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Monoclonal Antibodies with Orthogonal Azaspiracid Epitopes

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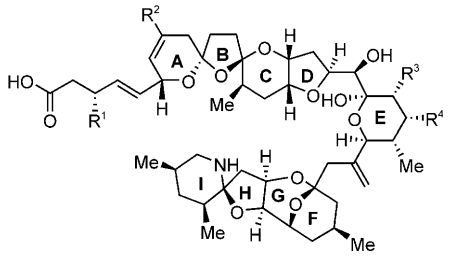
An incident of human illness with diarrhetic shellfish poisoning (DSP)-like symptoms reported in the Netherlands in 1995 was eventually traced back to mussels, *Mytilus edulis*, harvested from Killary Harbour, Ireland.[1] The causative toxins of this outbreak were found to be the azaspiracids (**1**–**11**, Table 1),[2]

Table 1. Structure of azaspiracids-1 through -11 (**1**–**11**).



	R^1	R^2	R^3	R^4
1: azaspiracid-1	H	H	Me	H
2: azaspiracid-2	H	Me	Me	H
3: azaspiracid-3	H	H	H	H
4: azaspiracid-4	OH	H	H	H
5: azaspiracid-5	H	H	H	OH
6: azaspiracid-6	H	Me	H	H
7: azaspiracid-7	OH	H	Me	H
8: azaspiracid-8	H	H	Me	OH
9: azaspiracid-9	OH	Me	H	H
10: azaspiracid-10	H	Me	H	OH
11: azaspiracid-11	OH	Me	Me	H

which have been proposed to originate from the dinoflagellate *Protoperidinium crassipes*. Through heroic efforts, and with minute amounts of material, the Yasumoto and Satake group proposed a structure for azaspiracid-1;[4] however, total synthesis found the originally proposed structure to be incorrect,[5] and degradative and synthetic efforts found the structure to be as depicted in Table 1.[6] Containing a trioxadispiroacetal system fused onto a tetrahydrofuran ring (ABCD domain) and an azaspiro ring system fused onto a 2,9-dioxabicyclo[3.3.1]-nonane system (FGHI domain), the azaspiracids represent a unique toxin both in terms of structure and toxic effects.[1,7]

While initially postulated to resemble diarrhetic shellfish poisoning, further study demonstrated that azaspiracid poisoning (AZP) has become a widespread problem throughout Europe,[8] with the potential to become a worldwide phenomena. Indeed, various analogues of azaspiracid have since been detected in waters off of western Europe, Morocco, and eastern Canada.[2] As a result, detection methods have been sought to ensure the safety of the seafood in which the azaspiracids are often found. Currently, the EU regulatory limit for azaspiracids is 0.16 μg per g of total shellfish tissue,[9] meaning a limit of detection for a given method should be less than that level, yet retain the ability to test for all members of the azaspiracid family. The current methods for detecting and quantifying azaspiracids are mass spectrometry and mouse toxicity assays,[10] but these methods are less desirable due to the required amounts of azaspiracid needed as a reference, or the use of animals. Additionally, studies have suggested that the azaspiracid reference test has, at best, a 50% chance of detecting the toxin at the EU regulatory limit.[2] Immunodiagnostics provide an alternative platform that can be readily and cost effectively implemented in high-throughput screening scenarios. Recently, the Forsyth and Miles groups have reported the isolation of polyclonal ovine antibodies from a synthetic hapten consisting of the azaspiracid FGHI domain that could recognize the parent toxin.[11] However, since these antibodies are polyclonal, a detection kit using solely this polyclonal sera is not ideal (vide infra). We hypothesized that monoclonal antibodies with distinct recognition epitopes could be generated by using the entire azaspiracid molecule for immunization. These antibodies would serve as the foundation of a general azaspiracid detection method for all members of this class of marine toxins while not requiring authentic samples of the molecule as a standard.

Monoclonal antibodies with orthogonal epitopes can be employed in antibody-based capture assays (that is, "sandwich" assays). These assays require two distinct antibody populations for detection. The first antibody is immobilized onto the solid support and serves to capture the desired analyte out of solution, while the second is conjugated to a suitable reporter enzyme (for example, horseradish peroxidase or alkaline phosphatase) that allows for secondary signal amplification through substrate turnover (Scheme 1). It is desirable for the first antibody to be monoclonal as all bound antigen is uniformly displayed; this maximizes the recognition and, consequently, the

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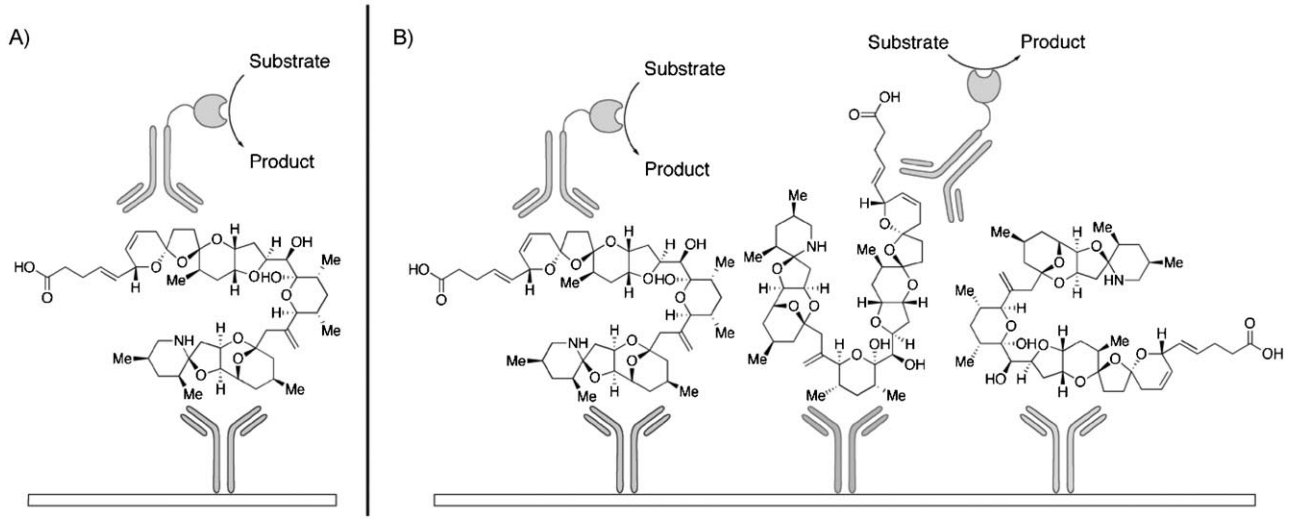

Scheme 1. Schematic depiction of antibody-based azaspiracid detection systems. A) Assay in which the capture antibody (bottom) is monoclonal and, thus, all azaspiracid molecules are uniformly oriented so as to maximize the analytical signal. B) System in which the capture antibody (bottom) is polyclonal. In this system, multiple conformations of the molecule can be displayed to the detection antibody, compromising the output signal, particularly in cases where the capture and detection antibodies share a common epitope (far right).

output signal that results from addition of the secondary detection antibody. The secondary antibody need not be monoclonal, but a monoclonal antibody can further enhance the analytical signal provided that the capture and detection antibodies have orthogonal epitopes.

Small molecules such as the azaspiracids are inherently nonimmunogenic and therefore require covalent coupling to a carrier protein to elicit an immune response. In many cases, coupling of a small molecule to carrier proteins is achieved through the addition of a nonnative functionality that can be chemically reacted with the side chains of the carrier (e.g., Lys residues). This requirement can be difficult to fulfill in the case of complex natural products, particularly when a semisynthetic route for the preparation of modified material is not readily apparent. Fortunately, examination of the azaspiracid structure reveals that no modification of the parent molecule is required for coupling, as the terminal carboxylic acid can be exploited in standard *N*-hydroxysuccinimide (NHS)-based coupling procedures. By using this handle, we coupled synthetically prepared azaspiracid-1[6] to bovine serum albumin (BSA) and keyhole limpet hemocyanin (KLH) through carrier protein lysine residues; the former immunoconjugate was to be used in antibody screening procedures, while the latter was used to immunize mice for antibody production.[12]

Surprisingly, mice immunized with **1**-KLH were found dead ~1 day after immunization. The toxic effects were reproducible, with all animals ($n=4$) succumbing from immunization across two groups. This finding was particularly intriguing as, to our knowledge, there are no reported examples of potent toxicity resulting from administration of the immunoconjugate of a toxic molecule. Indeed, it is this exact premise that has formed the basis of many immunodiagnostics, and immunization has been previously utilized for the generation of antibodies against a wide range of potentially toxic molecules, with immunoassays for various pesticides providing an illustrative example.[13] There are three scenarios which could be applied to this phenomenon: slow release of noncovalently bound azaspiracid from the carrier protein, in vivo degradation of the azaspiracid hapten into a pharmacologically active fragment, or toxicity resulting from the action of the azaspiracid immunoconjugate itself. To address the former possibility, all proteins were sterile filtered and extensively dialyzed multiple times prior to injection, yet this did not diminish the toxicity. Dialyzed immunoconjugates were then purified by size exclusion chromatography and analyzed for **1** by ESI-TOF MS. Only trace levels of **1** were detected, correlating to an injection of ~75 ng of uncoupled **1** in any single immunization. Given that the known lethal dose of **1** is ~4 μg for a 20 g mouse, the observed immunoconjugate toxicity cannot be attributed solely to residual azaspiracid-1 from the coupling reaction.

The molecular weight of KLH ranges from 4.5×10^5–1.3×10^7 Da as a result of aggregation state, and as such, it is extremely difficult to determine the exact number of azaspiracid molecules present on this protein. The extent of immunoconjugate coupling is generally estimated by MS analysis of the corresponding BSA immunoconjugate; this provides a lower limit for hapten copy number. In the case of **1**-BSA, it was determined that six azaspiracid moieties were coupled. Using this number, it was estimated that at least 1.2 μg of azaspiracid-1 was administered with each injection, albeit covalently conjugated to a carrier protein that by itself has no reported toxicity. It is likely that at least ten times this amount of azaspiracid was injected as KLH is known to display hundreds of surface lysine residues available for coupling. Thus the quantity of toxin immobilized on KLH does exceed the lethal dose. While this phenomenon is certainly of interest, we turned our attention away from the mechanism of toxicity and instead focused on vaccination strategies that would allow for anti-**1** antibody isolation.

Immunoconjugate toxicity is an unprecedented phenomenon and presents a significant roadblock to the procurement of monoclonal antibodies. Additionally, azaspiracid has been shown to result in necrosis of B and T lymphocytes in lymphoid organs, including the spleen, thymus, and Peyer's patches;[14b] this raises concern that the generation of high antibody titers would be difficult, if not impossible. We speculated that if the dose per injection could be reduced, titers against azaspiracid-1 still could be obtained; thus, we next turned to alternative immunization protocols. Empirically, we determined that a tenfold reduction in the amount of **1**-KLH in each injection (10 μg immunoconjugate/mouse/injection) mixed with Ribi adjuvant system (RAS) could be tolerated by mice without toxicity; however, even though the pronounced mortality was abrogated with this lower dose, the animals continued to display signs of reaction to the conjugate (for example, fur ruffling).

At this dose, appreciable titers could be observed after 21 days (1:1600–1:3200 per mouse) by employing a standard regimen of initial immunization, followed by a single booster injection in RAS at day 14. While the titer obtained was not suitable for monoclonal antibody production, this data did validate our hypothesis that a dose reduction could result in antibody titers against **1**-KLH. In order to increase the titer, the mice were boosted again with 10 μg of **1**-KLH in a suspension of alum adjuvant at day 35, and then bled at day 42. At this stage, antibody titers had increased slightly (1:3200–1:6400 per mouse). After an additional month, animals were again boosted with 10 μg of **1**-KLH in alum, at which point the titers had ceased to increase. At this time, spleens were harvested and used to generate antibody-secreting hybridomas.

Despite the low dose immunization protocol required by the toxicity of the immunoconjugate, a relatively large panel of 67 antibodies were obtained (Table 2). Roughly half of these antibodies showed good titer against the **1**-BSA immunoconjugate ($\geq$1:6400); this demonstrates the overall specificity for the hapten across the entire panel. In general, most antibodies within the panel bound both **1** and **2** with high affinity ($K_{d\,app} <$ 1 μM) with the tightest binding observed for mAbs 6B11 and 9E8, both of which bound azaspiracid-1 with a relative binding affinity of 0.2 μM (Table 2). These two antibodies also displayed good binding for azaspiracid-2, as expected based upon the structural similarity of these two molecules (Table 2).

Table 2. Ten tightest binding monoclonal antibodies procured from murine immunization of 1-KLH.

mAb	Isotype	$K_{d\,app}$ **1**[a] (μM)	$K_{d\,app}$ **2**[a] (μM)	$K_{d\,app}$ **12**[a] (μM)
2G2	κγ2α	0.6	0.6	9.4
2H3	κγ2α	0.6	0.6	2.4
2H6	κγ2α	0.3	0.8	>50
6B11	κγ2α	0.2	0.3	18.8
8F4	κγ2α	0.6	0.6	2.4
9A12	κγ2α	0.6	0.8	>50
9E8	κγ2α	0.2	0.6	>50
10G2	κγ2α	0.3	0.4	50
14H7	κγ1	0.6	0.8	4.7
15D7	κγ2α	0.6	0.8	>50

[a] $K_{d\,app}$ determined by competition ELISA. In all cases, the ELISA plate was coated with **1**-BSA and the desired small molecule competing antigen was added at a range of concentrations from 50 nM to 50 μM. $K_{d\,app}$ values are defined as the concentration of competing antigen that results in half-maximal OD_{400}.

In order to select antibodies with orthogonal azaspiracid epitopes, each antibody must be characterized so as to identify a pair that can bind simultaneously to antigen. With most small molecules, this is an extremely difficult task as the sheer size of the antibody molecule inherently prevents a second antibody from binding due to steric hindrance. In the case of azaspiracid, we felt that provided the epitopes of the two antibodies did not overlap, the molecule was of sufficient size to have one antibody bind to some portion of the ABCDE ring system, while a second antibody could recognize the FGHI ring system. To test this hypothesis, the panel of monoclonal antibodies was screened for binding to the FGHI fragment **12** of azaspiracid by competition ELISA (Figure 1). This region was selected

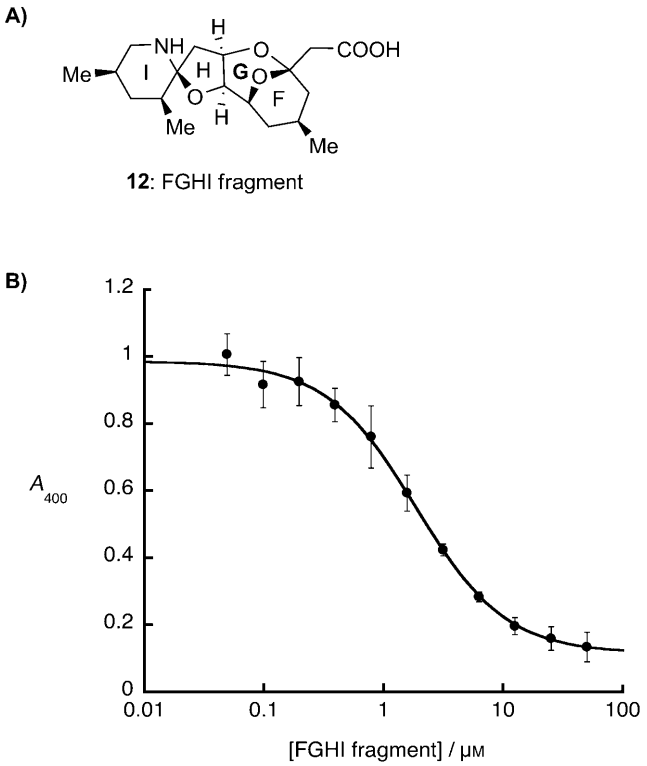

Figure 1. A) Molecular structure of FGHI fragment **12** used in competition ELISA screening. B) Representative competition ELISA data using the FGHI fragment and mAb 8F4.

for study as it is conserved across all known members of the azaspiracid class of molecules and has previously been shown to be immunogenic;[11] this suggests that this could be a dominant epitope within the panel, and antibodies with this epitope could serve as capture reagents in a sandwich assay. Binding of a given monoclonal antibody to the FGHI fragment implies that the epitope of the antibody is at least partially contained within the FGHI ring system, whereas weak or no

binding indicates the epitope must reside within the ABCDE domain. Screening of the ten antibodies that bound **1** or **2** the tightest showed that six antibodies possessed a FGHI epitope, while four others possessed an epitope that was comprised of some portion of the ABCDE domain (Table 2).

Given the differences between the molecular structure of **12** and **1**, we cannot discount the possibility that the nonnative carboxylic acid prevents binding of a given mAb to **12**, thereby biasing our results. Therefore, the epitopes of these antibodies were also assessed in a direct binding assay where **12** was covalently bound to amino-modified microtiter plates through the carboxylic acid moiety to generate an electronically neutral amide linkage. In agreement with the competition ELISA experiments, mAbs 2H6, 9A12, and 9E8 showed extremely poor binding to the FGHI epitope presented by **12**, while mAb 2H3 possessed the highest titer for **12**. Interestingly, while mAb 15D7 was deemed a poor binder by competition ELISA, the direct binding assay showed high titer for **12**, indicating that the carboxylic acid moiety does indeed interfere with the ability of this mAb to bind its antigen.

The results of this study demonstrate the application of the total synthesis of complex and scarce natural products to the preparation of reagents that have utility in clinically relevant diagnostics. Despite the relatively small size of the azaspiracids, antibodies were generated that have nonoverlapping epitopes, a primary requirement in the development of a sandwich ELISA-based detection system. Furthermore, the ability to select antibodies against multiple regions of this small molecule from a single immunization also argues that the azaspiracid molecular structure has sufficient in vivo stability to be recognized and processed by the immune system. By using the entire natural product as the hapten, antibodies could be generated from a single fusion against mutually exclusive epitopes. These antibodies can now be utilized in a combinatorial fashion for the development of an optimized and efficient assay for a potent marine toxin that poses a threat to public health. The results of these studies will be reported in due course.

Experimental Section

Immunoconjugate preparation: Azaspiracid-1 (5 mg) was treated with 3-sulfo-*N*-hydroxysuccinimide (1.3 equiv) and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (1.3 equiv) for 24 h in DMF. This reaction was then added to solutions (5 mg mL^{-1}) of KLH and BSA in PBS (50 mM, pH 7.4), respectively, and allowed to react 24 h at 4 °C. After reaction, unreacted azaspiracid was removed by dialysis (50000 molecular weight cut-off filter) against PBS.

Murine immunizations: All studies were reviewed and approved by the Scripps Institutional Animal Care and Use Committee. A group of two mice (129GIX+, eight weeks of age) were injected intraperitoneally with **1**-KLH (10 μg) in PBS which had been premixed with Ribi adjuvant system (RAS, 100 μL, 200 μL total volume). After two weeks, all mice received a booster injection identical to the initial immunization. Mice received two additional booster injections of **1**-KLH (10 μg) in PBS that had been premixed with alum (100 μL, 200 μL total volume) at days 35 and 70. One month later, an injection of **1**-KLH (10 μg) was administered intravenously, and the animals were then sacrificed and the spleens removed. Hybridoma generation followed from this point as per literature procedures.[12]

Competition ELISA experiments: An ELISA plate (96-well, CoStar, New York, USA) was coated overnight at 4 °C with **1**-BSA or **1**-KLH (125 ng) and then blocked with blocking buffer (4% skim milk powder in PBS, 50 μL) for 1 h at 37 °C. Typically, a 1:1000 dilution (25 μL) of the desired monoclonal antibody in blocking buffer containing serial dilutions of competing antigen was then added and incubated 1 h at 37 °C. After washing, a 1:1000 dilution (25 μL) of a goat-anti-mouse/horseradish peroxidase conjugate in blocking buffer was added and incubated for 1 h. The plate was developed with the colorimetric reagent 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid (ABTS) for 20 min and the absorbance measured on an ELISA plate reader at 400 nm. The $K_{d\,app}$ was defined as the concentration of competing antigen that resulted in one-half of the optical density measured in the absence of competing antigen.

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