Aerucyclamides A and B: Isolation and Synthesis of Toxic Ribosomal Heterocyclic Peptides from the Cyanobacterium *Microcystis aeruginosa* PCC 7806

Cyril Portmann,[†] Judith F. Blom,[‡] Karl Gademann,*[,†] and Friedrich Jüttner*[,‡]

Chemical Synthesis Laboratory, SB-ISIC-LSYNC, Swiss Federal Institute of Technology (EPFL), CH-1015 Lausanne, Switzerland, and Limnological Station, Institute of Plant Biology, University of Zürich, CH-8802 Kilchberg, Switzerland

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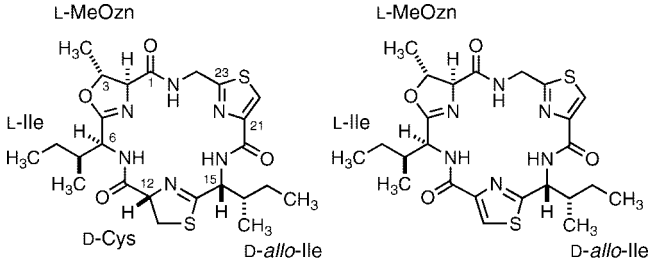
Two new modified hexacyclopeptides, aerucyclamides A and B, were isolated from the toxic freshwater cyanobacterium *Microcystis aeruginosa* PCC 7806. The constitution was assigned by spectroscopic methods, and the configuration determined by chemical degradation and analysis by Marfey's method combined with chemical synthesis. Synthetic aerucyclamide B was obtained through oxidation of aerucyclamide A (MnO_2, benzene). The aerucyclamides were found to be toxic to the freshwater crustacean *Thamnocephalus platyurus*, exhibiting LC_{50} values for congeners A and B of 30.5 and 33.8 μM, respectively.

Cyanobacteria are considered as prolific producers of bioactive metabolites,[1] producing many classes of natural products including alkaloids,[2] terpenoids,[3] polyketides,[4] depsipeptides,[5] and peptides.[5] The structural diversity of the latter is very high, including both linear and cyclic structures, nonproteinogenic amino acids, and modifications such as glycosylation, lipidation, and sulfatation. Well-known examples of cyanobacterial peptides include the microcystins,[6] which are considered to be the active principles in many toxic strains, as well as cyanopeptolins,[7] anabaenopeptins,[8] and aeruginosins.[9] Another structurally intriguing class is represented by the cyclamides (from cyanobacteria[10] or marine animals[11]), which are composed of cyclic peptides involving heterocyclic amino acids. We report the isolation, structural characterization, synthesis, and biological evaluation of aerucyclamides A (**1**) and B (**2**), toxic constituents of the cyanobacterium *Microcystis aeruginosa* PCC 7806.



Aerucyclamide A (**1**) Aerucyclamide B (**2**)

Results and Discussion

The aqueous methanolic extract of *Microcystis aeruginosa* PCC 7806 contained two lipophilic compounds eluting above 40% aqueous CH_3CN on a C_{18} RP-HPLC. The major peak, aerucyclamide A (**1**), displayed an exact mass of *m/z* 557.1982, which supports the molecular formula $C_{24}H_{34}N_6O_4S_2Na$ (calcd 557.1981) for the Na adduct. This elemental composition was corroborated by ^{15}N- and ^{34}S-labeled aerucyclamide A (**1**), in which LC-MS established the presence of six N atoms and two S atoms. The ^1H NMR spectrum (DMSO-d_6, 600 MHz) displayed the typical pattern of a peptide. Three NH resonances were present, of which two appeared as doublets and one as a pseudotriplet. Further characteristic signals pointed to the presence of two Ile residues, one Gly and one Thr residue, in this hexapeptide. Additionally, a CH_2 of a Ser or Cys residue was observed as well as a sharp aromatic singlet. All spin systems could be assigned by COSY and TOCSY experiments, and ^{13}C chemical shifts were assigned by HSQC and HMBC experiments. These data corroborated the presence of two NH-Ile and one NH-Gly fragments. The Thr residue, missing the NH resonance, was assigned as methyloxazoline (MeOzn), and the missing two amino acids of this cyclic hexapeptide were assigned as a thiazoline (Tzn) and thiazole (Tzl), on the basis of the presence of two sulfur atoms and characteristic signals. The sequence was clearly established by HMBC long-range correlations. Correlations from H-2 of MeOzn to C-5 of Ile, from H-6 of Ile to C-11 of Tzn, from H-12 of Tzn to C-14 of Ile, from H-15 of Ile to C-20 of Tzl, from H-22 of Tzl to C-23 of Gly, and from both H-24 protons of Gly to C-1 of MeOzn established the sequence of aerucyclamide A as *cyclo*-(−Gly-Tzl-Ile-Tzn-Ile-MeOzn−)−. It is interesting to note that a long-range *J* coupling between the C(α)−H of the methyloxazoline and the Ile residue was observed. Similar *J* couplings were already described for other cyclamides with oxazoline ring systems.[10a,11a,d]

The constitution of aerucyclamide B (**2**) was assigned utilizing the same methods. High-resolution mass spectroscopy suggested the formula $C_{24}H_{33}N_6O_4S_2$, which was corroborated by ^{15}N- and ^{34}S-labeling studies, establishing the presence of six N atoms and two S atoms. The difference of 2 H atoms of compound **2** when compared to **1** suggests that aerucyclamide B (**2**) was an oxidative derivative of **1**. This was corroborated by the ^1H NMR spectrum of **2**, which displayed a high level of similarity when compared to **1**, differing in the disappearance of the resonances assigned to the C(α)−H and the C(*β*)−H_2 atoms of the thiazoline and the appearance of a second aromatic singlet at 8.38 ppm, suggesting the replacement of the thiazoline by a thiazole residue. This was corroborated by 2D NMR experiments, as COSY, HSQC, and HMBC allowed for the assignment of all resonances in their respective spin systems. HMBC spectroscopy allowed for establishment of the sequence through a series of long-range correlations: from H-2 of the MeOzn to C-5 of Ile, from NH-2 of Ile to C-11 of the Tzl, from H-13 of Tzl to C-14 of the Ile, for NH-4 of the Ile to C-20 of the Tzl, from H-22 of Tzl to C-23 of the Gly, and from the NH-6 of Gly to C-1 of the MeOzn. Only one correlation between the two quaternary carbons C-11 and C-12 of the thiazole could not be observed, but according to the molecular formula,

* To whom correspondence should be addressed. Tel: ++41 21 693 93 15. Fax: ++41 21 693 97 00. E-mail: karl.gademann@epfl.ch (K.G.). Tel: ++41447154325. E-mail: juttner@limnol.uzh.ch (F.J.).

[†] Swiss Federal Institute of Technology.

[‡] University of Zürich.

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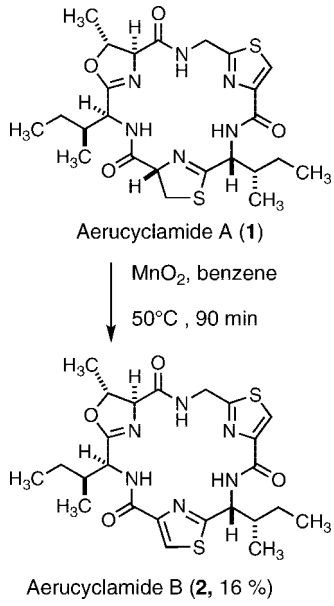
Scheme 1. Synthesis of Aerucyclamide B (**2**) through Oxidation of Aerucyclamide A (**1**)



aerucylamide B (**2**) was required to be tetracyclic and therefore C-11 and C-12 needed to be connected, thus closing the macrocyclic ring.

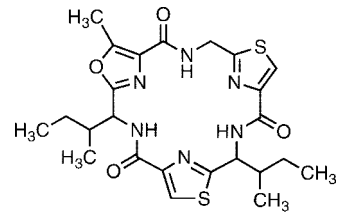
We investigated next whether aerucyclamide B (**2**) can be obtained through oxidation of aerucyclamide A (**1**). Therefore, a survey of conditions reported in the literature was conducted,[12] which led to the identification of MnO_2 as a mild and selective oxidant. Treatment of aerucyclamide A with MnO_2 in benzene at 50 °C for 90 min led to the formation of aerucyclamide B (**2**). Purification by RP-HPLC gave synthetic aerucyclamide B (**2**) in 16% yield at 50% conversion. The synthetic material was shown to be identical to a natural sample of **2** by HPLC co-injection. This transformation was crucial for the present work for two reasons: (1) Identity of synthetic and natural samples requires the configuration of the MeOzn and Ile residues of aerucyclamides A and B to be identical (key to the determination of the configuration, *vide infra*), and (2) this procedure allowed for the synthesis of larger amounts of aerucyclamide B (**2**) for structure determination.

The configuration of aerucyclamides A (**1**) and B (**2**) was assigned after chemical degradation, derivatization of the resulting amino acids, and analysis by GC or HPLC using chiral stationary phases or Marfey's method, respectively. Aerucyclamide A (**1**) was flash hydrolyzed (6 N HCl solution, 105 °C, 90 min), and L-Thr and D-Cys were assigned by FDAA derivatization.[13] In addition, a 1:1.5 mixture of L-Ile and D-*allo*-Ile was obtained. The differentiation of L-Ile and L-*allo*-Ile and D-Ile and D-*allo*-Ile was achieved using GC on chiral stationary phases, as both L- and D-amino acids were not resolved with their *allo*-epimers utilizing Marfey's method. This left the exact position of both Ile epimers to be assigned through chemical correlation (*vide infra*).

Aerucyclamide B (**2**) was hydrolyzed (6 N HCl solution), and the amino acids L-Thr and L-Ile were unambiguously assigned. It is well-known that amino acids adjacent to thiazole rings are not liberated under normal hydrolysis conditions;[14] therefore, the L-Ile amino acid must be adjacent to the more hydrolytically labile oxazoline ring system. As aerucyclamide B (**2**) can be obtained through oxidation of aerucyclamide A (**1**, Scheme 1), the second Ile in aerucyclamide B (**2**) must be D-*allo*-Ile. This was definitely corroborated by ozonolysis of **2** followed by hydrolysis, which is known to break thiazole, thiazoline, and oxazoline rings and thus liberate all amino acids.[15] After this degradation process, L-Thr, L-Ile, and D-*allo*-Ile were obtained. This experimental evidence unambiguously established the configuration of aerucyclamide B (**2**) as shown in Scheme 1. Through correlation by chemical synthesis, the configuration of aerucyclamide A (**1**), and in particular the position of the epimeric Ile residues, was unambiguously assigned as shown in Scheme 1 for **1**.

Aerucyclamides A (**1**) and B (**2**) were tested for grazer toxicity against the sensitive freshwater crustacean *Thamnocephalus platyurus* following published procedures.[16] Aerucyclamides A (**1**) and B (**2**) were found to be toxic, with LC_{50} values of 30.5 and 33.8 μM, respectively. Comparison of these measured toxicity values suggests that **1** and **2** are by a factor of 2 less toxic than nostocyclamide, a similar cyclopeptide isolated from *Nostoc*,[10c,16] and by roughly 1 order of magnitude less toxic than several microcystins.[17] It is interesting to note that the presence of toxins in addition to microcystins in *Microcystis* has been reported.[18] The aerucyclamides therefore constitute a second class of toxins observed in *Microcystis*, albeit with reduced toxicity. It is unclear at present whether this toxicity of **1** and **2** is ecologically relevant, in particular as several other toxins such as microcystin-LR and [Asp^3]-microcystin-LR, as well as other highly bioactive compounds, the cyanopeptolins A−D [7,19] are present in *M. aeruginosa* PCC 7806. The aerucyclamides A (**1**) and B (**2**) are members of a class of currently over 20 similar cyclopeptides that have been obtained either from cyanobacteria[10] or from sea-hares or ascidians.[11] The cyclamides isolated from animal sources are thought to be of cyanobacterial origin as well and are suggested to be either produced by symbionts or obtained through dietary uptake.[11a] Aerucyclamide A (**1**) is a rare example of a cyclamide that features oxazoline, thiazoline, and thiazole moieties in one compound, whereas most of the compounds reported to date include higher oxidized thiazole or oxazole rings. Interestingly, for all cyclamides reported to date, the available biological data fail to shed light on the ecological significance, as, in many cases, only moderate toxicity has been determined.

The biosynthetic origins of the aerucyclamides have recently been investigated. After the present work was finished, a report by Dittmann and co-workers appeared describing the sequencing of a gene cluster responsible for cyclamide production in *Microcystis aeruginosa* NIES298 and PCC 7806.[20] These studies established ribosomal peptide synthesis with hypervariable cassettes in a strongly conserved genetic background featuring tailoring enzymes, which is similar to recent studies on similar octapeptide derivatives, the patellamides produced by symbiotic *Prochloron* spp.[21] From the genetic data of *M. aeruginosa* PCC 7806, four peptide sequences could be obtained: ATVSIC, FTGCMC, ITGCIC, and ITGCIC.[20] The genetically encoded sequence ITGCIC can be regarded as the linear peptide precursor to both aerucyclamides A and B, and elaboration of this precursor is carried out by the corresponding enzymes Mca A−D, F, and G in *Microcystis*. Dittmann and colleagues used the genetic data to predict "cryptic metabolites", and they proposed the fully oxidized peptide **3** to be present in *M. aeruginosa* PCC 7806.[20] However, the isolation and characterization of the reduced derivatives **1** and **2** supports the notion that aerucyclamides A (**1**) and B (**2**) might be the actual metabolites produced via ribosomal peptide synthesis.



3

Experimental Section

General Experimental Procedures. NMR spectra were acquired on a Bruker DRX-600 equipped with a cryoprobe and referred to residual solvent proton and carbon signals (δ_H 2.50, δ_C 39.5 for DMSO-d_6). Accurate mass ESI spectra were recorded on a Micromass (ESI) Q-TOF Ultima API. 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-MS instrument (Fison Instruments, GC 8000 Top, MD 800).

Sources and Cultivation of Cyanobacteria. *M. aeruginosa* PCC 7806 was obtained from the Pasteur Culture Collection of Cyanobacteria, Paris, France. It was grown in a 20 L batch reactor (medium: $BG11_0$ + 2 mM $NaNO_3$ + 10 mM $NaHCO_3$) with continuous aeration and a light/dark cycle of 12:12 h. The biomass was isolated by centrifugation and subsequently kept frozen until extraction. For the labeling experiments, *M. aeruginosa* PCC 7806 was grown in Erlenmeyer flasks on a cyanobacterial medium as described[22] at 20 °C under continuous light from fluorescent tubes (7 μmol m^{-2} s^{-1}) and axenic conditions. To obtain a high percentage of labeled aerucyclamides, nitrate or sulfate in the growth medium was replaced by labeled anions. The final concentrations of $^{15}NO_3^-$ and $^{34}SO_4^{2-}$ in the medium were 4.0 and 0.4 mM, respectively. ^{15}N-Labeled sodium nitrate (chemical enrichment 98%; chemical purity 98%) was obtained from Cambridge Isotope Laboratories, Andover, MA) and Na_2SO_4 (99.3 at. % ^{34}S) from C.E. Saclay, Gif-Sur-Yvette, France.

Extraction and Isolation. Freshly thawed biomass was extracted three times with 60% MeOH; the extract was separated from the biomass by centrifugation. MeOH was removed from the combined extracts by evaporation under reduced pressure; the mixture was then dried by lyophilization. The resulting powder was dissolved in 90% MeOH and centrifuged to remove remaining particles. The solution was then subjected to a C_{18} SPE column (5 g, conditioned with 90% MeOH), and the column was eluted with 90% MeOH. The combined fractions were dried under reduced pressure. The resulting powder was dissolved in 70% MeOH, and the compounds were isolated using multiple C_{18} RP-HPLC runs (Phenomenex Gemini C_{18} 5 μm; 150 × 10 mm). The gradient CH_3CN/H_2O at a flow of 5 mL/min was the following: 30% CH_3CN to 35% over 10 min and 35% to 60% from 10 to 30 min. The column was then washed for 10 min with 100% CH_3CN and stabilized for the next cycle in 10 min with 30% CH_3CN. Aerucyclamides A and B (**1**, **2**) eluted at 20.3 and 23.2 min with 0.5% and 0.05% yield from crude extract, respectively.

Aerucyclamide A (1): colorless, amorphous solid; UV (CH_3CN/water, 48/52) λ_{max} 203, 237; ^{1}H and ^{13}C NMR data (DMSO-d_6), see Table 1; HRESI-QqTof-MS *m/z* $[M + Na]^+$ 557.1982 (calcd for $C_{24}H_{34}N_6O_4S_2Na$, 557.1981).

Aerucyclamide B (2): colorless, amorphous solid; UV (CH_3CN/water, 51/49) λ_{max} 204, 240; ^{1}H and ^{13}C NMR data (DMSO-d_6), see Table 2; HRESI-QqTof-MS *m/z* $[M + H]^+$ 533.2006 (calcd for $C_{24}H_{33}N_6O_4S_2$, 533.2004).

Hydrolysis of 1 and 2. Each compound (0.1 mg, 0.2 μmol) was hydrolyzed in a sealed tube with 6 N HCl (0.3 mL) at 105 °C for 1 h. The solution was concentrated to dryness under a stream of N_2.

TFA Derivatives and GC Analysis. To determine the configuration of the Ile enantiomers, the hydrolysates of aerucyclamides A and B and the four authentic standards were acylated with trifluoroacetic acid anhydride (Fluka, Buchs, Switzerland) at 80 °C for 1 h and quenched in MeOH. Analyses were conducted on a Chirasil-Val column (Permabond-L-Chirasil-Val; 25 m, 0.25 mm, Marchery-Nagel, Düren, Germany) under the following conditions: 2 min at 80 °C, 80 to 180 °C at the rate of 8 °C min^{-1}, 10 min at 180 °C. The retention times (min) of the Ile enantiomeres on the Chirasil-Val column were as follows: D-*allo*-Ile (7.84), L-*allo*-Ile (7.97), D-Ile (8.03), L-Ile (8.15).

Marfey's Analysis of 1 and 2. The hydrolyzed compound was treated with 60 μL of a solution of 1% N_α-(2,4-dinitro-5-fluorophenyl)-L-alanineamide (FDAA) in acetone and 60 μL of 6% triethylamine in a sealed vial at 50 °C for 1 h. To the mixture was added 60 μL of 5% acetic acid, and the mixture was dried under a stream of N_2. The resulting solid was dissolved in 200 μL of MeOH and analyzed by RP-HPLC using an Agilent Zorbax SB-C18 (3.55 μm 150 × 2.1 mm). Mobile phase A was 5% acetic acid and B was CH_3CN/MeOH (9:1). The linear gradient started with 5% B to reach 50% over 50 min. The column was washed for 10 min with 100% B, followed by 20 min of 100% CH_3CN. The column was stabilized before the next injection with 5% B for 20 min and maintained at 50 °C. The flow was set at 0.2 mL/min.

Table 1. NMR Spectroscopic Data (600 MHz, DMSO-d_6) for Aerucyclamide A (**1**)

C/H no.	δ_C	δ_H (*J* in Hz)	HMBC[a]
1	169.2, qC		
2	72.6, CH	4.27, dd (5.4, 1.6)	1, 4, 5
3	79.0, CH	5.00, m	1, 5
4	20.5, CH_3	1.31, d (6.4)	2, 3
5	166.1, qC		
6	50.7, CH	4.56, ddd (1.6, 2.3, 9.3)	5, 7, 8, 9, 11
7	37.7, CH	1.68, m	
8	15.0, CH_3	0.54, d (6.9)	6, 7, 9
9	22.9, CH_2	0.80, m	7, 8, 11
		0.62, m	7, 8, 11
10	11.3, CH_3	0.47, dd (7.4, 7.4)	7, 9
NH (2)		7.30, d (9.3)	5, 6, 11
11	169.3, qC		
12	77.3, CH	5.25, dd (9.9, 8.0)	11, 13, 14
13	35.8, CH_2	3.74, dd (9.9, 11.2)	11, 12, 14
		3.62, dd (8.0, 11.2)	11, 12, 14
14	172.9, qC		
15	53.7, CH	5.05, dd (9.4, 3.3)	14, 16, 17, 18, 20
16	39.4, CH	1.84, m	
17	13.9, CH_3	0.88, d (6.8)	15, 16, 18
18	25.9, CH_2	1.47, m	15, 16, 17, 19
		1.18, m	15, 16, 17, 19
19	11.7, CH_3	0.95, dd (7.4, 7.4)	16, 18
NH (4)		8.05, d (9.4)	14, 15, 20
20	159.4, qC		
21	148.3, qC		
22	124.7, CH	8.25, s	20, 21, 23
23	167.1, qC		
24	39.7, CH_2	4.89, dd (16.4, 6.9)	1, 23
		4.31, dd (16.4, 3.8)	1, 23
NH (6)		8.61, dd (6.9, 3.8)	1

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

Ozonolysis of 1 and 2. Each compound (0.1 mg, 0.2 μmol) in 0.5 mL of CH_2Cl_2 was ozonolyzed at room temperature for 5 min. The solvent was removed under a stream of N_2. Hydrolysis and Marfey's analysis were carried out as described above. The configuration was determined by comparison with the retention time of derivatives from commercially available amino acids. The retention time for each amino acid derivative was obtained by extracting the mass of the derivative in the HPLC-MS chromatogram. Retention times (min) for the standard amino acid derivatives were the following: L-Thr 26.4; L-*allo*-Thr 27.0; D-Thr 32.1; D-*allo*-Thr 30.0; L-Ile 44.7; L-*allo*-Ile 45.6; D-Ile 52.2; D-*allo*-Ile 52.1; L-Cys (di-FDAA derivative) 45.7; D-Cys (di-FDAA derivative) 49.

Aerucyclamide A (**1**) from hydrolysis−Marfey D-Cys; from hydrolysis−TFA L-Ile and D-*allo*-Ile; from ozonolysis−hydrolysis−Marfey L-Thr, a co-injection with L-*allo*-Thr was carried out.

Aerucyclamide B (**2**) from hydrolysis−TFA L-Ile; from ozonolysis−hydrolysis−Marfey L-Ile, D-*allo*-Ile, L-Thr a co-injection with L-*allo*-Thr was carried out.

Oxidation of 1 to 2. Aerucyclamide A (**1**, 1.7 mg, 30.2 μmol) in benzene (1.5 mL) was stirred with MnO_2 (4 mg, 460 μmol) at 50 °C for 1.5 h. Benzene was removed under a stream of N_2. MeOH was added, and the mixture was filtered with a syringe filter (0.25 μm). The mixture was analyzed by HPLC, indicating 50% conversion. Compounds **1** and **2** were separated by semipreparative HPLC as described above to afford aerucyclamide B (**2**, 0.28 mg, 16%).

24 h Acute Grazer Toxicity Bioassay. Aerucyclamides A (**1**) and B (**2**) were tested in a 24 h acute toxicity assay performed with *Thamnocephalus patyurus* (Thamnotoxkit F; G. Persoone, State University of Ghent, Belgium). The assay was performed in a multiwell plate using instar II−III larvae hatched from cysts. For every aerucyclamide, six different concentrations ranging from 1 to 100 μM were

Table 2. NMR Spectroscopic Data (600 MHz, DMSO-d_6) for Aerucyclamide B (**2**)

C/H no.	δ_C[a]	δ_H (J in Hz)	HMBC[b]
1	169.0, dC		
2	72.2, CH	4.38, dd (7.5, 1.6)	1, 4, 5
3	79.8, CH	4.97, dq (7.5, 6.3)	1, 5
4	20.3, CH_3	1.44, d (6.3)	2.3
5	166.3, qC		
6	51.0, CH	4.68, m	5
7	37.4, CH	1.97, m	
8	14.3, CH_3	0.74, d (7.1)	6, 7, 9
9	23.9, CH_2	1.32, m	7, 8
		1.11, m	7, 10
10	11.2, CH_3	0.79, dd (6.9, 6.9)	7, 9
NH (2)		8.40, d (7.9)	5, 11
11	158.3, qC		
12	146.8, qC		
13	125.0, CH	8.37, s	12, 14
14	169.5, qC		
15	53.3, CH	5.70, dd (9.2, 3.2)	14, 16, 17
16	41.9, CH	1.99, m	
17	13.3, CH_3	0.80, d (7.2)	16, 18
18	25.5, CH_2	1.54, m	16, 17, 19
		1.10, m	16, 17, 19
19	11.3, CH_3	0.96, dd (7.3, 7.3)	16, 17, 18
NH (4)		8.36, d (7.2)	20
20	159.0, qC		
21	147.5, qC		
22	125.0, CH	8.38, s	20, 21, 23
23	166.2, qC		
24	40.0, CH_2	4.86, dd (5.0, 17.8)	23
		4.68, dd (4.4, 17.8)	23
NH (6)		8.33, dd (4.4, 5.0)	1, 23

[a] Chemical shifts were determined using HSQC and HMBC. [b] HMBC correlations are given from the proton(s) stated to the indicated carbon atom.

tested in triplicate. The nonlinear regression analysis was done with Graph Pad Prism, Version 4.0 (Graph Pad Software, Inc.; San Diego, CA).

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Supporting Information Available: Copies of spectra and additional data are provided. This material is available free of charge via the Internet at http://pubs.acs.

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