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Bioguided discovery and pharmacophore modeling of the mycotoxic indole diterpene alkaloids penitrems as breast cancer proliferation, migration, and invasion inhibitors

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Abstract

Marine-derived fungi have proven to be important sources of bioactive natural organohalides. The genus *Penicillium* is recognized as a rich source of chemically diverse bioactive secondary metabolites. This study reports the fermentation, isolation and identification of a marine-derived *Penicillium* species. Bioassay-guided fractionation afforded the indole diterpene alkaloids penitrems A, B, D, E and F as well as paspaline and emnidole SB (**1–7**). Supplementing the fermentation broth of the growing fungus with KBr afforded the new 6-bromopenitrem B (**8**) and the known 6-bromopenitrem E (**9**). These compounds showed good antiproliferative, antimigratory and anti-invasive properties against human breast cancer cells. Penitrem B also showed a good activity profile in the NCI-60 DTP human tumor cell line screen. The nematode *Caenorhabditis elegans* was used to assess the BK channel inhibitory activity and toxicity of select compounds. A pharmacophore model was generated to explain the structural relationships of **1–9** with respect to their antiproliferative activity against the breast cancer MCF-7 cells. The structurally less complex biosynthetic precursors, paspaline (**6**) and emindole SB (**7**), were identified as potential hits suitable for future studies.

Introduction

Breast cancer is the most common cancer among women in the United States, other than skin cancer.¹ It is the second leading cause of cancer death in women, after lung cancer. In 2012 alone, about 226,870 new cases of invasive breast cancer in women were diagnosed and 39,510 deaths have been reported so far.¹ Resistance to chemotherapy is a major problem in the management of breast cancer, where many of the initially responsive tumors relapse and develop resistance to diverse chemotherapeutic agents.² Consequently, there is a

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[†]Electronic Supplementary Information (ESI) available: [HPLC chromatograms, ¹H and ¹³C NMR spectra for **8** and **9** and mean-60 graphs for compounds **1**, **2** and **4** from the National Cancer Institute screen against the 60-cell line panel]. See DOI: 10.1039/b000000x/

continuous demand for new chemical scaffolds for the management and treatment of various forms of breast cancer.

Halogenated secondary metabolites, while rare in terrestrial plants, are common in marine organisms due to the abundance of chloride and bromide ions in seawater.^{3,4} Organisms that have the ability to form halogenated compounds have been found among various species of algae, bacteria, fungi, sponges, molluscs, coelenterates and several marine worms.⁵ Many halogenated marine natural products show important biological activities of interest to the pharmaceutical industry.^{5,6} Examples of halogenated natural products that are potent anticancer agents include the γ -lactone salinosporamide A (currently in Phase I clinical trials), macrocyclic lactone polyether spongistatin, indolocarbazole rebeccamycin, and enediyne calicheamicin **1**.

In our laboratory, a number of sponge and sediment specimens collected at the Kuwaiti Arab Gulf coast were screened for the presence of fungal associates. The aim was to identify novel marine-derived fungal secondary metabolites with antiproliferative, antimigratory and anti-invasive activities against metastatic breast cancer. Several fungi were cultured and isolated. Of these, a fungus was identified as *Penicillium commune* isolate GS20 and selected for further investigation based on the bioactivity of its extract. Several marine-derived *Penicillium* species have been recently isolated from a variety of substrates such as molluscs, sponges, algae and sediments.⁷ *Penicillium janthinellum* Biourge, isolated from marine sediments collected from the Sea of Japan, afforded three new indole diterpene alkaloids, shearinines D–F, which were shown to induce apoptosis in human leukemic HL-60 cells.⁸ These metabolites are analogues of paspalinine and janthitrems B–G previously discovered from terrestrial fungi.⁹

This study reports the bioassay-guided fractionation of a marine-derived fungal extract from a newly-identified *Penicillium commune* isolate GS20. Preparative RP-HPLC of bioactive fractions afforded five known penitrems **1–5** and two of their biosynthetic precursors, paspaline (**6**) and emindole SB (**7**). One new (6-bromopenitrem B, **8**) and one previously reported (6-bromopenitrem E, **9**) analogues were isolated upon supplementing the fermentation broth of the growing fungus with KBr. Compounds **1–9** (Fig. 1) were evaluated *in vitro* for their antiproliferative activity against the human breast cancer cell lines MCF-7 and MDA-MB-231 in the MTT assay, as well as antimigratory and anti-invasive effects against MDA-MB-231 cells in the wound-healing and Cultrex® BME Cell Invasion assays, respectively. BK channel inhibition and toxicity of select compounds were evaluated using the nematode *Cenorhabditis elegans*. A pharmacophore model for the antiproliferative activity against MCF-7 cells was generated using PHASE module of Schrödinger molecular modeling software. Paspaline (**6**) and emindole SB (**7**) showed good antiproliferative and antimigratory activities without any BK channel inhibitory effect, thus posing as interesting hits for future studies.

Results and discussion

1. Fungal material

1a. Source—A number of sponge and sediment specimens collected from the Kuwaiti coast of the Arab Gulf were screened for fungal associates in search for novel compounds with antiproliferative, antimigratory and anti-invasive activities against human breast cancer cell lines *in vitro*. Several filamentous fungi were isolated, purified and subjected to 7-day small-scale fermentation. Preliminary chemical analyses (using TLC and ¹H NMR) and biological screenings (MTT, WHA) were used to prioritize fungal cultures for further investigations.¹⁰ The fungal strain GS20 was selected for this study based on the

antiproliferative activity of its total extract against the human breast cancer cell line, MCF-7 (Fig. 2) as well as promising chromatographic and spectroscopic profiles.

1b. Taxonomic identification—Both phenotypic and 18S-rDNA sequence analyses were used for taxonomic identification. The phylogeny of GS20 was inferred by sequence comparison of its amplified internal transcribed spacer (ITS) region (GenBank accession number JX217744) with published sequences obtained from GenBank.^{11,12} The most related sequences were aligned, and a neighbor-joining tree was calculated. GS20 was identified as *Penicillium commune* isolate GS20 (Fig. 3).

1c. Fermentation, extraction and isolation of fungal metabolites—Solid-phase fermentation¹³ was initially used for harvesting *P. commune* isolate GS20. Several media types were individually tested of which yeast malt agar (YMA) was selected for its support of the best fungal growth and extract yield. Culture plates were streaked from 7-day old inoculum^{14,15} and incubated for 28 days. Once collected, fungal mass was freeze-dried, crushed, and successively extracted with (CH₃)₂CO-CH₂Cl₂ (1:1). Bioassay-guided fractionation of the organic extract led to the identification of a bioactive fraction for which preparative RP-HPLC (see Experimental Section and Figs. SI1 and SI2 in Supplementary Information) afforded the known indole diterpene alkaloids penitrems A, B, D, E, and F (**1–5**) and their biosynthetic precursors paspaline (**6**) and emindole SB (**7**).^{16,17}

Indole diterpene alkaloids are a large, structurally diverse group of fungal secondary metabolites, with a common core structure comprising of a cyclic diterpene skeleton derived from geranylgeranyl diphosphate (GGPP) and an indole group derived from indole-3-glycerol phosphate, a precursor of tryptophan (Fig. 4).^{9,18} The structural diversity observed within this group of metabolites is achieved by additional prenylations along with diverse ring regio- and stereoselective substitutions.^{9,18}

Penitrems and related metabolites possess a number of biological effects including insect feeding deterrence, modulation of insect and mammalian ion channels, inhibition of specific enzymes as well as tremorgenic activity, which is their most dramatic effect.^{9,18,19} Penitrems are also potent inhibitors of the high conductance calcium-activated potassium channels (BK channels).⁹ More recently, Abdullaev *et al* reported the antiproliferative effects of paxilline and penitrem A (**1**) against a number of human glioblastoma cells.²⁰ Furthermore, Sheehan *et al* demonstrated the anticonvulsant effects of paxilline when intraperitoneally administered to picrotoxin-treated mice suffering tonic-clonic seizures.²¹ Paspaline (**6**) is non-tremorgen and is devoid of BK channel inhibition.^{9,18} It was found to cause slight inhibition of acyl-CoA:cholesterol *O*-acyltransferase (ACAT).⁹ No specific biological activity has been reported for emindole SB.⁹

2. Generation of new halogenated derivatives

To determine whether the fungal biosynthetic machinery can produce other halogenated compounds, KBr and KF were separately added to 3-day old cultures at 1 and 2 mM final concentrations. Accordingly, the new 6-bromopenitrem B (**8**) and the known 6-bromopenitrem E (**9**)²² were identified with the aid of LC-MS which showed the characteristic mass pattern (M and M+2 at 1:1 isotopic peak intensity) for monobrominated compounds. No fluoride derivative was identified, which was consistent with literature reports indicating the predominance of chlorination, followed by bromination, and the scarcity of iodination and fluorination in nature.⁵

Compound **8** showed a molecular ion peak at *m/z* 662.2475 [M+H]⁺ suggesting the molecular formula C₃₇H₄₄BrNO₅. The ¹H and ¹³C NMR data of **8** (Tables 1 and 2) were

nearly identical to those of **2**, except at C-6 and surrounding atoms where differences in chemical shifts are highly indicative of a 6-bromo substitution (See Supplementary Information (Figs. SI3 and SI4) for full NMR data for **2**). The aromatic methine pair of doublets H-6 and H-7 in **2** (δ_{H} 6.62 and 6.94, respectively) was replaced in **8** by a proton singlet H-7 (δ_{H} 7.35), indicating C-6 substitution. Moreover, the aromatic methine carbon C-6 in **2** (δ_{C} 120.1) was replaced with a quaternary aromatic carbon at δ_{C} 113.0 in **8** and a downfield shift was observed for C-7 (δ_{C} +3.6 and δ_{H} +0.41). Furthermore, C-4 downfield (δ_{C} +2.2) and C-5 upfield (δ_{C} -1.2) shifts were also observed in **8**. These observations are consistent with a bromo substitution at C-6. The CD spectra of **2** and **8** were found to be identical indicating similar stereochemical orientation. Therefore, **8** was determined to be 6-bromopenitrem B.

The tentative structure of 6-bromopenitrem E (**9**) was previously reported by Mantle *et al* without any spectral details.²² The ESIMS spectrum showed a molecular ion peak at m/z 676.2 $[\text{M}-\text{H}]^-$ and an isotopic peak at 678.2 $[\text{M}-\text{H}+2]^-$ with a 1:1 relative abundance, indicating a single bromo substitution. Thus, the molecular formula of **9** was determined to be $\text{C}_{37}\text{H}_{44}\text{BrNO}_6$. The ^1H and ^{13}C NMR data of **9** (Tables 1 and 2) were almost identical to those of **4** with subtle differences that further confirmed the 6-bromo substitution in a similar fashion detailed in **8** (See Supplementary Information, Figs. SI5 and SI6, for full NMR data of **4**). The CD spectra of **4** and **8** were found to be identical, indicating a similar stereochemical orientation. Therefore, **9** was determined to be 6-bromopenitrem E.

3. Biological activity

3a. MTT assay—The MTT assay is a safe, sensitive, *in vitro* assay that allows for the measurement of cell proliferation in a quantitative colorimetric fashion by utilizing the ability of metabolically active (viable) cells to reduce the MTT reagent to insoluble purple formazan crystals.²³ The human mammary carcinoma cell lines MCF-7 and MDA-MB-231 were both used in the MTT assay. At least four concentrations per compound were tested and used to calculate an IC_{50} (Table 3). Penitrem B (**2**) showed the highest antiproliferative activity against the human breast cancer cell line MCF-7. Replacing the 23, 24-epoxide in **2** with a 23,24 system (**3**) resulted in a marginal decrease in the activity, as evident from the IC_{50} values: 5.5 and 8.3 μM , respectively.

Introduction of chlorine atom at C-6 in **5**, or hydroxyl group at C-15 in **4** also decreased the activity (Table 3). Interestingly, however, the presence of both substitutions in **1** did not seem to compromise the activity in an additive fashion as would be expected.

In MDA-MB-231 cells, on the other hand, the effect of C-6 and C-15 substitutions on the antiproliferative activity of penitrems **1–5** showed a different pattern. The presence of both C-6 chloro and C-15 hydroxyl in **1** imparted the highest activity, whereas the absence of either substitution resulted in a noticeable reduction in activity (Table 3): the absence of C-6 chloro in **4** resulted in a three-fold reduction in activity, whereas the absence of C-15 hydroxyl in **5** only reduced the activity by two-fold. It is interesting to note, however, that the absence of both substituents in penitrem B (**2**) did not severely compromise the activity as would be predicted. Again, replacing the 23, 24-epoxide with a double bond as in **3** decreased the activity. The 6-bromo substitution did not produce significant effects on the antiproliferative activity against both cell lines as evident from the comparable activity of **8** and **9** to that of their chlorinated counterparts **5** and **1**, respectively (Table 3).

Paspaline (**6**) and emindole SB (**7**) are the simplest indole diterpene alkaloids isolated so far and would represent the minimal structural requirements for the activity of this class of secondary metabolites. Hence, it was interesting to see their bioactivity profile. Both

compounds showed an antiproliferative profile comparable to that of penitrems; with paspaline (**6**) almost twice as active as emindole SB (**7**) against MDA-MB-231 cells (Table 3).

3b. Wound-healing assay (WHA)—The WHA is a simple method for the study of directional cell migration *in vitro*.²⁴ The ability of compounds **1–5** to inhibit the migration of highly metastatic human breast cancer cells, MDA-MB-231 across a wound inflicted in a cell monolayer was evaluated (Fig. 5, Table 3). Subtoxic concentrations were used and for penitrems, the highest antimigratory effect was achieved with compounds **1–3**. Concomitant presence or absence of C-6 and C-15 substitution did not affect the activity while individual absence of C-6 chloro (in **4**) or C-15 hydroxyl substitution (in **5**) resulted in two- or three-fold reduction in activity, respectively. No influence was imparted by the substitution of the 23, 24-epoxide with a double bond. The type of halogen (Br instead of Cl) did not affect the antimigratory potential of these compounds (Table 3). It was interesting to note that paspaline (**6**) was almost as potent as penitrem A (**1**), and superior to emindole SB (**7**), in this assay.

3c. Cell invasion assay—The Cultrex[®] BME cell invasion assay kit was used to assess the anti-invasive properties of **1–9**. This assay offers a flexible, standardized, high-throughput format for quantifying the degree to which invasive cells penetrate a barrier consisting of basement membrane components *in vitro* in response to chemo-attractants and/or inhibiting compounds.²⁵ Two subtoxic doses (7.5 and 15.0 μM) of each compound were tested (Fig. 6). Penitrem A (**1**) showed the highest activity; inhibiting >75% of cell invasion at 15 μM . All other penitrems (**2–5**) were either moderately active (**2**, ~40% inhibition and **3**, ~50% inhibition) or inactive (**4** and **5**) at 15.0 μM . This pointed the importance of both C-6 chloro and C-15 hydroxyl to the anti-invasive activity since the absence of either substitution deteriorates the activity. Substitution of the 23, 24-epoxide (**2**) with a double bond (**3**) did not have any significant effect. 6-bromopenitrem B (**8**) showed significant anti-invasive effect that was higher than that of its chlorinated counterpart (**5**) whereas **9** was inactive. This shows the importance of the type of halogen on the anti-invasive activity of penitrems. Both paspaline (**6**) and emindole SB (**7**) were inactive in this assay (Fig. 6).

3d. Growth inhibition effect of compounds 1, 2, and 4 against the National Cancer Institute 60-human cancer cell lines—The growth inhibitory activity of **1**, **2**, and **4** was also evaluated by the National Cancer Institute's Developmental Therapeutics Program.^{26–28} This disease-oriented screening program utilizes 60 different human tumor cell lines (NCI60), representing leukemia, melanoma and cancers of the lung, colon, brain, ovary, breast, prostate, and kidney.²⁷ Compounds were tested at a single concentration of 10 μM , and the percent growth of the treated 60 cell lines was determined and presented in the form of mean graphs (See Supplementary Information, Figs. SI7–SI9).^{26,28} These mean graphs provide insight into the differential cell growth inhibition produced by each compound and show a characteristic fingerprint pattern which possesses a significant correlation to the compound's chemistry. Compound **2** showed the lowest mean growth percent (mean-60) of 58.95, corresponding to 41.05% growth inhibition (Fig. SI8), and was retested at five different concentrations; 100, 10, 1, 0.1, and 0.01 μM .²⁹ It displayed a selective activity against all cancer cells representing leukemia (Fig. SI8).

3e. *Caenorhabditis elegans*: Assessment of BK channel inhibition and toxicity—The BK channel inhibitory potential (as a marker for tremorgenicity) of compounds **7–9** as well as the toxicity of compounds **1–9** were studied using the nematode *C. elegans* as an *in vivo* model. The use of this model has several advantages including the availability of the complete genome sequence of the nematode *C. elegans*, coupled with the worm's size,

growth rate, ease of culturing, short life cycle and the realization that many of the basic physiological processes and stress responses that are observed in higher organisms (e.g., humans) are highly conserved. This makes this genetically tractable model a remarkable opportunity to dissect and identify numerous cellular processes *in vivo*.^{30–32}

The BK channel inhibition in *C. elegans* is associated with an abnormal behavior of locomotion of worms in terms of increased reversals, i.e., the number of times a worm stops and reverses its direction, which can be easily assessed and quantified.³³ The BK channel inhibitory potential of compounds **1–6** is well documented,^{9,18} and the model was used to assess BK channel inhibition of **7** and **9**, for which there are no reports in this regard. Briefly, 30–40 synchronized L1 stage larvae of the wild-type N2 strain were added to *E. coli* OP50 preseeded NGM plates containing 1 μ M of each test compound.³³ Plates were incubated at 20°C and the number of reversals was counted for at least 10 worms per treatment 42 hours (L4 larval stage) post exposure. The *C. elegans* *slo-1* knockout strain NM1968 *slo-1(js379)V*, which shows the phenotype of increased reversals, was used as a positive control. Penitrem A (**1**), the most potent tremorgen of all penitrems, causes a reversal pattern comparable to that of the knockout strain (Fig. 7).³³ 6-Bromopenitrem E (**9**) was equipotent to penitrem A (**1**) in inhibiting BK channels, indicating no specific halogenation preference for the activity. Emindole SB (**7**), however, did not inhibit the BK channels and the number of reversals exhibited by emindole SB-treated worms was comparable to that of the vehicle (DMSO) control (Fig. 7).

Concentrations of 10 and 50 μ M of each of **1–9** were evaluated for toxicity to *C. elegans* and compared with a vehicle control.³² Close observations for developmental abnormalities were performed for one week after exposure. These experiments did not reveal any generalized effects on the nematode's viability and reproduction.

4. Pharmacophore modelling

A pharmacophore model was generated using PHASE module of Schrödinger molecular modelling software³⁴ to elucidate the 3D structural features of the penitrems contributing to their antiproliferative activity against MCF-7 cells. Numerous common pharmacophore models with different combination of variants were generated. Only top scoring models (survival, survival-inactive and post-hoc scores) showing the best alignment of all actives were inspected.^{35–37} A good model should provide superior alignment with active compounds, discriminate between active and inactive compounds and display the lowest possible relative conformational energy values.³⁴ Accordingly, model ADDHRR was selected. This model identifies one hydrogen bond acceptor atom (A), two hydrogen bond donor atoms (D), one hydrophobic site (H) and two aromatic regions (Figs. 8a and 8b) as common to all active compounds and essential for the antiproliferative activity. Interestingly, paspaline (**6**) and emindole SB (**7**) showed good alignment with these features (Fig. 8c). This indicates that the structural complexity and bulkiness of the western side of the penitrem skeleton is not essential for activity. Though active (IC₅₀ ~10 μ M, Table 3), emindole SB (**7**) lacked one of the donor sites implying that it might not be a determinant for activity.

Conclusion

This study unequivocally demonstrates the antiproliferative, antimigratory, and anti-invasive potential of this class of indole diterpene alkaloids, previously viewed as hazardous mycotoxins. To our knowledge, this represents the first report of the antimigratory and anti-invasive potential of these compounds. The structurally less complex biosynthetic precursors, paspaline (**6**) and emindole SB (**7**), retain the pharmacophoric features that contribute to the activity profile of penitrems. However, they do not inhibit BK channels,

thus eliminating any plausible associated toxicity (mainly tremorgenicity) and rendering them as potential scaffolds for future SAR studies.

Experimental

General experimental procedures

Optical rotations were measured on a Rudolph Research Analytical Autopol III polarimeter in CH₃OH. Circular dichroism experiments were carried out at room temperature on a Jasco J-815 spectropolarimeter (JASCO Inc., Easton, MD) flushed with nitrogen. Spectra were collected from 350–225 nm using a 1 mm path length of a rectangular quartz cell. Each spectrum was the average of eight scans taken at a scan rate of 50 nm/min with a spectral bandwidth of 0.1 nm. The concentration of compounds used was 0.5 mM prepared in CH₃OH. IR spectra were recorded on a Varian 800 FT-IR spectrophotometer. The ¹H and ¹³C NMR spectra were recorded in *d*₆-acetone, using (CH₃)₄Si as an internal standard, on a JEOL Eclipse NMR spectrometer operating at 400 MHz for ¹H NMR and 100 MHz for ¹³C NMR. The HREIMS experiments were conducted at Louisiana State University on a 6200-TOF LCMS (Agilent) equipped with multimode source (mixed source that can ionized the compounds alternatively by ESI and APCI). The ESIMS dereplication experiments were conducted with a 3200 Q-trap LC/MS/MS system (Applied Biosystems, Foster City, CA) using Analyst version 1.4.1 software (MDS Sciex; Toronto, Canada) where analytes were ionized using electrospray ionization (ESI) interface operated in the negative mode. The analysis was conducted using Q₁ scan and mass scan range was *m/z* 50–700 (0.15s/scan). UltraClean™ Microbial DNA Isolation Kit was purchased from MO BIO Laboratories, Inc. (Carlsbad, CA). TLC analysis was carried on pre-coated Si Gel 60 F₂₅₄ 500 μm TLC plates (EMD Chemicals), using *n*-hexane–EtOAc (60:30) as mobile phase and *p*-anisaldehyde/H₂SO₄ in ethanol as visualizing reagent. For column chromatography, Si gel 60 (Natland International Corporation, 230–400 mm) and gradient *n*-hexane–EtOAc solvent system were used. Analytical HPLC analyses were conducted on a Shimadzu HPLC system (Columbia, MD) using a 5 μm Luna-C18 column (150 × 4.6 mm id; Agilent, Santa Clara, CA) at an isocratic elution (CH₃OH–H₂O; 80:20) with UV detection set at 230 and 296 nm, an injection volume of 20 μL and a 1.0 mL min^{−1} flow rate. Preparative HPLC was conducted on a Dionex Summit III [P680 pump, UVD340U detector, thermostatted column compartment TCC-100] system (San Diego, CA) using Cosmosil 5C18-AR-II (20 × 250 mm) column, isocratic elution (CH₃CN–H₂O; 80:20) with UV detection at 230 and 296 nm, an injection volume of 2 mL and a flow rate of 5 mL min^{−1}.

Biological materials

Several sponge and sediment specimens were collected from the Kuwaiti coast of the Arab Gulf in June 2006 and evaluated for the presence of culturable fungal associates. Frozen specimens were triturated with sterile sea salt solution, diluted and streaked on different solid media containing 1% chloramphenicol, namely sea salt agar, marine agar and tryptic soy agar. Plates were incubated at 28°C and followed up for fungal growth. Individual fungal colonies were isolated and re-plated until pure colonies were obtained. For this study, the fungal strain GS20 was selected based on chemical diversity and bioactivity of total extract. Different media types (marine agar, tryptic soy agar, potato dextrose agar and yeast malt agar) were tested to select the optimal one for this particular fungal strain. Yeast malt agar (YMA) was finally selected for its support of optimal fungal growth and extract yield.

DNA Isolation

GS20 grown on yeast malt agar (YMA) at 28°C for 7 days was inoculated into sterile compound medium- (20 g dextrose, 5g each of K₂HPO₄, peptone, yeast extract, and NaCl in 1L H₂O) and incubated for 4 days in the dark at room temperature (20–21°C). On day

four, the fungal mass was collected and subjected to a pulverization process. Microbeads were used to help in the mechanical disruption of fungal cell membranes, thus maximizing DNA release. DNA extraction followed using UltraClean™ Microbial DNA Isolation Kit according to manufacturer protocol.³⁸ Extracted DNA was stored at -20°C.

DNA Amplification and sequencing

The resulting *g*DNA was used as a template to amplify fungal ITS-rDNA region using the primers ITS1 (5'-TCC GTA GGT GAA CCT GCG-3') and ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3').¹¹ The reaction mixture contained 10 µL of 5 x Phusion® HF reaction buffer (NEB), 2.5 µL of DMSO, 1 µL of 10 mM dNTPs, 0.5 µL of 50 pmol forward primer, 0.5 µL of 50 pmol reverse primer, 1 µL of fungal *g*DNA, 0.5 µL of Phusion® high-fidelity DNA polymerase (2 U/µL, NEB), and 34 µL of water. PCR conditions were as follows: initial denaturation (95°C for 5 min); 30 cycles of denaturation (95°C for 45 s), primer annealing (55°C for 45 s), and elongation (72°C for 45 s), with a final elongation step at 72°C for 10 min. PCR product was purified from gel (0.8% agarose in Tris-acetate-EDTA buffer) using QIAquick® Gel Extraction Kit (Qiagen) and used directly for sequencing using ITS1 and ITS4 primers (DNA Sequencing & Services, University of Dundee).

Phylogenetic analysis

The ITS sequence obtained (571 bp, GenBank accession no. JX217744) was compared to available sequences in GenBank using the BLAST algorithm. Sequences were edited, aligned and imported into MEGA 4.¹² A neighbor-joining (NJ) tree was calculated using pairwise genetic distances on the basis of all substitutions with the Jukes-Cantor distance parameter. The quality of the branching patterns was assessed by bootstrap resampling of the data sets with 1000 replications.

Fermentation, extraction and isolation of fungal metabolites

To prepare the inoculum, *P. commune* isolate GS20 was grown on yeast malt agar (YMA) for 7 days at 28°C after which a spore suspension was prepared and stored in 10% aqueous glycerol at -80°C for subsequent use in experiments. Initial experiments aimed at selecting the most proper media type and amount for fungal growth and metabolite production. Three media types were tested: Czapek-Dox-0.5% yeast extract (CDB-YE), 2% yeast extract-4% sucrose (YES), and yeast malt broth (YMB). YES was eventually established as the optimal media type for this fungal strain. The amount of media per flask was then evaluated and it was concluded that media occupying 40% of flask volume proved optimal. Hence, for all experiments, cultures were grown under static conditions in YES media (40% of flask volume) for 28 days at room temperature (20–21°C). Static conditions are extremely important as shaking can severely compromise the production of penitrems.¹⁴ At the end of the incubation period, mycelia were harvested, freeze-dried, weighed, crushed and sonicated in (1:1) (CH₃)₂CO:CH₂Cl₂ for at least 2h, followed by soaking overnight. The solvent was then collected and a fresh amount was added and extraction continued for several more hours. Since ~97% of penitrems are formed in the mycelium in stationary cultures, all liquid media were discarded after autoclaving.¹⁴ Extracts were combined and filtered through anhydrous Na₂SO₄, dried, and subjected to MPLC on normal phase Si gel 60 (230–400 mm) using *n*-hexane-EtOAc gradient elution to afford several fractions. Penitrem-rich fractions identified by TLC, MS and ¹H NMR were eluted starting at 8.5:1.5 *n*-hexane-EtOAc. Fractions were combined and subjected to preparative RP-HPLC, affording five penitrems: A (1, R_t 18 min), B (2, R_t 28 min), D (3, R_t 26 min), E (4, R_t 15 min) and F (5, R_t 38 min), paspaline (6, R_t 63 min) and emindole SB (7, R_t 71 min) (Figs. SI1 and SI2).

Feeding studies

KBr and KF were dissolved in sterile distilled water. Each solution was separately divided into 3 equal doses and aseptically added to the fungal cultures starting on day-3 after inoculation. Incubation continued for 28 days after which mycelia were harvested and treated as described earlier.

6-Bromopenitrem B (8)—White solid; $[\alpha]_{\text{D}}^{25} -47.6$ (*c* 0.02, CH₂Cl₂); IR $_{\text{max}}$ (CH₂Cl₂) 3756, 3460, 3055, 2986, 1425, 1263 and 896 cm⁻¹; ¹H and ¹³C NMR (Tables 1 and 2); HREIMS *m/z* 662.2475 [M+H]⁺ (calcd for C₃₇H₄₅BrNO₅, 662.2481).

6-Bromopenitrem E (9)—White solid; $[\alpha]_{\text{D}}^{25} -67.6$ (*c* 0.02, CH₂Cl₂); IR $_{\text{max}}$ (CH₂Cl₂) 3461, 2986, 2306, 1425, 1264 and 896 cm⁻¹; ¹H and ¹³C NMR (Tables 1 and 2); ESIMS *m/z* 676.2 [M-H]⁻ (calcd for C₃₇H₄₃BrNO₆, 676.2274).

Cell culture

Breast cancer cell lines, MCF-7 and MDA-MB-231, were purchased from ATCC (Manassas, VA). Both cell lines were grown in RPMI 1640 medium (GIBCO-Invitrogen, NY) supplemented with 10% fetal bovine serum (FBS) and glutamine (2 mmol/L), penicillin G (100 U/mL), and streptomycin (100 µg/mL) and incubated at 37°C in a humidified incubator under 5% CO₂.

A stock solution of each compound was prepared in DMSO at a concentration of 25 mM for all assays. Appropriate media (serum-free, 0.5% FBS or 5% FBS) was used to prepare compounds at their final concentrations for each assay. The vehicle (DMSO) control was prepared by adding the maximum volume of DMSO used in preparing test compounds to the appropriate media type such that the final DMSO concentration never exceeded 0.2%.

MTT-proliferation assay

The antiproliferative effect of compounds **1–9** was tested in culture on the human breast cancer cell lines, MCF-7 and MDA-MB-231. Cells in exponential growth were plated in a 96-well plate at a density of 10×10^3 (MCF-7) and 8×10^3 (MDA-MB-231) cells per well, and allowed to attach overnight at 37°C under 5% CO₂ in a humidified incubator. Complete growth medium was then replaced with 100 µL of either RPMI serum-free medium (GIBCO-Invitrogen, NY) for MCF-7 cells or RPMI media supplemented with 5% FBS for MDA-MB-231 cells, containing various doses of the specific test compound and incubation resumed at 37°C under 5% CO₂ for 72 h. Control and treatment media were then removed and replaced with fresh media and 50 µL MTT solution (at 1 mg mL⁻¹) were added to each well and re-incubated for 4 h. At the end of the incubation period, the color reaction was stopped by removing the media and adding 100 µL DMSO to dissolve the formazan crystals formed. Incubation at 37°C was resumed for up to 20 minutes to ensure complete dissolution of crystals. Absorbance was determined at 570 nm with an ELISA plate reader (BioTek, VT, USA). The IC₅₀ value for each compound was calculated by nonlinear regression (curve fit) of log (concentration) versus % survival, implemented in GraphPad Prism version 5.0 (GraphPad Software, La Jolla, CA, USA). The number of cells at the end of the 72-hour incubation period in the vehicle (DMSO) control was 33×10^3 for MCF-7 and 28×10^3 for MDA-MB-231. The % cell survival was calculated as follows: % cell survival = (Cell No._{treatment}/Cell No._{DMSO}) × 100%.

Wound-healing assay (WHA)

MDA-MB-231 cells were plated in sterile 24-well plates and allowed to form a confluent cell monolayer per well (>95% confluence) overnight. A wound was then inflicted in each cell monolayer using a sterile 200 μ L pipette tip. Media was removed and cells were washed twice with PBS and once with fresh RPMI media supplemented with 0.5% FBS. Test compounds at the desired concentrations were prepared in fresh media (0.5% FBS) and were added to wells in triplicate. The incubation was carried out for 24 h, after which media was removed and cells were washed, fixed and stained using Diff-Quick™ staining (Dade Behring Diagnostics, Aguada, Puerto Rico). Cells which migrated across the inflicted wound were counted under the microscope in at least five randomly selected fields (magnification: 400X).

Cultrex® BME cell invasion assay

About 50 μ L of basement membrane extract (BME) coat (1X) was added per well. After overnight incubation at 37°C in a 5% CO₂, 50,000/50 μ L of MDA-MB-231 cells in 0.5% FBS RPMI medium was added per well to the top chamber. Test compounds were prepared at 6X the desired concentrations (45 and 90 μ M) and 10 μ L of each compound was added in triplicate to achieve the final test concentrations (7.5 and 15 μ M). 150 μ L of RPMI medium was then added to the lower chamber. Media contained 10% FBS and penicillin/streptomycin as well as fibronectin (1 μ L/mL) and *N*-formyl-met-leu-phe (10 nM) as chemo-attractants. Plates were re-incubated at 37°C in 5% CO₂ for 24 h after which the top and bottom chambers were aspirated and washed with wash buffer supplemented with the kit. 100 μ L of cell dissociation/calcein-AM solution was added to the bottom chamber and incubated at 37°C in 5% CO₂ for 1 h. The cells internalize calcein-AM, and the intracellular esterases cleave the acetomethylester (AM) moiety to generate free calcein. Fluorescence of the samples was determined at ^{excitation} 485 nm and ^{emission} 528 nm using an ELISA plate reader (BioTek, VT, USA). The numbers of cells that invaded through the BME coat were calculated using a standard curve. For the vehicle (DMSO) control, the total number of cells which actually invaded the BME coat was 17512. % invasion of different treatments were compared using One-Way-ANOVA and individual treatments were then compared to the DMSO control using Dunnett's test implemented in PASW Statistics 18. A *p*-value <0.05 was considered statistically significant.

Caenorhabditis elegans strains

The wild-type N2 Bristol strain of *C. elegans* and the *slo-1* knockout strain NM1968 *slo-1(js379)V* (*Caenorhabditis* Genetics Center, University of Minnesota) were used. Worm stocks were maintained on nematode growth medium (NGM) plates preseeded with OP50 *E. coli* (*Caenorhabditis* Genetics Center) as a food source at 20°C.³⁰ Stock solutions were prepared in DMSO. Proper dilutions to achieve final concentrations were conducted in NGM agar after autoclaving and at ~50°C. Final DMSO concentration was 28 mM, which does not have any effects on *C. elegans* locomotion.³³ All plates were seeded with ~100 μ L *E. coli* OP50.

Locomotion assay: Number of reversals count

The setup for the reversal assay was as follows: 30–40 synchronized L1 stage larvae of N2 and NM1968 *slo-1(js379)V* strains were transferred to NGM plates seeded with *E. coli* OP50 and containing 1.0 μ M of each compound or DMSO (vehicle control). Plates were incubated for 42 h at 20°C. At the end of the incubation period, L4 larvae were individually transferred to a plain un-seeded NGM plate and allowed to crawl for one minute to acclimatize and allow for the removal of any residual bacteria that may be adherent to the worm. Then the reversals were counted for 3 minutes. Numbers of reversals in different

treatments were compared using One-Way-ANOVA and individual treatments were then compared to the DMSO control using Dunnett's test implemented in PASW Statistics 18. A p -value <0.05 was considered statistically significant.

Toxicity evaluation

About 30–40 synchronized L1 stage larvae of the wild type N2 strain were transferred to NGM plates seeded with *E. coli* OP50 and containing each compound (at 10 and 50 μ M) or DMSO (vehicle control). Plates were incubated at 20°C and were observed starting at 42 h post exposure. All plates were followed up for up to 7 days and assessed for viability as well as effects on reproduction.

Pharmacophore modeling

Pharmacophore modeling was carried out using PHASE (version 3.5, 2013) module of Schrödinger suite implemented in Maestro (Maestro 9.3.5, 2012) molecular modeling package. PHASE identifies the spatial arrangement of functional groups that are common and essential for the biological activity of the compounds. The structures of compounds were built using the structure drawing tool and prepared using LigPrep (version 2.5, 2012). ConfGen was used for conformer generation by applying OPLS-2005 force field method with implicit GB/SA distance-dependent dielectric solvent model at cutoff root mean square deviation (RMSD) of 1 Å. Conformers not within 10 kJ/mol of the global minimum were automatically discarded. A maximum of 1000 conformers and 100 minimization steps were set. An activity threshold value was then assigned to divide compounds into a set of active and inactive ones. PHASE supplies a standard set of six built-in features/sites: hydrogen bond acceptor (A), hydrogen bond donor (D), hydrophobic group (H), negatively-charged group (N), positively-charged group (P) and aromatic ring (R). The maximum number of sites was set to 6 and minimum to 4. Resulting pharmacophore hypotheses were then scored using default weights of scoring parameters for both active (survival score) and inactive ones (survival-inactive scores). To further assist in ranking hypotheses, post-hoc score was generated in which an activity reward was added for hypotheses that utilize the most active compound as the reference.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Penitrem structures. Penitrems A-F (1–5) and two of their biosynthetic precursors, paspaline (6) and emindole SB (7) were isolated from a new marine-derived *Penicillium commune* isolate GS20. Supplementing the fermentation broth of the growing fungus with KBr afforded the new 6-bromopenitrem B (8) and the known 6-bromopenitrem E (9).

Fig. 2.
Dose response curve for the total extract of the fungal strain GS20 obtained in the MTT assay against the breast cancer cell line MCF-7. Error bars indicate SEM for $n=3$.

Fig. 3. Neighbor-joining phylogenetic tree based on ITS-rDNA sequences of fungal isolate GS20 and related sequences from GenBank. Accession numbers are between brackets.

Fig. 4.

Biosynthesis of the indole diterpene alkaloids via the mevalonate and the tryptophan pathways. IPP: isopentenyl diphosphate; FPP: farnesyl diphosphate; GGPP: geranylgeranyl diphosphate.¹⁸

Fig. 5.
WHA using MDA-MB-231 cells after 24 h exposure to (a) vehicle (DMSO) control, and (b) 7.5 μ M penitrem A (**1**).

Fig. 6. Anti-invasive activities of compounds 1-9 against the human breast cancer cell line MDA-MB-231 using the Cultrex® BME Cell Invasion assay kit. Error bars indicate the SEM of $n=3$ per compound. * $P < 0.05$ compared to the vehicle control group.

Fig. 7.

C. elegans as an *in vivo* model to assess the BK channel inhibitory effect of **1**, **7**, and **9**. Wild type N2 strain treated with 1 μ M of each compound is compared with DMSO (vehicle control) and the *slo-1* mutant NM1968 (*js379*)V. Error bars indicate SEM for at least $n=10$.

* $P < 0.05$ compared to their respective DMSO-treated control group.

Fig. 8. Common pharmacophore generation using PHASE: (a) Common pharmacophoric features identified by ADDHRR hypothesis. All distances are in Å unit. (b) Alignment of penitrems **1–5**, **8** and **9** to the pharmacophore. (c) Alignment of paspaline (**6**) and emindole SB (**7**) to the pharmacophore.

Table 1¹H NMR spectroscopic data of compounds **8** and **9**.^a

Position	H, mult. (J in Hz)	
	8	9
6	-	-
7	7.35, s	7.40, s
10	3.65, d (15.6) 3.19, d (16.0)	3.60, d (16.0) 3.26, dd (15.6, 1.4)
12	3.18, m	2.95, m
13	2.45, m 2.27, m	2.38, m 2.23, m
14	2.12, m	2.47, m
15	3.87, dd (9.6, 9.6)	-
18	4.76, d (8.2)	4.91, d (8.2)
19	2.61, m	2.62, m
20	1.91, m 1.77, m	1.91, m 1.79, m
21	1.73, m 1.46, m	1.76, m 1.45, m
24	3.55, brs	3.56, brs
25	4.01, brs	4.02, brs
26	4.01, brs	4.02, brs
28	4.27, d (9.2, 8.7)	4.27, dd (9.2, 8.7)
29	2.21, m 2.06, m	2.18, m 2.05, m
30	2.57, m 1.56, m	2.59, m 1.53, m
33	5.05, brs 4.86, brs	5.00, brs 4.86, brs
34	1.09, 3H, brs	1.05, 3H, brs
35	1.69, 3H, brs	1.73, 3H, brs
36	1.52, 3H, brs	1.69, 3H, brs
38	5.08, brs 4.89, brs	5.05, brs 4.86, brs
39	1.19, 3H, brs	1.20, 3H, brs
40	1.39, 3H, brs	1.38, 3H, brs

^aIn (CD₃)₂CO, J in Hz. 400 MHz for ¹H NMR.

Table 2¹³C NMR spectroscopic data of compounds **8** and **9**.^a

Position	C	
	8	9
2	153.1, qC	153.6, qC
3	118.6, qC	119.8, qC
4	130.2, qC	132.7, qC
5	126.1, qC	126.2, qC
6	113.0, qC	113.9, qC
7	112.9, CH	114.2, CH
8	121.9, qC	121.9, qC
9	138.5, qC	139.5, qC
10	37.6, CH ₂	37.1, CH ₂
11	148.2, qC	148.7, qC
12	34.2, CH	46.3, CH
13	25.6, CH ₂	23.7, CH ₂
14	51.5, CH	51.9, CH
15	38.9, CH	80.3, qC
16	74.6, qC	75.3, qC
18	71.1, qC	71.6, qC
19	58.2, CH	57.9, CH
20	17.7, CH ₂	17.7, CH ₂
21	29.7, CH ₂	29.7, CH ₂
22	77.3, qC	77.4, qC
23	65.2, qC	65.3, qC
24	61.0, CH	61.0, CH
25	65.4, CH	65.4, CH
26	73.8, CH	73.8, CH
28	71.1, CH	71.1, CH
29	28.0, CH ₂	28.0, CH ₂
30	26.0, CH ₂	26.1, CH ₂
31	42.7, qC	42.7, qC
32	48.9, qC	49.2, qC
33	106.6, CH ₂	106.4, CH ₂
34	17.8, CH ₃	19.5, CH ₃
35	27.9, CH ₃	30.2, CH ₃
36	18.9, CH ₃	18.9, CH ₃
37	142.4, qC	142.4, CH ₂
38	110.8, CH ₃	110.8, Qc
39	18.0, CH ₃	18.1, CH ₃
40	20.2, CH ₃	20.5, CH ₃

^a_{In} (CD₃)₂CO, *J* in Hz. 100 MHz for ¹³C NMR. Carbon multiplicities were determined by APT or PENDANT experiments, C = quaternary, CH = methine, CH₂ = methylene, CH₃ = methyl carbons.

Table 3

Antiproliferative and antimigratory activities of compounds **1–9** against human breast cancer cell lines.

Compound	Antiproliferative activity (IC ₅₀ , μ M)		Antimigratory activity (IC ₅₀ , μ M)
	MCF-7	MDA-MB-231	MDA-MB-231
1	11.9	9.8	8.7
2	5.5	13.7	10.3
3	8.3	29.7	9.2
4	17.5	25.4	20.3
5	15.0	13.8	35.0
6	12.8	12.4	7.6
7	10.1	21.3	19.0
8	19.3	18.8	30.3
9	16.7	8.5	9.6