Balgacyclamides, Antiplasmodial Heterocyclic Peptides from Microcystis aeruguinosa EAWAG 251

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Balgacyclamides, Antiplasmodial Heterocyclic Peptides from *Microcystis aeruginosa* EAWAG 251[1]

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[1] *Dedicated to Prof. Dr. Otto Sticher, of ETH-Zürich, Zürich, Switzerland, for his pioneering work in pharmacognosy and phytochemistry*

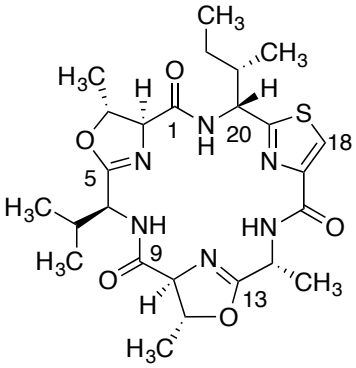
Abstract

The isolation and structural characterization of three new heterocyclic and macrocyclic peptides, balgacyclamides A–C from *Microcystis aeruginosa* EAWAG 251 is reported. The constitutions were determined by 2D-NMR methods and mass spectrometry and the configurations were assigned after ozonolysis and hydrolysis by HPLC-MS methods using Marfey's method as well as GC-MS using authentic standards. Balgacyclamides A and B were active against *Plasmodium falciparum* K1 in the low micromolar range, while displaying low toxicity to rat myoblasts.

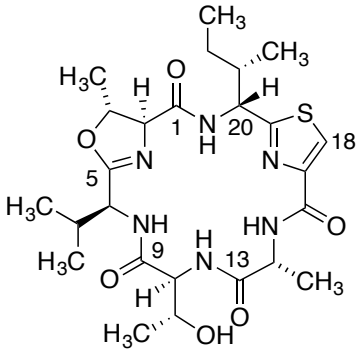
Cyclic peptides and derived macrocycles have recently received significant interest in a variety of fields, ranging from biosynthesis studies to drug discovery and development,[1] The potentially large, but structurally often well defined conformational space sampled, combined with the variety of amino acids renders peptides ideally suited to interact with many receptors or to interfere with protein/protein interactions.[1] Cyanobactins constitute a particular subset of cyclic peptides produced by cyanobacteria and can be defined as cyclic, ribosomally produced peptides (RiPP)[2] containing heterocyclic modifications.[3] They have been found not only in the producing organism, but also in other animals where they have been suggested to be of symbiotic or dietary origin.[4] A particular subset, the cyclamides are structurally well defined macrocyclic hexapeptides, where often hydrophobic residues flanked by heterocyclic rings such as oxazol(ine) or thiazol(ine) residues are observed.[5-8] While cyclamide biosynthesis has been investigated,[2-4,5m] the bioactivity of these compounds both in their ecological niche as well as in a therapeutic setting has received much less attention. Early assumptions on the bioactivity suggested a role as allelochemicals,[7] which has been supported by algicidal effects.[7,8] Another seemingly unrelated activity is related to multidrug-resistance reversing activity, which has been reported for several members of this family.[9] Most interestingly, Gerwick and coworkers reported significant antimalarial activity for the venturamides.[10] Independently, we have determined antimalarial activity for the aerucyclamides[11] that displayed improved activity when compared to an earlier study.[10] The best compound in this series, aerucyclamide B displayed an antimalarial IC_{50} value of 0.7 μM against the *Plasmodium falciparum* K1 strain with a high selectivity over the L6 rat myoblasts cell line. Aerucyclamide B was taken up as a lead structure for a medicinal chemistry follow-up program by Serra and coworkers that built on the first successful total synthesis of this compound.[12] Whereas these authors were able to briefly delineate the first structure activity

relationships, the role of the different units on activity remain unclear. The current main hypothesis suggests that the degree of unsaturation of the heterocyclic rings is correlated with biological activitiy,[10–12] with higher unsaturation leading to more potent compounds. While this hypothesis is supported by several examples[10–12] (e.g. oxidation of an thiazoline to an thiazole in aerucyclamide A and B led to 10 times increased activity),[11b] more compounds are needed to corroborate these observations. In this study, we report on the isolation of new cyclamides from cyanobacteria, and evaluate their antimalarial properties in the *P. falciparum* K1 assay.

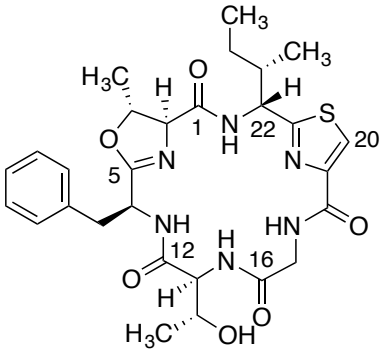
The balgacyclamides were isolated from aqueous methanolic extracts of *Microcystis aeruginosa* EAWAG 251 (identical to PCC 7820). These compounds were purified by a combination of C_{18}-SPE, semi-preparative and analytical reversed-phase HPLC methods. The structure determination proved to be challenging due to the low isolation yield of 0.55 mg, 0.15 mg, and 0.80 mg per 15 liters of culture, for the balgacyclamides A (**1**), B (**2**) and C (**3**) respectively.



Balgacyclamide A (**1**)



Balgacyclamide B (**2**)



Balgacyclamide C (**3**)

Balgacyclamide A **(1)** displayed an exact mass of *m/z* 533.2552, which supports the molecular formula $C_{25}H_{37}N_6O_5S$ for the $[M + H]^+$ pseudomolecular ion. The ^1H spectrum (DMSO-d_6, 500 MHz) displayed the typical pattern of a peptide (Table 1). Three NH resonances were present as doublets. Based on characteristic signals and COSY correlations, the fragments NH-Ala, NH-Val

and NH-Ile were assigned. HSQC and HMBC experiments were used to assign the ^{13}C chemical shifts. Furthermore, two Thr residues missing the NH resonance were assigned as methyloxazoline (MeOzn) moieties. The last fragment was assigned as a thiazole (Tzl) based on its characteristic signals (H-18 Tzl, singlet, ∂_H 8.30 ppm, C-18 Tzl ∂_C 126.4 ppm).[13] The sequence of the amino acids was challenging to determine because of the presence of resonance overlap in the ^{1}H and ^{13}C spectra. HMBC long-range correlations were measured in two different solvents, DMSO-d_6 and $CDCl_3$, to establish the sequence (Figure 1; additional NMR data in $CDCl_3$ are available in the Supporting Information). From the HMBC spectrum in DMSO-d_6, correlations from H-2 of MeOzn1 to C-5 of Val and from NH-6 of Ile to C-1 of MeOzn1 were unambiguous. Additionally, a long range *J* coupling of 2.0 Hz was observed between the C(α)-H of the MeOzn1 and the C(α)-H of the Val residue. These long range *J* couplings are frequently found in cyclamides with oxazoline ring systems.[11,14] From the HMBC spectrum recorded in $CDCl_3$, correlations from NH-2 of Val to C-9 of MeOzn2 and from H-18 of Tzl to C-19 of Ile established the partial sequence -Tzl-Ile-MeOzn1-Val-MeOzn2- (Figure 1). The position of Ala was not directly established by the NMR data, but based on the molecular formula, balgacyclamide A (**1**) is required to be tetracyclic, therefore the peptide had to be macrocyclic. As a result, the sequence of the peptide was assigned as cyclo-(-MeOzn1-Val-MeOzn2-Ala-Tzl-Ile-)-.

Table 1. NMR Spectroscopic Data (500 MHz, DMSO-d_6) for Balgacyclamide A (**1**).

C/N no	δ_C, type	δ_H (*J* in Hz)	HMBC [a]
1	168.9, C		
2	72.4, CH	4.41, dd (6.8, 2.0)	1, 4, 5
3	79.3, CH	4.95, dq (6.5, 6.4)	1, 5
4	20.4, CH_3	1.37, d (6.4)	2, 3
5	166.0, C		
6	50.6, CH	4.48, ddd (9.0, 2.5, 2.0)	5, 7, 8’
7	30.8, CH	1.98, m	
8	18.1, CH_3	0.62, d (6.9)	6, 7, 8’
8’	15.1, CH_3	0.35, d (6.9)	6, 7, 8
NH (2)		7.35, d (9.0)	5, 9
9	169.7, C		
10	72.7, CH	4.33, d (6.7)	9 or 13, 11, 12
11	81.1, CH	4.79, dq (6.7, 6.3)	9 or 13
12	21.0, CH_3	1.43, d (6.3)	9 or 13, 10, 11
13	169.7, C		
14	42.6, CH	4.73, dq (7.7, 6.9)	13, 15, 16
15	19.3, CH_3	1.47, d (6.9)	14
NH (4)		8.30, d (7.7)	
16	159.1, C		
17	148.0, C		
18	124.6, CH	8.30, s	17, 19
19	169.7, C		
20	54.0, CH	5.23, dd (8.4, 5.7)	19, 21, 22
21	40.4, CH	1.92, m	

22	14.2, CH_3	0.93, d (6.8)	20, 21, 23
23a	25.1, CH_2	1.44, m	
23b		1.04, m	21
24	11.1, CH_3	0.88, dd (7.4, 7.4)	21, 23
NH (6)		8.21, d (8.4)	1

[a] HMBC correlations are given from proton(s) stated to the indicated carbon atom.

Balgacyclamide B **(2)** displayed an exact mass of *m/z* 551.2657, which supports the molecular formula $C_{25}H_{39}N_6O_6S$ for the $[M + H]^+$ pseudomolecular ion. Its molecular formula suggests that **2** is an H_2O adduct of balgacyclamide A (**1**). The constitution of **2** was obtained utilizing the same methods as for balgacyclamide A (**1**). As for **1**, NH-Ala, NH-Val and NH-Ile were identified, but instead of two MeOzn in **1**, one MeOzn and one NH-Thr were present accounting for the difference in the molecular formula. The sequence was established by HMBC long-range correlations (Figure 1). Correlations were observed from H-2 of MeOzn to C-5 of Val, from H-6 of Val to C-9 of Thr, from H-10 of Thr to C-13 of Ala, from H-14 of Ala to C-16 of Tzl, from H-18 of Tzl to C-19 of Ile and from H-20 of Ile to C-1 of MeOzn. As the ^{13}C chemical shifts of the two carbonyls and of the thioimidate were close to each other (C-1 MeOzn ∂_C 169.7, C-9 Thr ∂_C 169.4 and C-19 Ile ∂_C 169.6), the sequence was confirmed by ROESY correlations. Correlations were observed between H-4 of MeOzn and H-6 of Val, between NH-2 of Val and H-10 and H-11 of Thr, between NH-3 of Thr and H-14 and H-15 of Ala and between NH-6 of Ile and H-2 of MeOzn. As for cyclamide **1**, a long range *J* coupling of 1.6 Hz was observed between the C(α)-H of MeOzn and the C(α)-H of the Val residue. Only the link between C-16 and C-17 of Tzl could not be established by the different NMR experiments. Again, according to the molecular formula, balgacyclamide B (**2**) was required to be tricyclic and therefore C-16 and C-17 needed to be connected, thus closing the macrocyclic ring of cyclo-(-MeOzn-Val-Thr-Ala-Tzl-Ile-)-.

Balgacyclamides A(**1**) and B(**2**) differ in only one structural variation, *i.e*. in the opening of the MeOzn residue to Thr. We think that this is not an artifact of the isolation procedure, as acidic conditions were avoided throughout the workup, and the presence of balgacyclamide B (**2**) was detected in the original extract.

Table 2: NMR Spectroscopic Data (500 MHz, DMSO-d_6) for Balgacyclamide B (**2**).

C/H no.	δ_C, type	δ_H (*J* in Hz)	HMBC [a]	ROESY
1	169.7, C			
2	73.4, CH	4.34, dd (6.2, 1.6)	1, 4, 5	4
3	78.0, CH	4.84, dq (6.2, 6.2)	1, 2, 5	
4	20.0, CH_3	1.29, d (6.2)	2, 3	
5	165.7, C			
6	51.1, CH	4.43, ddd (8.6, 3.7, 1.6)	5, 7, 8, 8', 9	4, 8, 8'
7	30.3, CH	1.95, m	5, 6, 8, 8'	8, 8'
8	17.8, CH_3	0.67, d (6.9)	6, 7, 8'	
8'	15.8, CH_3	0.53, d (6.9)	6, 7, 8	
NH (2)		6.95, d (8.6)	5, 6, 9	6, 7, 8, 8', 10, 11
9	169.4, C			
10	58.5, CH	4.23, dd (8.7, 3.5)	9, 11, 13	12
11	65.0, CH	4.19, m	12	12
12	20.0, CH_3	1.07, d (6.3)	10, 11	
OH		4.76, d (5.6)	10, 11, 12	12
NH (3)		8.47, d (8.7)	10, 11, 13	10, 11, 12, 14, 15, OH, NH(2)
13	172.4, C			
14	47.8, CH	4.80, m	13, 15, 16	

15	17.4, CH_3	1.30, d (6.5)	13, 14	
NH (4)		8.22, d (7.9)	13, 14, 16	14, 15, NH(2)
16	159.6, C			
17	147.9, C			
18	123.6, CH	8.22, s	17, 19	
19	169.6, C			
20	54.4, CH	5.13, dd (8.5, 5.5)	1, 19, 21, 22, 23	22, 23b
21	39.5, CH	1.91, m	19, 20, 22, 23, 24	24
22	14.1, CH_3	0.90, d (6.8)	20, 21, 23	
23a	25.2, CH_2	1.44, m	20, 21, 22, 24	
23b		1.11, m	20, 21, 22, 24	
24	10.9, CH_3	0.88, dd (7.3, 7.3)	20, 21, 23	
NH (6)		8.43, d (8.5)	1, 20	2, 6, 20, 21, 23a, 24

[a] HMBC correlations are given from proton(s) stated to the indicated carbon atom.

Balgacyclamide C **(3)** displayed an exact mass of *m/z* 585.2512, which supports the molecular formula $C_{28}H_{37}N_6O_6S$ for the $[M + H]^+$ pseudomolecular ion. The six fragments MeOzn, NH-Phe, NH-Thr, NH-Gly, Tzl and NH-Ile were identified as described above. HMBC spectroscopy allowed the establishment of the sequence through a series of long-range correlations: from H-2 of MeOzn to C-5 of Phe, from H-6 of Phe to C-12 of Thr, from NH-3 of Thr to C-16 of Gly, from H-17 of Gly to C-18 of Tzl, from H-20 of Tzl to C-21 of Ile and from NH-6 of Ile to C-1 of MeOzn, which established the sequence as cyclo-(-MeOzn-Phe-Thr-Gly-Tzl-Ile-)-.

When comparing balgacycmide C (**3**) to its congeners, the replacement of a Val by a Phe residue and of the Ala by a Gly residue becomes apparent. Again, the Thr residue is present, which we attribute to the original metabolite (corroborated by its presence in the extract).

Table 3: NMR Spectroscopic Data (500 MHz, DMSO-d_6) for Balgacyclamide C (**3**).

C/H no.	δ_C, type	δ_H (*J* in Hz)	HMBC [a]	ROESY
1	169.0, C			
2	73.8, CH	4.28, dd (7.6, 1.7)	1, 3, 4, 5	4
3	78.3, CH	4.88, dq (7.6, 6.4)	1, 2, 5	
4	20.3, CH_3	1.36, d (6.4)	2, 3	
5	165.0, C			
6	48.0, CH	4.76, m	5, 7, 8, 12	7a
7a	36.7, CH_2	2.98, dd (13.6, 5.2)	5, 6, 8, 9/9’	
7b		2.86, dd (13.6, 6.0)	5, 6, 8, 9/9’	
8	135.4, C			
9/9’	129.0, CH	7.01, d (7.4)	7, 9/9’, 11	3, 6, 7b, OH
10/10’	127.4, CH	6.94, dd (7.4, 7.4)	8, 9/9’, 10/10’	3, 6, 7b, OH
11	126.0, CH	6.82, t (7.4)	9/9’	
NH (2)		7.45, d (7.5)	12	9/9’, 6, 13, 14
12	169.2 C			
13	58.8, CH	4.19, dd (9.0, 4.5)	12, 14, 15	15
14	65.4, CH	4.09, m	12	15
15	19.8, CH_3	0.92, d (6.3)	13, 14	
OH		4.91, d (5.4)	13, 14, 15	14, 15
NH (3)		7.81, d (9.0)	13, 16	9/9’, 13, 14, 15, 17a, OH, NH(2)
16	169.6, C			
17a	42.9, CH_2	4.39, dd (15.3, 7.5)	16, 18	
17b		3.63, dd (15.3, 3.3)	16, 18	
NH (4)		8.26, dd (7.5, 3.3)		17a, 17b, NH(2), NH(3), NH(6)
18	159.9, C			
19	147.5, C			

20	123.7, CH	8.29, s	18, 19, 21	
21	168.6, C			
22	54.1, CH	5.18, dd (8.1, 5.2)	21, 23, 24, 25	23, 26
23	39.8, CH	1.92, m		26
24	13.9, CH_3	0.84, d (6.9)	22, 23, 25	
25a	25.0, CH_2	1.45, m	22, 23, 24, 26	
25b		0.98, m	22, 23, 24	
26	11.2, CH_3	0.87, dd (7.4, 7.4)	23, 25	
NH (6)		7.98, d (8.2)	1, 22	2, 22, 23, 24, 25a

[a] HMBC correlations are given from proton(s) stated to the indicated carbon atom.

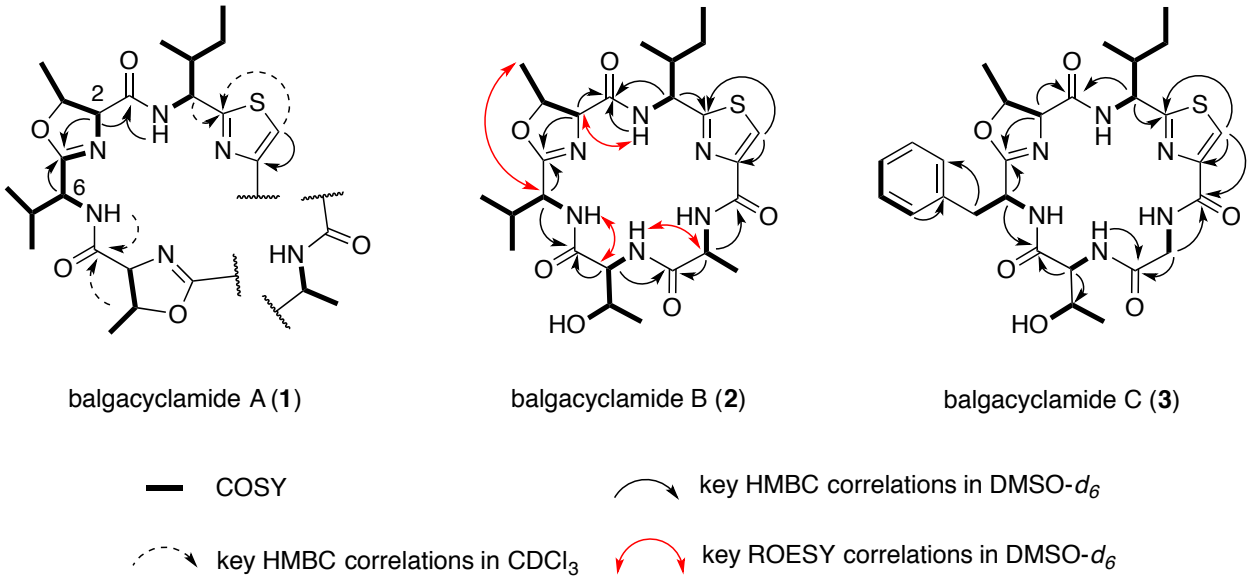


Figure 1: Key COSY, ROESY and HMBC correlations in balgacyclamides A (**1**), B (**2**) and C (**3**). Note the observed long-range *J* coupling between H-2 and H-6 in both balgacyclamides A (**1**) and B (**2**).

The configurations of balgacyclamides A-C (**1-3**) were determined by the same strategy as for the aerucyclamides.[11] First, the compounds were ozonized to avoid the epimerization of the Ile

residues that are next to the thiazole residues, as it is known that amino acids next to thiazole or oxazole residues are prone to epimerization during hydrolysis.[15] The compounds were then hydrolyzed, and half the amount of the hydrolyzed compounds was derivatized with the Marfey's reagent[16] while the other half was derivatized with trifluoroacetic acid anhydride. The Marfey's derivatives were analyzed by HPLC-MS to establish the configurations of the Ala, Thr, Val and Phe amino acids by comparing the retention times with authentic derivatized amino acids as standards. Co-injection of authentic and natural samples was performed when necessary. The TFA derivatives were analyzed by GC-MS to establish the configuration of the Ile amino acids. It was necessary to perform a co-injection analysis with Ile standards to differentiate between the D-Ile and D-*allo*-Ile residues. This methodology established the presence of D-Ala, L-Thr, L-Val and D-*allo*-Ile for balgacyclamides A and B (**1** and **2**) and L-Thr, L-Phe, D-*allo*-Ile for balgacyclamide C (**3**). These experiments therefore established the structures of balgacyclamides A-C (**1**–**3**).

Balgacyclamides A (**1**) and B (**2**) were evaluated for their antiparasite activity. Compounds **1** and **2** displayed micromolar IC_{50} activity (9.0 and 8.2 μM respectively) against the chloroquine-resistant strain K1 of *P. falciparum*, whereas **1** and **2** showed only moderate activity against other parasites, IC_{50} = 59 and 51 μM respectively against *Trypanosoma brucei rhodesiense* STIB 900 and IC_{50} = 28 μM for **2** against *Leishmania donovani* MHOM-ET-67/L82. Balgacyclamides A and B also displayed selectivity with respect to their cytotoxicity to the L6 rat myoblast cell line, for which no activity was detected up to 150 μM. These results are in agreement with previous antiparasite evaluations of cyclamide compounds. Venturamides A and B displayed IC_{50} values of 8.2 and 5.2 μM against *P. falciparum,*[10] while aerucyclamides A, B, C and D displayed IC_{50} values of 5.0, 0.7, 2.3 and 6.3 μM respectively.[11] It is interesting to note that opening of the oxazoline ring in **1** did not result in significantly lower bioactivity.

Table 4. Biological Evaluation of Balgacyclamides A (**1**) and B (**2**) against *Plasmodium falciparum* K1, *Trypanosoma brucei rhodesiense* STIB 900, *Trypanosoma cruzi* Tulahuen C4, *Leishmania donovani* MHOM-ET-67/L82 and rat myoblast cells L6 (IC_{50} values are reported as μM concentrations).

	P.f.[a]	*T. b. rhod.*[b]	*T. cruzi*[c]	*L. donovani*[d]	L6[e]
1	9.0	59	>150	>150	>150
2	8.2	51	>150	28	>150

[a] *Plasmodium falciparum* K1

[b] *Trypanosoma brucei rhodesiense* STIB 900

[c] *Trypanosoma cruzi* Tulahuen C4

[d] *Leishmania donovani* MHOM-ET-67/L82

[e] Rat myoblast L6 cells

In conclusion, we have reported the isolation and structure determination of balgacyclamides A (**1**), B (**2**) and C (**3**) from *Microcystis aeruguinosa* EAWAG 251. Balgacyclamides A (**1**) and B (**2**) were evaluated for their antiparasite activity and displayed micromolar IC_{50} activity against *P. falciparum* with good selectivity compared to their cytotoxicity.

Experimental Section

General Experimental Procedures. UV experiments were obtained with a Shimadzu UV-1650PC double-beam spectrophotometer using a 1 cm path length cell. ECD spectra were

measured in MeOH with a Chirascan™ CD spectrometer in a 0.1 cm path length cell. NMR spectra were acquired on a Bruker Avance 500, a Bruker Avance III TCI 1.7 mm MicroCryoProbe, Bruker DRX-600 and Bruker Avance II 800 spectrometers, all equipped with cryoprobes. The spectra were referred to residual solvent proton and carbon signals (δ_H 2.50, δ_C 39.5 for DMSO- d_6 and δ_H 7.26, δ_C 77.2 for $CDCl_3$). Accurate mass ESI spectra were recorded on a Micromass (ESI) Q-TOF Ultima API instrument. HPLC purification and analyses were performed on a Dionex HPLC system equipped with a P680 pump, an ASI-100 automated sample injector, a TCC-100 thermostated column compartment, a PDA-100 photodiode array detector, a Foxy Jr. fraction collector, and a MSQ-ESI mass spectrometric detector. The determination of the Ile enantiomers was carried out on a GC-FID instrument (Thermo Focus with a Thermo AS 3000 autosampler).

Sources and Cultivation of Cyanobacteria. *Microcystis aeruginosa* EAWAG 251 was obtained from the Eawag (Swiss Federal Institute of Aquatic Science and Technology) Culture Collection of Cyanobacteria, currently at the University of Basel. This strain is identical as the *M. aeruginosa* PCC7820 from the Pasteur Culture Collection of Cyanobacteria, Paris, France. The strain was grown in a 20 L batch reactor (Zehnder medium[17]) with continuous aeration and a light/dark cycle of 12:12 h. The biomass was isolated by centrifugation and subsequently kept frozen until extraction.

Extraction and Isolation. The biomass was extracted first with 100% MeOH and then twice with 60% MeOH. The extract was separated from the biomass by centrifugation. MeOH was removed from the combined extracts by evaporation under reduced pressure and the mixture was then dried by lyophilization. The resulting powder was dissolved in 80% MeOH and centrifuged to remove remaining particles. The solutions were then sequentially loaded to a C_{18} SPE column

(Supelco Discovery DSC-18, 10 g, conditioned with 10% aqueous MeOH), and eluted with 30%, 60%, 80%, and 100% aqueous MeOH solutions. MeOH was removed from the combined fractions by evaporation under reduced pressure and the mixture was then dried by lyophilization. The resulting oil from the 80% MeOH fraction was redissolved in 80% MeOH, and the compounds were isolated using multiple C_{18} RP-HPLC runs (Phenomenex Gemini C_{18} 5 μm; 150 × 10 mm). The gradient MeOH/H_2O at a flow of 5 mL/min was 35% MeOH to 100% over 30 min. The column was then washed for 10 min with 100% MeOH and stabilized for the next cycle over 10 min with 35% MeOH. MeOH was removed under reduced pressure and the compounds were then dried with a stream of N_2. Balgacyclamides A, B and C eluted at 17.4, 13.2 and 14.3 min respectively. Balgacyclamide B and C were further purified by HPLC using multiple runs on an analytical column C_{18} RP-HPLC (Phenomenex Gemini C_{18} 5 μm; 150 × 4.6 mm). The gradient CH_3CN/H_2O at a flow of 1 mL/min was 30% CH_3CN to 100% over 60 min. The column was then washed for 10 min with 100% CH_3CN and stabilized for the next cycle over 10 min with 30% CH_3CN. Balgacyclamide B and C eluted at 10.0 and 11.2 min respectively. The compounds were dried as described above. The isolation yields of the balgacyclamides A (**1**), B (**2**) and C (**3**) were 0.55 mg, 0.15 mg and 0.80 mg per 15 liters of culture, respectively.

Balgacyclamide A (1): colorless, amorphous solid; UV (CH_3CN) λ_{max} (log ε) 224 nm (3.54); ECD (MeOH, c = 3.5 x 10^{-4}) λ_{max} ($\Delta\varepsilon$) 244 (1.92), 207 (13.5); ^{1}H and ^{13}C NMR data (DMSO-d_6), see Table 1; ^{1}H and ^{13}C NMR data ($CDCl_3$), see supporting information; HRESI-QqTof-MS m/z 533.2552 $[M + H]^+$ (calcd for $C_{25}H_{37}N_6O_5S$, 533.2546).

Balgacyclamide B (2): colorless, amorphous solid; UV (CH_3CN) λ_{max} (log ε) 237 nm (2.61); ECD (MeOH, c = 5.9 x 10^{-3}) λ_{max} ($\Delta\varepsilon$) 231 (0.33), 209 (0.77); ^{1}H and ^{13}C NMR data (DMSO-d_6), see Table 2; HRESI-QqTof-MS m/z 551.2657 $[M + H]^+$ (calcd for $C_{25}H_{39}N_6O_6S$, 551.2652).

Balgacyclamide C (3): colorless, amorphous solid; UV (CH_3CN) λ_{max} (log ε) 242 nm (2.74); ECD (MeOH, c = 8.4 x 10^{-4}) λ_{max} ($\Delta\varepsilon$) 248 (0.45); ^{1}H and ^{13}C NMR data (DMSO-d_6), see Table 3; HRESI-QqTof-MS m/z 585.2512 $[M + H]^+$ (calcd for $C_{28}H_{37}N_6O_6S$, 585.2495).

Ozonolysis of Compounds 1–3: Each compound (0.1 mg, 0.2 μmol) was dissolved in 0.3 mL of dry CH_2Cl_2, ozonized at room temperature for 5 min and the solvent was removed under a stream of N_2.

Hydrolysis of Compounds 1–3: Each ozonized compound was hydrolyzed in a closed microwave tube by adding 0.5 mL of freshly prepared 6 N HCl at 108 °C for 20 h. The solvent was concentrated by a stream of N_2 and removed by lyophilization and then the residue was dissolved in 0.06 mL of H_2O.

Marfey's Analysis of Compounds 1–3: Half of the solution of the hydrolyzed compounds was treated with 0.03 mL of a 1% *N*-(2,4-dinitro-5-fluorophenyl)-L-alaninamide (FDAA) solution in acetone and 0.03 mL of 6% Et_3N in H_2O at 50°C for 1 h in a closed HPLC vial. The reaction was quenched with 0.03 mL of 5% acetic acid and the solvent was removed by lyophilisation. The residue was dissolved in 0.03 mL of MeOH and analyzed by RP-HPLC using an Agilent Zorbax SB-C18 column (3.55 μm 150 x 2.1 mm). The mobile phases were A, 5% acetic acid in H_2O, and B, CH_3CN/MeOH (9:1). The column was stabilized for 20 min with 5% of B, then the gradient went from 5-50% in 50 min and the column was washed with 100% of B for 20 min. The flow was set at 0.25 mL/min and the temperature of the column oven at 50 °C.

By comparison of the retention times of the derivatized commercially available amino acids the configurations can be determined except for isoleucine. The retention times (min) for the derivatized standard amino acids were: L-Ala 28.25; D-Ala 32.21; L-Val 35.89; D-Val 40.64; L-

Phe 41.01; D-Phe 46.39; L-Thr 21.09; L-*allo*-Thr 22.22; D-*allo*-Thr 24.73; D-Thr 28.89; L-Ile 40.47; L-*allo*-Ile 40.45; D-Ile 47.38 and D-*allo*-Ile 47.46.

TFA Derivatives and Chiral-Phase GC Analysis of 1, 2 and 3: 0.03 mL of the hydrolyzed compound solution was transferred to a GC vial and the solvent was evaporated. The residue was treated with 0.03 mL of trifluoroacetic acid anhydride at room temperature for 12 h and quenched with 0.03 mL of MeOH. The configuration of isoleucine was determined after analyses on a chiral-phase GC column (Permabond-L-Chirasil-Val; 25 m, 0.25 mm, Macherey-Nagel,) with the following conditions: 2 min at 80 °C, 80 to 180 °C at rate of 8 °C min^{-1} and 10 min at 180 °C with a split ratio 10:1, at a flow of 1 mL/min and an FID detector at 250 °C.

The four commercially available stereoisomers of isoleucine were derivatized with the same method as presented above and the retention times (min) were: D-*allo*-Ile 6.20, L-*allo*-Ile 6.35, D-Ile 6.39 and L-Ile 6.45.

Configuration of Compounds 1–3:

Balgacyclamide A (**1**): from ozonolysis–hydrolysis–Marfey D-Ala, L-Thr (confirmed by a co-injection with L-*allo*-Thr), L-Val and D-Ile or D-*allo*-Ile and from ozonolysis–hydrolysis–TFA D-*allo*-Ile (confirmed by a co-injection with D-Ile).

Balgacyclamide B (**2**): from ozonolysis–hydrolysis–Marfey D-Ala, L-Thr, L-Val and D-Ile or D-*allo*-Ile and from ozonolysis–hydrolysis–TFA D-*allo*-Ile (confirmed by a co-injection with D-Ile).

Balgacyclamide C (**3**): from ozonolysis–hydrolysis–Marfey L-Thr, L-Phe and D-Ile or D-*allo*-Ile and from ozonolysis–hydrolysis–TFA D-*allo*-Ile (confirmed by a co-injection with D-Ile).

Biological Assays:

Plasmodium falciparum, *Trypanosoma b. rhodesiense* and in vitro cytotoxicity assays were performed according to procedures previously described.[11b]

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Supporting Information Available: Copies of NMR spectra and the different HPLC and GC experiments for the determination of configuration are provided. This material is available free of charge via the Internet at http://pubs.acs.org.

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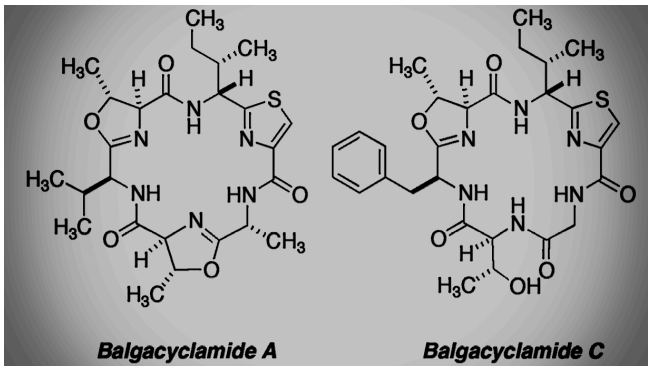
Balgacyclamide A
Balgacyclamide C