Cyclopeptides natural products as Herbicides and Inhibitors of Cyanobacteria. Synthesis of Versicotides E and F

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Abstract: The first total synthesis of cyclopeptides versicotide E and F, natural products produced by marine fungus *Aspergillus versicolor LZD-14-1*, was achieved in good yield by solid phase peptide synthesis (SPPS) of their linear precursors and solution phase cyclization. All the versicotides A-F were evaluated as herbicides and inhibitors of cyanobacterial growth. Versicotides A, B, D, E and F showed a significant inhibition of Rye grass seed's radicle growth at a concentration of 67µg/mL. Versicotides A, B and D also inhibited seed germination and leaf development. On the other hand, Versicotides D and F caused a relevant reduction on *Microcystis aeruginosa* population when cultures on exponential growth were incubated with 40 µg/mL solutions of these compounds. Evaluation of the concentration of microcystins after these treatments showed that versicotide D inhibited the production of microcystins in a comparable extent to the positive control, colistine. These results indicate versicotides, with versicotides D and F as top hits, could be considered as lead candidates in the development of bioherbicides able to mitigate the environmental impact that the evolution of agriculture has had on water quality.

Introduction

A large number of herbicides are currently available to prevent the growth of weeds in a variety of crops.[1] Unfortunately, the intensive use of synthetic herbicides in the last 50 years has led to environmental and ecological impact.[2] Moreover, the use of fertilizers has contributed to produce eutrophication with important impact in the aquatic environment. These agricultural practices, several hydrological alterations and the global warming promote cyanobacteria blooms, which have increased globally in intensity, frequency and duration during recent years.[3] Cyanobacteria produce cyanotoxins, such as microcystins, that can cause a range of adverse effects to the liver, digestive and nervous system to animals and humans, and in some cases even mortality. [4], [5], [6] In addition, Cyanobacteria produce taste and odorous compounds, which impair recreational lakes and the use of reservoirs for drinking water,[7] therefore, having a relevant social and economic impact.

Cyanobacterial blooms are currently a great threat to some of the world's largest and most important water bodies, such as the Baltic Sea in Europe, Río de la Plata in South America, Lake Victoria in Africa, Lake Erie in USA, Lake Taihu in China, Lake Biwa in Japan, and many other ecologically and economically important lakes, rivers and estuaries.[8] In Uruguay, cyanobacteria were detected in many agro-industrial regions as Uruguay River and Río Negro River, in artificial dams and in many south beaches.[9],[10] In particular, during 2019 summer, one of the most important blooms in the Uruguayan coast comprised 500 km of the Rio de la Plata and reached the Atlantic Ocean. [11]

As the effectiveness of the commercially available herbicides is decreasing,[12] the development of new ones is of great importance. In this context, products originated from living organisms and natural metabolites, have emerged as an alternative for sustainable agriculture.[13] Thus, microorganisms, plant extracts and metabolites would be used as bioherbicides to control the growth of weeds. Some of the advantages of these practices are the reduction of classic herbicides, minimizing their environmental impact.

A significant number of natural products derived from amino acids, among them, cyclopeptides, have been described with herbicidal, fungicidal or insecticidal activity.[14] For example, Tentoxin (**1**), Figure 1, a cyclotetrapeptide isolated from *fungus Alternaria alternata*, produces chlorosis on a variety of soybean and corn weeds. [15], [16] Furthermore, it was reported that the presence of anabaenopeptins, [17] (Figure 1, **2**), cyclic peptides derived from cyanobacterial blooms, produces lysis of *Microcystis aeruginosa* cells.[18] Hence, it was proposed that anabaenopeptins could possibly control cyanobacterial population.

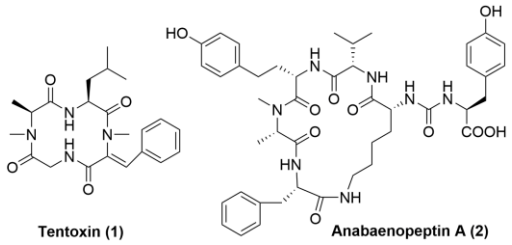


Figure 1. Natural products with phytotoxic or cyanobactericidal activity

Natural products from marine environments have been attracting special interest for their structural diversity.[19] Particularly, marine fungi produce unique secondary metabolites by gene modifications. Among natural products, cyclopeptides are promising candidates in the search for bioactive compounds,[20]

and it was described that the presence of N-methyl groups on them remarkably improves their cell permeability. [21]

Versicotides are cyclopeptides natural products produced by marine fungi. [22], [23] Versicotides E (**3**) and F (**4**), Figure 2, were isolated from *Aspergillus versicolor* LZD-14-1, along with versicotide D (**5**) and the previously known versicotides A (**6**) and B (**7**). The presence of two rigid β-amino acids, anthranilic (Anth) units, one proline (Pro) and one N-methyl- alanine (NMe-Ala) in **3** and **4** add further constraints to these cyclopeptides. On the other hand, versicotide D (**5**) is a cyclotetrapeptide containing two N-methyl amino acids and hydrophobic and aromatic amino acids, reminiscent to the structure of Tentoxin (**1**).

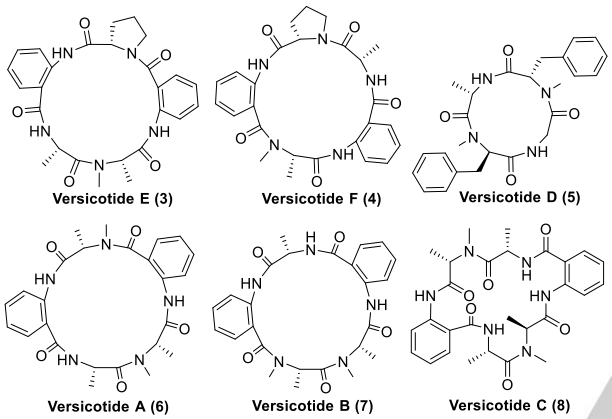


Figure 2. Versicotides A-F

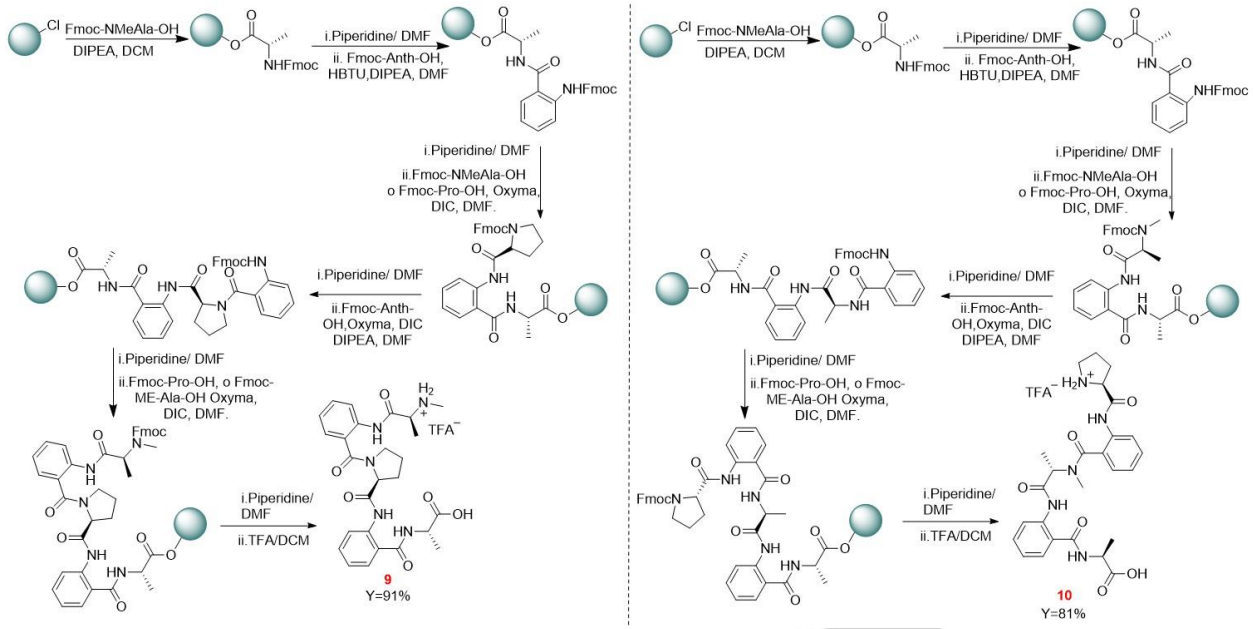
Previous exploration of the potential biological activities of versicotides proved versicotides D-F prevent foam cells formation in RAW264.7 cells by the reduction of lipid accumulation through the regulation of cholesterol efflux and influx.[22] Furthermore, we have reported micromolar activity of versicotide D (**5**) against *P. falciparum* and low cytotoxicity on HEPG2 cells.[24],[25] Meanwhile versicotides A and B showed no cytotoxicity against murine leukemia P388, human hepatoma cell line BEL-7402 and human leukemia cell HL-60; versicotides A–C showed no activity against cancer cell lines A-549, HeLa and SMMC-7721. The synthesis of those natural products containing Anth, represents an interesting challenge due to the low nucleophilia of Anth's aromatic amine. Furthermore, recently our group explored the synthesis of versicotides A-C, and many attempts to the preparation of the linear precursors were hampered by rearrangements in which Anth can be involved. Finally, we synthesized and reported the preparation and biological evaluation of versicotide A-D. [24], [25] Based on anthranilic acid derivatives that have been investigated as insecticides,[26] fungicide,[27] and herbicide,[28] in this work, we decided to prepare versicotide E (**3**) and F (**4**) and explore the bioactivity of the entire family of versicotides and their open precursors, as bioherbicides and inhibitors of cyanobacterial growth. In addition, to assess the possible cell breakage and toxin release, the concentration of microcystins in the culture medium is determined.

Results and Discussion

Synthesis

Following the procedures previously established by us for the synthesis of versicotides A-C and D, the linear precursors of versicotides E and F were synthesized on solid phase employing 2-chlorotrytil chloride resin (2-CTC), and Fmoc strategy (**Scheme 1**). The linear sequence of each one was chosen taking into account the low nucleophilicity of Anth's amine. Based on this, Anth amine was not chosen as the N-terminal in order to maximize the expected outcome of the cyclization reaction. The linear sequence chosen for the precursors of versicotides E and F were NHMeAla-Anth-Pro-Anth-Ala-OH (**9**) and Pro-Anth-NMeAla-Anth-Ala-OH (**10**), respectively.

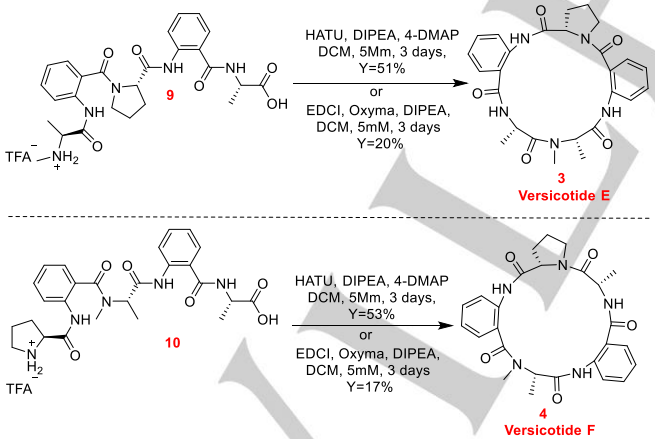
We noticed the preparation NHMeAla-Anth-Pro-Anth-Ala-OH (**9**) and Pro-Anth-NMeAla-Anth-Ala-OH (**10**) sequences would be challenging for Solid Phase Peptide Synthesis (SPPS) due to the probable high steric hindrance related to the couplings to aromatic amines and secondary amines. Our first efforts to obtain these peptides employing HBTU for coupling to



Scheme 1. Solid phase synthesis of linear precursor of Versicotide E (**9**) and F (**10**)

primary amines, HATU for secondary amines and a combination of DIC and Oxyma for couplings involving the aromatic amine of Anth, led to several incomplete couplings. Therefore, to avoid possible incomplete couplings due to steric hindrance, DIC+oxyma were also used for couplings to a secondary amine (Pro or NMeAla).

For both peptides **9** and **10**, the coupling of the last amino acid required recoupling, even when DIC+oxyma was used as coupling reagents. However, the linear precursors of versicotide E and F were obtained in good yield and purity (91 and 81% yield, respectively).



Scheme 2. Macrolactamization of 9 and 10

The cyclization reactions were performed in solution phase, Scheme 2, under high dilution conditions, for two days. Both HATU and the combination of Oxima+EDCI were tested as coupling regents, with the former giving higher yields.

^{1}H-NMR of **3** and **4,** and LC-MS data, match those from the naturally occurring compounds, versicotide E and F respectively. **3** shows many conformers in DMSO; the same behaviour was observed at the ^{1}H-NMR spectra of the natural product Versicotide E.[29] **4** shows a single conformer at the ^{1}H-NMR in $CDCl_3$, while multiple conformers can be observed at the ^{1}H-NMR in DMSO-*d6*. The chemical shifts of the main conformer match those reported for versicotide F.

Phytotoxicity evaluation

Versicotides E (**3**) and F (**4**), along with the previously reported versicotides A-D and their linear precursors were evaluated for their influence on germination, leaf development and radicle length growth of Rye grass (*Lolium multiflorum*) seeds. A primary screening for possible hits of phytotoxic activity was carried out at a concentration of 67μg/mL. Serial experiments were conducted using the agar germination methodology,[30] where the tested compounds and the respective controls were placed in glass Petri dishes (6 cm diameter), in 3 replicates per treatment. Ten Rye grass seeds were previously sterilized by immersing them in 70% alcohol for 10 seconds and distributed in the Petri dish on the agar in such a way as to ensure that they remained submerged in the solution, and were placed to germinate in a growth chamber (20°C, day/night temperature). DMSO was used as negative control and the herbicide *S*-metolachlor (2.1 μg/mL) as positive control.

The variables germinated plants and plants with developed leaves of the total number of plants placed to germinate were analyzed by fitting a generalized linear model, given that they had a binomial distribution. Glinmix procedure of the SAS statistical package was used. The results are showed as percentage of inhibition.

Inhibition of seed germination. The cyclopeptides versicotide C (**8**), E (**3**) and F (**4**) showed no significant impact on seed germination and leaf development when compared to the DMSO treatment. On the contrary, versicotides A (**6**), B (**7**) and D (**5**) significantly inhibited seed germination, showing a percentage of inhibition above 65% (Figure 3a). The statistical analysis of the different treatments showed a p-value =<0.0001. This indicates a significant difference between the treatments with versicotides

A (**6**), B (**7**) and D (**5**), and the rest of the compounds, which did not exceed a 25% inhibition rate. Meanwhile, no significant differences (P≤0.05) were found between treatments with these three compounds. However, at the percentage level it is possible to visualize that versicotide B (**7**) was the product with the highest inhibition percentage level.

Leaf development. The compounds versicotide A (**6**), B (**7**), F (**4**), and E (**3**) showed a significantly greater inhibitory effect on leaf development in comparison with the rest of the compounds (p-value =<0.0001) (Figure 3b). Versicotide C (**8**) showed the lowest inhibition percentage (9%), and versicotide D (**5**) exhibited an intermediate behavior between all treatments (56% inhibition).

These results show that, although versicotides E and F allowed the seeds to germinate (visible radicle), these seeds were not able to continue with their correct development.

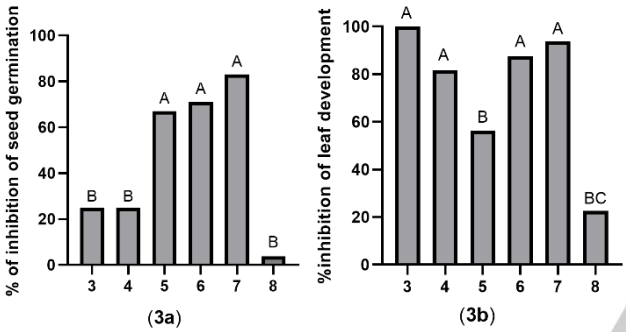


Figure 3. Left (**a**) Percentage of inhibition of seed germination in relation to the DMSO treatment (set as 0% inhibition). Right (**b**) Percentage of inhibition of leaf development in relation to the DMSO treatment (set as 0% inhibition). Compounds are identified at the "X" axis by their corresponding number. Different letters (A or B) over the bars indicate significant differences

Radicle growth inhibition. Figure 4 shows radicle growth inhibition, calculated as the percentage between the treatment with each compound and radicle length growth for the negative control (DMSO). The effect of the treatments on the variable root length was studied by carrying out comparisons of the means using a Tukey test (p-value < 0.05) in INFOSTAT. As it can be depicted, versicotides A (**6**), B (**7**), D (**5**), E (**3**) and F (**4**) showed a relevant inhibitory effect. Meanwhile, Versicotide C showed an inferior activity (49% of inhibition).

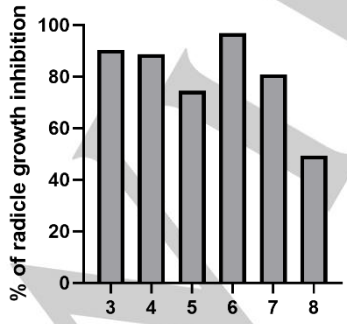


Figure 4. Percentage of radicle growth inhibition for each compound. Root length of the negative control (DMSO) is considered 0% inhibition.

Taking into account the results obtained for the three evaluated variables, all compounds but not Versicotide C showed phytotoxic effect on Rye grass seeds at the concentration studied. Versicotides A (**6**) and B (**7**) had a significant effect on all three variables, while versicotides E (**3**) and F (**4**) did not inhibited germination but affected the further development of the seeds. On the other hand, versicotide D (**5**) showed a relevant effect on seed germination and further radicle growth, but had lower effect on leaf development.

Evaluation of cyanobacteria inhibition

In a primary screening *Microcystis aeruginosa* strain, not axenic and previously isolated from Río de la Plata, Uruguay, was used. The strain was grown in BG11 medium at a temperature of 25ºC and light intensity of 60 µmol photon $m^{-2}s^{-1}$ with 16:8 light: dark photoperiod. A volume of the original culture grown in exponential phase was diluted in BG11 medium to achieve an initial chlorophyll *a* of 65 µg L^{-1}. The diluted culture was divided in aliquots of 25 mL then placed in sterile Greiner culture flasks of 50 mL with filter to allow gas exchange. Each treatment was run in duplicates during 10 days. In vivo chlorophyll *a* was evaluated fluorometrically at initial and final time (PhycoLA, BBE, Moldaenke, Germany). Colistine (30 µg/mL) and DMSO were used as positive and negative control, respectively.

As it is showed in Figure 5, incubation with versicotides F (**4**) and D (**5**) at 40 µg/mL concentration, showed a very significant decrease on cyanobacteria population, while versicotide A (**6**) was inactive and versicotides B (**7**), C (**8**) and E (**3**) induced some extent of decrease of chlorophyll *a*.

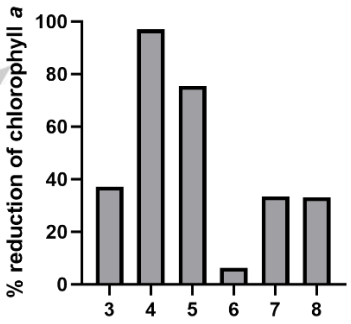


Figure 5. Percentage inhibition of the cyanobacterium *Microcystis aeruginosa* growth. The negative control (DMSO) is considered 0% inhibition.

Based on these results versicotides E (**3**) and F (**4**) were selected to determine their GI_{50} (growth inhibition 50) value. A diluted *Microcystis* culture (65 µg chlorophyll *a* L^{-1}) was incubated with different concentrations of **3** and **4** in the range from 5.5 to 80 µg/mL. Figure 6 and **7** show the concentration-response curves generated on GraphPad software using a 4 parameter Hill's equation for **3** and **4**, respectively. GI_{50} was calculated as 29.3 µg/mL for **3**. In the case of **4** GI_{50} is estimated < 5.5 µg/mL.

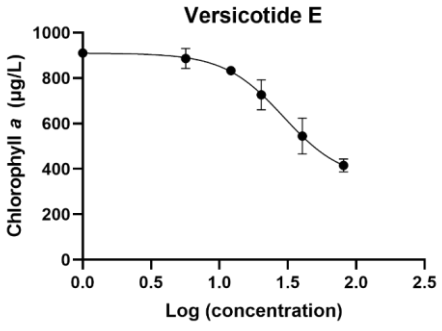


Figure 6. Concentration response curve for Versicotide E (**3**). Hill's equation parameters: Top= 910, Bottom= 350,4, IC50=29,33, Hills slope -2.000.

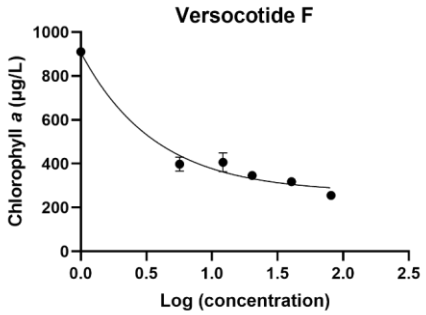


Figure 7. Concentration response curve for Versicotide F (**4**)

Determination of microcystins

Since the compounds could promote cell lysis of the cyanobacteria and release of toxins, we also determined the concentration of microcystins in the culture medium. Samples of the *Microcystis* cultures incubated with the synthesized verticotides dissolved in DMSO, as well as positive (colistine) and negative controls (DMSO) were freezed and thawed three times to lyse the cells and release the intracelular toxins. After filtration (0.22 µm) samples were analyzed by ELISA, using a llama nanobody (clon A2),[31] and calibration curves were prepared with MC-LR (Abraxis) in the concentration range: 0.2 – 2.5 µg/L. The percentage of absorbance of each standard with respect to the absorbance of the Zero (%A/Ao) was plotted versus the MC-LR concentration and log-linear fitted by GraphPad prism 7 software.

Versicotides A (**6**), B (**7**), C (**8**) and F (**4**) induced mild effects in the concentration of microcystins (% of inhibition in the range 13-27%), and Verticotide E (**3**) had practically no effect (5.5% decrease). The most relevant decrease in the concentration of microcystins was shown by Versicotide D (**5**). Moreover, its inhibitory effect at 40 µg/mL (82.8%) was very close to that of colistine at 30 µg/mL (86%).

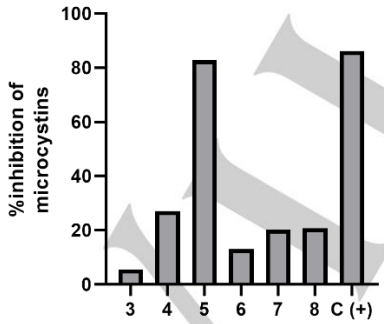


Figure 8. Percentage inhibition of microcystins from *Microcystis aeruginosa* after incubation with Versicotides A-F. The positive control (C(+)) is the treatment with colistine.

Conclusion

Natural products versicotides E (**3**) and F (**4**) were successfully synthesized by SPPS of their linear precursor and solution-phase macrolactamization. After evaluating the phytotoxicity and ability to inhibit cyanobacterial growth of versicotides A-F family, all of them, except versicotide C, showed activity at one or more of the evaluated features. In particular, versicotides D and F have stand out as promising hits to develop novel herbicides able to mitigate cyanobacterial blooms. In the case of verisicotide F, it does not prevent the germination of Rye grass seeds but hampers it further development, and has shown a strong inhibitory effect on cyanobacterial cultures. However, it did not show an important decrease on the concentration of microcystins. On the other hand, versicotide D combined the ability to prevent seeds to germinate and development, with the capacity to diminish the cyanobacterial population of a culture in exponential growth with a substantial depletion of the concentration of microcystins in the media.

Experimental Section

Solid Phase Peptide Synthesis of linear precursors (9) and (10). I) Resin loading: 500 mg of 2-Chlorotrityl chloride resin (2-CTC) resin were added to a syringe peptide synthesis vessel. The resin was swelled in CH2Cl2 (3 x 5 min). A solution of first protected amino acid Fmoc-AA-OH (1 eq. for 0.8 mmol/g loading) and DIPEA (3 eq.) in CH_2Cl_2 was added and the resin was shaken 10 minutes. Then, an extra 7.0 eq. of DIPEA were added and shaking was continued for 50 min. MeOH (0.8 mL/g of resin) was added to the previous mixture in order to cap unreacted functional groups on the resin, and shaken for 10 min. After filtering, the resin was washed with CH_2Cl_2 (x3), MeOH (x3), CH_2Cl_2 (x3), DMF (x3).

II) Removal of NHFmoc group: The resin was washed with DMF (x3) and Fmoc protecting group was removed by treating the resin with piperidine-DMF solution (1:4) for 1, 5 and 5 minutes successively. In exceptional cases deprotection step was accomplish by a single treatment with piperidine-DMF solution for 5 minutes, in order to prevent side reactions.

III) Coupling of subsequent N-Fmoc protected amino acids to primary or secondary amines: After removal of NHFmoc protecting group as previously described, the resin was washed with DMF (x3), CH_2Cl_2 (x3) and DMF (x3). A solution of Fmoc-AA-OH (3 eq.) and DIPEA (6 eq.) in DMF was added to the resin, followed by a solution of HBTU, for coupling to primary amines, or HATU (2.9 eq.) in DMF, in case of coupling to an N methylated amino acid. The mixture was stirred for 60 min. After the coupling was completed, the resin was washed with DMF (×3) and CH_2Cl_2 (x3). Deprotection and coupling cycles were repeated with the appropriate amino acids to provide the desired compound. Completion of the coupling was monitored by colorimetric assays; Kaiser test in case of primary amines and Chloranil test for secondary amines. Coupling procedure was repeated in case of positive results.

IV) Coupling of subsequent N-Fmoc protected amino acids to Anthranilic acid: After removal of NHFmoc protecting group as previously described, the resin was washed with DMF (x3), CH_2Cl_2 (x3) and DMF (x3). A solution of Fmoc-AA-OH (5 Eq.), Oxyma Pure (5 Eq.), and DIC (5 Eq.) was added to the vessel. The mixture was stirred for 60 min. Then, the resin was washed with DMF (×3) and CH_2Cl_2 (x3).

V) Cleavage: The peptide was cleaved from the resin by treatment with 1% TFA in CH2Cl2 for 2-3 minutes at room temperature followed by filtration and collection of the filtrate in MeOH. The treatment was repeated three times and then the resin washed with CH2Cl2 (x5) and MeOH (x3). Solvents were removed in vacuo to obtain the crude peptide. LC-MS was used to identify the desired product.

Cyclo-[NMeAla-Anth-Pro-Anth-Ala] (3, Versicotide E): The trifluoroacetate salt of NHMeAla-Anth-Pro-Anth-Ala-OH (1) was obtained following the general SPPS procedure. Global yield was 91% (191 mg, 0.31 mmol) as a white solid. ESI-MS m/z calc. for $C_{26}H_{32}N_5O_6$ ([M+H]+) 510.23, found 510.35. Macrocyclization reaction was performed starting from the trifluoroacetate salt of linear peptide NHMeAla-Anth-Pro-Anth-Ala-OH (200 mg, 0.33 mmol), in diluted conditions (5 mM, 3 day) using HATU (190 mg, 0.5 mmol), DIPEA (170 uL, 0.99 mmol.), 4-DMAP

(catalytic) in dried CH_2Cl_2 at room temperature during 1-3 days. The reaction mixture was washed with HCl 5% and then with saturated aqueous $NaHCO_3$, dried over $MgSO_4$, filtered and concentrated in vacuo. Further purification by flash chromatography using $CHCl_3$/MeOH 5% as mobile phase, rendered the desired macrocycle as a white solid in 51 % yield (81 mg, 0.17 mmol). **Rf**= 0.35 in $CHCl_3$: MeOH (10:0.5). **^{1}H-NMR** (400 MHz, DMSO-*d*^{6}) δ 1.32 – 1.40 (m, 6H), 1.78 – 1.88 (m, 2H), 1.92 – 2.03 (m, 2H), 2.97 (s, 3H), 3.60 – 3.76 (m, 1H), 4.75 – 4.84 (m, 1H), 4.99 – 5.09 (m, 1H), 5.12 (q, J = 6.8 Hz, 1H), 7.09 – 7.32 (m, 4 H), 7.36 – 7.43 (m, 2H), 7.45 – 7.54 (m, 2H), 7.59 (d, J = 7.4 Hz, 1H) 7.72 – 7.61 (m, 43H), 7.80 (s, 34H), 8.09 (d, J = 7.5 Hz, 1H), 8.47 (d, J = 8.4 Hz, 1H), 12.04 (s, 1H).**ESI-MS** *m/z* calc. for $C_{26}H_{30}N_5O_5$ ($[M+H]^+$) 492.2, found 492.4.

Cyclo-[Pro-Anth-NMeAla-Anth-Ala] (4, Versicotide F): The trifluoroacetate salt of **Pro-Anth-NMeAla-Anth-Ala-OH (2)** was obtained following the general SPPS procedure. Global yield was 81% (283 mg, 0.45 mmoles) as a yellow solid. **ESI-MS** *m/z* calc. for $C_{26}H_{32}N_5O_6$ ($[M+H]^+$) 510.2 found 510.2.

Macrocyclization reaction was performed starting from the trifluoroacetate salt of linear peptide NHMeAla-Anth-Pro-Anth-Ala-OH (100 mg, 0.20 mmol), in diluted conditions (5 mM, 3 day) using HATU (110 mg, 0.3 mmol), DIPEA (104 µL, 0.6 mmol.), 4-DMAP (catalytic) in dried CH_2Cl_2 at room temperature during 1-3 days. The reaction mixture was washed with HCl 5% and then with saturated aqueous $NaHCO_3$, dried over $MgSO_4$, filtered and concentrated in vacuo. Further purification by flash chromatography using $CHCl_3$/MeOH 5% as mobile phase, rendered the desired macrocycle as a white solid in 53 % yield (53 mg, 0.11 mmol). **Rf**= 0.35 in $CHCl_3$:MeOH (10:0.5). ^{1}H-RMN (400 MHz, DMSO-*d*^{6}), confórmero mayoritario δ 1.38 (d, J=7.1Hz, 3H), 1.58 (d, J=6.7 Hz, 3H), 1.75 – 1.90 (m, 1H), 1.90 – 2.00 (m, 1H), 2.15 – 2.23 (m, 1H), 2.24 – 2.39 (m, 1H), 2.75 (s, 3H), 3.41 – 3.53 (m, 1H), 3.80 – 3.87 (m, 1H), 4.12 – 4.20 (m, 1H), 4.55 (d, J=7.6 Hz, 1H), 4.90 (q, J=6.7 Hz, 1H), 7.12 – 7.23 (m, 2H), 7.39 –7.58 (m, 3H), 8.03 (dd, J=8.0, 1.2 Hz, 1H), 8.50 – 8.62 (m, 1H), 8.70 (d, J=8.3 Hz, 1H), 9.17 (d, J=4.8 Hz, 1H), 10.10 (s, 1H), 12.68 (s, 1H).**ESI-MS** *m/z* calc. for $C_{26}H_{30}N_5O_5$ ($[M+H]^+$) 492.2, found 492.4.

Phytotoxicity assays. Serial experiments were conducted using the Agar germination methodology, where the tested compounds and the respective controls were placed in glass Petri dishes (6 cm diameter), in 3 replicates per treatment. Ten Ryegrass seeds were germinated in a growth chamber (20°C, day/night temperature). The seeds were previously sterilized by immersing them in 70% alcohol for 10 seconds. When distributed in the Petri dish on the agar, the seeds were placed in such a way as to ensure that they remained submerged in the solution. Agar-water solution was prepared at 0.3%, 3 g of Agar was placed in 1 liter of deionized water and the solution was autoclaved at 100°C for 45 minutes. Once the agar medium had cooled to approximately 60°C, the solutions were prepared.The negative control - DMSO control, was prepared by adding 100 µl of DMSO per plate in ml15 of agar and then the seeds were distributed as mentioned above. A control without DMSO was also carried out to check that the product was not altering the correct development of the Ryegrass seeds. For this test, 15 ml of agar was placed in each Petri dish and, before it solidified, the seeds of the species evaluated were placed on top. The herbicide treatment, positive control - Control S- Metolachlor (960 g/l), was carried out for a conversion of 1/8 of the dose of 1 L/ha of commercial product. For this purpose, a stock solution of S-Metolachlor was prepared by placing 0.28 ml of the herbicide in a volumetric flask and topping up to 1000 ml. 25 ml of this stock solution was taken, placed in a volumetric flask and brought to 200 ml, thus generating the 1/8x solution of S-Metolachlor. A volume of 3 ml of 1/8x herbicide solution was mixed with 45 ml of the agar solution to bring ml16 into each Petri dish. This ensured that there was 1 ml of 1/8x S- Metolachlor solution per plate: 0.28 µl of herbicide solution. Seeds were arranged in the same way. Using the same method, the agar media corresponding to each plate (15 ml) were mixed with the cyclopeptides diluted in µl100 of DMSO. Germination, root length and leaf development were evaluated 12 days after preparation.

Cyanobacterial culture. *Microcystis aeruginosa* strain, not axenic and previously isolated from Río de la Plata, Uruguay, was grown in BG11 medium at a temperature of 25ºC and light intensity of 60 µmol photon $m^{-2}s^{-1}$ with 16:8 light:dark photoperiod. The strain was inspected periodically for bacterial contamination. A volume of the original culture grown in exponential phase was diluted in BG11 medium to achieve an initial chlorophyll *a* of 65 µg L^{-1}. The diluted culture was divided in aliquots of 25 mL then placed in sterile Greiner culture flasks of 50 mL with filter to allow gas exchange. Each treatment was run in duplicates during 10 days. Optical density was followed periodically and measured in a dark chamber with a LED light source and a 2π visible light sensor (LI-192 quantum sensor Li-Cor, USA). In vivo chlorophyll *a* was evaluated fluorometrically at initial and final time (PhycoLA, BBE, Moldaenke, Germany).

Analysis of Microcystins Samples of the Microcystis cultures incubated with the synthesized Verticotides dissolved in DMSO, as well as positive (colistine) and negative controls (DMSO) were freezed and thawed three times to lyse the cells and release the intracelular toxins. After filtration (0.22 µm) samples were analyzed by ELISA, using a llama nanobody (clon A2), as described in Pírez-Schirmer, et al., 2017. Briefly, samples and standards (150 µL) were previously mixed in a dilution plate with 34 µL of 1 M Tris buffer pH 7.5 containing 0.27 M NaCl; 0.27 M EDTA and 1% BSA. Then, 50 µL of each mixture were dispensed by triplicate in an ELISA plate previously coated with 100 µL/well of a 60 µg/L MC-LR-Bovin Seroalbumin conjugate solution (MC-LR-BSA) in saline phosphate buffer (PBS) and blocked with 0.5% gelatin in PBS. The samples were incubated with 50 µL of the nanobody solution (7 ng/mL). After incubation and washing steps, the bound nanobody was detected with a streptavidin-peroxidase conjugate (Pierce, 1/10.000) and a peroxidase enzyme substrate solution (H2O2 and 3,3',5,5'-Tetramethylbenzidine-TMB) in acetate buffer pH 5.5. After 15 min, the enzyme reaction was stopped by the addition of 50 µL of 2 N H_2SO_4, and the absorbance was read at 450 nm using a Fluostar Optima Reader (BMG, Ortenberg, GE). Calibration curves were prepared with MC-LR (Abraxis) in the concentration range: 0.2 – 2.5 µg/L. The percentage of absorbance of each standard with respect to the absorbance of the Zero (%A/Ao) was plotted versus the MC-LR concentration and log-linear fitted by GraphPad prism 7software. Quality controls included Certified Microcystin-LR Reference Standard (National Research Council of Canada) and blanks. To evaluate recovery samples were fortified with 1 µg/L MC-LR and analyzed, the results were in the range 88-130%.

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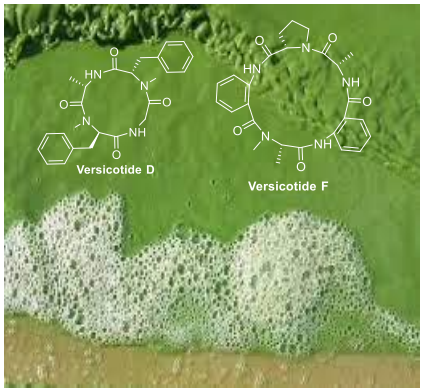

The first total syntheses of cyclopeptides versicotide E and F, containing anthranilic acid, are reported. Evaluation of the herbicide activity, cyanobacteria inhibition and microcystins concentration of all versicotides A-F demonstrated that several of them could be considered as lead candidates in the development of bioherbicides to mitigate the environmental impact that the evolution of agriculture has had on water quality.

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