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Synthetic Haptens and Enantioselective Monoclonal Antibodies to the Cyanotoxin Anatoxin-a

Guillermo Quiñones-Reyes⁺, Consuelo Agulló, Josep V. Mercader, Antonio Abad-Somovilla,^{*} Antonio Abad-Fuentes^{*}

Abstract: Early warning systems for monitoring toxic events may benefit from the availability of monoclonal antibodies enabling the sensitive and specific detection of anatoxin-a, a cyanotoxin involved in numerous cases of animal poisoning due to toxic algal blooms in freshwaters. Through the synthesis of three functionalized derivatives of anatoxin-a, we have succeeded in generating the first-ever reported immunoreagents (bioconjugates and antibodies) suitable for the development of immunoanalytical approaches aimed at rapid and onsite detection of this harmful cyanotoxin.

Cyanobacteria are ubiquitous photosynthetic prokaryote organisms that under favorable conditions can multiply rapidly giving rise to so-called algal blooms.^[1] These blooms primarily occur in fresh and estuarine waters, and they are currently considered more frequent and intense than in the past due to eutrophication caused by human activity and global warming.^[2] Cyanobacteria massive proliferation, when dominated by species able to produce secondary toxic metabolites (cyanotoxins), poses a serious threat to the environment and to the health of people, domestic animals, livestock, and wildlife.^[3] The main exposure routes to cyanotoxins are through contaminated water, although the consumption of fish and bivalve mollusks,^[4] or the presence of undesired cyanobacteria species in dietary supplements,^[5] are deemed emerging risks.

Anatoxin-a (2-acetyl-9-azabicyclo[4.2.1]non-2-ene) is a tropane-related bicyclic alkaloid which incorporates an α,β -unsaturated methyl carbonyl residue (Figure 1). The structure of this cyanotoxin was elucidated in the seventies from a strain of *Anabaena flos-aquae* found in an algal bloom that caused the death of cattle in 1961.^[6] At that time, the cyanotoxin was named Very Fast Death Factor because, when injected intraperitoneally into mice, animals died within minutes. Its high potency as a neurotoxin is due to the fact that it is an agonist of acetylcholine, irreversibly binding to the nicotinic acetylcholine receptor at the neuromuscular level.^[7] Many cases of animal deaths that are linked to anatoxin-a as a result of the intake of contaminated water have been reported so far.^[4,8] In some geographical areas, the problem is recurrent and incidents are recorded every year.^[9]

Antibody-based methods are common for the analysis of major cyanotoxins.^[10] In fact, the USEPA has recently validated an immunoassay for the determination of microcystins and

nodularins (Method 546). Unlike these cyanotoxins, no immunochemical method has been reported so far for anatoxin-a despite this aim was pursued in several research projects worldwide.^[11] Therefore, it has long been recognized the need for rapid and reliable antibody-based methodologies for anatoxin-a monitoring.^[12] The reasons why the generation of antibodies to anatoxin-a has remained elusive so far include the photoinstability of the molecule, its susceptibility to suffer Michael additions with sulphur compounds, its sensitivity to high pH, and its very small size – with a molecular weight of 156, anatoxin-a is one of the smallest cyanotoxins.

Figure 1. Structures of anatoxin-a, homoanatoxin-a, designed haptens, and the corresponding *N*-hydroxysuccinimide esters. All haptens are racemic compounds, but only the (1*R*,6*R*)-enantiomer is shown for simplicity.

The generation of antibodies to small chemicals like anatoxin-a strongly relies on the availability of adequate functional derivatives of the molecule, so-called haptens. Hapten design must be guided by the principle of maintaining the integrity of the framework as well as the characteristic groups of the parent molecule, through incorporating the linker chain at the site that induces the lowest possible changes in the electronic and conformational properties. The only documented attempt to synthesize a functionalized derivative of anatoxin-a that could be potentially useful for the production of antibodies to this cyanotoxin was reported in 2009, even though those authors did not go further.^[13] In the present communication, we describe the preparation of three haptens with a carboxylated linker located at different positions of the molecular framework through a convergent synthetic strategy that has allowed us to generate the first-ever described high-affinity monoclonal antibodies to anatoxin-a.

In designing haptens for anatoxin-a, the presence of two reactive functional groups needs to be carefully considered; on the one hand, the bridging secondary amino group of the bicyclic system, with basic and nucleophilic capacity to interact with acid or electrophilic centers;^[14] and, on the other hand, the

[*] G. Quiñones-Reyes, Prof. Dr. C. Agulló, Prof. Dr. A. Abad-Somovilla
Department of Organic Chemistry, University of Valencia, Doctor
Moliner 50, 46100 Burjassot, Valencia (Spain)
E-mail: antonio.abad@uv.es
Dr. J. V. Mercader, Dr. Antonio Abad-Fuentes
Institute of Agrochemistry and Food Technology (IATA), Spanish
National Research Council (CSIC), Agustí Escardino 7, 46980
Paterna, Valencia (Spain)
E-mail: aabad@iata.csic.es

[+] Present address: Unidad Académica de Ciencias Químicas,
Universidad de Zacatecas, Zacatecas (Mexico).

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α,β -unsaturated ketone moiety, potentially susceptible to easily experiencing Michael-like addition reactions with appropriate nucleophiles present *in vivo*.^[15] Accordingly, we initially designed haptens ANc and ANm as potentially good mimetics of the anatoxin-a structure (Figure 1). As the toxin itself, these haptens are protonated at the bridging nitrogen atom under physiological conditions. However, as desired, a lower reactivity of the conjugated double bond against nucleophiles was expected, particularly in hapten ANc, because of the weakly electron-withdrawing nature of the ketoxime ether moiety,^[16] but also in ANm due to steric factors. As in anatoxin-a,^[17] a transoid relationship exists between the C=C and C=O conjugated π systems of hapten ANm which places the flexible carboxylated alkyl chain in the neighborhood of the double bond,^[18] thus potentially lowering its reactivity towards nucleophilic addition. Besides these two haptens, a third derivative was also designed, hapten ANn (Figure 1), in which the linker chain, in this case a linear carboxylated chain of four carbon atoms, is attached to the bridge nitrogen atom through a C–N bond that maintains the basic character of the nitrogen atom. This hapten allows an alternative and complementary presentation of the anatoxin-a molecular framework to the immune system with respect to haptens ANc and ANm.

We initiated the synthetic work with the preparation of hapten ANm. Its synthesis was undertaken in eight steps from 1,2-epoxy-5-cyclooctene (Schemes S1 and S2). With this hapten in hand, we addressed the preparation of the required protein bioconjugates via the active ester method. However, all attempts to transform hapten ANm into the corresponding *N*-hydroxysuccinimidyl ester (ANm–NHS ester), or other active esters, failed.^[19] In view of this result, we used a modified approach for the preparation of ANm–NHS ester that involved the activation of the carboxylic acid group immediately before removal of the *N*-Boc protecting group, thus avoiding that the nucleophilic bridge amino group coexisted with the active ester moiety. This new strategy also started with the preparation of the bicyclic ketone **4**, synthesized as described by Kanne (Scheme 1).^[20] After replacement of the benzyl group in **4** with a Boc group, the carbonyl group of **5** was transformed into the corresponding enol triflate **6**, which underwent a palladium-catalyzed Sonogashira cross-coupling with methyl hex-5-ynoate to afford the enyne derivative **7**. This compound was converted into the α,β -unsaturated-ketone **8** by regioselective hydration of the acetylenic triple bond, thus completing the elaboration of the fully functionalized skeleton of hapten ANm. The synthesis of the intended *N*-hydroxysuccinimidyl ester was accomplished in three steps from **8**. First, basic hydrolysis of the methyl ester moiety of **8** to obtain acid **9**, followed by carbodiimide-mediated (EDC) esterification with NHS to give active ester **10** and, finally, chemoselective cleavage of the *tert*-butyl carbamate amine protecting group, using trifluoroacetic acid, to directly yield the trifluoroacetate-ammonium salt of ANm–NHS ester. The synthesis of this hapten was realized in ten steps from **1**, with a global yield of 27%.

The *N*-hydroxysuccinimidyl ester of hapten ANc, also obtained as the trifluoroacetic acid salt, was prepared from the

same key enol triflate intermediate **6**, following a similar strategy for the incorporation of the acetyl moiety at the C-2 position of the homotropene nucleus (Scheme 1). This involved the Pd(0)-catalyzed cross-coupling of **6** with ethynyltrimethylsilane followed by basic methanolic desilylation to obtain enyne **12**, whose triple bond was regioselectively hydrated to the corresponding carbonyl group, thus completing the incorporation of the acetyl group at C-2, to give *N*-Boc anatoxin-a (**13**). Then, the carbonyl group was condensed with 2 (aminooxy)acetic acid under basic conditions to give the (carboxymethoxy)imino derivative **14**, which was transformed into the trifluoroacetic acid salt of the desired succinimidyl active ester, ANc–NHS·CF₃CO₂H, as described above for hapten ANm. The synthesis of the active ester of hapten ANc required eleven steps from **1** and proceeded in 21% overall yield.

The preparation of the *N*-hydroxysuccinimidyl ester of hapten ANn had to be obviously approached using a different strategy: we first prepared the hapten with the free carboxylic group, followed by its activation. The synthesis of hapten ANn started from enyne **12**, previously obtained as an intermediate of the synthesis of hapten ANc–NHS (Scheme 1). It initially involved the incorporation of the linker chain by means of an *N*-alkylation reaction, which was done by cleavage of the *N*-Boc group of **12** with trifluoroacetic acid to give the trifluoroacetate ammonium salt **16**, followed by treatment with *tert*-butyl 4-bromobutanoate in basic medium. The transformation of the ethynyl group of the *N*-alkylated enyne **17** into the acetyl moiety was carried out by hydration reaction of the triple bond, in the same way as described above for the transformation of **12** into **13**, to give the *N*-alkylated anatoxin-a derivative **18**. Finally, the synthesis of hapten ANn was completed by removal of the *N*-Boc protective group with trifluoroacetic acid, which directly provided compound **19**, the trifluoroacetate ammonium salt of hapten ANn. Globally, the synthesis of hapten ANn from **1** involved twelve synthetic steps, with an overall yield of about 16%. Various methods for the activation of the carboxylic group of **19** were evaluated. Among all of the tested procedures, the most efficient one involved the use of EDC·HCl and *N*-hydroxysuccinimide in CH₂Cl₂. However, all attempts to isolate the desired compound ANn–NHS were unsuccessful, so the crude reaction mixture obtained by vacuum evaporation of the solvent had to be directly used in this case for the preparation of protein conjugates.

Immunizing bioconjugates with BSA and coating bioconjugates with OVA were readily prepared by mixing a DMF solution of the obtained hapten active esters with a buffered solution of the corresponding carrier protein. After purification of the bioconjugates by size exclusion chromatography, the number of incorporated hapten molecules was determined by MALDI-TOF/MS. The obtained hapten-to-protein molar ratios (MR) for the immunogens were 16.1, 14.5, and 8.1 for BSA–ANc, BSA–ANm, and BSA–ANn, respectively, whereas the MRs for the coating bioconjugates were 7.6, 8.0, and 3.4 for OVA–ANc, OVA–ANm, and OVA–ANn, respectively (Figure 2).

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Scheme 1. Synthesis of ANm-NHS, ANc-NHS and ANn-NHS esters. Reagents and conditions: [a] Pd(OH)₂/C, Boc₂O, EtOAc, H₂ 60 psi; [b] i. KHMDS, THF, -78 °C. ii. N-(5-Chloro-2-pyridyl)bis(trifluoromethanesulfonimide) (Comins' reagent); [c] Methyl hex-5-ynoate, PdCl₂(PPh₃)₂, CuI, Et₃N, DMF, rt; [d] HgO, BF₃·Et₂O, cat CCl₃CO₂H, MeOH, rt; [e] LiOH, THF-H₂O, rt; [f] EDC·HCl, NHS, CH₂Cl₂, rt; [g] CF₃CO₂H-CH₂Cl₂ (1:1), rt; [h] Ethynyl-trimethylsilane, PdCl₂(PPh₃)₂, CuI, Et₃N, DMF, rt; [i] K₂CO₃, MeOH, rt; [j] HgO, BF₃·Et₂O, cat CCl₃CO₂H, MeOH, rt; [k] H₂NOCH₂CO₂H·½ HCl, Py, EtOH, rt; [l] Br(CH₂)₃CO₂tBu, Cs₂CO₃, MeCN, 50 °C; [m] PTSA, acetone, rt.

While the obtained MR values for the conjugates of haptens ANc and ANm were as expected, the attained loading values for hapten ANn conjugates were rather low, which was probably due, in part, to the one-pot procedure employed for coupling, but also

to partial hydrolysis of the *N*-hydroxysuccinimidyl ester moiety during the conjugation reaction. We believe that the competitive hydrolysis of the active ester – commonly taking place during conjugation in aqueous medium – may have been facilitated in

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this case by the participation of the amino/ammonium bridge group, which can intramolecularly catalyze the hydrolysis reaction of the active ester group.^[21] Finally, we also coupled haptens ANc-NHS and ANm-NHS to the enzyme HRP, and MR values of 2.4 and 3.6 were obtained (Figure S1).

Figure 2. MALDI-TOF mass spectra (singly charged ions) of proteins (blue) and the corresponding conjugates with haptens ANc (green), ANm (orange) and ANn (cyan). A) BSA conjugates; B) OVA conjugates.

Three groups of four mice each were immunized with BSA-ANn, BSA-ANc, or BSA-ANm. Good immune responses were found with bioconjugates BSA-ANm and BSA-ANc, whereas low antibody titers were induced by the bioconjugate BSA-ANn. Accordingly, hybridoma generation was carried out only with splenocytes from mice immunized with the bioconjugates BSA-ANm and BSA-ANc. After screening more than 5000 cell culture supernatants from mice immunized with conjugate BSA-ANc, we could not identify hybridomas that secreted antibodies significantly recognizing anatoxin-a. However, from mice immunized with bioconjugate BSA-ANm, five hybridomas able to

tightly bind the cyanotoxin were found from two independent cell fusions. Monoclonal antibodies from these five unique cell lines were characterized by competitive ELISA in two formats, the conjugate-coated indirect format and the antibody-coated direct format. Remarkably, these five antibodies were able to bind anatoxin-a with nanomolar affinity values in the two ELISA formats (Table 1).

Table 1. Characterization of the monoclonal antibodies to anatoxin-a by competitive ELISA

mAb	Indirect ELISA ^[a]		Direct ELISA ^[b]	
	A_{max}	IC_{50} (nM)	A_{max}	IC_{50} (nM)
#38	0.71 ± 0.05	3.11 ± 0.28	1.64 ± 0.24	2.70 ± 0.46
#39	0.85 ± 0.15	3.84 ± 0.12	1.01 ± 0.21	3.96 ± 0.32
#44	0.92 ± 0.17	2.65 ± 0.58	1.20 ± 0.40	2.40 ± 0.60
#325	0.80 ± 0.05	3.41 ± 0.42	1.31 ± 0.23	2.88 ± 0.97
#417	0.83 ± 0.03	6.88 ± 0.06	1.00 ± 0.49	6.38 ± 3.23

[a] Microplates were coated with bioconjugate OVA-ANm at 0.1 $\mu\text{g/mL}$, except for the mAb#39 for which coating was carried out at 0.01 $\mu\text{g/mL}$. Antibodies were used at 30–100 ng/mL ($n=3$). [b] Microplates were coated with each antibody at 1 $\mu\text{g/mL}$, and the enzyme tracer HRP-ANm was used at 300 ng/mL ($n=3$).

Figure 3. Recognition of anatoxin-a and homoanatoxin-a enantiomers by mAb#44 using direct competitive ELISA. Inhibition curves are the mean of 3 independent experiments performed in different days. The binding properties of the rest of antibodies were very similar, so they are not depicted for clarity.

Homoanatoxin-a is a structural analogue of anatoxin-a with an extra methylene group at the pendant C-2 substituent (see Figure 1). Because of this structural similarity, homoanatoxin-a and anatoxin-a share almost identical toxicological properties, although the first one has been less extensively studied.^[22] In addition, both cyanotoxins exist in two enantiomeric forms, being the dextrorotatory (+) forms the only ones that are naturally found. In order to evaluate antibody selectivity, the two enantiomers of both cyanotoxins, previously synthesized in our laboratory,^[23] were tested against the five monoclonal antibodies for their ability to recognize them. Remarkably, all of the antibodies were able to bind the (+)-enantiomers with an affinity at least 100 times higher than the (–)-enantiomers, thus emphasizing the great enantioselectivity of the obtained monoclonal antibodies (Figure 3). It is also worthwhile to mention that all of the antibodies bound (+)-homoanatoxin-a with the same affinity than (+)-anatoxin-a.

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If we have a look to the structures of both cyanotoxins and hapten ANm, this finding was rather expected because the structure of hapten ANm is equally congruent with both cyanotoxins.

In summary, the generation of monoclonal antibodies exhibiting affinity to the natural enantiomers of both anatoxin-a and homoanatoxin-a in the low nanomolar range are reported herein for the first time. Among the three functionalized derivatives of the cyanotoxin synthesized for this purpose, hapten ANm emerged as the only one capable of attaining such a goal. This technological achievement paves the way to the development of diverse immunoanalytical systems for the rapid and on-site analysis of these potent biological toxins in environmental waters, thus contributing to the safety of water supplies, to human and animal health, and to the knowledge about the worldwide incidence of cyanobacterial harmful algal blooms and their toxic consequences.

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Conflict of interest

The authors declare no conflict of interest. The immunoreagents herein reported are available upon request for evaluation and research purposes. These developments have been patented (WO2017085339A1) and an exclusive license has been granted to Abraxis, Inc (Pennsylvania, USA) for the production and commercialization of immunoanalytical methods for anatoxin-a.

Keywords: hapten design • rapid methods • biotoxin • antibodies • immunoassays

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Guillermo Quiñones-Reyes, Consuelo Agulló, Josep V. Mercader, Antonio Abad-Somovilla,* Antonio Abad-Fuentes*

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Not any hapten works for the generation of anti-anatoxin-a monoclonal antibodies. A conveniently functionalized derivative of anatoxin-a allowed the long-standing goal of producing the first-ever reported immunoreagents (bioconjugates and antibodies) suitable for the point-of-care detection of this harmful cyanotoxin.

Synthetic Haptens and Enantioselective Monoclonal Antibodies to the Cyanotoxin Anatoxin-a

Toxic algae blamed for elk deaths in northeastern New Mexico