Brunsvicamides A−C: Sponge-Related Cyanobacterial Peptides with *Mycobacterium tuberculosis* Protein Tyrosine Phosphatase Inhibitory Activity

Daniela Müller,[†] Anja Krick,[†] Stefan Kehraus,[†] Christian Mehner,[†] Mark Hart,[‡] Frithjof C. Küpper,[‡] Krishna Saxena,[||] Heino Prinz,[§] Harald Schwalbe,[||] Petra Janning,[§] Herbert Waldmann,[§] and Gabriele M. König[†,*]

Institute for Pharmaceutical Biology, University of Bonn, Nussallee 6, 53115 Bonn, Germany, Culture Collection of Algae and Protozoa, Dunstaffnage Marine Laboratory, Scottish Association for Marine Science, Dunbeg, Oban, Argyll, PA37 1QA, Scotland, United Kingdom, Department IV - Chemical Biology, Max-Planck-Institute of Molecular Physiology, Otto-Hahn-Strasse 11, 44227 Dortmund, Germany, and Center for Biomolecular Magnetic Resonance, Institute for Organic Chemistry and Chemical Biology, Johann Wolfgang Goethe Universität Frankfurt, Marie-Curie-Strasse 11, D- 60439 Frankfurt, Germany

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The cyanobacterium *Tychonema* sp. produces the new cyclic hexapeptides brunsvicamide A−C (**1**−**3**). Brunsvicamide B (**2**) and C (**3**) selectively inhibit the *Mycobacterium tuberculosis* protein tyrosine phosphatase B (MptpB), a potential drug target for tuberculosis therapy for which no inhibitors are known to date. Brunsvicamide C contains an N-methylated *N*′-formylkynurenine moiety, a unique structural motif in cyclic peptides. The new peptides are related to the sponge-derived mozamides, supporting the suggestion that secondary metabolites of certain marine invertebrates are produced by associated microorganisms. Thus, microorganisms phylogenetically related to symbionts of marine invertebrates can be judged as a means to supply "marine-like" compounds for drug development.

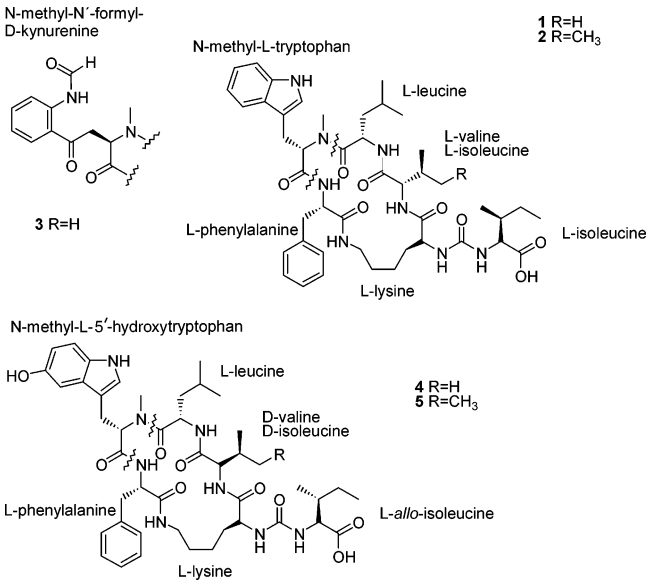
Introduction

Approximately one-third of the world's population is infected with *Mycobacterium tuberculosis*, the microorganism that causes tuberculosis (TB[a]).[1] Because of the increasing occurrence of resistant mycobacteria, new targets and drugs for therapeutic interventions are urgently required.[2] *Mycobacterium tuberculosis* protein tyrosine phosphatase A (MptpA) and MptpB are two enzymes excreted by mycobacterial cells and related to the survival of mycobacteria in their host macrophages.[3] MptpA and MptpB are discussed as new drug targets, since *M. tuberculosis* strains with disrupted *mptpB* genes were impaired in their ability to survive in guinea pigs.[4] Recently the first and only inhibitors for MptpA were described, including cyclic depsipeptides of the stevastelin family.[5]

The chemistry of cyanobacterial secondary metabolites is dominated by structurally novel bioactive peptides and depsipeptides,[6] some of which, e.g. cyclic peptides of the microcystin class, were found to inhibit protein phosphatases.[7] Based on these findings, the current project focused on the evaluation of cyanobacterial peptides as selective phosphatase inhibitors and resulted in the identification of the extremely selective MptpB inhibitory activity of novel cyclic peptides.

From an ecological point of view, it is of interest that compounds **1** and **2** show close similarity to the mozamides isolated formerly from a sponge belonging to the family Theonellidae.[8] Compound **3**, despite being clearly a mozamide/brunsvicamide derivative, contains the unusual amino acid residue *N*-methyl-*N*′-formylkynurenine, to date not yet encountered in cyclic peptides.

Scheme 1



Results

Molecular characterization based on its 16S rDNA sequence (GenBank accession number DQ072163) clearly placed the investigated cyanobacterial sample within the order Oscillatoriales, with morphological characteristics similar to those of *Tychonema* spp. (formerly *Oscillatoria*). Because of the general taxonomic uncertainties surrounding the order Oscillatoriales, pending a more detailed revision of the whole phylogenetic group, a more definite taxonomic identification is not attempted at this stage.

Preliminary investigations using ^{1}H NMR and MALDI-TOF spectroscopic measurements of *Tychonema* extracts indicated the presence of several peptidic compounds with a molecular weight between 700 and 1800 Da. After large scale cultivation of the cyanobacterial strain in a photobioreactor with inorganic medium, liquid−liquid extraction of the methanol extract of the

* To whom correspondence should be addressed. Phone: +49 228 733747. Fax: +49 228 733250. E-mail: g.koenig@uni-bonn.de. Internet: http://www.uni-bonn.de/pharmbio/queen/GAWK.html.
† University of Bonn.
‡ Scottish Association for Marine Science.
§ Max-Planck-Institute of Molecular Physiology.
|| Johann Wolfgang Goethe Universität Frankfurt.

[a] Abbreviations: Mptp A/B, *Mycobacterium tuberculosis* protein tyrosin phosphatase A/B; *N*-MeTrp, *N*-methyltrypthophan; nrps, nonribosomal peptide synthetase; SSU-rDNA, small subunit rDNA; TB, tuberculosis.

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Table 1. Spectral Data for Compound **1**[a]

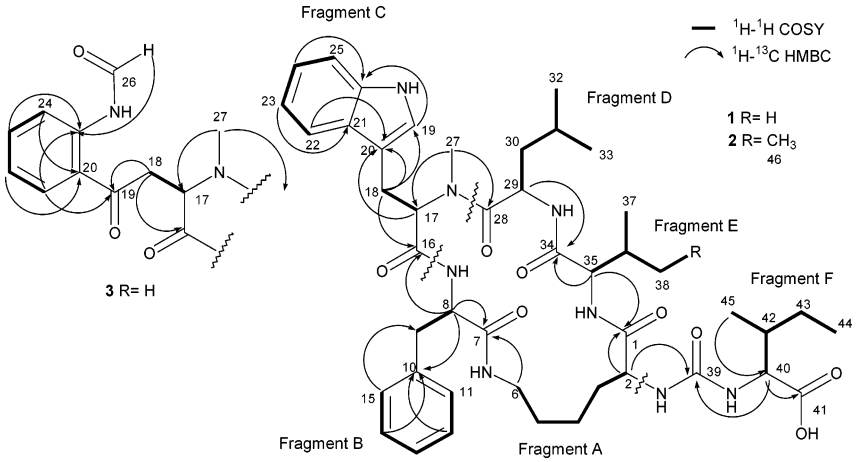
amino acid	no.[b]	^{13}C[c] (δ in ppm)	^{1}H[c] (δ in ppm, mult., J in Hz)	HMBC[e]	NOESY/ROESY
L-lys	1	176.3 (C)[d]			
	2	56.2 (CH)	4.08 (1H, dd, J = 3.8, 4.1)	1, 3, 4, 39	3, 5
	3	32.6 (CH_2)	1.71 (1H, m); 1.85 (1H, m)	1, 2, 4, 5	2, 4
	4	21.6 (CH_2)	1.33 (1H, m); 1.51 (1H, m)	3, 5, 6	
	5	29.3 (CH_2)	1.61 (2H, m)	4, 6	2, 6
	6	40.2 (CH_2)	3.03 (1H, dt, J = 13.6, 4.4); 3.77 (1H, m)	4, 5, 7	4, 5
L-phe	7	174.2 (C)			
	8	56.7 (CH)	4.79 (1H, dd, J = 2.5, 13.4)	7, 9, 10, 16	9, 11, 12, 14, 15
	9	39.1 (CH_2)	2.92 (1H, brt, J = 13.4); 3.49 (1H, dd, J = 2.5, 13.4)	7, 8, 10, 11, 15	8, 12, 14
	10	139.3 (C)			
	11	130.2 (CH)	7.19 (1H, d, J = 7.6)	9, 12, 13, 14	8, 9, 27, 37
	12	129.8 (CH)	7.29 (1H, t, J = 7.6)	10, 11, 15	8, 9, 27, 37
	13	127.7 (CH)	7.22 (1H, t, J = 7.6)	11, 15	
	14	129.8 (CH)	7.29 (1H, t, J = 7.6)	10, 11, 15	8, 9, 27, 37
	15	130.2 (CH)	7.19 (1H, d, J = 7.6)	9, 12, 13, 14	8, 9, 27, 37
N-Me-trp	16	172.2 (C)			
	17	63.1 (CH)	4.95 (1H, dd, J = 2.8, 11.0)	8, 16, 18, 20, 27, 28	
	18	23.4 (CH_2)	2.96 (1H, dd, J = 11.0, 15.5); 3.37 (1H,m)	16, 17, 19, 20, 21	17, 19, 22, 27, 29
	19	124.6 (CH)	6.89 (1H, s)	17, 18, 20, 21, 26	17, 18, 27, 30, 33
	20	110.9 (C)			
	21	128.7 (C)			
	22	119.1 (CH)	7.56 (1H, d, J = 7.9)	20, 21, 24, 25, 26	17, 18, 27, 33
	23	120.1 (CH)	7.04 (1H, brt, J = 7.9)	21, 25	
	24	122.6 (CH)	7.12 (1H, brt, J = 7.9)	22, 26	33
	25	112.7 (CH)	7.36 (1H, d, J = 7.9)	21, 23	32, 33
	26	137.8 (C)			
N-Me	27	29.0 (CH_3)	2.08 (3H, s)	17, 28	11, 12, 14, 15, 17, 18, 19, 22, 29
L-leu	28	175.4 (C)			
	29	49.2 (CH)	4.32 (1H, dd, J = 2.2, 12.0)	28, 30, 31	17, 18, 30, 31, 33
	30	38.9 (CH_2)	1.05 (1H, m); −0.54 (1H, dt, J = 2.2,12.3)	28, 29	19, 29, 31, 32, 33
	31	24.7 (CH)	1.41 (1H, m)	32, 33	29, 30, 32, 33
	32	20.1 (CH_3)	0.11 (3H, d, J = 6.6)	30, 31, 33	25, 29, 30, 31, 33
	33	23.1 (CH_3)	0.42 (3H, d, J = 6.3)	30, 31, 32	22, 23, 24, 25, 30, 31, 32
L-val	34	174.5 (C)			
	35	61.4 (CH)	3.73 (1H, d, J = 9.8)	1, 34, 36, 38	36, 37, 38
	36	31.1 (CH)	2.12 (1H, m)	35, 37	11, 12, 14, 15, 35, 37, 38
	37	19.7 (CH_3)	1.14 (3H, d, J = 6.9)	35, 36, 38	11, 12, 14, 15, 35, 36
	38	20.8 (CH_3)	1.04 (3H, d, J = 6.3)	35, 36, 37	35
urea	39	159.8 (C)			
L-ile	40	61.1 (CH)	4.12 (1H, d, J = 3.8)	39, 41, 42, 43, 45	42, 45
	41	179.8 (C)			
	42	39.4 (CH)	1.94 (1H, m)		40, 45
	43	25.6 (CH_2)	1.14 (1H, m); 1.52 (1H, m)	40, 42, 44, 45	45
	44	12.4 (CH_3)	0.92 (3H, t, J = 7.6)	42, 43	
	45	16.8 (CH_3)	0.96 (3H, d, J = 6.6)	40, 43	40, 42, 43

[a] MeOD, 500 MHz. [b] Position of carbon atom. [c] Assignments are based on extensive 1D and 2D NMR measurements (HMBC, HSQC, COSY). [d] Implied multiplicities determined by DEPT. [e] Numbers refer to carbon resonances.

bacterial pellet, and reversed phase (RP-C18) HPLC separations, three new cyclic hexapeptides were isolated (**1**−**3**).

The molecular formula of brunsvicamide A (**1**) was determined as $C_{45}H_{64}N_8O_8$ by HRMALDI-TOF indicating 18 elements of unsaturation within the molecule. Six α-proton signals between δ_H 3.7 and 5.0 in the ^{1}H NMR spectrum suggested that compound **1** is a peptide consisting of six amino acid residues (Table 1). The presence of peptidic bonds was confirmed by absorption bands at 1633 cm^{-1} in the IR spectrum. The ^{13}C NMR spectrum revealed signals for seven methyl, eight methylene, 13 methine, six α-CH, and 11 quaternary carbons. Using the results of ^{1}H−^{1}H COSY and HMBC measurements it was possible to establish all amino acid residues (Scheme 2). ^{1}H−^{1}H COSY correlations allowed us to deduce the presence of a spin system beginning at CH-2 and continuing through to the N-substituted CH-6 (δ_H 3.03 and 3.77, δ_C 40.2), characteristic for a lysine residue (fragment A). ^{1}H and ^{13}C NMR data indicated the presence of two aromatic moieties. The first one was assumed to be part of a phenylalanine residue due to ^{1}H−^{1}H couplings between the five aromatic methine groups CH-11, CH-12, CH-13, CH-14, and CH-15 and between α-CH-8 and H_2-9. ^{1}H−^{13}C long range (HMBC) couplings between C-13 and H-11 and H-15 and between C-10 and H-8, H-12, and H-14 confirmed the presence of phenylalanine as one of the two aromatic amino acids (fragment B). The second aromatic moiety consisted of a 1,2-substituted benzene ring as deduced from the ^{1}H−^{1}H coupling pattern of H-22, H-23, H-24, and H-25. HMBC correlations, especially between H-19 (δ_H 6.89) and C-21 and C-26, were indicative of an indole moiety, allowing to deduce fragment C as tryptophan. Correlations between H-17 (δ_H 4.95) and *N*-methyl carbon C-27 (δ_C 29.0) determined the tryptophan moiety to be N-methylated. The ^{1}H NMR spectrum of **1** contained several unusual highfield resonances assigned to one of the protons of the methylene group CH_2-30 (δ_H −0.54), the methyl group CH_3-32 (δ_H 0.11), and methyl group CH_3-33 (δ_H 0.42). H_2-30 showed ^{1}H−^{1}H COSY correlations to H-29 and H-31. Furthermore, ^{1}H−^{1}H COSY correlations between H_3-32 and H-31 as well as between H_3-33 and H-31 could be observed. ^{1}H−^{1}H COSY data thus clearly delineated a leucine residue (fragment D). The large ^{1}H NMR shieldings of H-30, H_3-32, and H_3-33 of the leucine residue can be explained by their spacial proximity to the indole nucleus of *N*-methyltryptophan and the resulting diamagnetic anisotropy due to ring current effects. ROESY cross-peaks seen between H-19 and H_2-30 and H_3-33, and between H_3-33 and H-22, H-24 and H-25, also suggest an adjacent position of leucine and *N*-methyltryptophan.

Scheme 2



H-36 coupled with the protons of the two methyl groups CH_3-37 and CH_3-38 and likewise with the α-CH-35, which established the presence of a valine residue (fragment E). The α-CH-40 proton coupled with H-42, and H-42 with H_2-43, which itself showed couplings to methyl CH_3-44. The methyl group CH_3-45 was connected with methine CH_2-42 by reason of ^{1}H−^{1}H COSY correlations between their corresponding proton signals. Hence the fourth amino acid was assigned as an isoleucine moiety (fragment F).

Fragments A−F were connected via diagnostic HMBC correlations. The HMBC spectrum showed H_2-6 to be coupled with carbon C-7. Hence, the amino acid lysine (fragment A) was bound to carbonyl C-7 of phenylalanine via its ϵ-amino group. The connection between phenylalanine (fragment B) and *N*-methyltryptophan (fragment C) was proven due to coupling between H-8 and carbonyl carbon C-16. HMBC correlation between H-17 and carbonyl carbon C-28 indicated the methylated tryptophan moiety (fragment C) to be linked to the leucine residue (fragment D). The α-proton H-35 exhibited long-range CH couplings to carbonyl carbon C-1. According to these data, the valine residue (fragment E) was connected with lysine (fragment A). The α-proton H-40 showed an HMBC correlation with the free carboxyl carbon C-41. Therefore, isoleucine (fragment F) was determined not to be part of the cyclic structure. HMBC correlation between H-40 and C-39 and between H-2 and C-39 were consistent with a ureido unit between the isoleucine and the lysine residue. The missing linkage between leucine and valine followed by deduction and became also apparent from HMBC data of compound **3**. Hence, the sequence of the cyclic pentapeptide was established as cyclo-(Lys-Phe-*N*-MeTrp-Leu-Val) with an ureido-isoleucine unit forming the side chain.

Chiral GC-MS analysis of the acid hydrolysate showed phenylalanine, leucine, and valine to be L-configured. Ozonolysis followed by acid hydrolysis gave evidence for the L-configuration of the degradation product of *N*-methyltryptophan, i.e. *N*-methylaspartic acid. Hydrazinolysis cleaved the ureido linkage, and after acid hydrolysis showed lysine and isoleucine to be L-configured. Thus, brunsvicamide A (**1**) was determined to be a 19-membered cyclic hexapeptide with all-L-configuration possessing an ureido linkage.

HRMALDI-TOF measurement of brunsvicamide B (**2**) revealed a molecular formula of $C_{46}H_{66}N_8O_8$. From ^{1}H, ^{13}C, and DEPT 135 NMR data (Table 2), it became apparent that compound **2** showed high similarity to compound **1**. The only difference between the two peptides was an additional methylene group (δ_H 1.22 and 1.65, δ_C 25.9) in compound **2**. Amino acid analysis of the hydrolysate of **2** suggested the presence of phenylalanine (Phe), *N*-methyltryptophan (*N*-MeTrp), leucine (Leu), lysine (Lys), and isoleucine (Ile) residues. Interpretation of 1D and 2D NMR spectra of **2** confirmed the existence of a second isoleucine moiety (fragment E), which replaced the valine moiety present in compound **1**. The connectivity of the amino acids in compound **2** was shown to be equal to that of compound **1** by HMBC correlations. In the ^{13}C NMR spectrum the carbon resonance for the free carboxyl-carbon C-41 was missing; its presence, however, followed from the molecular formula and the result of hydrolysis studies. Chiral GC-MS analyses of compound **2** proved all amino acids to possess the L-configuration.

The molecular formula $C_{45}H_{64}N_8O_{10}$ for brunsvicamide C (**3**) was established by HRMALDI-TOF, requiring compound **3** to have 18 degrees of unsaturation. Spectral data (Table 3) indicated close structural similarity between **1** and **3**. According to the molecular formula, however, compound **3** possessed two additional oxygen atoms. ^{1}H−^{1}H COSY and ^{1}H−^{13}C HMBC data clearly showed compound **3** to have five amino acid residues, i.e. lysine, phenylalanine, leucine, valine, and isoleucine, in common with compound **1**. Also the connection of these amino acids, including the ureido unit, was identical. From HMBC spectra, it was evident that leucine was connected with valine via carbonyl carbon C-34 on one side, whereas a not yet identified amino acid resided on the other side.

^{1}H NMR data gave reason for the proposal that compound **3** differed from compound **1** with respect to the tryptophan moiety. A signal for CH-19 was missing in the ^{1}H NMR spectrum of compound **3** and no unusually highfield shifted signals, i.e. as seen for H-30, H_3-32, and H_3-33 in compound **1**, were present. The ^{13}C NMR spectrum of **3** showed, when compared with that of **1**, resonances for two additional carbonyl groups, i.e. carbonyl C-19 (δ_C 202.4) and C-26 (δ_C 162.2). Additionally, a downfield resonance for an aldehyde proton H-26 (δ_H 8.43), corresponding to the carbon signal at 162.2 ppm, indicated the presence of a formyl moiety. ^{1}H−^{1}H couplings between the four aromatic methine groups CH-21 through to CH-24 and HMBC correlations identified the aromatic moiety as benzylic. The linkage between the carbonyl carbon C-19 and the aromatic carbon C-20 was inferred from long-range CH couplings between H-21 and C-19. HMBC correlations between H_2-18 and C-19 and ^{1}H−^{1}H COSY correlations between H_2-18 and α-proton H-17 finally defined a kynurenine substructure. Furthermore, CH long-range couplings between the aldehyde proton H-26 and the aromatic carbon C-25 showed the benzyl ring of the kynurenine moiety to be N-formylated. A remaining HMBC cross-peak

Table 2. Spectral Data for Compound **2**[a]

amino acid	no.[b]	^{13}C[c] (δ in ppm)	^{1}H[c] (δ in ppm, mult., J in Hz)	HMBC[e]	NOESY/ ROESY
L-lys	1	176.3 (C)[d]			
	2	56.3 (CH)	4.08 (1H, m)	1, 3	3, 4
	3	32.6 (CH_2)	1.75 (1H, m); 1.84 (1H, m)	1, 5	2, 4, 6
	4	21.6 (CH_2)	1.37 (1H, m); 1.54 (1H, m)	6	2
	5	29.3 (CH_2)	1.62 (2H, m)		2, 6
	6	40.2 (CH_2)	3.04 (1H, m); 3.81 (1H, m)	4, 5, 7	4
L-phe	7	174.2 (C)			
	8	56.7 (CH)	4.78 (1H, dd, J = 2.9, 12.5)	7, 16	9, 11, 15
	9	39.1 (CH_2)	2.93 (1H, m); 3,52 (1H, m)	7, 8, 10, 11, 15	8, 11, 36
	10	139.3 (C)			
	11	130.2 (CH)	7.21 (1H, d, J = 7.3)	13, 15	8, 9, 27
	12	129.8 (CH)	7.30 (1H, t, J = 7.3)	10, 11, 15	27, 30
	13	127.7 (CH)	7.25 (1H, t, J = 7.3)	11, 15	
	14	129.8 (CH)	7.30 (1H, t, J = 7.3)	10, 12, 14	27, 30
	15	130.2 (CH)	7.21 (1H, d, J = 7.3)	13, 11	8, 9, 27
N-Me-trp	16	172.6 (C)			
	17	63.1 (CH)	4.98 (1H, dd, J = 2.7, 11.3)	16, 27, 28	29
	18	23.4 (CH_2)	2.98 (1H, m); 3.38 (1H, m)	17, 19, 20, 21	17, 19, 22, 27, 29
	19	124.6 (CH)	6.91 (1H, s)	17, 18, 20, 21, 26	17, 18, 27, 30, 33
	20	111.0 (C)			
	21	128.7 (C)			
	22	119.1 (CH)	7.58 (1H, d, J = 7.5)	20, 21, 24, 25, 26	17, 18, 19, 27, 33
	23	120.1 (CH)	7.07 (1H, brt, J = 7.5)	21, 25	32, 33
	24	122.6 (CH)	7.14 (1H, brt, J = 7.5)	22, 26	
	25	112.7 (CH)	7.39 (1H, d, J = 7.5)	21, 23	32, 33
	26	137.8 (C)			
N-Me	27	29.0 (CH_3)	2.08 (3H, s)	17, 28	11, 12, 14, 15, 18, 19, 22
L-leu	28	175.4 (C)			
	29	49.3 (CH)	4.34 (1H, m)	28, 30	17, 18, 30, 31, 33
	30	38.9 (CH_2)	1.09 (1H, d, J = 6.9); −0.57 (1H, t, J = 12.7)		17, 19, 29, 31, 32, 33, 35
	31	24.7 (CH)	1.36 (1H, m)		29, 30, 32, 33
	32	20.1 (CH_3)	0.11 (3H, d, J = 6.9)	30, 31, 33	25, 29, 30, 31, 33
	33	23.1 (CH_3)	0.42 (3H, d, J = 6.9)	30, 31, 32	22, 23, 29, 30, 31, 32
L-ile	34	174.7 (C)			
	35	59.5(CH)	3.86 (1H, d, J = 10.0)	1, 34, 36, 37, 38	
	36	36.7 (CH)	2.30 (1H, m)		9
	37	14.4 (CH_3)	0.96 (3H, m)		
	38	25.9 (CH_2)	1.22 (1H, d, J = 5.9); 1.65 (1H, m)		35, 46
	46	10.6 (CH_3)	0.92 (3H, m)		35, 38
urea	39	159.7 (C)			
L-ile	40	60.7 (CH)	4.19 (1H, m)	42, 43, 45	44
	41	Not observed			
	42	39.6 (CH)	1.98 (1H, m)		
	43	25.6 (CH_2)	1.18 (1H, m); 1.50 (1H, m)		44
	44	12.4 (CH_3)	0.97 (3H; m)		40, 43
	45	16.8 (CH_3)	0.98 (3H, m)		2

[a] MeOD, 500 MHz. [b] Position of carbon atom. [c] Assignments are based on extensive 1D and 2D NMR measurements (HMBC, HSQC, COSY). [d] Implied multiplicities determined by DEPT. [e] Numbers refer to carbon resonances.

between α-CH-17 and H_3-27 gave evidence for the *N*′-formylkynurenine residue to be N-methylated. Thus, using ^{1}H−^{1}H COSY and HMBC data it was possible to establish an *N*-methyl-*N*′-formylkynurenine unit as the only difference between compound **3** and **1**.

Comparative chiral GC-MS analysis of the acid hydrolysate clarified that all amino acids, except the *N*-methyl-*N*′-formylkynurenine moiety, have the L-configuration. To determine the absolute stereochemistry of the latter, the peptide was treated with ozone resulting in the formation of *N*-methylaspartic acid, which after hydrolysis and derivatization was deduced as D-configured.

The inhibitory activity of peptides **1**−**3** was assessed toward a panel of protein phosphatases, including CDC25a, VHR, PTP1b, SHP2, and MptpA and MptpB. CDC25a and VHR are typical dual specificity phosphatases. The CDC25a phosphatase family is critical for cell cycle control.[28−30] VHR is a physiological regulator of ERKs, which belong to the mitogen-activated protein (MAP) kinase family.[31−33] PTP1b is a prototypical tyrosine phosphatase and acts as a key negative regulator of insulin-receptor activity. PTP1b inhibitors are expected to enhance insulin sensitivity and act as effective therapeutics for the treatment of type 2 diabetes, insulin resistance, and obesity.[34−36] SHP2, a potential target for the treatment of infectious diseases, is an intracellular protein tyrosine phosphatase. It is ubiquitously expressed and exerts positive functions in receptor tyrosine kinase signaling.

Brunvicamides were inactive (IC_{50} > 100μM) toward five of these phosphatases. The *Mycobacterium tuberculosis* enzyme MptpB was, however, potently inhibited by brunsvicamide B (**2**) (IC_{50} 7.3 μM) and brunsvicamide C (**3**) (IC_{50} 8.0 μM). Brunsvicamide A (**1**) showed only weak inhibitory properties (IC_{50} 64.2 μM). Because of the replacement of L-valine with L-isoleucine in compound **2**, the basic structures of brunsvicamide A (**1**) and B (**2**) only differ by a methyl group (C-46). The preferred conformation as estimated by molecular modeling using the Cerius^2 4.0 (MSI) software package in conjunction with NOE effects, however, showed major differences between the two compounds, mainly concerning the spatial orientation of the urea-bound side chain (see Supporting Information). In contrast to this, the preferred conformation of compound **3**, despite the D-configuration of the *N*-methyl-*N*′-formylkynurenine moiety, is very similar to that of compound **1**, regarding the eastern part of the molecule (C2 − C44).

Table 3. Spectral Data for Compound **3**[a]

amino acid	no.[b]	^{13}C[c] (δ in ppm)	^{1}H[c] (δ in ppm, mult., J in Hz)	HMBC[e]	NOESY/ROESY
L-lys	1	176.4 (C)[d]			
	2	56.3 (CH)	4.18 (1H, m)	1, 3, 4, 39	3
	3	32.4 (CH_2)	1.78 (1H, m); 1.95 (1H, m)	1, 2	2
	4	21.6 (CH_2)	1.41 (1H, m); 1.59 (1H, m)		
	5	29.0 (CH_2)	1.67 (1H, m); 2.07 (1H, m)		
	6	40.2 (CH_2)	3.07 (1H, m); 3.78 (1H, m)	4, 5, 7	
L-phe	7	174.2 (C)			
	8	57.1 (CH)	4.69 (1H, d, $J = 13.4$)	7, 9	
	9	39.2 (CH_2)	2.98 (1H, brt, $J = 13.4$); 3,45 (1H, brd, $J = 13.4$)	7, 8, 10, 11, 15	
	10	139.3 (C)			
	11	130.3 (CH)	7.20 (1H, d, $J = 8.2$)	9, 13	8, 9, 26, 36, 37
	12	129.8 (CH)	7.29 (1H, t, $J = 8.2$)	10	8, 37
	13	127.7 (CH)	7.22 (1H, t, $J = 8.2$)		
	14	129.8 (CH)	7.29 (1H, t, $J = 8.2$)	10	8, 37
	15	130.3 (CH)	7.20 (1H, d, $J = 8.2$)	9, 13	8, 9, 26, 36, 37
Me-formylkynurenine	16	171.3 (C)			
	17	58.2 (CH)	5.72 (1H, brs)	16, 18, 27, 28	
	18	40.0 (CH_2)	3.94 (1H, m); 3.98 (1H, d, $J = 8.5$)	16, 17, 19	21
	19	202.4 (C)			
	20	124.5 (C)			
	21	132.3 (CH)	8.14 (1H, d, $J = 7.6$)	19, 23, 25	18, 22
	22	124.9 (CH)	7.29 (1H, brt, $J = 7.6$)	20, 24, 25	23, 24
	23	135.8 (CH)	7.64 (1H, dd, $J = 7.6$, 8.2)	21, 25	22
	24	122.9 (CH)	8.57 (1H, d, $J = 8.2$)	20, 22	22
	25	139.9 (C)		21, 22, 23	
	26	162.2 (CH)	8.43 (1H, s)	25	11, 15, 23
N-Me	27	29.6 (CH_3)	2.00 (3H, s)	17, 28	18
L-leu	28	174.5 (C)			
	29	49.7 (CH)	5.02 (1H, brs)	30, 34	17, 31
	30	41.5 (CH_2)	1.53 (1H, m); 1.74 (1H, m)	29, 31, 33, 34	
	31	25.9 (CH)	1.80 (1H, m)		
	32	12.4 (CH_3)	0.95 (3H, d, $J = 7.3$)	30, 31	
	33	22.6 (CH_3)	0.95 (3H, d, $J = 7.3$)	30	
L-val	34	174.5 (C)			
	35	61.5 (CH)	3.88 (1H, d, $J = 9.8$)	1, 34, 36, 38	36, 37, 38
	36	31.2 (CH)	2.20 (1H, m)	35, 37, 38	11, 15, 35, 37, 38
	37	19.8 (CH_3)	1.20 (3H, d, $J = 6.7$)	35, 36	11, 15
	38	20.8 (CH_3)	1.08 (3H, d, $J = 6.4$)	35, 36	
urea	39	159.9 (C)			
L-ile	40	61.0 (CH)	4.18 (1H, m)	39, 41, 42, 43, 44	43, 44
	41	179.6 (C)			
	42	39.3 (CH)	1.99 (1H, m)		
	43	25.6 (CH_2)	1.15 (1H, m); 1.53 (1H, m)	40, 42, 44	
	44	16.8 (CH_3)	0.98 (3H, d, $J = 7.3$)	42, 43	40
	45	23.5 (CH_3)	0.99 (3H, d, $J = 7.0$)	40	

[a] MeOD, 500 MHz. [b] Position of carbon atom. [c] Assignments are based on extensive 1D and 2D NMR measurements (HMBC, HSQC, COSY). [d] Implied multiplicities determined by DEPT. [e] Numbers refer to carbon resonances.

Discussion

Brunsvicamides A and B (**1**, **2**) are cyclic hexapeptides composed of all L-amino acids. The structure of compound **3** differs from that of compounds **1** and **2** mainly concerning the *N*-methyl-*N*′-formylkynurenine moiety, which is D-configured and putatively derived from tryptophan through oxidation. The presence of D-amino acids in cyanobacterial peptides is rather common and mediated by epimerization domains in the respective biosynthetic enzymes.[37] *N*-Methyl-*N*′-formylkynurenine, as found in **3**, is a unique structural motif in cyclic peptides. To date, kynurenine as a building block of natural products is restricted to a very few nonpeptidic secondary metabolites, i.e. the cyanobacterial alkaloids fontonamide and hapalonamide A and G,[9] and the marine gastropod-derived monodontamides.[10,11]

We formerly reported the peptide leucamide A from the sponge *Leucetta microraphis*[12] which resembles cyanobacterial metabolites such as westiellamide[13] and tenuecyclamide.[14] The structures of cyanobacterial compounds **1** and **2** of the current report are closely related to the sponge-derived mozamide A and B (**4**, **5**)[8] and differ from these *Theonella* metabolites solely in the absence of a hydroxyl function in position 5 of the tryptophan moiety and in the stereochemistry of the amino acids valine and isoleucine. The cyanobacterial strain investigated here belongs to the order Oscillatoriales. Cyanobacteria of this order are frequently found as associated microorganisms of sponges, one of the best investigated example being *Dysidea herbacea* and *Oscillatoria spongeliae*.[15]

On the basis of our literature search, the occurrence of peptides featuring an ureido bond in sponges seems to be confined to the genus *Theonella*. The mozamides (**4**, **5**) as well as keramamide A and konbamide, both of which were isolated from *Theonella* spp., were all assumed to be produced by microorganisms.[16,17] Indeed, a keramamide-like metabolite, ferintoic acid, was later obtained from *Microcystis aeruginosa*.[18] Here we report the isolation of mozamide-related peptides from a cyanobacterium, adding further support to the hypothesis that, in some cases, microorganisms, i.e. cyanobacteria, are the true biosynthetic origin of sponge metabolites. It is noteworthy that the mozamide analoga **1**−**3** were obtained from a terrestrial cyanobacterium, which demonstrates that the biosynthetic potential is more related to phylogeny than habitat.

The first unambiguous proof for the cyanobacterial origin of cyclic peptides originally isolated from a marine invertebrate was recently published.[19] Patellamide A and C were initially

obtained from the ascidian *Lissoclinum patella*; the biosynthetic gene cluster, however, was located in the animal's cyanobacterial symbiont *Prochloron didemni*. Surprisingly, patellamides were found to be ribosomal peptides and after their identification were heterologously expressed in *Escherichia coli*. Judging from the structural features of the brunsvicamides, a nonribosomal biosynthesis of these peptides may be presumed. We thus screened *Tychonema* sp. DNA for *nrps* (non ribosomal peptide synthetase) gene fragments. Although various PCR conditions and numerous primer pairs were used, no *nrps* gene fragments could be amplified. Taking this result and the ribosomal origin of patellamides into account, the possibility of the brunsvicamides being synthesized ribosomally also has to be considered.

Marine natural products are most valuable pharmacological tools and drug candidates. A problem often encoutered in marine pharmacology is the lack of sufficient amount of compound to further their clinical development. As our results point out, biologically active natural products with structures related to marine metabolites are encountered in microorganisms phylogenetically related to marine invertebrate symbionts. The supply problem for marine natural products could thus, at least in part, be approached by making use of these easy to cultivate microorganisms. Additionally, the cloning of biosynthetic gene clusters from these organisms opens the possibility of heterologous gene expression. Currently, the biosynthetic gene clusters responsible for brunsvicamide production are under investigation.

Infections with *Mycobacterium tuberculosis* are a serious threat for the world's population, especially due to the increasing spread of drug-resistant mycobacteria.[2] Therefore, the development of new therapeutic agents for the treatment of tuberculosis is considered to be of utmost importance. In light of this urgent demand, Mptps were proposed as new drug targets for tuberculosis therapy.[3] The brunsvicamides are potent inhibitors for MptpB, in particular brunsvicamide B and C (**2**, **3**), which inhibit the enzyme in the lower micromolar range. These peptides do not interact with other phosphatases, a fact which illustrates the high selectivity of **2** and **3** toward the mycobacterium-derived enzyme. Based on the structures of **2** and **3**, currently a library of peptides is being synthesized with the aim of deducing structural features responsible for the observed activity, to improve the potency, and to obtain sufficient amount of compounds for in vivo testing.

Experimental Section

General Experimental Procedures. HPLC was performed on a Merck-Hitachi system equipped with a L-6200A pump, a L-4500A photodiode array detector, a D-6000A interface with D-7000 HSM software, and a Rheodyne 7725i injection system. ^{1}H, ^{13}C, COSY, NOESY, ROESY, HSQC, and HMBC NMR spectra were recorded in CD_3OD either using a Bruker Avance 300 DPX or 500 DRX spectrometer operating at 300 or 500 MHz for proton and at 75 or 125 MHz for ^{13}C, respectively. Spectra were referenced to residual solvent signals with resonances at $\delta_{H/C}$ 3.35/49.0 (CD_3OD). Chiral GC-MS analyses were accomplished on a Perkin-Elmer AutoSystem XL gas chromatograph linked to a Perkin-Elmer Turbomass mass spectrometer using an Alltech Capillary Chirasil-Val column. UV and IR spectra were measured on Perkin-Elmer Lambda 40 and Perkin-Elmer Spectrum BX instruments. Optical rotations were obtained using a Jasco DIP 140 polarimeter. HRMALDI-TOF spectra were recorded on a Applied Biosystems Voyager-DE STR with α-cyano-4-hydroxycinnamic acid as matrix.

Biological Material. The cyanobacterial strain was isolated from a sample collected from a pond of a sugar factory in Wierthe, Germany, and deposited in the Culture Collection of Algae and Protozoa (CCAP 1462/13) in Oban (Scotland, United Kingdom). The cyanobacterium was cultivated in a 30 L photobioreactor (planctotec system Pluto) with BG-11 medium (Sigma Aldrich, C3061 BG-11). During cultivation at 25 °C, the culture was constantly illuminated with white fluorescent light (Osram L 58W/11-869). A continuous stream of sterile air was bubbled through the culture. The pH was titrated to a value between 7 and 10 using an adequate volume of carbon dioxide.

Molecular Characterization. Cyanobacterial genomic DNA was extracted from 1 mL of culture using a method based on cetyltrimethylammonium bromide purification (CTAB).[20] The polymerase chain reaction (PCR) was used to amplify the small subunit rDNA (SSU-rDNA) gene from chromosomal DNA using the primer pair 27F (AGAGTTTGATCMTGGCTCAG) and 1492R (ACGGCTACCTTGTTACGACTT).[21] PCR was carried out on an MJ Research PTC200 DNA Engine thermocycler and used 1 U of Taq polymerase (Abgene, UK) in a 50 μL reaction containing a final concentration of 1.8 mM Mg^{2+}, 20 mM $(NH_4)_2SO_4$, 75 mM Tris-HCl (pH 8.8), and 0.01% Tween 20. Cycling parameters were as follows: 94 °C for 2 min, 26 cycles of 55 °C for 30 s, 72 °C for 2.5 min, and 94 °C for 10 s, followed by 72 °C for 10 min. The PCR products were purified through Centricon-PCR Ultrafilters (Millipore, UK) and sequenced in both directions using 27F and 1492R primers and ABI−PRISM Big-Dye terminator chemistry (Applied Biosystems) according to the standard protocols. Sequence reactions were electrophoresed on an ABI 377 DNA sequencer (Applied Biosystems), and resulting sequences were aligned and manually checked for consistent base-calling, using Sequence Navigator (Vers. 1.0.1, Applied Biosystems).

SSU rDNA sequences were compared with representative SSU rDNA sequences of cyanobacterial type strains and most closely related strains and environmental sequences as identified by BLAST-n analysis[22] and aligned using the software program ClustalX.[23] Genetic identity was further determined using Ribosomal Database Project II Sequence Match.[24] Genetic identity was in most instances assigned to the closest taxonomically described bacterial sequence in the RDP II and listed in Table 2 according to Bergey's taxonomic outline of the prokaryotes.[25]

Phylogenetic inference was performed on visually corrected alignments using PAUP* 4.0.[26] All ambiguous alignment positions were masked from the analysis. The method of maximum likelihood (ML) using the estimated rates of transition/transversion and alpha shape parameters (estimated from the data set following heuristic searching) were used to infer each phylogeny. Bootstrap support for each inferred tree was established following resampling of 1000 data sets based on neighbor-joining analysis.[27]

Isolation Procedure. The lyophilized biomass from 42 L of culture (240 g) was exhaustively extracted with 8 L dichloromethane, 6 L ethyl acetate, and 4 L methanol in three steps. In this manner 4.4 g of dichloromethane extract, 2.7 g of ethyl acetate extract, 20.6 g of methanol extract, and 6.1 g of methanol/water extract were obtained. ^{1}H NMR and MALDI-TOF spectroscopic measurements of the methanol extract indicated the presence of several peptidic compounds. Twelve grams of the methanol extract was dissolved in water and repeatedly extracted with diethyl ether (1.5 L) and butanol (4.5 L) resulting in 3.0 g of butanol extract with a high peptidic content. The crude butanol extract was fractionated by reversed phase HPLC (Macherey-Nagel, Nucleodur 100, C_{18}, 5 μm, 250 × 10 mm) using a gradient elution from 2:3 MeOH/H_2O to MeOH in 30 min, 2 mL/min, to yield six fractions. Further reversed phase HPLC separation of fraction 2 (49.9 mg) (Knauer C_{18} Eurospher-100, 5 μm, 250 × 8 mm, MeOH/H_2O 65/35) led to the isolation of brunsvicamide A (17 mg, 0.1% of crude methanol extract) and B (7 mg, 0.04% of crude methanol extract). Fraction 6 (12.7 mg) was purified by HPLC (Knauer C_{18} Eurospher-100, 5 μm, 250 × 8 mm) employing MeOH/H_2O (63:37), 0.8 mL/min as eluent to yield brunsvicamide C (4.5 mg, 0.05% of crude methanol extract).

Brunsvicamide A (1): white amorphous solid; $[\alpha]_D^{24}$ −102.2° (*c* 0.95, MeOH); UV (MeOH) λ 300−200 nm (br); λ_{max} 219 nm (ε 38889), 282 nm (ε 5069); IR (ATR) ν_{max} 3276, 2960, 1633,

1541, 1455, 1405, 1340, 1251, 1179, 1113, 1043 cm^{-1}; ^1H and ^{13}C NMR data (see Table 1); HRMALDI *m/z* 867.4764 (calcd for $C_{45}H_{64}N_8O_8Na$, $[M + Na^+]$ 867.4739).

Brunsvicamide B (2): white amorphous solid; $[\alpha]_D^{24}$ −52.4° (*c* 0.37, MeOH); UV (MeOH) λ 300−200 nm (br); λ_{max} 204 nm (ϵ 20733), 219 nm (ϵ 14976), 281 nm (ϵ 2663); IR (ATR) ν_{max} 3300, 2933, 1707, 1634, 1539, 1456, 1266, 1106, 1025 cm^{-1}; ^1H and ^{13}C NMR data (see Table 2); HRMALDI *m/z* 859.5063 (calcd for $C_{46}H_{67}N_8O_8$, $[M + H^+]$ 859.5076).

Brunsvicamide C (3): white amorphous solid; $[\alpha]_D^{24}$ −76.3° (*c* 0.42, MeOH); UV (MeOH) λ 300−200 nm (br); λ_{max} 204 nm (ϵ 21764), 230 nm (ϵ 15921), 261 nm (ϵ 7206); IR (ATR) ν_{max} 3305, 2936, 1653, 1417, 1110, 1022 cm^{-1}; ^1H and ^{13}C NMR data (see Table 3); HRMALDI *m/z* 899.4692 (calcd for $C_{45}H_{64}N_8O_{10}Na$, $[M + Na^+]$ 899.4638).

Amino Acid Analysis by Chiral GC. Peptides A, B, and C (0.2 mg each) were dissolved in 6 N HCl (1 mL) and heated to 115 °C for 15 h. The acid was removed using a stream of nitrogen, and the dry hydrolysate was treated with a mixture of ^{i}PrOH (500 μL) and acetyl chloride (150 μL) at 110 °C for 1 h. The surplus reagent was evaporated to dryness under N_2, and the residues were treated with pentafluoropropionic anhydride (0.4 mL) in CH_2Cl_2 (0.4 mL) at 110 °C for 15 min. After the mixture had cooled to room temperature, the excess reagent was again removed under N_2. The derivatized amino acids were redissolved in EtOAc (100 μL) and analyzed by GC-MS using an Alltech Capillary Chirasil-Val column (25 m × 0.25 mm; 0.16 μm; program rate: column temperature held at 50 °C for 3 min; 50 °C to 180 °C at 4 °C/min; flow: 0.6 mL/min; Inj: 250 °C, for N-Me-Asp analysis the program rate was as follow: initial column temperature 70 °C, 70 °C to 100 °C at 0.5 °C/min, 100 °C to 180 °C at 2 °C/min, flow: 0.5 mL/min; Inj: 200 °C). The retention times of the *N*-pentafluoropropionyl isopropyl ester derivatives of the amino acids were compared with those of standards that had been derivatized in the same manner. Thus the presence of L-valine, L-leucine, and L-phenylalanine was established for brunsvicamides A and C and L-isoleucine instead of valine in brunsvicamide B. (Standards: D-val 13.47 min, L-val 13.95 min, D-ile 15.81 min, L-ile 16.28 min, D-leu 17.04 min, L-leu 17.92 min, D-phe 27.33 min, L-phe 27.65 min; brunsvicamide A:, L-val 13.98 min, L-leu 17.98 min, L-phe 27.69 min; brunsvicamide B: L-ile 16.31 min, L-leu 17.98 min, L-phe 27.98 min; brunsvicamide C: L-val 13.94 min, L-leu 17.93 min, L-phe 27.66 min).

Hydrazinolysis. Amounts of 0.25 mg of each peptide was dissolved in 750 μL of hydrazine and heated at 110 °C for 18 h. Hydrazine was removed under high vacuum and the reaction mixture relyophilized repeatedly with water to remove the last traces of reagent. The resulting mixture was derivatized as described above. Chiral GC-MS analysis revealed the presence of L-isoleucine in each of the three peptides (standard: L-ile 16.28 min; brunsvicamide A: 16.24 min; brunsvicamide B: 16.27 min; brunsvicamide C: 16.29 min). L-Lysine was also identified in brunsvicamides A, B, and C by comparison with retention times of standards in chiral GC-MS analysis (standard: D-lys 34.38 min, L-lys 34.66 min; brunsvicamide A: 34.58 min; brunsvicamide B: 34.56 min; brunsvicamide C: 34.54 min).

Ozonolysis and Acid Hydrolysis. Amounts of 0.5 mg of each of the three peptides was dissolved in 4 mL of methanol. A stream of ozone in oxygen was bubbled through each solution for 15 min while cooling the solution with ice. The reaction was quenched with 10 drops of 30% H_2O_2. After 1 h the methanol was removed under a stream of nitrogen. The resulting residue was subjected to amino acid analysis as outlined below. For compound **1** and **2** the presence of *N*-methyl-L-aspartic acid (standard: 63.77 min, brunsvicamide A: 63.78 min; brunsvicamide B: 63.76 min) was determined by chiral GC-MS analysis, while for compound **3** *N*-methyl-D-aspartic acid (standard: 63.67 min, brunsvicamide C: 63.67 min) was detected.

Cloning, Expression and Purification of MptpA and MptpB. Plasmids (Dr. Anil Koul) containing the *mptpA* and *mptpB* gene were used as templates and both phosphatase genes were amplified by PCR and cloned into the *Nde*I/*Bam*HI site of pET16bTev. The resulting expression plasmid pETPtpA/B was used for transformation of *E. coli* strain Bl21 (DE3) (Novagen). Cells were grown in the presence of 100 μg/mL ampicillin at 25 °C until the OD_{600} reached 0.6 and then recombinant protein production was initiated by adding 1 mM IPTG (isopropyl β-D-thiogalactopyranoside). After 4 h induction cells were harvested and stored at −80 °C until usage.

Cell pellets were thawed and resuspended in 25 mM HEPES pH 8.0, 200 mM NaCl, 10 mM β-mercaptoethanol. Cells were lysed by sonification, and the cell debris was removed by centrifugation at 20000*g* for 20 min at 4 °C. The supernatant was applied to a Ni-NTA FastFlow column (Qiagen, Hilden, Germany) following the manufacturers recommendations. Further purification was achieved by gel filtration chromatography with a Hi Load 26/60 Superdex 75 column (Pharmacia Biotech) equlilibrated in 25 mM HEPES pH 7.4, 200 mM NaCl, 10 mM DTT, 5% glycerol.

Enzyme Assays. The enzymes CDC25a, VHR, PTP1b, and SHP2 were obtained and tested as described.[38] All enzyme assays were performed by means of an automated system consisting of a Zymark SciClone ALH 500 in conjunction with a Twister II and a Bio-Tek Power Wave 340 reader. The reaction volume was 10 μL. The reaction was started by the addition of 5 μL *p*-nitrophenyl phosphate to 5 μL of a solution containing the respective enzymes which had been preincubated for 10−15 min with different concentrations from 2-fold dilution series of inhibitors.

Reaction velocity was determined from the slope of the absorbance change at 405 nm and related to control values in absence of the inhibitor. IC_{50} values were calculated from linear extrapolations of reaction velocity as a function of the logarithmic of concentration. This nonbiased approach did not allow for the determination of IC_{50} values larger than 100 μM. The overall experimental error, including the water content of DMSO stock solutions and weighing errors, is approximately 50% of the respective IC_{50} values.

All buffered solutions contained 2 mM DTE (1,4-dithio-D,L-threitol added on the day of the experiment from 100 mM stock) and 0.025% (v/v) of the detergent NP-40 (Calbiochem 492015). The buffers consisted of 50 mM Tris, 50 mM NaCl, 0.1 mM EDTA in the case of CDC25A, or 25 mM HEPES, 50 mM NaCl, 2.5 mM EDTA in the case of PTP1b, MptpA, and MptpB, or 25 mM MOPS, 5 mM EDTA in the case of VHR. The 2-fold dilution series were obtained from 10 μL of a buffered enzyme solution containing 200 μM of inhibitor. Of this, 5 μL was removed and mixed with 5 μL of buffered enzyme solution resulting in a 2-fold dilution. This step was repeated five times. A volume of 5 μL of the final dilution was removed, so that each well consisted of 5 μL of buffered enzyme inhibitor mix. After addition of 5 μL *p*-nitrophenyl phosphate, the concentrations for the enzyme reaction were 50 mM in the case of CDC25A or 1 mM for all other phosphatases. The inhibitor concentrations were 100, 50, 25, 12.5, 6.25, or 3.125 μM, respectively. For all enzymes, their concentration was adjusted to an initial absorbance change of 1−2 OD_{405}/h. All reactions were performed as quadruplets from identical manual dilutions (1:10 in buffer from 10 mM stock solutions in DMSO).

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Supporting Information Available: Phylogenetic tree based on 16S rDNA sequences including the investigated cyanobacterium

of the genus *Tychonema*, chiral GC-MS analysis of acid hydrolysate of brunsvicamides,^{1}H NMR, ^{13}C NMR, 2D NMR spectra, HPLC data (purity) and molecular modeling of compounds **1**−**3**. This material is available free of charge via the Internet at http://pubs.acs.org.

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