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Isolation and Structure Elucidation of Thiazomycin

—A Potent Thiazolyl Peptide Antibiotic from *Amycolatopsis fastidiosa*

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Abstract Thiazolyl peptides are a class of rigid macrocyclic compounds richly populated with thiazole rings. They are highly potent antibiotics but none have been advanced to clinic due to poor aqueous solubility. Recent progress in this field prompted a reinvestigation leading to the isolation of a new thiazolyl peptide, thiazomycin, a congener of nocathiacins. Thiazomycin possesses an oxazolidine ring as part of the amino-sugar moiety in contrast to the dimethyl amino group present in nocathiacin I. The presence of the oxazolidine ring provides additional opportunities for chemical modifications that are not possible with other nocathiacins. Thiazomycin is extremely potent against Gram-positive bacteria both *in vitro* and *in vivo*. The titer of thiazomycin in the fermentation broth was very low compared to the nocathiacins I and III. The lower titer together with its sandwiched order of elution presented significant challenges in large scale purification of thiazomycin. This problem was resolved by the development of an innovative preferential protonation based one- and/or two-step chromatographic method, which was used for pilot plant scale purifications of thiazomycin. The isolation and structure elucidation of thiazomycin is herein described.

Keywords antibiotics, thiazolyl peptide, protein synthesis inhibitors, natural products

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Introduction

The discovery of penicillin and other antibacterial agents of microbial origin to treat infections from pathogenic bacteria is arguably one of the greatest achievements of the science and medicine of mid twentieth century [1, 2]. Incremental improvements to those discoveries led to development of new and improved antibiotics, which continue to serve humanity well. However, bacteria are adapting to these antibiotics rendering them ineffective, and leading to the emergence of resistance. Fortunately the frequency of resistance is quite variable amongst different bacterial species and the strains that are resistant to one antibiotic are susceptible to many other. A most frightening situation would be when bacterial strains become resistant to multiple antibiotics leading to the limited or no treatment options. Although these situations have started to emerge (*e.g.*, refractory methicillin resistant *Staphylococcus aureus* and others), fortunately they remain rare. In order to treat such infections and avoid an epidemic to occur, new treatment options must be made available by discovery of new structural chemotypes that inhibit growth of bacteria

by new modes of action (*e.g.*, platensimycin [3, 4] and platencin [5, 6]).

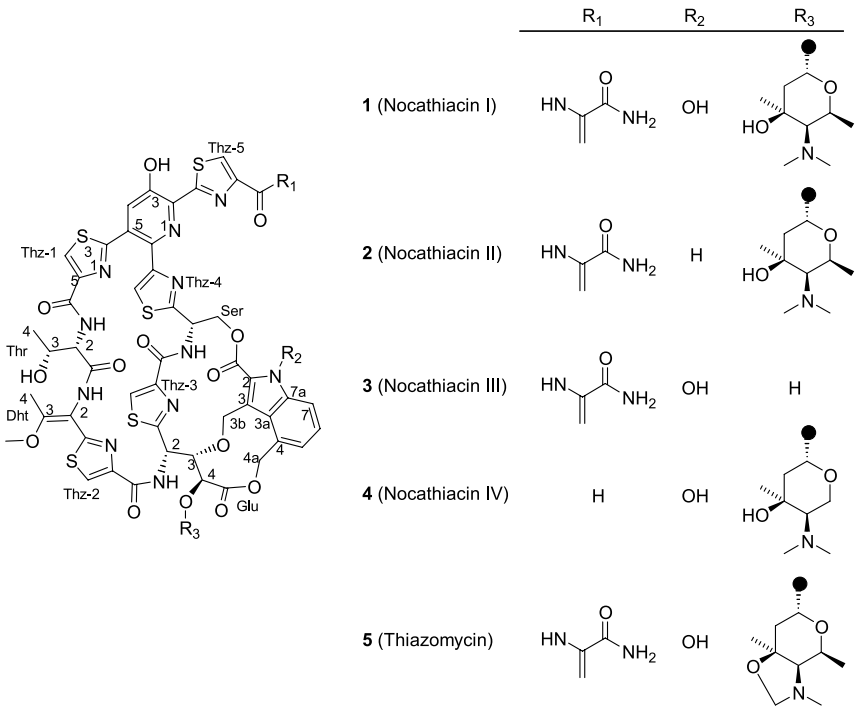
Thiazolyl peptides are a class of naturally occurring antibiotics produced by prokaryotic organisms. The discovery of these compounds dates back to late forties (*e.g.*, micrococcin in 1948) and early fifties (*e.g.*, thiostrepton in 1954) [7]. These compounds have been shown to exhibit some of the most potent *in vitro* activities, but they have not been developed as clinical agents due to poor physicochemical properties, most notably low aqueous solubility. However, key attributes of this class of natural products, such as highly potent activity and new modes of action, prompted us to study them further. Our research laboratories studied thiostrepton in the early eighties without making much progress in terms of development [8, 9]. Significant advances in biology and chemistry have taken place since then. Upon closer examination of this chemical class, compounds such as glycothiohexide α [10, 11], S54832A-I [7], MJ347-81F4A and B [12] and nocathiacins [13, 14] appeared more attractive than the rest because they contained aminoglycosidic residue, which inherently provided potential for improvement of aqueous solubility. This was illustrated by chemical modifications of nocathiacin I [13~23]. Therefore, we initiated an investigation to discover new thiazolyl peptides with chemical functionalities that can provide additional opportunities for chemical modifications, which could lead to compounds with better physical and pharmaceutical properties. Such compounds could be amenable to development as new antibiotics without cross resistance to existing antibiotics. To accomplish our goal, we acquired known producers of thiazolyl peptides and related cultures from internal and external sources and subjected them to both biological and chemical screening.

LCMS screening of the extracts of *Nocardia* sp. ATCC 202099 (renamed *Amycolatopsis fastidiosa*) indicated the presence of new thiazolyl peptides. Chromatographic fractionation of an extract led to the isolation of four known compounds nocathiacins I~IV (**1**~**4**) and a new thiazolyl peptide, thiazomycin (**5**). The isolation, structure elucidation, and large scale production of thiazomycin are herein described. Full biological characterization of thiazomycin is reported in the accompanying paper [24].

Results and Discussion

Revised Classification of the Producing Strain

The strain MA7332 (ATCC 202099) grows well at 28°C in a good range of agar media such as Yeast Malt Extract, Oatmeal, Glycerol Asparagine, Inorganic Salts Starch and Trypticase Soy agars, in the form of wrinkled yellowish small colonies. Microscopically the strain forms a sterile and extensively branched substrate mycelium that, even after long incubations, shows no traces of hyphal fragmentation or maturation of rudimentary aerial hyphae, morphological traits not corresponding to members of *Nocardia* spp. The taxonomic position of the producing



organism, originally identified as a *Nocardia* sp., was re-evaluated on the basis of new molecular data obtained in the course of this study. A phylogenetic study based on 16S rDNA sequences (GenBank accession EU072442) from representative members of the families *Nocardiaceae*, *Pseudonocardiaceae* and *Actinosynnemataceae* shows the relatedness of the strain with the type species *A. fastidiosa* ATCC31181, a relationship highly supported by

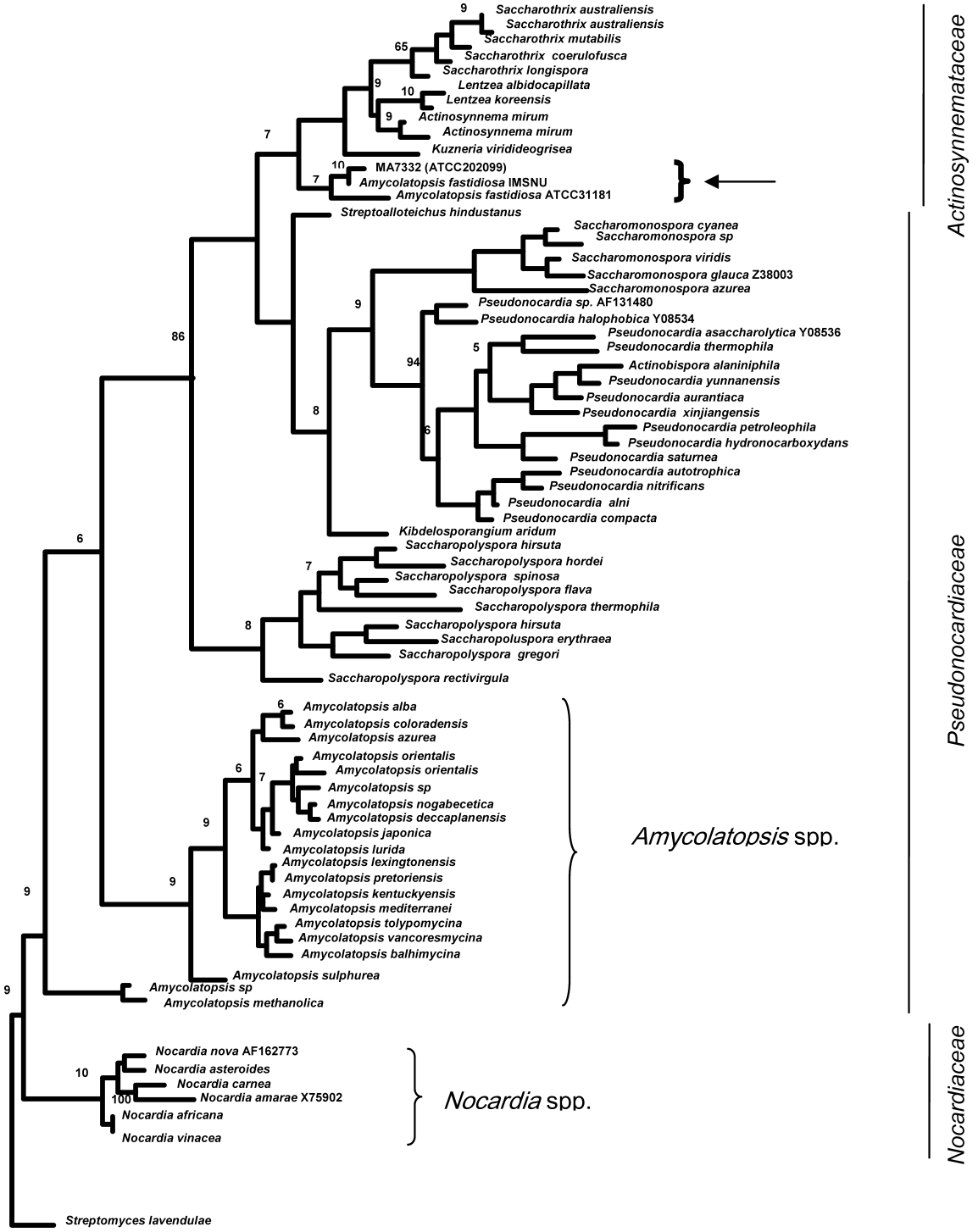


Fig. 1 Neighbor joining phylogenetic tree based on almost complete 16S rDNA sequences of the strain MA7332 and species of the families *Nocardiaceae, Actinosynnemataceae* and *Pseudonocardiaceae*.

The numbers above branches indicate bootstrap support (%) based on analysis of 1000 replicates. Only values above 50% are shown.

bootstrap values (Fig. 1). This new molecular data supports a re-evaluation of the taxonomic position of the strain MA7332 that should not longer be associated to strains of the genus *Nocardia*, and therefore we propose for its re-classification as a new member of the species *A. fastidiosa*. Nevertheless taking into account the external position of the type species *A. fastidiosa* with regard to the other members of the family *Pseudonocardiaceae*, and its closest relationship to members of the family *Actinosynnemataceae*, additional studies are needed to resolve the taxonomic position of this clade within the family *Pseudonocardiaceae*.

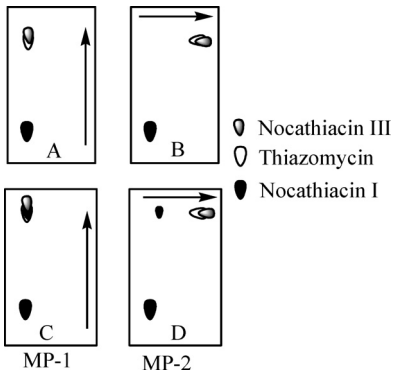


Fig. 2 2D TLC method to detect the differential protonation of nocathiacin I.

Top panel: TLC plates A and B containing feed with complete protonation of nocathiacin I, bottom panel: TLC plates C and D containing feed with incomplete protonation of nocathiacin I, Mobile phases: (MP-1) 10% MeOH in CH_2Cl_2, (MP-2) 10% MeOH in CH_2Cl_2+1% AcOH.

Initial Isolation of Thiazomycin

Initial isolation of thiazomycin was accomplished by ethyl acetate and acetone extractions of the fermentation broth followed by successive chromatographic steps on silica gel, Sephadex LH20, and RP HPLC. This process allowed the purification of 17.5 mg of thiazomycin from 18 liters of fermentation broth.

5 was present as a minor metabolite (1～2 mg/liter) in the fermentation broth that contained a 10～100 fold excess of **1** (100～200 mg/liter) and **3** (50～100 mg), along with minor amounts of **2** (2～5 mg/liter) and **4** (1～5 mg/liter). This presented significant challenges to its purification in larger quantities. The purification was complicated, not only due to its low concentration, but also due to its order of chromatographic elution with respect to other major thiazolyl peptides produced. In the elution profile, thiazomycin was sandwiched between the two major metabolites, **1** and **3**. Both **1** and **5** contain a basic tertiary nitrogen in the amino sugar residue, which was fortunately absent in **3**. **1** possesses a dimethyl amino group whereas **5** possesses an oxazolidine ring. The differences in the pKa of the basic nitrogens of these compounds were sufficient for differential protonation to occur in the presence of certain acids, which in turn led to significant alternations in their chromatographic properties.

Of all the acids tested, TFA selectively protonated **1** in the presence of **3**, **5**, and other related compounds most efficiently (Fig. 2) thus allowing for differential protonation to occur and development of the purification method. The success of the differential protonation method depended on the complete protonation of **1**. Partial protonation of **1** complicated the purification process due to co-elution of un-protonated **1** with **5**, which necessitated the careful monitoring of the extent of protonation of **1**. To accomplish this, a two dimensional TLC system was used. In this system, fully protonated **1** remained at the baseline of the TLC plate (Fig. 2, top panel, plates A and B); whereas partially protonated or un-protonated **1** moved with **5** without any resolution after the first development (plate C). One percent acetic acid used in the development solvent in the second dimension protonated the un-protonated **1**, thereby resolving it from the thiazomycin (plate D). Therefore if the protonation of **1** in the sample was complete, there would be no **1** present at the baseline after the development in the second dimension. The TLC spots were visualized under UV light. Interestingly, TFA in the mobile phase caused retardation of the mobility of **5** from **3** and **1**. This observation allowed us to develop a one-step purification of **5** using silica gel chromatography in which the crude extract was acidified with TFA and pre-adsorbed on to silica gel before charging on the silica gel column.

(a) One-step Purification

In a typical experiment, a methyl ethyl ketone extract of the broth was concentrated to an aqueous layer leading to precipitation of thiazolyl peptides which were collected and washed with hexane or heptane. The precipitate was dissolved in methylene chloride-methanol containing 1.0% TFA, adsorbed on to a minimum amount of silica gel, and chromatographed on to a dry-packed column pre-equilibrated with methylene chloride. The optimal ratio of the feed to silica gel was about 1 : 25 (wt/wt). The column was then eluted with various proportions of methanol in methylene chloride, followed by 1.0% NH_4OH to separate **3**, **5** and **1** (Fig. 3).

(b) Two-step Purification-pilot Plant Scale

Although the aforementioned method was acceptable for the purification of **5** at the laboratory scale, the use of chlorinated solvent and the pre-adsorption on silica gel before loading made it unsuitable for use in the pilot

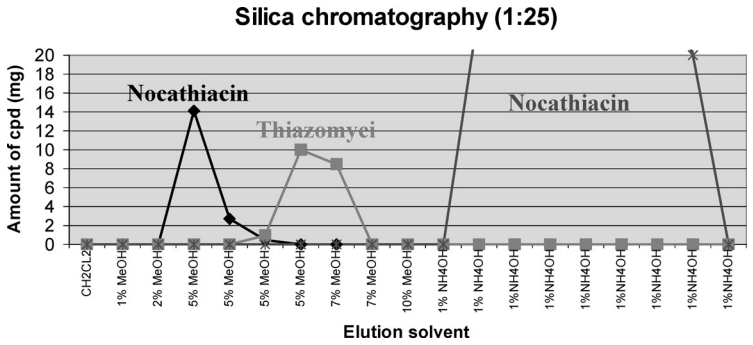


Fig. 3 Elution profile of thiazomycin and nocathiacins from the silica gel column.

plant or in the factory. After extensive studies, THF was identified as a replacement solvent for methylene chloride which necessitated insertion of a second step and a two-step chromatographic method was developed for the purification. The precipitate from the concentration of the MEK extract was dissolved in THF, concentrated to dryness and re-dissolved in a mixture of TFA-THF and charged directly to a silica gel column (capture step), which was equilibrated with TFA acidified THF. After loading, the column was washed with THF, and thiazomycin was eluted with 1.0% NH_4OH in THF. The rich cut was similarly rechromatographed on a small polishing column. This led to highly pure **5** with THF stabilizer, which was removed by washing with acetone to yield >95% pure **5**.

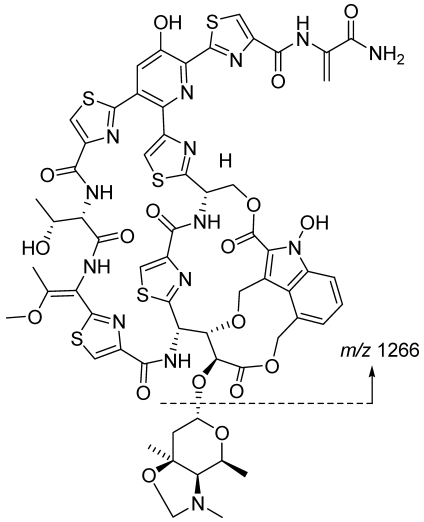


Fig. 4 Key ESI-MS fragmentation of thiazomycin.

Structure Elucidation

HRESIFT-MS analysis of **5** indicated a molecular ion at *m/z* 1435.2707 (M+H) and provided a molecular formula $C_{61}H_{58}N_{14}O_{18}S_5$ which was two hydrogen atoms less than the formula of **1**. The mass spectrum produced a fragment ion at *m/z* 1266, an ion also produced by **1**, suggesting an identical core structure for both compounds (Fig. 4). These data indicated that the structural differences resided in the amino sugar residue. The complete set of ^{1}H-, ^{13}C-, COSY, HMQC and HMBC data was recorded in DMSO and in a mixed solvent consisting of $CDCl_3/CD_3OD$ 5 : 1. The mixed solvent system produced much better resolution and sharpness of the ^{1}H-NMR signals and yielded a better data set which was used in the structure elucidation (Table 1). Both the ^{1}H- and the ^{13}C-NMR spectra of **5** supported the presence of the tricyclic thiazolyl peptide motif core of **1**. Comparison to the ^{1}H-NMR spectral data of **5** with that of **1** suggested the presence of only one amino-methyl group (δ_H 2.7, s) in **5** and showed the presence of a pair of self coupled doublets (δ_H 4.25 and 5.13) with *J* values of 5.5 Hz correlating to a methylene δ_C 83.4 ppm in the HMQC spectrum. The new methylene group displayed HMBC correlations to the NMe-4 (δ_C 40.0) and C-3 (δ 81.7) (Table 1) indicating the presence of the oxazolidine ring. The ^{1}H- and ^{13}C-NMR spectra were assigned by a comparison with corresponding spectra of **1** and verified by HMQC and HMBC experiments. The NMR spectra of **5** in the mixed solvents were highly dependent on the concentration of solute and the ratio of the two solvents. In the mixed solvents, many NH protons were slow to exchange and could be easily detected but most exchanged over time and were not useful for HMBC. Reduction of **5** with sodium cyanoborohydride in acidic conditions produced **1** in high yield which was compared directly (co-HPLC, ^{1}H-NMR, HRLCFT-MS) with an authentic sample of **1** isolated from the fermentation broth. Thus the relative and absolute configuration of **5** is confirmed to be identical to **1**.

The aqueous solubility of **5** (<0.1 mg/ml) was slightly poorer than **1** (<0.2 mg/ml) in water. These compounds

Table 1 ^{1}H- and ^{13}C-NMR assignments of thiazomycin (**5**) in $CDCl_3$ - CD_3OD (5 : 1)

Position	δ_C	δ_H, *J*, Hz	HMBC C→H
Thz-1 (N)			
Thz-1 (C_2)	164.9	—	Thz1-H-4, Pyr-H-4
Thz-1 (C_4)	125.4	8.35, s	
Thz-1 (C_5)	148.8	—	Thz1-H-4
Thz-1 (CO)	159.5	—	Thz1-H-4
Thr (CO)	167.4		
Thr (NH)		7.84, d, 6.0	
Thr (C_2)	56.1	4.15, m	Thr-H_3-4
Thr (C_3)	64.3	2.87, m	Thr-H_3-4
Thr (C_4)	17.8	1.13, d, 6.0	
Dht (NH)			
Dht (C_2)	110.0		Dht-H_3-4
Dht (C_3)	159.3		Dht-H_3-4, OMe
Dht (C_4)	13.4	1.84, s	
Dht (OMe)	55.6	3.77, s	
Thz-2 (N)			
Thz-2 (C_2)	162.1	—	Thz2-H-4
Thz-2 (C_4)	124.0	7.89, s	
Thz-2 (C_5)	145.5		Thz2-H-4
Thz-2 (CO)	160.3		Thz2-H-4, Glu-H-2
Glu (NH)		8.36, d, 10.0	
Glu (C_2)	48.5	5.91, dd, 10.0, 1.0	Glu-H-4
Glu (C_3)	81.1	3.87, dd, 10.0, 1.0	Glu-H-2, Glu-H-4, Indole-H-3b
Glu (C_4)	69.6	4.36, d, 10.0	
Glu (CO)	171.4		Glu-H-4, Indole-H-4[a]
Thz-3 (N)			
Thz-3 (C_2)	166.1		Thz3-H-4
Thz-3 (C_4)	124.9	8.19, s	
Thz-3 (C_5)	149.5		Thz3-H-4
Thz-3 (CO)	161.4		Thz3-H-4
Ser (NH)		8.05, d, 10	
Ser (C_2)	48.8	5.63, dd, 11.0, 6.5	Ser-H-3
Ser (C_3)	64.3	4.37, br d, 11.0	
		5.22, dd, 11.0, 6.0	
Thz-4 (N)			
Thz-4 (C_2)	168.8		Thz4-H-4, Ser-H-3, Ser-H-2
Thz-4 (C_4)	120.1	7.67, s	
Thz-4 (C_5)	154.9		Thz4-H-4
Pyr (C_2)	134.2		Pyr-H-4
Pyr (C_3)	151.1		Pyr-H-4
Pyr (C_4)	126.5	7.57, s	
Pyr (C_5)	129.8		Pyr-H-4
Pyr (C_6)	143.7		Pyr-H-4
Thz-5 (N)			
Thz-5 (C_2)	169.1		Thz5-H-4, Pyr-H-4 (w)
Thz-5 (C_4)	125.5	8.18, s	
Thz-5 (C_5)	149.4		Thz5-H-4
Thz-5 (CO)	158.4		
Deala (NH)			
Deala (C_2)	132.7		Deala-H-3

Table 1 Continued

Position	δ_C	δ_H, *J*, Hz	HMBC C→H
Deala (C_3)	104.3	5.59, s 6.53, s	
Deala (CO)	165.9		Deala-H-3
Deala (NH_2)			
Indole (CO)	161.1		Ser-H-3
Indole (OH)			
Indole (C_2)	126.6		Indole-H-3b
Indole (C_3)	109.7		Indole-H-3b
Indole (C_{3a})	119.1		Indole-H-3b; H-4a, H-5
Indole (C_{3b})	65.8	4.12, d, 10.0 4.78, d, 10.0	
Indole (C_4)	127.5		Indole-H-3b, H-4a, H-6
Indole (C_{4a})	67.9	4.90, d, 12.5 5.95, d, 12.5	Indole-H-5
Indole (C_5)	123.6	7.08, d, 7.0	Indole-H-4[a]
Indole (C_6)	124.9	7.32, t, 7.0	
Indole (C_7)	112.1	7.69, d, 7.0	Indole-H-5
Indole (C_{7a})	135.1		Indole-H-6
Sug (C_1)	93.2	5.04, dd, 7.5, 5.5	Glu-H-4, Sug-H-5, H-2
Sug (C_2)	35.1	1.90, dd, 15.0, 7.5 2.40, dd, 15.0, 5.5	Sug-C3-CH_3
Sug (C_3)	81.7		Sug-CH_3-3, Sug-H-2, H-4, O–CH_2–N
Sug (C_3-Me)	24.2	1.43, s	Sug-H-2, H-4
Sug (C_4)	71.7	3.05, br s	Sug-CH_3-3, Sug-H-2, N-Me
Sug (C_4-NMe)	40.0	2.70, s	Sug-H-4, O–CH_2–N
Sug (C_5)	62.7	3.91, m	
Sug (C_6)	15.7	0.74, d, 7.0	
O–CH_2–N	83.4	4.25, br d, 5.5 5.13, br d, 5.5	N-Me

turned out to be unstable in phosphate buffer at pH ranges 3∼7. The presence of an oxazolidine ring in the amino-sugar portion of **5** provides additional opportunities for chemical modifications that could lead to derivatives with increased water solubility and improved pharmaceutical properties. Although this compound was produced as a minor congener of **1**, it is reasonable to expect that the production titer and ratio of the two compounds could be favorably improved by mutation and fermentation media manipulations.

In summary, we have described the isolation and structure elucidation of a new thiazolyl peptide that is a highly potent antibiotic. A very efficient isolation procedure was developed to isolate this minor metabolite in the presence of large amounts of structurally-related metabolites.

Experimental

General Procedure

All reagents were obtained from Sigma-Aldrich and were used without further purification. The NMR spectra were obtained on a Varian Inova 500 or 600 MHz spectrometers operating at 500 or 600 MHz for ^{1}H and 125 or 150 MHz for ^{13}C nuclei. Residual solvent signal was used as a reference. Optical rotations were obtained on a Perkin-Elmer 241 Polarimeter, and IR spectral data were obtained on a Perkin-Elmer Spectrum One spectrometer. High-resolution mass spectra were obtained on a Thermo Finnigan LTQ-FT with the standard Ion Max API source (without the sweep cone) and ESI probe.

Producing Organism and Phylogenetic Analysis

The producing organism was obtained from the American

Type Culture Collection, Maryland, with accession number 202099. It has been deposited in the Merck culture collection with accession number MA7332. DNA isolation and 16S rDNA sequencing were performed as described previously [25]. Sequence alignments were done using CLUSTAL W [26] and phylogenetic analysis was done in PHYLIP [27] using the neighbor joining algorithm. The analysis was bootstrapped using 200 replicates.

Fermentation Conditions of ATCC 202099 (MA7332)

A 1.0 ml frozen vegetative stock culture of ATCC 202099 (MA7332) was used to inoculate 50 ml of the seed medium, in a 250-ml non-baffled Erlenmeyer flask, containing the following components per liter of water: soluble starch (Sigma #S-9765), 20 g; dextrose, 5.0 g; N–Z amine (Kerry Bio-Science, Hoffman Estates, IL), 3.0 g; Bacto yeast extract (Becton Dickinson), 2.0 g; Pharmamedia (Traders Protein, Memphis, TN), 5.0 g; calcium carbonate, 1.0 g. The culture was incubated at 32°C on a rotary shaker operating at 220 rpm for 3 days. Twenty ml of the resulting culture was used to inoculate 500 ml of seed medium, in a 2-liters non-baffled Erlenmeyer flask, containing the same components as for the 50 ml culture listed above. The culture was incubated at 32°C on a rotary shaker operating at 180 rpm for 1 day. The resulting 500 ml culture was used to inoculate 20 liters of media, in a 30-liters fermenter, containing the following components per liter of water: dextrose, 20 g; soy peptone-type SL (Marcor), 5.0 g; primary dried yeast (PDY) Yeast Products Inc., 10 g; Allophosite (aluminum silicate) (Sigma #A-3561), 5.0 g; P2000 anti-foam (polymeric material made by Dow Chemical, Midland MI that prevents foaming), 1.0 ml. The production fermentation was operated at a temperature of 32°C, at a back-pressure of 5 psi, and an agitation rate of 300 rpm. Air was sparged through the fermenter at 10 slpm and pH was controlled at 7.0 with 10% NaOH and 5.0% H_2SO_4. The fermenter was operated for 13 days, at which time the culture was harvested and compounds were extracted.

Fermentation Conditions for Mutant Strains

The *Amycolatopsis fastidiosa* strain used in this study was a descendant of ATCC 202099 generated from a classical mutation/strain improvement program which produced increased amounts of **5** compared to the wild-type strain. A 2 liters aliquot of frozen inoculum, prepared essentially as outlined by Junker *et al.* [28], was thawed and transferred to a 280-liters fermenter containing 180 liters of SNOC seed medium (2.0% soluble starch Stadex-60 (Staley), 0.3% NZ Amine-A (Quest Int.), 0.2% yeast extract #106 (Springer), 0.5% cotton seed flour (this is the same as Pharmaedia), 0.3% $CaCO_3$, 0.5% glucose added post-sterilization, pre-sterilization pH 7.0, 30 minutes sterilization at 122～124°C). The fermentation conditions for the seed fermentation were: temperature, 32°C; airflow, 100 lpm; agitation (Rushton-style impellors), 100 rpm; back pressure, 0.7 kg/cm^2.

After 3～4 days of seed fermenter cultivation, 30 liters aliquots of broth were transferred to several 800-liters fermenters containing 600 liters of NOC-AW-2500 production medium (0.4% Hy-Case SF (Quest Int.), 0.5% yeast extract # 106 (Springer), 0.5% soy peptone (Marcor), 1.0% glucose added post-sterilization, 3.5% fructose added post-sterilization, 0.2% P2000 to control foaming, pre-sterilization pH 7.0, 45 minutes sterilization at 122～124°C). The production fermentation conditions were: temperature, 32°C; airflow, 150 lpm; agitation (axial flow-style impellors), 100 rpm; back pressure, 0.7 kg/cm^2. pH was controlled at 7.0±0.1 through the automatic addition of 25% sulfuric acid or 25% sodium hydroxide. Dissolved oxygen was controlled at 50% of saturation through an automatic increase in agitator speed up to 250 rpm. Production fermentations were run for 8～12 days and approximately 25 g of **5** were produced in 3000 liters of whole broth.

Preliminary Isolation of Thiazomycin

A 18 liters batch of fermentation broth (pH 5.0) was extracted twice with 18 and 16 liters each of ethyl acetate by shaking overnight at room temperature and filtered through celite. The ethyl acetate layers were separated from the aqueous phase, pooled and concentrated under reduced pressure to give 7.1 g of solid. The cell mass with celite was extracted twice with 8 liters of acetone each. Acetone extracts were pooled with ethyl acetate extracts and concentrated to dryness to produce 15.6 g of solid, which was placed in a sintered funnel and washed with hexane (4×150 ml), suspended in 250 ml of 1 : 1 methylene chloride - methanol and filtered. This extract was adsorbed into 35 g of Sephadex LH20 and charged on a 2 liters Sephadex LH20 column packed in 1 : 4 hexane - methylene chloride. The column was eluted with two column volumes of 1 : 4 hexane - methylene chloride, followed by a stepwise gradient of varying amounts of methanol in methylene chloride. One g of the solid eluting with hexane - methylene chloride was dissolved in 50% methanol - methylene chloride and adsorbed on to 5.0 g of silica gel 60 (230～400 mesh, E-M Scientific, Germany). It was dried under vacuum and purified using vacuum liquid chromatography on 100 g of silica gel 60 (230～400 mesh, E-M Scientific, Germany) in sintered funnel (20 cm ID). This was eluted with 0.5 liters of chloroform, followed

by 1 liter of 10% methanol - chloroform, 0.5 liters of 20% methanol - chloroform, 1 liter of 50% methanol - chloroform and finally washed with 5.0% ammonium hydroxide in 50% methanol - chloroform. Fraction from the 10% methanol - chloroform elution was found to contain **5**. It was concentrated under reduced pressure and lyophilized to yield 650 mg of solid material.

The 650 mg fraction from the silica chromatography was purified by thirteen identical repetitive runs of preparative HPLC using Zorbax SB Phenyl [(21×250 mm) eluting with a 36 minutes gradient of 40～50% acetonitrile - water containing 0.1% TFA]. **5** eluted at Rt=32～33 minutes. It was lyophilized to yield 42 mg of light yellow powder which was further purified by two preparative HPLC's using a shallower gradient [Zorbax SB Phenyl (21×250 mm eluting with a 42 minutes gradient of 40～50% acetonitrile - water containing 0.1% TFA]. **5** eluted in fractions at Rt=33～37 minutes. The fractions containing **5** were pooled and lyophilized to yield 17.5 mg pale yellow powder. Structure and the purity were confirmed by MS, NMR and HPLC analysis. UV (MeOH) λ_{max} (log ε) 222 (4.77), 288 (4.40), 358 (4.08) nm, IR (ZnSe) ν_{max} 3389, 3096, 2929, 1669, 1638, 1535, 1476, 1424, 1387, 1320, 1253, 1203, 1229, 1099, 1038, 1016 cm^{-1}, $[\alpha]_D^{23}$ +49.09 (*c* 1.1, MeOH), HRESI-MS (*m/z*) 1435.2707 (calcd for $C_{61}H_{58}N_{14}O_{18}S_5$+H, 1435.2719), 1266.1610 (calcd for $C_{52}H_{44}N_{13}O_{16}S_5$, 1266.1633); For ^{1}H- and ^{13}C-NMR see Table 1.

One Step Purification of Thiazomycin

A larger-scale fermentation broth (3000 liters) was extracted with an equal volume of MEK by shaking for 3 hours and concentrated to a mostly aqueous layer. The precipitated thiazolyl peptides were collected by filtration and washed with water and hexane. One gram of the mixture of thiazolyl peptides was dissolved in a 10 ml of a 9 : 1 mixture of CH_2Cl_2 - MeOH, acidified with 0.3 ml TFA, and adsorbed on to 1.3 g of silica gel. Solvents were removed under reduced pressure. The dried powder was charged on a dry packed silica gel column (25 g, 1×4″) which was pre-equilibrated in CH_2Cl_2 and eluted at 17 ml/minute with a step gradient of MeOH in CH_2Cl_2. Elution with 2～5% and 5～7% MeOH - CH_2Cl_2 separated **3** and **5** respectively. **1** was eluted with 1.0% NH_4OH in 10% MeOH - CH_2Cl_2 (Fig. 3).

Two Step Purification of Thiazomycin (Pilot Plant Scale)

The fermentation broth was extracted with an equal volume of MEK overnight at room temperature. The aqueous layer was removed and discarded and the organic layer was washed with an equal volume of water. The organic extract was concentrated 5×(40°C, 30 torr vacuum). One volume of *n*-heptane was added to the concentrate to precipitate all thiazolyl peptides. The precipitation was tri-phasic. A solid phase settled between the aqueous and organic phases. The organic phase was removed from the batch and discarded. One half volume of THF was added to the aqueous/solid phase. Dissolution of the thiazolyl peptides in the THF led to two layers. The lower aqueous layer was discarded, and the THF layer that contained ～80% of mixtures of thiazolyl peptides was concentrated to give 800 *g* solid containing 23 g of **5**. This was purified by two successive silica gel steps as detailed below.

Capture Step

Silica gel (3.4 kg, 60 Å; 37～63 μm) was slurried with 1.0% TFA in tetrahydrofuran, packed into a prep-scale column (7.5 liters bed volume), and was equilibrated with 14 liters of 1.0% TFA in THF. Crude thiazolyl peptide solids (800 g, 23 g **5**) were dissolved in 36.1 liters of tetrahydrofuran and 6 liters of 1 : 1.5 mixture of TFA : THF (cooled to room temperature) were added to the batch. After the feed was acidified, the batch was filtered through a Buchner funnel to remove any solids, and the solution was fed to the column at 500 ml/minute flow rate. On completion of the feed loading, the column was washed with 6.9 column volumes of 1.0% TFA in THF (52.1 liters). Following the wash, **5** was eluted from the column with 7.5 column volumes of 1.0% NH_4OH (56.3 liters) in THF. Elution fractions 4～7 were combined to produce the rich cut (15.1 liters; 20.1 g **5**; 84% yield). The ratio of **5** and **1**～**3** in the rich cut was 1.5 and 0.014. The mass balance across the column was 90%. The rich cut was concentrated to solids on a rotary evaporator to yield 52.8 g of solids (95% thiazolyl peptide purity).

Polishing Step

Silica gel (1.4 kg, 60 Å; 230～400 mesh) was slurried with 1.0% TFA in THF, packed into a prep-scale column (3.1 liters bed volume), and was equilibrated with 6 liters of 1.0% TFA in THF. Solids (52.6 g) from the concentrated rich cut were dissolved in 720 ml of 1.0% TFA in THF. The feed was placed directly on top of the silica and was allowed to adsorb onto the silica gel using gravity. Once the feed was adsorbed onto the silica gel bed, the column was washed with 4 column volumes of 1.0% TFA in THF (12 liters). **5** was then eluted with 9.5 column volumes (30.2 liters) of 0.125% NH_4OH. Fractions 4～10 were pooled as the thiazomycin rich-cut (13.3 liters) and concentrated under reduced pressure to produce 18.2 g of **5** (90% yield) which was completely free of **1** and **3** impurities. The purity

of **5** was over 90% and the only impurity was THF stabilizer which was removed by triturating with heptane and acetone.

Conversion of Thiazomycin to Nocathiacin I

To a solution of thiazomycin (3.0 mg) in MeOH (0.2 ml) at room temperature was added 5 μl of concentrated HCl, followed by $NaCNBH_3$ (1.0 mg). The reaction mixture was stirred at room temperature for 1 hour. Purification by similar RP HPLC methods as described earlier gave the desired product (2.0 mg), identical in all aspects (co-HPLC, ^{1}H- and ^{13}C-NMR) with the natural **1**.

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