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Forazoline A, a Novel Marine-Derived Polyketide with Antifungal *In vivo* Efficacy**

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Abstract

Forazoline A, a novel antifungal polyketide with *in vivo* efficacy against *Candida albicans*, was discovered using LCMS-based metabolomics to investigate marine invertebrate-associated bacteria. Forazoline A had a highly unusual and unprecedented skeleton. Acquisition of ^{13}C - ^{13}C gCOSY and ^{13}C - ^{15}N HMQCNMR experiments provided the direct carbon-carbon and carbon-nitrogen connectivity, respectively; this represents the first example of determining direct ^{13}C - ^{15}N connectivity for a natural product. Using yeast chemical genomics, we propose that forazoline A works via a new mechanism of action with a phenotypic outcome of disrupting membrane integrity.

Keywords

antifungal agents; chemical genomics; natural products; NMR spectroscopy; structure elucidation

Fungal infections result in over 1.5 million deaths annually worldwide and cost \$12 billion to treat. ^[1] *Candida* spp. are the most common fungal infections, especially in intensive care units, in solid-organ transplant patients, and in blood and marrow transplant patients. ^[2] Of the more than one hundred known *Candida* spp., *Candida albicans* is the most common cause of fungal-born human disease. ^[2] *C. albicans* causes various infections, including candidiasis, which affects about 400,000 people per year, with an astonishingly high mortality rate between 46 and 75%. ^[2]

While amphotericin B has remained the standard for treatment of severe systemic fungal infections, it suffers from low solubility and is associated with dose limiting toxicity. ^[3] The high mortality rate ^[2] combined with the continued rise in fungal resistance to current therapeutics ^[4] demonstrates the ever-present need for new therapeutics.

As part of a drug discovery program to discover novel antifungal therapeutics, we analyzed a collection of marine invertebrate-associated bacteria using LCMS-based metabolomics in conjunction with disc diffusion assays. Metabolomics methodology has shown great promise for streamlining the discovery of novel natural products ^[5] and overcoming historic barriers such as the high rate of rediscovery of known compounds. ^[6]

Using metabolomics-based strategies that we previously published,^[5] we analyzed LCMS profiles of 34 marine-derived bacterial extracts by principal component analysis (PCA) and identified strain WMMB-499, an *Actinomadura* sp. cultivated from the ascidian *Ecteinascidia turbinata* (Herdman, 1880), as a metabolic outlier. After fermentation and subsequent purification, WMMB-499 was found to produce three distinct classes of novel compounds. The first class, halogenated electrophilic polyketides halomadurones A-D that activate the Nrf2-ARE pathway, was recently described.^[7] Forazoline A and B, described here, represented the second novel class; the third class was represented by a potent antibiotic, still under study. Forazoline A (**1**) was the lead antifungal agent with a highly unusual and unprecedented structure. Forazoline A (**1**) demonstrated *in vivo* efficacy against the fungal pathogen *Candida albicans* and works via a putative novel mechanism. Forazoline B (**2**), a brominated analog, was also produced to aid in structure elucidation.

Analysis of forazoline A (**1**) by HRMS supported the molecular formula of $C_{43}H_{69}ClN_4O_{10}S_2$. The MS isotopic distribution suggested the presence of one chlorine atom and two sulfurs. HRMS of forazoline A (**1**) in CD_3OD and D_2O revealed the presence of two exchangeables. Acquisition of a 1H - ^{15}N HSQC allowed us to conclude that one of the exchangeables was an enamine (δ_H 14.58, δ_N 170.3). To determine the location of the other exchangeable proton, forazoline A (**1**) was acetylated.^[8] Two major products, mono and bis-acetyl analogs were formed, and acquisition of 1D and 2D NMR determined that the other exchangeable proton was an OH at C-11.

Extensive 1D and 2D NMR data^[8] (Table S14) were analyzed to establish the majority of the planar structure. However, the structure elucidation presented several challenges. Therefore, we unambiguously determined the carbon backbone by fermentation with uniformly-labeled ^{13}C glucose and acquisition of a ^{13}C - ^{13}C gCOSY that we previously demonstrated as a useful approach for determining the carbon-carbon connectivity.^[9] Fermentation of WMMB-499 with ^{13}C -labeled glucose yielded forazoline A (**1**) with approximately 75% ^{13}C incorporation. The ^{13}C - ^{13}C gCOSY was acquired in 30 minutes on 7.0 mg forazoline A (**1**) and allowed complete assignment of the carbon backbone.

To help confirm the location of the chlorine atom, the amount of KBr in fermentation medium ASW-A was increased from 0.1 g/L to 10 g/L to produce a brominated analogue, forazoline B (**2**). HRMS of forazoline B (**2**) supported the molecular formula of $C_{43}H_{69}BrN_4O_{10}S_2$. A comparison of the 1H and ^{13}C NMR shifts of forazoline A (**1**) and B (**2**) showed that the chemical shifts of H-28, H-31, C-28, and C-31 shifted downfield, and C-29 shifted upfield in forazoline B (**2**).^[8] No other significant changes in chemical shifts between forazoline A (**1**) and B (**2**) existed, and we concluded that the halogen atom was located at C-29.

Due to the unique nature of the thiazoline type rings, we pursued direct determination of ^{13}C - ^{15}N connectivity, which had not been achieved for a natural product. Fermentation of 250 mL of WMMB-499 with $^{15}NH_4Cl$ and uniformly-labeled ^{13}C -glucose, provided ^{13}C - and ^{15}N -doubly labeled forazoline A (**1**). A ^{13}C - ^{15}N HMQC ($^1J_{CN}$ 15 Hz) was then developed and acquired to determine the ^{13}C - ^{15}N connectivity. The spectrum revealed that N-43 (δ 140.2 ppm) was attached to C-42 (δ 166.5) and C-38 (δ 76.2 ppm), and N-37 (δ 302.6 ppm) was attached to C-36 (δ 170.3 ppm) and C-34 (δ 76.3 ppm); the spectrum also confirmed the presence of two *N*-dimethyl groups. The downfield shift of N-37 and the downfield shift of C-36, combined with the ^{13}C - ^{15}N correlations, confirmed the thiazoline.

While analysis of the ^{13}C - ^{13}C COSY and ^{13}C - ^{15}N HMQC rapidly provided the majority of the planar structure, additional data were necessary to complete structure elucidation. The presence of the sulfoxide was indicated by an absorption in the IR spectrum at 1060 cm^{-1} . Additionally, having accounted for all carbon substituents, logically, the remaining oxygen was likely attached to sulfur. Apratoxin, isolated from the marine cyanobacterium *Lyngbya majuscula*,^[10] had a thiazoline ring; more recently, apratoxin sulfoxide, which contains a thiazoline moiety with a sulfoxide, was isolated.^[11] The ^{13}C NMR shift of the thiazoline methylene in apratoxin and apratoxin sulfoxide was 37.6 ppm and 58.3 ppm, respectively. The ^{13}C NMR shift of the thiazoline methylene (C-35) in forazoline A (**1**) was 38.8 ppm, similar to that of apratoxin and indicating that the sulfoxide was most likely not part of the thiazoline moiety in forazoline A (**1**). To support this hypothesis, low-energy conformers of the two possible regioisomers were determined with Spartan 10^[12] followed by DFT NMR calculations (Gaussian 09^[13]) that were analyzed with the DP4 probability method; the DP4 method uses a mathematical algorithm to compare calculated and experimental NMR shifts and determine which structure fits the experimental data better.^[14] It should be noted that these modeling studies were done after much of the configuration had been established as outlined below. Each of the two possible sulfoxide diastereomers was also investigated using Spartan 10 and Gaussian 09.^[8] After comparing the calculated ^{13}C chemical shifts of the two structures and the corresponding set of diastereomers with the observed chemical shifts of forazoline (**1**), the DP4 probability method predicted a 100.0% probability that the sulfoxide was attached to C-40 and C-42. Notably, for the structure with the sulfoxide in the thiazolinemioity, DFT calculations resulted in a ^{13}C chemical shift of 58.6 ppm for C-35, nearly identical to the methylene in apratoxin sulfoxide (Figure 1). Meanwhile, DFT calculations for forazoline A (**1**) predicted a ^{13}C chemical shift of 43.3 ppm at C-35. Thus, the location of the sulfoxide in forazoline A (**1**) was supported by chemical shifts of known compounds and DFT calculations.

The configuration of forazoline A (**1**) was determined by a combination of NOE studies, coupling constant analysis, extensive molecular modeling, and DFT calculations. We began by determining the relative configuration of the two sugars by analyzing NOE correlations and coupling constants as well as comparing ^{13}C NMR shifts of sugars in known compounds. We then assigned the configuration of the chlorine-containing cyclohexane ring using a combination of NOE correlations and coupling constants (Figure 2). The configuration of the heterocyclic system (C-34 to C-42) was then investigated by molecular modeling and double-pulsed-field-gradient-selective-excitation (DPFGSE) NOE studies; the DPGFSE NOE experiment is advantageous to traditional 1D NOE experiments in that it provides a cleaner spectrum greatly improving distance estimates. ^[15] In parallel, the configuration between C-9 and C-11 was assigned based on extensive molecular modeling and careful NOE studies. In addition, the configuration of the sulfoxide was determined by molecular modeling and DFT calculations. ^[8] Thus, convergent studies with NOE's, molecular modeling, and DFT calculations, which is detailed in the Supporting Information, allowed relative configurational assignment of forazoline A (**1**).

Forazoline A (**1**) and B (**2**) demonstrated *in vitro* activity against *C. albicans* K1 with a minimum inhibitory concentration of 16 $\mu\text{g/mL}$. *In vivo* studies were pursued due to the relatively high aqueous solubility ($\sim 5 \text{ mg/mL}$). Forazoline A (**1**) demonstrated *in vivo* efficacy in neutropenic (immunocompromised) mice in a disseminated candidiasis model against *Candida albicans* K1. ^[16] Mice were treated with forazoline A (**1**) at concentrations of 2.5, 0.78, and 0.125 mg/kg. After 8 hours, the mice treated with the compound showed a decrease in greater than 1 $\log_{10}$ cfu/kidney (1.5 ± 0.12) reduction in organism burden compared to control mice. No toxic effects from the compound were apparent.

Chemical genomic profiling with the yeast, *Saccharomyces cerevisiae*, was used to investigate the mechanism of action of forazoline A (**1**). This method has been used to explore the mechanism of action for bioactive compounds, including natural products. ^[17] Forazoline A (**1**) was screened against over four thousand deletion mutant yeast strains, genomic DNA was extracted, and mutant-specific DNA barcodes were amplified and sequenced by Illumina sequencing. Forazoline A (**1**) sensitive and resistant mutants were determined by quantification of DNA-barcodes, providing a chemical genomic profile, which was used to evaluate the mechanism of action.

Forazoline A (**1**) gave a distinct chemical genomic profile at 250 $\mu\text{g/mL}$. The top sensitive mutant strains ($P < 0.0001$) were significantly enriched for genes involved in phospholipid translocation (GO: 0045332, $P = 0.0009$). This enrichment was driven by sensitive mutants with deletions of the genes *LEM3* and *FPK1*. Lem3p forms a complex with Dnf1p/Dnf2p that is responsible for maintaining phospholipid asymmetry in membranes while Fpk1p is a Ser/Thr protein kinase that regulates Lem3p-Dnf1p/Dnf2p (Dnf1p is a phospholipid translocase). ^[18] These data suggest that forazoline A (**1**) either directly affects phospholipids or interacts with a protein target that complements the activity of the Lem3p complex. An important aspect of these data was that *LEM3* was not among the most sensitive strains for caspofungin, fluconazole, or amphotericin, suggesting that forazoline A (**1**) has a unique mechanism of action from known antifungal agents.

Among the top mutant strains resistant to forazoline A (**1**) ($P < 0.0001$), we saw significant enrichment for genes involved in negative regulation of chromatin silencing at rDNA (GO: 0061188, $P = 0.002$), driven by mutants of *SDS3* and *DEP1*, which encode proteins involved with the regulation of phospholipid biosynthesis. Dep1 is a transcriptional modulator involved in the regulation of phospholipid biosynthesis. [19]

We then compared the chemical genomic profile of forazoline A (**1**) to existing chemical genomic datasets [20] and our unpublished dataset and found its profile significantly correlated with that of papuamide B and tyrocidine B ($P < 0.0001$). Both of these compounds act by damaging cellular membranes and causing cell leakage. [21,22] Taken together, these data suggested that forazoline A (**1**) affected membrane integrity. On the other hand, the top 50 sensitive mutant strains for papuamide B, for example, did not contain deletions of *LEM3* or *FPK1*, [20] indicating that forazoline A (**1**) likely has a different mechanism of action than papuamide B, which targets phosphatidylserine.

To investigate the membrane integrity of yeast cells treated with forazoline A (**1**), we evaluated membrane permeability. Forazoline A (**1**) caused a dose dependent permeabilization of fungal membranes after 4h of treatment (Figure 3) but was less potent than amphotericin. Since chemical genomics suggested that forazoline A (**1**) had a different mechanism compared to amphotericin, we further evaluated that hypothesis through synergy studies. Forazoline A (**1**) showed synergy when tested with amphotericin indicating a parallel and/or complementary mechanism of action. The data indicated that membrane integrity was affected by forazoline A (**1**), but additional studies will be necessary to fully unravel the details surrounding mechanism of action.

Given the rising resistance to antifungal agents, there is a pressing need for new antifungal agents with novel mechanisms of action. Forazoline A (**1**), a complex, novel natural product from a marine-derived *Actinomadura* sp., represents a new class of antifungal natural products. Forazoline A (**1**) demonstrated *in vivo* efficacy – comparable to that of amphotericin B – in a mouse model of *C. albicans* and no toxicity. Additionally, combination treatment of forazoline A (**1**) and amphotericin B demonstrated a synergistic effect *in vitro*. A chemical genomic approach suggested that forazoline A (**1**) affects cell membranes, possibly through dysregulation of phospholipid homeostasis. While additional studies are necessary to better characterize the mechanism of action, forazoline A (**1**) represents a promising antifungal agent with a new mechanism of action compared to current clinically approved agents.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
DFT calculated ^{13}C NMR shifts.

Figure 2.
Key ROESY correlations and coupling constants.

Figure 3.

Forazoline compromises membrane integrity in a dose dependent manner. Forazoline caused cell permeability after 4h of treatment, and membrane damage increased with the concentration. Amphotericin B (AMB) was included as a positive control which causes cell leakage by binding ergosterol in fungal membranes. (Mean $\pm$ S.E.)