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Discovery, Total Synthesis, and SAR of Anaenamides A and B: Anticancer Cyanobacterial Depsipeptides with a Chlorinated Pharmacophore

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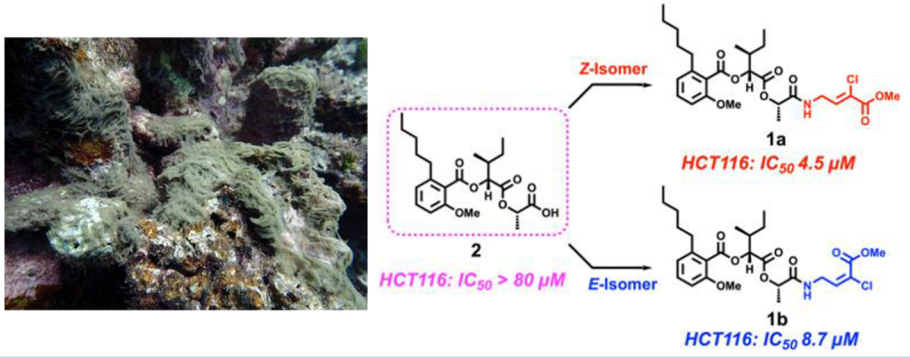

ABSTRACT: New modified depsipeptides and geometric isomers, termed anaenamides A (**1a**) and B (**1b**), along with the presumptive biosynthetic intermediate, anaenoic acid (**2**), were discovered from a marine cyanobacterium from Guam. Structures were confirmed by total synthesis. The alkylsalicylic acid fragment and the C-terminal α-chlorinated α,β-unsaturated ester are novelties in cyanobacterial natural products. Cancer cell viability assays indicated that the C-terminal unit serves as the pharmacophore and that the double-bond geometry impacts the cytotoxicity.

Marine cyanobacteria continue to be a source of natural products, producing a wealth of diverse biologically active compounds, in particular, unique peptides and depsipeptides.[1,2] Recently, our group described a family of alkylphenols produced by an undescribed gray filamentous cyanobacterium from the genus *Hormoscilla*.[3] This discovery highlighted the biosynthetic capacity of filamentous cyanobacteria, further prompting our search for novel and therapeutically relevant specialized metabolites. Here, a morphologically related green filamentous cyanobacterium, *Hormoscilla* sp. (16S rDNA; GenBank MT218338),[4,5] yielded a structurally distinct family of natural products, which we termed anaenamides. Their general scaffold is distinguished by two α-hydroxy acid residues, flanked by an alkylated salicylic fragment and an unusual α-chlorinated α,β-unsaturated (E/Z) ester. Their inherent novelty, with respect to cyanobacteria, further emphasized the untapped chemical diversity associated with understudied marine organisms.

The cyanobacterium was collected from the Anae Island reef system in Guam. The freeze-dried material was exhaustively extracted with EtOAc–MeOH (1:1) followed by H_2O–MeOH (1:9) to give nonpolar and polar extracts, respectively. The two extracts were combined, partitioned against H_2O and EtOAc, and subjected to repeated rounds of normal and reversed-phase chromatography yielding two isomerically pure compounds, anaenamide A (**1a**) [colorless, solid, $[\alpha]^{25}_D$ −46 (c 0.29, $CHCl_3$)] HRMS (ESI) m/z $[M + H]^+$ calcd for $C_{27}H_{39}NO_8{}^{35/37}Cl$, 540.2364/542.2334, found 540.2349/542.2322) and anaenamide B (**1b**) [colorless, solid, $[\alpha]^{25}_D$ −38 (c 0.06, $CHCl_3$)]; HRMS (ESI) m/z $[M + H]^+$ calcd for $C_{27}H_{39}NO_8{}^{35/37}Cl$, 540.2364/542.2334, found 540.2351/542.2330) (Figure 1). Reconciliation of the observed ^1H and ^{13}C NMR resonances for **1a** and **1b** with the HSQC spectra indicated the presence of six methylenes (C-8 to C-11, C-4′ and C-4‴), three methines (C-2′, C-3′, and C-2″), four methyl groups (C-12, C-5′, C-6′, and C-3″), three contiguous aromatic signals (C-4 to C-6), one isolated olefinic proton, and two *O*Me groups (C-13 and C-1‴). Additionally, the ^{13}C NMR spectrum indicated four nonprotonated sp^2 carbons (C-2, C-3, C-7, and C-2‴), and four carbonyls (δ_c 169.4, C-1; 169.0 C-1′; 170.5, C-1″; and 162.3, C-1‴). Analysis of the

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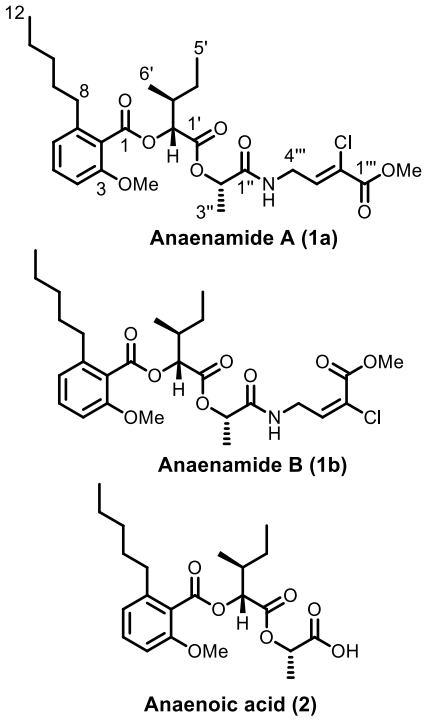

Figure 1. Structures of anaenamides A (**1a**), B (**1b**), and anaenoic acid (**2**).

COSY and HMBC spectra revealed the presence of the alkyl salicylic acid, 2-hydroxy-3-methylpentanoic acid (Hmpa), and lactic acid residues (Tables S1 and S2). Additionally, strong NOESY correlations (Table S2) between the 13-OMe (δ_H 3.79) to H-4 (δ_H 6.76) and 8-methylene (δ_H 2.57) to H-6 (δ_H 6.84) further confirmed the substitution pattern of the aromatic ring. The partial structure of the terminal amino ester of **1a** and **1b** was determined based on similar 2D NMR correlation arguments (Tables S1 and S2) and connected to the core scaffold based on a strong HMBC from NH (δ_H 7.09 in **1a** and **1b**) to the lactic acid carbonyl (δ_C 170.5 170.4 respectively, C-1′′). The distinct methoxy resonances observed in the ^{1}H NMR spectra for **1a** (δ_H 3.71) and **1b** (δ_H 3.76) were assigned as conjugated methyl esters based on: (i) the relatively low-field carbonyl chemical shifts (i.e., **1a** δ_C 162.3, and **1b** δ_C 162.7) and (ii) observed HMBC correlations from the methoxy protons to their respective carbonyls. Assignment of the chlorine atom to the quaternary C-2′′′ carbon satisfied the molecular formulas for **1a** and **1b** and was consistent with the observed shifts and 2D correlations (Figure 2A). The configuration of the olefin in **1a** was first determined based on a weak NOESY correlation between the methyl ester (δ_H 3.71) and the H-3′′′ proton (δ_H 7.01). Additionally, the H-3′′′ proton in **1b** (δ_H 6.43) was found to resonate upfield relative to **1a** due to shielding by the vinyl chloride. In combination, these data support the *Z* and *E* configurations for **1a** and **1b**, respectively (Figure 2B).

Further chemical investigation of another column fraction of the crude extract indicated the presence of an additional natural product, and possible biosynthetic intermediate to **1a** and **1b**, anaenoic acid (**2**) [colorless, solid, $[\alpha]^{25}_D$ −35 (*c* 0.15, $CHCl_3$); HRMS (ESI) *m*/*z* $[M + Na]^+$ calcd for $C_{22}H_{32}O_7Na$, 431.2045, found 431.2041. Based on the molecular formula and 2D NMR data, **2** was found to only differ from **1a** and **1b** by the lack of the amide-linked α,β-unsaturated ester. In order

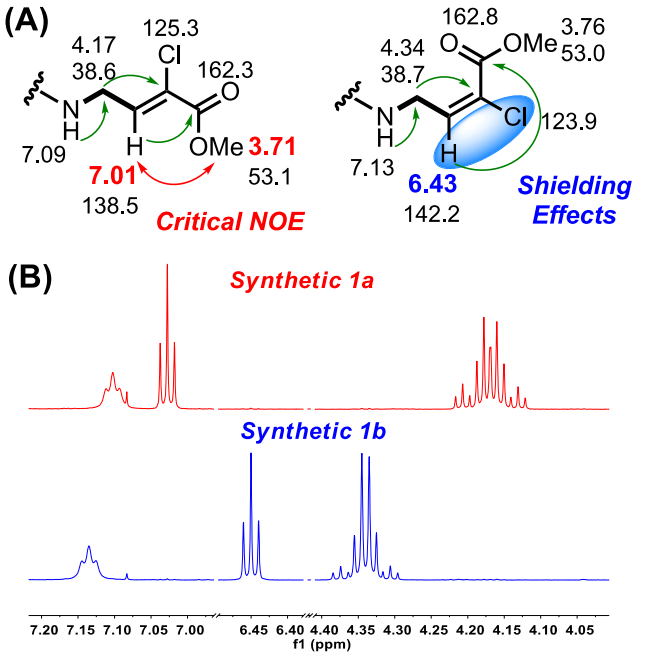

Figure 2. (A) Elucidation of the α-chlorinated α,β-unsaturated ester double bond configuration in natural **1a** and **1b** based on critical COSY (bold lines), HMBC (green arrows), and NOESY (red double arrow). (B) Spectral comparison of select α-chlorinated α,β-unsaturated ester proton resonances for synthetic **1a** and **1b**. Note the clear shift differentials due to shielding effects.

to confirm the presence of a free carboxylic acid, **2** was reacted with trimethylsilyldiazomethane yielding anaenoic acid methyl ester. New signals in the ^{1}H and ^{13}C NMR spectra of the product were consistent with an additional methoxy group (i.e., δ_H 3.75 and δ_C 52.4).

The absolute configurations of the amino acid residues of **1a**, **1b**, and **2** were determined via three independent acid hydrolyses (6 M HCl, 110 °C, 12 h) in combination with chiral HPLC. The retention times of the acid hydrolyzate components were compared to authentic standards and indicated that **1a**, **1b**, and **2** contained L-lactic acid and (2*R*,3*S*)-Hmpa. In order to further confirm these assignments and access sufficient quantities of material for biological testing, synthetic routes to all three natural products were devised and executed.

As **1a** and **1b** are geometric isomers, their presumptive biosynthetic intermediate **2** served as a synthetic handle for chemical divergence, promoting a linear synthesis (Scheme 1). Boc-protected intermediates of **3a** and **3b** could be obtained in one pot via HWE olefination starting from **4** and **5**.[6,7] Further dissection of **2** to amino acid derivatives **6** and **7** accounted for the three chiral centers.[8] The alkylated aromatic fragment **8** was found to be identical to an intermediate described in the total synthesis of the micacocidin family of natural products.[9−11]

Synthesis of fragment **8**, as previously described,[7] commenced with the lithium-directed acetylation of **9** with ethyl chloroformate and subsequent substitution of the tertiary amine to yield benzyl chloride **10** (Scheme 2). In order to install the remaining four carbons (i.e., C-9−C-12) of the alkyl chain, **10** was converted to the phosphorus ylide and reacted with crotonaldehyde under standard Wittig conditions yielding **11** as a mixture of dienes. Further reduction and hydrolysis of

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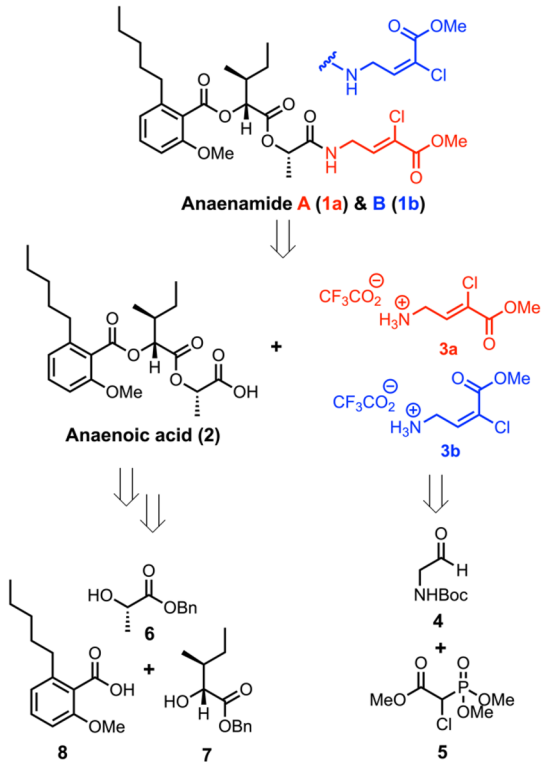
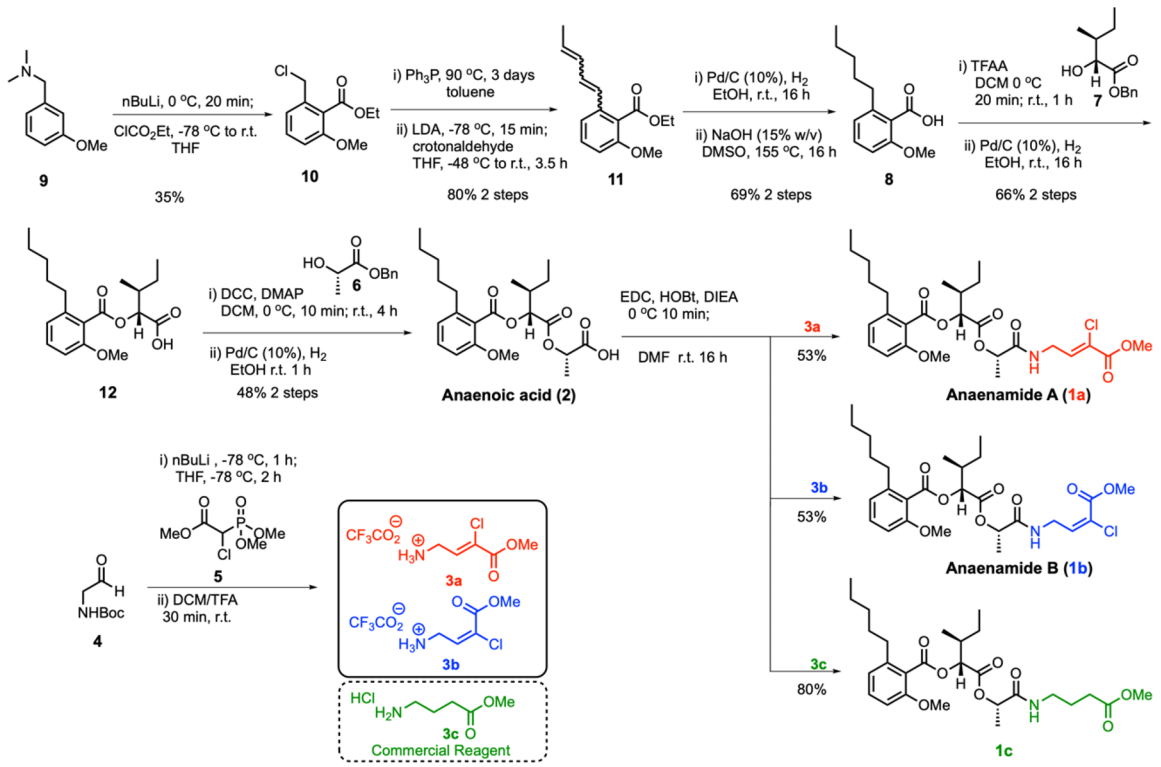
Scheme 1. Retrosynthetic Analysis of Anaenamide Scaffold



11 furnished the first fragment of the scaffold on gram scale. To our surprise, the esterification of **8** with **7** was notably challenging and did not occur under standard conditions (e.g., Yamaguchi esterification, Steglich coupling, and acid chloride activation).[12,13] We attributed the difficulty of forging this bond to (1) unfavorable ring electronics due to the ortho alkyl/OMe substituents and (2) steric clash between the bulky R groups on the branched amino acid and alkyl headgroup.[14] Utilizing trifluoroacetic anhydride (TFAA), we were able to access **12** on a half-gram scale with 66% yield (two steps). Anaenoic acid (**2**) was synthesized from **12** and **6** under standard DCC/DMAP coupling conditions and subsequent deprotection to the free acid. Boc Intermediates of **3a** and **3b** were first synthesized via HWE olefination from commercially purchased **4** and freshly prepared **5**, and the products were subsequently deprotected under acidic conditions.[7] Utilizing **2** as a point of chemical divergence, intermediates **3a** and **3b** were found to react in the presence of EDC/HOBt to yield anaenamide A (**1a**) and anaenamide B (**1b**). The ^{1}H and ^{13}C NMR data for the three isolated natural products and the synthetic compounds perfectly matched (Figures S16−S21). Additionally, optical rotations for the synthetic compounds were consistent with the isolated natural products, confirming the correct absolute configuration.

Since marine cyanobacteria are prolific producers of cytotoxic anticancer agents[15−19] with different mechanisms of action, including templates for payloads of FDA-approved antibody−drug conjugates,[19,20] we subjected the three compounds initially to cancer cell viability assays. Compounds **1a** and **1b** were first tested alongside **2** for antiproliferative activity to HCT116 colorectal cancer cells (Figure 3). While **2** only displayed slight activity at the highest concentration tested (~20% inhibition at 80 μM), **1a** and **1b** showed low micromolar activity, implicating the C-terminal residue as the pharmacophore. Additionally, we observed a SAR due to double-bond geometry as **1a** elicited 2-fold increased potency relative to **1b** (IC_{50} 2.8 vs 4.8 μM). We sought to further explore this SAR via compound **1c**, which notably lacked the

Scheme 2. Total Synthetic Route to 1a−c and 2



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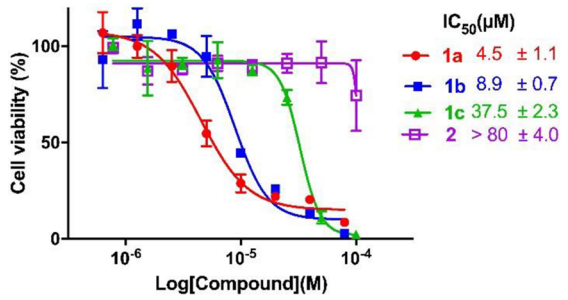

Figure 3. Dose−response curves for synthetic **1a**, **1b**, **1c**, and **2** in HCT116 cell viability assays using MTT. Error bars represent standard deviations of mean IC_{50} values based on three technical replicates.

halogenation and α,β-unsaturation (Scheme 2). Ablation of these structural features resulted in a ∼10-fold decrease in cytotoxicity. While this compound was still marginally active (IC_{50} 37.5 μM), we hypothesize that at higher concentrations either alternative cytotoxic mechanisms are observed in this cell line or that the presence of the conjugated π system reinforces target engagement. The unsaturated C-terminal unit could impact inherent binding affinity or selectivity via covalent or noncovalent interactions. Compound **1c** may later function as a control in our mechanistic studies.

On the basis of their structural features, the anaenamides are likely biosynthesized from a PKS/NRPS hybrid pathway containing undescribed cyanobacterial enzymology (Scheme S1). Putatively, we propose that the initiation module in the biosynthesis of **1a-2** includes a fatty acid AMP ligase domain and type 1 PKS, yielding the alkyl salicylic acid residue.[21,22] Interestingly, the same 5-carbon alkyl salicylic acid fragment observed in the anaenamides also serves as an intermediate in the biosynthesis of the micacocidins, and originates from an iterative type 1 PKS.[10,11] Studies of cyanobacterial lipopeptides (e.g., hassallidins and puwainaphycins) and their gene clusters have uncovered similar FAAL/PKS initiation domain motifs.[23−25] As (2*R*,3*S*)-HMPA is a derivative of D-*allo*-isoleucine, the first NRPS module would include the corresponding adenylation, and α-ketoreductase domains.[26] The L-lactic acid residue could be directly incorporated from a single NRPS module or derived from L-alanine via a 2-oxopropanoic acid intermediate. Given the high abundance of anaenoic acid (**2**) (0.13% dry weight), it is our opinion that **2** is a biosynthetic intermediate for **1a** and **1b**. The four-carbon backbone of the chlorinated α,β-unsaturated ester could originate from glycine and acetate. This chemistry is exemplified by the BaeJ gene, coding for the PKS/NRPS bacillaene, where glycine resides are modified and extended by downstream PKS modules.[27,28] As this functional group has never been reported in cyanobacteria, our efforts to characterize the underling cellular machinery is ongoing.

In summary, we have described the isolation of the anaenamide family of natural products (**1a**, **1b**, and **2**) from a green filamentous cyanobacterium *Hormoscilla* sp. These new compounds were successfully synthesized, confirming our structural assignments. Our strategic linear synthesis will provide a platform for the rapid generation of chemically diverse, biological probes, as demonstrated by the success of **1c**. While **1a** and **1b** displayed moderate cytotoxicity against HCT116 cancer cells, our results have demonstrated that the halogenated α,β-unsaturated ester moiety serves as the pharmacophore for the cytotoxic activity. We hypothesize that this highly unusual modification could function as a Michael acceptor, driving the observed SAR. In light of our identification of the halogenated α,β-unsaturated ester as a synthetically "tunable" pharmacophore, our ongoing work will explore the biological impacts of C-terminal residue functionalization.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.orglett.0c01281.

Experimental procedures, NMR Tables S1 and S2, ^{1}H, ^{13}C, COSY, HMBC, 2D NOESY, and HSQC NMR spectra of natural **1a**, **1b**, and **2** in addition to their synthetic equivalents and intermediates in $CDCl_3$, and Scheme S1 (PDF)

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Notes

The authors declare no competing financial interest.

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