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A New Structural Theme in the Imidazole-Containing Alkaloids from a Calcareous *Leucetta* Sponge

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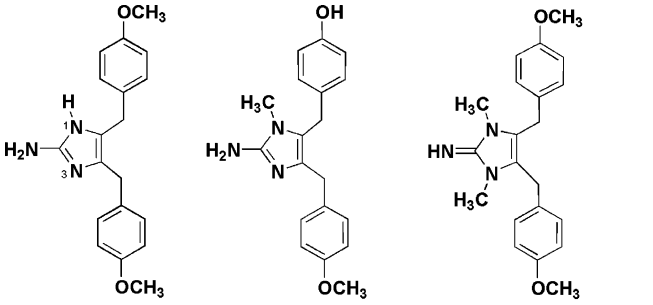
Further study of the Fijian sponge *Leucetta* sp., a source of (+)-calcaridine A (**4**) and (−)-spirocalcaridines A (**5**) and B (**6**), has yielded (−)-spiroleucettadine (**8**), the first natural product to contain a fused 2-aminoimidazole oxalane ring along with the known compounds *N,N*-dimethylnaamidine D (**3**) and isonaamine B (**7**). NMR analysis allowed the unambiguous 2D structural assignment of **8**, and its relative stereochemistry was determined by ROESY data. Good antibacterial activity was observed for **8** against *Enterococcus durans* with an MIC of less than 6.25 μg/mL.

Introduction

Natural products from sponges continue to dominate the literature of new molecular structures discovered annually from marine sources.[1] While biologically active sponge-derived alkaloids are prominently represented and attract much attention, they are not the largest category of natural products encountered. In this regard, the study of calcareous sponges can be rewarding as they are pan-oceanic and appear to be an unending source of unusual alkaloids.[2] In a recent report,[3] we commented on the accumulated chemical understanding of two Calcerea genera, *Leucetta* and *Clathrina*,[4] and their associated *Notodoris* nudibranchs.[5] Their alkaloids consist of compounds with a 2-aminoimidazole core, and these structures span four structural categories defined by the substitution position of a *p*-oxybenzyl moiety and the presence or absence of an imidazoledione group.[3]

The extent to which the central imidazole of such marine natural products occurs with additional methylation is an issue not previously considered. The ubiquitous compound from *Leucetta*, naamine D (**1**),[6] represents a possible biosynthetic starting point for such less common structures. The spectroscopic properties of **1** are consistent with the 1,3-conjugated tautomer shown here, and a similar situation prevails for the ***N-1*** monomethylated analogue, naamine A (**2**).[7] Some bismethylated derivatives are known, and each example contains the methylation pattern of *N*,*N*-dimethylnaamine D **(3)**,[3] whose imidazole ring must exist as the tautomer shown. Enigmatically, a biosynthetic possibility where the guanidinium nitrogen is methylated has not been encountered.



The intent of this study was to examine calcareous sponges from our repository for additional alkaloids to expand on the structural motifs discussed above. One member of this series, naamidine A (with the 2-amino H of **1** replaced by a dehydro hydantoin), is viewed as a potential molecular probe owing to its modest antitumor activity and its unique action on the mitogen-activated protein kinase pathway.[8] Thus, our goals were (a) to probe for additional polymethylated amino imidazoles, and (b) to obtain new fused polycyclic imidazoles. One of the sponges chosen for this effort was a globular shaped *Leucetta* sp. abundant in the coral reefs of Fiji and the subject of our previous report on the first nonorganome-

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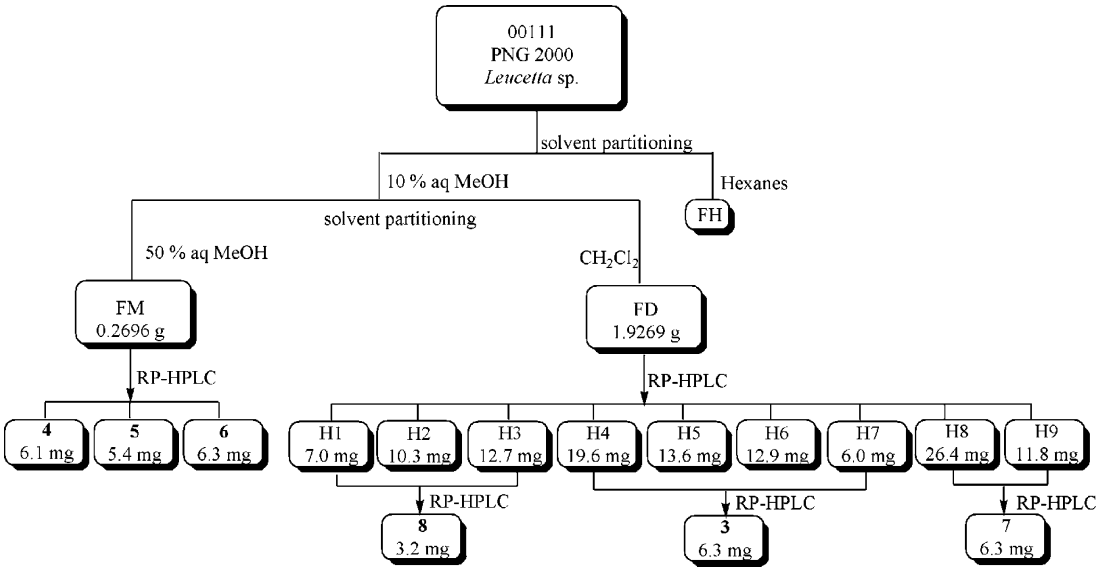
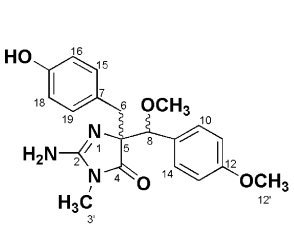
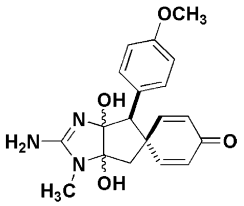
SCHEME 1. Isolation of Compounds 3-8.



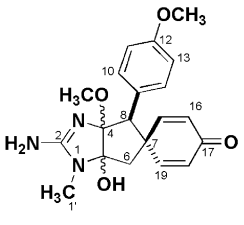
tallic chiral 2-aminoimidazoles (+) calcaridine A **(4)**, (−)-spirocalcaridine A **(5)**, and (−)-spirocalcaridine B **(6)**.[9] Outlined below is the isolation accompanied by the description of physical and biological properties of the known *N*,*N*-dimethylnaamidine D **(3)**[3] and isonaamine B (**7**)[10] along with the new alkaloid, (−)-spiroleucettadine **(8)**.



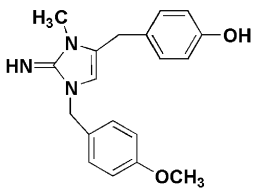
(+)-calcaridine A (**4**)



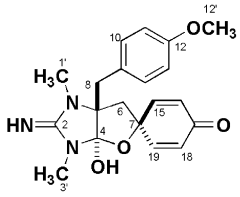
(-)-spirocalcaridine A (**5**)



(-)-spirocalcaridine B (**6**)



isonaamine B (**7**)



(-)-spiroleucettadine (**8**)

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Results and Discussion

Obtaining mass spectrometry data for the compounds to be isolated in this study was considered to be important for either rapid dereplication or a concise approach to total structure elucidation. The structures shown above have a $C_{19}H_{21}N_3O_2$ core, and the heteroatom count along with the presence or absence of diagnostic ^{13}C NMR resonances constitutes a concise way to distinguish between known and new structural types. For example, the physical and spectroscopic signatures of a 2-amino imidazole ring are a count of N_3, and a ^{13}C NMR quaternary peak in the range of δ 150−160. Secondarily, *p*-oxybenzyl groups can be pinpointed by the presence of three or more oxygen atoms along with additional sets of ^{13}C resonances including (a) quaternary carbons centered at δ 158, (b) methine peaks between δ 112−118, and (c) methylene carbons near δ 28. These preceding considerations guided our reinvestigation of the *Leucetta* sp. (coll. no. 00111) obtained from several sites south of Viti Levu, Fiji.

The outline of Scheme 1 provides an overview of the isolation work employed herein and also in the previous study of the *Leucetta* sample (coll. no. 00111).[9] Analysis of the formulas of the trio of compounds encountered from the methanol solvent partition fraction (labeled FM) of the earlier study illustrate the application of the MS and NMR data discussed above. The reversed-phase HPLC separation afforded three compounds immediately recognized as being unique on the basis of the combination of MS atom estimate of four oxygen atoms and the absence of NMR resonances in the key regions noted above. These compounds included (+)-calcaridine A (**4**), $C_{20}H_{23}N_3O_4$; (−)-spirocalcaridine A (**5**), $C_{19}H_{21}N_3O_4$; and (−)-spirocalcaridine B (**6**), $C_{20}H_{23}N_3O_4$. Probing for similar trends during this project facilitated dereplication of the structure analysis on the additional three compounds isolated.

The NMR data obtained on the CH_2Cl_2 solvent partition material (labeled FD) indicated that follow-up by preparative reversed-phase HPLC would be worthwhile. As shown in Scheme 1, there were nine fractions obtained

TABLE 1. NMR Data[a] of (−)-Spiroleucettadine (8) in MeOH-d_4

atom no.	δ_C (type)	δ_H mult, J (Hz)	gHMBC (δ_H to δ_C)	ROESY[b]
2	159.5 (C)			
4	102.5 (C)			
5	82.5 (C)			
6	46.9 (CH_2)	2.18d, 13.5	4, 5, 19	15
		2.05d, 13.5	5, 7, 15, 19	15
7	77.1 (C)			
8	37.1 (CH_2)	3.16d, 14	5, 6, 9, 10, 14	10, 14
		3.17d, 14	5, 6, 9, 10, 14	10, 14
9	127.5 (C)			
10	132.8 (CH)	7.45d, 8.5	8, 11, 14	15, 16, 8
11	112.7 (CH)	6.86d, 9	12, 13	11, 12′, 15
12	158.9 (C)			
13	112.7 (CH)	6.86d, 9	11, 12	12′, 15
14	132.8 (CH)	7.45d, 8.5	8, 10, 13	15, 16, 8
15	151.0 (CH)	5.92dd, 10.1, 2.7	7	10, 11, 13, 14, 19, 6, 6′
16	126.9 (CH)	5.93dd, 10.1, 2.2	17	10, 14
17	185.4 (C)			
18	125.9 (CH)	5.99dd, 10.1, 2.2	7	
19	150.1 (CH)	6.96dd, 10.1, 2.7	17	15, OH
1′	24.5 (CH_3)	2.38s	5	OH
3′	24.7 (CH_3)	2.77s	2, 4	
12′	54.2 (CH_3)	3.76s	12	11, 13
OH[b]		7.80s, 1H		1′, 19

[a] Measured at 500 MHz (^{1}H) and 125 MHz (^{13}C). [b] DMSO-d_6.

from the first round of HPLC. An ESI-MS peak at *m/z* 352 ($M + H^+$) corresponding to a candidate molecular formula of $C_{21}H_{25}N_3O_2$ (a difference of C_2H_4 versus **1**) was observed for fractions 4−7. These were combined and further purified by HPLC to afford the known compound *N,N*-dimethyl naamidine D (**3**).[3] The ESI-MS of fractions 8 and 9 showed a major component at *m/z* 324 $[M + H]^+$ (corresponding to the molecular formula of **1**) while NMR revealed the presence of only one OCH_3 group. After further purification and spectroscopic examination, this compound was concluded to be isonaamine B (**7**).[10] Finally, the larger mass of the ESI-MS peak at *m/z* 370 $[M + H]^+$ of fractions 1−3 provisionally matched the formulas of **4** and **6**. However, purification of the combined fractions yielded a compound whose properties were different than these compounds and it was eventually characterized as (−)-spiroleucettadine (**8**).

The structure elucidation of **8** began with confirming its molecular formula, $C_{20}H_{23}N_3O_4$ requiring 11 unsaturations, from the HR-ESI-MS *m/z* 370.17213 $[M + H]^+$. Based on arguments outlined above, a 2-amino imidazole ring was initially envisioned but the ^{13}C NMR vinylic resonances expected at δ 122 were missing as shown in Table 1. The substructure **F** shown in Figure 1 was visualized to rationalize the presence of a guanidine group (δ_C 159.5, C-2) substituted with two methyls. This proposition was justified based on characteristic *N*CH_3 signals at δ_H 2.38 and 2.77, along with the gHMBC correlation from *N*CH_3-3′ to C-2. The next step in the structure elucidation was to engage in a side-by-side comparison of the proton NMR data of (−)-spiroleucettadine (**8**) with that of (−)-spirocalcaridine B (**6**). There were two identical spin systems between these two compounds as follows. (1) The AA′XX′ spin system for the *p*-methoxy-disubstituted benzene ring of **8** was identified by symmetrical doublets at δ_H 7.45 and 6.86 and a methoxy singlet at δ_H 3.76 and (2) an AB spin system of **8** was present at δ_H 2.18 and 2.05 for the diastereotopic ring protons H-6/6′. One obvious difference

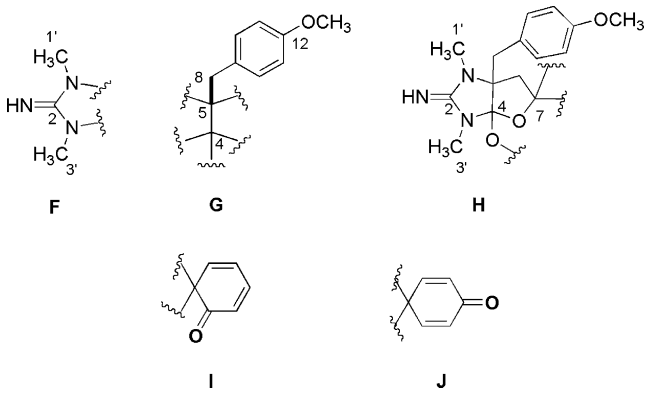

FIGURE 1. Substructures for **8**.

in the ^{1}H NMR of **8** compared to that of **6** was the isolated AB spin system at δ_H 3.16 and 3.17 that was assigned to the diastereotopic benzylic protons H-8/8′ and confirmed by gHMBC correlations to C-9 (δ_C 127.5), C-10, and 14 (δ_C 132.8). The remaining spin system in **8** was a complicated four-proton pattern in the aromatic region.

The above data along with additional 2D NMR measurements supported the assembly of additional substructures. The very low-field resonances of C-4 and C-5 suggested that these two aliphatic quaternary carbons must be attached to electron withdrawing groups as shown in substructure **G**. The bond between them was justified by the gHMBC correlations from *N*CH_3-1′ and *N*CH_3-3′ to C-5 (δ_C 82.5) and C-4 (δ_C 102.5), respectively. By analogy to **6**, C-5 (δ_C 82.5) was proposed to be attached to a nitrogen and the much lower field C-4 (δ_C 102.5) was assumed to have two attached oxygens and one nitrogen. This array could be further expanded to include the *p*-methoxy benzyl moiety based on gHMBC correlations observed from H-8/8′ to C-5 and *N*CH_3-1′ to C-5 (δ_C 82.5). Substructures **F** and **G** along with two other carbons were then merged into **H**. This was based upon gHMBC correlations from H-6 to C-4 and C-5 and from H-6′ to

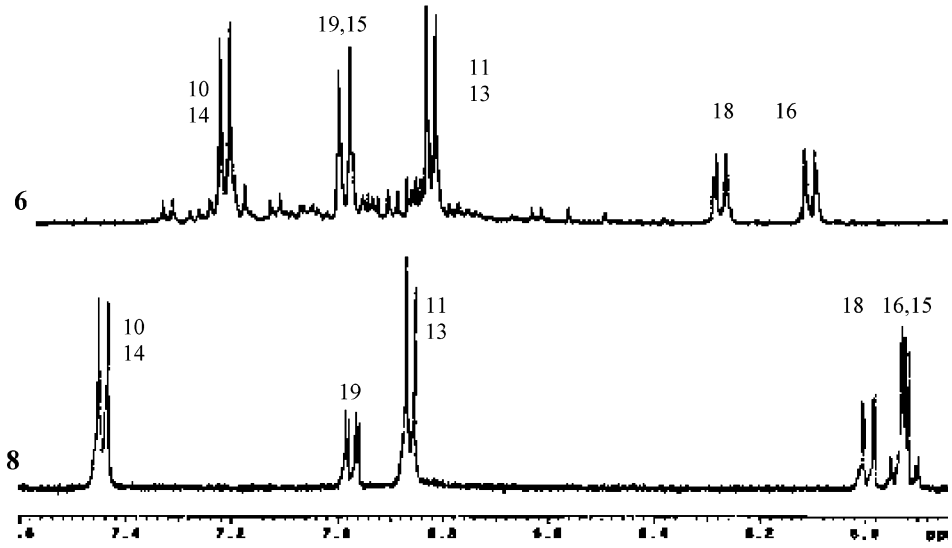

FIGURE 2. ^{1}H NMR spectra of **6** (top) and **8** (bottom) showing the double-bond region.

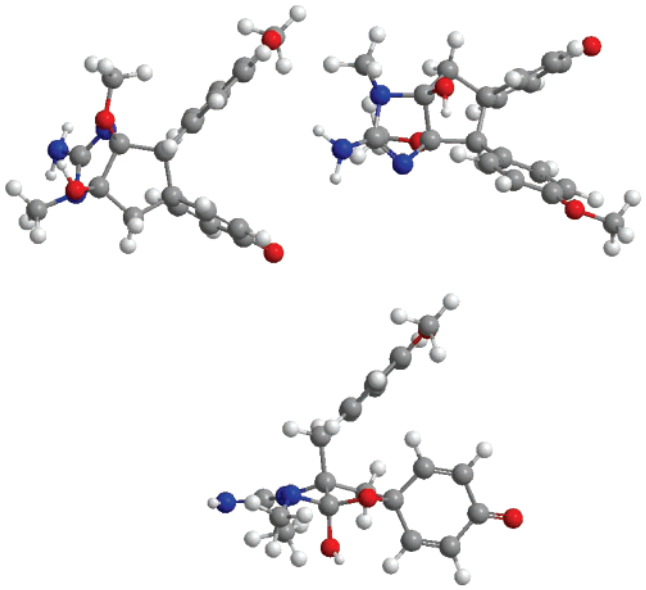

FIGURE 3. Energy-minimized structures of **6** (above: *cis*-left, *trans*-right) and **8** (below).

C-5 and C-7. While the carbon frameworks of **8** and **6** were obviously different they both possessed a spirocyclic ring junction at C-7. Finally, in **8** the low-field shift of C-7 at δ_C 77.1 required an attached oxygen, thereby completing the bicyclic 2-imino[2,3-*d*]imidazole moiety.

The remaining four degrees of unsaturation of **8** were explained by a spiroquinone functionality, which could be present as **I** or **J**. Initially, our early structures favored **I** because the four ^{1}H resonances were distinctly different than that of **6** as shown in Figure 2. The resonances of **6**, characteristic of **J**, appeared as a nicely resolved four spin system with large vicinal couplings (10 Hz) between the two sets of diastereotopic AB protons (β-Hs at δ_H 6.98 and 6.97 and α-Hs at δ_H 6.28 and 6.12). By contrast the spiroquinone protons of **8** appeared (Figure 2) as an unusual pattern consisting of a doubled doublet at δ_H 6.96 (H-19), a doubled doublet at δ_H 5.99 (H-18), and a symmetrical, complex multiplet at δ_H 5.93 (H-16) and δ_H 5.92 (H-15). Careful analysis of the vicinal *J*'s among these four protons revealed just two identical large coupling values (10.1 Hz, see Figure S3 (Supporting Information)) consistent with **J** and not with **I**. The similar ^{13}C shifts for the spiroquinone moiety of **6** and **8** further supported this conclusion. This finding was also in accord with key gHMBC correlations from H-15 (δ_H 5.92) and H-18 (δ_H 5.99) to C-7 and correlations from H-6/6′ to C-15 and C-19 and with the UV λ_{max} observed at 227 nm for **8**.

Computer modeling results shown in Figure 3 provided important insights into the different ^{1}H NMR shifts observed (see Figure 2) for the identical spiroquinone rings concluded to be present for **6** and **8**. These results also explained the unusual ^{1}H−^{1}H ROESY correlations observed in **8** from only one of the spiroquinone protons H-15 (δ_H 5.92) to the aromatic protons of the *p*-methoxy benzyl group H-10/14 (δ_H 7.45) and H-11/13 (δ_H 6.86). The minimized structure of **6** has a π-stacking arrangement between the cyclohexa-2,5-dienone and the para-disubstituted benzene rings for both the *cis*-fused and *trans*-fused isomers. In contrast, these two rings in **8** adopt an orthogonal arrangement with H-15 proximal to the center of the other aromatic ring, consistent with its 1 ppm upfield shift relative to that of H-19.

The relative stereochemistry at the chiral and single prochiral centers of **8** was established from ^{1}H−^{1}H ROESY data. These included correlations from the OH (δ_H 7.80 in DMSO-d_6) to the multiplet δ_H 6.96 (DMSO-d_6) along with the previously mentioned correlation from H-15 (δ_H 5.92) to H-10/14 (δ_H 7.45) and H-11/13 (δ_H 6.86). To verify that the OH was correlating to H-19 a solvent system of DMSO-d_6/benzene-d_6 (4:1) was employed. Its ^{1}H−^{1}H ROESY spectrum showed a correlation between the OH (δ_H 7.93) and H-19 (δ_H 6.98) and not H-11/13 (δ_H 6.91) (see Figure S6, Supporting Information). Furthermore, the computer-minimized structure of *cis*-fused **8** indicated an OH to H-10/14 distance of 2.52 Å as compared to OH to H-11/13 distance of 4.71 Å. Thus, the ^{1}H−^{1}H ROESY correlation was most likely due to the interaction between the former set, but this was not observed. In the case of *trans*-fused structure of **8**, our

calculations showed OH to H-19 distance of 2.41 Å. Thus, the OH and the *p*-methoxy benzyl group must be assigned as trans. Last, the absolute stereochemistry was determined using ORD-CD spectroscopy. The spectrum of **8** showed a weak positive Cotton effect (Figure S9, Supporting Information). This indicates a 4*R* and 5*R* stereochemistry when the structure was imbedded into an octant model (Figure S10, Supporting Information); however, the weak absorption was too ambiguous to conclusively support our assigments.

Our evaluation of the biological properties of the compounds isolated herein is still ongoing. To date, (−)-spiroleucettadine (**8**) demonstrated modest antibacterial activity against *E. coli* and *Staphylcoccus epidermitis*, with minimum inhibitory concentrations (MICs) of 200 μg/mL observed against each. Alternatively, **8** showed good antibacterial activity against *Enterococcus durans* with an MIC of <6.25 μg/mL compared with that of vancomycin (<0.625 μg/mL) and penicillin (12.5 μg/mL).

Conclusions

The discovery of (−)-spiroleucettadine (**8**) accomplished both aims of this research: (a) to expand understanding about the structural possibilities of poly-methylated imidazoles alkaloids and (b) to discover new fused polycyclic 2-aminoimidazoles. The fused spirobicyclic ring system of **8** is unprecedented and has a distant relationship to aculeatins, which have been observed in the rhizomes of *Amomum aculeatum* ROXB,[11] and gymnastatins from sponge-derived fungi.[12] In addition, the bicyclic 2-aminoimidazole oxalane core of **8** is represented in just one other natural product family, the slagenins derived from an *Agelas* sponge with a [5,5] *cis*-fused ring system.[13] However, in contrast to the slagenins, (−)-spiroleucettadine (**8**) contains a *trans*-fused bicyclic ring structure. *Trans*-fused bicyclic [5,5] ring systems are rare. Such ring systems have been isolated from the plants *Artemisia santolinifolia*[14] and *Lantana camara*.[15] While the kealiquinones,[16] also obtained from *Leucetta* sponges, possess a polycyclic fused 2-amino imidazole ring system they are quite different in overall structure and biogenetic formation in comparison to **8**. It appears that an *N*-methyl derivative of (+)-calcaridine A (**4**) may be a biosynthetic precursor of **8** as these structures are formally related by an intramolecular cyclization. Alternatively, **3** appears to be biosynthetically derived from methylation of naamine A (**1**), while **7** has a similar relationship to isonaamine A.

Experimental Section

Isolation. The sponge was preserved in the field according to our standard procedure and transported back to the laboratory at ambient temperature. The preserved sponge (6.2 kg wet wt) was extracted with MeOH three times, and the resulting oil was partitioned between hexanes and 10% aqueous methanol. The methanol layer was adjusted to 50% aqueous methanol and partitioned with CH_2Cl_2. The CH_2Cl_2 layer was evaporated in vacuo to yield a brown oil. The oil was subjected to reversed-phase HPLC using a gradient solvent system of 10% to 100% MeOH/H_2O over 60 min to afford nine fractions. Fractions 1−3 were combined and purified to yield **8** (3.2 mg). Fractions 4−7 were also combined and chromatographed on a reversed-phase column to afford **3** (6.3 mg) and fractions 8 and 9 were further purified to yield compound **7** (6.3 mg) as shown in Scheme 1.

Antibacterial Assay. Three different bacterial strains were employed including *Eschericia coli*, *Staphylococcus epidermitis* (ATTC no. 12228), and *Enterococcus durans* (ATTC no. 11576). Minimum inhibitory concentrations (MIC) against these three bacteria were measured using a micro broth dilution test in 96-well microtiter plates with 0.2 mL per well. The maximum concentration of **8** used was 400 μg/mL, and this was serially diluted down to 6.25 μg/mL. The microtiter plates were inoculated with 0.1 mL of overnight cultures that were diluted and adjusted to give concentrations of 10^5−10^6 CFU/mL (per well) to give a final volume of 0.2 mL. The 96-well microtiter plates were then incubated at 37 °C overnight for 24 h. A growth control was included to demonstrate the viability of the inoculum in each assay plate. Penicillin G and vancomycin were included as positive controls, and DMSO was also used as a negative control. The MIC values were determined by visual inspection as the minimum concentration of compound that gives 100% inhibition of bacterial growth.

***N,N,*-Dimethylnaamidine D (3):** light brown amorphous solid; HRESIMS $[M + H]^+$ obsd 352.2018 calcd for $C_{19}H_{22}N_3O_2$ 352.2025. NMR data are in accordance with literature values.[3]

Isonaamine B (7): light yellow powder; HRESIMS $[M+H]^+$ obsd 324.1684 calcd for $C_{19}H_{22}N_3O_2$ 324.169. NMR data are in accordance with literature values.[10]

(−)-Spiroleucettadine (8): yellow powder; UV (MeOH) λ_{max} 227 nm (ϵ 2407); $[\alpha]_D$ − 27.1 (*c* 0.38, MeOH); ^{1}H NMR (MeOH-d_4, 500 MHz) and ^{13}C NMR (MeOH-d_4, 125 MHz), see Table 1 and the Supporting Information; HRESIMS $[M + H]^+$ obsd 370.1721, calcd for $C_{20}H_{24}N_3O_4$ 370.1761.

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Supporting Information Available: General experimental procedures; NMR spectral (^{1}H, ^{13}C NMR, gHMBC, ROESY, and ORD CD) data of **8**. This material is available free of charge via the Internet at http://pubs.acs.org.

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