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Pinpointing Pseurotins from a Marine-Derived *Aspergillus* as Tools for Chemical Genetics Using a Synthetic Lethality Yeast Screen

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A new compound of mixed polyketide synthase–nonribosomal peptide synthetase (PKS/NRPS) origin, 11-*O*-methylpseurotin A (**1**), was identified from a marine-derived *Aspergillus fumigatus*. Bioassay-guided fractionation using a yeast halo assay with wild-type and cell cycle-related mutant strains of *Saccharomyces cerevisiae* resulted in the isolation of **1**, which selectively inhibited a Hof1 deletion strain. Techniques including 1D and 2D NMR, HRESIMS, optical rotation, *J*-based analysis, and biosynthetic parallels were used in the elucidation of the planar structure and absolute configuration of **1**. A related known compound, pseurotin A (**2**), was also isolated and found to be inactive in the yeast screen.

Embarking on the use of high-throughput phenotypic screens based on the concept of chemical genetics offers the possibility of discovering potent and specific bioactive agents. The advantage of employing natural products in this effort is that small molecules rich with both heteroatom functionality and chirality could emerge as hits. Although using eukaryotic model systems for phenotypic screens, such as fertilized *Danio rerio* (zebrafish) embryos, has attracted attention,[1,2] we favored utilizing the budding yeast *Saccharomyces cerevisiae* as a convenient and broadly applicable platform.[3,4] This was the basis of a collaborative program begun recently to explore libraries emphasizing natural products. As an important first step, we developed and employed a high-throughput yeast halo screen based on paired wild-type and cell cycle mutant strains. This was used to first test 3104 compounds from an NCI library, 167 marine sponge crude extracts, and 149 crude marine-derived fungal extracts against wild-type yeast.[5] One of the active sponge extracts afforded crambescidin-800 through bioassay-guided isolation. A next step was to simultaneously re-evaluate selected marine fungal samples for activity against both wild-type and a set of deletion strains that are synthetically lethal with a variety of cell cycle and cytokinesis genes. Synthetic lethality is a phenomenon whereby an inviable phenotype is generated by genetic or chemical inactivation of two genes that, when disrupted individually, do not result in cell death.[6] Identifying synthetically lethal combinations is a useful way to probe the interaction and function of biochemical pathways. Reported herein are the active constituents of the crude extract hit from *Aspergillus fumigatus* that showed selective lethality toward the *hof1*Δ. In addition, its crude extract fractions were rescreened against 15 additional deletion strains.

We began the isolation work mindful of the enormous previous attention given to the natural products isolated from *Aspergillus* cultures. In fact, *Aspergillus* represents one of the most studied marine-derived fungal genera, as at least 74 new molecular structures were reported since 1996, with 77% of these described within the last 4 years.[7] This pattern was not surprising, as roughly 1000 compounds have also been discovered from terrestrial *Aspergillus* strains.[8] Two of the most notable compounds from marine-derived *Aspergillus* sources are asperazine,[9] an unsymmetrical diketopiperazine dimer discovered in our laboratory that displayed selective cytotoxicity toward leukemia cells, and the tropolactones,[10] meroterpenoids isolated by the Fenical group that exhibited inhibition of human colon carcinoma cells at micromolar levels.

The *Aspergillus fumigatus* (coll. no. 030402d) was separated from deep water marine sediment collected in Vanuatu in 2003. Following a 24 L culture, the crude extracts were screened against 16 different *S. cerevisiae* haploid deletion strains whose deletions are involved in cell cycle control and cytokinesis. The greatest activity differential versus the wild-type strain was obtained against the Hof1 (*hof1*Δ) haploid deletion strain. Bioassay-guided fractionation employing the *hof1*Δ strain ultimately afforded the new secondary metabolite 11-*O*-methylpseurotin A (**1**) as the active constituent. Subsequently, known pseurotin A (**2**) was also isolated and found to be inactive against the *hof1*Δ strain.

The structure elucidation of **1** proceeded by determining the molecular formula, $C_{23}H_{27}NO_8$, by HRESIMS, followed by identifying its prominent structural features via ^{1}H and ^{13}C NMR data. Another important strategy was the survey of similar structures located in the literature through database searches. The molecular formula was consistent with *m/z* 446.1852 $[M + H]^+$ (Δ 4.1 mDa of calcd) and required 11 unsaturations. The ^{1}H and ^{13}C NMR data of **1** indicated the presence of (1) a monosubstituted benzene ring, (2) a disubstituted *cis* double bond ($J = 11.0$ Hz), (3) two *O*-methyl groups, (4) one allylic and one aliphatic methyl group, and (5) three carbonyls as one α,β-unsaturated ketone and two ester residues. Collectively these moieties accounted for eight of the 11 degrees of unsaturation, indicating two additional rings were also present. A literature search based on the molecular formula with the hits pruned for fungal-derived natural products gave no results. Next, an expanded literature search using a mass range of 445.0–445.3 Da provided seven fungal-derived hits. Of these, pseurotin E[11] (**3**), $C_{22}H_{23}NO_9$, with mass 445.1 Da, was the only one with three rings. It was also the most similar to the experimental NMR data for **1** with two exceptions: the C-14 ketone of **3** was replaced with a methylene (δ 20.8) in **1** and 11-OH of **3** was an *O*-Me (δ 55.4) in **1**. These new features in **1** were confirmed by 2D NMR data in Table 1 including (a) gCOSY correlations between H_3-15 and H-14a and H-14b and (b) gHMBC correlations from 11-*O*-Me to C-11. The second *O*-Me group was placed at C-8 with a gHMBC correlation from 8-*O*-Me to C-8. Subsequently, known pseurotin A[12,13] (**2**) was also isolated (Figure S1, Supporting Information) and its identity was confirmed via comparison of ^{13}C and ^{1}H NMR data to that in the literature.[13]

The absolute configuration of pseurotin A (**2**) had been previously established (5*S*, 8*S*, 9*R*, 10*S*, 11*S*, 12*Z*) by X-ray analysis of the

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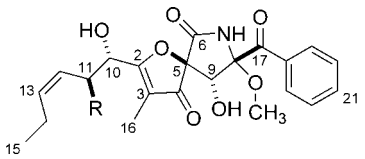
Table 1. ^{13}C (125 MHz), ^{1}H (500 MHz), and 2D NMR Data for 11-*O*-Methylpseurotin A (**1**) in MeOH-d_4 with δ_C of Pseurotin A (**2**) in Acetone-d_6[13]

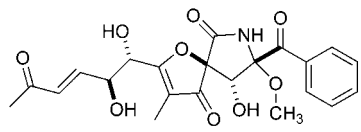
position	δ_C of **2**[c]	δ_C	δ_H; mult. (*J* Hz) int.	gCOSY	gHMBC (H to C)
2	167.3	167.2			
3	113.6	112.6			
4	197.9	198.0			
5	92.7	92.3			
6	187.7	187.3			
7-NH[a]			8.05; brs, 1H		
8	92.4	92.0			
8-*O*-Me	52.2	51.0	3.34; s, 3H		C-8
9	75.3	75.0	4.53; s, 1H		C-4, C-6, C-8, C-17
9-OH[a,b]			3.75; brs, 1H		
10	72.8	69.7	4.57; d (7.0), 1H	H-11	C-2, C-3, C-11, C-12
10-OH[a,b]			4.35; brs, 1H		
11	69.7	77.3	4.30; ddd (9.5, 7.0, 1.0), 1H	H-10, H-12	C-2, C-10, 11-*O*-Me, C-13
11-*O*-Me		55.4	3.28; s, 3H		C-11
12	129.0	125.1	5.32; ddt (11.0, 9.5, 1.5), 1H	H-11, H-13	C-14
13	135.9	138.4	5.79; dtd (11.0, 7.5, 1.0), 1H	H-12	C-14
14	21.8	20.8	2.20; dpd (15.0, 7.5, 1.5), 1H	H-15	C-12, C-13, C-15
			2.12; dpd (15.0, 7.5, 1.5), 1H	H-15	C-13, C-15
15	14.5	13.1	0.98; t (7.5), 3H	H-14	C-13, C14
16	5.8	4.3	1.73; s, 3H		C-2, C-3, C-4
17	196.2	195.8			
18	134.7	133.5			
19/23	131.3	130.2	8.36; brdd (8.5, 1.5), 2H	H-20/22	C-17, C-19/23, C-21
20/22	129.2	128.1	7.50; dd (8.5, 7.5), 2H	H-19/23, H-21	C-18, C-19/23, C-20/22
21	134.7	133.7	7.65; tt (7.5, 1.5), 1H	H-20/22	C-19/23, C-20/22

[a] Measured in MeCN-d_3. [b] Interchangeable. [c] In acetone-d_6.

dibromo derivative.[14] There have been two separate stereoselective syntheses further confirming the correctness of that assignment.[15,16] The 3D features shown for **1** are in direct analogy to those of **2**. The absolute configuration of the five stereocenters and the *Z* C-12/C-13 double bond of **1** were assigned on the basis of comparable data with **2** including ^{13}C NMR chemical shifts, ^{1}H–^{1}H coupling constants, similar specific rotation values, and the expectation about their parallel biosynthetic origins. The ^{13}C NMR shifts of the functionally similar allylic C-14 of **1** (δ 20.8) versus that of **2** (δ 21.8) confirmed the *Z* configuration, further solidified by the observed $^{3}J_{12-13}$ of 11.0 Hz in **1** (lit. for **2** = 10.8[17]) versus 16.0 Hz for the *trans* bond of **3**.[11] Comparable specific rotations were obtained for **2** ($[\alpha]^{28}_D$ −0.8 (*c* 0.2, MeOH), lit. value $[\alpha]^{20}_D$ −5 (*c* 0.5, MeOH)) and **1** ($[\alpha]^{28}_D$ −1.8 (*c* 0.2, MeOH)). A mixed polyketide synthase–nonribosomal peptide synthetase biosynthetic pathway of pseurotin A (**2**) has been documented; therefore, it is doubtful that **1** would be produced in a distinct manner.[18]



11-*O*-methylpseurotin A (**1**) R = OCH_3
pseurotin A (**2**) R = OH



pseurotin E (**3**)

The final issue deserving comment was that **1** could have been an isolation artifact derived from **2**. This possibility was ruled out on the basis of several observations. Each of the prior reports of **2** employed MeOH during extraction, and despite the five other known analogues of pseurotin A (**2**), none have been reported with methylation at the C-11 alcohol. The stability of **2** to the isolation conditions was tested by dissolving 1.0 mg in MeOH-d_4 with 0.1% TFA-*d* for a period of 1 month. This solution was regularly examined by ESIMS. The expected +4 Da mass shift corresponding to the incorporation of the four exchangeable protons was observed; however the $+CD_3$ – H mass shift (+17 Da) for the substitution of an OH with an *O*-Me-d_3 was not observed.

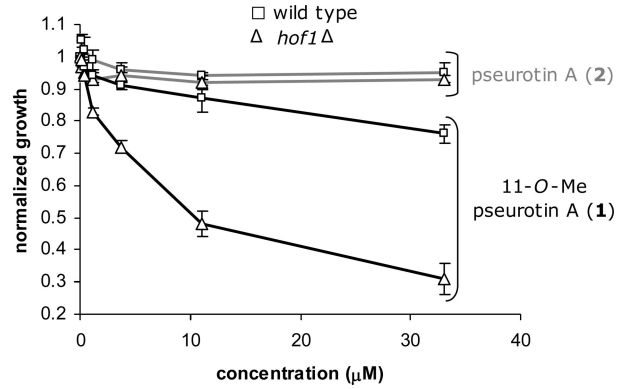


Figure 1. Concentration-dependent cell survival curve for 11-*O*-methylpseurotin A (**1**) and pseurotin A (**2**) against wild-type (□) and mutant *hof1*Δ (Δ) strains of *S. cerevisiae*. Growth is normalized against untreated control (1 = 100% growth, no inhibition), error bars are 1 standard deviation for triplicate values.

As noted previously, a bioassay-guided fractionation employing wild-type and *hof1*Δ strains of *S. cerevisiae* in a yeast halo assay led to the isolation of 11-*O*-methylpseurotin A (**1**). The activity results are illustrated in Figure 1, which displays cell growth normalized to an untreated control for compounds **1** and **2** in the *hof1*Δ and wild-type strains. Compound **2** was inactive against both strains, while **1** exhibited a 9 mm halo diameter against *hof1*Δ yeast and was less toxic to wild-type yeast with a halo of 4 mm.

Hof1 is an SH3 domain-containing protein that is localized to the bud neck and is required for cytokinesis. It regulates actomyosin ring dynamics and septin localization and interacts with the formins, Bni1p and Bnr1p, and with Cyk3p, Vrp1p, and Bni5p.[19–22] It is synthetically lethal with other cytokinesis-related genes, including CYK3 and BNI5, and the type II myosin-encoding gene MYO1, which is also required for cytokinesis and cell separation.[23]

Compounds with selective toxicity to the *hof1*Δ strain are therefore likely to target pathways parallel to Hof1 and may inhibit cytokinesis. We are currently working to identify the cellular target(s) of compound **1**.

The densely functionalized 1-oxa-7-azaspiro[4.4]nonane core with phenyl ketone and C_6-aliphatic appendages is not a commonly observed natural product. To date the nine members of this group consisting of the pseurotin family of compounds (A–E, F1 and F2, azaspirene, and synerazol, Chart S1, Supporting Information) are isolated from only three genera of fungi. Minor structural variations center primarily on the presence or absence of oxygen atoms at the side-chain carbons C-10 to C-14 and/or C-17, plus the variation of OH versus *O*-Me at C-8. The variation of the oxygen functionality at these key carbons may be responsible for the astounding host of biological activities that have been noted for these compounds. They include inhibition of chitin synthase (pseurotin A and F2)[17] and monoamine oxidase (pseurotin A)[24] and functioning as apomorphine antagonists (pseurotins A, D, F1, and F2).[25,26] Two other compounds in this family with misleading, unrelated, names are also modified at the C-10 and C-11 positions, azaspirene (double bond)[27] and synerazol (epoxide)[28] (Chart S1, Supporting Information), which respectively inhibit angiogenesis and the growth of *Candida albicans*. In this instance, modification of the C-11 alcohol of **2** has resulted in a differential ability to interact with the Hof1 related network of proteins. The protein cascades that govern cytokinesis play a vital role in cell cycle regulation and in the proliferation of cancer cells. The elucidation of cytokinesis pathways is currently incomplete; thus, 11-*O*-methylpseurotin A (**1**) may prove useful as a tool to further the study of these processes.

Experimental Section

General Experimental Procedures. Optical rotations were measured on a Jasco DIP-370 polorimeter as per instructions. The NMR data for **1** and **2** were recorded at 500 and 600 (^{1}H) and 125 (^{13}C) MHz on Varian UNITYplus or INOVA spectrometers. High-resolution mass spectra were acquired with a bench top Mariner ESI-TOF-MS. Semipreparative RP-HPLC was performed using MeCN and H_2O with 0.1% formic acid and a 5 μm ODS column.

Biological Materials. The *Aspergillus fumigatus* (coll. no. 030402d) was isolated from sediment collected at >33 m depth in Vanuatu in 2003. The strain was taxonomically identified by molecular (ITS and D1/D2 regions of rDNA) and morphological methods at the University of Texas Fungus Testing Laboratory. It is maintained as a cryopreserved glycerol stock at UCSC.

Culture Conditions. The 24 L culture of 030402d was grown in 3.5% Czapek-Dox media made with filtered Monterey Bay seawater, pH adjusted to 7.3, with ca. 50 g of prewashed XAD-16 resin added to each liter prior to autoclaving. Cultures were inoculated and shaken at 150 rpm for 21 days at room temperature.

Yeast Halo Assay. This assay has been fully described.[29] Briefly, media were inoculated with 400 μL of an overnight yeast culture (haploid strain BY4742, *hof1*Δ, and deletion strains for Cyk3, Shs1, Bnr1, Bni5, Bni4, Cla4, Sct4, Gin4, Elm1, Cdc10, Dbf20, Sic1, Top1, and wild type), poured into an OmniTray, and cooled for 30 min. Compound DMSO stocks (10 mM) plated in 384-well polypropylene trays were transferred into the solidified agar using notched pins that deliver 200 nL (±8%) each with a pin-tool robot. Absorbance readings were taken of the agar plates using a plate reader at 544 nm. Growth of the *hof1*Δ versus the wild-type yeast was compared to identify selective agents.

Isolation of 11-*O*-Methylpseurotin A and Pseurotin A. The 24 L culture material was transferred to a glass column with a cotton plug, and the broth was drained. The residual mycelia and resin were washed with H_2O, and the extract was eluted with MeOH followed by CH_2Cl_2. The resulting crude extract was partitioned between hexanes (CXH), CH_2Cl_2 (CXD), and MeOH (CXM) per our standard procedure.[30] The CXD (3.5 g) was applied to a Si gel column using a stepwise gradient of 20 to 40% EtOAc in CH_2Cl_2 followed by 100% MeOH then 100% MeCN, affording 10 fractions (Figure S1, Supporting Information). Fraction S12-19 (313.6 mg) was subjected to RP-HPLC first with 40–90% MeCN in H_2O, to generate five fractions. H4 (18.3 mg) contained semipure **1**, which was further purified by RP-HPLC (50–100% MeCN in H_2O), affording five fractions with pure **1** in H3 (5.8 mg). Si gel fraction S20-25 (104.7 mg) was subjected to RP-HPLC (50–65% MeCN in H_2O), affording pure **2** (H3, 19.4 mg).

11-*O*-Methylpseurotin A (1): pale yellow solid; $[\alpha]^{28}_D$ −1.8 (*c* 0.2, MeOH); ^{1}H and ^{13}C NMR data, see Table 1; HRESIMS *m/z* 446.1852 $[M + H]^+$ (calcd for $C_{23}H_{28}NO_8$, Δ 4.1 mDa).

Pseurotin A (2): pale yellow solid; $[\alpha]^{28}_D$ −0.8 (*c* 0.2, MeOH); ^{1}H and ^{13}C NMR matched reported values.[13]

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Supporting Information Available: A chart with the structures of compounds in the pseurotin family, an isolation scheme, and ^{1}H, ^{13}C, gHMBC, and gCOSY NMR spectra for 11-*O*-methylpseurotin A (**1**) are available free of charge via the Internet at http://pubs.acs.org.

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