3H, *d*), 1.26 (3H, *t*).

Synthesis of **5** *from* **3**. 2-Methyl-6-carboxy-1,4-naphthoquinone (2 g) was dissolved in thionylchloride (10 ml) and refluxed for 1 hr at 80°. The thionylchloride was evapd. The residue was dissolved in dry Et_2O and added to a mixt. of Mg (200 mg) in dry EtOH (6.5 ml) and CCl_4 (350 μl) and methylmalonic acid diethyl ester (1.45 g) in EtOH (3 ml) and Et_2O (6 ml). The soln was cooled (0°) and stirred for 1 hr and kept at 4° overnight. To the cooled soln conc. H_2SO_4 (7 ml) in H_2O (100 ml) was added dropwise and the mixture extracted with Et_2O (150 ml × 7). The Et_2O was washed (H_2O) and dried (Na_2SO_4), filtered and evapd. The residue was purified on a silica 60 column with toluene–dioxane–HOAc =(95:25:4) as the solvent system.

The ester derivative was then hydrolysed. The product was suspended in H_2O (20 ml) and flushed with N_2 (1 hr). NaOH (2 M, 20 ml) was added and the reaction mixt. kept for 10 min at room temp. HCl (2 M, 22 ml) was added and the mixt. extracted with EtOAc (50 ml × 6), dried with Na_2SO_4, filtered and evapd. Eventually the resulting carbonyl compound was reduced to **5** by $NaBH_4$. The residue (*vide supra*) was dissolved in MeOH (3 ml) and added to a soln of $NaBH_4$ (2 g) in H_2O. The mixt. was stirred at room temp. (4 hr), HCl (1 M) added, extracted with EtOAc and dried over Na_2SO_4. After evapn the product was purified by prep. TLC on silica gel (toluene–dioxane–HOAc=95:25:4) R_f of **5** 0.42. Yield 1.26% (32 mg). UV λ_{max}^{MeOH} ε: 334 (2594), 256 (17 956), 251 (15 430). IR ν^{KBr} cm^{-1}: 2920–3500 (OH), 1708 (C=O), 1662 (C=O), 1652 (C=O), 1622 (C=C aro), 1598 (C=C aro), 1458 (CH aro), 945, 899, 891, 865, 805, 694. MS *m/z* 274 ($[M]^+$, 2%), 217 (19%), 201 ($[M]^+ - C_3H_5O_2$, 100%), 173 (48%), 145 (20%), 133 (72%), 115 (65%), 105 (84%), 91 (9%), 74 (21%). ^1H NMR (90 MHz) $CDCl_3$ δ (ppm vs. TMS): 8.1 (1H, *d*), 8.03 (1H, *d*), 7.7 (1H, *dd*), 6.8 (1H, *q*), 3.5–4.4 (1H, *broad*), 4.9 (1H, *d*), 2.9 (1H, *m*), 2.2 (3H, *d*), 1.1 (3H, *d*).

Spectroscopic techniques. EIMS were recorded on a Kratos AEI MS 30 (70 eV). FABMS were recorded on a ZAB-HF spectrometer (VG Analytical). Axenomycins were dissolved in a small amount of MeOH. The matrix contained either 1-mercapto-2,3-propanediol or a mixt. of dithioerythritole and dithiothreitol (1:3). Ionization was carried out with Xenon at 7 kV.

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