Tutuilamides A–C: Vinyl-Chloride Containing Cyclodepsipeptides from Marine Cyanobacteria with Potent Elastase Inhibitory Properties

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ABSTRACT: Marine cyanobacteria (blue-green algae) have been shown to possess an enormous capacity to produce structurally diverse natural products that exhibit a broad spectrum of potent biological activities, including cytotoxic, antifungal, antiparasitic, antiviral and antibacterial activities. Using mass spectrometry-guided fractionation together with molecular networking, cyanobacterial field collections from American Samoa and Palmyra Atoll yielded three new cyclic peptides, tutuilamides A–C. Their structures were established by spectroscopic techniques including 1D and 2D NMR, HR-MS, and chemical derivatization. Structure elucidation was facilitated by employing advanced NMR techniques including non-uniform sampling in combination with the 1,1-ADEQUATE experiment. These cyclic peptides are characterized by the presence of several unusual residues including 3-amino-6-hydroxy-2-piperidone and 2-amino-2-butenoic acid, together with a novel vinyl chloride-containing residue. Tutuilamides A-C show potent elastase inhibitory activity together with moderate potency in H-460 lung cancer cell cytotoxicity assays. The binding mode to elastase was analyzed by X-ray crystallography revealing a reversible binding mode similar to the natural product lyngbyastatin 7. The presence of an additional hydrogen bond with the amino acid backbone of the flexible side chain of tutuilamide A, compared to lyngbyastatin 7, facilitates its stabilization in the elastase binding pocket and possibly explains its enhanced inhibitory potency.

INTRODUCTION

Cyanobacteria produce a wide range of cyclic depsipeptides that contain an unusual 3-amino-6-hydroxy-2-piperidone (Ahp) moiety; these peptides are generally referred to as cyanopeptolin-like peptides or Ahp-cyclodepsipeptides.[1–3] The general structure includes an amino acid chain of variable length of which six amino acid residues form a macrocyclic ring. Most cyanopeptolin-like peptides contain the Ahp residue and an ester linkage between the β-OH group of L-threonine and the carboxyl group of the C-terminal amino acid. Additionally, among these Ahp containing peptides, there are more than 20 compounds that contain a highly conserved hexadepsipeptide core bearing an additional 2-amino-2-butenoic acid (Abu) moiety adjacent to the Ahp ring together with a highly variable side chain (Table 1).

Ahp-containing peptides have frequently been observed from both marine and freshwater cyanobacteria as well as other bacteria, and over 200 are now known.[3] However, peptides containing the Abu moiety are generally found in marine bacteria, with the exception being stigonemapeptin which derives from a freshwater

Table 1. Abu and Ahp-containing peptides isolated from Cyanobacteria.

Compound name	Year	Collected from	Isolated organism
Dolastatin 13[5]	1989	East Africa, Indian Ocean	*Dolabella auricularia* (sea hare)
Symplostatin 2[6]	1999	Guam, Pago Bay	*Symploca hydnoides*
Somamide A+B[7]	2001	Fiji, Somo Somo	*Lyngbya majuscula* / *Schizothrix* sp.
Lyngbyastatin 4[8]	2007	South Florida, Atlantic coast	*Lyngbya confervoides*
Lyngbyastatin 5–7[9]	2007	South Florida, Fort Lauderdale / Florida Keys, Summerland Key	*Lyngbya confervoides*
Lyngbyastatin 8–10[10] / Bouillomide A+B[11]	2009	Guam, Tumon Bay	*Lyngbya semiplena*
Molassamide[12]	2010	Florida, Molasses Reef	*Dichothrix utahensis*
Stigonemapeptin[4]	2012	Wisconsin, North Nokomis Lake	*Stigonema* sp. (freshwater)
Symplostatin 5–10[13]	2013	Guam, Cetti Bay	red *Symploca* sp.
Kurahamide[14]	2014	Japan, Kuraha	*Lyngbya* sp. assembly

cyanobacterium (Table 1).[4] Interestingly, the Abu moiety in stigonemapeptin is *(E)*-configured whereas the Abu moie-ties derived from marine sources are consistently *(Z)*-configured.X-ray crystallography of the elastase complex formed with lyngbyastatin 7 (**4**) revealed that these Abu-cyclodepsipeptides act as substrate mimics.[13] The Abu moiety was shown to occupy the S1 substrate binding pocket and engage in a non-covalent interaction. Additionally, a study of the binding mode of scyptolin showed that the Ahp moiety occupies a crucial part of the active site pocket and thereby prevents hydrolysis.[15] It was suggested that the Abu moiety increases the potency of these elastase inhibitors and that the pendant side chain is responsible for modulating their activity and selectivity.[13]

In the current work, we report the isolation of three new members of this Ahp and Abu-containing family of peptides, named tutuilamides A (**1**) and B (**2**) based on the location of collection of the source cyanobacterium, *Schizothrix* sp., along with tutuilamide C (**3**) isolated from a *Coleofasciculus* sp. These structures were assembled by a combination of NMR, mass spectrometry (MS) and chromatographic analysis following acid hydrolysis and chiral derivatization, and feature an unusual vinyl chloride-containing residue never previously observed in this structure class. The new natural products, as well as two semisynthetic derivatives, were evaluated for serine protease inhibition and all of the cyclic species were found to be highly potent. The crystal structure of elastase in complex with tutuilamide A revealed extensive binding interactions in the substrate binding pocket, as has been shown previously with lyngbyastatin 7 (**4**). However, we identified additional hydrogen bond interactions between tutuilamide A and elastase that did not occur in the lyngbyastatin 7 co-crystal structure. These may be responsible for the increased potency of tutuilamide A compared to lyngbyastatin 7.

RESULTS AND DISCUSSION

Our discovery strategy to locate natural products with novel structural frameworks includes MS^2-based metabolomics (Molecular Networking) for strain selection and dereplication as well as chromatographic methods for isolation driven by structural features. Cyanobacterial colonies of the genus *Schizothrix* sp. and *Coleofasciculus* sp. were collected by hand from the main island of Tutuila in American Samoa in 2016 and Palmyra Atoll in 2008, respectively, using SCUBA gear. The crude CH_2Cl_2-MeOH (2:1) extract was initially fractionated using vacuum-liquid chromatography (VLC) as well as solid phase extraction (SPE) for further analysis by MS and NMR. This approach revealed the presence of peptides with unusual features and led to the HPLC isolation of tutuilamides A and B from *Schizothrix* sp and tutuilamide C from *Coleofasciculus* sp. In addition, we identified related peptides such as symplostatin 2 together with several derivatives of dolastatin 13 that have yet to be characterized. Tutuilamide A (**1**) and B (**2**) share the same cyclic peptide core and possess the unusual Ahp and Abu moieties. They differ by a single side chain residue wherein isoleucine is replaced by valine (Figure 1). Meanwhile, tutuilamide C (**3**) is an analog of **2** that lacks an alanine moiety, but bears an additional methylene unit in the aliphatic side-chain. Moreover, they are the first cyanopeptolins to possess a vinyl chloride residue in the side chain. Vinyl chloride functionalities in cyanobacteria have been shown to biosynthetically result from a unique cassette of enzymes that involve polyketide synthase (PKS) beta branch formation along with radical-based chlorination of an intermediate, such as in jamaicamide A.[16]

High-resolution electrospray ionization mass spectrometry (HR-ESI-MS) of tutuilamide A (**1**) displayed an ion peak at *m/z* 1043.4616 $[M + Na]^+$ (calculated for $C_{51}H_{69}ClN_8O_{12}Na$, 1043.4616, Δ = 0.0 ppm), consistent with the molecular formula $C_{51}H_{69}ClN_8O_{12}$ containing 21 double-bond equivalents. The isotope pattern for the molecular ion cluster indicated the clear presence of one chlorine atom. The ^1H NMR spectrum of **1** in DMSO-d_6 exhibited signals characteristic of a peptide including seven α-proton signals at δ 4.65 (overlap), 4.89 (1H, dd, *J* = 11.5, 2.2 Hz), 4.73 (1H, dd, *J* = 11.4, 4.0 Hz), 3.79 (1H, m), 4.67 (overlap), 4.40 (1H, t, *J* = 7.8 Hz), and 4.36 (1H, p, *J* = 7.2 Hz),

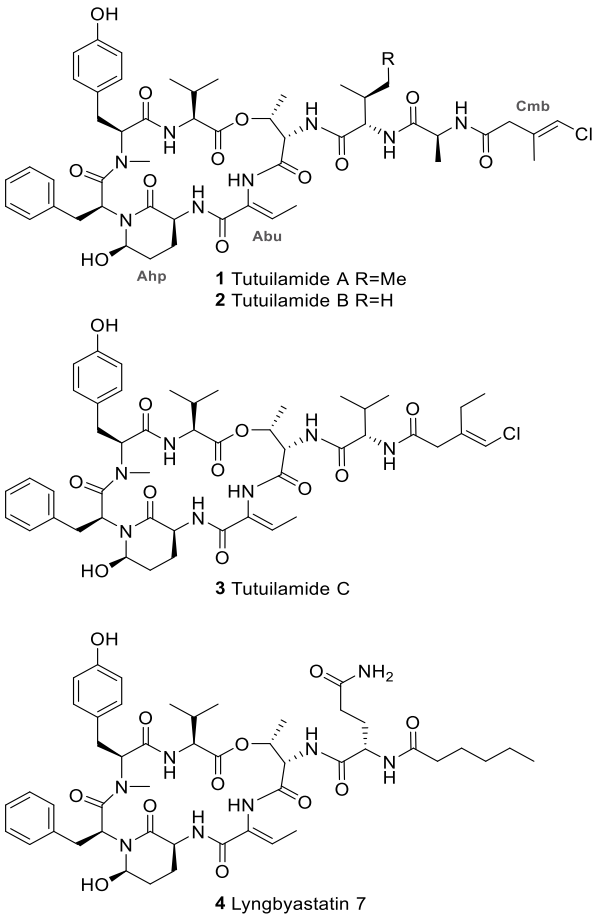


Figure 1. Chemical structure of tutuilamide A (**1**), B (**2**) and C (**3**) together with the related lyngbyastatin 7 (**4**).[9]

along with six amide NH signals at δ 7.53 (1H, t, *J* = 8.4 Hz), 7.22 (1H, broad), 9.23 (1H, broad), 7.91 (1H, broad), and 8.21 (1H, d, *J* = 7.4 Hz) (Table S1). Additionally, a downfield pair of triplets at δ 7.18 (2H, t, *J* = 7.2 Hz) and 7.15 (1H, t, *J* = 7.2 Hz) and a doublet at δ 6.83 (2H, d, *J* = 7.2 Hz) were characteristic of a phenyl group; similarly, two doublets at δ 7.00 (2H, d, *J* = 8.3 Hz) and 6.78 (2H, d, *J* = 8.3 Hz) were characteristic for a *para*-substituted phenol group. The proton NMR spectrum also showed a singlet at δ 2.77 (3H, s), indicative of an *N*-methyl amide, together with eight partially overlapping methyl signals at δ 0.75 (3H, d, *J* = 6.8 Hz), δ 0.80 (3H, t, *J* = 7.5 Hz), δ 0.84 (3H, d, *J* = 6.8 Hz), δ 0.88 (3H, d, *J* = 6.8 Hz), δ 1.19 (3H, d, *J* = 7.0 Hz), δ 1.21 (3H, d, *J* = 6.6 Hz), δ 1.48 (3H, d, *J* = 7.1 Hz), and δ 1.72 (3H, d, *J* = 1.2 Hz).

The HSQC spectrum revealed the presence of six methylene groups (δC-3_{Tyr} 32.5, δH-3_{Tyr} 3.11/2.69; δC-3_{Phe} 35.2, δH-3_{Phe} 2.88/1.83; δC-3_{Ahp} 21.9, δH-3_{Ahp} 2.42/1.58; δC-4_{Ahp} 29.2, δH-4_{Ahp} 1.72/1.58; δC-4_{Ile} 23.9, δH-4_{Ile} 1.43/1.08; δC-2_{Cmb} 42.5, δH-2_{Cmb} 2.97), two methines (δC-3_{Val} 30.5, δH-3_{Val} 2.07; δC-3_{Ile} 36.5, δH-3_{Ile} 1.81), two oxygenated methines (δC-5_{Ahp} 73.7, δH-5_{Ahp} 5.08; δC-3_{Thr} 71.7, δH-3_{Thr} 5.53), and two vinylic methines (δC-3_{Abu} 131.5, δH-3_{Abu} 6.52; δC-4_{Cmb} 114.3, δH-4_{Cmb} 6.10).

A detailed analysis of the 2D NMR data (HSQC, HMBC, and DQF-COSY) established the presence of valine, N-methyl-tyrosine, phenylalanine, threonine, isoleucine and alanine together with an Ahp, an Abu and a 4-chloro-3-methylbut-3-enoic acid (Cmb) residue. The Ahp residue was identified by COSY correlations following the sequential spin system starting at the α-proton at δ3.79 (H-2_{Ahp}) followed by two methylene protons at δ 2.42/1.58 (H-3_{Ahp}), another set of methylene protons at δ 1.72/1.58 (H-4_{Ahp}), and an oxygenated methine at δ 5.08 (H-5_{Ahp}). The ring closure was based on HMBC correlations from the α-proton at δ 3.79 (H-2_{Ahp}), the methylene protons at δ 2.42 and 1.58 (2H-3_{Ahp}) and the oxygenated methine proton at δ 5.08 (H-5_{Ahp}) to the carbonyl carbon at δ 168.5.

The Abu structure was based on a COSY correlation between the methyl protons at δ 1.48 (Me-4_{Abu}) and a vinylic methine proton at δ 6.52 (H-3_{Abu}) as well as HMBC correlations from both proton signals to carbon resonances at δ 129.8 (C-2_{Abu}) and 162.6 (C-1_{Abu}). The structure of the remaining C_5H_6ClO residue was deduced as follows. Long-range COSY correlations between the vinylic methine proton at δ 6.10 (H-4_{Cmb}), the methyl protons at δ 1.72 (Me-5_{Cmb}) and the methylene protons at δ 2.97 (2H-2_{Cmb}) gave an unclear picture of the residue structure. Additionally, HMBC correlations from all three of these proton signals to a quaternary carbon at δ 133.9 (C-3_{Cmb}) and a carbonyl signal at δ 168.4 (C-1_{Cmb}) left the position of the double bond unresolved as four-bond ($^4J_{CH}$) interactions are occasionally observed in the proximity of double bonds. To differentiate between a 4-chloro-3-methylbut-3-enoic acid and a 4-chloro-3-methyl-2-enoic acid residue, a 1,1-ADEQUATE spectrum with non-uniform sampling (NUS) was recorded which showed a two bond correlation from the methylene protons at δ 2.97 (2H-2_{Cmb}) to the carbonyl signal at δ 168.4 (C-1_{Cmb}), thereby confirming this as a 4-chloro-3-methylbut-3-enoic acid residue. Finally, an NOE correlation between the associated amide proton at δ 8.21 (NH_{Ala}) and the methylene protons at δ 2.97 (2H-2_{Cmb}) helped to confirm the identity and location of the residue. The sequence of residues was established as Val-N-MeTyr-Phe-Ahp-Abu-Thr-Ile-Ala-Cmb through HMBC correlations between consecutive α-protons to carbonyl carbons of adjacent residues together with NOE correlations between α-protons and amide protons (Figure 2). The ring closure between the C-terminal valine and the β-hydroxy group of threonine was established based on NOE correlations from the valine α-proton at δ 4.65 (H-2_{Val}) to the threonine methine and methyl protons at δ 5.53 (H-3_{Thr}) and δ 1.21 (Me-3_{Thr}), respectively.

The second and considerably less abundant new compound isolated from this extract, tutuilamide B (**2**), showed a HR-ESI-MS molecular ion peak at *m/z* 1029.4445 $[M + Na]^+$ (calcd for $C_{50}H_{67}ClN_8O_{12}Na$, 1029.4459, Δ = 1.36 ppm), thus having one less methylene unit than **1**. Comparison of NMR features for **2** (Table S2) showed it to be highly similar in all regards to **1**, with the primary difference being that the triplet methyl from the Ile residue at δ 0.80 (Me-5_{Ile}) in **1** was replaced by an additional doublet methyl at δ 0.82 (Me-5_{Val2}) in **2**, thus revealing **2** to be the valine analog of **1**.

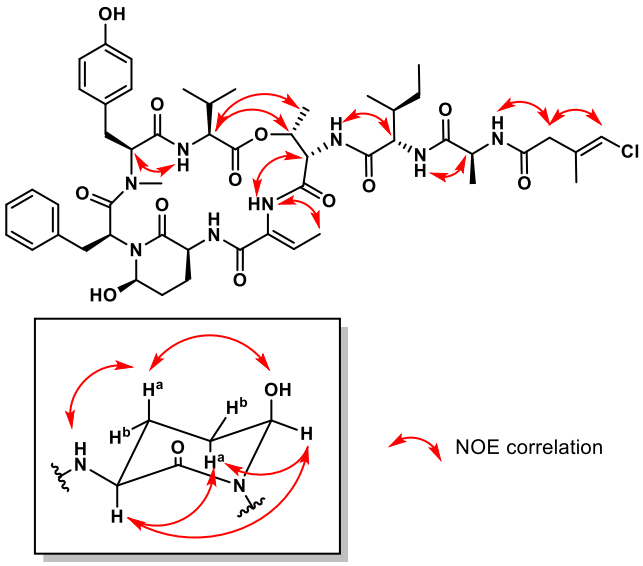


Figure 2. Selected NOE correlations for tutuilamide A (**1**). Inset shows selected correlations observed for the Ahp residue.

The absolute configurations of the amino acid residues in both natural products were determined by LC-MS analysis of the 1-fluoro-2,4-dinitro-phenyl-5-D-alanineamide (D-FDAA) derivatives derived from the acid hydrolyzate of **1** and **2** (Marfey's method, Table S3).[17] This revealed that all of the amino acid residues in both compounds were of L-configuration, as is the case for all other cyanopeptolin-like peptides. In addition, PDC oxidation followed by acid hydrolysis liberated L-glutamic acid from the Ahp residue, as determined by Marfey's analysis, therefore establishing the configuration of C-2_{Ahp} as *S*. To determine the relative stereochemistry between C-2_{Ahp} and C-5_{Ahp} in **1**, a combination of proton-proton coupling constants and NOE correlations was used (Figure 2). The relative conformation of the Ahp ring was determined based on the H-2_{Ahp} to H-3_{Ahp} coupling constant; a large value was observed ($^3J_{H\text{-}2,H\text{-}3a}$ = 12.3 Hz), indicating that these two deshielded protons were axially oriented.[18] NOE correlations between H-2_{Ahp}, H-$4a_{Ahp}$ and H-5_{Ahp} as well as between H-$3a_{Ahp}$, OH_{Ahp} and NH_{Ahp} revealed the relative configuration of the two stereocenters, and therefore established the configuration of C-5_{Ahp} as *R*. The highly comparable NMR data sets for this Ahp residue for compounds **1–3** (see Supporting Information, Tables 1 and 2) strongly suggests that they are all of the same relative configuration, and likely of the same absolute configuration. The geometry of the Abu olefinic bond was determined as *Z* based on an NOE correlation in DMSO-d_6 between the methyl protons at δ1.48 (Me-4_{Abu}) and the amide proton at δ 9.23 (NH_{Abu}). Finally, a 1D NOE experiment with selective irradiation of the vinylic proton at δ 6.07 (in methanol-d_4 to avoid overlapping signals with OH_{Ahp}) established the *E* geometry of the Cmb residue due to a correlation to the methylene protons at δ 3.03 (2H-2_{Cmb}).

Subsequently, a third tutuilamide was isolated from a *Coleofasciculus* sp. extract (**3**). The HR-ESI-MS of tutuilamide C showed a precursor ion at *m/z* 972.4252 $[M + Na]^+$ (calcd for $C_{48}H_{64}ClN_7O_{11}Na$, 972.4255, Δ = 0.31 ppm) and the NMR data were highly similar to that of **2**, except for the lack of the alanine moiety resonances and possessing an additional methylene in the side-chain (Table S2). HMBC, TOCSY and ROESY correlations corroborated the same connectivities observed between the amino acid residues as for **1** and **2**. Because the NMR data of **2** and **3** were highly similar, we propose that they are of the same relative configuration at comparable stereocenters. This hypothesis is additionally supported by the observation that all Abu and Ahp-containing peptides reported to date are composed only of L-amino acids.[7–14]

Inhibition of serine proteases with tutuilamide analogs. Studies have shown that Ahp-Abu-containing cyclic hexadepsipeptides inhibit serine proteases such as elastase by binding to the active site in a substrate-like manner. Therefore, 5 μM to 85 pM of **1**, **2** and **3** were incubated with porcine pancreatic elastase and potency was directly compared to lyngbyastatin 7 (**4**) and symplostatin 2. Compounds **1** and **2** were found to be the most potent in this group, inhibiting elastase with IC_{50}'s of 1–2 nM (Table 2, Figure S2 and S3). Compound **3** and symplostatin 2 had IC_{50} values of ~5 nM, while **4** was the weakest inhibitor with an IC_{50} of 11.5 nM.

Elastase is a member of the S1 family of serine proteases that forms a double β-barrel structure. Trypsin and chymotrypsin are also members of the S1 family and therefore we determined if tutuilamide A, B and C can also inhibit these structurally related enzymes. When bovine trypsin was incubated with up to 20 μM of these compounds, no inhibition was detected. This enzyme has a strong preference for binding to peptide inhibitors containing basic amino acids such as arginine and lysine, both of which are absent from these natural products. When incubated with chymotrypsin the IC_{50} values ranged from 542 nM to 1014 nM. Although the potency of these compounds is considerably weaker for chymotrypsin than elastase, the relative potency of the three tutuilamide analogs differs between enzymes. Chymotrypsin appears to have a preference for valine as found in **2** and **3** over isoleucine found in compound **1**, resulting in a 2-fold reduction in potency. This inhibition profile is consistent with several other Ahp-Abu-containing cyclic hexadepsipeptides that preferentially inhibit elastase over chymotrypsin and trypsin.[19]

Outside of the S1 family, commonly studied serine proteases are from the S8 family. These enzymes have no sequence or structural homology with S1 family serine proteases and include the bacterial and fungal subtilisin enzymes commonly used in laundry detergents and the mammalian kexin family of enzymes.[20] When **1** and **2** were incubated with proteinase K, a representative S8 protease isolated from the soil fungus *Engyodontium album*, we observed IC_{50}s of 103.7 and 87.6 nM, respectively.

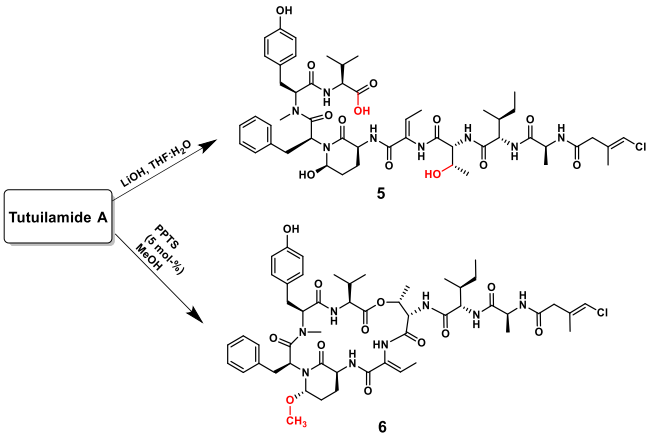
Table 2: Potency of compounds for select serine proteases following 30 minute incubations

	IC_{50} values (nM) with 95% Confidence Interval Values			
Name (Analog Number)	**Elastase**	**Chymotrypsin**	**Trypsin**	**Proteinase K**
Tutuilamide A (**1**)	1.18 (0.33 – 1.97)	1014 (853.0 – 1227)	>20,000	103.7 (84.6 – 129.2)
Tutuilamide B (**2**)	2.05 (1.68 – 2.46)	576.6 (447.0 – 767.3)	>20,000	87.6 (65.2 – 122.2)
Tutuilamide C (**3**)	4.93 (2.72 – 7.48)	542.0 (445.0 – 671.2)	>20,000	>5,000
Lyngbyastatin 7 (**4**)	11.50 (9.25 – 14.28)	Not Done	Not Done	1574 (806.0 to >5,000)
Symplostatin 2	5.41 (4.30 – 6.69)	Not Done	Not Done	216.5 (155.2 – 326.0)
Tutuilamide A-linear (**5**)	3,994 (2363 – 11,735)	Not Done	Not Done	>5,000
Tutuilamide A-methyl (**6**)	1.83 (1.56 – 2.12)	Not Done	Not Done	98.6 (94.4 – 100.1)

However, **3** had more than a 50-fold weaker inhibition for this enzyme (IC_{50} >5 μM), suggesting that longer peptides have higher potency for this enzyme. When comparing the potency of symplostatin 2, an 8-mer cyclic hexadepsipeptide to the 7-mer peptide **4**, the IC50 value decreased 7.3-fold for the shorter peptide inhibitor. These data show that changes in peptide length and amino acid composition of these cyclic hexadepsipeptides result in differences in potency and selectivity for serine proteases.

In addition, we determined the cytotoxic activity of compounds **1–3** towards the H-460 human lung cancer cell line. The compounds show moderate activity with IC_{50}s of 0.53 ± 0.04 μM, 1.27 ± 0.21 μM and 4.78 ± 0.45 μM, respectively (Figure S1).

Synthesis of semi-synthetic analogs of 1. A limited structure-activity relationship study on tutuilamide A was performed to explore the structural features related to the elastase and proteinase K inhibitory activity (Scheme 1). In the first semi-synthetic analog of **1**, the depsipeptide ester was hydrolyzed using $LiOH/H_2O$ to obtain the acyclic analog **5**. This analog failed to inhibit proteinase K activity at a concentration of 5 μM while potency for elastase was decreased 2,600-fold when compared to the cyclic tutuilamide A (**1**).



Scheme 1. Synthesis of semi-synthetic analogs **5** and **6** of tutuilamide A (**1**)

These data demonstrate the importance of the cyclic structure to inhibit these serine proteases. In addition, the hemi-aminal moiety in **1** was methylated with methanol in the presence of pyridinium *p*-toluenesulfonate (PPTS) to generate analog **6**. This modification is predicted to improve the metabolic stability of the Abu moiety without affecting the potency of the compound; indeed, analog **6** showed excellent single digit nanomolar potency to elastase (Table 3).

Rationalizing the binding of tutuilamide A to elastase. We next determined the crystal structure of porcine pancreatic elastase in complex with **1** in order to compare the binding mode of the depsipeptide with related molecules. The complex crystallized in space group $P\ 2_1\ 2_1\ 2_1$, and data was collected to 2.2 Å. The structure was determined by molecular replacement using the published elastase apo structure (PDB ID: 1LVY) as a search model (Figure 3A). Full details of the data collection and refinement statistics can be found in Table S4. Compound **1** was found to occupy the same binding pocket as lyngbyastatin 7 (**4**), explaining the observed identical mode of action of tutuilamide A compared to other Abu-bearing cyclic depsipeptides, with the Abu moiety and the N-terminal residues occupying the S1-S4 pockets (Figure 3B).[13,15] However, compared to **4**, the carbonyl group of the flexible Cmb moiety of **1** forms an additional hydrogen bond with the backbone amide of Arg217 (Figure 3B and Figure S4). This additional interaction could explain the slightly more potent inhibitory activity of tutuilamides compared to lyngbyastatin 7 in that this interaction would further stabilize the binding of tutuilamide A to the elastase binding pocket.

The potency of semi-synthetic analogs **5** and **6** was evaluated in the context of our co-crystal structure. As shown for **4**, the cyclic core of **1** also forms a network of direct and water mediated inter- and intramolecular hydrogen bonds (Figure S5). Therefore, one would expect any changes to the cyclic nature of these compounds to have a negative impact on their potency. This is indeed the case as shown by the observed nearly 3,400-fold decrease in potency of acyclic analog **5.** The hemiaminal moiety of **1** is facing a hydrophobic pocket formed by Leu 63 and Phe 65 (Figure S5). This pocket is large enough to

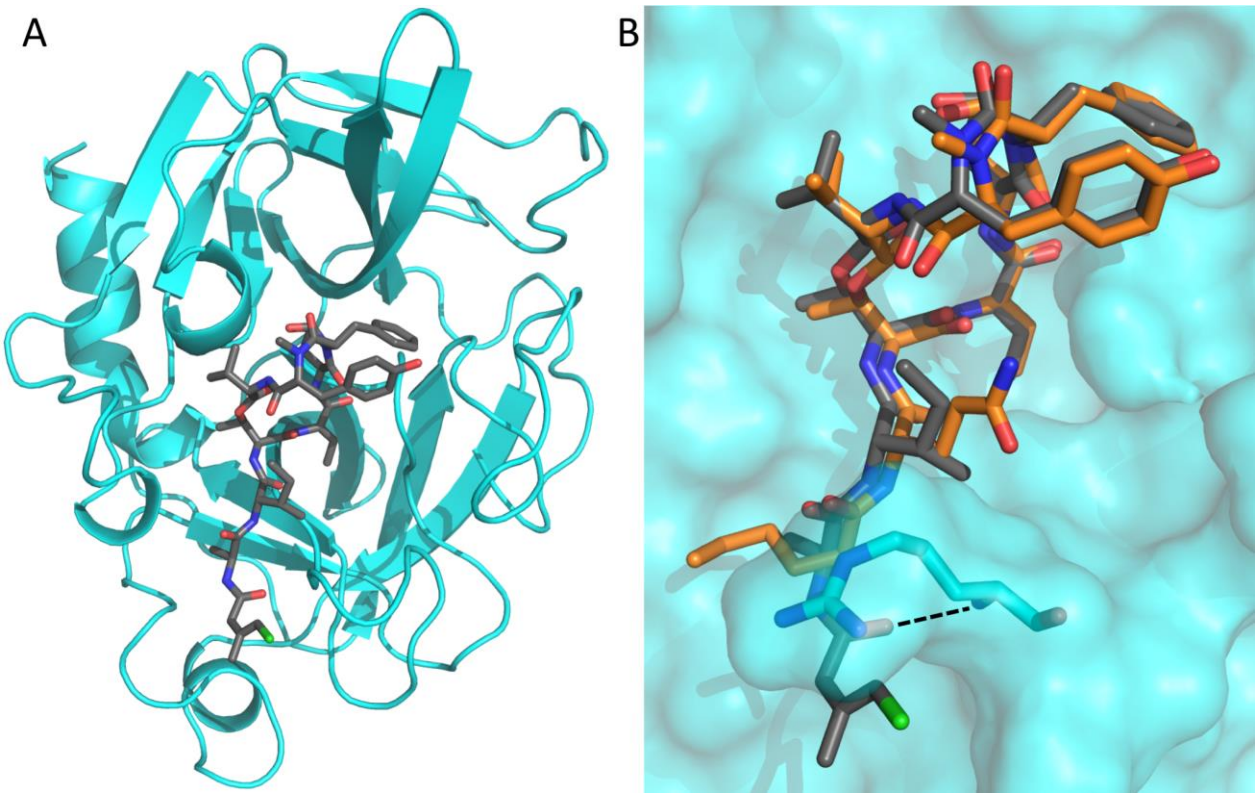


Figure 3. A Cartoon representation of PPE (cyan) in complex with **1** (shown as grey sticks). **B** Comparison of **1** (grey) and **4** (PDB: 4GVU; orange) binding to PPE (cyan surface). The side chains of PPE residues Ser216 and Arg217 are shown as sticks. The additional hydrogen bond found in the PPE-**1** complex structure is indicated by a dashed line.

accommodate methylated analog **6**, without resulting in a steric clash with the binding pocket (Figure S5). Given the size of the pocket, even larger hydrophobic functional groups at this position may be accommodated. This could result in an enhancement of hydrophobic interactions, thereby further stabilizing the compound in the active site. However, further optimization of this group must be pursued cautiously, as the oxygen atom of the hydroxy or methoxy group is involved in intramolecular hydrogen bonding with the neighboring residues, and thus facilitates the formation of a *cis* peptide bond (Figure S5). In turn, this contributes to the rigidity of the cyclic core making these compounds less sensitive to protease activity.[13,15,21,22]

CONCLUSION

Tutuilamides A–C are new representatives of the family of Ahp-cyclodepsipeptides (cyanopeptolin-like peptides) isolated from Cyanobacteria. They share the cyclic peptide core, including the distinctive 2-amino-2-butenoic acid (Abu) residue, with a group of cyclic depsipeptides mainly isolated from marine cyanobacteria; however, they also contain an unprecedented 4-chloro-but-3-enoic acid moiety. The predicted biosynthesis of these new cyclic lipopeptides suggests initiation of the pathway by a polyketide synthase which produces an intermediate β-ketobutyrate moiety. This is expected to undergo transformation by a β-branch cassette of genes that also introduces a chlorine atom via radical chemistry, as shown in the case of jamaicamide A biosynthesis.[16]

The new compounds are potent inhibitors of porcine pancreatic elastase and fungal proteinase K. Direct comparison to the potent elastase inhibitor lyngbyastatin 7 revealed that the new compounds have 2 to 4-fold increased potency. Structural analysis of tutuilamide A in complex with the porcine pancreatic elastase confirms the same binding mechanism of lyngbyastatin 7 with an additional strong hydrogen bond (2.8 Å) between the Cmb carbonyl group and the backbone amide group of elastase residue R226; this additional hydrogen bond appears to stabilize the ligand in the binding pocket and may explain the increased potency of tutuilamides A over lyngbyastatin 7.

ASSOCIATED CONTENT

Supporting Information. The supporting information contain a detailed description of the experimental procedures, tabulated ^{1}H and ^{13}C NMR data for compound **1** in methanol-d_4, ^{1}H NMR, ^{13}C NMR, ^{1}H-^{1}H COSY, ^{1}H-^{1}H NOESY, ^{1}H-^{13}C HSQC, ^{1}H-^{13}C HMBC and 1,1-ADEQUATE spectra in DMSO-d_6 as well as 1D-NOE in methanol-d_4 for compound **1**, ^{1}H NMR, ^{1}H-^{1}H COSY, ^{1}H-^{13}C HSQC and ^{1}H-^{13}C HMBC spectra in DMSO-d_6 for compounds **2** and **3,** dose response curves for elastase and H-460 cancer cell assays. This material is available free of charge via the Internet at http://pubs.acs.org.

Accession Codes. Coordinates were deposited in the Protein Databank with accession number 6TH7.

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All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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