Synthetic and Analytical Studies Aimed at Molecular Recognition Applications

by

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Certifies that this is the approved version of the following thesis:

Synthetic and Analytical Studies Aimed at Molecular Recognition Applications

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Philip D. Magnus

Dedication

To everyone who has taken a chance on adventure…I'll see you soon

Acknowledgements

I would not be able to start this thesis without thanking everyone who has helped me along the way. Mom, Dad and Sarah, always remember there is nothing worth sharing like the love that let us share our name. Megan, we're on a road to nowhere, thank you so much for taking a chance. Justin, you are a true friend, my beard will miss you the most. Chance, coffee won't taste the same without your company.

I would also like to thank Dr. Eric Anslyn for his guidance and advice and for giving me the freedom to help me find my path in life. Also, all of the Anslyn group members I knew during my stay, I could not have picked a better group.

Abstract

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The University of Texas, 2010

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The creation of small molecule libraries for binding into the NS1A protein of influenza A viruses and the development of an indicator displacement assay for the differentiation of fatty acids are reported herein. Using Mitsunobu chemistry, a variety of structures based on hydroquinone, resorcinol and 2,7-dihydroxynaphthalene cores were synthesized. Both polar and non-polar functional groups were added to diversify the cores to help understand which molecule binds best to the protein. Because of poor protein binding, the focus of the project moved to a new lead compound, epigallocatechin-3-gallate (EGCG). EGCG showed promise in computational studies and efforts towards the synthesis of the epigallocatechin core were undertaken.

Using a fluorescent indicator displacement assay (IDA), a sensing system for fatty acids was developed. The system consisted of bovine, rabbit, and human serum albumins as host molecules, while the fluorescent indicators were fluorescein, 2-anthracene carboxylic acid, and 1-anilino-8-naphthalene sulfonic acid. Fatty acids were able to be differentiated from one another based on their carbon chain length and the degree of unsaturation. The IDA was then subjected to a complex mixture of fatty acids, in the form of edible oils. The oils (extra virgin olive, hazelnut, peanut, sunflower and canola) with different fatty acid profiles were able to be differentiated from each other using principal component analysis.

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Chapter 1: Synthesis of Small Molecule Libraries

1.1 Introduction

Influenza viruses are the cause of highly contagious respiratory disease in humans. Influenza A is responsible for deadly pandemics in the past century. For example, the Spanish Flu of 1918 (Influenza A subtype H1N1) killed an estimated 20 to 40 million people worldwide.[1] With the ease of global transportation today, the next flu pandemic could have devastating effects.

The H1N1 subtype is believed to have been transmitted to humans directly from avian species through adaptive mutations.[2] The World Health Organization defines a pandemic virus as, (1) the emergence of a disease new to the population, (2) the agent infects humans, causing serious illness, and (3) the agent spreads easily and is sustainable among humans. H1N1 acquired the necessary mutations to fulfill these conditions and the result was one of the worst pandemics in human history.

Since the sixteenth century, the human population has endured on average 2-3 pandemics per century. Avian Influenza A viruses of the subtype H5N1 are prime candidates for the next pandemic. In 1997, the first human cases of H5N1 infection were reported in Hong Kong and six of the eighteen patients perished. Since 2003, 378 cases of infection have been reported resulting in 238 deaths.[3] Early cases of human infection occurred in East Asia, but recent reports show the virus has traveled as far south as Indonesia and as far west as Egypt.

At present, H5N1 satisfies two of the three conditions necessary for a pandemic: the virus is new to the population (first isolated in 1997) and it infects humans and causes

serious illness (>50% mortality rate). Fortunately, it still lacks the ability to efficiently and sustainably spread to humans.

Two classes of antivirals are used to treat and prevent influenza infections. Rimantadine and amantadine are adamantane derivatives that inhibit the pH-gate function of the M2 ion channel which results in an inhibition of viral replication.[4] Oseltamivir and zanamivir act as inhibitors of neuraminidase, an enzyme used to cleave progeny virus particles from the host. Inhibiting neuraminidase function prevents the release of new viral particles into the host organism.[5]

Resistance to the adamantanes has recently been reported for more than 95% of viruses in Thailand and Vietnam.[6] Although much less prevalent, cases of oseltamivir resistant viruses have been reported.[7] With virus resistance mounting, there is a need to explore and discover new targets for antiviral activity.

One candidate for drug targeting is non-structural protein 1 (NS1A). NS1A is a multi-functional protein that participates in both protein-RNA and protein-protein interactions.[8] The N-terminus consists of amino acid residues 1-73 and forms a symmetric homodimer with a six chain helical fold and is the binding site for host double stranded-RNA. Binding host ds-RNA protects the virus from the antiviral state induced by IFN-β and thus allows for viral replication. Sequestering ds-RNA is thought to attenuate the virus by blocking the activation of the 2,5-OAS/RNaseL pathway.[9]

Double stranded RNA binding relies solely on two arginine residues at position 38 and 38' in the NS1A homodimer structure[9]. The α2/α2' helices form anti-parallel tracks which give complementary interactions with A-form ds-RNA. X-ray

crystallography and NMR data show that these tracks are highly conserved suggesting that NS1A-RNA binding is necessary for virus survival. Hence, this site is a good candidate for antiviral development. The anti-parallel tracks form a channel that contains a deep hydrophobic pocket at its center which has been explored as a suitable site for drug development.[10]

Virtual screening of the NS1A binding domain resulted in the discovery of small molecule scaffolds based on hydroquinone and 2,7 dihydroxynaphthalene cores (Figure 1) as well as epigallocatechin-3-gallate (EGCG), an extract from green tea. These compounds showed promise of binding into the hydrophobic pocket of NS1A.

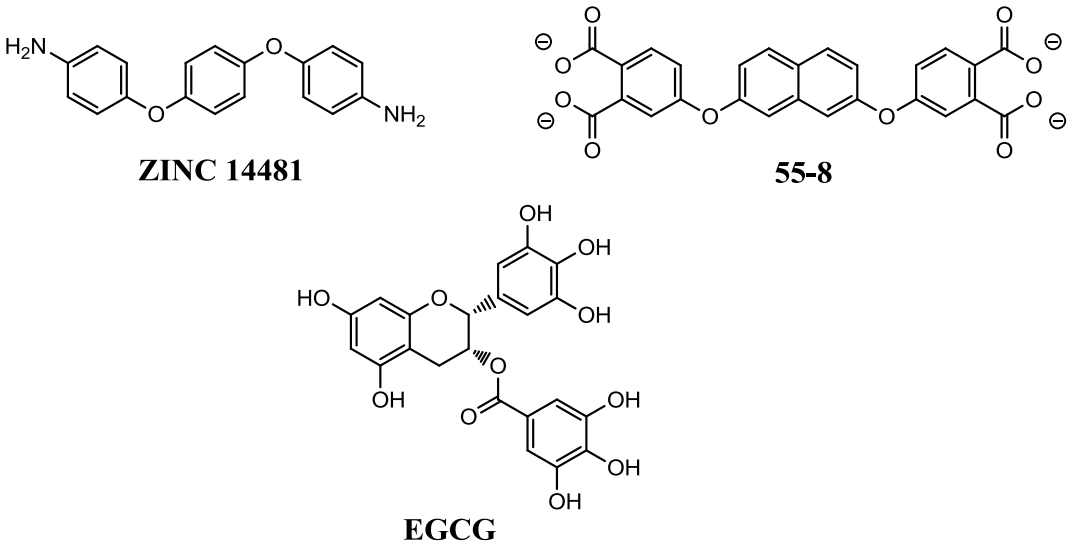
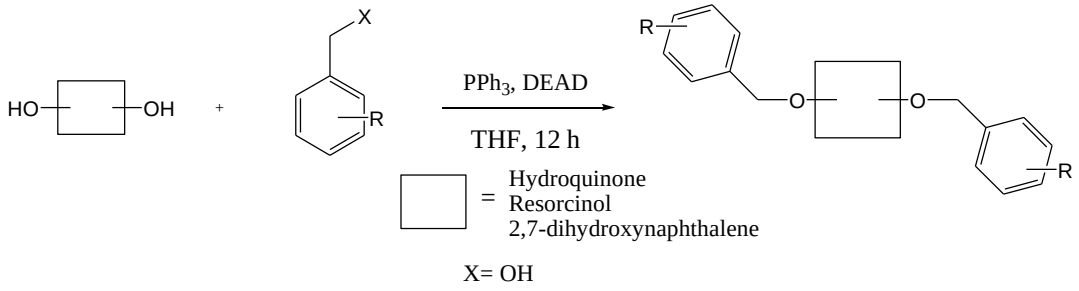


Figure 1. Virtual screening hits for NS1A RNA binding domain.

1.2 Results and Discussion

To create the desired small molecule library (Scheme 1), Mitsunobu chemistry was chosen to couple commercially available benzylic alcohols to three bis-phenolic

cores: hydroquinone, resorcinol, and 2,7 dihydroxynaphthalene. The Mitsunobu reaction was chosen because of its mild conditions. With the virtual screening hits as inspiration, the main focus of the synthetic work was to create diethers substituted with amino and carboxylate groups.



Scheme 1. General conditions for Mitsunobu reaction.

Due to acidity, carboxylic acid functionalities would compete with phenolate nucleophiles leading to unwanted esters; therefore, esterified benzylic alcohols were used and then hydrolyzed with methanol and acid to the corresponding acid. Both methyl 4-(hydroxymethyl) benzoate (Figure 2, A) and diethyl 5-(hydroxymethyl) isophthalate (Figure 2, B) were used as reactive benzyl alcohols.

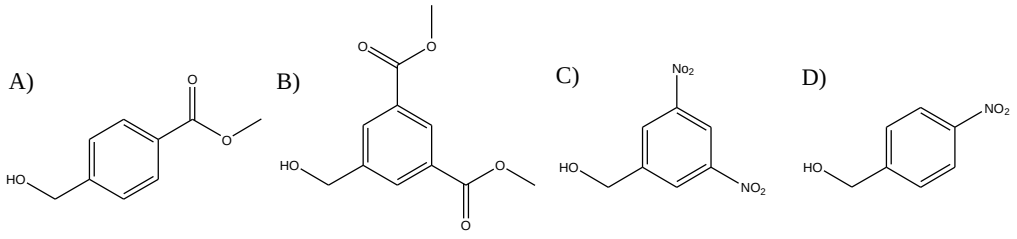


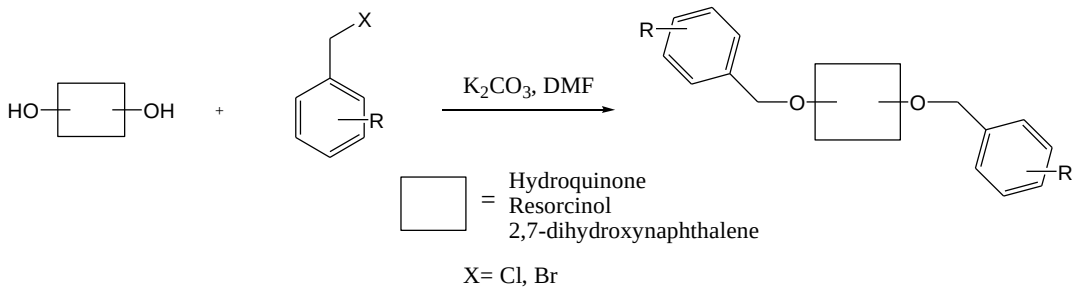
Figure 2. a) methyl 4-(hydroxymethyl) benzoate b) diethyl 5-(hydroxymethyl) isophthalate c) 3,5-dinitrobenzyl alcohol d) 4-nitrobenzyl alcohol

Previous experience in the Anslyn lab showed that the Mitsunobu reaction failed to produce the desired product when the benzyl alcohol was functionalized with an amine. To develop a scaffold resembling the virtual hit ZINC 14481, nitro groups were substituted for amines: these groups would then be reduced to amines after coupling (compounds C and D).

Using standard Mistunobu reagents: triphenyl phosphine (PPh_3) and diethylazodicarboxylate (DEAD) the reaction of various benzylic alcohols led to the desired diethers only in extremely low yields, between 5 and 15%. Diisopropylazodicarboxylate (DIAD) was used as an alternative reagent to DEAD, but failed to produce the desired ethers. Other attempts to optimize the reaction conditions led to no appreciable increase in the yield of the desired product.

To test reaction conditions, phenol was reacted with methyl 4-(hydroxymethyl) benzoate under the same conditions (DEAD, PPh_3). The reaction produced the corresponding ether in 76% yield after chromatography, verifying that the synthetic methodology was sound. In addition to low yields, the reaction requires a time-consuming work-up to remove excess starting reagents and by-products. Thus, a new synthetic path was chosen based on the Williamson ether synthesis.

Unlike the Mitsunobu reaction, the Williamson synthesis is run under harsher conditions. A strong base and high temperatures over long reaction times are used to drive the formation of ethers through an S_N2 pathway (Scheme 2). Also in this case, esters and nitro groups were used as convenient precursors of carboxylic acids and amines.



Scheme 2. General conditions for Williamson Ether Synthesis

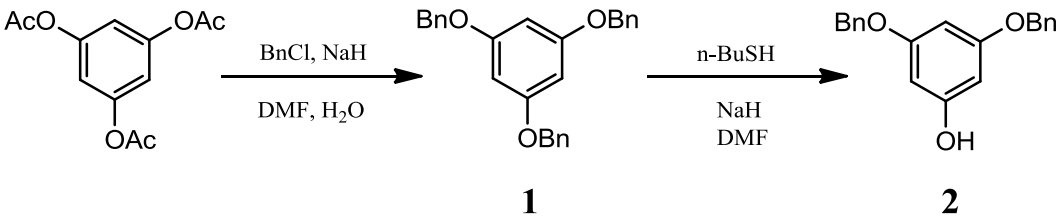
Reactions using 4-nitrobenzyl chloride with hydroquinone were unsuccessful; the desired product was never recovered. The failure of this reaction was unexpected, but a literature search revealed the cause of the problem. The electron withdrawing effects of the chloride and para-substituted nitro group increase the acidity of the benzylic position. The result is deprotonation at the benzylic position instead of the expected nucleophilic.[11] The esterified benzyl alcohols used in the Mitsunobu reaction were tosylated and then reacted under the same Williamson conditions. No significant yield of product was isolated.

These results were highly unexpected from the Williamson synthesis. To test the reaction conditions, phenol was reacted with benzyl alcohol to give the corresponding ether. Benzyloxybenzene was recovered in 80% yield.

More recently, the focus of our synthetic efforts shifted away from the symmetrical hydroquinone and naphthalene cores to the creation of a library of molecules based on the epigallocatechin core. EGCG is available commercially, but only in small quantities and it is expensive. Using the work of Chan *et al.*[12], efforts towards the synthesis of the epigallocatechin core were made. With the epigallocatechin core,

reactions at the 3-position with a variety of functional groups will create a diverse library that will allow for further studies into how EGCG and its analogs bind into the NS1A protein.

Scheme 3 shows the synthetic route used to create one of the starting materials for the synthesis of the core. The doubly protected phenol, **2**, was synthesized in two steps from the starting material phloroglucinol triacetate. First, the substrate was protected with benzyl chloride in the presence of sodium hydride and water in DMF. This product, **1**, was recovered in a 79% yield and was scaled up as high as ten grams. Next, the single deprotection was attempted with ethanethiol and sodium hydride in DMF, but in only 30% yield. In an attempt to improve the yield, n-butanethiol was used according to a previous report.[13] This reaction was successful and the singly deprotected phenol was recovered in 82% yield.

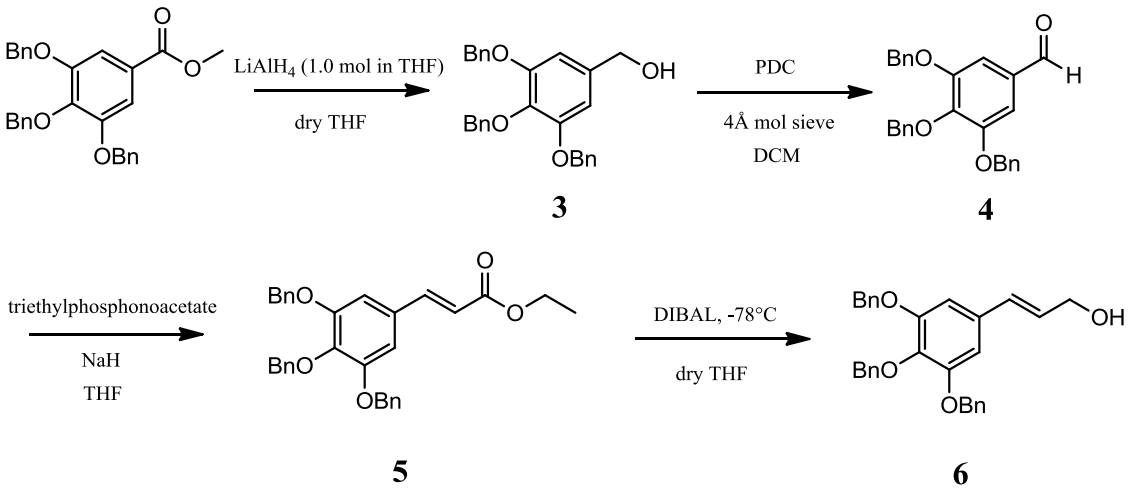


1 **2**

Scheme 3. Synthetic route towards 3,5-bis(benzyloxy)phenol

Cinnamyl alcohol was created in four steps starting with 3,4,5-tris(benzyloxy)methyl benzoate, Scheme 4. Using a 1.0M solution of $LiAlH_4$ in THF, the methyl ester was successfully reduced to benzyl alcohol, **3**, in 83% yield. Oxidation of **3** to aldehyde **4** was performed with pyridinium dichromate in the presence of molecular sieves and resulted in a 91% yield. Next, **4** was allowed to react with

triethylphosphonoacetate and NaH giving the resulting α,β unsaturated ester in quantitative yield. Finally, reduction of the ester with DIBAL gave the desired cinnamyl alcohol product, **6**, in 69% yield.



Scheme 4. Synthetic route towards cinnamyl alcohol

1.3 Conclusion

A small molecule library based on the virtual screening hits was synthesized using Mitsunobu chemistry. Ultimately, the project moved away from these compounds as possible drug targets and towards epigallocatechin derivatives. The major starting materials of the epigallocatechin core were successfully synthesized and large quantities of these building blocks were produced laying the foundation for a derivitized epigallocatechin library.

Chapter 2: Development of an Indicator Displacement Assay

2.1 Introduction

Because of their biological importance, fatty acids are interesting targets for molecular recognition, yet the literature contains few reports on fatty acid detection.[14,15] It is the goal of this project to create a sensing system able to detect the subtle differences in fatty acid structure and to apply this sensing system to a complex mixture of fatty acids, more specifically, edible oils.

In recent years, the use of differential sensing has gained popularity in the world of molecular recognition. Instead of the high specificity and selectivity of receptors in the classic "lock and key" principle, differential sensing uses an array of receptors with different binding characteristics. These arrays of receptors can be used in either single analyte or multi-analyte sensing (Figure 3).[16]

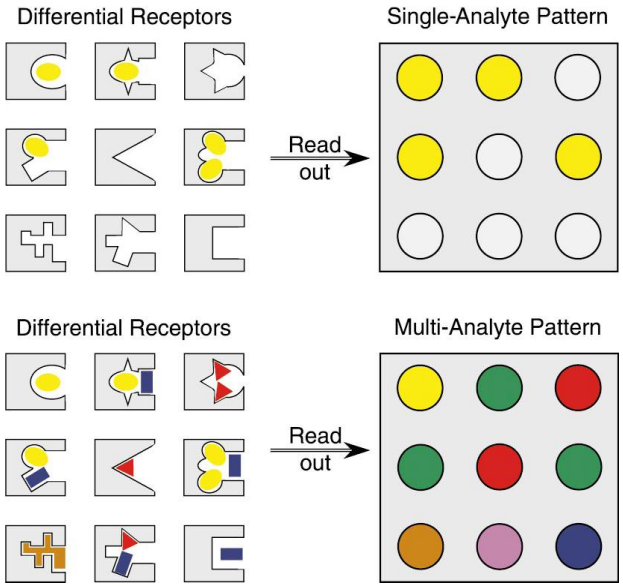


Figure 3. Differential sensing of single (top) and multi-analyte (bottom) systems

In both single and multi-analyte sensing, the analytes of interest interact with each receptor uniquely resulting in a composite signal that can be interpreted by pattern recognition software. These cross-reactive arrays were inspired by the mammalian senses of taste and smell.

Recent work in the Anslyn lab utilized differential sensing with various serum albumin proteins to target terpenes.[17] In this study, bovine, rabbit and human serum albumin proteins were used in conjunction with the fluorescent probe PRODAN and a hydrophobic additive to detect the subtle differences in terpene structure (Figure 4).

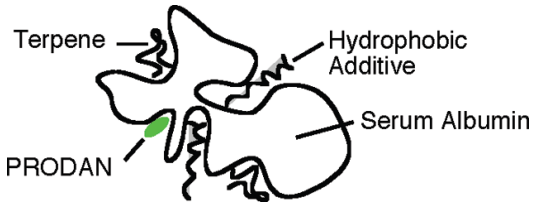


Figure 4. Differential sensing ensemble for terpenes.

Using a 96-well plate assay, the sensing ensemble successfully differentiated between five different terpene structures. Complex mixtures of terpenes were also tested and successfully differentiated from one another. This previous study in differential sensing is the inspiration for this project.

A common sensing ensemble used in the Anslyn group is an indicator displacement assay (Figure 5).[18] Here, an indicator reversibly binds a host molecule. Upon introduction of a competing analyte, the indicator is displaced as the analyte binds the host. The now free indicator in solution has a different optical signal than the bound indicator and this change can be measured by instrumental methods (i.e. UV-Vis or fluorescence). Coupled with a pattern recognition approach, we can use the differential

binding of various fatty acids to various host/indicator complexes to create a fingerprint of complex fatty acid solutions.

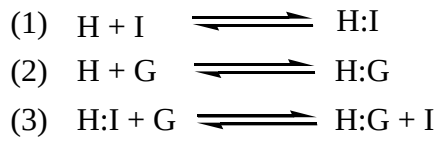


Figure 5. Equlibria involved in an indicator displacement assay

To increase the chances of successful differentiation, our sensing ensemble was built around three different serum albumin proteins (bovine, rabbit and human) and three different indicators. This allowed us to work with nine different host/indicator complexes and gave us the diversity we desired.

2.2 Results and Discussion

To develop a working IDA, suitable indicators must be chosen for the system. A suitable fluorophore must show an appreciable change in optical signal from when it is bound to the serum albumins to when it is free in solution. Another criterion for the selected fluorophore was its resistance to a change in solvent. Because of the low solubility of fatty acids in water, stock solutions of fatty acids were dissolved to the appropriate concentration in solutions of 100% ethanol. Control experiments were performed where ethanol was added in increasing concentration to the host/indicator complex. To this end, the fluorophores in Figure 6 were screened for binding into the serum albumins.

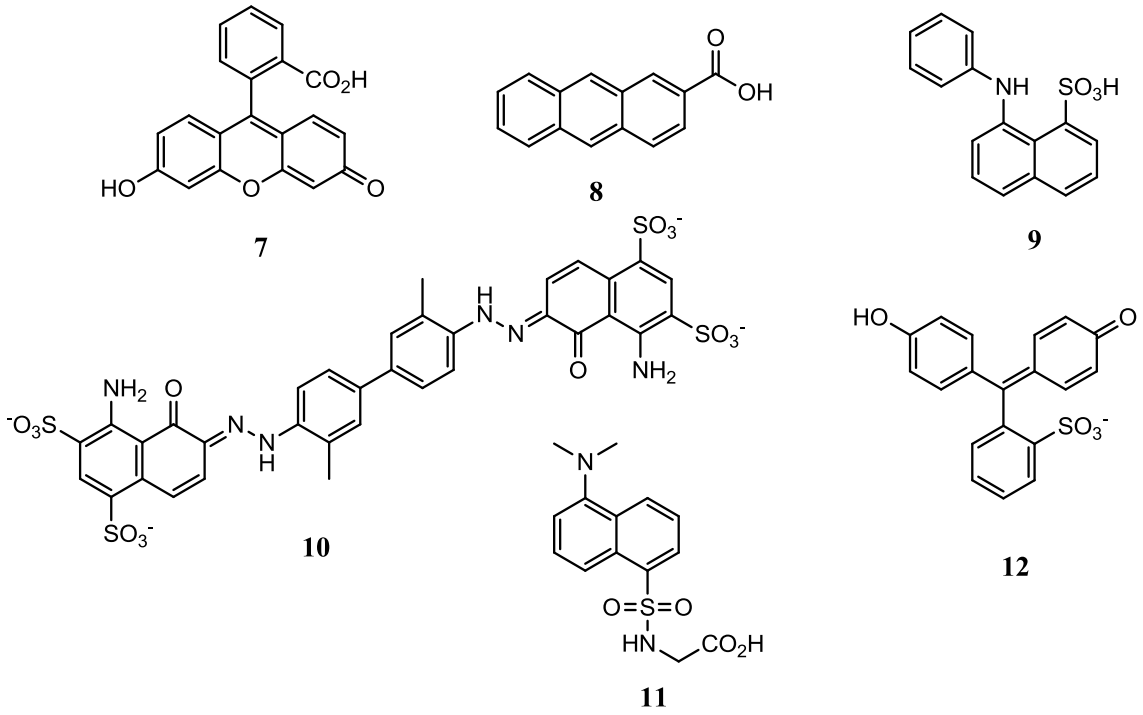


Figure 6. Indicators screened for binding serum albumins

Overall, six fluorophores were screened including fluorescein (**7**), 2-anthracene carboxylic acid (**8**), 1-anilino-8-naphthalene sulfonic acid (**9**), Evans Blue (**10**), dansyl glycine (**11**), and phenol red (**12**). Of the six tested, the three best performing fluorophores were fluorescein, 2-anthracene carboxylic acid (2-AC) and 1-anilino-8-naphthalene sulfonic acid (1,8-ANS). Figure 7 shows the differential binding of these fluorophores binding into the serum albumins.

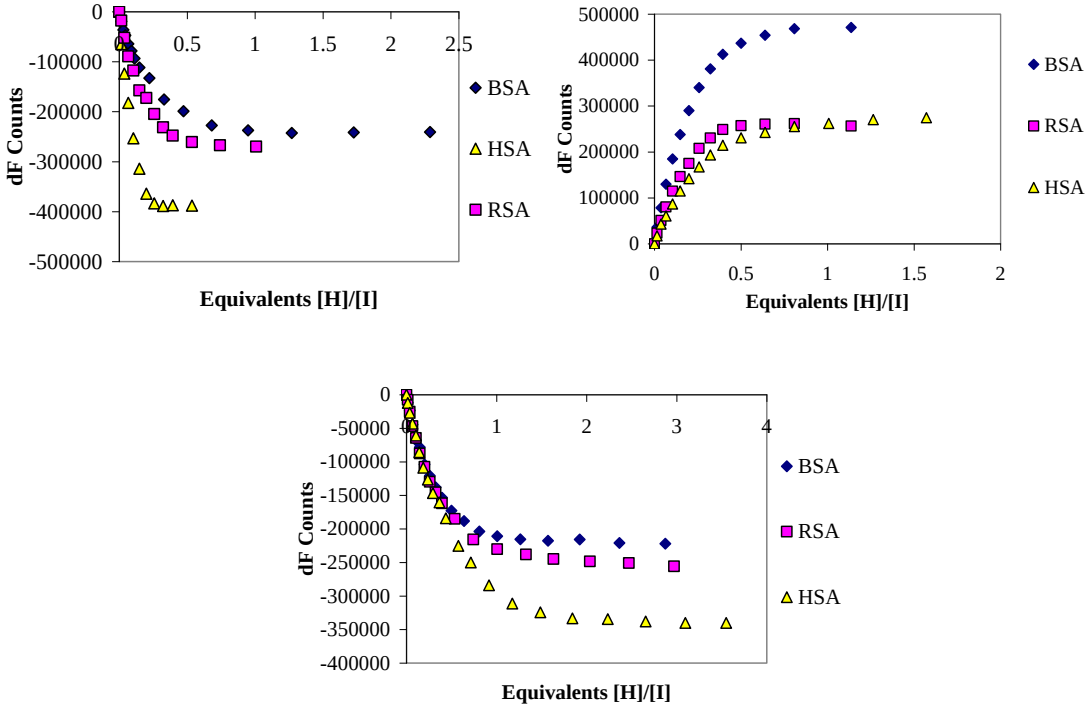


Figure 7. Indicators (clockwise from left) 2-AC, 1,8-ANS and fluorescein binding bovine serum albumin, rabbit serum albumin and human serum albumin.

With three differentially binding fluorophores, the next step was to test if our system responded to the addition of fatty acids. Stearic, palmitic, and linoleic acids were chosen as test fatty acids because of their shape and degree of unsaturation. Stearic (C18) and palmitic (C16) acids are saturated and linear, only differing in carbon chain length while linoleic is eighteen carbons in length but contains two double bonds (Figure 8).

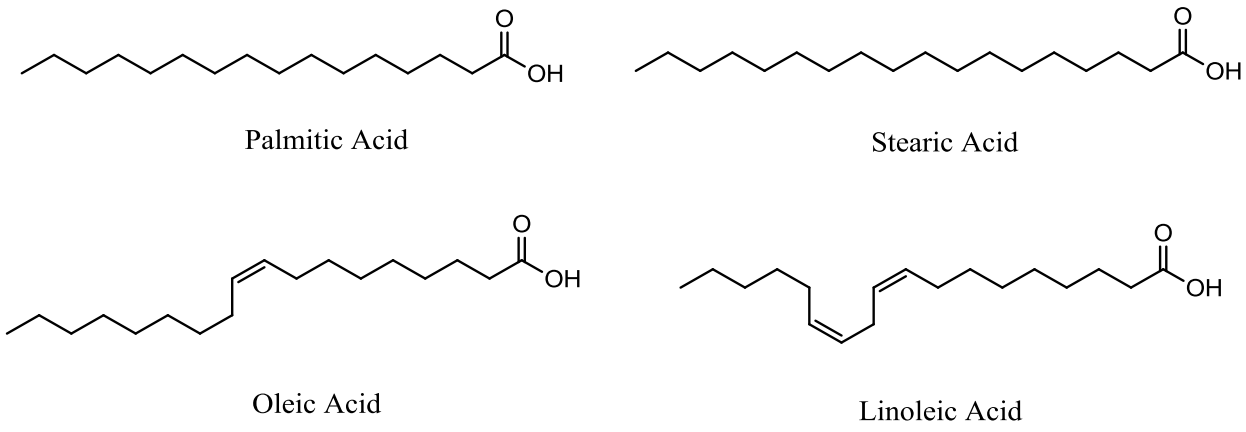


Figure 8. Fatty acids differentiated in sensing ensemble.

As shown in Figure 9, the fatty acids selected all showed differential binding when introduced to the serum albumin/indicator complex. For a test system, serum albumin and 2-AC were used as the host indicator complex for the fatty acid displacement titrations. The interesting shape of the displacement curves is believed to be the result of the fatty acid binding into serum albumin binding pockets that do not contain any indicator. The first equivalents of fatty acid show little effect on the indicators. Only at higher fatty acid concentrations does the displacement of indicator begin to occur.

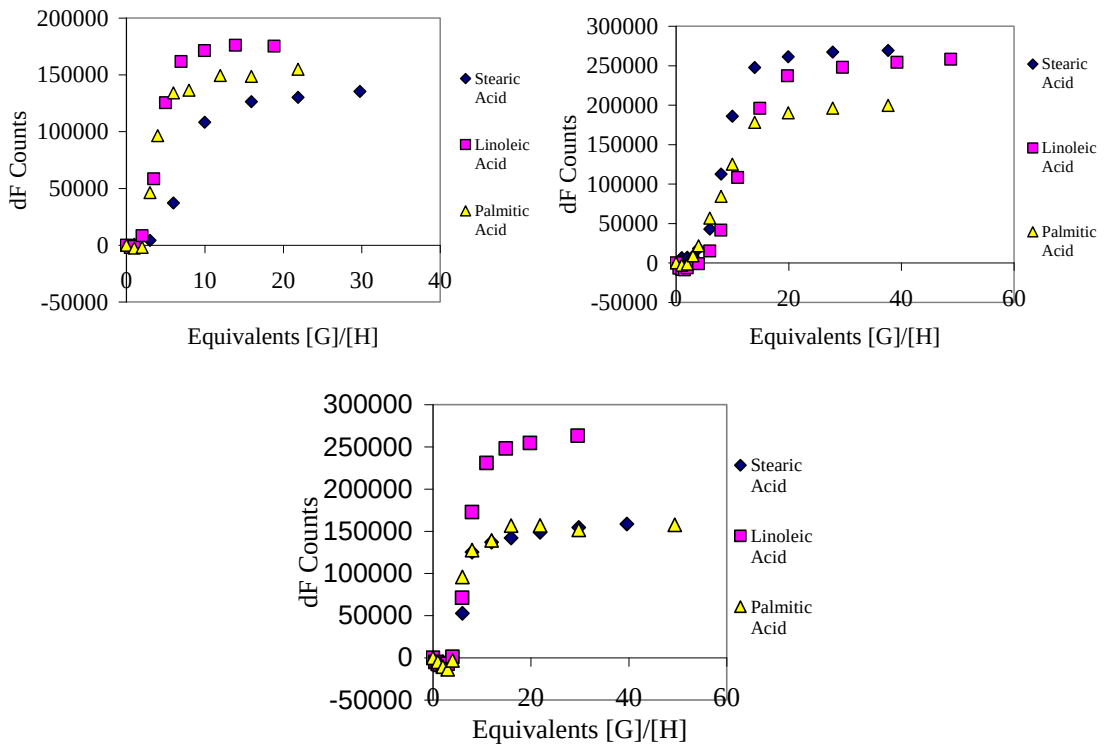


Figure 9. Displacement of 2-AC from serum albumins (clockwise from left: BSA, RSA and HSA) with various fatty acids.

With a working IDA system, the project was transitioned to a 96-well plate. The concentration of the serum albumin/indicator complex was determined by the binding curves from our fluorimeter studies. Fluorescein/serum albumin complexes were set at a 1:1 ratio while a 2:1 ratio was used for the 2-AC and 1,8-ANS/serum albumin complexes. These ratios were chosen because at these concentrations roughly 90% of the indicator is bound into the serum albumin. A 96-well plate was run to verify the reproducibility between the fluorimeter and plate reader. In all cases, the change in fluorescence mirrored that of the experiments performed in cuvettes. For example, in the case of

fluorescein, HSA had the greatest fluorescence change followed by RSA and finally BSA. This trend was seen in both cuvette titrations and on the plate reader.

Next, we introduced four fatty acids to our ensemble of serum albumins and indicators. Stearic, palmitic, linoleic and oleic acids were each tested with our system and the resulting PCA plot is shown in Figure 10. It is clearly seen that our sensing system is able to differentiate between all four of the fatty acids. The largest separation occurs between the saturated and unsaturated fatty acids. It is interesting to note the sensitivity of this system. Through the PCA plot, we are able to differentiate between two fatty acids that are different by only a two carbon chain length (stearic and palmitic).

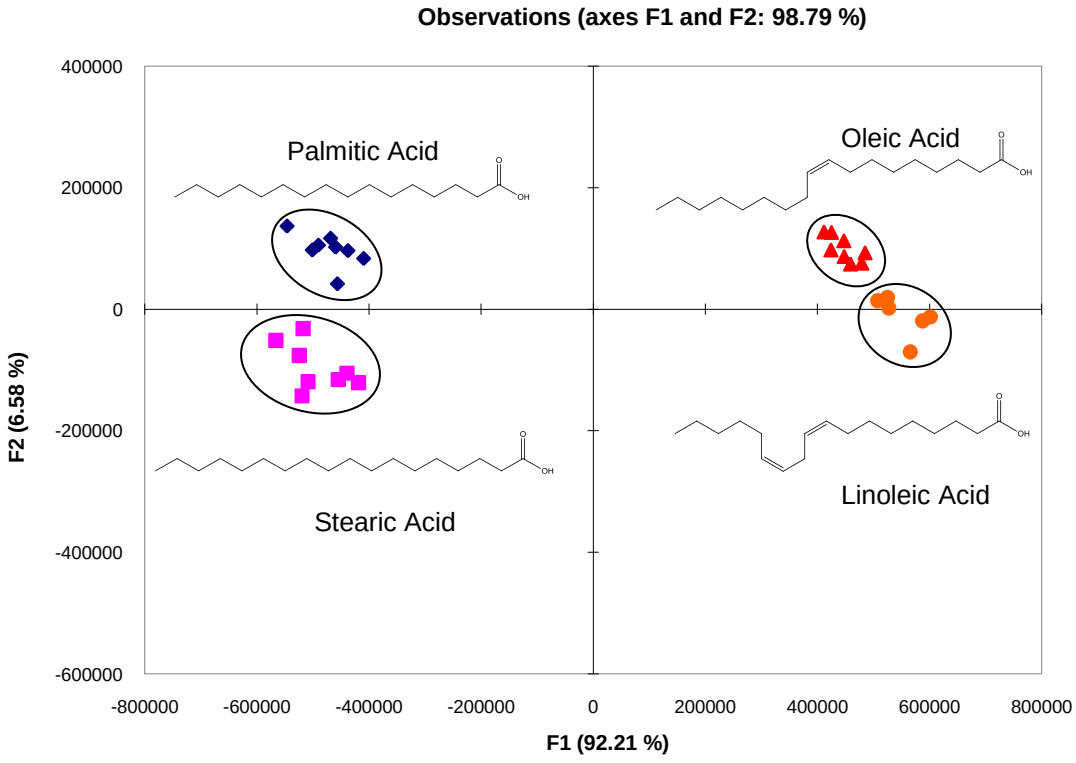


Figure 10. PCA plot 1 of sensing system when exposed to various fatty acids

We wanted to extend the scope of our system to more complex mixtures of fatty acids. An interesting analyte for this system are edible oils. Each oil contains a different fatty acid or lipid fingerprint and it was our goal to differentiate between various oils. For our study, hazelnut, extra virgin olive oil, peanut, safflower, and canola oil were chosen. The PCA plot in Figure 11 shows that our system was able to differentiate between various types of oils.

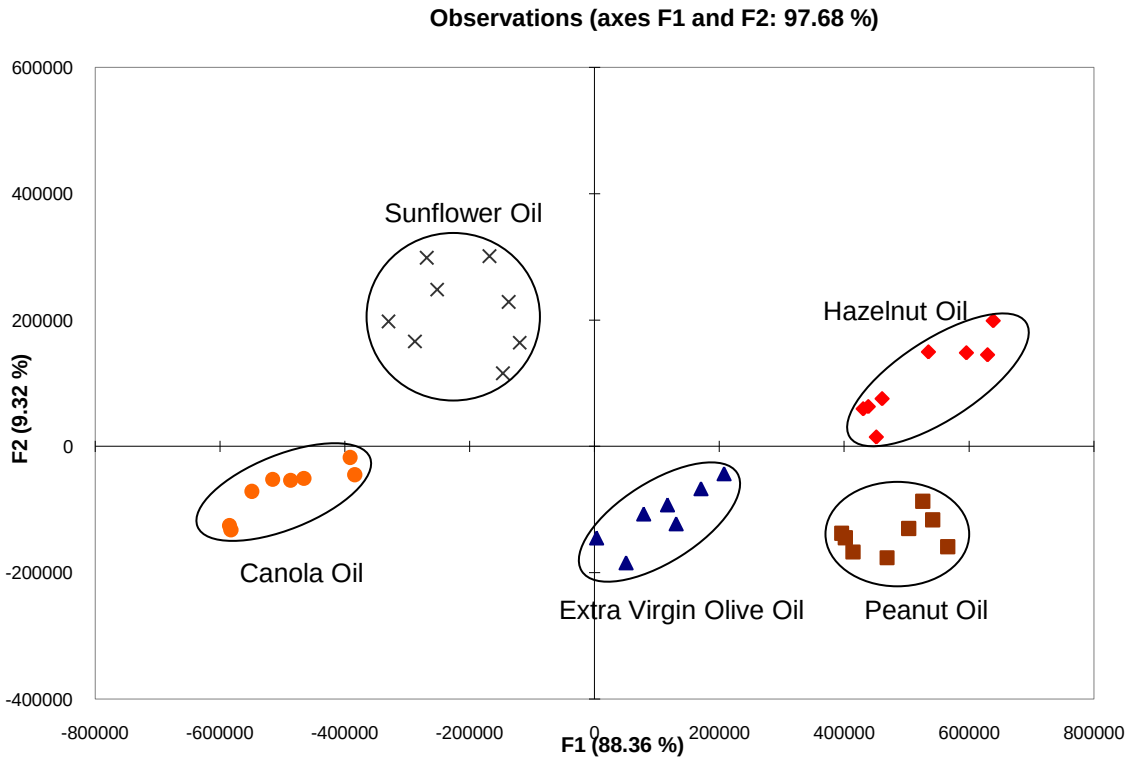


Figure 11. PCA plot 2 showing the differentiation of various edible oils using a serum albumin/indicator sensing system.

2.3 Conclusion

An indicator displacement assay sensing ensemble using serum albumin proteins and fluorescent indicators was developed to sense the subtle structural differences in fatty acids. Our system was able to differentiate between the various fatty acids based on their carbon chain length, shape, and degree of unsaturation. Complex mixtures of fatty acids, in the form of edible oils, were able to be differentiated with minimal sample preparation.

Chapter 3 Experimental

3.1 Synthesis of small molecule libraries

General Procedures

All NMR spectra were recorded using a Varian Unity Plus 400 MHz spectrometer and agreed with previous literature reports. Mass spectrometry samples were analyzed in CI mode by the University of Texas Mass Spectrometry facility. All solvents used were obtained from dry stills.

General Procedure for the Mitsunobu Reaction:

To an oven dried 100-mL round-bottom flask containing an oven dried stir bar, bis-phenolic core (1.82 mmol) was added with triphenylphosphine (5.45 mmol) and substituted benzylic alcohol (5.45 mmol). The flask was stoppered with a rubber septum, purged with N_2 and 40 mL of dry THf was added by cannula. The solution was cooled to 0°C under N_2 atmosphere. After 15 minutes, DEAD (5.45 mmol) was added slowly over 5 minutes with a syringe. The solution was allowed to warm to room temperature in an ice bath and stirred for 12 hours. TLC was used to monitor the reaction (7:3 Hexanes:Ethyl Acetate).

Upon reaction completion, as determined by TLC, THF was removed *in vacuo* and the residue was dissolved in 10 mL methylene chloride. To oxidize excess triphenylphosphine, 30% aqueous hydrogen peroxide was added and the mixture stirred for 15 minutes. TLC was used to follow the disappearance of triphenylphosphine to the more polar triphenylphosphine oxide. The organic layer was separated from aqueous hydrogen peroxide and dried with $MgSO_4$, then filtered. The solvent was removed using

rotovap. The resulting residue was placed directly onto a silica gel column and a gradient column was run starting at 90:10 Hexane:Ethyl acetate solvent mixture and ending at 65:35 Hexane: Ethyl acetate. Product fractions were collected and the solvent was removed *in vacuo*.

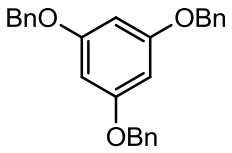
General Procedure for Williamson Reaction

To an oven-dried 100 mL round-bottom flask with oven-dried stir-bar, bis-phenolic core (1.82 mmol) was added and dissolved in 40 mL dry DMF. K_2CO_3 (42.4 mmol) was added and the flask was stoppered. The solution was allowed to stir for 24 hours under a continuous stream of nitrogen.

After completion, 50 mL of water was added to the reaction flask and the aqueous layer was extracted with ethyl acetate 3x50 mL. The organic layer was washed with water and saturated sodium chloride in consecutive additions. The organic layer was dried with $MgSO_4$, filtered and the solvent removed using a rotovap.

EGCG Core

1,3,5-tris(benzyloxy)benzene (1)

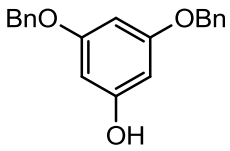


1

Phloroglucinol triacetate was added to a 100 mL round-bottom flask with 20 mL of DMF. Sodium hydride (28.3 mmol, 60% solid) was added and the reaction flask was cooled to 0°C. Next, benzyl chloride (14.3 mmol) was added slowly to the cooled

solution. Upon addition of benzyl chloride, water (11.9 mmol) was added by syringe to solution still at 0°C. The reaction was allowed to warm to room temperature as it stirred overnight. For work-up, the reaction was diluted with 50 mL ethyl acetate and the organic layer was washed twice with water and once with saturated sodium chloride. The organic layer was dried with $MgSO_4$, filtered and the solvent was removed on the rotovap. Crude solid was washed sequentially with methanol to afford a white solid. ^1H NMR ($CDCl_3$) 7.42-7.31 (m, 15H), 6.27 (s, 3H), 5.00 (s, 6H).

3,5-bis(benzyloxy)phenol (2)

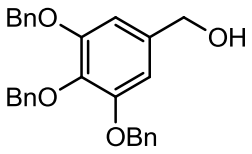


2

In a 100 mL round-bottom flask, n-butanethiol (1.69 mmol) was added to a solution of NaH (3.21 mmol, 60% solid) in dry DMF at 0°C. This solution was stirred at 0°C for 90 minutes. Then, 1,3,5-tris(benzyloxy)benzene (3.2 mmol) was dissolved in a portion of dry DMF and added slowly to the reaction vessel via an addition funnel. A reflux apparatus was assembled and the reaction was stirred at 150°C overnight. Upon completion, 10 mL of ethyl acetate was added to the reaction flask and the organic layer was extracted with 1N HCl. Next, the organic layer was washed with water, 4x20 mL. The organic layer was dried with $MgSO_4$, filtered and solvent removed with rotovap. The crude product was purified with silica gel column chromatography. An eluting solvent gradient was used as follows: 100:0 Hexane:Ethyl acetate to 80:20 Hexane:Ethyl acetate

to 70:30 Hexane: Ethyl acetate to 60:40 Hexane: Ethyl acetate. An orange solid was recovered as the product. ^{1}H NMR ($CDCl_3$) 7.36-7.26 (m, 10H), 6.25 (t, 1H), 6.11 (d, 2H), 5.00 (s, 4H), 1.69 (s, 1H).

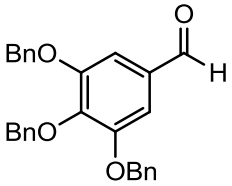
3,4,5-tris(benzyloxy)benzyl alcohol (3)



3

To a solution of $LiAlH_4$ (2.64 mmol, 1.0 M in THF) in dry THF, 3,4,5-tris(benzyloxy)benzoic acid methyl ester was added at room temperature and stirred for one hour. The reaction was followed on TLC (8:2 Hexane: Ethyl acetate). Upon disappearance of the top spot (the methyl ester), the solution was cooled to 0°C and a 1:1 mixture of THF and water was added. The reaction was quenched with 10% H_2SO_4 and the mixture was extracted 3x with diethyl ether. The organic layer was washed with saturated sodium bicarbonate and saturated sodium chloride and dried with $MgSO_4$. The resulting heterogenous mixture was filtered and the organic layer removed by rotovap to afford a white solid. ^{1}H NMR ($CDCl_3$) δ 7.52-7.25 (m, 15H), 6.67 (s, 2H), 5.11 (s, 4H), 5.06 (s, 2H), 4.56 (s, 2H), 1.91 (s, 1H).

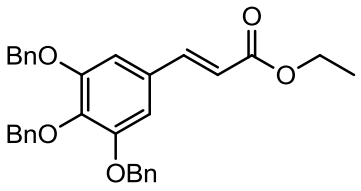
3,4,5-tris(benzyloxy)benzaldehyde (4)



4

3,4,5-tris(benzyloxy)benzyl alcohol (1.1 mmol) was stirred at room temperature under an argon atmosphere in 10 mL of DCM with 4 Å molecular sieves (0.3 g). PDC (0.55 mmol) was added to the reaction mixture which was stirred overnight. Diethyl ether (~30 mL) was added to the reaction mixture and was filtered through a layer of silica gel. The silica gel was washed with 20 mL portions of diethyl ether and the solvent was removed in a rotovap. A white solid was recovered. ^{1}H NMR ($CDCl_3$) 9.81 (s, 1H), 7.44-7.36 (m, 15H), 7.19 (s, 2H), 5.17 (s, 6H).

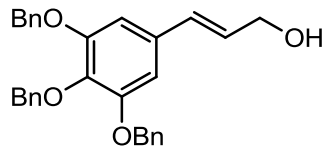
Ethyl (E)-3,4,5-tris(benzyloxy)cinnamate



5

3,4,5-tris(benzyloxy)benzaldehyde (0.706 mmol) was dissolved in dry THF and triethylphosphonoacetate (0.819 mmol) was added. The solution was placed in an ice bath and cooled to 0°C. Sodium hydride (0.819 mmol, 60% solid) was added slowly to the cooled solution which was then allowed to warm to room temperature and stirred for two hours. After two hours, concentrated sodium bicarbonate was added to the reaction mixture. The organic layer was separated from the aqueous layer which was extracted once more with ethyl acetate. The organic layers were combined, dried with $MgSO_4$, filtered and solvent was removed with a rotovap to afford a white solid. ^{1}H NMR ($CDCl_3$) δ 7.56 (d, 1H), 7.45-7.25 (m, 15H), 6.83 (s, 2H), 6.29 (d, 1H), 5.13 (s, 4H), 5.11 (s, 2H), 5.26 (q, 2H), 1.35 (t, 1H).

(E)-3,4,5-tris(benzyloxy)cinnamyl alcohol



6

The α,β-unsaturated ester (0.52 mmol) was dissolved in dry THF and placed in a dry ice/acetone bath (-78°C) under an argon atmosphere. DIBAL (2.6 mmol) was added dropwise via syringe and the reaction was stirred for one hour at -78°C and then for one hour at room temperature. The reaction was followed on TLC using an 8:2 Hexane:Ethyl acetate solvent mixture. Upon disappearance of starting material, the reaction was cooled to 0°C in an ice bath. 50 mL of hexanes and 5 mL of saturated sodium sulfate were added and stirred until a solid had formed. The solid was filtered and washed with ethyl acetate. The organic layer was dried with $MgSO_4$, filtered, and removed with a rotovap. The resulting crude product was purified on a silica gel column with a 50:50 Hexane:Ethyl acetate solvent mixture. ^{1}H NMR ($CDCl_3$) δ 7.45-7.25 (m, 15H), 6.68 (s, 2H), 6.48 (d, 1H), 6.18 (dt, 1H), 5.11 (s, 4H), 5.06 (s, 2H) 4.29 (d, 2H), 1.6 (s, 1H).

3.2 Development of an Indicator Displacement Assay

General Procedures

Fluorescence titrations were recorded at room temperature using a PTI QuantaMaster Fluorimeter. 96-Well plate measurements were performed on a Bio Tek Synergy 2 Multi-Mode Microplate reader. PCA plots were created using XLSTAT 2009.

Fluorescence Titrations

All serum albumins (HSA, RSA and BSA) were purchased in fatty acid free fractions from Sigma Aldrich. The excitation and emission of the indicators are as follows: Fluorescein; λ_{ex}=490 nm, λ_{em}=521 nm. 2-anthracene carboxylic acid; λ_{ex}= 386 nm, λ_{em}= 421 nm. 1-anilino-8-naphthalene sulfonic acid; λ_{ex}= 350 nm, λ_{em}=470 nm.

All fatty acids were purchased from Sigma Aldrich. Each fatty acid stock was dissolved to the appropriate concentration (Stearic and Palmitic acid= 40 mM, Linoleic acid= 20 mM) in 100% ethanol. This stock solution was added directly to the host/indicator complex in a quartz cuvette suitable for fluorescence measurements. The total ethanol concentration added to the aqueous solution was less than 1% of the total volume.

Edible oils were purchased at a local grocery store. To standardize the concentration of stock solutions, it was assumed that each oil was pure oleic acid. Oleic acid was chosen because it is the main free fatty acid in extra virgin olive oil. Each edible oil stock was brought to a concentration of 10 mM in 100% ethanol. These edible oil stocks were added directly to the various host/indicator aqueous complexes and the total ethanol addition was less than 1% of the total volume.

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Vita

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