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

II. Isolation, Characterization, and Structure Determination

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A new group of thiazolyl peptide antibiotics, the nocathiacins, was isolated from cultured broth of *Nocardia* sp. The major analogs nocathiacins I~III (**1**~**3**) were purified using silica gel and Sephadex LH-20 chromatography techniques. The structures of nocathiacins I~III were determined by spectroscopic (2D-NMR, MS^n) methods, and share structural similarities to glycothiohexide-α (**4**).

In the course of screening for novel antibiotics in microbial fermentations, a strain of *Nocardia* sp. (ATCC 202099) was selected for further evaluation. This investigation led to the discovery of the nocathiacins, a new group of thiazolyl peptide antibiotics[1]. The nocathiacins were isolated from cultured broth of *Nocardia* sp. and are highly potent against Gram-positive bacteria *in vitro* as well as *in vivo*. In the preceding paper[2], we reported the taxonomy of the producing strain, fermentation, and biological activities of nocathiacins I~III. This paper describes the isolation, characterization, and structure determination of nocathiacins I~III.

Results

Isolation of Nocathiacins I~III

The initial discovery of the nocathiacin antibiotics was facilitated by the use of *Enterococcus faecium* as the test organism in an antibacterial screen[1,2]. The following procedures were used for the preparative scale isolation of nocathiacins I~III.

Nocathiacin I (1)

A crude ethyl acetate extract (137 g) derived from a 1950-liter fermentation[3] was subjected to silica gel vacuum liquid chromatography (VLC). The extract was pre-adsorbed onto 75 g of silica gel 60 (63~200 μm, EM Separations) and applied to a 14×17 cm fritted filter funnel packed with 500 g of LiChroprep silica gel 60 (25~40 μm; EM Separations). Elution using house vacuum was carried out with hexane - chloroform 1 : 1 (2 liters), chloroform (2 liters), followed by chloroform with increasing percentages of methanol - water 10 : 1 (*i.e.* chloroform - methanol - water 99 : 1 : 0.1 (2 liters), 98 : 2 : 0.2 (6 liters), 97 : 3 : 0.3 (4 liters), 95 : 5 : 0.5 (6 liters), 93 : 7 : 0.7 (2 liters) and 90 : 10 : 1 (4 liters). The chromatography was monitored by silica gel TLC (chloroform - methanol - water 90 : 10 : 1; long wavelength UV and/or ceric sulfate spray for detection) and/or C18 HPLC. The nocathiacin I-enriched chloroform - methanol - water 95 : 5 : 0.5 fraction (27.2 g) was further purified in 10 g batches using Sephadex LH-20 chromatography (400 g, 5×100 cm column, chloroform - methanol 10 : 4 eluant), affording nocathiacin I (**1**), 16.5 g.

Nocathiacin II (2)

A crude chloroform - methanol 1 : 1 extract (39.3 g) derived from 4 kg (wet) cell mass recovered from a 475-liter fermentation[3] was subjected to silica gel VLC. The residue was preadsorbed onto 20 g of Merck LiChroprep silica gel 60 (25~40 μm) and applied to a 7×14 cm fritted filter funnel packed with 107 g of silica gel. Elution using

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house vacuum was carried out with hexane (500 ml), followed by hexane - chloroform 3 : 1, 1 : 1, chloroform, and chloroform with increasing percentages of methanol - water 10 : 1 (*i.e.* chloroform - methanol - water 99 : 1 : 0.1, 98 : 2 : 0.2 (4 times), 97 : 3 : 0.3, 95 : 5 : 0.5, 90 : 10 : 1, 80 : 20 : 2 (500 ml each). Immediately following elution of nocathiacin I (1.2 g) with chloroform - methanol - water 98 : 2 : 0.2 and 97 : 3 : 0.3, nocathiacin II was eluted with chloroform - methanol - water 95 : 5 : 0.5 and 90 : 10 : 1. Key to the removal of non-related polar impurities was a second, silica gel VLC step using a chloroform - methanol - acetic acid step gradient. In this manner, the nocathiacin II was retained on the silica gel upon elution with chloroform, chloroform - acetic acid 99 : 1, chloroform - methanol - acetic acid 95 : 5 : 1 and 90 : 10 : 1. Following removal of the major impurities, the nocathiacin II (0.9 g) was recovered from the silica gel with chloroform - methanol - ammonium hydroxide 90 : 10 : 1. A final silica gel VLC step using a chloroform - methanol - water step gradient as previously described was used to afford nocathiacin II (**2**, 200 mg).

Nocathiacin III (**3**)

The ethyl acetate extract (8.6 g) derived from a 40-liter fermentation[2] was initially subjected to silica gel VLC using a chloroform - methanol - water step gradient as previously described. The chloroform - methanol - water 95 : 5 : 0.5 fraction containing both nocathiacins I (**1**) and III (**3**) (3.5 g) was further subjected to silica gel column chromatography using a chloroform - methanol - acetic acid linear gradient. This method exploits the basic amino sugar present in **1**. The addition of acetic acid to the chromatographic eluant markedly increases the polarity of **1** relative to **3** on silica gel, presumably through protonation of the *N,N*-dimethylamino group. The aglycone is unaffected by the addition of acetic acid and readily elutes from the column. Accordingly, elution was begun with chloroform - acetic acid 99 : 1 with a linear gradient (2 liters total run) to chloroform - methanol - acetic acid 95 : 5 : 1. A total of twenty 100-ml fractions were collected. Fractions 12~15 contained the aglycone (**3**, 720 mg) and were pooled. A final purification step using Sephadex LH-20 (chloroform - methanol 10 : 4 eluant) afforded nocathiacin III (**3**, 490 mg). The nocathiacin I (**1**, 1.7 g) was recovered from the silica gel upon elution with chloroform - methanol - ammonium hydroxide 90 : 10 : 1.

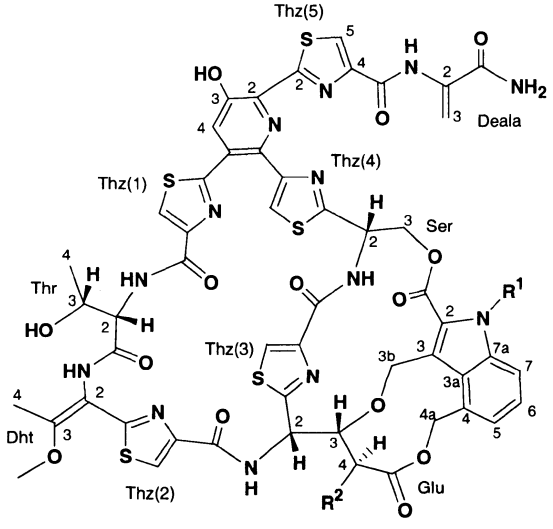
Characterization and Structure Determination

Nocathiacin I (**1**)

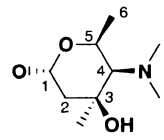
The major component of the nocathiacin antibiotic complex was isolated as a yellow amorphous solid. It is soluble in DMSO and chloroform-methanol mixtures, poorly soluble in chloroform, ethyl acetate, acetonitrile, methanol, and practically insoluble in water. The measured mass of **1** as determined by HR-ESIMS ($[M+H]^+$ at *m/z* 1437.2850) was consistent with the formula $C_{61}H_{60}N_{14}O_{18}S_5$. Further confirmation was provided by elemental analysis. The above formula requires 39 degrees of unsaturation in the molecule. The UV spectrum revealed characteristic absorption maxima: λ_{max} (MeOH) 222, 290, 364 nm ($\log \varepsilon$ 4.89, 4.52, 4.17). In addition, the compound in solution and on a TLC plate exhibited intense yellow-green fluorescence under long wavelength UV light, indicative of a highly conjugated chromophore. The IR spectrum showed strong amide carbonyl absorptions (1693, 1670, 1640 cm^{-1}) and weak ester absorptions (1740, 1721 cm^{-1}), consistent with a depsipeptide structure. Noteworthy resonances in the ^1H NMR spectrum were 3 methyl signals (δ 0.55, 1.13, 1.40), one vinyl methyl (δ 1.96), an *N,N*-dimethylamino group (δ 2.49) and an *O*-methyl group (δ 3.87). Also present were 6 non-exchangeable downfield (*i.e.* aromatic) one-proton singlets (δ 7.84, 7.87, 8.23, 8.49, 8.52, 8.61), and an *exo* methylene pair of singlets (δ 5.72, 6.34). The ^{13}C NMR spectrum revealed 60 carbon signals, of which there were 31 quaternary, 18 methine, 5 methylene and 6 methyl resonances. Of the 31 observed quaternary resonances, 27 were in the δ 126~172 range, again suggesting a peptide with a high degree of aromatic character. There were two groups of methine resonances [δ 112~128 (9); δ 49~80 (8)], one anomeric carbon (δ 95.1), and 4 methylene carbon signals (δ 40~68). There was one *exo* methylene carbon (δ 103.7), one *O*-methyl (δ 56.2), one *N,N*-dimethylamino carbon signal (δ 44.4, 2 carbons), and 4 C-methyl signals (δ 13.1, 17.9, 18.0, 30.6). Collectively, the molecular formula, UV, IR and NMR spectral data revealed that the nocathiacins are in the thiazolyl peptide class (*i.e.* nosiheptide group) of antibiotics[4,5]. In particular, there is significant overlap between the NMR spectra of nocathiacin I and published data for glycothiohexide-α (**4**) a previously reported nosiheptide-like thiazolyl peptide[6~8].

The ^{13}C and ^1H NMR chemical shift assignments for nocathiacin I (**1**) were based on 2D NMR (COSY, HMQC and HMBC data; Table 1). The ^{15}N chemical shift assignments for **1** were based on ^1H-^{15}N HMQC and

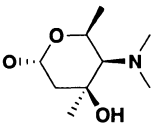
Fig. 1.



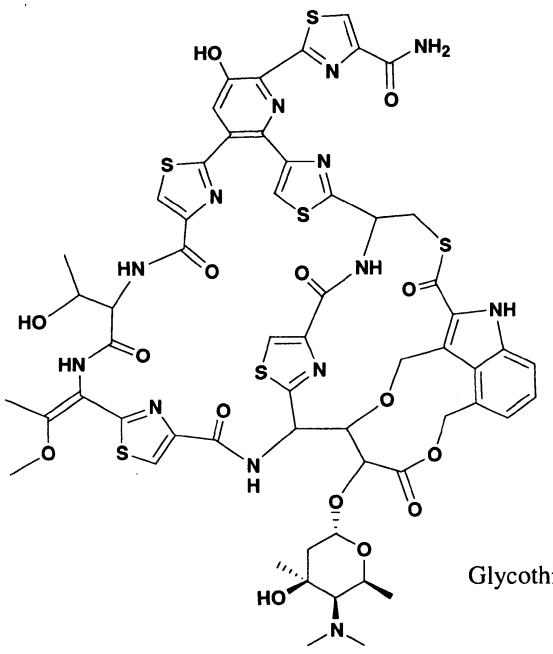
Nocathiacin I (**1**) R^1=OH, R^2 =



Nocathiacin II (**2**) R^1=H, R^2 =



Nocathiacin III (**3**) R^1=OH, R^2 = OH



Glycothiohexide α (**4**)

HMBC data, and were in agreement with published ^{15}N NMR data for nosiheptide[9] and sulfomycin-I[10]. Common structural elements in **1** and **4** include (in counter clockwise order): a 2,5,6-trithiazolyl-3-hydroxypyridine moiety, a threonine, (*E*)-dehydrothreonine (Dht) residue, thiazole [Thz(2)], a 3,4-dioxyglutamic acid (Glu) residue, an *N*,*N*-dimethylamino sugar moiety, an indole-2-carbonyl moiety, an aminoethyl residue, and an additional thiazole [Thz(3)] linked back to the Glu residue, completing the tricyclic peptide motif. Nocathiacin I (**1**) is thus a close structural analog of glycothiohexide-α (**4**), with the following significant differences.

Dehydroalanine Side Chain

A dehydroalanine side chain moiety, also found in nosiheptide[5,9], was indicated by a geminal pair of an *exo* methylene moiety (δ_H 5.72, 6.34; δ_C 103.7). These protons

Table 1. Nocathiacin I (**1**): NMR data (500 MHz, DMSO-d_6).

Position	^{13}C ppm	^{1}H ppm (mult, *J* (Hz))	^{15}N ppm	HMBC (^{13}C - ^{1}H correlations)	HMBC (^{15}N - ^{1}H correlations)
Thz(1)-N			315.1		7.32
Thz(1)-C2	164.0			8.61, 7.87	
Thz(1)-C4	149.6			8.61	
Thz(1)-C5	126.3	8.61 (1H, s)			
Thz(1)-CO	159.1			8.61, 7.32, 4.20	
Thz(2)-N			286.8		8.23, 5.04*
Thz(2)-C2	163.2			8.23	
Thz(2)-C4	145.7			8.23	
Thz(2)-C5	125.5	8.23 (1H, s)			
Thz(2)-CO	160.5			8.56, 8.23, 5.66	
Thz(3)-N			311.2		5.66
Thz(3)-C2	167.4			8.49, 5.66, 3.99	
Thz(3)-C4	148.8			8.49	
Thz(3)-C5	125.6	8.49 (1H, s)			
Thz(3)-CO	160.3			8.49, 7.86, 5.70	
Thz(4)-N			318.1		7.84
Thz(4)-C2	168.1			7.84, 5.70, 5.19, 4.49	
Thz(4)-C4	154.6			5.70, 7.84	
Thz(4)-C5	119.4	7.84 (1H, s)		5.70	
Thz(5)-N			298.8		10.03
Thz(5)-C2	167.0			8.52, 7.87	
Thz(5)-C4	149.7			10.03, 8.52, 6.34w, 5.72w	
Thz(5)-C5	127.2	8.52 (1H, s)			
Thz(5)-CO	158.6			10.03, 8.52	
Pyr-N			311.2		7.87, 7.84
Pyr-C2	135.3			8.52, 7.87	
Pyr-C3	151.9			7.87	
Pyr-C4	127.1	7.87 (1H, s)			
Pyr-C5	130.0			8.61	
Pyr-C6	142.5			7.87, 7.84	
Thr-CO	167.7			9.07, 7.32, 4.20, 2.42	
Thr-NH		7.32 (1H, m)	110.7		4.20
Thr-C2	55.5	4.20 (1H, m)		7.32, 5.04, 1.13	
Thr-C3	65.2	2.42 (1H, m)		5.04, 4.20, 1.13	
Thr-C3-OH		5.04 (1H, m)			
Thr-C4	17.9	1.13 (3H, s br)		4.20	
Dht-NH		9.07 (1H, s)	119.4		
Dht-C2	109.7			9.07, 8.23, 1.96	
Dht-C3	161.6			9.07, 3.87, 1.96	
Dht-C4	13.1	1.96 (3H, s)+		9.07	
Dht-OMe	56.2	3.87 (3H, s)			
Glu-NH		8.56 (1H, d, (8.3))	110.5		5.66, 3.99
Glu-C2	50.2	5.66 (1H, d, (8.8))		8.56, 8.49, 4.27	
Glu-C3	79.3	3.99 (1H, d, 9.5))		8.56, 5.66, 4.27, 4.10	
Glu-C4	70.6	4.27 (1H, d, 9.7))		4.91, 3.99	
Glu-CO	171.8			5.97, 4.27, 3.99	
Sug-C1	95.1	4.91 (1H, d, 4.4))		4.27, 3.74, 1.92, 1.77	
Sug-C2	40.0	1.77 (1H, d, 14.1)) 1.92 (1H, m)		1.40	
Sug-C3	67.5			4.91, 3.74, 2.08, 1.92, 1.77, 1.40	
Sug-C3-Me	30.6	1.40 (3H, s)		2.08, 1.92, 1.77	
Sug-C4	68.4	2.08 (1H, s br)		2.49, 1.40, 0.55	
Sug-C4-NMe_2	44.4	2.49 (6H, s)	16.9	2.08	3.74
Sug-C5	66.0	3.74 (1H, m)		0.55	
Sug-C6	18.0	0.55 (3H, d, 6.5))		3.74	
Indole-N-OH		10.71 (1H, s br)	177.9		10.71, 7.69
Indole-C2	126.3			4.73, 4.10	
Indole-C3	111.2			4.73, 4.10	
Indole-C3a	119.8			7.69, 7.14, 5.97, 5.01, 4.73, 4.10	
Indole-C3b	64.5	4.10 (1H, d, 10.5)) 4.73 (1H, d, 10.5))		3.99	

Table 1. (Continued).

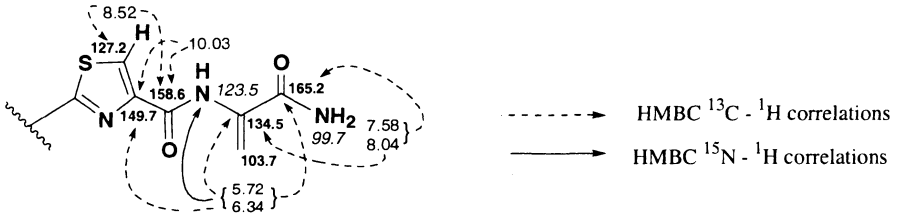
Position	^{13}C ppm	^{1}H ppm (mult, J (Hz))	^{15}N ppm	HMBC (^{13}C - ^{1}H correlations)	HMBC (^{15}N - ^{1}H correlations)
Indole-C4	128.1			7.69, 7.32, 5.97, 5.01	
Indole-C4a	67.5	5.01 (1H, d, 12.6)) 5.97 (1H, d, 12.2))		7.14	
Indole-C5	123.1	7.14 (1H, d, 7.0))		7.69, 7.32, 5.97, 5.01	
Indole-C6	124.0	7.32 (1H, m)		7.14	
Indole-C7	112.7	7.69 (1H, d, 8.4)		7.14, 5.97w	
Indole-C7a	135.0			7.69, 7.32, 7.14, 5.97w, 5.01w	
Indole-CO	161.1			5.19, 4.49	
Ser-NH		7.86 (1H, m)	110.6		5.70, 4.49
Ser-C2	49.8	5.70 (1H, m)		7.84, 5.19, 4.49	
Ser-C3	63.3	4.49 (1H, d, 11.0)) 5.19 (1H, m)		7.84	
Deala-NH_2		7.58 (1H, s br) 8.04 (1H, s br)	99.7		
Deala-CO	165.2			10.03, 8.04, 7.58, 6.34, 5.72	
Deala-C2	134.5			10.03, 7.58, 6.34, 5.72	
Deala-C3	103.7	5.72 (1H, s) 6.34 (1H, s)		10.03	
Deala-NH		10.03 (1H, s)	123.5		6.34, 5.72

† Irradiation of Dht-C4 (δ 1.96) by selective 1D NOESY enhanced the NH (δ 9.07) and OCH_3 (δ 3.87) signals, indicating the *E* substitution pattern for the Dht residue.

* Possible H-bond coupling.

w=weak coupling.

Fig. 2. Dehydroalanine side chain.



in turn show long-range heteronuclear correlations with δ_C 134.5 (Deala-C2), δ_C 165.2 (Deala CO) and δ_N 123.5 (Deala *N*-H), and with the outer thiazole ring, δ_C 149.7 [Thz(5)-C4]. The primary amide protons (δ_H 7.58, 8.04) show couplings with δ_C 134.5 (Deala-C2), δ_C 165.2 (Deala CO), as indicated in Fig. 2. The observed ^{15}N chemical shifts, δ 99.7 (Deala-NH_2) and δ 123.5 (Deala-NH) are in excellent agreement with published data for nosiheptide and sulfomycin-I[9,10].

Ester Linkage to *N*-Hydroxyindole Moiety

A significant difference between nocathiacin I (**1**) and glycothiohexide-α (**4**) was the presence of a downfield methylene carbon (δ 63.3) (nocathiacin I) in place of a δ 30.1 methylene resonance (glycothiohexide-α)[8]. These methylene protons (δ 4.49, 5.19) show long-range correlations to δ 49.8 (Ser-C2), δ 168.1 [Thz(4)-C2] and δ 161.1 (Indole-CO). Consideration of the molecular formula (*i.e.* oxygen, sulfur count) also supports the presence of an ester linkage in nocathiacin I, compared to a thioester linkage in glycothiohexide-α. Finally, we are left with one oxygen atom to account for the molecular formula. Accordingly, an OH group was assigned to the indole nitrogen, such as found in another nosiheptide-like thiazolyl peptide S-54832[11]. This assignment was supported by ^{15}N-

Fig. 3a. Indole substructure of nocathiacin I (**1**).

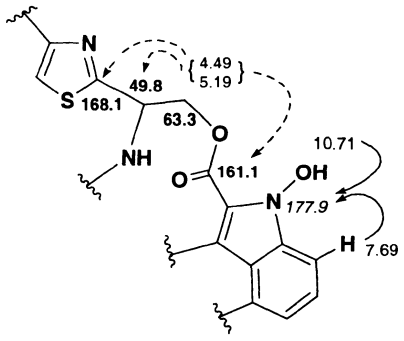


Fig. 3b. Indole substructure of nocathiacin II (**2**).

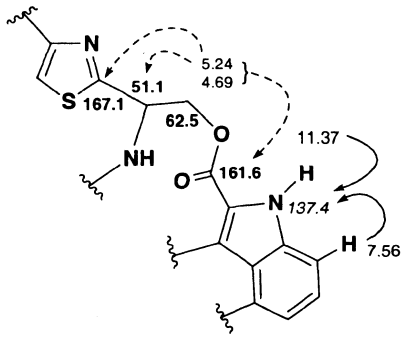
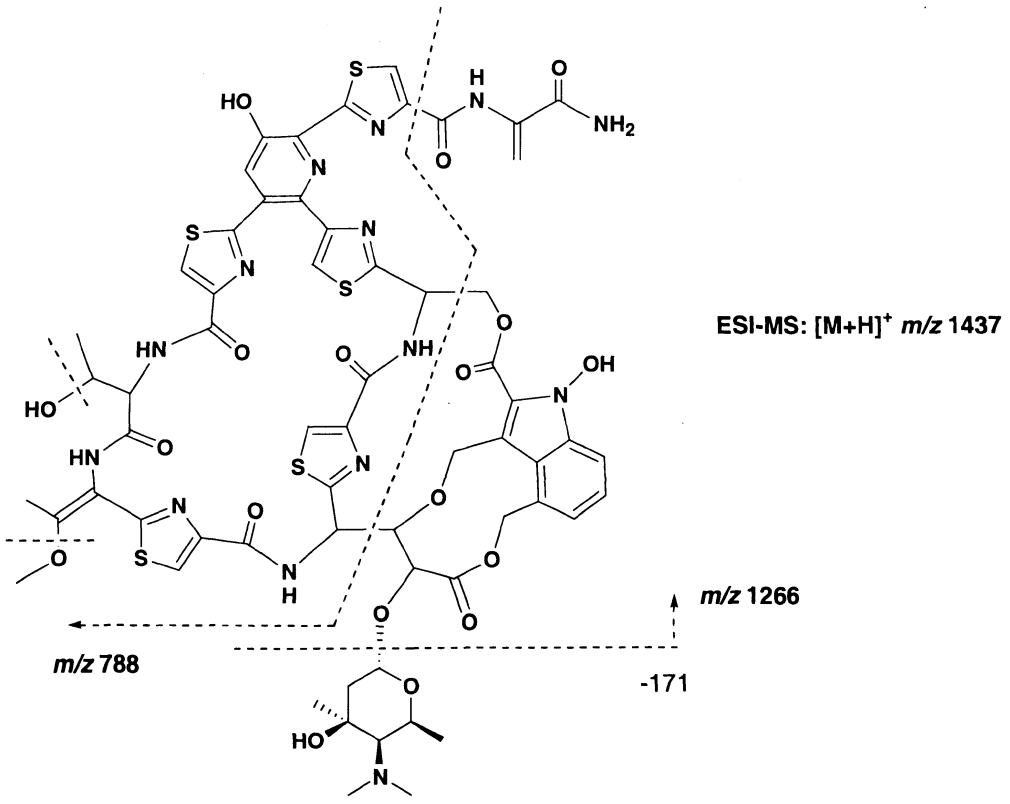


Fig. 4. Nocathiacin I (**1**): possible MS^n fragmentation displaying proposed core.



^{1}H HMBC data, where the *N*-hydroxyindole nitrogen (δ 177.9) showed a 2-bond coupling with a broad OH signal (δ 10.71), and a 3-bond coupling with indole-H7 (δ 7.69) (Fig. 3a).

Additional structural confirmation for nocathiacin I (**1**) was provided from ion-trap mass spectrometry, electrospray ionization, using MS and MS^n measurements. Under typical MS/MS conditions on triple quadrupole instruments, facile loss of 171 daltons (*i.e.* amino sugar moiety), resulting in an ion at *m/z* 1266 (aglycone) was observed. Additional fragmentation ions at *m/z* 1248, 1204, 1186, 991 and a proposed core ion at *m/z* 788 (Fig. 4) were obtained through multi-stage MS^n experiments utilizing various precursor ions (Table 4).

Table 2. Nocathiacin II (**2**): NMR data (500 MHz, DMSO-d_6).

Position	^{13}C ppm	^{1}H ppm (mult, *J* (Hz))	^{15}N ppm
Thz(1)-N			313.8
Thz(1)-C2	164.1		
Thz(1)-C4	150.1		
Thz(1)-C5	126.2	8.62 (1H, s)	
Thz(1)-CO	158.2		
Thz(2)-N			288.7
Thz(2)-C2	163.0		
Thz(2)-C4	145.9		
Thz(2)-C5	125.3	8.22 (1H, s)	
Thz(2)-CO	160.6		
Thz(3)-N			311.9
Thz(3)-C2	167.1		
Thz(3)-C4	148.9		
Thz(3)-C5	125.6	8.50 (1H, s)	
Thz(3)-CO	161.0		
Thz(4)-N			318.8
Thz(4)-C2	167.2		
Thz(4)-C4	154.2		
Thz(4)-C5	119.8	7.80 (1H, s)	
Thz(5)-N			299.3
Thz(5)-C2	166.6		
Thz(5)-C4	149.7		
Thz(5)-C5	127.3	8.54 (1H, s)	
Thz(5)-CO	158.6		
Pyr-C2	135.5		
Pyr-C3	152.1		
Pyr-C4	126.8	7.94 (1H, s)	
Pyr-C5	129.8		
Pyr-C6	142.0		
Thr-CO	168.1		
Thr-NH		7.06 (1H, d, 7.6)	108.7
Thr-C2	55.0	4.22 (1H, m)	
Thr-C3	66.0	2.12 (1H, m)	
Thr-C3-OH		4.86 (1H, d br)	
Thr-C4	17.9	1.05 (3H, d br)	
Dht-NH		9.10 (1H, s)	119.9
Dht-C2	109.8		
Dht-C3	161.3		
Dht-C4	13.0	1.99 (3H, s)	
Dht-OMe	56.1	3.89 (3H, s)	

Position	^{13}C ppm	^{1}H ppm (mult, *J* (Hz))	^{15}N ppm
Glu-NH		8.59 (1H, d, 8.3)	110.8
Glu-C2	50.6	5.68 (1H, d, 8.2)	
Glu-C3	79.0	4.02 (1H, d, 9.1)	
Glu-C4	70.7	4.35 (1H, d, 9.5)	
Glu-CO	171.9		
Sug-C1	95.0	4.93 (1H, m)	
Sug-C2	40.4	1.79 (1H, d, 14.1) 1.94 (1H, m)	
Sug-C3	67.5		
Sug-C3-Me	30.6	1.41 (3H, s)	
Sug-C4	68.4	2.08 (1h, s br)	
Sug-C4-NMe_2	44.4	2.51 (6H, s)	17.2
Sug-C5	65.8	3.74 (1H, m)	
Sug-C6	17.9	0.55 (3H, d, 6.0)	
Indole-NH		11.37 (1H, s br)	137.4
Indole-C2	126.6		
Indole-C3	116.2		
Indole-C3a	124.1		
Indole-C3b	64.0	4.10 (1H, d, 9.8) 4.93 (1H, m)	
Indole-C4	127.9		
Indole-C4a	67.6	5.00 (1H, d, 12.3) 6.02 (1H, d, 12.0)	
Indole-C5	122.8	7.12 (1H, d, 6.8)	
Indole-C6	123.7	7.25 (1H, t, 7.5)	
Indole-C7	115.8	7.56 (1H, d, 8.3)	
Indole-C7a	136.8		
Indole-CO	161.6		
Ser-NH		7.69 (1H, d, 10.7)	110.0
Ser-C2	51.1	5.77 (1H, m)	
Ser-C3	62.5	4.69 (1H, d, 11.0) 5.24 (1H, m)	
Deala-NH_2		7.61 (1H, s br) 8.06 (1H, s br)	99.8
Deala-CO	165.2		
Deala-C2	134.5		
Deala-C3	103.7	5.74 (1H, s) 6.37 (1H, s)	
Deala-NH		10.04 (1H, s)	123.4

Nocathiacin II (**2**)

The second, major component of the nocathiacin antibiotic complex was also obtained as a yellow amorphous solid. The measured mass of **2**, again determined by HR-ESIMS ($[M+H]^+$ at *m/z* 1421.2964) was consistent with the formula $C_{61}H_{60}N_{14}O_{17}S_5$. Additional confirmation was provided by elemental analysis. The molecular formula for **2** is thus consistent with an analog having one less oxygen than that of **1**. The UV spectrum, λ_{max}(MeOH) 220, 295, 364 nm (log ε 5.01, 4.67, 4.36) essentially overlapped that of **1**. The ^{1}H and ^{13}C spectra of nocathiacin II (**2**) were also similar to those of **1**. The NMR chemical shift assignments **2** were based on 2D NMR (COSY, HMQC and HMBC data) (Table 2). The indole nitrogen in nocathiacin II (**2**) is unsubstituted (*i.e.* NH group). This is supported by ^{15}N-^{1}H 2D NMR data, where the indole nitrogen resonance (δ 137.4) showed a 3-bond coupling with indole-H7 (δ 7.56), and a 1-bond coupling with a broad NH singlet (δ 11.37) (Fig. 3b). In further support of this assignment, the ^{15}N chemical shift reported for the indole nitrogen (NH) in nosiheptide is δ 137.3 (referenced to NH_4Cl)[9]. In the MS^n spectra, facile loss of 171 daltons (amino sugar) yielded a prominent aglycone ion at *m/z* 1250. MS^n fragmentation of the ion at *m/z* 1250 generated major substructure ions at *m/z* 1232, 1206, 1188, 1171, 1156, 1139, 1060, 975, and a proposed

Table 3. Nocathiacin III (**3**): NMR data (500 MHz, DMSO-d_6).

Position	^{13}C ppm	^{1}H ppm (mult, *J* (Hz))
Thz(1)-C2	164.4	
Thz(1)-C4	149.6	
Thz(1)-C5	126.4	8.63 (1H, s)
Thz(1)-CO	159.3	
Thz(2)-C2	163.0	
Thz(2)-C4	146.0	
Thz(2)-C5	125.3	8.20 (1H, s)
Thz(2)-CO	160.4	
Thz(3)-C2	167.8	
Thz(3)-C4	148.8	
Thz(3)-C5	126.3	8.51 (1H, s)
Thz(3)-CO	160.4	
Thz(4)-C2	168.1	
Thz(4)-C4	154.7	
Thz(4)-C5	119.5	7.83 (1H, s)
Thz(5)-C2	166.6	
Thz(5)-C4	149.6	
Thz(5)-C5	126.4	8.52 (1H, s)
Thz(5)-CO	158.8	
Pyr-C2	135.7	
Pyr-C3	152.9	
Pyr-C4	127.1	7.83 (1H, s)
Pyr-C5	129.9	
Pyr-C6	141.6	
Thr-CO	167.8	
Thr-NH		7.38 (1H, d, 7.2)
Thr-C2	55.7	4.16 (1H, m)
Thr-C3	65.3	2.55 (1H, m)
Thr-C4	18.0	1.17 (3H, s br)
Dht-NH		8.95 (1H, s)
Dht-C2	109.8	
Dht-C3	161.2	
Dht-C4	13.2	1.98 (3H, s)
Dht-OMe	56.2	3.87 (3H, s)
Glu-NH		8.38 (1H, d, 8.8)
Glu-C2	49.5	5.90 (1H, m)
Glu-C3	81.4	3.74 (1H, d, 9.5)
Glu-C4	67.9	4.02 (1H, d, 9.2)
Glu-CO	174.5	
Indole-C2	126.4	
Indole-C3	111.3	
Indole-C3a	119.7	
Indole-C3b	64.5	4.12 (1H, d, 10.3) 4.70 (1H, d, 10.3)
Indole-C4	128.6	
Indole-C4a	67.3	5.02 (1H, d, 12.5) 5.90 (1H, m)
Indole-C5	123.1	7.17 (1H, d, 7.0)
Indole-C6	124.2	7.33 (1H, t, 7.7)
Indole-C7	112.5	7.69 (1H, d, 8.4)
Indole-C7a	135.0	
Indole-CO	168.1	
Ser-NH		7.92 (1H, d, 10.9)
Ser-C2	49.7	5.75 (1H, m)
Ser-C3	63.5	4.52 (1H, d, 11.1) 5.23 (1H, m)
Deala-NH_2		7.63 (1H, s br) 8.09 (1H, s br)
Deala-CO	165.3	
Deala-C2	134.5	
Deala-C3	103.7	5.75 (1H, s) 6.39 (1H, s)
Deala-NH		10.06 (1H, s)

common core ion at *m/z* 788, in agreement with a deshydroxy analog of **1** (Table 4). Additionally, nocathiacin II (**2**) prepared semi-synthetically from nocathiacin I (**1**) was identical in terms of LC/MS and NMR spectral data to that of the natural product[12].

Nocathiacin III (Aglycone) (**3**)

A minor component of the nocathiacin antibiotic complex was again obtained as yellow amorphous solid. The measured mass of **3**, determined by HR-ESIMS ($[M+H]^+$ at *m/z* 1266.1615) was consistent with the formula $C_{52}H_{43}N_{13}O_{16}S_5$, *i.e.* the aglycone of **1**. In support of this assignment, the 2D NMR data for nocathiacin III (**3**) revealed the same core structural elements present in **1**. Furthermore, the amino sugar methyl proton NMR signals [δ 1.40 (3H, s), 2.49 (6H, s), 0.55 (3H, d)] observed in **1** were notably absent from the NMR spectrum of **3**. In addition, the UV spectrum, λ_{max} (MeOH) 224, 290, 364 nm ($\log \varepsilon$ 4.85, 4.52, 4.11) essentially overlapped that of **1**. MS^n fragmentation of **3** yielded ions at *m/z* 1248, 1222, 1204, 1186, 991, and proposed core ion at *m/z* 788, as observed for **1** (Table 4). The ^{1}H and ^{13}C chemical shift assignments for nocathiacin III (**3**) are summarized in Table 3.

Discussion

The structures of nocathiacins I~III (Fig. 1) were determined by spectroscopic (2D-NMR, MS^n) methods, and share structural similarities to glycothiohexide-α (**4**). In addition, nocathiacin I contains structural elements from nosiheptide (dehydroalanine side chain) and S-54832 (ester linkage, *N*-hydroxyindole moiety). During the course of our investigation and early development of the nocathiacin antibiotics, a new antibiotic MJ347-81F4-A from *Amycolatopsis* sp. was disclosed by T. OTANI *et al.* The general structure of the major analog nocathiacin I is

Table 4. Summary of MS^n data for nocathiacins I~III.

Nocathiacin I (**1**, MW 1436) MS^n (major ions)

MS^1 *m/z* 1437 $[M+H]^+$
MS^2 *m/z* 1437→ *m/z* 1266 [M+H-171 (amino sugar)](Aglycone)
MS^3 *m/z* 1437→ *m/z* 1266→ *m/z* 1248, 1204, 1186
MS^4 *m/z* 1437 → *m/z* 1266 → *m/z* 1248 → *m/z* 1204, 1186
MS^4 *m/z* 1437 → *m/z* 1266 → *m/z* 1204 → *m/z* 1186, 991, 788 (core)

Nocathiacin II (**2**, MW 1420) MS^n (major ions)

MS^1 *m/z* 1421 $[M+H]^+$
MS^2 *m/z* 1421→ *m/z* 1250 [M+H-171 (amino sugar)](Aglycone)
MS^3 *m/z* 1421→ *m/z* 1250→ *m/z* 1232, 1206, 1188
MS^4 *m/z* 1421 → *m/z* 1250 → *m/z* 1188 → *m/z* 1171, 1156, 1139, 1060, 975
MS^5 *m/z* 1421 → *m/z* 1250 → m/z 1188 → *m/z* 1171 → *m/z* 1153, 1138, 955, 788 (core)

Nocathiacin III (**3**, MW 1265) MS^n (major ions)

MS^1 *m/z* 1266 $[M+H]^+$
MS^2 *m/z* 1266→ *m/z* 1248, 1222, 1204, 1186
MS^3 *m/z* 1266→ *m/z* 1204→ *m/z* 1186, 1154, 991
MS^4 *m/z* 1266 → *m/z* 1204 → *m/z* 1186 → *m/z* 1169, 1155, 1058, 788 (core)

identical to that reported for MJ347-81F4-A[13]. Spectral data and stereochemical details, however, were not reported for the latter. Two new analogs produced in the *Nocardia* sp. (ATCC 202099) fermentation are the indole-NH (*i.e.* des-OH) analog (**2**), and the aglycone (**3**) of nocathiacin I (**1**). The solution conformation and absolute configuration of **1** were subsequently determined by NMR spectroscopy and chiral capillary electrophoresis studies[14]. The absolute configurations for co-occurring analogs **2** and **3** were not independently determined. Given, however, that the CD curves for nocathiacins I~III are very similar, it is assumed that **2** and **3** (*i.e.* aglycone portion) have the same absolute configuration as **1**. The nocathiacins represent a new group of antibiotics with both highly potent *in vitro* and *in vivo* activities. A critical goal for the further development of this chemotype lies in the preparation of potent, stable, aqueous soluble analogs. Further studies describing methods to improve the pharmaceutical properties of these natural products will be reported elsewhere.

Experimental

Analytical Instrumentation

NMR chemical shift assignments of nocathiacins I~III were obtained on a Bruker DRX 500 MHz spectrometer in DMSO-d_6 at 28°C, operating at 500.13 MHz for proton, 125.76 MHz for carbon and 50.69 MHz for nitrogen, respectively. The instrument was equipped with a 5-mm TXI cryo probe. Proton and carbon chemical shifts are reported in ppm relative to DMSO at δ_H 2.49 and δ_C 39.6. Nitrogen chemical shifts were observed indirectly from ^1H-^{15}N HMQC and HMBC experiments, reported relative to NH_4Cl, δ 27.3 (referenced to liquid ammonia[10]). For the NMR data presented in Tables 1~3, the numbering system for glycothiohexide-α was adopted[8].

Low-resolution MS measurements were performed with a Finnigan SSQ7000 single quadrupole mass spectrometer, using the positive electrospray ionization mode. MS/MS measurements were conducted in the positive electrospray

ionization mode with a Finnigan TSQ7000 triple quadrupole mass spectrometer using argon collision gas or a Finnigan LCQ ion-trap mass spectrometer. High-resolution MS data were determined with a Finnigan MAT900 magnetic sector mass spectrometer, positive electrospray ionization mode, polypropylene glycol reference. The UV spectra were obtained using a Hewlett-Packard 8452A diode array spectrophotometer. IR measurements were taken on a Perkin Elmer 2000 Fourier Transform spectrometer. CD data were recorded with a Jasco J-720 spectropolarimeter. Fluorescence data were obtained using a Spex Fluorolog spectrofluorimeter. HPLC (C18) conditions: APEX 5 μ ODS column, 0.46 i.d.×15 cm (Jones Chrom. Inc.). The mobile phase[15] consisted of 0.01 M potassium phosphate buffer pH 3.5 (solvent A) and acetonitrile (solvent B) with the following 32-minute step gradient: 15% B (initial 3 minutes), 15 to 40% B (3 minutes), 40% B (6 minutes), 40 to 55% B (7 minutes), 55 to 85% B (3 minutes), 85% B (6 minutes), and 85 to 15% B (4 minutes). The flow rate was 1.2 ml/minute; UV detection (254 nm).

The pKa measurements were determined using a pION Model GLpKa analyzer in the pH range 3~11. Where the ionizable groups are part of a UV chromophore, a spectrophotometric pH titration was used to determine the pKa's. Additionally, potentiometric pH titrations were used to determine the pKa's. Three or more different percentages of methanol in water were used as solvent in which the pKa was measured; pKa *vs.* percent methanol was plotted and the pKa at 0% methanol was extrapolated. The spectrophotometric and potentiometric pKa values agreed in all cases to within 0.5 pKa unit or less, and the values reported are averages of the results obtained by the two methods.

Nocathiacin I (**1**)

$C_{61}H_{60}N_{14}O_{18}S_5$; HR-ESIMS ($[M+H]^+$ *m/z* 1437.2850, calcd 1437.2892); ESI-MS^n ions: see Table 4; Elem. *Anal.*: C 49.35, H 4.31, N 13.07, S 10.68; KF moisture 2.65; Calcd for $C_{61}H_{60}N_{14}O_{18}S_5$+2.1($H_2O$): C 49.66, H 4.39, N 13.29, S 10.87; UV λ_{max} (MeOH) 222, 290, 364 nm ($\log \varepsilon$ 4.89, 4.52, 4.17); IR ν_{max} (KBr): 3392, 3108, 2932, 1740, 1721, 1694, 1670, 1640, 1533, 1478, 1420, 1384, 1320, 1250, 1207, 1128, 1091, 1037, 1014, 751 cm^{-1}; CD λ nm ($\Delta\varepsilon$) (MeOH) 212 (+34.1), 239 (−50.5), 267 (+20.8), 307 (−8.7), 364 (+5.5); HPLC (Rt) 25.6 minutes; pKa data: 5.8, 8.3, 8.9; Fluorescence $\lambda_{max\,(em)}$ (MeOH-H_2O 1:1) 511 nm @λ_{ex} 350 nm; ^{1}H-, ^{13}C-, ^{15}N-NMR data (DMSO-d_6): see Table 1.

Nocathiacin II (**2**)

$C_{61}H_{60}N_{14}O_{17}S_5$; HR-ESIMS ($[M+H]^+$ *m/z* 1421.2964, calcd 1421.2943); ESI-MS^n ions: see Table 4; Elem. *Anal.*: C 49.24, H 4.54, N 13.11, S 10.43; KF moisture 3.85; Calcd for $C_{61}H_{60}N_{14}O_{17}S_5$+3.1($H_2O$): C 49.59, H 4.52, N 13.27, S 10.85; UV λ_{max} (MeOH) 220, 295, 364 nm ($\log \varepsilon$ 5.01, 4.67, 4.36); IR ν_{max} (KBr): 3387, 1725, 1656, 1534, 1478, 1423, 1318, 1249, 1192, 1087, 1014, 886, 793, 751; CD λ nm ($\Delta\varepsilon$) (MeOH) 211 (+41.5), 235 (−56.1), 258 (+21.0), 277sh (+13.4), 307 (−10.0), 364 (+5.6); HPLC (Rt) 21.1 minutes; ^{1}H-, ^{13}C-, ^{15}N-NMR data (DMSO-d_6): see Table 2.

Nocathiacin III (**3**)

$C_{52}H_{43}N_{13}O_{16}S_5$; HR-ESIMS ($[M+H]^+$ *m/z* 1266.1615, calcd 1266.1628); ESI-MS^n ions: see Table 4; UV λ_{max} (MeOH) 224, 290, 364 nm ($\log \varepsilon$ 4.85, 4.52, 4.11); IR ν_{max} (KBr): 3382, 1720, 1667, 1643 sh, 1534, 1510, 1477, 1420, 1250, 1207, 1126, 1015, 750; CD λ nm ($\Delta\varepsilon$) (MeOH) 212 (+38.7), 238 (−47.6), 266 (+21.2), 306 (−11.4), 362 (+5.1); HPLC (Rt) 19.3 minutes; pKa data: 6.2, 8.4; Fluorescence $\lambda_{max\,(em)}$ (MeOH-H_2O 1:1) 519 nm @λ_{ex} 350 nm; ^{1}H-, ^{13}C-NMR data (DMSO-d_6): see Table 3.

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References

1) LEET, J. E.; H. A. AX, D. R. GUSTAVSON, D. M. BROWN, L. TURNER, K. BROWN, W. LI & K. S. LAM (Bristol-Myers Squibb Company): Nocathiacin Antibiotics. U.S. 6,218,398 B1, April 17, 2001
2) LI, W.; J. E. LEET, H. A. AX, D. R. GUSTAVSON, D. M. BROWN, L. TURNER, K. BROWN, J. CLARK, H. YANG, J. FUNG-TOMC & K. S. LAM: Nocathiacins, new Thiazolyl peptide antibiotics from *Nocardia* sp. I. Taxonomy, Fermentation, and Biological Activities. J Antibiotics 56: 226~231, 2003
3) KO, R.; P. CINO & D. CAZZULINO: Unpublished results
4) BERDY, J.: Thiazolyl peptides. Handbook of Antibiotic Compounds Vol. IV, Part I. *Ed.*, J. BERDY *et al.*, pp. 389~417, CRC Press Inc., 1980
5) PASCARD, C.; A. DUCRUIX, J. LUNEL & T. PRANGE: Highly modified cysteine-containing antibiotics. Chemical structure and configuration of nosiheptide. J. Am. Chem. Soc. 99: 6418~6423, 1977
6) STEINBERG, D. A.; V. S. BERNAN, D. A. MONTENEGRO, D. R. ABBANAT, C. J. PEARCE, J. D. KORSHALLA, N. V. JACOBUS, P. J. PETERSEN, M. J. MROCZENSKI-WILDEY, W.

M. MAIESE & M. GREENSTEIN: Glycothiohexides, novel antibiotics produced by "*Sebekia*" sp., LL-14E605. I. Taxonomy, fermentation, and biological evaluation. J. Antibiotics 47: 887~893, 1994

7) NORTHCOTE, P. T.; D. WILLIAMS, J. K. MANNING, D. B. BORDERS, W. M. MAIESE & M. D. LEE: Glycothiohexide α, a novel antibiotic produced by "*Sebekia*" sp., LL-14E605. II. Isolation and physical-chemical characterization. J. Antibiotics 47: 894~900, 1994

8) NORTHCOTE, P. T.; M. SIEGEL, D. B. BORDERS & M. D. LEE: Glycothiohexide α, a novel antibiotic produced by "*Sebekia*" sp., LL-14E605. III. Structural elucidation. J. Antibiotics 47: 901~908, 1994

9) MOCEK, U.; A. R. KNAGGS, R. TSUCHIYA, T. NGUYEN, J. M. BEALE & H. G. FLOSS: Biosynthesis of the modified peptide antibiotic nosiheptide in *Streptomyces actuosus*. J. Am. Chem. Soc. 115: 7557~7568, 1993

10) MARTIN, G. E.; F. W. CROW, B. D. KALUZNY, J. G. MARR, G. D. FATE & T. J. GILBERTSON: Partial assignment of the ^{15}N NMR spectrum of sulfomycin-I at natural abundance. Magn. Reson. Chem. 36: 635~644, 1998

11) KELLER-JUSLEN, C.; M. KUHN & H. D. KING (Sandoz Ltd.): Antibiotics, Pharmaceutical Compositions and Methods of Use. U.S. 4,478,831, October 23, 1984

12) CONNOLLY, T. P. *et al.*: Personal communication

13) SASAKI, T.; T. OTANI, H. MATSUMOTO, N. UNEMI, M. HAMADA, T. TAKEUCHI & M. HORI: MJ347-81F4 A & B, novel antibiotics from *Amycolatopsis* sp.: Taxonomic characteristics, fermentation, and antimicrobial activity. J. Antibiotics 51: 715~721, 1998

14) CONSTANTINE, K. L.; L. MUELLER, S. HUANG, S. ABID, K. S. LAM, W. LI & J. E. LEET: Conformation and absolute configuration of nocathiacin I determined by NMR spectroscopy and chiral capillary electrophoresis. J. Am. Chem. Soc. 124: 7284~7285, 2002

15) HOOK, D. J.; C. F. MORE, J. J. YACOBUCCI, G. DUBAY & S. O'CONNOR: Integrated biological-physicochemical system for the identification of antitumor compounds in fermentation broths. J. Chromatogr. 385: 99~108, 1987