Notes

Neopyrrolomycins with Broad Spectrum Antibacterial Activity

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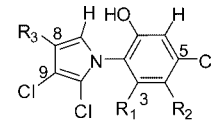
Three new antibiotics, neopyrrolomycins B (**1**), C (**2**), and D (**3**), with potent activity against Gram-positive pathogens were discovered. They exhibited MIC values < 1 μg/mL versus a number of resistant strains. The compounds were obtained from the ethyl acetate extracts of a *Streptomyces* sp. after purification by column chromatography and RP-HPLC. Their structures were elucidated using X-ray crystallography (**1**) and NMR spectroscopy (**2** and **3**).

Resistance to currently available antibiotics has become a widely recognized crisis in the medical community. The severity of the problem is exacerbated by the fact that many pharmaceutical companies abandoned antibacterial research in the 1980s and 1990s. The result is an empty pipeline of new drugs to battle the emerging resistant strains. Currently, many companies and researchers are refocusing their attention toward natural products, which have an excellent track record of producing effective antibacterial drugs. Natural products, particularly those produced by microbial fermentation, were the direct source or inspiration for virtually all antibiotics used today and remain the richest source for new antibiotic classes. In an effort to address this urgent need, Albany Molecular Research, Inc. (AMRI) began a screening campaign to identify novel antibiotics from our natural product resources. Our extensive library consisting of over 280 000 samples was screened for activity against *Escherichia coli* (ATCC 1411). The resulting subset of samples was then fractionated on an HPLC system employing UV, ELSD, and MS detectors. The eluted fractions were collected into a 96-well microtiter plate and submitted for bioassay. Active compounds were subsequently dereplicated on the basis of MS and UV data.

One of the hits resulting from our efforts originated from the ethyl acetate extract of a *Streptomyces* species (AMRI-33844). LCMS data for the active components returned a combination of UV spectra and molecular weights that did not have any matches in the Dictionary of Natural Products database. This triggered the scale-up fermentation of the active strain at the six-liter scale and subsequent purification of the active compounds. The resulting ethyl acetate extract was partitioned between hexane and aqueous methanol and then fractionated over silica gel followed by RP-HPLC to yield **1**, **2**, and **3** as light yellow solids.

FT-ICR HRMS for **1** gave a molecular formula of $C_{10}H_3Cl_6NO$ on the basis of a mass of 361.8273 for the $(M - H)^-$ peak (calcd 361.8268). The ^{1}H NMR spectrum showed only two singlets in the aromatic region. This left one proton unaccounted for, which was presumed to be exchangeable. The ^{13}C NMR spectrum similarly showed 10 resonances corresponding to substituted aromatic rings between δ_C 110 and 155. HMBC provided four correlations from a signal at δ_H 7.15 to carbon resonances at δ_C 122.8, 124.5, 136.4, and 155.9, while HSQC data established the corresponding resonance at δ_C 117.5. The remaining proton at δ_H 6.87 gave an HSQC correlation with a carbon at δ_C 120.1 and two HMBC cross-peaks with carbons at δ_C 110.1 and 115.9, which partially comprised a second ring system. The NMR data for these compounds is summarized in Table 1. Despite its small size, only a partial picture of the entire structure could be constructed due to the lack of correlations between the ring systems and the large number of possible arrangements of substituents. Consequently, X-ray crystallography was employed to provide an unambiguous answer. Suitable crystals were obtained by dissolving the sample in methylene chloride and adding a few drops of toluene. Data reduction yielded the structure shown (Figure 1).



	R_1	R_2	R_3
1	Cl	Cl	Cl
2	Cl	H	Cl
3	H	Cl	Cl
4	Cl	Cl	H

Compounds **2** and **3** shared the same molecular weight of 328. This corresponded to the substitution of a proton for one of the chlorine atoms as compared with **1**, resulting in a formula of $C_{10}H_4Cl_5NO$ for both. The appearance of one additional resonance in the ^{1}H NMR spectrum for **2** and **3** was consistent with this assignment. For **2**, the ^{1}H NMR spectrum showed that two protons shared a 2.2 Hz coupling, suggesting a *meta* substitution pattern on the benzene ring. The NMR data were unchanged in the pyrrole portion of the structure, which further corroborated the hypothesis that the extra proton was on the benzene ring. HMBC data revealed cross-peaks between these two coupled protons as well as a shared correlation with the carbons at the C-2 and C-5 positions. This established the extra proton on the C-4 position of the benzene ring for **2**. For **3**, the ^{1}H NMR spectrum had three singlets. The ^{13}C NMR spectrum was the same as **1** for the pyrrole portion of the molecule, indicating the extra proton was once again on the benzene ring. The HMBC spectrum showed that the extra proton shared all four correlations with the C-6 position, which appeared unchanged. Therefore, the only explanation for this observation would be a *para* substitution pattern with the additional proton at C-3.

Compounds **1**−**3** are very closely related to neopyrrolomycin (**4**).[1] The pyrrolomycins are a small group of antibiotics characterized by a pyrrole ring, which is usually attached to a benzene ring

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Table 1. ^{1}H and ^{13}C NMR Data for **1**, **2**, and **3**[a]

	1		2		3	
position	δ_C	δ_H (mult.)	δ_C	δ_H (mult., *J* in Hz)	δ_C	δ_H (mult.)
1	152.0		153.9		150.6	
2	122.3		121.1		123.6	
3	134.5		134.9		129.9	7.28 (s)
4	124.2		122.1	7.10 (d, 2.1)	124.4	
5	136.2		136.9		134.8	
6	116.7	7.16 (s)	115.9	7.00 (d, 2.1)	118.8	7.18 (s)
7	117.8	6.62 (s)	118.0	6.64 (s)	118.3	6.71 (s)
8	113.9		113.7		113.4	
9	111.1		111.0		111.0	
10	115.4		115.5		115.2	

[a] 500 and 125 MHz, respectively; measured in $CDCl_3$; chemical shift (δ) in ppm relative to $CDCl_3$ signal at δ 7.24 and 77.0 ppm; abbreviations: s, singlet; d, doublet.

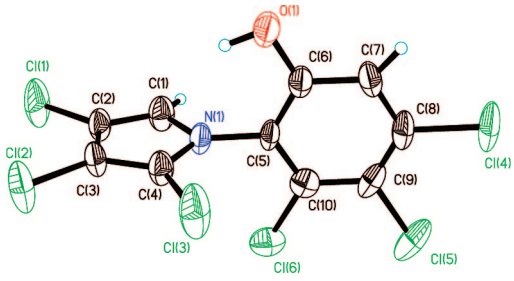

Figure 1. ORTEP representation of **1**.

Table 2. Antibacterial Activities of Isolated **1**, **2**, and **3** (MIC_{90}, μg/mL)

	E.coli[a] 25922	E.coli 1411	Kleb[b] 4352	MRSA[c] 43300	MSSA[d] 6538	MSSA 29213	MBC[e] 6538	Pseud[f] 27853	Strep[g] 49619	VRE[h] 700221
1	16	16	16	0.125	0.25	0.5	2	16	1	0.5
2	16	16	16	1	2	2	8	32	8	2
3	16	16	16	2	2	2	8	32	8	4

[a] *Escherichia coli*. [b] *Klebsiella pneumoniae*. [c] Methicillin-resistant *Staphylococcus aureus*. [d] Methicillin-sensitive *Staphylococcus aureus*. [e] Minimum bactericidal concentration. [f] *Pseudomonas aeruginosa*. [g] *Streptococcus pneumoniae*. [h] Vancomycin-resistant *Enterococcus faecium*.

through a variety of couplings.[2−7] They are also noteworthy for their high degree of halogenation. These small molecules typically have between three and five chlorine and/or bromine atoms attached. Syntheses of **4** and related structures have been reported.[8−11] Neopyrrolomycin (**4**) is unique among the pyrrolomycin class of compounds in that it is the only example where the pyrrole and benzene moieties are connected directly through the pyrrole nitrogen. Compounds **1**−**3** differ from **4** in either the number or arrangement of the chlorine atoms. A second deviation is in the three-dimensional configuration of **1** compared to **4**. The X-ray crystallographic data for **1** were reminiscent of the X-ray data in the literature for **4**. In both cases the two rings appear perpendicular to each other. However, the benzene ring of **1** was rotated 180° compared to **4**. This could explain the opposite optical rotation for the two molecules. We observed a value of +4.5°, while the literature reports −4.8°. X-ray data were not obtained for **2** and **3**, so it is not known if they share the same characteristic. However, optical rotation values for the two compounds were each zero, which may suggest a more random orientation.

Compounds **1**−**3** all displayed good activity against an internal panel of pathogens (Table 2). Compound **1** was more potent than **2** and **3** against Gram-positive organisms, particularly against *Staphyloccus* and *Enterococcus* species. All three exhibited some activity against Gram-negative strains. Compound **1** subsequently was submitted for testing against a wider panel and exhibited remarkable activity against numerous resistant Gram-positive strains (Table 3).[12] The activity was 2 orders of magnitude greater than

Table 3. Antibacterial Activities of **1** and Selected Standards against a Panel of Pathogens

		MIC_{90} (μg/mL)		
organism	phenotype	**1**	VAN[a]	CIP[b]
Staphylococcus aureus 100 (ATCC[c] 29213)	MSSA[d]	0.25	2	0.5
Staphylococcus aureus 1137	MRSA[e]	0.12	1	>2
Staphylococcus aureus 2170	MRSA	≤0.06	1	>2
Staphylococcus aureus 2012	VISA[f]	≤0.06	16	>2
Staphylococcus aureus 2018	VISA	≤0.06	8	>2
Staphylococcus aureus 1725	LRSA[g]	≤0.06	2	>2
Staphylococcus aureus 1651	LRSA	≤0.06	1	>2
Staphylococcus aureus 2144	CA[h]	0.12	0.12	>2
Staphylococcus epidermidis 1597	MSSE[i]	≤0.06	2	0.25
Staphylococcus epidermidis 1452	MRSE[j]	≤0.06	4	>2
Staphylococcus epidermidis 495		≤0.06	2	0.5
Enterococcus faecalis 846	VRE[k]	≤0.06	>64	>2
Enterococcus faecium 843	VSE[l]	≤0.06	2	>2
Enterococcus faecium 1254	VRE	≤0.06	>64	>2
Streptococcus pneumoniae 866	PSSP[m]	16	0.5	>2
Streptococcus pneumoniae 940	PRSP[n]	1	0.25	>2
Streptococcus pneumoniae 748	PRSP	1	0.5	1
Streptococcus pneumoniae 376	Quin-R[o]	2	0.2	>2
Streptococcus pneumoniae 379	Quin-R	16	0.5	>2
Streptococcus pneumoniae 933	MDR[p]	1	1	2
S. pneumoniae 1195 (ATCC 49619)		2	0.25	1
Streptococcus pyogenes 723		>16	0.5	0.5
Streptococcus agalactiae 2033		16	0.5	1
Moraxella catarrhalis 557		≤0.06	>64	0.03
Haemophilus influenzae 1742	ampR[q]	0.5	>64	0.008
H. influenzae 1224 (ATCC 49247)		0.25	>64	0.15
H. parainfluenzae	2319 (ATCC 7901)	1	64	0.15
Escherichia coli 102 (ATCC 25922)	QC strain	8	>64	0.008
Escherichia coli 2269	ESBL[r]-prod	8	>64	>2
Klebsiella pneumoniae 2239		16	>64	0.03
Klebsiella pneumoniae 2262	ampC, MDR[s]	8	>64	>2
Serratia marcescens 1635		16	>64	0.06
Pseudomonas aeruginosa 1473		16	>64	1

[a] Vancomycin. [b] Ciprofloxacin. [c] American Type Culture Collection. [d] Methicillin-resistant *Staphylococcus aureus*. [e] Methicillin-susceptible *Staphylococcus aureus*. [f] Vancomycin-intermediate *Staphylococcus aureus*. [g] Linezolid-resistant methicillin-resistant *Staphylococcus aureus*. [h] Community-acquired. [i] Methicillin-susceptible *Staphylococcus epidermidis*. [j] Methicillin-resistant *Staphylococcus epidermidis*. [k] Vancomycin-resistant *Streptococcus pneumoniae*. [l] Vancomycin-resistant *Streptococcus pneumoniae*. [m] Penicillin-susceptible *Streptococcus pneumoniae*. [n] Penicillin-resistant *Streptococcus pneumoniae*. [o] Quinolone-resistant *Streptococcus pneumoniae*. [p] Multiple-drug resistant. [q] Ampicillin-resistant. [r] Extended spectrum beta-lactamase. [s] ampC beta-lactamase, multidrug-resistant.

vancomycin[13] or ciprofloxacin[14] in many instances. The cytotoxic concentration required to inhibit the growth of 90% of the HepG2 cell line (CC_{90}) was measured for all three compounds. Compound **1** affected HepG2 cell viability after 48 h at 8 μg/mL, while **2** and **3** were both active at 43 μg/mL, indicating some toxicity against mammalian cells. Against a normal human dermal fibroblast (NHDF) cell line, the three compounds displayed CC_{90} values of 16 μg/mL for **1** and 43 μg/mL for **2** and **3**.

Experimental Section

General Experimental Procedures. ^{1}H and ^{13}C NMR as well as COSY, HSQC, and HMBC spectra were recorded using a Bruker DRX 500 NMR spectrometer in $CDCl_3$ at 500 MHz for ^{1}H and 125 MHz for ^{13}C NMR. Infrared data were obtained on a Perkin-Elmer Spectrum One. Isolation was carried out using an Agilent 1100 series HPLC system controlled by Chemstation software. The purification employed a Phenomenex Luna C_{18} column (50 × 250 mm; 15 μm). Silica gel (230−400 mesh, EM Science) was used for column chromatography. Mass spectrometry was performed on a Sciex API150 EX single quadrupole with an ionspray ionization source operating in negative mode. High-resolution mass spectra were gathered on a Bruker APEX III 47e Fourier transform (ion cyclotron resonance) mass spectrometer [FT(ICR)]MS at the Mass Spectrometry Center, University of Washington, Seattle, WA. The X-ray crystallographic data were collected

using a Bruker SMART CCD based diffractometer equipped with an Oxford Cryostream low-temperature apparatus operating at 193 K. The data were obtained with graphite-monochromated Mo Kα radiation ($\lambda = 0.71073$ Å). The structure was solved by the direct method using the SHELXS-97 program.[15] All non-hydrogen atoms were refined anisotropically. Hydrogens were found by difference Fourier methods and refined anisotropically.

Isolation of Producing Organism and Its Phylogenetic Affiliation. The *Streptomyces* sp. AMRI-33844 was isolated from a soil sample collected from the Brawley Wash watershed in Arizona. The culture was isolated by spread-plating on diluted ATCC 172 agar medium plates and incubating in the dark at 28 °C for 10 days. Observed colonies were transferred to and maintained on starch-casein agar plates and used to start the fermentation. For DNA extraction, the strain was grown on trypticase soy broth (TSB, Sigma) for 7 days at 28 °C, and the total genomic DNA preparation was carried out following a literature procedure.[16] PCR amplification of the 16S rRNA gene of the antibiotic producing strain was performed using two primers, StrepF (5′-ACGTGTGCAGCCCAAGACA-3′) and StrepR (5′-ACAAGCCCTG-GAAACGGGGT-3′) (IDT).[17] The PCR mixture consisted of 30 pmol of each primer, 100 ng of chromosomal DNA, 200 μM dNTPs (Invitrogen), and 2.5 units of Taq polymerase (Invitrogen) in 50 μL of polymerase buffer. The amplification was carried out for 30 cycles at 94 °C for 1 min, annealing at 53 °C for 1 min and extension at 72 °C for 2 min. The PCR reaction mixture was analyzed by agarose gel electrophoresis, and the remnant mixture was purified using QIA quick PCR purification reagents (Qiagen). The 16S rRNA gene was sequenced on both strands by ACGT, Inc.[18] The Blast program (www.ncbi.nlm-.nih.gov/blst) was used to assess the DNA similarities. Multiple sequence alignments were performed using BioEdit software.[19] Crystallographic data have been deposited in CIF format with the Cambridge Crystallographic Data Centre (deposition number 715693). Copies of the data can be obtained, free of charge, on application to the Director, CCDC, 12 Union Road, Cambridge CB1 1EZ, UK (fax: +44(0) 1223-336033 or e-mail: deposit@ccdc.cam.ac.uk).

Fermentation. The fermentation procedure utilized was a two-step process, in which a suspension of spores and mycelium was inoculated into 250 mL flasks containing 30 mL of a nutrient seed medium having the following composition per liter: 20 g D-glucose (Mallinckrodt), 15 g pharmamedia (Traders protein), 5 g yeast extract (Difco), 4 g $CaCO_3$ (Sigma), 3 g $(NH_4)_2(SO_4)$ (Mallinckrodt), and 0.03 g $ZnSO_4 \cdot 7H_2O$ (Sigma), adjusted to pH 6.5 prior to autoclaving. After inoculation, the flasks were incubated on a rotary shaker at 250 rpm (2 in. throw) and 28 °C for 2 days. Aliquots of 1 mL of the seed culture were then used to inoculate 300 × 250 mL flasks containing 30 mL of a production medium with the following composition per liter: 25 g soluble starch (Sigma), 15 g soybean meal, 2 g dry yeast (Red Star), and 4 g $CaCO_3$ (Sigma), adjusted to pH 6.2 prior to autoclaving. Following inoculation, the production flasks were incubated on a rotary shaker at 250 rpm and 28 °C for 6 days. The fermentation flasks were then harvested, and the fermentation mixture from each flask was pooled into a single extraction vessel for extraction (~3 L) with an equal volume of ethyl acetate.

Extraction and Isolation. Cultures were harvested by extracting with an equal volume of EtOAc. The organic layer was dried over Na_2SO_4 and evaporated to give a dark brown semisolid (2.6 g). The majority of this material (2.4 g) was partitioned between hexane and 90% aqueous MeOH. The aqueous MeOH fraction (1.0 g) was further fractionated over a silica gel column. The compounds of interest eluted with 100% $CHCl_3$ as a yellow band. This fraction (480 mg) was purified by RP-HPLC on a Phenomenex Luna C_{18} column (50 × 250 mm, 15 μm) using CH_3CN and H_2O each containing 0.05% TFA. The compounds were eluted using a gradient of 50:50 going to 0:100 over 15 min. This yielded **1** as a light yellow solid (174 mg). Compounds **2** and **3** were further purified on a Phenomenex Luna C_{18} column (10 × 250 mm, 10 μm) using CH_3CN and H_2O each containing 5 mM NH_4OAc to yield 9 and 3 mg, respectively.

Neopyrrolomycin B (1): light yellow solid; mp 117−120 °C; $[\alpha]_D$ +4.5 (*c* 0.001, $CHCl_3$); UV (CH_3CN) λ_{max} 208, 224 (sh), 291 (sh), 297 nm; IR ν_{max} (film) 3516, 3140, 2923, 2852, 1722, 1561, 1457, 1407, 1342, 1204, 1153, 965, and 841 cm^{-1}; ^1H and ^{13}C NMR data, see Table 1; ESMS *m/z* 362 $(M - H)^-$; HR FTICR-MS $[M - H]^-$ *m/z* 361.8273 [calcd for $C_{10}H_2NOCl_6$, 361.8268].

Neopyrrolomycin C (2): light yellow solid; UV (CH_3CN) λ_{max} 207, 224 (sh), 281 (sh), 288 nm; IR ν_{max} (film) 3510, 3376, 3144, 1727, 1586, 1496, 1416, 1337, 1154, 952, and 831 cm^{-1}; ^1H and ^{13}C NMR data, see Table 1; ESMS *m/z* 328 $(M - H)^-$.

Neopyrrolomycin D (3): light yellow solid; UV (CH_3CN) λ_{max} 207, 224 (sh), 294 nm; IR ν_{max} (film) 3523, 3140, 2946, 1735, 1595, 1499, 1479, 1399, 1341, 1128, 990, 918, and 884 cm^{-1}; ^1H and ^{13}C NMR data, see Table 1; ESMS *m/z* 328 $(M - H)^-$.

Antibacterial Assays. The agents tested were the natural product-derived antibiotics **1**, **2**, and **3**, vancomycin (VAN), and ciprofloxacin (CIP). Organisms used in this study included strains from the ATCC collection and clinical isolates. Minimum inhibitory concentrations (MICs) were determined by using the microdilution method described by CLSI.[20]

Cell Culture. Human hepatocellular carcinoma (HepG2) and normal human dermal fibroblast (NHDF) cell lines were purchased from ATCC and Lonza, respectively. The HepG2 cell line was maintained in MEM (minimal essential medium) with 10% heat-inactivated FBS (fetal bovine serum) (Invitrogen), 0.10 mM nonessential amino acids solution (Invitrogen), and 1 mM sodium pyruvate (Invitrogen) at 37 °C and 5% CO_2 humidified atmosphere. The NHDF cell line was maintained at 37 °C and 5% CO_2 humidified atmosphere using FGM-2BulletKit (Lonza) based on Lonza's recommendations with no more than 16 cell passages.

Cytotoxicity Assay. HepG2 and NHDF cells were seeded at 1.25×10^5 cells/mL 24 h prior to the addition of compounds. The compounds were resuspended in DMSO and diluted in assay media for a concentration testing range of 0.6 to 128 μg/mL at a final DMSO concentration of 1%. After incubation at the indicated compound concentrations for 48 h, cell viability was determined using the CellTiter Glow Luminescent kit from Promega following the manufacturer's instructions. Cytotoxicity was calculated as a percentage of the DMSO control. Additional control compounds were tested against HepG2 and NHDF cell lines using established protocols.[21,22]

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Supporting Information Available: A full report from the laboratory performing the X-ray crystallographic analysis, including all experimental parameters, is included. This material is available free of charge via the Internet at http://pubs.acs.org.

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