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Structure revision of trichotoxin, a chlorinated polyketide isolated from a *Trichodesmium thiebautii* bloom

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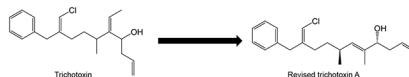
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Abstract

NMR-guided fractionation of the lipophilic extract of *Trichodesmium thiebautii* filaments led to the isolation of a phenyl-containing chlorinated polyketide (**1**) and an alkyne-containing analogue (**2**). Comparison of spectroscopic and spectrometric data of **1** with the data of the previously reported trichotoxin, strongly suggested that these metabolites were identical and supports a structural revision of trichotoxin and its designation as trichotoxin A. In addition, we report the isolation and characterization of the alkyne-containing analogue trichotoxin B (**2**). Absolute configuration of **1** and **2** is proposed based on spectroscopic comparison to a close structural analog.

Graphical Abstract



Keywords

Cyanobacteria blooms; *Trichodesmium thiebautii*; polyketide; trichotoxin

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Supplementary Data

Supplementary data: Table S1. NMR data of trichotoxin A (**1**) and the original trichotoxin. Table S2. Comparison of ¹H NMR and NOE data of **1** and the phenyl alkene compound. Table S3. Comparison of measured ¹³C NMR data for **1**, **2** and the phenyl alkene compound. Figures S1-S7. ¹H NMR, ¹³C NMR, HSQC, HMBC, COSY, TOCSY and NOESY of **1**. Figures S8-S-14. ¹H NMR, ¹³C NMR, HSQC, HMBC, COSY, TOCSY and NOESY of **2**. Figure S15. ¹H NMR for **1** recorded in CD₃CN. Figure S16. ¹³C NMR for **1** recorded in CD₃CN. The supplementary material associated with this article can be found in the online version.

Cyanobacteria continue to be a notable microbial source for the isolation of secondary metabolites displaying diversity with respect to chemical structures and biological activities.^{1,2} *Trichodesmium thiebautii* Gomont *ex* Gomont is a marine filamentous cyanobacterium ecologically relevant both for its nitrogen-fixing capability³ and its seasonal blooms.⁴ However, little is known about its secondary metabolite profile due to an inability to culture unialgal strains and difficulties in acquiring environmental material from offshore blooms. While homogenized cells, filtrates, aging cultures, and crude preparations of *Trichodesmium* filaments have shown toxicity to copepods,^{5,6} there are only a few examples of biologically active pure molecules isolated and characterized from *Trichodesmium* strains.^{7,8} One such molecule, trichotoxin, was isolated from an environmental collection of *T. thiebautii* from the western Gulf of Mexico collected in July and August of 2005.⁹ This compound showed some cytotoxicity against both GH4C1 cells and Neuro-2A cells. The present work details the isolation of a molecule (**1**) from a *T. thiebautii* collection from the western Gulf of Mexico (Padre Island) in 2014, which possessed a vinyl chloride functionality. A comparison of molecular features to the previously reported trichotoxin suggested a structural revision of the originally reported molecule. In addition, we report the characterization of an alkyne-containing analog, trichotoxin B (**2**). We propose the revision of trichotoxin based on analyses of NMR spectroscopic data and biosynthetic precedence with respect to cyanobacterial secondary metabolism. These molecules are recognizably derived from a polyketide synthase (PKS) biosynthetic system and appear to be repeatedly isolable from *T. thiebautii* blooms over time.

Results and Discussion

In an effort to evaluate the secondary metabolite composition of *Trichodesmium thiebautii* blooms in the Gulf of Mexico, cyanobacterial filaments from an environmental collection were extracted and fractionated and isolation was guided by examination of ¹H NMR spectra, ultimately leading to the purification of an optically active colorless oil (**1**) (Figure 1). HRESIMS analysis of **1** gave an [M+Na]⁺ of *m/z* 341.1647, suggesting a molecular formula of C₂₀H₂₇ClO and a requirement of 7 degrees of unsaturation. Analysis of ¹H and ¹³C NMR spectra of **1** showed the presence of a monosubstituted benzene ring (positions 12-17; (δ_C 138.2, 129.0, 128.5, 126.5, 128.5, 129.0 and δ_H 7.15, 7.29, 7.22, 7.29, 7.15 respectively). The benzylic methylene protons (δ_H 3.35) were substantially deshielded and showed HMBC correlations to the aromatic system (C-12, δ_C 138.2; C-13/17, δ_C 129.0) as well as a moderately polarized olefin (C-10, δ_C 142.3; C-18, δ_C 113.8). The polarization of the C-10/C-18 double bond and the singlet methine proton (H-18, δ_H 5.81) was consistent with the presence of a vinyl chloride functionality. Moderately deshielded allylic methylene protons (H₂-9, δ_H 2.06) showed HMBC correlations to the vinyl chloride moiety and COSY correlations to diastereotopic methylene protons (H-8a, δ_H 1.42; H-8b, δ_H 1.25). The H-8 methylene protons showed a COSY correlation to a methine proton (H-7, δ_H 2.33). The H-7 methine showed COSY correlations to an olefinic proton (H-6, δ_H 5.14) and a methyl group (H-19, δ_H 0.94). The H-6 olefin and an olefinic methyl (H-20, δ_H 11.7) both showed HMBC correlations to a quaternary carbon (C-5, δ_C 135.7). An oxymethine proton (H-4, δ_H 4.01) showed HMBC correlations to C-5, C-20 and a methylene carbon (C-3, δ_C 40.0). The H₂-3 protons (δ_H 2.27) showed HMBC correlations to a second polarized olefin (C-2, δ_C 135.0;

C-1, δ_C 117.5) supporting a terminal alkene functionality and satisfying the final degree of unsaturation. Thus, the planar structure of **1** was a linear polyketide hallmarked by a vinyl chloride functionality and terminal alkene. Key HMBC and COSY correlations are shown in Figure 2.

An NOE correlation between the H-18 methine (δ_H 5.80) and H-13 (δ_H 7.15) of the benzene ring supported a *Z* configuration of the vinyl chloride (Figure 2). An *E* configuration of the C-5/C-6 olefin was supported by examining the chemical shift of the olefinic methyl (H-20, δ_H 11.7) and the NOE correlation between H-7 and H-20.

This revised structure of trichotoxin (**1**) is a chlorinated analog of a phenyl alkene compound isolated from a mixture of three Florida sponges (Figure 1).¹⁰ While the authors note that this linear phenyl alkene molecule is an unusual find in sponges, unlike more prevalently isolated terpenoids and amino acid derivatives,¹⁰ this phenyl alkene does have structural features consistent with an underlying cyanobacterial biosynthetic architecture such as the vinyl group at C-10,¹¹ and the terminal alkene.¹² Metagenomic and other genetic methods have provided strong support for the microbial production of onnamide,¹³ swinholide A¹⁴ and chlorinated peptides¹⁵ in sponge-microbe assemblages.

The absolute configuration of **1** is proposed to be identical to that of phenyl alkene based on a comparison of optical rotation values, relative stereochemistry and ¹³C NMR chemical shift identity of the stereogenic centers (Table S2 and Table S3). The absolute configuration of the phenyl alkene compound was determined using a modified Mosher's protocol and *J*-coupling analysis.¹⁰

HRESIMS analysis of **2** gave an $[M+Na]^+$ of *m/z* 345.1960, suggesting a molecular formula of C₂₀H₃₁ClO and a requirement of 5 degrees of unsaturation. Analysis of 1D and 2D NMR spectra clearly showed **2** was an analogue of **1** and the planar structure was identical from C-1 to C-10 of the carbon backbone, including branched methylation, the presence of the vinyl chloride and the relative double bond configuration (cf. Table 1 and Table 2). Moderately deshielded allylic methylene protons (H-11, δ_H 2.05) showed HMBC correlations to C-10 (δ_C 142.7). Bidirectional HMBC and COSY correlations showed the presence of an aliphatic chain consisting of 5 methylene groups (positions 11-15) between the C-10 quaternary carbon and the C-16 quaternary carbon (δ_C 84.0) (Figure 2). A COSY correlation between H-17 (δ_H 1.95) and H-15 (δ_H 2.19) and the chemical shift of C-17 (δ_C 68.2) strongly supported an alkyne functionality, satisfied the final two degrees of unsaturation, and completed the planar structure of **2** (Figure 1). We propose the absolute configuration of **2** by analogy to **1** and phenyl alkene compound based on ¹³C NMR data and identical relative configuration (cf. Table 2 and Tables S2 and S3).

Support for revised structure - NMR analysis.

The significant structural differences between **1** and the original trichotoxin structure center on positions 4-6 and the olefinic methyl at position 20. The *cis* orientation of the vinyl methyl functionality and the secondary alcohol in the original trichotoxin structure should result in an upfield shift of C-4 (δ_C 65-70),^{11,16} The originally reported chemical shift of C-4 was considerably downfield (δ_C 76.7). Additionally, comparing ¹H NMR splitting

patterns of the vinyl methyl of the original trichotoxin structure to the exocyclic vinyl methyl present in the jasnudiflosides,¹⁷ we would predict that the H-6 splitting pattern would be a quartet, while the splitting pattern of H-20 would be a doublet or doublet of doublets. However, examining the ¹H NMR splitting patterns of the original trichotoxin structure, H-6 is a doublet, suggesting it is adjacent to a methine proton and H₃-20 is a singlet,⁹ indicating it is adjacent to a quaternary carbon, and strongly supporting that H-6 and H₃-20 are not coupled. In the original structure elucidation of trichotoxin, Schock et al., reported HMBC correlations from H₃-20 to C-4 and C-6. Examining the HMBC data in the present work, H₃-20 showed strong correlations to C-4, the quaternary carbon C-5, and C-6. The original work reported a COSY correlation between H₃-20 and H-6. This correlation may have been assumed to indicate a vicinal relationship between the H-20 methyl and H-6 methine. We did observe a weak COSY correlation between H₃-20 and H-6. However, we considered this correlation to represent an allylic correlation as is often observed in sesquiterpenoid molecules.¹⁸ The revised trichotoxin A structure (**1**) shows consistency with respect to predicted ¹³C chemical shifts, ¹H NMR splitting patterns, and vicinal ¹H-¹H coupling (Table 1). An authentic sample of the original trichotoxin no longer exists, thus trichotoxin A and the original trichotoxin were not able to be analyzed by a chromatographic comparison. An optical rotation was not recorded for the original trichotoxin. However, both compounds were isolated from *T. thiebautii* blooms. Additionally, a comparison of ¹H and ¹³C NMR signals provides strong support for these structures as identical chemical entities (Table S1).

Support for revised structure - polyketide biosynthesis.

We predict that the revised trichotoxin structure (**1**) would be generated from a PKS biosynthetic pathway extending acetate units from a proposed phenyl acetic acid starter unit in **1** and a 7-octynoate unit in **2**. The carbonyl of the starter unit would be modified into a vinyl functionality by the action of an HMG-CoA synthase cassette¹⁹ followed by chlorination by a halogenase in a similar fashion to that observed in the biosynthesis of jamaicamide.²⁰ This would be followed by the incorporation of four acetate units. The first fully reduced; the second subjected to ketoreduction and dehydration; the third subjected to ketoreduction and fourth fully reduced. Methylation at C-19 and C-20 would likely originate from S-adenosyl methionine (SAM) catalyzed by methyltransferases or they could come from the incorporation of propionate units into the growing polyketide chain instead of acetate units. We predict that the terminal alkene results from the action of a decarboxylating thioesterase as has been observed in the curacin A pathway.^{21,22} A cultured *T. thiebautii* strain originally isolated from the Sargasso Sea did show the presence of PKS genes²³ and further analysis of *T. thiebautii* bloom metagenomes may yield important information with respect to the biosynthetic capacity for polyketide production from this cyanobacterium.

The biosynthesis of the original trichotoxin structure would follow a similar pattern for the starter unit and the first acetate extension. The following acetate extension could be modified by the HCS cassette to generate the vinyl functionality followed by methylation, and the reforming of the double bond. If the terminal alkene is created by the action of a decarboxylating thioesterase then the penultimate extender unit in the biosynthesis of the original trichotoxin structure would need to possess three carbon atoms. There are examples

of compounds in which three carbon units, derived from the pool of glycolytic intermediates, are incorporated into growing polyketide chains.²⁴ However, these three carbon units are generally incorporated via an ester linkage, which is not present in this original structure.

While the revised structure (**1**) in this report is more consistent with conventional polyketide biosynthesis, an argument from biosynthesis alone cannot provide enough evidence for structural revision without analyzing a pure strain via stable isotope feeding studies or garnering genetic information from the biosynthetic gene cluster. However, when the NMR data and biosynthetic precedence are used in an orthogonal manner, the argument for revision is strongly supported. Interestingly, we have isolated the same metabolite from *T. thiebautii* blooms that occurred years apart from each other, making spatial and temporal secondary metabolomics studies of these blooms an intriguing prospect.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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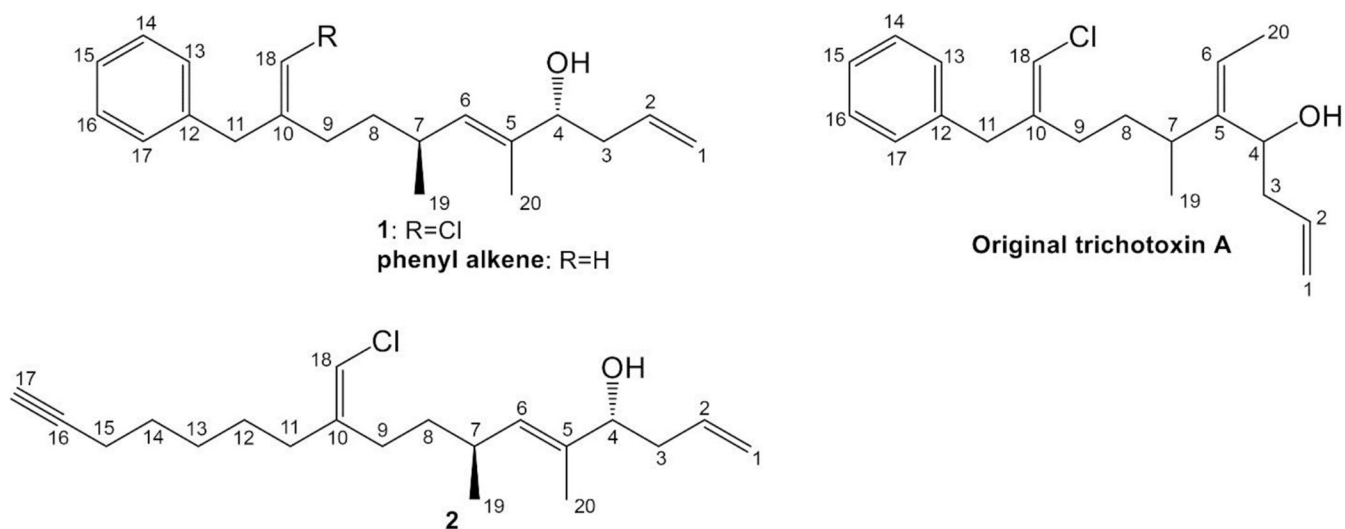


Figure 1. Structures of compound **1**, **2**, the original trichotoxin⁹ and phenyl alkene¹⁰ compound.

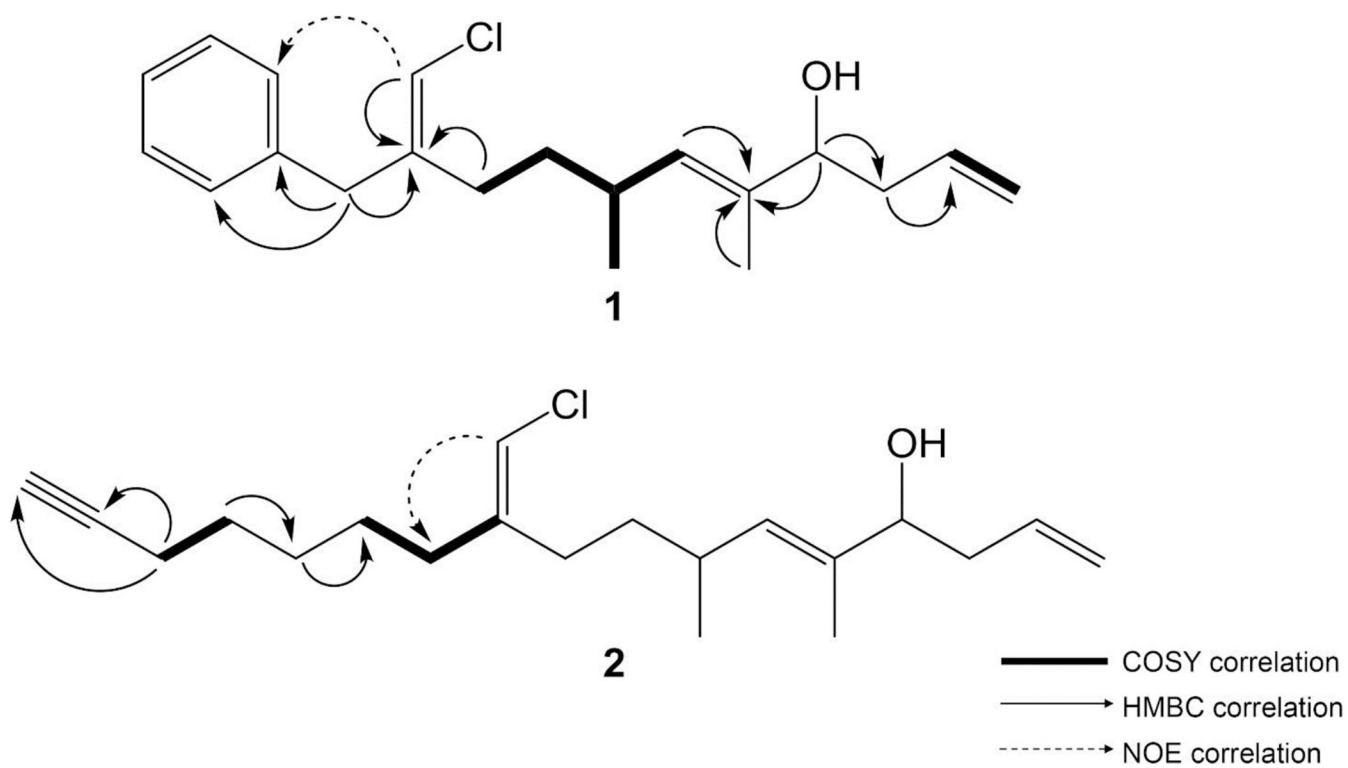


Figure 2.
Selected HMBC, COSY and NOE correlations for trichotoxin A (1) and B (2).

Table 1.NMR data for trichotoxin A (**1**)^a (800 MHz, CDCl₃)

position	δ_C type	δ_H (J in Hz)	HMBC	COSY
1a	117.5, CH ₂	5.10, dd (17.0, 1.9)	C-2, C-3	H-2
1b		5.05, dd (10.0, 1.9)	C-3	H-2
2	134.9, CH	5.73, m	C-3, C-4	H-1, H-3
3	40.0, CH ₂	2.27, m	C-1, C-2, C-4, C-5	H-2, H-4
4	76.7, CH	4.01, t (6.7)	C-2, C-3, C-5, C-6, C-20	H-3
5	135.7, qC			
6	132.7, CH	5.14, d (9.4)	C-4, C-7, C-8, C-19, C-20	H-7, H-20
7	32.2, CH	2.33, m	C-5, C-6, C-8, C-9, C-19	H-6, H-8a, H-8b H-19
8a	34.6, CH ₂	1.42, m	C-6, C-7, C-9, C-10 C-20	H-7, H-8b, H-9
8b		1.25, m	C-6, C-7, C-9, C-10 C-20	H-7, H-8a, H-9
9	28.2, CH ₂	2.06, t (8.6)	C-7, C-8, C-11, C-10, C-18	H-8a, H-8b
10	142.3, qC			
11	41.2, CH ₂	3.35, s	C-9, C-10, C-12, C-13, C-17, C-18	H-18
12	138.2, qC			
13/17	129.0, CH	7.15, d (7.6)	C-11, C-15	H-14, H-16
14/16	128.5, CH	7.29, t (7.6)	C-12	H-13, H-15, H-17
15	126.5, CH	7.22, t (7.6)	C-13, C-17	H-14, H-16
18	113.8, CH	5.81, s	C-9, C-10, C-11	H-11
19	20.9, CH ₃	0.94, d (6.6)	C-6, C-7, C-8	H-7
20	11.7, CH ₃	1.59, s	C-4, C-5, C-6, C-7, C-8, C-19	H-6

^aTrichotoxin A (**1**): colorless oil; α -²²D-2.5 (MeOH, *c* 0.20); UV (MeOH) λ_{\max} (log ϵ) 209 nm (5.5); (¹H NMR (800 MHz, CDCl₃) and ¹³C NMR (200 MHz, CDCl₃), see Table 1; HRESIMS *m/z* 341.1647 [M+Na]⁺ (calcd for C₂₀H₂₇ClONa, 341.1639).

Table 2.

NMR data for trichotoxin B (**2**)^a (800 MHz, CDCl₃)

position	δ_C , type	δ_H (J in Hz)	HMBC	COSY
1a	117.6, CH ₂	5.13, dd (17.0, 1.9)	C-2, C-3	H-2
1b		5.08, dd (10.0, 1.9)	C-3	H-2
2	134.8, CH	5.78, m	C-3, C-4	H-1a, H-1b, H-3
3	40.0, CH ₂	2.32, m	C-1, C-2, C-4, C-5	H-2, H-4
4	76.7, CH	4.06, t (6.7)	C-2, C-3, C-5, C-6, C-20	H-3
5	135.7, qC			
6	132.8, CH	5.23, d (9.4)	C-7, C-8, C-19, C-20	H-7
7	32.3, CH	2.38, m	C-8, C-19	H-6, H-19
8a	34.8, CH ₂	1.43, m	C-6, C-7, C-9, C-10, C-19	H-7, H-8b, H-9
8b		1.28, ovlp	C-6, C-7, C-9, C-10, C-19	H-7, H-8a, H-9
9	28.3, CH ₂	2.11, m	C-7, C-8, C-10, C-18	H-8a, H-8b
10	142.7, qC			
11	34.7, CH ₂	2.05, t (7.1)	C-10, C-12, C-13, C-18	H-12
12	27.1, CH ₂	1.41, ovlp	C-11, C-13	H-11
13a	28.3, CH ₂	1.52, ovlp	C-12, C-15, C-16	H-13b
13b		1.40, ovlp	C-12	H-13a
14a	28.2, CH ₂	1.53, ovlp	C-12, C-13, C-15, C-16	H-14b, H-15
14b		1.40, ovlp	C-12, C-13	H-14a
15	18.3, CH ₂	2.19, td (7.1, 2.7)	C-14, C-16, C-17	H-14a
16	84.0, qC			
17	68.2, CH	1.95, t (2.7)		H-15
18	112.0, CH	5.75, s	C-9, C-10, C-11	
19	20.9, CH ₃	0.98, d (6.6)	C-6, C-7, C-8	H-7, H-8a
20	11.5, CH ₃	1.64, s	C-4, C-5, C-6	H-6

^aTrichotoxin B (**2**): colorless oil; α -²²D-9.2 (MeOH, *c* 0.10); UV (MeOH) λ_{\max} (log ϵ) 204 nm (3.5); ¹H NMR (800 MHz, CDCl₃) and ¹³C NMR (200 MHz, CDCl₃), see Table 2; HRESIMS *m/z* 345.1960 [M+Na]⁺ (calcd for C₂₀H₃₁ClONa, 345.1956).