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Identification of Portimine B, a New Cell Permeable Spiroimine That Induces Apoptosis in Oral Squamous Cell Carcinoma

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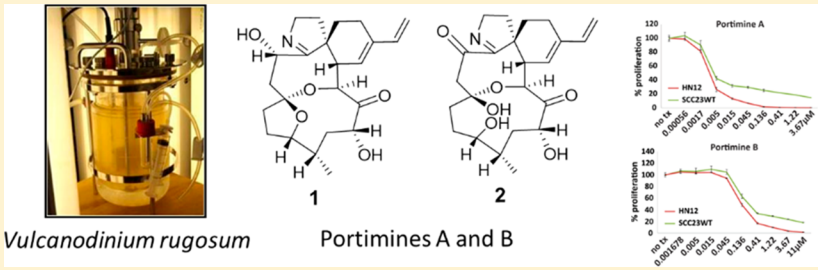
Supporting Information



ABSTRACT: Spiroimines are a class of compounds produced by marine dinoflagellates with a wide range of toxicity and therapeutic potential. The smallest of the cyclic imines, portimine, is far less toxic than other known members in several animal models. Portimine has also been shown to induce apoptosis and reduce the growth of a variety of cancer cell lines at low nanomolar concentrations. In an effort to discover new spiroimines, the current study undertook a metabolomic analysis of cultures of cyclic imine-producing dinoflagellates, and a new analog of portimine was discovered in which the five-membered cyclic ether is open. Further scrutiny with human oral cavity squamous cell carcinoma (OCSCC) cell lines revealed that the open ring congener was less potent than portimine A but could still lead to the accumulation of apoptotic gene transcripts, fragment genomic DNA, and reduce cancer cell proliferation in the range of 100−200 nM.

KEYWORDS: *Portimine, spiroimine, Vulcanodinium rugosum, dinoflagellate, apoptosis, OCSSC, natural products*

Spiroimine toxins are a growing family of compounds produced by members of several genera of harmful algal bloom-forming marine dinoflagellates. They include compound families described as gymnodimines,[1] spirolides,[2] pinnatoxins,[3] prorocentrolides,[4] pteriatoxins,[5] spiro-prorocentrimines,[6] and the recently described portimine.[7] Like many dinoflagellate compounds, spriroimine toxins are macrocycles containing cyclic ether rings and a characteristic five- to seven-membered cyclic imine with a spiro-linkage to a six-membered cyclohexene pharmacophore. Portimine is the only identified member with a five-membered cyclic imine or an eight-membered cyclic ether ring.[7]

The spiroimine family of toxins have gained notoriety as a potential threat to human health through ingestion of contaminated seafood. While no episodes of human intoxication have been linked to cyclic-imine contaminated foodstuffs, animal studies have shown that some of these "fast-acting" toxins can be fatal when injected or ingested even at low quantities (μg/kg).[2,8,9] A mechanism of action for the toxicity of these compounds has been put forth whereby they might lead to respiratory paralysis through the inhibition of muscular and neuronal nicotinic acetylcholine receptors.[10] As has been observed with other natural products, subtoxic concentrations of several cyclic imines have demonstrated efficacy in preclinical pathophysiologic rodent models and human cell lines. Gymnodinime A and 13-desmethylspirolide were able to cross the blood-brain barrier and improve protein expression profiles and behavioral tasks in murine Alzheimer's disease models.[11,12] The smallest member of the cyclic imine family, portimine, displays the least in vivo toxicity in animal models with an IP LD_{50} of 1570 μg/kg and no effects observed

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at 500−700 μg/kg compared to other spiroimines such as desmethyl spirolide C (LD_{50} = 7 μg/kg), pinnatoxin F (LD_{50} = 16 μg/kg), and gymnodimine (LD_{50} = 96 μg/kg).[7] The cytotoxicity of portimine was apparent in cancer cells and was nearly 100 times more potent than pinnatoxin (∼3 nM vs 1 μM for pinnatoxin F).[7] A recent report provided important mechanistic insight into the antiproliferative nature of these compounds with a series of experiments that identified the ability of portimine to induce BCL2-dependent apoptosis in Jurkat acute T cell leukemia cells.[13]

A comparative semitargeted metabolomics investigation, with known spiroimine producers housed in the UNC Wilmington Marine Biotechnology (MARBIONC) Algal Resources Collection (ARC), was performed to assess the breadth of spiroimine production across dinoflagellates and to identify new congeners. These examinations focused on three strains of disparate spiroimine producing genera, *Karenia selliformis*, a producer of gymnodimines;[14] *Alexandrium ostenfeldii*, a producer of both gymnodimines and spirolides;[15] and *Vulcanodinium rugosum*, the producer of portimine.[7] Principal component analysis (PCA) of the three species and a corresponding dendrogram visualization of the metabolomics data highlighted a distinct subset of metabolites unique to a strain of *V. rugosum* isolated from a 2004 ballast water collection in Port Tampa Bay Florida (Figure 1A,B).[16] This strain has been shown to produce portimine but not the structurally related pinnatoxins coproduced by other *V. rugosum* strains.[17] Further interrogation of the *V. rugosum* metabolite profile through S-plot analysis of the cell-specific compounds vs the media components indicated that portimine is one of the major secondary metabolites produced (Figure 1C). An additional mass feature was identified from this analysis that eluted closely with portimine, and the HRMS data suggested a related compound. Based on these data, the *V. rugosum* culture was scaled up in 4 × 10L photobioreactors for purification, structural characterization, and cytotoxicity profiling in oral cavity squamous cell carcinoma (OCSCC) cells.

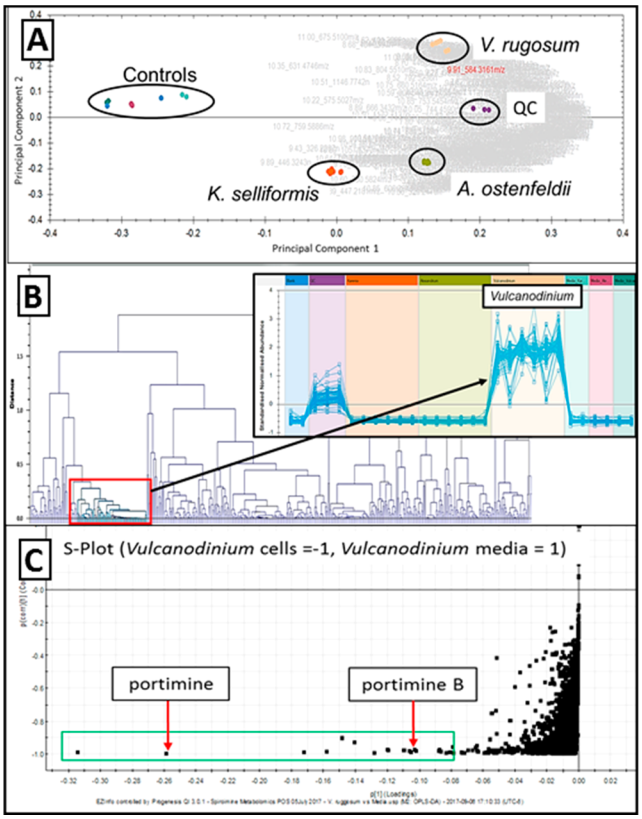

Figure 1. (A) PCA plot of MARBIONC ARC cyclic imine producers. (B) Dendrogram analysis metabolites from the ARC cultures with and expansion of abundance profiles for *V. rugosum* specific mass features. (C) S-Plot expansion of *V. rugosum* cell-specific mass features (mass-retention pairing represented by black dots) with portimines A and B highlighted.

Portimine B **2** (Figure 2) was isolated as a white powder. The molecular formula was established by HRESIMS as

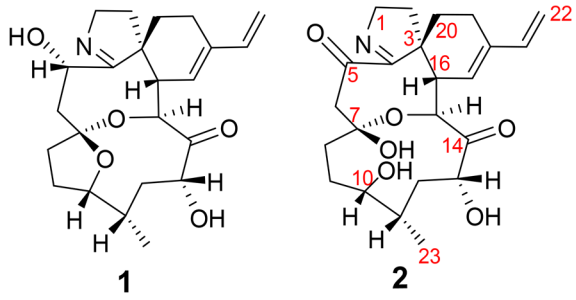

Figure 2. Structures of portimine A (**1**) and portimine B (**2**).

$C_{23}H_{31}NO_6$ (*m/z* 418.2230 $[M + H]^+$; calcd for $C_{23}H_{32}NO_6$ 418.2230, Δ = 0.0 ppm), requiring nine degrees of unsaturation. This is 16 amu greater than portimine, indicating the addition of an oxygen (**1**, $C_{23}H_{31}NO_5$) while retaining the same degrees of unsaturation.

Detailed analysis of 1D and 2D NMR spectra, and comparison with published data, revealed that much of the structure of **2** was similar to **1**, including the spiroimine ring system and the conjugated exomethylene (S1).[7] Several apparent differences were critical in assigning the planar structure of this new compound. Specifically, the hydroxy methine at position 5 (δH/δC 4.50, 65.6) observed in **1** was no longer present in **2**. Instead, a carbonyl (δC 201.4) appearing in the HMBC NMR spectrum of **2** was assigned to this position (S1). This substitution was further corroborated by changes in the chemical shift and the splitting pattern of the adjacent methylene at position 6 (S1).

Based on the molecular formula, an additional oxygen remained to be accounted for while maintaining the calculated degrees of unsaturation. Examination of the 1D and 2D NMR data indicated that one of the sprioketal rings was now open and that an additional hydroxyl was present. Key in determining which of the two possible rings had opened was an HMBC correlation from the hydroxymethine (δH/δC 4.63, 72.4) at position 15 to the hemiketal carbon at position 7 (δC 107.2), which would only be possible if the eight-membered ring was still intact. Thus, it became evident that the five-membered ether ring was no longer present in **2** and that hydroxyls were located on carbons 7 and 10 (Figure 2B).

Comparison with NOESY data (from **2**) with assignments for portimine indicated that all of the correlations for portimine were present in **2**, suggesting they shared a similar 3D structure (Table S1). The stereoisomeric configurations at carbons 7 and 10 were modeled in Chem3D relative to the assumed configuration of carbon 16, and under the constraints

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of the observed NOESY correlations, to confirm these assignments.[7]

The fact that **1** and other cyclic imine toxins are bioavailable has been established in preclinical vertebrate-derived cell models that include amphibian,[18,19] murine,[20] and human cancer.[13,21] The ability of any compound to passively permeate lipid membranes is an important measure of potential bioavailability and toxicity, a concept that becomes increasingly important when a substance is studied in a preclinical setting. Parallel artificial membrane permeability assays (PAMPA) were employed to more thoroughly explore the pharmacokinetic properties of **1** and **2**. A 1 mg/mL solution of a semipurified crude extract of *V. rugosum* was incubated in the donor compartment of a 96-well PAMPA plate for 6 h. The relative concentrations of **1** and **2** were quantitated by UPLC-HRMS qTOF-MS for the donor and acceptor wells via the extracted ion chromatograms (supplemental) and compared to verapamil (highly permeable) and prednisone (low permeability) controls (tested at 100 μM). Compounds **1** and **2** displayed passive permeability similar to verapamil, with **2** slightly lower than **1** (Table 1). These data suggest that the

Table 1. PAMPA Assays with the Percentage of the Indicated Compound Identified with UPLC-HRMS qTOF-MS in the Acceptor Well after 6 h

compound	permeability
verapamil	54 (±2)
prednisone	12 (±3)
portimine A (**1**)	54 (±4)
portimine B (**2**)	35 (±5)

portimine scaffold, in terms of permeability, has a promising pharmacokinetic profile and support the continued investigation of **1** and **2** in preclinical models of human diseases.

Oral cavity squamous cell carcinoma (OCSCC) is an underfunded and understudied major cancer[22] that annually claims approximately 10,000 lives in the USA. Despite a trending increase in purely synthetic and biological drugs, 1/3 of all cancer drugs approved by the FDA between 1981 and 2014 were natural products or natural product derivatives.[23] Work from our laboratory has demonstrated that OCSCC cell lines are sensitive to a variety of natural products isolated from aquatic microbes.[24,25] The OCSCC cell lines UMSCC23 and HN12 were cultured with increasing concentrations of purified (98% by UPLC-PDA) **1** and **2**. ATP-based luminescent proliferation assays revealed dose-dependent inhibition over a 36 h period, with **1** demonstrating substantially reduced IC_{50} values compared to **2** (Figure 3A). A previous report that **1** could induce apoptosis in human leukemic T cell lymphoblasts[13] prompted the evaluation of apoptotic transcripts in cDNA libraries generated from **1**- and **2**-treated OCSCC cultures. The experimentally determined IC_{50} for each compound was applied over a 36 h period. RT-qPCR analysis revealed an accumulation of pro-apoptotic *GADD45α*, *NOXA*, and *DR5* and antiapoptotic *BCL2* (Figure 3B). In general, increases in gene expression were observed at earlier time points for **1**. During homeostasis, BCL2 holds proapoptotic proteins (i.e., BAX and BAK) in an inactive state. The initiation of intrinsic (mitochondrial-mediated) apoptosis is preceded by increased levels of NOXA and other BH3-only BCL2 family members which then titrate BCL2 away from BAX and BAK. The release of BCL2 allows cytochrome *c* to

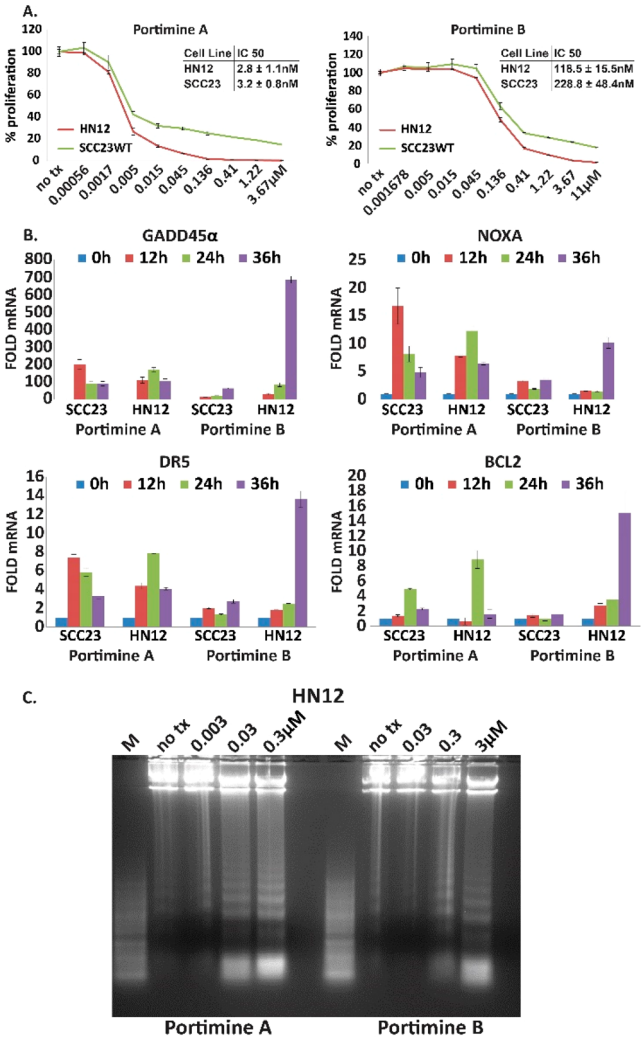

Figure 3. Portimines induce apoptosis and reduce OCSCC proliferation. (A) ATP-based proliferation assays after 48 h exposure to **1** (left) and **2** (right); error bars indicate SD. (B) Taqman RT-qPCR analysis of cDNA libraries generated from OCSCC cell lines treated with the IC_{50} (determined in A) for each compound; error bars represent SEM from three identical experiments performed in triplicate. (C) Electrophoretic resolution of genomic DNA harvested from HN12 cells exposed to **1** (left) or **2** (right) for 48 h.

rove from the space between the inner and outer mitochondrial membranes to the cytosol, leading to apoptosome formation and the sequential cleavage (activation) of caspases 9 and 3. The ability of **1** and **2** to activate both apoptotic and antiapoptotic genes indicates that parallel antithetical responses are commenced whereby apoptosis is initiated by *NOXA*, and an attempt to survive the challenge is provided by *BCL2*. Consistent with this notion, BCL2 overexpression could almost entirely ameliorate the apoptotic effects of **1**.[13] Finally, DNA laddering has been used previously to demonstrate the ability of natural products to induce apoptosis in oral cancer cells.[25−27] The IC_{50} for **1** and **2** as well as a semilog dose (above and below) were used to treat HN12 cells for 48 h. Electrophoretically resolved genomic DNA demonstrated dose-dependent laddering, a hallmark of apoptosis (Figure 3C). These data indicate that **1** and **2** induced apoptosis and reduced the proliferation of oral cancer cells.

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The current study has identified a previously unreported spiroimine isolated from *V. rugosum* collected off the coast of Florida, USA. The compound is structurally related to portimine and is designated herein as portimine B. A notable feature of this new congener is the opening of one of the sprioketal rings and the addition of a hydroxyl group at the same location. We hypothesize that increased polarity and decreased rigidity resulting from this feature were contributing factors to the observation that **2** was observed to be less potent than **1** with respect to the activation of apoptosis and the reduced proliferation in OCSCC cultures. Selwood et al. reported that the LD_{50} for mice injected with intraperitoneal portimine was 1570 μg/kg and noted this to be much lower than other imine shellfish toxins.[7] When considered in the light of the current study, and the anticancer activity of **1** noted in leukemia cell cultures,[13] further preclinical studies with *in vivo* cancer models and drug combinations are warranted to more fully appreciate what the translational value of this very potent class of polycyclic ethers might be.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmedchemlett.8b00473.

Experimental design, NMR spectra, and mass spectra (PDF)

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS

OCSCC, oral cavity squamous cell carcinoma; RT-qPCR, real time quantitative polymerase chain reaction; ER stress, endoplasmic reticulum stress; UPR, unfolded protein response

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