Biosynthetic Capacities of Actinomycetes. 4[†]

Echinoserine, a New Member of the Quinoxaline Group, Produced by *Streptomyces tendae*

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A new member of the quinoxaline group antibiotics has been detected by HPLC-diode-array screening. The main compound produced by *Streptomyces tendae* strain Tü 4031 showed a high degree of similarity in the UV-visible spectral region with echinomycin and their structural similarity was confirmed by structure elucidation using electron tandem mass spectrometry and 2D nuclear magnetic resonance. The new compound, named echinoserine, is a non-cyclic form of echinomycin, but it is not a biosynthetic precursor. Echinoserine is less antibiotically active than echinomycin.

Streptomycetes still represent an inexhaustible source for new secondary metabolites. However, efficient screening methods are required to detect this pool of new structures. We use the HPLC-DAD method as a non-target screening strategy. Culture filtrates and raw extracts are analyzed by reversed-phase HPLC and diode-array detection (DAD)[2], and the UV-visible spectra of the compounds from biological samples are compared with those of reference compounds stored in an UV-visible absorbance spectral library database. An identification of a metabolite is given, when retention time and UV-visible spectrum are identical.

The culture filtrate of *Streptomyces tendae* strain Tü 4031, isolated from a soil sample of Alice Springs (Australia), was screened by this HPLC-DAD method. We detected an interesting compound showing a UV spectrum very similar to that of echinomycin[3,4], but having a different retention time. Echinomycin is a cyclic depsipeptide with a thioacetal crossbridge, and is characterized by the presence of two quinoxaline-2-carboxylic acid moieties[5,6]. It is highly active against Gram-positive bacteria, cytotoxic to mammalian cells in culture, and shows significant antitumor activity[7~10]. HPLC-MS analysis indicated non-identity of the compound with echinomycin, with molecular masses of 1136 and 1101 respectively, being found.

In this paper we describe taxonomy of the producing strain, fermentation and isolation, as well as characterization, structural determination and biological activity of this new quinoxaline compound, named echinoserine.

Taxonomy

Strain Tü 4031 was isolated from a soil sample taken in Alice Springs, Northern Territory, Australia. The presence of LL-diaminopimelic acid in the peptidoglycan, the characteristic pattern of satured *iso*- and *anteiso* fatty acids and the formation of chains of spores in the aerial mycelium allows strain Tü 4031 to be assigned to the genus *Streptomyces*.

Strain Tü 4031 formed chains of spores of the spiral type with 2~5 extended turns mixed with straight, flexous spore chains. The number of spores in a chain

[†] See ref 1.

We dedicate this publication to Professor SATOSHI ŌMURA in honour of his 60th birthday.

Table 1. Cultural characteristics of strain Tü 4031.

Medium	Growth	Aerial mycelium	Reverse side colour	Soluble pigment
Yeast extract-malt extract agar (ISP 2)	Good	Gray	Beige to dark brown	None
Oatmeal agar (ISP 3)	Good	Gray	Beige brown to dark brown	None
Inorganic salts-starch agar (ISP 4)	Good	Light gray	Brownish black	None
Glycerol-asparagine agar (ISP 5)	Good	White to light gray	Whitish gray, yellowish brown	None
Oatmeal-nitrate agar*	Good in close connection with the agar	Light gray	Gray	None

* per liter H_2O: oatmeal 3 g, KNO_3 0.2 g, K_2HPO_4 0.5 g, $MgSO_4$ 0.2 g, agar 15 g (pH 7)

was in a range of 10~50. The surface of the spores was smooth as revealed by electron microscopy. The mass colour of mature sporulating aerial mycelium was gray. The colour of the substrate mycelium changed depending on the culture medium and the age of the culture from whitish gray over pale orange to dark brown. Melanoid pigments were produced on peptone-yeast extract iron agar (ISP 6).

Glucose, fructose, sucrose and rhamnose were utilized as carbon sources. Raffinose and cellulose were not utilized. The culture characteristics of strain Tü 4031 are summarized in Table 1. According to our morphological and physiological results with strain Tü 4031 in comparison with the type strain of *Streptomyces tendae* IMET 40459 (ISP 5101) and the data in the literature[11,12] we propose to assign strain Tü 4031 as a strain of *Streptomyces tendae* Ettlinger, Corbaz and Hütter. According to Locci[13], *Streptomyces tendae* is a subjective synonym of *Streptomyces rochei* Berger, Jampolsky and Goldberg.

Fermentation and Isolation

Streptomyces tendae Tü 4031 was cultivated in a 10-liter stirred tank fermentor. The complex medium for the production of echinoserine consisted of mannitol 2% and soybean meal 2% in tap water (pH 7.5). The batch fermentation required an incubation period of 96 hours, whereby the echinoserine production reached a concentration of 40 mg/liter.

The isolation of echinoserine required four steps of purification. Polystyrene resin chromatography with Amberlite XAD-16 was used to reduce the culture filtrate volume and to enrich the compound. The compound was then extracted with 1-butanol and subjected to exclusion chromatography with Sephadex LH 20. Finally, pure echinoserine was obtained after preparative reversed-phase HPLC using 10-micron Nucleosil-100 C-18 material with H_2O-MeOH gradient elution.

Characterization

Biological raw extracts from *Streptomyces tendae* Tü 4031 were screened by the HPLC-DAD method and our HPLC-UV-Vis database[2]. The main compound, echinoserine, showed a high degree of similarity in the UV-visible spectrum to that of the reference compound echinomycin by a match factor of 997. The maximum matching value is 1000 indicating identical spectra[14]. However, the retention time of echinoserine (7.68 minutes) differed from that of echinomycin (10.32 minutes), thus excluding identity of both compounds. This suggested that echinoserine belongs to the family of quinomycin antibiotics. The HPLC elution profile of the culture filtrate extract and UV spectrum of echinoserine is shown in Fig. 1.

Structure of Echinoserine

The UV-spectrum showed absorption maxima at 250 nm and 320 nm and was almost identical to the spectrum of echinomycin. This led to the conclusion that echinoserine and echinomycin contain the same chromophoric group.

The amino acids in the acidic hydrolysate of echinoserine were determined as their *N*-trifluoroacetyl/*n*-propyl derivative by gas chromatography (GC) on chiral stationary phase Chirasil-Val[15]. The presence of L-Ala and D-Ser was thus established together with a non-proteinogenic amino acid which was identified as *N*-methyl-valine (*N*-Me-Val) derivative by GC mass spectrometry. The combined use of homonuclear (TOCSY) and heteronuclear (HSQC, HMBC) 2D-NMR techniques allowed the detection of all residues which were found in echinomycin. The chromophore moieties were assigned to two quinoxaline-2-carboxylic acid

Fig. 1. HPLC analysis of culture filtrate extract from *Streptomyces tendae* Tü 4031 monitored at 210 nm and UV spectrum of echinoserine (7.68 minutes).

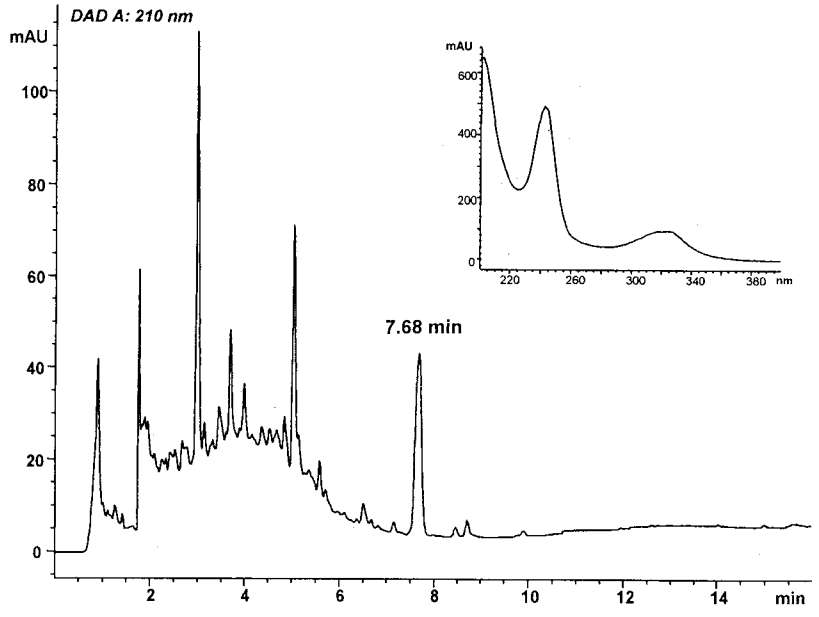
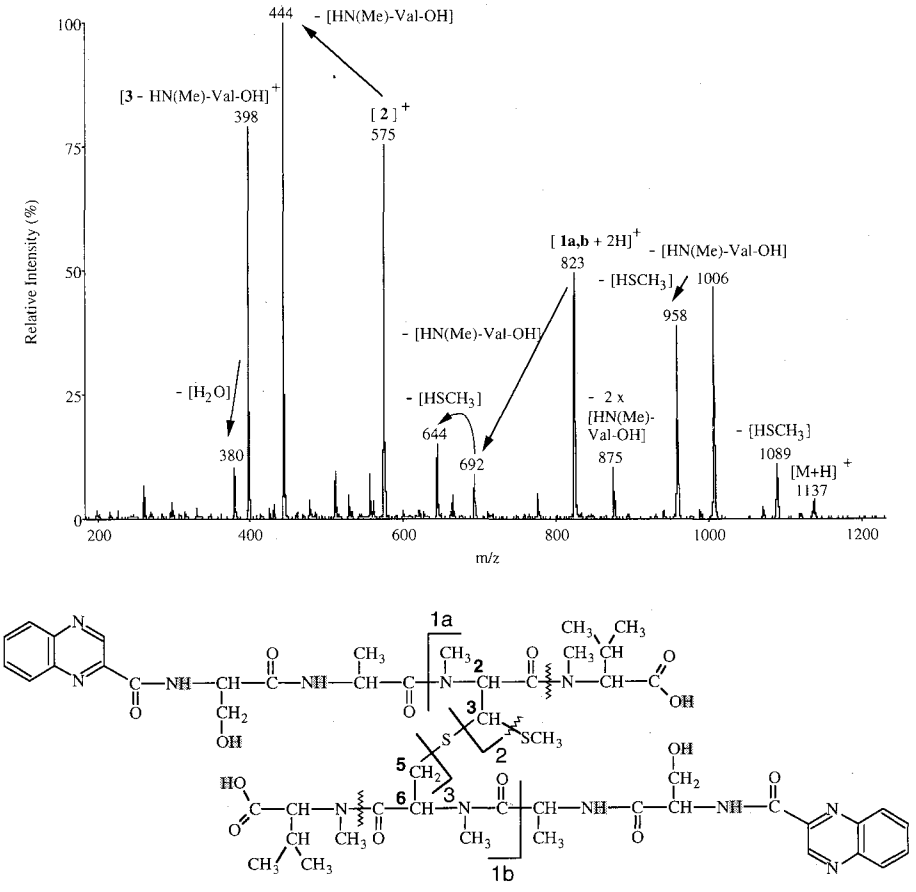
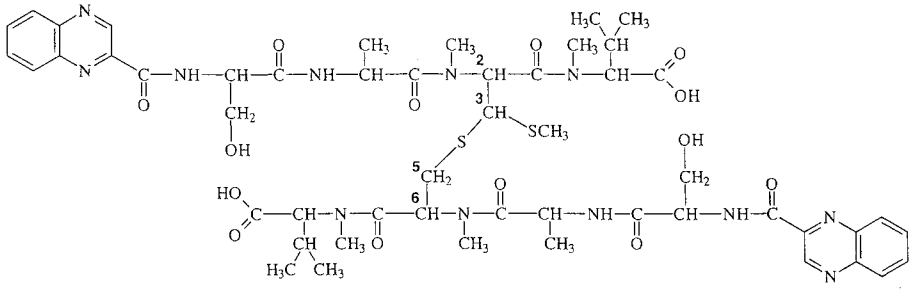


Fig. 2. Electrospray mass spectrum of echinoserine.



residues by their ^{13}C-chemical shifts. Two L-alanine and two D-serine amino acid residues were found. Furthermore one *N*,*N*′-dimethyl-3-thiomethoxy-lanthionine (*N*-Me-Tme-Lan) and two *N*-Me-Val residues were identified by their ^{13}C-chemical shifts and characteristic contacts in the HMBC spectrum (*N*-CH_3/C^2H, *S*-CH_3/

Fig. 3. Structure of echinoserine.



C^3H, C^3H/C^5H_2, N'-CH_3/C^6H). Remarkably, the resonances of the *N*-Me-Tme-Lan and the two *N*-Me-Val residues are split into four signals, respectively. Since echinoserine appears according to MS (see below) this may be caused by different configurations of the stereo centers and/or a conformational diversity of the molecule affecting the *N*-Me-Tme-Lan and the *N*-Me-Val residues in particular. The sequence of the two peptide chains could partly be derived from HMBC contacts between adjacent residues. In contrast no connectivities between the *C*-termini and the side chains of the D-serine residues could be detected.

The relative mass of echinoserine was determined by electrospray mass spectrometry (ESI-MS) to be 1136.2. This is 36 Daltons higher than the mass of echinomycin. The mass spectrum of echinoserine showed a significantly stronger fragmentation than that of echinomycin under identical operation conditions of the ESI source. The daughter ion (MS-MS) spectrum of the protonated molecular ion and the assignment of the main fragment ions is shown in Fig. 2. The fragment ions at *m/z* 1006 and 875 are formed by the loss of one and two HN(Me)-Val-OH residues, respectively, indicating that the *C*-termini of both N(Me)-Val residues in the echinoserine molecule are free. This finding was confirmed by two further experiments. First, echinoserine was methylated with diazomethane. The ESI-MS of this reaction showed a mass signal at *m/z* 1165.5, which is the $[M+H]^+$ ion of doubly methylated echinoserine. As expected, the daughter ion spectrum of this ion showed a signal at *m/z* 1020.5 correlating with cleavage of HN(Me)Val-OMe. Secondly, we measured the number of exchangeable protons (protons bound to heteroatoms) of echinoserine (data not shown). In a solution of $[^2H]_4$-MeOH the $[M+Na]^+$ ion was shifted from *m/z* 1160 to *m/z* 1168. This indicates the exchange of eight protons by deuterium, which is in agreement with the structure shown in Fig. 3, having four nitrogen bound and four oxygen bound protons.

Table 2. Antimicrobial activities of echinoserine and echinomycin, inhibition zone [mm].

Organism	Echinoserine (1 mg/ml)	Echinomycin (1 mg/ml)
Arthrobacter aurescens ATCC 13344	12	33
Bacillus subtilis ATCC 6051	8	26
Agrobacterium tumefaciens ATCC 15955	8	25
Escherichia coli K12	8	25
Proteus mirabilis ATCC 29906	9	26
Pseudomonas fluorescens ATCC 13525	9	29

The sequential assignment of the amino acids D-Ser and L-Ala was not possible from the NMR data. Daughter ion spectra of the fragment ions at *m/z* 444 and 398 showed a signal at *m/z* 244 (data not shown). This signal corresponds to the *N*-terminal fragment quinoxalinyl-Ser produced by cleavage of the peptide bond between the D-Ser and the L-Ala residue.

From these results we conclude that echinoserine has a structure similar to echinomycin but with both lactone-bonds hydrolysed as shown in Fig. 3.

Biological Properties

The biological activity of echinoserine and echinomycin were compared at a concentration of 1 mg/ml by the agar plate diffusion assay. While echinomycin is highly active against Gram-positive and Gram-negative bacteria at this concentration, echinoserine shows only weak activity against a few bacteria grown on chemically defined media as shown in Table 2. No antibacterial activity was found on complex media. Echinoserine can be considered as a less active analogue of echinomycin.

Discussion

Screening for new structures from natural sources

is always plagued by the problem of how to avoid the tedious isolation and structure elucidation of already known compounds. The screening method using reversed-phase HPLC, multiwavelength monitoring by diode-array detection and computerized comparison of the data with a UV-visible absorbance spectral database has been successful applied in the discovery of new secondary metabolites in culture filtrates and in extracts of culture filtrates[1,2,16]. This screening method is simple, economical and reliable. It allows identification of known metabolites in culture filtrates or raw extracts at an early stage of investigation. HPLC-MS analysis is capable of providing additional specific information upon those compounds which have been characterized by HPLC-DAD in the first screening stage.

Using this screening method we found a new secondary metabolite, echinoserine, produced by *Streptomyces tendae* Tü 4031. Echinoserine represents a non-cyclic form of echinomycin, in which both lactone-links of the cyclic molecule are hydrolised. This is reflected by the higher molecular mass of echinoserine and the different retention times on reversed-phase chromatography. Echinoserine is a natural occuring analogue of echinomycin. In addition to this main compound, we also detected a compound with one lactone-link together with small amounts of the antibacterially active echinomycin in the culture filtrate. However, echinoserine does not represent a precursor of echinomycin nor a degradation product, because it was not possible to convert echinoserine into echinomycin, and *vice versa* (results not shown). Echinoserine also differs from echinomycinic acid which was obtained by the alkaline hydrolysis of echinomycin[4].

In addition, we also screened the echinomycin producer *Streptomyces echinatus* and, interestingly, echinoserine was detected in relatively high amounts (30 mg/liter) in the culture filtrate. It has not been detected to date, because of its lower antibacterial activity compared to echinomycin. This shows impressively the advantages of the simple but efficient HPLC-DAD screening method.

Experimental

Microorganisms

Strain Tü 4031 was isolated from a soil sample collected in Alice Springs, Australia, and identified according to HÜTTER[11] and SHIRLING and GOTTLIEB[12] as a strain of *Streptomyces tendae*. It is deposited in the culture collection of our institute.

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

Fermentation

Strain *Streptomyces tendae* Tü 4031 was cultivated in a 10-liter fermentor (Biostat E, Braun Diessel Biotech) using a production medium consisting of: mannitol 2% and soybean meal 2% in tap water (pH 7.5). The fermentor was inoculated with 5 vol% of shaking cultures grown for 48 hours in the same medium. The fermentation was carried out at 27°C for 96 hours with an aeration rate of 0.5 v/v/m and an agitation of 300 rpm. The echinoserine production was monitored by reversed-phase HPLC.

Isolation

Hyflo Super-cel (2%) was added to the fermentation broth which was separated by multiple sheet filtration into culture filtrate and mycelium cake. The culture filtrate was passed through an Amberlite XAD-16 column. Impurities were washed out with H_2O-MeOH (40:60) and echinoserine was desorbed with MeOH. After removing the organic solvent by evaporation *in vacuo*, the aqueous residue was adjusted to pH 4 and extracted with 1-butanol. After concentration of the organic extract to dryness, the residue was dissolved in a small amount of MeOH and purified on a Sephadex LH 20 column using methanol as eluent. The echinoserine containing fractions were combined and concentrated to dryness.

Pure echinoserine was obtained by preparative reversed-phase HPLC using a stainless steel column (250 × 16 mm) filled with 10 µm Nucleosil-100 C-18, and linear gradient elution with H_2O-MeOH, starting from 50% MeOH to 80% MeOH within 15 minutes and a flow rate of 20 ml/minute. 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 240 nm by a Gilson spectrophotometer Mod. 116 equipped with a preparative cell.

Biological Assay

Antimicrobial activity was tested by agar plate diffusion assay. 10 µl of the test solution in a concentration of 1 mg/ml were applied onto filter discs (6 mm diameter), and the test plates were incubated for 24 hours at 37°C and 27°C, respectively. The following organisms were used as test organisms: *Arthrobacter aurescens* ATCC 13344, *Bacillus brevis* ATCC 9999, *Bacillus subtilis* ATCC 6051, *Clostridium pasteurianum* DSM 525, *Micrococcus luteus* ATCC 381, *Staphylococcus aureus* ATCC 11632, *Streptomyces viridochromogenes* Tü 57, *Agrobacterium tumefaciens* ATCC 15955, *Escherichia coli* K12, *Proteus mirabilis* ATCC 29906, *Pseudomonas fluorescens* ATCC 13525 and *Candida albicans* CBS 2718. These organisms were grown in complex and chemically defined media, respectively. The complex medium consisted of: nutrient broth 0.8% and NaCl 0.5% in deionized water (pH 7.3), whereas the chemically defined medium contained (per liter): glucose 5 g, tri-Na-citrate·$2H_2O$ 0.5 g, $(NH_4)_2SO_4$ 1 g, KH_2PO_4 3 g, K_2HPO_4 7 g, $MgSO_4 \cdot 7H_2O$ 0.1 g (pH 6.8). *Streptomyces viridochro-*

mogenes was grown in malt extract 1%, glucose 0.4% and yeast extract 0.4% in tap water (pH 7.3), and *Candida albicans* was grown in the same medium at pH 5.5.

HPLC-Analysis

The chromatographic system consisted of a Hewlett-Packard HP 1090M liquid chromatograph equipped with a built-in diode-array detector, 486/66 MHz computer (32 MB ram, 240 MB hard disk) and HP 3D-DOS ChemStation software rev. 2.02. Multiple wavelength monitoring was performed at 210 nm, 240 nm, 300 nm and 360 nm, and a reference wavelength at 550 nm. The spectrum range was from 200 to 600 nm, step 2 nm and sampling interval of 640 mseconds.

A sample of the fermentation broth was centrifuged (10 minutes, $13000 \times g$), 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 was packed with 7 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.

Mass Spectrometry

Echinoserine was dissolved in methanol - 0.1% aqueous formic acid (1 mg/ml). Electrospray mass spectra were recorded on a triple-quadrupole mass spectrometer API III equipped with a nebulizer-assisted electrospray (ionspray) source[17] (Sciex, Thornhill, Ontario, Canada). The solution was continuously infused with a medical infusion pump (Modell 22, Harvard Apparatus, Southnatick, U.S.A.) at a flow rate of 5 μl/minute. Positive ion ESI-mass spectra were acquired with a step size of 0.1 u and 0.5 u respectively, and a dwell time of 0.5 or 1 msecond. The potential of the spray was held at 4.9 kV. The spectra were recorded with an orifice voltage of +50 V or +60 V. Tandem mass spectra were performed using argon as collision gas with a target gas thickness of about 1.6×10^{14} atoms/cm^2 and a collision energy of 30 eV. By applying an orifice voltage of +100 V, fragmentation of the molecular ion in the transport region of the source was obtained. The MS-MS spectra of these fragment ions were measured with a target gas thickness of about 6×10^{14} atoms/cm^2 and a collision energy of 80 eV. Cesium iodide was used for calibration of both quadrupoles. For HPLC-MS a setup as previously described was used[18].

NMR Spectroscopy

High-resolution NMR spectra were recorded at 305 K on a Bruker AMX 600 spectrometer (Bruker Physics, Karlsruhe, Germany) using a selective proton probe for TOCSY[19] and an inverse triple resonance probe for HSQC[20] and HMBC[21]. A 5 mmol solution of echinoserine in $[^2H]_4$-MeOH was used for all measurements. Pulsed field gradients were applied in HSQC and HMBC for coherence selection and solvent suppression[22]. In the TOCSY, the OH resonance of the solvent was suppressed by presaturation. TOCSY and HSQC were acquired in phase-sensitive absorption mode with quadrature detection in both dimensions using the TPPI method. Generally 512 experiments with 32 to 128 scans and a data size of 2 K complex points were collected for each two-dimensional experiment. For the TOCSY experiment the clean TOCSY pulse sequence[23] with a mixing time of 64 mseconds was used. The HMBC was optimized for $J = 8$ Hz. A low pass ^1J-filter was implemented in the pulse sequence to suppress one-bond correlations. Data processing consisted of zerofilling to 4 K (F2) and to 1 K in the F1 dimension and apodisation with a shifted squared sine-bell weighting function.

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