Title

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Authors

Amagata, Taro
Whitman, Stephanie
Johnson, Tyler A
et al.

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Sponge Derived Terpenoids with Selectivity towards Human 15-lipoxygenase versus Human 12-Lipoxygenase

Taro Amagata,[†‡] Stephanie Whitman,[†] Tyler Johnson,[†‡] Chad C. Stessmann,[†‡] Jennifer Carroll,[†‡] Christopher Loo,[†‡] Jon Clardy,[§] Emil Lobkovsky,[§] Phillip Crews[†‡] and Theodore R. Holman,*[†]*

[†,‡] Department of Chemistry and Biochemistry and Institute for Marine Sciences, University of California Santa Cruz, Santa Cruz, CA 95064

[§]Department of Chemistry, Cornell University, Ithaca, NY 14853

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* To whom correspondence should be addressed

Tel: 831-459-2603, Fax: 831-459-2935, E-mail: phil@chemistry.ucsc.edu

Tel: 831-459-2603, Fax: 831-459-2935, E-mail: holman@chemistry.ucsc.edu

Abstract

Introduction

The discovery of novel lipoxygenase (LO) inhibitors has garnered wide enthusiasm due to the fact that lipoxygenase is a key biological player in a variety of diseases [add ref]. 5-lipoxygenase is involved in asthma [add ref], 12-lipoxygenase in psoriasis [add ref] and 15-lipoxygenase in atherosclerosis [add ref], to name a few. These facts have lead our labs into the area of LO inhibitor discovery from marine sponge extracts. Previously, few LO inhibitors from marine sources have been reported [add ref]. The only compounds found from marine sponge organisms are the brominated phenols [add ref] and terpenoid compounds [add ref], both from our labs. These compounds were discovered through screening of our marine natural product library, which contains ≈ 10,000 crudes extracts and ≈ 1,000 isolated compounds. The inhibitors we have isolated can be classified into two distinct groups, redox active, which reduce the iron site, and non-redox active, which bind to either the active site or the allosteric site[add ref]. Of the latter class, we previously demonstrated that jaspic acid (**8**) and subersic acid (**9**) are potent non-redox active inhibitors and that their structural differences account for a 10-fold difference in their IC_{50} values against 15-HLO [add ref]. We speculated that this difference could be related to the smaller hydrophobic tail of **8** versus **9**, which could fit in the active site better. This result indicated that marine sponges could be a rich source of lipoxygense inhibitors that would lead us to further important structure/activity conclusions.

In the current paper, we have continued screening our sponge-derived natural product library for LO inhibitors, however, we have expanded the search to include both 15-HLO and 12-HLO. As we have stated, specific isozymes of lipoxygenase affect different disease states and it is therefore critical to discover selective inhibitors to specific lipoxygenases if we wish to develop LO therapeutics for a particular disease [add ref]. We have therefore revisited our previously reported inhibitors and report herein their selectivity towards 12-HLO versus 15-HLO. In

addition, we have discovered 10 additional compounds as novel LO inhibitors, most of which were previously known in the literature yet unknown as LO inhibitors. One however, hyrtenone A (**1**), is a novel structure and is one of our puupehenones sub-class of LO inhibitors. The selective, inhibition properties of these compounds are reported herein, along with interpretations of their structure/function activity.

Results and Discussion

The isolation and characterization of the novel compound, hyrtenone A (**1**) was achieved through the following manner. The methylene chloride partitioned fraction of the sponge, *Hyrtios* sp. (coll. no. 99140) collected at Papua New Guinea appeared to contain unsaturated polycyclic mero-terpenoids, because of the observation of ^{1}H NMR signals based on approximately 10 singlet methyls in the upfield δ 0.8 – 1.3 and several sp^{2} singlet methines in the downfield δ 5.8 – 6.8. This extract was purified by employing a combination of silica gel column chromatography and reversed phase HPLC afforded hyrtenone A (**1**) and known compounds, puupehenone (**2**)[add ref TA1] and 21-chloropuupehenone (**3**)[add ref TA1].

Hyrtenone A (**1**) had the molecular formula $C_{24}H_{34}O_5$ (*m/z* 403.2484 $[M+H]^+$, calcd for 403.2479) established by high resolution electorospray ionization time-of-flight mass spectrometory (HRESI-TOFMS). The ^{1}H and ^{13}C NMR spectra of **1** (Table 1) were very similar to those of **2**. Comparing the NMR data of **1** to those of **2**, one sp^{3} methine neighboring an oxygen atom (δ_H 4.86, δ_C 81.4) and one sp^{3} quaternary carbon neighboring an oxygen atom (δ_C 79.9) were observed instead of the proton and carbon signals of the Δ^{20}-olefin in **2**. As new signals, one sp^{3} methylene (δ_H 2.21, 2.45, δ_C 47.1), one hemiacetal methine (δ_H 5.03, δ_C 104.0) and one methoxyl group (δ_H 3.45, δ_C 55.5) were observed. These signals were assigned as the partial structure A from analyses of ^{1}H-^{1}H gCOSY and gHMBC correlations (Figure 1).

Moreover the carbonyl carbon (δ_C 197.6) shifted downfield by ca. 15 ppm comparing to C-19 (δ_C 182.0) of **2**. The above observations and the similarity of carbon chemical shifts of C-1 – C-17 to **2** indicated that the partial structure A substituted at C-20 and C-21 on **2** as supported by HMBC correlations (Figure 1). Therefore, the planar structure was established.

INSERT TABLE 1 AND FIGURE 1

The stereochemistry of **1,** except for E ring, was deduced to be same as **2**, of which absolute stereochemistry were determined [add ref TA2], because **1**, **2** and **3** were produced via same biosynthetic pathway as supported by agreement of the carbon chemical shifts of C-1 – C-17 between **1** and **2**. Attempts to elucidate the stereochemistry of the E ring by NOESY experiments were failed. However, since **1** was able to obtain as single crystals by recrystallization, an X-ray crystal structure analysis was carried out. The result obtained allowed assignment of the absolute stereochemistry of all the asymmetric centers including C-20, C-21 and C-23 (5*S*, 8*S*, 9*R*, 10*S*, 20*R*, 21*S*, 23*R*) (Figure 2).

INSERAT FIGURE 2

These pure compounds, along with the other puupehenone derivatives and the previously discovered LO inhibitors [add ref], were then tested for lipoxygenase activity (Table 2). The inhibitors can be crudely divided into two categories; redox active and non-redox active [add ref]. The redox active compounds are potent because they reduce lipoxygenase to its in-active, ferrous form [add ref]. Lipoxygenases are a class of non–heme iron enzymes that catalyze the incorporation of dioxygen into 1,4–cis,cis–pentadiene containing fatty acids (e.g. linoleic and arachidonic acids) to form hydroperoxide products [add ref]. The essential iron atom is in the

active, ferric oxidation state which oxidizes the substrate to the pentadienyl radical. The radical is subsequently attached regio- and stereo-specifically to form the product [add ref]. The redox active inhibitors of this paper reduce the ferric form of LO to the inactive ferrous form [add ref]. These redox active inhibitors include jaspiquinol (**7**) (12-IC_{50} = 4.5 μM, 15-IC_{50} = 0.3 μM), previously reported as a 15-HLO inhibitor [add ref], halisulfate 1 (**11**) (12-IC_{50} = 1.0 μM, 15-IC_{50} = 0.9 μM) [add ref] and hydro-halisulfate 1 (**12**) (12-IC_{50} = 2.1 μM, 15-IC_{50} = 0.5 μM) [add ref]. All of these redox inhibitors are more potent against 15-HLO, with the greatest selectivity towards 15-HLO belonging to **7**. Interestingly, if the sulfate is removed from HS1, the IC_{50} for 12-HLO gets worse while the IC_{50} for 15-HLO improves, indicating that 12-HLO inhibition has a greater requirement for polarity on the inhibitor than 15-HLO. The well studied LO inhibitor, nordihydroguarierate acid (**10**) also manifests a greater selectivity towards 15-HLO (12-IC_{50} = 5.1 μM, 15-IC_{50} = 0.11 μM) [add ref], which could be indicative of either a structural difference or a change in their reduction potentials.

The other category of non-redox compounds include igernellin (**14**), halenaquinol sulfate (**15**), **8**, **9**, and the puupehenone derivatives. **14** and **15** are relatively inactive as inhibitors, most likely due to their rigid structure (**14**) and low polarity (i.e. low solubility) (**15**), while **8** and **9** are very potent. The relative inhibition of 12-HLO between **8** (12-IC_{50} = 0.7 μM, 15-IC_{50} = 1.4 μM) and **9** (12-IC_{50} = 13.5 μM, 15-IC_{50} = 14.5 μM) mirror the results of 15-HLO with **8** being more potent against both 12- and 15-HLO, than **9**. We had previously speculated that this difference in inhibition between **8** and **9** for 15-HLO was due to the location of the bicyclic ring relative to the polar head group. The present results suggest that this may also be the cause of the difference of inhibition against 12-HLO between JA and SA and indicates similar inhibitor binding constraints for both 12-HLO and 15-HLO. This is reasonable since it has previously been shown that 15-HLO can be converted to a 12-HLO by reducing the depth of the hydrophobic pocket and not the polar head pocket [add ref]

The puupehenone family of inhibitors shows remarkable differences in IC_{50} results although their structural changes are relatively minor. Methoxy-puupehenone (**4**) (12-IC_{50} = 13.2 μM, 15-IC_{50} = 1.7 μM), methoxy-puupehenol (**6**) (12-IC_{50} = 8.1 μM, 15-IC_{50} = 1.0 μM), 21-chloropuupehenone (**3**) (12-IC_{50} = 0.7 μM, 15-IC_{50} = 0.8 μM) and puupehenone (**2**) (12-IC_{50} = 8.3 μM, 15-IC_{50} = 0.76 μM) demonstrate comparable inhibition towards 15-HLO, however, vary dramatically towards 12-HLO. 21-chloropuupehenone (**3**) has comparable inhibition against 15-HLO and 12-HLO, while methoxy-puupehenone (**4**), methoxy-puupehenol (**6**) and puupehenone (**2**) are roughly 10-fold less potent against 12-HLO than 15-HLO. This is an interesting result because the strength of inhibition for 21-chloropuupehenone (**3**) increases 10-fold against 12-HLO versus puupehenone, solely based on the addition of a chloride to carbon 21. However, methoxy-puupehenone (**4**) and methoxy-puupehenol (**6**), all of which have additional steric bulk on carbon 20, do not increase their potency towards 12-HLO over that of puupehenone. Obviously, increasing the steric bulk with a chloride next to carbon 20 enhances the affinity towards 12- versus 15-HLO, while masking the oxygen on carbon 20 with a methyl group does not. Di-methoxy-puupehenone is approximately 10-fold less potent towards 12- and 15-HLO (12-IC_{50} = 80 μM, 15-IC_{50} = 7.2 μM), relative to methoxy-puupehenol, most likely due to the added bulk of the methoxide on carbon # (Please help phil or taro). Hyrtenone A (**1**) is the worst puupehenone derivative toward 15-HLO (15-IC_{50} = 59 μM), yet, it is a better inhibitor towards 12-HLO than di-methoxy-puupehenone (**5**) (12-IC_{50} = 31 μM). This indicates that the additional bulk near carbon 20 of the molecule enhances its selectivity towards 12-HLO over 15-HLO and correlates well with the improved 12-HLO selectivity of 21-chloropuupehenone (**3**), which has a chlorine in the same region. The only compound of the puupehenones derivatives that is redox active is puupehenone itself (against SLO), which is most likely due to its free phenol. The other derivatives do not reduce SLO even though some of them have free phenol alcohols, suggesting

that their alcohol groups are not in the correct position to achieve an inner sphere reduction of the iron.

Conclusions

As stated in the introduction, it is imperative that any lipoxygenase inhibitor used therapeutically must be selective against a particular isozyme in order to target a particular disease. The current collection of LO inhibitors demonstrated in this paper are predominately selective against 15-HLO. From this data, we can hypothesize a number of structural differences between the inhibitors that affects their selectivity. The inhibitors that target 12-HLO are more polar than 15-HLO. In addition, they ……EXPAND!!

Experimental Section

General Experimental Procedures. The NMR spectra were recorded at 500 and 125.7 MHz for ^{1}H and ^{13}C, respectively. UV/vis measurements were performed with a diode array detector. HPLC was performed with columns of 5µm ODS.

Biological material, Collection and Identification. Samples of the sponge, coll no. 99140 (1 kg wet wt) were collected from Gulf of Papua ca. 250 km west off the coast of Kwikila, Papua New Guinea at a depth of 30 m in December 1999 (S 10˚09.538', E 145˚33.886' and S 10˚11.907', E 145˚38.112'). This sponge was identified as *Hyrtios* sp.[add ref TA 13 and 14] (Family; Thorectidae, Order; Dictyoceratida), by Dr. M. C. Diaz (UCSC, IMS). The voucher samples of the sponge and the underwater photo are available from the Crews lab.

Extraction and Isolation. The sponges (coll. no. 99140) were soaked in MeOH three times. The residue obtained was partitioned between hexanes and MeOH–H_2O (9:1). After the water was added to adjust the solution of MeOH:H_2O to 1:1, it was extracted with CH_2Cl_2 three

times to give the crude extract (15.0 g). The extract (5.0 g) was applied to a silica gel column with a hexane–EtOAc gradient as the eluent to afford 12 fractions [F1 (758.8 mg), F2 (672.4 mg), F3 (428.6 mg), F4 (151.1 mg), F5 (210.3 mg), F6 (141.1 mg), F7 (223.8 mg), F8 (84.0 mg), F9 (88.7 mg), F10 (14.6 mg), F11 (51.0 mg), F12 (1.29 g)]. F4 from hexane–EtOAc (9:1 and 4:1) eluatses was purified by HPCL using MeOH–H_2O (4:1) the eluent to afford **1** (4.4 mg), **2** (17.1 mg) and **3** (5.2 mg), respectively.

Hyrtenone A (1): obtained as colorless crystals from MeOH; Mp. 104 – 105˚C; $[\alpha]_D^{28}$ – 119.4˚ (*c* 0.06, $CHCl_3$); UV (MeOH) λ_{max} (log ε) 302 nm (4.39); ^1H and ^{13}C NMR data, see Table 1.

X-ray crystal structure analysis for 1. Suitable crystals were obtained from MeOH by the vapor diffusion method. This crystal (0.60 x 0.60 x 0.05 mm^3) was mounted on a Bruker SMART diffractometer (MoKα; -100˚C). A hemisphere of data was taken using a narrow-scan routine (1406 frames, 0.3˚ steps ω-scan, exposure time was 30 s/frame, $2\theta_{max}$ = 49.42˚). Raw data were integrated with the Bruker SAINT+ program[add TA 3] to yield a total 24193 reflections, of which 7503 were independent (R_{int} = 7.61 %, completeness 99.7 %) and 1793 with I > 2σ(I). Data were collected for absorption using SADABS program (min. and max. transmission are 0.7500 and 0.9957, respectively)[add ref TA4]. The structure was solved by direct methods [add ref TA5] and refined by full matrix least square on F^2 techniques [add ref TA5] using anisotropic displacement parameters for all non-hydrogen atoms. All hydrogen atoms were found in the difference Fourier map and refine isotropically. At final convergence, R_1 = 5.29 % and GOF = 1.022 for 829 parameters. Additional information about these data includes crystal data and structure refinement in Table S1, atomic coordinates in Table S2, bond length and angles in Table S3, anisotropic displacement parameters in Table S4, hydrogen coordinates in Table S5.

Library compounds: Puupehenone (**2**) and 21-chloropuupehenone (**3**) were identified by comparison of their spectral data to published data. [add ref TA1] (+)-(5*S*, 8*S*, 9*R*, 10*S*)-20-

Methoxypuupehenone (**4**),[add ref TA6] (+)-(5*S*, 8*S*, 10*S*)-20-methoxy-9, 15-ene-puupehenone (**5**),[add ref TA6] (+)-(5*S*, 8*S*, 10*S*)-20-dimethoxypuupehenol (**6**),[add ref TA6] jaspaquinol (**7**),[add ref TA7] jaspic acid (**8**)[add ref TA7] suberic acid (**9**)[add ref TA8] and halenaquinol sulfate (**14**)[add ref TA9] were characterized by our group. The details including spectral data are mentioned in the literature. Halisulfate 1 (**11**)[add ref TA10] and its alcohol (**12**),[add ref TA10] igernellin (**13**)[add ref TA11] and halenaquinol sulfate (**14**)[add ref TA12] were also identified by comparison of their spectral data to published data.

Plasmid Construction, Expression and Purification of 6-His tagged 12-HLO and 15-HLO. Human 15-lipoxygenase with a 6-His tag on the N-teminus (15-HLO) was constructed as follows. The previously published plasmid, pFastBac1-15HLO [add ref], was treated with SalI, NcoI and alkaline phosphatase to release an ≈950 fragment. A PCR fragment was then generated with the following two primers which generated an ≈ 1200 bp fragment (5'-ACgC-gTC-gAC-ATg-CAC-CAC-CAT-CAC-CAT-CAC-ggT-CTC-TAC-CgC-ATC-CgC-gTg-3'and 5'-T-AAT-TTC-CAg-ggT-gTA-TCg-CAg-g-3'). This PCR fragment was then cut with SalI and NcoI and ligated into the digested pFastBac1-15HLO plasmid. This plasmid, pFB/6His-15HLO, was then checked for orientation by restriction digest and the PCR fragment sequenced to confirm the correct sequence and codon alignment.

Human 12-lipoxygenase with a 6-His tag on the N-teminus (12-HLO) was constructed as follows. The plasmid pcDNA/6His-12LX [add ref] (generously provided by Dr. C. Funk) was cut to produce an XbaI/XbaI fragment of the 6His-12LX gene. This fragment was then inserted into pFastBac1 (GibcoBRL), treated with XbaI/alkaline phosphatase. This plasmid, pFB/6His-12HLO, was then checked for complete insertion and correct orientation.

Both plasmids were then transposed into a recombinant FastBac bacmid with the help of DH10Bac cells (GibcoBRL) and then transfected into SF9 cells, as described in the product literature for pFastBac1 (GibcoBRL). The virus was subsequently amplified to ≈2x10^{10} plaque

forming units (pfu). This virus was then added to SF9 cells (≈2x10^6 cells/ml) at a concentration of ≈2x10^7 pfu/ml and allowed to shake for 72 hours. The cells were then harvested and dounced as previously published [add ref].

12-HLO and 15-HLO were purified by loading the cell extracts onto a 20 ml column of high-flow his-bind (Novagen) and eluted with a 0-400 mM imidazole gradient. The collected fractions contained 95% purified protein and were frozen at –80˚ C with glycerol added (20% glycerol for 12-HLO and 10% glycerol for 15-HLO). Subsequent thawing produced enzymatic activity for both 12-HLO and 15-HLO at level comparable to the parameters published previously for these enzymes [add ref].

Lipoxygenase Assay. The enzyme activity was determined by direct measurement of the product formation following the increase of absorbance at 234 nm (25 mM Hepes (pH 8), ≈ 3 □M arachidonic acid for 12-HLO and 25 mM Hepes (pH 7.5), ≈ 3 □M linoleic acid for 15-HLO). All reactions were performed in 2 ml of buffer, ≈ 200 nM enzyme 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 was fit to a simple saturation curve and the inhibitor concentration at 50 % activity was determined (IC_{50}). The inhibitors were typically dissolved in methanol at a concentration of ≈ 1 mg/ml. The reduction of SLO by addition of inhibitors was determined using fluorescence, as previously published [add ref].

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Supporting Information Available. ^{1}H and ^{13}C NMR spectra and X-ray data for hyrtenone A (**1**). This material is available free of charge via the Internet at http://pubs.acs.org.

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