Isolation and Characterization of 8-Demethoxy Steffimycins and Generation of 2,8-Demethoxy Steffimycins in *Streptomyces steffisburgensis* by the Nogalamycin Biosynthesis Genes

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Streptomyces steffisburgensis (NRRL 3193, ATCC 27466) is described as a steffimycin producer. Steffimycin belongs to the anthracycline group of aromatic polyketide antibiotics. The structural analysis of the products accumulated by the wild type ATCC 27466 strain revealed three different forms of 8-demethoxy steffimycin suggesting the loss of C-8 hydroxylation/methylation activity. In our approach to generate new anthracycline molecules, we used this strain as a host in gene cloning. The genes encoding the polyketide ketoreductase and aromatase enzymes of nogalamycin biosynthesis caused the production of 2-demethoxy steffimycins in *S. steffisburgensis*.

Streptomyces steffisburgensis was discovered in 1967[1] and found to produce an anthracycline antibiotic, steffimycin **5** (Fig. 1).[2] The structure of steffimycin differs notably from the well known daunomycin and aclacinomycin groups of anthracyclines since its aglycone has the opposite stereochemistry in ring A (7*S*, 9*S*), a methyl group instead of an ethyl group at C-9 and methoxy groups at C-2 and C-8. Most anthracyclines contain an aminosugar, whereas the sugar attached to steffimycinone, the aglycone of steffimycin, is 2-*O*-methylrhamnose. Presumably, the absence of an aminosugar influences the biological activity since, in contrast to most anthracyclines, steffimycin does not have significant antitumor activity.

Daunomycin and doxorubicin, the members of the daunomycin group of anthracyclines, are widely used cytostatic agents in cancer chemotherapy. For this reason the biosynthetic studies of anthracyclines have been mainly focused on the daunomycin group. As a result, the molecular genetics of daunomycin biosynthesis is almost completely resolved, since the genes responsible for most biosynthetic steps have been cloned and identified.[3~5] The early steps in the anthracycline pathway proceed as described for the other aromatic polyketides.[6]

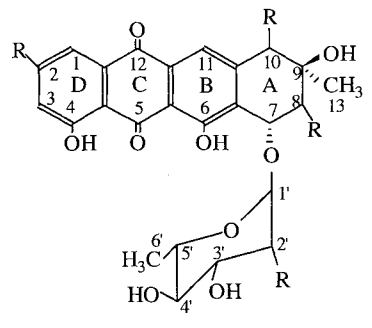
Here we report the characteristics of the products of *S. steffisburgensis* ATCC 27466 in the culture conditions described below. The products did not contain a methoxy group at C-8, even though this is characteristic of the steffimycins isolated previously from *S. steffisburgensis* or *S. elgreteus*.[2,7,8] In our approach to find new anthracyclines by the hybrid antibiotic technique,[9] we have cloned the gene cluster from the nogalamycin producer, *S. nogalater* and characterized some of the genes by expression studies and sequence analysis.[10,11] The products generated by introducing the nogalamycin ketoreductase and aromatase genes into *S. steffisburgensis* are 2-demethoxy steffimycins.

Materials and Methods

Bacterial Strains and Plasmids

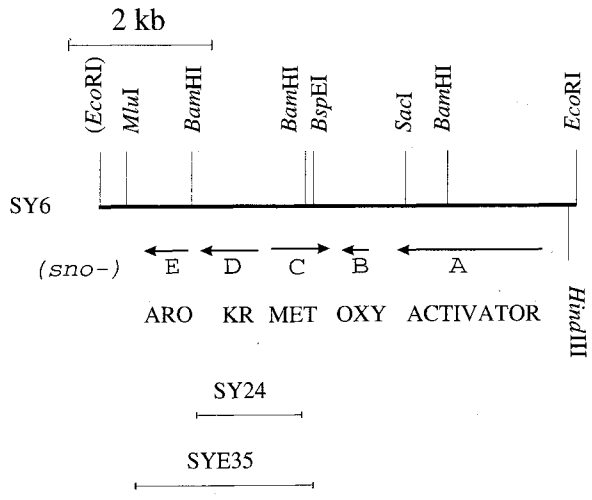
S. lividans TK24 was provided from John Innes Centre

Fig. 1. Structures of 8-demethoxy steffimycins (**1**, **2**, **3** and **4**) and steffimycin (**5**).



1	2-R=OCH_3	10-R=H, H	2'-R=OCH_3	8-R=H, H
2	2-R=OCH_3	10-R==O	2'-R=OCH_3	8-R=H, H
3	2-R=OCH_3	10-R=H, H	2'-R=OH	8-R=H, H
4	2-R=H	10-R=H, H	2'-R=OH	8-R=H, H
5	2-R=OCH_3	10-R=O	2'-R=OCH_3	8-R=OCH_3

Fig. 2. Restriction map of the insert in pSY6 (SY6) and the subclones, pSY24 and pSYE35 prepared from it.



The *Eco*RI-site on the left side is from λEMBL4-DNA and does not include in genomic DNA of *S. nogalater* (*sno*). Abbreviations: ARO = aromatase, KR = polyketide keto-reductase, MET = methyl transferase, OXY = mono-oxy-genase.

(Norwich, UK) and was used for cloning DNA. *S. steffisburgensis* ATCC 27466 was purchased from American Type Culture Collection and was grown on ISP4 agar (Difco) at 28°C for four to seven days. For longer term storage the strain was maintained as a glycerol stock at −70°C. The plasmids used in this study were pSY6[10], pSY24[11] and pSYE35 (this work). pSY6 contains nogalamycin biosynthesis genes for an activator, (SnoA), an oxygenase (OXY), a methyl transferase (MET), a polyketide ketoreductase (KR) and an aromatase (ARO). pSY6 was made by cloning a 6 kb *Eco*RI fragment of the λ-clone (λSno3) carrying a genomic DNA of *S. nogalater* in pIJ486.[12] pSY24 contains a complete KR gene and was made by cloning a 2 kb *Bam*HI fragment of pSY6 in pIJ486.[11] pSYE35 contains nogalamycin KR and ARO biosynthesis genes and was made by subcloning the *Bsp*EI–*Mlu*I-fragment from pSY6 into the plasmid pIJE486, which carries the *erm*E promoter[13] inserted in the polylinker of pIJ486. The *S. steffisburgensis* strains carrying pSY6, pSY24 or pSYE35 were designated as Sst/pSY6, Sst/pSY24 and Sst/pSYE35, respectively. The restriction map of the pSY6 insert and the subclones used in this study are shown in Fig. 2.

Cloning Procedures

Standard techniques for gene cloning were used.[14,15] The expression clones pSY6, pSY24 and pSYE35 were made in *S. lividans* TK24. TSB (Difco) medium supplemented with thiostrepton (10 μg/ml) was used to culture TK24 transformants for plasmid isolation. The plasmids were introduced into *S. steffisburgensis* by protoplast transformation.[14] The plasmid containing strains were cultured in the presence of thiostrepton (10 μg/ml in liquid media and 50 μg/ml in solid media).

Fermentation and Isolation of Compounds

E4-medium containing glucose 10 g, soluble starch 10 g, Pharmamedia (Traders protein) 5 g, yeast extract 2 g, NaCl 1 g, $CaCO_3$ 3 g and Trace element solution[14] 250 μl in 1 liter tap water, pH 7.2 was used for anthracycline production. The culture on ISP4-agar was used to inoculate 60 ml of E4-medium in a 250 ml Erlenmeyer flask and growth was allowed to continue for 3 to 5 days in a shaker (330 rpm, 30°C). The 60 ml seed culture was transferred into a 10 liter fermenter filled with E4 medium. A typical fermentation was carried out for five days at 28°C at 500 rpm with aeration 15 liter/minute. Cells and supernatant were separately extracted with MeOH - dichloromethane (1 : 3). The combined extracts were vacuum concentrated and precipitated from acidic EtOAc - hexane (1 : 10). HPLC-chromatogram of the precipitate indicated three major compounds and several minor ones both in the wild type and Sst/pSY6. The whole precipitated fraction was loaded onto a silica-flash-column and eluted with $CHCl_3$ - AcOH (10 : 0.1) with an increasing amount of methanol. Separate fractions were further purified on an oxalic acid modified silica column, eluting with $CHCl_3$ - EtOAc - MeOH - AcOH (250 : 50 : 5 : 0.5).

Spectroscopic and Other Methods

^1H NMR and ^{13}C NMR spectra were taken on a JEOL JNM-GX 400 spectrometer using 5 mm tubes with DMSO-d_6 as solvent, and both ^1H NMR and ^{13}C NMR samples were referenced to TMS. UV-spectra were recorded on a Pharmacia biochrom 4060 spectrophotometer in methanol. Analytical TLC was done with precoated Kieselgel 60 F_{254} plates. HPLC was done on a Merck Hitachi instrument (L-6200A/L-4250) using a Merck LiChrocart RP-18 column.

Results

Colony Morphology and Production Behavior of *S. steffisburgensis*

S. steffisburgensis (NRRL3193, ATCC 27466) was designated as the type strain and characterized as a

steffimycin producer.[1] We have, however, found some variable features of the type strain both in the steffimycins production capability in the E4 medium and in the colony morphology. The major products (**1**, **2**, **3**) were not produced in the same proportion in the wild type culture. Nevertheless, we considered compound **1** as the main product in the complex, since its portion was about 50% in a typical fermentation. The temporal appearance of the compounds was irregular, making it almost impossible to follow the conversion of these three forms of 8-demethoxy steffimycins (compound **1** is 8-demethoxy steffimycin C[8], **2** is 8-demethoxy steffimycin and **3** is 8,2′-demethoxy steffimycin C).

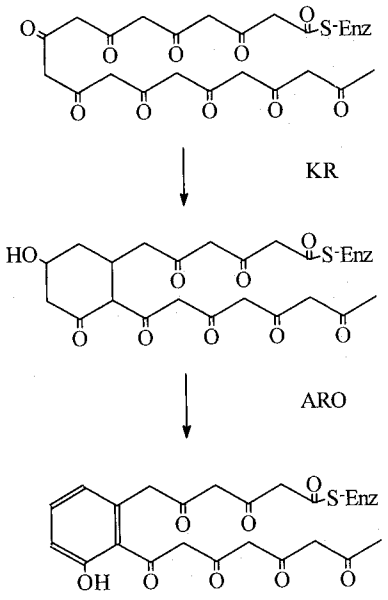
On ISP2 agar the strain sporulates poorly, whereas on ISP4 it sporulates well. However, three different phenotypes were detected on ISP4. Blue and white spore-bearing aerial hyphae was noticed in the colonies. Based on our studies it is possible that the colonies possessing white spores were changed into blue. In any case, the blue spore pigment developed later than the white aerial hyphae. After several rounds of cultivation some bald colonies, which did not possess aerial mycelia, were also grown on agar. These colonies were not a suitable inoculate for steffimycin production, since no colored compounds were obtained from the culture broth.

Expression of the Nogalamycin Biosynthesis Genes in *S. steffisburgensis*

S. steffisburgensis was transformed quite efficiently with the plasmid preparations isolated from *S. lividans* TK24 (10^4 transformants/μg plasmid DNA). Several separate clones were used to study the formation of hybrid products. The transformants were picked up on ISP4-agar supplemented with thiostrepton and used to inoculate 60 ml of E4-medium. The altered product profile as compared to that of the wild type was first observed in the crude extract of Sst/pSY6 culture broth. The structural analysis of the products revealed the presence of 2,8-demethoxy steffimycins, suggesting that the absence of the methoxy group at C-2 (**4**, 2,8,2′-demethoxy steffimycin C) was caused by the genes for nogalamycin biosynthesis.

In nogalamycin biosynthesis, KR reduces position 2 and based on the action of an ARO, a hydroxyl group is removed resulting in the first aromatic ring (D). According to the proposed biosynthetic pathway of anthracyclines (Fig. 3), we concluded that the *sno* genes for KR and ARO are responsible for these modifications. That is why the clone, Sst/pSYE35, carrying the genes

Fig. 3. The formation of a proposed monocyclic anthracycline intermediate.



The enzymes responsible for the reactions are ketoreductase (KR) and aromatase (ARO).

for aromatase and ketoreductase expressed from the *erm*E promoter was prepared. As expected, the production profile was similar to that of Sst/pSY6, thus demonstrating that the genes *sno*D and *sno*E encoding KR and ARO, respectively, caused the absence of the methoxy group at position 2 in the hybrid compound. Sst/pSY24 carrying the gene for KR, failed to generate 2,8-demethoxy steffimycins.

Structure Elucidation of 8-Demethoxy Steffimycins

Compounds **1**, **2** and **3** had similar UV-spectra in methanol (maximal absorptions at 280 and 440 nm) referring to an anthraquinone ring system. Compound **4** showed an otherwise similar spectrum except maximal absorptions were shifted 10 nm to lower wavelengths. All compounds changed color in basic conditions indicating the presence of enolic hydrogens.

Table 1 lists the chemical shift values of the identified compounds. At 400 MHz all of the multiplets were well separated and all couplings could be resolved. The ^{1}H NMR spectrum of **1** has chemical shifts of 7.43, 7.19 and 6.88 ppm arising from aromatic protons. Two higher field signals were mutually coupled with 2 Hz, suggesting that they are 1,3 to each other. Signals at 3.94 and 3.40 ppm corresponded to two methoxyl groups, with one being attached to an aromatic carbon. Aliphatic regions showed AB- and ABX-system, arising from the protons of the ring A of the aglycone. They were assigned to

Table 1. ^{13}C and ^{1}H NMR spectral data for compounds **1**~**3**, and ^{1}H NMR spectral data for compound **4**. ^{13}C chemical shifts in brackets may be interchanged with other resonances nearby.

Site	Compound **1**		Compound **2**		Compound **3**		Compound **4**
	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H
1	1H, 7.18, d, 2.5	(107.6)	1H, 7.19, d, 2.6	(108.1)	1H, 7.16, d, 2.5	(107.7)	1H, 7.71, dd, 8.3, 1.2
2	—	(166.1)	—	(166.6)	—	(166.2)	1H, 7.81, t, 8.3, 8.3
2-OMe	3H, 3.94, s	56.4	3H, 3.94, s	56.5	3H, 3.92, s	56.4	—
3	1H, 6.88, d, 2.5	(106.6)	1H, 6.88, d, 2.6	(106.1)	1H, 6.86, d, 2.5	(106.6)	1H, 7.38, dd, 8.3, 1.2
4	—	(164.4)	—	(164.5)	—	(164.4)	—
4-OH	1H, 12.97, brs	—	1H, 11.92, brs	—	1H, 12.06, brs	—	1H, 12.06, brs
4a	—	(110.5)	—	(110.3)	—	(109.9)	—
5	—	190.2	—	189.8	—	190.3	—
5a	—	120.1	—	(118.3)	—	(120.1)	—
6	—	(161.2)	—	(160.6)	—	(161.2)	—
6-OH	1H, 12.78, s	—	1H, 12.81, brs	—	1H, 12.70, brs	—	1H, 12.72, brs
6a	—	(131.3)	—	(132.8)	—	(131.5)	—
7	1H, 4.91, dd, 6.3, 5.0	(69.4)	1H, 5.13, dd, 4.9, 3.4	(69.8)	1H, 4.88, t, 5.1, 4.6	(70.7)	1H, 4.92, t, 6.4, 4.6
8A	1H, 2.13, dd, 13.6, 6.3	(43.1)	1H, 2.50, dd, 14.6, 4.9	41.4	1H, 2.11, dd, 13.7, 5.1	(43.1)	1H, 2.13, dd, 13.8, 6.4
8B	1H, 1.98, dd, 13.6, 5.0	—	1H, 2.26, dd, 14.6, 3.4	—	1H, 1.98, dd, 13.7, 4.6	—	1H, 1.98, dd, 13.8, 4.6
9	—	(67.3)	—	(69.7)	—	(67.4)	—
10A	1H, 2.96, d, 13.6	(44.4)	—	199.3	1H, 2.96, d, 17.3	(44.4)	1H, 2.98, d, 16.9
10B	1H, 2.76, d, 13.6	—	—	—	1H, 2.75, d, 17.3	—	1H, 2.75, d, 16.9
10a	—	146.5	—	(136.3)	—	146.6	—
11	1H, 7.45, s	(113.2)	1H, 8.03, s	(115.0)	1H, 7.40, s	(113.2)	1H, 7.46, s
11a	—	(134.7)	—	(134.9)	—	(134.9)	—
12	—	180.9	—	180.7	—	181.0	—
12a	—	(131.5)	—	(135.0)	—	(131.5)	—
13	3H, 1.28, s	28.4	3H, 1.44, s	25.8	3H, 1.28, s	28.6	3H, 1.29, s
1′	1H, 5.18, d, 1.3	100.2	1H, 5.28, d, 1.4	100.4	1H, 5.02, d, 1.2	(103.5)	1H, 5.04, d, 1.2
2′	1H, 3.26, dd, 3.3, 1.4	80.9	1H, 3.29, dd, 3.2, 1.5	80.8	1H, 3.60, dd, 3.6, 1.3	(70.7)	1H, 3.60, dd, 3.6, 1.3
2′-OMe	3H, 3.40, s	58.5	3H, 3.41, s	58.6	—	—	—
3′	1H, 3.38, dd, 9.6, 3.4	(72.1)	1H, 3.39, dd, 9.2, 3.2	(72.0)	1H, 3.90, dd, 9.6, 3.6	(72.0)	1H, 3.90, dd, 9.5, 3.6
4′	1H, 3.17, t, 9.6, 9.1	(72.5)	1H, 3.20, t, 9.6, 9.2	(72.2)	1H, 3.20, t, 9.6, 9.2	(71.8)	1H, 3.20,t, 9.5, 9.3
5′	1H, 3.58, dq, 9.6, 6.2	(70.5)	1H, 3.64, dq, 9.2, 6.2	(70.5)	1H, 3.64, dq, 9.2, 6.3	(70.6)	1H, 3.60, dq, 9.3, 6.3
6′	3H, 1.19, q, 6.2	17.9	3H, 1.22, q, 6.2	(17.9)	3H, 1.22, q, 6.3	17.9	3H, 1.19, q, 6.3

protons at 10 and 8, respectively. The triplet found at 4.90 ppm represented the X-part of position 8 and was identified as position 7. A singlet corresponding to three protons at 1.28 ppm indicated the presence of a methyl group at position 9. The stereochemistry of the ring A was not unequivocally established, but comparison with auramycinone suggested it to be (7*S*,9*S*). The doublet at 5.20 ppm was identified as the anomeric proton of the sugar ring. A small coupling, less than 2 Hz, between protons 1′ and 2′ established the glycosidic linkage to be in an alpha form. All substituents on the sugar ring were equatorial, except the axial 4′-hydroxyl. The sugar ring was identified as 2-*O*-methyl-L-rhamnose. These asignments were confirmed with the COSY-spectrum.

Of the 27 carbons in the ^{13}C-spectrum, 13 were identified as aliphatic and 14 as aromatic. The most prominent features in spectra were signals due to two anthraquinone carbonyls (190 and 180 ppm), three aromatic carbons bound to an OR-group (166, 164 and 161 ppm), an anomeric carbon (100 ppm), a sugar ring carbon 4′ (81 ppm), and two methoxy groups (58 and 56 ppm). All measured chemical shift values were in good accordance with earlier data.[7]

Compounds **2**, **3** and **4** were closely related to **1** and their structures were derived mainly by comparison of their spectroscopic data with those of **1**. Compound **2** had a carbonyl group at position 10 instead of a methylene group in **1**. In the ^{13}C-spectrum of **2** appearance of a new carbonyl resonance at 200 ppm and disappearance of methylene resonance at 43 ppm were observed. The proton spectrum showed major changes in chemical shifts from protons at positions 11 (1 ppm downfield), 9-Me (0.2 ppm downfield) and 8 (0.4 ppm downfield).

The structure of **3** was close to **1**; it has a hydroxyl group at position 2′ instead of the methoxyl in **1**. In the proton spectrum the methoxy resonance of **1** at 3.4 ppm was absent, and the signal of the proton 2′ was shifted 0.35 ppm downfield. Small changes in chemical shifts and couplings throughout the sugar ring were observed, as

well. Otherwise the spectrum was quite similar to that of **1**.

Compound **4** indicated changes in the ring D, and three adjacent protons were observed. Otherwise the spectrum of **4** matched with that of **3**.

Discussion

We have presented the structures of steffimycin-like anthracyclines lacking a methoxy group at C-8 and the hybrid compounds possessing no substituent at C-2 as is typical for many anthracyclines. The fact that no steffimycin with a methoxy group at C-8 was obtained is presumably due to the lack of expression of the gene responsible for the hydroxylation and methylation of C-8. This could be due to, for example, a mutation of the gene responsible for adding a hydroxyl group to position 8 or to the cultural conditions if in the growth conditions we used, the biosynthesis did not proceed completely. On the basis of the presence of **1**~**4** in the culture broth, the last steps in the proposed reaction sequence in the steffimycin pathway are methylation of C-2′, modification of position 10 and an addition of a methoxy group at position 8.

In the formation of many aromatic polyketides it is common to see a concomitant action of the KR and ARO enzymes.[16] KR acts on C-9 (corresponding C-2 in anthracyclines) of the nascent polyketide chain (Fig. 3) and subsequently ARO catalyzes dehydration and cyclization of the first carbocyclic ring.[16] However, if a reduction does not take place, the ring closure and aromatization is dictated either by the minimal PKS or by another enzyme. This model has been derived from studies on tetracenomycin biosynthesis.[6] Though steffimycin biosynthesis has not been studied well, it is probable that it proceeds as described for tetracenomycin C and the first ring closure of a hypothetical unreduced decaketide intermediate takes place between the different carbon atoms in steffimycin biosynthesis. The *S. steffisburgensis* gene responsible for the cyclization or aromatization of the first ring (D) did not, however, accept a reduced polyketide as a substrate, since no detectable amount of 2,8-demethoxy steffimycins was obtained in the Sst/pSY24 (*snoD*) fermentation. Nevertheless, the other enzymes of steffimycin pathway are able to use 2-deoxy-intermediate as a substrate since the final glycosylated products were formed.

Acknowledgments

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