Expanding the Strategies in Natural Product Studies of Marine-Derived Fungi: A Chemical Investigation of *Penicillium* Obtained from Deep Water Sediment

Jeffrey T. Gautschi,[†] Taro Amagata,[†] Akiko Amagata,[†] Frederick A. Valeriote,[‡] Susan L. Mooberry,[§] and Phillip Crews*[,†]

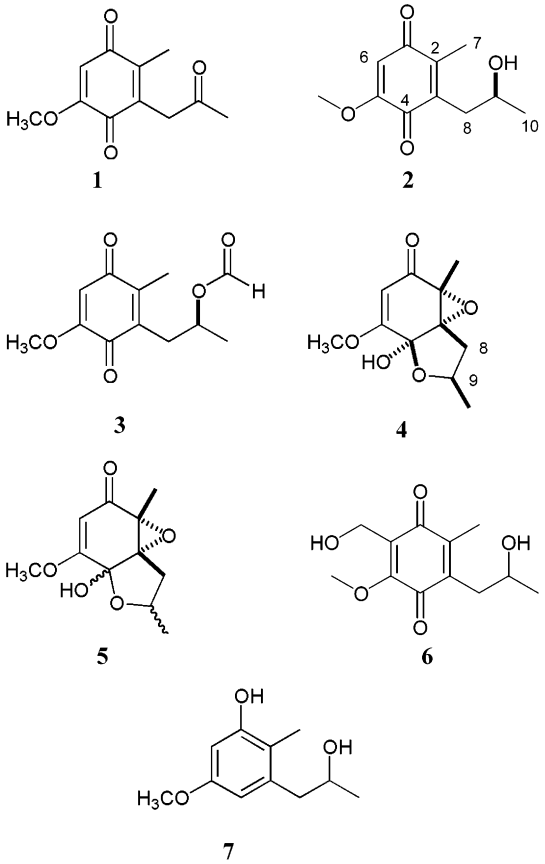
Department of Chemistry and Biochemistry & Institute of Marine Sciences, University of California, Santa Cruz, California 95064, Josephine Ford Cancer Center, Detroit, Michigan 48202, and Department of Physiology and Medicine, Southwest Foundation for Biomedical Research, San Antonio, Texas 78245

Received August 21, 2003

Three previously unknown pentaketides, (+)-formylanserinone B (**3**), (−)-epoxyserinone A (**4**), and (+)-epoxyserinone B (**5**), along with two known fungal pigments, anserinones A (**1**) and B (**2**), were isolated and identified from a deep water (−4380 ft), marine-derived saltwater fungal culture. Two other minor constituents, hydroxymethylanserinone B (**6**) and deoxyanserinone B (**7**), were also isolated, but not completely purified. The structures of **3**−**7**, each expanding the dense functionalization of the anserinones, were determined by dereplication and spectroscopic analysis. Bioactivity was explored in two separate cell-based assays. Leukemia selectivity was greatest with **2** and **3**, while **1**−**3** exhibited modest activity against the MDA-MB-435 cell line.

In the early 1990s we began exploring marine-derived filamentous fungi grown in saltwater culture as a source for a new generation of secondary metabolites. Two early proof-of-principle results emerged quickly showing that research on sponge-derived fungi was rewarding. These premier discoveries included Kitagawa's isolation of trichoharzin,[1] from the culture of a *Mycale* sponge-derived salt obligate strain *Trichoderma harzianum*, and our report of the chloriolins A−C,[2] produced during the saltwater culture of an unidentified fungus from *Jaspis splendens*. Further research along these lines continued in our laboratory and also began in many others, resulting in a host of significant additional discoveries. These include unique structures we described of sponge-derived fungal metabolites, such as the chlorocarolides A and B[3] obtained from saltwater cultures of *Aspergillus*, asperizine[4] isolated from another *Aspergillus* strain, and epoxysorbicillinol[5] derived from *Trichoderma longibrachiatum*. Parallel discoveries of sponge-derived fungi by others included the gymnastatins from *Gymnasella dankaliensis*,[6] the petrosifungins from a *Penicillium*,[7] the aspergillones from *Aspergillus*,[8] and the pandangolides from an unidentified fungus.[9]

The basis for the research described below was twofold. First, we wanted to extend the strategy of using the disk diffusion assay as a mechanism to prioritize fungal cultures for further investigation. The value of such an approach was recently illustrated in our study of cytotoxic trichoverroids and their macrolide analogues produced by saltwater culture of *Myrothecium verrucaria*.[10] Second, we wanted to begin study on the saltwater culture of fungi obtained from extreme environments, such as deep water.[11] Biological aspects of fungi from marine environments as deep as −13 500 ft have been described.[12] In fact, it is noteworthy that convincing evidence was published more than two decades ago showing fungi can be obtained from deep water.[13,14] Unknown when our research began was the extent to which deep water material could be a source of chemically prolific fungal cultures. Interestingly, deep water sponges are now recognized as a source of novel metabolites that may be of microbial origin.[15] There is growing evidence that deep water bacteria can be a source of unique natural products. These include the account of cytotoxic macrolactins A−F[16] from an unidentified deep water bacteria and more recently by the description of the salinosporamides from a novel genus *Salinospora*.[17] We now describe the first isolation of compounds from a deep water, marine-derived saltwater fungal culture.[18] Compounds isolated and identified include the known anserinone A (**1**) and (+)-anserinone B (**2**),[19] along with new



* To whom correspondence should be addressed. Tel: (831) 459-2603. Fax: (831) 459-2935. E-mail: phil@chemistry.ucsc.edu.
[†] University of California.
[‡] Josephine Ford Cancer Center.
[§] Southwest Foundation for Biomedical Research.

10.1021/np030388m CCC: $27.50
Published on Web 02/05/2004

Table 1. ^{1}H (500 MHz) and ^{13}C (125.7 MHz) NMR Data ($CDCl_3$) for **3**−**7**

	3		**4**		**5**		**6**[a]		**7**	
no.	δ_C	δ_H (mult., *J* in Hz)	δ_C	δ_H (mult., *J* in Hz)	δ_C	δ_H (mult., *J* in Hz)	δ_C	δ_H (mult., *J* in Hz)	δ_C	δ_H (mult., *J* in Hz)
1	187.3		192.0		192.0		187.1		155.2	
2	143.8		60.9		61.2		143.4		115.0	
3	138.3		71.3		71.1		136.2		139.2	
4	181.9		94.9		95.6		182.5		108.4	6.37 (d, 2.5)
5	158.4		168.3		168.4		158.4		158.3	
6	107.6	5.91 (s)	99.0	5.38 (s)	99.8	5.34 (s)	106.5		100.1	6.31 (d, 2.5)
7	12.9	2.11 (s)	12.7	1.50 (s)	13.1	1.53 (s)	11.0	2.03 (s)	11.2	2.15 (s)
8	32.8	2.87 (dd, 13.5, 5.0)	31.3	2.66 (dd, 13.5, 8.0)	31.5	2.44 (dd, 12.5, 9.5)	33.0	3.07 (m)	43.7	2.80 (dd, 13.5, 5.0)
8′		2.78 (dd, 13.5, 8.5)		1.86 (dd, 13.5, 5.5)		2.29 (dd, 13.0, 6.5)		2.50 (m)		2.70 (dd, 13.5, 9.0)
9	69.9	5.18 (m)	74.5	4.75 (m)	72.9	4.41 (m)	75.5	4.76 (m)	68.3	4.01 (m)
10	20.6	1.33 (d, 6.5)	23.7	1.35 (d, 6.5)	21.8	1.54 (d, 6.0)	20.5	1.47 (d, 6.5)	23.0	1.28 (d, 5.5)
11	56.2	3.81 (s)	56.7	3.80 (s)	56.8	3.80 (s)	55.0	3.74 (s)	55.4	3.76 (s)
12	160.0	7.95 (s)					51.0	3.87 (s)		

[a] ^{13}C signals derived from ^{1}H−^{13}C gHMBC in CD_3OD.

analogues (+)-formylanserinone B (**3**), (−)-epoxyserinone A (**4**), (+)-epoxyserinone B (**5**), hydroxymethylanserinone B (**6**), and deoxyanserinone B (**7**).

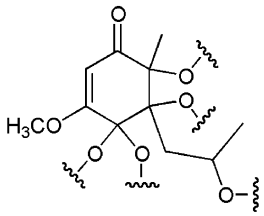
Results and Discussion

This research began by using the soft agar-based bioassay system alongside LCMS analysis[20] of small-scale, saltwater fungal cultures (150 mL) from three separate libraries of deep sea collections. The next step was to rank the results by assessing the cytotoxicity data for selectivity and to use established dereplication methods to evaluate the LCMS data.[21] We were able to identify a dozen or more fungal cultures as producing compounds with unique molecular formulas and interesting cytotoxicity profiles. Both phenotypic and 28S DNA sequence analyses were used to obtain taxonomic identification. The material chosen for this investigation consisted of a deep water sediment sample collected at −4380 feet (−1335 m) that provided a filamentous fungus (UCSC coll. no. 004181), which appeared to be a mixed culture of two *Penicillium corylophilum* strains. The mixed sample was used for the bulk culture. The liquid broth was separated from the mycelia, thoroughly extracted with EtOAc, and partitioned using our standard Kupchan-type solvent partition scheme. Resulting mixtures were profiled by LCMS and screened in a disk diffusion soft agar colony formation assay.[22] Data from LCMS suggested the bioactive mixtures consisted primarily of a series of related, low molecular weight compounds.

Examination of the semipure extract by analytical LCMS revealed that it was a complex mixture of 10 compounds, not all of which were isolated. We focused on those that were most easily purified, and the most abundant component was determined to be (+)-anserinone B (**2**), a yellow oil previously described as a pigment from the terrestrial coprophilious fungus *Podospora anserina*.[19] Comparison of our ^{13}C and $[\alpha]_D$ data to the literature assured **2** to be (+)-anserinone B. Long-range (4 Hz delay) gHMBC was used to reaffirm the placement of the OCH_3 at C-5 as opposed to C-6, on the basis of the relative intensity of the contour peaks from the OCH_3 to C-5 (100%), C-6 (43%), C-4 (21%), and C-1 (11%).[23] With this component identified, attention was next shifted to constituents whose masses shifted from the MW of **2** by −2, +16, and +28 amu. The detection of anserinone A (**1**), the known oxidized analogue of (+)-anserinone B (**2**), was therefore straightforward, and eventual isolation and spectral comparison (UV, ^{1}H NMR, HRESITOFMS) confirmed its structure.

The first novel analogue to be isolated, (+)-formylanserinone B (**3**), the formate ester of (+)-anserinone B (**2**), was deciphered beginning with a molecular formula of $C_{12}H_{14}O_5$ (*m*/*z* 261.0750 $[M + Na]^+$, calcd 261.0734) as deduced by HRESITOFMS. Comparison of the ^{13}C NMR data of **3** with that of **2** showed nearly identical carbon shifts for both of the conjugated ketones, the four olefinic carbons, the single methylene and single OCH_3, and finally the two methyl groups. Additionally, a carbon shift found at δ_C 160.0, together with the corresponding δ_H 7.95 singlet, suggested a formyl substituent on the anserinone framework (see Table 1). Upon closer comparison of ^{13}C and ^{1}H NMR data for **3**, as compared to **2**, the small downfield shift difference at C-9 and the small upfield shift difference at Me-10, together with the large downfield shift at H-9, made the formyl ester apparent. Correlation of the formic proton at δ_H 7.95 with the methine carbon at δ_C 69.9 by ^{1}H−^{13}C gHMBC confirmed this placement. As formic acid was used in the final HPLC purification of **3**, its origins were suspect. Analysis of ^{1}H NMR data from parent fractions, however, clearly revealed shifts corresponding to the formylester signals of **3**, indicating it is not an artifact. The similarities in $[\alpha]_D$ data between **3** and **2** suggested matching *S* stereochemistry at the lone stereocenter.

The densely functionalized anserinone analogues, (−)-epoxyserinone A (**4**) and its epimer (+)-epoxyserinone B (**5**), were next isolated, each as a white powder. The molecular formula $C_{11}H_{14}O_5$ was established for both **4** and **5** via HRESITOFMS (*m*/*z* 249.0736 $[M + Na]^+$ and 249.0756 $[M + Na]^+$, calcd 249.0733, respectively). Chromatographic separation, optical rotation, identical ^{13}C NMR data, and nearly identical ^{1}H NMR data for **4** and **5** (see Table 1) indicated that these were diastereomers. Characterization of **4** preceded that of **5** and began with the supposition that the only spin system, an A_2BX_3, suggested that the C-8 to C-10 array was similar between **4** and **2**. In the case of **4**, however, the aliphatic oxygenated methine multiplet was shifted downfield by 0.9 ppm, and the shift difference of diastereotopic methylene protons was more pronounced. Also similar to **2** were shifts in **4** corresponding to a single oxygenated olefinic carbon, an olefinic methine, a single OCH_3, and two methyls. Differing from **2**, however, compound **4** contained only one carbonyl signal at δ_C 192.0 and a singlet at δ_C 94.9, suggestive of a hemiacetal functionality. The two remaining shifts were oxygenated aliphatic carbon singlets. Substructure **A** was proposed, where two of the five oxygenated connection points must be the same oxygen atom in order to satisfy the molecular formula and



A

unsaturation number. Among the different permutations, an epoxide functionality at C-2 and C-3 and the five-membered ring connecting the oxygen at C-9 to C-4 explained the shift differences between **4** and **2**. The planar structure of **5** was established by comparison of its similar spectroscopic properties to those of **4**. Final confirmation of structure was provided through HMBC experiments of **5** (see Figure 1). Most notable is the correlation from H-9 to C-4, verifying the oxolane moiety. Interestingly, the upfield δ_C 192.0 shift is corraborated in other conjugated α-keto epoxy moieties, such as that in epoxysorbicillinol.[5a]

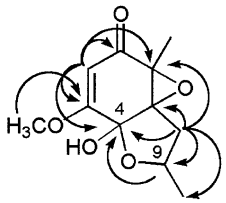


Figure 1. Selected $^1H-^{13}C$ gHMBC NMR correlations for (+)-epoxyserinone B (**5**).

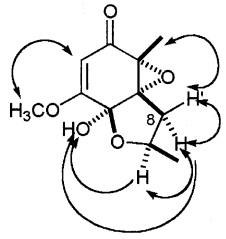


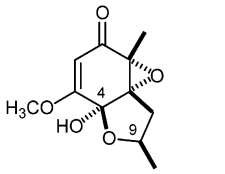
Figure 2. Selected $^1H-^1H$ NOE NMR correlations for (−)-epoxyserinone A (**4**).

With the constitutional structures of **4** and **5** defined, we began to decipher their relative stereochemistry. This first involved collecting data from $^1H-^1H$ NOE experiments in *p*-dioxane-d_8, followed by molecular mechanics calculations in which the E_{min} structures possessed a flattened six-membered ring. In the case of compound **4**, irradiation of H-8 (H-8′ is defined as the more upfield proton) revealed NOEs to H-9 and the hydroxyl proton, while irradiation of H-8′ shows correlations to Me-7 and Me-10 (see Figure 2). Conversly, irradiation of H-9 gave an NOE to H-8 and the hydroxy group. Thus, H-8, H-9, and the hydroxy group are defined as α, while H-8′, Me-7, and Me-10 are *β*. Together with the constraint that the epoxy functionality cannot be *trans*, these data reveal compound **4** as the 2*R**, 3*S**, 4*S**, 9*S** structure of the four possible relative stereoisomers (see Figure 3). The likely biogenetic relationship of **4** to compound **2** suggests that the absolute stereochemistry of the C-9 position is the same as that determined for compound **2**, namely, the *S* configuration.[19]

A similar approach was taken to establish the relative stereochemistry of the diastereomer **5** from the three remaining possible relative stereoisomers (***ii***, ***iii***, and ***iv*** in Figure 3). Again, $^1H-^1H$ NOE experiments were pursued. Selective irradiation of H-8 showed an NOE to Me-7, while H-8′ showed an NOE to Me-10; however, enhancements to or from the hydroxyl proton were not observed for **5**. Coupling constants for protons attached to C-8 and C-9 in the E_{min} structures were calculated for comparison to experimental values, but did not provide new insight (see Figure 3). As with compound **4**, probable biogenetic relationships of **5** and **2** argue for *S* stereochemistry at the C-9 position. Further investigation was not pursued, and the relative stereochemistry at the C-4 and C-9 positions could not be decided.

Careful examination of NMR data for the remaining HPLC fractions revealed two impure constituents, hydroxymethylanserinone B (**6**) and deoxyanserinone B (**7**). Compound **6** was present in a 1 mg fraction as a 1:1 mixture with **1**, while **7** was a 6:1 mixture with **2**. Both ^1H and ^{13}C signals for **6** and **7** could be identified, and data are presented in Table 1. The ^1H NMR of **6** displayed the A_2BX_3 system characteristic of the anserinones. Of the three remaining ^1H NMR shifts, two were characteristic of the anserinone framework, namely, a methoxy group (δ_H 3.74, 3H) and a vinylic methyl (δ_H 2.03, 3H). The downfield singlet of **2** (δ_H 5.90) was absent in the ^1H NMR of **6**, which argued for placement of a hydroxymethyl at C-6. Spectroscopic data from long-range (J = 4 Hz) $^1H-^{13}C$ gHMBC confirmed the structure (see Figure 4).

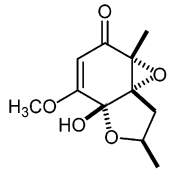
The final compound identified was deoxyanserinone B (**7**). The A_2BX_3 spin system suggested, as above, the presence of another anserinone analogue. Continuing the theme of the anserinone framework, **7** exhibited ^1H and ^{13}C NMR shifts corresponding to a second downfield methyl singlet (δ_C 11.2, δ_H 2.15) and a methoxy group (δ_C 55.4, δ_H 3.76). Conspicuously absent, however, were any ^{13}C NMR shifts corresponding to carbonyl groups. Furthermore, two coupled aromatic doublets (δ_H 6.37 and 6.31, J = 2.5 Hz) appeared instead of the familiar singlet for H-6 in **1**−**5**. Corresponding to the additional downfield proton signal



i

R*S*S*S*

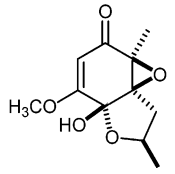
9*,8*	angle	*J*calc
αα	34°	7.0 Hz
αβ	88°	0.9 Hz



ii

R*S*R*S*

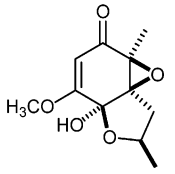
9*,8*	angle	*J*calc
αα	290°	6.2 Hz
αβ	153°	10.9 Hz



iii

S*R*R*S*

9*,8*	angle	*J*calc
αα	40°	4.3 Hz
αβ	163°	11.6 Hz



iv

S*R*S*S*

9*,8*	angle	*J*calc
αα	6°	9.6 Hz
αβ	117°	4.0 Hz

Figure 3. Calculated dihedral angles and coupling values for the E_{min} structures of the four possible *cis*-epoxy relative stereoisomers of the epoxyserinone structure.

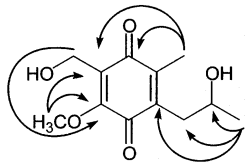


Figure 4. Selected $^{1}H-^{13}C$ gHMBC long-range ($J = 4$ Hz) correlations for hydroxymethylanserinone B (**6**).

was the additional δ_C 108.4 shift. Collectively, these data pointed to an anserinone-like structure containing the familiar $CH_2CH(O-)CH_3$ alkyl, the downfield shifted methyl, the methoxy, two aromatic protons with a meta relationship, and no carbonyls. Twelve possible trisubstituted phenols fitting these criteria were envisioned, and the experimental ^{13}C NMR shifts matched most closely to the predicted ^{13}C NMR shifts of the final structure of **7**; however, 2D NMR experiments were not performed due to loss of the sample.

Pentaketides **1**−**5** obtained from the deep water fungal culture were evaluated for cytotoxicity in two separate cell-based bioassays: a disk diffusion soft agar colony formation assay[22] and a sulforhodamine B (SRB)[24] assay (see Table 2). All compounds except **4** showed significant inhibition of murine cancer cell lines. Selective inhibition of murine leukemia cells over murine solid tumor cells was observed for both **2** and **3**; however, significant differentials between the murine leukemia and normal cell line were not observed for any compounds tested. Against human leukemia and solid tumor cell lines, only compounds **2** and **3** showed significant inhibition, and only **2** exhibited human leukemia selectivity. In accordance with that previously reported,[19] overall cytotoxicity for (+)-anserinone B (**2**) was higher than anserinone A (**1**) in the disk diffusion assay. Compound **3** approximated the zones of inhibition of **2**, while **4** and **5** showed less cytotoxicity. A similar activity trend was observed in the SRB assay, which employed the MDA-MB-435 cell line to evaluate inhibition of proliferation. Dose−response curves were generated and are consistent with the results obtained in the disk diffusion assay. Compounds **1**−**3** caused cytotoxicity with IC_{50} values ≤ 4 μg/mL (see Table 2), while the epoxide compounds **4** and **5** displayed no significant inhibition of growth at concentrations up to 10 μg/mL. Interestingly, **2** was slightly less active than **1** against MDA-MB-435. In an attempt to gain insight into the mode of action, **1**−**3** were also evaluated in a phenotypic cytoskeletal screen; however, changes in microtubules or microfilaments were not observed. Thus, a mode other than cytoskeletal disruption is responsible for cell inhibition.

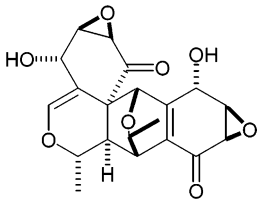
It is surprising that a deep water, marine-derived mixed fungal culture produced the same pentaketides, anserinones A (**1**) and B (**2**), as the phylogenetically distant, terrestrial-derived, *Podospora anserina*. The previously undescribed pentaketides **3**, **4**, **5**, and **6** add to the growing list of polyketides produced by saltwater cultures of marine-derived fungi from our laboratory. Earlier discoveries include epoxysorbicillinol,[5a] and the novel antibiotic seco-curvularin,[25] which is related to the pentaketide curvulin.[26] Recent discoveries of fungal pentaketides appear rare. According to a review of new fungal pigments,[27] the anserinones A (**1**) and B (**2**) were only two of six novel pentaketides reported between 1996 and 1999. Furthermore, comprehensive reviews of marine natural products[28] contain little mention of novel fungal pentaketides. The biological activity of the functionally dense pentaketides **3**, **4**, and **5** is interesting but not compelling for further study in cell-based cytotoxicity screens. The new epoxy-

Table 2. Zone Unit Measurements and Differentials in the Disk Diffusion Soft Agar Colony Formation Assay[a] and the Sulforhodamine B (SRB) Assay[c]

		soft agar disk diffusion assay[a]									
		cytotoxicity					leukemia selectivity				SRB assay[c]
compound	(mg/mL)[b]	Z_{L1210}	Z_{C38}	Z_{CFU-GM}	Z_{CEM}	Z_{H116}	Z_{H125}	$Z_{CEM-H116}$	$Z_{CEM-H125}$	$Z_{L1210-C38}$	$Z_{L1210-CFU-GM}$	MDA-MB-435 IC_{50} (μg/mL)
anserinone A (**1**)	6.0	**300**	**300**	150	n.t.	50	50	n.t.	n.t.	0	150	2.22 ± 0.12
(+)-anserinone B (**2**)	11.6	**700**	**400**	**650**	**650**	**400**	**400**	**250**	**250**	**300**	50	3.60 ± 0.37
(+)-formylanserinone B (**3**)	4.0	**550**	250	**600**	**550**	**400**	**350**	150	200	**300**	−50	2.90 ± 0.10
(−)-epoxyserinone A (**4**)	6.8	50	200	0	n.t.	0	0	n.t.	n.t.	−150	50	> 10
(+)-epoxyserinone B (**5**)	8.0	250	**400**	**400**	n.t.	200	50	n.t.	n.t.	−150	−150	> 10

[a] Measured in zone units: 200 zone units = 6 mm. Murine cell lines: L1210 (lymphocytic leukemia), C38 (colon adenocarcinoma), CFU-GM (normal bone marrow). Human cell lines: H116 (colon tumor), H125 (lung non-small cell carcinoma), CEM (lymphocytic leukemia). Zone unit values ≥ 300 are considered active and are bold. Murine leukemia selectivity is measured by the zone unit differential between L1210 and C38 ($Z_{L1210-C38}$), or L1210 and CFU-GM ($Z_{L1210-CFU-GM}$). Human leukemia selectivity is measured by the zone unit differential between CEM and H116 ($Z_{CEM-H116}$), or CEM and H125 ($Z_{CEM-H125}$). Zone unit differential values ≥ 250 are considered selective and are bold. [b] Concentration of 15 μL aliquot applied to disk. [c] Full dose−response curves in triplicate generated using MDA-MB-435 cells incubated with test compounds for 48 h.

serinones **4** and **5**, however, are reminiscent not only of our previously disclosed epoxysorbicillinol[5a] but also of the recently described epoxyquinol (**8**).[29] The antiangiogenic properties of **8** suggest evaluation of **4** and **5** in such assays is warranted. Finally, we conclude that the unique ecological niche of the deep sea should continue to be explored by marine natural product investigators.



8

Experimental Section

General Experimental Procedures. Optical rotation measurements were averaged from five separate [α] values. The NMR spectra were recorded at 500 and 125.7 MHz for ^{1}H and ^{13}C, respectively. High-resolution mass spectra were obtained using an ESITOF instrument. The LCMS was performed using an analytical HPLC outfitted with photodiode array (PDA), evaporative light scattering (ELS), and mass spectrometry (ESITOFMS) detectors. Analytical and semi-preparatory HPLC were performed with C-18 columns of 5 μm. Molecular modeling used MMX force fields within PC Model v.7.0.

Collection and Taxonomic Identification. The fungi (strain nos. a004181 and b004181) were isolated from a −4380 ft sediment grab at the seventh marine station (GPS coordinates: 18°15.2′ S, 178°38.5′ E) along a transect between Rewa river mouth, Fiji, and Matuka until the 2000 m isobath was reached. Collection occurred on the fourth joint expedition of the National Fisheries University of Japan and the University of the South Pacific, May 12, 1999. The sediment grab was immediately sampled using a sterile syringe, plated in triplicate on seawater KMV media with antibiotics, and incubated at 24 °C. Following transport back to UCSC, strains were subcultured as previously described.[30] Taxonomic analysis of fruiting body formation at University of Texas Health Science Center at San Antonio originally identified this fungus as *Penicillium citrinum*; however, upon closer inspection by Maren Klich of the United States Department of Agriculture, the taxonomic analysis was amended. When plated, the isolate was observed to have two sectors. One was identified as *P. corylophilum*, while the other possessed characteristics between *P. corylophilum* and *P. citrinum* and was thus named *P.* cf. *corylophilum*. Additionally, the fungal culture was submitted for taxonomic identification to Accugenix, a division of Acculabs, INS.[31] Comparative DNA analysis performed against the MicroSeq database using the sequence derived from the D2 expansion segment of the large subunit rRNA gene could identify the fungus only as a *Penicillium* sp. due to a genetic distance (GD) greater than 1% with the nearest species (*P. citrinum*, 1.25% GD; *P. solitum*, 2.49% GD). This fungus is maintained in a cryopreserved state at UCSC.

Disk Diffusion Soft Agar Colony Formation Assay. An in vitro cell-based assay using murine cells L1210 (leukemia), C38 (colon), and CFU-GM (normal), and human cells H116 (colon), H125 (lung), and leukemia (CEM), assessed cytotoxicity of original extracts, solvent partition fractions, and pure compounds. Samples are dissolved in 250 μL of DMSO, and a 15 μL aliquot is applied to a cellulose disk in an agar plate containing cells. After a period of incubation, a zone of cell colony inhibition, where 200 zone units (zu) = 6 mm, is quantified (Z). Activity for a given sample is defined by an antiproliferation zone 300 zu or greater. Selectivity is measured by the zone unit differentials between two cell lines. Solid tumor selectivity for human cells can be defined as $Z_{H116\ (or\ H125)} - Z_{CFU-GM} \geq 250$, or $Z_{H116\ (or\ H125)} - Z_{CEM} \geq 250$. Likewise, for murine cells, solid tumor selectivities are $Z_{C38} - Z_{CFU-GM} \geq 250$, or $Z_{C38} - Z_{L1210} \geq 250$. Leukemia selectivities for human cells are $Z_{CEM} - Z_{CFU-GM} \geq 250$, or $Z_{CEM} - Z_{H116\ (or\ H125)} \geq 250$. Leukemia selectivities for murine cells are $Z_{L1210} - Z_{CFU-GM} \geq 250$, or $Z_{L1210} - Z_{C38} \geq 250$. Cytotoxic compounds are those exhibiting activity but with no significant differential between tumor and normal cells.

Sulforhodamine B (SRB) Assay. Inhibition of MDA-MB-435 cell proliferation and cytotoxicity were measured using the SRB assay. Cells were plated in 96-well plates and allowed to attach and grow for 24 h. The compounds or vehicle (ethanol) was added and incubated with the cells for 48 h. Following drug exposure, the cells were fixed with 10% trichloroacetic acid, then the cell layer was stained with 0.4% SRB. The absorbance of the SRB solution was measured at 560 nm. Dose−response curves were generated, and the IC_{50} values, the concentration of compound required to inhibit cell proliferation by 50%, were calculated from the linear portion of log-dose−response curves.

Culture Conditions. The mixed fungal strains were grown in 1.5% malt extract broth in 20 L of filtered Monterey Bay seawater adjusted to pH 7.3 with shaking at 150 rpm for 28 days at room temperature, approximately 25 °C.

Extraction and Isolation. The culture was vacuum filtered and the broth was extracted three times with equal volumes of ethyl acetate to yield a crude extract (E, 1.2 g) that was partitioned between 10% aqueous MeOH and hexanes. The methanol layer was collected and the polarity adjusted to 50% aqueous MeOH before further partitioning against CH_2Cl_2. All partitions were profiled using LCMS. The CH_2Cl_2-soluble extract (EFD, 689 mg) was dried and chromatographed on a Sephadex-LH20 gravity column with 100% MeOH as the eluent. Again, all fractions were profiled using LCMS. The second fraction (S2, 78.6 mg) was further purified via C-18 column HPLC using a linear gradient of 43:57 MeOH−H_2O with 0.1% formic acid to 100% MeOH over 1 h to yield compounds **1** (2.0 mg), **2** (7.2 mg), **4** (1.6 mg), **5** (1.3 mg), and a 1:1 mixture of **1**:**6** (1.2 mg). The third Sephadex fraction (S3, 223.7 mg) was also purified via reversed-phase HPLC using a 45:55 MeOH−H_2O with 0.1% formic acid to 100% MeOH linear gradient over 1 h to yield compounds **1** (8.0 mg), **2** (26.0 mg), **3** (10.2 mg), **4** (2.6 mg), **5** (2.5 mg), and a 6:1 mixture of **2**:**7** (1.6 mg).

(+)-Formylanserinone B (3): amber crystals; $[\alpha]\ ^{27}_D$ +11.6° (*c* 0.2 MeOH); UV (CH_2Cl_2) λ_{max} (log ϵ) 360 nm (2.67), 270 nm (3.93); ^{1}H and ^{13}C NMR, see Table 1; HRESITOFMS *m*/*z* 261.0750 $[M + Na]^+$ (calcd for $C_{12}H_{14}O_5Na$, 261.0734).

(−)-Epoxyserinone A (4): white powder; $[\alpha]^{27}_D$ −115° (*c* 0.1 MeOH); UV (CH_2Cl_2) λ_{max} (log ϵ) 250 nm (3.90); ^{1}H and ^{13}C NMR, see Table 1; HRESITOFMS *m*/*z* 249.0775 $[M + Na]^+$ (calcd for $C_{11}H_{14}O_5Na$, 249.0733).

(+)-Epoxyserinone B (5): white powder; $[\alpha]\ ^{27}_D$ +245 (*c* 0.2 MeOH); UV (CH_2Cl_2) λ_{max} (log ϵ) 250 nm (3.33); ^{1}H and ^{13}C NMR, see Table 1; HRESITOFMS *m*/*z* 249.0775 $[M + Na]^+$ (calcd for $C_{11}H_{14}O_5Na$, 249.0733).

Hydroxymethylanserinone B (6): yellowish oil (impure, 1:1 mixture with **1**); ^{1}H and ^{13}C NMR, see Table 1; ESITOFMS *m*/*z* 241.1100 $[M + H]^+$ (calcd for $C_{12}H_{17}O_5$, 241.1071).

Deoxyanserinone B (7): yellowish powder (impure, 1:6 mixture with **2**); ^{1}H and ^{13}C NMR, see Table 1; ESITOFMS *m*/*z* 391.2044 $[2M - H]^-$ (calcd for $C_{22}H_{31}O_6$, 391.2126).

Acknowledgment. This work was supported by the National Institutes of Health RO1 CA47135 (P.C. and F.A.V.) and the William Randolph Hearst Foundation (S.L.M.). Additional financial support at UCSC was from equipment grants from NSF BIR-94-19409 (NMR) and a supplement to NIH CA52955 (ESITOFMS). Special thanks to L. Rahbaek of the University of the South Pacific for assistance in sample collection and initial agar plate field innoculations, National Fisheries University of Japan for assistance in sample collection, P. Wenzel for molecular modeling assistance, and finally, D. Sutton from the University of Texas Health Sciences Center

at San Antonio and M. Klich of the U.S. Department of Agriculture for detailed taxonomic analysis of spore formation.

Supporting Information Available: ^{1}H NMR of **3**−**7** and the parent crude fraction to **3**, ^{13}C NMR of **3**−**5** and **7**, an expansion of the ^{1}H−^{13}C gHMBC NMR of **5**, and selected ^{1}H−^{1}H NOE NMR irradiations of **4**. This material is available free of charge via the Internet at http://pubs.acs.org.

References and Notes

(1) Kobayashi, M.; Uehara, H.; Matsunami, K.; Aoki, S.; Kitagawa, I. *Tetrahedron Lett.* **1993**, *34*, 7925−7928.

(2) Cheng, X.-C.; Varoglu, M.; Abrell, L.; Crews, P.; Lobkovsky, E.; Clardy J. *J. Org. Chem.* **1994**, *59*, 6344−6348.

(3) Abrell, L. M.; Borgeson, B.; Crews, P. *Tetrahedron Lett.* **1996**, *37*, 2331−2334.

(4) (a) *isol:* Varoglu, M.; Corbett, T. H.; Valeriote, F. A.; Crews, P. *J. Org. Chem.* **1997**, *62*, 7078−7079. (b) *syn:* Govek, S. P.; Overman, L. E. *J. Am. Chem. Soc.* **2001**, *123*, 9468−9469.

(5) (a) *isol:* Sperry, S.; Samuels, G. J.; Crews, P. *J. Org. Chem.* **1998**, *63*, 10011−10014. (b) *syn:* Wood, J. L.; Thompson, B. D.; Yusuff, N.; Pflum, D. A.; Matthäus, M. S. P. *J. Am. Chem. Soc.* **2001**, *123*, 2097−2098.

(6) (a) *isol:* Amagata, T.; Doi, M.; Ohta, T.; Minoura, K.; Numata, A. *J. Chem. Soc., Perkin Trans. 1* **1998**, *21*, 3585−3599. (b) *syn:* Gurjar, M. K.; Bhaket, P. *Heterocycles* **2000**, *53*, 143−149.

(7) Lang, G.; Steffens, S.; Schaumann, K.; Bringmann, G. *3rd Euro Conference on Marine Natural Products*, 2002, Poster Abstracts, Abstract P67, p 67.

(8) Ebel, R.; Lin, W. H.; Edrada, R. A.; Proksch, P. *3rd Euro Conference on Marine Natural Products*, 2002, Lecture Abstracts, p 47.

(9) Smith, C. J.; Abbanat, D.; Bernan, V. S.; Maiese, W. M.; Greenstein, M.; Jompa, J.; Tahir, A.; Ireland, C. M. *J. Nat. Prod.* **2000**, *63*, 142−145.

(10) Amagata, T.; Rath, C.; Rigot, J.; Tarlov, N.; Tenney, K.; Valeriote, F. A.; Crews, P. *J. Med. Chem.* **2003**, *46*, 4342−4350.

(11) There is not a general agreement on the use of the term "deep water"; however, we prefer the adoption of −300 ft below sea level or deeper based on the limitations of nondecompression, nonrebreather, scientific diving procedures, and to be consistent with current literature on marine natural products from deep water organisms.

(12) The sparse literature of deep water fungi includes: (a) Raghukumar, C.; Raghukumar, S.; Sharma, S.; Chandramohan, D. *Oceanograph. Ind. Ocean* **1992**, *1*, 3−9. (b) Lorenz, R.; Molitoris, H. P. *Mycol. Res.* **1997**, *101*, 1355−1365. (c) Nagahama, T.; Hamamoto, M.; Nakase, T.; Takami, H.; Horikoshi, K. *Anton. Leeuw. Int. J. G.* **2001**, *80*, 101−110. (d) Chandramohan, D. *J. Mar. Biotechnol.* **1997**, *5*, 73−81. (e) Nagahama, T.; Hanamoto, M.; Nakase, T.; Horikoshi, K. *Anton. Leeuw. Int. J. G.* **2001**, *80*, 317−323. (f) Raghukumar, C.; Raghukumar, S. *Aquat. Microb. Ecol.* **1998**, *15*, 153−163. (g) Abe, F.; Horikoshi, K. *Extremophiles* **1998**, *2*, 223−228. (h) Gonda, K. E.; Jendrossek, D.; Molitoris, H. P. *Hydrobiologia* **2000**, *426*, 173−183.

(13) Kohlmeyer, J.; Kohlmeyer, E. *Marine Mycology The Higher Fungi*; Academic Press: New York, 1979; pp 41−44.

(14) Johnson, T.; Sparrow, F. *Fungi in Oceans and Estuaries*; Verlag von J. Cramer: New York, 1970; pp 9−12.

(15) (a) El Sayed, K. A.; Yousaf, M.; Hamann, M. T.; Avery, M. A.; Kelly, M.; Wipf, P. *J. Nat. Prod.* **2002**, *65*, 1547−1553. (b) Gunasekera, S. P.; McCarthy, P. J.; Longley, R. E.; Pomponi, S. A.; Wright, A. E. *J. Nat. Prod.* **1999**, *62*, 1208−1211. (c) Wirsen, C. O.; Molyneaux, S. J. *Appl. Environ. Microbiol.* **1999**, *65*, 5314−5321. (d) Gunasekera, S. P.; McCarthy, P. J.; Longley, R. E.; Pomponi, S. A.; Wright, A. E.; Lobkovsky, E.; Clardy, J. *J. Nat. Prod.* **1999**, *62*, 173−175. (e) Gunawardana, G. P.; Kohmoto, S.; Burrens, N. S. *Tetrahedron Lett.* **1989**, *30*, 4359−4362.

(16) Gustafson, K.; Roman, M.; Fenical, W. *J. Am. Chem. Soc.* **1989**, *111*, 7519−7524.

(17) Feling, R. H.; Buchanan, G. O.; Mincer, T. J.; Kauffman, C. A.; Jensen, P. R.; Fenical, W. *Angew. Chem., Int. Ed.* **2003**, *42*, 355−357.

(18) An extensive literature search revealed two natural product reports of *non*-saltwater fungal cultures of *Aspergillus fumigatus* from a sample of deep water sediment (−2280 ft): (a) Cui, C.-B.; Kakeya, H.; Osada, H. *J. Antibiot.* **1996**, *49*, 527−540. (b) Cui, C.-B.; Kakeya, H.; Osada, H. *Tetrahedron* **1996**, *52*, 12651−12666.

(19) Wang, H.; Gloer, K. B.; Gloer, J. B. *J. Nat. Prod.* **1997**, *60*, 629−631.

(20) For similar strategies, see: (a) Edrada, R. A.; Heubes, M.; Brauers, G.; Wray, V.; Berg, A.; Gräfe, U.; Wohlfarth, M.; Mühlbacher, J.; Schaumann, K.; Sudarsono; Bringmann, G.; Proksh, P. *J. Nat. Prod.* **2002**, *65*, 1598−1604. (b) Osterage, C.; Schwibbe, M.; König, G. M.; Wright, A. D. *Phytochem. Anal.* **2000**, *11*, 288−294. (c) Höller, U.; Wright, A. D.; Matthée, G. F.; König, G. M.; Draeger, S.; Aust, H.-J.; Shulz, B. *Mycol. Res.* **2000**, *104*, 1354−1365.

(21) Stessman, C. C.; Ebel, R.; Corvino, A. J.; Crews, P. *J. Nat. Prod.* **2002**, *65*, 1183−1186.

(22) (a) Sperry, S.; Valeriote, F. A.; Corbett, T. H.; Crews, P. *J. Nat. Prod.* **1998**, *61*, 241−247. (b) Valeriote, F.; Grieshaber, C. K.; Media, J.; Pietraszkewicz, H.; Hoffmann, J.; Pan, M.; McLaughlin, S. *J. Exp. Ther. Oncol.* **2002**, *2*, 228−236.

(23) Braun, S.; Kalinowski, H.-O.; Berger, S. *150 and More Basic NMR Experiments*, 2nd ed.; Wiley-VCH: Weinheim, Germany, 1998; pp 489−492, and references therein.

(24) Skehan, P.; Storeng, R.; Scudiero, D.; Monks, A.; McMahon, J.; Vistica, D.; Warren, J. T.; Bokesch, H.; Kenney, S.; Boyd, M. R. *J. Natl. Cancer Inst.* **1990**, *82*, 1107−1112.

(25) Abrell, L. M.; Borgeson, B.; Crews, P. *Tetrahedron Lett.* **1996**, *37*, 8983−8994.

(26) Turner, W.; Aldridge, D. *Fungal Metabolites II*; Academic Press: New York, 1983; p 96.

(27) Gill, M. *Nat. Prod. Rep.* **1999**, *16*, 301−317.

(28) (a) Faulkner, D. J. *Nat. Prod. Rep.* **1999**, *16*, 155−198. (b) Faulkner, D. J. *Nat. Prod. Rep.* **2001**, *18*, 1−49. (c) Faulkner, D. J. *Nat. Prod. Rep.* **2002**, *19*, 1−48.

(29) Kakeya, H.; Onose, R.; Koshino, H.; Yoshida, A.; Kobayashi, K.; Kageyama, S.-I.; Osada, H. *J. Am. Chem. Soc.* **2002**, *124*, 3496−3497.

(30) Varoglu, M.; Crews, P. *J. Nat. Prod.* **2000**, *63*, 41−43.

(31) Microseq Microbial Identification System by Applied Biosystems (www.appliedbiosystems.com) and Accugenix (www.accugenix.com).

NP030388M