Toxicity of the Aeruginosin Chlorosulfopeptides Investigated 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 in crustacean toxicity the well-known toxin microcystin LR, has been synthesized for the first time. In addition, three congeners with different permutations of the chloride and sulfate groups have been prepared, thereby allowing for 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, while NMR spectroscopic evidence suggests that the chloride substituent modulates the conformation, which influences protease inhibitory activity.*

Harmful algal blooms are increasingly prevalent in many freshwater and marine ecosystems and their massive occurrence poses a severe threat to drinking water supply, fisheries and recreational areas alike.[1] Research on the most prevalent cyanobacterial toxin microcystin provided important insight into toxicity and molecular mechanisms,[2] which led the world health organization to set threshold values considered safe for drinking water.[2] Over the last years, many additional cyanobacterial strains lacking microcystin production have been discovered, which resulted in the characterization of new compounds considered to be toxic to aquatic organisms.[3] However, their mechanism of action and a molecular understanding 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] While chlorosulfolipids such as danicalipin have been intensively studied by chemical synthesis with regard to their structural requirements for toxicity,[5] research on chlorosulfopeptides has so far only investigated 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 via chemical synthesis followed by *in vivo* toxicity studies. In particular, we were interested to (1) synthesize and characterize peptides with all permutations of sulfate and chloride groups present (Figure 1), and (2) evaluate the role of these functional units on
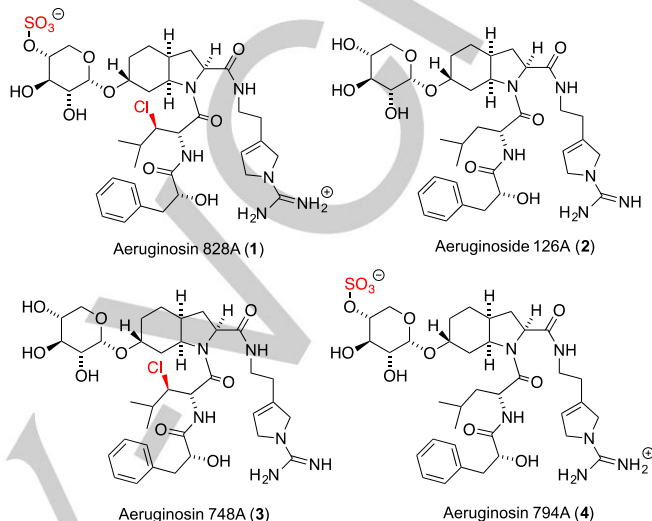

Figure 1. Aeruginosin 828A (**1**), aeruginoside 126A (**2**) and synthetic analogues aeruginosin 748 (**3**) and 794 (**4**).
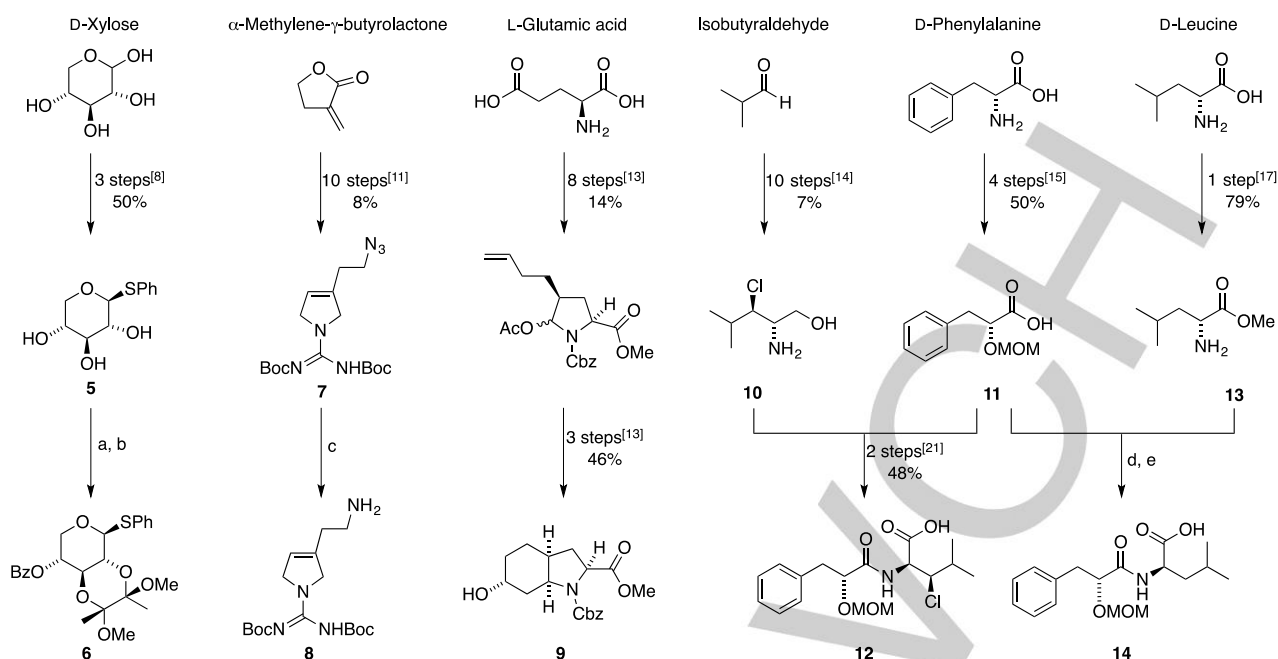
toxicity. In this communication, we report the successful synthesis of four natural products and putative congeners that allow for toxicity studies in the crustacean *Thamnocephalus platyurus*.

As the first two targets for the synthesis, we chose the naturally occurring chlorosulfopeptide aeruginosin 828A (**1**) bearing a sulfated xylose residue and a chloroleucine unit, and aeruginoside 126A (**2**),[6] which is devoid of sulfate and Cl groups. Interestingly, chlorosulfopeptide **1** was isolated from a toxic *Planktothrix* strain lacking microcystin production, whereas peptide **2** originated from a *Planktothrix* strain capable to produce microcystins.[7] These observations lend support to the hypothesis that chlorosulfopeptide **1** is restoring the toxic phenotype of the cyanobacterium, whereas compound **2** would be less toxic. Additional experimental support corroborating this hypothesis was provided by Blom and co-workers, who reported potent toxicity of **1**.[4] Surprisingly, toxicity of peptide **2** was not reported, which provided an additional stimulus for the work reported herein. To further evaluate the hypothesis, and to study the role of structure for toxicity, we chose aeruginosins 748A (**3**) and 794A (**4**) as targets for synthetic analogues, as **3** is containing the chloroleucine but has no sulfate group and **4**, which features the sulfate group but is lacking the Cl atom.

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Scheme 1. Synthesis of the different building blocks. Reagents and conditions: a) benzoyl chloride, Me$_2$SnCl$_2$, DIPEA, THF/H$_2$O (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$_2$Cl$_2$, 25 °C, 81%; e) LiOH, THF/H$_2$O (5:3), 25 °C, 98%. DIPEA = *N,N*-diisopropylethylamine; THF = tetrahydrofuran; CSA = camphorsulfonic acid; PyBOP = (benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate.

We first started with the preparation of the required building blocks for the various chlorosulfopeptides (Scheme 1). The O-4-position of the sulfate group on the xylosyl moiety required orthogonal protection with respect to positions O-3 and O-2, which led us to use a base labile group at O-4 and an acid labile group at O-2 and O-3 position, respectively. The synthesis of the xylose moiety was achieved starting from the known xyloside **5** which was obtained over three synthetic steps from commercially available D-xylose.[8] Regioselective protection of the OH-group at O-4 position with a benzoyl (Bz) protecting group was achieved with 81% yield using benzoyl chloride and Me$_2$SnCl$_2$ as catalyst.[9] The 2-OH and 3-OH groups were protected with the butane diacetal (BDA) group using 2,3-butanedione, trimethyl orthoformate and camphorsulfonic acid.[10] While initial attempts of the BDA protection always led to a mixture of isomers, optimization of the reaction conditions led to the formation of the Xyl moiety **6** as a single diastereoisomer.

The 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. While Hanessian and co-workers chose a Staudinger reduction for the conversion of the azide to the amine, we opted for a catalytic hydrogenation reduction using Lindlar's catalyst.[12] This procedure led to an increased yield of 96% with no side product formation and remarkably leaving the dihydropyrrole untouched.

The L-choi subunit was synthesized following the aza-Prins route developed in 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** could be synthesized over ten steps and an overall yield of 7% from isobutyraldehyde according to a protocol which was used in the total synthesis of chlorodysinosin A.[15] Further coupling of chloroleucine derivative **10** with the MOM-protected phenyllactic derivative **11**[16] followed by the oxidation of the alcohol to the acid gave the Cleu/Pla building block **12**. For the deschloro derivatives of aeruginosin 828A a building block without the chlorine atom was synthesized via coupling of phenyllactic

derivative **11** with leucine methylester **13**.[17] Cleavage of the methyl ester yielded the final intermediate **14** in an excellent yield.

With all subunits in hand, we proceeded with the assembly of the different building blocks (Scheme 2). Our strategy comprised first the challenging α-xylosylation of the L-choi core unit **9**. Starting from building block **6** several donors containing a sulfate group as a protected trichloroethyl ester at O-4 position were prepared.[18] However, all attempts to use these donors containing the protected sulfate group for the glycosylation were not successful, as the electron withdrawing capability of the sulfate group led to a disarming of the different donors,[19] leading to either little conversion or favoring of the β anomer. We therefore decided to introduce the sulfate group at a later stage of the synthesis and focused on donors with the benzoyl protection group at O-4 and different leaving groups at the C-1 position instead. Out of the ones surveyed, xyloside **6** with thiophenol as leaving group, in combination with NIS as activator and AgOTf as promoter, showed most promising selectivity and reactivity.[20] After optimization, a α/β ratio of 5:3 and a good yield of 81% was achieved for the xylosylation. As a next step, removal of the Cbz-group was met with only little or no conversion in the catalytic hydrogenation reaction (Pd/C, Pd(OH)$_2$, and H$_2$)[11] Pleasingly, **15** was treated with PdCl$_2$ and Et$_3$N dissolved in triethylsilane according a protocol of Birkofer and co-workers,[21] which led to 88% yield for the desired free amine **16**.
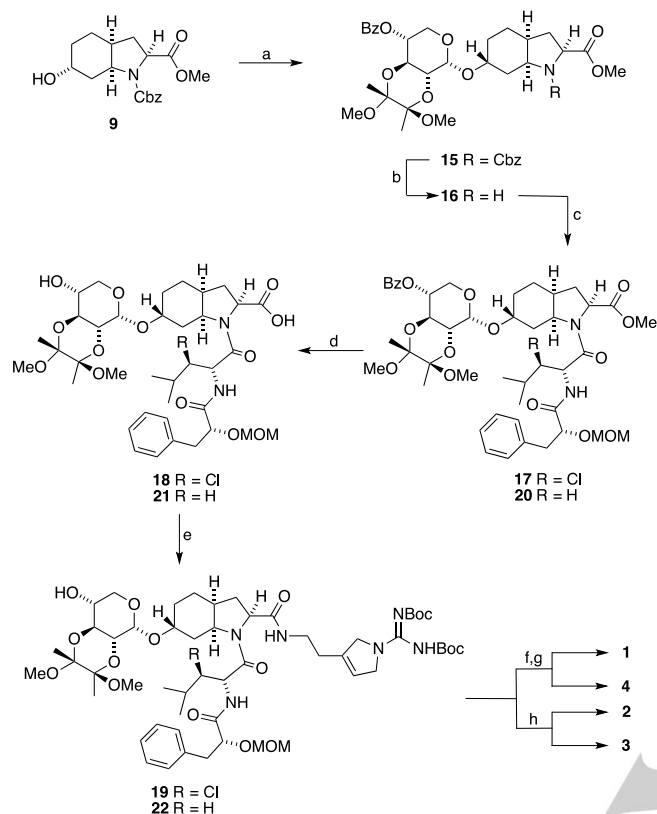
Scheme 2. Synthesis of aeruginosin 828A (**1**), aeruginoside 126A (**2**) and derivatives **3** and **4**. Reagents and conditions: a) **6**, NIS, AgOTf, Et$_2$O, 25 °C, 50%; b) PdCl$_2$, Et$_3$SiH, Et$_3$N, 25 °C, 88%; c) **12** or **14**, DMTMM, NMM, CH$_2$Cl$_2$, 0 °C to 25 °C, (**17**, 87% from **16**, **20**, 58% from **16**); d) 0.1 N LiOH, THF/H$_2$O (5:3), 25 °C, (**18**, 77% from **17**, **21**, 95% from **20**); e) **8**, PyBOP, 2,6-lutidine, CH$_2$Cl$_2$, 25 °C, (**19**, 60% from **18**, **22**, 64% from **21**); f) SO$_3$•pyridine, pyridine, 50 °C; g) CH$_2$Cl$_2$/TFA (10:1), 25 °C, (**1**, 72% from **19**, **4**, 75% from **22**); h) CH$_2$Cl$_2$/TFA (10:1), 25 °C, (**3**, 70% from **19**, **2**, 73% from **22**). NIS = *N*-iodosuccinimide; DMTMM = 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride; THF = tetrahydrofuran; PyBOP = (benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate; NMM = 4-methylmorpholine; TFA = trifluoroacetic acid.

The next step consisted in the coupling of amine **16** with the Cleu side chain **12**. Initial attempts with DEBPT as coupling reagent and 2,6-lutidine as base led to the elimination of HCl.[22] Similar behavior or racemization was already reported in the syntheses of other aeruginosins,[15],[23] as the secondary amine of the L-choi displays restricted reactivity, leading to 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, which is readily deprotonated leading to the elimination of HCl.[24] To overcome the problem of the elimination, first different bases like NMM, DIPEA or NaHCO$_3$ in combination with DEBPT as coupling reagent were tested, however without success. Also adjusting the temperature had no critical influence on the outcome of the reaction. We therefore started screening different coupling reagents (e.g. PyBOP, PyBrOP, HATU), identifying 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. Saponification of the methyl ester **17** was achieved in a 0.1 N aqueous LiOH solution to give acid **18**. The progress of the reaction was monitored by UPLC, 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 coupling reagent and 2,6-lutidine as base with a moderate yield of 60% to give tetrapeptide **19**. Sulfonation of the xylose moiety at O-4 position was performed using an excess of SO$_3$-pyridinium complex in pyridine. The global deprotection of the BOC, MOM and BDA groups was carried out in a 10% TFA/CH$_2$Cl$_2$ solution to obtain aeruginosin 828A (**1**) in 72% yield over two steps. Branching off intermediate **19**, the analog lacking the SO$_3$ group could be readily prepared by global acidic deprotection to give aeruginosin 748A (**3**) in 70% yield. Following the reaction sequence developed for the synthesis of aeruginosin 828A, we were also able to synthesize analogs aeruginoside 126A (**2**) and aeruginosin 794A (**4**). Coupling the Leu/Pla dipeptide **14** with L-Choi/Xyl **16** gave tripeptide **20** in a moderate yield. Saponification of **20** and further coupling with the Adc side chain **8** gave tetrapeptide **21**, from which aeruginoside 126A (**2**) and synthetic analog **4** could be synthesized in good yields. Comparison of the NMR spectra of the synthetic and the reported isolated aeruginoside 126A (**2**) showed slight differences in the chemical shifts. The distinction between the spectra might be derived from the water content in the NMR sample or caused by a residual counter ion from the HPLC purification. Similar behavior was already observed in our group.[26] However, definitive confirmation could only be performed by either mixing equimolar amounts of synthetic and isolated samples during the NMR experiments or by HPLC co-injection of both samples. Furthermore, the absolute configuration of the xylose moiety was not 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 a single or as a high excess of one rotamer (>50:1) in the ^{1}H-NMR spectrum. Analysis of the ROESY spectrum of **1** and **3** indicate the presence of *trans* rotamer, due to observed NOEs between Cleu H-2 and choi H-7, H-7' and H-7a, respectively. However, the intermediates devoid of the Cl group, including **2** and **4**, appeared as a rotameric mixture of around 4:1 of the L-choi-Leu peptide bond. The presence of two rotamers in **2** was already reported in the work of Dittmann and co-workers[6] and could be additionally confirmed for **2** and **4** by ROESY *exchange* crosspeaks between the methyl groups of both rotameric leucine units. The *trans* rotamer for **2** and **4** showed similar NOEs as described before, whereas the *cis* rotamer showed a NOE between Leu H-2 and L-choi H-2. The halogen atom therefore
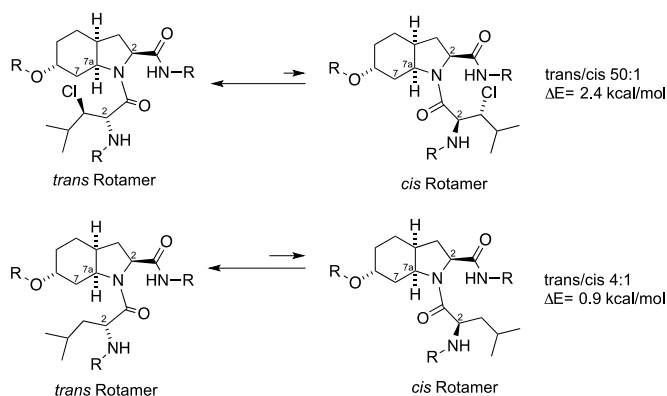

Figure 2. Amide isomer equilibrium of the L-choi-Cleu and the L-choi-Leu peptide bond, respectively.

appears to have a critical effect on the conformation of the different aeruginosins by restricting the torsion angle of the L-choi-Leu amide bond, a related phenomenon was observed earlier by Hanessian and co-workers for unnatural aeruginosin

hybrids. In addition, it has been then suggested that such conformational effects increase protease binding.[27]

Having the four derivatives prepared by chemical synthesis, we were interested to evaluate the role of the sulfate and chloride groups on bioactivity. 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 and 1.2 µM to 150 µM, respectively, were tested in an acute toxicity assay (24 h). For every concentration, 3-4 repetitions with 10-16 animals were used, and the mortality determined after 24 h by visual inspection of the animals (Figure 3).
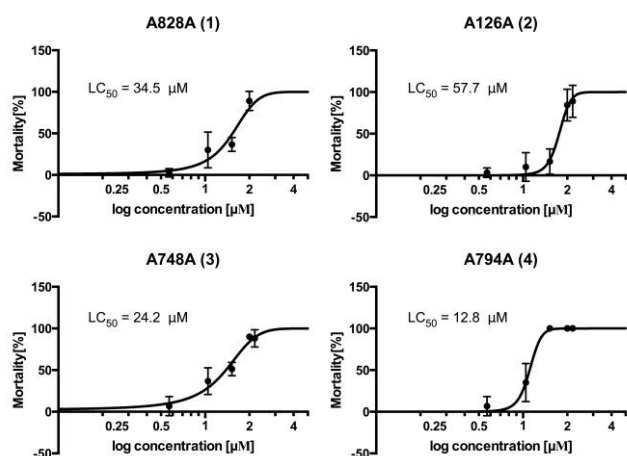

Figure 3. Mortality concentrations curves of A828A (**1**), A126A (**2**), A748A (**3**) and A794A (**4**). Acute toxicity was determined for *T. platyurus*.

Blom *et al.* reported a LC$_{50}$ value of 22.4 µM against *T. platyurus* for **1,** which is only slightly higher than the toxicity found for the known biotoxin microcystin.[28] Our assay with the synthetic aeruginosin 828A showed a comparable toxicity of 34.5 µM. Analogue **3**, which is lacking of the sulfate group, showed a toxicity of 24.2 µM, which is in the same range as **1**. Interestingly, the deschloro derivative **4** showed an increased and potent toxicity of 12.8 µM. As demonstrated above by NMR, the Cl atom has a strong impact on the conformation of the aeruginosins. It was also reported that the chlorine atom in the leucine moiety is important for the inhibition of enzymes such as thrombin.[13b],[27] For toxicity, however, this 'chlorine effect' is detrimental, likely due to the less restricted peptide bond between the L-choi and the leucine residue, which could result in an entropic penalty. Most interestingly, derivative **2**, without the sulfate group and the chlorine, showed a pronounced lower toxicity of 57.7 µM. Overall, the bioassays support the hypotheses that (1) the introduction of either Cl or sulfate groups leads to increased toxicity, and (2) the Cl group in combination with the sulfate moiety leads to an attenuation of toxicity. Both hypotheses can be supported by ecological observations: Chlorosulfopeptides such as **1** are produced in microcystin-deficient, yet still toxic cyanobacteria,[4] which supports the hypothesis that these chlorosulfopeptides restore the toxic phenotype. In contrast, the much less toxic congener **2** lacking both groups is found in a microcystin-producing strain.[7] Therefore, a cyanobacterium, upon loss of a gene for microcystin production, can restore toxicity by introduction of one sulfate group (switch 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**) is reported. With the developed synthetic route, aeruginoside 126A and the synthetic analogues aeruginosin 795A and aeruginosin 748A could be readily synthesized. Key features of the synthesis include the α-xylosylation of the L-choi core unit **9** and peptide coupling of amine **16** with the Cleu moiety **12**. The synthesized compounds were tested on their toxicity against *T. platyurus,* demonstrating that the sulfate group has a critical effect on the toxicity. This supports the hypothesis that chlorosulfopeptides are important for restoring the toxic phenotype in strains lacking microcystin production. This work therefore supports the conclusion that assessment of toxicity based on microcystin production alone should be revised.

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COMMUNICATION

Toxic brew: Four congeners of chlorosulfopeptides relevant to harmful algal blooms have been prepared by chemical synthesis, and the role of chloride and sulfate groups has been established by toxicity assays.
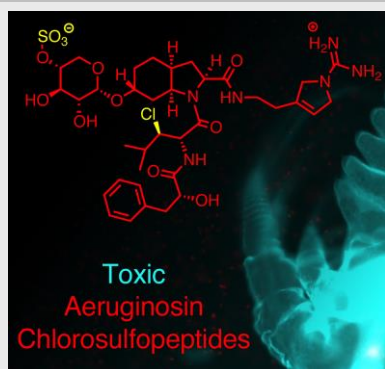
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