Biosynthesis of kendomycin: origin of the oxygen atoms and further investigations

Helge B. Bode and Axel Zeeck *

Institut für Organische Chemie, Universität Göttingen, Tammannstr. 2, D-37077 Göttingen, Germany

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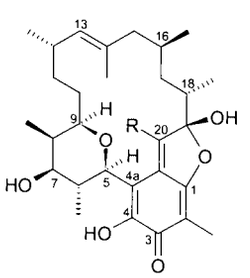
The origin of all oxygen atoms of the structurally unique polyketide antibiotic kendomycin **1** was confirmed by feeding [1-^{13}C,$^{18}O_2$]acetate, [1-^{13}C,$^{18}O_2$]propionate and $^{18}O_2$ to *Streptomyces violaceoruber* (strain 3844-33C) resulting in a more detailed insight into the biosynthesis of **1**. Further information about the biosynthesis of the starter unit in which a chalcone synthase (CHS) must be involved was obtained from comparison of recent literature data with the requirements of the kendomycin biosynthesis. The incorporation of acetate into the methylmalonyl extender units reported previously was investigated by additional feeding [2-^{13}C]malonic acid and [1,4-$^{13}C_2$]succinic acid to the strain. As a result, the coexistence of two independent pathways to methylmalonyl-CoA was demonstrated. Furthermore, feeding of *N*-acetylcysteamine and other thiols resulted in the formation of the new kendomycin derivatives **2** and **3** in good yields.

Introduction

Kendomycin **1** is a structurally unique polyketide isolated from different *Streptomyces* sp. with highly interesting biological activities.[1–4] In a previous publication about the biosynthesis we have described the origin of all carbon atoms of **1**, isolated from *Streptomyces violaceoruber* (strain 3844-33C).[1] The biosynthesis proceeds *via* a type I polyketide synthase (PKS) bearing some interesting features: the starter benzoic acid is derived from a chalcone synthase or a type II PKS from four acetate/malonate units resulting in the formation of a tetraketide which is further modified to the starter benzoic acid. Comparison of the biosynthesis of the starter unit with recent literature data encouraged us to propose a more detailed biosynthetic pathway. The macrocyclisation does occur *via* a mechanism similar to the aldol condensation found in fatty acid and polyketide biosynthesis resulting in a carbocyclic *ansa*-compound instead of the usual macrolactone. The methylmalonyl extender units of the *ansa*-chain showed a high level of incorporation after feeding of labelled acetate in previous experiments. Furthermore, the mechanism of the pyran ring formation was unclear. In this paper we present new insights into some of the mentioned aspects resulting from additional feeding experiments with [^{13}C] and [^{18}O] labelled precursors. The nature of the starter benzoic acid encouraged us to use precursor directed biosynthesis[5] as a tool to investigate the specificity of the loading domain of the type I PKS by feeding different benzoic acids and their corresponding *N*-acetylcysteamine thioesters to the strain. The formation of unexpected kendomycin derivatives is described.



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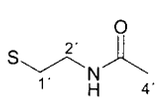
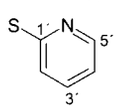
2 R = 

3 R = 

Results

Biosynthesis

Feeding of sodium [1-^{13}C,$^{18}O_2$]propionate to growing cultures of strain 3844-33C resulted in enriched kendomycin **1** (>95% ^{13}C and 60% ^{18}O incorporation) with significant α-isotopic shifts for C-7 (21 ppb), C-9 (26 ppb) and C-19 (12 ppb). Additional α-isotopic shifts for C-1 (12 ppb) and C-3 (34 ppb) were observed in enriched **1** (15% ^{13}C, 40% ^{18}O) after feeding of sodium [1-^{13}C,$^{18}O_2$]acetate. Cultivation of the strain in a $^{18}O_2$–$^{16}O_2$ atmosphere resulted in ^{18}O-enriched **1** (67% ^{18}O) with an α-isotopic shift for C-4 (14 ppb).

Previously the incorporation of acetate into the methylmalonyl extender units has been described.[1] In order to understand this result, we fed [2-^{13}C]malonic acid and [1,4-$^{13}C_2$]succinic acid to the strain. No incorporation of label after feeding of malonic acid into the methylmalonyl extender units was observed but high enrichments in the carbon atoms C-2, C-4, C-5, C-10, C-20 and C-20a were observed, as expected. Weak labelling of C-8, C-12, C-14, C-16, 14-CH_3 and 16-CH_3 might be a consequence of decarboxylation and incorporation of acetate (Table 1). ^{13}C-Enriched kendomycin **1**, obtained after feeding of [1,4-$^{13}C_2$]succinic acid showed the same incorporation pattern as described for the incorporation of [1-^{13}C]-propionate with good incorporation of label at C-7, C-9, C-13, C-15, C-17 and C-19 (Table 1). Fig. 1 summarises the labelling pattern derived from the described feeding experiments.

Addition of ancymidol and fermentation in an oxygen-rich atmosphere

Ancymidol (3 × 17 mg l^{-1}), a well-known inhibitor of P_{450}-dependent oxygenases,[6] was added to the strain during the

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Table 1 Carbon-13 distribution in **1** biosynthesized from labelled precursors ([D_6]acetone, 125.7 MHz) and ^{13}C-NMR data of **2** and **3** ($CDCl_3$, 75.5 MHz)

	1					
C-atom	δ_C (ppm)	[2-^{13}C] acetate[a]	[2-^{13}C] malonic acid[b]	[1,4-$^{13}C_2$] succinic acid[c]	**2** δ_C (ppm)	**3** δ_C (ppm)
1	168.6	0.3	0.0	0.4	166.9	168.2
2	104.2	7.0	7.3	0.4	104.2	105.6
3	182.1	0.3	0.0	0.5	181.1	181.5
4	146.8	4.4	5.4	0.5	147.6	148.4
4a	111.0	0.0	0.0	0.5	108.4	107.4
5	77.7	7.4	10.4	0.5	76.2	75.8
6	38.1	4.4	0.6	0.5	32.2	34.1
7	76.3	3.4	0.2	1.2	78.3	78.8
8	40.8	6.0	1.3	0.4	39.8	29.7
9	78.7	2.9	0.4	2.1	78.8	79.4
10	33.4	8.0	11.5	0.5	32.2	31.8
11	35.8	0.0	0.0	0.5	36.9	36.6
12	33.6	5.6	1.6	0.5	33.5	33.6[d]
13	129.9	3.6	0.9	2.2	132.1	132.8
14	132.1	6.3	2.4	0.5	131.5	131.8
15	46.1	2.7	0.6	1.7	45.3	45.3
16	26.5	4.4	1.5	0.4	28.8	29.6
17	39.7	3.6	0.7	1.8	32.8	33.8[d]
18	41.4	4.8	0.9	0.5	40.4	39.8
19	119.1	2.4	0.1	2.2	117.2	117.8
20	141.4	4.1	7.2	0.0	147.9	139.6
20a	130.2	5.5	6.6	0.7	132.2	136.7
2-Me	7.6	0.0	0.1	0.0	7.7	7.6
6-Me	13.3	6.2	0.9	0.2	14.0	13.3
8-Me	7.2	5.9	0.8	0.3	5.9	6.0
12-Me	22.7	5.0	0.5	0.2	20.7	20.2
14-Me	19.9	6.5	1.2	0.2	19.9	20.2
16-Me	19.7	6.6	1.3	0.2	19.7	19.4
18-Me	12.7	6.7	0.9	0.3	11.8	14.4
1′					37.7	159.2
2′					38.6	121.7
3′					171.5	148.8
4′					23.1	138.3
5′						122.7

[a] See ref. 1. [b] Relative to the natural abundance of C4a. [c] Relative to the natural abundance of 2-CH_3. [d] Assignments may be interchanged.

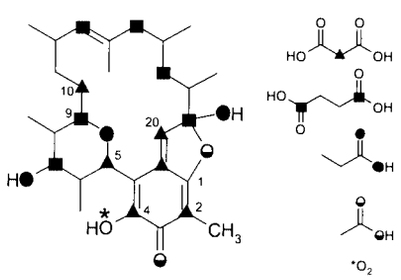


Fig. 1 Labelling pattern of kendomycin **1**.

Table 2 Mercapto compounds added to *S. violaceoruber* and yield of isolated compounds (mg l^{-1}); — = not detected; (+) = trace amounts

Compound	**1**	**2**	**3**
Control	70	—	—
Benzoic acid	70	—	—
3,5-Dihydroxybenzoic acid	70	—	—
Benzoyl-NAC thioester	—	110	—
3,5-Dihydroxybenzoyl-NAC thioester	—	125	—
Isobutyryl-NAC thioester	—	100	—
N-Acetylcysteamine (NAC)	(+)	100	—
2-Mercaptopyridine	60	—	38

production phase and the production rate of kendomycin compared to an untreated control was determined by HPTLC and HPLC. Ancymidol caused a reduction of the kendomycin production to about 80% but no additional compounds were detected. Cultivation of the strain in an airlift fermenter resulted in a four-fold increase of the production rate of **1** to about 280 mg l^{-1} (compared to 70 mg l^{-1} produced under the standard conditions in shaking flasks).

Feeding of mercapto compounds

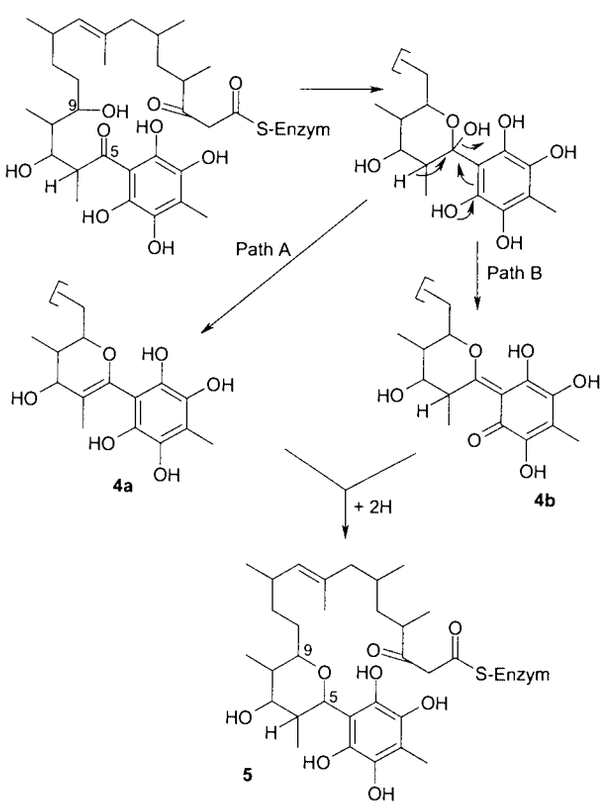
In order to investigate the potential of the well-known precursor-directed biosynthesis[5] to obtain kendomycin derivatives modified in the starter unit, we fed different benzoic acids and their corresponding thioesters to the strain (0.5 g l^{-1}) during the production phase (Table 2). No new kendomycins were detected when the free acids were fed, but a new yellow spot was visible on TLC in all experiments where *N*-acetylcysteamine thioesters have been added. Purification of this compound by subsequent column chromatography led to pure 20-(2-acetylaminoethylsulfanyl)kendomycin **2** (Table 2) in yields up to 125 mg l^{-1}. The same result was obtained when *N*-acetylcysteamine was added directly. The isolated yield of **2** was much higher than that of kendomycin **1** in control experiments (70 mg l^{-1}) and in most cases only traces of **1** were produced when *N*-acetylcysteamine compounds were added. After feeding the quite different compound 2-mercaptopyridine we were able to isolate 20-(pyridin-2-ylsulfanyl)kendomycin **3** (38 mg l^{-1}, Table 2) besides 60 mg l^{-1} of **1**. The structures of **2** and **3** were assigned unambiguously by 1D- and 2D-NMR spectroscopy (^{1}H–^{1}H COSY, HMQC, HMBC), the ^{13}C NMR data are given in Table 1.

Biological activity

The cytotoxic activity of the new kendomycin derivatives **2** and **3** was tested against three tumour cell lines (Table 3). Both compounds are much weaker than **1**. The antibacterial activity against *Bacillus subtilis*, *Staphylococcus aureus* and *Escherichia coli* was investigated using the agar diffusion assay and stem solutions with a concentration of 1 mg ml^{-1}. The new compounds are significantly more active than kendomycin **1** (Table 3). The antibacterial activity against multiresistant *Staphylococcus aureus* strains where **1** shows remarkable results has not been determined yet.

Discussion

The feeding experiments with ^{18}O labelled precursors confirm the origin of all oxygen atoms of kendomycin **1**. Only the hydroxy group at C-4 is derived from air oxygen whereas the other oxygen atoms are introduced *via* acetate/malonate (C-1, C-3) and methylmalonate (C-7, C-9, C-19). These results allow us to postulate a more detailed mechanism for the late biosynthesis of kendomycin **1**. Hemiacetal formation between the hydroxy group at C-9 and the carbonyl group at C-5 followed by elimination of water and reduction of the double bond results in the formation of the pyran ring. It still needs to be clarified whether the pyran ring formation does occur during growth of the polyketide chain or after macrocyclisation. Scheme 1 shows the formation of the pyran ring before



Scheme 1 Pyran ring formation of kendomycin biosynthesis.

the macrocyclisation occurs: Path A would follow a dehydration mechanism resulting in a double bond at C-5=C-6 (intermediate **4a**). Path B requires a phenolic hydroxy group in the *o*-position to the aliphatic side chain in the chromophore resulting in the *o*-quinone-like intermediate **4b** with the double bond at C-4a=C-5 after dehydration. In both cases reduction of these intermediates would result in the formation of compound **5** which after further modifications gives **1** as described previously.[1]

Table 3 Cytotoxic activity (μmol l^{-1}) and inhibition zones (mm, ∅ disc = 9 mm, c = 1 mg ml^{-1}) of **1**, **2** and **3**

		1	**2**	**3**
HMO2[a]	GI_{50}[d]	<0.1	5.5	2.3
	TGI[e]	0.2	7.3	6.4
HEP G2[b]	GI_{50}	<0.1	7.1	5.2
	TGI	0.2	>10	>10
MCF7[c]	GI_{50}	<0.1	>10	2.7
	TGI	0.5	>10	6.4
E. coli		15	21	22
S. aureus		15	22	18
B. subtilis		16	22	22

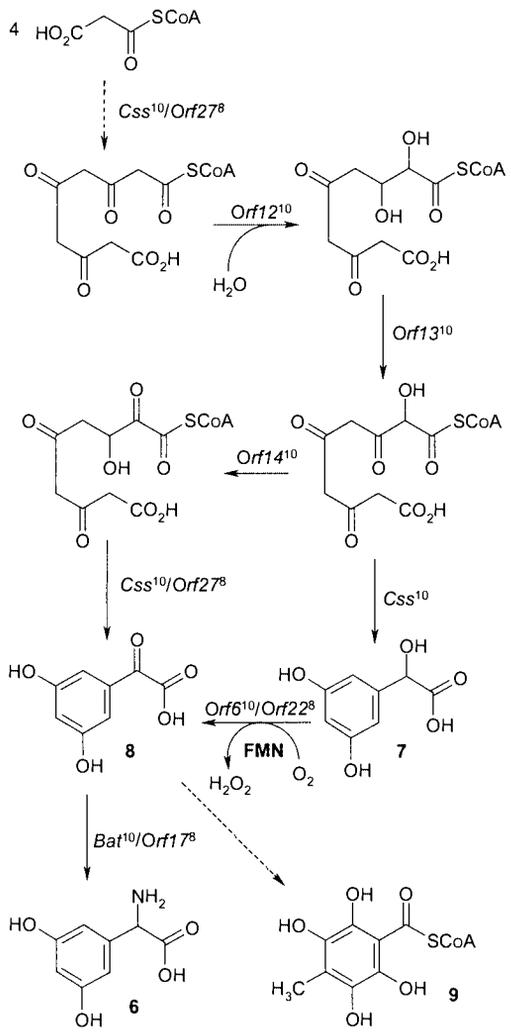
[a] Stomach adenocarcinoma. [b] Hepatocellular carcinoma. [c] Breast adenocarcinoma. [d] 50% growth inhibition. [e] Total growth inhibition.

In this context it seems remarkable that riboflavine could be isolated from those experiments where high titres of **1** have been obtained. Riboflavine (vitamin B_2), as part of flavine adenine dinucleotide (FAD) and flavine mononucleotide (FMN), is an important redox compound of almost all living organisms.[7] Addition of FMN, the direct precursor of FAD, to our strain during the production phase resulted in significant enhancement of the kendomycin production (data not shown). Whether it is used for the reduction step **4a**/**4b** to **5** or another oxidation step of the chromophore in the early or late biosynthesis remains open.

One possibility for the involvement of FAD/FMN in the biosynthesis of **1** follows from sequencing and analysis of the gene clusters of the two glycopeptides vancomycin[8] and balhimycin.[9,10] Both glycopeptides contain the unusual amino acid 3,5-dihydroxyphenylglycine **6** (DHPG) in their peptide backbone which is derived *via* a polyketide pathway[11,12] similar to the starter unit of kendomycin **1**. Pelzer *et al.* analysed the balhimycin gene cluster and found six genes that code for proteins that show high similarity to enzymes probably involved in the DHPG biosynthesis.[10] Scheme 2 summarises a possible biosynthesis of DHPG and the starter of the kendomycin biosynthesis deduced from these results *via* (3,5-dihydroxyphenyl)-2-hydroxyacetic acid **7** and (3,5-dihydroxyphenyl)-2-oxoacetic acid **8**. α-Oxidation of **8**, followed by hydroxylation and methylation would give the required starter **9** in the kendomycin biosynthesis.[1] One can speculate that the increase of the kendomycin production after addition of FMN is a result of an increased glycolate oxidase activity during the biosynthesis of the starter unit. Further experiments concerning the role of FMN/FAD in the biosynthesis of kendomycin **1** are in progress.

The important role of oxygen for the biosynthesis of **1** was demonstrated strikingly by the decrease of the production rate after addition of ancymidol and by the four-fold increase of the kendomycin production by cultivation in an oxygen-rich atmosphere. Furthermore we have found an easy way to improve the yield of this highly active secondary metabolite. The fact that no kendomycin precursors could be detected might point to the oxygenation of the starter benzoic acid prior to polyketide synthesis resulting in inhibition of the biosynthesis at the PKS state by non-hydroxylated starter molecules. This reaction might be catalysed by an oxygenase that oxidises the symmetric benzoic or phenylacetic acid in both *ortho* positions adjacent to the carboxy or carboxymethyl group.[13] Furthermore this mechanism can explain the 20% of kendomycin **1** produced despite ancymidol. It results from starter units hydroxylated prior to the addition of the inhibitor.

Feeding of succinic and malonic acid confirmed the complex biosynthetic network of the biosynthesis of methylmalonyl-CoA **14** in our strain. While [2-^{13}C]acetate led to **1** with significant labelling of the methylmalonyl extender units



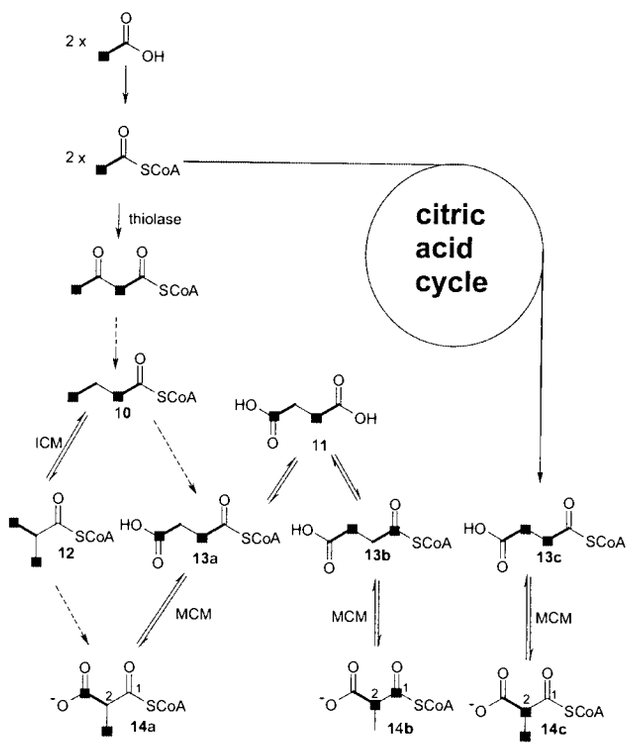
Scheme 2 Biosynthesis of 3,5-dihydroxyphenylglycine **6** and the starter benzoic acid **9** of the kendomycin biosynthesis. *Css*[10]/*Orf27*[8]: chalcone/stilbene synthase, *Orf12*[10]: enoyl-CoA reductase, *Orf13*[10]: hydroxyacyl-CoA dehydrogenase, *Orf6*[10]/*Orf22*[8]: glycolate oxidase, *Orf14*[10]: isomerase, *Bat*[10]/*Orf17*[8]: aminotransferase. Broken arrows indicate multistep reactions.

(Table 1), no malonate was incorporated. Thus we postulate at least two pathways coexist. One proceeds *via* succinyl-CoA **13c** derived from the citric acid cycle, the other *via* thiolase catalysed condensation of two acetyl-CoA molecules, successive reduction to butyryl-CoA **10**, oxidation to **13a** and isomerisation to **14a**. Alternatively **10** can be isomerised to isobutyryl-CoA **12** followed by subsequent oxidation to **14a** as described previously[14–16] (Scheme 3). Feeding of [2,4-$^{13}C_2$]-butyrate resulted in enriched **1** with an identical labelling pattern as observed after feeding of [2-^{13}C]acetate (data not shown). Therefore we conclude the complete cleavage of the butyrate to two acetyl-CoA molecules by β-oxidation. Additional transesterification of the possible isotopomers of **13** *via* succinate **12** would result in three different isotopomers of ^{13}C-enriched **14** from feeding of [2-^{13}C]acetate.

If one assumes equal enrichments of both [2-^{13}C]acetate derived carbon atoms of the different succinyl-CoA molecules due to the great excess of enriched precursors during the feeding experiments, we can determine the portion of each isotopomer of **14** from the enrichment of the methylmalonyl extender units. The overall enrichment of label at C-1 of **14** results from isotopomer **14b** only, the overall enrichment at C-2 results from **14b** and **14c** and the enrichment at C-3 results from **14a** and **14c**. The mean values of the enrichments (in %) for each methylmalonyl-CoA derived position (Table 4) are

Table 4 Enrichments of the three possible positions of the methylmalonyl extender units after feeding of [2-^{13}C]acetate and contributing isotopomers to these values (see Scheme 3)

C-1 position of the methylmalonyl extender units of **1** after feeding of [2-^{13}C]acetate	enrichment (%) $^{-}O_2C–CH_2–CH_3$		
C-7	3.4	4.0	6.2
C-9	2.9	6.0	5.9
C-13	3.6	5.6	5.0
C-15	2.7	6.3	6.5
C-17	3.6	4.4	6.6
C-19	2.4	4.8	6.7
mean value	**3.1**	**5.2**	**6.2**
contributing isotopomers	**14b**	**14b**	**14a**
		14c	**14c**



Scheme 3 Methylmalonyl-CoA biosynthesis in *S. violaceoruber* (strain 3844-33C) from acetate; MCM = methylmalonyl-CoA mutase; ICM = isobutyryl-CoA mutase. Broken arrows indicate multistep reactions.

3.1 (C-1 from isotopomer **14b**), 5.2 (C-2 from **14b** and **14c**) and 6.2 (2-Me from **14a** and **14c**). This means that the portion of isotopomer **14b** for enrichment at C-2 of methylmalonyl-CoA is also 3.1%. As a direct consequence of this observation the portion of **14c** is (5.2 − 3.1)% = 2.1%. Furthermore the portion of isotopomer **14a** is (6.2 − 2.1)% = 4.1%. Therefore we were able to estimate the overall ratio of the three isotopomers **14a**, **14b** and **14c** as 4 : 3 : 2 with the ratio of direct synthesized succinyl-CoA (**14a**/**14b**) to **14c** derived *via* the citric acid cycle being about 3.5 : 1. Although the values used in these calculations might not be correct in all details due to the natural variability of enrichment experiments, they nevertheless represent the coexistence of two different pathways for the biosynthesis of methylmalonyl-CoA. We were able to show for the first time, that both pathways are active simultaneously under the experimental conditions we used for the feeding experiments.

The isolation of two new mercapto substituted kendomycins by feeding of thiols or thioesters to the strain provides a simple and efficient way to new kendomycin derivatives. C-20 is the most electrophilic centre of **1**, thus the nucleophiles were bound in this position. Compounds **2** and **3** show an interesting

biological profile with greater antimicrobial activity but lower cytotoxicity than kendomycin **1**. Compound **2** was produced in amounts 1.5-fold higher than the production of unmodified **1** in control experiments. One question resulting from this observation is how *S. violaceoruber* protects itself against its own antibiotic that seems to be highly reactive towards various thiol nucleophiles that are usually present in living cells.

Experimental

General

Streptomyces violaceoruber (strain 3844-33C),[17] general methods and instrumentation have been described previously.[1] ESI-MS: VG Quattro (Micromass), HRESI-MS: MAT-95 (Thermoquest). [2-^{13}C]Malonic acid, sodium [2,4-$^{13}C_2$]butyrate and [$^{18}O_2$] were obtained from Campro Scientific. $CH_3{}^{13}C$-$^{18}O_2Na$ (99% ^{13}C, 76% ^{18}O) and $CH_3CH_2{}^{13}C^{18}O_2Na$ (99% ^{13}C, 65% ^{18}O) were prepared from the ^{13}C-labelled compounds (99% ^{13}C, Campro Scientific) by exchange with $H_2{}^{18}O$–HCl for 24 h at 100 °C. [1,4-$^{13}C_2$]Succinic acid was prepared as described previously from 1,2-dibromoethane and K^{13}CN in dimethyl sulfoxide[18] followed by hydrolysis with NaOH.[19] The product crystallised from acidified water and was used without further purification. Thioesters were prepared from the corresponding acids as described previously[20] and characterised by NMR and mass spectroscopy. Chemical shifts are expressed in δ values (ppm) using the solvent as internal reference ([D$_6$]acetone: $\delta_H = 2.04$, $\delta_C = 29.8$; $CDCl_3$: $\delta_H = 7.24$, $\delta_C = 77.0$; [D$_5$]pyridine: $\delta_H = 8.71$, $\delta_C = 149.9$). The cytotoxicity tests were carried out as described previously.[1]

Fermentation

For production of high titres of kendomycin **1** or for biosynthetic studies the following two-step protocol was used: a 1 cm^2 piece of agar was used to inoculate 100 ml of medium A (soybean meal 2%, mannitol 2%, deionised water, pH = 7.0 prior to sterilisation) in 300 ml Erlenmeyer flasks with three intrusions. These cultures were cultivated in a rotary shaker (250 rpm) at 28 °C for 3 d. For feeding experiments 5 ml of the seed culture was used to inoculate 100 ml of medium A in 300 ml Erlenmeyer flasks and the cultures were harvested after 48 h. For cultivation in an airlift fermenter (Bioengineering AG, Switzerland, 10 l volume, airation 40 l min^{-1}, 28 °C) 600 ml of the seed culture were used to inoculate 9.5 l of medium A.

Feeding experiments

Feeding experiments were carried out under the conditions described above. In general, precursors were administered to the fermentation as a sterile aqueous solution adjusted to pH 7.0. Pulse feeding protocols were followed in all cases with administration of the precursor at 28, 30, 32, 34, 36 and 38 h. The following amounts were added: [2-^{13}C]malonic acid, 3.1 mmol l^{-1}; [1,4-$^{13}C_2$]succinic acid, 1.5 mmol l^{-1}; sodium [1-^{13}C, $^{18}O_2$]acetate, 12 mmol l^{-1}; sodium [1-^{13}C,$^{18}O_2$]propionate, 5.2 mmol l^{-1}. For the incorporation of $^{18}O_2$ the following experiment was conducted: the cultures were grown until the start of the production phase (28 hours) as described above. After flushing the flasks with nitrogen for 5 minutes they were connected to an apparatus described previously.[21] Pure $^{18}O_2$ (1150 ml) was fed to the cultures over 28–44 h followed by addition of 300 ml $^{16}O_2$ for the last 4 h. The rate of oxygen consumption remained steady within a range of 40–50 ml h^{-1} (5 × 100 ml culture in 300 ml Erlenmeyer flasks with three intrusions) until the end of the cultivation after 48 h. Thiols and thioesters were added in four portions at 28, 32, 38 and 44 h in the following amounts as a solution in DMSO to two flasks: *N*-acetylcysteamine (NAC), 3,5-dihydroxybenzoic acid, 3,5-dihydroxybenzoyl-NAC thioester, benzoic acid, benzoyl-NAC thioester, isobutyryl-NAC thioester (all 0.5 g l^{-1}), 2-mercaptopyridine (100 mg l^{-1}). The overall concentration of DMSO in the culture broth was 1%.

Isolation and purification

The procedure described previously was optimised using the following protocol: the fermentation broth was centrifuged at 3300 rpm for 30 min and the mycelial pellet was lyophilised. When DMSO solutions were fed the whole culture broth was lyophilised. The dried mycelium was extracted with acetone in a Soxhlet extractor for 1 h, the solvent was evaporated *in vacuo* and the resulting crude extract was chromatographed on silica gel ($CHCl_3$–MeOH 9 : 1) to give an enriched fraction of kendomycin or its derivatives which were subjected to gel permeation chromatography on Sephadex LH-20 (acetone). The yield of **1** from feeding of labelled precursors averaged 20 mg l^{-1}, the yields of the new derivatives from feeding thiols and thioesters *etc*. are given in Table 2.

Addition of ancymidol and HPLC analysis

Ancymidol was added as a DMSO solution in three portions at 44, 50 and 54 h in a 70 h cultivation experiment to a final concentration of 100 mg l^{-1}. Centrifugation and extraction of the lyophilised mycelium as described above gave the crude extract that was diluted with acetonitrile–water 1 : 1 and used for HPTLC and HPLC analysis in comparison to an untreated control. The following equipment was used for HPLC analysis: Kontron P 322 (0.05–10 ml min^{-1}), Kontron Sa 360, Kontron DAD 440, Kontron Multiport RS 232, Kontron KromaSystem 2000, analytical 20 µl-sample volume. Pre-column Beckmann Ultrasphere ODS (5 µm, 4.6 × 45 mm), column Knauer Nucleosil 100 C18 (analytic, 5 µm, 3 × 250 mm). Solvents: A 0.1% H_3PO_4; B acetonitrile; gradient: 100% B to 20% B in 5 min, 20% B to 100% B in 20 min, 100% B for 5 min. Flow rate 0.5 ml min^{-1}. Detection 220, 254, 270 and 366 nm. R_t (**1**) = 24.13 min.

20-(2-Acetylaminoethylsulfanyl)kendomycin 2

Yellow powder; mp 116 °C; $[\alpha]_D^{20}$ −438 (*c* 1.13, MeOH); ν_{max} (KBr)/cm^{-1} 3417 (OH), 1655 (C=O) and 1600 (C=C); *m/z* (ESI) 626 ([M + Na]$^+$, 100%), 604 ([M + H]$^+$, 20) [*m/z* (HRESI) Found: [M + Na]$^+$ 626.31567. $C_{33}H_{49}NO_7SNa$ requires *M* 626.31274 (4.8 ppm error)]; *m/z* (EI) 484 ([M − R]$^+$, 60%), 466 ([M − R − H_2O]$^+$, 25), 446 (100) [*m/z* (HREI) Found: [M − R]$^+$ 484.2824. $C_{29}H_{40}O_6$ requires *M* 484.2824; R = 2-acetylaminoethylsulfanyl]; λ_{max}/nm (*c* 0.0113 in MeOH) 308 (log ε 3.92), 381 (4.33); λ_{max}/nm (MeOH + HCl) 308 (3.91), 381 (4.33); λ_{max}/nm (MeOH + NaOH) 220 (4.37), 283 (3.80), 379 (3.47), 540 (2.91); CD λ/nm (*c* 0.0113 in MeOH) 235 (Θ 7600), 242 (6100), 268 (37200), 287 (33800), 371 (−61000); δ_H (300 MHz, $CDCl_3$) 7.84 (br s, 19-O*H*), 6.61 (br s, N*H*), 5.05 (d, *J* = 10.0 Hz, 5-H), 4.83 (d, *J* = 9.0 Hz, 13-H), 3.79 (m, 7-O*H*), 3.53 (m, 2′-H_2), 3.41 (d, *J* = 8.0 Hz, 9-H), 3.36 (dd, *J* = 10.0, 3.5 Hz, 7-H), 2.79 (m, 1′-H_2), 2.75 (m, 6-H), 2.42 (m, 18-H), 2.12 (m, 15-H_a), 2.08 (m, 12-H), 1.99 (s, 4′-H_3), 1.89 (s, 2-CH_3), 1.79 (m, 8-H), 1.74 (m, 16-H), 1.53 (td, *J* = 13.0, 5.0 Hz, 11-H_a), 1.43 (m, 10-H_a, 15-H_b), 1.40 (s, 14-CH_3), 1.33 (d, *J* = 7.0 Hz, 18-CH_3), 1.30 (m, 17-H_a), 1.22 (m, 10-H_b, 11-H_b), 0.90 (d, *J* = 7.0 Hz, 8-CH_3), 0.88 (d, *J* = 7.0 Hz, 6-CH_3, 12-CH_3), 0.81 (d, *J* = 7.0 Hz, 16-CH_3), 0.48 (m, 17-H_b); δ_C (75.5 MHz, $CDCl_3$) see Table 1.

20-(Pyridin-2-ylsulfanyl)kendomycin 3

Yellow powder; mp 73 °C; $[\alpha]_D^{20}$ −233 (*c* 0.371, MeOH); ν_{max} (KBr)/cm^{-1} 3443 (OH), 1610 (C=O); *m/z* (ESI) 618 ([M + Na]$^+$, 100%) [*m/z* (HRESI) Found: [M + Na]$^+$ 618.28912. $C_{34}H_{45}NO_6SNa$ requires *M* 618.28653 (4.2 ppm error)]; *m/z* (EI) 484 ([M − R]$^+$, 5%), 87 (100) [*m/z* (HREI) Found: [M − R]$^+$

484.2824. $C_{29}H_{40}O_6$ requires M 484.2824; R = pyridin-2-ylsulfanyl]; λ_{max}/nm (c 0.0088 in MeOH) 244 (sh, log ε 3.94), 321 (4.18), 330 (4.15), 380 (3.62); λ_{max}/nm (MeOH + HCl) 242 (sh, 3.87), 321 (4.15), 332 (4.14), 367 (3.76); λ_{max}/nm (MeOH + NaOH) 220 (4.32), 241 (4.24), 288 (3.84), 544 (2.69); CD λ/nm (c 0.0088 in MeOH) 232 (Θ −4800), 241 (−5900), 257sh (3200), 284 (12500), 320 (−65600); δ_H (300 MHz, $CDCl_3$) 8.35 (m, 3′-H), 7.70 (td, J = 8.0, 2.0 Hz, 4′-H), 7.40 (d, J = 8.0 Hz, 5′-H), 7.18 (m, 2′-H), 4.95 (d, J = 9.0 Hz, 13-H), 4.92 (d, J = 11.0 Hz, 5-H), 3.63 (m, 9-H), 3.33 (dd, J = 10.0, 4.5 Hz, 7-H), 2.69 (m, 6-H), 2.46 (m, 18-H), 2.22 (m, 12-H, 15-H_a), 1.98 (s, 2-CH_3), 1.93 (m, 8-H), 1.80 (m, 16-H), 1.65 (m, 11-H_2), 1.53 (m, 10-H_a, 15-H_b), 1.51 (d, J = 1.0 Hz, 14-CH_3), 1.40 (m, 10-H_b), 1.32 (d, J = 7.0 Hz, 18-CH_3), 1.28 (m, 17-H_a), 0.98 (d, J = 7.0 Hz, 8-CH_3, 12-CH_3), 0.90 (d, J = 7.0 Hz, 16-CH_3), 0.82 (m, 17-H_b), 0.54 (d, J = 7.0 Hz, 5-CH_3); δ_C (75.5 MHz, $CDCl_3$) see Table 1.

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