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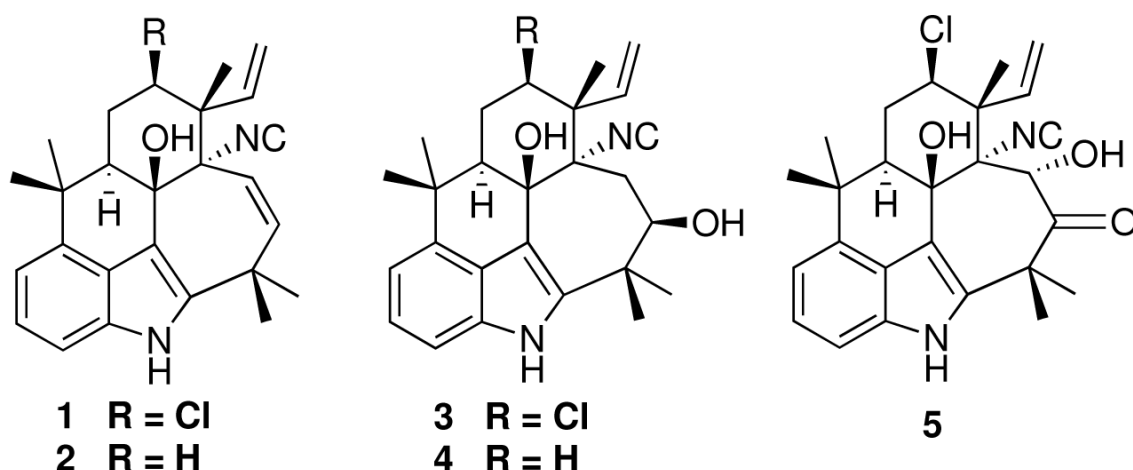
Antimicrobial Ambiguine Isonitriles from the Cyanobacterium

Fischerella ambigua

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Abstract



Five new antibacterial ambiguity K-O isonitriles (**1-5**) and nine previously described indole alkaloids were isolated from the cultured cyanobacterium *Fischerella ambigua* (UTEX 1903) by bioassay-guided fractionation. The planar structures of the new compounds were determined by spectroscopic analysis including MS, 1D- and 2D-NMR. X-ray crystallography was used to determine the absolute stereo-configuration of ambiguity K isonitrile. The isolates were evaluated for their antibacterial activities against a set of bacterial targets, including *Mycobacterium tuberculosis* and *Bacillus anthracis*. Ambiguine K and M isonitriles showed the most potent activity against *M. tuberculosis* with MIC values of 6.6 μ M and 7.5 μ M, respectively. Ambiguine A isonitrile showed the most potent activity against *B. anthracis* with a MIC of 1.0 μ M.

Cyanobacteria (blue-green algae) are known to be a rich source of secondary metabolites with diverse chemical structures and biological activities.¹⁻⁶ To date, over 1000 natural products of cyanobacterial origin have been reported.¹⁻⁶ These include many biologically active compounds with anticancer, antimicrobial, antiviral, and immunosuppressive activities.^{7,8} Branched filamentous cyanobacteria belonging to the order Stigonematales are known to produce antibacterial, antifungal and antialgal isonitrile containing indole alkaloids, such as

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Supporting Information available: A photomicrograph of *Fischerella ambigua* (UTEX 1903), tables with the complete NMR data of **1-5**, 1D NMR spectra of **1-5**, and ¹H NMR data of known compounds reported in this paper. This material is available free of charge via the internet at <http://pubs.acs.org>.

hapalindoles,⁹⁻¹⁴ ambiguine isonitriles,^{9,15,16} fischerindoles,¹⁷ and wetwitindolinones.^{18,19} These alkaloids all have tetra- or pentacyclic carbon skeletons derived from tryptophan and geranyl diphosphate.^{14,18} The ambiguine isonitriles contain an additional 1,1-dimethylallyl moiety at the 2 position of the indole ring, which often undergoes further cyclization to form the seven-membered ring observed in many ambiguine isonitriles.²⁰ Their promising biological activities and complex chemical structures have led to several synthetic efforts towards this class of alkaloids since Moore *et al.* originally discovered them from cyanobacteria.²⁰⁻³¹

In our preliminary studies the crude extract of *Fischerella ambigua* (Nageli) Gomont (UTEX 1903) showed significant antibacterial activity against *Mycobacterium tuberculosis* and *Bacillus anthracis*. Earlier investigations of *F. ambigua* (UTEX 1903), by Moore *et al.* led to the isolation of several ambiguine isonitriles and hapalindoles.¹⁵ Initial LC-MS dereplication indicated the presence of a number of previously described ambiguine isonitriles in the extract.³² In addition, the dereplication indicated the presence of several potentially novel ambiguine isonitriles. Bioassay-guided fractionation led to the isolation of fourteen antibacterial alkaloids including five new ambiguine isonitrile derivatives as well as nine previously described indole alkaloids.

Results and discussion

F. ambigua was mass cultured in Allen medium.³³ The freeze-dried biomass was extracted with CH₂Cl₂:MeOH (1:1) to yield a crude extract which showed significant antibacterial activity. Five new ambiguine isonitriles (**1-5**), and eight previously reported alkaloids, ambiguine A-C, E, F, and I isonitriles, hapalindoles G and H, were isolated using silica gel, Sephadex LH-20, and reversed phase chromatography. The chemical structures were identified by analysis of 1D and 2D NMR, MS, and X-ray data.

Ambiguine K isonitrile (**1**) was obtained as colorless needles (MeOH). The UV spectrum indicated the presence of an indole moiety [λ_{max} (log ϵ) 230 (2.72), 275 (2.11)]. The presence of an isonitrile moiety was evident from both ¹³C NMR (δ 158.2) and IR (ν_{max} 2125 cm⁻¹) data. The ESI mass spectrum of **1** exhibited a 3:1 ion cluster at m/z 443/445 [$M+\text{Na}$]⁺, indicating the presence of one chlorine atom. The HRMS gave a quasi-molecular ion [$M-\text{H}$]⁻ at m/z 419.18979 for the molecular formula of C₂₆H₂₉ClN₂O. Various features of the ¹H (Table 1) and ¹³C NMR (Table 2) chemical shift data suggested that **1** was an ambiguine isonitrile derivative.

Analysis of the ¹H, COSY (Figure 1), and HSQC spectra demonstrated the presence of a 1,2,3-trisubstituted aromatic moiety with signals at δ_{H} 6.98 (H-5), δ_{H} 7.07 (H-6), δ_{H} 7.09 (H-7), vinyl group signals at δ_{H} 6.06 (H-20), δ_{H} 5.43 (H-21E), δ_{H} 5.36 (H-21Z), and double bond signals at δ_{H} 5.73 (H-25) and δ_{H} 5.69 (H-26) as well as an isolated spin system consisting of three carbons: a methine at δ_{H} 4.47 (H-13), a methylene at δ_{H} 2.53 (H-14_{ax}) and δ_{H} 2.29 (H-14_{eq}), and a methine at δ_{H} 2.44 (H-15). In addition five methyl groups could be observed in the ¹H NMR spectrum (δ_{H} 1.57, H-28; 1.58, H-27; 1.62, H-19; 1.38, H-18; 1.51, H-17).

The ¹³C NMR spectrum displayed all 26 carbon resonances implied by the molecular formula. Thirteen of these signals were observed in the region from 100-160 ppm, eight of which were assigned to the indole moiety (δ_{C} 138.8, 111.0, 140.6, 114.2, 123.2, 108.3, 135.3, and 126.0). Another four carbons were attributed to the vinyl group (δ_{C} 143.2 and 118.7) and the double bond (δ_{C} 143.4 and 125.6), while the final deshielded resonance (δ_{C} 158.2) was assigned to the isonitrile moiety.¹⁵ Of the thirteen degrees of unsaturation required by the molecular formula, ten were accounted for by the indole ring, two double bonds, and the isonitrile moiety. Hence ambiguine K isonitrile was deduced to possess 3 additional rings.

The planar structure of ambiguiene K isonitrile was established by analysis of the correlations observed in the HMBC spectrum (Figure 1). The connectivity within the indole moiety was established by the correlations from H-5 to C-9, H-6 to C-4 and C-8, as well as H-7 to C-9. The *gem*-dimethyl group at C-16 was connected to C-4 based upon correlations observed from H₃-17 and H₃-18 to both C-4 and C-16. Further correlations from H₃-17 and H₃-18 to C-15 connected this portion to the H-15-H-13 fragment COSY coupled spin system. Three bond correlations from both H₃-19 and H-20 to C-11 and C-13 as well as from H-20 to C-12 revealed the position of the vinyl and C-19 methyl groups on C-12 and connected this fragment to C-13. In addition, correlations from H-26 to C-10, C-11, and C-12 as well as from H-25 to C-11 established a linkage of the Δ^{25} double bond to C-11. This double bond was in turn connected to C-2 of the indole via a second *gem*-dimethyl group as deduced by the correlations from H-25, H₃-27, and H₃-28 to C-2 and C-24.

Substitution with chlorine at position 13 was deduced by comparison of the chemical shift of H-13 (δ_{H} 4.47) and C-13 (δ_{C} 66.4) with those published for other ambiguiene isonitriles.^{15,16} Similarly, placement of the isonitrile group at C-11 (δ_{C} 71.7) and hydroxyl group at C-10 (δ_{C} 76.5) was based upon chemical shift comparison with published data.^{15,16} Although no correlations were observed to C-3 of the indole moiety in the HMBC spectrum, the connection between carbons C-10 and C-3 was established by considering the thus completed structure and the thirteen degrees of unsaturation required by the molecular formula, which necessitated a pentacyclic system.

The coupling constants between H-14_{ax} and H-13 (J = 12.5 Hz), as well as H-14_{ax} and H-15 (J = 13.1 Hz) suggested that both H-13 and H-15 were in the axial orientation. In addition, correlations observed in the 2D NOESY spectrum between H-15 and H-13, and H-13 and H-20 indicated all to be in the same plane. This was further confirmed by the correlation observed between H-14_{ax} and H₃-19. Together these observations established the relative configuration at positions 12, 13, and 15 as 12*R**, 13*R**, and 15*R**. The configuration of the remaining two stereo-centers (positions 10 and 11) was determined by X-ray crystallography, which also confirmed the assigned structure. The absolute configuration of **1** was determined on the basis of the Flack parameter value,³⁴ -.00(3), as 10*R*, 11*S*, 12*R*, 13*R*, and 15*R*. The ORTEP diagram is shown in Figure 2.

Ambiguiene L isonitrile (**2**) was obtained as a white amorphous powder. Again, the UV spectrum indicated the presence of an indole moiety [λ_{max} (log ϵ) 223 (3.47), 271 (2.91)] and the presence of an isonitrile moiety was evident from both ¹³C NMR (δ_{C} 157.2) and IR (ν_{max} 2127 cm⁻¹) data. The HRMS peak at m/z 387.24347 [M+H]⁺ suggested a molecular formula of C₂₆H₃₀N₂O for **2** and the absence of chlorine in the structure. Comparison of ¹H and ¹³C NMR spectra for **2** with those of **1** (Table 1 and 2) showed that they both were closely related ambiguiene isonitrile compounds. However, in the ¹H NMR spectrum of **2**, the resonance observed in **1** for the chlorinated methine group at δ_{H} 4.47 (H-13) was replaced by two signals at δ_{H} 1.52 (H-13_{eq}) and δ_{H} 1.84 (H-13_{ax}). The chlorinated C-13 methine resonance (δ_{C} 66.4) found in the spectrum for compound **1** was also absent in the DEPT spectrum for **2**, while an additional methylene carbon at δ_{C} 35.4 (C-13) was observed. Together these observations indicated that **2** lacked the chloro-substitution at position 13 observed in **1**. This conclusion was confirmed by analysis of COSY and HSQC data, which showed the presence of a CH₂CH₂CH spin system from C-13 to C-15. Based on all of the above information, as well as analysis of HMBC correlations, the planar structure of **2** was established as the deschloro derivative of ambiguiene K isonitrile.

The relative configuration of **2** was determined by comparison of chemical shifts and coupling constants with those of **1**, as well as correlations observed in the 2D NOESY spectrum. Similar to **1**, the coupling constant between H-14_{ax} and H-15 (J = 13.0 Hz) indicated that H-15 was in

the axial orientation, while the 2D NOESY correlations observed between H-13_{ax}, H-20 and H-15 suggested that all of these protons were in the same plane. This suggested a relative configuration of 12*R** and 15*R**. The relative configuration at C-10 (δ_C 75.8) and C-11 (δ_C 69.7) was assigned as 10*R**, 11*S** by comparison of chemical shifts with those of **1** (δ_{C-10} 76.5 and δ_{C-11} 71.7). Thus, ambiguine L isonitrile has the relative configuration of 10*R**, 11*S**, 12*R**, and 15*R**. This represents the same configuration as observed in ambiguine K isonitrile (**1**) at these positions and suggests the identical absolute configuration for ambiguine L isonitrile.

Ambiguine M isonitrile (**3**) was obtained as a white amorphous solid. Again, the UV spectrum indicated the presence of an indole moiety [λ_{\max} (log ϵ) 221 (2.64), 278 (1.92)] and the presence of an isonitrile moiety was evident from both ^{13}C NMR (δ_C 160.2) and IR (ν_{\max} 2130 cm^{-1}) data. The ESI mass spectrum of **3** exhibited a 3:1 ion cluster at m/z 439/441 $[\text{M}+\text{H}]^+$, indicating the presence of a chlorine. The HRMS peak at m/z 439.2143 $[\text{M}+\text{H}]^+$ coupled with NMR information established the molecular formula as $\text{C}_{26}\text{H}_{31}\text{ClN}_2\text{O}_2$. ^1H and ^{13}C NMR spectra (Tables 1 and 2) of compound **3** showed signals similar to those found in ambiguine K isonitrile (**1**). The major difference was the absence of the Δ^{25} double bond, which had been replaced by a -CHCH₂-spin system (CH-25 and CH₂-26). The chemical shift of H-25 (δ_H 3.97) and C-25 (δ_C 74.2) and consideration of the molecular formula placed a hydroxyl group at C-25. The linkage of the C-25-C-26 fragment to C-11 was determined by HMBC correlations from H-25 as well as H-26_{ax} and H-26_{eq} to C-10, C-11, and C-12. Similarly, the correlations from H-25, H₃-27, and H₃-28 to C-2 and C-24 established the connection of this moiety to C-2 of the indole via a *gem*-dimethyl group.

The relative configuration of the molecule was determined analogously to **1**. The coupling constant between H-14_{ax} and H-13 (J = 12.6 Hz), as well as H-14_{ax} and H-15 (J = 12.8 Hz) suggested that both H-13 and H-15 were in the axial orientation. This was confirmed by the 2D NOESY correlations observed between H-13 and both H-15 and H-20, indicating all to be in the same plane. This established the relative configuration as 12*R**, 13*R**, and 15*R**. The chemical shift of C-10 (δ_C 75.4) and C-11 (δ_C 74.4) were similar to those observed in ambiguine K isonitrile (see Table 2), indicating both compounds to have identical configuration at these positions. In addition, correlations observed in the 2D NOESY spectrum between H-26_{ax}, H₃-27 and H₃-19 suggested that they were in the same plane (the signals for H₃-19 and H₃-17 overlap at δ_H 1.54, but the observed correlation was assigned to H₃-19 due to the considerable distance between H-26_{ax} and H₃-17). The coupling constant for H-26_{ax} and H-25 (J = 10.9 Hz) indicated that H-25 was anti to H-26_{ax}, in agreement with the NOESY correlations observed between H-25 and H₃-28. Therefore, the relative configuration of C-25 was assigned as *R**. Thus, the relative configuration of ambiguine M isonitrile (**3**) was determined as 10*R**, 11*S**, 12*R**, 13*R**, 15*R**, and 25*R**. This is the same configuration as observed in ambiguine K isonitrile (**1**) at positions 10, 11, 12, 13, and 15. The shared biosynthetic pathway for **1** and **3** suggests the absolute configuration for ambiguine M isonitrile to be 10*R*, 11*S*, 12*R*, 13*R*, 15*R*, and 25*R*.

Ambiguine N isonitrile (**4**) was obtained as a white amorphous solid. The UV spectrum indicated the presence of an indole moiety [λ_{\max} (log ϵ) 229 (3.53), 270 (3.48)], while IR (ν_{\max} 2142 cm^{-1}) and ^{13}C NMR (δ_C 157.8) data showed the presence of an isonitrile moiety. The molecular formula was determined as $\text{C}_{26}\text{H}_{32}\text{N}_2\text{O}_2$ by HRMS (m/z 405.2534 $[\text{M}+\text{H}]^+$) and lacked chlorine in the structure. The ^1H and ^{13}C spectra (see Tables 1 and 2) for **4** indicated that it was the deschloro derivative of ambiguine M isonitrile (**3**). Comparison of ^1H NMR spectra showed that the signal at δ_H 4.51 (H-13) found in **3** had disappeared and was replaced by two signals at δ_H 1.55 (H-13_{eq}) and δ_H 2.02 (H-13_{ax}) in **4**. Similarly in the ^{13}C NMR spectra, the methine C-13 resonance for **3** (δ_C 67.2) had been replaced by a methylene carbon at δ_C

37.6 for **4**. The COSY and HSQC data also showed the presence of a CH₂CH₂CH spin system from C-13 to C-15.

The relative configuration of **4** was determined by comparison of chemical shifts and coupling constants to those of **1** and **3** (Tables 1 and 2). The coupling constant between H-14_{ax} and H-15 (J = 12.5 Hz) indicated H-15 was in the axial orientation. This was confirmed by inspection of the 2D NOESY experiment, where correlations were observed between H-15, H₃-17 and H-13_{ax}, as well as between H₃-19, H-13_{eq} and H-14_{ax}, indicating H-15 and H₃-19 to be in the opposite plane. The chemical shift of C-10 (δ_C 75.8) and C-11 (δ_C 73.5) were similar to those observed in ambiguine K isonitrile (see Table 2), indicating a 10*R**, 11*S** configuration. Similar to ambiguine M isonitrile, correlations were observed in the 2D NOESY spectrum between H-26_{ax}, H₃-19 and H₃-27, suggesting that they were in the same plane. The coupling constant for H-26_{ax} and H-25 (J =10.9 Hz) indicated that H-25 was anti to H-26_{ax}. Thus, the relative configuration of ambiguine N isonitrile (**4**) was determined as 10*R**, 11*S**, 12*R**, 15*R**, and 25*R**. Again, this is the same configuration as observed in ambiguine K isonitrile (**1**) at positions 10, 11, 12, and 15 and suggests the absolute configuration for ambiguine N isonitrile to be 10*R*, 11*S*, 12*R*, 15*R*, and 25*R*.

Ambiguine O isonitrile (**5**) exhibited a 3:1 ion cluster at m/z 451/453 [M-H]⁻ in the ESI MS. HRMS (m/z 451.1802 [M-H]⁻) determined the molecular formula as C₂₆H₂₉ClN₂O₃. The UV spectrum indicated the presence of an indole moiety [λ_{max} (log ϵ) 220 (3.44), 277 (2.83)]. ¹H and ¹³C NMR spectra showed signals indicative of an ambiguine isonitrile derivative (see Tables 1 and 2). The major differences as compared to ambiguine K-N isonitriles were observed in the seven-membered ring portion of the molecule. In particular, a new signal at δ_H 4.94 (H-26) appeared in ¹H spectrum for compound **5**, and was ascribed to a hydroxylated methine (δ_C 85.5, C-26). In addition, the ¹³C NMR spectrum indicated the presence of a ketone moiety (δ_C 209.3, C-25). HMBC correlations from H-26 to C-24 and C-25, and from H₃-27 and H₃-28 to C-24 and C-25 suggested the presence of a 1-hydroxy-3,3-dimethyl-2-propanone moiety as part of the seven membered ring system (Figure 3). The HMBC correlations from H-26 to C-12, C-11, and C-10 established the C-26 to C-11 connection, while the correlations from H₃-27 and H₃-28 to C-2 determined the connection of C-24 to C-2 of the indole moiety. Although no signal could be observed for the C-23 isonitrile in the ¹³C NMR spectrum, this substituent at C-11 was determined by a characteristic IR peak (ν_{max} 2123 cm⁻¹), the molecular formula, and comparison of the chemical shift of C-11 (δ 69.3) with other ambiguine isonitrile compounds reported in this paper.

Analysis of the coupling constants and correlations in the 2D NOESY spectrum established the relative configuration of **5**. The coupling constant between H-14_{ax} and H-13 (J = 12.9 Hz), as well as H-14_{ax} and H-15 (J = 12.9 Hz) suggested that both H-13 and H-15 were axial. This was confirmed by the correlations observed in the 2D NOESY spectrum between H-13, H-15 and H₃-20, which indicated that H-13, H-15 and H-20 were all in the same plane. The chemical shifts of C-10 (δ_C 77.8) and C-11 (δ_C 69.3) were similar to those observed for ambiguine K isonitrile (see Table 2), indicating that both compounds have identical at these positions. In addition, correlations observed in the 2D NOESY spectrum between H-26 and H₃-19, H-20 and H₂₁Z indicated these protons to be spatially close (integrated volumes suggested that H-26 was almost equidistant to all three protons). This requires H-26 to be in pseudoaxial position of the cycloheptene ring and the configuration of C-26 was assigned as *S**. In this configuration, OH group at C-26 is in pseudoequatorial orientation, minimizing steric interactions. In the opposite configuration (26*R**), multiple 1,3-diaxial interactions would be present, which would result in a ring twist thus placing H-26 farther away from H₃-19. The 26*S** configuration is also supported by the lack of NOE correlation between H-26 and H₃-27 (or H₃-28). In the 26*S** configuration, the interproton distance from H-26 to H₃-27 (or H₃-28) is increased due to the flattening of the C3-C24-C25 plane, similar as was observed

for 3,4-benzocycloheptanone by Bodennes and St.-Jacques.³⁷ Thus, the relative configuration of ambiguine O isonitrile was determined as 10*R**, 11*R**, 12*R**, 13*R**, 15*R**, and 26*S**.

All isolates were evaluated for inhibition of growth of *Mycobacterium tuberculosis* and *Bacillus anthracis* as well as Vero cell toxicity. In addition, the isolates were also evaluated against a set of microorganisms, *Staphylococcus aureus*, *Mycobacterium smegmatis*, and *Candida albicans*, to determine the spectrum of activity (Table 3). Several isolates were active against *M. tuberculosis*, the most active compound was ambiguine K isonitrile (MIC 6.6 μ M), while ambiguine A isonitrile showed the most potent activity against *B. anthracis* with a MIC of 1.0 μ M. Most ambiguine isonitriles showed moderate toxicity in the Vero cell assay (IC₅₀ ranging from 26.0 to >128 μ M). Interestingly, ambiguine I isonitrile (MIC 11.3 μ M) and hapalindole G (MIC 6.8 μ M) displayed over twenty-and fifty-fold higher inhibition against *M. tuberculosis* than *B. anthracis*, respectively, with no detectable cytotoxicity in a Vero cell assay. Most of the isolates also possessed strong antifungal activities, similar to levels previously reported for other ambiguine isonitriles and hapalindoles.^{16,35}

The co-occurrence of non-chlorinated and chlorinated ambiguine isonitriles in cyanobacteria of the order Stigonematales has been suggested to be due to some imperfection in the biosynthesis and the resulting arrays of compounds have been proposed to give an ecological advantage.¹⁶ In our study, we observed no distinct difference in the level of antimicrobial activity of non-chlorinated vs chlorinated ambiguine isonitriles, except for *B. anthracis*, where the chlorinated ambiguine isonitriles seem to possess somewhat lower MIC values than their non-chlorinated partner (i.e E vs I, K vs L, and M vs N).

Moore *et al.* have proposed that hapalindoles are biosynthesized by condensation of (Z)- β -ocimene and 3-((Z)-2'-isocyanoethenyl)indole.¹⁰ Ambiguine isonitriles have an additional isoprene unit attached to C-2 of the indole moiety. This isoprene moiety is often cyclized to form an additional seven-membered ring. In this study we observed substantial flexibility in the degree of oxidation of this seven-membered ring portion. Ambiguine K (**1**) and L (**2**) isonitriles, both possessing a C25-C26 double bond, can be considered the precursors for ambiguine M (**3**), N (**4**), and O (**5**) isonitriles as well as the previously reported ambiguine isonitriles containing a seven-membered ring. We only observed ambiguine D isonitrile in trace amounts (detected by LC-MS, data not shown) in this study. In contrast, ambiguine D isonitrile was found to be the major ambiguine isonitrile in this strain by Moore *et al.*¹⁵ We also did not observe ambiguine J isonitrile, the deschloro derivative of ambiguine D isonitrile recently found by Raveh and Carmeli¹⁶ in a *Fischerella* sp. from Israel.

Experimental section

General experimental procedures

Optical rotations were determined on a Perkin-Elmer 241 polarimeter. UV spectra were obtained on an Agilent 1100 HPLC system with a diode array detector. IR spectra were obtained on a Jasco FTIR-410 Fourier transform infrared spectrometer. ¹H and ¹³C NMR spectra were obtained on a Bruker Avance DRX600 MHz NMR spectrometer with 5mm CPTXI Z-gradient probe and a Bruker AVII900 MHz NMR spectrometer with 5mm ATM CPTCI Z-gradient probe, referenced to the corresponding solvent peaks. Low resolution ESI mass spectra were obtained on a ThermoFinnigan TSQuantum Triple Quadrupole Mass Spectrometer and an Agilent 1946A LC-MSD single quadrupole LC-mass spectrometer. High resolution ESI mass spectra were obtained on a Thermo Electron LTQ FT ICR mass spectrometer.

Biological Material

Fischerella ambigua was initially acquired from the Culture Collection of Algae at the University of Texas at Austin (UTEX 1903). The cyanobacterium was grown in a 2.8 L Fernbach flask containing 1 L of inorganic media (Allen media).³³ Cultures were illuminated with fluorescent lamps at 1.93 klx with an 18/6 hour light/dark cycle. The temperature of culture room was maintained at 22° C. After 6-8 weeks, the biomass of cyanobacteria was harvested by centrifugation and then freeze-dried.

Extraction and isolation

The freeze-dried biomass (2.14 g) from a total of 9 L of culture was extracted by repeated maceration with CH₂Cl₂:MeOH (1:1) to yield 691.1 mg of crude extract. The crude extract showed potent inhibitory activity against the TB pathogen *Mycobacterium tuberculosis* (MIC 2.7 µg/mL). A portion of crude extract (682 mg) was fractionated on silica gel using a gradient with increasing amount of MeOH in CH₂Cl₂ to afford 18 fractions. The TB active fractions 2 (MIC 1.5 µg/mL, 247.7 mg), 7 (MIC 2.7 µg/mL, 11.5 mg), and 8 (MIC 8.3 µg/mL, 9.0 mg) were fractionated as follows. Fraction 2 was further separated using Sephadex LH-20 with MeOH as an eluent to obtain nine fractions. Fraction 2-8 (160.2 mg) was subjected to reversed-phase HPLC (Alltima C18, 10 m, 250×10 mm, 4 mL/min) with a solvent gradient of MeOH-H₂O (85:15) to 100% MeOH over 20 minutes to afford ambiguiene A isonitrile (*t_R* = 12.8 min, 5.8 mg), ambiguiene B isonitrile (*t_R* = 9.8 min, 4.5 mg) and ambiguiene E isonitrile (*t_R* = 12.5 min, 13.5 mg), hapalindole H (*t_R* = 11.1 min, 14.0 mg), and a peak containing a mixture (*t_R* = 8.8 min). The mixture was further purified by RP-HPLC (Alltima C18, 10 µm, 250×10 mm, 4 mL/min) eluting with MeCN:H₂O (75:25) to afford **1** (*t_R* = 12.9 min, 2.8 mg) and **2** (*t_R* = 13.7 min, 2.2 mg), as well as hapalindole G (*t_R* = 11.2 min, 2.9 mg). Fractionation of Fr.2-7 using RP-HPLC (Alltima C18, 10 µm, 250×10 mm, 2 mL/min) and a solvent gradient of MeOH-H₂O (60:40) to 100% MeOH over 20 minutes led to two subfractions. Further chromatography of the first subfraction using RP-HPLC (Alltima C8, 5 µm, 250×10 mm, 3 mL/min) and a solvent gradient of MeOH-H₂O (80:20) to MeOH-H₂O (90:10) over 15 minutes afforded ambiguiene B isonitrile (*t_R* = 13.2 min, 0.7 mg) and ambiguiene C isonitrile (*t_R* = 12.7 min, 0.8 mg). The second subfraction was further fractionated using RP-HPLC (Alltima C18, 10 µm, 250×10 mm, 3 mL/min) and a solvent gradient of MeOH-H₂O (83:17) to MeOH-H₂O (90:10) over 20 minutes to give ambiguiene A isonitrile (*t_R* = 18.6 min, 0.7 mg) and ambiguiene I isonitrile (*t_R* = 16.7 min, 0.9 mg). The biologically active silica gel fraction 7 was fractionated using RP-HPLC (Alltima C18, 10 µm, 250×10 mm, 4 mL/min) and a solvent gradient of MeOH-H₂O (75:25) to 100% MeOH over 20 minutes to yield **3** (*t_R* = 14.1 min, 1.2 mg), **4** (*t_R* = 13.8 min, 1.0 mg), and **5** (*t_R* = 16.8 min, 0.4 mg). Silica gel fraction 8 was fractionated using the identical protocol as for fraction 7 to give ambiguiene F isonitrile (*t_R* = 14.8 min, 0.8 mg).

Ambiguiene K isonitrile (1)

colorless crystals (MeOH); [α]_D -94 (*c* 0.14, MeOH); UV (MeOH) λ_{max} (log ϵ) 230 (2.72), 275 (2.11) nm; IR (neat) ν_{max} 3416, 2965, 2125, 1631, 1452, 1377, 1323, 1086, 971, 927, 828, 755 cm⁻¹; ¹H NMR (see Table 1); ¹³C NMR (see Table 2); HRESIMS *m/z* 419.18979 [M-H]⁻ (calcd for C₂₆H₂₈ClN₂O, 419.18956).

Ambiguiene L isonitrile (2)

white amorphous powder; [α]_D -97 (*c* 0.13, MeOH); UV (MeOH) λ_{max} (log ϵ) 223 (3.47), 271 (2.91) nm; IR (neat) ν_{max} 3734, 3410, 2961, 2127, 1568, 1454, 1320, 1082, 1022, 971, 929, 719 cm⁻¹; ¹H NMR (see Table 1); ¹³C NMR (see Table 2); HRESIMS *m/z* 387.24347 [M+H]⁺ (calcd for C₂₆H₃₁N₂O, 387.24309).

Ambiguine M isonitrile (3)

white amorphous powder; $[\alpha]_D$ -21 (*c* 0.07, MeOH); UV (MeOH) λ_{\max} (log ϵ) 221 (2.64), 278 (1.92) nm; IR (neat) ν_{\max} 3385, 2130, 1645, 1444, 1015, 951 cm^{-1} ; ^1H NMR (see Table 1); ^{13}C NMR (see Table 2); HRESIMS m/z 439.21430 $[\text{M}+\text{H}]^+$ (calcd for $\text{C}_{26}\text{H}_{32}\text{ClN}_2\text{O}_2$, 439.21468).

Ambiguine N isonitrile (4)

white amorphous powder; $[\alpha]_D$ -12 (*c* 0.05, MeOH); UV (MeOH) λ_{\max} (log ϵ) 229 (3.53), 270 (3.48) nm; IR (neat) ν_{\max} 3735, 3367, 2928, 2142, 1684, 1652, 1540, 1510, 1456, 1081, 978, 867, 755 cm^{-1} ; ^1H NMR (see Table 1); ^{13}C NMR (see Table 2); HRESIMS m/z 405.25340 $[\text{M}+\text{H}]^+$ (calcd for $\text{C}_{26}\text{H}_{33}\text{N}_2\text{O}_2$, 405.25365).

Ambiguine O isonitrile (5)

white amorphous powder; $[\alpha]_D$ -17 (*c* 0.03, MeOH); UV (MeOH) λ_{\max} (log ϵ) 220 (3.44), 277 (2.83) nm; IR (neat) ν_{\max} 3387, 2924, 2123, 1649, 1433, 1303, 1018, 950, 755 cm^{-1} ; ^1H NMR (see Table 1); ^{13}C NMR (see Table 2); HRESIMS m/z 451.1802 $[\text{M}-\text{H}]^-$ (calcd for $\text{C}_{26}\text{H}_{29}\text{ClN}_2\text{O}_3$, 452.18667).

Ambiguine A isonitrile^{15,16}

colorless crystals (MeOH and MeCN); $[\alpha]_D$ -22 (*c* 0.22, MeOH); ^1H NMR (see Table 9 in Supporting Information).

Ambiguine B isonitrile^{15,16}

white amorphous powder; $[\alpha]_D$ -11 (*c* 0.08, MeOH); ^1H NMR (see Table 9 in Supporting Information).

Ambiguine C isonitrile¹⁵

white amorphous powder; $[\alpha]_D$ -6 (*c* 0.08, MeOH); ^1H NMR (see Table 9 in Supporting Information).

Ambiguine E isonitrile^{15,16}

white amorphous powder; $[\alpha]_D$ -83 (*c* 0.31, MeOH); ^1H NMR (see Table 9 in Supporting Information).

Ambiguine F isonitrile^{15,16}

white amorphous powder; ^1H NMR (see Table 9 in Supporting Information).

Ambiguine I isonitrile¹⁶

white amorphous powder; $[\alpha]_D$ -106 (*c* 0.23, MeOH); ^1H NMR (see Table 9 in supporting information).

Hapalindole G^{15,36}

white amorphous powder; $[\alpha]_D$ -41 (*c* 0.24, MeOH); ^1H NMR (see Table 9 in Supporting Information).

Hapalindole H^{15,36}

white amorphous powder (MeOH); $[\alpha]_D$ +186 (*c* 0.22, MeOH); ^1H NMR (see Table 9 in Supporting Information).

X-ray crystallographic analysis of ambiguine K isonitrile (1)

38 Single crystals for X-ray analysis were grown from a methanol solution. A colorless crystal with dimensions $0.02 \times 0.05 \times 0.20$ mm was used for the analysis. The diffraction data were collected on a Rigaku/MSR RAPID imaging plate area detector equipped with a normal focus sealed X-ray tube and Cu K α radiation. Crystal data: C₂₆H₂₉ClN₂O·CH₃OH, MW= 453.0, orthorhombic, space group C2 (5); $a = 18.730(3)$ Å, $b = 7.0493(11)$ Å, $c = 20.221(3)$ Å, $\beta = 110.242(9)$ Å, $V = 2505.0(9)$ Å³; $Z = 4$, $D_c = 1.20$ mg/m³; $\mu = 1.54$ mm⁻¹; $F(000) = 968$. Total collected reflections 8507, 3626 unique reflections ($R_{int} = 0.0497$); final $R1 = 0.0583$ for reflections with $I > 2\sigma(I)$; $R1 = 0.0804$, $wR2 = 0.1756$ for all unique data. The integration of intensities and refinement of unit cell parameters and orientation matrix were carried out using CrystalClear.³⁹ The WinGX package was used for completing the structure determination.⁴⁰ The structure was solved by direct methods, repeated cycling with least-squares refinement on F^2 and difference Fourier maps using SHELXL-97.⁴¹ All non-hydrogen atoms were refined with anisotropic Gaussian displacement parameters. The Flack parameter, $-0.00(3)$, was well-determined, and used to assign the absolute configuration of the structure as C(10) *R*, C(11) *S*, C(12) *R*, C(13) *R*, and C(15) *R*.

Bacillus anthracis—The fractions and compounds were tested with concentrations from 100 µg/mL to 48.8 ng/mL using the previously described method.⁴² The minimum inhibitory concentration (MIC) of each sample was calculated as the lowest concentration that prevents visible bacterial growth.

Mycobacterium tuberculosis—The inhibitory activity of fractions and compounds against *M. tuberculosis* was performed using the Microplate Alamar blue Assay (MABA).⁴³ Virulent H37Rv strain was used in the assay. The MIC value was determined as the lowest drug concentration effecting an inhibition of $\geq 90\%$.

Other organisms

The broth microdilution MIC method was used to test the activity of compounds against *Staphylococcus aureus*⁴⁴ and *Candida albicans*.⁴⁵

Cytotoxicity

The cytotoxicity of compounds were evaluated using green monkey kidney cells (Vero).⁴⁶ Cell viability was measured using the CellTiter 96 aqueous nonradioactive cell proliferation assay.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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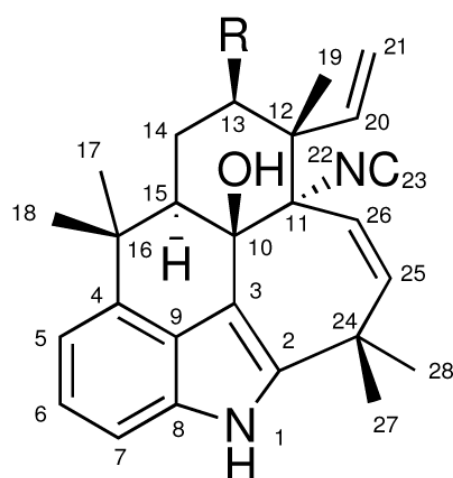
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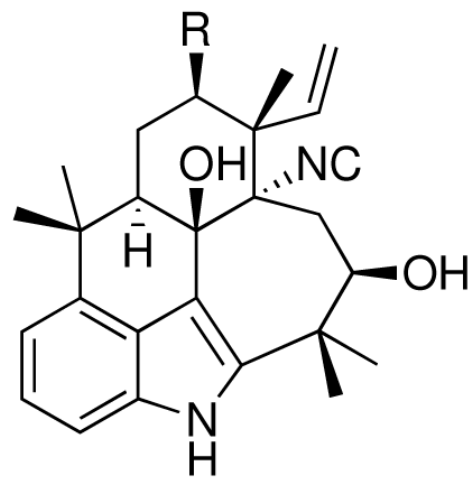
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data can be obtained, f. o. c., on application to the Director, CCDC, 12 Union Road, Cambridge CB2 1EZ, UK (fax: +44-(0)1223-336033 or e-mail: deposit@ccdc.cam.ac.uk).

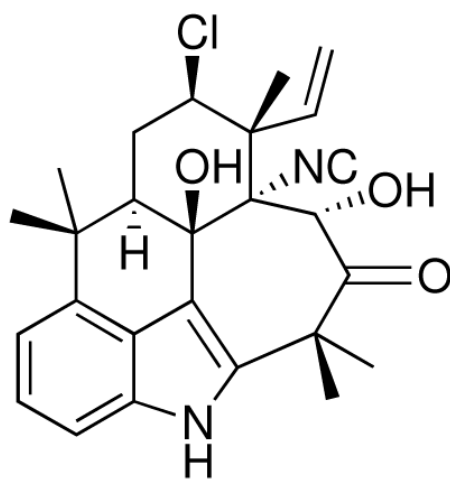
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1 R = Cl
2 R = H



3 R = Cl
4 R = H



5

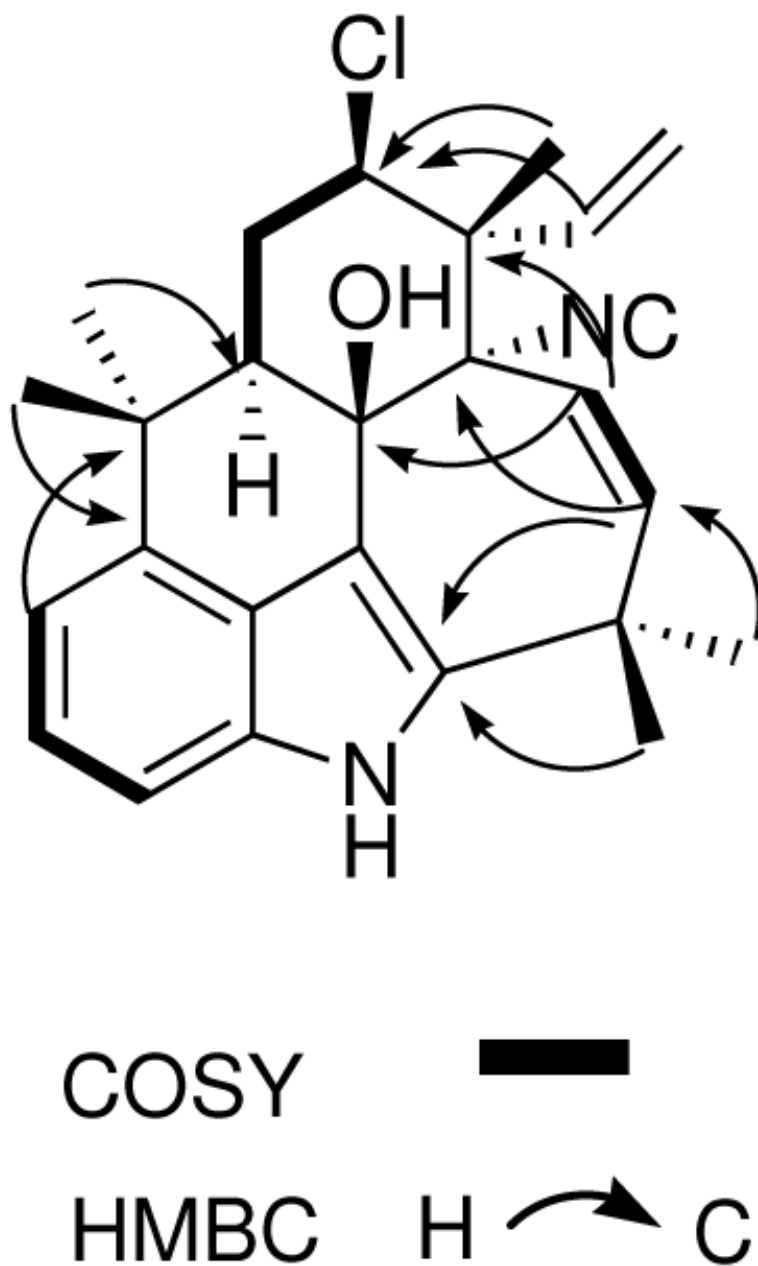


Figure 1.
Key HMBC and COSY correlations of ambigua K isonitrile (1)

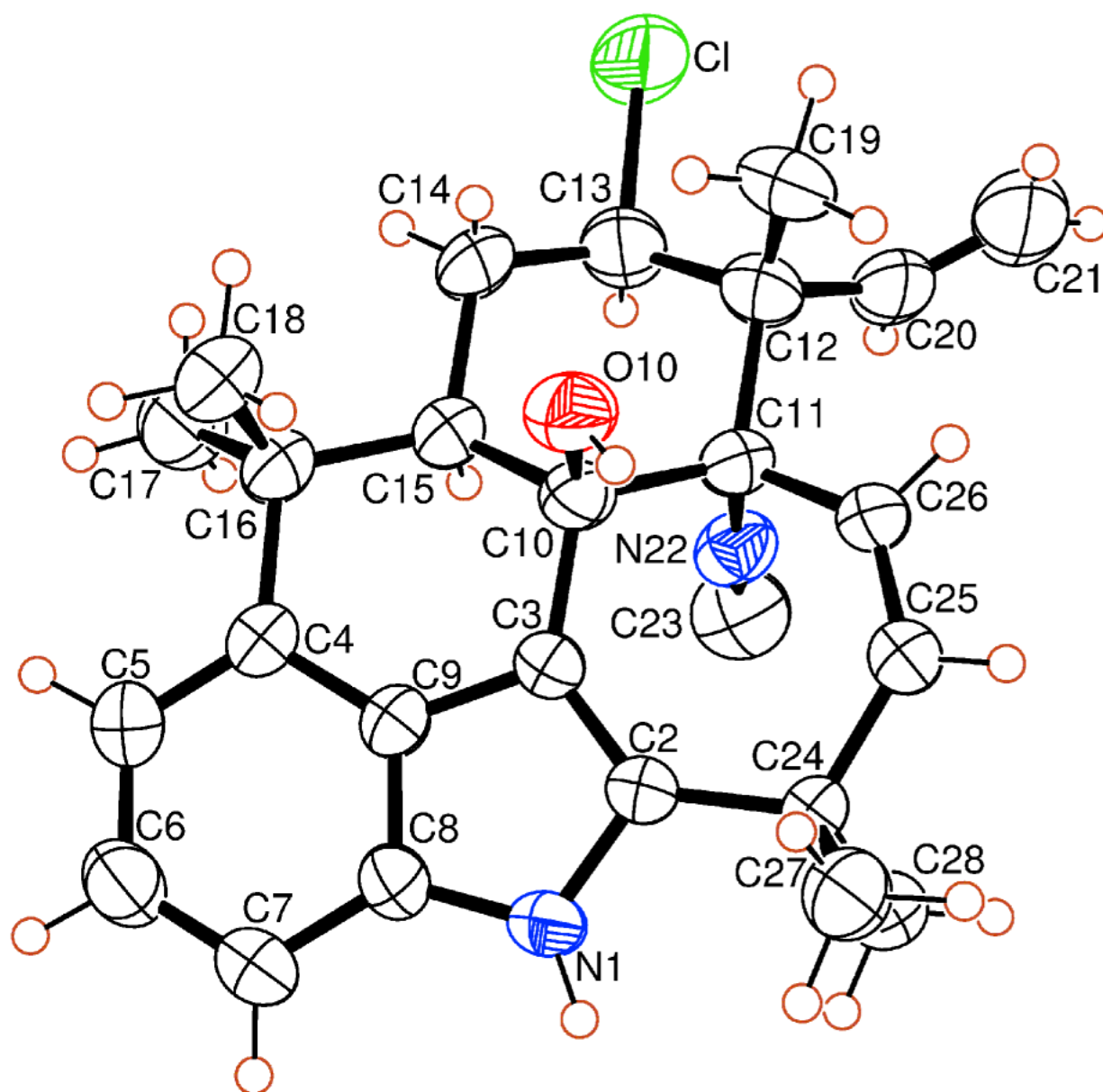
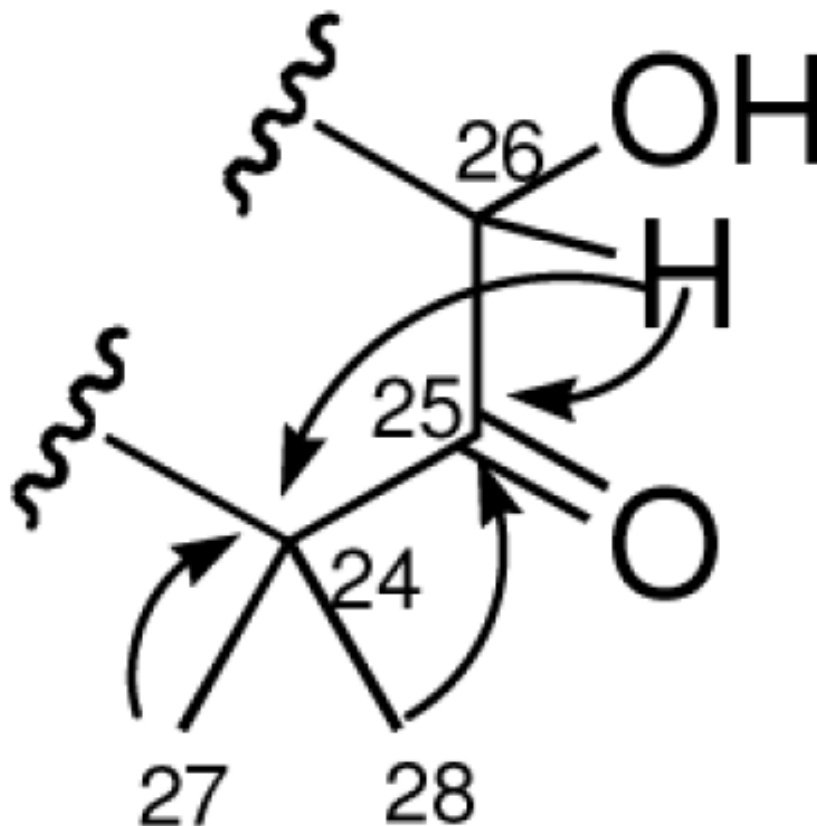


Figure 2.
The ORTEP diagram of ambigua K isonitrile (1)



HMBC H  C

Figure 3.
Structure and key HMBC correlations of the 1-hydroxy-3,3-dimethyl-2-propanone moiety

Table 1
¹H NMR data of compounds **1-5** (900 MHz) δ_H , mult., (*J* in Hz)

position	1 ^a	2 ^b	3 ^a	4 ^a	5 ^a
5	6.98, dd (7.0, 0.8)	6.94, d (7.2)	7.00, d (7.2)	6.98, d (7.3)	7.02, dd (6.8, 1.0)
6	7.07, t (7.5)	7.04, dd (7.2, 7.8)	7.08, dd (7.2, 7.9)	7.06, dd (7.3, 7.9)	7.14, dd (6.8, 6.8)
7	7.09, dd (7.9, 0.8)	7.09, d (7.8)	7.14, d (7.9)	7.12, d (7.9)	7.13, d (6.8)
				2.02, ddd	
13	4.47, dd (12.5, 3.7)	1.84, t (13.3) ^c	4.51, dd (12.6, 3.8)	(14.0, 13.5, 3.4) ^c	4.52, dd (12.9, 3.9)
		1.52, m ^d		1.55, ddd	
14 _{ax}	2.53, ddd (13.1, 12.6, 12.5)	2.01, ddd (13.3, 13.0, 12.4)	2.55, ddd (12.6, 12.6, 12.8)	2.17, dddd (14.0, 13.0, 12.5, 3.3)	2.66, ddd (12.9, 12.9, 12.6)
14 _{eq}	2.29, ddd (12.6, 3.7, 2.5)	1.77, d (12.4)	2.27, m	1.84, dddd (13.0, 3.3, 3.4, 2.4)	2.29, ddd (12.6, 3.9, 3.1)
15	2.44, dd (13.1, 2.5)	2.07, d (13.0)	2.48, dd (12.8, 2.2)	2.32, dd (12.5, 2.4)	2.37, dd (12.9, 3.1)
17	1.51, s	1.42, s	1.54, s	1.51, s	1.48, s
18	1.38, s	1.30, s	1.32, s	1.30, s	1.50, s
19	1.62, s	1.48, s	1.54, s	1.48, s	1.73, s
20	6.06, dd (17.4, 10.9)	6.11, dd (11.6, 17.6)	6.09, dd (17.4, 10.8)	6.21, dd (17.3, 11.1)	6.04, dd (17.4, 10.8)
21E	5.43, dd (10.9, 1.1)	5.19, br (11.6)	5.47, d (10.8)	5.26, dd (11.1, 1.6)	5.58, dd (10.8, 1.0)
21Z	5.36, dd (17.4, 1.1)	5.21, d (17.6)	5.34, d (17.4)	5.24, dd (17.3, 1.6)	5.45, dd (17.4, 1.0)
25	5.73, d, (12.6)	5.70, d (12.6)	3.97, dd (10.9, 1.8)	3.98, dd (10.9, 1.9)	
			3.06, dd (10.9, 12.9) ^c	3.01, m ^c	
26	5.69, d (12.6)	5.63, d (12.6)	2.07, dd (12.9, 1.8) ^d	2.00, dd (13.2, 1.9) ^d	4.94, s
27	1.58, s ^e	1.51, s ^e	1.29, s	1.30, s	1.62, s
28	1.57, s ^e	1.53, s ^e	1.61, s	1.61, s	1.83, s
NH		10.84, s			

^a in MeOH-d4

^b in DMSO-d6

^c axial

^d equatorial

^e assignments may be reversed

Table 2

¹³C NMR data of compounds **1-5** (226 MHz) δ_C, mult.

Position	1 (MeOH-d ₄)	2 (DMSO-d ₆)	3 (MeOH-d ₄)	4 (MeOH-d ₄)	5 (MeOH-d ₄)
2	138.8, qC	138.6, qC	139.8, qC	139.5, qC	134.2, qC
3	111.0, qC	111.3, qC	110.6, qC	111.8, qC	111.9, qC
4	140.6, qC	140.7, qC	140.1, qC	140.9, qC	140.5, qC
5	114.2, CH	113.8, CH	114.7, CH	114.5, CH	115.3, CH
6	123.2, CH	122.5, CH	123.0, CH	122.9, CH	123.7, CH
7	108.3, CH	108.1, CH	108.6, CH	108.4, CH	108.0, CH
8	135.3, qC	134.3, qC	134.9, qC	134.8, qC	135.7, qC
9	126.0, qC	125.6, qC	126.6, qC	126.7, qC	126.0, qC
10	76.5, qC	75.8, qC	75.4, qC	75.8, qC	77.8, qC
11	71.7, qC	69.7, qC	73.5, qC	73.5, qC	69.3, qC
12	50.4, qC	44.1, qC	51.4, qC	45.4, qC	50.7, qC
13	66.4, CH	35.4, CH ₃	67.2, CH	37.6, CH ₂	66.0, CH
14	30.2, CH ₂	18.2, CH ₂	30.5, CH ₂	19.1, CH ₂	31.0, CH ₂
15	50.5, CH	49.3, CH	50.0, CH	49.7, CH	50.5, CH
16	39.0, qC	38.2, qC	38.2, qC	38.0, qC	38.9, qC
17	28.5, CH ₃	29.2, CH ₃	28.9, CH ₃	28.9, CH ₃	27.2, CH ₃
18	26.5, CH ₃	27.0, CH ₃	26.4, CH ₃	26.6, CH ₃	30.3, CH ₃
19	13.8, CH ₃	20.7, CH ₃	13.0, CH ₃	19.2, CH ₃	13.0, CH ₃
20	143.2, CH	146.1, CH	143.6, CH	147.0, CH	143.0, CH
21	118.7, CH ₂	115.4, CH ₂	119.1, CH ₂	115.4, CH ₂	120.2, CH ₂
23 ^a	158.2, qC	157.2, qC	160.2, qC	157.8, qC	^a
24	39.8, qC	39.3, qC	41.9, qC	41.9, qC	49.5, qC
25	143.4, CH	143.1, CH	74.2, CH	74.6, CH	209.3, qC
26	125.6, CH	125.5, CH	35.4, CH ₂	35.3, CH ₂	85.5, CH
27	27.1, CH ₃ ^b	27.3, CH ₃ ^b	23.0, CH ₃	23.4, CH ₃	28.0, CH ₃
28	32.1, CH ₃ ^b	32.4, CH ₃ ^b	25.5, CH ₃	25.6, CH ₃	32.0, CH ₃

^aSignal not observed

^bassignments may be reversed

Table 3
MIC and IC₅₀ values of compounds from *Fischerella ambigua* against test organisms *in vitro*

Compound	M. t. ^a MIC (μM)	B. a. ^b MIC (μM)	S. a. ^c MIC (μM)	M. s. ^d MIC (μM)	C. a. ^e MIC (μM)	V. f. IC ₅₀ (μM)
Ambiguine K isonitrile (1)	6.6	7.4	4.6	23.7	<0.9	53.2
Ambiguine L isonitrile (2)	11.7	16.2	10.5	29.3	<1.0	44.6
Ambiguine M isonitrile (3)	7.5	28.5	4.7	25.8	1.1	79.8
Ambiguine N isonitrile (4)	27.1	30.9	5.5	48.8	<1.0	118.4
Ambiguine O isonitrile (5)	46.7	13.8	1.8	14.8	<1.0	80.7
Ambiguine A isonitrile	7.0	16.1	10.9	27.8	1.7	26.0
Ambiguine B isonitrile	21.0	3.6	7.4	59.6	<1.0	58.6
Ambiguine C isonitrile	61.2	>128	1.5	1.4	<0.9	78.3
Ambiguine E isonitrile	13.1	>128	8.9	59.7	1.7	42.6
Ambiguine F isonitrile	6.8	>128	>128	34.0	>128	57.9
Ambiguine I isonitrile	58.8	>128	>128	39.6	5.1	>128
Hapalindole G	0.1					>128
Hapalindole H						97.9
Rifampin		0.2				
Ciprofloxacin			1.4			
Gentamicin				0.7		
Moxifloxacin					0.03	
Ketoconazole						

^a *Mycobacterium tuberculosis*

^b *Bacillus anthracis*

^c *Staphylococcus aureus*

^d *Mycobacterium smegmatis*

^e *Candida albicans*

^f Vero Cells