Winter 1-1-2012

New Methods for the Construction and Characterization of Molecules on an Addressable Array

Bo Bi
Washington University in St. Louis

WASHINGTON UNIVERSITY IN ST. LOUIS

Department of Chemistry

Dissertation Examination Committee:

Kevin D. Moeller, Chairperson

John R. Bleeke

Suzanne Lapi

Garland Marshall

Joshua Maurer

John-Stephen A. Taylor

New Methods for the Construction and Characterization of Molecules on an Addressable Array

by

Bo Bi

A dissertation presented to the
Graduate School of Arts and Sciences
of Washington University in
partial fulfillment of the
requirements for the degree
of Doctor of Philosophy

December 2012

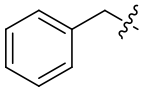
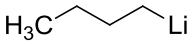
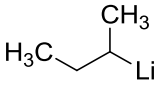
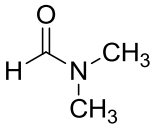
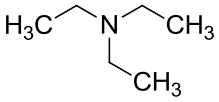
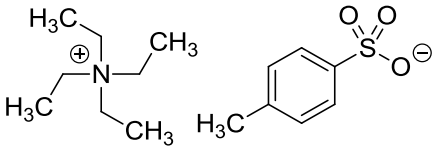
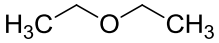
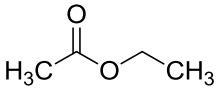
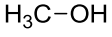
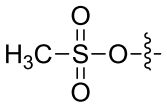
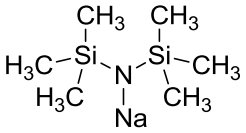
Saint Louis, Missouri

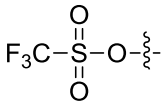
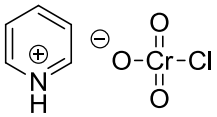
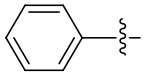
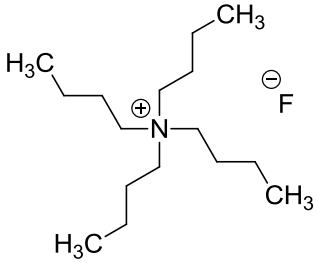
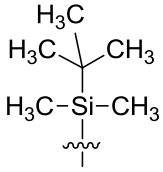
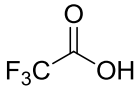
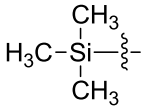
Table of Content

List of Schemes

List of Figures

List of Abbreviations

Abbreviation /Symbol	Full name	Structure
Bn	Benzyl	
n-BuLi	*n*-Butyllithium	
sec-BuLi	*sec*-Butyllithium	
DMF	Dimethylformamide	
Et_3N	Triethylamine	
Et_4NOTs	Tetraethylammonium *p*-toluenesulfonate	
Et_2O	Diethyl ether	
EtOAc	Ethyl acetate	
Me	Methyl	
MeOH	Methanol	
Ms	Mesylate	
NaHMDS	Sodium hexamethyldisilazide, Sodium bis(trimethylsilyl)amide	

Abbreviation /Symbol	Full name	Structure
OTf	Trifluoromethanesulfonate, triflate	
PCC	Pyridinium chlorochromate	
Ph	Phenyl	
TBAF	Tetra-*n*-butylammonium fluoride	
TBS, TBDMS	*tert*-Butyldimethylsilyl	
TFA	Trifluoroacetic acid	
THF	Tetrahydrofuran	
TMS	Trimethylsilyl	

Acknowlegement

First and foremost, I am deeply grateful to my advisor, Dr. Kevin Moeller, for his most patient guidance and generous supports in the past five years. His vast knowledge and greatest passion towards science has kept me motivated and brought me through all the challenges on the road of completing this thesis. In addition, he sets a role model in a lot more ways, where a reliable husband, a loving father, and a sincere friend are just a few of them.

Moreover, I would like to thank Prof. John-Stephen Taylor and Prof. John Bleeke for their helpful suggestions when serving in my thesis advisory committee. Also, I want to thank Prof. Joshua Maurer, Prof. Garland Marshall, and Prof. Suzanne Lapi for their time to serve in my examination committee and provide constructive feedback for my thesis.

Working in the Moeller group was certainly a very enjoyable experience. Dr. Honghui Wu, Dr. Ceng Chen, Dr. Feili Tang, Dr. Melissae Fellet, Dr. Laura Anderson, Dr. Hai-chao Xu, Dr. Libo Hu, Dr. Jennifer Bartels, Dr. Guoxi Xu, Dr. Alison Redden, Mr. John Campdell, Mr. Jake Smith, Mr. Matthew Graaf, Ms. Bichlien Nguyen, Ms. Sakshi Uppal, Mr. Robert Perkins, and Mr. Derek Rensing, thank them all for being the most supportive colleagues whom I can always get helping hands from and mess around with.

I also need to thank Prof. Kevin Moeller, Prof. John-Stephen Taylor, Prof. Vladimir Birman and Dr. Kit Mao for allowing me to work for them as a teaching assisstant and improve my knowledge, as well as teaching skills. Furthermore, I would like to acknowledge Prof. Richard Loomis, Dr. Ed Hiss, Dr. Rachel Linck, Ms. Norma Taylor, Ms. Jessica Owens, Ms. Phyllis Noelken, and Ms. Barbara Tessmer for their help with the administrative work. In addition, I want to thank Dr. Jeff Kao, Dr. André d'Avignon and the Gross group, particularly, Dr. Richard

Y-C. Huang for their help with NMR and Mass Spectrometry acquision and data processing.

All my friends at Washington University helped me adapted to the new culture and enriched my life in St. Louis. I would also like to extend my gratitude to all the people I have worked with in Graduate Student Senate, Toastmaster International, BALSA, St. Louis ACS YCC and Sigma-Aldrich. They made this journey unique and colorful.

A special appreciation goes to Ms. Xin Peng, my girlfriend and fellow graduate student. She accompanied me with her constant care and support on my trip from college to graduate school, from China to the US. This thesis could not have been possible and my life would not have been the same without her.

In the end, I would like to dedicate this thesis to my grandparents, parents and uncles. Their endless love sets the root for all the accomplishments in my life.

ABSTRACT OF THE DISSERTATION

New Methods for the Construction and Characterization of Molecules on an Addressable Array

By

Bo Bi

Doctor of Philosophy in Chemistry

Washington University in St. Louis, 2012

Professor Kevin D. Moeller, Chairperson

Microelectrode array has great potential as a platform for building "addressable" molecular libraries and being utilized to probe the ligand-receptor binding events in “real-time”. To achieve this objective, a complete study would generally include three phases: 1) to enable and expand site-selective chemistry on the array; 2) to understand structural information of the molecules loaded; and 3) to develop research protocols of electrochemical analysis on the device to monitor those molecular interactions. While 2), quality-control analysis, is a core task of this thesis, the studies in other phases that drove this work or derived from it, are also discussed in detail.

In Chapter 2, the thesis starts with the effort towards expanding site-selective reaction methodologies by using electrodes to tune Lewis-acid (Sc (III)) catalyzed reactions. A confining strategy that involves redox cycle of the catalyst is introduced, as well as three types of reactions presented. A new challenge arises when the structures resulted from Diels-Alder reactions and Povarov-type multi-component reactions on the array are only confirmed by a careful design of the controlled experiments and the fluorescent tags at the moment. In addition, the important

information regarding stereochemistry of those structures is missing. A new method to enable the complete characterization of the molecules on the array is required.

The new quality-control handle is derived from “safe-catch” linker. The linkers are attached to the array by means of an ester and contain either a protected amine or protected alcohol nucleophile that can be released using acid generated at the microelectrodes. By placing the substitutions on those linkers, it is allowed to collect the compounds generated from the site-selective chemistries and characterize with HPLC, LC-MS. Moreover, the arrays are kept intact during this procedure and could be recycled for reactions and analysis. In Chapter 3, a full story of the design, make and use of these linkers is to be discussed. The stereochemical question with respect to the reaction is thus answered and the role of the array in the reaction is revealed for the first time.

The work continues in Chapter 4 with a goal of developing a versatile tool to enable the quality control of not only the molecules but also the device before the signaling experiments. An amino-acid derived fluorescent linker is hereby prepared to replace the role of the tags used in the previous studies. The chemical reliability and compatibility of this fluorescent linker makes the first check possible before constructing libraries. Furthermore, it leads to a successful proof-of-principle signaling experiment in monitoring biotin-strepavidin interaction and a protocol to collect and analyze data from the Microelectrode Array. Lastly, the most recent efforts in combining features of chemically cleavable linkers and fluorescent linkers are introduced to conclude the chapter.

In the end, all progress and accomplishments are summarized in Chapter 5, followed by a couple of experiments proposed upon the linkers towards the construction of molecule libraries

and assessment of ligand-receptor bindings on the array.

Chapter One

Introduction

1.1 Small Molecule Microarrays

1.1.1 Molecule libraries

An understanding of ligand-receptor interactions is critical for the design and development of small molecule-drug candidates. However, gaining that understanding can require tremendous effort.

How does one find appropriate ligands for targeting a desired protein? One method is to assemble a library of molecules that can be used to probe the preferences of a protein receptor. The molecules in the library can be natural-product derivatives, small peptides, DNA fragments, etc. Such libraries can be studied in solution (micro-well plates), or on solid supports (molecular arrays).[1-4] In the later case, molecules are attached to a surface and screened for binding to the protein of interest.[5,6] The advantage of having so many different types of entities available within a compact surface is that it provides an efficient means for parallel screening of the library against multiple targets of choice, while consuming low quantities of both precious samples and analytes. The result is a high-throughput approach to the screening of molecular libraries that enables the rapid identification of compounds with the potential for further optimization as drug candidates.

1.1.2 Small molecule microarray

One application of microarray technology is the small molecule microarray (SSM), in which

thousands of small molecules are organized across a small planar surface.[7] Small molecule microarrays are increasingly used to identify specific chemical compounds that bind to a given receptor. A large number of review articles have been published in this area.[5,8-10]

One among many challenges facing the construction of a small-molecule array is the placement of the molecules in the library onto a uniform surface in a spatially isolated manner. This is done so that the binding of individual members of the library to a receptor of interest can be detected. Generally, there are two strategies for accomplishing this goal:[11]

1) The molecules in the library are printed onto a functionalized glass slide with a robotic spotter;
2) The molecules in the library are built on the surface.

The next challenge is to identify binding events. Typically, a screening method is needed to tell which molecules (sites) in the library bind to the receptor. The available screening methods generally fall into one of two groups based on the detection technique.[12]

Labeling methods: In these methods, the receptors (e.g. target proteins) are usually tagged with "reporter groups" such as fluorescent dyes and radioactive groups. In this way, the microarrays are incubated with the receptor of interest, often engineered to present a handle such as a His tag or GST. The binding event is marked with a fluorescence-labeled antibody to the handle. However, one of the major drawbacks of this type of signal is that it typically involves multiple washing steps that remove nonspecifically bound receptor and antibodies from the library. The washing steps can lead to the loss of potentially important information about the nature of the receptor being targeted because they wash away data associated with weaker binding events

Label-free detection methods: These methods include techniques like surface plasmon

resonance (SPR),[13-15] grating couplers, reflectometry, mass spectrometry, etc. The idea behind the methods is to avoid the loss of data by watching binding interactions occur in "real-time". The methods do have limitations when it comes to the construction, manipulation, and analysis of an addressable library. For example, surface plasmon resonance probes the binding event on surface by detecting the changes in the metal surface properties. To build the libraries for examination the molecules are assembled remotely and then placed on a surface. After that, there is no ability to synthetically further modify the molecules or recover them from the surface. This makes the technique time consuming with respect to the screening of larger libraries. The later method also puts an additional question upfront. With the tremendous effort often required to assemble a spatially addressed molecular library, we would ideally be able to recycle them for long-term use and multiple applications. How can we make sure an array-based small molecule library remains good over time? An answer to this question requires full characterization of the molecules in the library with respect to structure, stereochemistry, and purity. Such quality-control issues are a common concern with array-based small molecule libraries. If one cannot fully characterize the molecules in a library, then can one really trust the information gathered from its use?

With all of these things in mind, the potential use of microelectrode arrays as a platform for the evaluation of small-molecule libraries caught our group's attention.

1.2 Combimatrix Microelectrode Arrays and Setups

1.2.1 Combimatrix Microelectrode Arrays

Our lab works with two different microelectrode array formats that are available from

CustomArray, Inc. (formally CombiMatrix. Co.).[16,17] Photos of the arrays are shown below in **Figure 1.1**. These arrays are fabricated using complementary metal-oxide-semiconductor (CMOS) technology in order to afford collections of electrodes that are wired in parallel. The arrays are simple in design and consist of binary addressable X,Y-grid of wires. If a vertical wire and a horizontal wire are both employed at the same time, then the electrode located at the intersection is addressed.

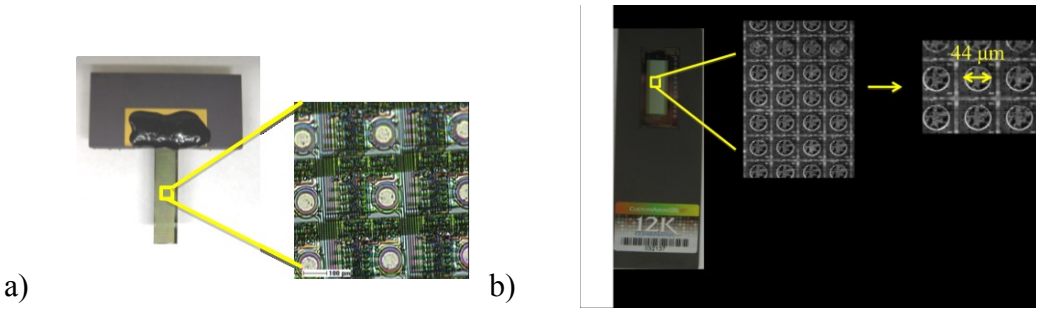


Figure 1.1 Two types of Microelectrode Arrays: a) 1K-array and its enlarged picture; b) 12K-array and its enlarged picture.

The simpler of the two arrays used in the group is a 1K-array containing 1,024 individually addressable microelectrodes per cm^2, **Figure 1.1a**.[17] The electrodes in these arrays are ~95 µm in diameter and range between 200-300 µm apart. These arrays are primarily used as a practice arrays. They are inexpensive, can be inserted into regular reaction vials, and have electrodes more geographically isolated from each other. Hence, the 1K-arrays provide an ideal platform for the discovery and optimization of new array-based reactions. One simply varies the reaction conditions employed in the same manner as any electrolysis reaction. However, the circuitry on the 1K-arrays do not allow for varying potentials. Hence, the arrays are not useful for the collection of analytical data.

For analytical experiments, the more modern 12K-arrays are required. These arrays are imbedded into a ceramic slide so that they can be slipped into the hardware required for

conducting cyclic voltammetry experiments on the arrays. The 12K-arrays contain 12,544 microelectrodes per cm^2, **Figure 1.1b.** The diameter of each electrode in a 12K-array is ~45 μm. The electrodes are 33 μm apart, or about 1/10 the distance between the electrodes found on a 1K array. Like the 1K-array, each electrode is individually addressable.

1.2.2 Electrochemical setups for the arrays

For the 1K-array, the ceramic chip that holds the array contains a pin system for addressing the electrodes. The pins are plugged into a socket board, **Figure 1.2**, leaving a free-standing array that can be inserted into reaction vessels.

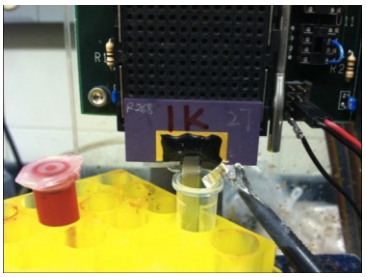


Figure 1.2: 1K-Microelectrode array inserted into a reaction vessel

Reaction solutions are typically made in an Eppendorf tube and then the array simply inserted into this tube. To pass current, a Pt counter-electrode is added to the tube. In this way, preparative reactions on a 1K-array are run like any other preparative electrolysis reaction with one of the electrodes in a typical experiment being replaced by the array. A computer program is then used to address the individual microelectrodes. The software allows us to "map" the pattern electrodes to be used in the experiment, as well as to modify both the current that is passed at the electrodes and the duration of the reaction. To run an oxidation versus reduction on the array is a simple matter of reversing the wiring of the cell.

Unlike the 1K-array, the electronics (ElectraSense reader, **Figure 1.3**) that addresses the 12K-arrays are compatible with sweeping a range of potentials, a capability that allows cyclic voltammetry experiments to be run on selected microelectrodes within the array.[18,19] Hence, the 12K-arrays are the ones used to probe ligand-receptor interactions. These experiments will be discussed further in the next section.

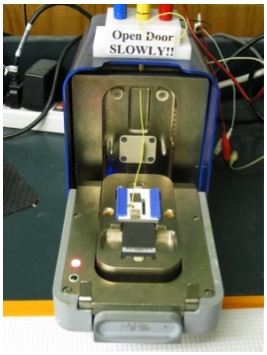


Figure 1.3: ElectraSense reader

For the 12K-arrays, the counter-electrode is platinum that is sputtered onto the cap (called the hyb-cap) that covers the surface (**Figure 1.4**). The Pt is located in a recessed area of the cap (0.75 cm^2) that is surrounded by an O ring that separates the Pt-electrode from the array. When the cap is placed over the array, it creates a space that can hold about 100 µL of solution. The distance between the array and the counter electrode can range from 650-800 µm. The cap is secured to the array using two plastic clips. A thin wire, epoxyed to the top of the hyb-cap, connects the sputtered platinum on the cap to the ElectraSense reader.

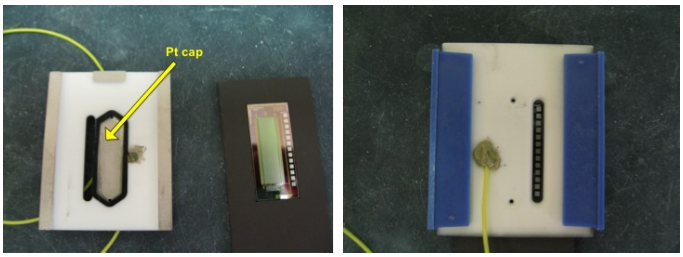


Figure 1.4: Hyb-cap as counter electrode for 12K-array

A newer version of the 12K-array surrounds the electrodes with a platinum grid. The grid can be used as the counter-electrode for electrochemical synthesis and voltammetry with these arrays. The grid can also be independently addressed using the reader. These arrays give much cleaner analytical data because the distance between the counter and working electrodes is rigorously maintained, and hence the resistance across the array is consistent.

Directly beside the electrode array are contact pads for a set of gold pins that are part of the ElectraSense Reader. The pins are then used to control the electrodes in the array with the use of a proprietary software package (available from CustomArray). Since the pads on the array are essential for addressing the array, they are protected from reaction solutions. For coating the array with a porous reaction layer (see below), the pads are covered with tape so that they are not exposed to the polymer solution during spin-coating of the polymer onto the array. After this step the tape is removed. For reactions on the array, the contact pads lie outside of the O-ring used to create space between the array and the cap and provide walls for the resulting reaction vessel above the microelectrode array.

As mentioned above, the ElectraSense reader allows the user to address the electrodes in the array using a personal computer. The reader contains an internal voltage source that can be used to set the potential drop across the cell. This source is used for electrochemical synthesis. The array is set either negative (reductions) or positive (oxidations) relative to the Pt-electrode on the

cap. For voltammetric measurements, the reader is used to activate selected electrodes on the array while an external potentiostat varies the potential drop across the cell. Unlike a "normal" CV with a reference electrode, the potential in this case does not represent an "absolute" number. Instead, the CV records the relative potential drop across the cell that leads to current flow. For this reason, the potentials measured on an array cannot be compared to literature potentials measured against an independent reference electrode.

The ElectraSense reader has six colored terminals that are used to make connections with the 12K-array and the Pt-counter electrode **Figure 1.5a**. For the purpose of this discussion, the white and orange ports will not be addressed since they are not employed for any of the reactions conducted in this thesis. As for the remaining ports, the blue one is connected to a positive potentiostat. It is used to apply a positive potential to the electrodes. Conversely, the black port is the ground. It is connected to the cathode and applies a negative potential (relative to the blue port). The last two terminals make connections with the array and the counter-electrode. The yellow terminal is connected to the Pt-cap while the red terminal is connected to the microelectrode array. Reduction and

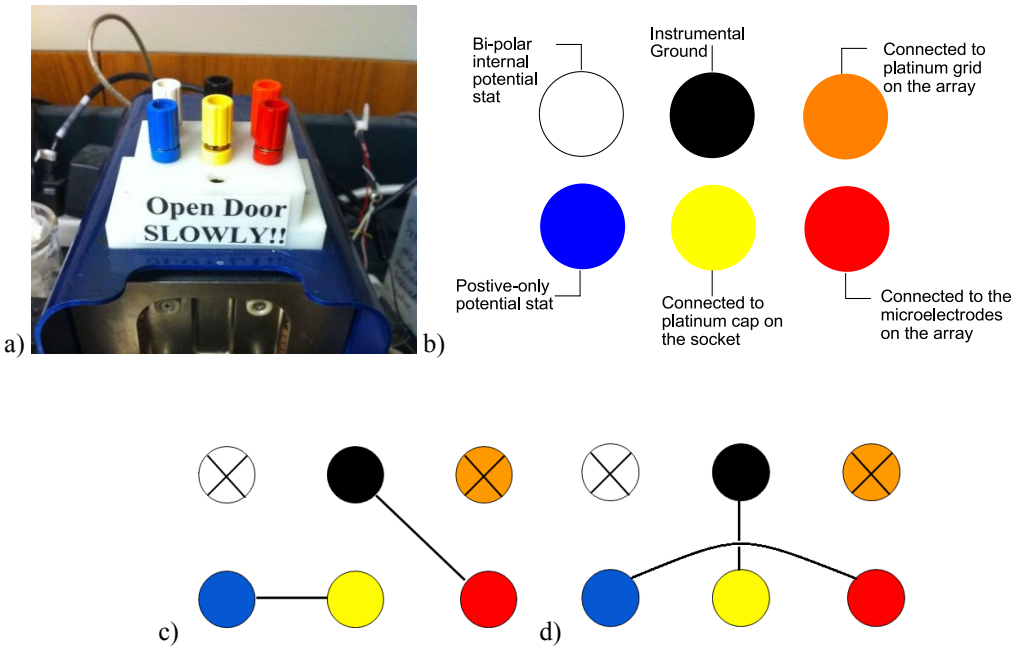


Figure 1.5 a) the terminals on the 12-K array; b) functions of those terminals; c) connections for a reduction; d) connections for an oxidation

oxidation reactions on the array are then controlled by how the terminals are connected. For a reduction reaction of the array, **Figure 1.5c**, the yellow and blue ports are connected, as are the red and black. This makes the counter electrode positive and the array negative. When an oxidation reaction on the array is desired, a connection is made between the black and yellow terminals, as well as between the blue and red terminals, **Figure 1.5d**.

To use the ElectraSense reader for electrochemical detection via cyclic voltammetry (CV), the reader is connected to an external potentiostat. A wire is used to connect the ground on the ElectraSense reader with the ground on the CV apparatus used. This provides a common ground for the two instruments. (**Figure 1.6**)

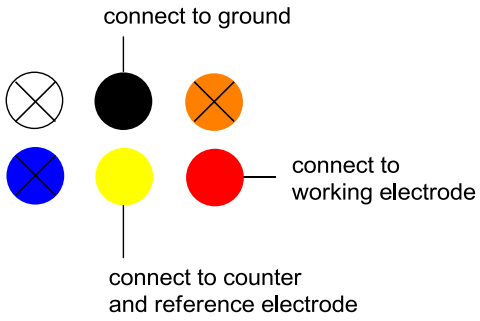


Figure 1.6: The terminal connections for the impedance experiments

The leads for the counter and the reference electrodes associated with the CV potentiostat are then both connected to the cap (yellow terminal) and the working electrode from the CV is connected to the array (red terminal). This leads to the CV potentiostat being "tricked" into recognizing the Pt-cap as both the counter and reference electrodes. The result is that the instrument sweeps potentials that represent the difference between the working electrodes in the array and the cap. Using the proprietary software to run the array, selected electrodes in the array are chosen to serve as working electrodes. The voltammogram is recorded using the CV-apparatus that measures the current that flows through the working electrodes relative to the potential at the electrode surface. With the newer version of the 12K arrays with the Pt-grid on the surface, the counter and the reference electrodes are both connected the grid on the array. The cap is then connected to ground, while working electrode for the CV-apparatus remains connected to the electrodes in the array.

1.3 Site-Selective Chemistry on Microelectrode Array

1.3.1 Surface coatings

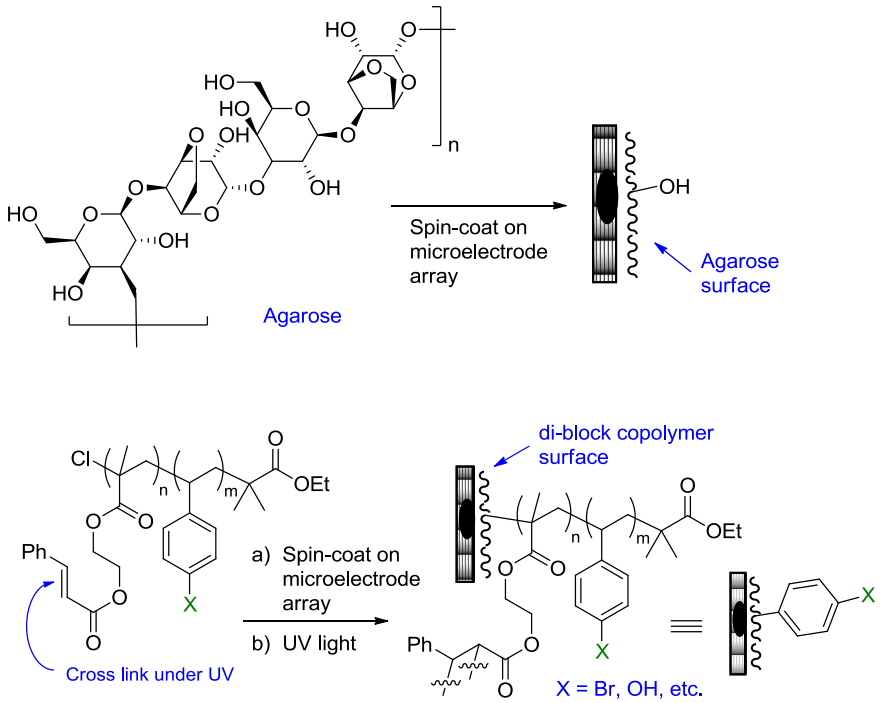
In order to run a reaction on an array, the array must first be coated with a porous polymer layer—typically agarose, sucrose, or a diblock copolymer by Dr. Libo Hu.[20] The porous reaction

layer provides attachment sites so that molecules can be fixed to the surface of the electrodes in the array. The porous reaction layer must be stable, inert with respect to the preparative and analytical chemistry conducted on the array, porous enough for small molecule reagents to migrate through the layer to the electrodes below, and relatively unreactive with respect to non-specific binding events with biological receptors.

All of the polymers used in the studies below were spin-coated onto the surface of the arrays. This was accomplished by pre-dissolving the polymers into solutions that would evaporate from the surface of the array and then dropping the resulting mixtures into the center of the spinning array. The array was spun with the use of a Laurell 400B-6NPP-400/LITE spin coater that was equipped with adapters to fit the arrays. **Scheme 1.1** shows two most common porous reaction layers used in our group.

Of the two, agarose is the least stable. It delaminates from the surface of the array with time, dissolves in a variety of solvents, and reacts with a number of the reagents used for site-selective synthesis. For this reason, agarose is mainly used as a "practice-polymer" for studying new reactions on the arrays. As a practice polymer, agarose is ideal since it can be removed easily from the arrays. That allows for recycle use of the arrays. In addition, the poly-hydroxylated surface limits the use of microelectrode arrays for monitoring the behavior of small molecules that are synthesized by using protected amine- and alcohol- functional groups, and the use of agarose has at times proven incompatible with biological studies.[21]

Scheme 1.1 Polymer layers on Microelectrode Array



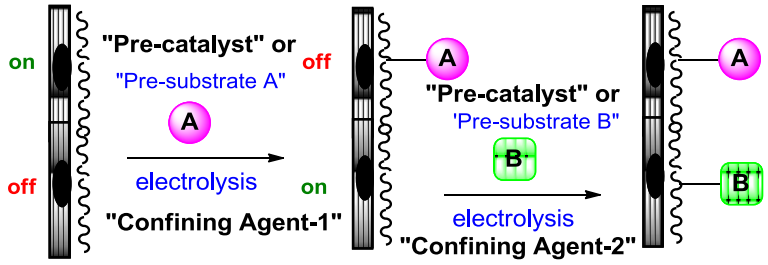
In 2009, Dr. Libo Hu *et al.* reported a more stable alternative to agarose that was constructed from a diblock copolymer. The polymer was comprised of a hydrophilic cinnamoyl-substituted polymethacrylate block for attachment to the surface of the array and a p-bromo-substituted polystyrene block for selective functionalization of the surface. Once the polymer was spin-coated onto an array, it was cross-linked by photochemically dimerizing the cinnamoyl groups in order to provide stability to the coating.

1.3.2 Confining strategy

Perhaps the major challenge associated with the construction or placement of molecules on a microelectrode array is the confinement of reactions to selected electrodes in the array. How does one conduct a chemical reaction at one electrode in an array without disturbing the

neighboring electrodes when there are over 12,000 of the electrodes in a 1 cm^2 area?

Scheme 1.2 General scheme of reaction confinement on Microelectrode Array



One solution to this problem is highlighted in **Scheme 1.2**. The illustration in the scheme shows two neighboring electrodes that can be individually manipulated: one being turned on while the other one left off. The array is immersed in a solution containing a chemical substrate for placement (Substrate A), a pre-catalyst or pre-reagent, and a "confining agent". Like most preparative electrolysis reactions, the reaction solution also contains an electrolyte that is used to lower the resistance of the cell. When the reaction starts, selected electrodes in the array are used to convert the pre-catalyst or pre-reagent into an active form that in turn will facilitate a reaction between the substrate and the porous reaction layer coating the electrodes. This is done by either oxidizing or reducing the pre-catalyst or reagent in order to reverse its chemical behavior. The new species generated in this fashion can be an acid, base, oxidant, reductant, transition-metal catalyst, Lewis-acid, nucleophile, electrophile, etc. By making the required catalyst or reagent at the electrode, it is generated exactly where it is needed on the array. The confining agent placed in solution is used to localize the active catalyst or reagent. It accomplishes this task by reacting with any of the electrochemically generated catalyst or reagent in solution before it migrates to a neighboring electrode. The reaction takes the catalyst or reagent back to its original inactive state

by either re-reducing or re-oxidizing it. This sets up a competition on the array between generation of the active catalyst or reagent at the electrodes and destruction of that same catalyst or reagent in solution. By repeating the same strategy with the different combinations of substrates (e.g. Substrate B) and confining agents (e.g. **Confining Agent-2**), a variety of compounds can be placed (or constructed) on the same array.

On the 1K arrays, the reactions are run by cycling the potential at the electrodes between on and off modes. This helps to control the rate of the reagent or catalyst being generated at the electrodes and thus aids the site-selectivity of the reactions. On the 12K-arrays, a similar level of control is obtained by pulsing the electrodes.

New efforts to apply this general strategy to the development of new microelectrode array-based synthetic methodologies will be further discussed in detail in Chapter 2.

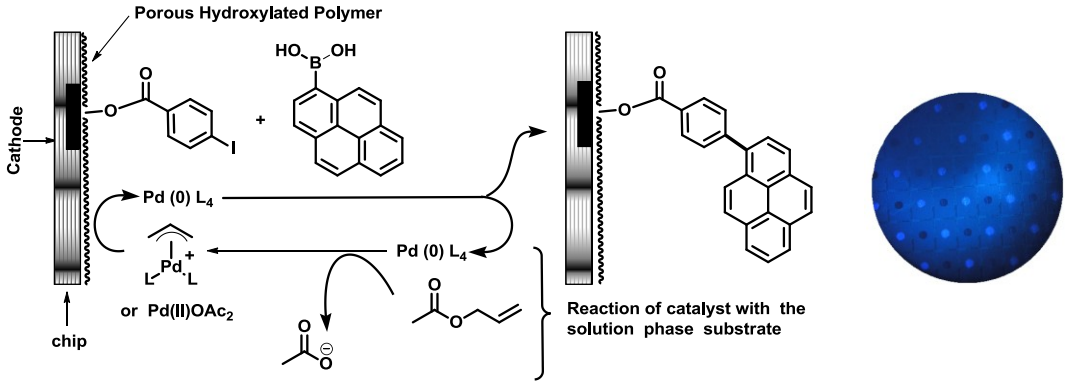
1.3.3 Examples of site-selective reactions on Microelectrode Arrays

Our group has made a number of breakthroughs in the construction of addressable libraries as platforms for biological assays on microelectrode arrays.[22] Early work illustrates how acyl-transfer reactions,[23] Heck reactions,[24] Wacker oxidations,[25] Suzuki reactions,[26] "click" reactions,[27] hetero-Michael reactions,[23] and reductive amination reactions[28] could be successfully accomplished on the arrays. The methods took advantage of the electrochemical generation of bases, Pd(0)-catalysts, Pd(II)-catalysts, oxidants, and Cu(I)-catalysts.

As an example, a site-selective Suzuki-reaction was conducted between either an aryl iodide attached to the surface of the array and an aryl boronic acid in solution or an aryl boronic acid attached to the surface of the array and an aryl bromide in solution. Both allyl acetate and air served as effective confining agents to quench the newly generated Pd(0) by oxidation before it could migrate away from the electrode of its origin.[28] (**Scheme 1.3**) This work was accomplished

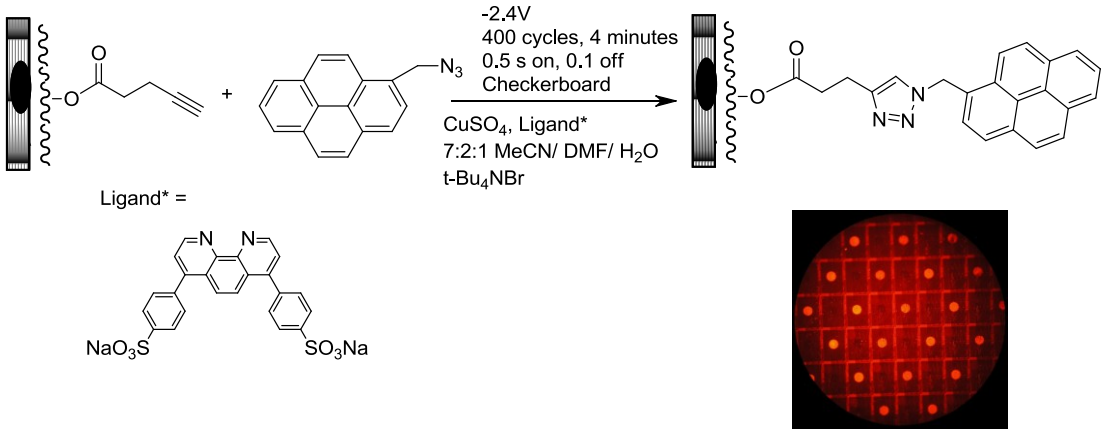
by Dr. Libo Hu in our group.

Scheme 1.3 The site-selective Suzuki reaction on Microelectrode Array



Another more recent example is the site-selective "Click-chemistry" developed by Dr. Jennifer Bartels.[29] As shown in **Scheme 1.4**, copper sulfate ($CuSO_4$) was added to the solution above an array. Selected electrodes were then used as cathodes in order to reduce the Cu(II) to Cu(I) and generate the catalyst needed for triggering a [3+2]-cycloaddition between an acetylene on the surface of the array and a solution-phase azide. In this case, the solution above the array contained oxygen. The oxygen oxidized the Cu(I)-catalyst in solution back to the inactive Cu(II) before it could diffuse away from the electrodes used for its generation. In this way, the dissolved oxygen served as the confining reagent for the reaction.

Scheme 1.4 The site-selective "Click chemistry" on Microelectrode Array



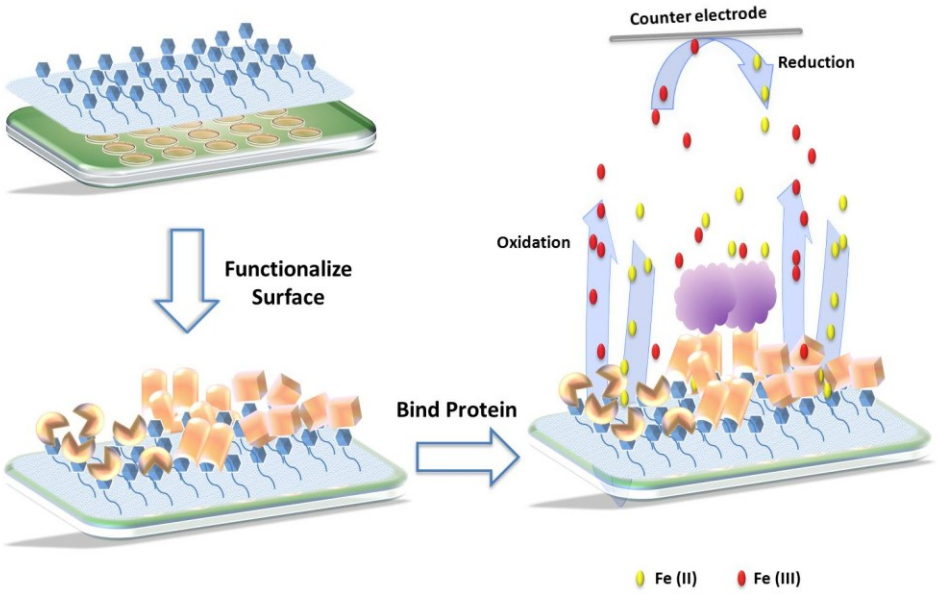
1.4 Probing Ligand-Receptor Interactions via Impedance Experiment

As discussed above, our motivation for developing microelectrode arrays as platforms for the analysis of molecular libraries stems from a need to detect weak-binding interactions in "real-time". To this end, the use of electrochemical impedance experiments to measure the binding interactions appears ideal.[30,31] In this experiment, the binding event is signaled by a redox couple in solution rather than a redox probe attached to the surface or one of the interacting partners. This is accomplished by treating a functionalized array (each unique member of a library by a unique addressable electrode in the array) with both the targeted receptor and a redox couple (illustrated with iron species in **Scheme 1.5**). The array is then used as an anode and the remote wire as a cathode. The reduced species in the redox couple then migrates through the porous reaction layer on the array to the electrodes below where it is oxidized. The oxidized species in the redox couple migrates to the cathode wire and is reduced, maintaining the initial balance of reduced and oxidized species. The result is a current that can be measured at each electrode in the array. When a binding event occurs between the receptor in solution and a ligand on the

surface of the array, the resulting complex impedes the redox couple from reaching the underlying electrode. This results in a measurable drop in current at the neighboring electrode.

The pioneering progress in probing ligand-receptor interactions via impedance experiment will be explicated in Chapter 4.

Scheme 1.5 General scheme of impedance experiment on Microelectrode Array



1.5 Quality Control Tools on Microelectrode Array

As discussed previously, one of the key issues that raise concerns about both this work and the use of any small-molecule library is the quality of the molecular library over time. How does one know that a small-molecule library contains the structures that we think it does? To solve this problem, a method for the characterization of molecules located by any electrode in a microelectrode array is needed.

Initial efforts along these lines took advantage of Time of Flight-Secondary Ion Mass Spectrometry (TOF-SIMS). This effort required the use of a linker to connect the molecule to be studied to the surface of the array that cleaved under the mass-spectrometry conditions faster than the polymer coating the array. Without the linker, only fragmentation of the polymer could be detected in the mass spectrometer. To this point, three such linkers have been developed in our lab.[29,32,33] One was designed to undergo a McLafferty fragmentation, one took advantage of a pyrene group as a cleavage-site, and in the most recent case one took advantage of a retro [3+2]-cycloaddition reaction. (**Figure 1.7**) While all of the linkers proved useful, they all suffered from two major disadvantages.

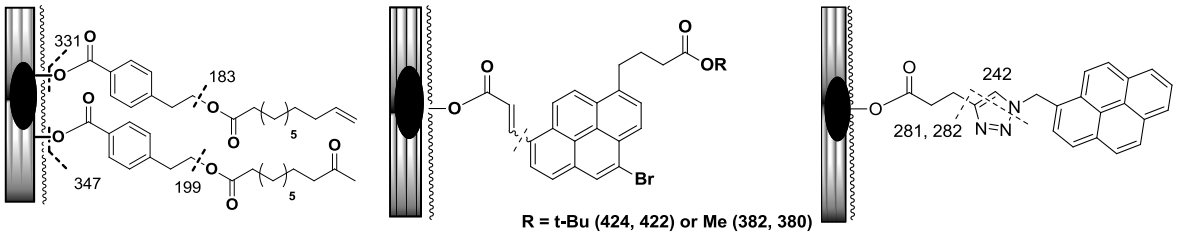


Figure 1.7: Illustrate three types of Mass Spectrometry cleavable linkers

First and foremost, TOF-SIMS as a characterization technique requires destruction of the array and its surface. This disallows the technique as a method for conducting quality control studies on a molecular library that one wants to reuse. Secondly, molecular weight information collected by mass spectrometry is not useful for characterizing either the stereochemistry or purity of a molecule. Both are necessary for taking full advantage of an observed ligand-receptor interaction.

With this in mind, we sought a mild, yet powerful tool for reclaiming molecules from the

surface of an array. This work will be discussed in detail in Chapter 3.

In addition, quality-control methods on an array must also allow us to check the quality of the microelectrode array itself. Microelectrode arrays address individual electrodes by simultaneously turning on two wires that meet at the electrode. Each individual wire addresses either a row or a column of electrodes on the array. When the arrays develop problems they typically arise because wires on the array become short circuited with other wires. With such a damaged array, attempts to use one wire lead to a number of wires being activated with each new wire addressing either a whole row or whole column of electrodes on the array. The result is that whole regions of the array are activated. Since the software that tests the quality of an array indicates the number of electrodes that are active, it does not identify this problem where all of the electrodes become active. Hence, linkers used on the array must be fluorescently active so that they can be used to assess the quality of the array. For example, with a fluorescent linker, the placement of a building block by every electrode in the array can be done in two steps each one using a checkerboard pattern. After the first, the array can be examined to show that the array is working uniformly well across its entire surface. Efforts to build a cleavable, fluorescent linker will be discussed in Chapter 4.

1.6 Scope of the Thesis

As mentioned earlier, our overall goal is to develop microelectrode arrays as a platform for monitoring ligand-receptor interactions in “real-time”. The work has focused on the development of synthetic methods for building addressable molecule libraries on an array and then detecting binding events that involve the molecules in the library.

With that backdrop, the primary focus of the research work highlighted in this thesis has been the development of new tools that will allow for quality control assessment of a molecular

library on an array. This primary effort has led to a number of supplemental studies as well.

In Chapter 2, a class of array-based reactions that utilize a site-selectively generated Lewis acid is described. The method allows for an array-based Diels-Alder reaction, a reaction that requires one to characterize both the structure and stereochemistry of the product generated. The reaction fueled efforts to build cleavable linkers on the arrays.

In Chapter 3, a new strategy based upon the Kenner "safety-catch" linker model will be discussed. The design and preparation of linker prototypes, experimental attempts to use various linkers, and the application of the linkers for solving structural questions on the arrays will be discussed.

In Chapter 4, a fluorescent label with modifiable functionalities will be discussed along with the efforts to use it in labeled linkers. Chemistry will show how the resulting linkers can be used to functionalize arrays in good quality prior to subsequent signaling experiments. The linkers are compatible with electrochemical-impedance experiments, an observation that allows us to effectively conduct proof-of-principle experiments that verify the utility of the arrays for probing molecular interactions in real-time. To conclude the chapter, ongoing efforts to build synthetically accessible, cleavable fluorescent linkers will be described.

In Chapter 5, a summary of the research accomplished is provided along with proposals that outline the potential use for the methodology discovered.

1.7 References

1. Lipshutz, R. J.; Fodor, S. P. A.; Gingeras, T. R.; Lockhart, D. J., "High density synthetic oligonucleotide arrays". *Nature Genetics* **1999,** *21*, 20-24.

2. Pirrung, M. C., "Spatially addressable combinatorial libraries". *Chemical Reviews* **1997,** *97* (2), 473-488.

3. Webb, M. S.; Miller, A. L.; Johnson, B. H.; Fofanov, Y.; Li, T. B.; Wood, T. G.; Thompson, E. B., "Gene networks in glucocorticoid-evoked apoptosis of leukemic cells". *Journal of Steroid Biochemistry and Molecular Biology* **2003,** *85* (2-5), 183-193.

4. Shih, S. R.; Wang, Y. W.; Chen, G. W.; Chang, L. Y.; Lin, T. Y.; Tseng, M. C.; Chiang, C.; Tsao, K. C.; Huang, C. G.; Shio, M. R.; Tai, J. H.; Wang, S. H.; Kuo, R. L.; Liu, W. T., "Serotype-specific detection of enterovirus 71 in clinical specimens by DNA microchip array". *Journal of virological methods* **2003,** *111* (1), 55-60.

5. Duffner, J. L.; Clemons, P. A.; Koehler, A. N., "A pipeline for ligand discovery using small-molecule microarrays". *Current Opinion in Chemical Biology* **2007,** *11* (1), 74-82.

6. Winssinger, N.; Pianowski, Z.; Debaene, F., "Probing biology with small molecule microarrays (SMM)". *Combinatorial Chemistry on Solid Supports* **2007,** *278*, 311-342.

7. Vegas, A. J.; Fuller, J. H.; Koehler, A. N., "Small-molecule microarrays as tools in ligand discovery". *Chemical Society Reviews* **2008,** *37* (7), 1385-1394.

8. Walsh, D. P.; Chang, Y. T., "Recent advances in small molecule microarrays: applications and technology". *Combinatorial Chemistry & High Throughput Screening* **2004,** *7* (6), 557-564.

9. Wu, H.; Ge, J.; Uttamchandani, M.; Yao, S. Q., "Small molecule microarrays: the first decade and beyond". *Chemical Communications* **2011,** *47* (20), 5664-5670.

10. Ma, H.; Horiuchi, K. Y., "Chemical microarray: a new tool for drug screening and

discovery". *Drug discovery today* **2006,** *11* (13-14), 661-668.

11. Shaginian, A.; Patel, M.; Li, M. H.; Flickinger, S. T.; Kim, C. H.; Cerrina, F.; Belshaw, P. J., "Light-directed radial combinatorial chemistry: Orthogonal safety-catch protecting groups for the synthesis of small molecule microarrays". *Journal of the American Chemical Society* **2004,** *126* (51), 16704-16705.

12. Kumaresan, P. R.; Lam, K. S., "Screening chemical microarrays: methods and applications". *Molecular bioSystems* **2006,** *2* (5), 259-270.

13. Cooper, M. A., "Optical biosensors in drug discovery". *Nature reviews. Drug discovery* **2002,** *1* (7), 515-528.

14. Homola, J., "Present and future of surface plasmon resonance biosensors". *Analytical and bioanalytical chemistry* **2003,** *377* (3), 528-539.

15. Pattnaik, P., "Surface plasmon resonance: applications in understanding receptor-ligand interaction". *Applied biochemistry and biotechnology* **2005,** *126* (2), 79-92.

16. Dill, K.; Liu, R. H.; Grodzinski, P., *Microarrays: preparation, microfluidics, detection methods, and biological applications*. Springer: New York, **2009**.

17. Dill, K.; Montgomery, D. D.; Wang, W.; Tsai, J. C., "Antigen detection using microelectrode array microchips". *Analytica Chimica Acta* **2001,** *444* (1), 69-78.

18. Roth, K. M.; Peyvan, K.; Schwarzkopf, K. R.; Ghindilis, A., "Electrochemical detection of short DNA oligomer hybridization using the CombiMatrix ElectraSense Microarray reader". *Electroanalysis* **2006,** *18* (19-20), 1982-1988.

19. Ghindilis, A. L.; Smith, M. W.; Schwarzkopf, K. R.; Roth, K. M.; Peyvan, K.; Munro, S. B.; Lodes, M. J.; Stover, A. G.; Bernards, K.; Dill, K.; McShea, A., "CombiMatrix oligonucleotide arrays: Genotyping and gene expression assays employing electrochemical detection".

Biosensors & Bioelectronics **2007,** *22* (9-10), 1853-1860.

20. Hu, L. B.; Bartels, J. L.; Bartels, J. W.; Maurer, K.; Moeller, K. D., "A New Porous Reaction Layer for Developing Addressable Molecular Libraries". *Journal of the American Chemical Society* **2009,** *131* (46), 16638-+.

21. Kesselring, D.; Maurer, K.; Moeller, K. D., "Microelectrode arrays and ceric ammonium nitrate: A simple strategy for developing new site-selective synthetic methods". *Journal of the American Chemical Society* **2008,** *130* (34), 11290-+.

22. Yoshida, J.; Nagaki, A., "Building addressable libraries as platforms for biological assays by an electrochemical method". *Angewandte Chemie International Edition* **2010,** *49* (22), 3720-3722.

23. Stuart, M.; Maurer, K.; Moeller, K. D., "Moving known libraries to an addressable array: a site-selective hetero-Michael reaction". *Bioconjugate Chemistry* **2008,** *19* (8), 1514-1517.

24. Tian, J.; Maurer, K.; Tesfu, E.; Moeller, K. D., "Building addressable libraries: the use of electrochemistry for spatially isolating a heck reaction on a chip". *Journal of the American Chemical Society* **2005,** *127* (5), 1392-1393.

25. Tesfu, E.; Maurer, K.; Ragsdale, S. R.; Moeller, K. D., "Building addressable libraries: The use of electrochemistry for generating reactive Pd(II) reagents at preselected sites on a chip". *Journal of the American Chemical Society* **2004,** *126* (20), 6212-6213.

26. Hu, L.; Maurer, K.; Moeller, K. D., "Building addressable libraries: site-selective Suzuki reactions on microelectrode arrays". *Organic Letters* **2009,** *11* (6), 1273-1276.

27. Bartels, J. L.; Lu, P.; Walker, A.; Maurer, K.; Moeller, K. D., "Building addressable libraries: a site-selective click-reaction strategy for rapidly assembling mass spectrometry cleavable linkers". *Chemical Communications (Cambridge, United Kingdom)* **2009,** (37), 5573-

5575.

28. Hu, L. B.; Maurer, K.; Moeller, K. D., "Building Addressable Libraries: Site-Selective Suzuki Reactions on Microelectrode Arrays". *Organic Letters* **2009,** *11* (6), 1273-1276.

29. Bartels, J. L.; Lu, P.; Walker, A.; Maurer, K.; Moeller, K. D., "Building addressable libraries: a site-selective click-reaction strategy for rapidly assembling mass spectrometry cleavable linkers". *Chemical Communications* **2009,** (37), 5573-5575.

30. Stuart, M.; Maurer, K.; Moeller, K. D., "Moving known libraries to an addressable array: A site-selective hetero-Michael reaction". *Bioconjugate Chemistry* **2008,** *19* (8), 1514-1517.

31. Tesfu, E.; Roth, K.; Maurer, K.; Moeller, K. D., "Buillding addressable libraries: Site selective coumarin synthesis and the "Real-Time" signaling of antiibody-coumarin binding". *Organic Letters* **2006,** *8* (4), 709-712.

32. Chen, C.; Lu, P.; Walker, A.; Maurer, K.; Moeller, K. D., "The use of a detectable, mass-spectrometry-cleavable linker for quality control on an addressable microelectrode array". *Electrochemistry Communications* **2008,** *10* (7), 973-976.

33. Chen, C.; Nagy, G.; Walker, A. V.; Maurer, K.; McShea, A.; Moeller, K. D., "Building addressable libraries: The use of a mass spectrometry cleavable linker for monitoring reactions on a microelectrode array". *Journal of the American Chemical Society* **2006,** *128* (50), 16020-16021.

Chapter Two

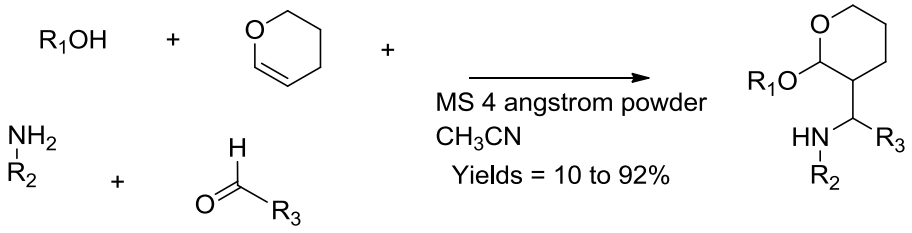
Site-Selective, Lewis-Acid Catalyzed Reactions on Microelectrode Arrays

2.1 Lewis-Acid Catalyzed Reactions

As discussed in Chapter 1, microelectrode arrays are potentially powerful tools for monitoring the binding of molecular libraries to biological receptors in "real-time". However, in order to capitalize on this potential, we must be able to synthesize the members of the molecular library in a manner that places each unique member of the library proximal to a unique, individually addressable microelectrode in the array. This is a daunting challenge since the arrays used for signaling contain up to 12,544 microelectrodes/cm^{-2}. Our group's initial studies along these lines were outlined in Chapter 1. These efforts focused on moving reactions like Pd-catalyzed Heck and Suzuki transformantions, Cu-catalyzed cycloaddition and coupling reactions, acid and base reactions, etc. These reactions can primarily be characterized as methods that allowed us to add things to the electrodes above an array. They are ideal for transferring a small library to the array, or adding newly synthesized molecules to an array-supported library of molecules. However, in an ideal scenario, we would also be able to build a library of molecules directly on the array in order to avoid the inefficiency associated with transferring even a moderate sized library to an array one member at a time. In connection with this idea, we sought to develop synthetic methods that would allow us to build the cyclic structures that typically form the core scaffold of a molecular library. To this end, the ability to employ a Lewis-acid

catalyst in a site-selective fashion was particularly intriguing. Lewis-acid catalysts are used to accelerate reactions, trigger cycloadditions, introduce stereocontrol elements into reaction transition states, and assemble reagents for multicomponent synthetic strategies. They have been used frequently to build functionalized ring skeletons. As an example, $Sc(III)OTf_3$ has been used to conduct the multicomponent synthesis of tetrahydrofuran derivatives (Scheme 2.1).[1] The products have three potential sites (R1, R2, R3) for diversification,

Scheme 2.1 Sc(III) catalyzed multicomponent reaction



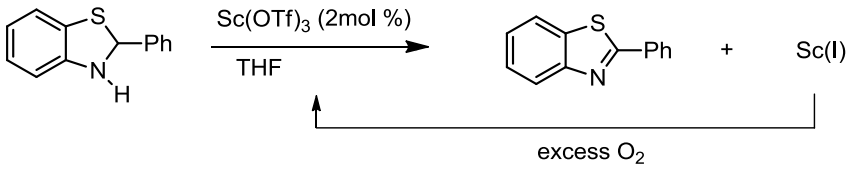
a situation that would allow the product to be built into a small to medium size molecular library. In a similar manner, $Sc(III)OTf_3$ can be used as a Lewis acid to catalyze the Diels-Alder reaction. Once again, the method is compatible with the construction of a central core-ring skeleton with sites that allow for diversification and the synthesis of a molecular library. But how can such Lewis-acid catalyzed reactions be run site-selectively on a microelectrode array?

2.2 Confining Strategies

The Sc(III)-catalyzed reaction illustrated in **Scheme 2.1** provided an excellent starting point for developing an array-based Lewis acid catalyst. As with any microelectrode-array-based method, the key questions that needed to be addressed were 1) how to site-selectively synthesize the desired reagent using the microelectrodes, and 2) how to confine the reagent to the area

surrounding a selected electrode once it had been generated. For the reaction illustrated in **Scheme 2.1**, this meant that we needed a method of generating an active Sc(III) catalyst at desired microelectrodes, and then a method for destroying the Sc(III) in the solution above the array. In principle, this scenario could be accomplished by using the microelectrodes to juggle Sc-oxidation states. If an inactive Sc(I)-reagent was introduced into the solution above a microelectrode array, then the desired Sc(III)-catalyst could be generated at selected microelectrodes in an array by using the electrodes as anodes to oxidize Sc(I) to Sc(III). In solution, a reducing agent would be needed to convert the Sc(III)-catalyst back to the inactive Sc(I), and in so doing keep the newly generated active Sc(III) from migrating to neighboring electrodes. The chemistry outlined in **Scheme 2.2** suggested that 2-phenylbenzthiazole might serve nicely as this solution-phase reducing agent.

Scheme 2.2 A strategy for confinement

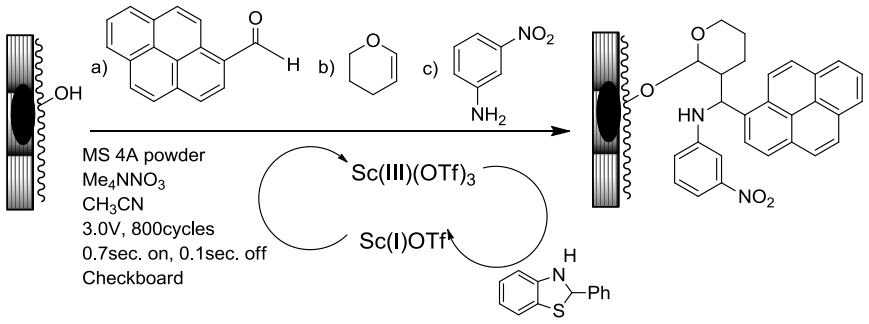


In the experiment illustrated, Sc(III) was used to mediate the oxidation of 2-arylbenzothiazole by oxygen.[2] Without the presence of the oxygen, the oxidation proceeds until the Sc(III) is consumed and then stops. The reaction completely converts the original Sc(III)-reagent to a Sc(I)-derivative. Hence for a microelectrode-array-based reaction, one could in principle run the reaction outlined in **Scheme 2.2** above an array and then use microelectrodes in the array in place of the oxygen in order to regenerate Sc(III) only where needed.

2.3 Site-Selective Lewis-Acid (Sc((III)) Catalyzed Reactions

In order to test whether such an approach can be used to site-selectively initiate reactions on a microelectrode array, a multicomponent reaction analogous to the chemistry illustrated in **Scheme 2.1** was designed (**Scheme 2.3**). In this reaction, the agarose coating on a microelectrode array was used as the alcohol component in the reaction. The aryl aldehyde to be used was a pyrene derivative. Hence, a successful multicomponent reaction would place a fluorescent group onto the surface of the array.

Scheme 2.3 Sc(III)-catalyzed tetrahydropyran synthesis



The reaction began by premixing a catalytic amount of $Sc(OTf)_3$ with an excess of 2-arylbenzothiazole in acetonitrile for 30 min. This was done to insure that all of the Sc(III) was reduced to Sc(I) prior to the start of the reaction. To this solution was then added the pyrene aldehyde, dihydrofuran, and *m*-nitroaniline along with tetramethylammonium nitrate as an electrolyte. A microelectrode array spin-coated with agarose was then submerged in the solution along with a remote Pt-wire that served as an auxiliary cathode. The Sc(III)-catalyst needed for triggering the multicomponent reaction was then produced at selected electrodes by cycling the electrodes on at a potential of +3.0 V vs. the remote Pt-wire cathode for a period of 0.7 s and

then off again for a period of 0.1 s. To generate the left image shown below in **Figure 2.1**, 800 such cycles were used in a checkerboard pattern on an array having containing 1024 electrodes in a 1cm^2 area (a 1K-array). The image was obtained by washing any excess pyrene aldehyde off of the chip following the reaction with excess amount of water and ethanol and then examining the array using a fluorescence microscope. In the image shown, the microelectrodes have a diameter of 95 microns. Clearly, the strategy for generating and confining a Sc(III) Lewis-acid catalyst on the 1K-array worked very well. In a similar manner (the operational differences were explicated in Chapter 1), the reaction was conducted on a 12K-array by pulsing current on selected electrodes. In this case, a checkerboard in a box pattern of electrodes was used as anodes. Once again, a high level of confinement was observed for the reaction (the right image in **Figure 2.1**).

Unlike one-step site-selective chemistry developed before, these fluorescent patterns were not enough for us to claim success due to the complex nature of the reaction. A couple of control experiments were thus conducted. First of all, on 1K-array, when the same conditions were applied with either dihydrofuran or *m*-nitroaniline being removed, there was no fluorescence observed on the array after the washing steps. It indicated 1) the pyrene aldehyde could not be coupled to the surface directly under the condition and 2) each of the other reagents did play a role to produce the final pattern.

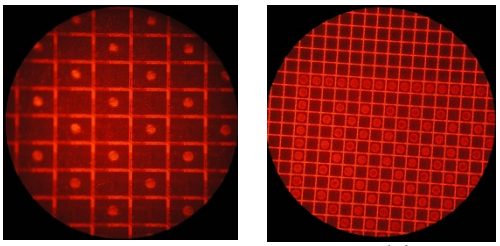


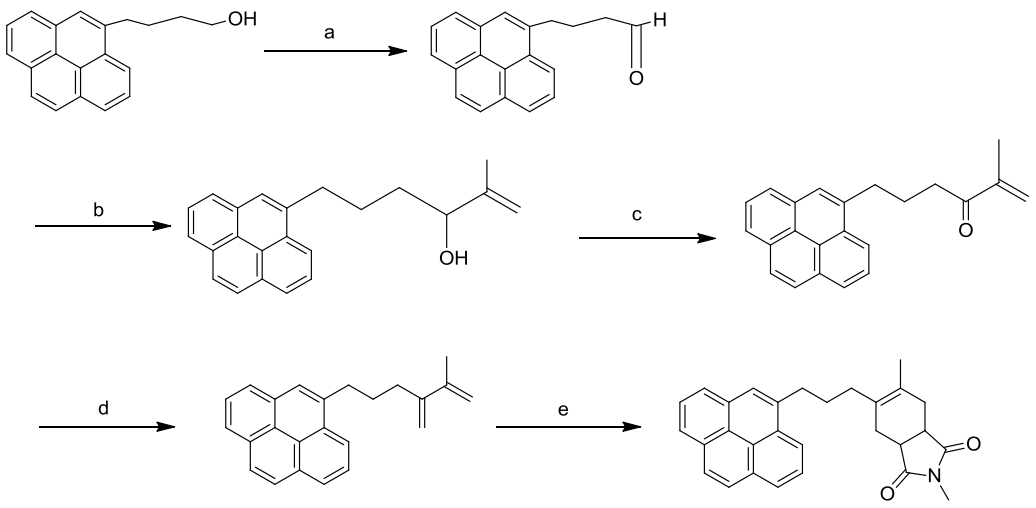
Figure 2.1 Povarov-type multicomponent reactions on Microelectrode Arrays (Left: 1K-array; Right: 12K-array)

Secondly, a parallel reaction was run in solution phase following the procedures reported. In this case, methanol was used to replace the role of agarose layer on the array. It generated the target products with 3 diastereomers and a combined yield of 63%. It is worth pointing out that 1 equivalent of Sc(III)OTf_3 was used in solution to shorten the reaction time to 18 hours. (A typical reaction time length in the literature was 48 hours with only 20% Sc(III)) On the array, the actual concentration of catalyst generated *in situ* was even much larger than that in solution. The reaction would be accelerated significantly. It is the reason why it usually requires a lot less time to finish reaction on the array. Above all, with that couple of experiments, we gained confidence in that the fluorescent pattern was actually produced by the site-selective multi-component reaction on the array.

With a successful confinement strategy for Sc(III) in place, attention was turned toward exploring the generality of the reaction. As mentioned above, Diels-Alder reactions have also been successfully catalyzed with Sc(III).[3] To determine the feasibility of a site-selective Diels-Alder reaction, a pyrene-labeled diene (**Scheme 2.4**) was prepared in four steps from 1-pyrenebutanol. As a control, the diene was treated with N-methylmaleimide and 10 mole% $Sc(OTf)_3$ in dichloromethane. The reaction afforded a 90% yield of the Diels-Alder adduct after 12 h at room temperature. The details of the reaction are presented in the experimental section.

The microelectrode array-based reaction started with the placement of the dienophile onto the array proximal to each of the microelectrodes. This was accomplished by first synthesizing an NHS-activated ester from a maleimide-functionalized hexanoic acid and then placing the resulting molecule onto the surface of an array using the Vitamin B_{12}

Scheme 2.4 Preparation of the diene and test of the Diels-Alder reaction in solution

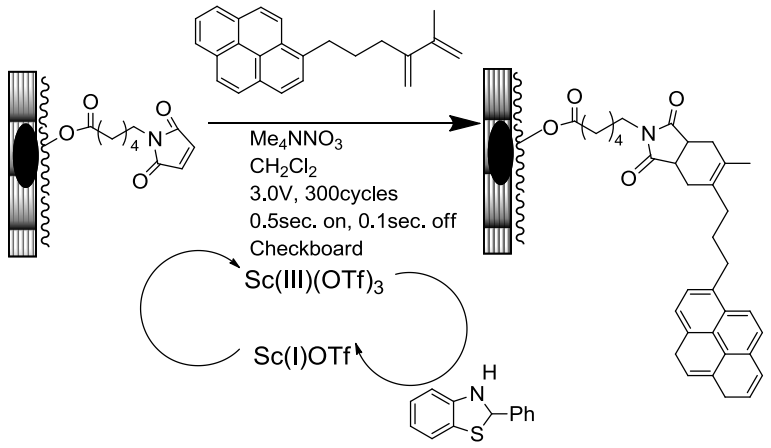


Reagents and Conditions: a) PCC, CH_2Cl_2, RT, 95%. b) Isopropenylmagnesium bromide, THF, 0ºC-RT, 84%. c) PCC, CH_2Cl_2, RT, 90%. d) CH_3PPh_3Br, Sodium bis(trimethylsilyl)amide, THF, 0ºC-RT, 74%. e) N-methylmaleimide, $Sc(OTf)_3$, CH_2Cl_2, RT, 77%.

mediated esterification chemistry developed previously. The dienophile was placed by every electrode in the array. The array was then treated with a premixed solution of $Sc(OTf)_3$ and 2-arylbenzothiozole in the same manner as described above. Into this mixture was added the pyrene-labeled diene and tetramethylammonium nitrate as an electrolyte.(**Scheme 2.5**) The reaction was conducted at selected electrodes in the array by turning the electrodes on at a potential of +3.0 V vs. a remote Pt wire for 0.5 s and then off again for 0.1 second. Three hundred such cycles were used.

Both a 1K- and a 12K-arrays were used. On the 1 K-array, a checkerboard pattern of microelectrodes was selected for the generation of the Sc(III)-catalyst. On the 12K-array, a checkerboard in a box pattern was selected (in a 12K-array the microelectrodes have a diameter of approximately 45 microns). Both arrays were imaged using a fluorescence

Scheme 2.5 Sc(III) catalyzed Diels-Alder reaction on Microelectrode Array



microscope following the reaction and washing of the array. The images are shown in **Figure 2.2**. In both cases, the Diels-Alder reaction was beautifully confined to the selected microelectrodes demonstrating that the conditions developed for the multicomponent reaction are also applicable to the Diels-Alder reaction.

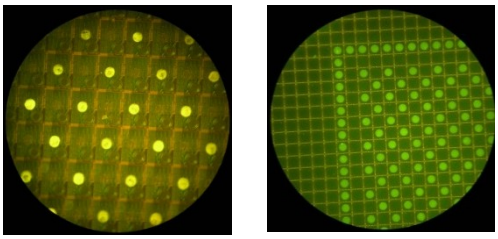
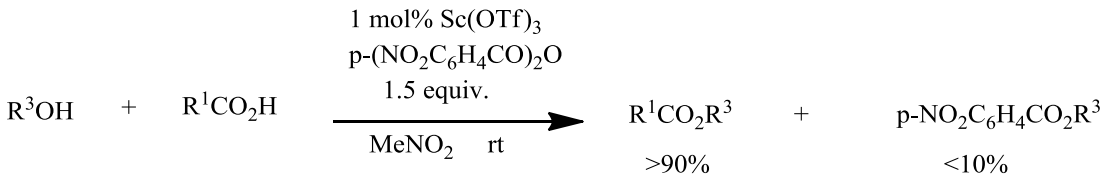


Figure 2.2 The Diels-Alder reactions on Microelectrode Arrays (Left: 1K-array; Right: 12K-array)

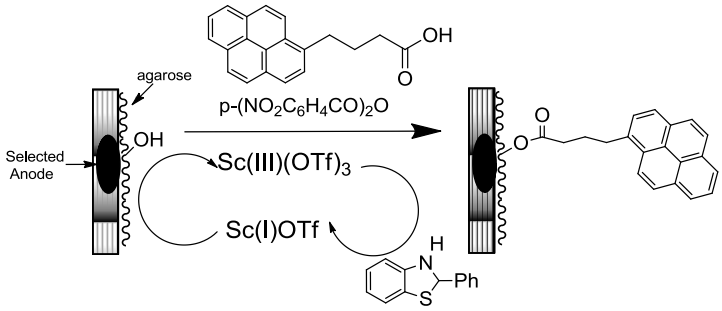
In principle, many other types of Lewis-acid catalyzed reactions can be performed in this manner. For example, consider the esterification reaction illustrated in **Scheme 2.6**, With the above Lewis-acid confining strategy, this acid-catalyzed esterification reaction would provide a complimentary approach to the base-catalyzed methods (see above) used previously for making ester linkages between a molecule and the surface of the array.

Scheme 2.6 A Sc(III)-catalyzed acyl-transfer reaction[4]



Prior to the moving this reaction to a microelectrode array, the same Sc(III)-catalyzed acyl-transfer reaction was tested on a pyrene-labeled carboxylic acid and ethanol in dichloromethane solution. With the presence of both $Sc(OTf)_3$ (10% equivalent) and para-nitrobenzoic anhydride, the desired ester was generated with 50% yield in only 2 hours. The control experiments were conducted by removing either $Sc(OTf)_3$ or para-nitrobenzoic anhydride from the reaction system, no new product was generated given the same length of time. Again, considering one main difference between the reactions on array and in solution is that the active metal species generated on the array is formed in much higher concentrations than typically seen in solution-phase reactions. In fact, on the array an excess of the "catalyst" is generated at any given electrode. As a result, it would take a lot less time for starting materials on the surface to reach "maximum conversion". (The maximum conversion would be assumed when extending reaction time would no longer increase the fluorescence intensity observed.)

Scheme 2.7 A Sc(III)-catalyzed acyl-transfer reaction on the array



Accordingly, a catalytic amount of $Sc(OTf)_3$ was premixed with excess 2-arylbenzothiazole in a dichloromethane solution for 30 minutes, followed by the addition of 1-pyrenebutyric acid and p-nitrobenzoic anhydride. A microelectrode array was spin-coated with agarose, dried, and then submerged in the solution. Sc(III) was produced at selected electrodes in the array by using them as anodes. On the 1K-array, this was accomplished by setting the electrodes to a potential of + 3.0 V relative to the remote Pt-counter electrode for 0.5 s followed by 0.1 s with the electrode off. This cycling was repeated 600 times. For 12K-microelectrode array, 0.1 mL solution was used to cover the whole chip, followed by imposing +2.4V for 60s in a boxed check-board pattern. The success of the reactions is shown in **Figure 2.3**. Clearly, a checkerboard pattern of electrodes was used on the 1K-array and a checkerboard in a box pattern used on the 12K- array. The Sc(III)-catalyzed acylation reaction was well confined in both cases. Two different filters (specifications of those filters are detailed in experimental section) were chosen for 1K-array and 12K-array because varies in the density of pyrene-derivatives on the area could lead to shift in wavelengths of excitation and emission. Our experience tells us a bright red pattern usually indicates a larger amount of pyrene-derivatives loaded on certain area than a blue one. It means that placing more material onto the 12K-array by increasing the length of the experiment would lead to a bright pattern under the same filter used for observing 1K-array. But such optimization does not make sense without a move to a more stable surface and away from our proof-of-principle practice coating (agarose).

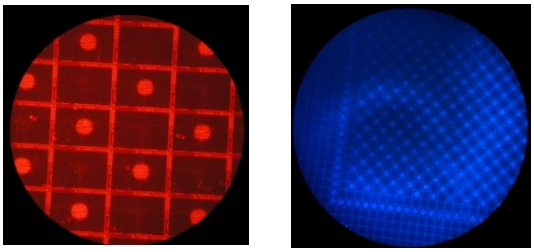


Figure 2.3 The esterification on Microelectrode Arrays (Left: 1K-array; Right: 12K-array)

2.4 A New Challenge

While the site-selective Sc(III)-catalyzed reactions appear to be very successful, they do lead to a very important question. The reaction products were only confirmed in two ways. First, one of the key components of the reaction was labeled with a fluorescent tag so that if the product formed it could be observed. Second, the surface reactions were repeated in solution by replacing the functional groups on surface with small molecules so that the product from the solution-phase reaction could be fully characterized. However, those methods do not address whether the presence of the surface alters the reaction. Does the surface reaction really give the same products as the solution-phase reaction?

For example, in the case of the multicomponent reaction the solution-phase reaction gives rise to a mixture of diastereomers. Is this true of the solid phase reaction, and if so was the ratio of products observed the same? The same question can be asked about the Diels-Alder reaction. Are endo/exo ratios obtained for a reaction on the surface of an array really the same as those obtained in solution, especially when it is well known that the use of a sterically bulky dienophile can influence the Diels-Alder reaction? In the initial experiments, we avoided this issue with the use of a simple substrate. However, if we are going to be building molecular scaffolds for use in molecular libraries, then stereochemistry of the products generated will be a critical issue. While mass spectrometry data (TOF-SIMS) can help verify the overall structure of

the product, stereochemical questions of this nature remained out of reach.

A second problem arises from the use of the fluorescent tag. While we certainly want a fluorescent group for quality control of the array surface, it is problematic to place that fluorescent group into the molecule being constructed. Will the presence of the fluorescent tag either influence the reaction used to assemble the scaffold, or the biological binding properties of the ligands being assembled? Clearly, characterization of product formation by tagging the product is not desirable. A better method for characterization is needed.

Third, neither method addresses the purity of the product obtained. A fluorescent spot does not tell us about percent conversion or yield. For quality control of a library, this information is essential.

In short, more accomplished synthetic methods that lead to the assembly of more complex molecules increases our need to fully characterize the molecules synthesized. The success of the Sc(III)-catalyzed reactions drove the next phase of the project.

2.5 Conclusion

In conclusion, Sc(III)-catalyzed reactions can be conveniently performed site-selectively on microelectrode arrays with the use of 2-arylbenzothiazole as a confining-agent. Three different reactions were shown to be compatible with the method developed. The reactions represent a new, general class of site-selective reactions that involve Lewis-acid catalysis, and their existence dramatically expands our synthetic capabilities on the arrays. For example, the ability to conduct cyclo-addition reactions on the arrays provides us with the technology needed to rapidly assemble functionalized, cyclic structures by any electrode in an array.

2.6 Experimental Procedures

2.6.1 General information of reagents and instruments

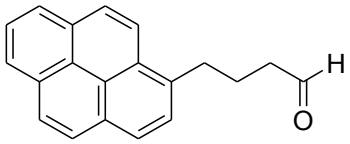
Chemical reagents and solvents were purchased from Sigma-Aldrich, Alfa Aesar, Mallinckrodt Baker, EMD Chemicals (Merck KGaA), and used without further purification unless otherwise noted. Dichloromethane (CH_2Cl_2) was distilled over calcium hydride. Anhydrous tetrahydrofuran (THF) was distilled over sodium benzophenone ketyl. Tetraethylammonium p-toluenesulfonate (Et_4NOTs) was dried in an oven before use. Flash chromatography was carried out by using the silica gel (200-400 mesh) purchased from Dynamic Adsorbent, Inc.

All proton and carbon-13 nuclear magnetic resonance (^1H and ^{13}C NMR) spectra were recorded by a Varian Inova 600, a Varian Inova 500, or a Varian Mercury 300 in a deuterated chloroform ($CDCl_3$) solvent with tetramethylsilane (TMS) as an internal standard unless otherwise noted. Infrared (IR) spectra were recorded by a Perkin Elmer Spectrum BS FT-IR instrument. High resolution electrospray ionization (ESI) mass spectra were obtained at the NIH/NCRR Mass Spectrometry Resource Facility at Washington University in St. Louis.

Fluorescence images were taken with a Nikon Eclipse E200 microscope connected to a Boyce Scientific, M-100 burner and a Nikon D5000 camera. CFW-BP01-Clinical-000 (Semrock) filter cube excitation 380-395 nm/emission 420-470 nm, ET-GFP (FITC/Cy2) (Chroma) filter cube excitation 450-490 nm, emission 500-550 nm and TxRed-A-Basic-000 (Semrock) filter cube excitation 540-580 nm, emission 590-670.

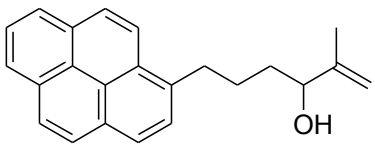
Microelectrode arrays were spin coated with a spin coater spin-coater MODEL WS-400B-6NPP/LITE.

2.6.2 Syntheses and compounds



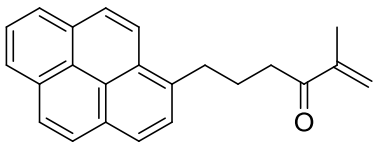
4-(pyren-1-yl)butanal (**2a**)

Pyridinium chlorochoromate 1.292 g (1.5 equiv.) was suspended in 20mL methylene chloride and 1.097 g (4 mmol) 1-pyrenebutanol in 20 mL methylene chloride was rapidly added at room temperature. Black insoluble reduced reagent deposited out. After 2-3 hours' the oxidation, followed by TLC was complete, the black reaction mixture was dilluted with 5 volumes of anhydrous ether. The solvent was decanted, and the black solid was washed twice with ether. Product was isolated simply by filtration of the organic extracts with silica gel, and evaporation of the solvent at reduced pressure. The crude material was then further purified with a silica gel column using an eluant of 10% ethyl acetate in hexane to afford 1.03 g (95%) of desired product as pale yellow needle-like crystal. ^{1}H NMR ($CDCl_3$/300MHz) δ 9.80 (t, J=1.2 Hz, 1H), 8.30 (d, J=9.6 Hz, 1H), 8.15 (m, 7H), 7.84 (d, J=7.8 Hz, 1H), 3.38 (t, J=7.2 Hz, 2H), 2.57 (t, J=8.4 Hz, 2H), 2.20 (m, 2H), ^{13}C NMR ($CDCl_3$/300MHz) δ 202.3, 135.6, 131.6, 131.0, 130.2, 128.9, 127.6, 127.4, 126.9, 126.0, 125.2, 125.1, 125.0, 123.3, 43.5, 32.7, 24.1, IR (KBr) 3040, 2941, 2876, 2821, 2721, 1722, 1603, 1182, 844, 759, 721, 682 cm^{-1}. HRESI MS m/z calculated for $(M+H)^+$ 273.1274, found 273.1276.



2-methyl-6(pyren-1-yl)hex-1-en-3-ol (**2b**)

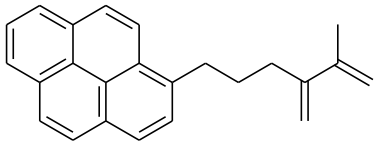
Isopropenylmagnesium bromide (21 mL of 0.5 M 3 equiv.) in tetrahydrofuran was cooled to 0℃ before dropwise addition of a dry tetrahydrofuran solution of 0.960 g (3.5 mmol) **2a**. The icebath was removed and stirring was continued at room temperature for 4 hours. The resulting mixture was cooled to 0 ^{o}C again and quenched with a saturated aqueous solution of ammonium chloride. The aqueous mixture was extracted three times with anhydrous ether (50 mL for each). The collected organic phase was dried over anhydrous sodium sulfate. Sillica gel column was utilized to isolate the crude product with 5% ethyl acetate in hexane. 0.924 g orange red oil was obtained with the yield of 84% after getting rid of solvent at reduced pressure. ^{1}H NMR ($CDCl_3$/300MHz) δ 8.27 (d, J=9.3 Hz, 1H), 8.02 (m, 7H), 7.86 (d, J=7.5 Hz, 1H), 4.94 (s, 1H), 4.84(s, 1H), 4.12 (t, J=6.0 Hz, 1H), 3.36 (t, J=7.5 Hz, 2H), 1.90 (m, 2H), 1.75 (t, J=6.6 Hz, 2H), 1.70 (s, 3H), ^{13}C NMR ($CDCl_3$/300MHz) δ 147.6, 136.9, 131.6, 131.1, 130.0, 128.8, 127.7, 127.4, 127.4, 126.7, 125.9, 125.2, 125.2, 125.0, 124.9, 124.8, 123.6, 111.3, 76.0, 35.0, 33.5, 28.0, 17.66, IR (KBr) 3390, 3039, 2938, 2862, 1416, 1182, 898, 842, 756, 718, 680 cm^{-1} HRESI MS m/z calculated for $(M+Na)^+$ 337.1568, found 337.1567, calculated for $(M+K)^+$ 353.1308, found 353.1306.



2-methyl-6-(pyren-1-yl)hex-1-en-3-one (**2c**)

Pyridinium chlorochoromate 0.808 g (1.5 equiv.) was suspended in 10mL methylene chloride and 0.781g (2.5 mmol) **2b** in 20 mL methylene chloride was rapidly added at room temperature. Black insoluble reduced reagent deposited out. After 1-2 hours' the oxidation, followed by TLC

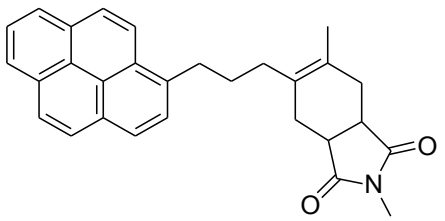
was complete, the black reaction mixture was dilluted with 5 volumes of anhydrous ether. The solvent was decanted, and the black solid was washed twice with ether. Product was isolated simply by filtration of the organic extracts with silica gel, and evaporation of the solvent at reduced pressure. The crude material was then further purified with a silica gel column using an eluant of 2.5% ethyl acetate in hexane to afford 0.702 g (90%) of desired product as white solid. ^{1}H NMR ($CDCl_3$/300MHz) δ8.33 (d, J=9.3 Hz, 1H), 8.12 (m, 7H), 7.86 (d, J=7.5 Hz, 1H), 5.90 (s, 1H), 5.73 (s, 1H), 3.38 (t, J=7.5 Hz, 2H), 2.83 (t, J=6.0 Hz, 2H), 2.20 (m, 2H), 1.89 (s, 3H), ^{13}C NMR ($CDCl_3$/300MHz) δ 202.1, 144.8, 136.5, 131.2, 130.2, 129.1, 127.8, 127.6, 127.6, 127.0, 126.1, 125.3, 125.2, 125.1, 125.0, 124.8, 123.8, 37.1, 33.1, 26.5, 18.0, IR (KBr) 3055, 2950, 2921, 1681, 1601, 1508, 1427, 1310, 1090, 917, 847, 756, 678 cm^{-1} HRESI MS m/z calculated for $(M+H)^+$ 313.1592, found 313.1591, calculated for $(M+Na)^+$ 335.1412, found 335.1411.



1-(5-methyl-4-methylenehex-5-enyl)pyrene (**2d**)

Into the tetrahydrofuran solution of 2.14 g methyltriphenylphosphonium bromide (3 equiv.), 6 mL of 1.0 M Sodium bis(trimethylsilyl)amide (3 equiv.) in tetrahydrofuran was added dropwise with the argon. The mixture solution was stirred for 30 minutes under the room temperature. The white sand-like solid suspended solution turned into orange clear one. 0.624 g (2.0 mmol) **2c** in 20 mL tetrahydrofuran was added into the system slowly with the argon. The stirring was continued for another 4 hours. 50 mL saturated ammonium chloride solution was

used to quench the reaction, followed by extracting the organic staffs with methylene chloride three times (30 mL for each time). The solution was dried with magnesium sulfate. Sillica gel column was utilized to isolate the crude product with hexane. 0.459 g pale orange oil was obtained with the yield of 74% after getting rid of solvent at reduced pressure. ^{1}H NMR ($CDCl_3$/300MHz) δ 8.27 (d, J=9.3Hz, 1H), 8.10 (m, 7H), 7.87 (d, J=7.8 Hz, 1H), 5.15 (s, 1H), 5.07 (s, 1H), 5.03 (s, 1H), 4.97 (s, 1H), 3.36 (t, J=7.8 Hz, 2H), 2.48 (t, J=7.8 Hz, 2H), 2.05 (m, 2H), 1.93 (s, 3H), ^{13}C NMR ($CDCl_3$/300MHz) δ 147.9, 142.9, 137.1, 131.7, 131.2, 130.0, 128.9, 127.8, 127.5, 127.4, 126.8, 126.0, 125.4, 125.3, 125.0, 124.9, 123.7, 112.9, 112.6, 34.0, 33.6, 31.1, 21.4, IR (KBr) 3039, 2937, 2937, 1596, 1488, 1457, 1416, 1181, 890, 841, 755, 707, 680 cm^{-1} HRESI MS m/z calculated for $(M+H)^+$ 311.1794, found 311.1795.

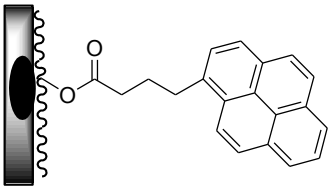


2,5-dimethyl-6-(3-(pyren-1-yl)propyl-3a,4,7,7a-tetrahydro-2H-isoindole-1,3-dione (**2e**)

Scandium triflate 0.492 g (0.1 equiv.) powder was added into 40mL anhydrous methylene chloride solution containing 0.310 g **2d**. Dissolve 0.333 g N-methylmaleimide (3 equiv.) into 10 mL anhydrous methylene chloride, which was transferred into diene solution with syringe drop by drop at the room temperature. The mixture solution was stirred for 12 hours. After being quenched by 50 mL brine, the aqueous layer was washed with 20 mL methylene chloride for three times, followed by being dried over anhydrous magnesium sulfate. The crude mixture was isolated by 20% ethyl acetate in hexane to afford 0.32 g target product with yield of 77%. ^{1}H

NMR ($CDCl_3$/300MHz) δ 8.12 (m, 8H), 7.83 (d, J=7.5 Hz, 1H), 3.21 (t, J=7.8 Hz, 2H), 2.95 (m, 2H), 2.88 (s, 3H), 2.46 (t, J=14.1 Hz, 2H), 2.18 (m, 4H), 1.83 (m, 2H), 1.67 (s, 3H) δ 180.5, 180.4, 136.7, 131.7, 131.1, 131.1, 130.0, 128.8, 128.2, 127.8, 127.5, 127.2, 126.8, 126.0, 125.3, 125.2, 125.1, 124.9, 123.5, 40.2, 40.0, 33.3, 33.2, 30.8, 29.9, 29.2, 25.1, 19.52, IR (KBr) 3041, 2940, 2857, 1698, 1433, 1382, 1283, 1182, 1150, 1020, 844, 722 cm^{-1} HRESI MS m/z calculated for $(M+H)^+$ 421.2036, found 421.2042

2.6.3 Chemistry on Microelectrode Arrays

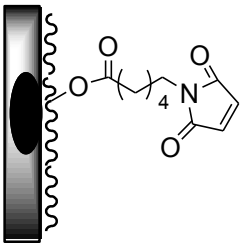


Example site-selective Sc(III) catalyzed esterification reaction on agarose coated 1K- and 12K- microelectrode arrays

A microelectrode array containing 1024 micro electrodes was spin coated at 2000rpm for 45 seconds with 4.5% agarose in the solvent of 90% N,N-Dimethylformamide and 10% water. After drying the polymer for 1 hour, the microelectrode array placed with a circuit was inserted into the methylene chloride solution containing the mixture of 0.0144 g (0.005 mmol) 1-pyrenebuyric acid, 0.0049 g $Sc(OTf)_3$ (0.2 equiv.), 0.0043 g 2-arylbenzothiolzole (0.4 equiv.), and 0.0047 g p-nitrobenzoic anhydride (3 equiv.), which had been premixed sealed for 30 minutes. A platinum wire was also inserted into the solution as counter electrode. A checkboard pattern was programmed with 600 potential cycles, +3.0V for 0.5 s and 0 V for 0.1 s for each. After being washed with de-ionized water and 95% ethanol, the picture was obtained with a fluorescence

microscope.

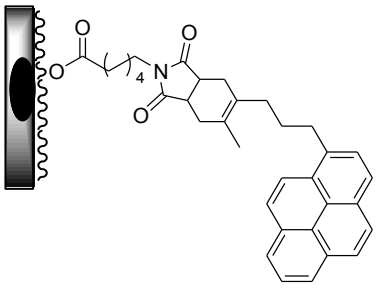
Onto 12K- chips, 0.1 mL of the above solution was added. The voltage was set to +2.4 V for 180 s in a small checkboard pattern.



Example coupling of the succinimide to agarose polymer on 1K-microelectrode arrays

A microelectrode array containing 1024 microelectrodes was spin-coated with 4% agarose at 3000 rpm for 45 seconds and allowed to dry for 1 hour. Then 7.8 mg of N-succinmidyl-6-maleimidocaproate was dissolved in DMF 100 μL. This was combined in a 1.5 mL Eppendorf tube with 3 mg of vitamin B_{12}, and 6 mg of tetramethylammmonium nitrate and 1.4 mL of methanol. The microelectrode array was immersed in this solution and a reduction was performed on every electrode on the microelectrode array (a wholeboard pattern). The potential was set to -2.4 V for 0.5 s and 0 V for 0.1 s, 300 cycles. The microelectrode array was removed from solution and washed by de-ionized water, and ethanol. The array was immediately carried on to the following oxidation reaction.

On 12K-chips, 0.1mL of the above solution was added to cover all electrodes. A small check-board pattern was imposed with -1.5 V for 60 s.



Example site-selective Sc(III) catalyzed Diels-Alder reaction on an agarose coated 1K-microelectrode array

A solution containing 0.0025 g $Sc(OTf)_3$ (0.1 equiv.), 0.0022 g 2-arylbenzothiolzole (0.2 equiv.) in 2.0 mL methylene chloride was premixed sealed for 30 minutes to let Sc(III) be reduced completely. 0.0016 g 4-(5-methyl-4-methylenehex-5-enyl)pyrene was added into the solution. The microelectrode array from the previous coupling reaction was immersed in this solution, being activated as anodes in a checkboard manner. The electrical potential cycle was set as: +3.0 V for 0.5 s and 0 V for 0.1 s. The cycle number was 300. The microelectrode array was washed following the previous procedures and visualized by the fluorescence microscopy.

For 12K-microelectrode array, 0.1 mL solution was used to cover the whole chip, followed by imposing +2.4 V for 60 s in a small check-board pattern.

2.7 References

1. Jimenez, O.; de la Rosa, G.; Lavilla, R., "Straightforward access to a structurally diverse set of oxacyclic scaffolds through a four-component reaction". *Angewandte Chemie-International Edition* **2005,** *44* (40), 6521-6525.

2. Itoh, T.; Nagata, K.; Ishikawa, H.; Ohsawa, A., "Synthesis of 2-arylbenzothiazoles and imidazoles using scandium triflate as a catalyst for both a ring closing and an oxidation steps". *Heterocycles* **2004,** *63* (12), 2769-2783.

3. Kobayashi, S.; Hachiya, I.; Araki, M.; Ishitani, H., "Scandium Trifluoromethanesulfonate (Sc(Otf)3) - a Novel Reusable Catalyst in the Diels-Alder Reaction". *Tetrahedron Letters* **1993,** *34* (23), 3755-3758.

4. Ishihara, K.; Kubota, M.; Kurihara, H.; Yamamoto, H., "Scandium trifluoromethanesulfonate as an extremely active Lewis acid catalyst in acylation of alcohols with acid anhydrides and mixed anhydrides". *Journal of Organic Chemistry* **1996,** *61* (14), 4560-4567.

Chapter Three

Chemically Cleavable Linkers on Microelectrode Arrays

3.1 Quality Control of an Addressable Molecular Library

The mission of turning microelectrode arrays into platforms that support addressable libraries requires a number of key steps. For example, site-selective reactions using a variety of mediators are needed in order to place or build molecules by individual microelectrodes in the array,[1-7] a stable porous reaction layer for attaching molecules to the surface of the electrodes in an array is needed,[8] and experiments that show the capability of the arrays for monitoring small molecule – receptor binding are needed (and will be discussed in details in the following chapter). In addition, there is the challenging task of quality control that is central to the use of any small-molecule library. Simply put, how does one know that the molecule built or placed on a microelectrode array is really the molecule one thinks it is?

When our effort was started, the group had shown that the use of a mass-spectrometry cleavable linker allows TOF-SIMS experiments to be used for this purpose.[9-11] Yet while this work was effective, it is also severely limited. First, the use of TOF-SIMS "sacrifices" the array, and second the use of mass spectrometry in general does not provide us with a handle to examine the purity or stereochemistry of molecules made on the arrays. Since we hope to use the arrays to probe the three-dimensional binding preferences of biological receptors, the inability to determine the stereochemistry of molecules on the arrays is particularly bothersome. Hence, an alternative approach that allows us to both fully characterize molecules on the array and retain the array for further biological studies is of utmost importance.

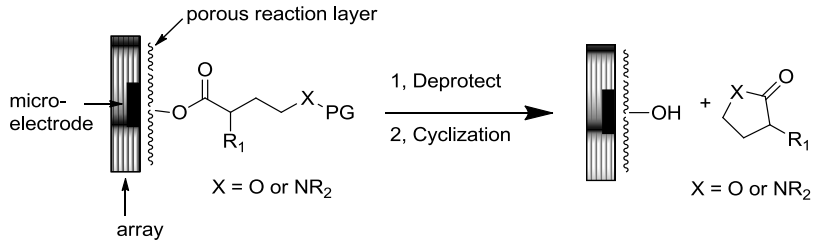
One approach to this problem would be to develop a strategy that allows us to site-selectively recover molecules located on the surface of any microelectrode in the array. To accomplish this requires a linker between a molecule and the surface of the electrode that can be cleaved using the microelectrode itself. Such a linker would require two main features: 1) it needs to be stable to the chemistry used to build molecules libraries, but readily cleavable under mild conditions when needed, and 2) since each microelectrode in the array has only 20-50 fmole of material on the polymer coating its surface, it is best if the linker contains a label to aid in detection of the product by instruments like HPLC. The same label can be used to quantify the relative amounts of material at the various microelectrodes in the array. Molecules obtained from the array using a linker that can be characterized by independent synthesis. Because reactions on microelectrode arrays can be run at larger scales either in solution, or on solid supports, using identical conditions, the independently synthesized molecules needed for the characterization can be prepared in a straightforward fashion.

3.2 "Safety-Catch" Linkers and Selective Cleavage Strategies

Our strategy capitalized on the use Kenner's "safety-catch" linker method used for biomolecules on solid surfaces.[12] In this approach, latent functionality (an alcohol or an amine) in a linker is masked while a desired target molecule is built on a solid support. The linker is tethered to the solid support with the use of an ester linkage. When the synthesis is complete, the functionality in the linker is unmasked using chemistry that is orthogonal to the methods used to synthesize the molecule. The newly released functional group reacts with the ester that attaches the linker to the solid support to form either a lactone or lactam, a transformation that cleaves the linker and the target molecule from the solid support. The strategy is illustrated for a potential array application in **Scheme 3.1**. In this example, the cleavage reaction was designed to form a

five-membered ring, a scenario that is consistent with rapid ring formation and hence rapid cleavage from the polymer coating the array.

Scheme 3.1 "Safety-Catch" strategy

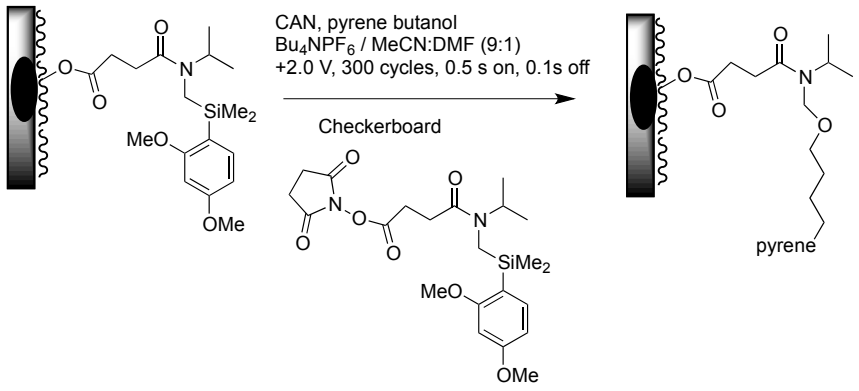


For this scenario to work, the linker needs to be functionalized with a substituent (R_1) on the α-carbon of the ester. The substituent will provide a handle for attaching fluorescence active groups, the small molecules that will serve as members of a targeted molecular library, or both. The use of a protected amine in this position would be particularly attractive because it would provide a second site (R_2) for locating a biological substrate or fluorescence label.

With a strategy in place, the search for protecting groups that can be removed with the site-selective array chemistry available began. Our first plan was to take advantage of the chemistry developed by Dr. Kesselring during his time in the group.[6] Dr. Kesselring showed that ceric ammonium nitrate (CAN) can be manipulated on an array by changing the oxidation states of the cerium ion between Ce(IV) and Ce(III). An example of this chemistry is shown in **Scheme 3.2**. In this chemistry, a site-selectively generated Ce(IV)-oxidant was used to trigger the formation of an N-acyliminium ion intermediate from a silyl-substituted amide on an array. The reactive intermediate was then trapped with a pyrene-labeled alcohol. The oxidant was confined to the

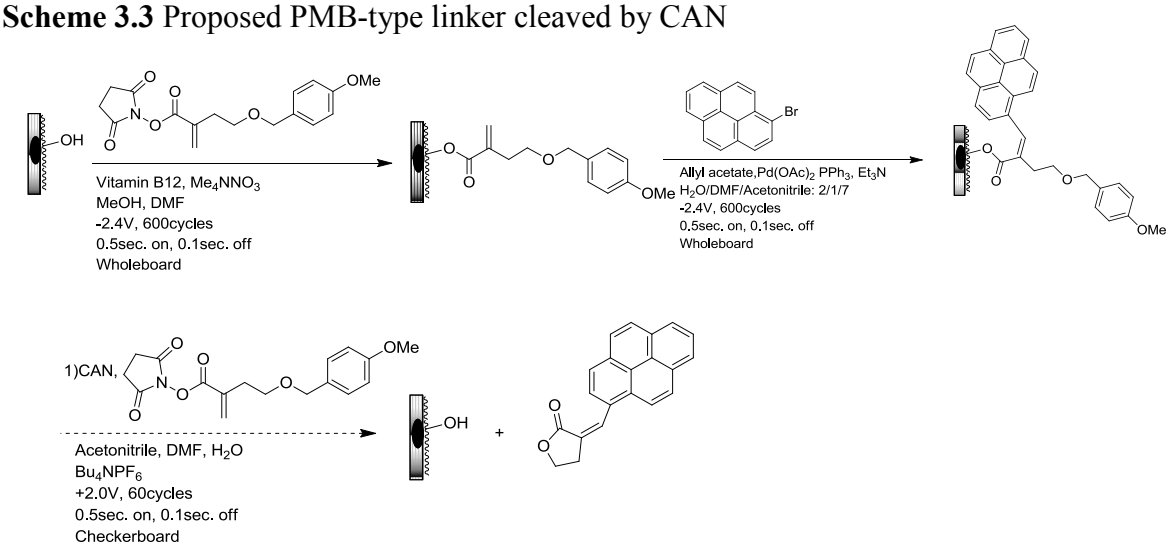
electrodes selected for the oxidation by simply placing some of the same substrate use on the array into solution to serve as a confining agent.

Scheme 3.2 Site-selective chemistry initiated by CAN on Microelectrode Array



In an attempt to capitalize on this chemistry, an alcohol group in a safety-catch linker was protected as a para-methoxylbenzyl ether moiety. A trial experiment to test the viability of this approach is shown in **Scheme 3.3**. In this case, oxidative cleavage of the protecting group would release the linker from the array. A pyrene group was placed on the linker so that the cleavage reaction could be monitored by fluorescence. This pyrene group was installed with the use of a Heck reaction[3] that took advantage of an olefin at the position alpha to the ester.

Scheme 3.3 Proposed PMB-type linker cleaved by CAN



Unfortunately, this first approach failed. The first two steps were successful, and led to a "full-board" pattern of fluorescence on the array when each electrode in the array was used. Alternatively, a chosen pattern of fluorescence could be obtained by selecting electrodes in the array. However, the cleavage reaction did not selectively remove molecules from the array. Instead, all of the fluorescent spots on the array faded. Fluorescence was lost even with very short reaction times. There clearly was a competing, uncontrolled reaction operating on the array that reduced fluorescence everywhere on the array. There were two possibilities for the observation. The first is that the Ce(III) is a good enough Lewis-acid to catalyze oxonium ion formation from the agarose polymer used to coat the array. A similar problem has been observed with both Pd(II) and Cu(II) reagents on the arrays.[11,13] A second possibility was that the pyrene itself was sensitive to oxidation – even with a Ce(III) reagent. If that were the case, then the pyrene would get oxidized everywhere without any need for the electrolysis required to generate Ce(IV). To investigate this suggestion, cyclic voltammetry was used to assess the relative oxidation potentials of the 4-methoxybenzyl group and the pyrene. The results are shown below in **Figure 3.1**.

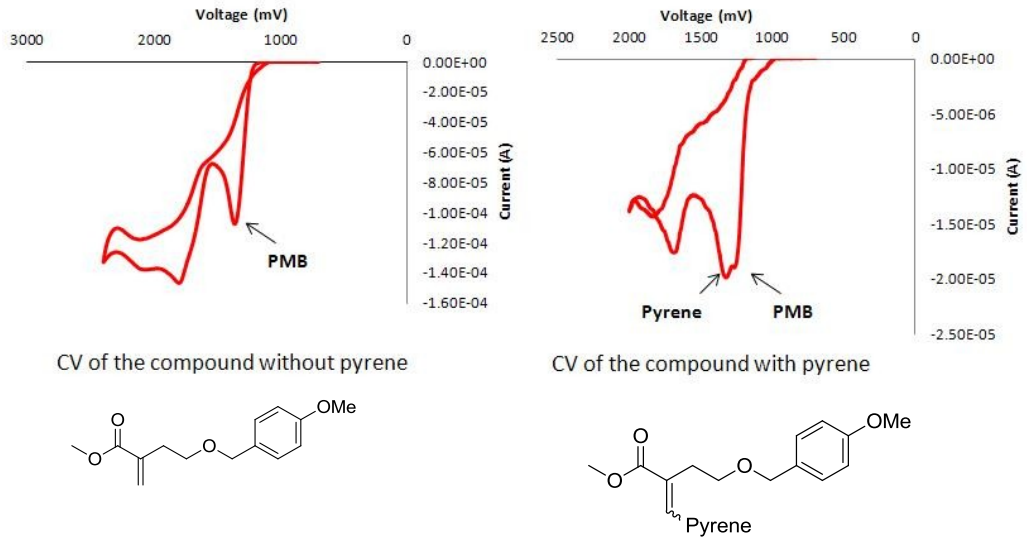


Figure 3.1 Cyclic Voltammetry of the PMB-linker structures

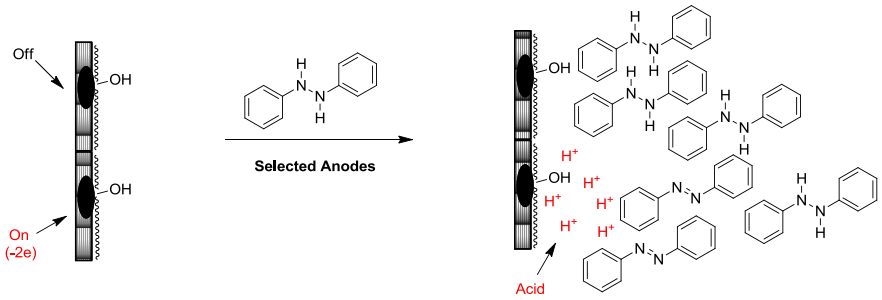
The linkers with and without the pyrene attached were compared. Both CV's were run using a Pt-disk working electrode, a Pt-wire auxiliary electrode, Ag/AgCl as reference electrode, 0.1 M $LiClO_4$ in CH_3CN as the medium, and a sweep rate of 100 mV/s. The data showed that the peaks for the two groups overlapped. Hence, the oxidation would struggle to differentiate the groups. While this data did not explain the non-selectivity of what was happening on the array, it did not bode well for the synthetic step being proposed. While the potential difference is large enough that one might be able to selectively oxidize the 4-methoxybenzyl ether with a very careful electrolysis, the chance of accomplishing the task with a Ce(IV) reagent was remote. This data exposed a "fatal" flaw with the strategy being taken. While an issue with Lewis-acid stability of the polymer can be readily fixed with the use of a more stable surface[8], a lack of selectivity in the oxidation cannot be easily addressed.

To address the above problem, one can consider two general possibilities. One is to change the fluorescent group being used. However, fluorescence tags typically are highly conjugated systems. Hence, the same problem with selectivity in the oxidation reaction might arise again.

The second option is to seek for an alternative protecting-group strategy that does not rely on an oxidation. A number of possibilities were available. Base, acid, Pd(0), and Cu(I) catalysts had all been used site-selectively on the arrays. Of these, base is typically used to make the ester that attaches the linker to the array, and Pd(0) and Cu(I) catalysts are commonly used in the construction of the small molecule libraries being assembled. That suggested the use of an acid-cleavable protecting group on the linker.

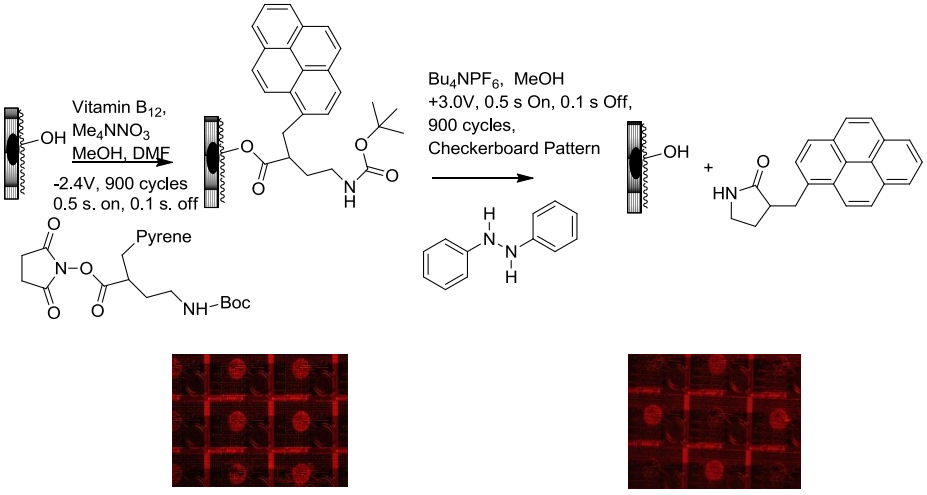
To this end, our collaborators at Combimatrix Company had found that 1,2-diphenylhydrazine is a great precursor for generating acid site selectively at electrodes in an array. This is done by oxidizing the hydrazine at the electrode in order to generate azobenzene and two equivalents of acid (**Scheme 3.4**).[5] Confinement of the acid to the microelectrodes selected for the reaction was accomplished by using excess 1,2-diphenylhydrazine in the reaction. The excess hydrazine served as a base to neutralize any acid escaping from the region of the array surrounding the selected microelectrodes. They took advantage of this reagent and realized a short peptide synthesis by selectively removing a t-Boc from the N-terminus of the chain.

Scheme 3.4 Protons generated site-selectively on Microelectrode Array



By directly incorporating this scheme into our linker strategy, we set out to determine if the cleavage reaction could be accomplished in a site-selective fashion. A Boc-protected linker **(Scheme 3.5**) was selected as an initial substrate. Pyrene was used as the fluorescent group for monitoring the reactions on the array, and an amine chosen as the masked nucleophile for release on the array. The experiment was begun by placing the activated-ester proximal to every microelectrode in an agarose-coated 1K-array.

Scheme 3.5 A Boc-protected chemistry cleavable linker on Microelectrode Array

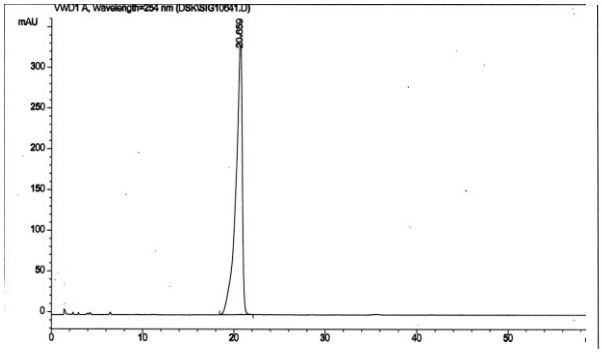


(1024 microelectrodes/cm^2). This was accomplished using the previously developed electrogenerated-base procedure.[4] The cleavage reaction was then performed by site-selectively generating acid on the array.[14] To this end, the array was submerged along with a remote Pt-wire into 1.5 mL of a methanol solution containing both 1,2-diphenylhydrazine and tetrabutylhexafluorophosphate electrolyte. A checkerboard-pattern of microelectrodes was then used as anodes to oxidize the diphenylhydrazine to form the desired acid. The oxidation was run by applying a potential of + 3.0 V to the selected microelectrodes relative to a remote Pt-wire in solution. The selected microelectrodes were turned on for a period of 0.5 s and off for a period of 0.1 s for a total of 900 cycles. The success of the reaction was determined by a loss of

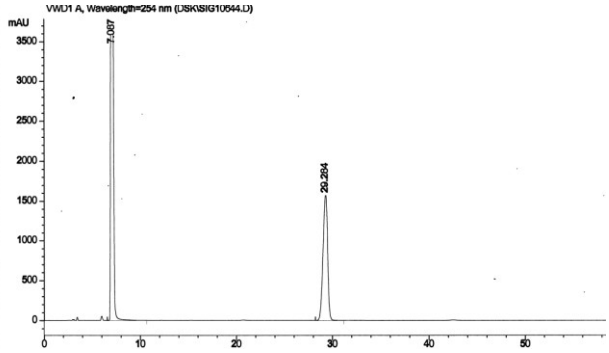
fluorescence by the selected microelectrodes. As can be seen in the image, a small amount of fluorescence did remain by the selected electrodes, but most of the substrate on the surface of the electrode was removed. No reaction was evident at the non-selected electrodes.

Of course, it is no longer adequate to simply say the reaction is working based upon a fluorescence-microscope image. The reason for the cleavable linker is so that we can obtain structural information. To prove that the molecule being released from the array was the expected lactam derivative, the compound was independently synthesized by mimicking the chemistry on the array in solution. This was done by treating the methyl ester derivative of the linker with trifluoroacetic acid to remove the *t*-Boc group and then triethylamine to trigger the cyclization. HPLC analysis was then used to compare the independently prepared standard with the material being removed from the array

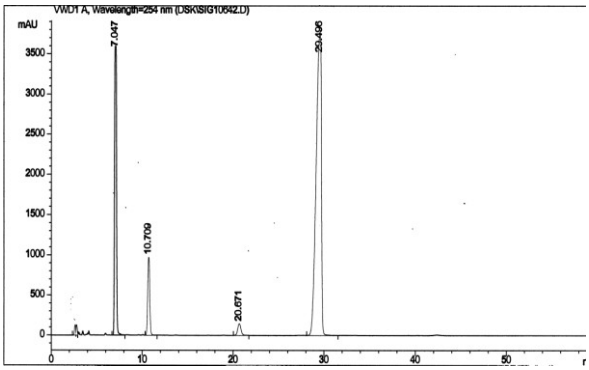
a)



b)



c)



d)

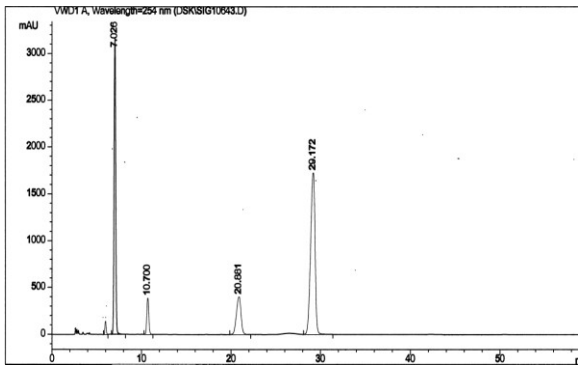


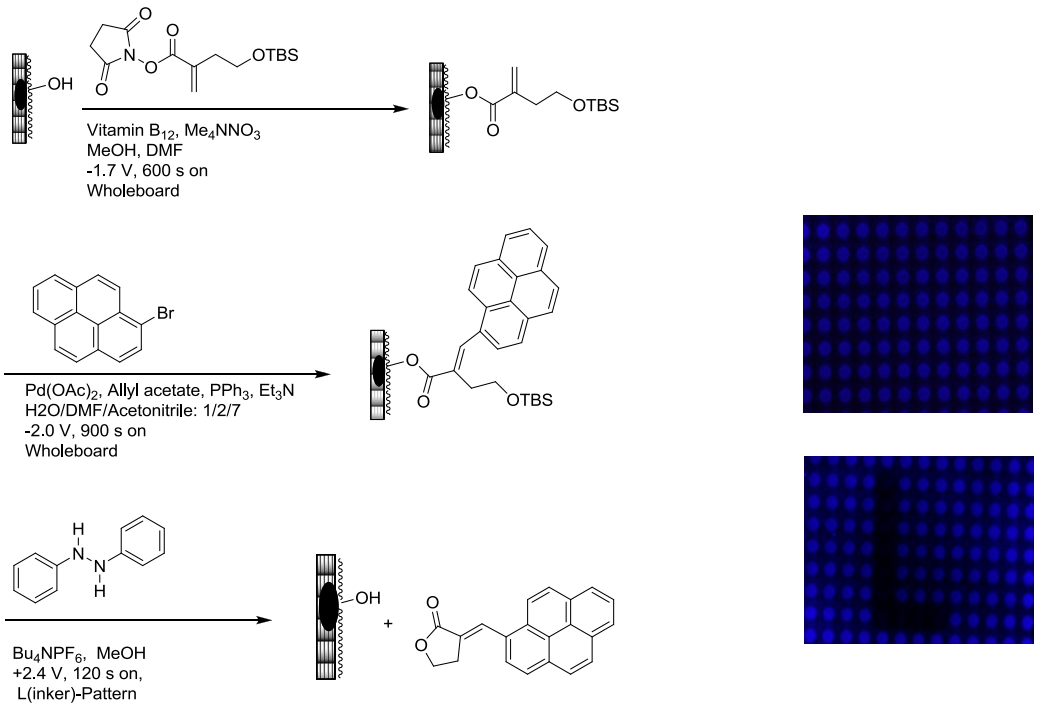
Figure 3.2 HPLC test results. The chromatograms were obtained using a Discovery® HS C18, 5µm; 25cm X 4.6mm column with a flow rate of 1mL/min. 70% methanol; 30% water was used as eluant with a 10.0 uL injection. A UV detector was used at 254nm. a) Independently synthesized lactam; b) The reaction solution prior to the electrochemical reactions; c) The reaction solution following the electrochemical reactions; d) Co-injection of c) and a).

(**Figure 3.2**). Four HPLC chromatograms are shown. **Figure 3.2b** shows the HPLC trace obtained for the reaction solution prior to the electrochemical cleavage reaction. The two peaks observed arise from the electrolyte used for the reaction (retention time = 7.05 min) and the excess 1,2-diphenylhydrazine (retention time = 29.5 min) used as the confining agent for the electrolysis. Clearly, the peak with a retention time of 20.6 min in **Figure 3.2c** is the lactam indicating that the electrochemical cleavage reaction on the array did give rise to the expected lactam. The peak at 10.7 min in **Figure 3.2c** is thought to be a side-product derived from hydrolysis of the agarose surface coating the array.

With the success of the nitrogen-nucleophile-based system, attention was turned back toward examining the use of the oxygen-nucleophile-based system and establishing the generality of the method, both in terms of the nucleophile and the type of microelectrode array used. As in the failed case before, I wanted to put the linker onto the array and then demonstrate that the linker was compatible with conducting reactions by the electrodes prior to releasing the molecule from the surface. The plan started by placing 4-*t*-butyldimethylsiloxy-2-methylidene butanoic acid by each of the microelectrodes in an agarose-coated 12-K (12,544 microelectrodes/ cm^2) array (**Scheme 3.6**). This was accomplished by again capitalizing on a base-catalyzed coupling reaction between an activated ester and the agarose surface coating the array. As in the earlier experiments, the potential was held constant relative to the counter electrode. Each enoate on the array was then used as a substrate for a Heck reaction with bromopyrene. This placed a

fluorescent group by each of the microelectrodes in the array. The strategy was intriguing because the same strategy could be used for adding a variety different fluorescent groups and/ or biological probes to the linker. Using this chemistry, the nature of the linker on the array can be varied without building each linker independently and then transferring them one at a time to the array. Instead, they can be built directly on the array.

Scheme 3.6 A lactone-based system



Cleavage of the linker from the array was accomplished using reaction conditions that were identical to those employed in the lactam case. In this case, an L-pattern (L for Linker) of electrodes was used to cleave the linker from the surface. The success of the reaction was monitored using a fluorescence microscope and can clearly be seen in the image provided. On the 12K-array, it does appear that the reaction did remove some of the groups on neighboring electrodes to the L-pattern. This problem can be addressed in the future by either further

optimization of the confinement conditions, or by spacing the members of a library on the array sufficiently far apart. For now, the latter strategy is considered fine since the libraries to be placed or made on the array will be smaller. Hence, there are a lot of extra electrodes.

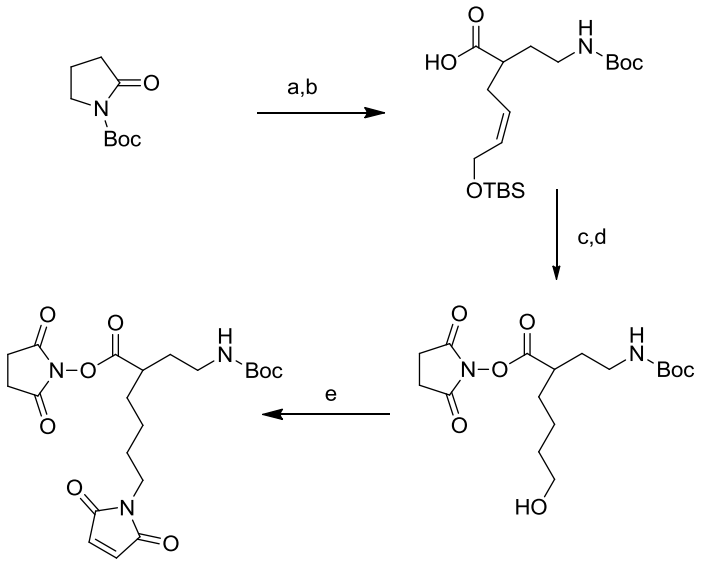
To conclude this part of work, a "safety-catch" linker strategy has been used to site-selectively cleave molecules from pre-selected, individual microelectrodes in a microelectrode array. Both amine and alcohol nucleophile strategies work well. The chemistry is compatible with arrays having either 1024 or 12,544 microelectrodes/ cm^2. The use of the "safety-catch" linkers will allow for characterization of the molecules built by the electrodes in the array, and in this way opens door for doing quality-control analysis of the molecules in addressable molecular libraries.

3.3 Characterization of Small Molecules Using "Safety-Catch" Linkers

The success of "safety-catch" with the simple model system suggested that the technique would prove useful for characterizing reaction products generated on an array. That suggestion refocused our attention on the array-based Diels-Alder reactions described in Chapter 2. The reactions are run by generating Sc(III) at selected electrodes in an array.[7] The Sc(III) serves as a Lewis-acid catalyst for the cycloaddition reaction. It is confined to the selected electrodes with the use of a 2-arylbenzothiozole in solution. The reaction led to beautiful fluorescence patterns on the arrays. But did the pretty spots really represent the product suggested, and if so what was the stereochemical outcome of the reaction? Typically, Lewis-acid catalyzed Diels-Alder reactions predominately lead to endo-product, but does the surface of the array alter this result? Since we hope to build libraries on the arrays and not just transfer molecules to the arrays following their synthesis, answers to questions of this nature are critical.

In order to answer questions about the Diels-Alder reaction, the linker needed to be modified with either a diene or a dienophile. The best way to make this change was to develop a synthetic approach to the linkers that would allow for the rapid incorporation of a variety of reactive groups. To this end, the chemistry outlined in **Scheme 3.7** was pursued. Based on the model systems highlighted in **Scheme 3.5**, a protected amine was selected as the masked nucleophile for the linker. In the earlier model systems, the use of a masked amine led to a cleaner cleavage reaction. The synthesis started with an alkylation of the t-Boc protected lactam. The alkylation proceeded best when a more reactive allylic bromide electrophile was used. The alkylated lactam was hydrolyzed, the acid converted into an activated N-hydroxy succinimide ester, and the olefin hydrogenated. The TBS protecting group was removed by methanolysis during the hydrogenation step. The dienophile needed for the Diels-Alder reaction was then added using a Mitsunobu reaction.

Scheme 3.7 Preparation of the dienophile

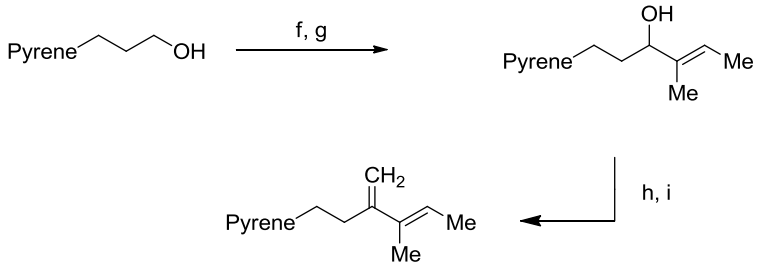


Reagents and Conditions: a) LDA, $BrCH_2CH=CHCH_2OTBS$, -78 ^oC, 84%. B) LiOH,

THF/H_2O (5:1), RT, 89%. c. N-hydroxysuccinimide, DCC, DMF, RT, 81%. d) H_2/ Pd-C, MeOH, RT, 76%. e) DEAD, PPh_3, neopentyl alcohol, maleimide, RT, 79%.

The diene needed for the array-based Diels-Alder reactions was prepared as outlined in **Scheme 3.8**. The diene was designed so that it contained the substituent needed to generate exo- and endo-products in the Diels-Alder reaction, the substituent to help populate the substrate conformation needed for the cycloaddition, and a fluorescent group for monitoring the progress of the reaction on an array.

Scheme 3.8 Preparation of the diene



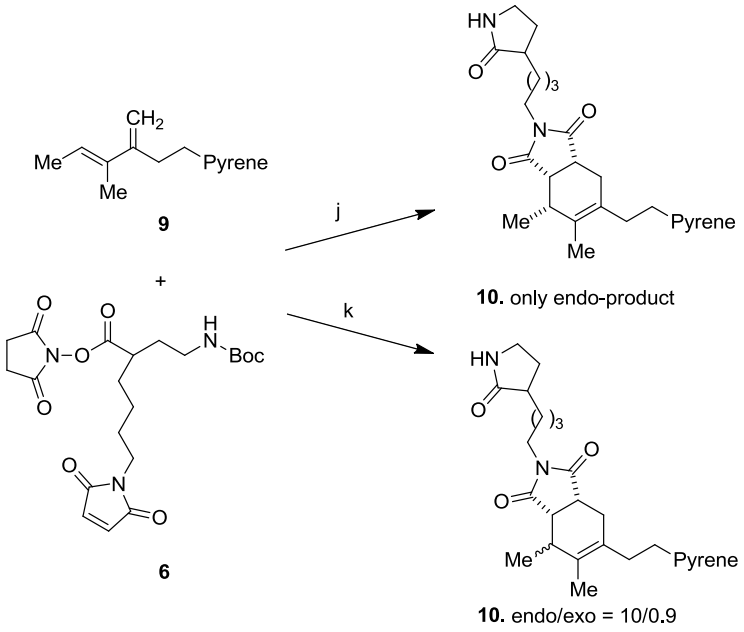
Reagents and Conditions: f) PCC, CH_2Cl_2, RT. g) (Z)-but-2-en-2-yllithium, THF, -78 ^oC. h) Dess-Martin Periodiane, CH_2Cl_2, RT. i) $Ph_3P{=}CH_2$, THF, RT, 46% from the alcohol.

The substrates for the Diels-Alder were used to conduct two solution-phase reactions (**Scheme 3.9**). The first utilized Sc(III) to catalyze the reaction. To this end, the activated ester containing the dienophile was treated with diene and 30 mole% of scandium(III) triflate in dichloromethane at room temperature. The mixture was stirred overnight. After washing the reaction with water, the reaction was dried over sodium sulfate, concentrated, and then treated

with a 7:3 dichloromethane and trifluoroacetic acid solution. Following this deprotection step, the reaction product was treated with triethylamine in order to deprotonate the ammonium salt and trigger lactam formation.

The reaction led to products that were isomeric with respect to the stereogenic atom on the lactam ring. Both products had endo-stereochemistry with respect to the Diels-Alder reaction. This stereochemistry was established using a NOESY experiment (**Figure 3.3**). By LC-MS, a single large peak was observed having the correct mass of the product.

Scheme 3.9 Diels-Alder reactions with different conditions in solution



Reagents and Conditions: j) $Sc(OTf)_3$, CH_2Cl_2, RT, TFA, CH_2Cl_2, Et_3N, RT, 71%. k) toluene, Reflux, TFA, CH_2Cl_2, Et_3N, RT, 68%.

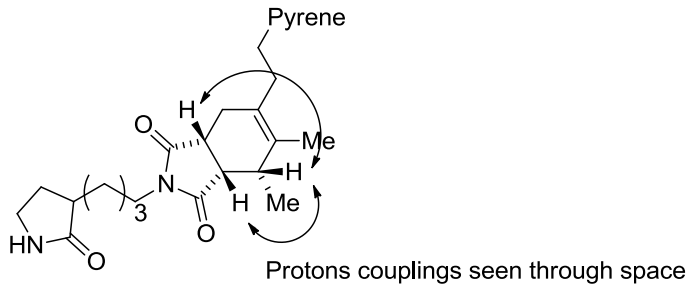


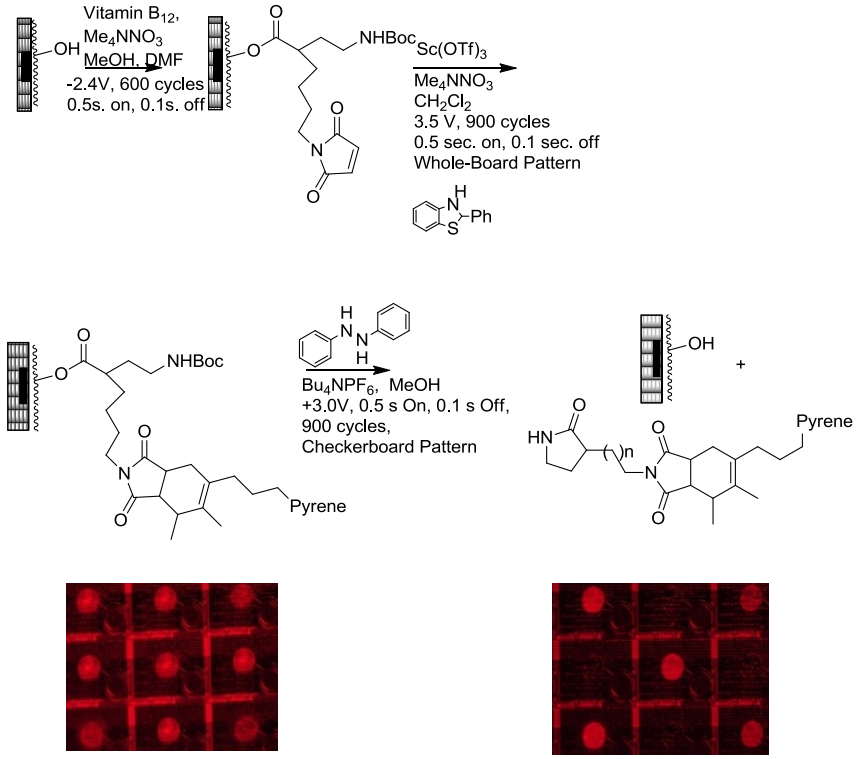
Figure 3.3 NOESY cross-peaks for assigning the endo-product

In the second solution-phase experiment, the Diels-Alder reaction was conducted thermally in the absence of the Lewis-acid. The rest of the conditions remained the same. This experiment was performed in order to generate a mixture of endo- and exo-stereoisomers and identify the exo-product. By LC-MS, two peaks having the correct mass for the product were observed in a ratio of 0.09:1 **(Figure 3.3)**. Co-injection with the product from the $Sc(OTf)_3$ catalyzed reaction showed that the major isomer formed was the endo-product.

With the independently synthesized products in place, attention was turned to the microelectrode-array reaction (**Scheme 3.10**). The process was started by using a solution of **6** in methanol/ DMF solvent to place the dienophile by each microelectrode in an array having 1024 microelectrodes·cm^{-2}. The reaction between the activated ester and the agarose polymer coating the surface of the array was catalyzed by base generated at the electrodes by the reduction of vitamin-B_{12}.[15] The Diels-Alder reaction was then conducted by treating the array with a Sc(I)-reagent solution made by premixing $Sc(OTf)_3$ and 2-arylbenzothiozole. To this mixture was added the diene and tetramethylammonium nitrate (electrolyte). The Diels-Alder reaction was then conducted at all of the microelectrodes in the array by setting each at a potential of +3.5 V vs. a remote Pt wire for 0.5 s and then turning each off again for 0.1 s. This was repeated for 600 cycles. Cycling the electrodes in this manner was done in order to control the rate at which Sc(III)

was generated. Faster generation of Sc(III) damaged the agarose surface. The success of the Diels-Alder reaction at each of the electrodes was ascertained with the use of a fluorescence microscope. The image of the array following the Diels-Alder reaction is shown on the left in **Scheme 3.10**.

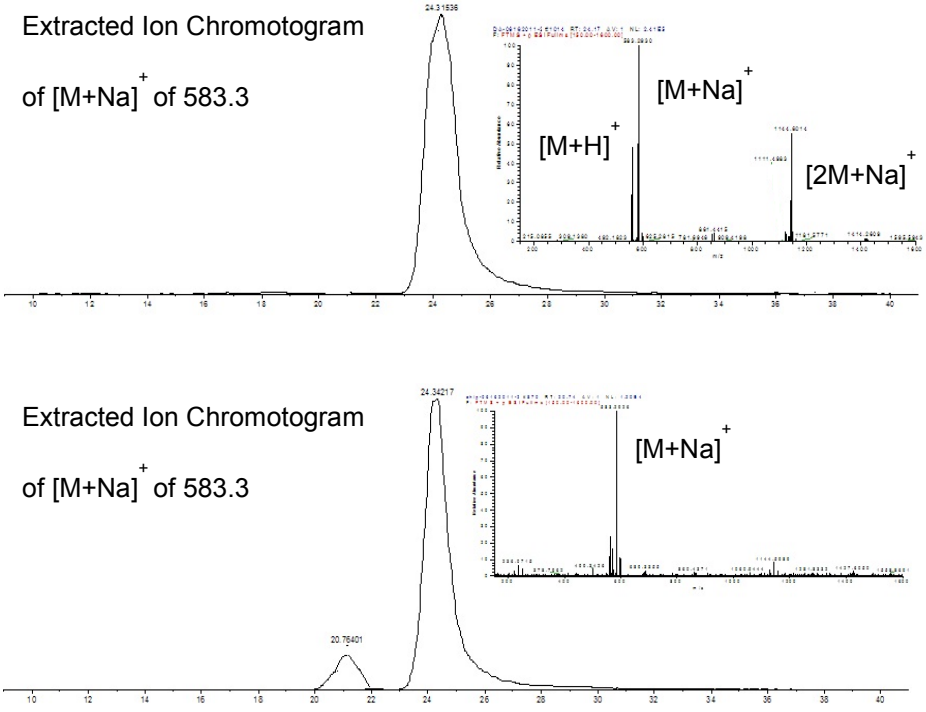
Scheme 3.10 Diels-Alder reactions with a "Safety-Catch" linker on Microelectrode Array



Characterization of the Diels-Alder product generated on the array then commenced by cleaving the product from one half of the electrodes. To do this, the array was submerged along with a remote Pt-wire into 1.5 mL of a methanol solution containing both 1,2-diphenylhydrazine and tetrabutylhexafluorophophate (electrolyte). As in the earlier reactions,[5] excess hydrazine was used as a confining agent for the acid generated at the electrodes. A checkerboard pattern of

electrodes was set at +3.0 V relative to the Pt-wire in solution for a period of 0.5 s and then off again for 0.1 s. The reaction was conducted for 900 such cycles. The solution above the array was then collected and the array imaged using a fluorescence microscope. The fluorescence image for the array following the cleavage reaction is shown on the right in **Scheme 3.10**.

The solution removed from the array was compared with the independently synthesized material using LC-MS. As in the case of the thermal solution-phase reaction, the array-based Diels-Alder led to both endo- and exo-products. The endo-product was again the major stereoisomer formed.



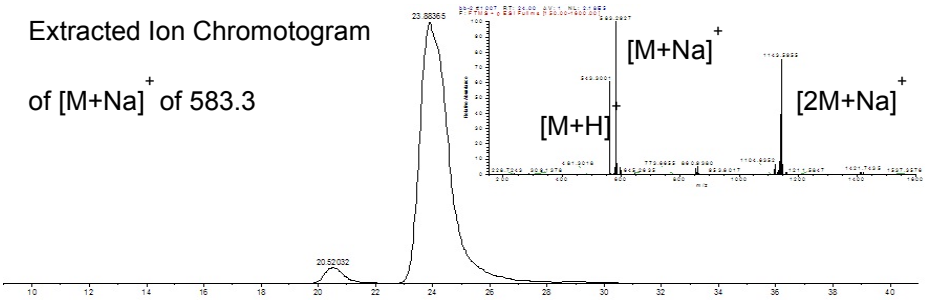


Figure 3.4 a) Diels-Alder product from the solution-phase reaction with $Sc(OTf)_3$ catalyst. b) Diels-Alder product from the solution-phase thermal reaction. c) Diels-Alder product removed from the microelectrode array as illustrated in **Scheme 3.10**

As can be seen in **Figure 3.4**, the ratio of exo- to endo-product was a little smaller (approximate ratio: 0.04/1) than that obtained for the thermal solution-phase reaction. However, the presence of the array did alter the stereochemical outcome of the Sc(III)-catalyzed reaction. The difference between the array-based reaction and the Sc(III)-catalyzed reaction in solution can be explained by noting that the stereochemistry of Diels-Alder reactions is typically controlled by two factors – stereoelectronic effects that favor the endo-product and steric effects that favor the exo-product. In the absence of the array, the current Sc(III)-catalyzed reaction is dominated by stereoelectronics leading to exclusively endo-product. However, when the array is present, the dienophile is larger. This increases the steric component of the reaction leading to some of the exo-product. In principle, the effect of the array can be reduced by increasing the length of the chain connecting the dienophile and the array. With the availability of a site-selectively cleavable linker, such an experiment is now possible.

3.4 Conclusion

One of the key challenges for any small molecule library is quality control. How do you know that the molecules in the library are what you think they are? This question is particularly

important for small molecule libraries that contain conformational probes. In such cases, both the composition and stereochemistry of the molecules need to be characterized. With the development of site-selectively cleavable "safety-catch" linkers reported here, we now have the ability to carry out such characterization studies for small molecule libraries that are placed on microelectrode arrays. This ability has been demonstrated with the characterization of a Diels-Alder reaction product synthesized on an array.

In addition, two features of the linker design are particularly attractive. First, the use of an electrochemically cleavable linker on a microelectrode array is extremely attractive because the same electrode used to monitor the binding properties of a molecule can also be used to isolate it for characterization. Second, the core structure of the linkers enables grafting a variety of functionalities on the side chain and thus great compatibility with reaction methodologies.

In this way, quality control efforts can be focused on the exact molecules giving rise to a biological signal of interest.

3.5 Experimental Procedures

3.5.1 General information of reagents and instruments

Chemical reagents and solvents were purchased from Sigma-Aldrich, Alfa Aesar, Mallinckrodt Baker, EMD Chemicals (Merck KGaA), and used without further purification unless otherwise noted. Dichloromethane (CH_2Cl_2) was distilled over calcium hydride. Anhydrous tetrahydrofuran (THF) was distilled over sodium benzophenone ketyl. Tetraethylammonium p-toluenesulfonate (Et_4NOTs) was dried in an oven before use. Flash chromatography was carried out by using silica gel (200-400 mesh) purchased from Dynamic Adsorbent, Inc.

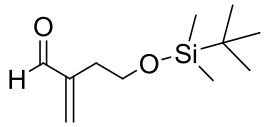
All proton and carbon-13 nuclear magnetic resonance (^{1}H and ^{13}C NMR) spectra were recorded by a Varian Inova 600, a Varian Inova 500, or a Varian Mercury 300 in a deuterated chloroform ($CDCl_3$) solvent with tetramethylsilane (TMS) as an internal standard unless otherwise noted. Infrared (IR) spectra were recorded by a Perkin Elmer Spectrum BS FT-IR instrument. High resolution electrospray ionization (ESI) mass spectra were obtained at the NIH/NCRR Mass Spectrometry Resource Facility at Washington University in St. Louis.

Fluorescence images were taken with a Nikon Eclipse E200 microscope connected to a Boyce Scientific, M-100 burner and a Nikon D5000 camera. CFW-BP01-Clinical-000 (Semrock) filter cube excitation 380-395 nm/emission 420-470 nm, ET-GFP (FITC/Cy2) (Chroma) filter cube excitation 450-490 nm, emission 500-550 nm and TxRed-A-Basic-000 (Semrock) filter cube excitation 540-580 nm, emission 590-670.

Analytical HPLC was performed on a HP 1100 instrument with a UV detector setting the working wavelength at 254nm. A Discovery® HS C18, 5µm, 25cm * 4.6mm column was employed. HPLC-Grade methanol and water were all purchased from Sigma-Aldrich USA and used as received. LC-MS samples were analyzed on a Thermo LTQ-FT hybrid mass spectrometer (Thermo Fisher, San Jose, CA). The instrument settings were: spray voltage, 3.5 kV; sheath gas flow rate, 8 arb; capillary temperature, 275 °C; capillary voltage, 35 V; and tube lens, 110 V.

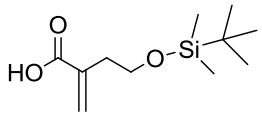
Microelectrode arrays were spin coated with a spin coater spin-coater MODEL WS-400B-6NPP/LITE.

3.5.2 Synthesis of the chemically cleavable linkers



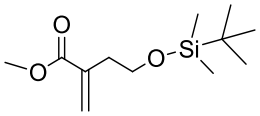
4-(tert-butyldimethylsilyloxy)-2-methylenebutanal (3a)

In a flask, 2.63 g (0.013 mol) of 4-(tert-butyldimethylsilyl)oxy-1-butanol was dissolved in 50 mL of DMSO and 11 mL (0.078 mol) of triethyamine under an atmosphere of argon. Sulfur trioxide pyridine complex (4.20 g, 0.026 mol) was added as a solid over 20 min with the solution being stirred at the room temperature. After stirring for another 2h, 1.54 g (0.016 mol) of N,N-dimethylmethyleneiminium chloride was added in one portion. After stirring overnight at rt, the reaction mixture was poured into a stirred biphasic mixture of cold saturated aqueous $NaHCO_3$ and diethyl ether. Once the bubbling stopped, the organic layer was separated and then washed with water and brine. The product was purified by chromatography through silica gel using 5% diethyl ether in hexane as eluant to afford 2.06 g (74%) of a yellow oil. ^{1}H NMR ($CDCl_3$/300MHz) δ 9.53 (s, 1H), 6.21 (AB q, 2H, Δγ=0.30 Hz, J=0.90 Hz), 3.70 (t, J=6.3 Hz, 2H), 2.47 (t, J=6.3 Hz, 2H), 0.87 (s, 9H), 0.02 (s, 6H), ^{13}C NMR ($CDCl_3$/300MHz) δ 194.5, 147.1, 135.8, 61.0, 31.6, 25.9, 22.6, 14.1, -5.4, IR (KBr) 2955, 2928, 2885, 2857, 2341, 1693, 1472, 1255, 1101, 925, 835, 776, 668 cm^{-1}. HRESI MS m/z calculated for $(M+Na)^+$ 237.1287, found 237.1285.



4-(tert-butyldimethylsilyloxy)-2-methylenebutanoic acid (3b)

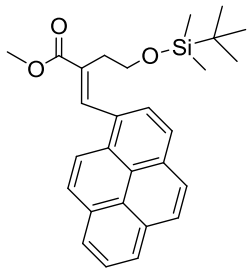
In a flask, 2.06 g (9.6 mmol) of **3a** was dissolved in 10 mL of t-butanol and 4 mL of 2-methyl-2-butene. The solution was gently stirred and maintained below 20 ^{o}C with an ice-water bath, another solution of 80% sodium chlorite and NaH_2PO_4 in 7 mL water was added dropwise over O.5 h. After 18h, the t-butanol was evaporated and the residue diluted with water. The solution was acidified with 50% H_3PO_4 in water from pH=7 to pH=3, and then the product extracted with dichloromethane and washed with 2 *N* aqueous citric acid and brine. The compound was further purified by chromatography using silica gel as the stationary phase and 20% diethyl ether in hexane as the eluant. 1.92g (87%) yellow crystal was collected. ^{1}H NMR ($CDCl_3$/300MHz) δ 6.04 (AB q, 2H, $\Delta\gamma$=0.62 Hz, J=1.5 Hz), 3.75 (t, J=6.6 Hz, 2H), 2.53 (t, J=6.6 Hz, 2H), 0.88 (s, 9H), 0.04 (s, 6H), ^{13}C NMR ($CDCl_3$/300MHz) δ 172.6, 137.0, 129.3, 61.9, 35.0, 25.9, 18.3, -5.4, IR (KBr) 3000, 2955, 2887, 2885, *2739*, 1697, 1628, 1439, 1256, 1101, 930, 836, 776 cm^{-1}. HRESI MS m/z calculated for $(M+Na)^+$ 253.1236, found 253.1239.



Methyl 4-(tert-butyldimethylsilyloxy)-2-methylenebutanoate (3c)

Compound **3b** (230.38 mg, 1 mmol) and 167 mg (2 mmol) sodium bicarbonate were dissolved into 10 mL of DMF. Iodomethane (0.25mL, 4 mmol) was added dropwisely at room temperature. After being stirred overnight, the reaction was quenched with saturated ammonium chloride and then extracted with ethyl acetate and washed with water and brine. 222.36 mg (91%) product was chromatographed through silica gel. The column was eluted with 5% diethyl ether in hexane. ^{1}H NMR ($CDCl_3$/300MHz) δ 5.90 (AB q, 2H, $\Delta\gamma$=0.58 Hz, J=0.90 Hz), 3.73 (s, 3H), 3.71 (t, J=6.6 Hz, 2H), 2.51 (t, J=6.6 Hz, 2H), 0.86 (s, 9H), 0.01 (s, 6H), ^{13}C NMR

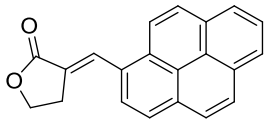
($CDCl_3$/300MHz) δ 167.7, 137.5, 127.1, 62.0, 51.9, 35.7, 26.1, 18.5, -5.2, IR (KBr) 2954, 2929, 2857, 1724, 1631, 1439, 1256, 1156, 1100, 929, 835, 776 cm^{-1}. HRESI MS m/z calculated for $(M+Na)^+$ 267.1393, found 267.1381.



(E)-methyl 4-(tert-butyldimethylsilyloxy)-2-(pyren-1-ylmethylene)butanoate (3d)

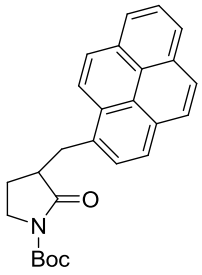
$Pd(OAc)_2$ (33.6 mg, 0.15 mmol), 322.4 mg of Bu_4NBr (1.0 mmol) and 157.4 mg of triphenyl phosphine (0.6 mmol) were mixed in a pre-dried and argon-filled 50 mL flask equipped with a condenser. Compound **1 c** (122.18 mg, 0.5mmol) was dissolved into a mixture of 9 mL DMF, 1 mL triethyl amine, and 1 ml water in a second flask. The solution was degassed by bubbling argon though it for 5 min, and then immediately transferred into the pre-mixed solids. After being stirred overnight at 70 ^oC, the reaction was quenched with 1 *M* HCl that was added dropwise until the pH of the solution was around 4. The organic compounds were extracted with dichloromethane three times and washed with brine. The solution was concentrated and then chromatographed through a silica gel column using a gradient eluant of 1%, 2% and 3% ethyl acetate in hexane to afford 133.9 mg (60%) of the pure product. ^1H NMR ($CDCl_3$/300MHz) δ 8.59 (s, 1H), 8.22-7.99 (m, 9H), 3.94 (s, 3H), 3.84 (t, J=6.6 Hz, 2H), 2.79 (t, J=6.6 Hz, 2H), 0.84 (s, 9H), -0.017 (s, 6H), ^{13}C NMR ($CDCl_3$/300MHz) δ 169.1, 140.8, 132.5, 132.0, 131.9, 131.5, 130.7, 129.7, 128.5, 128.3, 127.9, 127.5, 126.7, 126.1, 126.1, 125.3, 125.1, 124.6, 62.6, 52.7,

32.0, 26.5, 19.0, -4.8, IR (KBr) 2952, 2927, 2854, 1711, 1434, 1259, 1118, 1092, 837, 776 cm^{-1}. HRESI MS m/z calculated for $(M+Na)^+$ 467.2019, found 467.2009.



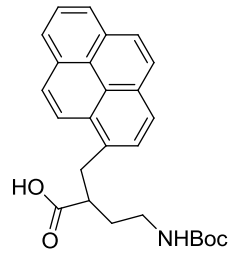
(E)-3-(pyren-1-ylmethylene) dihydrofuran-2(3H)-one (3e)

Compound **3d** (89.27 mg,0.2 mmol) was dissolved in 5 mL of methanol. One drop of HCl was added at room temperature. After being stirred for 8 h, the reaction was quenched with $CaCO_3$, and stirred until the bubbling stopped. The reaction mixture was filtered, the solvent removed by evaporation, and then the resulting residue diluted with ether and washed with 1% Na_2CO_3, water and brine. The organic layer was concentrated *in vacuo* and then chromatographed through silica gel using 20% ethyl acetate in hexane as eluant to afford 38.8 mg (65%) of the desired product. ^1H NMR ($CDCl_3$/300MHz) δ 8.64 (t, J=2.7 Hz, 1H), 8.44 (d, J=9 Hz, 1H), 8.27-8.03 (m, 8H), 4.52 (t, J=7.2 Hz, 2H), 3.36 (dt, J=2.7 Hz, 7.2Hz, 2H), ^{13}C NMR ($CDCl_3$/300MHz) δ 172.4, 137.6, 133.9, 132.2, 131.2, 130.7, 130.2, 128.8, 128.7, 127.2, 126.4, 126.0, 125.1, 125.6, 125.6, 124.6, 122.9, 65.6, 27.7, NOESY ($CDCl_3$ /600MHz) cross peaks between δ 3.56 and 4.52, 3.56 and 8.19, and 8.42 and 8.64 confirmed the configuration of the tri-substituted vinyl group, IR (KBr) 3400, 2916, 1743, 1636, 1559, 1540, 1218, 1183, 1028, 844, 668 cm^{-1}. HRESI MS m/z calculated for $(M+Na)^+$ 321.0886, found 321.0886.



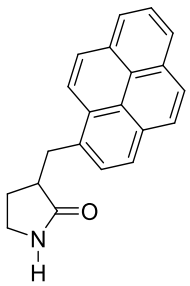
Tert-butyl 2-oxo-3-(pyren-1-ylmethyl) pyrrolidine-1-carboxylate (3f)

Diisopropyl amine (0.3 mL, 2.2 mmol) was dissolved in 20 mL of anhydrous THF. The solution was cooled to -78^{o}C and 1.25 mL (2 mmol) of a 1.6 *M* n-BuLi in hexane solution was added dropwisely. The mixture was stirred for 30 min before adding 370.4 mg (2 mmol) of 1-(tert-butoxycarbonyl)-2-pyrrolidinone. The reaction was stirred at -78^{o}C for another 30 minutes before slowly adding a solution of 1.18 g (4 mmol) 1-(bromomethyl)pyrene in 10 mL of cold anhydrous THF. The mixture was stirred at -78 ^{o}C for 7 h and then quenched with saturated ammonium chloride. The reaction was diluted with ethyl acetate and washed with water. The organic phase was concentrated *in vacuo* and then chromatographed through silica gel using a a gradient eluant from 15% to 20% ethyl acetate in hexane to afford 695.1 mg (87%) of the alkylated product. ^{1}H NMR ($CDCl_3$/300MHz) δ 8.32-7.85 (m, 9H), 4.22 (A in ABX, J_{AB}=13.8 Hz, J_{AX}=3.6 Hz, 1H), 3.73 (m, 1H), 3.47 (m, 1H), 3.18 (B in ABX, J_{AB}=13.8 Hz, J_{BX}=10.5 Hz, 1H), 3.06 (m, 1H), 1.84 (m, 2H), 1.56 (s, 9H), ^{13}C NMR ($CDCl_3$/300MHz) δ 175.7, 150.6, 133.3, 131.6, 131.0, 130.6, 129.2, 129.0, 128.0, 127.9, 127.6, 127.2, 126.2, 125.4, 125.2, 125.0, 124.6, 123.2, 83.2, 46.1, 44.7, 34.2, 28.3, 24.7, IR (KBr) 3468, 3041, 2977, 2930, 1779, 1744, 1712, 1366, 1314, 1249, 1151, 844, 722 cm^{-1}. HRESI MS m/z calculated for $(M+H)^+$ 400.4872, found 400.4868, calculated for $(M+Na)^+$ 422.4698, found 422.4691.



Methyl 4-(tert-butoxycarbonylamino)-2-(pyren-1-ylmethyl)butanoic acid (3g)

To a solution of 360 mg (0.9 mmol) **3f** in a mixture of 10 mL THF and 2 mL of water was added 60 mg (2.25 mmol) of LiOH. After stirring overnight, the reaction solution was concentrated *in vacou* and diluted with water. The aqueous solution was acidified with CH_3COOH to pH=4~5 and then extracted with ethyl acetate. The combined organic layers were washed with brine and dried over sodium sulfate. The solvent was removed by distillation under reduced pressure to afford 345.3 mg (92%) of the crude product. ^{1}H NMR ($CDCl_3$/300MHz) δ 8.30-7.80 (m, 9H), 4.66 (bs, 1H), 3.87-3.71 (m, 1H), 3.50-3.32 (m, 1H), 3.26-2.87 (m, 3H), 1.94-1.83 (m, 1H), 1.60-1.50 (m, 1H), ^{13}C NMR ($CDCl_3$/300MHz) δ 179.9, 156.6, 133.3, 131.7, 131.5, 131.1, 131.0, 130.5, 129.2, 128.3, 127.8, 127.6, 127.1, 126.1, 125.2, 125.1, 124.9, 123.3, 79.8, 36.8, 36.1, 36.0, 32.6, 28.5, IR (KBr) 3338, 3041, 2930, 2975, 1702, 1508, 1366, 1247, 1167, 844, 731cm^{-1}. HRESI MS m/z calculated for $(M+H)^+$ 418.5076, found 418.5075, calculated for $(M+Na)^+$ 440.4896, found 440.4891.



3-(pyren-1-ylmethyl) pyrrolidin-2-one (3h)

This compound was prepared in one of two ways:

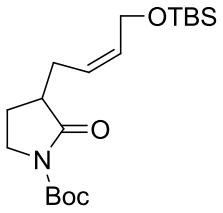
1) **3g** 41.9 mg was dissolved in 10 mL of DMF and then 150 mg (>2 equiv.) of sodium bicarbonate added. Iodomethane (0.23 mL, >4 equiv.) was added into the stirred suspended solution and the reaction stirred overnight. The reaction was quenched with saturated ammonium chloride, the layers separated, and the aqueous phase extracted with ethyl acetate. The combined organic layers were washed with water and brine, dried over $MgSO_4$, and concentrated *in vacuo*. The crude product was purified by chromatography through silica gel using 20% diethyl ether in hexane as eluant. The purified product was dissolved in a mixed solution of CH_2Cl_2: CF_3COOH (7:3), stirred for 30 min at room temperature, and an excess of triethylamine added. The mixture was stirred for an hour before being quenched with a mixture of saturated ammonium chloride and ethyl acetate. The pH of the solution was found to be about 5~6. This product was chromatographed through silica gel using ethyl acetate as eluant to afford 25.3 mg (85%) of the desired product.

2) Compound **3f** (40 mg, 0.1 mmol) was de-protected by treating it with a solution of CH_2Cl_2:CF_3COOH (7:3) for 30 min. The reaction was quenched with ice water, the layers separated, and the organic phase washed with sodium bicarbonate. The aqueous layers were extracted with ethyl acetate. The combined organic layers were concentrated *in vacuo* and the resulting residue chromatographed through a silica gel column to afford 27.8mg (93%) of the pure product.

^{1}H NMR ($CDCl_3$/300MHz) δ 8.40-7.88 (m, 9H), 6.40 (bs, 1H), 4.17 (A in ABX, J_{AB}=14.1 Hz, J_{AX}=3.9 Hz, 1H), 3.25 (m, 3H), 2.95 (m, 1H), 1.96 (m, 2H) ^{13}C NMR ($CDCl_3$/300MHz) δ

179.8, 134.0, 131.6, 131.1, 130.4, 129.1, 127.9, 127.8, 127.7, 127.1, 126.2, 125.4, 125.3, 125.2, 125.1, 125.0, 123.4, 43.2, 40.6, 34.3, 27.7, IR (KBr) 3219, 2998, 2987, 2875, 2874, 2778, 1644, 1504, 1438, 1422, 1332, 1258, 1119, 1073, 836, 779 cm^{-1}. HRESI MS m/z calculated for $(M+H)^+$ 300.1382, found 300.1383, calculated for $(M+Na)^+$ 322.1208, found 322.1202.

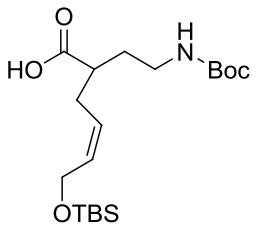
Preparation of the chemically cleavable linker with the dienophile attached



(Z)-tert-butyl 3-(4-(tert-butyldimethylsilyloxy)but-2-enyl)-2-oxopyrrolidine-1-carboxylate (3i)

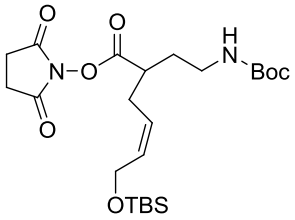
Diisopropyl amine (0.6 mL, 4.4 mmol) was dissolved in 20 mL of anhydrous THF. The solution was cooled to -78°C and 2.5 mL (4 mmol) of a 1.6 *M* n-BuLi in hexane solution was added dropwisely. The mixture was stirred for 30 min before adding 740.8 mg (4 mmol) of 1-(tert-butoxycarbonyl)-2-pyrrolidinone. The reaction was stirred at -78°C for another 30 minutes before slowly adding a solution of 1.06 g (4 mmol) (Z)-(4-bromobut-2-enyloxy)(tert-butyl)dimethylsilane in 10 mL of cold anhydrous THF. The mixture was stirred at -78 °C for 7 h and then quenched with saturated ammonium chloride. The reaction was diluted with ethyl acetate and washed with water. The organic phase was concentrated *in vacuo* and then chromatographed through silica gel using a an eluant of 15% ethyl acetate in hexane to afford 1.24 g (84%) product. ^1H NMR ($CDCl_3$/300MHz) δ 5.65-5.56 (m, 1H), 5.42-5.33 (m, 1H), 4.18 (d, J= 6.0 Hz, 2H), 3.75-3.68 (m, 1H), 3.57-3.48 (m, 1H), 2.63-2.40 (m, 2H), 2.28-1.98 (m, 2H), 1.75-1.57 (m, 1H), 1.49 (s, 9H), 0.85 (s, 9H), 0.02 (s, 6H) ppm; ^{13}C NMR ($CDCl_3$/300MHz)

δ 175.0, 150.2, 132.2, 126.4, 82.7, 59.1, 44.3, 43.5, 28.1, 27.9, 25.8, 23.5, 18.2, -5.2 ppm; IR (KBr) 2930, 2856, 1786, 1749, 1715, 1472, 1367, 1318, 1253, 1153, 1088, 837, 777 cm^{-1}. HRESI MS m/z calculated for $(M+Na)^+$ 392.2229, found 392.2229.



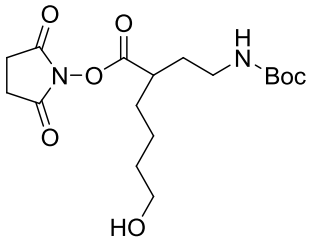
(Z)-2-(2-(tert-butoxycarbonylamino)ethyl)-6-(tert-butyldimethylsilyloxy)hex-4-enoic acid (3j)[16]

To a solution of 1.11 g (3 mmol) **3i** in a mixture of 20 mL THF and 5 mL of water was added 0.21 g (9 mmol) of LiOH. After stirring overnight, the reaction solution was concentrated *in vacuo* and diluted with water. The aqueous solution was acidified with CH_3COOH to pH=4~5 and then extracted with ethyl acetate. The combined organic layers were washed with brine and dried over sodium sulfate. The solvent was removed by distillation under reduced pressure to afford 1.03 g (89%) of the crude product. ^1H NMR ($CDCl_3$/300MHz) δ 10.60-9.50 (bs, 1H), 6.50- 6.09 (bs, 0.5H), 5.59 (m, 1H), 5.39 (m, 1H), 4.96-4.68 (bs, 0.5H), 4.21 (d, J= 6.0 Hz, 2H), 3.16 (m, 2H), 2.40 (m, 2H), 2.26 (m, 1H), 1.71 (m, 2H), 1.43 (s, 9H), 0.86 (s, 9H), 0.06 (s, 9H) ppm, ^{13}C NMR ($CDCl_3$/300MHz) 180.0, 132.4, 126.8, 79.6, 59.6, 43.0, 38.8, 32.0, 30.3, 28.6, 26.1, 25.8, 18.5, -4.9 ppm, IR (KBr) 3334, 2930, 2857, 1710, 1519, 1471, 1409, 1367, 1252, 1171, 1089, 837, 777, 667 cm^{-1}. HRESI MS m/z $[M+H]^+$ found 388.2506, within 2 ppm of the theoretical value.



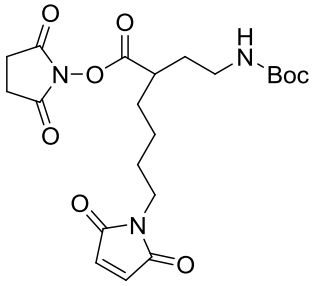
(Z)-2,5-dioxopyrrolidin-1-yl 2-(2-(tert-butoxycarbonylamino)ethyl)-6-(tertbutyldimthylsilyoxy) hex-4-enoate (3k)

A round bottom flask was charged with 968 mg **3j** (2.5 mmol), 316 mg N-hydroxyl succinimide (2.75 mmol), and 619 mg DCC (3 mmol). 7 mL DMF was then added into the flask to dissolve the solid mixture. After being stirred at room temperature overnight, the reaction mixture was poured into 5 % LiCl aqueous solution, followed by being extracted with EtOAc. The collected organic layer was further washed with saturated NaCl aqueous solution to get rid of most of DMF. The concentrated organic stuff was then loaded onto the chromatography. A solution of 30 % EtOAc in Hexane was employed to separate the crude products. 981 mg (81 %) pure product was yielded. ^{1}H NMR ($CDCl_3$/300MHz) δ 5.64 (m, 1H), 5.40 (m, 1H), 4.98 (bs, 1H), 4.18 (m, 1H), 3.18 (m, 2H), 2.79 (s, 4H), 2.73 (m, 1H), 2.41 (m, 2H), 1.84 (m, 2H), 1.38 (s, 9H), 0.85 (s, 9H), 0.02 (s, 6H) ppm, ^{13}C NMR ($CDCl_3$/300MHz) 170.6, 169.2, 156.2, 133.2, 125.6, 79.4, 59.4, 40.8, 38.3, 32.1, 30.0, 28.6, 26.1, 25.8, 18.5, -5.0 ppm, IR (KBr) 3305, 2730, 2657, 1749, 1701, 1456, 1409, 1297, 1202, 1089, 839, 734, 657 cm $^{-1}$. HRESI MS m/z of $[M+Na]^+$ found 507.2489, within 2 ppm of the theoretical value.



2,5-dioxopyrrolidin-1-yl 2-(2-(tert-butoxycarbonylamino)ethyl)-6-hydroxyhexanoate (3l)[17]

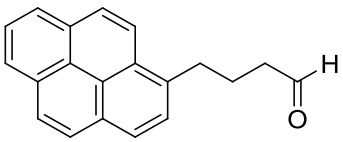
A 25 mL pear-shaped flask was pre-dried before being charged with 960 mg **3k** (2 mmol) and 10 mL anhydrous Methanol. The solution mixture was bubbled with Argon for 5 minutes. In another 25 mL round bottom flask, 780 mg Palladium, 10 % wt. on activated carbon was weighed under being protected with Argon. Then, the bubbled solution above was carefully transferred into the flask with Pd/C in it, under Argon as well. After being de-gassed, Hydrogen gas was slowly added into the system with a balloon attached. The mixture with the hydrogen-filled balloon was stirred for 5 hours before being filtered with celite pad, and washed with EtOAc. A chromotography with silica gel was used to purify the product. A solvent mixture of 50 % EtOAc in Hexane was used to wash 566 mg (76 %) pure compound off. ^{1}H NMR ($CDCl_3$/300MHz) δ 4.97 (bs, 1H), 3.64 (t, J= 6.0 Hz, 2H), 3.36-3.08 (m, 2H), 2.83 (s, 4H), 2.75 (m, 1H), 1.97-1.46 (m, 9H), 1.42 (s, 9H) ppm, ^{13}C NMR ($CDCl_3$/300MHz) 171.3, 169.6, 156.3, 79.4, 62.3, 40.7, 38.3, 32.5, 32.3, 31.8, 28.6, 25.8, 23.2 ppm, IR (KBr) 3385, 2976, 2939, 2868, 1810, 1780, 1738, 1697, 1520, 1366, 1208, 1170, 1067, 733, 647 cm^{-1}. HRESI MS m/z of $[M+Na]^+$, found 395.1784, within 2 ppm of the theoretical value.



2,5-dioxopyrrolidin-1-yl 2-(2-(tert-butoxycarbonylamino)ethyl)-6-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)hexanoate (3m)[18]

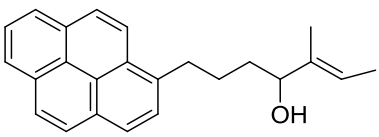
0.45 mL solution of 40 % Diethyl azodicarboxylate (1 mmol) in Toluene was mixed with 260 mg PPh_3 in 10 mL THF at room temperature, followed by being added in 372.4 mg (1 mmol) **3l**. 96 mg (1 mmol) and 44.5 mg (0.5 mmol) neopentyl alcohol were then poured into the mixture. The solution was left stirred overnight before being concentrated under reduced pressure. The mixture was separated by silica chromatography with 50 % EtOAc in Hexane. The pure compound was afforded as 357 mg (79 %). ^{1}H NMR ($CDCl_3$/300MHz) δ 6.68 (s, 2H), 4.92 (bs, 1H), 3.53 (t, J= 6.0 Hz, 2H), 3.33-3.12 (m, 2H), 2.83 (s, 4H), 2.71 (m, 1H), 1.94-1.56 (m, 7H), 1.43 (s, 9H) ppm, ^{13}C NMR ($CDCl_3$/300MHz) 171.1, 171.0, 169.0, 156.2, 134.2, 79.4, 40.8, 38.4, 37.6, 32.7, 31.7, 28.6, 28.4, 25.8, 24.2 ppm, IR (KBr) 3385, 2977, 2941, 2868, 1739, 1705, 1517, 1410, 1366, 1207, 1170, 1066, 829, 732, 695cm^{-1}. HRESI MS m/z of $[M+Na]^+$ found 474.1829, within 4 ppm of the theoretical value.

Preparation of the pyrene labeled diene



4-(pyren-4-yl)butanal (2a)

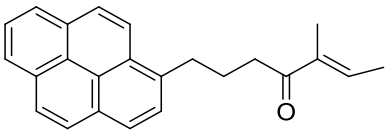
The spectra of the compound were in accordance with the one reported before.[7]



(E)-3-methyl-7-(pyren-1-yl)hept-2-en-4-ol (3n)

270 mg (2 mmol) (E)-2-bromobut-2-ene was dissolved into 10 mL THF under Argon,

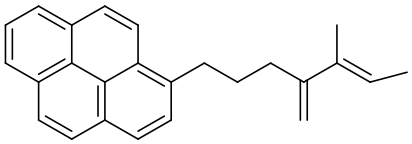
followed by being cooled down to -78°C. 2.4 mL t-BuLi in Pentane (4 mmol) was added in dropwise. After being reacted for 30 minutes, all **2a** prepared above in 2 mL THF was injected into the solution at -78°C under argon. The reaction was kept at this temperature for 10 more minutes, then warmed up to the room temperature. In 3 hours, several drops of water was firstly added in to quench the rest of t-BuLi before 5 % $NaHCO_3$ aqueous solution was poured in to quench the reaction. The organic stuff was extracted with EtOAc three times, followed by dried with Na_2SO_4. The solution was evacuated under reduced pressure to afford the crude product that was carried into the next step without purification.



(E)-3-methyl-7-(pyren-1-yl)hept-2-en-4-one (3o)

The crude product above was dissolved into 5 mL CH_2Cl_2. 12 mL Dess Martin Periodinane (0.3M) in CH_2Cl_2 was added in at the room temperature. The reaction mixture was left stirred for 2 hours before being quenched with 5 % $Na_2S_2O_3$ and 5 % $NaHCO_3$ aqueous solution, followed by being extracted with EtOAc. The organic layers were combined, dried with Na_2SO_4, and concentrated. The mixture was purified with a short column which was washed with 5% EtOAc in Hexane to elute the product (449 mg) off with a yield of 68 % from **2a**. ^{1}H NMR ($CDCl_3$/300MHz) δ 8.40-7.84 (m, 9H), 6.66 (q, J= 6.0 Hz, 1H), 3.37 (t, J= 9.0 Hz, 2H), 2.78 (t, J=6.0 Hz, 2H), 2.19 (m, 2H), 1.81 (d, J= 9.0 Hz, 3H), 1.79 (s, 3H), ^{13}C NMR ($CDCl_3$/300MHz) 202.1, 144.8, 136.5, 131.2, 130.8, 130.2, 129.1, 128.3, 127.8, 127.7, 127.6, 127.0, 126.1, 125.3, 125.2, 125.1, 125.0, 124.8, 123.8, 37.1, 33.4, 26.6, 17.9, 17.7, IR (KBr) 3056, 2950, 2922, 1681, 1602, 1508, 1428, 1310, 1090, 918, 848, 756 cm^{-1}. HRESI MS m/z of $(M+H)^+$ found 327.1666,

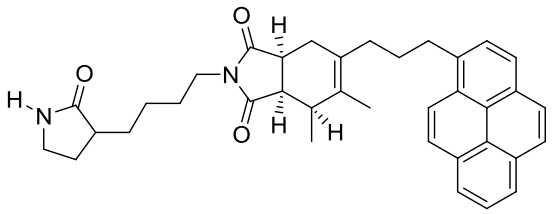
within 4 ppm of the theoretical value.



(E)-1-(5-methyl-4-methylenehept-5-en-1-yl)pyrene (3p)

Into the tetrahydrofuran solution of 1.07g methyltriphenylphosphonium bromide (3 equiv.), 3mL of 1.0M sodium bis(trimethylsilyl)amide (3 equiv.) in tetrahydrofuran was added dropwise with the argon. The mixture solution was stirred for 30 minutes under the room temperature. The white sand-like solid suspended solution turned into orange clear one. 326 mg (1.0mmol) **3o** in 10mL tetrahydrofuran was added into the system slowly under the argon. The stirring was continued for another 4 hours. 25mL saturated ammonium chloride solution was used to quench the reaction, followed by extracting the organic staffs with methylene chloride three times. The solution was dried with magnesium sulfate. Sillica gel column was utilized to isolate the crude product with Hexane. 230 mg pale orange oil was obtained with the yield of 71% after getting rid of solvent at reduced pressure. ^{1}H NMR ($CDCl_3$/300MHz) δ 8.30-7.80 (m, 9H), 5.66 (q, J= 6.0 Hz, 1H), 5.04 (s, 1H), 4.92 (s, 1H), 3.34 (t, J= 9.0 Hz, 2H), 2.47 (t, J= 9.0 Hz, 2H), 2.02 (m, 2H), 1.81 (s, 3H), 1.71 (d, J=6.0 Hz, 3H) ppm, ^{13}C NMR ($CDCl_3$/300MHz) 149.6, 137.3, 135.3, 134.1, 133.9, 131.7, 131.2, 130.0, 129.0, 128.7, 127.8, 127.3, 126.8, 126.0, 125.3, 125.0, 124.9, 123.7, 121.9, 110.3, 34.2, 33.6, 31.1, 14.4, 14.2 ppm, IR (KBr) 3435, 3042, 2936, 2862, 1602, 1586, 1433, 1181, 842, 743, 696, 501 cm^{-1}. HRESI MS m/z of $[M+H]^+$ found 325.1937, within 5 ppm of the theoretical value.

The Diels-Alder reactions between **3p** and **3m**



4,5-dimethyl-2-(4-(2-oxopyrrolidin-3-yl)butyl)-6-(3-(pyren-1-yl)propyl)-3a,4,7,7a-tetrahydro-1H-isoindole-1,3(2H)-dione (3q)

a. The $Sc(OTf)_3$ catalyzed route

45.2 mg (0.1 mmol) **3p**, 32.4 mg (0.1 mmol) **3m**, and 14.8 mg $Sc(OTf)_3$ were mixed together in a 25 mL round bottom flask. After being dissolved into 7 mL methylene chloride at room temperature, the reaction mixture was stirred overnight, followed by being quenched with water. The organic stuffs were extracted with methylene chloride three times. The combined organic layer was dried with sodium sulfate before getting rid of solvent at reduced pressure. The crude product was re-dissolved in a mixed solution of CH_2Cl_2: CF_3COOH (7:3), stirred for 30 min at room temperature, and an excess triethylamine added. The mixture was stirred for an hour before being quenched with a mixture of saturated ammonium chloride and ethyl acetate. The pH of the solution was found to be about 5~6. This product was chromatographed through silica gel using as eluant to afford 39.8 mg (71%) of the desired product.

b. The thermo-condition route

As reported above, 45.2 mg (0.1 mmol) **3p**, and 32.4 mg (0.1 mmol) **3m** were mixed together in a 25 mL round bottom flask. After being dissolved into 4 mL Toluene at room temperature, the reaction mixture was heated up to reflux with stirring being continued overnight. After being cooled down, the reaction solution was then evacuated to get rid of most of toluene. The mixture was diluted with water, followed by being extracted with methylene chloride three times. The

combined organic layer was dried with sodium sulfate before getting rid of solvent at reduced pressure. Again, the same as above, the crude product was re-dissolved in a mixed solution of CH_2Cl_2: CF_3COOH (7:3), stirred for 30 min at room temperature, and an excess triethylamine added. The mixture was stirred for an hour before being quenched with a mixture of saturated ammonium chloride and ethyl acetate. The pH of the solution was found to be about 5~6. This product was chromatographed through silica gel using 10 % methanol in ethyl acetate as eluant to afford 38.1 mg (68%) of the desired product.

^{1}H NMR ($CDCl_3$/300MHz) δ 8.22-7.84 (m, 9H), 3.38 (t, J= 6.0 Hz, 2H), 3.28 (t, J= 6.0 Hz, 2H), 3.18, (m, 2H), 2.98 (m, 1H), 2.89 (m, 1H), 2.49 (m, 2H), 2.30-2.16 (m, 4H), 2.11 (m, 1H), 1.87 (m, 2H), 1.73 (m, 2H), 1.65 (s, 3H), 1.58 (m, 1H), 1.45 (m, 2H), 1.25-1.21 (m, 5H) ppm, ^{13}C NMR ($CDCl_3$/300MHz) δ 182.9, 182.8, 181.3, 139.2, 134.7, 134.0, 133.5, 133.2, 133.1, 132.4, 131.2, 130.2, 129.9, 129.7, 129.2, 128.4, 127.7, 127.6, 127.5, 127.3, 125.9, 47.4, 43.2, 42.8, 42.6, 41.0, 40.9, 37.8, 36.2, 35.9, 32.9, 32.8, 31.1, 30.4, 30.1, 30.0, 27.2 ppm, COSY, HMQC, and TOCSY confirmed the structure, NOESY showed interactions among protons on newly built cyclic part, confirming the stereochemistry, IR (KBr) 3368, 2934, 2911, 1810, 1790, 1693, 1434, 1401, 1243, 1161, 846, 724 cm^{-1}. HRESI MS m/z calculated for $(M+H)^+$ 561.3117, found 561.3119. The theoretical value of $(M+H)^+$ 13C isotopic m/z 562.3145, found 562.3154, $(M+Na)^+$ 583.2939, found 583.2930.

3.5.3 Chemistry on Microelectrode Arrays

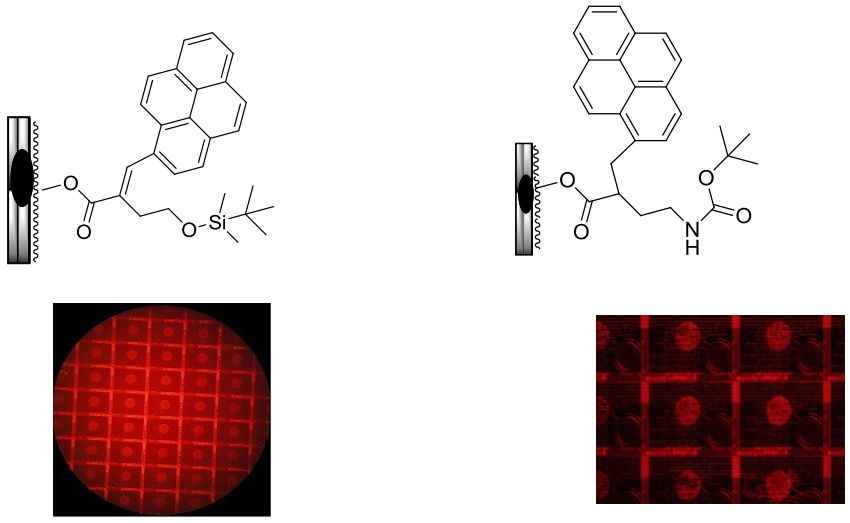


Figure 3.5 Example constructing the linkers on agarose coated 1K-microelectrode arrays

A microelectrode array containing 1024 electrodes cm^{-1} was spin coated at 2000 rpm for 45 seconds with 4% agarose in 90% N,N-dimethylformamide and 10% water. After drying the polymer for 1 hour, the microelectrode array was plugged into a circuit allowing the microelectrodes to be addressed and then inserted into a 1.5 mL Eppendorf tube containing 3 mg of vitamin B_{12}, 6 mg of tetramethylammonium nitrate, and 8 mg of the activated ester in 1.5 mL of methanol. A platinum wire was inserted into the solution as the counter electrode. A whole-board pattern was programmed with 600 potential cycles, -2.4 V relative to the Pt-wire for 0.5 s and 0 V for 0.1 s for each. When the reaction was complete, the array was removed from the solution and then washed with de-ionized water and 95% ethanol. The array was then imaged using a fluorescence microscope.

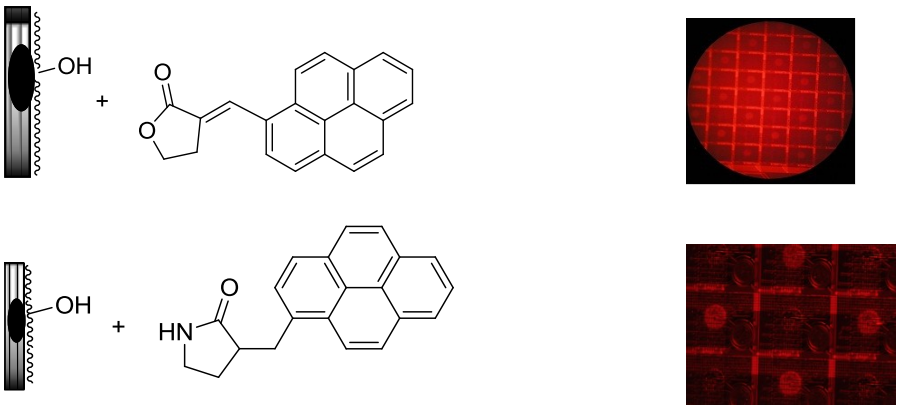


Figure 3.6 Example cleavage of the linkers off agarose coated 1K-microelectrode arrays

The microelectrode array with the linker built on the surface using the chemistry described above was inserted into the solution consisting of 50 mg 1, 2-diphenyhydrazine, 100 mg tetrabutyl-ammonium hexafluorophosphate, and 1.5 mL methanol. A platinum wire was inserted into the solution as counter electrode. A checker-board pattern of electrodes was then programmed for 900 potential cycles of on for 0.5 s at +3.0 V and off (0 V) for 0.1 s. After the reaction was complete, the array was removed from the solution and washed with de-ionized water and 95% ethanol. An image of the array was then obtained with a fluorescence microscope. The solution in the Eppendorf tube was analyzed utilizing HPLC.

Example constructing lactone-based linker on agarose-coated 12K-microelectrode arrays

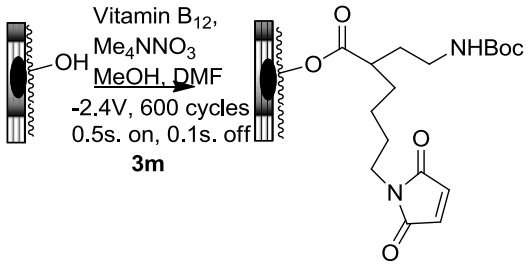
A microelectrode array containing 12468 microelectrodes cm^{-1} was spin coated at 2000rpm for 45 s with 3% agarose in 90% N,N-dimethylformamide and 10% water. After drying the polymer for 1 h, the microelectrode array was placed in a circuit to address the electrodes and then treated with a thin film of solution taken from a stock solution of 3 mg of vitamin B_{12}, 6 mg of tetramethylammonium nitrate, and 8mg of the activated ester in 1.5 mL of methanol. A

volume of 0.1 mL of above solution was used to cover the surface of the array. A whole-board pattern of electrodes was utilized for 600 s at -1.7 V vs a remote Pt-electrode. When the reaction was complete, the array was washed with de-ionized water and 95% ethanol. The array was then treated with 0.1 mL of a second solution derived from 20 mg 1-bromopyrene, 1 mg $Pd(OAc)_2$, 100 uL allyl acetate, 28 uL Et_3N, 1.5 mL acetonitrile, and several drops of DMF. The electrodes were turned on for 900 seconds at -2.0 V relative to the remote Pt-electrode. After being washed with deionized water and 95% ethanol, an image of the array was taken with a fluorescence microscope.

Example cleavage of the Linker off agarose-coated 12K-microelectrode arrays

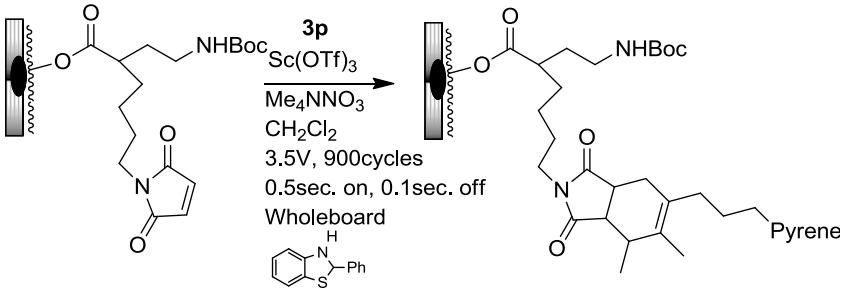
The microelectrode array with the linker built on the surface was treated with 0.1 mL of a solution made from 1.5 mL of acetonitrile, 50 mg of 1,2-diphenyhydrazine, and 100 mg of tetrabutylammonium hexafluorophosphate. An "L (inker)"-pattern was turned on for 120 seconds at +2.4 V relative to a remote Pt-electrode. After being washed with de-ionized water and 95% ethanol, the array was imaged using a fluorescence microscope.

Example characterize complex structures on microelectrode arrays using chemically cleavable linkers with LC-MS

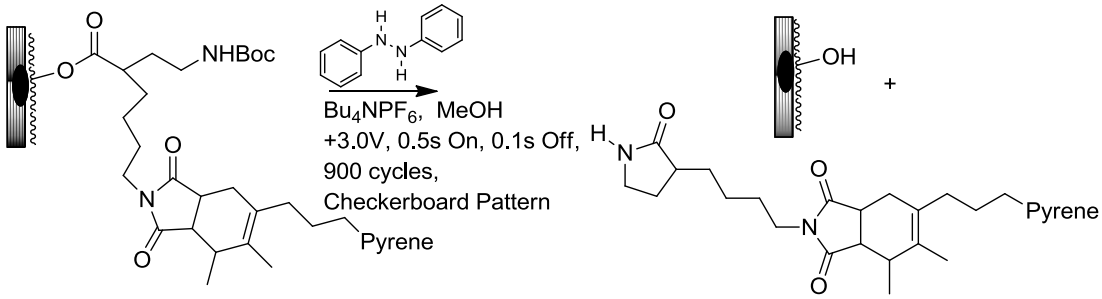


The Microelectrode Array that had been prepared as reported above was insterted into a 1.5

mL Eppendorf tube containing 3 mg of vitamin B_{12}, 6 mg of tetramethylammonium nitrate, and 8 mg **3m** in 1.5 mL of methanol. A whole-board pattern was programmed with 600 potential cycles, -2.4 V relative to the Pt-wire for 0.5 s and 0 V for 0.1 s for each. When the reaction was complete, the array was removed from the solution and then washed with de-ionized water and 95% ethanol.



The array with the dienophile mounted linker loaded was immersed in a solution containing 7.5 mg $Sc(OTf)_3$, 6.6 mg 2-arylbenzothiolzole and 10 mg **3p** in 2.0mL Methylene chloride, which was premixed sealed for 30 minutes to let Sc(III) be reduced completely. The array was then activated as anodes in a whole-board manner. The electrical potential cycle was set as: +3.5 V for 0.5 s and 0 V for 0.1 s. The cycle number was 900. The microelectrode array was washed following the previous procedures and visualized by the fluorescence microscopy.



The Microelectrode Array with the structure built by using the chemistry described above was inserted into the solution consisting of 50 mg 1, 2-diphenyhydrazine, 100 mg tetrabutyl-

ammonium hexafluorophosphate, and 1.5 mL methanol. A checker-board pattern of electrodes was then programmed for 900 potential cycles of on for 0.5 s at +3.0 V and off (0 V) for 0.1 s. After the reaction was complete, the array was removed from the solution and washed with de-ionized water and 95% ethanol. An image of the array was then obtained with a fluorescence microscope. The solution in the Eppendorf tube was collected for LC- MS tests.

3.5.4 General procedures of characterizing compounds using HPLC and LC-MS

Characterization of cleaved linkers using HPLC

Lactone: 10 uL of the solution collected from the cleavage was injected. The column was eluted with 50% methanol/ water for 60 min at a rate of 0.5 mL per minute. The lactone was characterized by coinjection with a known sample made from 1.0 mg of independently synthesized lactone and 1 mL of methanol. 10 uL of this solution was coinjected with the material from the array reaction.

Lactam: 10 uL of the solution collected from the cleavage was injected. The column was eluted with 70% methanol/ water for 60 minutes at a rate of 1.0 mL per minute. The lactam was characterized by coinjection with a known sample made from 1.0 mg of independently synthesized lactam and 1 mL of methanol. 10 uL of this solution was coinected with the material from the array reaction.

Characterization of cleaved linkers using LC-MS

Samples were diluted in 85 % MeOH, 15% H_2O and loaded onto a C18 column (Gemini C18, 5 μm, 110 A, 4.6 mm x 250 mm) (Phenomenex, Torrance, CA) followed by an isocratic elution at 85 % MeOH, 15% H_2O via an Agilent 1200 HPLC (Santa Clara, CA) with flow rate of 1

mL/min. The flow was split for a final flow rate of 200 μL/min prior to the ESI source.

3.6 References

1. Sperry, J. B.; Wright, D. L., "The application of cathodic reductions and anodic oxidations in the synthesis of complex molecules". *Chemical Society Reviews* **2006,** *35* (7), 605-621.

2. Hu, L. B.; Maurer, K.; Moeller, K. D., "Building Addressable Libraries: Site-Selective Suzuki Reactions on Microelectrode Arrays". *Organic Letters* **2009,** *11* (6), 1273-1276.

3. Tian, J.; Maurer, K.; Tesfu, E.; Moeller, K. D., "Building addressable libraries: The use of electrochemistry for spatially isolating a Heck reaction on a chip". *Journal of the American Chemical Society* **2005,** *127* (5), 1392-1393.

4. Tesfu, E.; Maurer, K.; Moeller, K. D., "Building addressable libraries: Spatially isolated, chip-based reductive amination reactions". *Journal of the American Chemical Society* **2006,** *128* (1), 70-71.

5. Maurer, K.; McShea, A.; Strathmann, M.; Dill, K., "The removal of the t-BOC group by electrochemically generated acid and use of an addressable electrode array for peptide synthesis". *Journal of Combinatorial Chemistry* **2005,** *7* (5), 637-640.

6. Kesselring, D.; Maurer, K.; Moeller, K. D., "Microelectrode arrays and ceric ammonium nitrate: A simple strategy for developing new site-selective synthetic methods". *Journal of the American Chemical Society* **2008,** *130* (34), 11290-+.

7. Bi, B.; Maurer, K.; Moeller, K. D., "Building Addressable Libraries: Site-Selective Lewis Acid (Scandium(III)) Catalyzed Reactions". *Angewandte Chemie-International Edition* **2009,** *48* (32), 5872-5874.

8. Hu, L. B.; Bartels, J. L.; Bartels, J. W.; Maurer, K.; Moeller, K. D., "A New Porous Reaction Layer for Developing Addressable Molecular Libraries". *Journal of the American Chemical*

Society **2009,** *131* (46), 16638-+.

9. Chen, C.; Nagy, G.; Walker, A. V.; Maurer, K.; McShea, A.; Moeller, K. D., "Building addressable libraries: The use of a mass spectrometry cleavable linker for monitoring reactions on a microelectrode array". *Journal of the American Chemical Society* **2006,** *128* (50), 16020-16021.

10. Chen, C.; Lu, P.; Walker, A.; Maurer, K.; Moeller, K. D., "The use of a detectable, mass-spectrometry-cleavable linker for quality control on an addressable microelectrode array". *Electrochemistry Communications* **2008,** *10* (7), 973-976.

11. Bartels, J. L.; Lu, P.; Walker, A.; Maurer, K.; Moeller, K. D., "Building addressable libraries: a site-selective click-reaction strategy for rapidly assembling mass spectrometry cleavable linkers". *Chemical Communications* **2009,** (37), 5573-5575.

12. Kenner, G. W.; Mcdermot.Jr; Sheppard, R. C., "Safety Catch Principle in Solid Phase Peptide Synthesis". *Journal of the Chemical Society D-Chemical Communications* **1971,** (12), 636-&.

13. Hu, L. B.; Stuart, M.; Tian, J.; Maurer, K.; Moeller, K. D., "Building Addressable Libraries: Site-Selective Use of Pd(0) Catalysts on Microelectrode Arrays". *Journal of the American Chemical Society* **2010,** *132* (46), 16610-16616.

14. Kesselring, D.; Maurer, K.; Moeller, K. D., "Building addressable libraries: Site-selective formation of an N-acyliminium ion intermediate". *Organic Letters* **2008,** *10* (12), 2501-2504.

15. Stuart, M.; Maurer, K.; Moeller, K. D., "Moving known libraries to an addressable array: A site-selective hetero-Michael reaction". *Bioconjugate Chemistry* **2008,** *19* (8), 1514-1517.

16. Hon, Y. S.; Chang, R. C.; Chau, T. Y., "The Studies of Metal-Ion Catalyzed Carbon-Hydrogen Insertion of Alpha-Alkoxy-Alpha'-Diazoketones Derived from Mandelic and Lactic

Acids". *Heterocycles* **1990,** *31* (10), 1745-1750.

17. Ikawa, T.; Sajiki, H.; Hirota, K., "Unexpected deprotection of silyl and THP ethers induced by serious disparity in the quality of Pd/C catalysts and elucidation of the mechanism". *Tetrahedron* **2004,** *60* (29), 6189-6195.

18. Walker, M. A., "A High-Yielding Synthesis of N-Alkyl Maleimides Using a Novel Modification of the Mitsunobu Reaction". *Journal of Organic Chemistry* **1995,** *60* (16), 5352-5355.

Chapter Four

Linkers and Signaling Studies

4.1 Introduction

In Chapter 3, the development of chemically cleavable linkers was discussed, as a method for determining the structure of molecules placed or synthesized on a microelectrode array. The linkers are structurally simple and compatible with a wide variety of chemical reactions that one might utilize on an array. However, there are two more features that are needed for such a linker to serve as an effective tool for use on a microelectrode array. It must be fluorescent, and it must be compatible with electrochemical signaling studies.

As introduced in Chapter 1, fluorescent linkers are important because they provide a means to assess the quality of not only molecules on an array, but also the array itself. Microelectrode arrays typically fail when a short circuit in their wiring permanently turns on all of the electrodes in a region of the array. When this occurs, a reaction on the array can no longer be confined in that region. It occurs at both the electrodes selected for generating the reagent of choice and every electrode accidently activated by the short-circuit. Since the software used to assess whether an array is working properly simply checks to see if all of the electrodes are active, it does not detect this problem. Hence, the placement of the initial substrate (and linker) on the array is the first opportunity one has to check to see if the array is working properly. A fluorescent linker is particularly useful in this regard. First, if places the linker by each electrode in the array by using two successive checkerboard patterns, then one can assess the quality of the array following the first step. Second, no analysis of the relative binding between molecules on

the surface of the array and a receptor in solution will be valid without knowing the quantity material placed by each electrode in the array. This is especially important since reactions rarely yield 100% conversion and the coating placed on the array will not be completely uniform. Hence, one cannot simply time the length of a reaction on an array and make the assumption that there is a direct correlation between reaction time and surface coverage. A fluorescent linker helps resolve these issues by allowing for quantification of the material placed by any electrode in the array.

With this in mind, an "ideal linker" for use on the arrays will contain sites for the attachment of both a fluorescent tag and the molecules to be monitored for biological activity.

4.2 Design of an "Ideal Linkers"

The first plan for a fluorescent label was to simply take advantage of the pyrene groups we had used on several previous occasions. As shown on the left of **Figure 4.1**, the first plan called for an approach nearly identical to the linker used in Chapter 3. In this case, a disubstituted pyrene would be placed in the chain connecting the linker and the molecule to be studied.[1-3] However, from the start this plan was worrisome. Construction of the disubsituted pyrene core of the linker required the bromination of a pyrenyl butanoic acid derivative, a reaction that led to a complex mixture of products, generated only low yields of those products, and could not be scaled-up.[4] Because of these synthetic problems, the compatibility of the linker with electrochemical signaling experiments on the arrays was not determined.

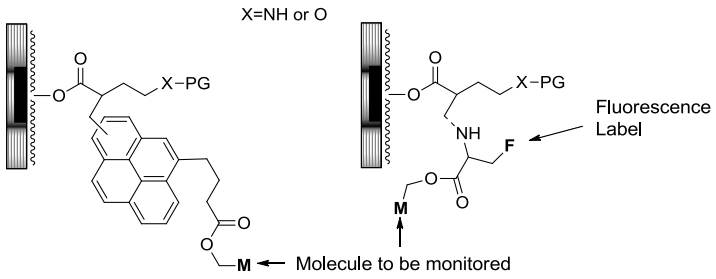


Figure 4.1 Designs of the ideal linkers: a) pyrene derivatives (left) and b) amino-acid derivatives (right)

A better approach was needed. It appears that a fluorescent amino acid derivative might provide a solution to this synthetic challenge (**Figure 4.1b)**. In this case the fluorescent group does not need to serve as a bridge between groups, and the C- and N- terminuses of the amino acid can be used to attach the chemically cleavable linker to the ligand of interests. The fluorescent label would remain unchanged through those reactions. The approach was intriguing because it would allow for variations in the fluorescent group to be made without requiring a change in the overall strategy. One more advantage about this fluorescence-tagged amino acid derivative is that it could also be utilized independent of the cleavable-linker strategy by directly attaching the C- terminus of the labeled amino acid to the array. This increases the versatility of the fluorescent label and provides an opportunity to quickly test of the compatibility of the label with the electrochemical signaling experiments. For this thesis, the fluorescent amino acid derivative will be referred to as the "fluorescent linker" whenever it is used independently from the cleavable linker portion of the "ideal linker" ("ultimate linker").

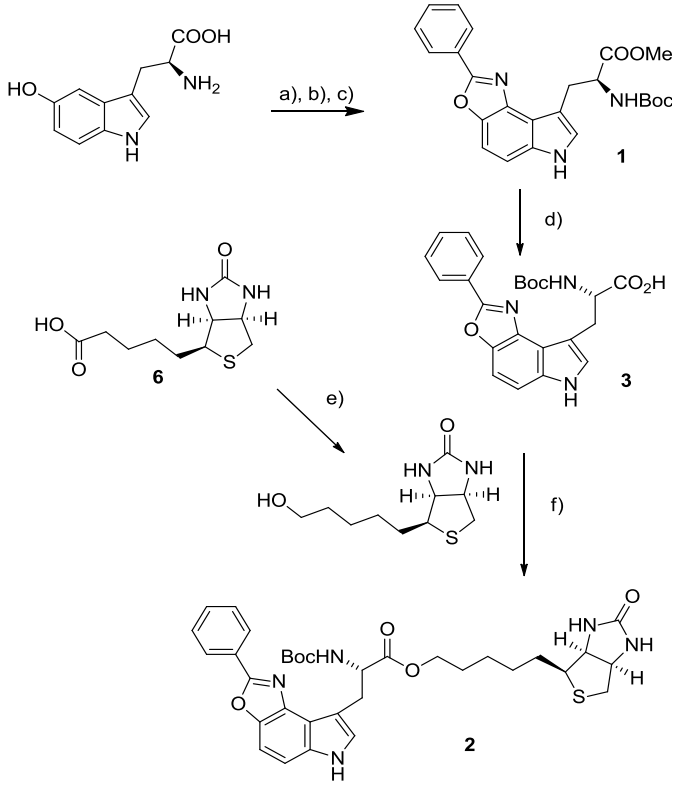
4.3 The Fluorescent Linker

The development of a fluorescent linker was faced with two key questions: first, what should the fluorescent group be, and second, is the fluorescent group and the amino-acid linker

compatible with the electrochemical impedance experiments employed for monitoring binding events on the arrays?

The choice of a fluorescent tag was based on the availability of the amino acid starting material and the simplicity of the synthetic route needed for its construction. With this in mind, we elected to take advantage of an oxidative strategy for synthesizing fluorescent groups from hydroxylated indole rings.[5] The synthesis started with 5-hydroxytryptophan (**Scheme 4.1**).

Scheme 4.1 Synthesis of the fluorescent linker



Reagents and Conditions: a) $SOCl_2$, MeOH, RT. b) Boc_2O, Na_2CO_3, Dioxane/H_2O, RT. c) MnO_2, Benzyl amine, dry THF, 0^oC-RT, 31% in three steps. d) LiOH, THF/MeOH/H_2O, RT, 92%. e) $LiAlH_4$, THF, 45^oC, 84%. f) DCC, DMAP, DMF, RT, 63%.

Following protection of the C-terminus and N-terminus of the amino acid, the fluorescent label was constructed from the hydroxyindol with the use of an oxidative cycloaddition. While

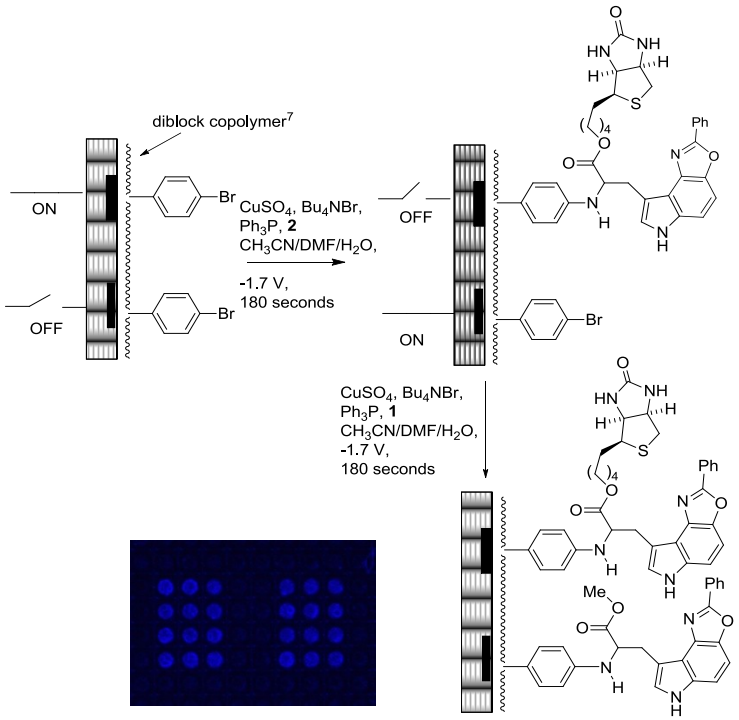
the yield of the cycloaddition was low, it provided a readily scalable, one-step synthesis of the fluorescent group that could be varied by simply changing the arylamine used. The skeleton of the fluorescent linker **1** was thus completed.

To test the chemistry and biology compatibilities of this structure, biotin was attached to the C-terminus of the amino acid derivative so that biotin/streptavidin interactions could be used to determine the compatibility of the linker with electrochemical-impedance experiments on the arrays. This was accomplished by saponification followed by esterification of the resulting acid with the alcohol.[6]

The compound **2** was then placed onto an array having 12,544 microelectrodes·cm^{-1} (**Scheme 4.2**). The array was coated with a diblock copolymer containing a cinnamoyl-functionalized methacrylate block and a poly-*p*-bromostyrene block.[7] The cinammoyl groups were photochemically crosslinked to provide the necessary stability for the surface. At this point, the *t*-Boc-protected amine in compound **2** was directly coupled to the poly-*p*-bromostyrene block of the polymer proximal to selected electrodes in the array by using a previously developed Cu(I)-coupling strategy.[7-10] To this end, the array was treated with a $CH_3CN/DMF/H_2O$ solution containing **2**, copper sulfate, triphenylphosphine, and tetrabutylammonium bromide. The electrodes selected for the reaction were set at a potential of – 1.7 V vs. the Pt-counter electrode for 180 s in order to reduce the copper sulfate to Cu(I) exactly where the catalyst was needed. Oxygen was used as a solution-phase oxidant in order to destroy the Cu(I)-catalyst before it could migrate to remote sites on the array. Initially, the electrolysis reaction was conducted at ten-separate sites on the array each containing a block of 12 microelectrodes. When the reaction was complete, the array was washed with water and ethanol. The reaction was then repeated, but in this second case compound **1** was used as the substrate along with a new set of electrodes.

Again, the reaction was run at ten-separate sites on the array each containing a block of 12 microelectrodes. The result was an array that contained both blocks of electrodes that were functionalized with **2** and blocks of electrodes that were functionalized with **1**. When the reactions were complete, the array was washed with water and ethanol and then imaged with a fluorescence microscope.

Scheme 4.2 Placing the fluorescent linker onto Microelectrode Array



The picture provided in **Scheme 4.2** shows two of the electrode blocks functionalized with the linker that had been attached to biotin. The image clearly shows that the array worked perfectly. The reaction was nicely confined to the selected electrodes, and roughly equal amounts of material were placed at each of the electrodes.

Interestingly, solution-phase experiments using these same reaction conditions showed that Cu(I) did not catalyze the addition of either a t-Boc protected nitrogen or an indole ring to an arylbromide. Only the amine could be coupled. It appears that the array reaction initially leads to removal of the t-Boc group and then a subsequent coupling between the amine and then the arylbromide polymer. The deprotection is not unexpected since the reaction on the array is an undivided cell electrolysis that generates acid at the anode and we have seen the result of acid catalyzed reactions previously.

4.4 Signaling Studies

With the functionalized array in hand, signaling experiments were conducted by randomly selecting three of the ten-electrode blocks functionalized with the linker plus biotin, three of the ten-electrode blocks functionalized with only the linker, and three unfunctionalized blocks of 12 electrodes each from the remainder of the array. The array was then submerged in a series of solutions containing streptavidin and 8 mM $K_4[Fe(CN)_4]_2/K_3[Fe(CN)_4]_3$ in 1X phosphate buffer saline (PBS). The solutions ranged in concentration of streptavidin from 10^{-6} to 10^{-20} M. The first solution used was the 10^{-20} M streptavidin solution. After submerging the array in this solution, a cyclic voltammogram was recorded for the iron in solution at each of the nine selected blocks of electrodes by sweeping the potential at the electrodes from -700 to 700 mV relative to the counter electrode.[11] A sweep rate of 400 mV/s was used. Following this experiment, the array was washed three times with a 10^{-19} M streptavidin solution and inserted into the next solution having 10^{-19} M streptavidin. This was done to insure a consistent concentration of 10^{-19} M streptavidin across the surface of the array. The cyclic voltammetry experiment was then repeated at all nine blocks of electrodes. The array was then washed three times with a 10^{-18} M streptavidin solution and inserted into the solution having 10^{-18} M streptavidin. The cyclic

voltammetry experiment was again repeated at all nine blocks of electrodes. This process was continued until a cyclic voltammogram for the iron in solution had been recorded at all nine blocks of electrodes for every concentration of streptavidin. The data recorded at one of the blocks of electrodes having biotin on the surface are shown in **Figure 4.2**. If one examines the current measured for iron at a potential of 25, mV, then it can be seen how the current at the block of electrodes dropped as the concentration of streptavidin increased. The presence of streptavidin causes a drop in current because it binds the biotin on the surface of the array and blocks iron from reaching the electrode below.

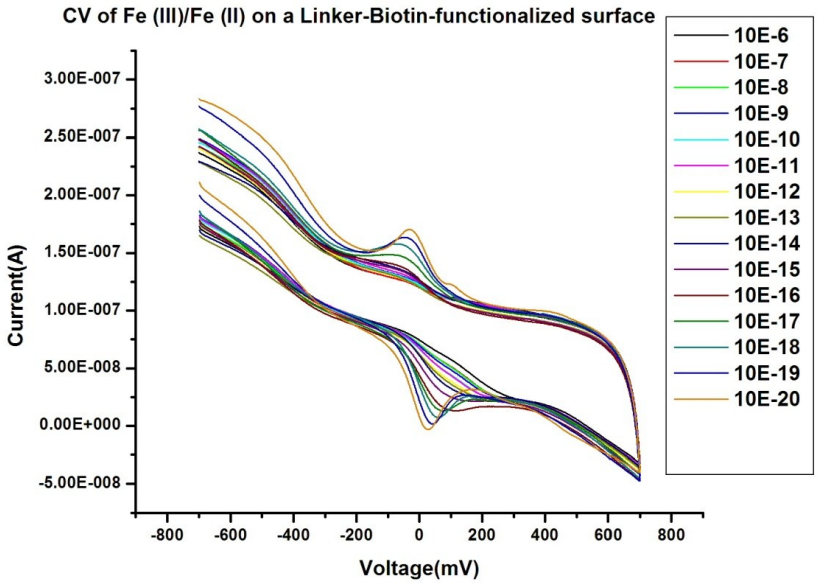


Figure 4.2 Sample voltammograms for one block of electrodes

The current measured at this potential was then used to compare all of the cyclic voltammograms measured at each concentration of streptavidin used. This data is summarized in **Figure 4.3**. Each data point in the figure is the average of the current measured for the three blocks of electrodes used at 25 mV. For example, the data point on the red line at a concentration

of 10^{-14} M streptavidin represents the average current measured at 25 mV for this concentration of receptor at the three blocks of electrodes

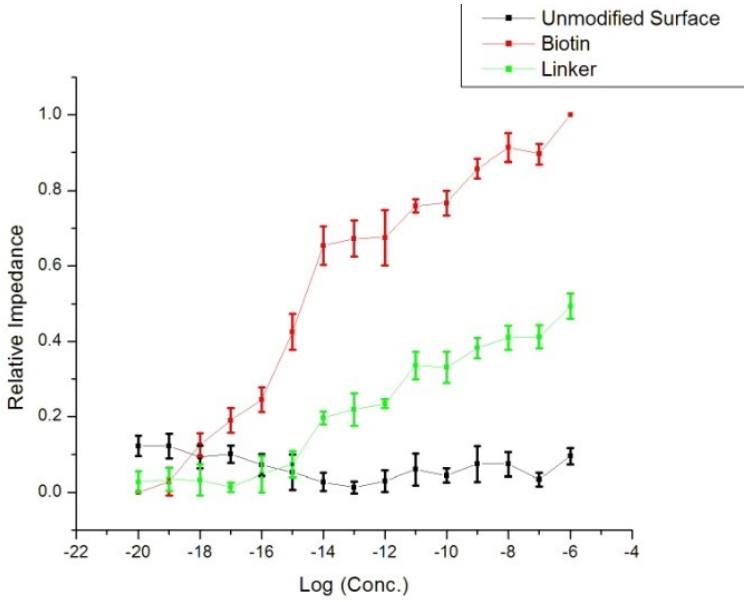


Figure 4.3 Summary of the CV data for all nine blocks of electrodes. Normalized curves are shown for the impedance measured electrodes functionalized with the fluorescent linker and biotin (red), electrodes functionalized with the fluorescent linker and no biotin (green), and unfunctionalized electrodes (black)

with the linker plus biotin on the surface. The point on the green line at this concentration represents the average current measured at 25 mV for the three blocks of electrodes with only the linker on the surface. The data has been normalized and the largest change in current (greatest impedance) observed scaled from zero to one. The largest current measured for each set of data was given a value of zero. In this way, the data directly reflects the binding of streptavidin to the surface of the array. The larger number on the vertical axis, the greater the binding of the receptor to the surface.

The black line in **Figure 4.3** summarizes the data for the three blocks of unfunctionalized

electrodes taken at random places on the array. This data shows that the streptavidin has no binding to the un-functionalized polymer coating the array. The green line in the Figure shows the data for the three blocks of electrodes functionalized with the linker (**1**) and no biotin. It indicates that the streptavidin does bind the surface once it has been functionalized. The red line shows the binding of streptavidin to the surface of the electrodes functionalized with the linker and biotin (**2**). The difference between the green and red lines shows that the presence of the biotin causes a significant increase in the binding of the streptavidin to the surface of the electrodes.[12] The nature of this difference can be seen clearly by taking the difference between the lines (**Figure 4.4**). Initially, the binding of streptavidin to the surface of the electrodes functionalized with biotin increases much more rapidly with increasing protein concentration than it does to the surface of the electrodes that are functionalized with only the linker. At higher concentrations of streptavidin this difference falls off and the rate of change at the two electrodes with increasing streptavidin concentration becomes almost equal (parallel slopes in **Figure 4.3**).

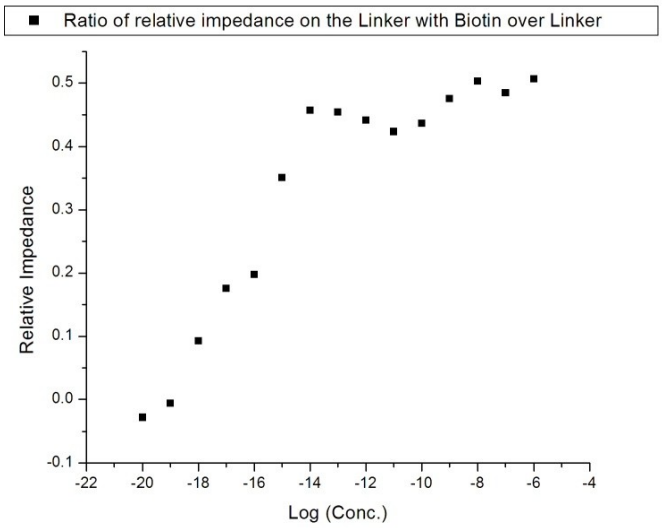


Figure 4.4 Difference data for the current drops associated with the electrodes functionalized with the linker plus biotin (red line in **Figure 4.3**) and the electrodes functionalized with only the linker (green line in **Figure 4.3**)

Since there is only about 20-50 fmole of material by each electrode in the array, at high concentrations of streptavidin the biotin becomes completely bound. Hence, any further binding of streptavidin at these sites results from non-specific binding of the streptavidin to the surface. The fact that at these concentrations the rate of change in the impedance at sites with biotin is the same as the rate of change for sites without the biotin indicates that the non-specific binding of streptavidin to the surface is roughly the same in both places. Hence, the difference in impedance between electrodes functionalized with biotin and electrodes functionalized with only the linker at low concentrations shows the binding of streptavidin to the biotin. Clearly, both the polymer surface coating the electrodes and the fluorescent linker are compatible with the electrochemical impedance experiments needed to monitor binding events on the arrays.

To this end, the use of an amino-acid-derived fluorescent linker has proven to be an effective and practical method for the placement of molecules onto the surface of a microelectrode array. The fluorescent linker itself is easy to synthesize and can be rapidly functionalized with different biological probes to be studied or substrates to be developed on the array's surface. It enables quality-control assessment of the array itself, is compatible with the analytical experiments used to probe binding events on the surface of the array, and is stable enough to allow for the multiple analytical experiments needed to evaluate binding events in a quantitative manner.[13]

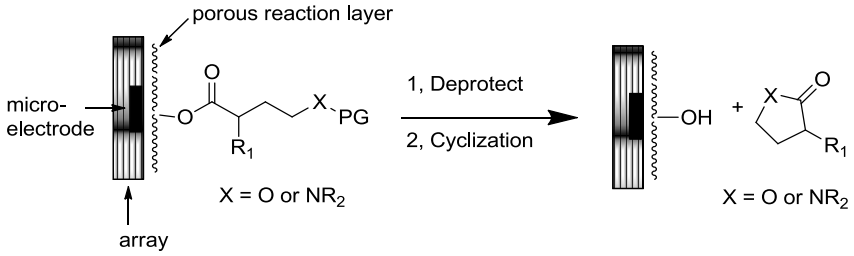
4.5 Efforts toward an "Ultimate Linker"

The success of making a biocompatible fluorescent structure clears a big obstacle on the way towards constructing an ideal linker that enables quality control of both the array and the molecules placed on its surface.

However, other lessons have been learned that suggested a change in strategy from the

simple design illustrated above in **Figure 4.1**. That design simply planned on incorporating the fluorescent-labeled amino acid into the cleavable linker strategy into the R_1 position shown in **Scheme 4.3**.

Scheme 4.3 Revisit of the chemically cleavable linker[14,15]



However, the long-term plan for the linker requires the linker to be versatile enough to accommodate a wide-range of receptor ligands attached to the end of R_1 in addition to the fluorescent group. For a larger library, these ligands would ideally be assembled on the array rather than synthesized independently and then transferred to the array one at a time. Hence, the linker must be compatible with multiple reaction methodologies. While the amino acid part of the fluorescent linker developed above should not be problematic in this regard, the same cannot be said of the fluorescent label itself. For example, we have already shown that the groups are not compatible with oxidation chemistry. Hence, if the fluorescent label is grafted onto the linker before the ligands are constructed, then it will limit the chemistry available for building the ligands.[10]

Moreover, we have shown in previous studies that the distance between the surface of electrodes and the molecule being studied can influence the subsequent signaling experiment run

on the array. Hence, in an ideal scenario we would be able to add a fluorescent label to the linker without increasing the distance between the molecule to be studied and the surface of the array.

Based upon the above considerations, a design of an "ultimate linker" is shown in

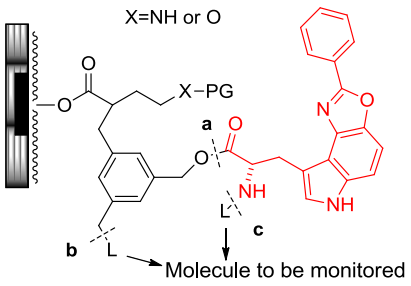
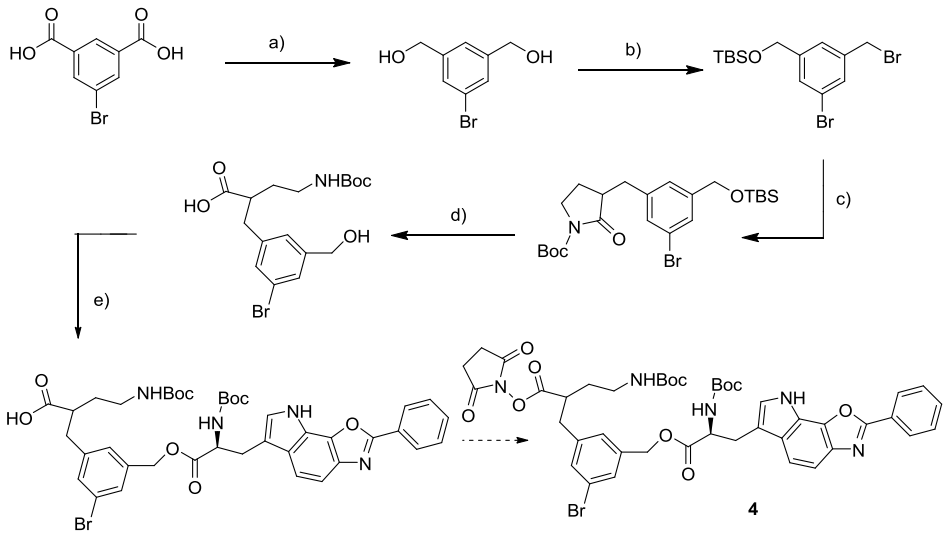


Figure 4.5 "Ultimate Linker"

Figure 4.5. In this example, an aromatic ring would be used as a central attachment point for the cleavable linker, the molecule to be monitored, and the fluorescent tag. Alternatively, the molecule to be studied could be added to the fluorescent group at site **c** in analogy to the initial fluorescent linker design. One of the major benefits of this linker design is that the fluorescent group can be added to site **b** of the linker after construction of a molecule to be studied at site **a**. In this way, the fluorescent tag can be introduced after any potentially incompatible chemistry is accomplished.

Scheme 4.4 Synthesis of the "Ultimate Linker"



Reagents and Conditions: a) $LiAlH_4$, THF, 0°C-RT, 91%. b) NaH, TBDMSCl, THF, 0°C-RT, CBr_4, PPh_3, CH_2Cl_2, 0°C, 84% in two steps. c) Isopropylamine, n-butyllithium, 1-(tert-butoxycarbonyl)-2-pyrrolidinone, THF, -78°C, 87%. d) LiOH, THF/H_2O, 0°C-RT, TBAF, THF, 0°C-RT, 71% in two steps. e) **3 in Scheme 4.1**, EDC, DMAP, DMF, 0°C-RT, diastereomer ratio: 1:1, 38% combined.

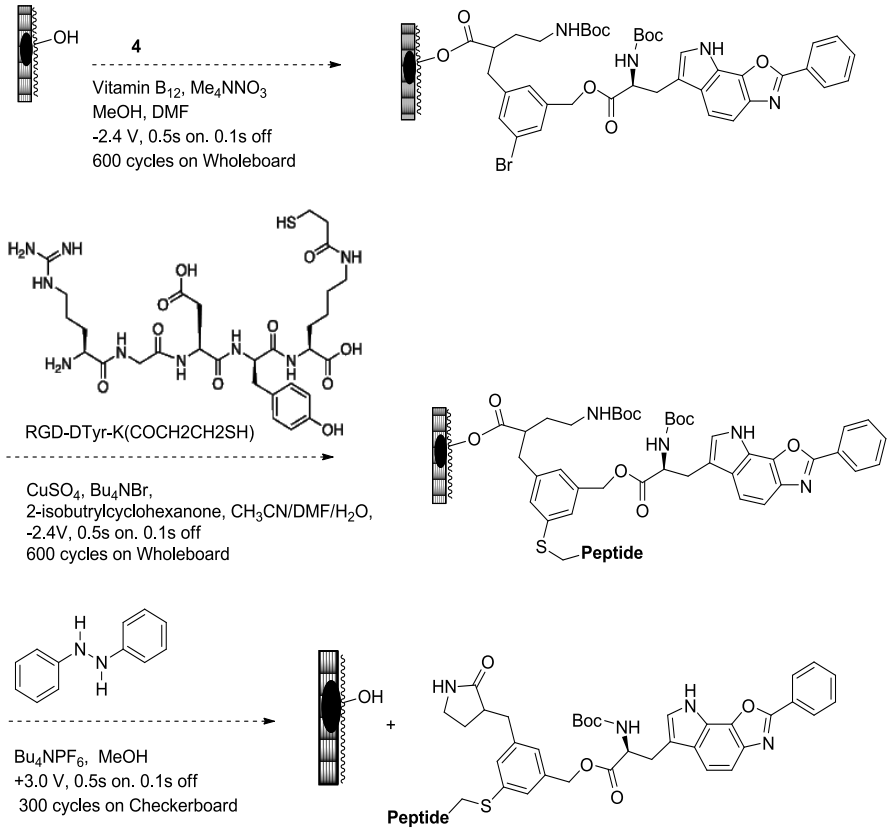
The preparation of a prototype linker began with 5-bromobenzen-1,3-dicarboxylic acid. The acids were reduced to form a diol with $LiAlH_4$. Protection of one of the two hydroxyl groups, followed by exchange of the remaining hydrodroxy group with a bromine led to a substrate for the same lactam-alkylation reaction used previously. The alkylation led to lactam **4e**. The lactam was saponified, and then the silyl protecting group removed from the benzylic alcohol. The acid alcohol was then coupled to the fluorescent linker synthesized in **Scheme 4.1** with the used of an EDC-based esterification reaction. The yield of the reaction was only 38%. The difficulty was perhaps the presence of the acid in the linker substrate that might compete with the acid of the fluorescent linker in the coupling reaction. The order of steps was undertaken in order to avoid problems with the ester linking the fluorescent group to the linker during opening of the lactam. Clearly, a different strategy will be needed in order to optimize the yield of the ultimate linker in the future. For example, it is possible that the acid of the linker can be attached to the solid

support coating the array before the TBS protecting group is removed from the benzyl alcohol and the fluorescent group added. However, for the present we wanted to be able to fully characterize the molecule to be placed on the array. So, the current synthetic route was followed. This route will conclude by converting the acid of the linker to a succinimide ester that can be used for placing the entire ensemble onto an array (**Scheme 4.4**). For these studies, the molecule to be examined for binding to a receptor can be added to the linker with the remaining aryl bromide either before or after the linker is placed onto the array.

The array-chemistry to be used will not be new.[14,15] The process will start by coating an array having 1024 microelectrodes·cm^{-2} with a di-block copolymer that contains hydroxyl functional groups.[7] The reaction between the activated ester and the polymer will be catalyzed by base generated by the reduction of vitamin-B_{12} at selected electrodes in the array. (**Scheme 4.5**)

To test the utility of the array for signaling studies, a penta-peptide with an imbedded RGD sequence and a thiol nucleophile will be added to the aryl bromide in the linker with the use of a Cu(I)-catalyzed addition. The method is related to the approach used to test the compatibility of the initial fluorescent linker with electrochemically signaling studies. In the original paper, the feasibility of the signaling experiment was determined by examining an RGD-peptide–integrin interaction. In this case, the linker used with hopefully allow for the binding study and then provide a handle for characterization of the molecule at the end in order to make sure that it has not changed. In terms of the detailed plans for the test, peptide RGD-DTyr-K(COCH2CH2SH) will be placed by

Scheme 4.5 Proof-of-principle study of "Ultimate Linker" on Microelectrode Array



submerging the functionalized 1-K array in a 7:2:1 acetonitrile to dimethylformade to water solution that contains the peptide, copper sulfate, a 1,3-dicarbonyl ligand for the copper, and tetrabutylammonium bromide as an electrolyte. All electrodes in the array will then be used as cathodes by setting them to a potential of -2.4 V relative to a remote Pt-anode. The electrodes are programmed for 600 cycles, each of which consists of 0.5s on and 0.1s off. Oxygen will be used in solution as a 'confining-agent" (oxidant for Cu(I)) in order to destroy the catalyst before it can migrate to electrodes not selected for the reaction.

After the signaling studies, the last step showed in **Scheme 4.5** will be conducted in order to reclaim the substrate from the surface of the electrodes. This chemistry will simply repeat the chemistry described earlier in Chapter 3. This final part of the study is currently underway, as

well as the impedance experiment demonstrating the compatibility of the "ultimate linker" with the signaling experiments.

4.5 Conclusion

The effort to assess the quality of molecules made on arrays and of the arrays themselves continues with the design and synthesis of a versatile linker that has a central aromatic ring. The aromatic ring can be used as a central linking platform for the addition of the safety catch linker, the molecule to be studied, and a fluorescent tag. The groups can be added in any order so that a sensitive fluorescent tag can be introduced after methodology to which it may not be compatible.

Along these lines, an amino-acid-derived fluorescent tag has been developed in order to replace the pyrene-based systems used previously. This fluorescent group is easy to prepare and to modify. In addition, the reliability of the based-catalyzed esterification strategy for attaching the linker the arrays makes it possible to check the condition of the device before any chemistry and biological analysis begins. The compatibility of the fluorescent linker with the signaling studies was accomplished with the use of the biotin-strepavidin interaction, as well as a set of general procedures for running the impedance experiments and processing the electrochemical data. [16]

Finally, the synthesis of the "ultimate linker" is underway. Only one step remains before the linker can be placed onto an array and its compatibility with electrochemical signaling studies examined.

4.6 Experimental Procedures

4.6.1 General information of reagents and instruments

Chemical reagents and solvents were purchased from Sigma-Aldrich, Alfa Aesar,

Mallinckrodt Baker, EMD Chemicals (Merck KGaA), and used without further purification unless otherwise noted. Dichloromethane (CH_2Cl_2) was distilled over calcium hydride. Anhydrous tetrahydrofuran (THF) was distilled over sodium benzophenone ketyl. Tetraethylammonium p-toluenesulfonate (Et_4NOTs) was dried in an oven before use. Flash chromatography was carried out by using silica gel (200-400 mesh) purchased from Dynamic Adsorbent, Inc.

All proton and carbon-13 nuclear magnetic resonance (^1H and ^{13}C NMR) spectra were recorded by a Varian Inova 600, a Varian Inova 500, or a Varian Mercury 300 in a deuterated chloroform ($CDCl_3$) solvent with tetramethylsilane (TMS) as an internal standard unless otherwise noted. Infrared (IR) spectra were recorded by a Perkin Elmer Spectrum BS FT-IR instrument. High resolution electrospray ionization (ESI) mass spectra were obtained at the NIH/NCRR Mass Spectrometry Resource Facility at Washington University in St. Louis.

Fluorescence images were taken with a Nikon Eclipse E200 microscope connected to a Boyce Scientific, M-100 burner and a Nikon D5000 camera. CFW-BP01-Clinical-000 (Semrock) filter cube excitation 380-395 nm/emission 420-470 nm, ET-GFP (FITC/Cy2) (Chroma) filter cube excitation 450-490 nm, emission 500-550 nm and TxRed-A-Basic-000 (Semrock) filter cube excitation 540-580 nm, emission 590-670.

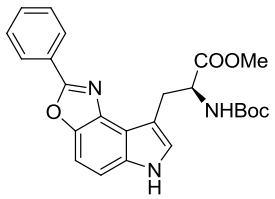
Microelectrode arrays were spin coated with a spin coater spin-coater MODEL WS-400B-6NPP/LITE.

An ElectraSense® reader (Available from CustomArray) was used to address the electrodes on the array using a personal computer. For voltammetric measurements, the reader is used to activate selected electrodes on the array and an external BAS 100B/W potentiastat controls the

potential sweep.

4.6.2 Syntheses and compounds

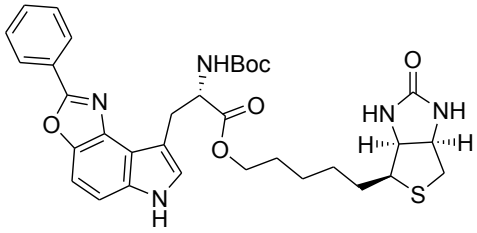
Synthesis of the Fluorescent Linker



(S)-methyl 2-(tert-butoxycarbonylamino)-3-(2-phenyl-6H-oxazolo[4,5-e]indol-8-yl)propanoate (4a)

To a solution of 5-hydroxy serotonin (650 mg, 3.0 mmol) in MeOH (12 mL) was added $SOCl_2$ (693 μL) at 0°C. After being stirred overnight, the reaction mixture was concentrated in vacuo. The residue was diluted with 20 mL of a dioxane / H_2O (2 / 1) solution, and the resulting mixture treated with Na_2CO_3 (4.5 eq.) and Boc_2O (1.1 eq.) at 0°C. After being stirred overnight, the reaction mixture was quenched aq. NH_4Cl. The mixture was extracted with EtOAc, the organic layer concentrated *in vacuo*, and the residue purified by column chromotagraphy through silica gel using Hexane / EtOAc (2 : 1) as eluant. The purified product was dissolved in dry THF (56 mL) and then MnO_2 (2.58 g, ca 10 eq.) and benzyl amine (2.58 mL, 10 eq.) added at 0°C.[5] After being stirred for two days, the mixture was filtered with through celite and washed with MeOH. The filtered solution was concentrated *in vacuo* and the residue was purified by chromatography though silica gel using a dichloromethane / EtOAc (10 : 1) mixture as eluant. The product was afforded in 31 % yield over the three steps. The spectal data for the product was as follows. ^1H NMR ($CDCl_3$/300MHz) δ 8.47 (broad s, 1H), 8.33 (m, 2H), 7.59 (m, 3H), 7.45 (m,

2H), 7.33 (m, 1H), 7.15 (m, 1H), 4.66 (m, X in ABX, 1H), 3.75 (s, 3H), 3.52 (m, A and B in ABX, 2H), 1.30 (bs, 1H), 1.15 (s, 9H) ppm, ^{13}C NMR ($CDCl_3$/300MHz) δ173.5, 156.5, 146.0, 134.3, 131.1, 129.0, 127.6, 124.7, 124.3, 124.1, 121.8, 119.1, 111.0, 109.2, 105.0, 100.1, 79.3, 74.9, 56.9, 52.3, 28.6, 28.3 ppm; IR (KBr) 3305, 2976, 2950, 1738, 1700, 1549, 1529, 1487, 1453, 1377, 1366, 1221, 1202, 1056, 995, 775, 709 cm^{-1}. HRESI MS m/z $[M+Na]^+$ found 458.1692, calcd. 458.1692.



(S)-5-((3aS, 4S, 6aR)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentyl 2-(tert-butoxycarbonylamino)-2-(2-phenyl-6H-oxazolo[4,5-e]indol-8-yl)acetate (4b)

Preparation of biotinol:[6]

Biotin (1.0 g, 4 mmol) was suspended in 15 mL of THF and then 4.8 mL of a 1 *M* solution of $LiAlH_4$ in THF added drop-wise. The mixture was heated to 45 ^{o}C for 3 h followed by the addition of a second portion of the $LiAlH_4$ solution (19.2 mL). Stirring was continued at 45 ^{o}C until the reaction was complete as evidenced by silica gel TLC (20% MeOH in acetone as eluant). The reaction was cooled to room temperature, and then water carefully dripped into the mixture until the bubbling stopped. The resulting mixture was concentrated under reduced pressure and then the resulting residue taken up in ethanol filtered, dried, and concentrated to afford 795 mg (84 %) of a white solid. The spectrum obtained for the product matched the literature. The product was carried on without further purification.

Hydrolysis of **4a** to form carboxylic acid **4c**:

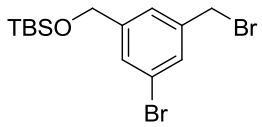
Compound **4a** (43.5 mg, 0.1 mmol) was dissolved in 5 mL of a 3/1/1 THF/ MeOH/ H_2O solution. To this mixture was added approximately 5 equiv. of LiOH. The reaction was allowed to run at room temperature for 3h after which is was concentrated *in vacuo*. The residual solution was carefully acidified with 10 % citric acid in water solution to a pH of 3 to 4. The organic material was then extracted with EtOAc and the combined organic layers dried and concentrated *in vacuo*. The resulting carboxylic acid was used in the next step without further purification.

Coupling of biotinol and acid **4c**:

Approximate 30 mg of biotinol (0.13 mmol), 45 mg of acid **4c** (0.11 mmol), 7 mg DMAP (0.5 equivalents), and 21 mg of EDC (1.1 equivalents) were dissolved in 3 mL of DMF at 0 ^{o}C. The mixture was allowed to warm to room temperature and stirred overnight. Following the reaction, the solution was acidified to a pH of 4 to 5, diluted with a 5% aqueous LiCl solution, and then extracted with EtOAc. The combined organic layers were dried, concentrated *in vacuo*, and then purified through a silica gel column using 5% MeOH in EtOAc as eluent. Product **4b** was formed in a 47% isolated yield (35.2 mg) after two steps.

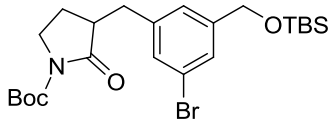
^{1}H NMR ($CDCl_3$/300MHz) δ 9.28 (s, 1H), 8.34 (m, 2H), 7.59-7.31 (m, 5H), 7.16 (m, 1H), 5.97 (bs, 1H), 5.40 (bs, 1H), 4.55 (m, 1H), 4.66 (m, X in ABX, 1H), 4.36 (m, 1H), 4.08 (t, J= 6 Hz, 2H), 3.53 (m, A and B in ABX, 2H), 3.14 (m, 2H), 2.90 (m, 2H), 2.72 (m, 2H), 1.66 (m, 2H), 1.64 (s, 9H), 1.21 (m, 4H) ppm; ^{13}C NMR ($CDCl_3$/300MHz) δ173. 6, 173.5, 163.7, 156.5, 146.0, 134.3, 132.1, 129.4, 127.6, 125.7, 124.3, 124.2, 121.8, 119.7, 111.3, 109.2, 104.7, 100.3, 79.3, 74.9, 61.9, 56.9, 55.4, 40.4, 33.9, 28.5, 28.3, 28.0, 24.7 ppm; IR (KBr) 3247, 2935, 2362, 1729, 1700, 1562, 1534, 1477, 1377, 1320, 1202, 1056, 995, 745 cm $^{-1}$. HRESI MS m/z of $[M+H]^{+}$

found 634.2361, calculated 634.2699.



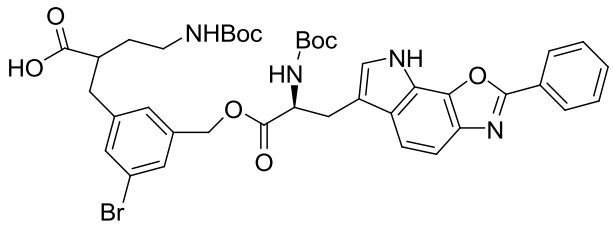
3-bromo-5-(bromomethyl)benzyl)oxy)(tert-butyl)dimethylsilane (4d)

Approximate 130 mg NaH (60% dispersion in mineral oil, 3 mmol) was suspended in 5 mL THF under argon at 0 ºC. It was added dropwisely to a THF solution containing 2.17 g (5-bromo-1,3-phenylene)dimethanol (10 mmol). After being stirred for 2 hours, a total of 450 mg TBDMSCl (3 mmol) was added to the solution at one time. The reaction was left slowly raised to room temperature and stirred overnight before being quenched by water and concentrated *in vacuo*. Organic compounds were extracted by ethyl acetate. After a flash chromatography with silica gel and 25% ethyl acetate in a mixed solvent with hexane, the product was dissolved 20 mL CH_2Cl_2. CBr_4 and PPh_3 (1 equiv. for each) were added to the solution at 0 ºC. After being stirred for about 4 hours, approximate 50 mL hexane was poured into the solution. The precipitation was filtered with celite pad. The solution was concentrated at reduced pressure before being purified with another flash chromatography. The product (0.99 mg, 84%) was afforded. ^{1}H NMR ($CDCl_3$/300MHz) δ 7.40 (s, 2H), 7.25 (s, 1H), 4.59 (s, 2H), 4.42 (s, 2H), 0.94 (s, 9H), 0.11 (s, 6H) ppm, ^{13}C NMR ($CDCl_3$/300MHz) δ144.3, 139.6, 130.4, 129.0, 125.1, 122.4, 63.9, 32.2, 25.9, 18.4, -5.3 ppm; IR (KBr) 2954, 2927, 2855, 1783, 1720, 1574, 1471, 1462, 1444, 1368, 1255, 1153, 1114, 1093, 817 cm^{-1}. HRESI MS m/z $[M+Na]^+$ 416.9688 found, 416.9684 calcd.



tert-butyl 3-(3-bromo-5-(((tert-butyldimethylsilyl)oxy)methyl)benzyl)-2-oxopyrrolidine-1-carboxylate (4e)

Diisopropyl amine (0.1 mL, 1.2 equiv.) was dissolved in 5 mL of anhydrous THF. The solution was cooled to -78^{o}C and 0.36 mL (1 equiv.) of a 1.6 *M* n-BuLi in hexane solution was added dropwisely. The mixture was stirred for 30 min before adding 108 mg (0.66 mmol) of 1-(tert-butoxycarbonyl)-2-pyrrolidinone. The reaction was stirred at -78^{o}C for another 30 minutes before slowly adding a solution of 230 mg (0.66 mmol) **4d** in 2 mL of cold anhydrous THF. The mixture was stirred at -78 ^{o}C for 7 h and then quenched with saturated ammonium chloride. The reaction was diluted with ethyl acetate and washed with water. The organic phase was concentrated *in vacuo* and then chromatographed through silica gel using a an eluant of 15% ethyl acetate in hexane to afford 286 mg (87%) product. ^{1}H NMR ($CDCl_3$/300MHz) δ 7.32 (s, 1H), 7.19 (s, 1H), 7.05 (s, 1H), 4.66 (s, 2H), 3.68 (m, 1H), 3.52 (m, 1H), 3.23 (A in ABX, J_{AB}= 13.8 Hz, J_{AX}= 3.9 Hz, 1H), 2.74 (m, 1H), 2.57 (B in ABX, J_{AB}= 13.8 Hz, J_{BX}= 9.9 Hz, 1H), 2.02 (m, 1H), 1.53 (m, 1H), 1.52 (s, 9H), 0.92 (s, 9H), 0.08 (s, 6H) ppm, ^{13}C NMR ($CDCl_3$/300MHz) δ174.7, 150.2, 144.0, 141.0, 130.2, 127.2, 125.1, 122.5, 82.9, 64.1, 45.3, 44.3, 36.0, 28.0, 25.9, 23.9, 18.4, -5.3 ppm; IR (KBr) 3349, 2954, 2930, 2885, 1698, 1682, 1651, 1573, 1519, 1446, 1367, 1252, 1165, 1114, 838 cm^{-1}. HRESI MS m/z $[M+Na]^+$ 522.1475 found, 522.1474 calcd.



2-(3-bromo-5-((((S)-2-((tert-butoxycarbonyl)amino)-3-(2-phenyl-8H-oxazolo[4,5-g]indol-6-yl)propanoyl)oxy)methyl)benzyl)-4-((tert-butoxycarbonyl)amino)butanoic acid (4f)

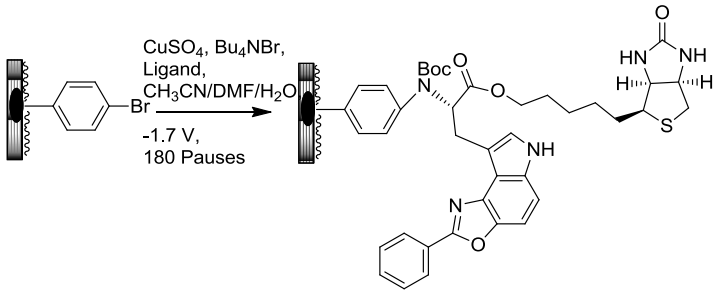
To a solution of 249 mg (0.5 mmol) **4e** in a mixture of 7 mL THF and 1.5 mL of water was added 80 mg (~ 2 equiv.) of LiOH. After stirring overnight, the reaction solution was concentrated *in vacuo* and diluted with water. The aqueous solution was acidified with CH_3COOH to pH=4~5 and then extracted with ethyl acetate. The combined organic layers were washed with brine and dried over sodium sulfate. The solvent was removed by distillation under reduced pressure before a flash chromatography with silica gel and 30% ethyl acetate in hexane to afford a pure product. The product was then dissolved in 4 mL dry THF. After being cooled down to 0 ºC, the solution was added 1 mL TBAF solution (0.6 M in THF). The reaction was stirred and allowed to warm up to the room temperature for 3 hours, before being quenched by water. The aqueous solution was acidified with CH_3COOH to pH=4~5 and then extracted with ethyl acetate. The combined organic layers were washed with brine and dried over sodium sulfate. The solvent was removed by distillation under reduced pressure before a flash chromatography with silica gel and 50% ethyl acetate in hexane to afford a pure product (**4g**, 143 mg, 71% in two steps).

Approximate 50 mg of **4g** (0.125 mmol), 130 mg of acid **4c** (0.3 mmol), 7 mg DMAP (0.5 equivalents), and 24 mg of EDC (1.1 equivalents) were dissolved in 3 mL of DMF at 0 ^{o}C. The mixture was allowed to warm to room temperature and stirred overnight. Following the reaction,

the solution was acidified to a pH of 4 to 5, diluted with a 5% aqueous LiCl solution, and then extracted with EtOAc. The combined organic layers were dried, concentrated *in vacuo*, and then purified through a silica gel column using 50% EtOAc in hexane as eluent. Product **4f** was formed in a 38% isolated yield (38 mg). ^{1}H NMR ($CDCl_3$/300MHz) and ^{13}C NMR ($CDCl_3$/300MHz) show a mixture of diastereomers. Analytical HPLC with the mixed solvent of acetonitrile and water (7:3) as the mobile phase indicates a diastereomer ratio of 1:1. HRESI MS m/z $[M+H]^+$ 805.2443 found, 805.2443 calcd.

4.6.3 Chemistry on Microelectrode Arrays

A microelectrode array containing 12544 electrodes/cm^2 was spin-coated at 1000 rpm for 45 seconds with 3 % poly(4-homostyrene)-block-(2-cinnamoyloxyethyl methacrylate) in 50% THF and 50% p-xylene. After the polymer coated surface was irradiated with UV light for 30 min, the microelectrode array was plugged into a circuit and the electodes controlled with the use of a PC (software available from CustomArray). The array was covered with a cap containing the Pt-counter electrode (Pt is sputtered onto the surface of the cap and connected to the circuit with a Pt-wire). The cap is separated from the array using a Kalrez spacer and the reaction run by flowing the reaction medium into the space between the array and the cap.



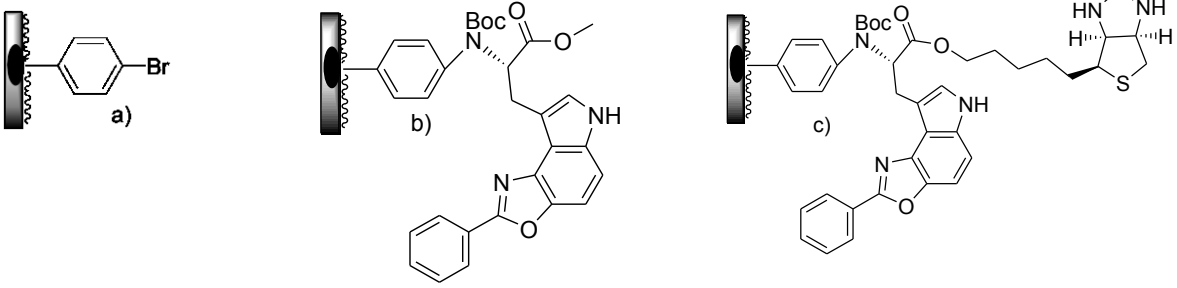
In this way, a polymer coated 12 K microelectrode array was placed under a solution made from 1.5 mL of CH_3CN/ DMF/ H_20 (7/ 2/ 1), 10 mg of **2**, 10 mg of tetrabuylammonium bromide

as an electrolyte, and 6 uL each of a 25 *mM* aqueous $CuSO_4$ solution and a 50 *mM* ligand (Ph_3P) in DMF solution. The reaction was run by setting selected microelectrodes in the array at -1.7 V relative to the Pt-electrode on the cap for 180 sec. As metioned in the text, the selected electrodes were comprised of ten blocks of 12 microelectrodes each. After the reaction, the array was rinsed with EtOH, a small amount of DMF, and EtOH again. The array was then visualized using a fluorescence microscope.

The same experiment was repeated a second time with the use of compound **1** as the substrate.

4.6.4 Probing of compatibility of the fluorescent linker with the signaling experiments

The result of the preparative work described above was an array with three types of surfaces for testing. There were electrodes coated with only the diblock copolymer (a), blocks of electrodes coated with only the fluorescent linker (b), and blocks of electrodes coated with the fluorescent linker plus biotin.

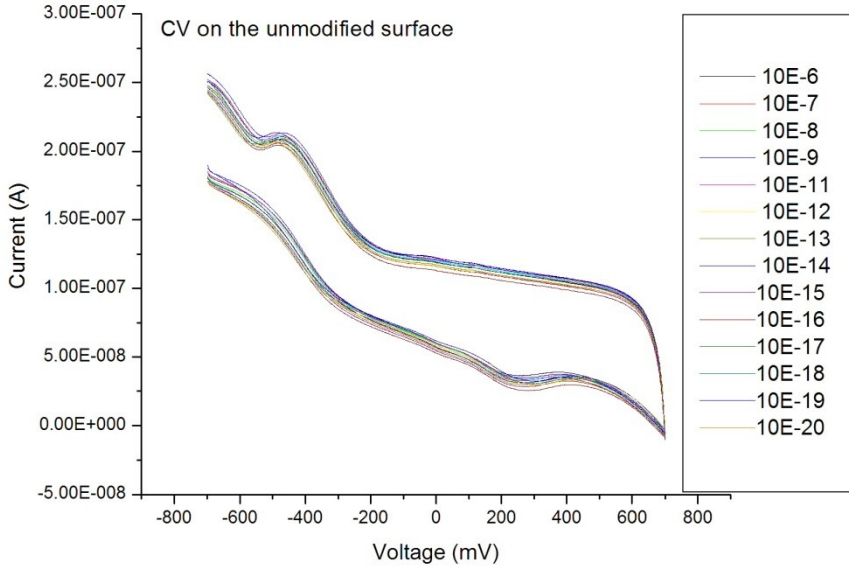


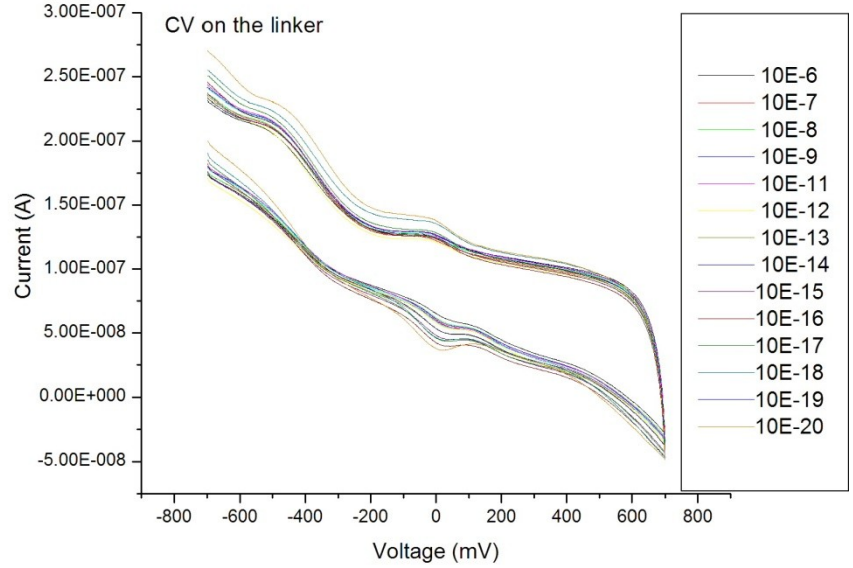
With the array ready to use, a series of strepavidin buffer solutions with gradient concentrations was needed. The preparation of these solutions started with 200 mL of 1x PBS (pH ≈ 7.4) aqeous solution. Approximate 131.5 mg $K_3[Fe(CN)_6]_3$ and 169.0 mg $K_4[Fe(CN)6]_2$ were dissolved in the PBS solution so that the redox couple had a concentration of 8 *mM*.

Commercially available strepavidin solid was then dissolved and diluted with the prepared Fe (III)/Fe (II) solution to form a 1.0 X 10^{-5} *M* stock solution. This stock solution was diluted with the 8 *mM* iron solution to make streptavidin solutions ranging in concentration from 1.0 X 10-6 *M* to 1.0 X 10-20 *M* (1 mL for each).

The electro-analysis began by running cyclic voltammetry tests various electrodes on the array using the Fe (III)/Fe(II) buffer solution without any protein added. The potential at the electrodes was scanned from –700 mV to +700 mV relative to the Pt-cap over the array at a scan rate of 400 mV/s. The oxidative current peaks were measured. The experiment looked at three of the ten blocks of electrodes functionalized with the fluorescent linker, three of the ten blocks funtionalized with linker plus biotin, and three blocks of twelve electrodes from random, unfunctionalized regions on the array. The array was then washed and the experiment repeated with the lowest concentration of streptavidin. After washing the array again, the experiment was repeated for a third time using the next highest concentration of streptavidin. This procedure was repeated until a CV had been recorded at each of the nine selected blocks of electrodes for each concentration of streptavidin. The data from these experiments was processed and analyzed using the approach described in the paper. The experiments were repeated on different arrays to make sure that reproducible results could be obtained.

4.6.5 Cyclic Voltammetries on Microelectrode Array





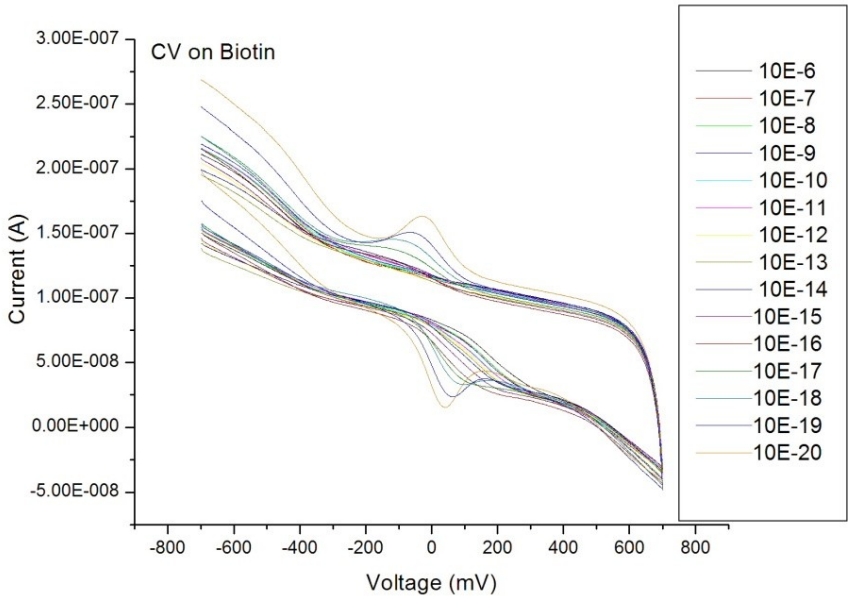


Figure 4.6 Sample Cyclic Voltammetries of Fe(II)/Fe(III) on Microelectrode Array

4.7 References

1. Dong, H.; Song, X. Z.; Lasanajak, Y.; Cummings, R. D.; Chaikof, E. L., "Facile construction of fluorescent peptide microarrays: One-step fluorescent derivatization of sub-microscale peptide aldehydes for selective terminal immobilization". *Analytical Biochemistry* **2010,** *398* (1), 132-134.

2. Orth, R.; Sieber, S. A., "A Photolabile Linker for the Mild and Selective Cleavage of Enriched Biomolecules from Solid Support". *Journal of Organic Chemistry* **2009,** *74* (21), 8476-8479.

3. Pendri, A.; Martinez, A.; Xia, J.; Shorr, R. G. L.; Greenwald, R. B., "Poly(Ethylene Glycol) Fluorescent Linkers". *Bioconjugate Chemistry* **1995,** *6* (5), 596-598.

4. Chen, C.; Lu, P.; Walker, A.; Maurer, K.; Moeller, K. D., "The use of a detectable, mass-spectrometry-cleavable linker for quality control on an addressable microelectrode array". *Electrochemistry Communications* **2008,** *10* (7), 973-976.

5. Yamagishi, Y.; Ashigai, H.; Goto, Y.; Murakami, H.; Suga, H., "Ribosomal Synthesis of Cyclic Peptides with a Fluorogenic Oxidative Coupling Reaction". *Chembiochem* **2009,** *10* (9), 1469-1472.

6. Bohrsch, V.; Serwa, R.; Majkut, P.; Krause, E.; Hackenberger, C. P. R., "Site-specific functionalisation of proteins by a Staudinger-type reaction using unsymmetrical phosphites". *Chemical Communications* **2010,** *46* (18), 3176-3178.

7. Hu, L. B.; Bartels, J. L.; Bartels, J. W.; Maurer, K.; Moeller, K. D., "A New Porous Reaction Layer for Developing Addressable Molecular Libraries". *Journal of the American Chemical Society* **2009,** *131* (46), 16638-+.

8. Jones, G. O.; Liu, P.; Houk, K. N.; Buchwald, S. L., "Computational Explorations of

Mechanisms and Ligand-Directed Selectivities of Copper-Catalyzed Ullmann-Type Reactions". *Journal of the American Chemical Society* **2010,** *132* (17), 6205-6213.

9. Kwong, F. Y.; Buchwald, S. L., "Mild and efficient copper-catalyzed amination of aryl bromides with primary alkylamines". *Organic Letters* **2003,** *5* (6), 793-796.

10. Kwong, F. Y.; Klapars, A.; Buchwald, S. L., "Copper-catalyzed coupling of alkylamines and aryl iodides: An efficient system even in an air atmosphere". *Organic Letters* **2002,** *4* (4), 581-584.

11. Murata, T.; Shimada, M.; Sakakibara, S.; Yoshino, T.; Masuda, T.; Shintani, T.; Sato, H.; Koriyama, Y.; Fukushima, K.; Nunami, N.; Yamauchi, M.; Fuchikami, K.; Komura, H.; Watanabe, A.; Ziegelbauer, K. B.; Bacon, K. B.; Lowinger, T. B., "Synthesis and structure-activity relationships of novel IKK-beta inhibitors. Part 3: Orally active anti-inflammatory agents". *Bioorganic & Medicinal Chemistry Letters* **2004,** *14* (15), 4019-4022.

12. Holmberg, A.; Blomstergren, A.; Nord, O.; Lukacs, M.; Lundeberg, J.; Uhlen, M., "The biotin-streptavidin interaction can be reversibly broken using water at elevated temperatures". *Electrophoresis* **2005,** *26* (3), 501-510.

13. Tanabe, T.; Bi, B.; Hu, L. B.; Maurer, K.; Moeller, K. D., "Building Addressable Libraries: Amino Acid Derived Fluorescent Linkers". *Langmuir* **2012,** *28* (3), 1689-1693.

14. Bi, B.; Huang, R. Y. C.; Maurer, K.; Chen, C.; Moeller, K. D., "Site-Selective, Cleavable Linkers: Quality Control and the Characterization of Small Molecules on Microelectrode Arrays". *Journal of Organic Chemistry* **2011,** *76* (21), 9053-9059.

15. Bi, B.; Maurer, K.; Moeller, K. D., "Building Addressable Libraries: The Use of "Safety-Catch" Linkers on Microelectrode Arrays". *Journal of the American Chemical Society* **2010,** *132* (49), 17405-17407.

16. Fellet, M. S.; Bartels, J. L.; Bi, B.; Moeller, K. D., "Site-selective chemistry and the attachment of peptides to the surface of a microelectrode array". *Journal of the American Chemical Society* **2012,** *134* (40), 16891-16898.

Chapter Five

Conclusion and Future Work

5.1 Conclusion

This work described in this thesis has laid the groundwork for the direct synthesis of small-molecule libraries on microelectrode arrays. It has done so by both demonstrating how core scaffolds can be constructed on the arrays using Lewis-acid catalyzed multicomponent reactions and Diels-Alder cycloaddition reactions. This chemistry took advantage of Sc(III) as the Lewis acid. Like all site-selective reactions run on an array, the key to success was finding a general "confining strategy" that allowed the reagent to be localized to specific, preselected electrodes in the array. In this case, Sc(I) was oxidized to Sc(III) at sites where the reaction was desired, and then the Sc(III) reduced back to Sc(I) in the solution above the array with the use of an arylbenzothiazole.

Of course, the accomplishment of more complex reactions on the array immediately raised issues with characterization of the product generated. This is a common problem with a small-molecule library. How does one conduct a quality control assessment of the molecules in the library? The major part of the thesis work described addressed this question along with a second issue that involved the quality of the array itself. The approach taken along these lines was a "molecular-linker" strategy.

To this end, a general and versatile "chemically cleavable linker" has been developed to check the quality of molecules on the arrays. In this approach, γ-amino acids or γ-hydroxyl acids with the nitrogen or the oxygen protected serve as the "linkers" to tie molecules for study to a

polymer coating the surface of the electrodes in the array. The linkers are attached to a polymer coating the surface of the electrodes in the array by making an ester linkage between the acid in the linker and an alcohol of the polymer. The molecules to be studied on the array are typically tethered to the α-position of the γ-amino acid or γ-hydroxy acid linker. The most successful linkers used an acid-cleavable protecting group on the amine or alcohol. In these cases, acid generated at a neighboring electrode in the array leads to cleavage of the protecting group. The newly de-protected amino or alcohol promptly attacks the ester linking the molecule to the surface of the array leading to the formation of either a lactam or lactone and cleavage of the ensemble from the array. The molecules released from the array in this manner can be collected and then analyzed by LC-MS or HPLC (with fluorescent tags enhancing absorption coefficients) and compared with independently synthesized material. By using this tool, the product from the Diels-Alder reaction could be completely characterized and the stereochemistry of the product assigned. A comparison between the molecules cleaved from the array and Diels-Alder products synthesized in solution demonstrated that the steric impact of the array led to the formation of some of the exo Diels-Alder product (only endo product had been obtained from the Sc(III)-catalyzed reaction in solution). Hence, for the first time the effect of the array on a chemical reaction could be assessed.

The work was particularly attractive because it allowed for analysis of the reaction products without destruction of the array. In direct contrast with TOF-SIMS studies, the arrays could then be reused.

In addition to enabling the characterization of molecules on the arrays, it is important that the linkers can be used to assess the quality of the microelectrode array itself. To this end, it is helpful if the linkers are fluorescent. In collaboration with Dr. Takamasa Tanabe, a former post-

doctoral scientist in our group, we developed a new amino-acid fluorescent linker for use on an array. This "fluorescent linker" has proven to be very effective. It is also being incorporated along with a chemically cleavable linker strategy in a new "ultimate linker" that is both easy to prepare and compatible with quality control analysis of both a molecular library and the array itself.

In order to test the viability of the linkers being developed, "real-time" signaling efforts that take advantage of electrochemical impedance experiments were undertaken. This work comprises the third part of the thesis. The first such study probed the effectiveness of the amino acid-based "fluorescent linker". In that study, a biotin-coupled fluorescent linker was placed proximal to selected electrodes in an array. In addition, electrodes functionalized with the linker minus the biotin group and electrodes not having any functionalization were examined. Measurements of the current associated with the oxidation of iron at each of the electrodes were made as the concentration of streptavidin in the solution above the array was varied. The experiments clearly showed the binding between the biotin and streptavidin as the event occurred. As part of these efforts, a new protocol for the signaling was developed that aided with the experiment setup and data analysis.

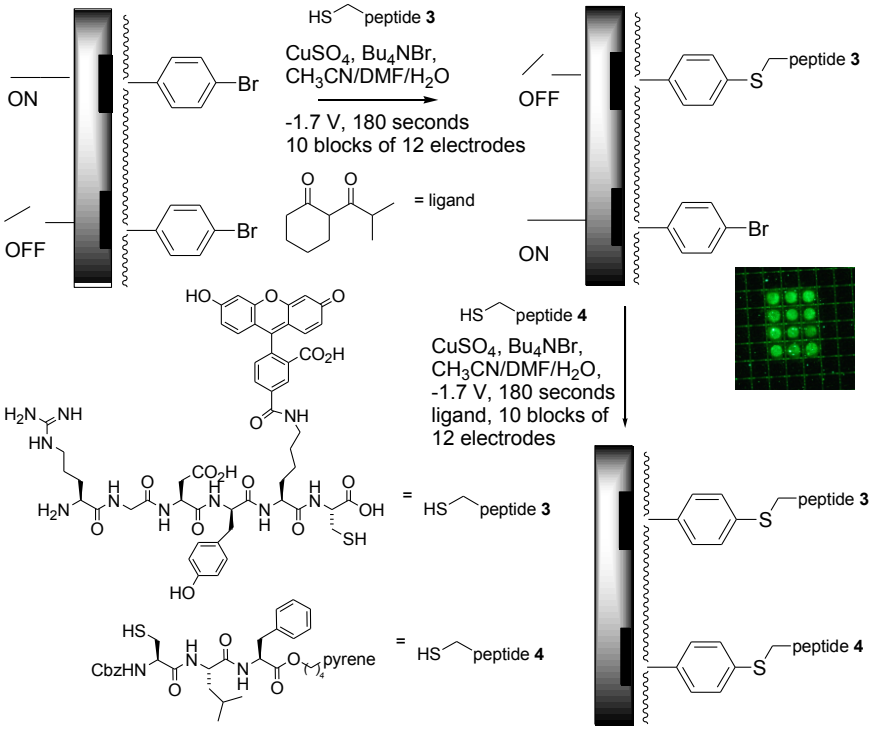
5.2 Future work

5.2.1 Application of the "Ultimate Linker"

As discussed in Chapter 4, we have built a linker that has all of the attributes needed. However, is the linker compatible with the signaling studies? In principle, an answer to this question can be obtained with the use of an RGD-peptide and integrin receptor. The chemistry for attaching an RGD-peptide to the arylbromide group on the ultimate linker has been optimized

and published by Dr. Fellet, Dr. Bartels, and coworkers.[1] A general scheme showing their work is illustrated in **Scheme 5.1**. Note how a cysteine in the peptides was coupled to an arylbromide in the presence of a fluorescent tag. In principle, the same approach can be used with the linker developed in Chapter 4 (the scheme proposed in **Scheme 4.5**).

Scheme 5.1 The Cu(I)-catalyzed selective attachment of peptides on the array[1]



One advantage of this new approach would be that the peptide itself would no longer require a fluorescent group because that group would be part of the linker. This should make the peptide easier (cheaper) to produce. The success of the coupling reaction would be ascertained by taking advantage of the chemical-cleavable linker. The linker once assembled on the array would be checked for its compatibility with electrochemical impedance experiments with the use of an integrin receptor.

5.2.2 Chemo-selectivity of coupling chemistry on Microelectrode Array

There is a remaining issue regarding Cu(I) and Pd(0)- catalyzed couplings: chemo-selectivity. For example, in **Scheme 5.1**, isobutyl 2-carboxycyclohexanone was chosen as the ligand to favor the thiol-end coupling over the amino-end in the same peptide.[2] This is in accordance with the solution studies done by other researchers like Buchwald group, [3,4] and our own observations on the arrays.[2] However, while we made observations about the use of the ligand and relative rates of couplings on an array based on fluorescence images, we had no way at the time to prove if a substrate having two different heteroatoms for coupling really behaved in the expected manner. With a cleavable linker strategy in place, that is no longer the case. Answering questions about the chemoselectivity of a Cu(I)-based coupling reaction on the arrays is important from not only a synthesis point of view, but also for understanding the binding data that comes from a particular molecule. Certainly, its binding efficiency might vary greatly depending on the manner in which it is bound to a surface.

To this end, we propose that molecules containing amines, alcohols, thiols, and other nucleophiles be placed onto arrays using a variety of Cu(I)-based catalysts and the chemoselectivity of the reactions determined using a cleavable linker strategy. The data from these studies can then be used to guide the attachment of future peptide mimetics to the arrays.

5.2.3 Better understanding of the impedance experiments on the array

The most recent signaling study also showed the impedance measurement of peptides-loaded surface on the array (**Figure 5.1**).[1]

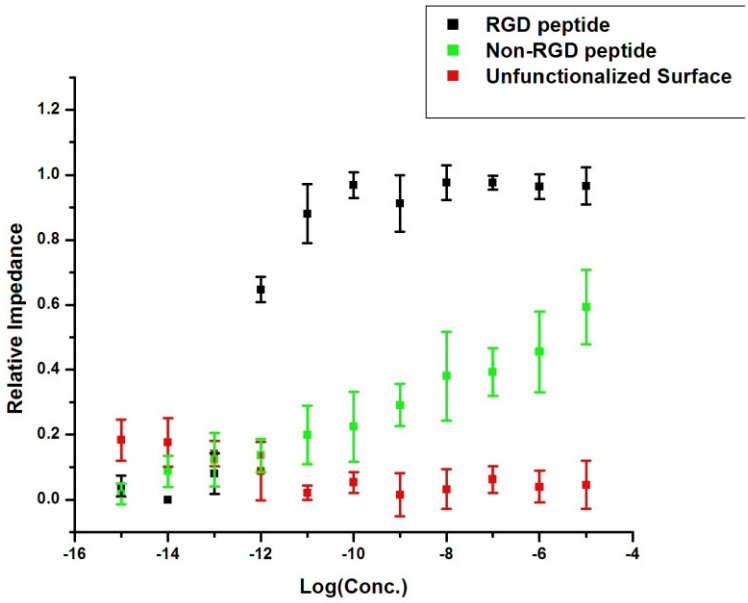


Figure 5.1 Summary of the CV data for all nine blocks of electrodes. Normalized curves are shown for the impedance measured electrodes functionalized with the RGD peptide (black), Non-RGD peptide (green), and unfunctionalized electrodes (red)

In that case, little impedance was observed at the electrodes functionalized with the peptide **4** indicating a low level non-specific binding to the functionalized surface of the array. The steady climb in impedance at the sites functionalized with **4** without any leveling off of the curve suggested that the non-specific binding that was observed was a result of the functionalized surface and not peptide **4**. There is only 10 to 50 fmole of peptide bound to the surface of the electrode so binding to the peptide itself would lead to a leveling off of the impedance with increasing concentration of the receptor. This leveling off of the impedance was observed with the binding of the receptor to peptide **3** (with RGD sequence). At the unfunctionalized electrodes no binding was observed indicating that functionalization of the surface did increase the level of non-specific binding of the integrin receptor to the polymer surface. The level of impedance observed for the electrodes functionalized with **3** relative to those functionalized with **4** clearly demonstrated that the integrin receptor recognized the RGD-peptide on the surface.

However, at this point, it is not known why the impedance measurement made on the array is more sensitive that the typical nanomolar binding constant associated with RGD-integrin binding. One idea is that the integrin binds to the surface of the array because of there are multiple copies of the ligand bound to the array. If it dissociates with one, then it can immediately bind to the next. In that way, the receptor is slow to diffuse away from the surface, a scenario that leads to larger impedances. To determine if this is the case, a careful quantitative measurement of how much of the RGD-peptide is placed by an electrode in an array is needed. By probing the same interaction with different amount of ligand on the surface of the array, we could test if there was an impedance dependence on surface concentration of the ligand.

The fluorescent linker would provide an ideal tool for this study because it can be used to quantify the amount of material placed on an array. In principle, the amount of material placed by an electrode is directly related to the length of time employed for the coupling reaction. Hence, concentration gradients of the RGD-peptide can in principle be placed on an array. The result of the study would be a better understanding of the impedance experiments run on the arrays and more insight into their potential use. For example, if the arrays really do amplify the signal for a binding event, then they can be used to monitor much weaker binding events.

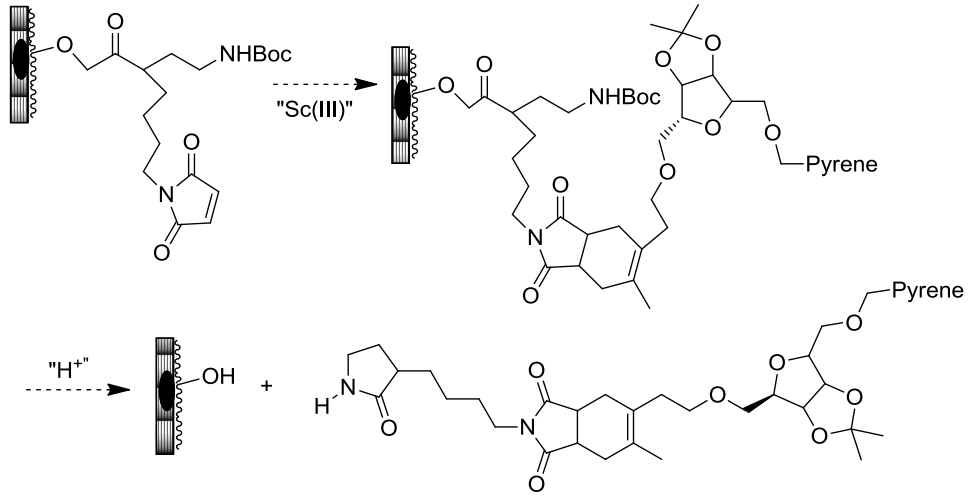
5.2.4 Diversify the chemically cleavable linkers and build molecule libraries

After several years of effort, we have developed a number of powerful synthetic methods for array-based applications, protocols for signaling studies on the arrays, and now tools for conducting quality control measurements on the arrays. It is time to start building molecule libraries and monitoring their binding behaviors.

As discussed in Chapter 3, one of advantages of the chemically cleavable linkers is that it is readily to be modified by replacing the substitutions on the side chain. These changes can be

made without needing to alter the synthetic route to the linker. In this way, the linker can be used to conduct quality control assessment of a variety of structures built with the site-selective chemistry developed. For example, Dr. Guoxi Xu in our group showed that a Diels-Alder reaction can be used to place C-glycosides onto the surface of an array.[5] This scheme could be adapted to the chemically cleavable linkers (**Scheme 5.2**) in order to make sure that the chemistry proceeded as planned.

Scheme 5.2 Example the quality control of the C-glycoside with the chemically cleavable linker



By using the similar strategies, a library of sugar monomer derivatives, peptides, etc. can be placed by different electrodes of the array with Lewis-acid, Cu(I), Pd(0) as catalysts in a convenient and convincing manner. Then the relative interaction of those ligands with receptors of interest can be probed in "real-time". Molecules that show a signal can then be reclaimed from the array using the same electrode used to record the signal in order to make sure that they remain the molecule that was intended for the study. The approach would enable the first true applications of microelectrode arrays for the analysis of a small molecule library.

5.3 References

1. Fellet, M. S.; Bartels, J. L.; Bi, B.; Moeller, K. D., "Site-selective chemistry and the attachment of peptides to the surface of a microelectrode array". *Journal of the American Chemical Society* **2012,** *134* (40), 16891-16898.

2. Bartels, J. L.; Lu, P.; Walker, A.; Maurer, K.; Moeller, K. D., "Building addressable libraries: a site-selective click-reaction strategy for rapidly assembling mass spectrometry cleavable linkers". *Chemical Communications* **2009,** (37), 5573-5575.

3. Shafir, A.; Lichtor, P. A.; Buchwald, S. L., "N- versus O-arylation of aminoalcohols: Orthogonal selectivity in copper-based catalysts". *Journal of the American Chemical Society* **2007,** *129* (12), 3490-+.

4. Lv, X.; Bao, W. L., "A beta-keto ester as a novel, efficient, and versatile ligand for copper(I)-catalyzed C-N, C-O, and C-S coupling reactions". *Journal of Organic Chemistry* **2007,** *72* (10), 3863-3867.

5. Xu, G. "Synthesis of C-Glycosides via Anodic Olefin Coupling Reaction and Building of Addressable C-Glycoside Libraries on Microelectrode Arrays." Ph.D. dissertation, Washington University in St. Louis, **2012.**