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Hapalindole-related Alkaloids from the Cultured Cyanobacterium *Fischerella ambigua*

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Abstract

Four new hapalindole-related alkaloids, namely fischambiguines A and B, ambiguine P, ambiguine Q nitrile as well as ambiguine G nitrile were identified from the cultured cyanobacterium *Fischerella ambigua* (UTEX 1903). The structures were determined by spectroscopic analysis including MS, 1D and 2D NMR and X-ray crystallography. The alkaloids possessed fused penta- and hexacyclic carbon skeletons. Fischambiguine B displayed a strong inhibitory activity against *Mycobacterium tuberculosis* with an MIC value of 2 μ M, with no detectable cytotoxicity in a Vero cell line.

Keywords

Fischerella ambigua; cyanobacterium; *Mycobacterium tuberculosis*; fischambiguine A; fischambiguine B; ambiguine P; ambiguine Q nitrile

1. Introduction

Cyanobacteria (blue-green algae) are ancient photosynthetic prokaryotic organisms that produce biological active secondary metabolites with diverse chemical structures (Burja *et al.*, 2001; Gerwick, 2001; Sielaff *et al.*, 2006; Singh *et al.*, 2005; Tan, 2007; Van Wagoner *et al.*, 2007; Yuzuru, 2003). Several recent reviews have discussed their potential as a source of pharmaceutical leads (Sielaff *et al.*, 2006; Tan, 2007; Williams, 2009). One family of compounds that has been explored for their pharmaceutical potential are isonitrile containing indole alkaloids, such as hapalindoles (Doan *et al.*, 2001; Huber *et al.*, 1998; Klein *et al.*, 1995; Moore *et al.*, 1987; Moore *et al.*, 1989), ambiguine isonitriles (Huber *et al.*, 1998; Raveh and Carmeli, 2007; Smitka *et al.*, 1992), fischerindoles (Park *et al.*, 1992) and wetwitindolinones (Jimenez *et al.*, 1999; Stratmann *et al.*, 1994) from branched filamentous cyanobacteria of the genera *Fischerella*, *Hapalosiphon*, *Westiella*, and *Westiellopsis*. Since the

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first report of hapalindoles in 1984 by Moore and coworkers (Moore *et al.*, 1984), over 60 natural products belonging to this family have been identified (Richter *et al.*, 2008). All these alkaloids have polycyclic carbon skeletons that derive from condensation of a tryptophan derivative and geranyl pyrophosphate (Moore *et al.*, 1984; Richter *et al.*, 2008; Stratmann *et al.*, 1994). The promising biological activities and intricate structures have led to several synthetic efforts for this class of alkaloids (Baran *et al.*, 2007; Baran and Richter, 2004; Baran and Richter, 2005; Chandra *et al.*, 2007; Fukuyama and Chen, 1994; Kinsman and Kerr, 2001; Muratake *et al.*, 1990; Muratake and Natsume, 1990a; Muratake and Natsume, 1990b; Reisman *et al.*, 2006; Richter *et al.*, 2008; Vaillancourt and Albizati, 1993).

The crude extract of cultured cyanobacterium *Fischerella ambigua* (UTEX 1903) showed significant inhibitory activities against *Mycobacterium tuberculosis* and *Bacillus anthracis* (Chlipala *et al.*, 2009). Our initial investigation of the biologically active components of *F. ambigua* led to the identification of fourteen alkaloids including five new ambiguine isonitrile derivatives and nine previously described indole alkaloids (Mo *et al.*, 2009). Continued investigation of this cyanobacterium resulted in the identification of four novel hapalindole-related indole alkaloids, fischambiguines A (**1**) and B (**2**), ambiguine P (**3**) and ambiguine Q (**4**) nitrile as well as one previously reported compound, ambiguine G nitrile (**5**) (Huber *et al.*, 1998). Herein, we reported the isolation, structural elucidation and biological activity of these compounds.

2. Results and discussion

The alkaloids were isolated from the CH₂Cl₂:MeOH (1:1) extract of the cultured cyanobacterium *F. ambigua* using a combination of silica gel, Sephadex LH-20 and reversed phase chromatography, as outlined in the experimental section. Their chemical structures were identified by analysis of MS, 1D and 2D NMR data and X-ray crystallography.

Fischambiguine A (**1**) was obtained as colorless crystals from MeOH and MeCN (1:1). HRMS measurement indicated a molecular formula of C₂₆H₃₀N₂O ([M-H]⁺ at *m/z* 385.2277 calcd for C₂₆H₂₉N₂O 385.2285). The UV spectrum showed absorptions characteristic of an indole moiety [$\lambda_{\max}$ (log ϵ) 229 (2.72), 277 (2.11) nm]. Various features of the ¹H and ¹³C NMR data (Table 1) indicated compound **1** to be related to hapalindole alkaloids.

The ¹H NMR signals (Table 1) observed at δ_{H} 6.95 (1H, d, *J*=7.1 Hz, H-5), 7.04 (1H, dd, *J*=7.8 and 7.1 Hz, H-6), and 7.10 (1H, d, *J*=7.8 Hz, H-7) were attributed to three consecutive aromatic protons of the indole moiety. The resonances at δ_{H} 6.53 (1H, dd, *J*=17.6 and 11.0 Hz, H-20), 5.26 (1H, dd, *J*=17.6 and 1.3 Hz, H-21 *E*) and 5.15 (1H, dd, *J*=11.0 and 1.3 Hz, H-21 *Z*) indicated the presence of a vinyl group, while two singlets at δ_{H} 5.87 and 5.76 were assigned as a sp² methylene group (C-26, δ_{C} 119.9) of an exomethylene moiety. In addition, five methyl groups were observed at δ_{H} 1.81 (3H, s), 1.71 (3H, s), 1.68 (3H, s), 1.45 (3H, s) and 1.38 (3H, s). The signals at δ_{H} 2.02 (H_{13ax}), 1.50 (H_{13eq}), 2.22 (H_{14ax}), 1.74 (H_{14eq}) and 2.22 (H₁₅) were ascribed to the CH₁₅CH_{14ax}CH_{14eq}CH_{13ax}CH_{13eq} spin system. Signal overlap made it impossible to calculate the coupling constants between H₂-14 and H-15, or between H₂-14 and H₂-13. The assignment of the *axial* and *equatorial* protons at C-13 and C-14 was determined by comparison of the ¹H NMR data with those reported for deschloro ambiguine isonitriles (Raveh and Carmeli, 2007; Smitka *et al.*, 1992), where the resonances of both H-13_{ax} and H-14_{ax} always appear in the lower field when compared with the *equatorial* proton for the same position. The chemical shifts for both H-14_{ax} and H-15 overlapped at δ_{H} 2.22 in the ¹H NMR spectra. However, based on a careful analysis of the HSQC spectrum, the accurate chemical shifts for H-14_{ax} and H-15 were determined as δ_{H} 2.222 and 2.217 ppm, respectively. The 0.005 ppm chemical shift difference (4.5 Hz) was resolved by the high-resolution of the

900 MHz NMR spectrometer, thus enabling the unequivocal assignment of the corresponding 2D NMR correlations of these two protons.

The deductions above were confirmed in the ^{13}C NMR spectrum for **1**, where 26 carbon resonances could be observed including thirteen in the 100–160 ppm region. These were assigned to the indole moiety (δ_{C} 140.8 s, 138.5 s, 136.6 s, 125.1 s, 123.3 d, 114.6 d, 112.2 s and 109.4 d), the vinyl group (δ_{C} 148.8 d and 113.0 t), the exomethylene group (δ_{C} 152.8 s and 119.9 t), and an isonitrile moiety (δ_{C} 157.4 s). This accounted for ten degrees of unsaturation, and three additional rings were required to satisfy the total of thirteen degrees of unsaturation implied by the molecular formula. Hence, fischambiguine A was pentacyclic.

The above deduced partial structures were connected by analysis of correlations observed in the HMBC spectrum. HMBC correlations from H-5 and H-7 to C-9, as well as H-6 to C-4 and C-8 established the structure of indole moiety. The *gem*-dimethyl group at C-16 was connected to C-4 based upon correlations observed from H₃-17 and H₃-18 to C-4 and C-16. Further correlations from H₃-17 and H₃-18 to C-15 connected this portion to the H-15/H₂-14/H₂-13 fragment deduced from the ^1H - ^1H COSY spectrum. 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 placed the vinyl and C-19 methyl groups at C-12 and connected this fragment to C-13. Meanwhile, the HMBC correlations from the protons of the exomethylene moiety H₂-26 to C-25 and C-11 suggested that this group was connected to C-11. Additional HMBC correlations from H₃-27 and H₃-28 to C-24 and C-25 indicated the second *gem*-dimethyl group to be adjacent to the Δ^{25} -double bond. This *gem*-dimethyl group was further connected to C-2 of the indole moiety based on HMBC correlations from both H₃-27 and H₃-28 to C-2, thus completing a six-membered ring E. The positions of the isonitrile group at C-11 (δ_{C} 74.6) and hydroxyl group at C-10 (δ_{C} 71.2) were established by comparison of the chemical shift with those published for ambiguine isonitrile compounds (Mo *et al.*, 2009; Raveh and Carmeli, 2007; Smitka *et al.*, 1992).

Correlations in the 2D NOESY spectrum for **1** between H-13_{ax}, H-20, and H-15, as well as between H-15 and H₃-18 indicated H-15 and H-20 to be in the α plane. This led to the assignment of the relative configuration as 12*R** and 15*R**. X-ray crystallography confirmed the gross structure and determined the relative configuration of fischambiguine A (**1**) as 10*R**, 11*R**, 12*R**, 15*R** (fig.1). The ORTEP diagram is shown in Fig. 2. Fischambiguine A (**1**) is the first hapalindole-related alkaloid to possess a six-membered E ring.

Fischambiguine B (**2**) was obtained as a white amorphous solid, and exhibited a 3:1 molecular ion cluster at m/z 435/437 [$\text{M}-\text{H}$][−] in the ESI MS. The molecular formula was determined as C₂₆H₂₉ClN₂O₂ by HRMS (m/z 435.1866 [$\text{M}-\text{H}$][−]). ^1H and ^{13}C NMR spectra of **2** (Table 1) indicated it to be closely related to fischambiguine A (**1**). However, in the ^1H and COSY NMR spectra of **2**, the signals observed for the H-15/H₂-14/H₂-13 spin system were replaced by resonances for a H-15/H₂-14/H-13 spin system. The chemical shift of H-13 (δ_{H} 4.25) and C-13 (δ_{C} 68.2) in **2** suggested a chlorine substitution at C-13. The presence of the hapalindole-core structure was established by analyzing 1D and 2D NMR spectral data (Table 1). The ^1H and ^{13}C NMR spectra of **2** also lacked the resonances observed for the exomethylene group ($\delta_{\text{H}-26}$ 5.87 and 5.76, $\delta_{\text{C}-26}$ 119.9 and $\delta_{\text{C}-25}$ 152.8) in fischambiguine A. These had been replaced by resonances at δ_{H} 3.35 and 2.67 (H₂-26) along with δ_{C} 43.4 (C-26) and δ_{C} 66.9 (C-25). These chemical shifts indicated both C-25 and C-26 to be adjacent to oxygen atoms. The molecular formula required two oxygen atoms in the structure: one was assigned to a hydroxyl group substituted at C-10 (δ_{C} 71.0) by comparison of ^{13}C NMR chemical shift to that in **1** ($\delta_{\text{C}-10}$ 71.2). Consequently, both C-25 and C-26 were connected to the same oxygen atom to form an epoxide ring. The HMBC correlations from H-26 (*pro S*) to C-11 and C-24, as well as from H₃-27 and H₃-28 to both C-25 and C-2 led to the construction of a spiro-epoxy

containing six membered ring E system. This hexacyclic structure accounted for all fourteen degrees of unsaturation required by the molecular formula.

The coupling constant between H-14_{ax} and H-13 ($J = 12.3$ 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, NOESY correlations observed from H-15 to H-13 and H₃-18, as well as from H-13 to H-20 indicated a relative configuration of 12*R**, 13*R** and 15*R**, identical to those found for **1**. Meanwhile, NOESY correlations observed from H-26 (*pro S*) to H₃-19, H₃-27 and OH-10 suggested that these all were in the β plane. Therefore, the relative configuration at positions 10 and 25 were assigned as 10*R** and 25*R**. The relative configuration at position 11 was determined as 11*S** by comparing ¹³C NMR chemical shifts of **2** with those for **1**. Based upon these results, the structure of fischambiguine B (**2**) was concluded as shown and the relative configuration was assigned as 10*R**, 11*S**, 12*R**, 13*R**, 15*R** and 25*R** (fig.1).

Ambiguine P (**3**) was obtained as a white amorphous solid. The HRMS at m/z 358.2151 [M-H][−] suggested a molecular formula of C₂₅H₂₉NO. ¹H and ¹³C NMR data indicated that this compound was related to ambiguine isonitriles. However, absence of the IR band typical for an isonitrile group at $\nu_{\max}$ 2100–2150 cm^{−1} and the absence of a ¹³C NMR resonance around δ_C 157 ppm suggested that **3** was lacking the isonitrile moiety found in other ambiguine isonitriles. This conclusion was also confirmed by the molecular formula, which indicated the presence of only one nitrogen atom, assigned to the indole moiety.

Fourteen of the 25 carbon resonances observed in the DEPTQ NMR spectrum of **3** appeared at >100 ppm (Table 2). Eight of these were assigned to the indole moiety (δ_C 142.1 s, 139.1 s, 134.9 s, 125.6 s, 123.5 d, 114.6 d, 109.6 d and 108.8 s). Another four carbon resonances were attributed to a vinyl group (δ_C 146.9 d and 114.6 t) and the Δ^{23} - double bond (δ_C 133.2 d and 128.6 d). The final two carbons (δ_C 133.5 s, C-10 and 133.9 s, C-11) were assigned to the tetrasubstituted Δ^{10} - double bond based on the HMBC correlations (Table 2).

The presence of the indole moiety and two fused hexacyclic rings were established by analysis of HMBC correlations as described above for fischambiguine B (**2**). In addition, correlations from H-23 to C-11 and from H-24 to C-10 established the linkage of the Δ^{23} -double bond at C-11. The Δ^{23} -double bond was in turn connected to C-2 of the indole *via* a *gem*-dimethyl group as deduced by the correlations from H-23, H₃-25 and H₃-26 to C-2. A hydroxyl group was placed on the quaternary carbon C-15 based on the carbon chemical shift (δ_C 77.2 ppm), as well as to satisfy the molecular formula.

The relative configuration of ambiguine P (**3**) was determined by analysis of chemical shifts and NOE correlations observed in the 2D NOESY spectrum. NOESY correlations observed between H-14_{ax} and H₃-18, as well as between H-20, H-14_{ax} and H-13_{eq} indicated all these protons to be in the α plane. A NOESY correlation between H₃-17 and 15-OH suggested that 15-OH and H₃-17 were oriented in the β plane. In this configuration, one of the methyl groups at C-16 (H₃-17) was pseudoequatorial and almost coplanar with the indole system, while the other methyl group (H₃-18) was pseudoaxial and below the indole ring. The high-field chemical shift of H₃-18 (δ_H 1.00) and strong NOE correlations between H₃-17 and H-5, as well as between H₃-18 and H-14_{ax} confirmed this configuration. Similarly, the methyl groups at C-22 were found to be in pseudoaxial (H₃-26, δ_H 1.02) and pseudoequatorial positions (NOE correlations between H₃-25 and NH). Furthermore, the NOESY correlations from H-21Z to H₃-18 and H₃-26 indicated that the vinyl group was orientated axially in the same plane with H₃-18 and H₃-26. Based on all of the above information, the relative configuration at position 12 and 15 was determined as 12*R** and 15*R** (fig.1).

Ambiguine Q nitrile (**4**) was obtained as a pale yellow amorphous solid. The HRMS peak at m/z 367.2170 [M-H][−] suggested the molecular formula as C₂₆H₂₈N₂. The IR band at $\nu_{\max}$ 2201

cm⁻¹ indicated the presence of a nitrile group. The ¹H NMR spectrum (Table 2) of **4** showed it to be related to ambiguine P (**3**) and ambiguine G nitrile (**5**) (Huber *et al.*, 1998) with signals for a 1,2,3-trisubstituted aromatic moiety (δ_{H} 7.19, 7.13 and 7.00), a vinyl group (δ_{H} 5.84, 5.18 and 5.09), five isolated methyl groups (δ_{H} 1.87, 1.55, 1.46, 1.02, and 1.00), and an isolated CH₂CH₂CH spin system from C-13 to C-15. The coupling constants between H-14 and H-13, as well as H-14 and H-15 could not be determined due to signal overlapped. The assignment of the axial and equatorial protons was accomplished by comparing the chemical shift of protons at C-13 and C-14 with those reported for deschloro ambiguine isonitriles (Huber *et al.*, 1998; Raveh and Carmeli, 2007; Smitka *et al.*, 1992). Although a ¹³C NMR spectrum could not be acquired successfully due to the limited quantity of compound (0.2 mg), all carbon chemical shifts could be determined indirectly by analysis of HSQC and HMBC spectral information.

The HMBC correlations from H-24 to C-10, C-11, and C-12 connected C-24 to C-11. C-24 was in turn connected to a nitrile-substituted sp² quaternary carbon (C-23) as deduced by HMBC correlation from H-24 to nitrile group carbon C-27 (δ_{C} 120.9). A *gem*-dimethyl group at C-22 was connected to C-23 based on the HMBC correlations from H-24 to C-22, and from H₃-25 and H₃-26 to C-22 and C-23. This *gem*-dimethyl group was further connected to C-2 of the indole moiety to form the seven membered ring moiety indicated by the HMBC correlations from H₃-25 and H₃-26 to C-2. Together these observations establish the planar structure of ambiguine Q nitrile (**4**) as deschloro ambiguine G nitrile. The relative configuration was determined by analysis of NOE correlations observed in the 2D NOESY spectrum. A correlation between H-14_{ax} to H₃-19 indicated both to be in the β plane, while H-13_{ax} and H-15 were in the β plane based on the NOESY correlations between H-13_{ax} and H-20, and H-15 and H₃-18. Together these observations established the relative configuration as 12*R** and 15*R** (fig.1), the same as reported by Moore and coworkers for ambiguine G nitrile (Huber *et al.*, 1998).

The previously reported compound ambiguine G nitrile (**5**) was identified by comparing the spectroscopic data with those reported in the literature. (Huber *et al.*, 1998)

All isolates, except compound **4**, were evaluated for antimicrobial activity against a series of microorganisms including *M. tuberculosis*, *B. anthracis*, *Staphylococcus aureus*, *M. smegmatis*, *Escherichia coli* and *Candida albicans*, as well as for Vero cell toxicity (Table 3). Although fischambiguine A (**1**) was not active against *M. tuberculosis* at 100 μ M, fischambiguine B (**2**) showed inhibitory activity against *M. tuberculosis* with MIC value of 2 μ M. Compound **2** showed lower antibiotic activity against the other microorganisms, and no detectable cytotoxicity in the Vero cell assay. The structural differences between these two compounds are in the chlorination at the C-13 position and the epoxidation of the Δ^{25} -double bond for fischambiguine B (**2**). In both our and Raveh and Carmeli's studies, the antimicrobial activity of nonchlorinated ambiguine isonitriles was found to be similar to their chlorinated counterparts (Mo *et al.*, 2009; Raveh and Carmeli, 2007). This suggests that the chlorine substitution at C-13 position may not be important for the antibacterial activity of ambiguine isonitriles. The same could be assumed for fischambiguines, since both fischambiguines and ambiguine isonitriles contain the same hapalindole core structure (Richter *et al.*, 2008). Ambiguine E isonitrile also contains an epoxy ring, which is fused with the seven-membered E ring. However, this compound showed 10-fold lower activity against *M. tuberculosis* than Fischambiguine B (Mo *et al.*, 2009). This would imply that the spiro-epoxy containing six membered ring E in the structure of fischambiguine B (**2**) is essential for the antituberculosis activity observed for this compound. Ambiguine P (**3**) showed weak antimicrobial activity against *C. albicans* (MIC 32.9 μ M), while ambiguine G nitrile inhibited the growth of *S. aureus* and *M. tuberculosis* with MIC value at 6.6 and 49.7 μ M, respectively. None of these compounds inhibited the growth of the gram-negative bacterium *E. coli*.

Fischambiguines A (**1**) and B (**2**) are structurally related to ambiguine isonitriles. In both structural classes, an isoprene unit is present at the 2 position of the indole moiety of a tetracyclic hapalindole. This isoprene unit may undergo further cyclization to form either a seven membered ring as found in many ambiguine isonitriles (Huber *et al.*, 1998; Mo *et al.*, 2009; Raveh and Carmeli, 2007; Richter *et al.*, 2008; Smitka *et al.*, 1992) or a six membered ring as reported here for fischambiguines. This novel cyclization pathway of the ring E portion further expands the array of related compounds produced by *F. ambigua*.

Ambiguine P (**3**) and ambiguine Q nitrile (**4**) are new cyanobacterial hapalindole-related alkaloids. Moore *et al.* suggested that the biogenesis of ambiguine G nitrile (**5**) probably involves the rearrangement of ambiguine E isonitrile (Huber *et al.*, 1998). This would imply that the precursor of ambiguine Q nitrile (**4**) could be the deschloro derivative, ambiguine I isonitrile. However, the co-occurrence of ambiguine P (**3**) and ambiguine Q nitrile (**4**) raised the concern that these compounds were artificial products produced during the purification. In order to evaluate this, 2.5 mg of ambiguine E isonitrile, the precursor Moore *et al.* suggested for ambiguine G nitrile was dissolved in 2 mL of CH₂Cl₂:MeOH (60:1) (the eluting solvent for **3** and **4**) and mixed with 250 mg silica gel. The mixture was sealed and placed at room temperature for 24 hours with occasional stirring. The solution was filtered and the solvent was evaporated under vacuum. Comparison of HPLC-UV chromatogram and ¹H NMR spectrum for the residue with those for ambiguine E isonitrile indicated that no ambiguine G nitrile or other decomposition products was produced during this treatment. In addition, the presence of the peaks corresponding to ambiguines P and Q nitrile in the LC-MS chromatogram of the crude extract also support that ambiguines P and Q nitrile are present in the cyanobacterium and are not products of our isolation protocol. Ambiguine P (**3**) is the first ambiguine derivative found without an isonitrile or nitrile functionality in the structure.

3. Conclusion

The bioassay-guided chemical investigation of cyanobacterium *Fischerella ambigua* led to the isolation of four new hapalindole-related alkaloids. Fischambiguines A (**1**) and B (**2**) contain a six-membered E ring, which suggests a new cyclization pathway in the biosynthesis of ambiguine isonitrile compounds. Ambiguine P (**3**) is the first ambiguine derivative found without an isonitrile or nitrile group. This compound is also the only ambiguine derivative bearing a hydroxyl group at C-15. Fischambiguine B (**2**) showed a inhibitory activity against *M. tuberculosis* with MIC value of 2 μM and no detectable cytotoxicity in the Vero cell assay.

4. Experimental

4.1. 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 using a Bruker Avance DRX400 MHz NMR spectrometer, a Bruker Avance DRX600 MHz NMR spectrometer with 5 mm CPTXI Z-gradient probe and a Bruker AVII900 MHz NMR spectrometer with 5 mm 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 and a Shimadzu LCMS IT-TOF. The X-ray analysis was carried out on a Rigaku/MSC RAPID imaging plate area detector equipped with a normal focus sealed X-ray tube and Cu Kα radiation.

4.2. 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) (Andersen *et al.*, 2005). 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.

4.3. 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, a portion of which (682 mg) was fractionated on silica gel using a gradient with increasing amount of MeOH in CH₂Cl₂ to afford 18 fractions. The TB active fraction 2 (MIC 1.5 µg/mL, 247.7 mg) was further fractionated using Sephadex LH-20 with MeOH as an eluent to obtain nine fractions. Fraction 2–8 (160.2 mg) was subjected to a reversed phase HPLC (Alltima C18, 10 µm, 250×10 mm, 4 mL/min) with a solvent gradient of 85% MeOH/15% H₂O to 100% MeOH over 20 minutes to afford compound **2** (*t*_R = 10.4 min, 0.5 mg) and a peak containing a mixture (*t*_R = 8.8 min). The mixture was further purified by the RP-HPLC eluting with acetonitrile:H₂O (75:25) to afford compound **1** (*t*_R = 14.6 min, 1.0 mg).

The freeze-dried biomass from a second 5 L of culture was extracted by repeated maceration with CH₂Cl₂:MeOH (1:1) to yield 178 mg of crude extract. The extract was fractionated on an open silica gel column using a gradient with increasing amount of MeOH in CH₂Cl₂ to afford 9 fractions. Fraction 3 (2.3 mg) was subjected to a reversed-phase HPLC (Alltima C8, 5 µm, 250×10 mm, 4 mL/min) with a solvent gradient of MeOH: H₂O (6:4) to MeOH:H₂O (9:1) over 15 minutes to afford compound **3** (*t*_R = 8.4 min, 0.4 mg) and **4** (*t*_R = 10.1 min, 0.2 mg), as well as ambiguine G nitrile (**5**, *t*_R = 9.6 min, 0.3 mg).

4.3.1 Fischambiguine A (1)—colorless crystals (MeOH:acetonitrile 1:1); [α]_D −6 (*c* 0.09 MeOH); UV $\lambda_{\max}$ (log ϵ) 229 (2.72), 277 (2.11) nm; IR $\nu_{\max}$ 3315, 2927, 2853, 2132, 1591, 1458, 1432, 1365 cm^{−1}; ¹H and ¹³C NMR (see Table 1); HRESIMS *m/z* 385.2277 [M-H][−] (calcd for C₂₆H₂₉N₂O, 385.2285).

4.3.2 Fischambiguine B (2)—white amorphous powder (MeOH); [α]_D −33 (*c* 0.13 MeOH); UV $\lambda_{\max}$ (log ϵ) 228 (2.50), 280 (1.89) nm; IR $\nu_{\max}$ 3390, 2928, 2124, 1590, 1462, 1365 cm^{−1}; ¹H and ¹³C NMR (see Table 1); HRESIMS *m/z* 435.1866 [M-H][−] (calcd for C₂₆H₂₈ClN₂O₂, 435.1839).

4.3.3 Ambiguine P (3)—white amorphous powder; [α]_D −37 (*c* 0.03, MeOH); UV $\lambda_{\max}$ (log ϵ) 235 (2.4), 280 (0.7), 325 (0.6) nm; IR $\nu_{\max}$ 3629, 2966, 1597, 1458, 1362 cm^{−1}; ¹H and ¹³C NMR (see Table 2); HRESIMS *m/z* 358.2151 [M-H][−] (calcd for C₂₅H₂₈NO, 358.2151).

4.3.4 Ambiguine Q nitrile (4)—yellow amorphous powder; [α]_D +13 (*c* 0.03, MeOH); UV $\lambda_{\max}$ (log ϵ) 230 (2.4), 275 (1.6), 390 (1.0) nm; IR $\nu_{\max}$ 3628, 3354, 2922, 2201, 1652, 1558, 1553, 1457 cm^{−1}; ¹H and ¹³C NMR (see Table 2); HRESIMS *m/z* 367.2170 [M-H][−] (calcd for C₂₆H₂₇N₂, 367.2167).

4.3.5 Ambiguine G nitrile (5)—yellow amorphous powder; [α]_D +35 (*c* 0.06, MeOH); Other spectroscopic data as previously reported (Huber, *et al.*, 1998).

4.4. Single-crystal X-ray crystallography of fischambiguine A (1)

Single crystals for X-ray analysis were grown from MeOH: MeCN (1:1). A colorless crystal with dimensions $0.02 \times 0.05 \times 0.20$ mm was used for analysis. Crystal data: $C_{26}H_{30}N_2O \cdot CH_3CN$, MW = 427.6, Monoclinic, space group $P2_1$ (No. 4); $a = 12.4994(9)$ Å, $b = 6.9773(6)$ Å, $c = 14.2759(11)$ Å, $\beta = 94.239(6)^\circ$, $V = 1241.63(17)$ Å³; $Z = 2$, $D_c = 1.144$ mg/m³; $\mu = 0.542$ mm⁻¹; $F(000) = 460$. $R_{int} = 0.082$; $R_1 = 0.095$, $wR_2 = 0.254$. The integration of intensities and refinement of unit cell parameters and orientation matrix were carried out using CrystalClear (1999). The WinGX package was used for completing the structure determination (Farrugia, 1999). The structure was solved by direct methods, repeated cycling with least-squares refinement on F^2 and difference Fourier maps using SHELXL-97 (Sheldrick, 1998). All non-hydrogen atoms were refined with anisotropic Gaussian displacement parameters. The Flack parameter was -1.4 (15) and the relative configuration of the structure was determined as C(10) R^* , C(11) R^* , C(12) R^* , and C(15) R^* . Crystallographic data (CCDC 722563) for this structure have been deposited with the Cambridge crystallographic Data Centre. Copies of the data can be obtained, free of charge, on application to the Director, CCDC, 12 Union Road, Cambridge CB2 1EZ, UK (fax: +44-(0)1223-336033 or deposit@ccdc.cam.ac.uk).

4.5. Treatment of ambiguine E isonitrile with silica gel

2.5 mg of ambiguine E isonitrile was dissolved in 2 mL of CH_2Cl_2 :MeOH (60:1), and then mixed with 250 mg silica gel. The mixture was sealed and placed at room temperature for 24 hours with occasional stirring. The solution was filtered to remove silica gel and the solvent was evaporated under vacuum. The HPLC-UV chromatograms and ¹H NMR spectra of the residue and ambiguine E isonitrile were obtained and compared.

4.6. Antimicrobial activity

4.6.1 Mycobacterium tuberculosis—The inhibitory activity of fractions and compounds against *M. tuberculosis* was tested using the Microplate Alamar blue Assay (MABA) (Falzari *et al.*, 2005). Virulent H37Rv strain was used in the assay. The MIC value was determined as the lowest drug concentration effecting an inhibition of $\geq 90\%$. Each evaluation was done as a single dose response and repeated once.

4.6.2 Bacillus anthracis—The fractions and compounds were tested using the previously described method (Athamna *et al.*, 2004). The MIC of each sample was calculated as the lowest concentration that prevents visible bacterial growth. Each evaluation was done as a single dose response and repeated once.

4.6.3 Other organisms—The microbroth dilution MIC method was used to test the activity of compounds against *Staphylococcus aureus* (Isenberg, 2002) and *Candida albicans* (Clinical and Laboratory Standards Institute, 2002). Each evaluation was done as a single dose response and repeated once.

4.6.4 Cytotoxicity—The cytotoxicity of compounds was evaluated using green monkey kidney cells (Vero) (Cantrell *et al.*, 1996). Cell viability was measured using the CellTiter 96 aqueous nonradioactive cell proliferation assay. Results were expressed as the mean value of duplicate data points.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
Structures of fischambiguines A (**1**) and B (**2**), ambiguine P (**3**), ambiguine Q (**4**) and G (**5**) nitriles

Figure 2.
ORTEP diagram of fischambiguine A (**1**)

Table 1

NMR spectroscopic data for compounds **1** in MeOH-*d*₄ and **2** in DMSO-*d*₆^a

Position	1		2		HMBC correlations	HMBC correlations
	δ_C , mult.	δ_H , mult. (<i>J</i> in Hz)	δ_C , mult.	δ_H , mult. (<i>J</i> in Hz)		
2	138.5, s		138.2, s			
3	112.2, s		110.3, s			
4	140.8, s		139.9, s			
5	114.6, d	6.95, d (7.1)	114.6, d	6.96, d (7.3)		C-7, 9, 16
6	123.3, d	7.04, dd (7.8, 7.1)	123.2, d	7.05, dd (7.9, 7.3)		C-4, 8
7	109.4, d	7.10, d (7.8)	109.7, d	7.15, d (7.9)		C-5, 9
8	136.6, s		135.6, s			
9	125.1, s		124.4, s			
10	71.2, s		71.0, s			
11	74.6, s		70.5, s			
12	44.3, s		49.6, s			
13 _{ax}		2.02, m				
13 _{eq}	41.4, d	1.50, m	68.2, d	4.25, dd (12.3, 3.6)		C-12, 19, 20
14 _{ax}	19.0, t	2.22, m ^b	30.0, t	2.42, ddd (13.1, 12.3, 12.2)		C-10, 13, 15
14 _{eq}		1.74, m		2.13, dd (12.2, 3.6, 2.3)		
15	49.3, d	2.22, m ^b	48.7, d	2.10, dd (13.1, 2.3)		C-16, 17, 18, 13
16	39.6, s		39.5, s			
17	26.3, q	1.38, s	26.8, q	1.34, s		C-18, 16, 15, 4
18	27.2, q	1.45, s	27.8, q	1.39, s		C-17, 16, 15, 4
19	20.5, q	1.81, s	12.9, q	1.62, s		C-11, 12, 13, 20
20	148.8, d	6.53, dd (17.6, 11.0)	143.3, d	6.16, dd (17.4, 10.8)		C-19, 12, 13, 11
21Z	113.0, t	5.26, dd (17.6, 1.3)		5.30, dd (17.4, 1.2)		
21E		5.15, d (11.0, 1.3)	119.5, t	5.44, dd (10.8, 1.2)		C-12, 20
23	157.4, s		162.7, s			
24	40.8, s		41.2, s			
25	152.8, s		66.9, s			
26Z	119.9, t	5.87, s				

Position	1		2	
	δ_C , mult.	δ_H , mult. (<i>J</i> in Hz)	HMBC correlations	δ_C , mult. δ_H , mult. (<i>J</i> in Hz) HMBC correlations
26E		5.76, s		
26 <i>pro S</i>				3.35, d (4.0) C-11, 24, 25
26 <i>pro R</i>			43.4, t	2.67, d (4.0) C-24, 25
27	32.1, q	1.68, s	C-28, 24, 2, 25	1.59, s C-28, 24, 2, 25
28	32.5, q	1.71, s	C-27, 24, 2, 25	1.15, s C-27, 24, 2, 25
NH				11.0, s C-3, 9, 2, 8
10-OH				5.16, brs C-3, 10, 15

^aFrequency: 900 MHz for ¹H and 226 MHz for ¹³C

^bThe chemical shifts of H-14_{ax} and H-15 in compound 1 were determined as 2.222 and 2.217 ppm, respectively. The 0.005 ppm difference between these two resonances can be resolved in 900 MHz NMR spectra. Therefore, the corresponding 2D NMR correlations were assigned unequivocally.

Table 2

NMR spectroscopic data for compounds **3** and **4** in MeOH-*d*₄^a

Position	3		4	
	δ_C , mult.	δ_H , mult. (<i>J</i> in Hz)	HMBC correlations	δ_C , mult. δ_H , mult. (<i>J</i> in Hz) HMBC correlations
2	142.1, s			138.0, s
3	108.8, s			111.0, s ^b
4	139.1, s			140.8, s
5	114.6, d	6.95, dd (7.2, 0.6)	C-7, 9, 16	114.2, d 7.00, d (7.1) C-7, 9, 16
6	123.5, d	7.07, t (7.15)	C-4, 8	124.3, d 7.13, t (7.6) C-4, 8
7	109.6, d	7.16, dd (8.0, 0.6)	C-5, 9	109.7, d 7.19, d (7.9) C-5, 9
8	134.9, s			134.9, s
9	125.6, s			125.7, s
10	133.5, s			138.7, s
11	133.9, s			134.2, s
12	42.5, s			40.8, s
13 _{ax}		1.95, ddd (14.3, 13.2, 2.7)		1.71, m
13 _{eq}	34.0, t	1.60, ddd (13.2, 3.7, 3.1)	C-12, 15, 20	37.2, t 1.72, m C-15, 19
14 _{ax}	27.1, t	2.15, ddd (14.3, 13.3, 3.1)		1.99, m
14 _{eq}		1.79, ddd (13.3, 3.7, 2.7)	C-12, 13, 10	20.3, t 2.03, m C-15, 16
15	77.2, s			48.4, d 2.92, m
16	45.9, s			40.4, s
17	18.6, q	1.53, s	C-18, 16, 15, 4	1.00, s
18	29.2, q	1.00, s	C-17, 16, 15, 4	1.55, s
19	28.9, q	1.23, s	C-11, 12, 13, 20	1.46, s
20	146.9, d	5.88, dd (17.4, 10.5)	C-19, 12, 11	5.84, dd (17.5, 10.7) C-19, 12, 11
21 _E	114.6, t	5.11, dd (10.5, 1.8)		5.18, dd (17.5, 1.2)
21 _Z		4.93, dd (17.4, 1.8)	C-12, 20	5.09, dd (10.7, 1.2) C-12, 20
22	36.7, s			36.2, s
23	133.2, d	5.40, d (11.4)	C-26, 22, 2, 11	108.8, s
24	128.6, d	5.93, d (11.4)	C-22, 12, 10	6.78, s
25	27.4, q	1.68, s	C-26, 23, 2	1.02, s

Position	3		4			
	δ_C , mult.	δ_H , mult. (<i>J</i> in Hz)	HMBC correlations	δ_C , mult.	δ_H , mult. (<i>J</i> in Hz)	HMBC correlations
26	26.4, q	1.02, s	C-25, 23, 2	25.6, q	1.87, s	C-25, 22, 23, 2
27				120.9, s		
OH ^b		3.97, brs ^b				
NH ^b		10.65, brs ^b			11.15, s	C-3

^aFrequency: 900 MHz for ¹H and 226 MHz for ¹³C

^bsignals were only observed in NMR spectra acquired in DMSO-d₆

Table 3

MIC and IC₅₀ values for compounds from *F. ambigua*

Compound	<i>M. tuberculosis</i> MIC (μM)	<i>B. anthracis</i> MIC (μM)	<i>S. aureus</i> MIC (μM)	<i>M. smegmatis</i> MIC (μM)	<i>C. albicans</i> MIC (μM)	<i>E. coli</i> MIC (μM)	<i>Vero Cells</i> IC ₅₀ (μM)
Fischambiguine A (1)	>100	ND	82.3	ND	15.3	>100	ND
Fischambiguine B (2)	2.0	28.7	19.4	23.4	ND	>100	>128
Ambiguine P (3)	>100	ND	>100	ND	32.9	ND	ND
Ambiguine G nitrile (5)	53.7	>100	6.6	49.7	>100	>100	40.8
Rifampin	0.10						97.9
Ciprofloxacin		0.07					
Gentamicin			0.65			0.45	
Moxifloxacin				0.26			
Ketoconazole					0.03		

ND: not determined