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Modular Strategies for Structure and Function Employed by Marine Cyanobacteria: Characterization and Synthesis of Pitinoic Acids

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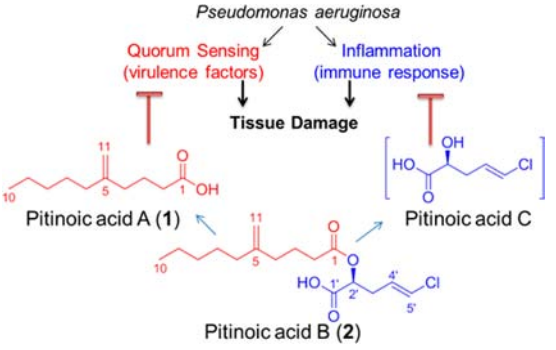
ABSTRACT



Novel bioactive lipids were identified from a Guamanian cyanobacterium, the *Pseudomonas aeruginosa* quorum sensing inhibitor pitinoic acid A (1) and the anti-inflammatory pitinoic acids B (2) and C. The structure of 2 was confirmed by synthesis, which also allowed for biological evaluation. Since 2 is an ester of pitinoic acids A and C, it represents a prodrug strategy to liberate dual biological activity for the management of *P. aeruginosa* infections and their associated inflammation.

Pseudomonas aeruginosa is a Gram-negative bacterium that causes opportunistic infections in different host tissues and organs. For example, cystic fibrosis (CF) infections and microbial keratitis (MK) are two of the most serious lung and eye infections, respectively, caused by this organism.[1,2] Bacterial quorum sensing (QS) has been shown to play a major role in bacterial pathogenesis, where the infection and tissue destruction are facilitated by several QS-regulated virulence factors including elastase (LasB) and the pigment pyocyanin.[2,3] Tissue damage is also aggravated by the host's defense response, where the release of pro-inflammatory mediators and the prolonged inflammatory response are other contributors to tissue pathology.[2,4−8] Accordingly, inhibiting QS pathways in *P. aeruginosa* and simultaneously controlling the associated inflammatory response is a promising strategy for the treatment of infections caused by this organism.

We explored a population of a marine cyanobacterium morphologically similar to *Lyngbya* sp. collected from a channel at the north end of Piti Bay at Guam, through NMR-guided fractionation. We isolated and characterized the novel lipids pitinoic acid A (**1**) (5-methylene decanoic acid)

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and its chlorinated ester, pitinoic acid B (**2**). The fatty acid **1** inhibits QS in *P. aeruginosa*, while the ester **2** prevents the induction of pro-inflammatory cytokine expression in LPS-induced THP-1 macrophages. In addition, we were able to identify the presence of pitinoic acid C, the alcohol moiety in **2**, in one HPLC fraction, which also maintained the anti-inflammatory effect detected for **2**. Based on this, **2** appears to be a nature-designed prodrug which could deliver two essential moieties with different bioactivities upon hydrolysis, the QS-inhibitory module pitinoic acid A (**1**) and the anti-inflammatory module pitinoic acid C.

The EtOAc–MeOH extract was subjected to fractionation. ^{1}H NMR profiles of the fractions showed the abundance of a simple fatty acid (**1**), which was purified by HPLC. The ^{1}H NMR spectrum of **1** showed typical peaks for fatty acids (Table 1). Additionally, NMR spectral analysis showed the presence of an exo-double bond along the fatty acid chain. The analysis of NMR spectra and MS data led to the characterization of pitinoic acid A (**1**) (Table 1, see Supporting Information for more details).

HPLC purification of another fraction yielded the ester **2**, where the ^{1}H and ^{13}C NMR spectra included all the peaks corresponding to **1** as well as peaks for five additional carbons and attached protons (Table 1). COSY, TOCSY and HMBC data led to the assignment of the additional portion as 2-hydroxy-pent-4-enoic acid, and a terminal chloride attachment was suggested by HRESIMS analysis including the isotopic cluster. The chemical shift of the carbonyl carbon in the decanoic acid part was shifted upfield compared to **1** (Table 1), and therefore the compound appeared to be a fatty acid ester, **2**. The geometry of the alkene was determined to be *E* based on the large vicinal coupling constant ($^3J_{H,H}$ 13.4 Hz) between the olefinic protons. The configuration at C2′ was assigned through ozonolysis followed by oxidative workup to yield the corresponding malic acid, which was then analyzed by chiral HPLC to reveal *S* configuration.

The abundance of **1** in this sample (~0.3% of total dry weight) and was sufficient for biological activity evaluation in several assays. However, the chlorinated ester **2** was only isolated in minute amounts (~900 μg) and required synthetic efforts to allow for biological evaluation.

Our initial retrosynthetic strategy relied on the obvious disconnection at the ester linkage to the fatty acid **1** and pitinoic acid C (Scheme 1, strategy 1). The selective introduction of the *E*-vinyl chloride in the alcohol moiety could be achieved through Takai-Utimoto olefination reaction.[9] However, trials starting from L-malic acid or L-aspartic acid were not successful (See Supporting Information for more details).

Our next retrosynthetic analysis (Scheme 1, strategy 2) aimed to primarily introduce the vinyl chloride terminus by Takai–Utimoto olefination to a C4 aldehyde with a terminal vicinal diol. The secondary alcohol could be coupled first to **1** before the primary alcohol at C1 could be converted to the carboxylic acid terminal. This attempt started from the commercially available acetonide

Table 1. NMR Spectroscopic Data for Pitinoic Acid A (**1**) and Pitinoic Acid B (**2**) in $CDCl_3$ (δ in ppm, J in Hz) at 600 MHz

	pitinoic acid A (**1**)			pitinoic acid B (**2**)		
C/H no.	δ_C	δ_H (J)	HMBC[a]	δ_C	δ_H (J)	HMBC[a]
1	180.1	–	2, 3	173.3	–	2, 3
2	33.6	2.35 t (7.4)	3, 4	33.4	2.40 dt (7.8, 6.7)	3, 4
3	22.5	1.77 m	2, 4	22.7	1.79 m	2, 4
4	35.0	2.06 t (7.5)	3, 6, 11	35.2	2.06 t (7.3)	2, 3, 6, 11
5	148.7	–	3, 4, 6, 7, 11	148.9	–	3, 4, 6, 7, 11
6	35.6	1.99 t (7.5)	4, 7, 11	35.8	1.99 t (7.6)	4, 7, 11
7	27.3	1.42 m	6, 8	27.4	1.42 m	6, 8
8	31.6	1.26 m	7, 9, 10	31.7	1.26 m	6, 7, 9, 10
9	22.4	1.31 m	7, 8, 10	22.6	1.32 m	8, 10
10	13.9	0.89 t (7.1)	8, 9	14.1	0.89 t (7.1)	8, 9
11	109.5	4.72 s, 4.75 s	4, 6	109.8	4.72 s, 4.75 s	4, 6
1′				173.5	–	2′
2′				70.7	5.09 t (5.6)	3′, 4′, 5′
3′				32.5	2.64 m	2′, 4′, 5′
4′				127.3	5.9 m	2′, 3′, 5′
5′				121.4	6.12 d (13.4)	3′, 4′

[a] Protons showing long-range correlation to indicated carbon.

(*S*)-2-(2,2-dimethyl-1,3-dioxolan-4-yl) ethanol (Scheme 2), where the terminal alcohol group was oxidized to the corresponding aldehyde **3** in 76% yield using PCC in CH_2Cl_2.[10] Takai–Utimoto olefination for **3** appeared to proceed with good selectivity ($E/Z \approx 8{:}1$) and reasonable yield (37%). Acetonide opening for **4** was done using DOWEX in MeOH[11] to give the 1,2-diol (**5**) with good yield (~95%). The primary alcohol in **5** was then protected as the TBDMS-ether (**6**, 47% yield), and the free secondary alcohol was coupled to **1** to furnish **7** (76% yield). The next step was to deprotect the primary alcohol and oxidize it to the corresponding carboxylic acid to obtain the final ester **2**. First attempts to deprotect the primary alcohol in **7** using TBAF[12] were faced with the challenge of acyl migration from the secondary to the primary alcohol. Minimal acyl migration was obtained by using a neutral mixture of TBAF and AcOH in THF and by limiting the reaction time to 2 h giving the free primary alcohol **8** in good yield (72%; 85% BRSM). Unreacted starting material was recovered and subjected to the same conditions again.

For the final oxidation step, we first pursued several mild oxidation attempts, since we were concerned about the

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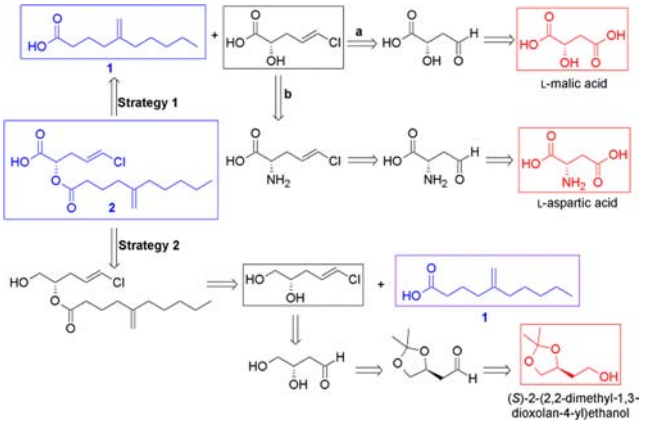
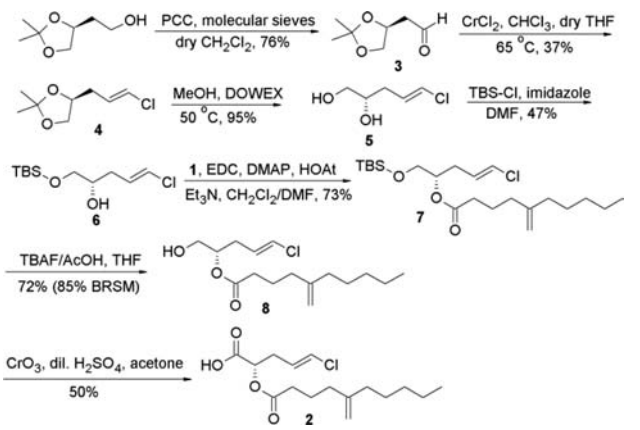
Scheme 1. Retrosynthetic Strategies to Obtain Pitinoic Acid B (**2**)[a]



[a] Natural products are shown in blue; starting points for each strategy are shown in red.

Scheme 2. Final Scheme for the Semisynthesis of Pitinoic Acid B (**2**)



stability of the ester linkage and the vinyl chloride functionalities under any harsh reaction conditions. The traditional PDC-mediated oxidation in DMF[13] was ineffective. Additionally, two-step oxidation protocols to the aldehyde and then to the acid were also inefficient to produce the desired carboxylic acid, including Dess-Martin oxidation followed by $NaClO_2$ or PDC treatment, and TEMPO-catalyzed oxidations with $NaOCl/NaClO_2$ system.[14] Notably, the exo-methylene group in the fatty acid part appeared to be affected by the $NaOCl/NaClO_2$ oxidizing system. One-pot deprotection of the silyl ether catalyzed by $Bi(OTf)_3$ followed by TEMPO-catalyzed oxidation[15] did not give desirable results either. Finally, Jones oxidation was the only method that gave the desired final product with a moderate yield (~50%). However, the essential HPLC purification to obtain the final pure product appeared to significantly lower the yield of the pure product (yield dropped to 20% after HPLC purification). The NMR spectral data and optical rotation for the synthetic compound matched those for the natural product **2** (Figure S28). The problems faced with the stability and purification of this chlorinated ester could provide one explanation for the low amount of the natural product that we were able to isolate from the cyanobacterium. However, a sufficient amount was successfully prepared to allow for further biological characterization.

One of the common biological roles of fatty acid compounds produced by bacteria in large quantities is the interference with quorum sensing (communication between bacteria in response to high population densities). This was shown in several previous reports,[16−19] including by our group where we reported the antiquorum sensing activity of lyngbyoic acid.[20] In *Pseudomonas aeruginosa*, the quorum sensing (QS) system integrates two chemically distinct classes of signal molecules which act on differnet QS pathways, the *N*-acylhomoserine lactones which act on the LasR-LasI pathway and the 4-quinolones which act on the *Pseudomonas* quinolone signaling pathway.[21,22] Lyngbyoic acid was found to inhibit QS through the LasR pathway, where the cyclopropyl ring appeared to be essential for this activity.[20] Based on structural similarity, **1** could also possess QS inhibitory activity similar to lyngbyoic acid, where the exomethylene group might also be influential for this biological activity. Accordingly, we tested **1** for its ability to interfere with quorum sensing in *P. aeruginosa* by monitoring its effect on the transcription and production of two virulence factors, the elastase LasB enzyme and the pigment pyocyanin. Compound **1** significantly reduced the transcript levels of *lasB* and the pyocyanin biosynthetic member *phzG1* after 6 h at 1 mM and 100 μM as assessed by RT-qPCR (Figure 1). Additionally, after 6 h, the levels of LasB and pyocyanin in the culture supernatants were also significantly reduced by **1** at 1 mM, as evaluated by an enzymatic assay for LasB and quantitative evaluation using UV absorbance for pyocyanin (Figure 1). Measuring the bacterial cell density (OD_{600}) showed that this fatty acid did not affect cellular growth at the tested concentrations.

The chlorinated ester **2** shares some structural features with the recently reported anti-inflammatory marine cyanobacterial fatty acid esters honaucins.[19] We initiated the biological characterization of **2** by testing its effect on LPS-induced inflammatory responses in human acute monocytic leukemia cell line THP-1 after differentiation to

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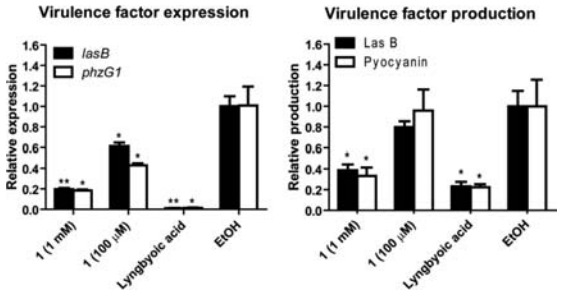

Figure 1. Quorum sensing inhibition by pitinoic acid A (**1**) in *P. aeruginosa*. Lyngbyoic acid (1 mM) was used as a positive control. **P* < 0.05, ***P* < 0.01 (*t*-test); *n* = 3. Results are calculated relative to the endogenous control *rpoD*. Data are presented as mean ± SD.

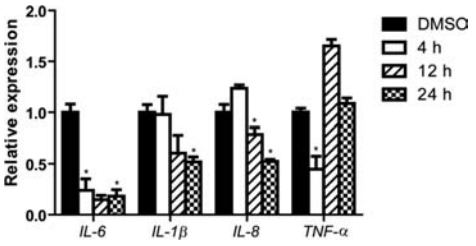

Figure 2. Effect of pitinoic acid B (**2**) (100 μM) on transcript levels of pro-inflammatory cytokines in differentiated THP-1 cells. **P*-value < 0.05 (*t*-test); *n* = 3. Results are calculated relative to the endogenous control *GAPDH*. Data are presented as mean ± SD.

macrophages. Compound **2** was able to decrease the transcript levels of the pro-inflammatory cytokines *TNF*-α and *IL-6* after 4 h of LPS stimulation at 100 μM (Figure 2).[23] Additionally, the anti-inflammatory effect was also sustained after 24 h, where the mRNA levels of *IL-6*, *IL-1β*, and *IL-8* were significantly reduced (Figure 2). Notably, *IL-6* was the most affected cytokine at all time points. Cell viability under the tested conditions was 73% based on MTT colorimetric assay.

Based on its structural features, we hypothesize that the chlorinated alcohol part in the ester **2** is the major contributor to its detected anti-inflammatory effect. This notion is consistent with the structural similarity of this moiety to other previously reported anti-inflammatory compounds and to the anti-inflammatory acid part in honaucins.[19] Additionally, using ^{1}H NMR and MS analysis, we were able to confirm the presence of this α-hydroxy acid in one of the minor impure HPLC fractions obtained during the purification of **2** (Figures S29, S30). Pitinoic acid C appeared to be the major compound in this HPLC fraction (Figure S30), but it was unstable since purification trials led to the loss of material. Accordingly, the impure fraction containing pitinoic acid C was tested for the anti-inflammatory effects. Indeed, this fraction was also able to reduce the levels of *IL-6* and *TNF*-α after 4 h at 15 μg/mL (Figure S31). Notably, **1** was also able to reduce the levels of those pro-inflammatory cytokines after 4 h at 100 μM (Figure S31). This is not surprising, since it has been shown that QS mediators could modulate the production of pro-inflammatory cytokines in mammalian cells.[24,25]

In conclusion, we identified a set of interesting marine bioactive lipids. The producing cyanobacterium appears to utilize a clever modular strategy to maintain the structure and function of those secondary metabolites. We suspect **2** to be a prodrug that utilizes the QS-inhibitor **1** as a protecting group to circumvent early reaction and deactivation of the α-hydroxy acid pitinoic acid C. One possible mechanism for the biological activity of the latter is dehydration to give a Michael acceptor fragment, which could be the reactive species. If true, then the protection of pitinoic acid C by esterification, as employed by this organism, is an optimum solution to avoid the early release of this reactive species. Also, if hydrolysis of **2** takes place *in vivo*, it will also release the antiquorum sensing acid part **1**. This could highlight **2** as a hybrid prodrug molecule with dual biological activity, which could be effective in the management of *P. aeruginosa* infections and associated inflammation. Further studies will allow for the detailed understanding of the mode of action of those molecules and the development of more potent analogues.

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Supporting Information Available. 1D and 2D NMR spectra for **1** and **2**, supporting figures including spectral data and characterization of synthetic intermediates, experimental procedures, and additional RT-qPCR data. This material is available free of charge via the Internet at http://pubs.acs.org.

The authors declare no competing financial interest.

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