Selection of DNA Aptamers for Mycotoxins

by

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A thesis submitted to the Faculty of Graduate and Postdoctoral Affairs in partial fulfillment of the requirements for the degree of

Master of Science

in

Chemistry: Chemical and Environmental Toxicology

Carleton University

Ottawa, Ontario

Abstract

Mycotoxins deoxynivalenol (DON) and patulin (PAT) are toxic fungal metabolites that contaminate food commodities worldwide. They pose health risks for humans and animals, and have a negative impact on the economy. Mycotoxin detection is vital to ensure public safety. Although traditional detection techniques for mycotoxin detection (e.g. LC-MS) have proven to be highly selective and sensitive, simpler, more rapid, and more cost-effective approaches are required. Aptamer-based techniques are a possible solution. Aptamers are oligonucleotides that can bind to molecules with high affinity and specificity, generated via an *in vitro* process known as SELEX. We attempted to generate aptamers for DON and PAT. The selection process for DON was challenging and it would be beneficial to continue SELEX, whereas the process for PAT was simpler thus sequences for potential aptamers were obtained. This work has contributed to the production of aptamer-based assays and biosensors for mycotoxin detection.

Acknowledgements

I made it to the end of my Master's degree but not without a lot of hard work, determination, and most importantly, the continued support from a long list of people.

First and foremost, I must acknowledge my supervisor Dr. Maria DeRosa. I know she had to exercise a lot of patience with me as I learned, and continue to learn, how to do research like a scientist. She brought out my strengths and helped me overcome my weaknesses. In addition to all that, she has provided me with learning and research opportunities that I will never forget. Her passion and enthusiasm are truly inspiring, and I am very grateful to have had the chance to work for her!

My lab mates – Eman, Erin, Phepa, Nadine, and Emily (in order of desk arrangement). Spending every day with you girls made those hard days in the lab livable. Our lunchtime breaks rarely passed without a good laugh. When my SELEX would go wrong for no apparent reason or when I would forget to clamp my gels before pouring, you would also get angry alongside me or give me encouraging words so I wouldn't quit. Of course, I can't forget the men of the lab; I've always enjoyed a good laugh with Christopher, Selvan, and Dr.Velu. If I ever needed a hand, they always willingly offered their time to help. A special bond was formed and I'm grateful for meeting all of you.

I must also thank two professors in particular; Dr. Miller and Dr. Ianoul. Dr. Miller, who helped guide me towards the research area of aptamers and mycotoxins, an area in which I have become very much interested. I will always be grateful for his words of advice and encouragement. Dr. Ianoul, my undergraduate supervisor, who gave me my first opportunity to work in a lab in my second year of University. Everything was new to me and I had a lot of learning to do. His

patience and guidance has shaped the research student I am today. But I must also thank the Ph.D student who walked me through all the experiments and answered all my questions patiently and with a smile; Amy, thank you for all your help!

I also need to thank my wonderful siblings. When you're around me, I forget about all my stresses and worries. I can always count on you for a good laugh and a fun time. Plus, you snap me back to reality, something I need often – writing a thesis is not the end of the world! Betty, I just have to think about how easy going you were when writing your thesis and I start to relax!

And of course, my grandparents; those here and in spirit, who have always encouraged me to pursue an education and at the same time reminded me to always make time for family.

A special note goes to my incredible parents- you have always been there for me and have shaped me into the person I am today. You offer a level of comfort that no one else can, especially during moments of stress and breakdowns (!). I'll never forget the small gestures such as bringing me espresso and treats while I worked, and giving me a free pass for not having to help clean the house during the busy periods with school work. You have done so much for me and my siblings, and continue to do so. I wouldn't be where I am today without you. I owe you everything and could never thank you enough. I love you so much.

Last but not least, my husband Justin; we've come a long way since we've met and I couldn't be happier with the way things have worked out. You've been so very patient and loving as I finished my undergraduate and now graduate degree. You've seen me at my worst, and somehow you still love me. As I finish writing this paragraph I end a chapter of my life, and I look forward to starting the next one with you in our new home. I love you.

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List of Abbreviations

ADON – Acetyldeoxynivalenol

ALISA – Aptamer-Linked Immobilized Sorbent Assay

APS – Ammonium Persulfate

ARfD – Acute Reference Dose

B.w – Body Weight

CD – Circular Dichroism

CDI – 1,1'-Carbonyldiimidazole

CEN – European Committee for Standardization

CGA – Chaetoglobosin A

CPG – Controlled Pore Glass

CTN – Citrinin

DADPA – Diaminodipropylamine

DMF – Dimethyl formamide

DMSO – Dimethyl Sulfoxide

DON – Deoxynivalenol

DSB – DON Selection Buffer

EDC – 1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride

ELAA – Enzyme-linked Aptamer Assay

ELISA – Enzyme-Linked Immunosorbent Assay

EU – European Union

FAO – Food and Agriculture Organization

FDA – Food and Drug Administration

FHB – Fusarium Head Blight

GC – Gas Chromatography

HPLC – High-Performance Liquid Chromatography

HRP – Horseradish Peroxidase

IPCS – International Programme on Chemical Safety

JECFA – Joint FAO/WHO Expert Committee on Food Additives

K_D – Dissociation Constant

k_{off} – Dissociation rate

k_{on} – Association rate

LD_{50} –Lethal Dose, 50%

LFD – Lateral Flow Device

MS – Mass Spectrometry

NHS – *N*-hydroxysulfosuccinimide

NMR – Nuclear Magnetic Resonance

PAT – Patulin

PEG – Polyethylene glycol

PCR – Polymerase Chain Reaction

PMTDI – Provisional Maximum Tolerable Daily Intake

SELEX – Systematic evolution of ligands by exponential enrichment

SPR – Surface Plasmon Resonance

TBE – Tris Borate EDTA

TEMED – Tetramethylethylenediamine

TLC – Thin-Layer Chromatography

UNEP – United Nations Environment Programme

UV – Ultraviolet

WHO – World Health Organization

Chapter 1 – Introduction

1.1 Aptamers

1.1.1 Properties and Structure

DNA is commonly recognized for its role as the biological molecule that stores hereditary and biological information. DNA is not generally seen as an affinity probe or molecular recognition tool used as drugs or biosensors, which is usually the territory of proteins. This is because the idea to use them that way was not really thought of or acted upon until the early 1990's; Craig Tuerk and Larry Gold[1] pioneered the use of synthetic oligonucleotides, termed aptamers, for binding to molecular ligands.

Aptamers (from the latin word *aptus* meaning "to fit" and from the greek work *meros* meaning "part") [2] are synthetic single-stranded oligonucleotides (DNA or RNA), which fold into 3-dimensional structures that bind to targets. These oligonucleotides do not bind their target by Watson-Crick base pairing but via other non-covalent interactions such as hydrogen bonds, Van Der Waals forces, and electrostatic and stacking interactions. Aptamers are capable of binding with high affinity and specificity to their target, thus their dissociation constants can be in the nanomolar range, and can discriminate for their specific target with high precision. In fact, aptamers can differentiate enantiomers and targets that differ from a single functional group[3]. Aptamers can theoretically bind to a wide range of molecular targets. These targets include small molecules such as mycotoxins[4], amino acids[5], metal ions[6], and larger molecules such as enzymes[7], viral proteins[8], whole cells[9], viruses[10], and bacteria[11].

There are common secondary and tertiary structures which aptamers can adopt. Depending on primary sequence and salt concentration, common secondary structures include bulge, hair-pin loop, g-quadruplex [12], short helical loops. An image with two of these structures is found in

figure 1.1. The combination of these structures dictates the 3-dimensional form which allows for binding to the target. Examples of tertiary structures include two types of coaxial stacking - pseudoknots[13] and kissing loops[14].

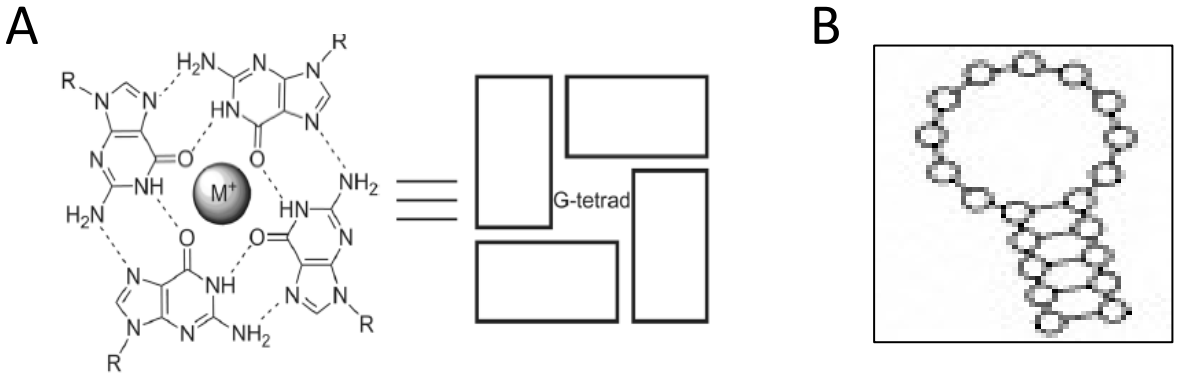


Figure 1. 1 Examples of possible aptamer secondary structures. A) G-tetrad stabilized by a cation in the center and Hoogsteen bonds between guanines. The stacking of G-tetrads forms a G-quadruplex. Reproduced with permission by John Wiley and Sons[12]; B) stem loop.

The secondary structures of aptamers in the absence of their targets can be predicted[15] by softwares such as Mfold and RNAStructure, this is due to the fact that DNA and RNA secondary structures result from strong interactions such as Watson-Crick and Hoogsteen base pairing as opposed to weaker interactions involved in tertiary structure folding[15]. Nonetheless, the tertiary structure is important to determine. The more information that is known about the structure of aptamers and the types of targets they bind to, the easier it would be to predict the 3-dimensional conformation. Methods for elucidating the tertiary structure of DNA and RNA include X-ray crystallography[16], circular dichroism (CD) spectroscopy[17], and nuclear magnetic resonance (NMR)[18].

1.1.2 Systematic Evolution by Exponential Enrichment (SELEX)

Aptamers are generated from an *in vitro* process known as the Systematic Evolution of Ligands by EXponential enrichement (SELEX); SELEX is a multi-step process in which strongly binding ligands are selected and amplified by polymerase chain reaction (PCR). All SELEX experiments begin with a large oligonucleotide library, which can consist of $10^{13} – 10^{16}$ unique sequences[19] of the same length consisting of a random region of nucleotides flanked by shorter regions of known sequence. The length of the random region ranges from 30 to 45 bases[20]. The longer the random region, the more variety of opportunities for folding and the higher chance of generating an aptamer for a specific target; however, increasing the length also means increasing the costs for production. Therefore, there is a balance between folding opportunities and cost. The short known regions are for use in PCR, amplifying the sequences after each round. It is the hope that at least one of these sequences binds strongly and selectively to the target of interest. SELEX is divided into three major steps: 1) Selection, where the pool is incubated with target; 2) Partitioning, where the sequences which bind the target are separated from the sequences which do not bind, and, the aptamer-target complex is separated; and 3) Amplification, where the sequences which bound are amplified for another round, creating a more refined library. An illustration of SELEX can be found in Figure 1.2. Many rounds can be performed for a single target.

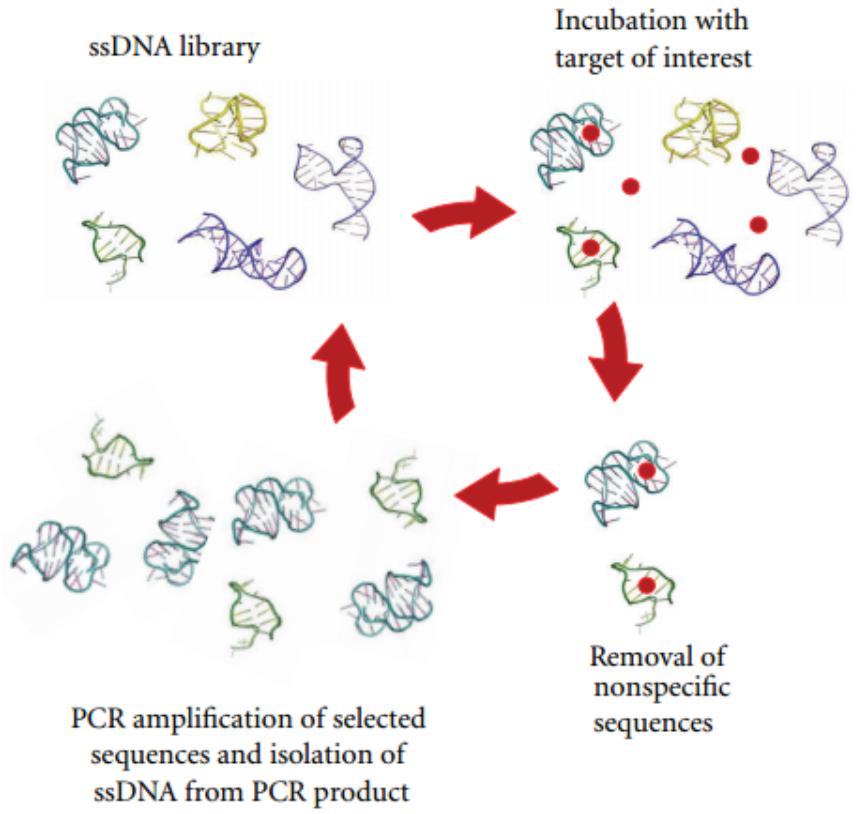


Figure 1. 2 An illustration of Systematic Evolution of Ligands by EXponential enrichment (SELEX) for selecting and amplifying DNA sequences which bind to a target of interest. Reproduced with permission via Creative Commons[21].

There are numerous methods of performing SELEX and a comparison of these modifications can be found in Table 1.1.

Table 1. 1 Different SELEX methods[19].

SELEX METHODS	PROS	CONS
Nitrocellulose filter binding	Selection is easily carried out in solution and no special equipment is required.	Many selection rounds required (up to 20) and high probability of non-specific binders.
Bead-based	Can be used for most targets (small molecules to cells) and not many rounds required (1-6).	Target or aptamer must be immobilized, less surface for binding.
Electrophoretic	Quick separation of bound and unbound aptamers.	Smaller DNA library can only be used (10^{12} sequences). Target must cause electrophoretic shift on aptamers.
Microarray-based	Target is free in solution and aptamer is immobilized. Can also be used as a large scale binding assay.	Aptamer sequences need to be known, therefore there is limited capacity for aptamer library ($<10^5$ sequences). Only for ssDNA and is for single target selections.
Microscopic	Selection can be done in one round.	Small DNA library can only be used ($<10^8$ sequences). Requires special equipment such as atomic force microscope, and requires immobilization of target or pool.

Table 1. 1 (Continued)

SELEX METHODS	PROS	CONS
Cell-SELEX	Aptamers could eventually be used as therapeutics.	Limited to targets found on the cell and non-specific binding is probable.
In Vivo SELEX	Aptamers selected *in vivo,*	Small DNA library can only be used (10^4 sequences). Non-specific binding is probable.
Small molecule SELEX	No immobilization of small molecule target required.	Many selection rounds required. Aptamer release from solid phase depends on change in DNA conformation from target induced binding.

SELEX can be modified in various ways for efficiency and to increase stringency of the selection process such that the quality of the resulting aptamer is high. Stringency is defined as creating standards of performance that are rigorous. In other words, increasing stringency is setting conditions which make it harder for DNA to bind to the target. Only sequences with high affinity would be able to bind in more stringent conditions. Several SELEX modifications and their descriptions can be found in Table 1.2.

Table 1. 2 Example modifications to the SELEX process[22].

TYPE OF SELEX	DESCRIPTION	REFERENCES
Negative SELEX	Pre-selection without the target. Removing non-specific binders from the pool.	23
Counter-SELEX	Exposing the pool to a molecule similar to the target to attract and remove non-selective or weak binders from the pool.	24
Blended SELEX	Addition of non-nucleic acid components to the sequences to extend aptamer properties.	25
Covalent SELEX	Sequences contain reactive groups which can bind covalently to a target protein.	26
Non-SELEX	The amplification stage is omitted.	27
EMSA SELEX	Electrophoretic mobility shift assay (EMSA) is used for partitioning.	28
Indirect SELEX	Selection target is not the actual binding partner of the aptamer, but binding occurs only in its presence.	29
Tissue SELEX	Method for generating aptamers which bind to complex targets found on tissues.	30

It is important to note that for SELEX to be efficient, target: DNA ratio must be taken into consideration. DNA concentration must exceed the amount of target available for selection to allow for competition among sequences; lower concentrations of target result in higher affinity aptamers[31].

1.1.3 Aptamer Stability

RNA and DNA aptamers are not very stable in serum due to their susceptibility to degradation by nucleases; yet their degradation can be seen as an advantage. Since they are short lived in serum and are cleared rapidly by the kidneys, they are considered non-toxic[32]. In addition, oligonucleotides are not recognized as foreign objects to the immune system, therefore they are also considered non-immunogenic[20].

Due to the absence of the 2' hydroxyl group on the deoxyribose sugar, DNA is more stable than RNA. As RNA is more susceptible to nucleases[33] it is generally required to modify RNA bases to increase stability, which can add to costs. Nucleases primarily attack pyrimidines, therefore protecting uracil and thymine is one option to lower nuclease susceptibility. Protection is carried out by replacing the 2' hydroxyl group with 2'-amino, 2'-fluoro, or 2'-O-methyl groups[32,34]. These modifications are particularly useful for aptamers applications in the therapeutic industry. One of the best known examples where an aptamer used as a drug is Pegaptanib sodium (Macugen). Approved in 2004 by the US Food and Drug Administration (FDA), Macugen is an RNA aptamer used to treat age-related macular degeneration, a condition which leads to loss of vision and which involves a specific isoform of vascular endothelial growth factor[32, 35]. The aptamer was modified with 2'-O-methylated purines and 2'-fluorine-pyrimidines to protect against endonucleases, and also with polyethylene glycol (PEG) and a 3'-dT for protection against exonucleases and to improve its pharmacokinetic properties. Before the modifications, the aptamer had a half life of 1.4 hr, whereas after the modifications, it was increased to 131 hr[32] The knowledge involved in getting Macugen to the shelves has paved the way for aptamers as therapeutics.

Although RNA is less stable than DNA, RNA is capable of folding into more conformations than DNA[32], making it more structurally diverse which leads to a higher chance of finding an aptamer for a target. However, the SELEX process for RNA aptamers involves extra steps, such as reverse transcription to and from DNA[19]; this makes selections with RNA less desirable as SELEX is already challenging and time-consuming.

1.1.4 Sequencing

Once the SELEX process has been completed, pools from selected rounds are sent for sequencing. The two most common methods of sequencing are by bacterial cloning (low throughput) and high throughput sequencing. The advantage of low throughput sequencing is the quick and easy preparation process, with results obtainable in a few days. The disadvantage, however, is cloning is performed with bacteria; the bacteria acquires a sequence at random, therefore the whole pool cannot be studied and one cannot easily compare sequences from other rounds. Since only a relatively small number of viable colonies are produced, one is limited in the amount of sequences obtained. The sequences which become available through cloning, which are likely the highest frequency sequences, may not be the best binders.

High throughput sequencing, on the other hand, is also relatively easy with minimal preparations required. A major advantage to this type of sequencing is that millions of sequences in the pool are revealed, thus multiple rounds can be compared to each other including the negative pool. Specific sequences which are found in the negative and positive pools of the final round of SELEX will likely be disregarded, as the replicate is an indication that there may have been contamination with non-specific binders in the positive pool. The ability to make a comparison between the sequences in the initial pool with the sequences of the middle and final selection

rounds is appealing, as the progress of enrichment is observable. Unfortunately, high throughput sequencing is much more expensive and requires substantially more time to receive results[36].

1.1.5 Binding Affinity (K_D)

A select number of sequences obtained from sequencing are chosen to test for their affinity to the target. The affinity is measured in terms of equilibrium dissociation constant, K_D, and can be represented by equation 1.1[37]

$$K_D = \frac{[A]\cdot[B]}{[AB]} \qquad \text{Equation 1.1}$$

where $[A]$ and $[B]$ are aptamer and target concentrations, respectively, and $[AB]$ is the aptamer-target complex. Binding is not all or nothing, which is why affinity can also be measured in terms of complex dissociation and association rates (equation 1.2)[37].

$$K_D = \frac{k_{off}}{k_{on}} = \frac{[A]\cdot[B]}{[AB]} \qquad \text{Equation 1.2}$$

where k_{off} and k_{on} are the complex dissociation and association rates, respectively. Equilibrium is reached when concentrations do not change. Different aptamers may have similar K_D's but different k_{on} or k_{off} values[37].

The K_D of aptamers is usually in the range of micromolar to picomolar, which is similar or even better than the affinity of antibodies to their antigens[38]. To measure the K_D, a fixed concentration of target is added to increasing concentrations of aptamer, or vice versa[37]. In the case where the initial aptamer concentration is constant and the target concentration increases, the amount of bound aptamer can be measured using equation 1.3.

$$f_a = \frac{[B]}{K_D + [B]} \qquad \text{Equation 1.3}$$

where f_a and [B] represent bound aptamer and target concentration, respectively. The equation forms a rectangular hyperbola, with one asymptote of f_a equal to 1, and K_D can then be measured using a non-linear regression analysis on the binding curve[37]. There are two types of methods for determining the affinity of the aptamer for the target; separation and mixture based[37]. Separation based methods include dialysis, ultrafiltration, gel and capillary electrophoresis, and HPLC, whereas mixture based methods include fluorescence polarization and anisotropy, UV-Vis absorption, circular dichroism, and surface plasmon resonance[37].

1.1.6 Aptamers vs. Antibodies

When it relates to affinity probes, antibodies have been the gold standard for years; monoclonal antibodies bind their targets with high selectivity and affinity. However, aptamers are becoming increasingly popular since their introduction in the early 1990s for a variety of reasons. Compared to antibodies, aptamers have the advantage by being synthesized *in vitro* as opposed to *in vivo*. Wherein the target of interest has to be placed into a living system to get an immune response and subsequently produce antibodies, aptamers are synthesized from nucleic acids produced in commercially available automated systems. The antibody production process is lengthy, and quite costly[39]. Oligonucleotide synthesizer systems are small enough to fit into an office-sized space, thus substantially reducing the manufacturing costs, with synthesis costs continually decreasing[39]. The *in vitro* process negates the use of animals. As well, once the sequence of an aptamer is determined, it can be easily continuously synthesized with little batch-

to-batch activity variation, which is unlike the case for antibodies[40]. Contrasting to antibodies, aptamers have a longer shelf life and are stable over a wide range of pH, ionic strength, and temperature, thus selections in non-physiological conditions are possible. Antibodies on the other hand are irreversibly denatured in conditions where temperature and/or pH are outside their narrow range of stability.

However, the fact that aptamers are composed of only four building blocks, compared to the twenty-two building blocks for proteins, is a disadvantage; the fewer the building blocks, the less variability and thus the less folding structures possible.

An important advantage of aptamers is the fact that they can be selected in conditions that match the same conditions as the assay they would eventually be used for, a luxury not possible with antibodies. For example, an aptamer that would be required to detect a target in basic conditions can be selected in basic media. Thus selections performed in this manner allows for enrichment of the library with aptamers that bind to the target in a particular set of conditions. However, a disadvantage of aptamers is that they are oligonucleotides and thus have an overall negative charge, making them highly hydrophilic. Therefore, they cross cell membranes inefficiently and are mostly restricted to extracellular applications such as the blocking of viral glycoproteins to prevent cell attachment and viral entry[41].

1.1.7 Applications

Due to their unique properties, aptamers can be used for a wide range of applications. Fields such as bio-technology, medicine (drug development), pharmacology (targeted therapy), cell biology (tracking), microbiology (virus and bacteria detection), and analytical chemistry

(chromatographic separation) are taking advantage of aptamers because they can be uniquely designed to meet specific goals[42].

A biosensor is a small analytical device involving a molecular recognition element with a physiochemical transducer[43]. The biological component interacts with a target of interest, and the transducer component. The reaction between the molecular recognition element and the target is controlled by affinity interactions. A biosensor can qualitatively or quantitatively measure target. It is the affinity and selectively of the recognition element which determines the success of the sensor. Antibodies are frequently used as a recognition element in biosensors; however the use of antibodies is limiting, as antibodies cannot be created for targets that are non-immunogenic[43]. Therefore, other recognition elements such as aptamers are being tested as an alternative. Biosensor which utilizes aptamers as the recognition element is named an aptasensor. Aptamers can be easily immobilized onto a solid support without affecting their affinity, and since they are stable in a wide range of temperatures, aptasensors can be constantly re-used in different environments. There are two main types of aptasensors; electrochemical and optical. Electrochemical aptasensors involve immobilizing the aptamer on an electrode surface. The aptamer-target binding is measured based on changes in the electrochemical current. These types of sensors are highly sensitive and inexpensive[43].

Aptamers can also be used in place of antibodies in ELISAs; an ELISA is one of the main mycotoxin and clinical diagnostic tests currently used[20]. The use of aptamers changes the name to aptamer-linked immobilized sorbent assay (ALISA) or enzyme linked aptamer assay (ELAA) [20]. One type of ALISA is the indirect competitive assay. The aptamer is linked to biotin while the target is coated onto microplates. The aptamer is added to the plate containing the target, and after several washes to remove any leftover aptamer, enzyme horseradish peroxidise (HRP)

bound to steptavidin is added. Following the addition of substrate for the enzyme HRP, a colorimetric chemical reaction takes place and the optical densities are measured. A direct competitive ELISA is similar, however the antibody is coated on the microplate instead of the target, and a second antibody for the target is bound to HRP. Biotin and streptavidin are sometimes used to immobilize the aptamer to the microplates[20].

An application of ALISA is for the detection of the mycotoxin ochratoxin A (OTA) in wine[44]. A direct and indirect competitive ALISA was performed. The direct assay was less time consuming than the indirect and was successfully used to detect low amounts of OTA. Therefore, not only was the test sensitive and specific due to the chosen aptamer, but also detection of OTA was quick and simple[44].

Lateral flow devices (LFDs) are commonly used these days for pregnancy tests, drug tests, and food pathogens tests. They are easy to use, inexpensive, portable, stable, and testing is quick[45]. As aptamers are gaining more interest, they are being tested as replacements for antibodies in these assays. An example is the aptamer-based test strip for the detection of OTA[45]; a nitrocellulose strip contains a sample pad on one end followed by a conjugate pad and two test strips downstream. The sample pad holds the sample that might contain the target of interest. The conjugate pad holds aptamer-modified gold nanoparticles (AuNPs), which OTA competes for with a DNA probe on the test strip line. If OTA is present, it will bind the AuNPs and thus the AuNPs cannot bind the DNA probe on the test line, resulting in the absence of red color. If OTA is not present, however, the AuNPs bind the DNA probe and a red color is observed. A control line further downstream from the test line holds another DNA probe which will bind the AuNPs regardless of concentration of OTA to ensure the test is valid. The test was able to successfully detect low levels of OTA[45].

1.2 Mycotoxins

1.2.1 General

Mycotoxins are low molecular weight compounds found to contaminate staple crops around the world, and which are known to affect the health of animals and humans. In fully developed countries such as Canada, the most agriculturally important mycotoxins negatively affect the agricultural food system economy, whereas in developing countries there is considerable loss of life[46,47]. It has been discovered that over 72% of cereal crops are contaminated with detectable amounts of mycotoxins, and 38% contained more than one type[48]. However, only about 25% of those are contaminated enough to have an effect on human/animal health[49]. Although roughly 10,000 metabolites produced from fungi have been discovered, not all are mycotoxins[50]. There are 400 types of mycotoxins, and they are divided into separate classes based on their general structure (Table 1.3.). However, the ones which have gained most attention as agriculturally important worldwide are aflatoxin, deoxynivalenol, ochratoxin, fumonisin, and zearalenone[48]. Other toxins found in grain include T-2, HT-2; however these are minor contaminants with the exception of abused (e.g. overwintered) grain[51,52].

Humans are mainly exposed to mycotoxins via ingestion; indeed it has been found that the intake of mycotoxins via food consumption is considerable[53]. Other routes of exposure are via dermal contact and the inhalation of air and dust containing the toxin. Mycotoxins pose acute health risks for humans and animals; the lethal dose to cause 50% mortality of test animals (LD_{50}) for deoxynivalenol and patulin is 46-78 mg/ kg and 29-55 mg/ kg, respectively, for rodents by oral administration[54,55].

Table 1. 3 Types of mycotoxins that are of most concern, including the fungus genera which produces the toxins, and the food commodities the toxin type infects[56].

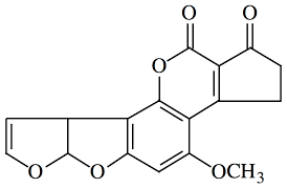
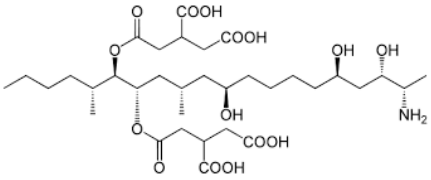
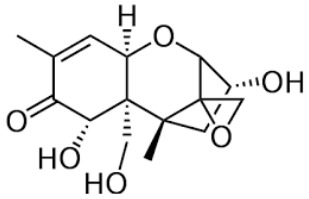
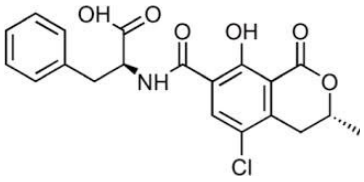
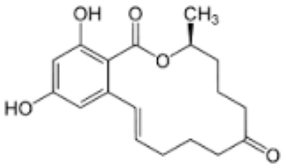
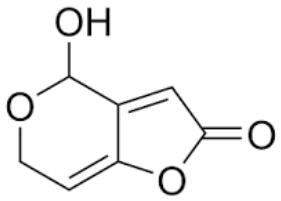
MYCOTOXIN TYPE	EXAMPLE STRUCTURE	FUNGUS GENERA	COMMODITY
AFLATOXIN		*Aspergillus*	Nuts, grains
FUMONISIN		*Fusarium*	Corn, wheat, cereals
TRICHOTHECENE		*Fusarium,*	Wheat, corn, cereals
OCHRATOXIN		*Aspergillus, Penicillium*	Grains, coffee, spices, fruit
ZEARALENONE		*Fusarium*	Corn, wheat, cereals

Table 1. 3 (Continued)

MYCOTOXIN TYPE	EXAMPLE STRUCTURE	FUNGUS GENERA	INFECTED COMMODITY
PATULIN		*Aspergillus,* *Penicillium*	Apples, pears

More recently in June 2013, Canada imported baby food from Russia which contained HT-2 (type A trichothecene) toxin. The product was re-called after several cases of human illness related to the consumption of this product were discovered[57].

The presence of mycotoxins in field and in storage cannot be completely eradicated; however the applications of certain practices are used in an attempt to control levels. For DON, these practices include[58], 1) crop rotation, such that pests do not become established in the soil over time; 2) variety resistance, although no crop is 100% resistant to diseases caused by the fungus, some varieties are more tolerant; 3) application of fungicides, and 4) tillage, to bury any debris carrying disease. The best way to control patulin levels is to monitor the quality of fruits used for production. The surface of damaged and rotten fruit is likely infected with patulin-producing fungi; by avoiding the use of spoiled fruit patulin levels can be reduced by 90%[59]. Patulin levels can further be decreased by washing the fruit. Unfortunately, however, apples with an open bottom end can be contaminated with patulin in the core of the fruit, thereby making contamination unobservable. Since it is not obviously rotten, the apple remains in the batch and contaminates the commodity[59].

1.2.2 Economic Impacts

Mycotoxin contamination is an ongoing and expensive problem that is negatively affecting the economy worldwide due to loss of human life, loss of livestock production and feed, regulatory costs, research costs, and health care and veterinary costs. Due to its prevalence and abundance, everyone in the food and feed supply chain is affected, from crop and animal producers, grain handlers and distributors, processors, to the consumers[60]. It has been estimated that mycotoxin contamination costs Canada and the US roughly $5 billion per year[60], without considering the cost of human health which is an additional $500 million. US researching costs for aflatoxin alone are estimated at $20-30 million per year. Also, the amount of food and products lost weighs in at around 1 billion metric tons[60].

Developing countries are at a greater disadvantage when it comes to mycotoxin contamination due to the fact that the resources for proper storage and handling are not widely available. In addition, the issue that is mycotoxin contamination is not taught to many and therefore is not known. For these reasons, mycotoxin contamination is more prevalent in developing countries. In fact, $900 million per year in losses due to aflatoxins are estimated for Indonesia, Phillippines, and Thailand together[60].

Another issue is the reduced crop value; producers cannot sell their crops as they are rejected by grain elevators and other buyers because their crops are too contaminated with mycotoxins. The grains are either destroyed or sold at lower prices. In developing countries, producers are sometimes forced to sell the high-quality crops and consume the lower-quality, contaminated crops[60].

1.2.3 Occurrence

As previously mentioned, mycotoxins are found worldwide. They are prevalent in different abundances according to regional climatic conditions. Only the occurrence of DON and patulin will be discussed.

A 2013 study by Schatzmayr and Streit[48] researched the global occurrence of these mycotoxins by collecting over 17,000 samples from America (North and South), Asia (South-East, South and North), Oceania, Europe (Northern, Central, Southern and Eastern), Middle East, and Africa. These samples include different types of cereals, soybean meal, corn gluten meal, dried distillers grains, and straw. The samples were tested for the most popular mycotoxins; aflatoxin, zearalenone, deoxynivalenol (DON), fumonisin, and ochratoxin A (OTA). DON was the most abundant in North Asia where 78% of the samples tested positive and the average concentration was 1,060 μg/kg[48]. However, North America where 68% of samples contained DON, the average concentration was the highest at 1,418 μg/kg[48].

In addition, 38% of the total samples were contaminated by more than one mycotoxin[48]. Multiple toxin contamination can be expected, since many fungi are able to produce more than one type of mycotoxin at a given time. Even if the levels of co-occurring mycotoxins are below their respective limit, additive or synergistic interactions may occur[48]. Therefore, the detection of multiple mycotoxins on the same commodity is a necessity.

As for patulin, a 2008-2009 study[61] involved 44 and 60 samples of apple juice and apple juice concentrate, respectively, which were collected across Canada and tested for patulin levels. 84% and 92% of these samples contained levels below the reporting limit of 10 ppb, respectively. The apple juice sample that was measured to have the highest amount of patulin contained 26.7 ppb

and for apple juice concentrate it was 24.0 ppb, thereby showing no difference in patulin concentration between the two types of samples. The Codex draft maximum level for patulin is 50 ppb, thus all samples were below the limit. Currently, patulin levels in Canada do not pose a risk, however it is still important to monitor the levels[61].

In the U.S., a study examined 493 samples obtained from Michigan apple cider mills for their patulin levels[62]. 18.7% of samples contained levels of patulin around 4 g/L and 2.2% of samples had levels of 50 g/L. Of the 159 samples of apple juice and cider obtained from grocery stores, 23% contained detectable levels of patulin, and 11.3% of those samples had concentrations of 50 g/L. However, some samples had high patulin levels of 2,700 g/L. As the U.S. Food and Drug Administration (FDA) action level for patulin is 50 g/kg, it is understood that most samples are below the limit but some samples are not, indicating that some apple cider and juice processors do not have sufficient controls over patulin concentrations in final products, and thus an improvement on the quality of fruit used in juice production is required to lower the levels of patulin[62].

A 2002 study by Majerus and Kapp[63] found that only 17.5% and 2.4% of food products in the European Union contained levels of PAT greater than 25 μg/kg and 50 μg/kg, respectively.

The 2013 study demonstrates that the abundance of a specific mycotoxin is different from country to country, and that geographic location has an effect on the production of mycotoxins. This report also provides valuable information as to which mycotoxin(s) can potentially cause problems, and which will need to be carefully monitored and controlled such that public and food safety is not compromised.

Although a great deal of effort has been expended by the scientific community to understand the effects of mycotoxin contamination and the different ways to control it, a viable solution has yet to be found. This justifies further research to address the potential rise in economic loss and health problems of humans and animals through the accurate detection of mycotoxin levels.

1.3 Thesis Objectives

The work presented in this thesis ultimately aims to provide aptamers as an alternative affinity probe to antibodies for the detection of mycotoxins in food and feed.

The aim will be to generate an aptamer that binds with high affinity to deoxynivalenol (DON) and patulin (PAT), respectively. Currently, an aptamer for patulin does not exist, and aptamers for DON were only recently generated. The selection process will be performed with SELEX; separately, DON and patulin will be conjugated to a solid support matrix and exposed to a DNA library consisting of many different sequences. It is the hope that at least one of these sequences binds to the target mycotoxin.

1.4 General Methods

1.4.1 DNA Synthesis

DNA was synthesized using standard phosphoramidite chemistry on a BioAutomation Mermade 6 (Plano, Texas) according to manufacturer's protocol. 1 μM controlled pore glass (CPG) columns of 1000 Å size were used. Upon synthesis completion, the columns were removed from the instrument and the beads transferred to a new 1.5 mL tube. 1 mL of ammonium hydroxide was added to the beads and incubated for 16 hr at 55ºC. The beads were centrifuged and the supernatant transferred to new clean tube. The beads were washed with 200 μL of deionized water four to five times, always keeping the supernatant. The DNA was then dried down by cryopumping in a Savant AES2010 SpeedVac overnight.

1.4.2 Polyacrylamide Gel Electrophoresis

To purify DNA pool and primers, 12% polyacrylamide gel electrophoresis (PAGE) gels were prepared. The following reagents were combined into a 250 mL beaker: 31.5 g urea, 23.5 mL acrylamide stock, 15 mL 5X TBE buffer (45 mM Tris, 45 mM boric acid, 1 mM EDTA), and 14 mL deionized water. The mixture was heated to 37°C with stirring, then filtered using Whatman No. 1 Filter paper and cooled to room temperature. The gel apparatus was assembled in the meanwhile. Next, a 10% solution of ammonium persulfate (APS) was prepared by adding 0.1 g of ammonium persulfate into 1 mL of deionized water. 450 µL of the APS solution, along with 35 µL of N,N,N',N'-tetramethylethylenediamine (TEMED), was added to the acrylamide solution and swirled before pouring into the gel plates and the combs added. Once the acrylamide polymerized, the combs were removed and the wells rinsed 3-5 times with deionized water. The gels were placed in the electrophoresis apparatus where the bottom and the electrode wire were covered with 1 x TBE running buffer. The system ran for 15 min at the appropriate current, and meanwhile the DNA samples were prepared; equal volumes of deionized water and formamide were added to the samples and vortexed for 30 seconds before being heated for 5 minutes at 90°C. Samples were then loaded into the gel wells and the system ran at 50 amps for 2-3 hours. Gels were imaged on a 20 x 20 cm TLC plate using an Alpha Imager Multi Image Light Cabinet (AlphaInnotech) on the epi-UV setting with excitation wavelength of 254nm. DNA bands of interest were cut out and placed into a 50 mL tube. The gel bands were broken up by, and then 25 mL of deionized water was added to the tube. The tube was incubated at 37 °C with shaking overnight.

1.4.3 Desalting

Desalting was performed with Amicon YM-3 Centrifugal Filter Devices with a 3kDa cut off. Filters were centrifuged with 500 µL deionized water at 13000 RPM for 25 min prior to the addition of the sample. The sample was then added to the filter tube, followed by washing 4-5 times with deionized water. Finally, the filter was inverted into a new clean tube and spun at 1000 RPM for 3 minutes to retrieve the DNA.

1.4.4 Polymerase Chain Reaction

Polymerase chain reaction (PCR) was carried out in a flow hood which was UV irradiated 1 hour prior. Each 150 µL reaction tube contained 50 µL FluMag buffer (100 mM KCl, 200 mM Tris, 2% Triton X-100), 8 µL of 25 mM $MgCl_2$, 2 µL dNTP, 0.5 µL of 200 mM FLU primer and POLY A primer, 5 units Taq Polymerase, 38 µL deionized water, and 1-2.5 µL of template DNA (in deionized water). A positive control with pool DNA and a negative control with deionized water were always included. 30-60 reaction tubes were prepared and placed in a Thermocyler (Eppendorf AG 22331 Hamburg, No. 5341 K). For these tubes were subjected to 10 min at 94˚C, 20 or 25 cycles of 1 min at 94˚C, 1 min at 47˚C, 1 min at 72˚C, 10 min at 72˚C after the last cycle, and finally remaining at 4˚C until samples removed. Samples were then combined and dried down in a Savant AES2010 SpeedVac overnight in preparation for polyacrylamide gel electrophoresis.

1.4.5 Cloning

DNA sample was amplified with unmodified primers, purified using PAGE, and desalted four times with deionized water. This sample was then used in the StrataClone PCR cloning kit (Agilent Technologies) according to manufacturer's protocol: 3 µL of Cloning Buffer, 2 µL of DNA sample, and 1 µL of Vector Mix were mixed together and incubated for 5 min at room temperature. The mix was then placed on ice before 2 µL was added to the competent cells. The cells were incubated for 20 min on ice, then heat-shocked for 45 s at 42ºC. 250 µL of LB broth at 37ºC was added to the cells, which were then incubated at 37ºC for one hour with gentle shaking. In the meantime, 40 µL of 2% X-gal dissolved in dimethylformamide (DMF) was spread onto the AMP-agar plate. 100 µL of the cells were then added to the plate. The plate was placed in an incubator at 37ºC overnight to let the colonies grow. The day after, the sequences were amplified by rolling circle method using the TempliPhi Amplification kit. The kit protocol was followed. 24 white colonies were scraped from the plate with a pipette tip and placed into individual tubes containing 5 µL of Sample Buffer (contains random hexamers which prime DNA synthesis randomly) and heated at 95°C for 3 minutes then cooled to room temperature. Separately, 5 µL of Reaction Buffer (contains salts and deoxynucleotides) was mixed with 0.2 µL of enzyme mix, and 5 µL of this mix was added to each of the cooled samples. Samples were incubated at 30C for 2-18 hours, and then the enzyme was deactivated by heating the samples at 65ºC for 10 min. The samples were cooled to 4°C before being sent to the University of Calgary University Core DNA Services for Full Service sequencing.

Chapter 2 - Development of DNA Aptamer for the Mycotoxin Patulin

2.1 Introduction

2.1.1 Properties and Structure of Patulin

Patulin (PAT) is a mycotoxin and polyketide secondary metabolite produced by certain species of *Penicillium*, *Aspergillus*, and *Byssochylamys*, particularly *Penicillium expansum*[59,64]. The chemical name for PAT is 4-hydroxy-4H-furo[3,2c]pyran-2(6H)-one and it belongs to its own class of mycotoxins[65]. PAT is water soluble and is stable in aqueous acidic media[66]. The structure of PAT is shown in Figure 2.1

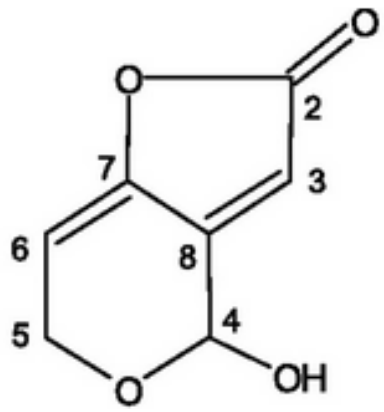


Figure 2. 1 Molecular structure of patulin with its carbons numerically labelled.

Patulin-producing fungi are common pathogens found post-harvest. *P. expansum*, the main producer of patulin, causes soft rot and the formation of blue pustules in fruits such as apples and pears[67], but more commonly in apples. Patulin is normally found in apple juice and apple cider but not in fermented apple products as patulin does not survive the fermentation process[65]. On the other hand, patulin is stable enough to survive heating and processing, thus cooking apple products does not reduce the amount of patulin [64,65]. It was found that after pasteurization, enzymatic treatment, microfiltration and evaporation processes the mean loss of patulin was only 39.6, 28.3, 20.1 and 28.4%, respectively[68].

Apple juice and cider are monitored for patulin levels because lower grade fruits that are not suitable for direct market retail are used for their production[69]. Patulin can also be found in cheese, however the levels of patulin in cheese do not pose a high risk; this is because patulin has high affinity for compounds containing sulfhydryl groups such as cysteine, of which cheeses generally contain a high content, resulting in the formation of adducts with reduced toxicity between patulin and cysteine[59,70].

2.1.2 Conditions for PAT Production

Patulin is produced in warm, humid conditions which make storing apples a challenge; even at 0ºC *P. expansum* growth can occur place, although rotting would occur more slowly[71]. At storage temperatures between 4ºC and 25ºC, the highest amount of patulin produced was detected[72].

Patulin contamination is abundant in fruit before harvest if the fruit is affected by insect damage or disease. The reality is that the amount of patulin contamination in apple juice and products is directly primarily related to the quality of the fruit at harvest, and secondly how well the fruit is stored before manufacturing[73].

2.1.3 Toxicity

Patulin was originally isolated for treating common colds and fungal skin infections[65]. However, these practices were quickly discontinued when it was discovered that PAT not only had antibacterial, antiviral, and antifungal properties but was toxic to plants and animals[65]. The toxicity of patulin *in vivo* stems from its high affinity to sulfhydryl groups, which leads to the inhibition of many enzymes by interfering with their active site[67,74]. Specifically, patulin inhibits

enzymes such as Na^{+}-K^{-} ATPase (plasma membrane) and biosynthetic enzymes such as RNA polymerase and aminoacyl-tRNA synthetases[70].

Patulin has been found to alter the functions of the intestinal epithelium barriers; it quickly induces a decrease of trans-epithelial resistance, which in turn disorganizes tight junctions[70]. Ultimately, this leads to inflammation, ulceration, and hemorrhages in animals. There is evidence to suggest that patulin and high concentrations of patulin -cysteine adducts are mutagenic and teratogenic[67,70]. There is ongoing debate as to whether patulin is carcinogenic since studies provide evidence that patulin is carcinogenic to rats; however, some studies used levels of patulin that are not relevant to human exposure[64]. In 1986, the International Agency for Research on Cancer classified patulin as a group 3 carcinogen i.e. a compound which there is not enough data to allow its classification[64].

2.1.4 Limits

Some rotten apples, or some at the initial stages of rotting, do get by the selection and cleaning phase and end up in apple products. Since apple products are popular, it would be beneficial to monitor the levels of patulin for public safety[64].

Since the risk of chronic exposure to patulin exists, the quality of apples used for processing and the levels of patulin are being closely monitored worldwide. The United Nations Environmental Programme (UNEP) and the World Health Organization (WHO) International Programme on Chemical Safety (IPCS) have proclaimed that humans have the right to food that is free from mycotoxin levels that could lead to health risks[47]. Therefore, when it comes to patulin, the Canadian standard (Health Canada) is set to a maximum acceptable limit to 50 ppb (µg/kg) in apple juice and unfermented apple cider[75]. Similarly, the Food and Drug Administration (FDA)

proposed maximum limits of 50 ppb for apple juice, apple juice concentrates, and apple juice products[59]. The European Union (EU) has set a limit of 50 ppb for adults, and 10 ppb for infants and small children[76]. The Scientific Committee on Food (SCF) has set a provisional maximal tolerable daily intake (PMTDI) of 0.4 µg/kg body weight (b.w.) per day[59].

2.1.4 Current Detection Methods

Currently, methods for detecting patulin include HPLC[68], TLC with a charge coupled device detector[77], UV spectroscopy[72], GC-MS[78], and, recently discovered, the anti-patulin ELISA kit[79]. Most of the aforementioned methods involve lengthy analysis times, and the use of expensive equipment and skilled technicians. Therefore a cheap, quick, and reliable method for detecting patulin would be ideal and may be found in aptamers.

2.2 Chapter Objectives

Patulin (PAT) is a mycotoxin found in apple juice and apple products, which are popular commodities consumed worldwide. Current methods for detecting the toxin are expensive and sometimes require skilled technicians. Therefore, the search for inexpensive and easy to use detection agents for this toxin is ongoing. Aptamers could be used as a possible solution for detection of patulin in food and feed and thus provide improved quality control. A library of different sequences of DNA is synthesized and subjected to patulin, which is supported on a solid phase column. The repeated process of exposing the DNA library to patulin and amplifying the sequences which bind is known as SELEX. The objective of this project is to generate a DNA aptamer for the mycotoxin patulin.

2.3 Statement of Contributions

Dr. Stefania Valenzano synthesized the DNA library. DNA sequencing was performed by the Sequencing Lab at the University of Calgary. Aptamer selection and cloning was performed by Annamaria Ruscito.

2.4 Materials and Methods

2.4.1 Materials

Patulin was purchased from Sigma-Aldrich (Canada) with ≥ 98% purity (TLC). Dynabeads® M-450 epoxy was purchased from Life Technologies Inc. (Canada). Propylene oxide was purchased from Acro Organics (New Jersey, USA). Phosphoramidites, modifiers, activator, deblock, capping, and oxidizing reagents for DNA synthesis were obtained from Glen Research. Acetonitrile and standard support columns were purchased from BioAutomation. Strataclone Cloning Kit was purchased from Agilent Technologies Inc (Ontario, Canada). Illustra TempliPhi 100 Amplification Kit was purchased from GE Healthcare Life Sciences (Quebec, Canada). MinuteMaid® apple juice was purchased in Ontario, Canada. Buffer salts were purchased from Sigma Aldrich. High Purity 5.0 argon was purchased from Praxair. Buffers were prepared with Millipore Milli-Q deionized water at 18 MΩcm.

2.4.2 Library and Primer Synthesis

DNA library (also called pool) and primers for PAT SELEX were synthesized by Dr. Stefania Valenzano using standard phosphoramidite chemistry on a BioAutomation Mermade 6 (Plano, Texas) with 1 μM controlled pore glass (CPG) columns of 1000 Å size. The pool, 71 bases in length, is composed of fixed primer binding regions which flank a random region: 5'-TGGTGGCTGTAGGTCA-N40-GAGCATCGGACAACG-3'. The forward primer is

fluorescently modified to fluorescently label binding DNA such that it can be monitored throughout the SELEX process, and the reverse primer is modified with a poly A tail to increase the strand size such that the double stands can be distinguished during separation with denaturing gel electrophoresis. The primers are: 1) ModPrimer1: 5'-6FAM- TGGTGGCTGTAGGTCA-3' and 2) ModPrimer2: 5'-poly-dA_{20}-HEG-CGTTGTCCGATGCTC-3'. The HEG link stands for hexaethylene glycol Spacer Phosphoramidite 18 (Glen Research) and serves to prevent the formation of the polyA tail complement during polymerase chain reaction (PCR). The pool was purified using 12% denaturing gel electrophoresis and desalted. For cloning, unmodified versions of the above primers were used: Primer1: 5'-TGGTGGCTGTAGGTCA-3' and Primer2: 5'-CGTTGTCCGATGCTC -3'.

2.4.3 SELEX Beads

In this study magnetic beads were used to immobilize the target molecule. The magnetic beads are uniform, monodispersed, non-porous, and 4.5 μm in diameter. They are superparamagnetic, composed of a mixture of maghemite (gamma-Fe_2O_3) and magnetite (Fe_3O_4). To coat the magnetic material, a polystyrene shell is used. These beads can be used to immobilize a variety of molecules, including mycotoxins, and can be re-used.

2.4.4 Column Preparation

The following protocol is a scaled down version from Invitrogen™ Life Technologies™ Dynabeads® M-450 Epoxy beads (Catalog no. 14011), modified for small molecule reaction.

Dynabeads ® were vortexed for 30 seconds, and then 1 mL was removed and placed in 2 mL glass vial. 1 mL of PBS Buffer #1 (22 mM $NaH_2PO_4 \cdot H_2O$, 76 mM Na_2HPO_4, pH 9.5) was added and the mixture was vortexed before being placed in a magnetic tube holder. The supernatant

was discarded and fresh buffer was added. The washing process was repeated 2 times. 900 µL of PBS Buffer #1 was used to rehydrate 0.5 mg PAT which was dried down in a Savant AES2010 SpeedVac, and the solution was added to the beads. The beads and PAT were incubated for 24 h at room temperature with gentle shaking. The tube containing the beads and PAT was placed in the magnetic tube holder and the supernatant was collected and set aside. The tube was removed and the beads were washed 3 times with 1 mL of PBS Buffer #2 (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 2 mM EDTA, pH 7.4) for 5 min with gentle shaking. Beads were stored in PBS Buffer #2 at 4ºC. Negative column consisted of non-reacted Dynabeads washed 5 times and stored in Buffer #2 at 4ºC.

2.4.5 Preparation for Testing the Magnetic Bead Reaction

To determine if the reaction with magnetic beads was successful, the same protocol was tested with propylene oxide in place of magnetic beads and analyzed by mass spectrometry (MS). 0.2 mg PAT in ethanol was mixed with 300 µL propylene oxide. The reaction was incubated overnight at room temperature with gently stirring. The reaction was then dried with argon gas and stored at -4ºC for 48 hours before being shipped for MS analysis.

2.4.6 Preparing Apple Juice Buffer

To perform selection in apple juice, the PBS Buffer # 2 was modified; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, and 2 mM EDTA was added to 50 mL of deionized water, followed by 50 mL of MinuteMaid® apple juice. The pH was neutralized to 7.4. The buffer was filtered and stored at room temperature. It was confirmed by the MinuteMaid® manufacturer that there is less than 50 ppb of PAT in the apple juice.

2.4.7 SELEX

The sequences in the DNA library for PAT SELEX each contain a random region of 40 nucleotides flanked by regions of known sequence. The flanked regions are for primer binding during PCR amplification after each round. DNA in PBS Buffer #2 was heated at 90ºC for 5 min, cooled at 4ºC for 10 min, and then warmed to room temperature for 10 min. The DNA was added to negative column beads and shaken for 30 min. DNA that did not bind to the beads was heated and cooled as before then added to the beads of the positive column. After 30 min of shaking, the negative and positive beads were washed five times with PBS Buffer #2. 90ºC heat and 6 M urea was used to elute DNA bound to the beads, and a total of five elutions were performed. Rounds 9 and 10 column incubations were performed with PBS Buffer #2 mixed with MinuteMaid® apple juice, pH 7.4. Samples were analyzed using Fluorolog Fluorescence Spectrophotometer (Horiba Jobin Yvon, USA) at an excitation wavelength of 490 nm and emission wavelength of 520 nm, the excitation and emission slit widths were 5 nm. Subsequently, the samples were desalted and amplified for the next round. The equation for percent recovery is shown below. Table 2.1 shows the changes made during the selection rounds.

$$\%Recovery = \frac{\int \text{elution} * 100\%}{\int \text{pool}}$$

Table 2. 1 Changes made to PAT SELEX procedure at specific selection rounds. Changes were carried to subsequent rounds unless stated otherwise.

SELECTION ROUND	CHANGES MADE TO PROCEDURE
1	Started with 5 nmol DNA and 100 µL beads. 500 µL volumes of washes and elutions were used. Elutions performed with heat and 6 M urea and placed on a shaker for 10 min.
2	Decreased amount of DNA pool from 500 pmol to 200 pmol. Decreased volume of beads from 100 µL to 40 µL. 200 µL volumes of washes and elutions were used.
3	Diluted beads of positive column 10X with beads of negative column.
5	During elutions, the tubes were placed on heat block at 90ºC and vortexed three times throughout incubation.
7	Decreased amount of DNA pool from 200 pmol to 100 pmol. Diluted beads of positive column 5X with beads of negative column.
11	SELEX performed in MinuteMaid® apple juice buffer.

Cloning was performed as described in section 1.4.5.

2.5 Results and Discussion

2.5.1 Column Test – UV Vis

After the reaction was complete, it was necessary to determine the amount of PAT that did not react to the beads; a UV-Visible spectrometer was used to detect the amount of PAT in the column washes. Due to the conjugated system between C6, C7, C8, and C3, PAT absorbs in the UV range, specifically at the wavelength λ_{max}=275 nm. The amount of PAT found in the washes was 0.04 mg, or 0.8% of the starting material, which is negligible. Therefore, almost all of PAT has reacted to the beads.

2.5.2 Column Test – MS

The column reaction was repeated with propylene oxide in place of the magnetic beads; this was to mimic the beads and then be able to use mass spectrometry on the entire reaction solution. The reaction and expected product are shown in Figure 2.2.

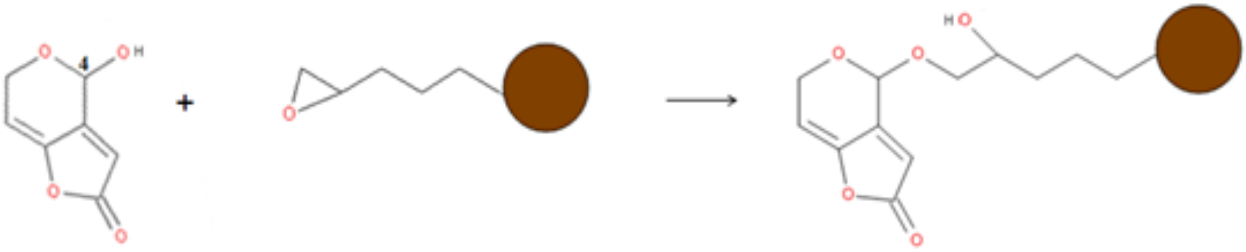


Figure 2. 2 Reaction of PAT with magnetic beads. The C4 hydroxyl on PAT reacts with the epoxide on the magnetic bead.

To test the purity of patulin, a MS spectrum was obtained (Figure 2.3A). The most prominent peak with a mass to charge ratio of 177, which is equivalent to patulin's molar mass (154 g/mol) bound to a sodium ion (23 g/mol). The correct mass and the absence of other peaks is consistent with patulin. To test for the success of the reaction, a MS spectrum was obtained for patulin reacted to propylene oxide via the C4 hydroxyl. The expected mass of the product is 212 g/mol. The resulting spectrum shows a peak at 213 g/mol, albeit it is not the most prominent peak (Figure 2.3B). The discrepancy between 213 and 212 is likely caused by protonation, thus the peak mass is consistent with the expected reaction product. It is important to note that the reaction solution was sent for analysis as a crude sample without purification, therefore other peaks could represent molecules which would be normally be washed away from the column. MS analysis has confirmed that patulin has reacted to the beads in the method expected.

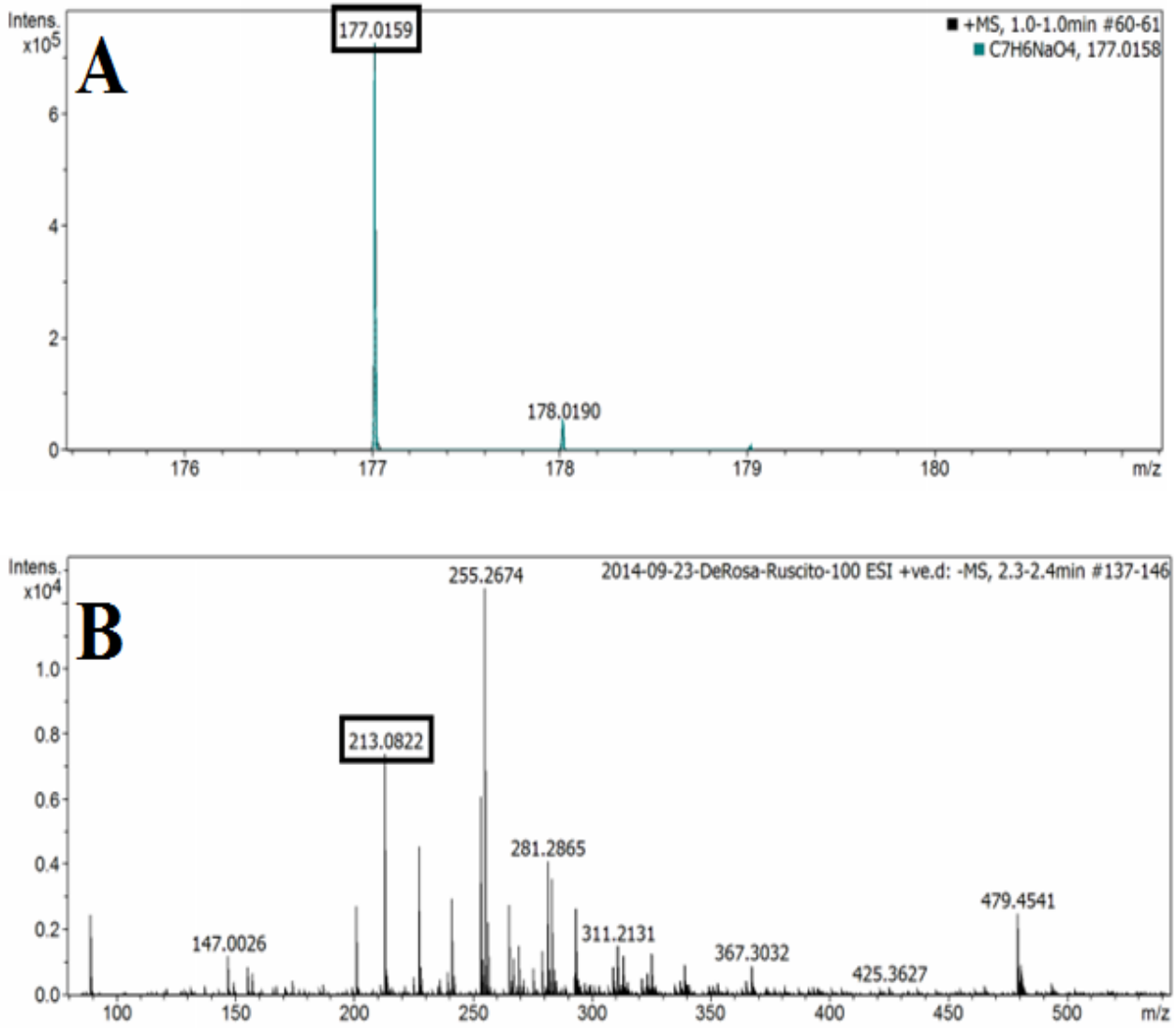


Figure 2. 3 Mass spectrometry spectrum of (A) PAT only; and (B) PAT+propylene oxide using a maXis impact mass spectrophotometer. Reaction solution B is crude; there was no purification prior to analysis. Mass-to-charge ratios of interest are boxed.

2.5.3 SELEX

Results

Selections were monitored by the fluorophore attached to the DNA, and twelve rounds of SELEX were performed for patulin.

Percent recovery of the positive column was initially high at 3.95%, and the recovery from the negative column was almost five times lower with a percentage of 0.75% (Figure 2.4). However, recovery from the positive column in the next round drastically decreased by a factor of 10 to 0.45% after the amount of DNA and the volume of the beads was lowered to increase stringency. This was particularly true after the beads of the positive column were diluted with beads of the negative column in round 4, the percent recovery of the positive column dropped to 0.10%.

Recovery appeared to be increasing in round 5 when the beads were heated during the entire elution incubation period, as opposed to using hot urea and placing them on a shaker. In round 6 the percent recovery dropped again. The recovery of the negative column was lower in the first two rounds, and then began to exceed that of the positive column for rounds 4 and 5. The ratio of percent recoveries from the positive and negative columns for rounds 4 and 5 were 1:3 and 1:1.2, respectively.

The percent recoveries of the positive column from rounds 7-9 were not measured due to instrument malfunction. The sequences recovered from the positive column were desalted and prepared for the next round.

Recovery from the positive column in round 11 was the highest since the initial round with a value of 2.25%. Recovery from the negative column in the same round was roughly three times lower, with a value of 0.79%.

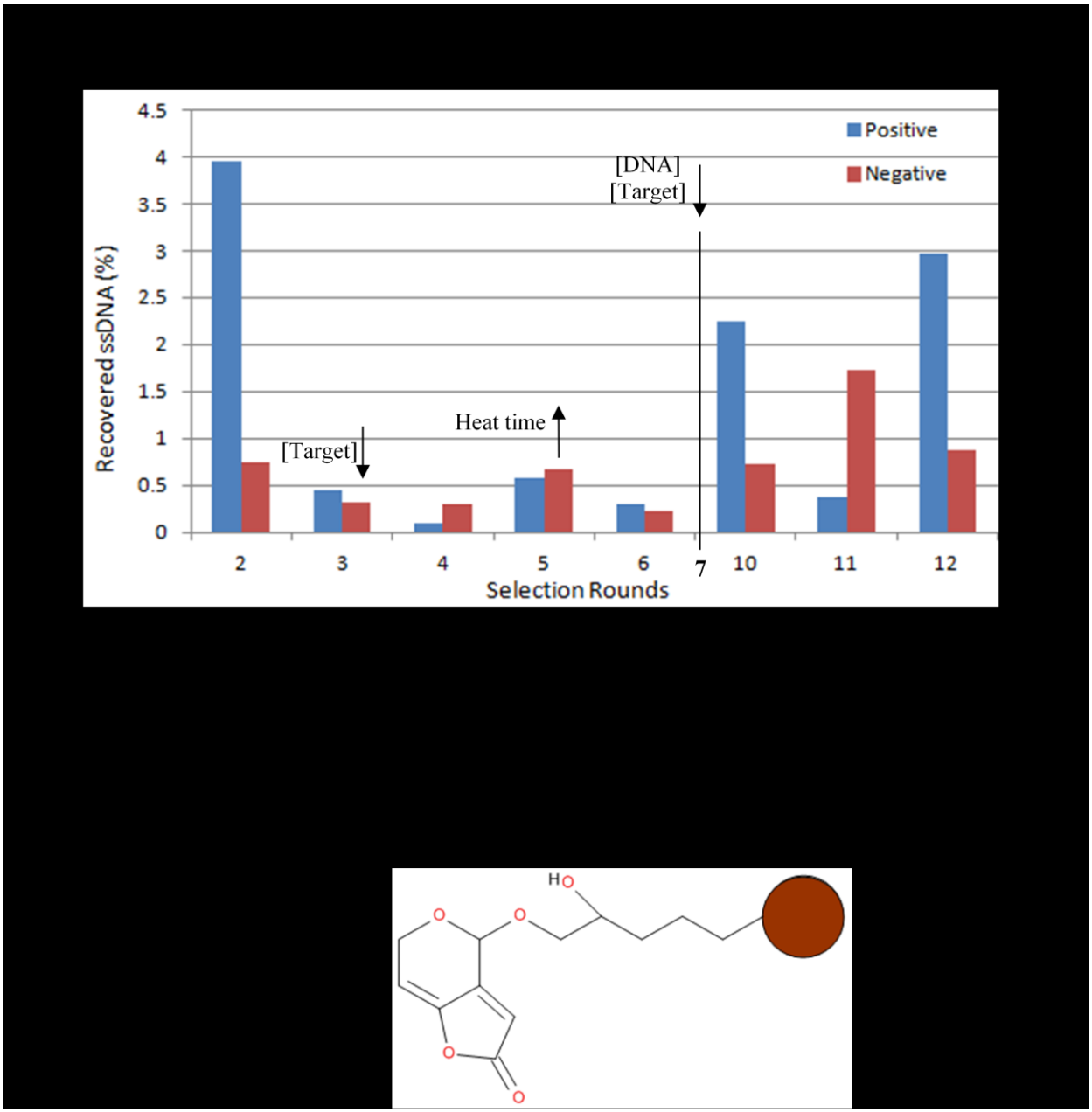


Figure 2. 4 Graph showing percent recovery of single-stranded DNA (ssDNA) in each round of PAT SELEX. Selection was monitored by the fluorophore attached to the DNA. The concentration of DNA, the column volume, and the wash/elution volume were lowered in round 2. Target concentration was lowered in round 3 and heat time was increased in round 5. In round 7, the DNA and target concentrations were lowered. The structure of patulin immobilized onto the magnetic bead in the positive column is shown. Percent recovered ssDNA in rounds 7-9 were not measured. Round 11 and 12 were executed in apple juice buffer instead of SELEX buffer. Positive and Negative refer to columns.

Finally, in rounds 11 and 12, the SELEX buffer was replaced with apple juice buffer. Initially, in round 11, the recovery from the positive column decreased to 0.37% and the negative column increased to 1.73% (1:1.5). When round 12 was performed, the direction of the column recoveries reversed; the positive column recovery increased to 2.96% whereas negative column recovery decreased to 0.88% (3:1).

The DNA pool from round 12 was then cloned using *E.coli* and twenty-four colonies were picked and sent for sequencing. Of the twenty-four, only seven usable sequences were obtained.

Discussion

For this experiment a DNA library was chosen. DNA is more stable than RNA, and in addition DNA does not require the extra steps of transcription for PCR and reverse transcription to continue from one round to another[19].

Twelve rounds of SELEX were performed for the mycotoxin patulin (PAT). A library of DNA oligonucleotides is exposed to a target molecule, patulin, and during selection, the sequences which bind to the target are separated from the ones that do not bind, and hence aptamers are discovered. Sequences are monitored via the fluorophore attached to the DNA. The amount recovered from each column is measured as the percentage of recovered single-stranded DNA. Evidence of pool enrichment is the increased percent recovery from the positive column and decreased percent recovery from the negative column.

The initial round was performed with non-fluorescent DNA pool; the pool was synthesized but at that point is not fluorescent until it is amplified using fluorescently labelled primers in PCR. A concentrated amount of pool is required for the first round, which is easy to obtain after

synthesis, but more difficult to obtain after PCR, therefore the first round was left unmonitored until the subsequent rounds.

High recovery is observed in the second round from the positive column and low recovery from the negative column, indicating that the pool contains binders to patulin; however the sequences are likely binders of low affinity with a few high affinity binders, therefore the pool requires enrichment. Recovery from both columns drastically lowered in round 3, due to the decrease in available DNA and patulin attached to the beads, then further decreased due to the dilution of the positive column with beads from the negative column in the next round; this dilution is due to the awareness that there was a larger excess of target than DNA. With excess target, all sequences can bind to the target and there would not be any competition among them; consequently the pool would not enrich with high affinity binders.

In rounds 4 and 5, the recovery from the negative column exceeded the recovery from the positive column. Since it is the beginning of the selection process, the high recovery from the negative column is likely the result of the pool enriching; after each round, an increasing amount of DNA sequences are being removed from the pool due to the negative selection which removes more sequences binding non-specifically to the target. As such, the decrease was not cause for much concern. The recovery from the positive column in round 5 increased from the previous round, demonstrating potential enrichment.

Rounds 6-9 ultimately shows gradual increase in percent recovery from the positive column. In round 6, the recovery of the negative column decreased to a percentage below that of the positive column, which is ideal. To avoid the recovery from the negative column exceeding that of the

positive, it was decided to decrease stringency for the remaining rounds by decreasing the concentration of the beads.

In round 10 the recovery from the positive column is much greater than the negative column, and since this is further along in the course of selection, it is reasonable to assume that the pool is composed of more high affinity binders to patulin than non-specific or low affinity binders.

In round 11, the selection conditions were altered. Rather than pure selection buffer as a medium, apple juice buffer was used. This change is to mimic conditions in which the aptamer would ultimately be used in to detect patulin. Not all DNA sequences will bind just as well in apple juice as in buffer, likewise, sequences which did not bind very well in buffer may bind well in apple juice. This manipulation is one of the advantages of aptamers over other affinity probes such as antibodies. The aptamer can be selected in the same conditions in which it will be used in, resulting in an aptamer that binds to the target with high affinity in working conditions. Recovery from the positive column in round 11 lowered considerably from the previous round due to the change in conditions; sequences which do not fold properly are washed and removed from the pool. The pool was obviously enriched with sequences that bound well to the target in those conditions as the recovery from the positive column in round 12 increased to a percentage close to but higher than before the change in conditions with apple juice buffer. The recovery from the negative column was also much lower in the last round than the previous round, indicating less weak binders now present in the pool.

Counter-selections for PAT were not performed. The loss of high affinity binders to gain slightly better selectivity is not sensible in this case. However, potential molecules that are suitable for PAT counter-selections are citrinin (CTN- Figure 2.5, left) and chaetoglobosin A (CGA- Figure

2.5, right). CGA and CTN are mycotoxins produced from *P. expansum* in addition to patulin[80]. CGA contains a moiety that is structurally similar to PAT, and CTN is a small molecule with similar structure and functional groups as PAT, thus both may bind to the future aptamer if the aptamer is not selective enough.

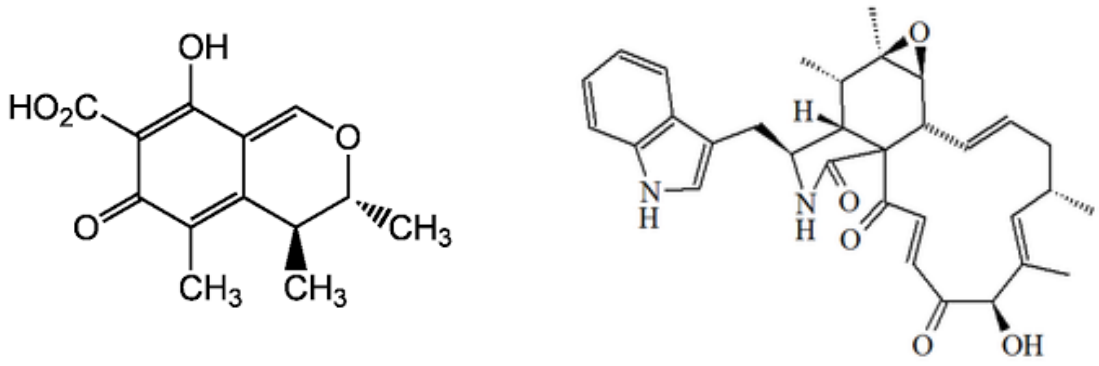


Figure 2. 5 Molecular structure of citrinin (CTN- left) and chaetoglobosin A (CGA).

The final step in this selection process was sequencing. Round 12 was chosen for low throughput sequencing of the aptamers contained in the pool. The pool was cloned in *E.coli*, and 24 colonies were chosen.

Of the twenty-four colonies chosen, only seven contained usable aptamer sequences, with one of those colonies containing sequences short of two nucleotides in the random region. The loss of two nucleotides likely occurred during synthesis of the pool, which would not be observable by PAGE due to the small difference in mass, or by incomplete amplification during PCR, since PCR is susceptible to mutations; nonetheless, the sequence might still be useful.

Each sequence had its secondary structure predicted by RNAStructure (temperature at 298 K) Structures are shown in Figure 2.6 and 2.7.

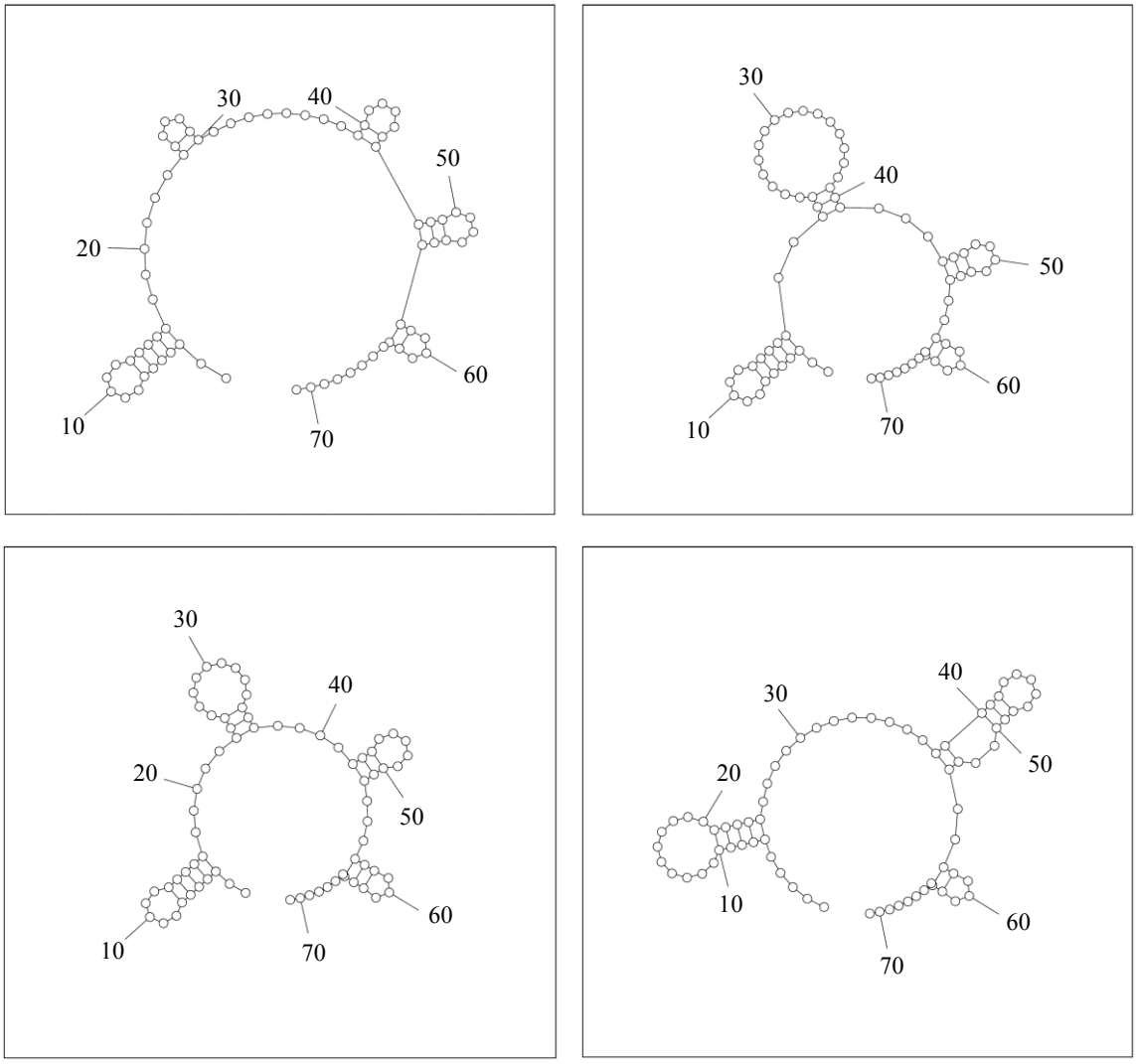


Figure 2. 6 Secondary structure of 4 of the 7 sequences cloned as predicted by RNAstructure. Each sequence is composed of 71 nucleotides, including primers.

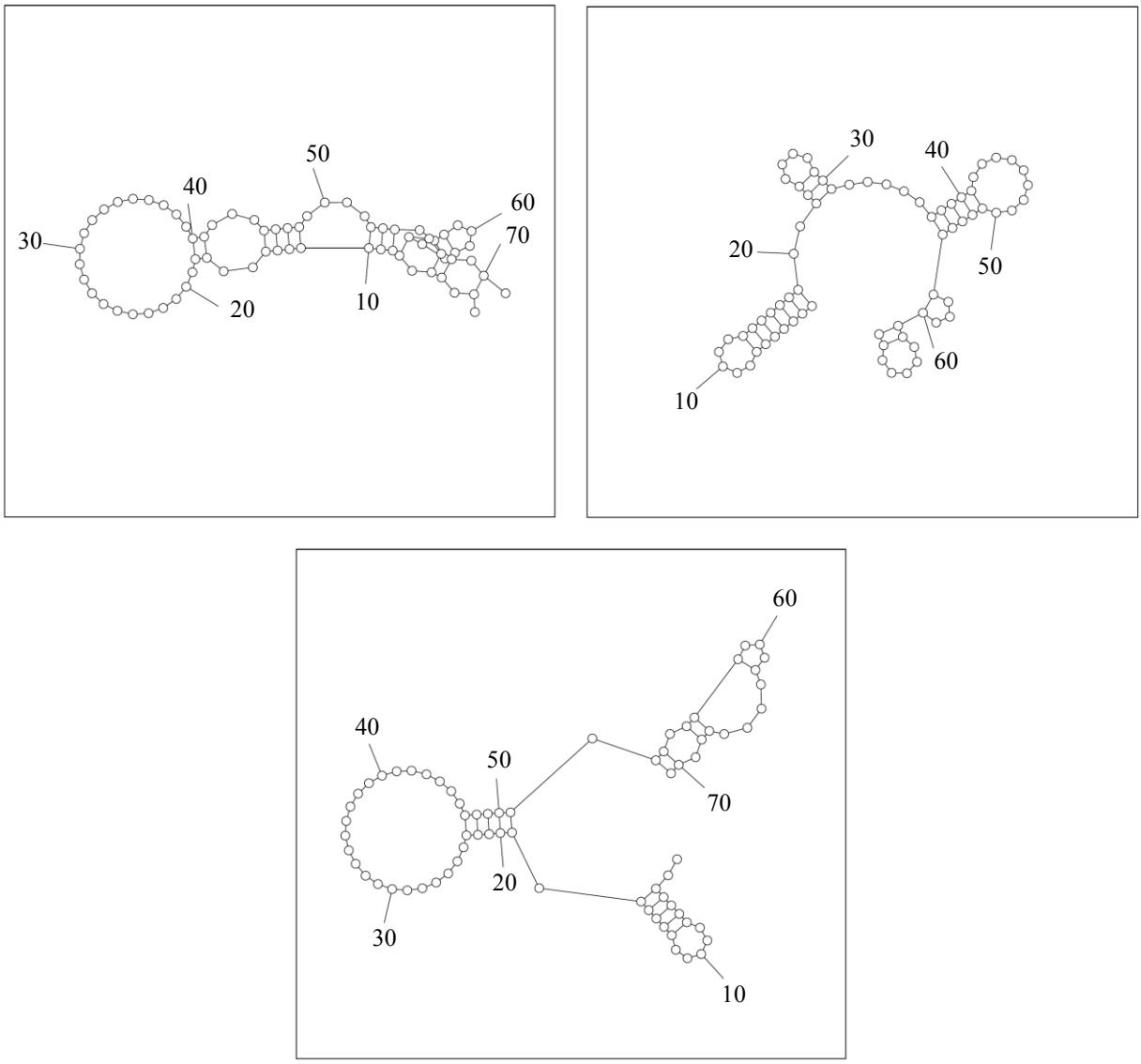


Figure 2. 7 Secondary structure of the remaining 3 of the 7 sequences cloned as predicted by RNAstructure. Each sequence is composed of 71 nucleotides, including primers, except for the top right structure which has 69 (missing two nucleotides in random region).

All sequences contain stem loops of different lengths and sizes. At this point, it is not known if these sequences have high affinity to the target, nor is it known which part of the sequences would bind. RNAstructure software does not predict tertiary structures and as a result, the tertiary structure of the sequence is also unknown. These matters will be studied in the near future.

Various methods of SELEX are used to generate aptamers for small molecules as outlined in Table 1.2, many of which have resulted in successful aptamers for small molecules, yet an aptamer for patulin has not been described. There are a several possible reasons for this: 1) the molecule is much smaller compared to the individual strand of DNA such that the change in mass upon forming a complex is minor, thus separation after the selection stage is difficult; 2) by immobilizing the target to a solid support matrix, an area of the target which could have bound potential aptamers is no longer accessible, thereby limiting which type of sequences can bind; 3) if the molecule is attached to the column, sequences can bind to the column rather than the target, since the column material volume is greater than the amount of target present; 4) even if a potential aptamer is generated, it may have low binding affinity to the target free in solution due to modifications subjected for immobilization. An example is with the rhodamine aptamer, which binds with higher affinity to rhodamine when the aptamer is immobilized on a solid support matrix than when it is free in solution[81]; and 5) the amount of column material is proportionately much larger compared to the amount of the target, thus high specific binders can bind to the column material itself and can increase the possibility of carrying over non-specific sequences to the next rounds, even if a negative selection is utilized.

For the first few rounds, conditions are not very stringent and a high amount of DNA is used; this is because the aptamer pool may originally have a low frequency of certain types of sequences that may not be recovered if conditions are too stringent, and these low frequency sequences can potentially bind the target with equal or better affinity than the high frequency sequences[82,83].

Further into the selection process, when the low affinity binders have become more frequent, conditions are changed to increase stringency[82]. In this chapter, the conditions changed to

increase stringency include higher DNA to target ratio, and the switch from selections performed in buffer to selections performed in apple juice. Studies have shown that decreasing the amount of target during selection increases stringency on the selection and results in high affinity aptamers[82,84]. By increasing stringency, the pool becomes enriched with better binding sequences. It is suggested to be as stringent as possible, as aptamers of better quality will be obtained in the end than those obtained from less stringent conditions[83].

The dissociation constant (K_D) of the cloned sequences will be determined in future studies, however it is interesting to note that there is a relationship between the equilibrium binding and the length of the pool and target incubation. The patulin concentration is diluted as selection proceeds and is incubated with the pool for 30 min. Compared to other selections which have an incubation time of 60 min[85, 86], the incubation period for this selection is relatively short. Short incubation periods and dilute concentrations of target usually result in the favouring of aptamers with high reactivity and low K_D values[83]. The lower the K_D value, the higher the affinity of the aptamer for the target.

The components of the buffer used for the SELEX experiments were based on what would make the DNA bind better to the target and more structurally stable. Sodium and potassium are monovalent ions which are used for stabilizing DNA[87]. Since the DNA backbone is polyanionic, positive ions are required to block the repulsions of the negative charges which would otherwise inhibit the structure from folding compactly with a target[88]. In addition, potassium is used to stabilize sequences with G-rich regions that might fold into G-quadruplex tertiary structures[89].

2.6 Conclusion

The mycotoxin patulin (PAT) is found to contaminate apples and apple products. In this chapter, a method for generating an aptamer for PAT was developed. Currently, there is no aptamer for PAT. The selection progression looked promising even after increasing stringency by performing the selections in apple juice, as the recovery gradually increased over the 12 rounds. After the final round of SELEX, the pool was cloned and sequenced. 7 potential aptamers have emerged from the pool and their secondary structure predicted. The affinity of these sequences to PAT will be determined in future work. The pool will also be sent for high throughput sequencing such that more sequences may be uncovered.

Chapter 3 - Development of DNA Aptamer for the Mycotoxin Deoxynivalenol

3.1 Introduction

3.1.1 Properties and Structure of Deoxynivalenol

Deoxynivalenol (DON) or Vomitoxin, is an epoxy sesquiterpenoid mycotoxin and secondary metabolite produced by fungi in the genus *Fusarium*, specifically *F. graminearum* and *F. culmorum*. The chemical name of DON is 12,13-epoxy-3α,7α,15-trihydroxytrichothec-9-en-8-on[90], and is part of over 200 toxins belonging to a class known as the trichothecenes. Trichothecenes are small, amphipathic molecules that can move passively across cell membranes[91]. The distinguishing part of trichothecenes is the tricyclic 12,13-epoxytrichothec-9-ene core structure, and based on the different substitutions on the core structure, the trichothecenes are divided into four groups[91]. These groups consist of Type A, Type B, Type C, and Type D trichothecenes, and an example of a mycotoxin belonging to each group is T-2 toxin, nivalenol, crotocin, and roridin A, respectively[91]. DON, together with DON's acetyl derivatives 3-acetyl DON (3-ADON) and 15-acteyl DON (15-ADON), belongs in the type B group, which differs from the other groups by the carbonyl at the C8 position[91]. Although T-2 toxin is the most toxic of the aforementioned trichothecenes, DON is most prevalent worldwide[91]. The structure of DON is shown in Figure 3.1.

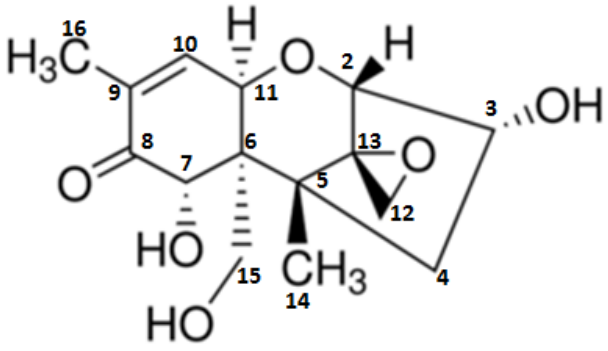


Figure 3. 1 Molecular structure of deoxynivalenol (DON) with numerically labelled carbons.

DON is the most abundant mycotoxin found to invade crops such as wheat, oats, and barley[52]. It can bypass several manufacturing processes and is heat resistant, which is why it is often found in foods even after cooking and processing[92].

Aside from standard farming practices outlined in section 1.2.1 there is not much that can be done to prevent DON contamination, especially as weather has the largest impact on fungal growth and consequently mycotoxin production. DON cannot be completely eradicated from fields as it clings to fungi spores and can contaminate other crops[93].

3.1.2 Conditions for DON Production

It is estimated that the average temperature is increasing every year and the resulting outcome is increased moisture in the air. Extra moisture can lead to shifts in the type of mycotoxin that becomes more abundant, as was the case with zearalenone. Nearly 10 years ago, zearalenone was the most abundant mycotoxin in corn in Canada, however due to increasing average temperature, DON has become the most abundant[51].

The crop diseases Fusarium Head Blight (FHB) and pink ear rot are caused by infection with *Fusarium graminearum* in wheat and barley, and corn, respectively[51]. The diseases occur when weather conditions are warm and damp during the flowering stage of these crops[51]. For farmers, the mentioned crop diseases result in reduced marketability of their crops, and having to lower their prices in order to sell. For processors, it results in having to look for other sources of grains for manufacturing purposes which are less contaminated[94].

3.1.3 Toxicity

Human and animal exposure to DON is usually chronic and is primarily through ingestion; however inhalation and dermal contact are also possible. Symptoms associated with increasing DON exposure include feed refusal and vomiting in animals[91,95]. Generally, DON acts by inhibiting protein synthesis in eukaryotes[98]. It prevents the formation of the peptide bond by peptidyltransferase in the ribosomal subunit, thereby inhibiting polypeptide chain initiation or elongation. A ribotoxic stress response is then activated resulting in the increased expression of pro-inflammatory genes or apoptosis[96,97,98]. Eventually it leads to the production of high levels of oxidative stress due to the production of free-radicals.

More specifically, DON induces vomiting by interfering with serotonin, a neurotransmitter produced mainly in the gastrointestinal tract involved in appetite and vomiting[99,100]. Serotonin activates vomiting by binding to receptors on vagal afferent which in turn sends stimuli to the medulla oblongata which directs autonomic and motor neurons. DON can also cause anorexia by altering neuroendocrine and cytokine signaling in the gut-brain axis. More specifically, DON increases the production of proinflammatory cytokines interleukin-1β, interleukin-6 and tumor necrosis factor-α in the brain which in turn reduce food intake[95]. The functional groups on DON essentially responsible for its toxicity are the three hydroxyls on C3, C7, and C15[100].

The acetyl derivatives of DON are always found with DON contamination although the concentrations are much lower[94]. All three toxins have been shown to exhibit similar acute toxicities after oral, and intraperitoneal and subcutaneous injection; however 3-ADON is considered similarly toxic to DON whereas 15-ADON is less toxic than DON[94].

3.1.4 Limits

Since contamination with DON in crops cannot be prevented, nor can it be eliminated during manufacturing, DON levels are being closely monitored worldwide for public safety. The United Nations Environmental Programme (UNEP) and the World Health Organization (WHO) International Programme on Chemical Safety (IPCS) have proclaimed that humans have the right to food that is free from mycotoxin levels that could lead to health risks[47]. When it comes to DON, the Canadian standard (Health Canada) is set to a maximum acceptable limit of 1 ppm (mg/kg) for un-cleaned soft wheat in baby food and 2 ppm for un-cleaned soft wheat in non-staple foods[75]. Likewise, the Food and Drug Administration (FDA) proposed maximum of limits 1 ppm on finished wheat products for human consumption, 10 ppm for cattle and chickens, and 5 ppm for grains and grain products for humans (composing ≤40% of diet), and for swine and other animals (composing ≤ 20% of diet)[101,102]. The FDA has not set a maximum limit for the amount of DON in wheat intended for milling as the levels of DON get reduced during the manufacturing processes, therefore imposing limits is deemed impractical at that point. The European Union (EU) has set a stricter limit of 0.2 ppm for infants and small children and 1.75 ppm for unprocessed durum wheat/oats/maize[76]. The limits of certain countries can be found in Table 3.1.

Table 3. 1 DON limits in various countries[76].

COUNTRY	FOODSTUFF	DON (PPM)
Canada	Uncleaned soft wheat in non-staple foods	2
	Uncleaned soft wheat in baby foods	1
China	Corn, wheat, barley, cereals	1
India	Wheat	1
Japan	Wheat	1.1
US	Finished wheat products	1

Finally, the Scientific Committee on Food[103] has set a provisional maximal tolerable daily intake (PMTDI) of 1 μg/kg body weight (b.w.) per day. Recently, the limits were extended to the acetyl derivatives of DON (3-ADON and 15-ADON) by the Joint FAO/WHO Expert Committee on Food Additives (JECFA)[103] due to the fact that they become deacetylated *in vivo*. They have also set the acute reference dose (ARfD) for DON and its acetyl derivatives at 8 μg/kg b.w[103]. Since DON can also be converted into a sugar conjugate named DON glucoside and then subsequently converted to DON when consumed, DON-glucoside is being monitored and may be included in the PMTDI[51,103]. The sugar-conjugate is undetectable during DON analysis.

3.1.5 Current Methods of Detection

Fungal infection does not always cause crop damage, and therefore mycotoxin contamination is not always obvious[47]. Since DON is toxic and widespread, there is a need to test for DON contamination in field, during storage, and after storage. There are official analytical methods for mycotoxin detection, validated by the Association of Official Analytical Chemists

International and the European Committee for Standardization[47]; main methods include high performance liquid chromatography (HPLC), often coupled to a mass spectrometer for peak analysis, and immuno-based methods such as enzyme-linked immunoassay (ELISA). The latter is used if quick analyses are needed. These methods are reliable, accurate, and sensitive. However there are downfalls; HPLC is an expensive instrument, and analysis generally requires sample purification, and ELISAs involve antibodies which have a short shelf life and must be refrigerated[44]. Therefore, other methods are employed such as thin-layer chromatography (TLC), which is another quick detection method which is reliable and inexpensive. Nevertheless, TLC is not suitable for quantification of DON, and the preparation of standards can be expensive. Another method which is widely used is gas chromatography (GC) [104], however, the instrument is expensive and also requires a skilled technician to perform the analysis. Surface Plasmon Resonance (SPR) [105] and UV spectroscopy [106] are also used as reliable analysis methods.

3.2 Chapter Objectives

Deoxynivalenol (DON) is a mycotoxin found in wheat and corn. Current methods for detecting the toxin are expensive, have a short shelf life, and sometimes require a skilled technician. Therefore, the search for inexpensive and specific detection agents for this toxin is ongoing. Aptamers could be a possible solution for the detection of DON in food and feed, thus providing improved quality control. The objective of this project is to generate a DNA aptamer for the mycotoxin DON. To generate an aptamer for DON, a library of different sequences of DNA is synthesized and subjected to DON, which is supported on a solid phase column. The repeated process of exposing the DNA library to DON and amplifying the sequences which bind is known as SELEX. Aptamers for DON did not exist until a thesis authored by Jakob Eifler[107] released in

July 2014 claimed to have successfully generated aptamers for DON. The thesis will be a valuable reference for comparison of my DON aptamers.

3.3 Statement of Contributions

Library synthesis and the initial three rounds of selection were performed by Dr. Maureen McKeague. DNA pool sequencing was performed by the Sequencing Lab at the University of Calgary. Primer synthesis, aptamer selection, and cloning were performed by Annamaria Ruscito.

3.4 Materials and Methods

3.4.1 Materials

Deoxynivalenol (DON) was purchased from Sigma-Aldrich (Canada) with ≥ 98% purity (TLC). Also purchased from Sigma-Aldrich (Canada) were 1-butaneboronic acid, succinic anhydride, hydroxylamine hydrochloride, pyridine-d_5, pyridine, and 1,1′-Carbonyldiimidazole (CDI). Propylene oxide was purchased from Acro Organics (New Jersey, USA). Ultra High Purity 5.0 argon was purchased from Praxair. Ground corn, oats (malted), barley, distiller's grain, and wheat samples were provided by Trilogy Analytical Laboratory (Washington, MO). CarboxyLink Coupling Resin (immobilized diaminodipropylamine, DADPA, 50% slurry), EDC, sulfo-NHS, and suflo-NHS acetate were purchased from Thermo Scientific Pierce (Illinois, USA). Dynabeads® M-450 epoxy was purchased from Life Technologies Inc. (Canada). Oligo d(T)25 beads were purchased from New England Biolabs (Massachussets, USA). Phosphoramidites, modifiers, activator, de-block, capping, and oxidizing reagents for DNA synthesis were obtained from Glen Research. Acetonitrile and standard support columns were purchased from BioAutomation. Unmodified primers for cloning were obtained from Integrated

DNA technologies (IDT) – Iowa, USA). Strataclone Cloning Kit was purchased from Agilent Technologies Inc (Ontario, Canada). Illustra TempliPhi 100 Amplification Kit was purchased from GE Healthcare Life Sciences (Quebec, Canada). The ELISA kit was purchased from Helica (California, USA). Buffer salts were purchased from Sigma Aldrich. Buffers were prepared with Millipore Milli-Q deionized water at 18 MΩcm.

3.4.2 Library and Primer Synthesis

DNA library (also called pool) and primers for DON SELEX were synthesized by Dr. Maureen McKeague and Annamaria Ruscito, respectively, using standard phosphoramidite chemistry on a BioAutomation Mermade 6 (Plano, Texas) with 1 μM controlled pore glass (CPG) columns of 1000 Å size. The pool, 81 bases in length, is composed of fixed primer binding regions which flank a random region:

5'- ATCCAGAGTGACGCAGCA-N45-TGGACACGGTGGCTTAGT -3'. The forward primer is fluorescently modified to fluorescently label binding DNA such that it can be monitored throughout the SELEX process, and the reverse primer is modified with a poly A tail to increase the strand size such that the double stands can be distinguished during separation with denaturing gel electrophoresis. The primers are: 1) ModPrimer1: 5'-6FAM-ATCCAGAGTGACGCAGCA-3' and 2) ModPrimer2: 5'-poly-dA_{20}-HEG-ACTAAGCCACCGTGTCCA-3'. The HEG link stands for hexaethylene glycol Spacer Phosphoramidite 18 (Glen Research) and serves to prevent the formation of the polyA tail complement during polymerase chain reaction (PCR). The pool was purified using 12% denaturing gel electrophoresis and desalted. For cloning, unmodified versions of the above primers were used: Primer1: 5'-ATCCAGAGTGACGCAGCA-3' and Primer2: 5'-ACTAAGCCACCGTGTCCA-3'.

3.4.3 SELEX Beads

In this study, two types of beads were used for immobilization of the target molecule; agarose and magnetic beads. The agarose beads are convalently linked to a diamino-dipropylamine (DADPA) spacer which contains a primary amine at the end. These beads are porous and stable. The magnetic beads are uniform, monodispersed, non-porous, and 4.5 μm in diameter. They are superparamagnetic, composed of a mixture of maghemite (gamma-Fe_2O_3) and magnetite (Fe_3O_4). To coat the magnetic material, a polystyrene shell is used. Both beads can be used to immobilize a variety of molecules, including mycotoxins, and can be re-used.

3.4.4 Column for Selection Rounds 1-3 (Column 1)

The protocol for this reaction was modified from Vatèle *et al*[108]. For the first three rounds, the positive column was created using CDI chemistry. 2 mg DON was added to 1.0 mg CDI and 20 μL reagent grade acetone. The acetone is used to convert the alcohol into a reactive carbamate. The reaction was left to shake for 7 h. In the meanwhile, 5 mL DADPA slurry was washed with a mixture of 0.2 M $NaHCO_3$, pH 10, and acetone in a gradient, beginning with 0% up to 30% acetone in 5% increments. The slurry was suspended in 5 mL of 0.2 M $NaHCO_3$ before the DON-CDI reaction was added and left to react for three days at room temperature. Afterwards, the slurry was washed five times with 0.1 M $NaHCO_3$, pH 8.5. 100 mg sulfo-NHS acetate was dissolved in 3 mL of 0.1 M $NaHCO_3$ and added to the slurry, reacting for an hour with shaking in order to quench un-reacted amine groups present on the DADPA beads. The mixture was then washed ten times with DON Selection Buffer (DSB) containing 20 mM HEPES, 125 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$, pH 7.6. Lastly, the negative column was created by

reacting sulfo-NHS acetate to 5 mL of unmodified DADPA beads and washed the same way as the positive column.

3.4.5 Column for Selection Rounds 4-8 (Column 2)

The protocol for this reaction was modified from Casale *et al*[109]. 4 mg DON dissolved in acetone in a 2 mL glass vial was dried into a film with argon gas and rehydrated with 200 µL pyridine-d_5. 17 mg 1-butaneboronic acid was added and the mixture was stirred overnight at room temperature. The next day, 200 µL of 1.7 M succinic anhydride dissolved in pyridine-d_5 was added while stirring. The glass vial was sealed and placed in a boiling water bath for 90 min. The pyridine solution was dried into a film with argon gas and refrigerated for 48 hours.

The following protocol is from ThermoScientific – EDC Instructions: Procedure for Two-Step Coupling of Proteins Using EDC and NHS or Sulfo-NHS (Catalog no. 77149). This protocol is performed to react DON with DADPA beads.

5 mL of DADPA beads was washed ten times and suspended in coupling buffer (Phosphate Buffered Saline, PBS - 100 mM sodium phosphate, 150 mM NaCl, pH 7.2). The DON film was rehydrated with 2 mL of activation buffer (0.1 M MES, 0.5 M NaCl, pH 6.0). 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and sulfo-N-hydroxysulfosuccinimide (NHS) were equilibrated to room temperature. Then 0.7 mg EDC (2 mM) and 2.2 mg sulfo-NHS (5 mM) were added to DON. The solution was left to react for 15 min at room temperature. Afterwards, 2-mercaptopethanol, added to a final concentration of 20 mM, was used to quench the EDC. The solution was added to the DADPA beads and allowed to react for 2 hours with gentle shaking at room temperature. To quench the reaction, hydroxylamine hydrochloride was added to a final concentration of 10 mM. The beads were washed another five times with PBS, then washed three

times with methanol. The methanol evaporated from the beads with argon gas between each wash. Finally, the beads were washed ten times with DSB and stored at 4ºC.

The same procedure was followed for the negative column, only that DON was omitted in the reaction. No methanol washings were performed.

3.4.6 Enzyme-Linked Immunosorbent Assay (ELISA) for Column 2

The washings from column 2 reaction were collected and tested with a Helica DON ELISA kit. The test is to determine the amount of DON unbound and washed away from the beads. The manufacturer's protocol was followed. The collected washings were diluted 300 times with deionized water before being added to the microplate well. The reaction signal was measured using an Asys Hitech GmbH Expert plus UV ELISA plate reader with a 450 nm filter.

3.4.7 Column for Selection Rounds 9-10 (Column 3)

The following protocol is a scaled down version from Invitrogen™ Life Technologies™ Dynabeads® M-450 Epoxy beads (Catalog no. 14011), modified for small molecule reaction.

0.5 mg DON in 200 µL acetonitrile was transferred to a 2 mL glass vial with 5.5 mg 1-butaneboronic acid. The solution was left stirring for 48 h, and meanwhile Dynabeads ® were vortexed for 30 seconds before removing 1.5 mL and washing four times with acetonitrile. The volume of the DON solution was increased to 1.7 mL, equal parts pyridine and acetonitrile, before being added to the magnetic beads. The bead-DON solution was left to shake for 48 h at room temperature. After removing the supernatant, the beads were washed three times with a 1:1 solution of pyridine: acetonitrile, then four times with a new DON selection buffer (DSB-137

mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 2 mM EDTA, pH 7.4). The beads were left suspended in the new DSB and stored at 4ºC.

To open the epoxides on the beads of the negative column, such that they do not resemble the epoxides on DON, a modified protocol by Reeve and Sadle[110] was followed. 1 mL magnetic beads were suspended in 1 mL DSB, pH 4, while a solution of DSB composed of 10% methanol was made. Beads were incubated with methanol mixture for 5 min. Incubation with DSB and methanol was repeated three times. Beads were then washed three times with DSB and stored at 4ºC.

3.4.8 Testing the Magnetic Bead Reaction for Column 3

To determine if the reaction with magnetic beads in the positive column was successful, the conjugation protocol was tested with propylene oxide in place of magnetic beads and analyzed by mass spectrometry (MS). 0.2 mg DON in acetonitrile was added to 0.5 mg 1-butaneboronic acid and left to react for 24 h with gentle stirring. A 1:1 solution of pyridine: acetonitrile was added to the reaction mixture followed by 300 µL propylene oxide. The reaction was incubated overnight at room temperature with gently stirring. The reaction was then dried with argon gas and stored at -4ºC for 48 hours before being shipped for MS analysis.

3.4.9 Isolating PolyA DNA

The following protocol is a modified version from New England Biolabs - NEBNext Magnetic Oligo d(T)25 (Catalog no. S1419S).

To ensure all polyA-modified sequences were removed from the pool, the pool DNA was incubated with oligo d(T) - magnetic beads. 15 µL of Oligo $d(T)_{25}$ beads were washed twice with

100 µL 1 M NaCl and then re-suspended in 50 µL. DNA pool dried down on a Savant AES2010 SpeedVac was rehydrated in 50 µL deionized water and added to the tube with the beads. The beads were heated at 90ºC for 5 min, and then cooled to 4ºC. Once the temperature reached 4ºC, the beads were incubated for 5 min at room temperature. The tube with the beads was placed in a magnetic rack for 2 min at room temperature. The supernatant was set aside and the tube removed from the rack. The beads were washed twice with 200 µL of 500 mM NaCl to remove unbound DNA, pipetting up and down six times. The tube was placed back into the magnetic rack for 2 min at room temperature. The supernatant was set aside and the tube removed from the rack. The beads were washed twice with 200 mM NaCl and then with deionized water. DNA from supernatant was dried down on the SpeedVac and purified with polyacrylamide gel electrophoresis.

3.4.10 Phenol-Chloroform Extraction and Ethanol Precipitation

To purify eluted DNA from the supernatant of grain mix containing proteins and lipids, phenol/chloroform extraction and ethanol precipitation were performed. Prior to the extraction, the eluted DNA from SELEX was desalted five times and filtered three times with a Spin-X cellulose acetate microcentrifuge filter tube. A 1:1 volume of phenol/chloroform: DNA was added to the DNA and vortexed vigorously for 10 sec. The solution was centrifuged for 1 min at 14800 *g*. The top aqueous layer containing DNA was transferred to a new tube. A solution containing 50 µL of 3 M NaCl and 1 mL of anhydrous ethanol was made and chilled on dry ice. Once chilled, 100 µL of the DNA solution was added to the ethanol/salt solution and placed on dry ice for 1 hr. The solution was then centrifuged at -9ºC at 14.8 g for 30 min, followed by cooling over dry ice for 10 min. This process was repeated a second time. The supernatant was

removed, leaving a pellet containing salt and DNA. The pellet was dried overnight by cryopumping on a Savant AES2010 SpeedVac and subsequently desalted.

3.4.11 Preparing Grain Supernatant for SELEX

0.5 g of ground distiller's grain, corn, malted oats, barley, and wheat were mixed in a 50 mL tube. 6 mL of deionized water was added, and the tube was sealed and placed on a shaker for 10 min. The tube was taken out of the shaker and left at room temperature overnight to let particulate matter settle. The supernatant was removed and filtered through a Whatman No. 1 filter paper, then through a 0.45 μm syringe filter, and after that through a Spin-X cellulose acetate microcentrifuge filter tube. The supernatant was heated at 90ºC for 10 min, and then filtered two more times through a Spin-X tube. The solution was stored at 4ºC.

3.4.12 SELEX

The sequences in the DNA library for DON SELEX each contain a random region of 45 nucleotides flanked by regions of known sequence. The flanked regions are for primer binding during PCR amplification after each round.

DNA in DSB was heated at 90ºC for 5 min, cooled at 4ºC for 10 min, and then warmed to room temperature for 10 min. The DNA was added to the beads of the negative column and shaken for 30 min. DNA that did not bind to the beads was heated and cooled as before then added to the beads of the positive column. After 30 min of shaking, the beads of the negative and positive column were washed five times with DSB. 90ºC heat and 6-8 M urea was used to elute DNA bound to the beads, and a total of five elutions were performed. Round 10 column incubations were performed with the supernatant of a grain mix. Fluorescence measurements were obtained

using Fluorolog Fluorescence Spectrophotometer (Horiba Jobin Yvon, USA) at an excitation wavelength of 490 nm and emission wavelength of 520 nm. The excitation and emission slit widths were 5 nm. Subsequently, the samples were desalted and amplified for the next round. The equation for percent recovery is shown below. Table 3.2 shows the changes made during the selection rounds. The equation below estimates the amount of sequences that bound to the column matrix:

$$\%Recovery = \frac{Felution * 100\%}{Fpool}$$

Where *Felution* and *Fpool* are the fluorescence intensities of the eluted sequences and the initial DNA library, respectively.

Table 3. 2 Changes made to DON SELEX procedure at specific selection rounds. Column numbers 2 and 3 represent columns made with DADPA beads + succinic anhydride and magnetic beads, respectively. Changes were carried to subsequent rounds unless stated otherwise.

SELECTION ROUND	COLUMN NUMBER	CHANGES MADE TO PROCEDURE
4	2	Decreased number of washes from 7 to 5. Increased amount of pool DNA from 200 pmol to 500 pmol.
6		Increased urea concentration to 8 M from 6 M.
7		Decreased amount of pool DNA from 500 pmol to 200 pmol.
8		Re-protected negative column with sulfo-NHS acetate.
9	3	New DON Selection Buffer (DBS). Diluted beads of positive column 10X with beads of negative column, then decreased volume of beads from 100 µL to 40 µL. During elutions tubes were placed on heat block at 90ºC and vortexed three times throughout elution incubation instead of being shaken the entire time.
10		Selection performed in supernatant of mixed grains.

3.5 Results and Discussion

3.5.1 Immobilization Strategy

DON was immobilized onto a solid support matrix for the selection of DON-specific aptamers from a DNA library. Immobilizing DON allows for easy separation of sequences that do not bind to DON from those that do bind. While there are advantages associated with this method, such as simple and quick separation, there are also some issues; non-specific sequences may bind to the solid support matrix and, if used, the linker between the target and the solid support matrix. Less specific sequences will bind partly to DON and partly to the solid support matrix and/or linker. In addition, high affinity binders which would bind to the part of DON that is immobilized may

not be accounted for. To minimize these problems as much as possible, three chemistries were investigated and further modifications were made.

In the first column, carbonyldiimide (CDI) chemistry was performed. DON reacted with CDI, forming an intermediate that would then bind to the primary amine on the bead. Un-reacted amines are blocked to prevent any further reactions and to avoid non-specific binding.

The second column used succinic anhydride as a linker between DON and the DADPA bead. The DON-free column also contained succinic anhydride, such that sequences that would bind to the linker instead of DON are removed. Finally, the last column involved the reaction of DON and the epoxy group on magnetic beads.

The epoxides in the DON-free column were opened with methanol to avoid losing sequences that would bind to the epoxide moiety of DON. Two columns use the hydroxyl on C3 for reaction and the other uses the hydroxyl on C15; C7 hydroxyl is poorly reactive compared to the hydroxyl on C3 and C15 due to the presence of the carbonyl at C8[111].There is less steric hindrance at the C3 hydroxyl position than at the others. In addition, antibodies for DON are produced by conjugating DON via either the C3 or C15 hydroxyl [109,111]. 1-butaneboronic acid was used for the columns which immobilized DON on the hydroxyl of the C3 carbon to protect the C7 and C15 hydroxyls from reacting. However, it was discovered later with the column 2 that 1-butaneboronic acid separates from diols in the presence of aqueous solution such as buffer[112]. Therefore, the reaction for this column was performed in pyridine and acetonitrile in column 3.

3.5.2 Column 1

To perform SELEX with DON, the positive column had to be created. DON SELEX was initiated with a column design by Vatèle *et al*.[108] Using CDI chemistry, DON is theoretically reacted to amine groups on the beads only through the C3 hydroxyl (Figure 3.2).

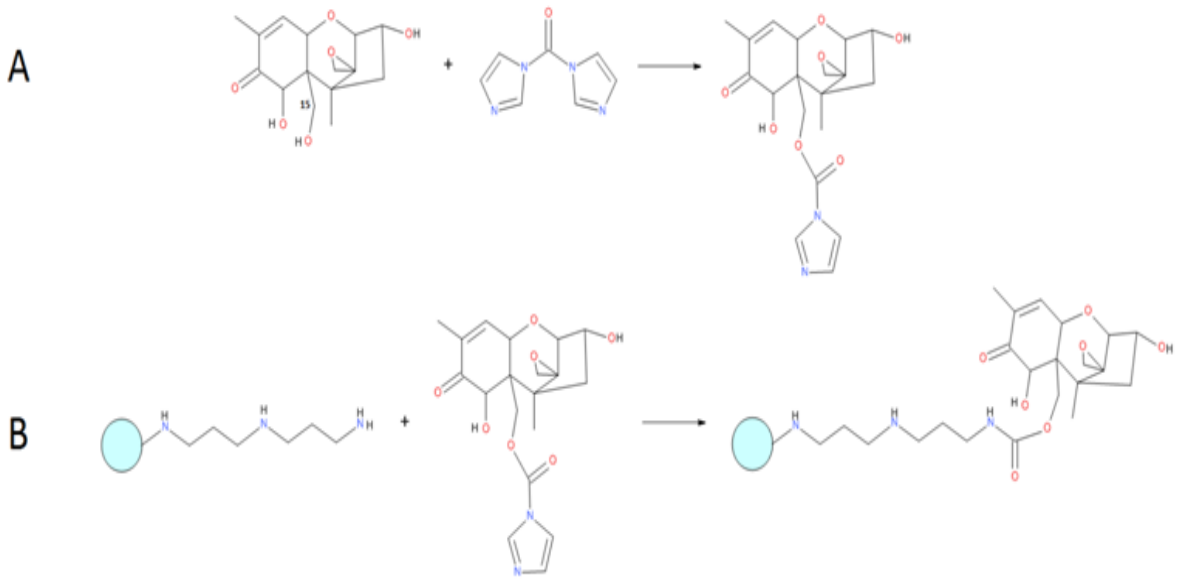


Figure 3. 2 Reaction of DON with carbonyldiimidazole (CDI) and DADPA beads for column 2. Steps involve (A) DON reaction with CDI on C15 hydroxyl, and then (B) reaction with primary amine on DADPA beads. Unreacted amines were protected with sulfo-NHS acetate.

Mass spectrometry analysis of the DON and CDI reaction (Figure 3.2A) showed a different product (Figure 3.3).

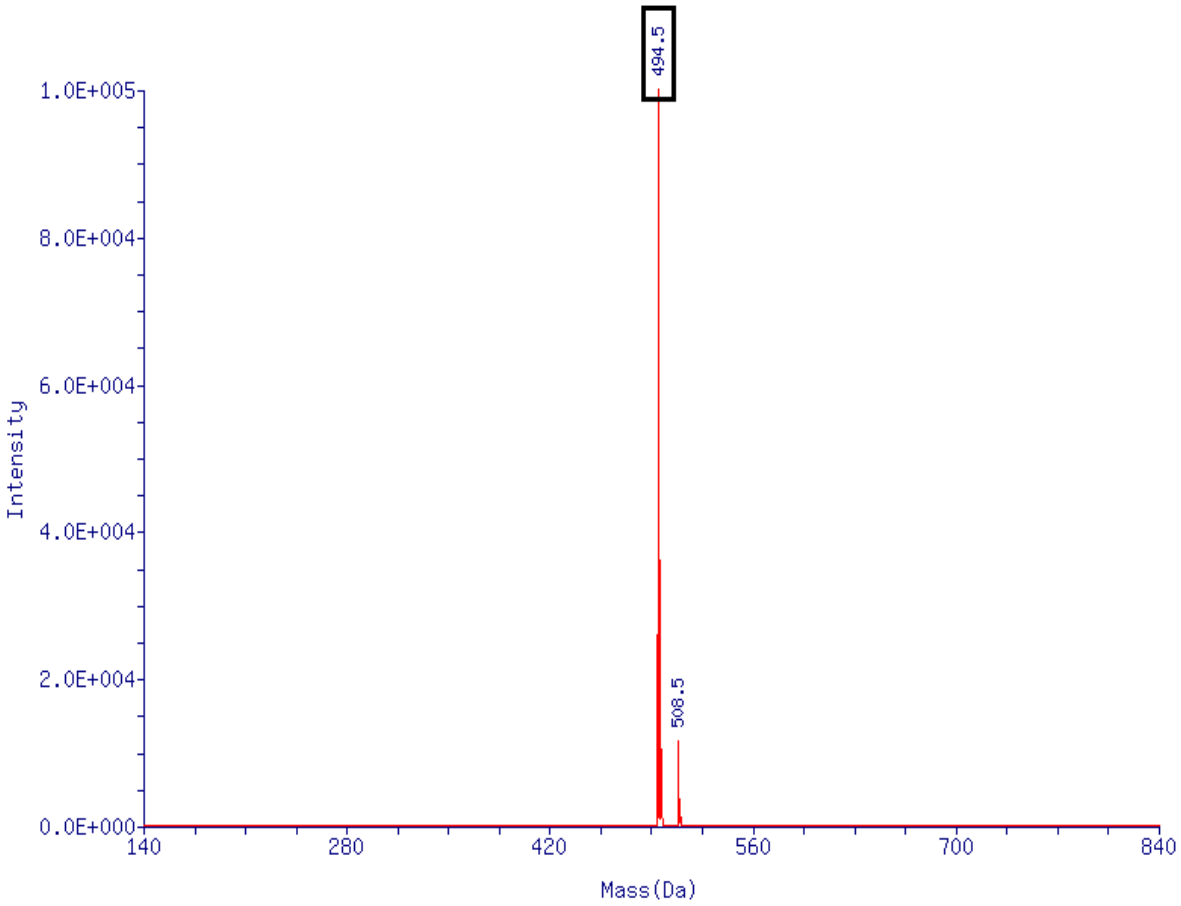


Figure 3. 3 Mass spectrometry spectrum of DON+CDI. Reaction solution is crude; there was no purification prior to analysis. Most prominent peak has a mass-to-charge ratio of 494.4 g/mol (boxed).

The two acceptable intermediate molecules following the reaction of DON with CDI are DON with an imidazole functional group and DON with the hydrolyzed form of the imidazole, as seen in Figure 3.4 (A-B). The former and later intermediates have a mass of 390 and 340 g/mol, respectively, and would react with one amine group on the bead. These masses, however, were not found in the mass spectrometry spectrum. Rather, the most abundant peak represented a mass-to-charge ratio of 494.5 g/mol which is likely the molecule shown in Figure 3.4C.

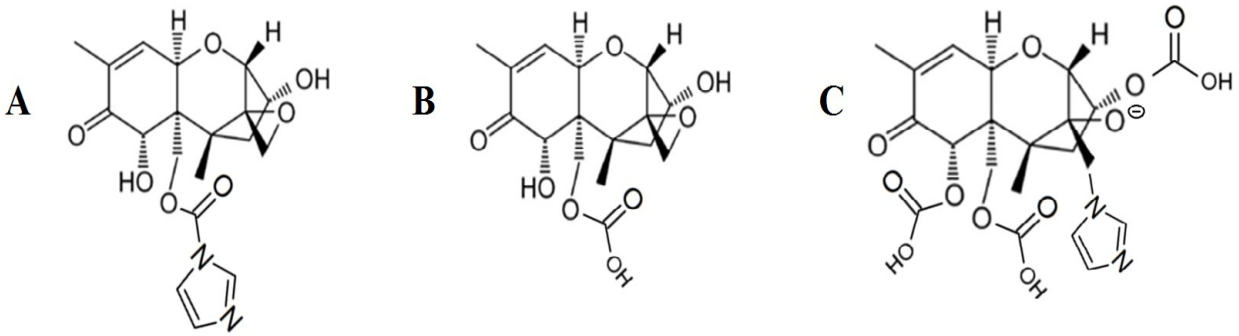


Figure 3. 4 Molecular structure of two expected DON+CDI intermediates (A-imidazole group and B-hydrolyzed imidazole) and the actual intermediate (C). Actual structure based on mass spectrometry results.

As shown, the actual product is not only modified at all three hydroxyls with the hydrolyzed imidazole form but the epoxide has also been opened and has an imidazole group attached to one end. These four modifications would have resulted in multiple beads binding to one molecule, and the opening of the epoxide makes this molecule essentially unrecognizable as DON. The epoxide on DON is a distinguishing functional group. If the molecule in Figure 3.4C were used in the positive column the resulting aptamer would likely not recognize free DON. As the plan for the aptamer is to be used in a sensor to detect free DON in food and feed, the whole purpose of generating an aptamer which binds with high affinity to DON would be negated.

As a result of these findings, the column was no longer used for SELEX; however the DNA pool which was exposed to this column for the first three rounds was used in the rounds that followed. We hypothesized that round 3 is still early enough in the selection process that the DNA pool is not yet enriched with sequences which would bind a specific target, thus it would still contain high affinity binders for DON, and in addition would be void of some unspecific sequences which bind to the solid phase, such as tube walls and the beads themselves.

3.5.3 Column 2

The protocol for the second column, modified from Casale *et al.*[109], appeared more promising (Figure 3.5).

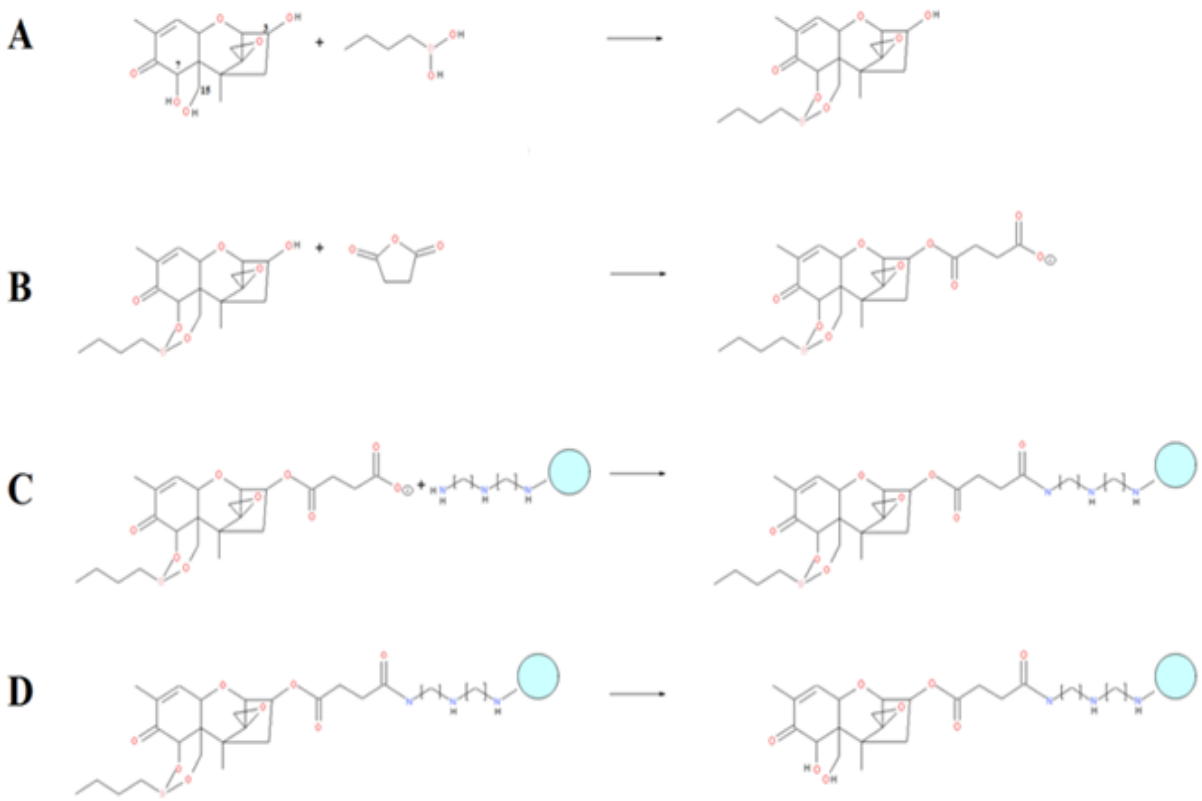


Figure 3. 5 Reaction of DON with DADPA beads for column 2. Steps involve (A) protection of C7 and C15 hydroxyls with 1-butaneboronic acid, and then (B) the reaction of the C3 hydroxyl with succinic anhydride; (C) Reaction of carboxyl group on succinic anhydride with amine on beads; and finally (D) C7 and C15 hydroxyls de-protected with methanol.

To ensure purity of the sample a mass spectrometry spectrum was taken of DON before column formation (Figure 3.6). According to the spectrum in Figure 3.6, the peak at 319 is consistent with DON+Na^+.

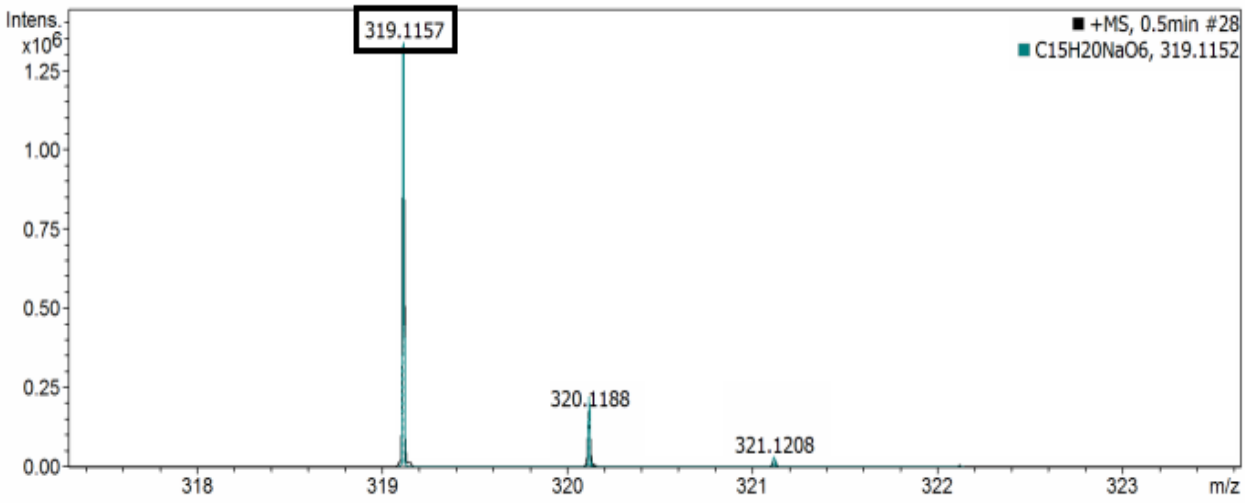


Figure 3. 6 Mass spectrometry spectrum of DON+Na^+ peak with mass-to-charge ratio of 319 (boxed). Spectrum obtained using a maXis impact mass spectrophotometer.

It is likely that the reaction to immobilize DON to the bead was successful; 1-butaneboronic acid likely bound to the specific hydroxyls on C7 and C15 as boronates have an affinity for 1,3-diols[112], and the boronic acid was added in excess. Succinic anhydride would have reacted with the remaining hydroxyl, opening the strained ring structure of succininc anhydride and exposing a carboxylic acid arm to react with the amine on the beads.

3.5.4 Enzyme-Linked Immunosorbent Assay

An ELISA test was performed on the column washings. The washings would contain a concentration of DON which did not react with the beads. With the measured average absorbance of 1.136 ± 0.139, the concentration from the standard curve (Figure 3.7) multiplied by the dilution factor was found to be 10960.465 ng/mL. Therefore, it was found that 6.85% (0.274 mg) of the starting material was present in the washings, implying that 93% of the initial amount of DON reacted with the beads.

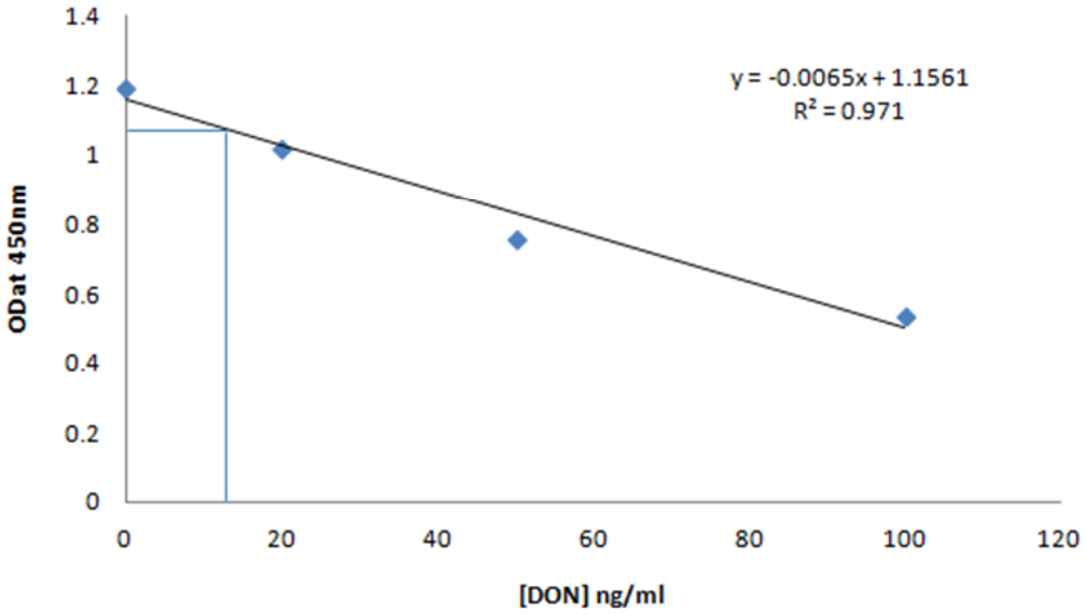


Figure 3. 7 DON standard curve results from ELISA test. Curve used to determine the concentration of DON in washes of positive column. Absorption wavelength at 450 nm.

3.5.5 Column 3

A different and less complicated method of reacting DON to the beads was discovered using magnetic beads with epoxide groups attached. This method does not involve a linker between DON and the bead, which may simplify the selection process. The removal of the linker arm, such as succinic anhydride in the previous column, means that there is no opportunity for DNA to bind to it, essentially leaving DON as the only target to bind to. The use of magnetic beads is more convenient and quick compared to Spin-X tubes; removal of the supernatant is as easy as placing the tube with the beads in a magnet to bring to the beads to one side, leaving the supernatant free of beads without the hassle of using a microcentrifuge to separate the beads and the supernatant using a nitrocellulose filter. Also, much less of target is required for conjugation; 1.6 mg and 5 mg of DON is required for conjugation with 5 mL of magnetic and sepharose

beads, respectively. Reacting DON to the magnet beads was a simple two-step process, first involving the protection of two hydroxyls on DON with 1-butaneboronic acid, and subsequently the reaction of the free remaining hydroxyl with the epoxide group on the bead, all at room temperature.

After the reaction was complete, it was necessary to determine the amount of DON on the beads; a UV-Visible spectrometer was used to detect the amount of DON in the column washes. Due to the conjugated system between C10, C9, C8, and the oxygen from C8, DON absorbs in the UV range, specifically at the wavelength λ_{max}=218 nm. The amount of DON found in the washes was 0.54 mg, or 77.7% of the starting material. Although this value may lead to believe that the reaction was unsuccessful and that there is not enough DON attached to the beads for SELEX, one must remember that it is the DNA: DON ratio that is of most importance; as long as the amount of DNA exceeds the amount of DON on the beads, SELEX can be efficient.

Analysis by mass spectrometry (MS) was employed to verify that DON was not modified further than expected. The reaction and expected product are shown in Figure 3.8.

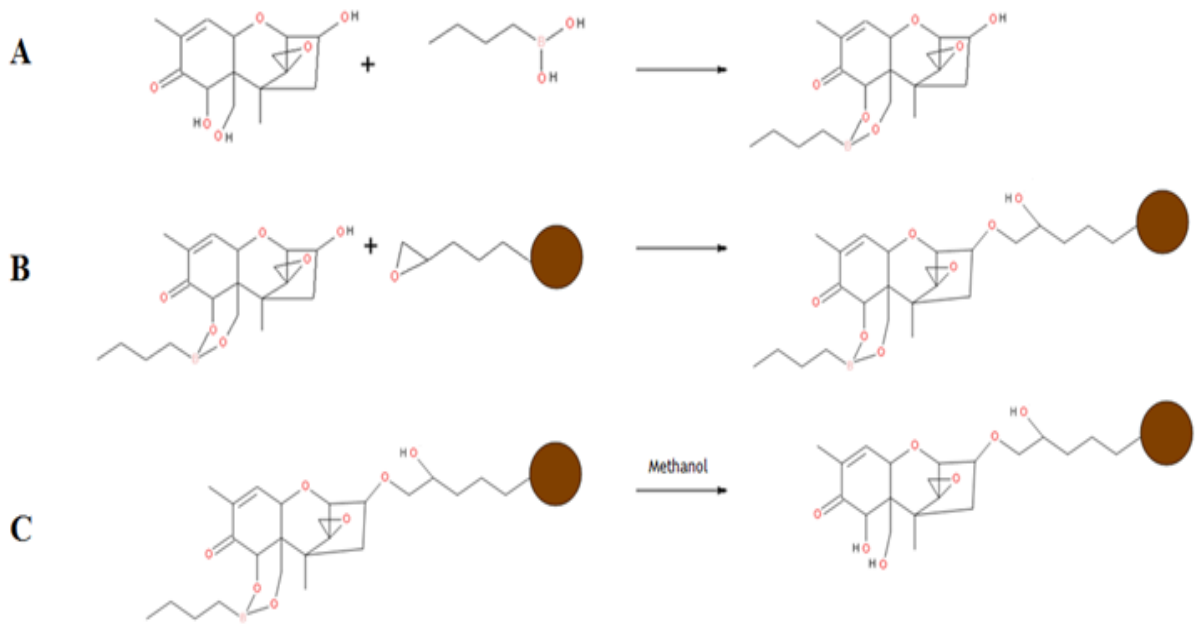


Figure 3. 8 Reaction of DON with magnetic beads for column 3. Steps involve (A) protection of C7 and C15 hydroxyls with 1-butaneboronic acid, and then (B) the reaction of the C3 hydroxyl with the epoxide on the magnetic bead. (C) C7 and C15 hydroxyls are de-protected with methanol.

The column reaction was repeated with propylene oxide in place of the magnetic beads; this was to mimic the beads and allow for analysis of the entire reaction solution with MS. The spectrum of DON alone is found in Figure 3.9A. The mass-to-charge ratio of the molecule of interest is 353, which would represent DON attached to propylene oxide via the C3 hydroxyl. The resulting spectrum shows a peak at 353, albeit it is not the most prominent peak (Figure 3.9B). However, it is important to note that the reaction solution was sent for analysis as a crude sample without purification, therefore other peaks could represent molecules which would likely be washed away from the column. With confirmation that DON is on the beads and is attached the way it was intended, SELEX was resumed.

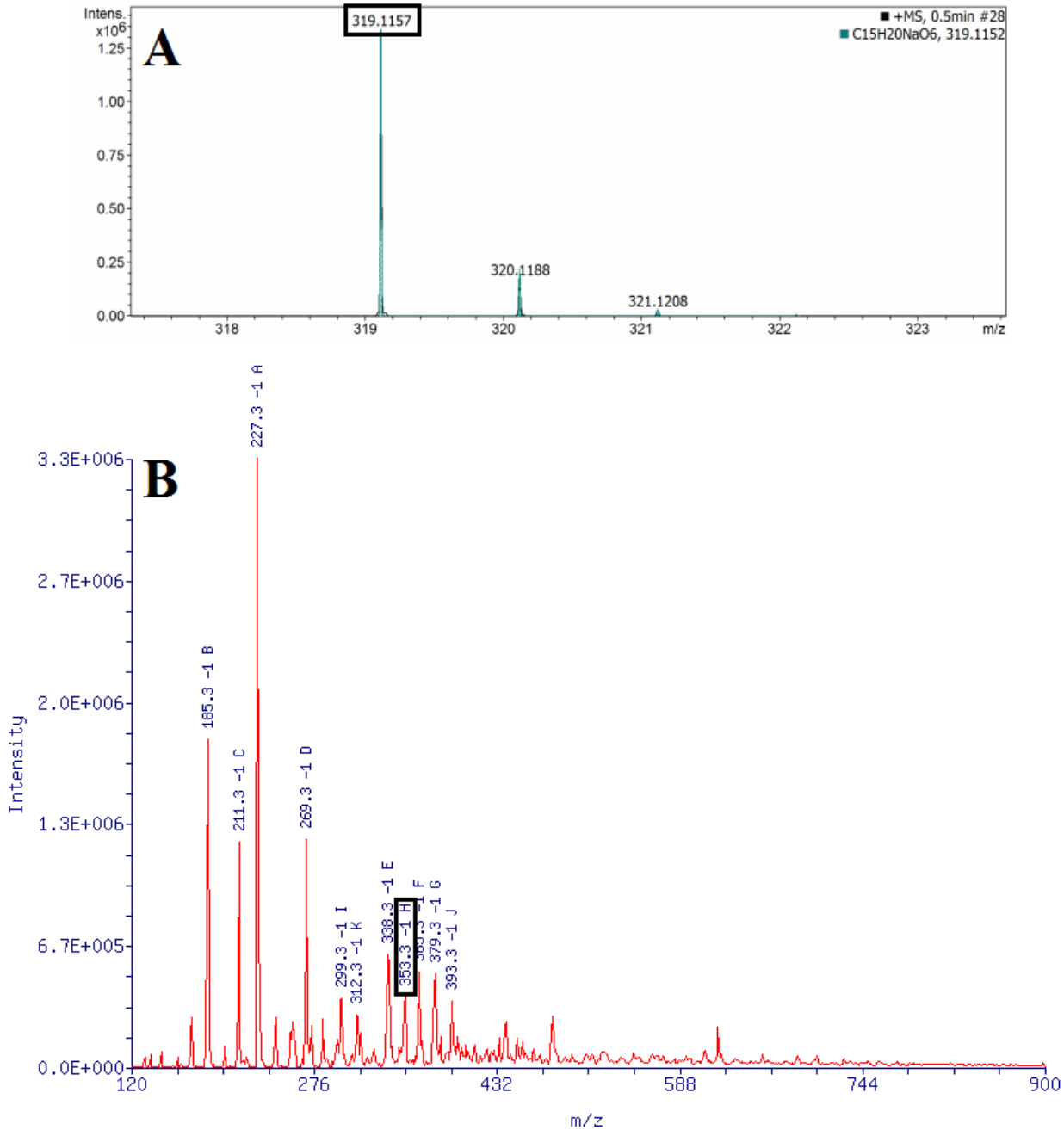


Figure 3. 9 Mass spectrometry spectrum of (A) DON+Na^+ peak with mass-to-charge ratio of 319 (boxed) using a maXis impact mass spectrophotometer, and (B) Crude solution of DON+1-butaneboronic acid+propylene oxide; there was no purification prior to analysis. Mass-to-charge ratio of interest is 353 g/mol (boxed).

3.5.6 SELEX

Results

A DNA library was chosen for this experiment as DNA is more stable than RNA, and DNA does not require the extra steps of transcription for PCR and reverse transcription to continue from one round to another[19].

The selection bar graph is shown in Figure 2.13. DNA recovery in rounds 1-3 began to increase, reaching 8% on round 2, showing promising pool enrichment. However, after recognizing that the CDI chemistry for the column by Vatèle *et al.* was not optimal for the selections, a new column was fabricated using a modified protocol from Casale *et al.* This protocol involved the protection of two hydroxyls on DON, with the remaining hydroxyl left to react with succinic anhydride which subsequently reacted with the amine on the DADPA linker.

After changing column matrix and increasing the DNA concentration from 200 to 500 pmol in round 4, the percent recovery decreased from about 5% to 0.72%, a factor of 5. This is attributed to the change in conditions. The recovery of the positive column gradually increased as selections went on until round 7. After changing the urea concentration from 6 M to 8 M in round 7, the percent recovery increased considerably. Recovery from round 7 was 100%, which is doubtful, especially since the percent recovery of the negative column from rounds 4-8 was 100% (not shown in figure), however, higher recovery than 37% (round 6) is expected. The concentration of DNA was then lowered to 200 pmol, resulting in a lower positive column recovery in round 8 compared to the previous 3 rounds. The negative column recovery remained at 100%.

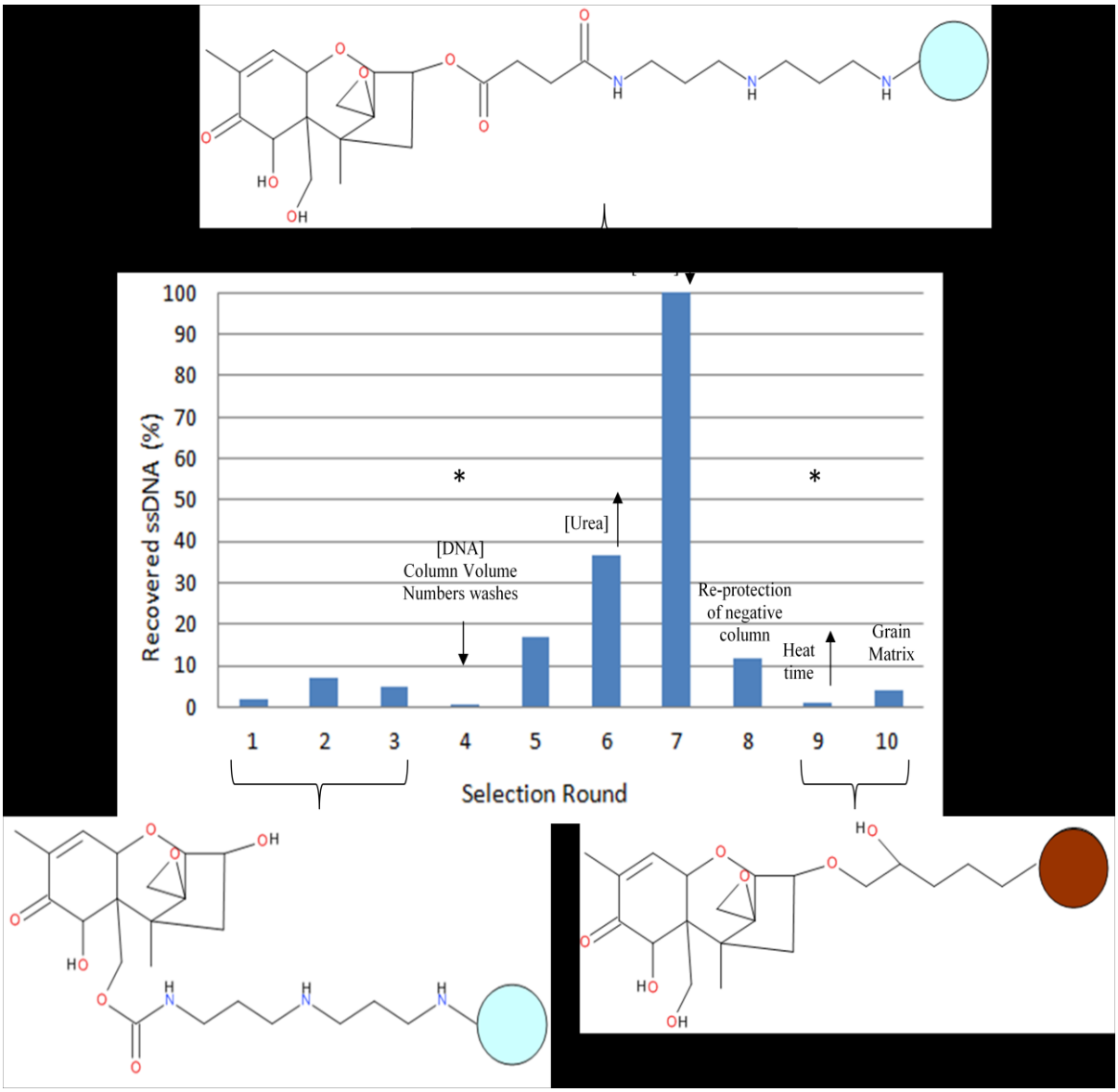


Figure 3. 10 Graph showing percent recovery of single-stranded DNA (ssDNA) in each round of DON SELEX. Selection was monitored by the fluorophore attached to the DNA. Asterisks represent the beginning of selections with a new column. Rounds 1-3 were performed with positive column involving CDI; rounds 4-8 performed with positive column involving succinic anhydride; rounds 9-10 performed with positive column involving magnetic beads. In round 4, the DNA concentration, column volume, and number of washes was decreased. In round 6, the urea concentration for the elutions was increased. Round 7 was performed with a lower DNA concentration. The negative column was re-protected with sulfo-NHS acetate in round 8. In round 9, the heat time for the elutions was increased. Round 10 was executed in grain supernatant instead of SELEX buffer. Recoveries from negative column not shown.

Another column was fabricated for the remaining two rounds in attempt to lower the recovery of the negative column. This new protocol involved protection of two hydroxyls on DON and the remaining hydroxyl reacting with an epoxide on the magnetic bead. The recoveries from the positive and negative columns of round 9 were both below 100%, at 1.2% and 0.14%, respectively. Positive column recovery decreased substantially compared to other rounds; yet, it was still greater than recovery from the negative column, which was expected. Selection in round 10 was performed in grain supernatant instead of buffer. The effect resulting from the change in conditions was an increase in recovery for both columns, from 1.2% to 4.1% for the positive column and from 0.14% to 4.8% for the negative column. The recovery of the negative column was slightly greater than the recovery of the positive column (4.8% vs. 4.1%).

Ten rounds of SELEX were performed for the mycotoxin deoxynivalenol (DON). A library of DNA oligonucleotides is exposed to a target molecule, DON, and during selection the sequences which bind to the target are separated from the ones that do not bind, and hence aptamers are generated. Sequences in the positive (containing DON) and a negative column are monitored via the fluorophore attached to the DNA. The amount recovered from each column is measured as the percentage of recovered single-stranded DNA. Evidence of pool enrichment is the increased percent recovery from the positive column and decreased percent recovery from the negative column.

Discussion

The initial three rounds of SELEX were performed by former colleague Maureen McKeague with column 1. The resulting pool was then used for selections performed with column 2. The pool still contained high affinity binders and had been through several negative selections, such

that it was already free of some non-specific binders. It is critical to remove non-specific binders such that the pool can be enriched with sequences which bind only to the target. Negative selection for column 1 and 2 (rounds 3-8) both consisted of DADPA beads with amine groups blocked by sulfo-NHS acetate, resulting in un-reactive acetyl groups in place of reactive amine groups. Additionally, since the *pKa* of amines is higher than the pH of the buffer there was no concern about positively charged groups attracting the negatively charged DNA.

The percent recovery of the positive column steadily increased from rounds 4-7, with round 7 having 100% binding. However, the percent recovery of the negative column from rounds 4-8 was 100% (not shown in figure). Ideally the percent recovery should be higher in the positive column than the negative, and the positive column recovery should only be increasing as the negative decreases as this would imply that the pool is becoming enriched with sequences that bind strongly to the target. However, sometimes a percent recovery slightly higher in the negative column than the positive column is not cause for too much concern; weak and non-specific binders are being removed from the pool, leaving only strong and high affinity binders for the next round

After two rounds of relatively low recovery from the positive column and over 100% recovery in the negative column (rounds 4 and 5), it was decided to increase the concentration of urea during elution. It is possible that not all of the sequences binding to DON, especially those binding very strongly, were being eluted. Urea functions by competing with the H-bonds between the aptamer and the target, resulting in the separation of the aptamer from the target. Therefore, by increasing the urea concentration, more sequences should have eluted. In fact, the percent recovery after round 5 increased substantially; partly due to the elution and perhaps also due to enrichment of the pool.

There could be a couple of explanations for the high negative column recovery; one explanation is that the fluorescent DNA in the pool is self-quenching due to the high concentration. The quenching would lead to the effect of having added a much smaller amount of DNA pool but recovering the same fluorescence as before. Therefore, the percent recovery would be greater than 100%.

To ensure that all the sequences were being eluted from the positive column, the concentration of urea was increased in round 6. By increasing the concentration to 8M, there is more denaturant interfering more strongly with the sequences binding to the target. This change likely played a part in the higher recovery in the next round.

Round 7 percent recovery for the positive column was 100%; this is unlikely to be the actual value as there was recovery in the negative column as well. However, it may be possible that there is not enough competition between the DNA and the available amount of DON on the column. DNA to target ratio is an important aspect in SELEX; with lower concentrations of DNA than target, every sequence can bind to DON given an appropriate amount of incubation time, meaning there no competition among sequences. On the other hand, if there is too much DNA, high affinity binders may be washed away due to not having the chance to bind as a result of molecular crowding[16]. Therefore, the amount of DNA was reduced such that target concentration was higher, thereby increasing stringency and allowing competition among sequences in the pool. The result of lowering the concentration was evident in the following round, where the percent recovery for the positive column decreased. Although the percent recovery for the positive was more controlled, the negative was still too high. In effort to reduce the percent recovery from the negative column, the amines on the negative column were re-blocked with sulfo- NHS acetate. It is possible that not all the amines sufficiently capped with an

acetyl group and thus a percentage of the amines were probably exposed, allowing sequences to bind electrostatically. Unfortunately, the next round did not show any improvement and the recovery was still 100% (data not shown), therefore, the search for another type of column was initiated.

After PCR amplification, the sequences are double-stranded; one strand is modified with fluorescein (aptamer) and the other with a polyA tail. These strands are separated and the polyA-modified strands discarded prior to SELEX. If not, the fluorescent strand, a possible aptamer, would be unavailable to bind DON as it will be in duplex form and will be washed away during selection. Therefore, as a preventative measure prior to using column 3, the pool underwent purification using poly(dT) magnetic beads. These beads bind the polyA tail of the specific modified strands, leaving the fluorescent sequences in the supernatant. Although PAGE is used for separating the two types of sequences, low concentrations may not always be detectable.

As mentioned, round 9 was performed with new columns; the positive column involved DON reacted to an epoxide on a magnetic bead, and the negative column consisted of methanol in place of DON such that the epoxides are open. If the epoxides were intact in the negative column, sequences which would bind to DON via its epoxide would be screened out before ever coming into contact with DON.

Percent recovery for the positive column was low compared to previous rounds, however, this is normal as the pool is subjected to a change in the environment (different beads, linkers, buffer). Enriched sequences which bound less specifically were removed in the negative column, leaving less DNA to be exposed to DON in the positive column. The sequences which bound strongly to DON were likely the sequences which remained. It is also important to note that the percent

recovery of the negative column also decreased from previous rounds, and is lower than the positive column recovery. This situation is ideal, as it provides evidence that the pool has more specific binders than non-specific binders.

Finally, round 10 was performed in the grain supernatant; instead of selecting for aptamers in buffer, they were selected in the supernatant of a mixture of powdered grains which would normally be contaminated with DON. The grains provided were tested for DON contamination, and it was found that DON levels were below the limit of detection of the instrument; this is important because if DON were present in detectable concentrations, the DNA pool would bind to DON in solution as well as to DON supported by the matrix, but the sequences bound to free DON would not be measurable or accounted for. Also, it would be possible that aptamers binding to immobilized DON would be removed on the beads and bind to free DON, resulting in lower percent recovery and ultimately less strong-binding aptamers.

One of the advantages of SELEX is the ability to select for aptamers in the environment conditions they will likely be exposed to[18], this way the best aptamer for that condition may be chosen. Aptamers do not all behave similarly in different conditions, therefore, this type of selection is often necessary for best results in the long run.

Compared to round 9, the percent recovery for the positive and negative column in round 10 increased; more sequences bound to the positive column, but more sequences were also excluded after binding to the negative column. Although the environment has changed in respect to the solution the pool is exposed to, the beads and their linkers have not changed, allowing for pool enrichment. At the same time, it appears that some sequences have bound better to the negative column than they would have in buffer conditions, resulting in the loss of non-specific or weak

binders. More sequences have possibly been washed away in round 9 than in round 10 which would explain increased percent recovery in negative and positive columns, likely due to the change in beads.

The percent recovery from the negative column was greater than that of the positive column in round 10 (result not shown). This result can be expected since the conditions have changed from buffer to grain supernatant. Fewer sequences would be able to fold properly or in the same manner as before, resulting in less effective aptamers. Nonetheless, strong binders are expected to have been recovered from the positive column.

The ability to perform counter-selections with molecules similar to the target is an advantage with SELEX; less selective sequences will bind to the similar molecule while the more selective ones are carried on to the next round, ultimately resulting in a pool with aptamers of high selectivity to the target of interest. Possible mycotoxins for the counter-selection in DON SELEX would be DON's acetyl derivatives (3-ADON and 15-ADON) and nivalenol. Counter-selection with the derivatives is more practical as they have been known to cross-react in ELISA tests, leading to higher than actual results; the impact of this could lead to a false positive for DON, which would have devastating impact on farmers, for example, whose actually safe product would likely be rejected by grain elevators and perhaps destroyed. On the other hand, one may argue that if the acetyl derivatives do cross-react, leading to false high concentrations of DON, the product is not safe in any case, thus discarding the product is safer. After all, the acetyl derivatives are about as toxic as DON which is why JECFA has recommended the same provisional maximum tolerable daily intake (PMTDI) as DON[103]. Nivalenol on the other hand is more toxic than DON; however, it is not common in Canada. The acetyl derivatives can interfere with ELISAs performed worldwide. There are numerous DON antibodies reported in the

literature[109,111] that when tested against similar mycotoxins, these antibodies have been shown to bind selectively to DON. However, some of these antibodies bind 3-ADON[111]. This confirms the idea that aptamer counter-selections should be performed with the acetyl derivatives of DON to avoid cross-reactivity.

Counter-selections with 3-ADON was planned for this SELEX; unfortunately due to time constraints, it was not performed. However, in future work, SELEX for DON will be continued and the counter-selection will be performed.

The next step in this selection process was sequencing. Round 10 was chosen for low throughput sequencing of the aptamers contained in the pool. The pool was cloned in *E.coli*, and twenty-four colonies were chosen and sent for sequencing. Regrettably, no sequences were obtained despite the presence of colonies. A possible reason for this outcome is contamination in the template which results in loss of resolution. The pool has been prepared and sent for high throughput sequencing as well, therefore the results that will be obtained will give an idea as to why low throughput sequencing was unsuccessful.

As outlined in Table 1.2, there are various methods modifying SELEX to generate an aptamer for a target molecule, yet an aptamer for DON has only recently been successfully generated. In July 2014, the Ph.D thesis dissertation by Jakob Eifler[107] claimed to have generated aptamers for DON. The selection process was performed with the DNA library bound reversibly to an affinity column and DON free in solution is added. When DNA is bound to DON, the DNA undergoes a conformational change resulting in the separation of the strand from the column. After cloning sequences from a specific round, the affinity of the cloned sequences was measured using a binding assay similar to the aptamer selection method; rather than binding a library of sequences to the solid phase, only the specific clone was bound. DON free in solution was added to the

clones and the eluted sequences were analyzed. A negative control was also performed with a solution devoid of DON. The affinity was measured based on the amount of sequences eluted with DON vs. with the negative control, and which sequences were most DON-dependent. 14 sequences were found to have affinity for DON. Eventually, when sequences from this chapter are sequenced and their affinity for DON studied, the sequences from the thesis of Jakob Eifler may serve as valuable comparisons.

There was difficulty in performing SELEX on DON for several reasons: 1) DON was modified such that it can react to the solid phase, therefore the pool is exposed to a modified target as opposed to the preferred option of an unmodified target. The modification can result in the exclusion of potential aptamers which bind that area of the molecule; 2) potential aptamer sequences obtained from SELEX may not bind well to the target due to the structure differences of the immobilized versus free target. And finally, 3) targets containing many bonds that rotate have more degrees of freedom and can thus result in poorer binding affinity[21].

The amount of pool is high at first and conditions are less stringent because certain sequences are present in few copies; the pool may be saturated with certain types of sequences while some others are present at lower frequencies. The sequences present at lower frequencies may bind the target just as well or with higher affinity than the sequences which saturate the pool, but may not be recovered[82,83]. After a few rounds, the amount of pool is reduced but must be enriched, as it is assumed that low affinity binders outnumber the high affinity binders[82]. To enrich the pool, selection modifications resulting in increased stringency is required. It is advantageous to be as stringent as possible, even if the selection process will take longer. In the end, the sequences will be of better quality than those selected in less stringent conditions[83]. In this chapter, the conditions that changed to increase stringency include augmenting the DNA to target ratio and

urea concentration, and switching from selections performed in buffer to selections performed in the supernatant of a grain mix. Studies have shown that decreasing the amount of target during selection increases stringency on the selection and results in high affinity aptamers[82,84].

Although the dissociation constant (K_D) of the cloned sequences to the target have not yet been studied, there is a relationship between the equilibrium binding and the incubation period of the pool with the target. The concentration of DON was reduced along the selection process, and was incubated with the pool for 30 min, which is a relatively short time period compared to other selections which incubate for 60 min[85,86]. Short incubation periods with dilute target concentrations commonly lead to the generation of aptamers with high reactivity and low K_D values[83].

The components of the buffer used for the SELEX experiments were based on what would make the DNA more structurally stable and bind better to the target. Sodium and potassium are monovalent ions which are used for stabilizing DNA[87]; the DNA backbone is polyanionic and thus the absence of positive ions to block the repulsions of the negative charges would inhibit the structure from folding compactly with the target. As well, potassium is used to stabilize sequences with G-rich regions that fold into G-quadruplex tertiary structures[89]. Magnesium is a divalent cation which also functions to stabilize the DNA backbone; on the other hand there is evidence to suggest that higher affinity aptamers generally do not rely much on magnesium compared to lower affinity binders[113], thus only a small amount was added. Finally, the addition of calcium can increase the affinity of the aptamer to the target by inducing a conformation change in the DNA[114].

3.6 Conclusion

Deoxynivalenol (DON) is a mycotoxin found in various commodities including wheat and corn. In this chapter, methods for generating an aptamer for DON were developed. The progression of the selection was initially hopeful, despite the challenges and setbacks. The high recovery from the negative column that remained throughout several rounds of the selection process was of concern. The struggle of conjugating DON to the matrix without modifying DON such that it is too different from the original was ongoing throughout the selection, as DON is a complicated molecule. Nevertheless, even after increasing stringency by performing the selections in the supernatant of a grain mix, a decent recovery was obtained from the positive column. The pool was not successfully sequenced; however the results from high throughput sequencing should be able to shed a light on the motive for the absence in signal. In future work, once the aptamers are sequenced successfully, their affinity to DON will be determined and compared to the sequences of aptamers generated by Jakob Eifler[107].

Chapter 4 – Contributions to Knowledge

The detection of mycotoxins deoxynivalenol (DON) and patulin (PAT) is vital to ensure public safety, especially since contamination of food commodities is not detectable by simple visual inspection. Although traditional detection techniques for mycotoxin detection such as liquid chromatography - mass spectrometry have proven to be highly selective and sensitive, simpler, more rapid and more cost-effective approaches are required. Aptamer-based assays and biosensors are a possible solution. Therefore, we aimed to generate aptamers for DON and PAT.

From these selections for these mycotoxins, we found that using magnetic beads for immobilizing the target to a solid support matrix was better than using DADPA beads. A linker between the target and the beads was not necessary and less target was required which decreased the costs associated with buying the extra material. The use of the magnetic beads also provided a simpler method for immobilizing the target as only a few steps were required.

The last few selections for each target were performed in the conditions in which the potential aptamers would likely be used in, thus many sequences that bind only in standard conditions were removed. Changing the conditions increased stringency during selection that will ultimately result in better binding aptamers.

While selections with DON must be continued in order to obtain potential aptamer sequences, sequences for patulin have been obtained and their secondary structures predicted. Future work will entail measuring the affinity of these sequences to patulin to determine if a potential aptamer that binds with high affinity exists, as well as performing high throughput sequencing to uncover more sequences. Currently, an aptamer does not exist for patulin.

The work in this thesis has contributed to the development of DON and patulin aptamers, which bring the science community steps closer to the production of an aptamer-based biosensor for accurate detection of mycotoxin levels.

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