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Piperazimycins: Cytotoxic Hexadepsipeptides from a Marine-Derived Bacterium of the Genus *Streptomyces*

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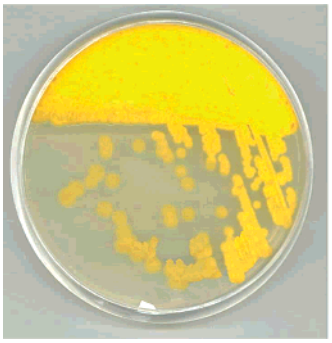

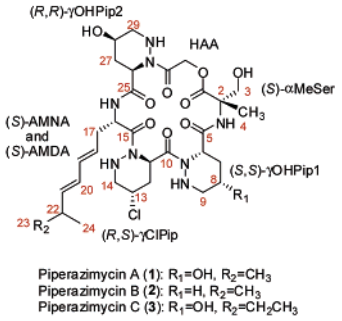

Three potent cancer cell cytotoxins, piperazimycins A−C (**1**−**3**), have been isolated from the fermentation broth of a *Streptomyces* sp., cultivated from marine sediments near the island of Guam. The structures of these cyclic hexadepsipeptides were assigned by a combination of spectral, chemical, and crystallographic methods. The piperazimycins are composed of rare amino acids, including hydroxyacetic acid, α-methylserine, γ-hydroxypiperazic acid, and γ-chloropiperazic acid. The novel amino acid residues 2-amino-8-methyl-4,6-nonadienoic acid and 2-amino-8-methyl-4,6-decadienoic acid were found as components of piperazimycins A and C, respectively. When screened in the National Cancer Institute's 60 cancer cell line panel, piperazimycin A exhibited potent in vitro cytotoxicity toward multiple tumor cell lines with a mean GI_{50} of 100 nM.

Introduction

Although great progress has been made in the treatment of cancer through the administration of chemotherapeutic agents, the need for new chemical entities with antitumor properties continues. This becomes evident when one considers that, despite intense effort, mortality rates for many types of cancers are still significant.[1] As part of an ongoing research program to discover new anticancer agents from marine sediment-derived actinomycetes, we identified a crude fermentation extract that showed potent cytotoxicity to the human colon carcinoma cell line HCT-116 (GI_{50} = 76 ng/mL). Bioassay-guided fractionation led to the isolation of three cyclic peptides, piperazimycins A−C (**1**−**3**, Chart 1), which were responsible for the overall cytotoxicity of the extract.

(1) Jemal, A.; Murray, T.; Ward, E.; Samuels, A.; Tiwari, C.; Ghafoor, A.; Feuer, E.; Thun, M. *CA-Cancer J. Clin.* **2005**, *55*, 10−30.

Results and Discussion

Piperazimycin A (**1**) was isolated and purified by EtOAc extraction of the whole microbial culture broth, followed by bioassay-guided fractionation (HCT-116 colon carcinoma) via flash, C_{18} reversed phase column chromatography, and C_{18} reversed phase HPLC (57% CH_3CN:H_2O). In the purified form, piperazimycin A (**1**) was obtained as a white powder that analyzed for the molecular formula $C_{31}H_{47}N_8O_{10}{}^{35}Cl$ by HR-FABMS $[M + H]^+$ *m/z* 727.3186 (calcd for $C_{31}H_{48}N_8O_{10}{}^{35}Cl$, 727.3176) and NMR spectral data. Interpretation of HSQC and DEPT NMR spectral data enabled the assignment of all protons to their respective carbons, with the exception of eight exchangeable ^1H NMR signals that were assumed to be attached to heteroatoms. The chemical shifts of these ^1H NMR signals suggested, although did not confirm, the presence of three alcohols, two amides, and three secondary amines (Table 1). This information, in combination with the ^{13}C NMR spectra, which revealed six carbonyl carbons within the amide/ester

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TABLE 1. NMR Spectral Data for 1 Recorded in $CDCl_3$ (500 MHz) at 25 °C[a]

unit	position	δ_H mult (*J* in Hz)	δ_C, DEPT	HMBC	COSY
αMeSer C═O	1		173.6, C		
α	2		63.0, C		
β	3a	4.04[b] dd (11.5, 4.0)	64.2, CH_2	C1, C2, C4	H3b, OH-1
	3b	4.14[b] dd (11.5, 11.5)		C1, C2, C4	H3a, OH-1
αMe	4	1.57 s	18.3, CH_3	C1, C2, C3	
NH-1		7.57 s		C1, C2, C3, C4, C5, C6	
OH-1		3.24[b] dd (11.5, 4.0)			H3ab
γOHPip1 C═O	5		172.4, C		
α	6	5.15 dd (7.0, 2.0)	51.2, CH	C5, C7, C8, C10	H7ab
β	7a	2.01[c] m	28.5, CH_2	C5, C6, C8, C9	H6, H7b, H8
	7b	2.26[c] m		C8, C9	H6, H7a, H8
γ	8	3.75[c] bs	58.9, CH	C6, C7, C9	H7ab, H9ab, OH-2
δ	9a	2.86 ddd (14.5, 13.0, 2.0)	53.8, CH_2	C7,C8	H8, H9b, NH-2
	9b	3.05 dd (14.5, 3.0)		C7,C8	H8, H9a, NH-2
NH-2		4.59 dd (13.0, 3.0)		C8,C10	H9ab
OH-2		6.27 d (7.0)		C7,C8	H8
γClPip C═O	10		174.4, C		
α	11	5.81 dd (7.0, 2.5)	51.0, CH	C10, C12, C13, C15	H12ab
β	12a	2.00[c] m	35.7, CH_2	C10, C11, C13, C14	H11, H12b, H13
	12b	2.40 ddd (10.0, 4.0, 3.0)		C11, C13, C14	H11, H12a, H13
γ	13	3.92 dddd (13.5, 11.0, 4.0, 4.0)	51.3, CH	C11, C12, C14	H12ab, H14ab
δ	14a	2.78 td (13.5, 10.5)	53.9, CH_2	C12, C13	H13, H14b, NH-3
	14b	3.30 ddd (10.5, 4.0, 3.0)		C12, C13	H13, H14a, NH-3
NH-3		5.20 dd (13.5, 3.0)		C11, C13, C14, C15	H14ab
AMNA C═O	15		172.2, C		
α	16	5.28 ddd (6.0, 6.0, 4.0)	50.4, CH	C15, C17, C18, C25	H17ab, NH−3
β	17a	2.48 ddd (14.5, 7.0, 4.0)	36.2, CH_2	C15, C16, C18, C20	H16, H17b, H18
	17b	2.52 ddd (14.5, 8.5 ,6.0)		C15, C16, C20	H16, H17a, H18
γ	18	5.37 ddd (15.5, 8.5, 7.0)	124.8, CH	C16, C17, C21	H17ab, H19
δ	19	5.94[c] m	141.5, CH	C17, C20, C21, C22	H18, H20
ϵ	20	5.92[c] m	134.3, CH	C17, C18, C19, C21, C22	H19, H21
Z	21	5.57 dd (14.5, 6.5)	126.7, CH	C20, C22, C23, C24	H20, H22
H	22	2.28[c] m	31.0, CH	C19, C21, C23, C24	H21, H23, H24
Θ	23	0.97 d (7.0)	22.2, CH_3	C19, C22, C24	H22
H-Me	24	0.97 d (7.0)	22.3, CH_3	C19, C22, C23	H22
NH-4		7.60 d (6.0)		C15, C16, C25, C26	H16
γOHPip2 C═O	25		169.5, C		
α	26	4.93 dd (7.0, 2.0)	51.0, CH	C25, C27, C28	H27ab
β	27a	2.01[c] m	28.5, CH_2	C25, C26, C28, C29	H26, H27b, H28
	27b	2.16 ddd (15.0, 4.5, 2.5)		C25, C26, C28, C29	H26, H27a, H28
γ	28	3.75 bs	58.6, CH	C26, C27, C29	H27ab, H29ab, OH-3
δ	29a	2.81 ddd (14.5, 13.0, 2.0)	53.5, CH_2	C28	H28, H29b, NH-5
	29b	2.99 dd (14.5, 2.5)		C27, C28	H28, H29a, NH-5
NH-5		4.37 dd (13.0, 2.5)		C26, C28, C29, C30	H29ab
OH-3		6.47 d (7.0)		C27, C28	H28
HAAC C═O	30		169.1, C		
	31a	4.40 d (15.5)	63.0, CH_2	C1, C30	H31b
	31b	5.49 d (15.5)		C1, C30	H31a

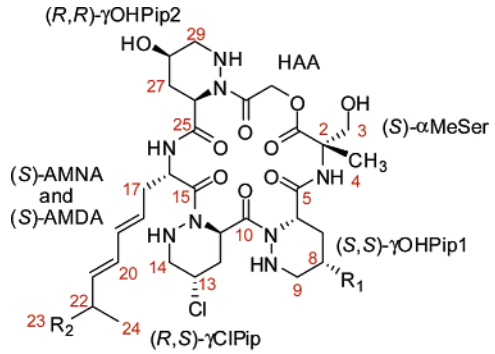
[a] All multiplicity obtained by interpretation of *J*-resolved NMR spectral data unless otherwise noted. [b] Multiplicity determined from ^1H NMR in $CDCl_3$ (500 MHz) at 25 °C. [c] Unresolved in homonuclear 2D*J* analyses

range, suggested **1** was composed of six amino acids. The possibility that **1** was a depsipeptide (also possessed an ester linkage) was inferred by these data, but not rigorously indicated until additional NMR data were obtained. Subsequent analysis of HSQC, COSY, and HMBC NMR spectral data allowed five amino acids and one hydroxy acid to be assembled, accounting for all of the atoms in **1**. The specific assignments made from analysis of the 1D and 2D NMR spectra of **1** are as follows:

A hydroxyacetic acid (HAA) residue was assigned following the observation of a pair of oxygenated methylene ^1H NMR signals (H_2-31) that exhibited exclusively geminal coupling [d, J = 15.5 Hz]. COSY NMR correlations were observed only within the H_2-31 geminal pair and the only HMBC NMR correlations observed from H_2-31 were to carbonyl carbons at positions C-1 and C-30 (Table 1). An α-methylserine residue was then assigned following the observation of COSY NMR correlations originating from another set of oxygenated methylene ^1H NMR signals (H_2-3) to an unassigned ^1H NMR signal (OH-1), indicating that a hydroxyl group was attached to C-3 [δ_C 64.2]. HMBC NMR correlations observed from both H_2-3 and a secondary amide proton signal (NH-1 [δ_H 7.57 (s)]) to a carbonyl carbon (C-1) and a quaternary carbon (C-2) implied the presence of a modified serine residue. Further HMBC NMR correlations from H_2-3 and NH-1 to a methyl singlet (H_3-4), coupled with HMBC NMR correlations from H_3-4 to C-1, C-2, and C-3, indicated methylation of C-2 and confirmed this residue as α-methylserine (αMeSer).

The 2-amino-8-methyl-4,6-nonadienoic acid (AMNA) residue in **1** was assigned following analysis of ^{13}C and ^1H NMR chemical shifts, ^1H multiplicity, *J* values, and interpretation of correlations observed in COSY and HMBC NMR spectral data (Table 1). A gem-dimethyl configuration for the C-23 and C-24 terminal methyl groups was proposed based on COSY NMR correlations from the overlapping methyl doublets H_3-23 [δ_H 0.97 (d, J = 7.0 Hz)] and H_3-24 [δ_H 0.97 (d, J = 7.0 Hz)] to the methine proton signal, H-22. Interpretation of a network of

CHART 1. Structures of Piperazimycins A−C (1−3)



Piperazimycin A (**1**): R_1=OH, R_2=CH_3
Piperazimycin B (**2**): R_1=H, R_2=CH_3
Piperazimycin C (**3**): R_1=OH, R_2=CH_2CH_3

COSY and HMBC NMR correlations originating from H-22 established the connectivity to C-16 and the presence of a conjugated diene with unsaturation at C-20 and C-18. HMBC NMR correlations observed from the amide proton, NH-4 [δ_H 7.60 (d, J = 6.0 Hz)], to C-16 and the carbonyl carbon C-15, coupled with the observation of HMBC NMR correlations from H-16 and H_2-17 to C-15 established the novel AMNA residue.

Combined spectral analyses allowed three substituted piperazic acids in **1** to be defined. The first, a rare, but known γ-hydroxypiperazic acid (γOHPip1) residue, was identified following interpretation of COSY and HMBC NMR correlations originating from the methine proton signal H-6, which established connectivity from the carbonyl carbon C-5 to the secondary ϵ-amine (NH-2) (Table 1). COSY NMR correlations from H-8 to an unassigned proton signal (OH-2) indicated that an alcohol was attached to C-8 [δ_C 58.9]. The observation of an HMBC NMR correlation from NH-2 to C-6, that could only be rationalized if the residue was cyclic, at first, suggested a γ-hydroxyproline residue. Closer examination of the COSY NMR data revealed that H-6 was not coupled to NH-2 as would be expected in this amino acid. On this basis and on the proportionally larger number of nitrogen atoms present in **1**, this group was assigned as a γ-hydroxypiperazic acid (γOHPip1) moiety. Comparison of the ^{13}C and ^{1}H NMR chemical shift values found in this study to those previously reported for γOHPip amino acids[2,3] supported this structural assignment. The structures of two other modified piperazic acid units, another γ-hydroxypiperazic acid (γOHPip2) residue, and one γ-chloropiperazic acid (γClPip) were subsequently proposed following analysis of ^{1}H and ^{13}C NMR chemical shift values and comparison to those observed in γOHPip1 (Table 1).

The sequence of amino acids in **1** was initially determined by interpretation of key correlations observed in the HMBC NMR spectra (Figure 1). Specifically, HMBC NMR correlations observed from the αMeSer amide proton (NH-1) to the γOHPip carbonyl carbon (C-5) established the connectivity of those two residues. Additional HMBC NMR correlations from the γOHPip1 α proton (H-6) and secondary amine (NH-2) to the γClPip carbonyl carbon (C-10) allowed these two residues to be connected. Additional HMBC NMR correlations from the

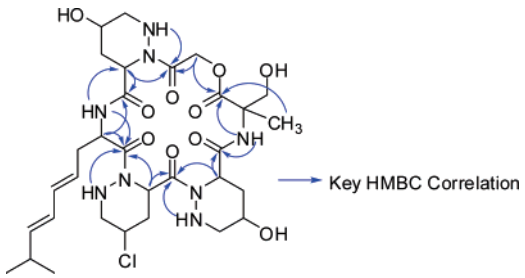


FIGURE 1. Key HMBC correlations used to establish the amino acid sequence of piperazimycin A (**1**).

γClPip α proton (H-11) and secondary amine (NH-3) to the AMNA carbonyl carbon (C-15) indicated that the AMNA residue was attached to the γClPip moiety. Further HMBC NMR correlations from the AMNA α proton (H-16) to the γOHPip2 carbonyl carbon (C-25) and from the AMNA amide proton (NH-4) to the γOHPip2 α carbon (C-26) established the position of the γOHPip2 residue in **1**. HMBC NMR correlations from the γOHPip2 α proton (H-26) and secondary amine (NH-5) protons to the HAA carbonyl carbon (C-30) were then used to establish the connectivity of the γOHPip2 and HAA residues. By using this approach, all partial structures in **1** could be accounted for. Observation of an HMBC NMR correlation from the oxygenated methylene at position H_2-31 to the αMeSer ester carbonyl carbon (C-1) suggested the cyclic lactone nature of **1**, thus accounting for the single remaining degree of unsaturation inherent in the molecular formula.

Support for the initially hypothesized depsipeptide structure was also obtained by a characteristic IR absorption (1737 cm^{-1}) indicative of an ester functionality. The ester linkage in **1** was then confirmed by sodium methoxide methanolysis to yield the methyl ester **4** (LRESIMS $[M + Na]^+$ m/z 781.4). Subsequent analysis of 1D and 2D NMR spectra [see Table S1 in the Supporting Information] showed the presence of a new methoxyl substituent [δ_H 3.75 (s); δ_C 53.6] in the ^{1}H NMR spectrum of **4**. HMBC NMR correlations from the methoxyl group protons to the αMeSer carbonyl carbon (C-1) indicated the presence of a methyl ester, as would be expected following methanolysis of the ester linkage in **1**. Further support was obtained by the observation of an additional hydroxyl group (OH-4) in the ^{1}H NMR spectra of **4**. COSY NMR correlations observed from H_2-31 to OH-4 indicated that the alcohol was attached to C-31, hence confirming that this is the site of lactonization in **1**.

The amino acid sequence of **1**, assigned on the basis of HMBC correlations (Figure 1), was fully supported by the ESI-MS/MS fragmentation pattern of **4** (Figure 2). Key fragmentations include m/z 745, which indicated cleavage of chlorine from γClPip, and fragment ions at m/z 723, 595, 430, and 284, which resulted from successive cleavage of amide bonds between HAA/γOHPip2, γOHPip2/AMNA, AMNA/γClPip, and γClPip/γOHPip1, respectively.

Piperazimycin B (**2**) was isolated as a white powder that analyzed for the molecular formula $C_{31}H_{47}N_8O_9{}^{35}Cl$ by HR-FABMS $[M + H]^+$ m/z 711.3231 (calcd for $C_{31}H_{48}N_8O_9{}^{35}Cl$, 711.3227) and comprehensive analysis of NMR data [see Table S2 in the Supporting Information]. The ^{1}H and ^{13}C NMR spectra of **2** were highly analogous to those of **1** and consistent with a cyclic hexadepsipeptide; however, major differences were observed in the γOHPip1 region. The most notable difference appeared at the γ-position of the residue where the oxygenated

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(3) Leet, J.; Schroeder, D.; Golik, J.; Matson, J.; Doyle, T.; Lam, K.; Hill, S.; Lee, M.; Whitney, J.; Krishnan, B. *J. Antibiot.* **1996**, *49*, 299−311.

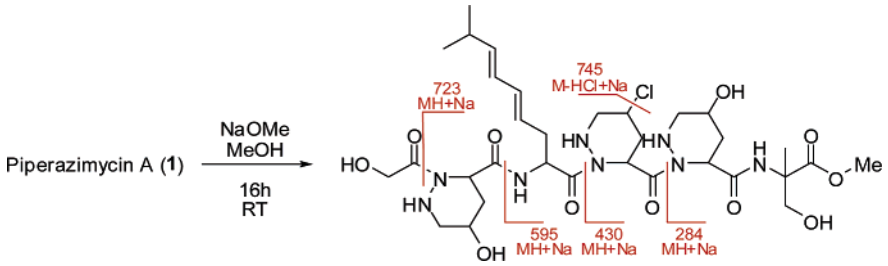

FIGURE 2. MS/MS fragmentation pattern of the methanolysis product **4**.

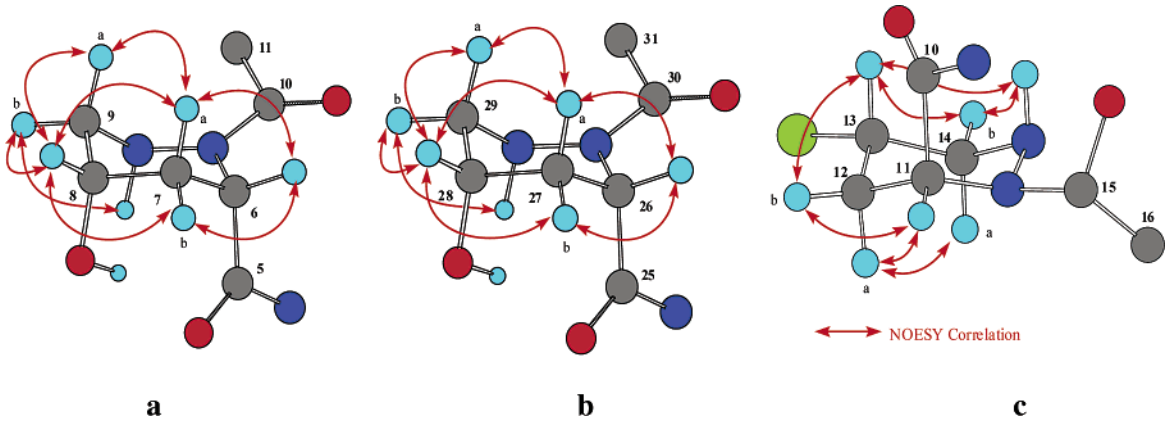

FIGURE 3. Key NOESY correlations used to establish the relative stereochemistries of the γOHPip1 (a), γOHPip2 (b), and γClPip (c) residues of piperazimycin A (**1**).

methine of **1** (H-8) had been replaced by a methylene group (H_2-8) thus forming an unsubstituted piperazic acid (Pip) unit in **2**. NMR analyses utilizing a combination of ^{13}C NMR, ^{1}H NMR, DEPT, HSQC, COSY, and HMBC experiments, coupled with examination of the molecular formula, allowed **2** to be assigned as the γOHPip1 desoxy congener of **1**.

Piperazimycin C (**3**) was isolated as a white powder that analyzed for the molecular formula $C_{32}H_{49}N_8O_{10}{}^{35}Cl$ by HR-FABMS $[M + H]^+$ *m/z* 741.3337 (calcd for $C_{32}H_{50}N_8O_{10}{}^{35}Cl$, 741.3333) and comprehensive analysis of NMR data [see Table S3 in the Supporting Information]. The ^{1}H and ^{13}C NMR spectra of **3** were highly analogous to those of **1** and consistent with a cyclic hexadepsipeptide; however, major differences were observed in the AMNA region. The most notable difference appeared at the θ-position of the AMNA residue where the methyl group in **1** (H_3-23) had been replaced by an ethyl group in **3**, thus forming 2-amino-8-methyl-4,6 decadienoic acid (AMDA), a novel amino acid residue. Analyses utilizing a combination of ^{13}C, ^{1}H, DEPT, HSQC, COSY, and HMBC NMR experiments, coupled with examination of the molecular formula, allowed **3** to be assigned as the AMDA congener of **1**.

Following assignment of planar structures for **1**−**3** efforts were focused on determining the relative stereochemistry of **1**. The conjugated diene of the AMNA residue was determined to be 18*E*,20*E* by ^{1}H NMR selective decoupling experiments and interpretation of *J* values. Decoupling the ^{1}H NMR signal at H_2-17 converted the ^{1}H NMR signal at H-18 from a doublet of doublets of doublets (J = 15.5, 8.5, 7.0 Hz) to a broad doublet (J = 15.5 Hz) indicating the *E* configuration of the C-18, C-19 olefin. Likewise, decoupling the H-22 multiplet converted the ^{1}H NMR signal for H-21 from a doublet of doublets (J = 14.5, 6.5 Hz) to a doublet (J = 14.5 Hz), indicating that the C-20, C-21 olefin was also *E*.

The relative stereochemistry and chair conformation of the γOHPip1, γOHPip2, and γClPip1 rings were established through 1D NOESY NMR experiments (Figure 3). Specific 1D NOESY NMR correlations observed between the γOHPip1 α-methine proton (H-6) and both protons at position H_2-7 indicated H-6 was equatorial. An additional NOESY correlation observed from H-7a to H-9a indicated a 1,3-diaxial interaction suggesting a chair conformation for the γOHPip1 ring. NOESY correlations from H-8 to H-7ab and H-9ab illustrated that H-8 was equatorial and the C-8 hydroxyl group axial. The secondary amine proton (NH-2) exhibited a NOESY correlation to only H-9b indicating the proton at position NH-2 was also axial. The γOHPip2 residue was assigned the same conformation as γOHPip1 after identical NOESY NMR correlations were observed between the respective γOHPip2 protons. The γClPip residue was also assigned the same overall conformation as the γOHPip1 and γOHPip2 residues; however, an important exception in the configuration at the γ-position was noted after NOESY NMR correlations were observed from H-13 to only H-12b and NH-3 indicating that H-13 was in an axial position and the C-13 chlorine in an equatorial position.

The absolute stereochemistry of **1** was defined by a combination of spectral and chemical methods. The configuration of the αMeSer residue was determined as *S* by acid hydrolysis and application of Marfey's method,[4,5] using standards of known configuration (Table 2). Absolute configurations of the γOHPip1, γOHPip2, and γClPip residues could not be determined from

(4) Marfey, P. *Carlsberg Res. Commun.* **1984**, *49*, 591−596.
(5) Bhushan, R.; Brückner, H. *Amino Acids* **2004**, *27*, 231−247.

TABLE 2. Retention Times for the Amino Acids from 1 as Their FDAA Derivatives

residue	mass FDAA derivative ESI-LRMS $[M + H]^+$ *m/z*	t_R, min L-FDAA	t_R, min D-FDAA
(*R*)-αMeSer[a]	372.3	28.7	23.8
αMeSer[b]	372.3	23.6	NA
AMNA[b,c]	440.2	59.3	63.8

[a] Standard of known configuration. [b] From acid hydrolysate of **1**. [c] From hydrogenate of **1**.

any hydrolysate by the Marfey method even after multiple variations to both the hydrolysis and derivatization conditions. Previous studies have shown that reductive cleavage of piperazic acid to ornithine was possible via catalytic hydrogenation[6] and that the absolute configuration of ornithine could be determined by Marfey's analysis.[7] Consequently, we attempted reductive cleavage of the various γOHPip and γClPip N−N bonds by catalytic hydrogenation. Despite significant effort utilizing both $Pt(IV)O_2$ and Pd/C we were not able to characterize any reduced γOHPip or γClPip residues (reduction was only observed at the conjugated diene of the AMNA residue). In a separate experiment, acid hydrolysis of **1**, followed by $Pt(IV)O_2$-catalyzed hydrogenation, derivatization with FDAA, and application of the advanced Marfey method[8,9] led to the assignment of the absolute stereochemistry at C-16 of the reduced AMNA residue as *S* (Table 2).

Determination of the absolute stereochemistry of the γOHPip units was then attempted by derivatizing **1** with (*R*)- and (*S*)-MTPACl and applying the modified Mosher method.[10] Subsequent interpretation of $\Delta\delta_{S-R}$ values for the tri-MTPA derivative [see Table S4 in the Supporting Information] yielded inconclusive results and no clear assignment of absolute configuration could be made by this method.[11]

Because of the aforementioned difficulties in determining the absolute stereochemistry of the γOHPip and γClPip residues of **1** by chemical methods, efforts were focused toward crystallization and a small crystal was ultimately obtained from methanol. The crystal structure of piperazimycin A (Figure 4) confirmed the connectivity and relative stereochemistry assigned by NMR methods. However, due to the poor quality of the crystal, the absolute configuration, potentially determined by anomalous scattering from the chlorine atom, was not feasible. However, given that the absolute configuration of C-2 (*S*) and C-16 (*S*) had been determined by the Marfey method (Table 2), the overall absolute configuration of **1** could be assigned as 2*S*,6*S*,8*S*,11*R*,13*S*,16*S*,26*R*,28*R*.

We note that the γOHPip residue has been found in the chair conformation[12,13] with the alcohol in the axial position for each case where the stereochemistry has been reported.[6,12−14] Further, MTPA derivatives of axial alcohols have been shown to be

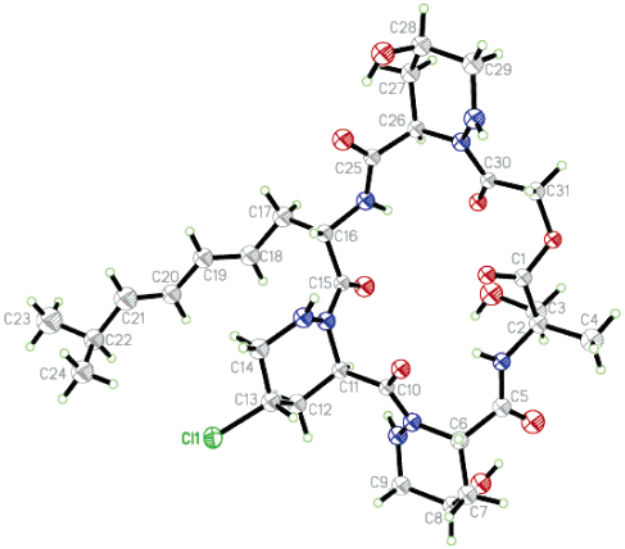

FIGURE 4. Computer-generated plot of the final X-ray structure of **1** depicting relative stereochemistry only.

susceptible to steric compression thus causing significant deviations from the assumed conformation.[15] Deviations caused by steric compression lead to irregular anisotropic shielding effects, which are reflected in $\Delta\delta_{S-R}$ values with little or no definitive pattern, exactly as we observed in this study. Therefore, it is concluded that the Mosher method would be ineffective in determining the absolute configuration of the γ-position in the γOHPip residue. It is also interesting to note that the absolute configurations of the γOHPip1 and the γOHPip2 residues of **1** were (*S*,*S*) and (*R*,*R*), respectively. While both the (*R*,*R*) and (*S*,*S*) γOHPip residues have been found independently in himastatin[3] and monamycin,[16] respectively, to the best of our knowledge, this report represents the first time in which the (*R*,*R*) and (*S*,*S*) enantiomers have been found in the same molecule.

Assignment of the absolute configurations of **2** and **3** was approached by comparison of their respective CD spectra [see Figure S1 in the Supporting Information] to that of **1**. The planar structures of **1** and **2**, which differ only in the presence of an alcohol at position C-8 in **1**, exhibited nearly identical CD behavior [Cotton effects: **1**, λ 235 (Δε −5.7),; **2**, λ 240 (Δε −7.0)]. Piperazimycins A and C, **1** and **3**, which differ only in substitution at C-23, also exhibit very similar CD spectra [Cotton effects: **1**, λ 235 (Δε −5.7); **3**, λ 236 (Δε −4.5)]. Interpretation of the CD data suggests, but does not rigorously confirm that the configurations of all the identical centers in **1**−**3** are the same. The absolute configuration of the center at C-22 in **3** could not be suggested from these experiments.

Examination of the literature revealed a number of studies in which bioactive piperazic acid containing cyclic peptides had

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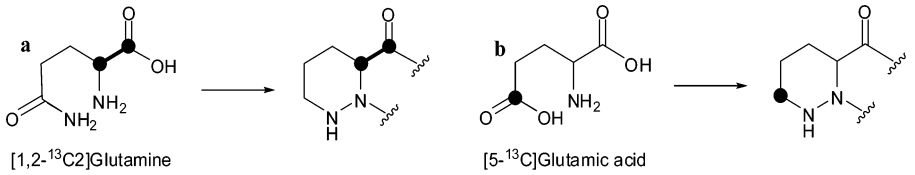

FIGURE 5. Incorporation of glutamine (a) and glutamic acid (b) into piperazic acid rings as reported by Umezawa et al.[32]

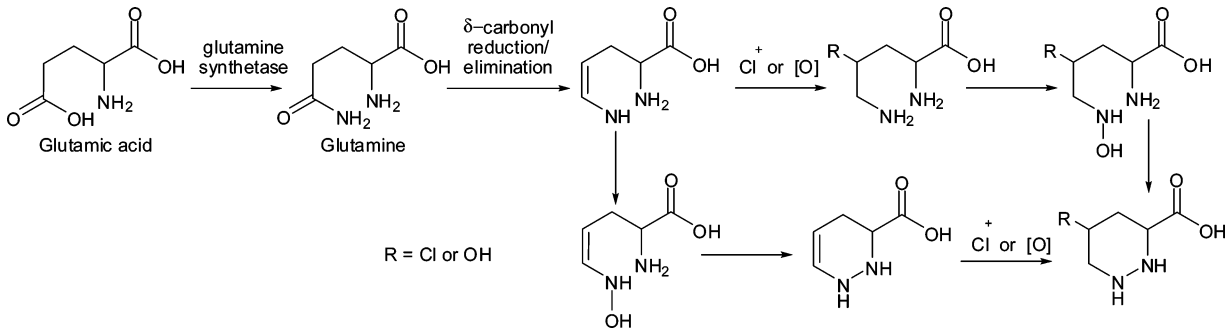

FIGURE 6. Hypothetical biosynthetic pathways to γ-substituted piperazic acids.

been isolated. Examples include antitumor antibiotics,[17−19] tuberculostatics,[20] anti-inflammatory agents,[21] and anti-HIV agents.[22,23] Originally identified by Hassall and co-workers in the monamycin series of antibiotics,[16] molecules containing piperazic acids have since been isolated from the culture broth of numerous *Streptomyces* spp.[2,3,13,24,25] and also from strains of the genus *Actinomadura*.[19,26] Because of the wide spectrum of biological activity and interest in piperazic acids, these moieties have also been the subject of two excellent reviews by Ciufolini and Xi concerning both their chemistry[27] and conformational properties.[27,28]

Review of the literature reveals a molecule named sohbumycin, reported in both a paper and two patents, that shares the same molecular formula as piperazimycin A (**1**).[29] Unfortunately, no structural information was reported for this molecule, and because of the age of this report the published spectral data do not allow for a precise comparison.

While synthetic routes to γ-substituted piperazic acids have been developed,[30] to the best of our knowledge the biosynthetic mechanisms by which piperazic acids are formed are poorly understood. Original work by Arroyo using [2-^{14}C]piperazic acid suggested that the piperazic acids, as well as γOHPIP and γClPip moieties, were incorporated into monamycin as intact residues.[31] Further studies by Arroyo indicated that ornithine was not a precursor to piperazic acids, as no labeled ornithine could be incorporated into the piperazic acid ring. Subsequent studies by Umezawa[32] showed that L-[1,2-$^{13}C_2$]glutamine was incorporated into the carbonyl group and α-carbon and DL-[5-^{13}C]glutamic acid was incorporated into the δ-carbon of piperazic acid residues (Figure 5). Additional work by Parry, defining the biosynthesis of the *Streptomyces*-derived natural product valanimycin, has suggested that N−N bond formation occurs via the reaction of a hydroxylamine with an amine to yield a hydrazine.[33] Parry and Tao also suggested that the hydroxlamine is likely formed by a PIP specialized flavoprotein monooxygenase, as NADH and FAD are required for its formation.[34] It is possible that piperazic acids could be formed from glutamine following reduction of the δ-carbonyl and conversion of either the α- or γ-amine to the hydroxylamine. A subsequent intramolecular cyclization would result in formation of the piperazic acid ring (Figure 6). In the case of glutamic acid, the same scheme could be envisioned following conversion of glutamic acid to glutamine via a glutamine synthetase. Addition of hydroxyl or chloro groups to the γ-position of the piperazic acid ring, as is the case in **1**, could occur at any stage after reduction of the δ-carbonyl and elimination of water to

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form the enamine. Substitution at the now nucleophilic γ-position most likely occurs via oxygenase and halogenase enzymes.

Biological activities for the piperazimycins A−C (**1**−**3**) were initially evaluated in vitro against the human colon carcinoma cell line HCT-116. All compounds exhibited significant cytotoxicity with an average GI_{50} = 76 ng/mL for each. Piperazimycin A (**1**) also showed potent biological activity when evaluated against the oncologically diverse 60 cancer cell line panel at the National Cancer Institute, with mean values for all cell lines of GI_{50} = 100 nM (TGI = 300 nM, LC_{50} = 2 μM [see Figures S2 and S3 in the Supporting Information]. Overall, piperazimycin A exhibited a nearly 3-fold more potent activity against solid tumors (average LC_{50} = 13.9 μM) than against the leukemia cell lines tested (average LC_{50} = 31.4 μM). Within the solid tumors, **1** was most active against the melanoma (average LC_{50} = 0.3 μM), CNS (average LC_{50} = 0.4 μM), and prostate cell lines (average LC_{50} = 0.6 μM) cancers. Piperazimycin A was also active against the colon cancer group (average LC_{50} = 1.2 μM), renal cancers (average LC_{50} = 9.5 μM), ovarian cancers (average LC_{50} = 10.3 μM), non-small cell lung cancers (average LC_{50} = 12.9 μM), and breast cancers (average LC_{50} = 55.8 μM). Although some selectivity was observed, the general cytotoxicity noted in the 60 cell line panel indicates a general mode of cell toxicity. This result does not support further development of these agents for the treatment of cancer. Further studies ongoing at the National Cancer Institute will provide additional information to answer this question.

Experimental Section

Isolation, Identification, and Cultivation of Strain CNQ-593. Strain CNQ-593 was isolated from a sediment sample collected at a depth of approximately 20 m in January 2002 near the island of Guam. The strain was identified as a *Streptomyces* sp. based on greater than 99% 16S rRNA gene sequence homology with other members of the genus. The strain was cultured in 2.8 L Fernbach flasks (40 × 1 L) in a seawater-based marine medium (chitosan 0.2%, kelp powder 0.2%, menhaden meal 0.2%, fish solubles 0.4%, starch 0.5%) and shaken at 230 rpm at 27 °C. After 8 days of cultivation, the whole culture was extracted with EtOAc in the manner described below.

Extraction and Compound Isolation. The whole culture media (40 L) was repetitively extracted with an equal volume of EtOAc and dried in vacuo to yield 4.2 g of dry extract. The extract was fractionated by C_{18} reversed-phase column chromatography utilizing a step gradient beginning with 100% H_2O and continuing in 10% CH_3CN increments to 100% CH_3CN, followed by a final wash with 50% MeOH/50% CH_2Cl_2. Bioassay guided fractionation (HCT-116 colon carcinoma cell line) showed the cytotoxic constituents were present in the 70% CH_3CN fraction (66.5 mg, amorphous white powder). The powder was solubilized in DMSO and separated via HPLC by loading 6.5 mg aliquots onto a C_{18} (5 μm; 10 × 50 mm) semi-prep column (ELSD detection). Separation was achieved by using 57% CH_3CN in H_2O, at 3.0 mL/min, employing a step gradient to 75% CH_3CN at 20 min. Piperazimycins A−C (**1**−**3**) eluted at 15, 25, and 21 min, respectively.

Piperazimycin A (1): amorphous white powder; $[\alpha]_D$ −45 (*c* 0.3, $CHCl_3$); IR (NaCl) ν_{max} 3317, 3252, 2965, 2916, 1737, 1655, 1631, 1533, 1434, 1238, 984 cm^{-1}; ^1H and ^{13}C NMR (500 MHz, $CDCl_3$) see Table 1; crystal information (see below); UV λ_{max} CH_3CN (ε) 233 nm (15 600); CD (MeOH) λ 216 (Δε 4.5), 233 (Δε −7.1); FABHRMS $[M + H]^+$ *m/z* 727.3186 (calcd for $C_{31}H_{48}N_8O_{10}{}^{35}Cl$, 727.3176).

Piperazimycin B (2): amorphous white powder; $[\alpha]_D$ −91 (*c* 0.08, $CHCl_3$); IR (NaCl) ν_{max} 3415, 3252, 2965, 2916, 1737, 1680, 1631, 1524, 1434, 1238, 984 cm^{-1}; ^1H and ^{13}C NMR (500 MHz, $CDCl_3$) see Table S2 in the Supporting Information; UV λ_{max} CH_3CN (ε) 233 nm (19 600); CD (MeOH) λ 225 (Δε 3.4) λ 240 (Δε −7.0); FABHRMS $[M + H]^+$ *m/z* 711.3231 (calcd for $C_{31}H_{48}N_8O_9{}^{35}Cl$, 711.3227)

Piperazimycin C (3): amorphous white powder; $[\alpha]_D$ +25 (*c* 0.08, $CHCl_3$); IR (NaCl) ν_{max} 3260, 3252, 2965, 2916, 1729, 1647, 1631, 1549, 1426, 1254, 992 cm^{-1}; ^1H and ^{13}C NMR (500 MHz, $CDCl_3$) see Table S3 in the Supporting Information; UV λ_{max} CH_3CN (ε) 233 nm (19 700); CD (MeOH) λ 215 (Δε 2.7) λ 237 (Δε −4.2); FABHRMS $[M + H]^+$ *m/z* 741.3337 (calcd for $C_{32}H_{50}N_8O_{10}{}^{35}Cl$, 741.3333)

NaOMe Methanolysis of 1. To 1.8 mg of **1** were added 2 mL of MeOH and 54 mg of NaOMe (0.5 M). The reaction was stirred at room temperature for 18 h, neutralized with 6 N HCl, dried, and partitioned between hexane/H_2O. The hexane fraction, which contained the methyl ester **4** (LRESIMS $[M + Na]^+$ *m/z* 781.4), was purified by HPLC with use of a semi-prep column [C_{18} (5 μm; 100 × 50 mm)] and isocratic conditions (50% MeOH in H_2O) at 3.0 mL/min.

Acid Hydrolysis of 1. To 3.0 mg of **1** was added 3.0 mL of 6 N HCl and the reaction mixture was stirred at 110 °C for 16 h. The total reaction mixture was then dried in vacuo. The hydrolysate was directly used in the absolute stereochemistry determination of (*S*)-αMeSer. These same conditions were used to form the hydrolysate that was subsequently hydrogenated and used to determine the absolute stereochemistry of the (*S*)-AMNA residue.

Hydrogenation of 1. To 1.7 mg of the acid hydrolysate of **1** were added 170 μL of EtOH, 100 μL of MeOH, and ca. 10 mg of $Pt(IV)O_2$. The solution was purged with H_2, sealed, and then stirred at room temperature for 8 days at 47 psi H_2. The catalyst was then filtered and the solvent removed in vacuo.

Preparation of (*R*)-αMeSer FDAA Standards. To 0.5 mg of (*R*)-αMeSer were added 50 μL of H_2O, 20 μL of 1 N $NaHCO_3$, and in separate experiments 0.05 mg of L-FDAA or L+D-FDAA in acetone. Following incubation at 80 °C for 10 min, the reaction mixture was cooled, quenched with 20 μL of 2 N HCl, dried under N_2, and redissolved to 5 mg/mL in MeOH. Analysis was achieved by reversed phase HPLC [C_{18} (0.5 μM)], using a 10−100% CH_3CN in H_2O gradient over a 120 min period (trifluoroacetic acid was added to all buffers to a final concentration of 0.1 M). Elution was monitored by 210, 254, and 340 nm UV detection and ionization achieved in positive mode with a capillary voltage of 6000 V. Formic acid (0.1 N) was introduced to the ionization chamber during each run at a flow of 0.1 mL/min. The separation and ionization conditions stated above were identical for all Marfey's analysis reported herein and all elution times can be found in Table 2.

Marfey's Derivatization of 1: Determination of (*S*)-αMeSer. To 0.65 mg of the acid hydrolysis product of **1** were added 65 μL of H_2O, 26 μL of 1 N $NaHCO_3$, and in separate experiments 0.065 mg of L-FDAA or L+D-FDAA in acetone. Following incubation at 80 °C for 10 min, the reaction mixture was cooled, quenched with 26 μL of 2 N HCl, dried under N_2, and redissolved to 5 mg/mL in MeOH.

Marfey's Derivatization of 1: Determination of (*S*)-AMNA. To 0.5 mg of the hydrogenated acid hydrolysis product of **1** were added 50 μL of H_2O, 20 μL of 1 N $NaHCO_3$, and 0.05 mg of L-FDAA or L+D-FDAA in acetone. Following incubation at 80 °C for 10 min, the reaction mixture was cooled, quenched with 20 μL of 2 N HCl, dried under N_2, and redissolved to a concentration of 5 mg/mL in MeOH.

Tris-MTPA Ester of Piperazimycin A (1). To 1.0 mg of **1** was added 1.0 mL of anhydrous CH_2Cl_2 and the solution was mixed for 10 min at 25 °C under an atmosphere of argon. Triethylamine

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(2 μL) was then added, and the resulting mixture was stirred at 25 °C for 15 min. Six small crystals of DMAP were then added and the resulting mixture was stirred for 1 h. Twenty microliters of (*R*)- or (*S*)-MTPACl was then added, and the mixture was stirred at 25 °C for 1 h. Five-hundred microliters of pyridine was then added, and the reaction mixture was stirred for 16 h at 25 °C. The respective (*R*)- or (*S*)-Tris-MTPA esters of **1** were independently purified by HPLC, using a semi-prep column [C_{18} (5 μm; 10 mm $\times$ 50 mm)] with a linear gradient (10−100% CH_3CN in H_2O) over 45 min, followed by isocratic (100% CH_3CN) conditions for 20 min.

Crystallization Method. Approximately 3.0 mg of **1** was placed in a polyspring glass insert with 100 μL of MeOH and sealed inside an amber vial. The septum of the vial was pierced with a 21 gauge needle and the solution was evaporated for 16 h at 25 °C. The resulting solution contained numerous small, clear crystals (mp 222−225 °C).

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Supporting Information Available: All supplemental information noted in the text, 1D and 2D NMR spectral data for **1**−**4**, and HRMS data for **1**−**3**. This material is available free of charge via the Internet at http://pubs.acs.org.

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