Algal Blooms

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Investigating the Toxicity of the Aeruginosin Chlorosulfopeptides by Chemical Synthesis

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Abstract: *Harmful algal blooms are becoming more prevalent all over the world, and identification and mechanism-of-action studies of the responsible toxins serve to protect ecosystems, livestock, and humans alike. In this study, the chlorosulfopeptide aeruginosin 828A, which rivals the well-known toxin microcystin LR in terms of crustacean toxicity, has been synthesized for the first time. Furthermore, three congeners with different permutations of the chloride and sulfate groups were prepared, thereby enabling toxicity studies without the risk of contamination by other natural toxins. Toxicity assays with the sensitive crustacean Thamnocephalus platyurus demonstrated that the introduction of a sulfate group leads to pronounced toxicity, and NMR spectroscopic evidence suggests that the chloride substituent modulates the conformation, which in turn might influence protease inhibition.*

Harmful algal blooms are increasingly prevalent in many freshwater and marine ecosystems, and their massive occurrence poses a severe threat to drinking water supplies, fisheries, and recreational areas alike.[1] Research on the most prevalent cyanobacterial toxin microcystin provided important insight into its toxicity and molecular mechanisms,[2] which led the World Health Organization (WHO) to set threshold values considered safe for drinking water.[2] Over the last years, many additional cyanobacterial strains that do not produce microcystin have been discovered, which resulted in the characterization of new compounds considered to be toxic to aquatic organisms.[3] However, their mechanisms of action and the molecular basis of the processes leading to death remain unclear for many structures.[3]

Chlorosulfopeptides such as aeruginosin 828A have recently emerged as harmful compounds restoring the toxic phenotype of microcystin-deficient bacteria (Figure 1).[4] Whereas chlorosulfolipids such as danicalipin have been intensively studied by chemical synthesis with regard to the structural requirements for toxicity,[5] research on chlorosulfopeptides has to date been restricted to compounds obtained by isolation from natural sources.[4] As contamination with other toxins or biologically active compounds cannot be ruled out for these isolated compounds, we sought to investigate their toxicity by chemical synthesis followed by in vivo toxicity studies. In particular, we were interested in 1) synthesizing and characterizing peptides with all permutations with regard to the sulfate and chloride groups present (Figure 1), and 2) evaluating the effect of these functional units on toxicity. Herein, we report the successful synthesis of four natural products and putative congeners, which then enabled toxicity studies with the crustacean *Thamnocephalus platyurus*.

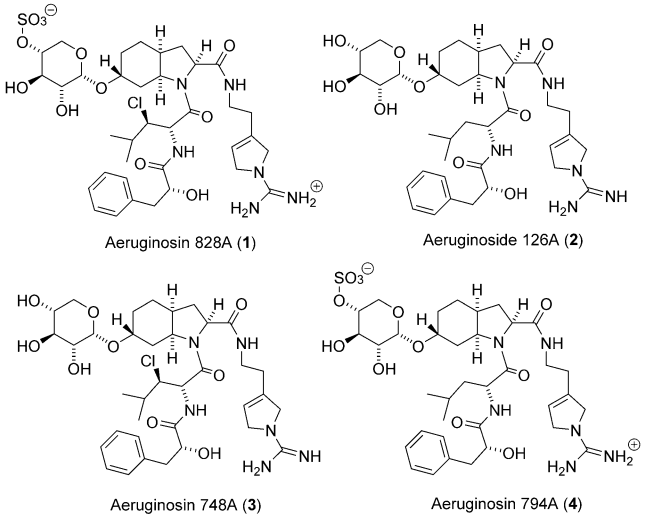

Figure 1. Aeruginosin 828A (**1**) and aeruginoside 126A (**2**) as well as the synthetic analogues aeruginosin 748A (**3**) and 794A (**4**).

As the first two targets for the synthesis, we chose the naturally occurring chlorosulfopeptide aeruginosin 828A (**1**), which bears a sulfated xylose (Xyl) residue and a chloroleucine (Cleu) unit, and aeruginoside 126A (**2**),[6] which is devoid of sulfate and chloride groups. Interestingly, chlorosulfopeptide **1** was isolated from a toxic *Planktothrix* strain lacking microcystin production, whereas peptide **2** originates from a *Planktothrix* strain that is capable of producing microcystins.[7] These observations support the hypothesis that chlorosulfopeptide **1** restores the toxic phenotype of the cyanobacterium, whereas compound **2** should be less toxic. Additional experimental support corroborating this hypothesis was provided by Blom and co-workers, who reported **1** to be highly toxic.[4] Surprisingly, the toxicity of peptide **2** had not been investigated, which provided an additional stimulus for the work reported herein. To further evaluate this hypothesis and to study the influence of the structure on toxicity, we chose the synthetic analogues aeruginosin 748A

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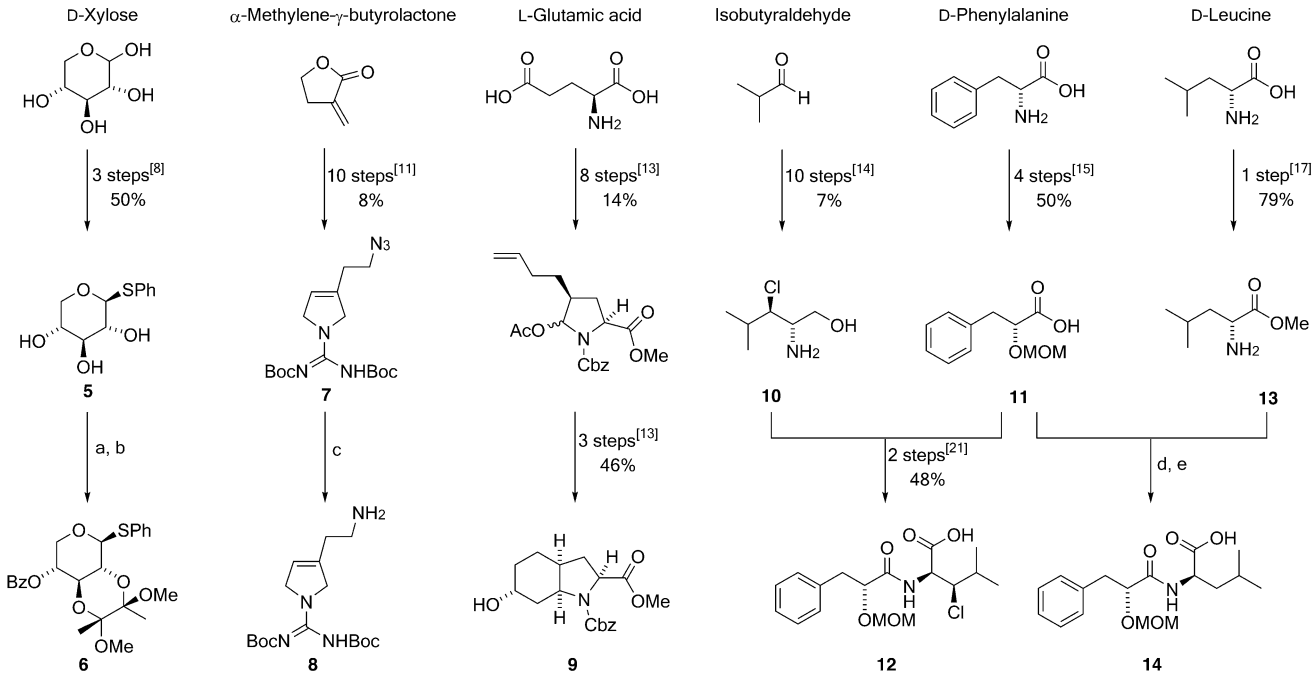
(**3**) and 794A (**4**) as targets, as **3** contains the chloroleucine moiety, but no sulfate group, and **4** features the sulfate group, but is devoid of chloride substitution.

We started with the preparation of the required building blocks for the various chlorosulfopeptides (Scheme 1). The selective installation of the sulfate group at the O4 position of the xylosyl moiety required orthogonal protection of the O4 position with respect to positions O2 and O3. Therefore, we used a base-labile protecting group for O4 and an acid-labile group for O2 and O3. The xylose moiety was synthesized starting from the known xyloside **5**, which was obtained from commercially available D-xylose in three steps.[8] Regioselective protection of the O4 hydroxy group with a benzoyl (Bz) protecting group was achieved in 81% yield using benzoyl chloride and Me_2SnCl_2 as catalyst.[9] The O2 and O3 hydroxy groups were protected as the butane diacetal (BDA) using 2,3-butanedione, trimethyl orthoformate, and camphorsulfonic acid.[10] Initial attempts of the BDA protection had always led to mixtures of isomers; however, after optimization of the reaction conditions, xylose derivative **6** could be obtained as a single diastereoisomer.

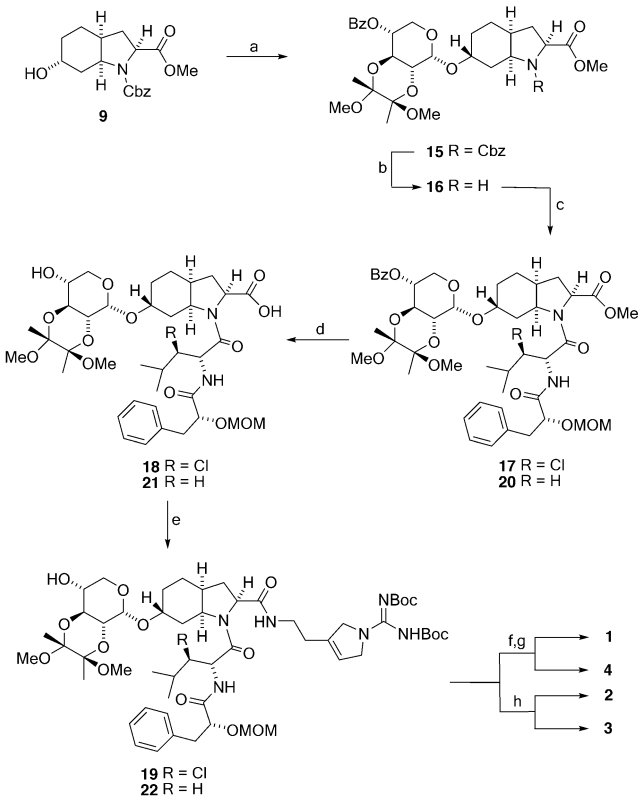
The 1-(*N*-amidino-Δ^3-pyrrolino)ethyl (Adc) side chain **8** was synthesized following a slightly modified procedure by Hanessian and co-workers,[11] which led to the intermediate dihydropyrrole **7** in ten synthetic steps and 8% overall yield from commercially available α-methylene-γ-butyrolactone. Whereas Hanessian and co-workers had chosen a Staudinger reduction for the conversion of the azide into the amine, we opted for a catalytic hydrogenation using Lindlar catalyst.[12] This procedure led to an increased yield of 96% with no side product formation while remarkably leaving the dihydropyrrole moiety untouched.

The L-2-carboxy-6-octahydroindole (L-Choi) subunit was synthesized following the aza-Prins route developed for the total synthesis of oscillarin,[13,14] which gave the L-Choi building block **9** in 6.4% yield over eleven synthetic steps. The chloroleucine derivative **10** was synthesized over ten steps and in an overall yield of 7% from isobutyraldehyde according to a method that was used in the total synthesis of chlorodysinosin A.[15] Peptide coupling of chloroleucine derivative **10** with the MOM-protected phenyllactic acid (Pla) derivative **11**[16] followed by oxidation of the alcohol to the acid gave the Cleu/Pla building block **12**. For the derivatives of aeruginosin 828A lacking the chloride substituent, a building block without the chlorine atom was synthesized through the coupling of phenyllactic acid derivative **11** with leucine (Leu) methyl ester (**13**).[17] Cleavage of the methyl ester gave the final intermediate **14** in excellent yield.

With all subunits in hand, we proceeded with the assembly of the different building blocks (Scheme 2). Our strategy started with the challenging α-xylosylation of the L-Choi core unit **9**. Starting from building block **6**, several donors containing a sulfate group protected as the trichloroethyl ester at the O4 position were prepared.[18] However, all attempts to use these donors with protected sulfate groups for the glycosylation were not successful, as the electron-withdrawing nature of the sulfate group reduced the reactivity ("disarming") of the different donors,[19] leading to either little conversion or the preferred formation of the β-anomer. We therefore decided to introduce the sulfate group at a later



Scheme 1. Synthesis of the different building blocks. Reagents and conditions: a) benzoyl chloride, Me_2SnCl_2, DIPEA, THF/H_2O (9:1), 25 °C, 81%; b) 2,3-butanedione, $HC(OCH_3)_3$, CSA, MeOH, 67 °C, 69%; c) Lindlar cat., H_2, MeOH, 25 °C, 96%; d) PyBOP, 2,6-lutidine, CH_2Cl_2, 25 °C, 81%; e) LiOH, THF/H_2O (5:3), 25 °C, 98%. Boc = *tert*-butoxycarbonyl, Bz = benzoyl, Cbz = carboxybenzyl, CSA = camphorsulfonic acid, DIPEA = *N*,*N*-diisopropylethylamine, MOM = methoxymethyl, PyBOP = (benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate, THF = tetrahydrofuran.



Scheme 2. Synthesis of aeruginosin 828A (**1**) and aeruginoside 126A (**2**) as well as derivatives **3** and **4**. Reagents and conditions: a) **6**, NIS, AgOTf, Et_2O, 25 °C, 50%; b) $PdCl_2$, Et_3SiH, Et_3N, 25 °C, 88%; c) **12** or **14**, DMTMM, NMM, CH_2Cl_2, 0→25 °C (**17**: 87% from **16**; **20**: 58% from **16**); d) 0.1 N LiOH, THF/H_2O (5:3), 25 °C (**18**: 77% from **17**; **21**: 95% from **20**); e) **8**, PyBOP, 2,6-lutidine, CH_2Cl_2, 25 °C (**19**: 60% from **18**; **22**: 64% from **21**); f) SO_3·pyridine, pyridine, 50 °C; g) CH_2Cl_2/TFA (10:1), 25 °C (**1**: 72% from **19**; **4**: 75% from **22**); h) CH_2Cl_2/TFA (10:1), 25 °C (**3**: 70% from **19**; **2**: 73% from **22**). DMTMM = 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride, NIS = *N*-iodosuccinimide, NMM = 4-methylmorpholine, TFA = trifluoroacetic acid.

stage of the synthesis and focused on donors with the benzoyl protecting group at O4 and different leaving groups at the C1 position instead. Among the donors surveyed, xyloside **6**, with thiophenol as the leaving group, in combination with NIS as activator and AgOTf as promoter, showed the most promising selectivity and reactivity.[20] After optimization, the xylosylation gave the desired product with an α/β ratio of 5:3 and a good yield of 81%. Attempts to remove the Cbz group by catalytic hydrogenation (Pd/C, $Pd(OH)_2$, and H_2)[11] resulted in little or no conversion. Pleasingly, when **15** was treated with $PdCl_2$ and Et_3N dissolved in triethylsilane according to a method developed by Birkofer and co-workers,[21] the desired free amine **16** was formed in 88% yield.

Next, we investigated the coupling of amine **16** with the Cleu side chain **12**. Initial attempts with 3-(diethoxyphosphoryloxy)-1,2,3-benzotriazin-4(3H)-one (DEBPT) as coupling reagent and 2,6-lutidine as base led to the elimination of HCl.[22] Similar behavior or racemization had already been observed in the syntheses of other aeruginosins.[15,23] These undesired side reactions are likely due to the poor reactivity of the secondary amine of the L-Choi group, which is responsible for long reaction times of several days for the coupling. The elimination can be explained by the formation of an oxazolone intermediate during the peptide coupling that is readily deprotonated, leading to the elimination of HCl.[24] To overcome the problem of elimination, different bases, such as NMM, DIPEA, or $NaHCO_3$, were tested in combination with DEBPT as the coupling reagent, but without success. Adjusting the temperature also had no critical influence on the outcome of the reaction. We therefore started screening different coupling reagents (e.g., PyBOP, bromotripyrrolidinophosphonium hexafluorophosphate (PyBrOP), and 2-(7-aza-1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU)), and found 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMTMM) to be the best promoter for the coupling.[25] With DMTMM as coupling reagent and NMM as base, the coupling was completed after only two hours, resulting in an excellent yield of 87% with no elimination of HCl. Hydrolysis of methyl ester **17** to acid **18** was achieved in 0.1N aqueous LiOH solution. The progress of the reaction was monitored by ultra-performance liquid chromatography (UHPLC), which allowed for timely quenching of the reaction, as longer reaction times again led to the elimination of HCl. Acid **18** was further coupled with the Adc unit **8** using PyBOP as the coupling reagent and 2,6-lutidine as the base to give tetrapeptide **19** in a moderate yield of 60%. Sulfatation of the O4 hydroxy group of the xylose moiety was achieved using an excess of SO_3–pyridinium complex in pyridine. Global deprotection of the Boc, MOM, and BDA groups was carried out in TFA/CH_2Cl_2 solution to obtain aeruginosin 828A (**1**) in 72% yield over two steps. When intermediate **19** was directly subjected to global deprotection under acidic conditions, aeruginosin 748A (**3**), which lacks the SO_3 group, was obtained in 70% yield.

Following the reaction sequence developed for the synthesis of aeruginosin 828A, we were also able to synthesize the analogues aeruginoside 126A (**2**) and aeruginosin 794A (**4**). Coupling of the Leu/Pla dipeptide **14** with L-Choi/Xyl **16** gave tripeptide **20** in moderate yield. Ester hydrolysis of **20** and further coupling with the Adc side chain **8** gave tetrapeptide **21**, which served as an intermediate for the synthesis of aeruginoside 126A (**2**) and synthetic analogue **4** in good yields. Comparison of the NMR spectra of synthetic and previously isolated aeruginoside 126A (**2**) showed slight differences in the chemical shifts. These differences might result either from different amounts of water in the NMR samples or from a residual counterion as a result of the HPLC purification. Similar observations have already been reported by our group for different natural products.[26] However, definitive proof that the synthetic and isolated samples are identical can only be obtained by recording NMR spectra of equimolar mixtures of the two samples or by HPLC co-injection. Furthermore, the absolute configuration of the xylose moiety had not been assigned during the isolation work, which, however, should result in larger differences in the NMR spectra in the case of isomeric xyloses.

It is interesting to note that the late-stage intermediates containing the Cleu side chain, including **1** and **3**, appeared as

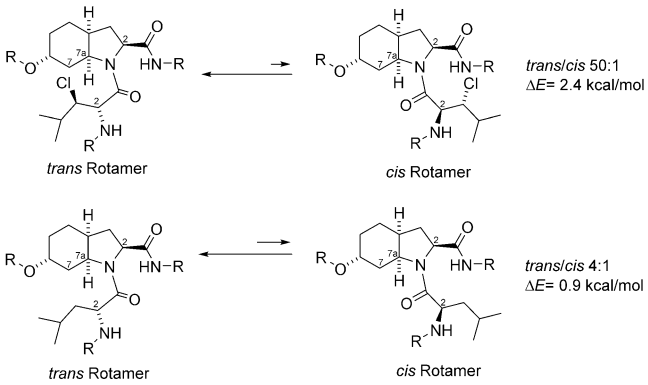


Figure 2. The amide bond rotamers of the L-Choi–Cleu and L-Choi–Leu units are in equilibrium.

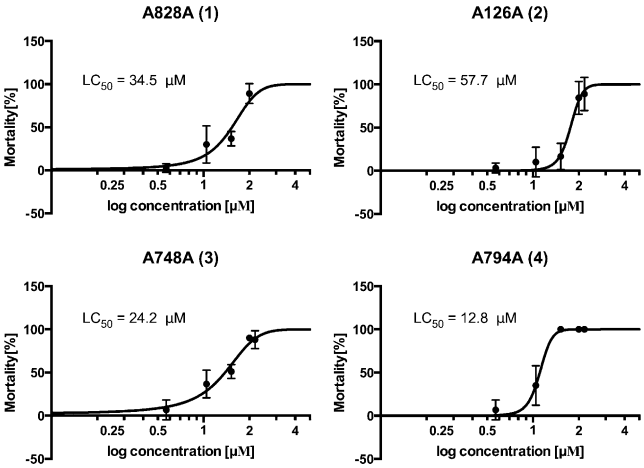


Figure 3. To study the acute toxicity of compounds **1**–**4** towards *T. platyurus*, the mortalities were determined as a function of the concentration of these compounds.

a single rotamer, or as a large excess of one rotamer (> 50:1), in the ^{1}H NMR spectra (Figure 2). Analysis of the ROESY spectra of **1** and **3** indicates that the *trans* rotamer dominates, as nuclear Overhauser effects (NOEs) were observed between Cleu H2 and Choi (H7, H7′, and H7a). However, the intermediates devoid of the chloride substituent, including **2** and **4**, appeared as rotameric mixtures (ca. 4:1) around the L-Choi–Leu peptide bond. The presence of two rotamers for **2** had already been reported by Dittmann and co-workers[6] and was additionally confirmed by the exchange cross-peaks between the methyl groups of both rotameric leucine units in the ROESY spectra of **2** and **4**. The *trans* rotamers of **2** and **4** showed similar NOEs as described before, whereas the *cis* rotamers gave NOEs between the Leu H2 and L-Choi H2 hydrogen atoms. The halogen substituent therefore appears to have a critical effect on the conformation of the different aeruginosins by restricting the rotation around the L-Choi–Leu amide bond; a related phenomenon has already been observed by Hanessian and co-workers for unnatural aeruginosin hybrids. Furthermore, these authors suggested that such conformational effects increase protease binding.[27]

With the four derivatives in hand, we were interested in evaluating the effect of the sulfate and chloride groups on bioactivity. The environmental toxicity was studied in standard assays with the sensitive freshwater crustacean *Thamnocephalus platyurus*. For this assay, six concentrations ranging from 0.41 μM to 100 μM for **1** and 1.2 μM to 150 μM for **2**, **3**, and **4** were tested in an acute toxicity assay (24 h). For every concentration, three to four experiments with 10 to 16 animals per experiment were conducted, and the mortality after 24 h was determined by visual inspection of the animals (Figure 3). Blom et al. had reported an LC_{50} value of 22.4 μM against *T. platyurus* for **1**,[4] which is only slightly higher than the toxicity of the known biotoxin microcystin.[28] Our assay with synthetic aeruginosin 828A showed a comparable toxicity of 34.5 μM. Analogue **3**, which lacks the sulfate group, showed a toxicity of 24.2 μM, which is in the same range as that of **1**. Interestingly, the dechloro derivative **4** showed an increased and potent toxicity of 12.8 μM. As demonstrated above by NMR spectroscopy, the chlorine substituent has a strong impact on the conformation of the aeruginosins. It has also been reported that the chlorine substituent in the leucine moiety is important for the inhibition of enzymes such as thrombin.[13b,27] For toxicity, however, this "chlorine effect" appears to be detrimental, which is likely due to the restricted rotation around the peptide bond between the L-Choi and the leucine residues, which could result in an entropic penalty. Most interestingly, derivative **2**, which contains neither the sulfate group nor a chlorine substituent, showed a significantly lower toxicity of 57.7 μM. Overall, the bioassays support the hypotheses that 1) the introduction of either chloride or sulfate groups leads to increased toxicity, and 2) the chloride group in combination with the sulfate moiety leads to an attenuation of toxicity. Both hypotheses are supported by ecological observations: Chlorosulfopeptides such as **1** are produced in microcystin-deficient, but still toxic cyanobacteria,[4] which supports the hypothesis that these chlorosulfopeptides restore the toxic phenotype. In contrast, the much less toxic congener **2**, which lacks both groups, is found in a microcystin-producing strain.[7] Therefore, a cyanobacterium can restore its toxicity by the introduction of one sulfate group upon loss of the gene for microcystin production (switching from **2** to **4**).

In conclusion, the first total synthesis of aeruginosin 828A (longest linear sequence: 18 steps, overall yield: 13 % from known building block **9**) has been reported. With the developed synthetic route, aeruginoside 126A and the synthetic analogues aeruginosin 794A and aeruginosin 748A were readily synthesized. Key features of the synthesis include the α-xylosylation of the L-Choi core unit **9** and the peptide coupling of amine **16** with Cleu moiety **12**. The synthesized compounds were tested with regard to their toxicity against *T. platyurus*, demonstrating that the sulfate group has a critical effect on toxicity. This finding supports the hypothesis that chlorosulfopeptides are important for restoring the toxic phenotype in strains that are incapable of microcystin production. This work therefore supports the conclusion that the toxicity of cyanobacteria should not be assessed in terms of microcystin production alone.

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