Antineoplastic Agents. 485. Isolation and Structure of Cribrostatin 6, a Dark Blue Cancer Cell Growth Inhibitor from the Marine Sponge *Cribrochalina* sp.[†,1a]

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Cribrostatin 6, a dark blue cancer cell growth inhibiting (P388 ED_{50} 0.3 μg/mL) constituent of the Republic of Maldives marine sponge *Cribrochalina* sp., has been assigned structure **3** on the basis of a combination of HRMS, high-field (500 MHz, HMBC, and GOESY experiments) ^{15}N, ^{1}H, and ^{13}C NMR, and X-ray crystal structure analyses. Cribrostatin 6 also was found to inhibit the growth of a number of pathogenic bacteria and fungi.

Marine porifera have continued to be an increasingly important source of new nitrogen heterocyclic compounds with significant biological activities. Recent examples include the cytotoxic constituents pateomine (*Mycale* sp.),[1b] a pyridine betaine (*Microcosmus vulgaris*),[2] topsentin B2 (*Rhaphisia lacazei*),[3] asmarine A (*Raspailia* sp.),[4] cyclic guanidines (*Monanchora* sp.),[5] the antiviral dragmacidin F (*Halicortex* sp.),[6] and our isolation and structure determination of cribrostatins 4 (**1**) and 5 (**2**) from the Republic of Maldives blue-colored sponge *Cribrochalina* sp.[7]

Earlier we had observed a number of biologically active blue- to black-colored fractions arising during P388 lymphocytic leukemia guided separations of a 195 g dichloromethane-soluble portion of the extract obtained from 350 kg (wet wt) of *Cribrochalina* sp. The cancer cell growth (P388) inhibitory dark-colored fractions were finally separated by a successive series of gel permeation and partition chromatographic techniques on Sephadex LH-20. That sequence was followed by high-speed countercurrent distribution using an Ito Coil-Planet centrifuge to afford 88 mg of a dark blue constituent (P388 ED_{50} 0.3 μg/mL), designated as cribrostatin 6 (**3**). Owing to difficulties in unequivocally deducing the structure of this interesting substance on the basis of spectral evidence, attempts were made at various times over a 10 year period to reach a correct solution and/or to produce crystals suitable for X-ray structure determination. We were eventually pleased to find that cribrostatin 6 (**3**) would crystallize from acetone following long cold storage of the solution. To follow is a summary of the spectral and X-ray crystallographic interpretation that completed a correct structural assignment for cribrostatin 6 (**3**) (see Table 1 and Figure 1).

The molecular formula of cribrostatin 6 (**3**) was established as $C_{15}H_{14}N_2O_3$ by HRMS, using an APCI inlet system. Inspection of the ^{1}H and APT NMR spectra indicated the presence of three methyls, one methylene, three methines, and eight quaternary carbons. Protonated carbons were assigned using a HMQC experiment. The APT spectrum indicated that four of the carbons were oxygenated and suggested the presence of the quinone. An HMBC experiment allowed placement of the C-9 ethoxy (H-13 to C-9) and C-8 methyl (H-12 to C-7, -8, and -9) groups and established the positions of the quaternary carbons at C-8 and -9 as well as the carbonyl carbons at C-7 and -10, which were assigned by analogy with known isoquinolinequinones such as the saframycins.[8] This accounted for five of the 10 degrees of unsaturation determined from the molecular formula. The nature of the B ring was established by $^{1}H-^{1}H$ COSY, which indicated the presence of a double bond. The HMBC spectrum showed connectivities from the proton at H-6 to C-6a, -7, and -10a as well as establishing the position of the double bond at $\Delta^{5,6}$, which was confirmed by HMBC correlations from H-5 to C-6 and -6a. The remaining three degrees of unsaturation and fragment $C_3H_4N_2$ suggested an imidazo partial structure for a C ring. The overall structure was determined by X-ray diffraction on a small needle-shaped crystal. Although the overall connectivity could be readily established, the low observed data-to-parameter ratio did not permit a clear distinction between structures **3** and **4**. Analogy to previous cribrostatin-related compounds[7,9] (cf. **1**, **2**) gave the location of one of the N atoms at position 4 with reasonable certainty. However, conclusive structural assignment as 9-ethoxy-3,8-dimethylimidazo[5,1-*a*]isoquinoline-7,10-dione (**3**) required further, more detailed analysis of earlier and new NMR data.

Examination of the HMBC spectrum showed correlations from H-5 to C-3 and C10b and implied placement of a nitrogen at position 4. A strong correlation from the remaining methine proton to C-3 suggested position 1, with the remaining nitrogen at position 2. An additional correlation from H-11 to C-3 located the remaining methyl group (δ 2.75p) at C-3. A DPFGSENOE (GOESY) experiment demonstrated NOE enhancement[10] between H-5 and H-11 that would be consistent with either structure **3** or **4**, but gave no indication of an enhancement between H-2 and H-11, which would be expected to exist in structure **4**. Measurement of $^{15}N-^{1}H$ HMBC showed two strong three-bond correlations from the methyl protons H-11 to both nitrogens N-2 and N-4. HMBC correlations were observed from H-5 and H-6 to N-4, but not N-2. H-1 showed weak

[†] Dedicated to the memory of Professor Thomas A. Connors, an extraordinarily effective advocate of new anticancer drug discovery and development, who passed away February 3, 2002 (b. 1934).

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Table 1. High-Field (500 MHz) NMR Assignments for Cribrostatin 6 (**3**) (3,8-dimethyl-9-ethoxyimidazo[5,1-*a*]isoquinoline-7,10-dione) in $CDCl_3$

position	^1H δ (#H, mult, *J*)	^{13}C and ^{15}N	HMBC[a]	COSY	GOESY[b]
1	8.29 (1H, s)	125.68	C-3		
2					
3		137.64			
4					
5	7.90 (1H, d, 7.5 Hz)	124.73	C-3, C-6, C-6a	H-6	H-6, H-11
6	7.26 (1H, d, 7.5 Hz)	107.73	C-6a, C-7, C-10a	H-5	
6a		125.00			
7		184.86			
8		130.06			
9		156.16			
10		180.58			
10a		123.49			
10b		123.87			
11	2.75 (3H, s)	12.57	C-3		H-5
12	2.06 (3H, s)	9.15	C-7, C-8, C-9		
13	4.40 (2H, q, 6.9 Hz)	69.64	C-9, C-14	H-14	
14	1.41 (3H, t, 6.8 Hz)	15.97	C-13	H-13	
N-4		189.5			
N-2		273.9			

[a] For HMBC, $J^1_{CH} = 140$ Hz and $J^n_{CH} = 8$ Hz. [b] The mixing time for the GOESY was 300 ms.

Table 2. Cribrostatin 6 (**3**) Inhibitory Activity (GI_{50}, μg/mL) against a Panel of Human Cancer Cell Lines

cell type	cell line	cribrostatin 6
pancreas-adenocarcinoma	BXPC-3	>1
breast-adenocarcinoma	MCF-7	0.21
CNS glioblastoma	SF-268	0.24
lung-NSC	NCI−H460	>1
colon-adenocarcinoma	KM20L2	>1
prostate	DU-145	0.38
mouse leukemia	P388	0.29

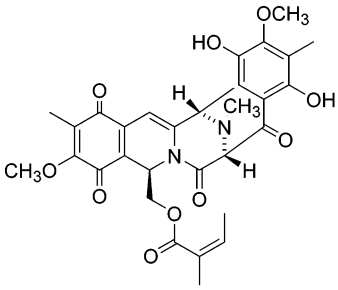
Table 3. Antimicrobial Activities of Cribrostatin 6

microorganism	minimum inhibitory concentration (μg/mL)
Candida albicans (ATCC 90028)	64
Cryptococcus neoformans (ATCC 90112)	2
Micrococcus luteus (Presque Isle 456)	16
Staphylococcus aureus (ATCC 29213)	16
Methicillin-resistant *S. aureus* (clinical isolate)	16
Enterococcus faecalis (ATCC 29212)	32
Vancomycin-resistant *E. faecalis* (clinical isolate)	32
Bacillus subtilis (clinical isolate)	2
Streptococcus pneumoniae (ATCC 6303)	0.5
Penicillin-resistant *S. pneumoniae* (clinical isolate)	2
Invasive *S. pneumoniae* (clinical isolate)	1
Group A *Streptococcus* (clinical isolate)	16
Stenotrophomonas maltophilia (ATCC 13637)	>64
Escherichia coli (ATCC 25922)	>64
Enterobacter cloacae (ATCC 13047)	>64
Neisseria gonorrhoeae (ATCC 49226)	0.0625

correlations to both N-2 and N-4. Only structure **3** is consistent with these results.

In addition to cancer cell growth inhibition of murine P388 lymphocytic leukemia and human cancer cell lines (Table 2), cribrostatin 6 exhibited antimicrobial activity against antibiotic-resistant Gram-positive bacteria and pathogenic fungi (Table 3). The only Gram-negative bacterium inhibited was *Neisseria gonorrhoeae*. Cribrostatin 2 (**6**) has an antimicrobial profile similar to cribrostatin 6,[7] while cribrostatins 1 (**5**), 3, 4, and 5 have antibacterial but not antifungal activities.[7] The cribrostatins warrant further investigation as antibacterial and antifungal agents.

Recently, two phosphorylated sterol sulfates were isolated from a *Cribrochalina* sp. and found to be membrane-type metalloproteinase (MT1-MMP) inhibitors.[11] That advance extends the structural variety of *Cribrochalina* genus cell growth regulatory constituents that so far range from acetylenic alcohols[12,13] to quinones (cf. **3**)[7,9] and peptides.[14]



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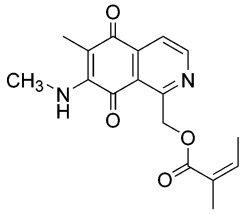
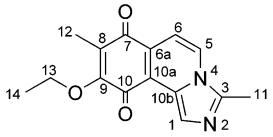
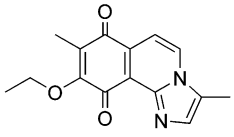


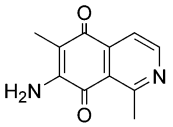
2, Cribrostatin 5



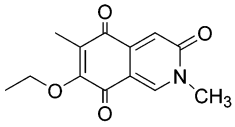
3, Cribrostatin 6



4



5, Cribrostatin 1



6, Cribrostatin 2

Experimental Section

General Experimental Methods. Except as noted, the general experimental procedures employed in our original investigations[7,9] of the *Cribrochalina* sp. were continued here. NMR spectra were recorded using a Varian Inova system equipped with a 5 mm triple resonance triaxial PFG probe at 500 MHz for ^1H and 125 MHz for ^{13}C and 50.65 MHz for ^{15}N. $^{15}N-^1H$ gradient HMBC experiments were performed on 2.2 mg of sample dissolved in 100 μL of $CDCl_3$ using a Shigemi 3 mm NMR tube susceptibility matched to $CDCl_3$, a Nalorac 3 mm $^1H\{^{15}N-^{31}P\}$ indirect-detection probe, and delays optimized for coupling constants of 90 Hz (one-bond) and 5 Hz (multiple-bond). The ^{15}N spectra were referenced to formamide (112 pm downfield of liquid ammonia).[15] The ^1H NMR and ^{13}C NMR spectra were referenced to residual solvent signals at 7.25 and 77.0 ppm for $CDCl_3$. HRMS data were obtained using

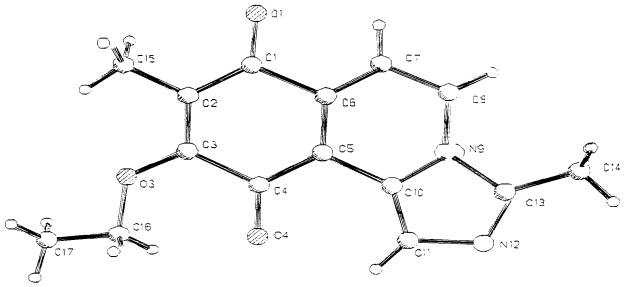


Figure 1. Solid-state structure of cribrostatin 6 (**3**).

a JEOL LCMate magnetic sector instrument in the APCI mode, calibrated using a polythylene glycol reference mixture. The X-ray data collection was accomplished using a Bruker AXS 6000 diffractometer.

Isolation of Cribrostatin 6 (1). The blue marine sponge *Cribrochalina* sp. was collected and extracted as previously described.[7,9] Fractionation of the extract, guided by the blue color and the screening results obtained using the murine P388 lymphocytic leukemia cell line, was carried out on columns of Sephadex LH-20, eluted successively with (a) CH_3OH; (b) $CH_2Cl_2-CH_3OH$ (3:2); (c) hexane−toluene−CH_3OH (3:1:1); and (d) hexane−i-PrOH−CH_3OH (8:1:1). In preparation for a separation using high-speed countercurrent distribution on an Ito Coil-Planet centrifuge, the blue fraction from the previous column was triturated with the upper (less polar) phase of the system hexane−EtOAc−CH_3OH−water (700:300:150:60), and the solution was filtered. The sparingly soluble material thus obtained (35.7 mg) proved to be the same as the solid isolated from the principal blue fraction from the countercurrent run (53 mg). The two were combined and recrystallized from acetone to afford dark blue needles: mp 169−171°C; P-388 ED_{50} 0.3 μg/mL; λ_{max} 203 (26,758), 266 (24,432), 323 (5597), 552 (1479); IR ν_{max} 2920, 1660, 1620, 1605, 1522, 1170 cm^{-1}; ^1H and ^{13}C NMR, see Table 1; LREIMS (*m*/*z*) 270, 242, 214, 185, 172, 157, 145, 116; HRMS ($APCI^+$) 271.10968 (calcd for $(M + H)^+$ ion $C_{15}H_{15}N_2O_3$, 271.10828; error 5.2 ppm).

Crystal Structure of Cribrostatin 6 (3).[16] A very small, dark blue needle obtained via slow evaporation of an acetone solution, with approximate dimensions of 0.05 × 0.05 × 0.20 mm, was mounted on the tip of a glass fiber. An initial set of cell constants was calculated from reflections harvested from three sets of 60 frames at 298(2) K on a Bruker 6000 diffractometer. Cell parameters indicated an orthorhombic space group. Subsequent data collection, using 30 s scans/frame and 0.396° steps in ω, was conducted in such a manner as to completely survey a complete hemisphere of reflections. This resulted in >93% coverage of the total reflections possible to a resolution of 0.83 Å. A total of 10 229 reflections were harvested from the total data collection, and final cell constants were calculated from a set of 332 strong, unique reflections. Subsequent statistical analysis of the complete reflection data set using the XPREP[17] program indicated the space group was $Pca2_1$.

Crystal data: $C_{15}H_{14}N_2O_3$, a = 15.414(15) Å, b = 11.532(11) Å, c = 7.201(7) Å, V = 1280(2) Å^3, λ = (Cu Kα) = 1.54178 Å, μ(Cu K) = 0.817 mm^{-1}, ρ_c = 1.403 g cm^{-3} for Z = 4 and M_r = 270.28, $F(000)$ = 568. After data reduction, merging of equivalent reflections and rejection of systematic absences, 1885 unique reflections remained (R_{int} = 0.5248), of which 315 were considered observed ($I_o > 2(I_o)$) and were used in the subsequent structure solution and refinement. An absorption correction was applied to the data with SADABS.[18] Direct methods structure determination and refinement were accomplished with the SHELXTL NT ver. V5.10[17] suite of programs. All non-hydrogen atoms for cribrostatin 6 (**3**) were located using the default settings of that program. Although the overall connectivity of the non-hydrogen atoms in quinone 3 could be readily established from the X-ray data, the low observed data-to-parameter ratio did not allow a completely unambiguous assignment of the two nitrogen atoms. The location of one of the N atoms at position 9 (Figure 1; X-ray numbering system) was known with reasonable certainty (due to analogy to previous cribrostatin-related compounds), and the position of the second N atom was less certain, with positions 11 and 12 both being likely candidates. Refinement of each of these possible isomeric structures (i.e., structure **3** or **4)** resulted in nearly identical residual R_1 values (0.0982 vs 0.1002, respectively). Although the former (**3**) was slightly favored by these results, the final, conclusive structural assignment was based on observed ^{15}N NMR experiments. Since the quality of data precluded the direct determination of hydrogen atom positions, the remaining hydrogen atom coordinates were calculated at optimum positions using the program SHELXL.[17] These latter atoms were assigned thermal parameters equal to either 1.2 or 1.5 (depending upon chemical type) of the U_{iso} value of the atom to which they were attached, then both coordinates and thermal values were forced to ride that atom during final cycles of refinement. All non-hydrogen atoms were refined anisotropically in a full-matrix least-squares refinement process. The final standard residual R_1 value for the model shown in Figure 1 was 0.0982 (for observed data) and 0.3817 (for all data). The corresponding Sheldrick R values were wR_2 of 0.2174 and 0.2741, respectively. The difference Fourier map showed insignificant residual electron density, the largest difference peak and hole being +0.255 and −0.252 e/Å^3, respectively. Final bond distances and angles were all within acceptable limits.

Antimicrobial Susceptibility Testing. Compounds were screened against bacteria and fungi according to established broth microdilution susceptibility assays.[19,20] The minimum inhibitory concentration was defined as the lowest concentration of compound that inhibited all visible growth of the test organism (optically clear). Assays were repeated on separate days.

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Supporting Information Available: X-ray details for cribrostatin 6 (**3**). This material is available free of charge via the Internet at http://pubs.acs.org.

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