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A Fluorescent-Labeled Microcystin-LR Terbium Cryptate

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Um novo composto fluorescente de microcistina-LR marcado com criptato de térbio foi obtido pela conjugação inicial da microcistina-LR com aminoetanotiol, seguido pela reação com o grupo éster do criptato de térbio. A formação do produto foi acompanhada por cromatografia líquida de alta eficiência (CLAE) em 238 nm e 310 nm. A presença de microcistina-LR na molécula marcada foi confirmada através de ensaio com enzima ligada a imunoabsorvente (ELISA) e por reação protéica com ácido bicincônico. O espectro de luminescência do criptato e da molécula conjugada também foram confirmados.

A new fluorescent labeled compound of microcystin-LR with terbium cryptate was obtained by initial conjugation of microcystins-LR with aminoethanethiol followed by the reaction with the ester group of terbium cryptate. The product formation was followed by high performance liquid chromatography (HPLC) at 238 nm and 310 nm. The presence of microcystin-LR in the labeled molecule was confirmed by enzyme linked immunosorbent assay ELISA and by protein reaction with bicinhonic acid. Luminescence spectra of cryptate and the conjugated molecule were carried through as well.

Keywords: terbium cryptate, microcystin-LR, cyanobacteria

Introduction

Microcystins are a group of stable heptapeptide hepatotoxins produced by freshwater cyanobacteria genera that lower the water quality leading to an increase in the risk of intoxication for animals and humans. Species of genera *Microcystis*, *Anabaena*, Aphanizomenon, Planktothrix (*Oscillatoria)*, and *Nostoc* are frequently described as producers of microcystins.[1] The eutrophication of ponds, rivers and other freshwater ecosystems allows cyanobacteria to flourish, leading to contamination of the water.[2]

Microcystins are potent inhibitors of protein phosphatases 1 (PP_1) and 2A (PP_{2A}), which are regulatory enzymes present in the cytosol of mammalian cells.[3] The general structure of microcystins is composed of cyclo-(D-Ala-X-D-MeAsp-Z-Adda-D-Glu-Mdha), where X and Z could be various kinds of L-amino acids; D-MeAsp is D-erythro-β-methylaspartic acid; Mdha is N-methyldehydroalanine; and Adda is the unusual amino acid 2S,3S,8S,9S-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4E,6E-dienoic acid.[4] The most extensively studied form is microcystin-LR that contains L-leucine and L-arginine in the two main variant positions.[3] More than 60 different analogues of microcystins have been described and their acute mouse i.p. toxicity (DL_{50}) varies between 50-600 μg kg^{-1}.[1]

Contamination of surface drinking water by microcystin-producing cyanobacteria is a probable cause of primary liver cancer.[5] Chronic oral ingestion of a crude extract of *Microcystis* suggests that microcystins can cause tumors after long-term exposure.[6] Human mortalities resulting from oral consumption of cyanotoxins have been suspected but not confirmed, due the difficulty of obtaining reliable data regarding vectors and the circumstances that would confirm the presence of cyanotoxins in human food

or water supplies.[7] Increasing care is being taken in the consumption of products contaminated with cyanobacteria toxins, because of the risks involved in consuming food that contains blue-green algae or water contaminated by their toxins.[8] Recently, an acute case of poisoning of humans undergoing haemodialysis with water contaminated with microcystin killed 76 of a total of 131 patients in the city of Caruaru in Northeast Brazil.[7,9,10] As a consequence, a microcystin limit of 1μg L^{-1} was established for drinking water in Brazil.[11]

Currently, the aim is to develop a fast, inexpensive and convenient analytical method for detecting microcystins. Immunological fluorescent methods using antigen, monoclonal or polyclonal antibody have become a well-known protocol.[12-16] Fluorescent-labeled reagents have been developed for biological applications. Kim *et. al.*[17] employed a one-step fluorescence immunochromatographic assay to quantify microcystins using Alexa Fluor 647. A fluorescent phosphatases enzyme inhibition assay for microcystin-LR was developed by Fontal *et al.*[3]

Complexes with a lanthanide ion, when excited by ultraviolet (UV) radiation, show long-lasting luminescence and are excellent light conversion molecular devices, LCMD's[18] applied in time-resolved microscopy,[19] fluorescent lighting,[20] UV dosimeters,[21] antirefelection coatings for solar cells,[22] and the labeling of biological assays.[23,24]

In the search for more sensitive markers, some chelates of lanthanides had already been obtained and tested in various biological assay models.[25-27] Time-resolved fluoroimmunometric assay (TR-FIA) is a method based on the luminescence emission that is measured after a delay has elapsed from a pulsed excitation, enabling the short-lived background fluorescence to be excluded.[28] Soini *et. al.*[29] labeled anti-rabbit IgG with europium chelate as a model to detect rabbit IgG for human smooth muscle myosin in a histological section; Bonin *et al.*[30] used a complex of europium and samarium for the detection of diphteria antitoxins in serum using the ELISA method and Xu *et al.*[31] labeled anti-*β*-LH and monoclonal anti-*β*-FSH antibodies with europium and samarium, respectively. The use of luminescent lanthanide as a substitute for labeled enzyme (e.g. horseradish peroxidase - HRP) in the ELISA method for microcystin detection has been described by Mehto *et al.*[28] This system uses microcystin-LR labeled with a europium chelate.

One of the classes of lanthanide complexes that is increasingly being used in immunological assays is the cryptates. These compounds are highly stable when dissolved in water, and have a high coefficient of molar absorption, a high quantum yield in water solution and

kinetic and thermodynamic stability.[32] Europium cryptate also has been used for labeling DNA[33] and antibodies,[34] and the Cis-Bio-Schering Company (France) has now put on the market a kit with trisbipyridine europium cryptate for labeling biomolecules. Terbium complex has been used in thin films as very efficient light converters under UV excitation, where the ligands act as antennas, absorbing UV radiation and efficiently transferring energy to Tb^{3+} emitters. The green emission, $^5D_4$ à $^7F_5$, transition is monitored as function of UV excitation.[18] The use of a terbium cryptate for labeling cyanotoxins, however, has not been investigated yet.

There is obviously a need to monitor cyanotoxins in water samples and products that may have previously been in contact with cyanobacteria.

This paper describes how a new luminescent terbium cryptate conjugate with microcystin-LR was synthesized and characterized.

Experimental

Materials and methods

The water used was purified by distillation or deionization followed by purification via Milli-Q water system (Millipore), producing water with 18.2 MΩ cm resistivity. All other reagents, where not specified, were of analytical or liquid chromatographic grade.

Terbium cryptate synthesis

The labels (a), (b) and (c) refer to the intermediate compounds shown in Figure 1. The synthesis of the cryptates was achieved by bromination of diethyl 2,6-dimethyl-3,5-pyridinedicarboxilate (99% Aldrich), via the initiator 1,1'-Azo (cyclohexanecarbonitrile) (98% Aldrich) and N-bromosuccinimide (NBS, 99% Aldrich) under reflux and irradiation by a 100 W incandescent lamp to give 2,6-dibromomethyl-3,5-diethyl-dicarboxilate (a). The bromi-nation of 6,6'-dimethyl-2,2'-bipyridine used benzoyl peroxide associated with NBS.[35,36] In this procedure the product 6,6'-Bis (bromomethyl)-2,2'-bipyridine (b) is obtained in the pure state after re-crystallization. The ring (c) was synthesized following the procedure described in the literature.[35,37] The subsequent step was the formation of the cryptate [Li⊂bpy.bpy.py(CO$_2$Et)$_2$]CO$_3^-$. The product (c) was added to CH$_3$CN$_{(aq)}$ in an atmosphere of N$_{2(g)}$. The product (a) was dissolved in an aqueous solution of Li$_2$CO$_3$ under reflux. The cryptate thus formed (d), was purified using HPLC. The inclusion of the terbium ion in the cryptate was achieved by single ionic change with Li$^+$ cation and the lanthanide (e).[38,39]

The lanthanide cryptate was purified by HPLC and characterized by UV-VIS and IR spectroscopies and mass spectrometry. The UV-VIS absorption spectrum for an ethanolic solution of terbium cryptate (10^{-5} mol L^{-1}) presented maximum intensities at 208 nm, 243 nm and 291 nm. The product analised by IR with KBr disk for transmission analysis showed the main bands at 3138.2 cm^{-1}, 2938.3 cm^{-1}, 1685.0 cm^{-1}, 1436.1 cm^{-1}, 1206.3 cm^{-1} and 1130.8 cm^{-1}.[38,39] The proposed formulae of the cryptates synthesized are also consistent with the mass spectrometry results: [Tb⊂bpy:bpy.py(CO_2Et)$_2$ $^{3+}$] (MS m/z 800 (M+)) and [Li⊂bpy:bpy:py(CO_2Et)$_2$]Br (MS m/z 728 (M+)).[38,39]

Microcystin-LR-terbium cryptate conjugation

First stage: conjugation of microcystin-LR to amino-ethanethiol. Microcystin-LR (Sigma) was dissolved in a mixture of 20 percent methanol and 80 percent 100 mmol L^{-1} phosphate buffer (pH 7.0) to a final concentration of 5.0 mg mL^{-1} and it was maintained at –20 °C. In the first stage of conjugation, 13.9 mg of 2-aminoethanethiol hydro-chloride (Sigma) was added to 60 μg of microcystin-LR in a molar excess of 3,000-fold. The reaction was carried out in a solution of 24 μL of dimethyl sulfoxide (Merck), 18 μL of purified water and 8 μL of 5.0 mol L^{-1} NaOH. The mixture was incubated for one hour at 50 °C under nitrogen,

followed by the addition of 62 μL of concentrated acetic acid and 496 μL of Trifluoracetic acid 0.1% (pH 1.5) as suggested by Moorhead *et al.*[40] Before the purification of conjugated microcystin-LR-aminoethanethiol, a mixture of equal parts of the reaction and microcystin-LR solution of the same concentration was injected to verify the retention times. The conjugate was purified by HPLC, frozen-dried (ModulyoD, ThermoSavant) and suspended in 200 μL of 50 mmol L^{-1} NaHCO$_3$, 0.15 mol L^{-1} NaCl buffer (pH 8.5).

Second stage: conjugation of microcystin-LR- amino-ethanethiol to terbium cryptate. The reaction was carried out in molar excess of 25:1 cryptate: microcystin-LR-aminoethanethiol for a 24-hour period (t_{oh} – t_{24h}). Purified terbium cryptate (1.7 mg) was dissolved in 200 μL of 50 mmol L^{-1} NaHCO$_3$, 0.15 mol L^{-1} NaCl buffer (pH 8.5) and mixed with 200 μL of the microcystin-LR-aminoethanethiol solution and incubated at ambient temperature for 24 hours.

High-Performance Liquid Chromatography (HPLC)

Microcystin-LR, microcystin-LR-aminoethanethiol, terbium cryptate and all reactions were analyzed using HPLC. The system consisted of a Shimadzu (Kyoto, Japan) LC-10ATvp pump and a column C$_{18}$ LichroCart with 5 μm
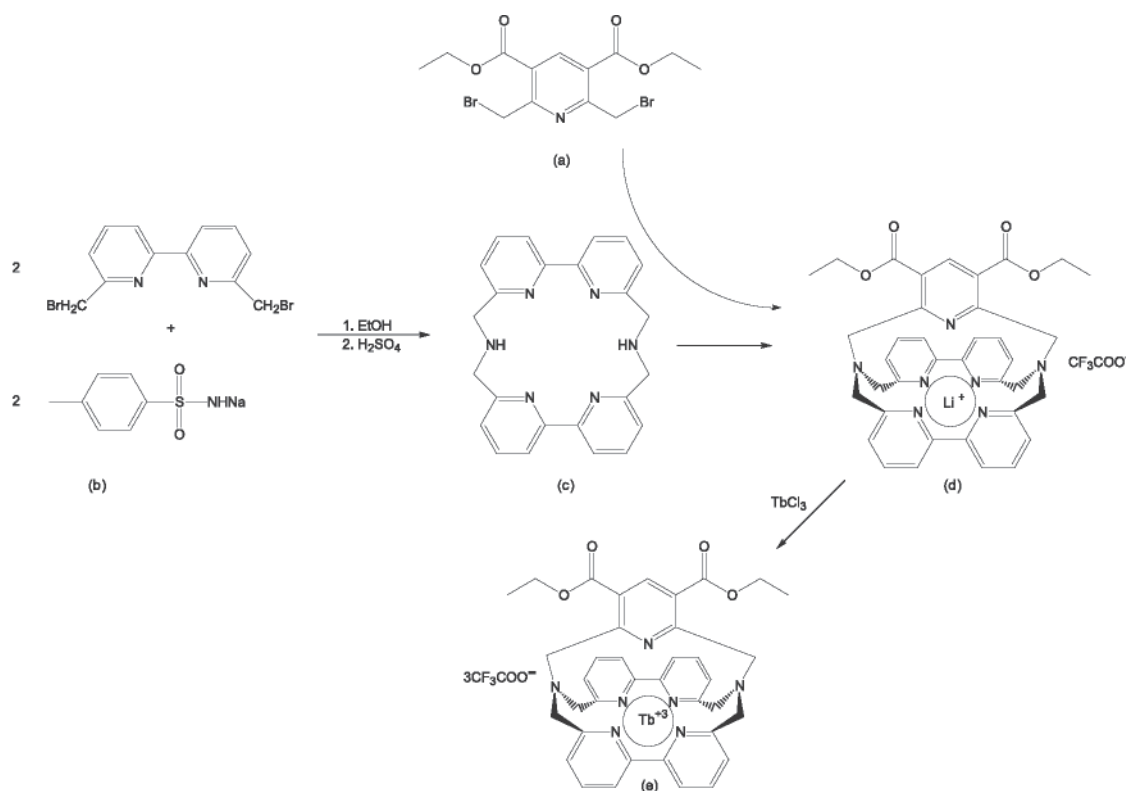

Figure 1. Schematic synthesis of terbium cryptate.

particle size, 250x4 mm (Merck) attached to a UV detector (SPDM-10Avp). Samples of 50 μL were injected and separation was accomplished using a reverse phase gradient and a mobile phase of acetonitrile: 1.0% (v/v) TFA at rate of flow of 1.0 mL min^{-1} at 40 °C. The linear gradient starting at 15% (v/v) acetonitrile and increasing to 100% over 35 min and maintained for 40 min. The acetonitrile was then reduced by 15% for 45 min and kept at this ratio until 55 min had elapsed and afterwards, a new injection was administered. Detector resolution was set at 1.0 nm and the reaction monitored at 238 nm and 310 nm. The fractions of terbium-microcystins-LR conjugation were collected, pooled, frozen-dried and suspended in 500 μL of purified water so as it could be used in the fluorescence assays (luminescence), ELISA and protein reaction.

Luminescence spectroscopy

The fraction corresponding to the terbium cryptate and the terbium-microcystin-LR conjugated, obtained at the end of the HPLC purification, were analyzed by emission spectroscopy. The luminescence spectra were measured at 300 K using a Jobin Yvon double monochromator model U-1000 Ramanor attached to a water-cooled RCA C310340-02 photomultiplier. In this case, a 450 UV Xe lamp was used as the source of excitation. The registering and processing of the signal were carried out using a Spectralink interface to an IBM microcomputer.

Purification of anti-microcystin-LR-KLH antibodies

The affinity column of protein G (HiTrap, Amersham, London, UK) was stabilized with 5 mL of 20 mmol L^{-1} phosphate buffer (pH 7.0). Nine milliliters of the rabbit serum anti-microcystin-LR-KLH were applied in portions of 0.5 mL and the column washed with 5.0 mL of the same buffer. The fraction of interest was extracted with 3.0 mL of 0.1 mol L^{-1} glicina-HCl buffer (pH 2.7) directly into a tube that contained 0.3 mL of 0.1 mol L^{-1} tris-HCl buffer (pH 9.0). The solution of IgG was reduced to a volume of approximately 200 μL using a Centricon tube of 30 KD. The final concentration of antibodies was determined by the total protein analysis.[41]

Competitive indirect ELISA

The assay used was developed by Karnikowski for analysis of microcystins.[42] The aqueous fraction of terbium-microcystins-LR conjugation and the fraction corresponding to the terbium cryptate obtained at the end of the HPLC purification were diluted in 1/10 in the same buffer used for microcystin-LR standards. A blank of water and buffer was utilized. All samples and standards were analysed three times. This assay is an indirect competitive enzyme immunoassay on a 96-well microtiter plate (Maxisorp, Nunc, Roskilde, Denmark) coated with microcystin-LR-ovoalbumin. The standard curve was achieved with microcystin-LR solutions of 0.16, 1.6, 5.0 and 10.0 μg L^{-1}. In summary, 50 μL of a solution of the equal parts of sample or microcystin-LR standard with the antibody were incubated for 90 min at 37 °C and pipetted into the well and incubated for 2 hours at ambient temperature. The wells were emptied and washed three times with 200 μL of 15 mmol L^{-1} phosphate buffer -0.05% Tween (pH 7.2). A 50 μL aliquot of polyclonal anti-rabbit IgG (whole molecule)–Peroxidase from goat (Sigma-Aldrich Co.) diluted 1 : 2,000 was added and incubated for another 90 min at 37 °C. The wells were washed again. An aliquot of 50 μL of substrate o-phenilenediamine (OPD, Sigma) was added to each well and incubated for 10 min at ambient temperature and protected from light. Finally, 50 μL of a 3.0 mol L^{-1} hydrochloric solution was added to stop the reaction and the absorbance immediately recorded at 492 nm using a microplate reader (EIA/ELISA reader mod. 2550, Biolab).

Protein analysis

The aqueous fraction of terbium-microcystin-LR conjugation and terbium cryptate obtained at the end of HPLC purification were tested for the protein reaction utilizing a bicinchonic acid protein assay kit (Pierce art. 23255).

Results and Discussion

Microcystin-LR-terbium cryptate conjugation

No peak was detected in the HPLC analysis at the wavelengths of 238 nm and 310 nm after injection of 50 μL of bicarbonate buffer and aminoethanethiol used for the conjugation of microcistin-LR to terbium cryptate. The microcystin-LR standard, when injected separately, showed a peak at 238 nm after 22.6 min and no peak at 310 nm. The chromatogram of a mixture of equal parts of microcystin-LR and microcystin-LR-aminoethanethiol after reaction (1 hour, 50 °C) showed two peaks at 238 nm, one within 22.6 min and another after 21.5 min respectively, showing good separation under analytical test conditions (Figure 2). The injection of the reactional mixture microcystin-LR-aminoethanethiol (t$_{24h}$) alone confirmed the appearance of only one peak after 21.5 min (data not shown).
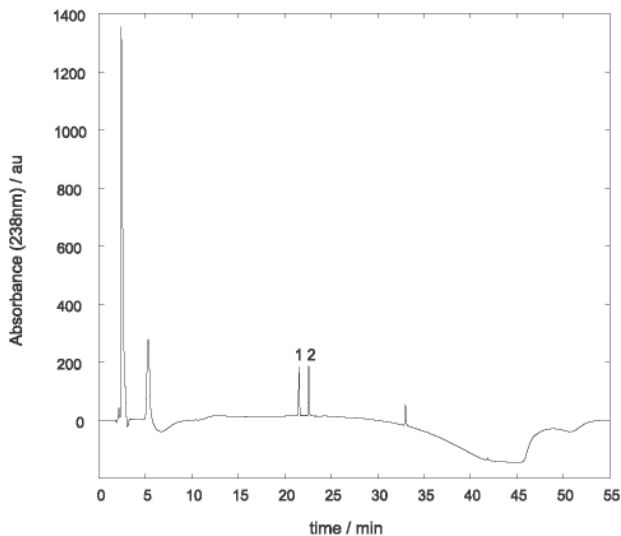
Figure 2. Chromatogram in gradient reversed-phase HPLC of mixture of reaction product: (1) microcystin-LR-aminoethanethiol and (2) microcystin-LR standard.
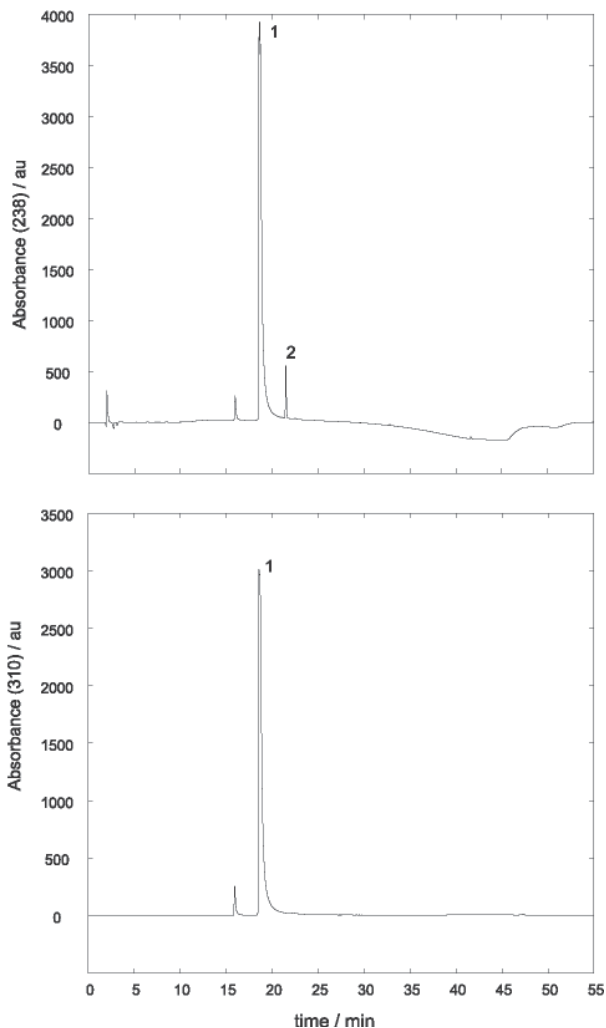

Figure 3. Chromatogram in gradient reversed-phase HPLC at the beginning of conjugation time (t_{oh}) of microcystin-LR-aminoethanethiol and terbium cryptate at 238 nm and 310 nm: (1) Terbium cryptate and (2) Microcystin-LR-aminoethanethiol.

Purified terbium cryptate dissolved in a NaHCO$_3$ buffer showed a main peak after 18.6 min at 238 nm and 310 nm (data not shown). The same retention times of the isolated products, terbium cryptate and microcystin-LR-aminoethanethiol, were observed at the beginning of the conjugation, t_{oh} (Figure 3). A small peak after 15.9 min was also observed at the beginning of the conjugation at 238 nm and also at 310 nm.

After 24 h of reaction (t_{24h}) a sharp peak was observed within 15.9 min and an slightly less intense peak after 18.6 min (Figure 4).
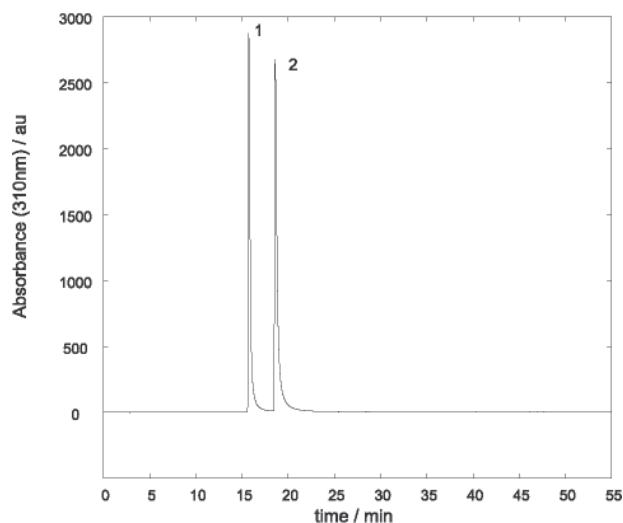

Figure 4. Chromatogram in gradient reversed-phase HPLC at the final conjugation time (t_{24h}) of microcystin-LR-aminoethanethiol and terbium cryptate at 310 nm: (1) Microcystin-LR-Terbium cryptate and (2) Terbium cryptate.

The luminescence spectra of the isolated fractions of terbium-microcystin-LR and terbium cryptate proved to be characteristic of metallic ion as represented in the Figure 5.

The standard ELISA curve used as standard showed a good correlation coefficient (r = 0.9346; t = 8.3; p < 0.01)
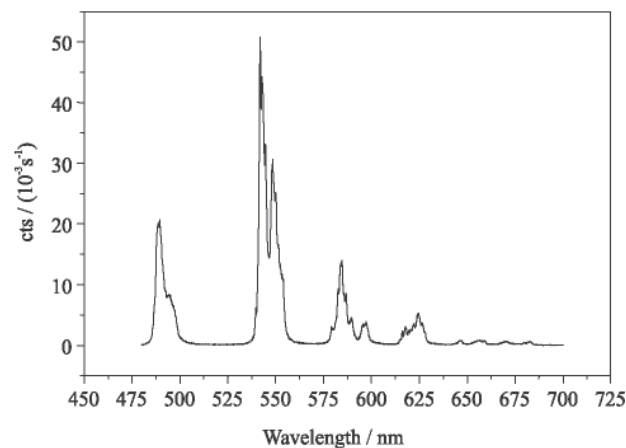

Figure 5. Emission spectra of microcystin-LR-terbium cryptate fraction obtained in gradient reversed-phase HPLC. Excitation wavelength: 310 nm.

during the interval from 0.16 μg L^{-1} to 10.0 μg L^{-1} (Figure 6). The fraction of microcystin-LR-terbium cryptate conjugation diluted 1/10, showed a 43.3% inhibition of antibody response while that corresponding to the terbium cryptate and the blank did not show inhibition.

The aqueous fraction of terbium-microcystin-LR conjugation was tested positive for protein using bicinchonic acid reaction, while the fraction of terbium cryptate did not.
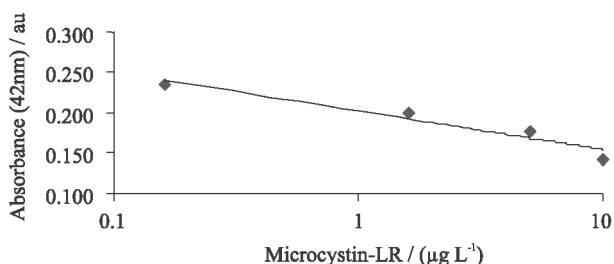

Figure 6. Semi-log graphic of competitive indirect ELISA of standard microcystin-LR using anti-microcystin-LR-KHL IgG on a microplate coated with microcystin-LR- chiken ovoalbumin (n = 3).

There is an increasing demand for more sensitive and inexpensive analytical methods to deal with lower detection limits and this has given rise to a need for more sensitive labeling.

A luminescent complex of microcystin-LR-terbium cryptate was developed for microcystin analysis.

The microcystin-LR was used because it is one of the most common microcystins[28] and is the most hepatotoxic among microcystins.[1] Moreover, it is recommended to evaluate its concetration in water in various countries, including Brazil,[11] Great Britain and other European countries[43] and the World Health Organization - WHO also recommended a provisional standard level of 1.0 μg L^{-1} for drinking water.[44]

The cryptate of terbium is very advantageous because terbium is not so sensitive to aqueous quenching and the efficient energy transference from ligand triplet state to the excited metallic state overcomes the difficulty of the use of terbium chelates in bioanalytical applications.[24] Terbium cryptate molecule is also of small dimension when compared with chicken egg albumin (44,000 Da), horseradish peroxidase (44,000 Da) or IgG antibodies (150,000 Da) - molecules normally used in immunoassays – thereby minimizing the possibility of steric hindrance. The cryptate as biomolecule labeling groups, in contrast of chelates, has the advantage of being directly conjugated without a displacement step.[33]

The microcystin-LR conjugation with aminoethanethiol was chosen because it is known that the reaction occurs with the amino acid methyldehydroalanine (Mdha) of the toxin[40,45] in a position that does not interfere with the main antigenic region of the molecule, the Adda amino acid.

The following reaction is proposed for the conjugation of toxin with terbium cryptate. It is based on the reaction presented by Metho *et al.*[28] for linking microcystin-LR with europium chelate (Figure 7).
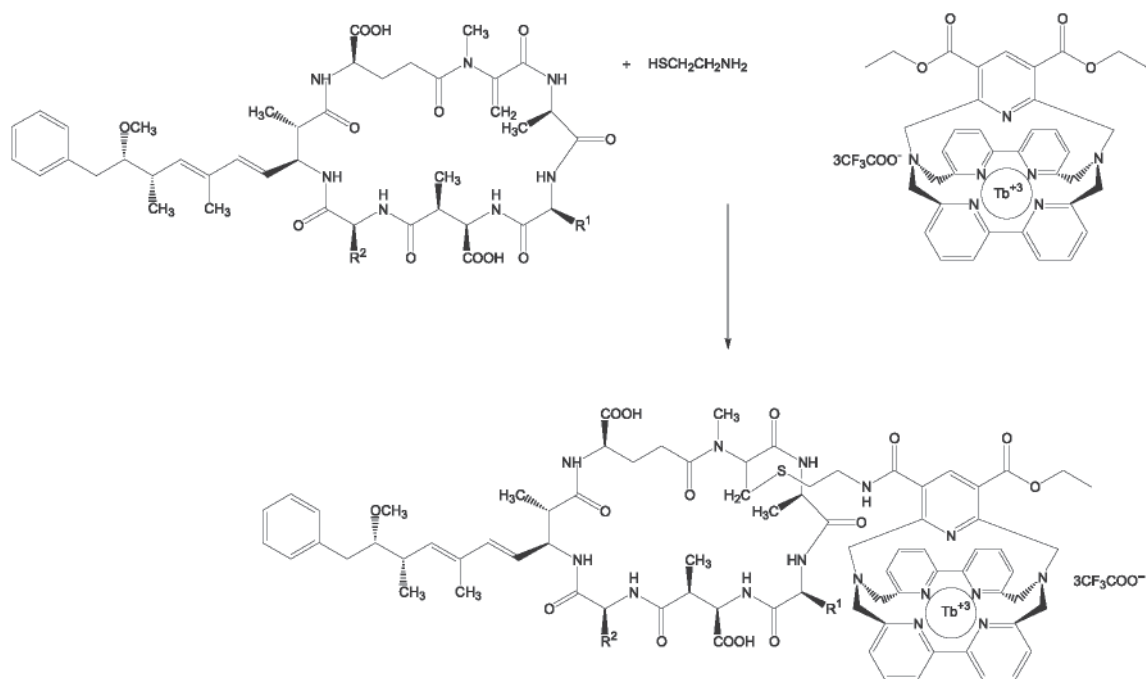

Figure 7. Microcystin-LR terbium cryptate conjugation reactions.

Microcystin-LR reacted, in its entirety, with the low molecular weight aminoethanethiol, as confirmed by HPLC: a single peak after 21.5 min (chromatogram not shown) was detected. Therefore, it could be successfully resolved from microcystin-LR standard (Figure 2) allowing it to be recovered for the subsequent reaction. According to Moorhead *et al.*,[40] the reaction of aminoethanethiol with the carbonyl group of the Mdha residue is stronger at a high pH value, producing more than 95% of intermediate product.

The link inhibition of antibody with microcystin-LR-chicken ovoalbumin, in the ELISA plate, by the conjugate (peak 1, Figure 4) demonstrates the immunoreagent capacity and confirms the microcystin presence in the structure. In accordance with Lopez *et al.*[34] trisbipyridine diamine europium cryptate was conjugated to antibody by the method utilized here without any loss of immunoreactivity. The structure of the hydrophobic amino acid Adda, which has the (E) form at the C-6 double bond is essential for determining antibody specificity[13] and was preserved because the reaction occurred selectively in the amino acid methyldehydroalanine.[40,45]

The presence of the toxin in this molecule is strengthened by a positive protein reaction in this fraction when using the bicinchonic acid method, whose sensitivity is for structures with up to four amino acids. The conjugation of the microcystin-LR is also confirmed by the luminescence spectra of isolated cryptate and the microcystin-LR-terbium cryptate fraction.

The terbium criptate used in microcystin-LR conjugation has a high emission quantum yield in aqueous solution (q_{298K} =25% at λ_{exc} = 320 nm),[38] which makes its use as, e.g, antigen in a ELISA competitive assay through TR-FIA method, that presents some advantages such as sensitivity, safety, convenience and ease for automation.[46]

Acknowledgments

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