

Using Enzyme Assays to Evaluate the Structure and Bioactivity of Sponge-Derived Meroterpenes

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Enzyme screening of crude sponge extracts prioritized a 2005 Papua New Guinea collection of *Hyrtios* sp. for further study. The MeOH extract contained puupehenone and four puupehenone analogues (**1**, **2**, **3**, **5**, and **7**) along with a new diastereomer, 20-epi-hydroxyhaterumadienone (**4**), and a new analogue, 15-oxo-puupehenic acid (**6**). The drimane terpene core of **4** and **6** was rapidly dereplicated, and the modified Mosher's method identified **4**, while 1D and 2D NMR techniques were used to solve **6**. These compounds plus noteworthy repository natural products and standards were tested against three lipoxygenase isozymes, human 5-, 12-, and 15-lipoxygenases. Significant potency and selectivity profiles were exhibited in the human 5-lipoxygenase assay by puupehenone (**1**) and jaspaginol (**9**) and structural factors responsible for activity identified.

In recent years our quest to exploit sponge-derived scaffolds has been guided by four types of screening paradigms. These range from (a) employing LC-MS to guide isolation of major and minor metabolites,¹ (b) using cell-based primary screens to detect compounds selective against solid tumors,² (c) probing for disruption of protein–protein interactions (PPI) between Bcl-2 family proteins with the pro-survival BH3-domain binding proteins,³ and (d) challenging enzyme targets relevant to anticancer therapeutic lead discovery.⁴ The work described herein initially began using paradigm (d) against histone deacetylase (HDAC). Our focus first involved a repository extract hit, suspected to contain meroterpenes that were active against class I HDAC enzymes; subsequently it shifted to profiling selected meroterpenoids challenged with human 5-lipoxygenase (5-hLO). The sponge extract of a *Hyrtios* sp. (coll. no. 05409 from Papua New Guinea) was active (IC₅₀ < 0.08 μg/mL) in the former screen, and a mini-library of meroterpenes displayed activity (at μM levels) in the latter. HDAC is recognized as a validated target in screening programs, and inhibitors of its various isoforms are showing promise as chemotherapeutics for solid tumor and hematologic malignancy.⁵ Especially significant is suberoylanilide hydroxamic acid (SAHA, Vorinostat, Zolinza), which represents the first FDA-approved drug to treat advanced cutaneous T-cell lymphoma via HDAC inhibition.⁶ Similarly, 5-hLO is emerging as a significant enzyme target for chemotherapeutic intercession in a number of diseases.⁷ One FDA-approved drug for the treatment of asthma via 5-hLO inhibition is zileuton (Zyflo).⁸ The goal of this project was (1) to isolate, characterize, and screen the bioactive constituents of the extract and (2) identify compounds from our repository demonstrating selectivity for 5-hLO in a panel including 12- and 15-hLO isozymes. Our findings are reported below.

Results and Discussion

Before launching the drive to characterize the constituents of the active *Hyrtios* MeOH extract, it was essential to map out an approach involving aggressive dereplication. Our two prior evaluations of the bioactive products of *Hyrtios* sp. collections, with similar morphological properties to those of the organism under investigation, utilized sponges from different Indo-Pacific sites

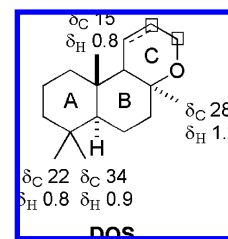


Figure 1. ABC ring drimane oxygenated shikimate (**DOS**) core (□ indicating non-terpene portion) with corresponding diagnostic CH₃ δ_C/δ_H NMR data.

including Papua New Guinea (coll. no. 99140)⁹ and Indonesia (coll. no. 95653).¹⁰ In both cases we observed these extracts to be prolific in their content of puupehenone analogues. In this regard, the haterumadienone¹¹ family represents another related biosynthetic skeleton. Overall, as first pointed out by Scheuer,¹² such compounds have conserved ABC rings comprised of a drimane fused to an oxygenated shikimate (**DOS**, Figure 1). We have found that such compounds can be quickly recognized in crude extracts by the NMR signature peaks for the four methyl groups attached to quaternary sites as illustrated in Figure 1. Two other important facts are as follows: (1) the absolute configuration of the **DOS** terpene core has been established as 5*S*, 8*S*, 9*R*, 10*S*¹³ and (2) the puupehenone family (see **1**) differs from the haterumadienone family (see **3**) by the size of the carbocyclic D ring, C₆ in the former and C₅ in the latter.

The above background plus our prior experience with extracts containing puupehenone analogues provided the basis for the design of the subsequent isolation and dereplication efforts of new and known puupehenone/haterumadienone compounds. Eventually we discovered that the D ring was the locus for unprecedented substitution patterns. The core **DOS** δ_{C,H} chemical shifts were used to interrogate each of the metabolites during the purification process, as shown in Figure 2 (see also Figure S18, Supporting Information). The known puupehenones were easily identified using the signature NMR data of Figure 1 accompanied by an analysis of their molecular formulas established by ESITOFMS. The CH₂Cl₂-soluble MeOH extract (coded FD) contained puupehenone (**1**) (*m/z* 329.2033 [M + H]⁺, C₂₁H₂₉O₃),¹² 15,20-dimethoxypuupehenol (**2**) (*m/z* 375.2456 [M + H]⁺, C₂₃H₃₅O₄),¹⁰ 20-hydroxyhaterumadienone (**3**) (*m/z* 317.2110 [M + H]⁺, C₂₀H₂₉O₃),¹⁴ the new diastereomer 20-epi-hydroxyhaterumadienone (**4**) (*m/z* 317.2113 [M + H]⁺, C₂₀H₂₈O₃), puupehedione (**5**) (*m/z*

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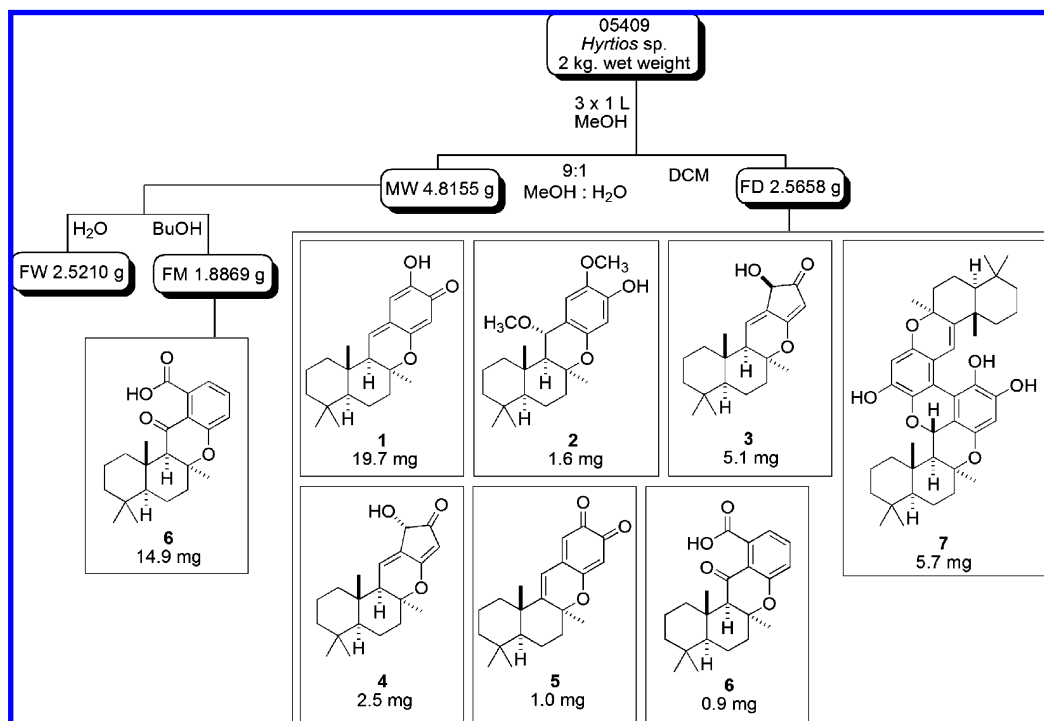
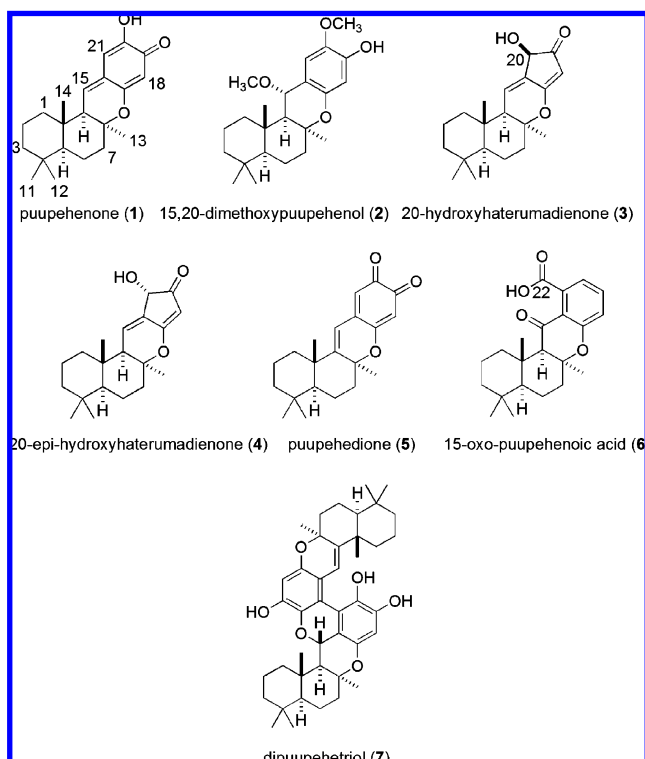


Figure 2. Isolation scheme for compounds 1–7.

327.1889 $[M + H]^+$, $C_{21}H_{27}O_3$),¹⁵ a new analogue identified as compound **6** (m/z 357.2107 $[M + H]^+$, $C_{22}H_{29}O_4$), and dipuuphetriol (**7**) (m/z 655.3922 $[M + H]^+$, $C_{42}H_{55}O_6$).¹⁶ Structures of the known compounds were established by rigorous comparison of their NMR and MS properties to those in the literature.



Initially a compound having the framework consistent with known 20-hydroxyhaterumadienone (**3**)¹⁴ was isolated from the FD fraction coded as L2 (28.4 mg, see Figure S18, Supporting Information). However, it was isolated as a mixture containing a new diastereomer (deduced by MS formula analysis as shown

above) ultimately concluded to be **4**. A clearer view of this circumstance was obtained by the 1H NMR spectrum of the sample obtained from a second chromatographic step, coded as FD L2 H5, shown in Figure 3, emphasizing the doubling of signals for H-15, H-18, and H-20, in relative proportions of 2:1. The final purification of this fraction utilized chiral chromatography to afford **3** (5.1 mg) and **4** (2.5 mg).

The low-field region of the 1H NMR spectrum of **3** and **4** showed the greatest difference in shifts between isostructural protons; therefore it was assumed that their configuration differences were at C-20 rather than at C-8 or C-9. If this assumption was correct, then we could determine the absolute configuration at C-20 in each diastereomer by application of the modified Mosher's method.^{17,18} Derivatization of **3** with (*R*)- and (*S*)-(+)-methoxy- α -trifluoromethylphenylacetyl chloride ((*R*) and (*S*)-MTPA-Cl) afforded *S*-MTPA-**3** (**3'**) and *R*-MTPA-**3** (**3''**), respectively, as seen in Figure 4, and a similar treatment of **4** provided **4'** and **4''**. Surprisingly, the straightforward analysis of $\Delta\delta^{SR}$ data shown in Figure 4 (also see Figure S13, Supporting Information) could not be completed until an error in the literature for **3a'** (= our **3'**) and **3a''** (= our **3''**) was corrected, as now shown in Figure 4.¹⁴ The error was resolved by accurate correlation, using gHMQC NMR data, of the low-field δ_H values for all four compounds (**3'**, **3''**, **4'**, **4''**), Figures S19–S23, Supporting Information) to their correct structural positions. The assembled data supported the conclusions that **3** possessed the 20*R* configuration and **4** was the new 20*S* analogue. Further, NOESY correlations observed between H-3 (ax) and H-5, H-5 and H-9, and H-9 and H₃-13 confirmed the same ABC ring relative configuration between **1** and **4**. These data, plus the fact that **3** and **4** were isolated with puupehenone (**1**), led to the assumption that all had identical absolute chirality for the ABC rings.

The second new compound to be isolated, **6**, was initially obtained in small amounts from FD partition chromatographic fractions (see Figure S18, Supporting Information), coded FD L5 H2 (0.6 mg) and FD L6 H2 (0.3 mg). A much larger sample was obtained from the more polar partition fractions (coded MW, leading to the fraction coded FM). It took two rounds of HPLC to yield FM P10 H9 (14.9 mg), clearly containing **6**, as evidenced

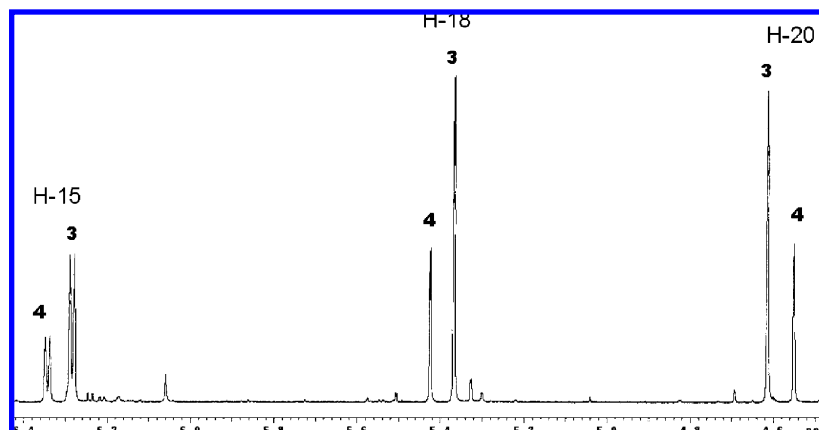


Figure 3. Downfield ^1H NMR (600 MHz, CDCl_3) spectra of FD L2 H5 with **3** and **4** in a 2:1 ratio, respectively.

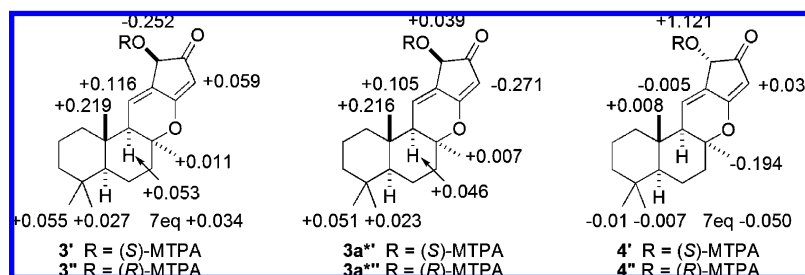


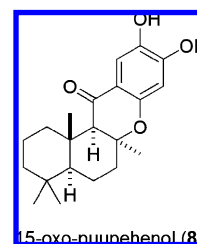
Figure 4. Modified Mosher's method analysis, $\Delta\delta^{SR}$, of **3**, **3a**, and **4**. *Literature data of $\Delta\delta^{SR}$ at C-18 and C-20 of **3a** were inadvertently switched in the original publication.¹⁴

Table 1. ^1H , ^{13}C , gCOSY, gHMBC, and gNOESY NMR Data of 15-Oxo-puuphehoic Acid (**6**) in CDCl_3 at 600 MHz (^1H) and 150 MHz (^{13}C)

pos #	δ_{C} , type ^b	δ_{H} , m., (J [Hz])	gCOSY	gHMBC (^1H to ^{13}C)	NOESY
1 ax	40.0, CH_2	1.21, m	2	2, 10	5, 9
1 eq		1.62, m			
2	18.1, CH_2	1.66, m	1, 3	1	
3 ax	41.4, CH_2	1.18, m	2	1, 4, 12	1ax, 5, 11
3 eq		1.42, m		2	
4	33.4, qC				
5	54.2, CH	0.91, dd (11.5, 2.5)	6	4, 6, 14	1ax, 9, 11
6	18.3, CH_2	1.44, m	5, 7	7	
7 ax	39.5, CH_2	1.57, ddd (14, 11, 2.5)	6	5, 9, 17 ^a	9
7 eq		2.26, dt (14, 2.5)		8, 13	
8	80.4, qC				
9	64.9, CH	2.04, s	1, 5, 10, 13, 15, 16	1ax, 5, 7 ax, 13	
10	38.7, qC				
11	33.7, CH_3	0.92, s		3, 5, 12	5, 9
12	21.9, CH_3	0.84, s		3, 11	
13	26.4, CH_3	1.22, s		7, 8, 9	9
14	15.4, CH_3	0.88, s		1, 9, 10	
15	196.2, qC				
16	119.2, qC				
17	161.0, qC				
18	121.3, CH	7.01, dd (8, 1.5)	19	15 ^a , 16, 17, 20	20
19	135.3, CH	7.48, dd (8, 7.5)	18, 20	16 ^a , 17, 20, 21, 22 ^a	19, 21
20	122.2, CH	7.27, dd (7.5, 1.5)	19	16, 17 ^a , 18, 19, 22	20
21	132.6, qC				
22	171.9, qC				

^a gHMBC at 600 MHz optimized for 5 Hz correlations in CDCl_3 . ^b Carbon type determined by a DEPT NMR experiment.

by the 1,2,3-trisubstituted benzene D ring ^1H NMR signals shown in Table 1, consisting of resonances at δ 7.48 (dd, 8, 7.5 Hz), 7.27 (dd, 7.5, 1.5 Hz), and 7.01 (dd, 8, 1.5 Hz). In addition, the NMR shifts (δ_{C} and δ_{H}) for **6** were nearly identical to those of the known 15-oxo-puuphehoic acid (**8**)¹⁵ for the ABC rings and their corresponding substituents. This observation established both the framework and relative configurations of all moieties in this portion of the structure. Also evident from a plot of $\Delta\delta_{\text{C}}$ shift differences for **6** versus **8** in Figure 5 was that the locus of structural differences seemed confined to the D ring. Supporting this conclusion were additional relationships for B, C, and D ring residues of **6** established through gHMBC correlations from H-9 (δ_{H} 2.04) to C-15 (δ_{C} 196.2) and to C-16 (δ_{C} 119.2).



The final step in establishing structure **6** involved defining the carboxylate residue and its environment relative to the D ring. The two possible sets of substructures are shown in Figure 6 and

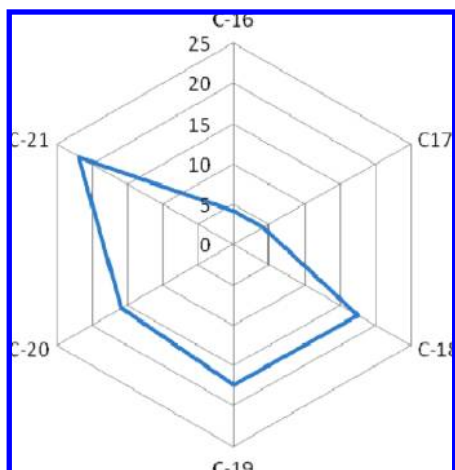


Figure 5. ^{13}C NMR $\Delta\delta$ shift differences between 15-oxo-puuephenoic acid (**6**) and 15-oxo-puuephenol (**8**), with $\Delta\delta_{\text{C}}$ differences < 2 ppm not included.

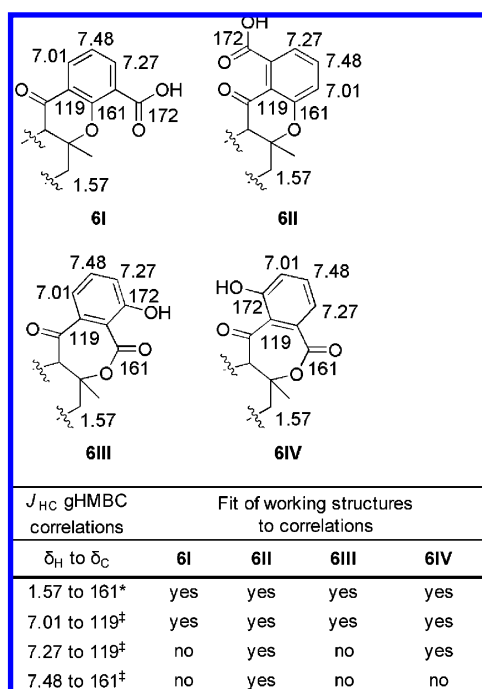


Figure 6. Analysis of working structures for the CD rings of **6** based on J_{HC} correlations expected for each working structure. * $^4J_{\text{HC}}$ and $^{\ddagger,3}J_{\text{HC}}$ correlations requiring $\delta_{\text{H/C}}$ placement as shown.

consisted of (a) **6I** and **6II** or (b) **6III** and **6IV**. The assignments of δ_{C} 's alongside each of the structures were based on gHMBC results rather than chemical shift analogies to model compounds. One anchor point for this process was the array consisting of the aromatic AMX pattern protons. A second element involved establishing the relative proximity of carbons separated by two to four bonds with specific H–C correlations including (a) from H-7ax (δ_{H} 1.57) to δ_{C} 161 and (b) from H-Ar (δ_{H} 7.27) to δ_{C} 172. Four distinct J_{HC} gHMBC correlations were used (see Figure 6 and Table 1), and only substructure **6II** was consistent with these data. Finally, the *trans-cis* fused ABC ring junction proposed above was confirmed by the NOESY data (shown in Table 1), and absolute configurations shown here for **6** were based on the biosynthetic analogy to **1**, noted above.

At completion of the purifications and characterizations of compounds **1–7** (see Figure 2), efforts were made to subject these samples to the HDAC inhibition assay. Unfortunately, the overall

Table 2. Comparison of hLO IC_{50} Values (μM) of Natural Products and Standards

compound	12-hLO	15-hLO-1	5-hLO	12-hLO/ 5-hLO	15-hLO/ 5-hLO
1	22 ± 3	6.0 ± 1	0.68 ± 0.1	32	9
3	>150	>150	>50	~3	~3
5	18 ± 2	7.0 ± 1	4.6 ± 2	4	1.5
6	>150	>150	>150	n.s.	n.s.
9	>50	19 ± 3	0.45 ± 0.08	>111	42
10	7.9 ± 0.8	4.7 ± 0.3	14 ± 2	0.6	0.3
11	31 ± 3	16 ± 3	18 ± 8	2	0.9
12	5.9 ± 0.8	1.1 ± 0.1	0.11 ± 0.03	54	11
13	active ^{a,b}	NA ^a	0.5 ± 0.1 ^a	>200	>200

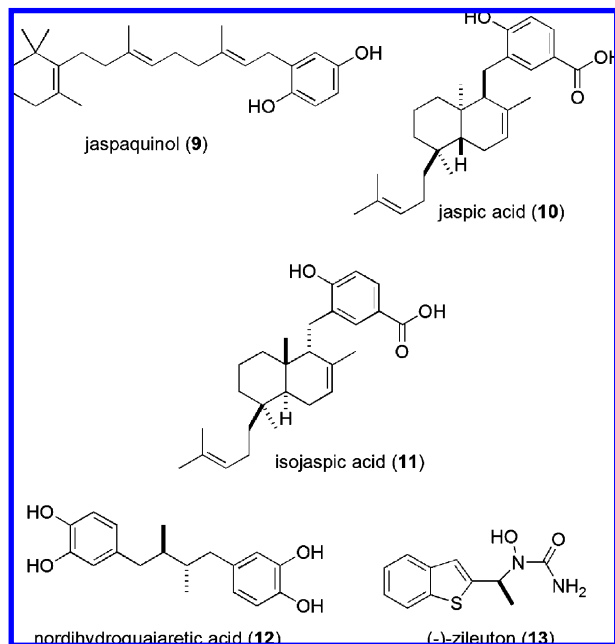
^a Data from literature.²⁰ ^b 19% inhibition at 100 μM .

outcome was incomplete due to the discontinuation of the assay by our therapeutics collaborators and disparate Biomol kit test results. Examples of the positive HDAC inhibition activities observed on chromatographic fractions are as follows: (a) the fraction coded L2 (28.4 mg), containing **1** (1.9 mg), **3** (5.1 mg), and **4** (2.5 mg), exhibited $\text{IC}_{50} = 7 \mu\text{g/mL}$; (b) the fraction coded L3 (30.0 mg), a source of **2** (0.7 mg) and a mixture of **3** and **4** (0.2 mg), exhibited $\text{IC}_{50} = 3 \mu\text{g/mL}$; (c) the fraction coded as L4 (52 mg), affording **1** (6.2 mg) and **2** (0.9 mg), exhibited $\text{IC}_{50} = 2 \mu\text{g/mL}$; and (d) the fraction coded L5 (151.8 mg), consisting of 95% pure puuephenone (**1**) (11.6 mg, from partial chromatography), exhibited $\text{IC}_{50} = 0.9 \mu\text{g/mL}$. Recognizing that there are possible complications from impurities, these data collectively indicate that the puuephenone core in **1** may be a reasonably active HDAC inhibitor. However, as noted above, no follow-up assay data were obtained on the pure compounds.

We then turned to selecting meroterpenoids from our repository to accompany the compounds isolated above in a further survey using a panel of hLOs. The goal was to identify compounds that might be especially potent and/or selective against the panel of three hLO isoforms. Two new developments were represented in our redesigned hLO evaluation. First, the lipoxygenase assay was altered to flag compounds that were promiscuous inhibitors. This involved the testing of effects in the presence of detergent (Triton X100). Second, a new assay arm involved 5-hLO in addition to 12- and 15-hLOs.

Overall, eight compounds were tested, and the results appear in Table 2. These are as follows: set I (puuephenone and analogues) **1** (previous IC_{50} [μM] 15-hLO = 0.8; 12-hLO = 8.3),⁹ **3**, **5**, and **6**; set II (sesterterpenes from the repository) jaspaquinol (**9**, previous IC_{50} [μM] 15-hLO = 0.3; 12-hLO = 4.5),⁹ jaspic acid (**10**, previous IC_{50} [μM] 15-hLO = 1.4; 12-hLO = 0.7),⁹ and isojaipic acid (**11**, previous IC_{50} [μM] 15-hLO = 5.3; 12-hLO = 3.1);¹⁹ and set III (standards) NDGA (**12**, previous IC_{50} [μM] 15-hLO = 0.6; 12-hLO = 42).⁹ The standard **12** exhibited the expected behavior of potency and selectivity ratios (54 and 11 shown in Table 2) in the hLO panel.^{20,21} The literature data for zileuton (**13**, Zylflo),²⁰ not tested here but an FDA-approved therapy for the treatment of asthma via 5-hLO inhibition, provides an important reference point. Encouraging potency and selectivity profiles were exhibited in the 5-hLO assay by puuephenone (**1**) and jaspaquinol (**9**). First, **9** exhibited outstanding selectivity for 5-hLO versus 12-hLO (ratio >111-fold) and very good selectivity versus 15-hLO (ratio = 42), while **1** exhibited very good selectivity for 5-hLO versus 12-hLO (ratio = 32) but less selectivity versus 15-hLO (ratio = 9). Second, we revisited our previous hypothesis and experimental evidence that suggested **1**, **9**, and **12** were reductive LO inhibitors.^{9,21} The new data obtained here support this hypothesis for **9** and **12**, but add an additional perspective of **1**. The difference in 5-hLO potency for **1** and its oxidized analogue **5** presents a pattern expected for reductive inhibitors and adds support to our previous hypothesis.^{20,21} However, the identical potency profile observed for this pair against 5-hLO and 12-hLO is unexpected and suggests additional mechanisms may be operating. Furthermore, the new data indicate that

structural factors beyond those present in the DOS framework of Figure 1 are essential for activity, as shown by the inactivity of **3** and **6**. The circumstance of isojaspic acid (**11**) being slightly less potent than jaspic acid (**10**) seems directly a function of the change in relative configuration of the fused decalene ring. Finally, in the cases of **1**, **9**, **10**, and **11**, the addition of Triton X100 lowers potency (data from this study [Table 2] versus our previous work noted above), possibly due to the formation of aggregates that nonspecifically inhibit the hLOs. As a different observation, **1** exhibited modest selectivity against solid tumor cells (human lung adenocarcinoma cells over human leukemic lymphoid cells) in the soft agar disk diffusion assay (DDA, Table S4, Supporting Information).



There are several conclusions to be drawn from this study. Interestingly, no new puupehenone analogues have been reported in the last five years. The new compound isolated here expands the list of such frameworks to a total count of 17.²² Widespread interest in puupehenones has driven a range of biological activity studies that have been accompanied by the development of synthetic strategies to access this structurally interesting chemotype (Table S1, Supporting Information).^{23–31} In the future we plan, by semisynthesis, to create potentially even more selective 5-hLO inhibitors by fusing the structures of puupehenone (**1**) and jaspaquinol (**9**) through their shikimate residues.

Experimental Section

General Experimental Procedures. The optical rotations were determined on a Jasco DIP 370 polarimeter. UV data were obtained on an Agilent 8453 UV/vis spectrophotometer, and IR data were collected on a Perkin-Elmer Spectrum One FTIR spectrometer. All NMR spectra were recorded in CDCl₃ at 600/150 MHz with a 5 mm triple resonance (HCN) cold probe. Chemical shifts are reported in ppm relative to CDCl₃ (δ_H 7.27 and δ_C 77.2). Low- and high-resolution mass measurements were obtained by ESI-TOF mass spectrometry. Preparative separation was carried out two ways: The first is utilizing a Shimadzu SCL-1014 system controller, with Shimadzu LC-8A pumps and a Phenomenex Synergy RP 10 μ m column, 250 \times 21.2 mm. Peak detection employed a Sedex 75 evaporative light scattering detector (ELSD) with fraction collection into disposable culture tubes (25 \times 150 mm) using a Gilson 215 liquid handler (Figure S14, Supporting Information). Concurrent MS data were collected on an Applied Biosystems Mariner Biospectrometry workstation. The second method involved using a Waters 600E system controller and pumps with a Prep LC 25 mm radial compression column using 25 \times 100 mm C18 Nova-Pak HR16 (6 μ m) cartridges. Peak detection utilized both ELSD and UV (254

nm). Semipreparative RP HPLC employed a Phenomenex Synergy Hydro-RP 4 μ m column, 10 \times 250 mm, and UV detection (254 nm). Chiral chromatography utilized a Diacel Chemical Industries Chiralcel OJ-RH 5 μ m column (150 \times 4.6 mm i.d.).

Animal Material. The *Hyrtios* sp. (UCSC coll. no. 05409, 2 kg wet weight) was collected by SCUBA in November 2005 at Pocklington Reef (10°48.074' S, 155°44.621' E), between 60 and 45 feet on a reef wall. The taxonomic identification of *Hyrtios* sp. (POR 3407) was performed by Dr. Nicole J. de Voogd, curator of the National Museum of Natural History in Leiden, The Netherlands. The animal had a pale yellow interior with a brown-yellow exterior, was dense and massive with tubes, and had a finely conulose surface with numerous complex oscules. These properties are comparable to those described in the literature.³² Pictures and voucher specimens are available from the corresponding author (P.C.).

Extraction and Isolation. The sponge material was preserved in the field according to our standard procedure,³³ transported back to the laboratory at ambient temperature, and stored at 4 °C until processing. Preliminary extraction under high pressure (1500 psi) at 100 °C (ASE apparatus) was performed on approximately 100 g of lightly chopped, dried sponge using hexanes (268 mg), then CH₂Cl₂ (245 mg), and last MeOH (602 mg). The MeOH extract demonstrated bioactivity in the HDAC enzyme assay (IC₅₀ < 0.08 μ g/mL), but the hexanes and CH₂Cl₂ extracts did not.

The remaining animal material was dried, cut into pieces, and extracted at room temperature by soaking overnight in MeOH (1 L) three times. The crude MeOH extract (7.8813 g, Figure S18, Supporting Information) was partitioned between MeOH–H₂O (9:1) (coded MW, 4.8155 g) and CH₂Cl₂ (coded FD, 2.5658 g). The ELSD-guided preparative RP separation of the FD partition afforded 20 \times 50 mL fractions (coded L1–L20) collected over a linear 45 min gradient (40–100% CH₃CN–H₂O with 0.1% formic acid (FA)) followed by 15 min elution with 100% CH₃CN (flow rate 20 mL/min). Semipreparative RP HPLC fractionation of the fraction coded FD L2 over a linear 25 min gradient (50–100% CH₃CN–H₂O with 0.1% FA) afforded **1** (1.9 mg, FD L2 H6) and a mixture of **3** and **4** (11.0 mg, FD L2 H5). This mixture was subjected to semipreparative RP chiral chromatography (60–80% CH₃CN–H₂O with 0.1% FA, 40 min) to separate **3** (5.1 mg, FD L2 H5 H2) and **4** (2.5 mg, FD L2 H5 H1). Fraction FD L3 was processed via RP HPLC (65–95% CH₃CN–H₂O, 0.1% FA, 30 min) into seven fractions (H1–H7), two of these included **2** (0.7 mg, FD L3 H6) and a mixture of **3** and **4** (0.2 mg, FD L3 H7). Fractionation of FD L4 (75–100% CH₃CN–H₂O with 0.1% FA, 30 min) gave nine fractions (H1–H9) by RP HPLC, affording **1** (6.2 mg, FD L4 H8) and **2** (0.9 mg, FD L4 H4). In a similar fashion FD L5 gave seven fractions (H1–H7, 85–100% CH₃CN–H₂O with 0.1% FA over 25 min) including **1** (11.6 mg, FD L5 H4) and **6** (0.6 mg, FD L5 H2). Next FD L6 gave **5** (1.0 mg, FD L6 H3) and **6** (0.3 mg, FD L6 H2) via RP HPLC over a 25 min linear gradient (85–100% CH₃CN–H₂O, 0.1% FA). Purification of fraction FD L16 (97–100% CH₃CN–H₂O with 0.1% FA over 10 min followed by 100% CH₃CN for 15 min) afforded **7** (5.7 mg, FD L16 H7). The process to obtain additional **6** (see Figure S18, Supporting Information) was as follows: MW (4.8155 g) was partitioned between 1:1 nBuOH–H₂O into FM (1.8869 g) and FW (2.5210 g), respectively, and FM was separated into 10 fractions via RP HPLC (40–100% CH₃CN–H₂O, 0.1% FA, 45 min, 10 mL/min). Next, fraction 05409 FM P10 (24.9 mg) was separated into 10 fractions (75–100% 8:2 MeOH–CH₃CN–H₂O, 36 min) to afford **6** (14.9 mg, FM P10 H9). In summary five known compounds were obtained and delimitated by comparing their properties to those in the literature: **1**,¹² **2**,¹⁰ **3**,¹⁴ **5**,¹⁶ and **7**.¹⁶

HDAC Assays. Fractions L1–L20 (see Figure S18, Supporting Information) were evaluated using the HDAC fluorimetric assay/drug discovery kit (AK500 kit, Biomol Research Laboratories, Plymouth Meeting, PA). The HeLa cell nuclear extract, which contained a mixture of nuclear factors and HDAC isozymes, was used as the source of HDAC enzymes. The final substrate concentration was 30 μ M, and the positive control was trichostatin A (50, 5, and 0.5 nM). The incubation (30 min, 37 °C) was terminated with the addition of 50 μ L of developer. The assay vessels were black, clear-bottom polystyrene 384-well plates, and data collection employed a Wallac Victor2 V 1420 multilabel HTS counter fluorescence plate reader with excitation wavelength 360 nm and emission wavelength 450 nm.

Disk Diffusion Assay. The soft agar disk diffusion assay was performed using published protocols.³⁴

20-Epi-hydroxyhaterumadienone (4): white solid (2.5 mg); $[\alpha]_D^{27}$ -62 (c 0.28, CHCl_3); UV (MeOH) λ_{max} (log ϵ) 201 (3.45), 284 (3.75) nm; IR (NaCl) ν_{max} 3328, 2922, 1563, 1421, 1196 cm^{-1} ; ^1H NMR and ^{13}C NMR (see Table S2); HRESIMS $[\text{M} + \text{H}]^+ m/z$ 317.2113 (calcd for $\text{C}_{20}\text{H}_{29}\text{O}_3$, 317.2111).

15-Oxo-puupehenic Acid (6): white solid (14.9 mg); $[\alpha]_D^{27}$ $+27$ (c 0.1, CHCl_3); UV (MeOH) λ_{max} (log ϵ) 216 (4.14), 265 (3.52), 331 (3.23) nm; IR (NaCl) ν_{max} 3492, 2925, 1679, 1594, 1476, 1437, 1285, 1020 cm^{-1} ; ^1H NMR and ^{13}C NMR (see Table 1); HRESIMS $[\text{M} + \text{H}]^+ m/z$ 357.2107 (calcd for $\text{C}_{22}\text{H}_{29}\text{O}_4$, 357.2060).

Reaction of 3 to Form 3' and 3''. To a solution of **3** (0.5 mg, 1.6 μmol) in pyridine (0.1 mL) was added (–)-*R*-MTPA-Cl (24 mg, 96 μmol). The mixture was stirred at rt for 2 h and quenched with MeOH (0.2 mL). Water (0.2 mL) was then added, and the mixture was extracted with EtOAc (1 mL \times 3). The organic phase was dried and concentrated *in vacuo*. CDCl_3 was added (300 μL) to **3'** and directly examined by ^1H (Figure S22, Supporting Information) and gHMQC (Figure S19, Supporting Information) NMR at 600 MHz in a CDCl_3 Shigemi tube. Using the same procedure described above, **3''** was obtained from the reaction between (+)-(*S*)-MTPA chloride (24 mg, 96 μmol) and **3** (0.25 mg, 0.8 μmol) in pyridine (0.1 mL) and evaluated with ^1H (Figure S22, Supporting Information) and gHMQC (Figure S20, Supporting Information) NMR.

Reaction of 4 to Form 4' and 4''. To a solution of **4** (0.25 mg, 0.8 μmol) in pyridine (0.1 mL) was added (–)-(*R*)-MTPA chloride (24 mg, 96 μmol). The mixture was stirred at rt for 2 h and quenched with MeOH (0.2 mL). H_2O (0.2 mL) was then added, and the mixture was extracted with EtOAc (1 mL \times 3). The organic phase was dried and concentrated *in vacuo*. CDCl_3 was added (300 μL) to **4'** and examined by ^1H and gHMQC NMR techniques in a Shigemi tube (CDCl_3 -type, 600 MHz, Figures S21 and S23, Supporting Information). Using the same procedure as described above, **4''** was obtained from the reaction between (+)-(*S*)-MTPA chloride (24 mg, 96 μmol) and **4** (0.25 mg, 0.8 μmol) in pyridine (0.1 mL) and evaluated by ^1H and gHMQC NMR (Figures S21 and S23, Supporting Information).

Overexpression and Purification of Human Reticulocyte 15-Lipoxygenase-1, Human Platelet 12-Lipoxygenase, and Human 5-Lipoxygenase. Human reticulocyte 15-lipoxygenase-1 (15-hLO-1) and human platelet 12-lipoxygenase (12-hLO) were expressed as N-terminally, His6-tagged proteins and purified to greater than 90% purity, as evaluated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis.^{9,35} Human 5-lipoxygenase (5-hLO) in a pET21 expression vector was received from the Tatulian Lab (University of Central Florida).³⁶ It was transformed into *E. coli* BL21-DE3 and grown overnight in LB medium (50 mL) with 0.1 mg/mL ampicillin at 25 °C. Next, the 50 mL growth mixture was separated into 7 \times 2 L flasks of media and grown at 30 °C until the OD_{600} reached 0.6. The cells were then induced with 0.25 mM IPTG, followed by lowering the temperature to 18 °C. The cells were harvested 16–20 h post-induction by pelleting at 5000g (10 min) and stored at -80 °C until purification.

The frozen cell pellet was resuspended in buffer A (10 mM β -mercaptoethanol, 0.1 mM EDTA, and 50 mM triethanolamine (pH 7.3)) and placed on ice. Cells were lysed using a power laboratory French press and cleared by spinning at 40000g (25 min). Proteins in the supernatant were precipitated at 50% saturated ammonium sulfate and spun at 40000g (25 min). Proteins in the salt pellet can be stored under liquid nitrogen with negligible loss of 5-hLO activity for over 6 months. The salt pellets were resuspended in buffer A for use in activity assays.

Lipoxygenase Assay. The enzyme activity was determined by direct measurement of the product formation following the increase of absorbance at 234 nm using a Perkin-Elmer Lambda 40 UV/vis spectrometer (25 mM HEPES (pH 8), 0.01% Triton X100, 3 μM arachidonic acid for 12-hLO, 25 mM HEPES (pH 7.5), 0.01% Triton X100, 3 μM arachidonic acid for 15-hLO-1, and 25 mM HEPES (pH 7.3), 0.3 mM CaCl_2 , 0.1 mM EDTA, 0.2 mM ATP, 0.01% Triton X100, 10 μM arachidonic acid for 5-hLO). All reactions were performed in 2 mL of buffer and constantly stirred with a rotating stir bar (22 °C). IC_{50} values were determined by measuring the enzymatic rate at a variety of inhibitor concentrations (depending on the inhibitor potency) and plotting their values versus inhibitor concentration. The corresponding data were fitted to a saturation curve using KaleidaGraph (Synergy), and the inhibitor concentration at 50% activity was

determined (IC_{50}). The inhibitors were dissolved in MeOH or DMSO and the corresponding control reactions subtracted from the inhibition data.

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Supporting Information Available: ^1H , ^{13}C , gCOSY, gHMBC, and gHMQC NMR data and MS data for **6** and ^1H and ^{13}C NMR and MS data for **4** along with an isolation scheme for **1**, **2**, **3**, **4**, **5**, **6**, and **7** and a table of puupehenone bioactivities are provided free of charge via the Internet at <http://pubs.acs.org>. Also free at this Web site is a database of select puupehenone bioactivities and optical rotations.

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