



Published in final edited form as:

J Nat Prod. 2009 June ; 72(6): 1178–1183. doi:10.1021/np900183v.

ISOLATION, SYNTHESIS AND BIOLOGICAL ACTIVITY OF APHROCALLISTIN, AN ADENINE SUBSTITUTED BROMOTYRAMINE METABOLITE FROM THE HEXACTINELLIDA SPONGE *APHROCALLISTES BEATRIX*

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Abstract

A new adenine substituted bromotyrosine derived metabolite designated as aphrocallistin (**1**) has been isolated from the deep-water Hexactinellida sponge *Aphrocallistes beatrix beatrix* Gray, 1858 (Order Hexactinosida, Family Aphrocallistidae). Its structure was elucidated on the basis of spectral data and confirmed through a convergent, modular total synthetic route that is amenable towards future analog preparation. Aphrocallistin inhibits the growth of a panel of human tumor cell lines with IC₅₀ values ranging from 7.5 to >100 μM and has been shown to induce G1 cell cycle arrest in the PANC-1 pancreatic carcinoma cell line. Aphrocallistin has been fully characterized in the NCI cancer cell line panel and has undergone *in vitro* ADME pharmacological profiling.

Hexactinellida sponges occur almost exclusively in deep-water marine habitats at depths greater than 200 meters. The skeleton of the hexactinellids is made entirely of silicon oxides giving them a “glassy” look and thus the common designation “glass sponges.” The lack of spongin leads to a fairly rigid body with limited resilience or flexibility making collection of these deep-water sponges using methods such as trawling difficult, as the sponges crush and fragment in the trawl. Although these sponges are fairly common, there is very little published on their chemistry. Thiel and co-workers have described the lipid complement of a series of hexactinellid sponges as part of a chemotaxonomic investigation¹⁻² and ergosta-4,24(28)-dien-3-one has been reported from an Antarctic hexactinellid.³ As part of our ongoing search for compounds with potential utility against cancer, a crude ethanol extract of the sponge *Aphrocallistes beatrix beatrix* Gray, 1858 showed moderate inhibition of the proliferation of the PANC-1 human pancreatic carcinoma and the DLD-1 human

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Supporting Information Available: Copies of the ¹H and ¹³C NMR spectra along with selected 2D NMR spectra (g-DQF-COSY, edited-g-HSQC, g-HMBC, g-NOESY) for the natural product; the ¹H NMR spectrum and LC-MS analysis of synthetic aphrocallistin; details of preparation of compound **6**; and details of the Pharmacological testing; are available free of charge via the Internet at <http://pubs.acs.org>

colon carcinoma cell lines. Bioassay-guided fractionation led to the purification of a novel compound which we have designated as aphrocallistin (**1**). This manuscript describes the isolation, structure elucidation, synthesis, cytotoxic activity and preliminary pharmacokinetics of aphrocallistin.

A sample of the sponge *Aphrocallistes beatrix beatrix* Gray, 1858 (Order Hexactinosida, Family Aphrocallistidae) was collected using the Johnson-Sea-Link manned submersible east of Fort Pierce, Florida on deep-water *Lophelia* coral mounds. The specimen used in this study was stored at -20°C immediately after collection until further work up. The frozen sponge was extracted exhaustively with EtOH and after concentration by distillation under reduced pressure, the extract was partitioned between EtOAc and H₂O. The EtOAc partition was further separated by vacuum column chromatography on a C-18 stationary phase using a step gradient of CH₃CN in H₂O. Active fractions were further purified by medium pressure liquid chromatography followed by HPLC to yield a fraction which ultimately contained aphrocallistin (**1**) as a light brown oil.

Low resolution ESIMS analysis suggested the presence of a single compound in the active fraction having a three ion cluster m/z 539/541/543 with typical ion abundance for a dibrominated compound. HRESIMS analysis showed a similar pattern and suggested a formula of C₂₀H₂₄Br₂N₆O₂ requiring 11 unsaturation equivalents in the molecule. Inspection of the ¹H and ¹³C NMR spectra revealed a series of resonances that appeared doubled and significantly more carbons than would be consistent with the molecular formula suggested by HRESIMS. The presence of two closely related compounds was suggested by a series of repeating resonances in the ¹H and DQF-COSY 2D-NMR spectra that appeared to constitute two 1,2-substituted ethyl groups; two *N*-methyl groups; and two acetyl methyls. Careful integration of the ¹H NMR spectrum allowed for the determination that the active fraction consists of two closely related compounds that were present in a ratio of 3:2. Nominally the integrated ¹³C NMR spectrum appeared to have twenty-nine carbon resonances, fifteen sp² hybridized and fourteen sp³ hybridized. Some of the resonances were obviously doubled while others were not. Two resonances were extremely broad suggesting either attachment to nitrogen or presence of a tautomeric species. As not all resonances in the ¹³C NMR spectrum were doubled, initial assignment of the resonances was difficult. Collection of an inverse gated carbon spectrum led to a ¹³C spectrum that could be readily integrated and allowed for the assignment of the carbons in the major, minor or in both compounds.

The NMR spectra of **1** exhibited resonances attributable to an *N*-methyl functionality [δ_{H} 2.98 (3H, s), δ_{C} 36.9 (CH₃)]; an acetyl functionality [δ_{H} 2.02 (3H, s), δ_{C} 20.0 (CH₃); δ_{C} 173.4 (qC)]; and an A₂X₂ spin system [δ_{H} 2.74 (2H, t $J=7.6$ Hz), δ_{C} 33.0 (CH₂); δ_{H} 3.50 (2H, t $J=7.6$ Hz), δ_{C} 50.1 (CH₂)] consistent with an ethyl amine functionality. Correlations in the HMBC spectrum between the *N*-methyl protons and the nitrogen bearing methylene carbon of the ethyl chain (C-2) and the amide carbonyl carbon (C-15) suggested the presence of an *N*-methyl amide in **1**. Correlations in the HMBC spectrum between H₂-2 and both C-14 and C-15; and H₃-16 to C-15 also support the presence of an *N*-methyl-*N*-acetyl-ethylamine in **1**. The presence of an aromatic singlet (δ_{H} 7.45) attributable to two hydrogen atoms along with carbon resonances (δ_{C} 134.4 and 118.9) each integrating for two carbon atoms suggested the presence of a 1,2, 4,6- symmetrically substituted aromatic ring in **1**. The ¹H resonance observed at δ_{H} 7.45 (H-5, H-9) had correlations to the ethyl carbon (C-3) as well as to C-4, C-8 and C-7 of the aromatic ring. The C-5 and C-7 substituents of the aromatic ring were assigned as bromine based upon comparison of the chemical shifts to related compounds. The chemical shift of C-6 suggested oxygen substitution (δ_{C} 152.7). The DQF-COSY spectrum showed the presence of a 3-aminopropan-1-ol group in **1** [δ_{H} 4.11 (2H, m), δ_{C} 71.9 (CH₂); δ_{H} 2.27 (2H, m), δ_{C} 30.5 (CH₂); δ_{H} 4.04 (2H, m), δ_{C} 40.4 (CH₂)].

A correlation observed in the HMBC spectrum from the methylene protons observed at δ_{H} 4.11 (H₂-10) to C-7 of the aromatic ring allowed for attachment of the propyl functionality to C-7 of the dibromophenyl ring through an ether linkage. The chemical shift of H₂-12 (δ_{H} 4.04) and C-12 (δ_{C} 40.4) were consistent with nitrogen substitution at C-12. Atoms 1–13 of **1** constitute a common bromotyrosine derived functionality observed in over 83 marine natural products and has been given the common name moloka'iamine.⁴ All data observed for N-1 through C-13 in aphrocallistin are consistent with those reported for moloka'iamine.

The remaining atoms to be incorporated into the structure are C₆H₅N₄. The ¹H, ¹³C and edited-g-HSQC NMR spectra showed the presence of three quaternary sp² hybridized carbons (δ_{C} 153.5, 148.9, 112.6); two methine sp² hybridized carbons (δ_{H} 8.64 (s), δ_{C} 149.7 (CH); δ_{H} 8.42 (s), δ_{C} 145.9 (CH)] and one methyl group [δ_{H} 4.06 (3H, s), δ_{C} 36.9 (CH₃)]. These atoms were connected together based upon chemical shift arguments, ¹J_{C-H} coupling constants and correlations observed in the ¹H-¹³C (Figure 1) and ¹H-¹⁵N HMBC spectra, resulting in the assignment of a 3-methyladenine (adenine numbering system) substituent attached to the molakai'imine moiety through N-13. In the HMBC spectrum, residual signals from the un-suppressed one bond ¹J_{C-H} for the two methine resonances could be observed and both were greater than 200 Hz suggesting that both carbons bear two nitrogen substituents.⁵ Both of the protons showed correlations in the HMBC spectrum to the same quaternary carbon observed at δ_{C} 148.9 (C-4') suggesting that both protons are three bonds away from this carbon. The *N*-methyl resonance observed at δ_{H} 4.06 showed HMBC correlations to C-2' and to C-4' placing it on N-3'. The H-2' proton showed HMBC correlations to a carbon observed at δ_{C} 153.5 (C-6') which in turn had coupling from H₂-12 linking the adenine functionality to the moloka'iamine functionality through N-13. The proton observed at δ_{H} 8.42 (H-8') showed long range coupling in the HMBC spectrum to the final quaternary carbon observed at δ_{C} 112.6 (C-5') which is consistent with the chemical shift reported for C-5 of an adenine functionality. The ¹H-¹⁵N HMBC experiment also supports this assignment with correlations observed between the H₃-17 methyl and a nitrogen observed at δ_{N} 148 (N-4'); H-2' and two nitrogens observed at δ_{N} 148 (N-4') and δ_{N} 226 (N-1'); H₂-11 to a nitrogen observed at δ_{N} 106 (N-13); and H₂-3, H₃-14 and H₃-16 all showing correlations to a nitrogen observed at δ_{N} 111. This completes the overall structure of aphrocallistin.

Aphrocallistin has a relatively unusual 3-methyladenine functionality that has not been observed in other marine natural products. It is also the first in the bromotyrosine derived compounds to have both acetyl and methyl substitution at N-1 of the moloka'iamine moiety. It is this functionality that leads to the 3:2 mixture in which the major component is *cis* across the peptide bond and the minor component is *trans* as determined by nOe measurement [the major has a strong nOe between the N-1 CH₃ and the *N*-acetyl methyl while the minor has a strong nOe between the *N*-acetyl methyl and H₂-2 methylene protons].

In order to confirm the structure and to obtain larger amounts of the compound for biological evaluation, a chemical synthesis of **1** was conducted. Aphrocallistin (**1**) can be regarded as a three-part construct of a tyramine derivative **2** and an adenine moiety **4** connected together by a three-carbon chain presumably stemming from 3-aminopropan-1-ol (**3**), a linker commonly found in many natural products (Figure 2A). Some prominent structural features in the molecule of **1** can be noted. The tyramine nitrogen is present as a tertiary amide, an attribute not common in nature, and the adenine piece is unusually functionalized at position #3 (adenine numbering) with a methyl group.

Aphrocallistin (**1**) can be assembled in a convergent biomimetic manner from tyramine **9** and the purine **14** via *S_NAr*-like chemistry. Initial efforts to prepare the tyramine derivative **7** en route to **9** via reductive methylation of tyramine followed by acetylation to form the

corresponding tertiary amide were unsuccessful. The two step approach did not lead to satisfactory results as a competing Pictet-Spengler like cyclization gave diminished yields of the desired secondary amine. This early challenge was easily circumvented by reversing the order of synthesis. Installation of the acetate functionality on the *O*-protected compound **5** was achieved following Okuda's method.⁶ Improvement of the protocol provided **5** in excellent yield. Treatment of **5** with sodium hydride (NaH) and subsequent addition of iodomethane (MeI) followed by conventional heating (refluxing DMF) did not provide the desired tertiary amide but returned the starting material, an outcome attributed to the high volatility of iodomethane and the extreme reaction conditions, high temperature, and long reaction time, to achieve alkylation. A microwave-assisted protocol (5 min at 130°C) led to a substantial improvement in the conversion however the reaction required repetition and resubmission of crude material to the reaction conditions for three cycles in order to obtain a good yield of product. Removal of the phenolic *O*-methyl group in the presence of boron tribromide delivered compound **7** in good yield (Figure 2B). With compound **7** in hand, we were able to prepare the left-hand side of aphrocallistin (compound **2**) via a reaction of **7** with bromine under acidic conditions.⁷ Linker **3** was then incorporated using a two-step procedure. Alkylation of the phenolic oxygen of **2** with the alkyl halide **8**, followed by unmasking of the primary amine functionality, set the stage for the final attachment of the purine moiety.

The purine moiety **14** was prepared following literature procedures^{8,9,10} which were adapted and optimized to achieve good conversion and high yields (Figure 2C). The synthetic route was initiated by preparing pyrimidin-4-one **12** in three steps. Commercially available starting materials **10** and **11** were heated at reflux in anhydrous EtOH in the presence of freshly prepared sodium ethoxide.⁸ The resulting cyclic nitroso compound was then reduced with sodium dithionite to the corresponding amino analog⁸ which was condensed with trimethyl orthoformate⁹ to provide **12** in good overall yield. Desulfuration of **12** in the presence of Raney Ni^{8,10} led to intermediate **13**. Reaction of the purin-6-one **13** with phosphorous pentasulfide¹⁰, alkylation on the sulfur residue¹⁰, and subsequent chloride displacement¹¹ formed **14**, a key intermediate for the final step of the synthesis of aphrocallistin (**1**).

The convergent synthesis of aphrocallistin (**1**) concluded with a nucleophilic aromatic substitution of purine **14** with the tyramine moiety **9** (Figure 2D).¹² Ultimately, 70 mg of **1** was prepared in eight linear steps from commercially available starting materials in good overall yield. Spectral analysis (¹H-NMR and LCMS) confirmed the synthetic material to be identical with the natural product. Aphrocallistin was evaluated in a panel of human tumor cell lines and gave IC₅₀ values ranging from 7.5 μM in the DLD-1 colon carcinoma cell line to >100 μM in the extremely resistant AsPc-1 pancreatic carcinoma cell line (Table 2). Cell cycle analysis of aphrocallistin in the Panc-1 pancreatic adenocarcinoma cell line showed significant cell cycle arrest at G1 at doses ≤ 46.5 μM. Interestingly aphrocallistin shows the greatest inhibition of tumor proliferation for cell lines having a p53 mutation, being about three times more potent against Panc-1 cells and about 10-times more potent against DLD-1 than Vero cells (cell line derived from normal kidney cells of an African Monkey). These cells lines can not initiate p21 modulated response and DNA repair mechanisms due to the loss of the p53 pathway. Aphrocallistin has been fully characterized in the NCI cancer cell line panel (see Supporting Information for full data) and showed significant selectivity of growth inhibition in the following cell lines: SR (leukemia), HCT-116 (colon), SK-MEL-5 (melanoma), T-47D and MDA-MB-468 (breast). In a study aimed at detecting whether aphrocallistin binds directly to DNA, no displacement of ethidium bromide was observed suggesting that it does not directly bind to DNA. Aphrocallistin was tested for antimicrobial activity against *Candida albicans*, *Pseudomonas aeruginosa* and methicillin-resistant

Staphylococcus aureus and did not show significant inhibition under the conditions used for the assay.

The pharmacological properties of aphrocallistin were evaluated in various *in vitro* assays. The solubility of the compound was assessed to be high at three physiologically relevant pH levels. It exhibited moderate permeability as measured by Parallel Artificial Membrane Permeation Assay at pH 6.2 and 7.4 and low effective permeability at pH 5.0. Aphrocallistin also showed moderate protein binding and excellent stability (3 h) at both test concentrations in human and mouse plasma. However, it was rapidly metabolized with 96.9% and 98.5% of the compound consumed within 60 min in both human and mouse liver microsomes, respectively. This suggests that aphrocallistin will be subject to significant first pass hepatic metabolism *in vivo*. Data is detailed in the Supporting Information.

General Experimental Procedures

NMR data used in the structure elucidation were collected on a JEOL ECA-600 spectrometer operating at 600.17 MHz for ^1H ; 150.9 MHz for ^{13}C and 60.8 MHz for ^{15}N (instrument reference set to liquid NH_3). The edited-g-HSQC was optimized for 140 Hz and the g-HMBC was optimized for 8 Hz. Chemical shifts are referenced to solvent e.g. CD_3OD δ_{H} observed at 3.31 ppm and δ_{C} observed at 49.0 ppm. Chemical shifts for ^{15}N was referenced to liquid NH_3 with long range $J_{\text{H,N}}$ optimized for 6 Hz. The HRFABMS were measured using a JEOL XYZ mass spectrometer. Flow cytometry was measured on a BD FACSCalibur.

All reactions involving air and moisture sensitive reagents and solvents were performed under a nitrogen atmosphere using standard chemical techniques. The following solvents DMF, CH_2Cl_2 , CH_3CN , 1,4-dioxane were purchased in Sure/Seal bottles from Sigma-Aldrich and were used "as is"; EtOAc, pyridine and EtOH were purchased and used fresh from Sigma-Aldrich. All organic reagents were used as purchased. Unless otherwise noted, a typical reaction work-up was performed by addition of H_2O and extraction with EtOAc; the organic phase thus obtained was dried over MgSO_4 and concentrated *in vacuo*. Microwave-assisted reactions were performed using a CEM Discover system. All NMR spectra for the synthetic materials were recorded on Bruker Avance II 400 MHz instrument. ^1H and ^{13}C chemical shifts are reported in δ values in ppm in the corresponding solvent. Analytical thin layer chromatography was performed on Partisil K6F silica gel 60 Å, 250 μm . Reverse phase purification and LCMS analysis of synthetic materials were completed on a Waters Autopurification system which consists of a 2767 sample manager, a 2545 binary gradient module, a system fluidics organizer, a 2489 UV/Vis detector, and a 3100 mass detector, all controlled with MassLynx software. A Sunfire Analytical C18 5 μm column (4.6 \times 50mm) and a Sunfire Preparative OBD 5 μm column (19 \times 50mm) were used for analytical and preparative LCMS respectively. All solvents used for chromatography on the synthetic materials were Fisher Chemical HPLC grade and the water was Millipore Milli-Q PP filtered.

Biological Material

A sample of a sponge identified as *Aphrocallistes beatrix beatrix* (Gray) [Phylum: Porifera, Class Hexactinellida, Order Hexactinosida, Family Aphrocallistidae] was collected by manned submersible at a depth of 725.4 m approximately 40 nautical miles east of Fort Pierce on deep-water *Lophelia* coral mounds (latitude 27° 39.430'N, longitude 79° 34.967'W). The sponge is vasiform with the appearance of folded plates and fingers. It is crispy in texture and friable. It was collected growing in association with yellow zoanths. A reference sample preserved in ethanol has been deposited in the Harbor Branch

Oceanographic Museum (catalog number 002:00022, DBMR number 20-V-04-1-005) and is available for taxonomic evaluation by those skilled in the art.

Isolation of Aphrocallistin

The frozen sponge (106 g) was diced and extracted exhaustively with EtOH (Pharmco 100%). The combined EtOH extracts were concentrated to dryness and the residue partitioned between EtOAc and H₂O. The EtOAc partition was concentrated to dryness to yield 0.493 g of an oil residue. The residue from the EtOAc partition was chromatographed by vacuum flash chromatography on a custom prepared RP-18 stationary phase using a step gradient of H₂O -CH₃CN-IPA as eluent. Column size was 150 mL. The eluent series is as follows: fraction 1: 100 mL of H₂O - CH₃CN (80:20 v/v); fraction 2: 100 mL of H₂O - CH₃CN (60:40 v/v); fraction 3: 100 mL of H₂O - CH₃CN (40:60 v/v); fraction 4: 100 mL of H₂O - CH₃CN (20:80 v/v); fraction 5: 100 mL of CH₃CN; Fraction 6: 100 mL of H₂O - CH₃CN:TFA (20:80:0.1 v/v); Fraction 7: 100% CH₃CN; Fraction 8: 100% IPA. Fractions 6 and 7 (84.4 mg) were combined and further purified by medium pressure liquid chromatography on a C-18 reversed-phase stationary phase using a Combiflash Companion (Isco) with the following gradient program [Solvent A: 5% CH₃CN in H₂O v/v; Solvent B: 100% CH₃CN; t= 0 min, A:B (100:0); t=1 min, A: B (100:0); t=11 min, A: B (70:30); t=17 min, A:B (0:100); flow = 15 mL/min; detected by UV absorption observed at 230 nm] to yield 40 mg of aphrocallistin.

Aphrocallistin (1): light brown oil; UV (EtOH) λ_{\max} (log ϵ) 285 (4.0); ¹H NMR (600.2 MHz, CD₃OD, see table 1); ¹³C NMR data (160.9 MHz, CD₃OD see Table 1); HRESIMS: *m/z* observed 539.042786 [M+H]⁺ (calculated for C₂₀H₂₅ Br₂ N₆ O₂ 539.040571).

Preparation of *N*-(4-Hydroxyphenethyl)-*N*-methylacetamide (7)

Acetamide **6** (550.0 mg, 2.85 mmol) was placed in a microwave tube and dissolved in DMF (14 mL). Sodium hydride (60% suspension in oil, 230.0 mg, 5.75 mmol, 2.0 equiv) was added at room temperature and the reaction mixture was stirred for 15 min until the bubbling ceased. Iodomethane (888 μ L, 14.23 mmol, 5.0 equiv) was added and the vessel was sealed and placed in the microwave reactor. The reaction was heated for 5 min at 130°C with stirring. After the heating was completed the vessel was uncapped and another portion of iodomethane was added to it followed by heating. This process was repeated once to assure full consumption of the starting amide. The reaction was worked up following the standard protocol to afford 590 mg (100%) of the desired tertiary amide as an off-white solid. The compound was used without further purification. *N*-(4-Methoxyphenethyl)-*N*-methylacetamide: ¹H NMR (400 MHz, CDCl₃) δ 7.13 (d, *J* = 8.6 Hz, 2H, minor), 7.06 (d, *J* = 8.6 Hz, 2H, major), 6.85 (d, *J* = 8.6 Hz, 2H, major), 6.83 (d, *J* = 8.6 Hz, 2H, minor), 3.788 (s, 3H, major), 3.786 (s, 3H, minor), 3.54 (dd, *J* = 7.6, 7.6 Hz, 2H, minor), 3.46 (t, *J* = 7.1 Hz, 2H, major), 2.93 (s, 3H, minor), 2.89 (s, 3H, major), 2.79 (t, *J* = 7.1 Hz, 2H, major), 2.78 (dd, *J* = 7.6, 7.6 Hz, 2H, minor), 2.06 (s, 3H, major), 1.85 (s, 3H, minor). ¹³C NMR (100 MHz, CDCl₃) δ 170.7 (minor), 170.5 (major), 158.5 (major), 158.2 (minor), 131.3 (major), 130.3 (minor), 129.83 (two carbons, minor), 129.81 (two carbons, major), 114.2 (two carbons, major), 114.0 (two carbons, minor), 55.35 (major), 55.32 (minor), 52.9 (major), 50.0 (minor), 37.0 (minor), 33.9 (major), 33.5 (minor), 32.9 (major), 22.1 (minor), 21.1 (major).

To a solution of *N*-(4-Methoxyphenethyl)-*N*-methylacetamide (1.05 g, 5.07 mmol) in CH₂Cl₂ (50 mL) at 0°C BBr₃ (1M, 25.33 mL, 25.33 mmol, 5.0 equiv) was added dropwise. The reaction mixture was slowly brought to room temperature and stirred for 2 h then was quenched with H₂O. The aqueous phase was extracted with EtOAc once. The combined organic layers were extracted with 1M NaOH. The aqueous layer was neutralized with 1M

HCl and extracted with EtOAc, dried (MgSO₄) and concentrated *in vacuo* to afford 870 mg (89%) of the unprotected phenol **7** as an off-white solid: ¹H NMR (400 MHz, CDCl₃) δ 8.10 (br s, 1H), 7.03 (d, *J* = 8.5 Hz, 2H, minor), 6.94 (d, *J* = 8.5 Hz, 2H, major), 6.740 (d, *J* = 8.5 Hz, 2H, major), 6.736 (d, *J* = 8.5 Hz, 2H, minor), 3.59 (dd, *J* = 7.3, 7.3 Hz, 2H, minor), 3.48 (dd, *J* = 6.3, 6.3 Hz, 2H, major), 2.97 (s, 3H, major), 2.91 (s, 3H, minor), 2.755 (dd, *J* = 6.3, 6.3 Hz, 2H, major), 2.752 (dd, *J* = 7.3, 7.3 Hz, 2H, minor), 2.03 (s, 3H, minor), 1.65 (s, 3H, major). ¹³C NMR (100 MHz, CDCl₃) δ 171.9 (major), 171.1 (minor), 155.9 (major), 155.1 (minor), 129.84 (major, two carbons), 129.75 (minor, two carbons; minor, one carbon), 128.9 (major), 115.8 (major, two carbons), 115.4 (minor, two carbons), 52.9 (major), 49.3 (minor), 36.4 (minor), 33.5 (major), 33.5 (major), 32.7 (minor), 21.7 (minor), 20.7 (major).

Preparation of *N*-(3,5-Dibromo-4-hydroxyphenethyl)-*N*-methylacetamide (**2**)

Phenol **7** (510.0mg, 2.64 mmol) was dissolved in glacial acetic acid (5.30 mL) and then treated with Br₂ (340 μL, 6.62 mmol, 2.5 equiv) dropwise at room temperature. The reaction went to completion within 3 h, as monitored by TLC and was quenched with 10% aq. Na₂S₂O₃. The product **2** was extracted with EtOAc, dried (MgSO₄) and concentrated *in vacuo* to provide 820 mg (88%) of tan colored material that was used without further purification: ¹H NMR (400 MHz, CD₃OD) δ 7.37 (s, 2H, minor), 7.36 (s, 2H, major), 3.54 (t, *J* = 7.0 Hz, 2H, minor), 3.50 (dd, *J* = 7.7, 7.7 Hz, 2H, major), 2.98 (s, 3H, major), 2.90 (s, 3H, minor), 2.78 (t, *J* = 7.0 Hz, minor), 2.71 (dd, *J* = 7.5, 7.5 Hz, 2H, major), 2.05 (s, 3H, major), 1.87 (s, 3H, minor). ¹³C NMR (100 MHz, CD₃OD) δ 173.3, 151.1 (major), 150.8 (minor), 134.6 (major), 134.1 (minor), 133.9 (two carbons, minor), 133.7 (two carbons, major), 112.3 (two carbons, minor), 112.2 (two carbons, major), 53.3 (minor), 50.5 (major), 37.2 (major), 33.8 (minor), 33.7 (minor), 32.8 (major), 21.7 (major), 21.0 (minor).

N-(4-(3-Aminopropoxy)-3,5-dibromophenethyl)-*N*-methylacetamide (**9**)

Phenol **2** (1.54g, 4.39 mmol) was dissolved in CH₃CN (22 mL) and treated with K₂CO₃ (910 mg, 6.58 mmol, 1.5 equiv) at room temperature. The resulting suspension was heated to 50°C and alkyl bromide **8** (1.04 g, 4.37 mmol, 1 equiv) in CH₃CN (22 mL) was added to it. The reaction was allowed to proceed overnight, then cooled to room temperature and worked-up following the standard protocol to give 1.78 g (80%) of the desired alkylated phenol as an off-white solid. In addition, the aqueous phase was neutralized with 1M HCl and further extracted with EtOAc (3 × 50 mL), dried (MgSO₄) and concentrated *in vacuo* to yield 120 mg of the unreacted starting material (8% recovery). The product was used without further purification: ¹H NMR (400 MHz, CDCl₃) δ 7.36 (s, 2H, major), 7.32 (s, 2H, minor), 4.04 (t, *J* = 5.8 Hz, 2H, minor), 4.03 (t, *J* = 5.8 Hz, 2H, major), 3.53 – 3.42 (m, 4H), 2.94 (s, 3H, minor), 2.93 (s, 3H, major), 2.77 (t, *J* = 7.7 Hz, 2H, minor), 2.75 (dd, *J* = 7.7, 7.7 Hz, 2H, major), 2.08 (s, 3H, major), 2.04 (m, 2H), 1.99 (s, 3H, minor), 1.44 (s, 9H). ¹³C NMR (100 MHz, CDCl₃) δ 170.7 (major), 170.4 (minor), 156.2, 152.1 (minor), 151.6 (major), 138.1 (major), 137.0 (minor), 133.1 (two carbons, major), 132.9 (two carbons, minor), 118.6 (two carbons, minor), 118.3 (two carbons, major), 79.3, 71.4, 52.2 (minor), 49.5 (major), 38.2, 37.1 (major), 33.8 (minor), 33.6 (minor), 32.6 (major), 30.2, 28.6 (three carbons), 22.1 (major), 21.3 (minor). *tert*-Butyl-3-(2,6-dibromo-4-(2-(*N*-methylacetamido)ethyl)-phenoxy)propylcarbamate (1.78 g, 3.50 mmol) was dissolved in 1,4-dioxane (40 mL) and the resulting solution was placed in an ice bath. Concentrated HCl (500 μL, 37%) was added dropwise and the bath was removed. The reaction was completed in 1.5 h as revealed by TLC. The pH of the solution was adjusted to 7 by adding 1M NaOH and extracted with EtOAc (3 × 100 mL), dried (MgSO₄) and concentrated *in vacuo* to yield 1.37 g (96%) of **9**: ¹H NMR (400 MHz, DMSO-*d*₆) δ 8.40 (br s, 2H), 7.63 (s, 2H, major), 7.53 (s, 2H, minor), 4.95 (br s, 1H), 3.98 (t, *J* = 5.9 Hz, 2H), 3.44 (m, 2H), 3.01 (t, *J* = 7.4 Hz, 2H), 2.93 (s, 3H, minor), 2.786 (s, 3H, major), 2.785 (m, 2H, minor), 2.69 (dd, *J* = 7.5, 7.5 Hz, 2H, major), 2.05 (m, 2H), 1.94 (s, 3H, minor), 1.86 (s, 3H, major). ¹³C NMR (100

MHz, DMSO- d_6) δ 169.5 (minor), 169.3 (major), 150.6 (major), 150.4 (minor), 139.0 (minor), 138.6 (major), 133.3 (two carbons, major), 132.9 (two carbons, minor), 117.3 (two carbons, major), 117.2 (two carbons, minor), 70.59 (major), 70.53 (minor), 51.0 (minor), 47.7 (major), 36.5, 35.7 (minor), 32.6 (major), 32.3 (minor), 31.4 (major), 28.4, 21.6 (minor), 20.9 (major).

Preparation of 3-Methyl-3H-purin-6(9H)-one (12)

Sodium metal (1.47 g, 64.0 mmol, 2 equiv.) was dissolved in absolute EtOH (60.0 mL) to prepare 1.0M sodium ethoxide in EtOH. Compound **10** (4.55 g, 32.0 mmol) was added to the solution at room temperature and stirred until all material dissolved. A solution of thiourea **11** (2.88 g, 32.0 mmol) in 42 mL of 2-methoxyethanol was combined with the reaction mixture and the resulting mix was refluxed for 5.5 h. After cooling to 0 °C, 1.0 M HCl was added dropwise until a pH of 2 was obtained. A blue-grey solid formed which was filtered, washed with small portion of ice-cold H₂O, and dried in oven overnight to yield 5.10 g (86%) of 6-amino-2-mercapto-1-methyl-5-nitrosopyrimidin-4(1H)-one. The compound was used without purification.

6-Amino-2-mercapto-1-methyl-5-nitrosopyrimidin-4(1H)-one (5.10g, 27.4 mmol) was dissolved in 100 mL 1.0 M NaOH and sodium dithionite (17.65 g, 101.4 mmol, 3.7 equiv.) was added. After 1.5 h at room temperature, the solids were filtered off and dried in oven to obtain 4.7 g of 5,6-diamino-2-mercapto-1-methylpyrimidin-4(1H)-one in quantitative yield. These steps were repeated to provide sufficient material for the last stage of the synthesis of **12**.

5,6-Diamino-2-mercapto-1-methylpyrimidin-4(1H)-one (7.74 g, 44.95 mmol) was dissolved in DMF (10 mL) and trimethyl orthoformate (73.76 mL, 674.18 mmol, 15.0 equiv.). The mixture was heated at reflux for 5 days. After full conversion of the starting material was achieved (LC-MS analysis) the reaction mixture was concentrated and the residual material was recrystallized from water to obtain 8.19 g (100%) of compound **12**: ¹H NMR (400 MHz, DMSO- d_6) δ 13.84 (br s, 1H), 12.47 (br s, 1H), 8.17 (s, 1H), 3.78 (s, 3H).

3-Methyl-3H-purin-6(9H)-one (13)

Raney nickel (16.0 g) was washed three times with 2-methoxyethanol, then suspended in minimal H₂O. A solution of purinone **12** (8.20 g, 45.0 mmol) in 1.0 M NaOH (55.0 mL) was added to the Raney nickel slurry. The reaction mixture was heated to 80 °C for 3 h until all starting material was consumed as determined by TLC. The room temperature mixture was filtered through a pad of Celite which was washed with H₂O and 2-methoxyethanol. The filtrate was concentrated *in vacuo*. 6.76 g of **13** was obtained which was used without purification: ¹H NMR (400 MHz, DMSO- d_6) δ 7.86 (s, 1H), 7.32 (s, 1H), 3.66 (s, 3H).

6-Chloro-3-methyl-9H-purin-3-um chloride (14)

To purinone **13** (100 mg, 666 μ mol) was added pyridine (5.45 mL) and phosphorus pentasulfide (454.5 mg, 2.04 mmol, 3.1 equiv.) and the reaction was refluxed for 4 h and then concentrated *in vacuo*. To the resulting brown solid, boiling water was added. Suspension formed which was allowed to cool to room temperature. The solids were filtered off and dried in oven overnight to deliver 56 mg (51%) of the corresponding purine-6(9H)-thione: ¹H NMR (400 MHz, DMSO- d_6) δ 13.65 (br s, 1H), 8.42 (s, 1H), 8.36 (s, 1H), 3.86 (s, 3H).

3-Methyl-3H-purine-6(9H)-thione (5.34 g, 32.13 mmol) was combined with 2.5 % aqueous NaOH (66.74 mL, 1.3 equiv.) and iodomethane (4.00 mL, 64.26 mmol, 2.0 equiv.) at room temperature. The resulting mixture was stirred for 2 h at room temperature during which

time a white solid precipitated. The precipitate was filtered off and dried to deliver 3.56 g (61%) of the methylthio product: ^1H NMR (400 MHz, DMSO- d_6) δ 8.84 (s, 1H), 8.15 (s, 1H), 4.11 (s, 3H), 2.74 (s, 3H).

Chlorine gas was bubbled through ice-cold anhydrous MeOH (2.5 mL). To this saturated solution, 3-methyl-6-(methylthio)-3*H*-purine (215.0 mg, 1.19 μmol) in MeOH (1.5 mL) was slowly added, after which Cl_2 was bubbled through the solution for an additional 10 min. The resultant precipitate was filtered, washed with cold benzene and dried *in vacuo* to afford a light yellow solid salt **14** (70 mg, 29%).

Preparation of Aphrocallistin (1)

A solution of amine **9** (139 mg, 341 μmol) in 1-butanol (5 mL) and Hünig's base (130 μL , 751 μmol) was cooled to 0 °C. To this solution, salt **14** (70 mg, 341 μmol) was added and stirred at 0 °C for 1 h after which the solution was warmed to room temperature and stirred for 16 h. The reaction was quenched with brine and the pH was adjusted to 9 with saturated Na_2CO_3 solution and extracted with EtOAc (3 \times 20mL), dried (MgSO_4), and concentrated *in vacuo*.

Salt formation

Crude **1** was dissolved into anhydrous methanol (5 mL) and TFA (100 μL) was added. The solution was stirred for 30 min at room temperature. The reaction was concentrated *in vacuo*, redissolved in MeOH (5 mL) and concentrated *in vacuo* (2 \times). The resulting yellow oil was purified via reverse phase HPLC to obtain 70 mg of **1-TFA** (31% unoptimized yield). Spectral analysis (^1H -NMR and LCMS- See Supporting Information) confirmed the synthetic material to be identical with the natural product.

Cytotoxicity assays

Aphrocallistin was analyzed as to its effects on proliferation of A549 human lung adenocarcinoma, AsPC-1 human pancreatic adenocarcinoma, PANC-1 human pancreatic carcinoma, BxPC-3 pancreatic adenocarcinoma, MIA PaCa2 human pancreatic carcinoma, DLD-1 human colorectal adenocarcinoma, NCI-ADR-RES (formerly MCF-7/ADR) human ovarian carcinoma and the Vero monkey kidney cell lines. The A549 (ATCC# CCL-185), PANC-1 (ATCC# CRL-1469), DLD-1 (ATCC# CCL-221), AsPC-1 (ATCC# CRL-1682), BxPC-3 (ATCC# CRL-1687), MiaPaca2 (ATCC# CRL-1420) and Vero (ATCC# CCL-81) cell lines were obtained from the American Type Culture Collection (Rockville, MD). The NCI/ADR-RES cell line was obtained from the NCI Cancer Cell Repository. Assays were run using protocols described previously.¹³ All samples were assayed a minimum of three times to derive the final IC_{50} value.

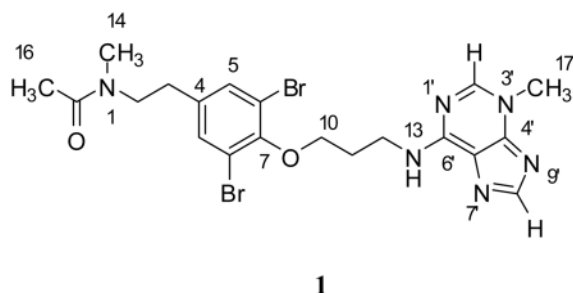
Cell Cycle Analysis

PANC-1 human pancreatic carcinoma cells were used as targets to observe the effects of aphrocallistin on the cell cycle. Cell cycle analysis was performed as follows: PANC-1 cells were incubated in tissue culture media (TCM = Roswell Park Memorial Institute (RPMI) medium 1640 supplemented with 100 U/mL penicillin, 100 mg/mL streptomycin, 60 mg/mL L-glutamine, 18 mM HEPES, 0.05 mg/mL gentamicin, 100 $\mu\text{g}/\text{mL}$ sodium pyruvate, 2.5 mg/mL glucose and 10% fetal bovine serum) at 37°C in 5% CO_2 in air in the presence or absence of varying concentrations of aphrocallistin, methanol (vehicle control) or paclitaxel (positive control) for 24 h. At the end of this incubation, cells were harvested, fixed in ethanol and stained with 0.02 mg/mL of propidium iodide (P.I.) together with 0.1 mg/mL of RNase A. Stained preparations were analyzed on a BD FACSCanto. 10,000 events were

collected per sample. Raw histogram data was further analyzed using the DNA analysis software ModFit (Verity, Topsham, ME).

Antimicrobial testing

The antimicrobial activity of **1** was determined through the use of broth dilution assays using the following strains identified by their ATCC catalog number: *Candida albicans* 44506; methicillin-resistant *Staphylococcus aureus* 700787; and *Pseudomonas aeruginosa* 27853. Assays were performed as standard microtiter assays in a total volume of 50 μ L at a seeding density of 10^3 /mL. *C. albicans* was grown in Sabouraud Dextrose Broth. The bacteria were grown in cation-supplemented Mueller-Hinton broth. Assays were performed at 37°C. The level of growth was determined after 24 h with the minimum inhibitory concentration being defined as the lowest concentration of the compound which completely inhibited growth of the test strain. **1** gave MICs of 250 μ g/mL against *C. albicans* and methicillin-resistant *S. aureus* and >500 μ g/mL against *P. aeruginosa*.



Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The work described in this paper was funded in part by NIH grant number CA-93455 and the State of Florida Fish and Wildlife Research Institute Drug Discovery from Florida's Oceans contract. We acknowledge a grant from HRSA which funded in part the purchase of the JEOL NMR and FACSCanto flow cytometer used in this work. We thank C. Cody at JEOL for high resolution mass spectral data; Dr. H. M. Reiswig of the Department of Biology, University of Victoria, Victoria, British Columbia, Canada for the sponge identification; and the Burnham Exploratory Pharmacology group (M. Vicchiarelli and A. Mangravita-Novo) for performing the in vitro ADME profiling assays. This is Harbor Branch Oceanographic Institute contribution number 1733.

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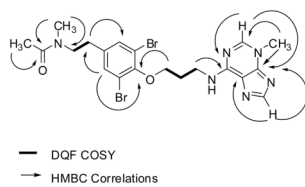


Figure 1.
Selected ^1H - ^{13}C HMBC correlations for aphrocallistin (**1**).

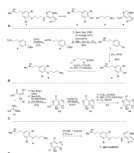


Figure 2.
(A) Retrosynthetic analysis of aphrocallistin (**1**); (B) Synthesis of tyramine moiety **9**; (C) Synthesis of purine **14**; (D) Final step in the synthesis of **1**.

Table 1

NMR Spectroscopic Data (600 MHz, CD₃OD) for Aphrocallistin (1)

Position	Aphrocallistin major rotamer				Minor rotamer			
	δ_C mult	δ_N	δ_H (<i>J</i> in Hz)	COSY	HMBC ^a	NOESY	δ_C	δ_H (<i>J</i> in Hz)
1		111						
2	50.1, CH ₂		3.50, t (7.6)	3	3, 4, 14, 15	3, 5, 9, 14	53.0	3.55, t (6.9)
3	33.0, CH ₂		2.74, t (7.6)	2	1, 2, 4, 5, 9	2, 5, 9	33.9	2.82, t (6.9)
4	140.0, qC						139.4	
5 and 9	134.4, CH		7.45, s		3, 6, 7, 5 or 9	2, 3	134.6	7.47, s
6 and 8	118.9, qC						119.1	
7	152.8, qC						153.1	
10	71.9, CH ₂		4.11, m	11	7, 11, 12	11	71.9	4.11, m
11	30.5, CH ₂		2.27, m	10, 12	10, 12, 13	10, 12	30.5	2.27, m
12	40.4, CH ₂		4.04, m	11	10, 11, 6'	11	40.4	4.04, m
13		106						
1'		227						
2'	149.7, CH		8.64, s		1', 3', 4', 6', 17	17	149.7	8.64, s
3'		149						
4'	149.2 br, qC						149.2	
5'	112.6 br, qC						112.6	
6'	153.5, qC						153.5	
7 ^b								
8'	145.6 br, CH		8.42, s		4', 5'		145.6	8.42, s
9 ^b								
14	36.9, CH ₃		2.98, s		1, 2, 15	3, 16	33.8	2.88, s
15	173.4, qC						173.3	
16	21.6 CH ₃		2.02, s		1, 15	14	20.9	1.84, s
17	36.8, CH ₃		4.06, s		2', 3', 4'	2'	36.8	4.06, s

^aHMBC correlations optimized for 8Hz are from proton(s) stated to the listed carbon or nitrogen.^bnot observed

Table 2

Cytotoxicity Data for Aphrocallistin (1) in a Panel of Tumor Cell Lines

Cell line	Aphrocallistin (1) IC ₅₀ μM ± SE
A549	15.9 ± 2.1
AsPC-1	>100
BxPC-3	70.9 ± 2.2
DLD-1	7.5 ± 2.0
MiaPaca-2	26.6 ± 2.0
NCI/ADR-Res	34.3 ± 2.0
PANC-1	22.8 ± 2.0
Vero	76.1 ± 2.0