

# Ungeremine and Its Hemisynthesized Analogues as Bactericides against *Flavobacterium columnare*

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**ABSTRACT:** The Gram-negative bacterium *Flavobacterium columnare* is the cause of columnaris disease, which can occur in channel catfish (*Ictalurus punctatus*). In a previous study, the betaine-type alkaloid ungeremine, **1**, obtained from *Pancreaticum maritimum* L. was found to have strong antibacterial activity against *F. columnare*. In this study, analogues of **1** were evaluated using a rapid bioassay for activity against *F. columnare* to determine if the analogues might provide greater antibacterial activity and to determine structure–activity relationships of the test compounds. Several ungeremine analogues were prepared by hydrochlorination of the alkaloid and by selenium dioxide oxidation of both lycorine, **7**, and pseudolycorine, **8**, which yielded the isomer of ungeremine, **3**, and zefbetaine, **4**, respectively. The treatment of lycorine with phosphorus oxychloride allowed the synthesis of an anhydrolycorine lactam, **5**, showing, with respect to **1**, the deoxygenation and oxygenation of C-2 and C-7 of the C and B rings, respectively. The results of the structure–activity relationship studies showed that the aromatization of the C ring and the oxidation to an azomethine group of C-7 of the B ring are structural features important for antibacterial activity. In addition, the position of the oxygenation of the C ring as well as the presence of the 1,3-dioxole ring joined to the A ring of the pyrrolo[de]phenanthridine skeleton also plays a significant role in imparting antibacterial activity. On the basis of 24-h 50% inhibition concentration (IC<sub>50</sub>) results, ungeremine hydrochloride, **2**, was similar in toxicity to **1**, whereas **5** had the lowest activity. Analogue **2** is soluble in water, which may provide the benefit for use as an effective feed additive or therapeutic compared to ungeremine.

**KEYWORDS:** alkaloids, Amaryllidaceae, bactericides, channel catfish, *Flavobacterium columnare*, *Pancreaticum maritimum*, ungeremine

## INTRODUCTION

Farm-raised channel catfish (*Ictalurus punctatus*) currently comprise the largest segment of the freshwater aquaculture industry in the United States, and Mississippi supplies over half of the farm-raised catfish processed annually. The two most common bacterial diseases of pond-raised catfish are columnaris disease and enteric septicemia of catfish (ESC), which cause large economic losses to producers of pond-raised catfish.<sup>1</sup> Columnaris disease and ESC are caused by the Gram-negative bacteria *Flavobacterium columnare* and *Edwardsiella ictaluri*, respectively.<sup>1</sup> Although both of these bacterial pathogens can be the cause of primary infections, columnaris disease can also develop as a secondary pathogen, becoming acute with rapid mortality at rates as high as 50–60% in pond populations.<sup>1</sup>

The most common management approaches used by producers to control *F. columnare* and *E. ictaluri* are the use of medicated feeds, attenuated vaccines, and nonantibiotic therapeutics. The U.S. Food and Drug Administration has approved Aquaflor (50% w/w florfenicol active ingredient) for treating ESC and columnaris disease, currently under conditional approval, in channel catfish.<sup>2</sup> However, the use of antibiotics has several concerns such as the potential development of antibiotic-resistant strains of *E. ictaluri* and *F. columnare* and the environmental impact from the use of antibiotic-laden

feeds in agriculture, which make the future use of medicated feed in catfish aquaculture uncertain.

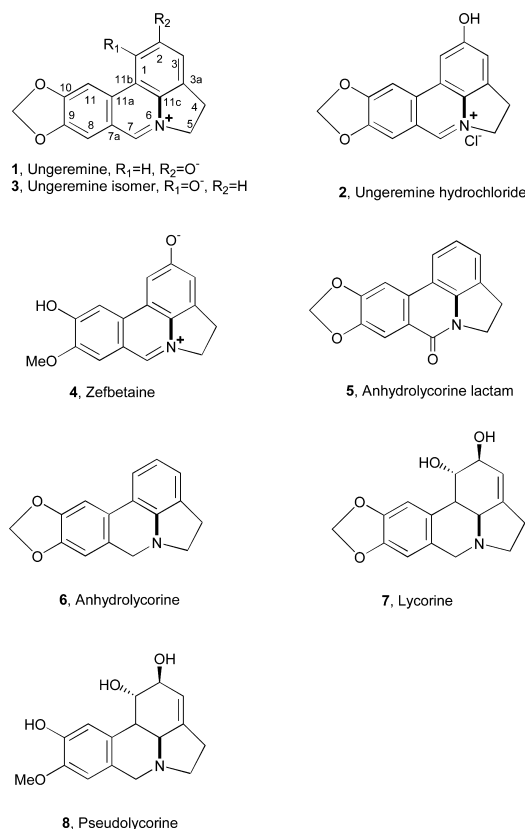
Plants remain a vastly unexplored source for antibacterial compounds, especially in the discovery of novel antibacterial compounds as alternatives to commercially available antibiotics currently used in aquaculture (e.g., Aquaflor). Previous research discovered that ungeremine had strong antibacterial activities against two isolates of *F. columnare* (ALM-00-173 and BioMed).<sup>3</sup> Ungeremine, **1** (Figure 1), is a betaine-type alkaloid isolated from *Pancreaticum maritimum* L. (family Amaryllidaceae),<sup>4</sup> and it can also be isolated from other species of Amaryllidaceae including *Ungernia minor*, *Crinum americanum*, *Crinum asiaticum*, and *Zephyranthes flava*.<sup>4</sup> In addition, ungeremine has been obtained as the main metabolite of the microbial degradation of lycorine, **7** (Figure 1), the main Amaryllidaceae alkaloid,<sup>5</sup> by an unidentified species of the bacterium *Pseudomonas* (strain ITM 311) that was isolated from the rhizosphere of *Sternbergia lutea* Ker Gawl.<sup>6</sup> This plant species is also the best source of lycorine.<sup>7</sup> Ungeremine was also obtained by selenium dioxide oxidation of lycorine by applying a modified protocol<sup>6</sup> with respect to those previously

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**Figure 1.** Chemical structures of ungeremine, 1, lycorine, 7, and respective analogues.

reported in a study on the actions of oxidizing agents on this alkaloid.<sup>8</sup>

While considering the economics of obtaining a sufficient amount of ungeremine by the oxidation of lycorine, previously isolated in large amounts from *S. lutea*,<sup>7</sup> and the possibilities of preparing ungeremine analogues from lycorine and other Amaryllidaceae alkaloids by using different oxidizing agents, we developed a structure–activity relationship (SAR) study. This study was performed in conjunction with the study to evaluate natural or hemisynthetic ungeremine analogues for potentially increased antibacterial activities and/or specificities against *F. columnare*. The results of the bioassay evaluation of the ungeremine analogues and the SAR study are reported here.

## MATERIALS AND METHODS

**General Experimental Procedures.** Melting point (mp) was measured with an Axioskop Zeiss (Oberkochen, Germany); elemental analyses were performed with a Perkin-Elmer (Norwalk, CT, USA) AA Analyst 700. IR spectra were recorded as glassy film on a Perkin-Elmer Spectrum One FT-IR spectrometer, and UV spectra were taken in MeCN solution on a Perkin-Elmer Lambda 25 UV–vis spectrophotometer.  $^1H$  and  $^{13}C$  NMR spectra were recorded at 600 and 125 MHz, respectively, in  $CDCl_3$ , on Bruker (Kalsruhe, Germany) spectrometers. The same solvent was used as internal standard. ESI MS spectra were recorded on an Agilent Technologies (Milan, Italy) 6120 quadrupole LC-MS. Analytical and preparative thin layer chromatography (TLC) were performed on silica gel plates (0.25 mm Kieselgel 60 and 0.5 mm F<sub>254</sub>; Merck, Darmstadt, Germany); the spots were visualized by exposure to UV light or by exposure to  $I_2$  vapors. Column chromatography was performed on 0.063–0.200 mm silica gel (Merck, Rahway, NJ, USA) or 40–63  $\mu m$ , 10  $\times$  240 mm, Lichroprep RP18 (Merck, Rahway, NJ, USA) working at a medium pressure of 3 bar. Pseudolycorine was generously supplied by Prof. H.

M. Fales, Department of Health, Education, and Welfare, Bethesda, MD, USA.

**Plant Materials.** Fresh bulbs of *P. maritimum* L. were collected from sandy hills on the northern coastal strip of Egypt (Baltim) during the flowering and fruit-producing stage. The plant was kindly identified by Professor Dr. N. El Hadidy, University of Cairo, Egypt. A voucher sample is deposited in the Department of Pharmacognosy, Faculty of Pharmacy, University of Alexandria, Egypt. The bulbs were dried by ventilation in an oven at 35 °C, minced, and stored at –20 °C. The plant *S. lutea* Ker Gawl, collected near Bari, Italy, during the weathering period, was identified by Prof. Arrigoni, Dipartimento di Biologia e Patologia Vegetale, Università di Bari, Italy, where a voucher specimen has been deposited. The fresh bulbs were dried, minced, and stored as reported for *P. maritimum*.

**Ungeremine, 1.** Initially, 1 was extracted with EtOH from the dried and minced bulbs of *P. maritimum* L. (1.6 kg) as previously reported.<sup>4</sup> The crude extract was submitted to different steps of extraction followed by chromatography as previously reported<sup>4</sup> to give 1 (15 mg) as brilliant orange needles, which were recrystallized from MeOH/ $C_6H_6$ ; mp was 267–271 °C, which is very close to the reference mp of 268–270 °C.<sup>4</sup> Compound 1 was also obtained by oxidation of lycorine using a modified procedure<sup>6</sup> with respect to that previously reported.<sup>8</sup> Briefly, a suspension of lycorine (375 mg) and sublimed  $SeO_2$  (250 mg) in EtOH (3 mL) was warmed on a steam bath to effect solution. After the precipitation of selenium metal, the reaction was heated under reflux for 4 h and filtered while hot. The partial evaporation of ethanol under vacuum left a yellow solid. The solution was dissolved in water and treated with 4 N NaOH to give yellow flocculi. The crude betaine was collected by centrifugation (10000 rpm for 45 min). Crystallization from EtOH yielded light orange needles of 1 (76 mg): IR  $\nu_{max}$  1649, 1615, 1592, 1449, 1410, 1013  $cm^{-1}$ ; UV  $\lambda_{max}$  nm (log  $\epsilon$ ) 369 (3.40), 287 (3.75), 261 (4.06); ESI MS (+)  $m/z$  266 [M + H]<sup>+</sup>. The  $^1H$  and  $^{13}C$  NMR data are very similar to those previously reported.<sup>4,6</sup>

**Ungeremine Hydrochloride, 2.** Compound 1 (20 mg) dissolved in water was treated with 1 N HCl (2 mL) at room temperature. A pale yellow precipitate was immediately generated and collected by centrifugation (10000 rpm  $\times$  30 min). The solid was dried as previously reported<sup>8</sup> at 137 °C to give derivative 2 as yellow needles (15 mg). Elemental analysis: C, 63.62; H, 4.06; Cl, 11.72; N, 4.60; O, 15.93 [calcd for  $C_{16}H_{12}ClNO_3$ , C, 63.69; H, 4.01; Cl, 11.75; N, 4.64; O, 15.91 (Fales et al.<sup>8</sup> found  $C_{16}H_{11}NO_3 \cdot HCl \cdot H_2O$ , C, 59.69; H, 4.33; N, 5.28)]. ESI MS (+)  $m/z$  266 [M + H]<sup>+</sup>.

**Ungeremine Isomer, 3.** According to a modified procedure with respect to that previously reported,<sup>8</sup> lycorine (250 mg) was dissolved in warm 10% of AcOH (10 mL), and then  $Hg(OAc)_2$  (700 mg) was added. The solution was heated on a steam bath for 3 min, and a dense precipitate of  $Hg(OAc)_2$  was formed. After 45 min, the reaction filtered hot and the 10% HOAc was removed by vacuum evaporation. The residue (676 mg) was purified by column RP-18 Lichroprep column, using as solvent  $H_2O/CH_3CN$  (8:2, v/v), giving three groups of homogeneous fraction. The residue (13.3 mg) of the second fraction showed the presence of two compounds, which were purified by preparative TLC on silica gel using as solvent BuOH/ $H_2O$ /AcOH (3:1:1, v/v/v), subsequently yielding 1 (9.7 mg,  $R_f$  0.49) and its isomer, 3 (2.7 mg,  $R_f$  0.42). Compound 3 had the following elemental analysis: C, 72.51; H, 4.23; N, 5.21; O, 18.13 (calcd for  $C_{16}H_{11}NO_3$ , C, 72.45; H, 4.18; N, 5.28; O, 18.09). ESI MS (+)  $m/z$  266 [M + H]<sup>+</sup>.

**Zefbetaine, 4.** According to a modified procedure with respect to that previously reported,<sup>4</sup> a suspension of pseudolycorine, 8 (15 mg), and sublimed  $SeO_2$  (9 mg) in EtOH (1 mL) was warmed on a steam bath to effect solution. After the precipitation of selenium metal, the reaction was heated under reflux for 4 h and filtered while hot. The solid residue (20 mg) left by evaporation of the ethanol was purified by preparative TLC on silica gel using as solvent  $CHCl_3/MeOH/EtOAc$  (2:2:1, v/v/v) giving 4 as an amorphous yellow solid (5 mg,  $R_f$  0.47): IR  $\nu_{max}$  2984, 1652, 1612, 1576, 1452, 1410, 1013  $cm^{-1}$ ; UV  $\lambda_{max}$  nm (log  $\epsilon$ ) 411 (3.21), 265 (3.59), 220 (4.00); ESI MS (+)  $m/z$  268 [M + H]<sup>+</sup>. The  $^1H$  and  $^{13}C$  NMR data are very similar to those previously reported.<sup>4</sup>

**Anhydrolycorine Lactam, 5.** According to a previously reported procedure,<sup>9</sup> a mixture of 200 mg of lycorine and 680 mg of POCl<sub>3</sub> (414  $\mu$ L) was refluxed at 100 °C for 15 min. The mixture was poured dropwise into ice-cold water (1 mL) to provide a complete solution. This solution was basified with 10% NaOH (50  $\mu$ L) and 10% Na<sub>2</sub>CO<sub>3</sub> (75  $\mu$ L) and then extracted with ethyl ether (3  $\times$  3 mL). The combined ether extracts were dried (Na<sub>2</sub>SO<sub>4</sub>), filtered, and evaporated under reduced pressure. The residue was dissolved in MeOH (2 mL), and then 10% KOH (200 mL) was added. After reflux for 1 h, the reaction mixture was diluted with H<sub>2</sub>O (204  $\mu$ L) and extracted (3  $\times$  5 mL) with a solution of C<sub>6</sub>H<sub>6</sub>/ethyl ether (10:3, v/v). The organic extracts were combined, dried (Na<sub>2</sub>SO<sub>4</sub>), filtered, and evaporated under reduced pressure. The residue (33 mg) was purified by preparative TLC on silica gel using as solvent solvent CHCl<sub>3</sub>/MeOH/EtOAc (2:2:1, v/v/v), yielding the derivative 5 (4.3 mg) as an amorphous solid: IR  $\nu_{\max}$  1630, 1610, cm<sup>-1</sup> [Hänsel and Thober<sup>10</sup> found IR (KBr) 3045, 2975, 2915, 2780, 1640, 1620, 1585, 1505, 1495, 1470, 900–700 cm<sup>-1</sup>]; UV (EtOH)  $\lambda_{\max}$  nm (log  $\epsilon$ ) 341 (3.88), 324 (3.88), 309 (3.88), 273 (4.24), 246 (4.54), 242 (4.58). The <sup>1</sup>H data are very similar to those previously reported.<sup>6</sup> Elemental analysis: C, 72.23; H, 4.60; N, 5.19; O, 17.94 (calcd for C<sub>16</sub>H<sub>12</sub>NO<sub>3</sub>, C, 72.17; H, 4.54; N, 5.26; O, 18.03). ESI MS (+)  $m/z$  266 [M + H]<sup>+</sup>.

**Lycorine, 7.** Lycorine was obtained by extraction with 1% H<sub>2</sub>SO<sub>4</sub> from dried bulbs of *S. lutea*. The free base obtained by alkalization of the acid extracts was crystallized as white prisms as detailed previously reported (11 g/kg of dried plant bulbs).

**Bactericide Bioassay.** Two isolates of *F. columnare* [ALM-00-173 (genomovar II) and BioMed (genomovar I)] were obtained from Dr. Covadonga Arias (Department of Fisheries and Allied Aquacultures, Auburn University, AL, USA). Purity of cultures of the *F. columnare* isolate was assured by streaking the bacteria for isolation onto modified Shieh (MS) agar plates (pH 7.2–7.4)<sup>11</sup> and then checking after incubation at 29  $\pm$  1 °C for 3–5 days that only one bacterial colony type was present. Prior to conducting the bioassay, we used single colonies of the test cultures to prepare the assay culture materials by culturing each isolate in 75 mL of modified Shieh broth (18 h for BioMed and 24-h for ALM-00-173) at 29  $\pm$  1 °C at 150 rpm on a rotary shaker. Test compounds were evaluated for antibacterial activity using a rapid 96-well microplate bioassay<sup>12</sup> of which procedures are briefly described below. Florfenicol and oxytetracycline HCl (Sigma-Aldrich, St. Louis, MO, USA), antibiotics that are utilized in medicated feed, were included as positive drug controls for each bioassay. In addition, control wells (no test compound or solvent added) and wells containing ungeremine were included in each assay. Technical grade methanol was used to dissolve the test compounds, except that technical grade methylene chloride was used to dissolve 5. Final concentrations of test compounds and each drug control in the microplate wells were 0.01, 0.1, 1.0, 10.0, 100.0, and 1000.0  $\mu$ M, and three replications were used for each dilution of each test compound and controls. To determine the 24-h 50% inhibition concentration (IC<sub>50</sub>) and minimum inhibition concentration (MIC) of each test compound, sterile 96-well polystyrene microplates (Corning Costar Corp., Acton, MA, USA) with flat-bottom wells were used to conduct the bioassay. Dissolved test compounds were added to microplate wells (10  $\mu$ L/well), and the solvent was allowed to completely evaporate at room temperature before 0.5 MacFarland bacterial culture<sup>12</sup> was added to the microplate wells (200  $\mu$ L/well). Microplates were maintained at 29  $\pm$  1 °C in an incubator. A SpectraCount microplate photometer (Packard Instrument Co., Meriden, CT, USA) was used to measure the absorbance (630 nm) of the microplate wells at time 0 and 24-h. The means and standard errors of absorbance measurements were calculated, graphed, and compared to controls to determine the 24-h IC<sub>50</sub> and MIC for each test compound.<sup>12</sup> The 24-h IC<sub>50</sub> and MIC results for each test compound were divided by the respective 24-h IC<sub>50</sub> and MIC results obtained for the positive controls florfenicol and oxytetracycline to determine the relative-to-drug-control florfenicol (RDCF) and relative-to-drug-control oxytetracycline (RDCO) values.

## RESULTS AND DISCUSSION

Ungeremine, 1 (Figure 1), was obtained from bulbs of *P. maritimum* L. and also by selenium dioxide oxidation of lycorine as previously reported.<sup>4,6</sup> Lycorine, 7 (Figure 1), was obtained as white prisms in large amount from bulbs of *S. lutea* as previously reported.<sup>7</sup> Compound 1 was converted into its hydrochloride, 2 (Figure 1), by treatment with HCl, whereas its isomer, 3 (Figure 1), was obtained by oxidation of 7 with Hg(AcO)<sub>2</sub> by applying a modified protocol with respect to that previously reported.<sup>8</sup> Zefbetaine, 4 (Figure 1), another betaine-type alkaloid previously isolated from *P. maritimum* L. and also from *Z. flava*,<sup>13</sup> was obtained by selenium dioxide oxidation of pseudolycorine, 8 (Figure 1), another Amaryllidaceae alkaloid<sup>5</sup>, by applying a modified protocol with respect to that previously reported.<sup>4</sup> The physical and spectroscopic data of 1, its analogues 2 and 3, and 4 were in full agreement with those previously reported<sup>4,6,8</sup> and confirmed by ESI MS as reported in detail in the corresponding experimental paragraphs. Compound 2 differs from 1 only by the presence of a quaternary protonated nitrogen atom that, depending on the pH, could be reversibly reconverted into the nonprotonated form. The isomer 3 differs from 1 substantially by the oxygenation of the aromatized C ring, the negatively charged oxygen being at C-1 in 3 instead of at C-2 as in 1. Finally, 4 differs from 1 only by the reductive opening of the dioxole ring joined to the A ring, which in 4 was converted so that the hydroxy and methoxy groups were bonded to C-9 and C-10, respectively. Finally, in the attempt to prepare anhydrolycorine, 6 (Figure 1), by reaction of 7 with POCl<sub>3</sub>, anhydrolycorine lactam, 5 (Figure 1) was obtained. Its physical and spectroscopic data were in full agreement with those previously reported.<sup>6,10,14</sup>

Compound 1 and its hemisynthetic analogues 2–5 were assayed against *F. columnare* [isolate ALM-00-173 (genomovar II) and isolate BioMed (genomovar I)] as reported in detail under Materials and Methods, and the results are reported in Table 1. The strong bactericidal activities of 1 previously observed<sup>3</sup> were confirmed again. The 24-h IC<sub>50</sub> results of 1 for *F. columnare* ALM-00-173 and *F. columnare* BioMed were 0.54  $\pm$  0.06 and 2.15  $\pm$  0.05  $\mu$ M, respectively. Genomovar II isolates such as *F. columnare* ALM-00-173 are more pathogenic for channel catfish.<sup>15</sup> For both isolates, the MIC of 1 was 1.0  $\pm$  0  $\mu$ M. As expected, 2 showed similar bactericidal activity as 1. The 24-h IC<sub>50</sub> results of 2 for *F. columnare* ALM-00-173 and *F. columnare* BioMed were 1.8  $\pm$  0 and 3.0  $\pm$  0.1  $\mu$ M, respectively, whereas the MIC results were 1.0  $\pm$  0 and 10.0  $\pm$  0  $\mu$ M, respectively. The 24-h IC<sub>50</sub> relative-to-drug control (RDC) values were slightly higher for 2 compared to 1. It is believed that 2 in solution resulted in conversion of the nitrogen atom into the nonprotonated form, which would thereby result in 2 being converted into 1. These results agreed with those previously observed when 7 and its hydrochloride salt were assayed for their ability to inhibit the ascorbic acid biosynthesis in potato tubers<sup>16</sup> and when they were assayed more recently for their anticancer activity on different tumor cell lines.<sup>17</sup>

The isomer 3 was significantly less active than 1 against both isolates of *F. columnare*, and this could substantially depend on the different position of the negatively charged oxygen atom on the C ring. In fact, it was bonded at C-2 in 1 and at C-1 in 3. The 24-h IC<sub>50</sub> results of 3 for *F. columnare* ALM-00-173 and *F. columnare* BioMed were 14.6  $\pm$  11.4 and 31.0  $\pm$  0  $\mu$ M,



Table 1. Evaluation of the Toxicities of Ungeremine and Its Analogues against *Flavobacterium columnare* Isolates

compound	ALM-00-173 <i>Flavobacterium columnare</i> isolate						BioMed <i>Flavobacterium columnare</i> isolate					
	24-h IC <sub>50</sub> <sup>a</sup>			MIC			24-h IC <sub>50</sub>			MIC		
	24-h IC <sub>50</sub> <sup>a</sup>	RDCF <sup>b</sup>	RDCO <sup>c</sup>	MIC <sup>d</sup>	RDCF	RDCO	24-h IC <sub>50</sub>	RDCF	RDCO	MIC	RDCF	RDCO
ungeremine, <b>1</b>	0.54 ± 0.06	0.28 ± 0.03	0.27 ± 0.03	1.0 ± 0	1.0 ± 0	1.1 ± 0	2.15 ± 0.05	0.91 ± 0.03	1.27 ± 0.03	1.0 ± 0	1.0 ± 0	1.1 ± 0
ungeremine hydrochloride, <b>2</b>	1.8 ± 0	0.9 ± 0	0.9 ± 0	1.0 ± 0	1.0 ± 0	1.1 ± 0	3.0 ± 0.1	1.3 ± 0.02	1.7 ± 0.03	10.0 ± 0	10.0 ± 0	10.8 ± 0
ungeremine isomer, <b>3</b>	14.6 ± 11.4	7.6 ± 5.9	7.2 ± 5.6	10.0 ± 0	10.0 ± 0	10.8 ± 0	31.0 ± 0	13.1 ± 0	18.2 ± 0	100.0 ± 0	100.0 ± 0	107.5 ± 0
zefbetaine, <b>4</b>	45.5 ± 4.5	18.4 ± 1.2	19.9 ± 4.7	100.0 ± 0	100.0 ± 0	107.5 ± 0	15.0 ± 2.0	6.0 ± 0.3	10.1 ± 0.1	10.0 ± 0	10.0 ± 0	10.8 ± 0
DHAL-HCl, <b>5</b>	37.0 ± 13.0	24.7 ± 8.7	19.3 ± 6.4	10.0 ± 0	10.0 ± 0	10.8 ± 0	195.0 ± 5.0	52.8 ± 1.5	63.7 ± 2.3	100.0 ± 0	100.0 ± 0	107.5 ± 0
lycorine, <b>7</b>	95.0 ± 0	49.2 ± 0	46.8 ± 0	100.0 ± 0	100.0 ± 0	107.5 ± 0	190.0 ± 0	80.5 ± 0	58.8 ± 0	100.0 ± 0	100.0 ± 0	107.5 ± 0

<sup>a</sup>Mean 50% inhibition concentration (μM, ± standard error of the mean) of growth after 24-h of incubation. <sup>b</sup>Relative-to-drug-control florfenicol; mean values above and closer to "1.0" indicate lower antibacterial activity, whereas mean values below "1.0" indicate the strongest activity and greater antibacterial activity compared with that of florfenicol. Standard error of the mean is the given. <sup>c</sup>Relative-to-drug-control oxytetracycline; mean values above and closer to "1.0" indicate lower antibacterial activity, whereas mean values below "1.0" indicate the strongest activity and greater antibacterial activity compared with that of oxytetracycline. Standard error of the mean is given. <sup>d</sup>Mean minimum inhibition concentration (μM) of growth after 24-h of incubation. Standard error of the mean is given. <sup>e</sup>7-Dehydro-7-hydroxyanhydrolycorinium chloride. <sup>f</sup>Values are derived from ref 18.

respectively, and MIC results were 10.0 ± 0 and 100.0 ± 0 μM, respectively (Table 1).

Compounds **4** and **5** were among the least active ungeremine analogues. For *F. columnare* ALM-00-173 and *F. columnare* BioMed, the 24-h IC<sub>50</sub> values of **4** were 45.5 ± 4.5 and 15.0 ± 2.0 μM, respectively, whereas the MIC values of **4** were 100.0 ± 0 and 10.0 ± 0 μM, respectively (Table 1). On the basis of the 24-h IC<sub>50</sub> results, **5** was less active against *F. columnare* BioMed than against *F. columnare* ALM-00-173, with 24-h IC<sub>50</sub> values of 195.0 ± 5.0 and 37.0 ± 13.0 μM, respectively. A previous study<sup>18</sup> determined that the 24-h IC<sub>50</sub> values of **7** for *F. columnare* ALM-00-173 and *F. columnare* BioMed were 95.0 ± 0 and 190 ± 0 μM, respectively, whereas the MIC value for both isolates was 100.0 ± 0 μM (Table 1). Therefore, **7** was determined to also be among the least active compounds.

These results showed that the 1,3-dioxole ring, lacking in **4** as consequence of its reductive opening, plays a significant role in providing bactericidal activity. The reduced activity of derivative **5** was due to the oxidation of H<sub>2</sub>C-7 to the carbonyl group generating the corresponding tertiary amide, which could probably be converted into the corresponding enol form under acidic conditions. The modifications of **5** determine the loss of the betaine nature present in **1**, which is a fundamental structural feature for the activity. These results are in disagreement with the results of previous structure–activity relationship studies such as the testing of antibiotic activity against *Corynebacterium fascians*<sup>6</sup> and the study on the inhibition of ascorbic acid biosynthesis in potato tubers using lycorine and several hemisynthetic derivatives and analogues, which demonstrated that the different oxidation state of C-7 did not affect the activity.<sup>19</sup> Instead, the lactam nature of C-7 as in **5** determined the strong reduction of activity when it was assayed in comparison with lycorine against *Plasmodium falciparum*.<sup>14</sup> This information may prove useful in future mode-of-action studies for **1** as related to its antibacterial activity against *F. columnare*.

Overall, none of the ungeremine analogues were more active than **1** against either isolate of *F. columnare*. However, **2** was nearly as active as **1** against both isolates on the basis of 24-h IC<sub>50</sub> results; **2** was less active than **1** against *F. columnare* BioMed, whereas **2** had the same activity as **1** against *F. columnare* ALM-00-173 on the basis of MIC results. Analogue **2** does provide water solubility to the parent compound ungeremine, which is an important consideration in terms of an adequate delivery mechanism (e.g., feed additive or therapeutic) to test fish during any future efficacy studies.

In conclusion, the results of the SAR studies showed that the aromatization of the C ring as well as the oxidation to azomethine group of C-7 of the B ring, which determines the betaine nature of ungeremine, are structural features important for the bactericidal activity. The position of the oxygenation of the C ring as well as the presence of the 1,3-dioxole ring joined to the A ring of the pyrrolo[de]phenanthridine skeleton also plays a significant role in providing antibacterial activity.

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## Notes

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