Carbazole Antibiotics Synthesis in a *Streptomyces tendae* Bald Mutant, Created by Acriflavine Treatment

Hartmut Grammel[a], Heinz Wolf[a], Ernst-Dieter Gilles[a], Felix Huth[b] and Hartmut Laatsch[b]

[a] Institut für Systemdynamik und Regelungstechnik, Universität Stuttgart, Pfaffenwaldring 9, D-70550 Stuttgart, Germany
[b] Institut für Organische Chemie, Universität Göttingen, Tammannstraße 2, D-37077 Göttingen, Germany

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Acriflavine treatment on *Streptomyces tendae* generated a bald mutant (*bld*-1) with an altered antibiotic pattern. The parental strain produced nikkomycins and juglomycins, whereas the mutant *bld*-1 was only capable of juglomycin synthesis. The existence of a mutant defective in morphogenesis and in nikkomycin biosynthesis suggests a common regulation of these processes. An interesting finding of this study is that mutant *bld*-1 produced two carbazole derivatives, hitherto never seen in cultures of the parental strain. It seems likely that the DNA intercalating dye acriflavine, by mutagenesis, had activated cryptic genes which are involved in carbazole synthesis. The two carbazole derivatives were identified as the neuronal cell protecting compounds CS-79B and carquinostatin A, recently isolated from a wild-type of *S. exfoliatus.* We found that both substances showed antibacterial activity.

Introduction

Sreptomyces species, compared to other bacteria, exhibit a remarkable capacity for morphological differentiation. This process is achieved by the development of an aerial mycelium growing on substrate mycelial colonies. Some of the aerial hyphae are then converted partly into chains of spores. Morphological differentiation in streptomycetes is usually accompanied by physiological differentiation. Production of secondary metabolites such as antibiotics coincides with the onset of aerial growth. Mutants defective in sporulation enable studies of the pleiotropic regulation of both processes (Chater and Bibb, 1997).

Streptomyces tendae strain Tü 901/8c produces at least two structurally unrelated antibiotics, the nikkomycin and juglomycin complexes (Hege-Treskatis *et al.*, 1992). Fermentation studies with *S. tendae* indicate that there are different conditions leading to either nikkomycin or juglomycin production (Hege-Treskatis *et al.*, 1992). This observation led us to examine the antibiotic production in a *S. tendae* mutant deficient in aerial mycelium (strain *bld*-1). Partial deficiencies were found in antibiotic production. Mutant *bld*-1 was capable of juglomycin production, but failed to synthesize nikkomycin. Surprisingly, mutant *bld*-1 produced two carbazole derivatives which had never been seen in cultures of the parental strain and are unrelated in structure to juglomycin and nikkomycin.

The two carbazol derivatives were identified as the neuronal cell protecting compounds CS-79B (Seto and Hayakawa, 1994) and carquinostatin A (Shin-Ya *et al.*, 1993), recently isolated from a wild-type of *S. exfoliatus.* We found that both substances showed antibacterial activity.

The present study compares the antibiotic production pattern of wild-type and mutant *bld*-1 of *S. tendae*, describes the identification of CS-79B and carquinostatin A, and reports antimicrobial spectra of these compounds.

Materials and Methods

Mutagenesis

S. tendae mutant *bld*-1 was generated by acriflavine treatment on the *S. tendae* parent strain Tü 901/8c. Spores were plated on YM agar (yeast ex-

Reprint requests to H. Grammel.
Fax: +49–711–685–6371.
E-mail: grammel@isr.uni-stuttgart.de.

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tract 0.4%, malt extract 1%, glucose 0.4%, agar 1.5%; pH 7.4) supplemented with 5 μg/ml acriflavine. After incubation at 27 °C for 7 days, colonies were visually screened for mutants deficient in aerial mycelium. *Bld*-mutants were isolated and purified. A mutant, the developmental block of which was conditional with respect to carbon source (*bld*-1) and the parental strain of *S. tendae* were used in this study.

Culture and growth conditions

S. tendae wild-type and mutant *bld*-1 were grown on minimal medium composed of 50 mM 2-morpholinoethanesulfonic acid (MES) pH 7.0, 30 g/l glucose (or mannitol), 25 mM $(NH_4)_2 \cdot SO_4$, 2.5 mM $K_2HPO_4 \cdot 3H_2O$, 20 mg/l $FeSO_4 \cdot 7H_2O$, 10 mg/l $ZnSO_4 \cdot 7H_2O$, 7 mg/l $MnSO_4 \cdot H_2O$, 230 mg/ml Na_3 citrate $\cdot 2H_2O$, 100 mg/l $CaCl_2 \cdot 2H_2O$, 1 g/l $MgSO_4 \cdot 7H_2O$, 1 g/l NaCl. For analyzing the antibiotic production pattern of mutant *bld*-1 and the parental strain, a culture in minimal medium (2% inoculum) was shaken in baffled erlenmeyer flasks (0.5 l in 2-l flask) for 7 days at 27 °C at 140 rpm on a rotary shaker. For production of the azole compounds, YM medium was used.

Analytical methods

The concentrations of nikkomycins X, Z, I and J in the culture fluid were measured by high-performance liquid chromatography (HPLC) as described in (Hege-Treskatis *et al.*, 1992). In the identical HPLC system, the amounts of juglomycins A and B and the two carbazole derivatives in the culture fluid were determined. As the proportions of the nikkomycins, juglomycins and carbazoles were approximately constant during fermentation, we depicted the sum of the respective antibiotics. Biomass was determined as the dry weight of washed cells collected from 5 ml samples by filtration.

Isolation of the carbazole derivates

10 l culture of mutant *bld*-1 were extracted with ethyl acetate. The extract was evaporated to dryness, the residue suspended in 50 ml of methanol and extensively extracted with cycloyhexane. The methanolic solution was subjected to column chromatography on Sephadex LH-20. The carbazoles containing fractions were evaporated to dryness and the residue was separated on silica gel plates with chloroform – methanol (19 : 1) to yield the two compounds. Both carbazoles were then purified by reversed-phase chromatography on a RP-18 column (Eurosphere 5 μm; 16 x 250 mm) employing a mixture of acetonitrile (63%) and water and a flow rate of 12.5 ml/min. The peak which occurred at 3.0-min retention time was carquinostatin A (yield 2.4 mg), the peak at 3.2-min retention time was CS-79B (yield 23 mg).

Physico-chemical properties of CS-79B: mp 143 °C dec.; MS 352.4 (ES); UV λ_{max} nm 223, 248, 428; IR ν_{max} cm^{-1} 3600, 3100, 3020, 2980, 1680, 1650, 1630, 1480; Rf value 0.15 (chloroform – 5% methanol).

Physico-chemical properties of carquinostatin A: mp 197 °C dec.; UV λ_{max} nm 231, 271, 423; IR ν_{max} cm^{-1} 3600, 3100, 3020, 2980, 1680, 1650, 1630, 1480; Rf value 0.30 (chloroform – 5% methanol).

Results

Antibiotic production patterns of S. tendae wild-type and mutant bld-1

S. tendae colonies which failed to develop aerial mycelia were isolated from nutrient agar containing acriflavine. Subsequently, these mutants were differentiated. As differentiation criteria, conditionality of developmental block with respect to carbon source was used. Mutant *bld*-1 which failed to develop normally on glucose minimal medium, but was unblocked on mannitol minimal medium, was chosen for further analysis.

Mutant *bld*-1 of *S. tendae* was phenotypically characterized by its ability to produce antibiotics. Fig. 1 shows batch fermentations of *S. tendae* wild-type and mutant *bld*-1 in minimal medium. Concentrations of nikkomycin, juglomycin and carbazole in the culture fluid were measured by high-performance liquid chromatography. Antibiotic production in *S. tendae* mainly occurred after vegetative growth estimated by monitoring biomass. However, in the fermentation of mutant *bld*-1 in glucose minimal medium (Fig. 1b), biomass continued to increase after the onset of antibiotic production. This may have been caused by accumulation of storage compounds such as glycogen (Brãna *et al.*, 1986).

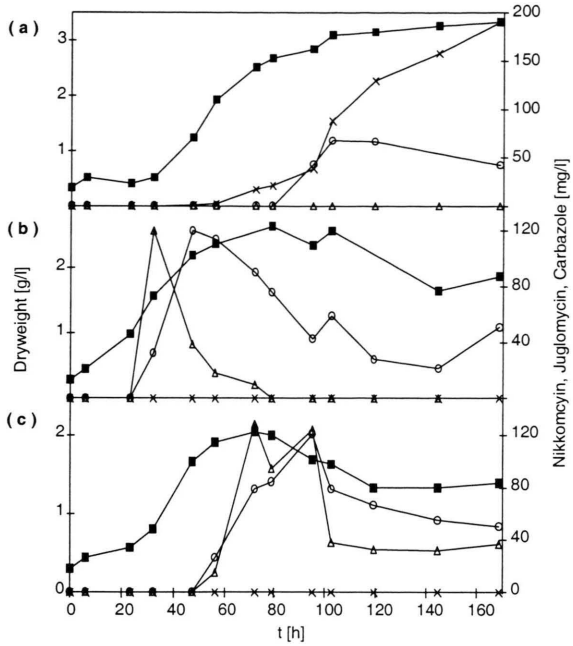

Fig. 1. Antibiotic production of *Streptomyces tendae*. (a) Wild-type in glucose minimal medium, (b) mutant *bld*-1 in glucose minimal medium, (c) mutant *bld*-1 in mannitol minimal medium.
Biomass (■), nikkomycin complex (x), juglomycin complex (○), carbazole complex (△).

In glucose minimal medium, *S. tendae* wild-type produced the two groups of antibiotics nikkomycins and juglomycins up to concentrations of 190 and 70 mg/l, respectively (Fig. 1a). However, partial deficiencies in antibiotic synthesis were found in cultivation of *S. tendae* mutant *bld*-1 under identical fermentation conditions (Fig. 1b). Mutant *bld*-1 was completely blocked for nikkomycin formation, but was still able to synthesize juglomycins up to concentrations of 120 mg/l (ratio of juglomycin A to B: 1.5 : 1). Surprisingly, mutant *bld*-1 produced two carbazole derivatives which had never been seen in cultures of the parent strain. The concentrations were approximately 120 mg/l (ratio of carquinostatin A to CS-79B: 1 : 10).

S. tendae bld-1 strain belongs to a class of *bld* mutants with a carbon source dependent aerial mycelium development (data not shown). This class is bald when grown on glucose but forms sporulating mycelia when grown on other carbon sources such as mannitol. Fig. 1b and 1c show that the antibiotic production pattern was the same with glucose or mannitol as carbon source. The carbon and nitrogen sources were not depleted in the cultures during fermentation (Fig. 1a-c).

Identification of the carbazoles as CS-79B and carquinostatin A

The ^{1}H NMR spectrum of CS-79B (Table I) exhibited signals between δ 6.9 and δ 7.7 indicating the presence of a 1,4,5-trisubstituted benzene ring. The doublet at δ 7.64 coupled with the double doublet at δ 6.99. The coupling constant of 2 Hz suggested a *meta*-position of the both protons. Further, the signal at δ 6.99 coupled with the doublet at δ 7.50 revealing by means of the cou-

Table I. ^{13}C and ^{1}H chemical shifts of CS-79B and carquinostatin A.

Position	CS-79B (**1**) ^{13}C	CS-79B (**1**) ^{1}H	Carquinostatin A (**2**) ^{13}C	Carquinostatin A (**2**) ^{1}H
1	142.3		139.7	
2	132.2		134.4	
3	184.4		183.6	
4	172.0		172.6	
4a	111.3		110.6	
4b	137.1		137.3	
5	119.0	7.64	119.2	7.65
6	125.7		125.9	
7	124.6	6.99	124.8	7.04
8	114.2	7.50	113.1	7.51
8a	136.5		135.4	
9a	145.5		146.1	
10	74.1	4.69	37.6	2.74
11	69.3	3.98	65.8	3.96
12	19.5	1.16	23.6	1.14
13	12.0	1.94	12.1	1.92
14	33.8	3.37	33.8	3.38
15	123.7	5.32	123.6	5.32
16	131.3		131.4	
17	25.4	1.72	25.4	1.72
18	17.6	1.72	17.6	1.72

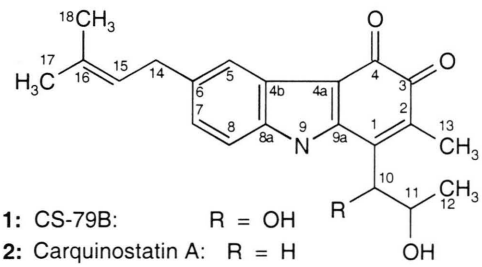

Fig. 2. Structures of CS-79B (**1**) and carquinostatin A (**2**).

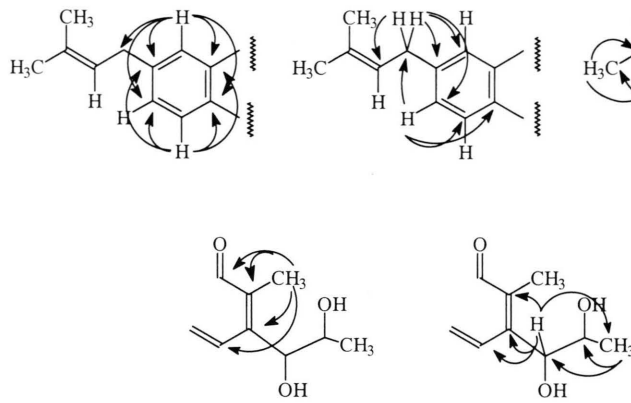

Fig. 3. HMBC C-H couplings (at 75.47 and 300 MHz) of CS-79B in [6] DMSO.

Table II. Antimicrobial activities of CS-79B and carquinostatin A (agar diffusion test).

Test organisms	CS-79B	Carquinostatin A
Agrobacterium tumefaciens DSM* 3069	10[1]	10[1]
Amycolatopsis mediterranei DSM 5908	–	–
Arthrobacter crystallopoites ATCC* 15481	7	16
Arthrobacter globiformis ATCC 8010	t[2]	10
Arthrobacter oxydans ATCC 14358	8	14
Arthrobacter pascens ATCC 13346	7	12
Bacillus migulanus ATCC 9999	–	–
Bacillus subtilis DSM 10	–	–
Brevibacterium sterolicum ATCC 21387	t	9
Corynebacterium glutamicum ATCC 14067	–	–
Curtobacterium flaccumfaciens ATCC 9682	–	–
Escherichia coli A 19	–	–
Escherichia coli H 560	t	t
Halobacterium halobium DSM 670	–	–
Klebsiella planticola DSM 3069	–	–
Micrococcus luteus DSM 348	–	–
Nocardioides simplex ATCC 19566	t	t
Nocardioides simplex DSM 20130	t	t
Pseudomonas saccharophila ATCC 15946	–	–
Lactobacillus casei DSM 20011	–	–
Streptomyces tendae Tü 901/8c* (vegetative cells)	–	–
Streptomyces tendae (spores)	t	t
Streptomyces tendae bld I (this work)	–	–
Xanthomonas oryzae ATCC 35934	–	–
Saccharomyces cerevisiae DSM 1334	–	–

[1] Inhibition zone [mm diameter], created by 10 µg agent added to the paper disk [6 mm diameter].
[2] t, trace amount of inhibition zone.
* ATCC, American Type Culture Collection, Rockville, MD, USA; DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany; Tü, Institut für Mikrobiologie/Biotechnologie der Universität, Tübingen, Germany.

pling constant an *ortho*-position of the both protons.

The substitution pattern of the phenyl ring was also confirmed by HMBC correlation (Fig. 3). The protons at C-5 and C-7 coupled with the methylene group at δ 33.8/3.37. The methylene group coupled with the adjacent double-bond C-atom. The two terminal methyl groups showed both ^2J and ^3J couplings. A second molecular fragment was obtained by the aid of ^2J and ^3J couplings of

the aliphatic methyl group with the two adjacent methine groups, which are bounded to oxygen. Further, C-10 coupled to the adjacent ring. This was confirmed by couplings of the methyl group of the ring.

With this structure, a search in AntiBase (Laatsch, 1997) pointed to compound CS-79B (Fig. 2) (Seto and Hayakawa, 1994), an antioxidative carbazole derivative, which was recently isolated from *Streptomyces exfoliatus* 2419-SVT2.

The ^{1}H NMR spectrum of carquinostatin A was very simular to that of CS-79B except for the absence of the hydroxy group at C-10. The search in AntiBase (Laatsch, 1997) led to carquinostatin A (Fig. 2) (Shin-Ya *et al.*, 1993), which was also isolated from *Streptomyces exfoliatus* 2419-SVT2.

Antimicrobial activity of CS-79B and carquinostatin A

As presented in Table II, the spectrum of antibacterial activity of both carbazole compounds was similar, except that CS-79 was somewhat less active than carquinostatin A. The antimicrobial activity was limited to *Arthrobacter* strains, *Agrobacterium tumefaciens* and *Brevibacterium sterolicum*. The minimal inhibitory concentrations (MICs) for the sensitive strains in the serial dilution assay ranged approximately from 1 to 10 µg/ml (Table III). The effects were bacteriostatic as jugded by subculturing of 1 µl culture on agar plates. The other strains tested were insensitive against both agents.

Discussion

Acriflavine treatment on *S. tendae* generated a bald mutant (*bld*-1) with an altered antibiotic pattern. The parental strain produced nikkomycin and juglomycin, whereas the mutant *bld*-1 was unable of nikkomycin synthesis. The existence of a mutant defective in morphogenesis and in nikkomycin biosynthesis suggests a common regulation of these processes. In contrast to nikkomycin, juglomycin synthesized in the mutant *bld*-1 seems to be rather a product of the primary metabolism than of the secondary metabolism. This interpretion was further substantiated by the earlier finding that conditions favouring vegetative growth of the wild-type also enhance juglomycin productivity in continous culture (Hege-Treskatis *et al.*, 1992). However, during the exponential growth in batch culture, no juglomycin was produced at all.

S. tendae mutant *bld*-1 belongs to a phenotypic class of bald mutants characterized by the fact that morphogenesis is conditional with respect to carbon source. Although strain *bld*-1 was unable to sporulate on glucose minimal medium agar, it sporulated reasonably well on mannitol minimal medium agar. However, the antibiotic synthesis was unconditional with respect to carbon source in the mutant *bld*-1. On solid or liquid mannitol minimal medium, the mutant was not restored to nikkomycin production. In this context, antibiotic synthesis does not seem to be linked to morphological development.

An interesting finding of this study is that *S. tendae* mutant *bld*-1 produced two carbazole derivatives, hitherto never seen in cultures of the parental strain. It seems likely that the DNA intercalating dye acriflavine, by mutagenesis, had activated cryptic genes which are involved in carbazole synthesis. The activation of cryptic genes has already been described: Ogura *et al.*, 1986 reported the induction of curromycin production with ethidium bromide in *S. hygroscopicus.*

In contrast to the carbazoles, however, curromycin was also produced by the parent strain under altered culture conditions.

CS-79B and carquinostatin A have recently been isolated from *S. exfoliatus* as potent neuronal cell protecting substances which also showed radical scavenging activity (Seto and Hayakawa, 1994; Shin-Ya *et al.*, 1993). In this study, both carbazoles have been demonstrated to be active against bacteria. The antibiotic activity, however, was limited to *Arthrobacter* strains, *Agrobacterium tumefaciens* and *Brevibacterium sterolicum.* The mechanism of action in the sensitive strains does not

Table III. Antibacterial activities of CS-79B and Carquinostatin A (serial dilution test).

Test organisms	MIC [µg/ml]: CS-79B	Carquinostatin A
Agrobacterium tumefaciens	1.25	1.25
Arthrobacter crystallopoites	>10	10
Arthrobacter globiformis	10	5
Arthrobacter oxydans	>10	10
Arthrobacter pascens	2.5	2.5
Brevibacterium sterolicum	>10	10

seem to be related to the radical scavenging activity of the two carbazoles, because butylated hydroxytoluene, which is a well known antioxidant, was not active against the sensitive strains at concentrations of 100 μg added to paper disks in the agar diffusion test (data not shown).

The mechanism of activation of carbazole production by acriflavine is not known. It seems likely that the DNA intercalating dye caused changes in *S. tendae* to induce the expression of genes for carbazole synthesis. Therefore, the characterization of the antibiotic induction needs extensive genetic analysis. Generally, bald mutants of streptomycetes may serve as useful sources for new antibiotics.

Acknowledments

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