DNA Methyl Transferase Inhibiting Halogenated Monoterpenes from the Madagascar Red Marine Alga *Portieria hornemannii*

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Three new halogenated monoterpenes, **2**, **3**, and **4**, along with the known compounds halomon (**1**) and two analogues, **5** and **6**, were isolated from the Madagascar red marine alga *Portieria hornemannii*. The structures of all three new compounds were determined by NMR spectroscopy in combination with mass spectrometric data analysis. Two of these monoterpenes (**1** and **2**) were low micromolar inhibitors of DNA methyl transferase-1.

Many species of red algae synthesize natural products that contain bromine, chlorine, and occasionally iodine atoms.[1] Halomon [6(*R*)-bromo-3(*S*)-bromomethyl)-7-methyl-2,3,7-trichloro-1-octene] was first isolated from the red alga *Portieria hornemannii* (Lynbye) collected in the Philippines in 1992.[2] Halomon exhibited strong differential cytotoxicity to brain-, renal-, and colon-derived cell lines in the National Cancer Institute's in vitro human tumor cell line screen.[2,3] On the basis of its unprecedented cytotoxicity profile, halomon was selected by the NCI for preclinical drug development.[2,4] However, research and development of halomon as an anticancer lead has been limited by the lack of a reliable natural source and failure to show in vivo effects.[5] Attempts to re-isolate halomon from *P. hornemannii* collected from Batan Island in the Philippines and other locations in the Pacific Ocean have been unsuccessful,[6] largely due to site-to-site and temporal variations in terpene content.[7] Chemical syntheses of halomon and analogues have been achieved although with some difficulties in regio- and stereocontrol.[8,9] Nevertheless, Thierry et al. reported a successful high-yielding total synthesis of halomon (13% overall) and a variety of analogues.[10]

Screening of the organic extracts of marine algae and cyanobacteria for mechanism-based anticancer agents has been productive and led to the discovery of new chemotypes showing antiproliferative properties.[11,12] In our ongoing efforts to discover and develop new marine natural product biomedicinals, we found that the organic extract of the Madagascar red alga, *P. hornemannii*, possessed a potent inhibitory activity to the DNA methyltransferase-1 (DNMT-1) isoform. DNMT-1 causes methylation of the cytosine phosphodiester-linked guanine dinucleotide (CpG) by catalyzing the transfer of a methyl group from *S*-adenosylmethionine to the 5′ position on cytosine residues residing at CpG sites. In many cancers, promoters of tumor suppressor genes are silenced by hypermethylation at CpG sites, and thus, the inhibition of DNMT-1 could potentially reverse tumor growth. Subsequently, the extract was subjected to bioassay-guided separation and resulted in the isolation of three new halogenated monoterpenes, **2**, **3**, and **4**, along with the known compounds halomon (**1**) and its analogues **5** and **6**. To our knowledge this is the first report of DNMT-1 enzyme inhibition by a halogenated monoterpene, including halomon.

Results and Discussion

P. hornemannii was collected in the south of Madagascar (Tolagniaro, Fort Dauphin) in March 1997. It was found growing on shallow reef rocks and was taxonomically identified by its distinctive branching pattern and pungent odor when crushed.

The organic extract of *P. hornemannii* was initially fractionated by normal-phase vacuum-liquid chromatography (NPVLC), and these fractions were tested for DNMT-1 inhibitory activity. The least polar fraction (fraction A eluting with 100% hexanes) was active in this assay at 10 μg/mL. Preparative normal-phase HPLC of fraction A yielded three subfractions, and two were found to inhibit DNMT-1 (fractions A1 and A3 at 4 and 8 μg/mL, respectively). Further fractionation of A3 by analytical NP-HPLC yielded halomon (**1**) and analogue **6** as the major components. Continued purification of A1 using analytical normal-phase HPLC led to seven fractions, A1a to A1g, two of which were new compounds, **2** and **3**. Further HPLC purification of fractions A1a and A1d led to the isolation of the new compound **4** and known compound **5**, respectively. The identity of halomon (**1**) was established by direct comparison of ^{1}H and ^{13}C NMR, HR-MS, and specific rotation with literature values.[1]

The LR-CIMS of **2** displayed a number of complex fragment ion clusters, indicating that it had several halogen atoms. The fragment ion cluster at *m*/*z* 247/249/251 indicated the presence of one bromine and one chlorine atom, whereas the fragment ion cluster at *m*/*z* 291/293/295 indicated the occurrence of two bromines. HR-CIMS of the peak at *m*/*z* 246.99022 $[M - Br]^+$ analyzed for $C_{10}H_{13}ClBr$, and thus compound **2** had a molecular formula of $C_{10}H_{13}ClBr_2$, consistent with three degrees of unsaturation.

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Table 1. ^{1}H NMR Data of Compounds **1**−**4** (400 MHz, $CDCl_3$)

position	**1**	**2**	**3**	**4**
1	5.68 (d, 2.3)	5.48 (d, 1.55)	5.49 (d, 1.65)	5.53 (d, 1.92)
	5.84 (d, 2.3)	5.68 (d, 1.55)	5.69 (d, 1.65)	5.70 (d, 1.92)
2				
3				
4	2.57 (ddd, 13, 9.3, 1.3)	2.62 (m)	2.80 (m)	2.70 (m)
	2.19 (t, 11.5)	2.62 (m)	2.50 (m)	2.75 (m)
5	2.57 (ddd, 13, 9.3, 1.3)	2.62 (m)	2.49 (m)	2.70 (m)
	2.02 (dddd, 9.3, 10.4, 11.5, 1.3)	2.62 (m)	1.89 (m)	2.75 (m)
6	4.08 (dd, 10.4, 1.3)		4.03 (dd, 11.3, 1)	
7				
8	1.71 (s)	1.80 (s)	1.69 (s)	1.84 (s)
9	3.89 (d, 11)	6.25 (s)	6.35 (s)	6.97 (s)
	3.83 (d, 11)			
10	1.85 (s)	1.95 (s)	1.81 (s)	1.90 (s)

Table 2. ^{13}C NMR Data of Compounds **1**−**4** (100 MHz, $CDCl_3$)

position	**1**	**2**	**3**	**4**
1	118.9	118.9	118.9	115.1
2	140.1	136.1	135.7	137.7
3	74	142.3	142.3	140.7
4	38.4	36.2	34.4	36.3
5	30.5	34.7	32.4	30.5
6	65.2	120.1	64.5	120.3
7	72.2	132.2	72.4	132.2
8	27.5	20.9	27.7	20.8
9	39.1	106.1	106.8	112.6
10	33.5	25.8	33.6	25.9

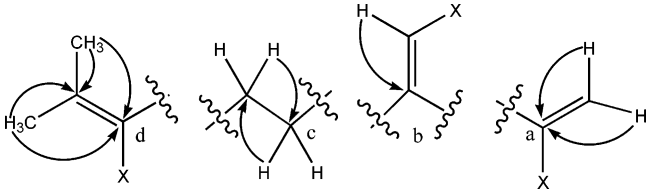

Figure 1. Partial structures **a**−**d** in compound **2** (arrows indicate HMBC correlations).

The ^{13}C NMR, DEPT, and multiplicity-edited HSQC of **2** characterized 10 carbon atoms and included six olefinic carbons (δ 106.1, 118.9, 120.1, 132.2, 136.1, 142.3), two methyl groups (δ 20.9, 25.8), and two methylene carbons (δ 34.7, 36.2) (Table 2). The ^{1}H NMR of **2** showed the presence of two three-proton singlets (δ 1.80, 1.95) assigned as geminal olefinic methyl groups, a four-proton multiplet (δ 2.62) assigned as protons belonging to two methylene groups, and finally, three olefinic protons composed of one singlet (δ 6.25) and two doublets (δ 5.48, 5.68) (Table 1). A multiplicity-edited HSQC revealed that the latter protons were attached to the same carbon (δ 118.9), while the singlet proton was attached to a carbon at δ 106.1. Thus, the remaining olefinic carbons were fully substituted.

The arrangement of these 10 carbons was established using HMBC and ^{1}H−^{1}H COSY experiments. The two protons attached to the carbon at δ 118.9 showed HMBC correlations to the carbon at δ 136.1, establishing partial structure **a** (Figure 1). The olefinic proton singlet at δ 6.25 displayed an HMBC correlation to the quaternary carbon at δ 142.3, indicating the connectivity of partial structure **b**. Strong HMBC correlations between the multiplet protons at δ 2.62 and carbons at δ 34.7 and 36.2 indicated that these two methylenes were adjacent (partial structure **c**). Finally, the methyl groups at δ 1.95 and 1.80 showed HMBC correlations to carbons at δ 132.2 and 120.1, thereby establishing partial structure **d**.

The connection between partial structures **a** and **b** was established by HMBC correlations (Figure 2) between the protons at δ 5.48 and 5.68 and the quaternary carbon at δ 142.3. Connectivities

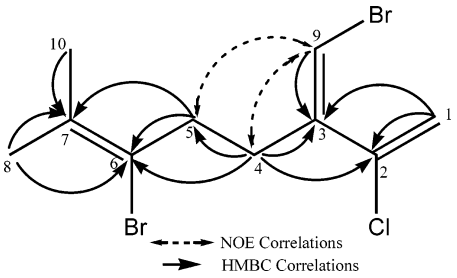

Figure 2. Key HMBC and selected NOE correlations for compound **2**.

between partial structures **c** and **b**, as well as **c** and **d**, were established by HMBC correlations between protons at δ 2.62 and quaternary carbons at δ 142.3/106.1 and 120.1/132.2, respectively. By ^{1}H−^{1}H COSY an allylic coupling was observed between the protons at δ 2.62 and the methyl group at δ 1.95, confirming the connectivity of partial structures **c** and **d**.

Given the proton and carbon count from the molecular formula and their assigned positions as described above, all of the halogen atoms were required to be positioned on double bonds. Carbon-13 NMR spectroscopy played an important role in the assignment of the regiochemistry. While both bromine and chlorine markedly deshield the carbon to which they are attached, the effect of chlorine is generally larger.[13] For example, the chemical shift of the C-2 quaternary carbon (δ 136.1) indicated that chlorine was attached, whereas the shift at the C-6 quaternary carbon (δ 120.1) indicated bromine was present. Similarly, the chemical shift at C-9 (δ 106.1) indicated that bromine was attached at this position. To determine the geometry of the double bond between C-3 and C-9, a series of 1D NOE experiments were performed (Figure 2). Irradiation of H-9 (δ 6.25) showed NOE enhancement in H-5 (δ 2.62) but not H-1, indicating that the double-bond geometry was *Z*, and thus completing the structure of compound **2**.

LR-GCMS analysis of **3** showed major fragment peaks at *m*/*z* $[M - Br - HCl]^+$ 247/249/251, with relative intensities indicative of one chlorine and one bromine atom, and *m*/*z* $[M - Br]^+$ 283/285/287, with relative intensities indicative of one bromine and two chlorine atoms. This indicated a molecular formula of $C_{10}H_{14}Cl_2Br_2$, which was confirmed by HR-CIMS (see Experimental Section). The two degrees of unsaturation inherent to the molecular formula of **3** were assigned to two olefinic bonds (Tables 1, 2). The ^{13}C NMR spectrum of compound **3** showed similarities to **2** at positions C-1 through C-4 and C-9 and similarities to compound **1** at positions C-5 through C-8 and C-10 (Table 2). For example, the ^{13}C NMR resonances for C-6 and C-7 in **2** were missing in **3**, and new halogen-bearing carbons were present as for **1**. Further, the ^{1}H NMR showed an additional signal at δ 4.03 by comparison with compound **2**. Multiplicity-edited HSQC showed that this proton was attached to the halogen-bearing carbon at δ 64.5, and an HMBC

correlation between this proton and the other halogen-bearing carbon at δ 72.4 revealed that these two carbons were arranged precisely as found in halomon (**1**).

The regiochemistry of compound **3** was established using ^{13}C NMR chemical shift analysis in comparison with **2** for C-2 and C-9 and with **1** for C-6 and C-7. Thus, bromine was attached to C-9 (δ 106.1), whereas chlorine was attached to C-2 (δ 135.7). Correspondingly, bromine was attached to C-6 (δ 64.5) and chlorine to C-7 (δ 72.4). The *Z*-geometry of the C-3/C-9 double bond was defined by a 1D NOE experiment in which irradiation of H-9 enhanced H-4.

Extensive GC-MS analyses showed that compound **4** also contained several halogen atoms. The fragment ion cluster at *m/z* $[M - Cl]^+$ 247/249/251 indicated the occurrence of one chlorine atom and one bromine atom, and thus, compound **4** had a molecular formula of $C_{10}H_{13}Cl_2Br$. The ^1H NMR spectrum of compound **4** was quite similar to that of compound **2**. The primary difference was the downfield proton chemical shift of H-9 to δ 6.97 in compound **4** from δ 6.25 in **2**. Coupled with the MS data and molecular formula, this chemical shift change was consistent with the replacement of the H-9 bromine atom with a chlorine atom. The chemical shift of C-9 (δ 112.6) confirmed this deduction. Thus, compound **4** was the 9-chloro analogue of metabolite **2**.

Halomon (**1**) and compounds **2**, **3**, and **6** were tested in a DNMT-1 enzyme inhibition assay. Halomon (**1**) and compound **2** were found to have comparable activities (1.25 and 1.65 μM, respectively), while compounds **3** and **6** were only weakly active (55 and 21.9 μM, respectively). Compounds **4** and **5** were not tested due to the small quantities isolated.

The biological halogenation of monoterpenes is believed to involve the formation of halonium ions and their subsequent Markovnikov addition to the olefinic bond of myrcene.[14] Vanadium bromoperoxidase (V-BrPO) catalyzes the oxidation of halides (Br^-, Cl^-) by the peroxo complex of V-BrPO.[15−17] The oxidized halogen can then react with an appropriate unsaturated organic substrate by an electrophilic mechanism (Br^+, Cl^+). The resulting carbocation intermediate may then either react with a nucleophilic halogen ion (Br^- or Cl^-) to form mainly saturated natural products such as halomon or lose a proton to regenerate the unsaturated species. In this Malagasy specimen of *P. hornemannii*, both of these mechanisms appear to be in operation.

Experimental Section

General Experimental Procedures. Optical rotations were measured on a JASCO P 1010 polarimeter. UV and FT-IR spectra were obtained employing Hewlett-Packard 8452A and Nicolet 510 instruments, respectively. All NMR spectra were recorded on a Bruker Avance DPX400 spectrometer using residual $CHCl_3$ as reference (δ_H 7.27; δ_C 77.0). HPLC separations were performed using Waters 515 HPLC pumps, a Rheodyne 7725i injector, and a Waters 996 photodiode array detector. LR and HR CI mass spectra were recorded on a Kratos MS50TC instrument, and GC/MS data were acquired on a Hewlett-Packard 5890 series II chromatograph with a Hewlett-Packard 5971 series mass selective detector.

Collection, Extraction, and Isolation. *Portieria hornemannii* was collected by hand using scuba in March 1997 from southern Madagascar (Bay of Fort Dauphin) (voucher specimen available from W.H.G. as collection number MFM-31/Mar/97-03). The alga was stored at −20 °C in 70% EtOH until workup. The alga (78.5 g dry wt) was extracted four times with CH_2Cl_2−MeOH (2:1) to give a crude organic extract (4.65 g). A portion of the extract (4.50 g) was fractionated on silica gel by NPVLC to give nine fractions using a stepwise gradient of hexanes−EtOAc and EtOAc−MeOH. Fraction A, eluting with 100% hexanes, was further chromatographed on preparative normal-phase HPLC (Phenomenex Maxsil 10 silica 10 μm, 500 × 10.0 mm, 100% hexanes) and yielded three fractions, A1 to A3. Fraction A1 was subjected to analytical normal-phase HPLC (Phenomenex Luna silica 10 μm, 250 × 4.60 mm, 100% hexanes) to yield successively 4.1 mg of **2**, 1.8 mg of **3**, and mixtures A1a and A1d. Fraction A1a was further purified on analytical reversed-phase HPLC (Phenomenex Sphereclone ODS 5 μm, 250 × 10.0 mm, 100% acetonitrile) to yield 1 mg of **4**. Fraction A1d was further purified on analytical normal-phase HPLC (Phenomenex Luna silica 10 μm, 250 × 4.60 mm, 100% hexanes) to yield 4.7 mg of **5** as the major component. Fraction A3 was subjected to analytical normal-phase HPLC (Phenomenex Luna silica 10 μm, 250 × 4.60 mm, 100% hexanes) to yield 2 mg of **1** and 2 mg of **6**.

Enzyme Bioassay. The DNMT-1 assay was carried out as a homogeneous scintillation proximity assay (SPA) in the following manner. Using a 50 mM Tris pH 8.0 buffer in a 96-well Isoplate (Wallac, 1450-51), preparations of 25 nM DNMT-1 were combined with test compounds, 200 nM biotinylated hemimethylated DNA substrate, and the methyl donor *S*-adenosyl-L-[methyl-3*H*]methionine (3H-SAM at 250 nM) and mixed for 2 h at 37 °C. Following addition of streptavidin-coated SPA beads, bead-associated methylated substrate was quantitated in a microplate scintillation counter.[18,19]

Compound 2: colorless oil; UV ($CHCl_3$) λ_{max} 252 nm (log ϵ 3.3); ^1H NMR (400 MHz, $CDCl_3$) and ^{13}C NMR (100 MHz, $CDCl_3$), see Tables 1 and 2; HMBC correlations, H-1/C-2,C-3,C-9; H-4/C-2,C-3,C-5,C-6,C-9; H-5/C-3,C-4,C-6,C-7; H-8/C-5,C-6,C-7,C-10; H-9/C-2,C-3,C-4; H-10/C-5,C-6,C-7,C-8; LR-CIMS fragment ions at *m/z* $[M - C_5H_5BrCl]^+$ 147(25)/149(30), *m/z* $[M - 2Br]^+$ 168(95)/ 170(25), *m/z* $[M - Br]^+$ 247(45)/249(60)/251(15), $[M - Cl]^+$ 290.9(19)/292.9(34)/294.9(17), *m/z* $[M]^+$ 325.9(1)/327.9(3)/329.9(1), and *m/z* $[M - Cl]^+$ 290.9(19)/ 292.9(34)/294.9(17); HR-CIMS *m/z* $[M - Br]^+$ 246.9902 (calcd for $C_{10}H_{13}{}^{35}Cl^{79}Br$, 246.9889).

Compound 3: colorless oil; $[\alpha]_D^{25}$ +1 (*c* 0.3, $CHCl_3$); UV ($CHCl_3$) λ_{max} 249 nm (log ϵ 3.3); ^1H NMR (400 MHz, $CDCl_3$) and ^{13}C NMR (100 MHz, $CDCl_3$), see Tables 1 and 2; HMBC correlations, H-1/C-2,C-3; H-4/C-2,C-3,C-5,C-6,C-9; H-5/C-3,C-4,C-6; H-6/C-5,C-7; H-8/C-5,C-6,C-7,C-10; H-9/C-2,C-3,C-4; H-10/ C-5,C-6,C-7,C-8; LR-GCMS fragment ion at *m/z* $[M - 2HBr - Cl]^+$ 167(100)/169(30), *m/z* $[M - HBr - Br]^+$ 203(30)/205(18)/207(3), *m/z* $[M - 2Cl-Br]^+$ 213-(5)/215(5), *m/z* $[M - Br - HCl]^+$ 247(7)/249(10)/251(2), and *m/z* $[M - Br]^+$ 283(6)/285(10)/287(5); HR-CIMS *m/z* $[M - Br]^+$ 282.96557 (calcd for $C_{10}H_{14}{}^{35}Cl_2{}^{79}Br$, 282.96559).

Compound 4: colorless oil; UV ($CHCl_3$) λ_{max} 251 nm (log ϵ 3.3); ^1H NMR (400 MHz, $CDCl_3$) and ^{13}C NMR (100 MHz, $CDCl_3$), see Tables 1 and 2; HMBC correlations, H-1/C-2; H-4/C-2,C-3,C-5,C-6,C-9; H-5/C-4; H-8/C-6,C-7,C-10; H-9/C-3; H-10/C-6,C-7,C-8; LR-CIMS at *m/z* $[M + Na + 2H]^+$ 307(17)/309(30)/311(19); LR-GC/MS fragment ions at *m/z* $[M - C_5H_5Cl_2]^+$ 147(30)/149(30), *m/z* $[M - Cl - Br]^+$ 168(100)/170(35), and *m/z* $[M - Cl]^+$ 247(45)/249(60)/251(14); HR-CIMS *m/z* $[M - Cl]^+$ 246.9901 (calcd for $C_{10}H_{13}{}^{35}Cl^{79}Br$, 246.9889).

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Supporting Information Available: ^1H NMR, ^{13}C NMR, $^1H-^1H$ COSY, HSQC, HMBC, and mass spectral data of compounds **2**, **3**, and **4**. This material is available free of charge via the Internet at http://pubs.acs.org.

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