BIOSYNTHETIC CAPACITIES OF ACTINOMYCETES. 2[#] JUGLOMYCIN Z, A NEW NAPHTHOQUINONE ANTIBIOTIC FROM *Streptomyces tendae*

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A new juglomycin-type antibiotic was identified by a HPLC-diode array screening technique in the culture filtrate of *Streptomyces tendae* Tü 901/8c. Juglomycin Z (**1**) differs from all other known juglomycin compounds by an additional methyl group in position 3 of the naphthoquinone ring system. Juglomycin Z is antibiotically active against Gram-positive and Gram-negative bacteria and against yeasts.

The discovery of new secondary metabolites demands high efficient screening strategies and can be realized both by target and non-target methods. Our screening strategy is based on standardized reversed-phase HPLC separation of culture filtrates and raw extracts, multiwavelength monitoring by diode array detection and comparison of the data by an UV-visible absorbance spectral library database[1]. In the course of our screening program a compound was detected in the culture filtrate of the nikkomycin producing organism *Streptomyces tendae* Tü 901 which showed a good correlation in the UV spectrum with juglomycins A (**5**) and B (**6**), but differed in the retention time from both reference compounds. Juglomycins A and B were first described as antibiotics produced by *Streptomyces diastatochromogenes*[2,3] and other strains of *Streptomyces species*[4]. They were rediscovered in *Streptomyces tendae* Tü 901[5,6], which produces nikkomycins (reviewed by FIEDLER *et al.*[7]), ketomycin, chlorothricin and bromothricin[1].

In this paper we describe the fermentation and isolation, as well as characterization, structural elucidation and biological activity of this new naphthoquinone-type antibiotic, named juglomycin Z (**1**).

Fermentation and Isolation

Batch fermentations of *Streptomyces tendae* Tü 901/8c were carried out in 25-liter fermentors equipped with an intensor system and 10-liter stirred tank fermentors using a complex medium that consisted of starch 2%, peptone 0.5% and meat extract 0.25% (pH 7.2). Juglomycin Z (**1**) production was strongly dependent on the type of the fermentor used. In an intensor system the production started 18 hours after inoculation, and maximal production of juglomycin Z was observed after a 23-hour incubation period and reached a concentration of 90 mg/liter, as shown in Fig. 1. At this time no juglomycin A was detectable, whose production started not before 40 hours incubation time and reached a maximum after 52 hours at a concentration of 36 mg/liter. Cultivation in a stirred tank fermentor showed a totally different course

[#] See ref 1.

Fig. 1. Batch-fermentation of *Streptomyces tendae* Tü 901/8c.
□ juglomycin Z (**1**), ○ dry weight.

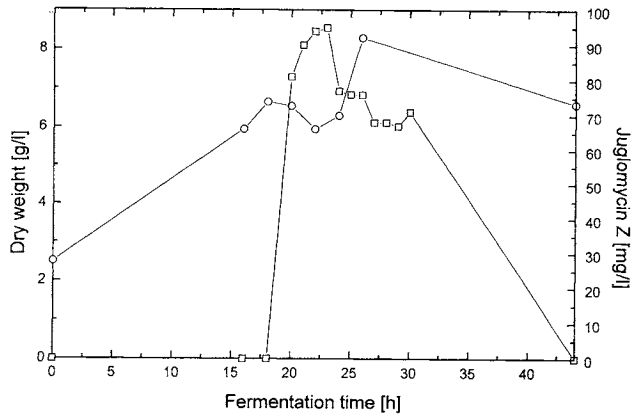
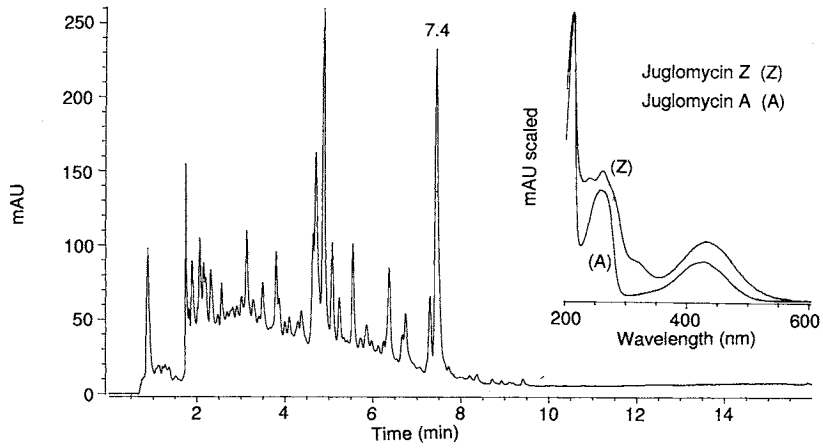


Fig. 2. HPLC analysis of culture filtrate from *Streptomyces tendae* Tü 901/8c monitored at 210 nm and overlayed UV-visible spectra from juglomycin Z (7.4 minutes) and juglomycin A (reference compound).



of juglomycin production. No jugomycin Z was observed during the whole fermentation period, whereas juglomycin A reached a maximum of 130 mg/liter after a 48-hour incubation period.

Isolation of juglomycin Z was carried out by extraction of the culture filtrate with ethyl acetate and chromatography on silica gel with CH_2Cl_2 - MeOH gradient elution. Pure juglomycin Z was obtained after preparative reversed-phase HPLC using 10-micron Nucleosil-100 C-18 material with H_2O - MeOH gradient elution.

Characterization

Juglomycin Z (**1**) was identified as a juglomycin-type compound by analyzing the culture filtrate of *Streptomyces tendae* using HPLC and our HPLC-UV-Vis database[1]. Whereas the retention time (7.4 minutes) differed from those of the juglomycins A (**5**; 6.9 minutes) and B (**6**; 6.5 minutes), the UV-visible spectrum showed similarity in the spectra of these reference compounds stored in the quinone library of the computer database. The resulting match factor of 940 indicated a structure similar to the juglomycins.

The maximal matching value is 1000, and identity is given in case of match factors in the range between 997 and 1000. All other 82 quinone reference compounds showed match factors below 900, and compounds stored in libraries of other natural substance classes showed no acceptable values. The HPLC elution profile of the culture filtrate and overlayed UV-visible spectra of juglomycins Z and A are shown in Fig. 2.

Structure of Juglomycin Z

Juglomycin Z (**1**) was obtained as a black amorphous powder and was found to be readily soluble with yellow colour in methanol, weakly soluble in chloroform and insoluble in *n*-hexane. The new metabolite was characterized spectroscopically, its molecular formula $C_{15}H_{14}O_6$ was determined by mass spectra (FAB-MS) and its structure was elucidated by comparing the ^1H, ^{13}C, ^1H-^1H correlation and ^1H-^{13}C shift correlation NMR data to those of known juglomycins[4,8]. The 5-hydroxy-1,4-naphthoquinone chromophore was indicated by IR carbonyl absorption bands at 1650 and 1630 cm^{-1}, an UV absorption maximum at 419 nm, which is shifted to 524 nm by sodium hydroxide, and the typical ^{13}C NMR signals especially of the quinone carbonyl groups at δ 186.6 (C-4) and 182.2 (C-1). The inspection of the ^1H NMR spectrum of 1 revealed the presence of the typical ABC spin system of the aromatic ring [δ_H 7.25, 7.63, 7.64]. A three-proton singlet at δ 2.67 suggested a methyl group attached to the quinone ring. Additionally a typical signal pattern, which corresponds to the one from juglomycin C (**3**)[8] proved to be a β-hydroxybutyric acid side chain. The assignment of the C-atoms to the ^{13}C NMR data and the regiochemistry followed from the $^nJ_{C,H}$ long range couplings (COLOC and HMBC spectra) of the methyl ester **2** are shown in Fig. 3.

The absolute configuration at C-3′ of juglomycin Z (**1**) was proven via the HELMCHEN method[9] developed for secondary alcohols. First, **1** was transformed into its methyl ester **2** by diazomethane. Second, **2** was esterified at the C-3′ hydroxy group with both enantiomers of 2-phenylbutyric acid[9]. The resulting diastereomeric 2-phenylbutyrates were analyzed by ^1H NMR ($CDCl_3$). Compared with **2** the (2″*R*) diastereomer of **4** showed high field shifts of 2′-H_2 ($\Delta\delta = -0.05$ ppm) and OMe ($\Delta\delta = -0.44$ ppm), and low field shifts of 4′-H_2 ($\Delta\delta = +0.04$ ppm)

Fig. 3. $^nJ_{C,H}$ correlations observed in **2** by COLOC and HMBC pulse sequences at 500 and 300 MHz.

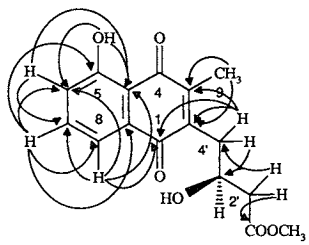
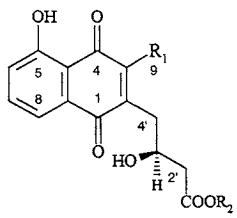
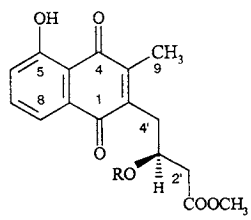


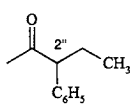
Fig. 4. Structures of juglomycins Z (**1**), Z-methyl ester (**2**), C (**3**), Z-methyl ester-3′-(2-phenylbutyrate) (**4**), A (**5**) and B (**6**).

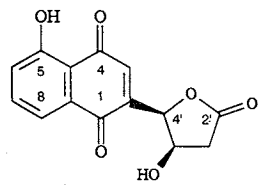


1 $R_1 = CH_3$, $R_2 = H$ (Juglomycin Z)
2 $R_1 = R_2 = CH_3$
3 $R_1 = R_2 = H$ (Juglomycin C)



4 R=





5 (Juglomycin A)
6 Enantiomer at C-4′ (Juglomycin B)

and 9-H_3 ($\Delta\delta = +0.23$ ppm). The (2″*S*) diastereomer of **4** revealed the opposite situation. This shielding/deshielding effects proved the (3′*S*) configuration of **1** and **2**, in accordance with that found for juglomycin C (**3**)[8] and the juglomycins A (**5**) and B (**6**)[4]. The juglomycin structures are summarized in Fig. 4.

Table 1. Antimicrobial activities of juglomycins Z and A.

Organism	MIC (μg/ml)	
	Juglomycin Z	Juglomycin A
Bacillus brevis ATCC 9999	0.1	1
Bacillus subtilis ATCC 6051	1	3
Streptomyces tendae Tü 901	30	10
Pseudomonas fluorescens ATCC 13525	10	1
Escherichia coli K12	3	3
Candida albicans CBS 2718	100	30

Biological Properties

Juglomycin Z showed an antimicrobial activity against Gram-positive bacteria (*Bacillus brevis*, *Bacillus subtilis*, *Micrococcus luteus*, *Staphylococcus aureus*, *Arthrobacter aurescens*, *Clostridium pasteurianum*, *Streptomyces viridochromogenes*), against Gram-negative bacteria (*Agrobacterium tumefaciens*, *Escherichia coli*, *Pseudomonas fluorescens*, *Pseudomonas mirabilis*) and against yeasts (*Saccharomyces cerevisiae*, *Candida albicans*), as tested by the agar-plate diffusion assay. No effect on fungi (*Botrytis cinerea*, *Mucor hiemalis*, *Paecilomyces variotii*, *Penicillium notatum*) was observed at concentrations of 1 mg/ml. The most sensitive organism was *Bacillus brevis* as determined by the broth dilution method, showing a 10-fold higher sensitivity against juglomycin Z compared with juglomycin A with a minimal inhibitory concentration of 0.1 μg/ml. The results are summarized in Table 1.

Discussion

Screening for new secondary metabolites can be performed by a high throughput of organisms or by analysis of only few, but potent organisms with high efficient methods that allow detection of preferably all compounds, which were produced by the organisms. Analyzing culture filtrates and raw extracts of microorganisms by HPLC and diode array detection, and comparing retention times and UV-visible spectra with data from known antibiotics and other metabolites stored in natural substance class libraries in a database, permit efficient screening for new secondary metabolites at a very early stage of investigations. This method can be used in chemical screening as well as in target screening programs thereby reducing cumbersome, intensive and costly investigations of previously characterized compounds.

This technique was successfully used for detection of new nikkomycin compounds in culture filtrates of wildtype strain and mutants of *Streptomyces tendae* Tü 901[10]. Because we have found that the biosynthetic capacity of this organism is not only limited to the production of these nucleoside-peptide antibiotics, but also to production of the macrolide antibiotics chlorothricin and bromothricin, production of ketomycin and of the naphthoquinone antibiotics juglomycins A (**5**) and B (**6**), we investigated the biosynthetic potency of *Streptomyces tendae* under altered cultivation conditions. All mentioned compounds were produced under slow growth conditions. When *Streptomyces tendae* was kept under conditions of fast growth, the strain produced neither nikkomycins nor ketomycin, but a lot of other compounds were detected by our HPLC technique[1], one of them was the new juglomycin Z (**1**). In which manner the polyketide pathway normally resulting in the juglomycin A and B is changed will be evaluated by feeding experiments using ^{13}C-labeled acetate, propionate and methionine.

Experimental

Microorganisms

Strain Tü 901/8c was obtained by subcloning the nikkomycin producing wildtype strain *Streptomyces tendae* Tü 901. Strain Tü 901/8c is characterized by a good sporulation and production of nikkomycins

Z, X, J and I[11].

The standard strains for testing the biological activity spectrum were obtained from the stock culture collection of our laboratory, from ATCC and CBS.

Fermentation

Strain Tü 901/8c was cultivated in 10-liter fermentors (Biostat E, stirred tank; Braun Diessel Biotech) and 25-liter fermentors (b-20, intensor system; Giovanola) using a production medium that consisted of starch 2%, peptone 0.5% and meat extract 1% in tap water (pH 7.2). The fermentors were inoculated with 5 vol-% of shaking cultures grown for 48 hours in a medium containing starch 2% and soy bean meal 2% in tap water (pH 6.8). The fermentors were kept at 27°C with an aeration rate of 0.5 v/v/m. Agitation was 300 rpm in case of 10-liter fermentors, and 1200 rpm in case of 25-liter fermentors.

Isolation

Hyflo Super-cel (2%) was added to the fermentation broth (25 liters) which was separated by multiple sheet filtration into biomass and culture filtrate. The mycelium was discarded. The culture filtrate (22 liters) was adjusted to pH 5 and extracted 3 times with ethyl acetate. The combined extracts were concentrated to dryness (4.57 g) and separated on a silica gel column (40 × 5 cm, SI 60, 63~200 μm; Merck) by linear gradient elution, starting with CH_2Cl_2 to a MeOH content of 15% within 3 hours and flow rate of 600 ml/hour, using a medium pressure pump and gradient forming unit (B-680; Büchi). The juglomycin Z containing fractions were combined and concentrated to dryness (1.1 g).

Pure juglomycin Z was obtained by preparative reversed-phase HPLC using a stainless steel column (250 × 16 mm; Grom) filled with 10 μm Nucleosil-100 C-18 particles, and linear gradient elution with H_2O - MeOH, starting from 60% MeOH to 70% MeOH within 20 minutes and a flow rate of 20 ml/minute, yielding 412 mg juglomycin Z. The preparative HPLC system consisted of two high-pressure pumps (Sepapress HPP-200/100; Kronwald), gradient unit (Sepacon GCU-311), and Valco preparative injection valve (6UW; VICI) with 5 ml-sample loop. The UV absorbance of the eluate was monitored simultaneously at 220 nm and 254 nm by a Gilson spectrophotometer Mod. 116 equipped with a preparative cell.

Biological Assays

The agar-plate diffusion assay was used to determine the antibacterial spectrum of juglomycins. The test solutions were applied to filter discs (6 mm diameter) and the test plates were incubated for 24 hours at 37°C.

The broth dilution method was used to determine the MIC of juglomycins. The antibiotics were dissolved in DMSO with a DMSO content in the cultures of not more than 5%. *B. brevis*, *B. subtilis*, *E. coli* and *P. fluorescens* were grown in nutrient broth 0.8% and NaCl 0.5% in tap water, *S. tendae* was grown in malt extract 1%, glucose 0.4% and yeast extract 0.4% in tap water (pH 7.3), and *C. albicans* was grown in the same medium at pH 5.5. 10^6 cells/ml or spores/ml, respectively, were used as inoculum of the complex media and growth inhibition was evaluated after incubation for 24 and 48 hours at 27°C and 37°C, respectively, on a rotary shaker.

HPLC-Analysis

The chromatographic system consisted of a Hewlett-Packard HP 1090M liquid chromatograph equipped with a built-in diode array detector, HP 79994B pascal-workstation (200 MB hard disk) and HP 79988A software rev. 5.3. Multiple wavelength monitoring was performed at 210 nm, 230 nm, 260 nm, 310 nm, 360 nm and 435 nm without reference wavelength; the spectrum range was from 200 nm to 600 nm, step 2 nm and sampling interval of 640 msec.

A sample of the fermentation broth was centrifuged (3 minutes, 12,000 × *g*), and 10 μl of the supernatant were injected onto an HPLC column (125 × 4.6 mm), fitted with a guard column (20 × 4.6 mm) which were packed with 5 μm Nucleosil-100 C-18 (Grom). The samples were separated by linear gradient elution; solvent A was 0.1% phosphoric acid, solvent B was acetonitrile. The linear gradient was from 0% to 100% solvent B in 15 minutes with a 1-minute hold at 100% B and a 5-minute post time at initial conditions, at a flow rate of 2 ml/minute.

The compare algorithm used in the UV-visible spectral library search are reviewed by SIEVERT &

DROUEN[12]. The spectral evaluation was performed both manually and by an automated search program. The program checks the purity of each peak and runs a library search.

Structure Elucidation

MP's were determined with a Reichert hot stage microscope. UV spectra were recorded using a Kontron Uvikon 860 spectrometer. IR spectra were recorded on a Perkin-Elmer 1600 FTIR spectrophotometer. The NMR spectra (δ in ppm relative to internal TMS) were determined using a Varian VXR-200 and a Bruker AMX-300. The FAB-mass spectra and the electron impact mass spectra (EI-MS, 70 eV) were obtained on a Varian MAT 8230 (Matrix: a-nitrobenzylalcohol) and on a Finnigan MAT 311 A (direct probe insert); high resolution with perfluorokerosine as a standard. A Perkin-Elmer Model 241 polarimeter was used for recording of the optical rotations.

Juglomycin Z (1)

MP >300°C; $[\alpha]_D^{20}$ +144 (*c* 0.025, MeOH); Rf 0.12 (TLC Silica gel 60, chloroform-methanol 9:1); IR ν_{max} (KBr) cm^{-1} 3440, 2930, 1710, 1660 sh, 1650, 1630; UV λ_{max} (MeOH) nm (ε) 209 (16,430), 239 (8,730), 255 (6,270), 419 (3,530); UV λ_{max} (MeOH+NaOH) nm (ε) 211 (16,400), 417 (2,730), 524 (2,820); ^{1}H NMR (200 MHz, $CDCl_3/CD_3OD$) δ 2.56 (2H, d, J=8.0 Hz, 2'-H_2), 2.67 (3H, s, 9-H_3), 3.09 (1H, dd, J=12.6, 8.0 Hz, 4'-H_a), 3.27 (1H, dd, J=12.6, 4.0 Hz, 4'-H_b), 4.28 (1H, ddd, J=8.0, 6.0, 5.0 Hz, 3'-H), 7.25 (1H, dd, J=8.0, 2.0 Hz, 6-H), 7.63/7.64 (2H, m, 7-H, 8-H); ^{13}C NMR (50.3 MHz, $CDCl_3$) δ 18.5 (q, C-9), 36.8 (t, C-4'), 42.0 (t, C-2'), 67.9 (d, C-3'), 115.8 (s, C-4a), 119.8 (d, C-8), 124.1 (d, C-6), 132.3 (s, C-8a), 136.7 (d, C-7), 148.4* (s, C-2), 149.6* (s, C-3), 161.6 (s, C-5), 174.5 (s, C-1'), 182.2 (s, C-1), 186.6 (s, C-4); FAB-MS *m/z* 290 (M+). (* means assignments interchangeable)

Juglomycin Z-methyl ester (2)

15 mg **1** was treated at 0°C with 3 ml etheral solution of diazomethane (3.6 M). After 10 minutes stirring at ambient temperature, the reaction mixture was evaporated *in vacuo* and the crude product was chromatographed on Silica gel SI 60 (column 20×1.5 cm, acetone-*n*-hexane 2:3) yielding 12 mg (76.3%) **2** as an orange amorphous powder; Rf 0.22 (TLC Silica gel 60, acetone-*n*-hexane 2:3), 0.92 (chloroform-methanol 9:1); MP 85°C; $[\alpha]_D^{20}$ +37.3 (*c* 0.01, MeOH); IR ν_{max} (KBr) cm^{-1} 3436, 2960 sh, 2928, 2856, 1713, 1625, 1538; UV λ_{max} (MeOH) nm (ε) 210 (23,570), 240 (8,990), 423 (3,630); UV λ_{max} (MeOH+NaOH) nm (ε) 208 (30,910), 425 (3,310), 522 (3,980); ^{1}H NMR (200 MHz, $CDCl_3$) δ 2.58 (1H, dd, J=15.5, 7.5 Hz, 2'-H_a), 2.63 (1H, dd, J=15.5, 5.5 Hz, 2'-H_b), 2.65 (1H, s, 9-H_3), 3.09 (1H, dd, J=12.6, 7.5 Hz, 4'-H_a), 3.27 (1H, dd, J=12.6; 4.5, 4'-H_b), 3.72 (3H, s, OMe), 4.28 (1H, m, 3'-H), 7.25 (1H, dd, J=8.0, 2.0 Hz, 6-H), 7.61 (1H, dd, J=7.8, 2.0 Hz, 8-H), 7.63 (1H, dd, J=8.0, 7.8 Hz, 7-H), 11.9 (1H, s, OH); ^{13}C NMR (125.7 MHz, $CDCl_3$) δ 18.4 (q, C-9), 36.0 (t, C-4'), 41.2 (t, C-2'), 51.8 (d, OMe), 67.7 (d, C-3'), 115.4 (s, C-4a), 119.5 (d, C-8), 124.0 (d, C-6), 131.8 (s, C-8a), 136.3 (d, C-7), 147.9* (s, C-2), 149.2* (s, C-3), 161.6 (s, C-5), 172.6 (s, C-1'), 182.0 (s, C-1), 186.3 (s, C-4); HREI-MS *m/z* 304.0445 (M+ calcd. for $C_{16}H_{16}O_6$: 304.0447); FAB-MS *m/z* 305 $(M+H)^+$, 306 $(M+2H)^+$.

Acknowledgments

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