

Application of chemical structure analysis methods to cyanobacterial toxins and synthesized biologically active compounds

theses of doctoral (PhD) dissertation

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1. INTRODUCTION

Identification of natural compounds with biological activity and determination of their concentrations as accurate as possible is one of the most important objective of environmental analysis. There are many ways to identify a chemical structures, NMR and mass spectrometry etc. can be used. One of the advantages of mass spectrometry among others is the very little amount of substance (pg/ml) needed for the analysis, therefore its application is more and more common in the analysis of toxic substances present in surface water. An excellent example for this type of application is the analysis of toxic water-blooms.

Water-bloom means the mass growth of planktonic organisms in surface waters, resulting in turbidity growth and intensive discolouring of the water surfaces. Water-blooms are called toxic when planktonic organisms release metabolites which show toxic effects in mouse test. Toxic water-blooms have been observed in many places of Earth. The phenomenon was rather rare before the 1950's, but recently it is typical for the eutrophic (nitrate and phosphate stressed) surface waters. Either prokaryotes or eukaryotes can cause toxic water-blooms. Cyanobacteria belong to the first group, their isolates can be either toxic (cyanotoxin) or non-toxic metabolites producing ones. The proliferation of toxic cyanobacteria is especially dangerous when it takes place in water bodies providing drinking water. Cyanotoxins cannot be removed usually by the common water purification methods. Moreover, the applied procedures could give rise to the lysis of cyanobacteria, thus increase the concentration of cyanotoxins in the drinking water. The cause of animal and human intoxications are usually the consuming of drinking water or intake of food containing the cyanobacteria with the toxic metabolites.

Toxic metabolites producing by cyanobacteria can be classified by their chemical structure and by action mechanism. By their chemical structure the following groups can be formed: (1) cyclic peptides (microcystins, nodularin), (2) alkaloids (anatoxin-a, anatoxin-a(s), aplysiatoxin, cylindrospermopsin, lyngbiatoxin) and (3) lipopolysaccharides. By their mechanism of action cyanobacterial toxins can be classified into four groups: (1) neurotoxins (anatoxin-a, anatoxin-a(s), saxitoxin, neosaxitoxin), (2) hepatotoxins (microcystins, nodularin), (3) cytotoxins (cylindrospermopsin) and (4) skin irritating and gastrointestinal toxins (lipopolysaccharides).

There are numerous methods for the detection of cyanotoxins: mouse test, enzyme activity tests, the *in vitro* plant test (BGST) and chemical methods. Among the chemical

methods the most widely used is the HPLC which can be combined with UV, diode array (PDA), MS and MS/MS detection. The most efficient method for detection is a HPLC system equipped with a mass spectrometer, not only because of its high sensitivity but the feature of providing information on chemical structure too. Capillary electrophoresis is one of the most efficient separation techniques. Thin-layer chromatography (TLC) can also be used to detect cyanotoxins.

Cylindrospermopsis raciborskii cyanobacterium was first isolated in 1912 in Java island and it was described as a typical tropical cyanobacterium. By now however the species have spread both in the tropical and the mediterranean area, and have been detected in European freshwaters as well. To compare cyanobacteria isolated in different areas *rpoC1*, STRR, *nifH*, *cpcBA*-IGS, 16S rRNS, ITS, M13, ERCl and IST1 sequences were used. The *rpoC1*, 16S rRNS and ITS sequences were found suitable to identify the species. In the studies of the isolates both straight and coiled filaments were observed. For the growth of *C. raciborskii* the optimal temperature range is 20-35 °C.

Besides cylindrospermopsin the organism can produce other cyanotoxins which are derivatives of cylindrospermopsin (7-epi-cylindrospermopsin, 7-deoxy-cylindrospermopsin) or neurotoxins (saxitoxin, gonyautoxin). The 7-epi-cylindrospermopsin is as toxic as the cylindrospermopsin, meanwhile the 7-deoxy derivative is not toxic. Numerous studies state, that the *C. raciborskii* organism can release not yet identified toxic metabolites, other than the above mentioned ones. According to our present knowledge, the cyanotoxin cylindrospermopsin is produced by the species *Cylindrospermopsis raciborskii*, *Aphanizomenon ovalisporum*, *Aphanizomenon flos-aquae*, *Raphidiopsis curvata*, *Lyngbya wollei* and *Umezakia natans*. For the synthesis of cylindrospermopsin supposedly the genes of the PKS (polyketide synthase) and the NRPS (nonribosomal peptide synthase) proteins are responsible.

Cylindrospermopsin (CYN) is a sulfur-containing uracil derivate containing a tricycloguanidine moiety with a molecular mass of 415 Da. Its structure was confirmed using NMR and MS techniques. For its detection mainly HPLC-PDA, HPLC-MS, HPLC-MS/MS and CE systems are used. In contrast to the neurotoxic, alkaloid-type cyanotoxins already mentioned, cylindrospermopsin, despite of its alkaloid character carries hepatotoxic properties. In mice test its LD₅₀ after 24 hours of exposition was 2.1 mg/kg, but after longer exposition times death was also observed, at lower dose. *In vitro* experiments proved that cylindrospermopsin inhibits the synthesis of glutathione and proteins. It is a non-competitive inhibitor of the uridine monophosphate synthase complex under *in vitro* circumstances.

Our laboratory proved by chemical approaches that *C. raciborskii* strain (BGSD 266), the Hungarian isolate of Lake Balaton does not produce CYN, the characteristic cyanotoxin of the species; however, the strain turned out to be toxic in plant and mouse tests.

The other cyanobacterial strain studied was *Microcystis aeruginosa*, which is the most common cyanobacterium in water-blooms. It is a unicellular organism that forms colonies containing thousands of cells. The outermost mucilage layer of its Gram-negative cell walls holds the cells in a gelatinous coating. Cyanotoxins produced by the organism are called microcystins which are cyclic heptapeptides with the common amino acid sequency D-Ala-X-D-MeAsp-Z-Adda-D-Glu-Mdha. Among the amino acids are both D and L ones, and in the X and Z positions are variable L-amino acids which provide to the cyanotoxin group a high degree of diversity.

Microcystins are chemically and thermally stable molecules, their degradation needs an appropriate enzyme system. The microcystins specifically inhibit the PP1A and PP2A-types protein phosphatase enzymes.

The only aim of *Microcystis* studies is the identification of cyanotoxins isolated from surface water badies of Kis-Balaton Reservoir and study other biologically active compounds, like the pterocarpan in the flavanoid group. Flavanoids belong to the group of pigment molecules giving the nature brilliancy. The attention was drawn to them by pioneering researches started in the 1890's. The collective name „flavanoid” is used regarding mainly the compounds having C₆-C₃-C₆ carbon skeleton (diphenylpropane skeleton), but homoisoflavanoids and rotenoids bearing C₁₅+C skeleton also belong to this group. Pterocarpan represent a valuable, pharmacologically active group of natural isoflavanoids. Many of their representatives through the considerable fungicide property can be included in the family of phytoalexins. The physical and chemical properties of pterocarpan have been studied in detail, but only a few data can be found in the literature on their behaviour in mass spectrometry. To perform such studies, the detailed analysis of their skeleton by mass spectrometry is essential; we aimed the examination of deuterated derivatives of pterocarpan.

2. AIMS

The aims of the study were:

1. To work out the culturing of *C. raciborskii* strain (BGSD 266), isolated from Lake Balaton in 1995, under laboratory circumstances and to optimize its mass production for the purpose of cyanotoxin purification.
2. To isolate the unknown cyanotoxin (plant growth inhibitor) produced by *C. raciborskii* (BGSD 266), a Hungarian isolate.
3. To identify the structure of the unknown cyanotoxin.
4. A preliminary study of the biological effects of the unknown cyanotoxin on mustard seedlings using polyacrylamide gelelectrophoresis.
5. To study the nutrient starvation (sulfur and phosphorous) of the strain.
6. To identify the structure of the cyanotoxins of *Microcystis aeruginosa* isolated from the water-bloom of Kis-Balaton Reservoir in 2001.
7. To synthesize the deuterio derivatives of the skeleton of the biologically active compounds pterocarpanes to study its behaviour in mass spectrometry.

3. MATERIALS AND METHODS

3.1. Isolation and culturing the cyanobacterial strains used in this work

In our study the strain *C. raciborskii*, isolated from Lake Balaton in 1995 and the strain *M. aeruginosa*, isolated from Kis-Balaton Reservoir in 2001 were studied. Those isolates were identified by colleagues of Department of Botany, University of Debrecen, using light microscope.

C. raciborskii was surface grown using Petri dishes containing dilute agar (0.1 %) and nutrient solution. Cultures were grown in shaken Erlenmeyer flasks (100 ml), air bubbled in 250 ml vessels and for mass production air bubbled in Erlenmeyer flasks (1 and 5 l). To maintain the strains, nitrate-free BG11 and Allen's medium were used.

3.2. Determination the growth of *C. raciborskii*

Growth was determined by measuring the A_{800} , chlorophyll-a content, protein content and dry mass of the culture grown in air bubbled vessels (250 ml). In paralell, the toxicity of the cultures was also determined.

3.3. Determination of toxicity using Blue-Green Sinapis Test, a plant test

It was previously demonstrated by our laboratory, that mustard seedlings are suitable for the detection of cyanotoxins; the method was named *Blue-Green Sinapis Test* (BGST). In the test, seeds of *Sinapis alba* L. are used. The length of the axial organs of the plant were used to describe the growth inhibitor property of the cyanotoxin or the crude cyanobacterial extracts.

3.4. Isolation of toxic metabolites

Cyanobacterial cells were lysed by freezing and thawing. The disrupted cells were extracted with 4-fold volume of 75 %(v/v) methanol overnight, the obtained suspension was centrifuged, then the supernatant evaporated. The toxic metabolites were purified using three columns, each contained different packing: a DEAE-52 Whatman ion exchanger, a Toyopearl HW-40 molecular sieve matrix and silicagel. After each step of chromatography the obtained fractions were examined by a UV-VIS spectrophotometer at 210 and 260 nm. In the last

separation step HPLC was used. The toxicity of the fractions were determined by mustard seedling test. The toxin content was tracked using thin-layer chromatography too. Based on BGST the most plant inhibitory compound was the subject of chemical structure analysis.

3.5. NMR analysis of the unknown compound

The ^1H , ^{13}C , HSQC (Heteronuclear Single Quantum Correlation), COSY (Correlation Spectroscopy), HMBC (Heteronuclear Multiple-Bond Correlation), NOESY (Nuclear Overhauser Effect Spectroscopy) and TOCSY (Total Correlation Spectroscopy) measurements were performed on a Bruker DRX-500 instrument operating at 500 MHz (^1H) and 125 MHz (^{13}C), using D_2O or $\text{d}_6\text{-DMSO}$ as solvents.

3.6. Mass spectrometric studies including the unknown compound

Mass spectrometric measurements, needed for the structure identification of the cyanotoxin, were performed in the Laboratory for Mass Spectrometry of the Chemical Research Center (Hungarian Academy of Sciences) and in the Laboratory for Mass Spectrometry of the Department of Applied Chemistry (University of Debrecen), using Waters Q-TOF Premier (ESI-Q-TOF) and Applied Biosystems API 2000 (ESI-Q₃) and Bruker Biflex MALDI-TOF instruments, respectively. Exact masses were determined using the ESI-Q-TOF instrument. Identification of the microcystins was carried out at the Department of Organic Chemistry, University of Debrecen, using FAB instrument.

3.7. IR analysis of the unknown compound

The IR spectrum was recorded at the Department of Organic Chemistry, University of Debrecen, on a Perkin Elmer 16 PC FT-IR instrument, using KBr pellet.

3.8. Examination of mustard seedlings treated with the cyanobacterial crude extract

An experimental system was set up as follows: the *C. raciborskii* cyanobacterium culture at stationary phase was centrifuged and the biomass was freeze-dried. Using the obtained dry substance a stock-suspension was prepared in water. Mustard seedlings were put on 1 % (m/v) agar which contained crude cyanobacterial extract in a given concentration. The

lengths and the wet mass of the plants at their 2nd, 3rd and 4th day were recorded, then they were grinded with quartz sand and plant extract was prepared from them. The effects of the crude cyanobacterial extract on seedlings were studied using denaturing (SDS) and native (ssDNase and protease) gelelectrophoresis techniques.

3.9. Examination of mustard seedlings treated with the pure cyanotoxin

Over the treatment of seedlings with the purified cyanotoxin we studied the toxic effect as a function of concentration. Based on preliminary experiments, concentrations 200, 400, 600, 800 and 1600 µg/ml were chosen. The length and wet mass data of the samples taken at day 3 (counted from the planting) were recorded and plant extract were prepared. The biological effects of the pure cyanotoxin on seedlings were studied using SDS, ssDNase and protease gelelectrophoresis.

3.10. Studies of the growth, cyanotoxin production and protein pattern of the sulfur- or phosphorous-starved *C. raciborskii*

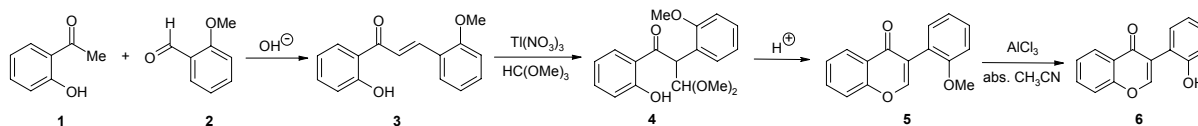
The *C. raciborskii* culture grown in nitrate-free BG11 medium was inoculated into sulfur- or phosphorous-free medium. The first contained equivalent amount of KCl instead of K₂HPO₄, the latter contained MgCl₂ instead of MgSO₄ and FeCl₃ instead of Fe₂(SO₄)₃. In the sulfur-free solution of micro-elements, Zn(NO₃)₂ and CuCl₂ substituted ZnSO₄ and CuSO₄, respectively. For the analysis of growth samples were taken from the cultures and chlorophyll-a content, protein content, dry mass content, A₈₀₀ and toxicity were measured. The alteration of protein patterns of the cells were studied by SDS gelelectrophoresis.

3.11. Isolation of cyanotoxins from *M. aeruginosa* water-blooms (Kis-Balaton Reservoir)

The collected biomass was lysed, extracted with methanol and purified on DEAE-cellulose column. The fractions were analysed by spectrophotometry and their toxicity examined using BGST. Toxic fractions were combined and further purified using a column packed with Toyopearl (HW-40) molecular sieves. At last, an HPLC was applied. The identification of the purified toxins were carried out by means of their mass spectra.

3.12. Synthesis of pterocarpan and its deuterated derivatives

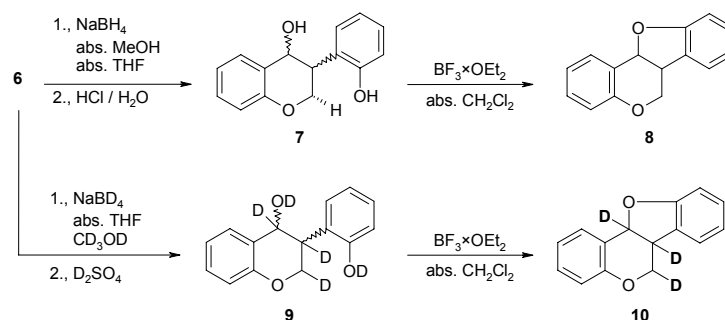
The synthesis of the pterocarpan was accomplished via a 6-step procedure starting from the readily available 2-hydroxyacetophenone (**1**) and salicylaldehyde methyl ether (**2**). First, the basic condensation of the starting materials gave rise to 2'-hydroxychalcone **3** whose oxidative rearrangement by $\text{Ti}(\text{NO}_3)_3$ in the presence of trimethyl orthoformate gave rise to β -ketoaldehyde dimethyl acetal **4**. From **4** 2'-methoxyisoflavanone **5** was prepared by acidic treatment. 2'-hydroxyisoflavone **6** was obtained by removal of the methyl protecting group with AlCl_3 in dry acetonitrile (Scheme 1).



Scheme 1

Reduction of **6** using NaBH_4 gave the *cis-trans* mixture of alcohol **7**. Ring closure reaction of **7** with $\text{BF}_3 \cdot \text{Et}_2\text{O}$ in dry dichloromethane gave the desired pterocarpan **8** (Scheme 2).

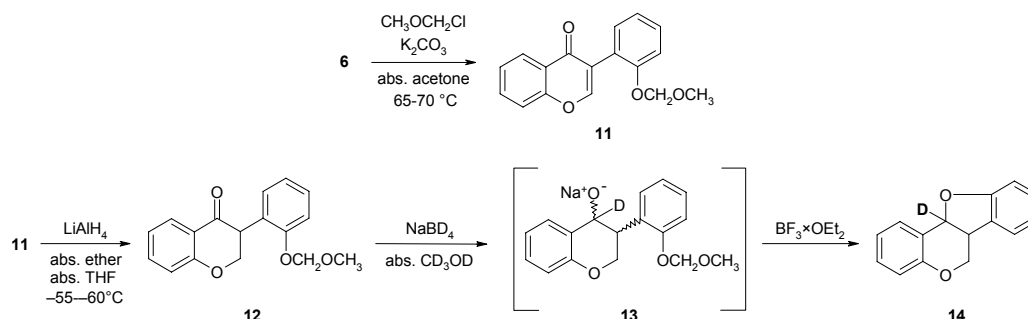
Synthesis of 6,6a,11a-tetradeutero-pterocarpan **10** was carried out using isoflavone **6**. Performing the reduction with NaBD_4 in the mixture of dry THF and CD_3OD , *cis-trans* mixture of tetradeutero-isoflavan-4-ol **9** formed which reacted with $\text{BF}_3 \cdot \text{Et}_2\text{O}$ to give the desired tetradeutero-pterocarpan **10** (Scheme 2).



Scheme 2

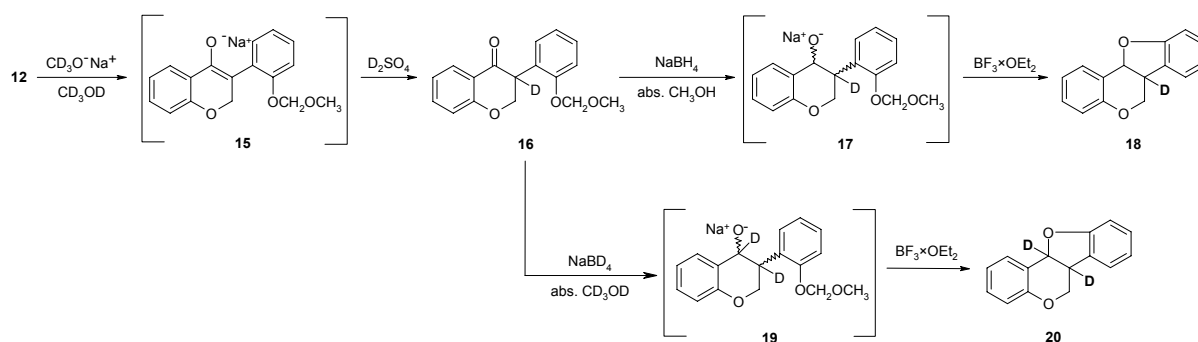
2'-hydroxyisoflavone **6** was chosen as the starting compound to synthesize the partially deuterated pterocarpan. Methoxymethyl group was introduced for the temporary protection of the hydroxyl group. The protected isoflavone **11** was reduced to isoflavanone **12**

with LiAlH_4 in the mixture of dry ether and dry THF, at low temperature. **12** then was reacted with NaBD_4 in CD_3OD , the reaction gave rise to *cis-trans*-4-deutero-alcoholate **13** which was treated with $\text{BF}_3 \cdot \text{Et}_2\text{O}$ to prepare 11a-deutero-pterocarpan **14** (Scheme 3).



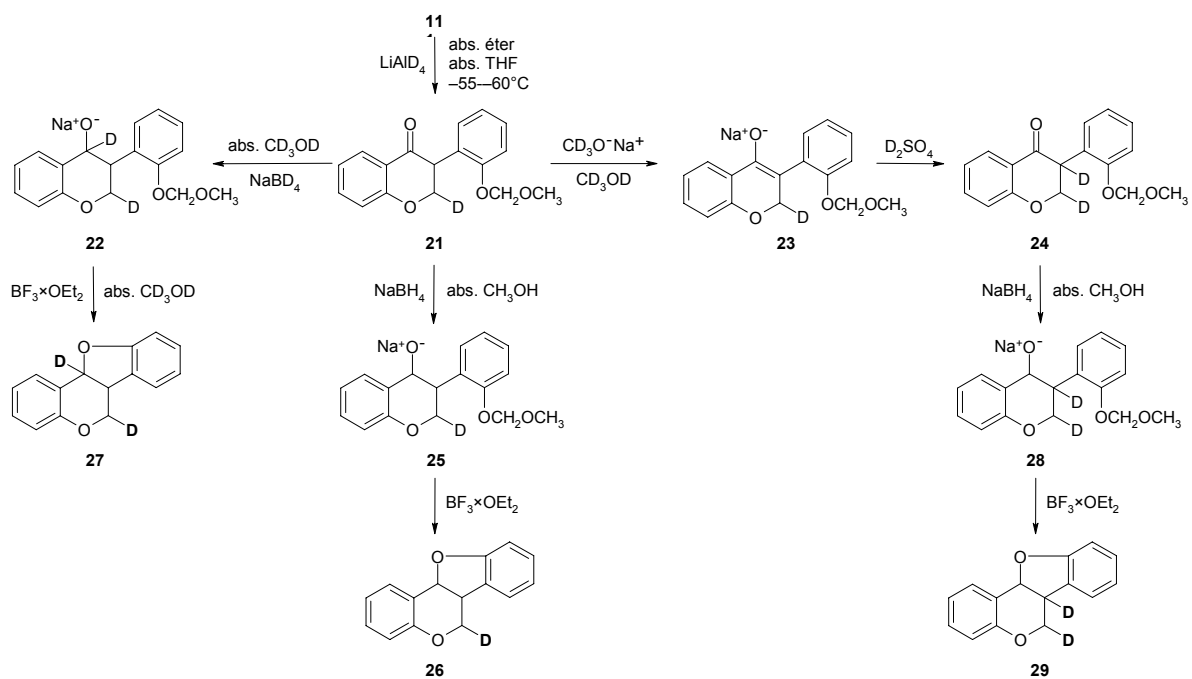
Scheme 3

6a-Deutero-pterocarpan **18** was synthesized from 2'-methoxymethyl-isoflavanone **12**. The H-3 hydrogen of **12** was changed to deuterium via the enol form **15** using NaOCD_3 , then D_2SO_4 . **16** obtained this way reduced and cyclised to 6a-deutero-pterocarpan **18** by the usual methods. **16** was used to prepare the 6a,11a-dideutero derivative **20**. Reduction of **16** with NaBD_4 in CD_3OD then cyclisation with $\text{BF}_3 \cdot \text{Et}_2\text{O}$ gave the desired 6a,11a-dideutero-pterocarpan **20** (Scheme 4).



Scheme 4

6-deutero-pterocarpan **25**, 6,11a-dideutero-pterocarpan **26** and 6,6a-dideutero-pterocarpan **28** was obtained from **11**. The chromone ring of **11** was reduced with LiAlD_4 , then the dihydro-benzo[b]furane moiety was built by methods already stated. The summary of these reactions are outlined in Scheme 5.



Scheme 5

The structures were supported by their NMR and MS spectra.

4. RESULTS AND DISCUSSION

4.1. The growth of the cyanobacterium *Cylindrospermopsis raciborskii* under laboratory circumstances

The growth of the organism was followed using a medium containing combined nitrogen (Allen+NaNO₃) and under nitrogen fixation conditions (BG11 without NaNO₃). Based on the data of A₈₀₀, protein and chlorophyll-a content, it can be stated that the lag phase lasts 3-4 days and after 2 weeks the culture turns into the stationary phase. Preliminary experiments proved that, in the case of inoculation less than 0.2 A₈₀₀ unit, the culture contains so less cells that self-shadowing does not take place. Thus, because of the high amount of light, the growth progress starts very slowly, if it starts at all. The nitrate-free medium, because it allows the growing of nitrogen fixing organisms only, is ideal for culturing and obtaining inoculum. For obtaining biomass, the combined nitrogen containing Allen medium proved to be suitable, because it supports a faster growth of the cyanobacterial cells and what is more, it results in higher mass.

4.2. Isolation of the plant inhibitory compound from *Cylindrospermopsis raciborskii*

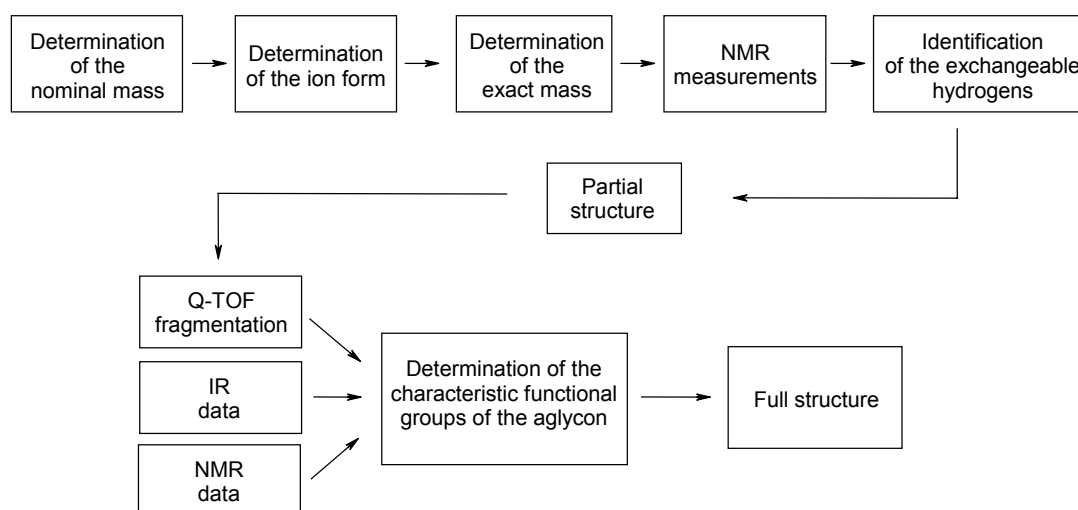
Our first goal was to isolate toxic, plant growth inhibitory compound(s), and chromatographic techniques were used for this purpose. We started from 40 grammes of biomass which was prepared as written in the chapter „Materials and methods”. The fraction containing the toxic substance(s) was applied to a DEAE anion-exchange column. Eluted fractions having plant growth inhibitory effect were purified on columns of Toyopearl molecular sieves. The fractions, which were judged to be toxic, were subjected to semipreparative HPLC. A more efficient purification of the crude cyanobacterial extracts was achieved by introducing a Silica gel 60 column. Using silica gel, the less polar impurities and pigments eluted first, the cyanotoxin component was eluted later in time, separatingly from the majority of the impurities. This technique allowed us to eliminate the ion-exchange step (DEAE column). In case of smaller quantities of substance this method proved to be so efficient, that toxic fractions from the silica gel column were directly suitable for HPLC. In case of higher quantities, the purification step with the Toyopearl column could not be

avoided. The whole purification process was tracked by thin-layer chromatography. As well, fractions were analysed for plant growth inhibition by mustard seedling test (BGST).

The fractions containing the most plant inhibitory compound was chosen as a subject of chemical structure analysis.

4.3. Chemical structure analysis of the purified unknown cyanobacterial metabolite of *Cylindrospermopsis raciborskii*

Methods below were used for the identification of the chemical structure of the purified toxic compound: NMR, IR, UV spectroscopy, mass spectrometry and molecule modelling. Interpreting the results parallelly, we found the structure via the steps outlined in Scheme 6.



Scheme 6

4.3.1. Determination of the nominal molecular mass

The purified toxic substance was subjected to mass spectrometric analysis using an ESI-Q₃ instrument. The mass spectrum showed one peak at 436 Da. To determine the type of the adduct, lithium, sodium and potassium compounds were added to a small amount of sample. This change resulted in two extra peaks in the spectrum: one at 420 Da (M+Li⁺) and an other at 452 Da (M+K⁺). This method supported that the peak at 436 Da was the sodium-adduct (M+Na⁺) and the unknown toxic compound has a molecular mass of 413 Da.

4.3.2. Determination of the exact molecular mass

For exact measurements of the molecular mass an ESI-Q-TOF instrument was used which operated in positive mode. The measurement was calibrated.

Exact mass of both the sodium- and the proton-adduct was independently determined. The results are summarized in Table 1.

Table 1.

	Measured mass (Da)	Formula	Calculated mass (Da)
Molecule + H ⁺	414,1617	C ₁₆ H ₂₄ O ₈ N ₅	414,1625
Molecule + Na ⁺	436,1448	C ₁₆ H ₂₃ O ₈ N ₅ Na	436,1444

Thus, formula of the isolated toxic metabolite is **C₁₆H₂₃O₈N₅**.

4.3.3. NMR methods for structure analysis of the unknown cyanobacterial compound

In parallel with the mass spectrometric studies, NMR measurements were also performed. These two techniques provided the basics of the structure investigation. The coupling network was mapped using two-dimensional COSY, TOCSY (ⁿJ_{HH}, n=2-5), HSQC (ⁿJ_{CH}, n=1) and HMBC (ⁿJ_{CH}, n=2-5) techniques. Theoretically, information provided by these methods could be enough for determining the constitution of the unknown molecule. However, as turned out with the progression of the work, because of the relatively small number of hydrogen atoms of the molecule, 2D NOESY measurements were necessary to determine protons which are close to each other in space (0.16-0.5 nm).

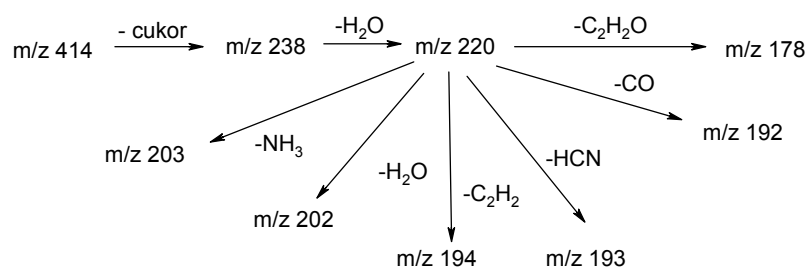
NMR spectra were recorded in both D₂O and d₆-DMSO, because the signs of exchangeable protons (–NH–, –NH₂, –OH) appear only in the latter solvent; in D₂O these protons are changed to deuterium.

By NMR data the following can be stated: the molecule contains 15 non-exchangeable protons, and 10 of the carbon atoms are primary or tertiary, 6 of them are secondary or quaternary. The HSQC spectra indicate that 11 carbon atoms carry hydrogen. Interpreting the ¹H-NMR couplings, the HMBC, TOCSY and NOESY spectra it can be stated that the isolated plant inhibitor contains a 3-*O*-methyl galactose moiety. To this galactose part, through its glycosidic hydroxyl group, an isopropyl group is linked which provide connection to the yet

unknown rest of the molecule. To clear up what this unknown part may be, data of hydrogen-deuterium exchange, high resolution MS/MS fragmentation, IR spectrum and NMR spectra were used. Thus, we found that the aglycon contains five carbon atoms; four of them do not carry hydrogen, but to one of them a hydrogen is connected which gave a singlet sign, therefore it is connected to either a heteroatom or a carbon with no hydrogen. There are five nitrogen atoms and three exchangeable protons in the aglycon which is connected back to the galactose ring via one of its hydroxyl group. Calculating the total number of double bonds and rings in the molecule we got the value of 6.

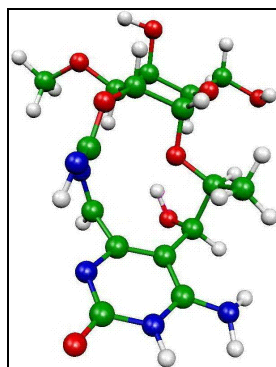
4.3.4. Mass spectrometric data of the isolated compound

By the high resolution mass spectra it can be stated, that during fragmentation the protonated molecule-ion loses its sugar moiety as a neutral molecule (m/z 238). The so formed protonated aglycon gives the ion with m/z 220 by losing one molecule of water. In the MS/MS spectrum of this ion (m/z 220) the following fragments were detected (Scheme 7):



Scheme 7

By MS/MS and IR data we found, that the aglycon part of the molecule contains hydroxyl and carbonyl groups, the latter in the form of amide. Thus, the structure in Scheme 8 could be suggested.



Scheme 8

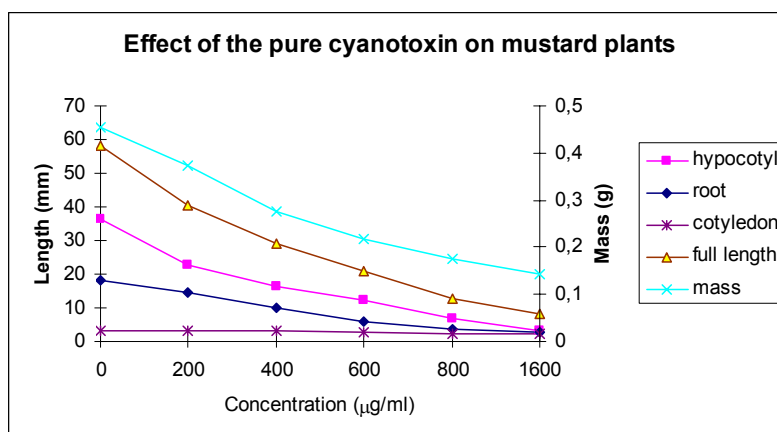
4.4. Analysis of the biological effects of the isolated cyanobacterial metabolite

4.4.1. Analysis of mustard seedlings exposed to the crude extract of *C. raciborskii*

Mustard seedling were used to study the biological effects of toxic cyanobacterial metabolites. Before each experiment with the purified cyanotoxin, preliminary experiments had been made with the crude extract of *C. raciborskii*. As we worked under axenic circumstances, the cause of growth inhibition was exclusively the crude extract or the pure toxin in the agar. We used etiolated plants, since rapid lengthening of the hypocotyl in dark causes the growth inhibition become more visible and, at the same time, allows a more accurate determination of the IC_{50} value. The pregerminated seeds were put on agar containing the crude extract in different concentrations, and samples were taken after 2, 3 and 4 days. The lengths and dry mass were evaluated to determine the plant growth inhibition. The crude extract inhibits the growth of roots and hypocotyls of the mustard plants. Besides that, no morphological changes of the plants were experienced. The IC_{50} values were between 800 and 1600 $\mu\text{g/ml}$ for the crude extract. The degree of toxicity manifested better in the length data than in the mass data. Results obtained after 3 day proved to be the most informative, thus it was expedient to use 3-day old samples for the studies with the newly isolated pure toxin, cylindrospermopsicyclin (CYC).

4.4.2. The effect of the pure cyanotoxin (cylindrospermopsicyclin) on mustard seedlings

As mentioned in chapter 4.4.1., 3-day old samples were used. By the measured length data the IC_{50} values were between 400 and 600 $\mu\text{g/ml}$, while regarding the wet mass data the value was 600 $\mu\text{g/ml}$. No growth inhibition was observed at lower concentrations. Necrotic spots did not appear at higher concentrations. At the highest studied cyanotoxin concentration the mustard plants started to grow and very few seedling remained in seed state. At high cyanotoxin concentrations thickening of the root could be observed.



Scheme 9

4.4.3. Alteration of protein pattern of cylindrospermopsicyclin and crude extract treated plants

Because of the growth inhibition induced by CYC treatment we suppose stress induced responses in mustard plants. We were curious to see whether plant treatment with the toxic metabolite would induce the alteration of the protein patterns in the mustard plants. By means of gelelectrophoresis, two new bands of protein (18.9 and 19.5 kDa) were detected in the plants treated with the crude extract. These bands were also visible in the case of the pure cylindrospermopsicyclin exposed plants, besides two bands at 20.1 and 21.7 kDa. The intensity of several bands were decreased compared to the control.

4.4.4. Polyacrylamide gelelectrophoresis of proteases

To measure the native protease activity SDS-PAGE containing 4 % of gelatine was used. This kind of protease activity gel is suitable to detect mainly cysteine proteases. Etiolated, 3-day old mustard plants were used in the experiment. Gels were incubated at two pH (basic and acidic). Before the treatment with the pure cyanotoxin, preliminary experiment with the crude extract had been done. From the control plant six protease isoenzymes (36.3, 38.6, 59.4, 66.9, 77.0 and 88.0 kDa) were detected. The crude extract of *C. raciborskii* inhibited the activity of two isoenzymes of molecular masses of 36.3 and 38.6 kDa active at acidic pH. In the case of the pure cyanotoxic treatment, decreasing in the activity of all the isoenzymes were observed.

4.4.5. The effect of the isolated cyanotoxin (cylindrospermopsicyclin) on mustard nucleases

In plants, under stress conditions hydrolytic enzymes, including single strand specific DNA degrading enzymes, nucleases are induced. Studying the effects of cylindrospermopsicyclin on ssDNases a decrease in the nuclease activity was obtained. A similar tendency was obvious to crude extract of *C. raciborskii* exposed mustard seedlings.

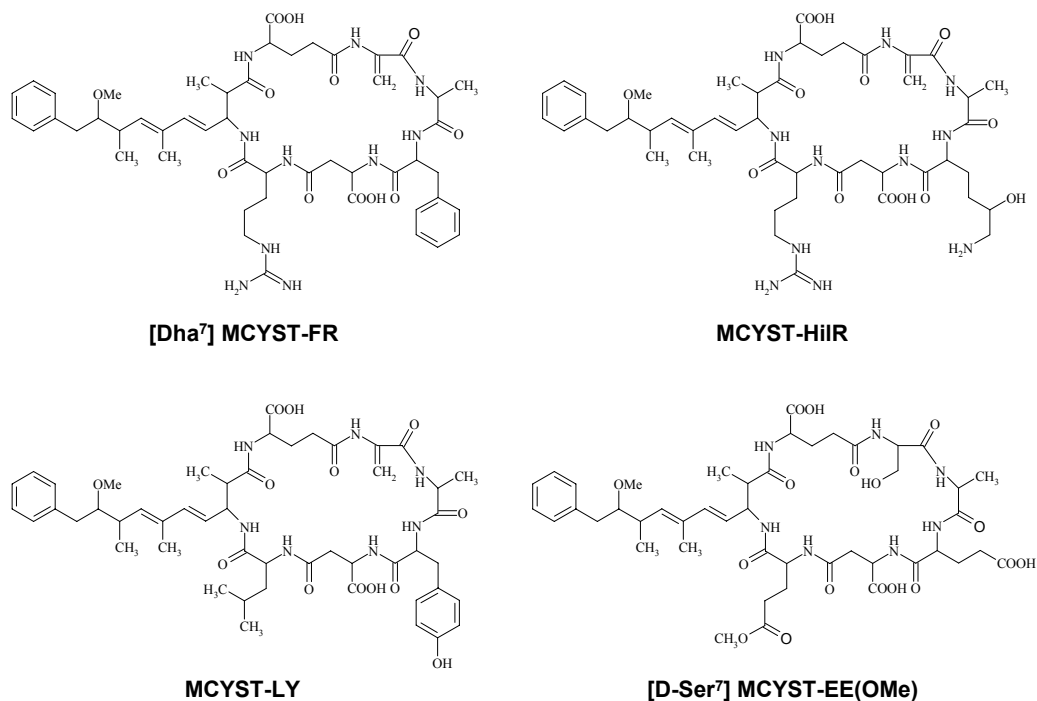
4.5. The effect of phosphorous and sulfur starvation on the growth and toxin production of cyanobacterium *C. raciborskii*

The availability of nutrients is recognised as major limitation for proliferation of phytoplankton in water habitats. Among others, phosphorous and sulfur were identified as main limiting nutrients, whose relative contribution varies in different habitats. How phosphorous and/or sulfur starvation interferes with cyanotoxin content no data are available. One may anticipate, that any perturbation of cyanobacterial metabolism results in alterations of cyanotoxin pool size, including cylindrospermopsicyclin. Indeed, it was found that due to the phosphorous and sulfur limitation the cultures virtually do not grow, the minimal degree of growing is due to the stored nutrients. The thin-layer chromatograms showed a significantly lowered amount of cylindrospermopsicyclin. The gelelectrophoretic studies of protein pattern of *C. raciborskii* indicated that nutrient starvation induces new proteins.

4.6. Determination of various microcystins in cyanobacteria isolated from Kis-Balaton Reservoir

In 2001 a water-bloom in Kis-Balaton Reservoir was observed. Plankton samples of this bloom were collected and concentrated by filtration and/or centrifugation. From the pellet obtained this way several microcystins were isolated. The steps of the purification were as follows: extraction with methanol, elution through DEAE cellulose column and elution through C-18 Sep-Pack Plus cartridges. Toxic fractions were further purified on a Toyopearl column. Mustard seedlings were used for the toxicity tests. The plants showed necrotic spots, what made the separation and recognition of the toxic fractions easier. The last step involved DAD-HPLC, by which the microcystins were easily identified by their

characteristic spectra. The structure of the isolated microcystins were determined by their MS spectra. The identified microcystins of the isolated *M. aeruginosa* are shown in **Scheme 10**:

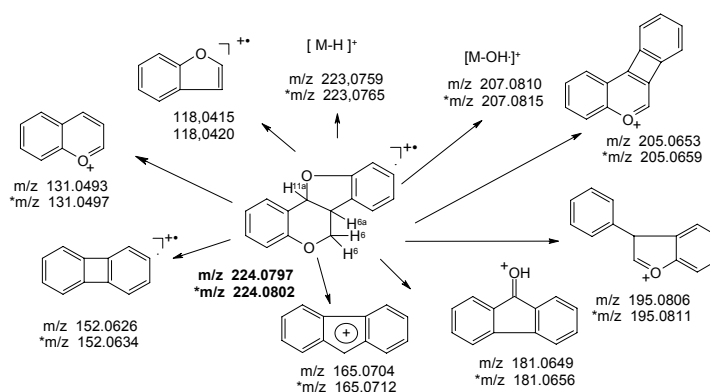


Scheme 10

4.7. Synthesis of pterocarpan and their mass spectrometric study

Pterocarpan and its derivatives have been synthesized, their structure and degree of deuteration have been proved by mass spectrometric studies.

The characteristic fragment ions and their high resolution data are shown in Scheme 11. (Values with asterisk are calculated, without asterisk are measured.)



By the mass spectra it could be observed that pterocarpanes resulted in molecule ions with high intensity, these ions gave the base peak of the spectra of each compound. The ion $[M-H]^+$ had the second largest intensity. This ion can be originated via the loss of either hydrogen H-6 or H-11a. The intensities showed the loss of H-6 to be more preferred. Fragment with m/z 207 is formed from the enol form of the molecule ion via loss of hydroxyl radical. Fragment with m/z 205 is formed from the same enol form via loss of water, then hydrogen radical. The intensity data of the deuterated derivatives showed that, if loss of both hydrogen and deuterium is possible, the latter is preferred. An elimination of a formaldehyde radical (COH^\bullet) gave rise to the ion with m/z 195. Fragment with m/z 181 is from the molecule ion which loses an ethylene oxide and a hydrogen radical. If the rearranged molecule ion eliminates an aldehyde radical, then an aldehyde molecule, the ion with m/z 165 is formed. Ion with m/z 152 arose from the molecule ion after rearrangement and elimination of acetaldehyde and carbon monoxide. Simultaneous cleavage of the bonds of benzofurane and chromanone rings gave the pyrrilium structure with m/z 131 and the benzofurane radical cation with m/z 118.

5. SUMMARY

Summarizing our results we conclude that culturing of the cyanobacterium *Cylindrospermopsis raciborskii* (BGSD 266) isolated from Lake Balaton can be successfully performed under laboratory circumstances.

From *C. raciborskii* we have isolated a new plant growth inhibitor, named to cylindrospermopsicyclin using chromatographic techniques. The purification method involves silica gel and the thin-layer chromatography introduced by us has significantly increased the efficiency of the isolation.

The chemical structure of the isolated plant growth inhibitor has been identified using structure determination methods (NMR and IR spectroscopy and MS). The newly isolated cylindrospermopsicyclin has molecular mass of 413 Da.

Experiments showed that the isolated metabolite inhibits the growth of the mustard plant with the IC₅₀ value of 600 µg/ml. By the protein gelelectrophoretic studies it can be concluded that the purified cyanotoxin induces new proteins. The cyanotoxin decreases the activity of the ssDNase and acidic protease of the mustard.

Nutrient starvation (deprivation of phosphorous or sulfur) of the strain *C. raciborskii* induces fall-off in the growth of the culture. The amount of cyanotoxin produced by the culture (determined by thin-layer chromatography) also decreased under nutrient starvation conditions.

In a separate study we have isolated four microcystins ([Dha⁷]MCYST-FR, MCYST-HiLR, MCYST-LY and [D-Ser⁷]MCYST-EE(OMe)) from the organism *Microcystis aeruginosa* isolated in 2001 from Kis-Balaton Reservoir. The structures of these microcystins have been identified by mass spectrometry.

We have successfully synthesized seven deuterated analogues of pterocarpan. By mass spectrometric studies the fragmentation pathway of the natural compound has been mapped.