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Brocaenols A−C: Novel Polyketides from a Marine-Derived *Penicillium brocae*

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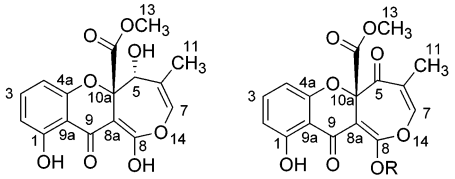
Abstract: Chemical investigation of a *Penicillium brocae*, obtained from a tissue sample of a Fijian *Zyzyya* sp. sponge, yielded two known diketopiperazines and three novel cytotoxic polyketides, brocaenols A−C. The brocaenols contain an unusual enolized oxepine lactone ring system that to the best of our knowledge is unprecedented in the literature. The structures were elucidated by using 2D-NMR methods including an INADEQUATE experiment. The absolute stereochemistry of brocaenol A was established by using a modified Mosher method. The taxonomy of the producing fungus was elucidated by using both morphological and rDNA sequence analysis.

Marine microorganisms have proven to be rich sources of secondary metabolites that have both unique structures[1] and potential as pharmaceutical leads.[2] In particular, fungi obtained from marine invertebrates have yielded novel metabolites with potent antibacterial and anticancer activities.[3] This potential has led us to investigate cultured filamentous marine-derived fungi for antibacterial activity and cytotoxic activity against HCT-116 human colon carcinoma cell lines.

One isolate (F97S76) was obtained from a Fijian *Zyzzya* sp. sponge and was investigated due to its remarkable activity against both methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant *Enterococcus faecalis*. Although the antibiotic activity was traced to the epicorazine family of diketopiperazines,[4] the extract yielded three novel polyketides, brocaenols A−C (**1**−**3**), that showed moderate activity against an HCT-116 human colon carcinoma cell line. The structure elucidation including absolute stereochemistry, the biological activity, and the taxonomy of the producing organism are presented here.



1 **2** R = H **3** R = CH_3

Brocaenol A (**1**) was isolated as a pale yellow amorphous solid with a molecular formula of $C_{16}H_{15}O_8$ for the $[M + H]^+$, established by HRFABMS. The ^1H NMR spectrum showed eight signals: two methyls, one oxygenated methine, one olefinic methine, three aromatic methines, and one hydrogen-bonded phenol. Brocaenol A (**1**) formed a diacetate upon treatment with pyridine and acetic anhydride, and the ^1H NMR spectrum showed that the diacetate still contained one exchangeable proton (δ 4.47). The trisubstituted benzene ring was established by using ^1H, ^{13}C, COSY, and HMBC NMR data. The ^{13}C shifts of the aromatic carbons were consistent with a 1,3 oxygen substitution and an aryl ketone as shown in substructure A, Figure 1. Additionally, the downfield shift of the phenolic proton (δ 11.93) indicated that the phenol was hydrogen bonded, further supporting the aryl ketone.

Another substructure, assigned by using COSY and HMBC data, contained an allylic alcohol and is shown as substructure B in Figure 1. COSY correlations showed that the oxygenated methine (H-5) was coupled to both the H-11 methyl and the H-7 olefinic proton. A COSY correlation between the H-11 methyl and the H-7 olefinic proton further supported the spin system assignment. An HMBC correlation between H-5 and the C-12 carbonyl indicated the position of the ester carbonyl.

Although the two substructures were elucidated, connecting the two proved difficult. In an attempt to fully elucidate the carbon backbone of brocaenol A (**1**), a 2-D INADEQUATE NMR experiment was performed by using a standard pulse sequence. The data were analyzed with NMR Analyst software.[5] With the exception of two carbons (C-13 and C-8), the entire backbone (Figure 2) was assembled from the INADEQUATE spectrum. Since C-13 is a methoxyl, no correlations were expected, but a correlation was expected for C-8. However, the intensity of this response would be reduced, since neither of the two carbons are protonated and little or no NOE would be expected. In addition the C-8/C-8a coupling constant would be expected to be unusually high since $^1J_{C-C}$ values increase with both increasing s-character and increasing electronegativity of bonded atoms. An approximate increase of 10 Hz for each oxygen would be expected and in this case would result in a coupling constant of 80 Hz or higher.[6] Since the INADEQUATE experiment was optimized for a $^1J_{C-C}$ value of 55 Hz, the standard coupling constant observed in benzene, it is not surprising that the C-8/C-8a correlation was not observed. Lack

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(1) Faulkner, D. J. *Nat. Prod. Rep.* **2002**, *19*, 1−48.
(2) Fenical, W. *Trends Biotechnol.* **1997**, *15*, 339−341.
(3) Jensen, P. R.; Fenical, W. In *Drugs From the Sea*; Fusetani, N., Eds.; Karger: Basel, 2000; p 6−29.
(4) Deffieux, G.; Filleau, M. J.; Baute, R. *J. Antibiot.* **1978**, *31*, 1106−1109. Deffieux, G.; Baute, M. A.; Baute, R.; Filleau, M. J. *J. Antibiot.* **1978**, *31*, 1102−1105.
(5) NMR Analyst; ScienceSoft, LLC: Salt Lake City, UT 84102.
(6) Wehrli, F. W.; Marchand, A. P.; Wehrli, S. *Interpretation of carbon-13 NMR spectra*; John Wiley & Sons Ltd.: New York, 1988.

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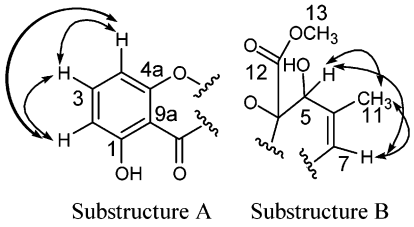

FIGURE 1. Partial structures for compound **1** showing COSY correlations.

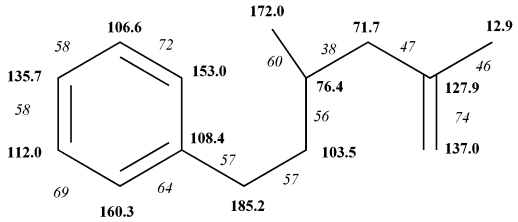

FIGURE 2. Carbon skeleton from INADEQUATE correlations with ^{13}C assignments and coupling constants in italics.

of attached protons also means that C-8 and C-8a have long T1 relaxation times. Measured values were 11.4 and 10.6 s, which could also hinder the signal intensity. The position of C-8, the position of the oxygens, and the formation of the tricyclic ring system were elucidated by careful analysis of the ^{13}C and HMBC data.

The ether linkage between C-4a and C-10a was supported by the ^{13}C chemical shifts of both carbons. Formation of the oxepine ring to complete the structure for brocaenol A (**1**) was supported by the ^{13}C chemical shifts of C-9, C-8a, C-8, and the C-11 methyl. First, the relatively high-field shift of the aryl ketone (δ 185.2) required an adjacent olefin.[7] Second, the shifts of C-8a (δ 103.5) and C-8 (δ 162.9) were consistent with a β-oxygenated enone system.[8] Last, the high-field chemical shift of the C-11 (δ 12.9) methyl supported C-7 oxygenation. These data indicated a seven-membered enol ether ring as shown for compound **1**. HMBC correlations from both C-8 and C-8a to H-7 further supported the ether bridge.

The absolute stereochemistry of brocaenol A (**1**) was determined by using a modified Mosher method.[9] Brocaenol A (**1**) was esterified with *R*- and *S*-methoxy-(trifluoromethyl)phenylacetyl (MTPA) chloride in pyridine to form the corresponding *S*- and *R*-MTPA esters, respectively. The $\Delta\delta^{RS}$ values were measured for H-7, H-11, and H-13 yielding +0.05, +0.17, and −0.17, respectively. On the basis of the model shown[9] in Figure 3, the configuration at C-5 was assigned as *R*.

The stereochemistry at position C-10a was confirmed by using a gHSQMBC[10] and CD. By measuring the $^3J_{C-H}$ between H-5 and C-12, the stereochemsitry at position C-10a could be determined. The measured value (5.2 Hz)

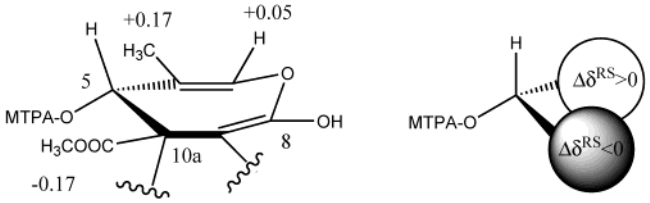

FIGURE 3. Model used to assign the configuration at C-5 with $\Delta\delta^{RS}$ values in ppm.

was compared to the coupling constants calculated for each of the two possible diastereomers. Coupling constants were calculated by using a modified Karplus equation[11] in which the dihedral angles were determined by molecular modeling. With use of CS Chem 3D Pro, conformations of each diastereomer were analyzed after energy minimization, using MM2 followed by semiempirical methods (AM1). When OH-5 and C-12 were syn, the dihedral angle was −108.2° and gave a coupling constant of 1.7 Hz. When OH-5 and C-12 were anti, the dihedral angle was −12.1° and gave a coupling constant of 6.5 Hz. Therefore, the relationship between OH-5 and C-12 was determined to be anti resulting in an *S*-configuration at C-10a. These results were also consistent with the CD. Brocaenol A (**1**) showed a negative Cotton effect at 304 nm, indicating *S*-configuration at C-10a based on CD spectra for secalonic acids[12] and nidulalin A,[13] both of which show a negative Cotton effect for an *S*-configuration.

Brocaenol B (**2**) was isolated as a white crystalline solid. A molecular formula of $C_{17}H_{14}O_8$ was established by HRCIMS. On the basis of the molecular formula and the ^1H NMR spectrum, brocaenol B (**2**) differed from **1** by an additional *O*-methyl and a loss of two protons. The ^{13}C and HMBC spectra indicated that brocaenol B (**2**) contained a ketone at position 5. Specifically, the downfield shift of C-7 as compared to **1** and correlations from H-7 and H-11 to a carbon at δ 197.2 (C-5) supported the presence of a ketone at position 5. An HMBC correlation between the methoxyl (C-15) at δ 4.06 and the carbon at δ 164.9 supported the methyl ether and its connectivity to the oxepine ring. Additional support for methylation of the enol came from the subsequent upfield shift of C-8a. On the basis of phenols[8] and NMR chemical shift prediction,[14] *O*-alkylation at position 8 would have a downfield effect on C-8 and an upfield effect on C-8a. The *S*-configuration at C-10a in **2** was determined by comparison of the CD spectrum to that of brocaenol A (**1**).

Brocaenol C (**3**) was isolated as an amorphous pale solid. HRCIMS gave a molecular formula of $C_{16}H_{12}O_8$. From the ^1H NMR data, it was clear that brocaenol C (**3**) was closely related to brocaenol B (**2**) as the chemical shifts were nearly identical (Table 2). On the basis of the molecular formula, brocaenol C (**3**) differed from brocaenol B (**2**) by one methyl and differed from brocaenol A

(7) Breitmaier, E.; Voelter, W. *Carbon-13 NMR Spectroscopy*; VCH: New York, 1990; p 144.
(8) Pretsch, E.; Buhlmann, P.; Affolter, C. *Structure determination of organic compounds: Tables of spectral data*, 3rd ed.; Springer: Berlin, Heidelburg, Germany, 2000.
(9) Seco, J. M.; Quinoa, E.; Riguera, R. *Tetrahedron: Asymmetry* **2001**, *12*, 2915−2925.
(10) Williamson, R. T.; Marquez, B. L.; Gerwick, W. H.; Kover, K. E. *Magn. Reson. Chem.* **2000**, *38*, 265−273.
(11) Breitmaier, E.; Voelter, W. *Carbon-13 NMR Spectroscopy*; VCH: New York, 1990; p 143.
(12) Anderson, R.; Buchi, G.; Kobbe, B.; Demain, A. L. *J. Org. Chem.* **1977**, *42*, 352−353. Cole, R. J.; Cox, R. H. In *Handbook of toxic fungal metabolites*; Academic Press: New York, 1981; pp 646−669.
(13) Kawahara, N.; Sekita, S.; Satake, M.; Udagawa, S.; Kawai, K. *Chem. Pharm. Bull.* **1994**, *42*, 1720−1723.
(14) GmbH, U. S., 1.1 ed.; Scientific Software Engineering: Hergiswil, 1999−2000.

TABLE 1. NMR Data for Brocaenol A (1) in $CDCl_3$

position	^{13}C	^{1}H	COSY	HMBC[a]
1	160.3			
2	112.0	6.76 (dd, 8.1, 0.8)	3, 4	1, 4, 9a
3	135.7	7.49 (t, 8.1)	2, 4	1, 4a
4	106.5	6.82 (dd, 8.1, 0.8)	2, 3	2, 4a, 9, 9a
4a	153.0			
5	71.7	4.94 (dd, 1.9, 0.7)	7, 11	8a, 6, 7, 10a, 11, 12
6	127.9			
7	137.0	6.53 (dq, 1.9)	5, 11	5, 6, 8, 11
8	162.9			
8a	103.5			
9	185.2			
9a	108.4			
10a	76.4			
11	12.9	1.71 (dd, 1.9, 0.7)	5, 7	4, 5, 6, 7, 8
12	172.0			
13	53.4	3.78 (s)		12
1-OH		11.93 (s)		1, 2, 9a

[a] Numbers refer to the carbon.

TABLE 2. NMR Data for Compounds 2 and 3

	compd **2**[a]			compd **3**[b]		
	δ_C	δ_H	HMBC[c]	δ_C	δ_H	HMBC
1	160.8			159.9		
2	113.1	6.82 (d, 8.6)	1, 4, 9a	109.6	6.51 (d, 8.4)	4
3	134.8	7.46 (t, 8.6)		132.4	7.27 (t, 8.4)	1, 4a
4	105.9	6.81 (d, 8.6)	2, 4a, 9a	106.1	6.59 (d, 8.4)	2, 4a
4a	152.5			154.5		
5	197.2			200.6		
6	113.8			113.8		
7	172.8	8.10 (q, 1.2)	5, 6, 10a	174.1	8.25 (bd, 1.3)	5, 6, 10a
8	164.9			165.4		
8a	96.8			110.3		
9	181.9			186.1[d]		
9a	108.5			107.2		
10a	85.1			89.5		
11	5.8	1.78 (d, 1.2)	5, 6, 7	4.8	1.73 (d, 1.3)	5, 6, 7
12	165.0			167.9		
13	53.8	3.81 (s)	12	52.8	3.72 (s)	12
15	57.0	4.06 (s)	8			
OH		12.19 (s)	1, 2, 9a			

[a] Collected in $CDCl_3$. [b] Collected in MeOH-d_6. [c] Numbers refer to the carbon. [d] Shift is from the ^{13}C spectrum collected in DMSO-d_6.

(**1**) by two protons. On the basis of these data, brocaenol C (**3**) was proposed to be the oxidized analogue of **1**. Assigning brocaenol C (**3**) proved problematic because the aryl ketone signal (C-9) was not observed in CD_3OD. Interestingly, this signal was observed at δ 186.1 in DMSO; however, now the C-5 carbonyl was missing. Nonetheless, an HMBC correlation was observed from the C-5 carbonyl to H-11 at δ_C 198.6. The difficulty in observing all 16 carbon signals could be due to either tautomerization or a combination of long T1 relaxation times and insufficient material.

The C-10a stereochemistry for compound **3** was determined to be *S* on the basis of the optical rotation. All three brocaenols (**1**−**3**) showed a positive rotation. The CD spectrum of brocaenol C (**3**) was not interpreted due to its complexity. The spectrum showed continuous oscillation with the highest intensity between 275 and 350 nm and is most likely due to tautomerization or rapid conformation conversion. Conversion of compound **1** to compound **3** was attempted to help confirm the structure of brocaenol C (**3**), but attempts with PCC and TPAP/NMO failed.

All three compounds were tested in an HCT-116 cell line with use of the MTT assay and showed moderate cytotoxicity. The IC_{50} values for **1**, **2**, and **3** were 20, 50, and >50 μg/mL, respectively.

Taxonomic characterization of the fungus was based on both morphology and sequence analysis of the ITS regions. Comparison with the personal collection of Stephen Peterson (National Center for Agricultural Utilization Research, US Department of Agriculture) revealed that the ITS region of F97S76 was identical with a newly described species *Penicillium brocae* (GenBank accession AF484396).[15] Additional details for the taxonomic characterization can be found in the Supporting Information.

This is the first reported chemical investigation of the recently described *Penicillium brocae*. Biosynthetically the brocaenols resemble terrestrial fungal metabolites such as the nidulalins[13] and the secalonic acids.[12] However, enolized oxepine lactones appear to be uncommon in natural products.

Experimental Section

General Experimental Procedures. Optical rotations were measured in a 5-cm cell in MeOH. All other general experimental details are the same as those reported in Bugni et al.[16]

Biological Material. Strain F97S76 was obtained from a tissue homogenate of a *Zyzzya* sp. sponge collected in Fiji. Strain F97S76 has been deposited in the Wyeth-Ayerst Culture Collection in Pearl River, NY.

Fermentation, Extraction, and Isolation. Culture F97S76 grown on Bennett's agar (10 g/L of dextrose, 1 g/L of beef extract [Difco], 1 g/L of yeast extract [Difco], 2 g/L of *N*-Z-Amine A [Quest International], 15 g/L of agar) was inoculated into 10 mL of potato dextrose broth (PDB, Difco), pH 7.0, in a 25 × 150 mm test tube and incubated at 22 °C, 160 rpm for 7 days. A second stage seed was prepared by transferring the 10 mL culture to a 250 mL Erlenmeyer flask containing 50 mL of PDB, which was incubated at 22 °C, 200 rpm for 4 days. Production fermentation was performed in a 2.8 L Fernbach flask containing 1 L of Czapek-Dox medium (Difco). Each flask was inoculated with 50 mL of second stage seed and incubated at 22 °C, 200 rpm for 7 days.

One liter of ethyl acetate was added followed by shaking at 200 rpm for 1 h. The aqueous and ethyl acetate phases were separated and the ethyl acetate removed by rotary evaporation.

The ethyl acetate extract (3.0 g) was dissolved in 110 mL of 10% H_2O/MeOH and extracted two times with 100 mL of hexanes. The hexane layers were combined, and the solvent was removed by rotary evaporation to yield 29.7 mg of hexanes soluble material. Water (42 mL) was then added to the MeOH portion and subsequently extracted four times with 100 mL of $CHCl_3$. The $CHCl_3$ layers were combined and the solvent removed by rotary evaporation to yield 526.7 mg of $CHCl_3$-soluble material.

The $CHCl_3$-soluble material was chromatographed in portions on Sephadex LH-20 (38 cm × 2 cm, 1:1 $CHCl_3$/MeOH) and monitored by TLC (SiO_2, 10:1 $CHCl_3$/MeOH). Fractions 8 through 12 (eluting between 98 and 122 mL) contained a mixture of compounds **1** through **3**. These fractions were combined and chromatographed with use of centrifugal countercurrent chromatography (7:20:34:20 Hex/$CHCl_3$/MeOH/H_2O, normal phase,

(15) Peterson, S. W.; Perez, J.; Vega, F. E.; Infante, F. *Mycologia* **2002**, in press.

(16) Bugni, T. S.; Abbanat, D.; Bernan, V. S.; Maiese, W. M.; Greenstein, M.; VanWagoner, R. M.; Ireland, C. M. *J. Org. Chem.* **2000**, *65*, 7195−7200.

800 rpm, flow 2 mL/min). Final purification of compounds **1** through **3** was performed by using normal phase HPLC (Rainin SiO_2 250 mm × 10 mm) with 5% IPA/hexanes and a 40 min linear gradient to 80% IPA. The solvents were removed under reduced pressure to yield 31.0 mg of brocaenol A (**1**), 13.1 mg of brocaenol B (**2**), and 3.4 mg of brocaenol C (**3**).

Brocaenol A (1): $[\alpha]^{25}_D$ +34 (*c* 0.008, MeOH); UV (MeOH) λ_{max} (log ϵ) 224 (4.49), 238 (4.52), 322 (3.94); CD_{MeOH} (*c* 3.14 × 10^{-4} M) $[\theta]_{267}$ +9888, $[\theta]_{304}$ −1815; IR ν 3481, 2954, 1741, 1648, 1606 cm^{-1}; ^1H, ^{13}C, COSY, and HMBC NMR data are listed in Table 1; HRFABMS *m*/*z* 335.0751 ($[M + H]^+$ calcd for $C_{16}H_{15}O_8$, 335.0767).

Brocaenol B (2): mp 186−190 °C; $[\alpha]^{25}_D$ +142 (*c* 0.009, MeOH); UV (MeOH) λ_{max} (log ϵ) 236 (3.73), 270 (3.36), 316 (3.15); CD_{MeOH} (*c* 2.89 × 10^{-3} M) $[\theta]_{275}$ +7665, $[\theta]_{314}$ −1386; IR ν 3416, 2928, 1738, 1698, 1646, 1604 cm^{-1}; ^1H, ^{13}C, and HMBC NMR data are listed in Table 2; HRCIMS (CH_4) *m*/*z* 346.0707 (M^+ calcd for $C_{17}H_{14}O_8$, 346.0688).

Brocaenol C (3): $[\alpha]^{25}_D$ +83 (*c* 0.0025, MeOH); UV (MeOH) λ_{max} (log ϵ) 224 (4.07), 244 (s) (3.80), 292 (3.82), 308 (s) 3.78; IR ν 3747, 2821, 1750, 1704, 1649, 1625, 1604 cm^{-1}; ^1H, ^{13}C, and HMBC NMR data are listed in Table 2; HRCIMS (isobutane) *m*/*z* 333.0607 ($[M + H]^+$ calcd for $C_{16}H_{13}O_8$, 333.0610).

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Supporting Information Available: Experimental details for the acetylation and MTPA esterifications of compound **1**, as well as ^1H and ^{13}C NMR spectra for brocaenols A−C (**1**−**3**). This material is available free of charge via the Internet at http://pubs.acs.org.

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