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Nocathiacins, New Thiazolyl Peptide Antibiotics from *Nocardia* sp.

I. Taxonomy, Fermentation and Biological Activities

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Thiazolyl peptide antibiotics, nocathiacin I, II and III, were identified in a culture of *Nocardia* sp. WW-12651 (ATCC 202099). They exhibit potent *in vitro* activity (ng/ml) against a wide spectrum of Gram-positive bacteria, including multiple-drug resistant pathogens such as methicillin-resistant *Staphylococcus aureus* (MRSA), multi-drug resistant *Enterococcus faecium* (MREF) and fully penicillin-resistant *Streptococcus pneumoniae* (PRSP), and demonstrate excellent *in vivo* efficacy in a systemic *Staphylococcus aureus* infection mice model.

Multidrug-resistant strains of many clinically important pathogenic bacteria, including methicillin-resistant *Staphylococcus aureus* (MRSA), *Streptococcus pneumoniae*, *Mycobacterium tuberculosis*, and *Enterococcus* strains are becoming a worldwide health problem[1,2]. There is an urgent need to discover new agents to treat patients infected with multidrug-resistant bacteria[3~5]. A new group of thiazolyl peptide antibiotics (designated herein as nocathiacins) was discovered in the course of screening for novel antibiotics in a multi-drug resistant *Enterococcus faecium* (MREF) assay. The nocathiacins I~III (Fig. 1) were isolated from culture broth of *Nocardia* sp. WW-12651 (ATCC-202099) and share structural similarities to the glycothiohexides[6~8], thiostrepton[9], nosiheptide[10], micrococcins[11], GE2270 A[12], cyclothiazomycin[13] and berninamycin[14]. MJ347-81F4-A[15], produced by *Amycolatopsis* sp., was reported to have the same general structure as that of nocathiacin I, but its spectral data and absolute configuration were not reported. This paper deals with the taxonomy of the producing strain, fermentation, and biological activities of nocathiacins I~III. The isolation and structural elucidation of these compounds will be reported elsewhere[16,17].

Materials and Methods

Microorganism

Strain WW-12651 was isolated from a soil sample collected in New Mexico, USA and was deposited with the American Type Culture Collection (ATCC) in Rockville, MD, with the accession number of ATCC 202099. The stock culture was maintained in 10% (W/V) glycerol and 5% (W/V) sucrose solution at −80°C. Other strains used in this study, including several clinical isolates, are in the Bristol-Myers Squibb Company culture collection.

Taxonomy

The cultural and physiological characteristics of WW-12651 strain were tested according to the methods of SHERLING and GOTTLIEB[18], SCHAAL[19] and LECHEVALIER and LECHEVALIER[20]. Microscopic studies were carried out on various ISP (International *Streptomyces* Project) media and observations were made at 7, 14, and 21 days of incubation at 28°C.

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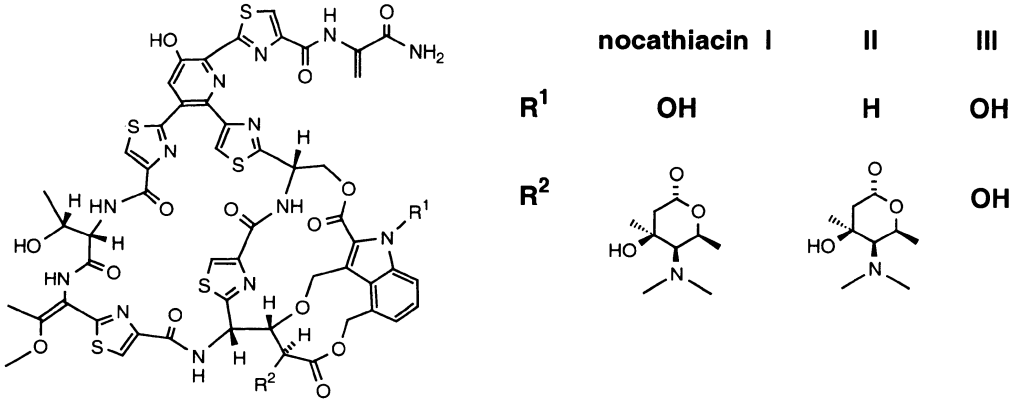
Fig. 1. Structures of nocathiacin I, II and III.



Production of Nocathiacin I, II and III in Shake Flask Culture

Four ml of WW-12651 frozen stock was inoculated into a 500-ml Erlenmeyer flask, containing 100 ml of seed medium consisting of soluble starch 2%, dextrose 0.5%, N-Z case 0.3%, yeast extract 0.2%, fish meat extract 0.5%, $CaCO_3$ 0.3%. The flask was incubated for 3 days at 32°C and at 250 rpm on a rotary shaker. Four ml of this seed culture was inoculated into each 500-ml flask containing 100 ml of production medium HYDN consisting of HY Yeast 412 1%, dextrose 2%, Nutrisoy 1% (pH adjusted to 7 before sterilization). The production culture was incubated at 32°C and at 250 rpm on a rotary shaker for 4~5 days.

For the production of nocathiacin III, the seed culture was incubated at 28°C and at 250 rpm on a rotary shaker for 3 days and the production cultures were incubated at 28°C and at 180 rpm on a rotary shaker for 2 days.

Production of Uniformly $^{13}C/^{15}N$-labeled nocathiacin I for Conformation and Absolute Configuration Determination

The seed culture was incubated at 28°C and at 250 rpm on a rotary shaker for 3 days. Five ml of this seed culture was transferred to a 15-ml sterile centrifuge tube. Supernatant was removed after centrifugation at 3000 rpm for 15 minutes, and the cells were washed with 5 ml sterile 0.9% NaCl solution and collected after centrifugation at 3000 rpm for 15 minutes. The washed cells were transferred to 100 ml sterile Celtone®-CN medium (^{13}C, >98%; ^{15}N, >98%; Martek Biosciences Corp.) in a 500-ml flask and incubated for 3 days at 28°C and at 250 rpm on a rotary shaker. Ten ml of the resulting broth was transferred to a 15-ml sterile centrifuge tube. Cells were collected after centrifugation at 3000 rpm for 15 minutes and transferred to 100 ml sterile fresh Celtone®-CN medium in a 500-ml flask. The cultures (a total of 9 flasks) were incubated at 28°C and at 250 rpm for 3 days. $^{13}C/^{15}N$-labeled nocathiacin I (36 mg) was isolated from the fermentation broth[16]. This labeled material was used in a detailed NOE study for determining the conformation and configuration of nocathiacin I[17].

Multi-drug Resistant *Enterococcus faecium* (MREF) Screening Assay

Enterococcus faecium strain A28152 was inoculated into Brain Heart Infusion Broth nutrient medium and incubated at 37°C with agitation. When optical density at 595 nm of the culture (measured with 0.2 ml aliquot in a well in a 96 well clear plastic flat-bottom plate) reached 0.2 to 0.4, the bacteria were harvested by centrifugation at $1000\times g$ for 10 minutes. The cell pellet was resuspended in Mueller-Hinton II growth medium and inoculated into molten Mueller-Hinton II growth media containing 1% Bacto agar at 48°C, to give an inoculated cell density of 1×10^7 cells/ml. Twenty-five ml of the cell suspension was poured into a sterile rectangular plate. A special sterile plastic lid that mated with the plate was placed on top of the plate while the medium was still molten. This lid contains plastic pegs arranged in the standard 8×12 format. The medium was allowed to gel at room temperature for 15 minutes, and then the pin lid was removed. Small concave impressions remain in the gelled media where the pins contacted the surface of the molten media. These serve as sample loading zones.

Tested samples were dissolved to a concentration of

300 μM in 100% dimethylsulfoxide (DMSO). A 6 μl volume of each sample was applied to individual sample loading zones on the plate and the plate was incubated at 37°C for 24 hours. Growth inhibition was detected as a clear circular zone surrounding the sample zone. DMSO alone did not cause any detectable growth inhibition under these conditions. Samples of chloramphenicol and DMSO were included on each plate as positive and negative controls, respectively.

Antibiotic Activity (*In Vitro*) Assay

The minimum inhibitory concentration (MIC) of antibiotic was obtained against a variety of bacteria using a conventional broth dilution assay in accordance with standards recommended by the National Committee for Clinical Laboratory Standards (NCCLS). The serial micro broth dilution method used Mueller-Hinton medium except for the *Streptococcus pneumoniae* which was tested in 50% Muller-Hinton medium and 50% Todd Hewitt medium. The final bacterial inoculum contained approximately 5×10^5 CFU/well and was run on microtiter plates. The volume of each well was 100 μl and the plates were incubated at 35°C for 18 hours in ambient air. The MIC was defined as the lowest drug concentration that prevented visible growth of the bacterium.

Antibiotic Activity (*In Vivo*) Assay

Nocathiacins were evaluated for antibiotic activity *in vivo*, in an acute systemic *S. aureus* infection model in mice. Adult female ICR mice (21 to 23 g; Harlan Sprague-Dawley, Inc., Indianapolis, Ind.) were inoculated intraperitoneally with a sufficient number of pathogens to kill 100% of the untreated infected animals within 24 hours. Each mouse received a $5\sim6\times10^6$ cfu overnight culture of *S. aureus* A15090 strain suspended in 7% sterile hog gastric mucin. Drug was prepared in a 10% DMSO/5% Tween 80/85% water vehicle and administered subcutaneously, twice daily at 1 and 4 hours after pathogen inoculation. The number of mice that survived in each experimental group was monitored up to 8 days after pathogen inoculation, and the 50% protective doses (PD_{50}s) of the drug-treated animals were determined by the Spearman-Karber nonparametric estimator method[21]. Each experimental group consisted of 10 animals and a minimum of three different concentrations of drug was evaluated per compound. The ranges of drug doses tested were 5 to 0.3 mg/kg of body weight per day given b.i.d. The statistical comparison of PD_{50}s was carried out using the Z test as described by HUBERT[21].

Analytical HPLC

The production of nocathiacins in the fermentation was monitored by a Hewlett Packard 1100 HPLC (equipped with a diode array UV detector) using a C-18 reversed-phase column (YMC ODS-AQ 4.6×150 mm) and UV absorption at 220 and 360 nm. The mobile phase consisted of 1 mM HCl in water (solvent A) and CH_3CN (solvent B) with the following step gradient: 15% B (3 minutes), 15~40% B (3 minutes), 40~70% B (9 minutes), 70~85% B (1 minute), 85% B (2 minutes), 85~15% B (1 minute). The flow rate was 1.2 ml/minute. The retention times of nocathiacin I, nocathiacin II, and nocathiacin III, were 9.7 minutes, 8.3 minutes and 13.7 minutes, respectively.

Results

Taxonomy Studies

Growth of strain WW-12651 on ISP4 medium developed as yellow cream colored colonies. The aerial mycelium was white and fragments extensively. Under light microscopy, spore chains were observed in the vegetative mycelium while few to no spore chains were seen in the cob web-like mycelium. The observed morphology classified this organism as a non-*Streptomyces* type. A light brown-orange reverse color was observed on salts-starch agar (ISP4). There were no diffusable pigments produced on any of the ISP media (ISP2, ISP3, ISP4, ISP5 and ISP7). Melanoid pigments were not formed on tyrosine agar (ISP7) and were not detected by the modified Arai and Mikami melanin formation test. The amino acid components of the cell wall were alanine, L-glutamic acid, aspartic acid, and the *meso*-diaminopimelic acid isomer. The sugar components of the cell wall were galactose, arabinose, and ribose. Carbon utilization studies showed that glucose, mannitol, sucrose, xylose and fructose (weak) were utilized for growth when incorporated into inorganic salts agar (ISP9) as sole carbon sources. Arabinose, inositol, raffinose, and rhamnose were not utilized for growth as sole carbon sources with ISP9. Based on the above characteristics and analysis of mycolic acids, this organism was characterized as a member of the genus *Nocardia*.

Production of Nocathiacins

Nocathiacin I and II were produced in the culture of WW-12651 at the incubation temperature of 32°C and the agitation rate of 250 rpm. Nocathiacin I was the major product in the fermentation, reaching its maximal concentration of about 150 μg/ml, after 4~5 days of

incubation. Nocathiacin II was detected under the same conditions with the peak titer of 10 μg/ml. These two compounds had identical UV spectra. In addition, HPLC analysis of the fermentation broth showed more than 10 minor peaks with the same or slightly different UV spectrum of nocathiacin I and II, indicating that other nocathiacin analogs were present in the fermentation. Nocathiacin III, the aglycone of nocathiacin I, was not detected under the above fermentation conditions. When the fermentation temperature was lowered from 32°C to 28°C and the agitation rate reduced from 250 rpm to 180 rpm, nocathiacin III was observed after 2 days of incubation and disappeared after 4~5 days. Accordingly, nocathiacin III was isolated with 2 day incubation at 28°C with a peak titer of 30 μg/ml.

Biological Activities

Nocathiacin I, II and III exhibit potent *in vitro* activity against a broad spectrum of Gram-positive bacteria (Table 1), including multiple-drug resistant (MDR) pathogens such as MRSA strains A27218 and A27223, MREF strain A24885 and fully penicillin-resistant *Streptococcus pneumoniae* A28272 (PRSP), and demonstrate excellent *in vivo* efficacy in a systemic *Staphylococcus aureus* infection mice model (Table 2).

Table 1. MIC values for nocathiacins I~III.

Organism	Strain #	MIC (μg/ml)		
		Nocathiacin I	Nocathiacin II	Nocathiacin III
Streptococcus pneumoniae	A9585	0.001	0.0005	≤0.002
Streptococcus pneumoniae (penicillin intermediate)	A27881	0.001	0.0005	≤0.002
Streptococcus pneumoniae (penicillin resistant)	A28272	0.001	0.0005	≤0.002
Streptococcus pyogenes	A9604	0.001	NT	NT
Enterococcus faecalis	A20688	0.03	0.015	0.03
Enterococcus faecalis	A27519	0.03	NT	0.03
Enterococcus faecium	A24885	0.015	0.015	0.03
Enterococcus avium	A27456	0.015	NT	0.03
Staphylococcus aureus (β-lactamase positive)	A15090	0.007	0.007	0.007
Staphylococcus aureus (ATCC 29213)	A24407	0.007	NT	0.007
Staphylococcus aureus (methicillin resistant)	A27218	0.003	NT	NT
Staphylococcus aureus (methicillin resistant)	A27223	0.003	0.003	0.007
Staphylococcus aureus + 50% calf serum	A27223	0.001	0.015	0.007
Staphylococcus epidermidis	A24548	0.003	0.003	0.007
Staphylococcus haemolyticus	A27298	0.03	0.015	0.007
Moraxella catarrhalis (β-lactamase positive)	A22344	0.06	0.25	0.06
Moraxella catarrhalis (β-lactamase positive)	A25409	0.06	NT	0.06

NT = Not Tested

Table 2. *In vivo* efficacy of nocathiacin I~III in a systemic *Staphylococcus aureus* infection mice model.

PD_{50} (mg/kg/day)			
Nocathiacin I	Nocathiacin II	Nocathiacin III	Vancomycin
0.8	0.62[a]	0.89	1.3

[a] obtained with a semi-synthetic sample[22].

Discussion

Drug-resistant pathogens are a growing concern, with some strains being resistant to multiple antibiotic agents. The emergence of bacterial resistance to β-lactam antibiotics, macrolides, quinolones, and vancomycin is becoming a major worldwide health problem. A significant problem in clinical practice is the increase in incidence of MRSA strains. MRSA infections have traditionally been treated with vancomycin. However, there are recent reports of emerging vancomycin resistance in some MRSA isolates. MREF continues to be problematic therapeutically due to intrinsic resistance to antimicrobial agents. Of particular concern is the emerging resistance worldwide of the important community acquired pathogen *Streptococcus pneumoniae* to penicillin and other antibacterials. Therefore there is an urgent need to discover new agents to treat patients infected with multiple drug-resistant bacteria.

Nocathiacin I, II and III exhibit *in vitro* nanomolar potency against a wide spectrum of Gram-positive bacteria, including MRSA, MREF and PRSP. Therefore nocathiacins represent promising new leads for combating the above emerging drug-resistant pathogens. Furthermore, nocathiacin I~III show excellent *in vivo* efficacy in a systemic *Staphylococcus aureus* infection mice model, with potency comparable to that of vancomycin in the same study. Nocathiacins are structurally related to nosiheptide, glycothiohexides, GE2270 A, micrococcins, cyclothiazomycin and berninamycin. Several of the above compounds have been reported to have potent antimicrobial activity against Gram-positive bacteria *in vitro*. However, relatively few thiazolyl peptides have been shown to have noteworthy *in vivo* activity. Nocathiacins represent a new class of thiazolyl peptides that demonstrate both exceptional *in vitro* and *in vivo* antibacterial activities.

Like nosiheptide[23], the aglycone portion of nocathiacins appear to be derived from amino acids. We speculated that the attachment of the amino sugar in nocathiacin I was at the late stage of biosynthesis. Nocathiacin III (aglycone) was not observed under the initial fermentation conditions, which we reasoned was due to high glycosidation rate at high fermentation temperature (32°C) and agitation rate (250 rpm) conditions. Indeed, nocathiacin III was detected at lower fermentation temperatures and lower agitation rates, and was consumed with prolonged incubation with concomitant increase in the titer of nocathiacin I, suggesting that nocathiacin III was a close precursor of nocathiacin I. Further biosynthetic studies are necessary to address the questions.

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