Chemoselective reactions and their applications in metabolite isolation and analysis.

Sadakatali Shokatali Gori
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CHEMOSELECTIVE REACTIONS AND THEIR APPLICATIONS IN

METABOLITE ISOLATION AND ANALYSIS

By

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A Dissertation Approved on

May 24, 2016

by the following Dissertation Committee:

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Dr. Xiang Zhang

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Dr. Xiao-An Fu
DEDICATION

This dissertation is dedicated to my loving and supportive family,

   my grandmother Aashiya S. Gori,

   and my mother Bismillah S. Gori
ACKNOWLEDGEMENTS

I want to express my deepest gratitude to Dr. Michael H. Nantz for taking me under his wing and allowing me to be a part of the Nantz Team. Throughout my graduate career, Dr. Nantz has guided me and nurtured my scientific temper with immense patience and poise. His uncanny ability to see through a problem and offer multiple inventive solutions has helped me overcome many challenges in my graduate research. Dr. Nantz pushed me to the limits of my ability and made me realize that the boundaries are just an illusion and can be overcome by thinking smart and working hard. His brilliance is paralleled only by his leadership, in that he ensures a fair work environment and creates opportunities for his students to seek out success in professional as well as personal lives. In addition to his contribution in my professional development, Dr. Nantz has greatly influenced my outlook towards life and over time has become a father figure and a confidant. I am forever indebted to him for his guidance and friendship.

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ABSTRACT

CHEMOSELECTIVE REACTIONS AND THEIR APPLICATIONS IN
METABOLITE ISOLATION AND ANALYSIS

Sadakatali Gori

May 24, 2016

Chemoselectivity is the preferential reaction of a reagent with a select functional group among other plausible reactions. This dissertation describes chemoselective reagents and methods explored to develop more accurate, efficient and high-throughput means to quantify metabolites containing either thiol, carboxylic acid or 1,2-diol moieties directly from biological mixtures.

Chapter 1 discusses how dysregulation of thiol- or carboxylic acid-containing metabolites is either the cause or effect of cellular dysfunction. Quantifying such metabolites is important in discovery of pathways to understand etiology of diseases. Chapter 1 also discusses the need for sustainable syntheses using renewable resources and describes the traditional methods for isolation of sugars from biomass hydrolyzates.

Chapter 2 describes the design, synthesis and use of the iodoacetamide-based reagents QDE and *QDE for selective capture and analysis of thiol metabolites. The identification of major thiol metabolites and quantification of glutathione in A549 cells
were realized using this technology. Total concentrations of [GSH] and [GSSG] in cultured cells of a human lung adenocarcinoma cell line (A549) were determined at 34.4 ± 11.5 nmole/mg protein and 10.1 ± 4.0 nmole/mg protein, respectively.

Chapter 3 presents attempts to prepare a bis(boron halide) reagent for capture of carboxylic acids via formation of a six-member cationic N,B,C,O heteroaromatic ring. Although the reagent proved to be impractical for use in metabolite analysis due to air/moisture sensitivity, we isolated a novel oxygen bridged bicyclic boron fluoride complex with potential applications in electroluminescent devices.

Chapter 4 outlines a method for chemoselective isolation of C5 sugars from dried distillers’ grains hydrolyzate that exploits the phenylboronic acid chelation of cis-1,2-diols followed by a transesterification protocol on the resultant diol-boronate adducts. A large scale demonstration of the process delivered crystalline xylose from hydrolyzate in 48% yield with recovery of >80% of all reagents/solvents used.

Chapter 5 describes the experimental protocols for the syntheses and use of the aforementioned chemoselective reagents.

To summarize, the present research illustrates how application of chemoselective reactions can lead to superior methods for isolation and identification of targeted metabolites in complex mixtures. The high reaction rate, selectivity and adduct stability afforded by chemoselective derivatization can be employed in a myriad of other research disciplines.
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1.1. INTRODUCTION TO METABOLOMICS

Metabolomics is the systematic study of low molecular weight substrates and byproducts of phenotypic metabolism. Roger Williams first reported in the late 1940s that metabolites observed in bodily fluids, such as saliva and urine, could be used to uncover pathology [1]. Technological advancements in analytical instrumentation during 1950–1970s paved the way for high throughput and quantitative analysis of metabolites. Horning et al. introduced the term “metabolite profiling” in 1971 when they used gas chromatography-mass spectrometry (GC-MS) to study metabolites in urine and tissue extracts [2]. The past decade has seen an exponential growth in MS-based metabolomics studies [3], the creation of large metabolomics databases [4], the establishment of the human metabolic database [5] and the advent of real-time metabolite profiling [6]. The accelerated growth of metabolomics research can be attributed to clear advantages over other “omics” fields, such as genomics, transcriptomics and proteomics. Proteins or genes far outnumber total metabolites in any given biological system; this makes metabolomic analysis less cumbersome when compared to other “omics” techniques.
The chemical entities generated by metabolic processes are a result of genomic, transcriptomic, and proteomic variability, therefore metabolomics provides the most integrated profile of such processes. Since low molecular weight metabolites are the closest link to human phenotype, metabolomics provides a simpler, wholesome and more relevant data set for analysis that becomes the basis for discovery of metabolic pathways and possibly even for pharmaco-therapeutic interventions (Figure 1.1)[7].

Metabolomics can be broadly divided into two categories: targeted and untargeted analysis. The goal of a study determines the category best suited for a specified experiment. Targeted metabolomics deals with analysis of a predetermined set of metabolites and focuses on a specific pathway (Figure 1.2, a). Such analysis is driven by questions arising from mechanism of action of these metabolites and effects on the phenotype due to their quantitative flux. Targeted metabolomics is frequently used for pharmacokinetic studies of drug metabolism and to quantitate effects of therapeutic interventions [8].

In contrast, untargeted metabolomics is an unbiased approach for global analysis of control and test samples and determination of metabolic differences between them (Figure 1.2, b). Such indiscriminate analyses result in highly complex and large data sets that require specialized software for analysis [9,10]. Although the data collection and analysis can be cumbersome for untargeted metabolomics, this approach leads to discovery of uncharacterized molecules and discovery of new pathways. Another potential application of targeted metabolomics, as described by Furlan et al., utilizes metabolomic analyses to identify and synthesize libraries of diverse semisynthetic
Figure 1.1. Generic flowchart for application based metabolomics [11].

molecules for development of biologically active compounds [12]. Metabolomic analyses of natural products have been utilized in phylogenetic assignment, biosynthetic transformation of natural products and detection of preclinical biomarkers [13].

This thesis focuses primarily on targeted metabolite isolation and analysis. Specifically, new approaches for analysis of polar thiols, lipidic carboxylates and biomass sugars (diols) are described. The significance of these metabolite classes as well as the reagents and techniques developed for their targeted analysis are discussed in the following subsections.
Figure 1.2. Differences in targeted and untargeted metabolomics [14].
1.2. THIOL METABOLISM AND DISEASES

Sulfur containing compounds are ubiquitous in cellular and extracellular matrices. The thiol functionality is essential for numerous metabolic processes to occur, such as redox homeostasis, oxidative stress responses [15–17], methylation [18,19] and cell division [20]. The protective roles of thiols in general and glutathione (GSH) in particular have been well documented in the literature. For example, excessive reactive O$_2$ species (ROS), such as lipid hydroperoxides and H$_2$O$_2$, are toxic to cells and are reduced via the glutathione peroxidase (GSHPrx) mechanism equations, 1, 2, 3 [21]. Glutathione disulfide (GSSG) is then reduced to GSH by NADPH via the action of glutathione reductase (equation 4). Alterations in these enzymes and GSH / GSSG levels have been implicated in cancers, aging and neurological disorders [22].

\[
\begin{align*}
H_2O_2 + Prx-S^- \rightarrow OH^- + Prx-SOH & \quad (1) \\
Prx-SOH + GSH \rightarrow Prx-SSG + H_2O & \quad (2) \\
Prx-SSG + GSH \rightarrow Prx-S^- + GSSG & \quad (3)
\end{align*}
\]

where Prx-SOH represents sulfenic acid intermediate of the enzyme that catalyzes oxidation of GSH.

\[
GSSG + NADPH + H^+ \rightarrow NADP^+ + 2GSH \quad (4)
\]

The thiol-disulfide reversible reaction is responsible for many molecular changes such as determination of tertiary structure of proteins, protection against oxidative radicals and regulation of thio-metabolites. Often glutathione exchanges with protein thiols in response to external stimuli and produces protein-glutathione mixed disulfides. These trans-sulfuration processes have effects on signaling pathways as shown in Figure
1.3. A diseased phenotype shows up/down regulation of these metabolites in response to stress which, if quantified, may allow discovery of novel mechanisms and open new venues to build diagnostic technologies.

Dysregulation of thiol metabolism is implicated in the occurrence of several diseases. For example, brain tissue contains only a millimolar concentration of glutathione [23] and it is more susceptible to oxidative damage than other tissues. The progression of Parkinson’s disease (PD) is associated with a depletion of GSH levels and an increase in ROS within the substantia nigra pars compacta [24,25]. In a clinical study, administration of reduced GSH to patients alleviated some symptoms of PD [26]. Synthesis of taurine is up-regulated in nearly all cancers [27–32], glutathione in brain and CNS cancers [33], cysteine in ovarian cancers [34,35], and cystamine in colorectal cancers [36]. GSH levels are depleted in plasma, epithelial lining fluid (ELF), peripheral blood mononuclear cells and monocytes in asymptomatic HIV-infected individuals and in AIDS patients [37]. Stunted GSH production in liver due to hereditary disorders or dietary deficiencies can disrupt detoxification functions of this organ and cause a number of pathologies. Mitochondrial GSH depletion exacerbates ROS damage in liver cells causing cirrhosis in alcoholics [38]. Patients with cystic fibrosis frequently have higher levels of lipid peroxidation byproducts [39,40], caused by progressing GSH deficiency. Thus changes in GSH:GSSG ratio is associated with either cause, effect or cure of a wide range of diseases. Consequently, evaluating perturbations in the GSH:GSSG ratio is vital for diagnosis and possibly medical intervention. One aim of this thesis work is to synthesize a thiol capture reagent that would facilitate sensitive, accurate and high-throughput
analysis of thiol metabolites. A major criterion in the design of such a probe is to determine the exact mass of the thiol metabolite as well as to serve as a quantification tool. For this purpose, we used MS as the principal tool for analyses. Therefore, we designed a reagent that would react rapidly with thiols and allow separation of the resultant adduct from the cellular matrix via extraction steps. Since the analytical approach was MS, the reagent was fitted with a cationic charge to enhance detection by MS. This technology is discussed in detail in Chapter 2 of this dissertation.

1.3. CHEMoselective Reactions for Thiol Metabolite Analysis

Owing to their importance as described above, thiol metabolites have been widely studied in the past decade. New technologies are being developed to exploit the ever-increasing capabilities of instruments, particularly with regard to improvements in instrument sensitivity and resolution. Efforts also seek to create processes that enhance the quality of samples generated for targeted studies. Instrumental analysis of thiols using MS or NMR can become complex unless a targeted set of metabolites is processed from a phenotype of interest via a purification technique. Such processes can be tedious, time consuming and can generate errors. To overcome such challenges a number of
chemoselective reagents/techniques have been developed that selectively tag thio-
metabolites for NMR, MS and colorimetric analysis. Thiol capture reagents generally are
categorized by the type of reaction they undergo with thiol metabolites, either alkylation
or conjugate addition.

**Halide Displacement.** One of the most common techniques to derivatize thiols
for analysis is to alkylate them via S_N2 halogen displacement. A halide, which serves as
the reactive site, is introduced on a substrate that assists in either colorimetric or mass
spectrometric (MS) analysis of the adduct. For example, monobromobimane 1 is a
commonly used fluorescence reagent that reacts with thiols via bromide displacement
[42]. The adduct fluorescence is then measured after washing off any unreacted reagent
to quantitate total thiol concentration in cellular matrices. Other such reactive motifs
include aromatic fluorides, such as 4-fluoro-7-sulfamoylbenzofurazan 2 [43],
iodoacetamides such as N-phenyl iodoacetamide 3 [44] and halopyridinium salts such as
2-chloro-1-methylpyridinium iodide 5 [45] (Figure 1.4). The halides in these reagents are
displaced by thiols to give the adducts which are analyzed using colorimetric / MS or NMR
spectrometry. The reaction of thiols with these reagents generally takes between 5 min
to 2 hours, at temperatures between 25°C and 60 °C usually at pH 7.5 or higher [46].
Rapid alkylation and high instrumental sensitivity have made these techniques very
popular over the past decade. However, especially for colorimetric techniques, the
fluorescence of the reagent itself and other cellular matrix compounds interfere with the
analysis. The high pH required for reaction can make the reagents less selective towards
thiols.
Figure 1.4. Thiol capture reagents bearing halogens.

**Fluorescence Dequenching.** The 2,4-dinitrophenyl sulfanyl (DNBS) moiety (Figure 1.5) contains an electron deficient phenyl ring. This electron deficiency causes photoinduced electron transfer (PET) to occur to quench fluorescence of an attached fluorophore. Upon reaction with thiols, the DNBS group is released from the fluorophore via desulfonation thereby causing fluorescence. This “on/off” switch approach is frequently exploited to quantify low concentrations of GSH and Cys in cells. Commercial DNBS reagents, such as fluorescein-DNBS 6, exploit this concept as shown in Figure 1.5. Although giving high sensitivity, a common issue with such probes is their degradation over time. Another fluorophore-DNBS reagent, compound 9, features a conjugated system with a $\pi$ donor acceptor manifold that is triggered upon amine release due to nucleophilic reaction of thiol on the DNBS motif. Once again, reaction with a thiol activates quantifiable fluorescence for analysis. Recently, more photostable and high yield 4,4-difluoro-4-bora-3a,4a-diaza-s-indacene (BODIPY) 10 based reagents have been
Figure 1.5. DNBS reagents and mechanism of fluorescence activation.

Scission of DNBS (indicated by red wavy line) liberates fluorescence-active group.

developed for fluorescence studies using the DNBS fluorescence quenching approach.

**Conjugate Addition.** Thiols readily add to Michael acceptors, such as N-ethyl maleimide (NEM, Figure 1.6). NEM is frequently used to block the redox flux by rapid alkylation of thiols (i.e. conjugate addition by RSH occurs rapidly, and once RSH has reacted, the thiol cannot undergo oxidation to RSSR). This blocking allows for accurate quantification of reduced thiols which might otherwise oxidize to disulfides. NEM is also widely used as the reactive motif in commercial fluorescence based thio-reactive probes such as ThioGlo 5 (14, Figure 1.6) that have shown a detection limit of 50 fM using HPLC analysis [47]. Detection is facilitated by PET-based quenching of fluorescence by the maleimide motif which is “switched off” on reaction with thiols. This increases the fluorescence intensity by up to 350 fold [48]. BODIPY based NEMs 15 work on the same principle and have been synthesized to create more photo-stable reagents. A somewhat
Figure 1.6. Michael addition-based reagents.

lesser known Michael addition type motif involves conjugated quinone 16. This reagent is only infrequently used for colorimetric analysis of thiols.

A variation of NEM where open chain Michael acceptors are used for thiol capture to induce intramolecular PET modulation of fluorescence also have been reported [49].
These probes are reported to have reaction times of milliseconds with detection limits as low as 0.5 nM. An example of an open conjugated Michael acceptor is shown by 17 & 18 (Figure 1.6). Although these reagents have very good reactivity towards thiols, their use is limited to fluorescence based quantitative analyses as they provide no molecular weight or structural information about new thiols that may exist within the cellular matrix.

Squaraine thiol probes are another class of Michael addition-based probes that are used in colorimetric analysis. Nucleophilic addition of thiols to π conjugated squarines (Scheme 1.1) disrupts conjugation resulting in changes fluorescence in visible and NIR regions. This change in UV absorption has been exploited to analyze thiols using near infrared (NIR) spectroscopy [50]. For example, a PET fluorescence quenching unit (pyrrole) that is switched off by thiol addition increases fluorescence of a previous fluorescence inactive molecule. Another strategy involves Michael addition ring opening reagents, that show colorimetric changes as shown in Scheme 1.2 [51].
**Scheme 1.1. Squarine based thiol reagent.**

**Scheme 1.2. Michael addition triggered ring opening.**

**RSSR Cleavage.** Thiol-disulfide transulfuration is one of the oldest techniques used for RSH analysis. For example, Ellman’s reagent (5,5’-dithiobis-2-nitrobenzoic acid or DTNB 25, Figure 1.7) introduced in 1959 [52] has 2 electron deficient phenyl groups...
Fluorescence probes based on disulfide cleavage.

linked by a disulfide bond. Upon reaction with RSH, the disulfide bond is cleaved releasing 5-thio-2-nitrobenzoate 27, which has a strong UV absorption [53]. The low stability of DTNB at higher pH values and undesired background absorption due to degradation has prompted synthesis and use of other innovative reagents, such as shown in Figure 1.7, based on the same principle. An interesting example is the Ratio-HPSSC probe 28 developed by Lin et al. (Figure 1.7). This probe consists of a tetrakis(4-hydroxyphenyl)porphyrin scaffold linked to coumarin via a disulfide bond. The fluorescence of coumarin is quenched via Förster resonance energy transfer (FRET) to the porphyrin ring due to overlapping emission and absorption bands of coumarin and
porphyrin, respectively. Disulfide bond cleavage triggers FRET disruption thereby restoring fluorescence of coumarin [54].

Drug release via substrate cyclization is a popular technique employed in drug delivery research. In recent years this technology has gained some popularity in analyses as well. A disulfide bond is linked to either a fluorophore or a low molecular weight standard via a carbamate linkage. Upon reduction the generated free thiol undergoes intramolecular cyclization via the carbonyl carbon and releases the fluorophore/standard [55] (Scheme 1.3). Either fluorescence measurement or mass spectrometric analysis of the released standard gives quantitative information of the metabolites.

```
\[
\begin{align*}
R\cdot S\cdot S\cdot O\cdot N\cdot \Phi & \quad \text{\text{R''-SH}} & \quad \text{\text{R''-S\cdot S\cdot R}} & \quad \text{\text{HN\cdot \Phi}} \\
\text{MS analysis}
\end{align*}
\]
\[
\Phi = \text{standard or fluorescence agent}
\]

Scheme 1.3. Cyclization release via disulfide cleavage.
```

Another technique that has gained much prominence in recent years employs Se–N bond cleavage to form Se–S bond for MS analysis [56]. Ebselen, 2-phenyl-1,2-benzisoselenazol-3(2H)-one 31 (Scheme 1.4), is considered as a mimetic of GSHPx [57]. The proposed mechanism for ebselen-catalyzed thiol oxidation involves Se-N bond cleavage by thiol RSH to produce the corresponding selenenyl sulfide Se-S.
Scheme 1.4. Reagents based on Se–N cleavage.

Both these reagents are reported to be exclusively selective to cysteine residues and have been shown to react faster than the Michael addition based reagents. These reagents are used for derivatization of thiols usually for LC-MS analyses. The naturally occurring isotopes of Se facilitate the identification of adduct molecules.

Stable isotopologues of iodoacetamides, as shown in Figure 1.8, are also used to trap thiols for analysis using LC-MS techniques [58]. Stable isotope adducts of thiols elute simultaneously and these isotopic peak pairs can be easily distinguished from unwanted cellular components facilitating rapid and accurate data analysis. These techniques have also been adopted in affinity separation studies where iodoacetamides act as thiol reactive motifs that are attached to a labeled linker and an affinity group [59]. The affinity group helps in separation of thiol adducts and labelling via light, and heavy (D, $^{13}$C, $^{15}$N) isotopes help resolve peaks in mass spectrometry. Pyridinium salts provide a non-
1.8. Stable isotope reagents for thiol capture and analysis by MS.

titratable positive charge that improves ionization in MS techniques thereby allowing easy
detection of low abundant thiol metabolites.

1.4. SIGNIFICANCE OF LIPID METABOLITES

Carboxylic acids are present in a wide range of metabolites that include small
hydrophilic molecules such as formic or acetic acid, hydrophobic molecules such as lipidic
fatty acids, bile acids, amino acids, prostanoids, etc. These CA metabolites constitute a
major part of the central carbon metabolism. This complex, enzyme mediated network
is of utmost importance in conservation of metabolic sub-pathways such as glycolysis,
gluconeogenesis, the pentose phosphate pathway, and the tri-carboxylic acid (TCA) cycle.
The keto- and poly-carboxylic intermediates of the TCA cycle act as substrates for the
biosynthesis of other biomolecules, such as amino acids. The TCA cycle is indirectly
responsible for production of usable energy in the form of adenosine triphosphate.

General CA metabolism can take place via glucuronidation, acyl-CoA thioester
formation or acyl-glutathione thioester formation [60] (Figure 1.9, 1.10).
Figure 1.9. Overview of the metabolism of carboxylic acids [60].
Figure 1.10. Three routes of chemical reactivity of carboxylic acid metabolites [60].
Pathway A shows the reaction of an acyl-O-glucuronide with a nucleophile, e.g., a functional group in a protein. Pathway B shows the base-catalyzed rearrangement of β-1-O-acyl glucuronide to the isomers 2-, 3- and 4-O-acyl glucuronide and subsequent reaction of the free aldehyde with an amino-group. Pathway C shows the reaction between a thio-ester and a nucleophile, e.g., a functional group in a protein.

Thus, analysis of indigenous CA-containing metabolites can reveal information about the pathology in cellular functions and lead to a possible cure. Hence, many techniques have been developed to analyze and quantify CA-containing metabolites.

1.5. CHEMOSELECTIVE REACTIONS FOR CARBOXYLIC ACID ANALYSIS

Carboxylic acid functional group is found in many indigenous metabolites as well as pharmaceutical drug substances. Often times these metabolites/drugs are implicated in dysregulation of cellular functions via cellular pathways that are integral in carboxylic acid metabolism. Thus, CAs have been widely analyzed using modern instruments and novel techniques to study their metabolism and to uncover possible mechanisms. CAs are extensively analyzed using mass spectrometry, however, they suffer from poor ionization as they can be observed only in negative ion mode. Negative mode in MS suffers from poor ionization and can be a hindrance in analysis of moderate to low abundant metabolites. To overcome this challenge a number of chemoselective reagents that switch the negative charge on CAs to positively charged adducts have been developed. These charge switch reagents facilitate better ionization and thus more sensitive analysis of CA metabolites while sometimes also rendering a large mass to low molecular weight CA metabolites for easier detection.
Derivatization of a CA requires activation of the carboxyl group followed by either an esterification or amidation step. For example, CAs can be activated using halopyridinium salts in the presence of a base (e.g. triethylamine). The activated acid is then treated with a high molecular weight amine, such as tris(2,4,6-trimethoxyphenyl) phosphonium propylamine bromide, to form an amide bond. The resulting adduct has a much larger molecular weight and a phosphonium cation that drastically increases detection by MS (Scheme 1.5) [61]. A comparative study of negative ion and positive ion MS on maleic, fumaric, salicylic and sorbic acid was done by Cartwright et al. to show the improvement in detection after charge switch derivatization using the above technique (instrument parameters shown in Table 1.1). Detection was performed by monitoring the appropriate precursor ion in quadrupole 1 and the most abundant fragment ion in quadrupole 3. For the underivatized acids, the most abundant fragment ion corresponded to the loss of 44 Da (assigned as CO₂) from the [M–H] precursor ion; for the derivatized acids the most efficient fragmentation was variable, so the most abundant fragment for each test analyte was used in the analyses.
Table 1.1. Common parameters for negative and position ion MS of CAs [61].

<table>
<thead>
<tr>
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<th>Carboxylic Acids</th>
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<tbody>
<tr>
<td></td>
<td>Maleic</td>
</tr>
<tr>
<td>ESI-MS/MS operating conditions of underivatized acids</td>
<td></td>
</tr>
<tr>
<td>Quadrupole 1 (m/z)</td>
<td>115</td>
</tr>
<tr>
<td>Quadrupole 3 (m/z)</td>
<td>71</td>
</tr>
<tr>
<td>Ionspray potential (IS) (V)</td>
<td>−4200</td>
</tr>
<tr>
<td>Declustering potential (V)</td>
<td>−25</td>
</tr>
<tr>
<td>Collision energy (eV)</td>
<td>−14</td>
</tr>
<tr>
<td>ESI-MS/MS operating conditions of derivatized acids</td>
<td></td>
</tr>
<tr>
<td>Quadrupole 1 (m/z)</td>
<td>688</td>
</tr>
<tr>
<td>Quadrupole 3 (m/z)</td>
<td>590</td>
</tr>
<tr>
<td>Ionspray potential (IS) (V)</td>
<td>5500</td>
</tr>
<tr>
<td>Declustering potential (V)</td>
<td>95</td>
</tr>
<tr>
<td>Collision energy (eV)</td>
<td>55</td>
</tr>
</tbody>
</table>

CA activation is also accomplished via 1H-benzotriazol tetramethyl uronium (HBTU) reagent coupling. This activated ester is then amidated using cholamine (Scheme 1.6) [62] to give a positively charged adduct. The positive charge allows for electrospray at low ionization potential (i.e. no fragmentation is needed for analysis), thus making the analyses more sensitive. Isotopically labelled cholamine is often used to facilitate easy identification of tagged CAs.
Guo and Li published analysis of CAs in human urine using LC-MS and isotopically labelled “lighter and heavier” versions of $p$-dimethylaminophenacyl bromide (DmAPBr) for derivatization [63] using triethanolamine as a catalytic base (Scheme 1.7). The reagent showed no reactivity with alcohols, thiols, amides, amines, ketones, or aldehydes, thus, confirming its selectivity for CAs. They created a library of 113 CA containing metabolites by labelling them with these reagents and noting their retention times in LC. As expected, MS analysis revealed each LC peak to be a pair of isotopologues. A challenge with cholamine based derivatization for LC-MS analysis is their solubility in organic solvents. Polar CAs either do not dissolve in organic solvents or cause precipitation in LC columns making analyses difficult also, reverse phase LC analysis would show all polar metabolites, thus making the data analysis cumbersome.
Scheme 1.7. DmAPBr for stable isotope coded CA analyses.

Scheme 1.8. Triphenylphosphine and DPDS activation of CA.

CAs are also derivatized using hydrazine based reagents, for example, CAs are activated using a mixture of triphenyl phosphine and 2,2'-dipyridyl disulfide (DPDS) and then derivatized using 2-hydrazinopyridine or 2-hydrazinoquinoline [64] (Scheme 1.8). The reagents are isotopically paired to facilitate MS analyses following chromatographic separation. The same activation technique has also been used for amidation with 2-picolyamine [65].
Scheme 1.9. DBD-PZ-NH$_2$ derivatization of lipidic CAs.

Tsukamoto and co-workers reported the use of 7-(N,N-dimethylaminosulfonyl)-4-(aminoethyl) piperazino-2,1,3-benzo diazole (DBD-PZ-NH$_2$) and its deuterated analog ($d_6$-DBD-PZ-NH$_2$) for the quantitation of lipidic acids using MS [66, 67] (Scheme 1.9). The carboxylic acids are activated using carbodiimides and then treated with isotopologues, thus resulting in paired adducts. These are chromatographed using LC and analyzed in MS via easy peak pair identification.

CAs are often activated by reaction with 2-halo pyridinium salts or 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide [68] and then treated with amines, alcohols or hydrazines (Scheme 1.10) to derivatize the activated acid metabolites [61,62,69]. This two-step derivatization process is not feasible for use in metabolomic studies due to formation of multiple by-products by either the activating or derivatizing reagent. Moreover, these reagents also suffer from low ionization in positive ion ESI-MS especially in case of hydroxy carboxylic acids. These derivatization techniques have been reported for HPLC/UV quantitation of biotin [70], organic acids [71–73], short-chain [74,75]/
Scheme 1.10. CA activation and derivatization.

straight chain/long-chain fatty acids [76, 77] and for quantification of $^{13}$C-pyruvate and $^{13}$C-lactate in dog blood by RP-HPLC/ESIMS [78].

The said methods have been improved upon analytically but the chemistry of derivatization process remains a concern due to the need of an activating reagent, elevated reaction temperatures and added byproducts, esp. for applications in MS. With this background in mind, we sought to develop a reagent that would react selectively with CAs to directly generate a positively charged adduct suitable for MS analysis without the aid of activation steps. This work is discussed in Chapter 3.

1.6. BIOMASS: ALTERNATIVE TO FOSSIL FUEL-BASED CHEMICALS

Unstable oil prices and negative effects of petroleum based products on the environment and the advantages of biomass towards sustainability of resources has accelerated the development and utilization of unused biomass. The ability to re-grow harvested biomass and recapture the carbon emitted to the atmosphere through photosynthesis allows the possibility of carbon neutrality. Abundant plant biomass
contains a number of important chemicals and has the potential to be a source of sustainable energy and feedstock for downstream chemical derivatives (Figure 1.11). The chemicals in biomass hydrolyzates have a huge potential for production of fuel alternatives and chemical diversification. However, lack of economization of these processes have thus far limited the use of this abundant resource (Figure 1.12). The utilization of biomass for chemical manufacture can significantly eliminate the harmful effects of fossil based chemicals on the environment.

Broadly, any organic matter derived from agricultural or forestry sources in a recurring fashion is called biomass. Such organic matter mainly consists of products similar in composition to fossil feedstock [80]. Carbohydrates, cellulose and hemicelluloses are the largest renewable sources of carbon [81]. Advanced technologies in fermentation and pyrolysis have given access to products such bio-oil, char and gases.
Figure 1.12. The 2015 energy flow chart of sources of energy production and usage in US [82].
Table 1.2. Commonly reported chemicals in biomass hydrolyzate.

<table>
<thead>
<tr>
<th>1) Acids</th>
<th>3) Monosaccharides</th>
</tr>
</thead>
<tbody>
<tr>
<td>- acetic acid</td>
<td>- xylose</td>
</tr>
<tr>
<td>- hydroxybenzoic acid</td>
<td>- arabinose</td>
</tr>
<tr>
<td>- dodecanoic acid</td>
<td>- glucose</td>
</tr>
<tr>
<td>- formic acid</td>
<td>- fructose</td>
</tr>
<tr>
<td>- hexadecanoic acid</td>
<td>- mannose</td>
</tr>
<tr>
<td>- levulinic acid</td>
<td>- galactose</td>
</tr>
<tr>
<td>- benzoic acid</td>
<td></td>
</tr>
<tr>
<td>- syringic acid</td>
<td></td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>2) Aldehydes</th>
<th>4) Furans</th>
</tr>
</thead>
<tbody>
<tr>
<td>- acetaldehyde</td>
<td>- 2,5-dimethyl furan</td>
</tr>
<tr>
<td>- formaldehyde</td>
<td>- furfuryl alcohol</td>
</tr>
<tr>
<td>- furfural</td>
<td>- 3-methyl-2-furanone</td>
</tr>
<tr>
<td>- vanillin</td>
<td>- 2-furan methanol acetate</td>
</tr>
<tr>
<td>- hydroxyl benzaldehyde</td>
<td>- hydroxy methyl furfural</td>
</tr>
</tbody>
</table>

| 5) Phenol, alcohols, etc.                  |                                |

Bio-oil is a highly dense, polar oxygenated liquid that can be used as a fuel and for the production of chemicals [83–90]. Also, the introduction and implementation of bio-refinery concept that integrates a variety of processing technologies to produce multiple bio-products from various biomass sources has been the driving force for a gradual shift towards renewable biomass resources for chemical manufacturing [91–93]. Such an approach will help maximize the value of biomass and minimize by-products [94,95].

Lignocellulose biomass is the source of many chemical entities that are bound within the cellular matrix of the plant material. Thus hydrolysis of biomass give access to a number of chemicals as aqueous solutions. C6 and C5 sugars constitute a major
proportion in biomass hydrolyzate. Much research is being done to convert these sugars into energy or transform them into important chemicals that have a wide range of applications.

1.7. TECHNIQUES FOR SUGAR ISOLATION FROM BIOMASS

Rising interest in biomass processing has led to many sugar isolation techniques. Nearly all techniques include a hydrolysis step to release monosaccharides followed by either their selective isolation or direct enzymatic conversion to hydrogen, ethanol, furfural, etc. Hydrolysis of lignocellulosic biomass releases a number of plant metabolites including xylose, arabinose and glucose and is usually accomplished by heating with strong mineral acids for several hours. Often, hydrolysis is followed with a concentration step to separate sugars from other hydrolyzate components to facilitate sugar isolation and to prevent side reactions. The hydrolysis and concentration steps cause deterioration of sugars and are reported to be toxic to the fermentation process. A plethora of techniques such as overliming, addition of nutrients to offset toxic inhibition, or ion exchange have been employed to overcome these challenges. However, these solutions not only raise the cost of sugar isolation but cause even more by-products in the aqueous sugar solutions. Large scale sugar separation is predominantly carried out via difficult chromatographic separations which also makes it expensive and unsustainable [96].

In recent years a reversible boron chelation approach has gained prominence for separation of sugars from the hydrolyzate mix [97]. One prominent boronic acid based technique uses ion pairing of the sugar chelated boronate anions with lipophilic cationic salts to extract sugars in organic phase followed by hydrolysis in an aqueous solution.
These techniques have been optimized to increase the concentration of xylose by 7 fold in a clean water solution. Some of the reagents used in such techniques and mechanism of action are shown in Figures 1.13 and 1.14.

The method involves use of lipophilic boronic acid in organic solvents such as Exxal®10 (primarily composed of isodecyl alcohol and other aliphatic alcohols) or Shellsol®2046 (composed of aromatic, naphthalenes and paraffinics) that chelates sugars across the interface of immiscible aqueous hydrolyzate solution and organic solvent at pH 9 or above. Once the anionic boronates are formed, the cationic lipid extractant (Aliquat 336) in organic solvent pair with them and isolate the chelated sugars in organic solution.
These boronic esters are then stripped in an acidic aqueous solution to give aqueous sugar solution. This reactive solvent technology is frequently used prior to fermentation steps for production of ethanol to avoid catalyst / enzyme poisoning. However, the process delivers an aqueous solution of sugars limiting its utility to chemical modification.

Another process to isolate sugars uses a technique where polymeric microporous membrane with impregnated lipophilic carriers are used for sugar extraction. These supported liquid membranes were impregnated with cationic lipids or lipophilic boronic acids (Figure 1.16) and placed in an aqueous buffered solution of glucose and fructose. After 24 hours the membranes were washed with sugarless buffered solution and an enzymatic assay was done to estimate sugar isolation. The reported mechanism for such isolation is said to be the hydrogen bonding interaction between sugar hydroxyls and chloride counter ion of the quaternary ammonium carriers [98]. As expected boronic acid carriers in such experiments have been demonstrated to be the most reactive and selective for sugar isolation. Leaching of the carriers into aqueous phase is a common problem with these techniques.

Among all the technology invented for chemosensing or isolating sugars from either a medical or biomass processing perspective, boronic acids seem to be the most effective. The pH dependent reversible chelation of sugars has been extensively used to separate sugars from other chemicals components within a given biological matrix. However, these processes deliver aqueous solutions of sugars which are dried using other physical techniques to give solid sugars. The energy intensive drying or crystallization
processes are time consuming and increase the cost of production especially when the hydrolyzates/biomass contain lower concentration of sugars.

Isolation of sugars in crystalline form would be expected to give access to a much wider diversity of chemical modifications for applications in polymer, health and biofuel industries. Chapter 4 of this thesis describes a sustainable sugar isolation method that delivers crystalline C5 sugars in dry, pure form.

1.8. CONCLUSION

As reviewed in this chapter, thiol metabolites are responsible for a vast number of biological functions in the human body. Owing to their versatility and importance in metabolic functions, the analysis of thiols has been targeted extensively in the past decade. It is also clear from the methods listed in this chapter that there is a need for chemoselective reagents that can rapidly react with thiol metabolites and render properties that might help in their qualitative as well as quantitative analysis. To fulfill
this criteria, Chapter 2 describes a derivatization technology that provides structural information as well as allows high throughput quantitative capabilities for the analyzed thiols.

In Chapter 3 we describe a new idea for analysis of CA containing metabolites. Whereas the work was proven to be unsuitable for CA analysis due to sensitivity constraints, it led to a novel bridged oxygen-containing boron fluoride species. We are currently exploring new synthetic routes to synthesize this complex.

Chapter 4 describes a chemoselective technique for isolation of C5 sugars from DDG hydrolyzate solution using a sustainable method. We developed a phenyl boronic acid to chelate xylose and arabinose in aqueous solutions and extracted the adducts into organic solvents. Subsequent transesterification precipitated crystalline, dry sugars that could be easily filtered off for easy collection.

The experimental procedures for all chemistry and protocols throughout this thesis are describe in Chapter 5. All spectral data is combined in Appendix 1.
CHAPTER 2

PROFILING THIOL METABOLITES AND QUANTIFICATION OF CELLULAR GLUTATHIONE

2.1. INTRODUCTION

2.1.1. Significance of Thiol Analyses

Sulfur ranks among the top seven most abundant elements in the human body. It enters the metabolic pathways principally as methionine, a thioether amino acid, via ingestion of plant and animal proteins. Besides metabolic and catalytic activities, metabolism of methionine leads to synthesis of higher thio-proteins via various transsulfuration pathways as shown in Chapter 1 [99]. These cellular thiols constitute a major portion of total body antioxidants and are primarily known for their role in detoxification [100], signal transduction [101], apoptosis and defense against reactive oxygen species [102,103]. Mammalian tissues are rich in protein thiols that can undergo chemical modifications. For example, protein cysteine (Cys) residues can be oxidized to free thiols, intra- or inter-protein disulfides, nitrosothiols and sulphenic, sulphinic or sulphonic acids (Figure 2.1).

Cellular stress causes oxidation of Cys to form mixed disulfides between protein thiol groups and low molecular mass thiols, particularly with glutathione (GSH) (Figure
Figure 2.1. Principal oxidative modifications of cysteiny1 residues and their reduction pathways [104].

Grx = Glutaredoxin, Trx = thioredoxin, Srx = sulfiredoxin, GSNO = nitrosoglutathione.

1.3, chapter 1). This type of thio-regulation protects the cells against higher oxidation states of the protein thiol, thereby preserving the reversibility of such modifications and preventing permanent damage to the cells [105]. When GSSG accumulates in cells, it can undergo disulfide exchange reactions with other protein thiols, leading to their S-glutathionylation which is indicative of pathophysiological conditions. S-Glutathionylated proteins have been investigated as possible biomarkers of oxidative/nitrosative stress in some human diseases, such as cardiovascular disorders [106], chronic renal failure [107],
gastro-intestinal diseases [108] and diabetes [109–111]. Thus, cellular thiol containing metabolites, such as cysteine and glutathione, are crucially important for maintaining and regulating redox homeostasis in addition to protein functions [112,113].

As described above, the intracellular levels of GSH and GSSG can be indicative of cell pathogenesis [114–116] and studying the ratio of GSH to GSSG as well as quantifying the dysregulation of GSH and GSSG levels are keys to understanding oxidative stress and possibly the etiology of many important human diseases.

2.1.2. Methods for RSH Analysis

Numerous reagents and techniques have been developed to analyze thiol metabolites/proteins, esp. glutathione. As discussed in chapter 1, Ellman’s reagent, bimane derivatives and protein based probes are among the most commonly used reagents to study thio-proteins. The limitations of these probes also have been widely reported in the literature [117,118]. The rapid and constant thiol-disulfide flux in cells cause discrepancies in analysis of such metabolites. Any meaningful investigation of thiol metabolites requires a freeze in redox perturbation of molecular thiols. This is best accomplished using thiol selective reagents that would react irreversibly to form stable thiol adducts (Figure 2.2). Such chemoselective (CS) reagents are usually chemically modified to suit a particular analytical technique, e.g. fluorescent detection [119,120], colorimetric assays [121–123], capillary electrophoresis [124], electrochemical detection [125], SALDI-MS [126] and chromatographic methods coupled with mass spectrometry (GC-MS [127], LC-MS [128]). These are designed to covalently modify (i.e., tag) glutathione for detection and quantification using UV absorption studies. Assays
using these probes are also useful for quantification of known metabolites using reference standards. However, they are unsuited for quantifying metabolites when no reference standards are available, or in cases where isotopic tracers are employed, to deduce metabolic pathways or flux; nor can they be used for elucidating new thiol structures. The accuracy of colorimetric probes is sometimes also diminished due to the interfering cellular matrix.

In recent years, proteomic / metabolomic studies of thiols have used isotopic reagents to alkylate thiol metabolites / proteins followed either by affinity chromatography (e.g., biotin-avidin [129]) or liquid chromatography prior to analysis by MS [130,131]. Such S-alkylation using reagent isotopologues in a 1:1 ratio allows simple yet effective discernment of thousands of extraneous peaks from the mass spectrum to isolate the peaks of thiol adducts. These methods are expressly designed to increase the dynamic range for low abundance metabolites or low-level degradation products and to reduce ionization suppression [132]. While chromatographic purification approaches are effective for thiol metabolite identification as well as for determination of GSH/GSSG concentrations, the reliance on separation techniques can be tedious for high-throughput
applications, and in some cases, unnecessary, particularly with the advent of ultra-high resolution MS instruments [133]. Thio-alkylation reactions for these processes are usually conducted at high pH (8-9), which compromises the chemoselectivity of the reagents causing alkylations of cellular amines, phenols and carboxylate salts leading to false positives in the identification of cellular thiols [134]. For applications in metabolomics, this can be a severe constraint due to interference across the many other metabolite classes that must also be analyzed for metabolic networks.

2.1.3. Introduction to QDE/*QDE

To tackle the above-mentioned challenges for thiol metabolite analysis and as part of our ongoing efforts to develop reliable CS approaches for metabolite profiling [135], we developed a chemoselective method for the identification and quantification of thiol metabolites by direct infusion Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR-MS). FT-ICR-MS allows detection of ions with an accuracy of 0.000005 amu (5 ppm) while not requiring fragmentation of ions. The instrument is very effective to discern complicated isotopic patterns and allows MS^n analysis for structural confirmation. This characteristic was well suited to accomplish metabolite analysis using isotopic reagents. Quarternary ammonium Dodecyldimethyl Ethyl- iodoacetamide (QDE) (Figure 2.3.) was synthesized and tested as a thiol-selective reagent for capture of thiols. QDE features a hydrophobic domain to facilitate extraction of QDE-thiol adducts from aqueous cell extracts to minimize matrix interference with MS for accurate quantification. Moreover, the probe contains a permanent positive charge (quaternary ammonium moiety) to enhance detection of even low abundant thiol adducts by [+] -ion electrospray
mass spectrometry. The ultra-high resolution and accuracy mass capabilities of FT-ICR-MS enable simultaneous detection of many thousands of metabolite ions while providing credible molecular formula information, thus circumventing the need for chromatographic separations for the majority of metabolites. MS of polar cellular extracts contain thousands of metabolites peaks indistinguishable from the thiol adducts. This poses a challenge in identification of thiols vs. other metabolites. Given our aim of targeted metabolomics analysis of thiols we further streamlined the detection of QDE-thiol adducts by developing a convenient protocol that employed both QDE and its $^{13}\text{CD}_3$ isotopologue in acetonitrile to lyse cells at pH 4 or 7.4 for effectively tagging GSH and other thiol metabolites as isotopic pairs. The MS of such cell extracts would identify thiols adducts by manifesting equi-intensity peak pairs for each thiol adduct. This protocol considerably simplified the data analysis of observed MS peaks. Another major criterion of this project was to use relatively mild conditions that did not degrade non-thiol metabolites for their simultaneous or subsequent detection.

We hypothesized that by direct and selective alkylation of cellular thiols using isotopologues and their subsequent MS analyses would minimize disulfide formation and allow for the identification of observed thiol metabolites. Since iodoacetamide moiety
can react with thiols at lower pH values (<5), the reaction would be selective to thiols when compared with other techniques while the metabolite m/z obtained from MS analyses would give mass and molecular formulae of the metabolites - also inaccessible when using colorimetric or fluorometric techniques. The ammonium salt in the reagent served as tool for better ionization of the adducts in MS analyses, thus providing better sensitivity for low abundant metabolites.

The major aims of this project are, a) to develop a synthetic route to QDE and a m/z + 4 isotopologue (*QDE); b) to test the reactivity and selectivity of QDE towards thiols; c) optimize the use of QDE/*QDE for analysis of thiols in cultured cells; d) to identify and confirm thiol metabolites in cultured cell extracts; and e) to use the reagents for quantification of thiols.

2.2. RESULTS AND DISCUSSION

2.2.1. CS Probe Design

With the help of background information on thiol derivatization and analysis and already having some research group experience with the design of a CS-reagent for carbonyls, we hypothesized that a reagent with the following four features would be ideally suited for high throughput and accurate analysis of thiols. First, a reactive moiety that would rapidly block any thiol-disulfide flux by irreversibly reacting with thiols. This functionality also had to be chemoselective towards thiols and not generate any byproducts. Second, a hydrophobic chain that would increase the solubility of thiol adducts in an organic solvent. This would allow separation of polar, non-thiol metabolites
from thiol adducts. Third, a permanent cationic motif to allow analysis of thiol adducts via soft ionization techniques, without the need for fragmentation. A permanent charge would also ensure good ionization of the adduct species even at lower ionization energy. And lastly, an isotopologue of the reagent to facilitate quick and easy discernment of thiol signals from other metabolite signals in MS. This feature would help reduce false positive hits. These design features closely mirror the rationale behind QDA, a CS probe for carbonyl metabolites [135] (Figure 2.4).

The propensity of cellular thiols to form disulfides under oxidative stress makes it a challenge to analyze these metabolites. As discussed in Chapter 1, many reagents have been developed that chemically modify thiol metabolites to render functionalization that may assist in identification of new metabolites and provide the ability to accurately quantify them. To overcome the thiol-disulfide perturbation an optimal reagent for reaction with thiol-containing substrates should include the ability to alkylate a thiol

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**Figure 2.4. Comparison of QDA and QDE reagents.**
moiety with high efficiency and selectivity while imparting stability to the resultant adduct for subsequent MS analysis. Some of the more common derivatization reagents currently used for thiol analysis are depicted in Figure 2.5.

\[ \text{IAA} \quad \quad \text{IAM} \quad \quad \text{MMTS} \]

\[ \text{R} = \text{CH}_3, \quad \text{NEM} \quad \quad \text{VP} \]

\[ \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \ quad
Figure 2.6. Peak pairing of thiol adducts when isotopologues QDE and *QDE are used.

One of the major challenges in any metabolite analysis is the occurrence of false positive peaks in MS due to extraneous ions from the cellular matrix. To eliminate these peaks, we synthesized *QDE, a $^{13}$C$_1^2$H$_3$-isotopologue of QDE by N-alkylation using labeled iodomethane. The presence of a ($m/z + 4$) peak for every thiol adduct in MS allows exclusion of false positives, and therefore a more precise analysis of the metabolites (Figure 2.6.). For example, the availability of an $m/z$ (QDE) and $m/z + 4.02188$ (*QDE) isotopic pair of reagents is key to rapid and automated identification of thiol adducts. Cells treated with a 1:1 mixture of QDE and *QDE can be expected to produce respective adducts in a similar ratio. Thus, the presence of an equi-intensity $m/z + 4.02188$ peak for any peak observed in the FT-ICR-MS spectrum of the organic phase extract is a primary
selection criterion to identify true adducts as opposed to underivatized cell components. Incorporation of stable isotopes into the downstream metabolites using precursors enriched with stable isotopes such as $^2$H, $^{13}$C or $^{15}$N provides vital clues regarding disease mechanisms through the tracing of isotope enriched metabolite products through the various metabolic pathways and through the measurement of fluxes [141]. Finally, the availability of an isotopically labeled probe enables the synthesis of labeled adducts for use as standards to quantify particular thiols (e.g., quantification of GSH using [*QDE-GSH] $^+$).

2.2.2. Synthesis of QDE and *QDE (Aim a)

QDE and *QDE were synthesized using steps described in Schemes 2.1. and 2.2. The thiol reactive reagents were synthesized from readily available chemicals. Amino phthalimide 1 was synthesized from $N,N$-dimethylethanolamine using a literature procedure (modified Mitsunobu reaction) [142]. Alkylation of the amine in 1 was accomplished by reaction with 1-iodododecane in acetonitrile. The crude ammonium phthalimide salt was easily collected by evaporating the solvent and subsequent washings with hexanes. $^1$H NMR spectroscopy of the crude material showed characteristic aliphatic methylene and methyl protons of the C$_{12}$ chain between $\delta$ 0.7 and 2.0 ppm confirming the alkylation. Hydrazinolysis using hydrazine monohydrate of the crude phthalimide revealed the amine. The formation of amine 2 was manifested with the precipitation of phthalhydrazide. On completion of the reaction, the solvent was evaporated and the solids were reconstituted in CH$_2$Cl$_2$ (DCM) whereby the undissolved solids(phthalhydrazide) were separated from soluble $N$-(2-aminoethyl)-$N,N$-dimethyl-
Scheme 2.1. Synthesis of QDE.

Reagents and conditions: a. 1-iodododecane, CH₃CN, 100 °C, 20 h; b. H₂NNH₂•H₂O, EtOH, 40 °C, 12 h, 83% (2 steps); c. chloroacetyl chloride, K₂CO₃, CH₂Cl₂, rt, 12 h; d. NaI, acetone, 65 °C (sealed tube), 20 h, 78% (2 steps).

Scheme 2.2. Synthesis of *QDE.

Reagents and conditions: a. 1-bromododecane, Na₂CO₃, EtOH, reflux, 24 h; b. phthalimide, PPh₃, DIAD, THF, 0 °C to rt, 12 h, 29% (2 steps); c. ¹³CD₃I, 50 °C (sealed tube), 3 h; d. H₂NNH₂•H₂O, EtOH, 40 °C, 12 h, 93% (2 steps); e. chloroacetyl chloride, K₂CO₃, CH₂Cl₂, rt, 12 h; f. NaI, acetone, 65 °C (sealed tube), 20 h, 32% (2 steps)
dodecylammonium iodide (2). DCM was evaporated to collect pale yellow solids in 83% yield (2 steps). $^1$H NMR spectral analysis of 2 confirmed the assigned structure (no aromatic protons); IR absorption at 3490 cm$^{-1}$ confirmed the presence of N–H and high resolution mass spectrum (HRMS) confirmed the mass of the compound to be 257.2954 (calculated mass, 257.2951).

With amine 2 in hand, we tried to prepare the iodoacetamide directly by treating 2 with iodoacetyl chloride in DCM in presence of K$_2$CO$_3$. After workup the product NMR revealed $\alpha$–methylene protons at $\sim$3.8 and $\sim$4.1 ppm indicating a mixture of chloro and iodoacetamide products. Presumably the halide exchange at the $\alpha$-carbon is problematic. To avoid this problem we first reacted amine 2 with chloroacetyl chloride and then treated the resultant chloroacetamide with NaI in acetone (Finkelstein reaction [143]) to give iodoacetamide QDE. The crude material was purified by column chromatography to obtain 78% yield over 2 steps. $^1$H NMR of the product showed $\alpha$–methylene protons overlapping the $N$-methylene protons (3.8 ppm). HRMS peak at 425.2025 m/z confirmed the product. The $^1$H, 2D-COSY and HRMS spectra of QDE are shown in Figure 2.7.

The synthesis of *QDE required a different order of reactions so as to install the $^{13}$CD$_3$ label. Ethanolamine was alkylated using bromododecane in ethanol to give the corresponding tertiary amine. The amino alcohol was obtained as a viscous liquid in 81% yield by Kugelrohr distillation. This amino alcohol was then subjected to a Mitsunobu protocol to deliver amine phthalimide 3. This product was confirmed by the characteristic protons of the phthalimide 3 at $\delta$ 7.83 and 7.77 ppm.
To introduce the isotopically labeled methyl group, phthalimide 3 was treated with $^{13}$CD$_3$I in DCM in a sealed tube. Precipitation indicated rapid alkylation of the amine. Quaternization of the amine was confirmed by the downfield shift of the $N$-methylene and $N$-methyl groups in $^1$H NMR at $\delta$ 3.5 and $\delta$ 3.2, respectively. Furthermore, introduction of the labelled carbon was confirmed by the coupling of the carbon observed in $^{13}$C NMR spectrum at 51.4 ppm.

The labeled phthalimide was then subjected to hydrazinolysis using hydrazine monohydrate as used previously in the QDE synthesis. The labelled amino ammonium salt 4 was dried to give a waxy solid in 93% yield. This amine salt was then subjected to amidation and halide exchange reaction using the established protocol to give *QDE in 35% yield over two steps. The somewhat low yield can be attributed poor separation of *QDE in the column. The $^1$H, $^{13}$C and HRMS spectra of *QDE are shown in Figure 2.8.
Figure 2.7. Spectral analysis of QDE.

a) 400 MHz $^1$H NMR spectrum of QDE in CDCl$_3$, b) 400 MHz 2D COSY $^1$H NMR spectrum of QDE in CDCl$_3$ and c) FT-ICR-MS of QDE
Figure 2.8. Spectral analysis of *QDE.

a) 400 MHz $^1$H NMR spectrum of *QDE in CDCl$_3$, b) 100 MHz $^{13}$C NMR spectrum of *QDE in CDCl$_3$ and c) FT-ICR-MS of *QDE
2.2.3. CS Probe Reactivity (Aim b)

To test the reactivity / selectivity of QDE towards thiols and to optimize those reaction conditions we examined several parameters for reaction of QDE with the representative biological thiols l-glutathione and l-cysteine (Scheme 2.3.). Reactions performed at pH 5.8 (potassium phosphate buffer in acetonitrile) with 3:1 molar excess of QDE to thiol showed exhaustive consumption of the thiol with the respective adduct formation as seen in FT-MS. However, at pH 8 under similar conditions we also noted the bisalkylation of GSH, presumably due to GSH amine alkylation at this pH (Figure 2.9). The reactivity of α-iodoacetamide reagents, such as QDE, with cellular amines [138,144], phenols and carboxylate salts has been reported previously under alkaline pH conditions [134]. Although at low pH the reaction of thiols with the α-iodoacetamide moiety is slow [136], the chemoselectivity is very high because amine and carboxylate alkylations are deterred. Consequently, we employed QDE/*QDE for parallel thiol derivatization at pH 4 (to improve chemoselectivity for the thiol group) and pH 8 (for exhaustive derivatization – quantification).

We repeated these reactions, divided the reaction mixtures into 5 aliquots and then extracted these aliquots with different solvents, viz. CH₂Cl₂, CHCl₃, n-BuOH, toluene and Et₂O. As expected we observed that n-BuOH was most effective solvent to extract [QDE-thiol]⁺ adducts from an aqueous phase — for example, more than 95% of the [QDE-Cys]⁺ adduct was recovered within the first two extractions. These results closely mirror our earlier observations [135] on the extraction of structurally analogous aminoxo probe (QDA)-carbonyl metabolite adducts.
Scheme 2.3. Reactions of QDE with GSH and Cys at pH 5.8 and 8.0.

Figure 2.9. Ion trap mass spectrum of QDE, GSH reaction at pH 8.
2.2.4. Profiling Thiol Metabolites in A549 Cells (Aim c)

Human lung adenocarcinoma cell line A549 was established in 1976 and since then it has been well established in the literature as an experimental system to study cancer biology [145]. Hence, we obtained cultured human adenocarcinoma A549 cells from our collaborative labs (Dr. Teresa Fan and Dr. Richard Higashi) for QDE/*QDE derivatization of cellular thiol metabolites as described in Figure 2.10. FT-MS spectra of the extracted thiol adducts was observed to be consistently and unusually lower than previously reported values. For example, Spadaro et al. reported 30.11 nmol/mg protein of GSH in A549 cells using an HPLC technique where oxidation of GSH to GSSG was prevented using an acidification technique [146]. To ensure complete alkylation of all thiols the ratio of QDE/*QDE to thiols was increased from 3:1 to 10:1. Surprisingly, increasing the amount of QDE/*QDE used per extract further increased the intensity gap between QDE and QDE-thiol adduct peaks. This could be explained only if the concentration of thiols was decreased due to disulfide formation during cell lysis and extraction.

To solve this problem, human lung adenocarcinoma A549 cells were lysed with equimolar mixtures of QDE and *QDE in acetonitrile at pH 4 and 7.4 and then analyzed by FT-ICR-MS as described in Chapter 5. This direct, in situ quench/derivatization approach allowed for the rapid S-alkylation of labile thiols prior to their oxidations to corresponding disulfides, thus showing an intensity increase for QDE-thiol adduct peaks and measuring true value of reduced thiols in cellular matrix. The profiling experiments were performed 4x for each pH condition using this direct quench technique.
2.2.5. Data Analysis (Aim d)

The FT-ICR-MS data was exported to a Microsoft Excel 2010 worksheet, any peaks below the noise threshold were removed and an in-house program (PREMISE [147]) was used to screen peaks with similar ion counts of \(m/z\) and \(m/z + 4.02188\). This provided a list of masses of QDE adducts that were subjected to mathematical computation to remove the mass of QDE (\(m/z\) of adducts – 297.29004) and to obtain exact masses of...
metabolites. A query of these exact masses provided a registry of metabolite molecular formulae which were uploaded to Human Metabolome Database (HMDB) for metabolite identification. We identified 12 metabolite masses that passed the filter criteria in at least 2 out of 4 experiments at a given pH, which are listed in Table 2.1. The structural assignments for the highest ion count metabolites (entries 1-3) are in agreement with the chemoselectivity of QDE, namely the 4/4 hit rate at pH 4 strongly supports the thiol assignments. Collision induced mass spectroscopy (CID MS/MS) analyses confirmed the structural assignments as cysteine, cysteinylglycine and glutathione (Figure 2.11.), respectively. These thiols have been documented previously as major thiol metabolites [148]. Their relative abundance is nearly ten-fold higher than that of the other thiol metabolites, as can be seen in a representative mass spectrum (Figure 2.11.) that shows the ion count matching of the blue QDE and red *QDE adduct pairs. Due to the high ion count of these metabolites, we were able to confirm the $^{34}$S isotope incidences at ~4% of the respective $^{32}$S adducts. The sodiated C-terminal peaks for the glutathione adduct were also observed.

Entries 4-6 (Table 2.1.) are low-to-moderate abundant metabolites that also were assigned as containing nucleophilic sulfur based on the observed reactivity with QDE at pH 4. Whereas hypotaurine (Figure 2.11.), a sulfinic acid intermediate of taurine biosynthesis [149], is not a thiol, it reacts similarly due to the nucleophilicity of the sulfinic sulfur atom [150]. The assignments of hypotaurine and homocysteine (entries 4 and 5) were confirmed using CID MS/MS analysis. An example of the comparison between fragmentation pattern of the metabolite adduct and the standard is shown in Figure 2.13.
Table 2.1. Metabolites identified using paired QDE/*QDE approach.\textsuperscript{a}

<table>
<thead>
<tr>
<th>Entry</th>
<th>Metabolite MF</th>
<th>pH 4.0\textsuperscript{b}</th>
<th>pH 7.4\textsuperscript{b}</th>
<th>Metabolite mass\textsuperscript{c}</th>
<th>Mass error\textsuperscript{d}</th>
<th>Ion Count\textsuperscript{e}</th>
<th>Metabolite assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>C\textsubscript{3}H\textsubscript{7}NO\textsubscript{2}S</td>
<td>4</td>
<td>4</td>
<td>121.01884</td>
<td>0.00091</td>
<td>1200443</td>
<td>cysteine</td>
</tr>
<tr>
<td>2</td>
<td>C\textsubscript{10}H\textsubscript{11}N\textsubscript{2}O\textsubscript{5}S</td>
<td>4</td>
<td>4</td>
<td>178.04046</td>
<td>0.00077</td>
<td>494591</td>
<td>cysteinylglycine</td>
</tr>
<tr>
<td>3</td>
<td>C\textsubscript{12}H\textsubscript{11}N\textsubscript{2}O\textsubscript{6}S</td>
<td>4</td>
<td>4</td>
<td>307.08321</td>
<td>0.00061</td>
<td>1492062</td>
<td>glutathione</td>
</tr>
<tr>
<td>4</td>
<td>C\textsubscript{3}H\textsubscript{7}NO\textsubscript{2}S</td>
<td>4</td>
<td>4</td>
<td>109.01886</td>
<td>0.00090</td>
<td>32595</td>
<td>hypotaurine</td>
</tr>
<tr>
<td>5</td>
<td>C\textsubscript{4}H\textsubscript{8}NO\textsubscript{2}S</td>
<td>3</td>
<td>4</td>
<td>135.03451</td>
<td>0.00090</td>
<td>23527</td>
<td>homocysteine</td>
</tr>
<tr>
<td>6</td>
<td>C\textsubscript{11}H\textsubscript{15}N\textsubscript{2}O\textsubscript{7}S</td>
<td>2</td>
<td>1</td>
<td>337.09397</td>
<td>0.00042</td>
<td>14746</td>
<td>tripeptide comprised of Cys, Gln and Ser</td>
</tr>
<tr>
<td>7</td>
<td>C\textsubscript{12}H\textsubscript{12}NO\textsubscript{2}S\textsubscript{2}</td>
<td>0</td>
<td>2</td>
<td>307.09109</td>
<td>0.00084</td>
<td>56570</td>
<td>succinylldihydrioloamide \textsuperscript{g}</td>
</tr>
<tr>
<td>8</td>
<td>C\textsubscript{5}H\textsubscript{9}NO\textsubscript{4}S</td>
<td>0</td>
<td>4</td>
<td>179.02439</td>
<td>0.00085</td>
<td>36528</td>
<td>S-carboxymethylcysteine</td>
</tr>
<tr>
<td>9</td>
<td>C\textsubscript{6}H\textsubscript{10}O\textsubscript{6}</td>
<td>0</td>
<td>2</td>
<td>178.04711</td>
<td>0.00064</td>
<td>82132</td>
<td>ketodeoxygluconic acid; deoxyglucuronic acid</td>
</tr>
<tr>
<td>10</td>
<td>C\textsubscript{8}H\textsubscript{13}NO\textsubscript{2}S</td>
<td>0</td>
<td>2</td>
<td>163.06593</td>
<td>0.00078</td>
<td>27324</td>
<td>S-(2-carboxypropyl)cysteamine; S-propylcysteine</td>
</tr>
</tbody>
</table>

**Thiol metabolites** (high ion count abundance)**f**

**Thiol or sulfinic metabolites** (moderate to low ion count abundance)**f**

**Carboxylic acid or amine metabolites**\textsuperscript{g}

**Unassigned metabolite**

\textsuperscript{a} Only metabolite adducts registering a minimum of 10K ion counts and occurring in at least 2 out of 4 experiments under either the pH 4 or 7.4 conditions are tabulated; \textsuperscript{b} number of times the metabolite adduct was observed in 4 experiments at the indicated pH; \textsuperscript{c} adduct m/z – (QDE – iodide) + H; \textsuperscript{d} actual metabolite mass computed from MF – measured metabolite mass (column at left); \textsuperscript{e} highest ion count observed at either pH condition; \textsuperscript{f} ion count abundance categorized either as high (>100K), moderate (>50K), or low (<50K); \textsuperscript{g} putative metabolite assignment(s); \textsuperscript{h} >5 molecular formulae fit this mass ± 0.0015 amu.
Figure 2.11. Confirmed thio-metabolites identified using paired QDE/*QDE approach.
(neutral configurations depicted, nucleophilic center circled).

Figure 2.12. Mass spectrum obtained from $n$-BuOH extract of A549 cells treated with QDE/*QDE.
(blue = [QDE-thiol]$^+$ adduct, red = [*QDE-thiol]$^+$ adduct) at pH 7.4. The three high ion count abundant [QDE-thiol]$^+$ adducts are identified.

The QDE-Cys adduct fragments first into the ammonium cation and Cys residue to give expected fragments of 214 and 205 m/z, respectively. Further MS/MS analysis of the cysteine containing fragment in both the standard and test sample gave nearly identical fragmentation pattern.
Figure 2.13. CID MS/MS/MS spectrum of QDE-Cys.

Top: QDE-Cys in derivatized cell extracts; Bottom: QDE-Cys Standard

Interestingly, the lack of adduct formation at pH 4 for the metabolites of entry 7 is consistent with the highly labile nature of succinylhydrolipoamide, an intermediate involved in the transfer of a succinyl group from oxoglutarate via the oxoglutarate dehydrogenase complex (α-ketoglutarate dehydrogenase complex) [151]. This adduct was registered only under pH 7.4 conditions at which thiol alkylation occurs readily, while at pH 4 this reaction occurred at a slower rate and presumably hydrolysis of the labile thioester bond in succinylhydrolipoamide prevented the buildup of the adducts to detectable levels. The lack of QDE adduct formation at pH 4 for the metabolites of entries 8-11 suggests that these are either highly labile metabolites or, more likely, non-thiol
metabolites. The most probable HMDB-based assignments for these entries are given except for entry 11 for which more than 10 possibilities exist. The value of performing thiol profiling at both pH 4 and 7.4 is further noted when comparing the reactivity of the entry 8 metabolite — the lack of adduct appearance at pH 4 suggests that the sulfur in this C_5H_9NO_4S metabolite is not a free sulfhydryl. Rather, adduct formation is likely a result of carboxylate alkylation, hence its putative assignment as the S-ether [152,153]. The same is true for the C_6H_{13}NO_2S metabolites of entry 10, also putatively assigned as S-alkylated thiols [154,155].

2.2.6. Quantification of Cellular Glutathione (Aim e)

In response to stress, cells undergo perturbation of the thiol-disulfide levels. Redox cycling of glutathione has been well established in the literature as one of the protective roles of this versatile antioxidant [100]. To understand this role of glutathione, we must accurately measure both the reduced (GSH) and oxidized (GSSG) glutathione. Oxidation of GSH due to stress during cell lysis makes it a challenge for it to be quantified accurately and any meaningful analysis of thiols would require a freeze in thiol-disulfide flux. To accomplish this, it is necessary to both quench any artifactual oxidation of GSH to GSSG by direct *in situ* derivatization as illustrated above, and to exhaustively reduce GSSG. For the latter, commonly used reducing agents include tris(2-carboxyethyl)phosphine hydrochloride (TCEP⋅HCl) [156,157], dithiothreitol [158], trialkylphosphines [159], NaBH_4 [160], and occasionally sulfites, cyanides or some enzymes. We selected the TCEP⋅HCl reagent (Scheme 2.4.) because it eliminates the complication of added thiol reagents, as with the use of dithiothreitol, and the possibility
Scheme 2.4. Use of TCEP•HCl for reduction of disulfides.

of over-reduction, as often the case with using NaBH₄. An excess of reducing agent is required to exhaustively reduce all disulfide bonds. Unfortunately, since TCEP•HCl is a phosphonium cation, adding excess of this reducing agent would present a very high intensity signal in MS, thereby causing ion suppression of the thiol adducts. We were gratified to find that TCEP•HCl reacted with the iodoacetamide group of QDE to form the [QDE-TCEP] adduct (m/z 547.3512) which was readily eliminated at the ion trap stage of the FT-ICR-MS thus enabling tuning of the ion abundance targets (called Automated Gain Control targets) to optimize the spectra [161,162]. Thus, after reduction of glutathione disulfide using TCEP•HCl, excess QDE was added to capture the newly reduced GSH as well as to consume the excess TCEP•HCl.
We synthesized an isotopic internal standard [*QDE-GSH]^+ for intensity normalization and quantification purposes. Increasing concentrations of [*QDE-GSH]^+ were added to the cellular extracts to establish calibration curves while nullifying interferences from cell matrices. This also accommodated for any sodiation of the adduct in the extracts being analyzed. We found the linear range for quantification to be between 0.04—5.00 μM of [*QDE-GSH]^+ with regression \((R^2)\) values consistently over 99% for three replicate experiments (Figure 2.14.). The quantification of GSH and GSSG was thus carried out within this range. Three plates were analyzed and each extract was quantified three times to ensure reproducibility of the quantification results. Total concentrations of [GSH] and [GSSG] were determined as 34.4 ± 11.5 nmole/mg protein and 10.1 ± 4.0 nmole/mg protein, respectively. Whereas the GSSG concentration in A549 cells has not been reported previously, our measured GSH concentration in this cell line agrees with the GSH concentration of 30.1 ± 1.5 nmole/mg protein recently reported by Spadaro et al. [146]. In contrast, the GSH concentrations measured in surgically resected human lung tumors of eight patients, also adenocarcinoma, averaged 8.8 ± 1.0 nmole/mg protein [163]. The lower value in this case is attributed to cancerous cell heterogeneity in tumor tissues and/or the rapid GSH/GSSG perturbations during cell lysis that were not taken into consideration. The direct in situ quench method shown here avoids such perturbations and allows for more accurate quantification.
Figure 2.14. Quantification of GSH and GSSG in A549 cells.

Linear ranges for detection of GSH (♦) and GSSG (▲) were established using [*QDE-GSH]* adduct as a standard. The [GSH] (■) and [GSSG] (●) concentrations were measured (n=3) from 145 μL aliquots taken from the n-BuOH supernatants at stages § and §§ (Figure 2.10.) for GSH and GSSG, respectively.
2.3. CONCLUSIONS

The synthesis and evaluation of a reagent for chemoselective capture of thiols is described. This research established an experimentally convenient approach to profile cellular thiols. Addition of the novel ammonium α-iodoacetamide reagent QDE and its m/z + 4 isotopologue *QDE directly to live cells followed by n-BuOH extraction and direct electrospray FT-ICR-MS analysis of the adduct mixture provides a registry of isotopologue pairs that is readily processed for compound identification. The probe design and experimental protocol coupled with the high mass accuracy and ultra-high resolution of FT-ICR-MS enable determination of metabolite molecular formulae once QDE-thiol adduct signals have been confirmed. Equally important is the enhanced chemoselectivity of QDE when cell extracts were reacted at pH 4, thereby better delineating thiol from non-thiol metabolites. Our analysis of A549 cell extracts revealed the major thiols to be glutathione, cysteine and cysteinylglycine. We also observed, for the first time, S-alkylated hypotaurine. Finally, QDE and *QDE were readily applied for thiol quantification, such as demonstrated for the quantifications of glutathione and glutathione disulfide. We showed this approach to be sensitive at the 40 nM range and linear in the concentration range of 0.04 to 5.0 μM with >99% accuracy. Fluorometric or colorimetric analysis described in chapter 1 provides limited quantitative information that is sometimes biased due to non-selective nature of such analyses. Our technique provides more information about the metabolites viz. m/z, molecular formulae, while allowing confirmation of the identity of the metabolites via MS/MS. We also demonstrated our quantitative analysis was comparable to untargeted HPLC technique
employed for GSH analysis by Spadaro et al. In summary, this thesis work enables high-throughput sample analyses and information for profiling cellular thiols as well as facilitates their quantification.

2.4. GRAPHICAL SUMMARY

2.5. FUTURE DIRECTIONS

Since publication of this work, several articles have been published describing oxidation behavior of thiol-containing amino acids with glutathione, quantitative analysis of thiols in biofluids, and antioxidant behavior of thiols [164–166]. Clearly the analysis of thiol metabolites is of great interest. Consequently, the described protocol is currently being streamlined for faster analysis by reducing the reaction times for thiol-adduct
formation. The extraction of adducts in n-BuOH to remove other components of the cellular matrix has now been replaced with a facile, micro-tip filtration technique developed by our collaborator, Dr. Fan. The goal of this research is to develop multiplexed analysis technology for use in high-throughput metabolite identification and quantification. A comparative study of healthy and diseased cells with demonstrable differences in metabolite intensities would pave the way for a diagnostic tool for the future. A better tool for analysis of the mass spectral data is being developed by Dr. Hunter Moseley’s lab at the University of Kentucky to hopefully expedite screening and accord structural identification with greater accuracy. We hope to extend this technology to understand seleno-cystine metabolism in cells.
CHAPTER 3

FORMATION OF CATIONIC $B_2NO_2C$ 6π HÜCKEL RINGS TO ANALYZE CARBOXYLIC ACIDS

3.1. INTRODUCTION

3.1.1. Significance of Carboxyl Metabolites

The carboxylic acid (CA) functional group is ubiquitous in a large number of essential metabolites such as fatty acids, keto-acids, bile acids, messenger molecules and breakdown products of hormones, etc. CAs are also integral in endogenous molecules such as mono-, di- and triglycerides and in fatty acylations of proteins. These acids range from very hydrophilic small-chain fatty acids (formic and acetic acid being the smallest) to hydrophobic long-chain fatty acids, prostanoids, bile acids, etc. The chemical reactivity of the CA group in metabolites has a cascade effect on metabolic pathways that often leads to formation of covalently bound protein adducts.

Carboxylic acid intermediates play an important role in central carbon metabolism that feeds several metabolic subpathways, such as glycolysis, gluconeogenesis, pentose phosphate pathway, and the tricarboxylic acid (TCA) cycle [167]. These processes are responsible for production of usable cell energy in the form of adenosine triphosphate, synthesis of essential biomolecules (e.g., amino acids) and to facilitate cellular responses
Table 3.1. Carboxylic acid drugs associated with toxicity.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Structure</th>
<th>Observed Toxicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benoxaprofen (racemic mixture)</td>
<td><img src="image" alt="Structure" /></td>
<td>Cholestatic hepatotoxicity, Skin rash, photosensitivity</td>
</tr>
<tr>
<td>Clofibrate</td>
<td><img src="image" alt="Structure" /></td>
<td>Elevated aminotransferases, cholestasis, hepatitis, muscle injury</td>
</tr>
<tr>
<td>Clometacin</td>
<td><img src="image" alt="Structure" /></td>
<td>Elevated aminotransferases, hepatitis, cirrhosis, renal injury</td>
</tr>
<tr>
<td>Diclofenac</td>
<td><img src="image" alt="Structure" /></td>
<td>Elevated aminotransferases, hepatitis, jaundice, liver cell necrosis, skin rash, anaphylactic shock</td>
</tr>
<tr>
<td>Fenbufen</td>
<td><img src="image" alt="Structure" /></td>
<td>Elevated aminotransferases and alkaline phosphatases, hepatitis, hepatic necrosis, skin rash</td>
</tr>
<tr>
<td>Hypoglycin</td>
<td><img src="image" alt="Structure" /></td>
<td>Hypoglycaemia, steatosis</td>
</tr>
<tr>
<td>Ibufenac</td>
<td><img src="image" alt="Structure" /></td>
<td>Elevated aminotransferases, jaundice, hepatic necrosis</td>
</tr>
<tr>
<td>Tolmetin</td>
<td><img src="image" alt="Structure" /></td>
<td>Hepatotoxicity</td>
</tr>
<tr>
<td>Zomepirac</td>
<td><img src="image" alt="Structure" /></td>
<td>Anaphylactic shock, renal failure</td>
</tr>
</tbody>
</table>

to external stimuli [168–172]. Perturbations in CA concentrations of a specific pathway can indicate defects in cellular metabolism and therefore pathology. Identification of carboxylic acids, and quantification of alterations in their biological concentration during physiological and pathological processes is essential to discovering their mechanistic roles in diseases [173–181].
Over the past century many CA-containing drugs have been introduced and then withdrawn from the drug market due to rare but serious adverse reactions (Table 3.1) [182]. Of particular interest are drugs that belong to the nonsteroidal anti-inflammatory drug class. Many of these have been proven to cause a number of liver ailments, jaundice, skin rash, eczema, etc. Bioactivation of the CA groups to reactive metabolites in these drugs can affect critical proteins, cellular functionality or may induce an immune response, eliciting adverse effects that in serious cases can be fatal [183]. Hence, analysis of CA metabolites would be beneficial in understanding and possibly even resolving some of these dysfunctions.

3.1.2. Methods for Carboxylic Acid Analysis

CAs are predominantly analyzed using gas chromatography coupled to mass spectrometry (GC-MS) after their chemical derivatization with reagents such as boron trifluoride / butanol [184], hexamethyldisilazane [185], N-methyl-N-tert-butylmethyldimethylsilyl trifluoroacetamide [186] or N-methyl-N-trimethylsilyl trifluoroacetamide [187,188]. Another common technique for analysis of organic CAs is anion exchange chromatography [189] or ion exclusion chromatography [190] coupled to mass spectrometry. Generally, these techniques suffer from poor ionization in negative mode MS and thus it is advisable to derivatize carboxylic acid metabolites with a charge-switch probe.

Nearly all CA reagents require some form of activation for derivatization. For example, reagents based on amidation or esterification of CAs usually require activation via halopyridinium salt or carbodiimide followed by subsequent reaction with an amine.
or alcohol (Scheme 3.1). This mode of activation is often inefficient and many analytes do not efficiently react and are lost in the process. Furthermore, the amine or alcohol coupling reactions often require harsh reaction conditions that eventually lead to byproducts and degradation of metabolites [191]. Other techniques include fluorescence measurements and isotopic labelling via chemical derivatization. However, these techniques also face the same challenges listed above.

3.1.3. Novel Carboxylic Acid Reagent

To introduce a new technique for analysis of CAs we sought to develop a reagent capable of rapidly chelating CAs to create a cationic species that could be analyzed using FT-ICR-MS. The Lewis acidity of boron and its affinity for oxygen is well reported in the
literature [192] and seemed ideally suited for design of a reagent that would chelate both oxygen atoms of the CA moiety. The innovative angle to this research was the idea to exploit formation of a novel boron-based heteroaromatic nucleus that would render stability to the CA adduct and simultaneously impart a positive charge. As discussed below, such an approach to generate a positive charge as a consequence of aromatization could be the key to mass spectrometric analysis of CAs (Figure 3.1).

We hypothesized that a reagent with two boron halides linked with a heteroatom would chelate carboxylic acids. The formation and stabilization of such molecule would be driven by aromatization of the ring structure and the resulting molecule would have a cationic charge similar to that in a pyrylium ion (frequently used for MS analysis of amines). Such chelation of CAs and the resultant charge switch due to formation of aromatic cation would thus enhance their ionization in MS for analyses. Thus we set out to design a boron based CA reagent for metabolomic analysis with the following aims.
Our main aims for this project included: a) design of a boron-based reagent that would chelate CAs to impart a positive charge for MS analysis; b) to explore aromaticity as a driving force for CA complexation; c) to synthesize the reagent and test its reactivity towards carboxylate salts; and d) to test the reagent with biological extracts to provide proof of concept for metabolomics analyses applications.

3.2. RESULTS AND DISCUSSION

3.2.1. Carboxyl Probe Design (Aims a/b)

As discussed above, existing technologies for analysis of carboxylic acids suffer from added interfering ions and multiple reaction steps. To exploit the resolving power (200,000 and mass accuracy of 0.5 ppm at 400 m/z) and high sensitivity (<40 nM solutions) of modern MS instruments we hoped to develop a new chemical technique for derivatization of carboxylic acids. The pre-eminent requirement of such a new technology would be a clean, salt-free conjugation without byproduct formation at any stage or the need to add subsequent reagents, such as coupling partners. Other requirements include good ionization potential in MS and easy or even direct analysis of adduct mixtures. We postulated that a reagent with a boron halide functionality would be ideal for initial reaction of carboxylate salt to form an adduct. A reagent with two such motifs joined by a suitable linker (i.e. electron donating) could promote ring formation and could possibly be thermodynamically driven (Figure 3.2, a). The adduct, as postulated in Figure 3.1., would have two boron atoms, and 2 oxygens with lone pair electrons that could be delocalized forming a 6π-Hückel consistent ring. A suitable linker atom with a p-orbital
and two electrons would be needed to achieve aromaticity (Figure 3.2, b). Such delocalization would suggest aromatic character that could provide some measure of stabilization for the assembly (Figure 3.2, c). Thus, we hoped to exploit the Lewis acidity of boron, its affinity towards oxygen and the inherent stability of aromatic assemblies by making a reagent that would bind carboxylic acids under mild reaction conditions to form stable adducts suitable for MS analysis.

Our first idea was to use azadiboriridines (ADB) (structure a, Scheme 3.2.) as carboxyl tagging reagent. ADBs are three-membered rings that are isoelectronic with the \( \text{C}_3 \)-ring skeleton of the aromatic cyclopropenyl cation. ADBs follow Hückel’s rule and are
classified as being aromatic [193,194]. Interestingly, the B–B bond of azadiboriridine exhibits basic character towards acidic protons [195]. For example, CA-derived proton can add to the B–B bond to form a BHB bond (3 centered 2 electron bond – indicated by a triangle within the three membered ring, as in intermediate b, Scheme 3.2). The resultant electron deficient species b from Scheme 3.2 reacts with the carboxylate anion to give c, Scheme 3.2. [196]. The added carboxylate then contributes a second lone pair of electrons to cleave the B–B bond to yield 2,4,5,6-tetra-alkyl-6-hydro-1-oxa-3-oxonia-5-azonia-4,6-diborata-2,4-cyclohexadiene d, Scheme 3.2. Adduct d can have enantiomeric structures due to 1, 5 sigmatropic migration of the hydride as shown in [197]. This migration is fast at room temperature with respect to NMR time scale. These molecules have been previously synthesized and characterized using $^1$H, $^{13}$C and $^{11}$B NMR [196,198,199].

We hypothesized that oxidation of d would provide a net cationic adduct e, that is isoelectronic with benzene. Theoretically, this molecule satisfies all rules of aromaticity and we believe this structure will be stable in non-reactive, organic solvents, such as Et$_2$O or hexane. Importantly, the compound is cationic only when reacted with carboxylates, which would be ideal for selective MS applications. We initially thought to accomplish this net oxidation by adding a strong acid (e.g., trifluoroacetic acid, methanesulfonylic acid, trifluoromethane sulfonic acid, etc.) to react with the boron hydride. We expected that the resulting aromatic product would be stable to the non-nucleophilic counter ion. Ab initio studies by Sundeberg et al. [200] on reduction of ketones/aldehydes using aminoboranes and oxazaborolidines have shown significantly lower energy of formation
of cyclic structures that are analogous to our own proposed structure. However, this technique would entail the additional steps we sought to avoid, such as adding an oxidizing agent to consume the hydride.

We opted instead to explore open chain boron halide reagents as our CA-chelating reagents. A simpler more stable reagent was considered at this stage where the carboxylate oxygens would displace halide atoms (Figure 3.2). To prevent reactions at the nitrogen and boron sites, substituent groups were selected to be bulky alkyl or aryl groups. We envisioned that the halide substitution would be facilitated by a non-nucleophilic salt (e.g. AgBF₄) that would not only bind the halides causing dissociation and also provide a counter-ion for the synthesized cationic aromatic product. This design however posed another challenge. A preliminary DFT study showed that the resulting adduct would not be planar, and thus not aromatic, for such bulky groups.
A further refinement was made to the reagent design to allow flexibility for planarization of the molecule while still providing bulk for preventing reactions at boron or nitrogen. Hence, the t-butyl groups were replaced with phenyl rings that provide steric cover while allowing the flexibility for formation of a planar, aromatic system (Figure 3.3.). Preliminary DFT studies on a number of molecules by our collaborator, Mr. Brady Garabato indicate all structures with N,B,O,C constitution were stable and planar if t-butyl groups were replaced by sterically accommodating groups such as phenyl, N,N dimethyl or i-Pr. All structures were optimized in the gas phase at the DFT level using the B3LYP exchange correlation functional, and the 6-311G(d,p) basis set for all atoms. Hence, this reagent design was adopted for exploring CA chelation. Although, we expect the bis(chlorophenylboryl) mesitylene amine (BCMA) would be reactive towards other functional groups (viz., amino acids, alkenes), a carboxylate adduct would form an aromatic structure with a net positive charge and thus be more readily detected by MS. For example, other cationic aromatic aromatic molecules such as pyrylium salts are frequently used to enhance ionization of amine metabolites for easy MS detection [201].
Hence, we hypothesize these molecules would be detected in positive ion MS with far greater sensitivity and thus serve to profile the CA fraction of a mixture.

3.2.2. Reagent Synthesis (Aim c)

Considering the air / moisture sensitivity of BCMA, the entire synthesis was carried out under argon atmosphere. The general synthesis steps are as shown in Scheme 3.4. Diethyl amine was stannylated using a procedure published by Wright et al. [202] where the amine is deprotonated using n-BuLi in hexanes and diethyl ether at –78 °C and then treated with trimethyl stannyl chloride at reflux for 2 hours. The crude reaction mixture was centrifuged and filtered to remove the precipitated lithium chloride salts and the (diethylamino)trimethylstannane was used in the next trans-stannylation step without further purification.

Trans-stannylation was accomplished using a procedure described by Diemer et al. [203]. A mixture of crude (diethylamino)trimethylstannane and 2,4,6-trimethylaniline were reacted at 150 °C for 5 days, whereby the diethylamine generated over time was

Figure 3.3. Phenyl based CA reagent.

![Figure 3.3. Phenyl based CA reagent.](image-url)
distilled as it formed, thus driving the trans-stannylation (from diethyl amine to mesitylene amine) reaction.

After 5 days the product was purified via a short path distillation at 170-176 °C under 30 Torr vacuum [199]. Pure mesitylbis(trimethylstannyl)amine was isolated in excellent yield. $^1$H NMR analysis of the material confirmed the product and showed 4 singlets; 18 protons at 0.12 ppm (6 x Sn–CH$_3$), 6 protons at 2.13 ppm (2 x Ph–CH$_3$, ortho), 3 protons at 2.22 ppm (Ph–CH$_3$, para) and 2 protons at 6.79 ppm (2 x Ph–H, meta).

The last step for synthesis of BCMA was to substitute the trimethyl tin groups on mesitylbis(trimethylstannyl)amine with chlorophenyl boryl groups. This synthesis was also attempted by Diemer et al. but the product was not isolated due to its lability [204]. Mes–N(SnMe$_3$)$_2$ in dichloromethane (DCM) was added dropwise to a solution of Ph–BCl$_2$
Figure 3.4. Stacked NMR spectra for BCMA, AMB reaction progression.

$^{11}$B NMR spectra of 1) PhBCl$_2$, 2) BCMA, 3) BCMA synthesis using heat and 4) $^{11}$B NMR of a mixture of bridged oxygen boron complex and BF$_3$•aniline.

in DCM with stirring at room temperature. Boron NMR after overnight stirring showed a signal at 60 ppm (Figure 3.4.) which was not listed in the published reports. Published NMR data by Diemer et al. references 3 major peaks; $\delta = 55.0$ ppm for Ph–BCl$_2$, 47 ppm for Mes–N(BPhCl)$_2$ and a signal at 37.2 ppm that was tentatively assigned for Mes–N(BPhCl)(SnMe$_3$).

Upon stirring for 12 days, we observed the $^{11}$B NMR signal at 47 ppm on analysis of the reaction mixture; however, the peak at 60 ppm was still present. We surmised this
signal corresponds to intermediate Mes–N(BPhCl)(SnMe₃), which did not convert to BCMA. We applied heat to expedite the synthesis of BCMA; this however resulted in formation of a byproduct, Mes–N=B–Ph (ArNB)(¹¹B NMR, δ = 34 ppm). To avoid formation of ArNB, the reaction was carried out at room temperature. To isolate pure BCMA from the in situ formed Me₃SnCl, we applied high vacuum to the crude reaction mixture. This too caused the formation of ArNB. Diemer et al. also reported the detection of ArNB when BCMA was dried under vacuum in their attempt to synthesize a similar molecule [203]. Thus, the crude reaction mixture was used to test the reaction of BCMA with an aromatic carboxylate.

We also synthesized mesityl(trimethylsilyl)amine in the hopes that trimethylsilyl chloride would be easier to remove after the reaction with Ph–BCl₂ [205]. However, the silylamine reagent did not react with Ph–BCl₂, either at room temperature or heating at 80 °C, even after 5 days of stirring. This was confirmed via ¹H NMR of the crude reaction mixture.

3.2.3. BCMA Reactivity and Unexpected Results

Although BCMA was contaminated with Me₃SnCl, we explored its tendency to form the targeted heteroaromatic ring on exposure to a test CA. We first prepared silver ρ-methoxybenzoate (AMB). AMB was dried 48 hours under vacuum and at 60 °C to remove all moisture. A suspension of AMB in DCM was added dropwise to a solution of BCMA in DCM and stirred overnight. To assist displacement of boron-chloride by the CA, AgBF₄ was added 15 minutes after benzoate addition (Scheme 3.5). Table 3.2 summarizes the results of our study to prepare BCMA-AMB. The addition of AMB and AgBF₄ to the
BCMA solution resulted in formation of precipitates. An aliquot from the reaction mixture was analyzed by $^{11}$B NMR spectroscopy and showed an upfield shift of the signals at 60 ppm and 47 ppm (BCMA) to 2 major signals at $-8$ and $-7$ ppm, respectively. Although there is no literature on the target heteroaromatic boron complex, these NMR signals were in agreement with shifts expected for $^{11}$B with the expected substituents.

Liquid/liquid diffusion crystallization was set up using a DCM solution of the product (~200 to 500 mg/mL) and hexanes. Slow diffusion of hexanes into the DCM solution caused formation of colorless crystals over a period of 5 days. These were subjected to X-ray analysis and found to be a byproduct of AMB and AgBF$_4$, as shown in Figure 3.5. Another aliquot of the same reaction mixture was sampled for X-ray analysis after 5 days of stirring at room temperature, but showed same product as mentioned above. The reaction was repeated with one modification, the reaction mixture was heated at 80 °C and sampled overnight. After 5 days, we observed the same products as were present after 16h. Other attempts were made to obtain X-ray quality crystals of the products of the reaction. The reaction was performed in chlorobenzene instead of DCM and by stirring simultaneous reactions, one at room temperature and another at 80 °C. Both were sampled for X-ray quality crystals at 16h and 5 days.
**Table 3.2. Attempted reaction conditions for synthesis of aromatic BCMA-AMB.**

<table>
<thead>
<tr>
<th>No.</th>
<th>Starting Materials (all reagents were added in equimolar amounts)</th>
<th>Solvent</th>
<th>Temp. °C</th>
<th>Time</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>BCMA, AMB, AgBF₄</td>
<td>CH₂Cl₂</td>
<td>25</td>
<td>16h to 5d</td>
<td>BF₃•aniline crystals</td>
</tr>
<tr>
<td>2</td>
<td>BCMA, AMB, AgBF₄</td>
<td>CH₂Cl₂</td>
<td>reflux</td>
<td>16h</td>
<td>dark soln., rxn mix. dried</td>
</tr>
<tr>
<td>3</td>
<td>BCMA, AMB, AgBF₄</td>
<td>chlorobenzene</td>
<td>25</td>
<td>16h to 5d</td>
<td>bridged oxygen complex</td>
</tr>
<tr>
<td>4</td>
<td>BCMA, AMB, AgBF₄</td>
<td>chlorobenzene</td>
<td>80</td>
<td>16h</td>
<td>dark soln., no crystals</td>
</tr>
<tr>
<td>5</td>
<td>BCMA, AMB</td>
<td>CH₂Cl₂</td>
<td>25</td>
<td>16h to 5d</td>
<td>no crystals</td>
</tr>
<tr>
<td>6</td>
<td>BCMA, AMB</td>
<td>CH₂Cl₂</td>
<td>reflux</td>
<td>16h</td>
<td>no crystals</td>
</tr>
<tr>
<td>7</td>
<td>BCMA, AMB</td>
<td>chlorobenzene</td>
<td>25</td>
<td>16h to 5d</td>
<td>no crystals</td>
</tr>
<tr>
<td>8</td>
<td>BCMA, AMB</td>
<td>chlorobenzene</td>
<td>80</td>
<td>16h</td>
<td>no crystals</td>
</tr>
</tbody>
</table>

**Figure 3.5. Byproducts of BCMA and AMB reaction.**

Crystallizations were carried out using the crude reaction mixtures and using hexanes via liquid-liquid diffusion technique. After 5 days, both colorless and yellow-colored crystals were harvest for X-ray analysis. The yellow crystals were found to be labile and disintegrated upon exposure to air. Several attempts to analyze these crystals using various X-ray crystallographic methods resulted in convoluted information. X-ray analysis of the colorless crystals showed an unexpected but interesting byproduct (BOBF), as shown in Figure 3.5.

Although, BF₃•aniline complexes are known [206], the bridged oxygen boron fluoride complexes have only been reported by Binder *et al.* [207]. They obtained the
bridged complex via another route (Scheme 3.6). Binder et al. used alkoxydifluoroborane 1 and acetic anhydride to form intermediate 3a. This intermediate rapidly converts into intermediate 3b upon loss of an acetate group (possibly via nucleophilic fluoride addition to the carboxylate carbon), which then reacts with acetic anhydride to give product 4 and acetylfuoride.

For the formation of BOBF, we hypothesize that the presence of Me₃SnCl in the BCMA mixture promotes the formation of BF₃ from AgBF₄. BF₃ then complexes with AMB, and the resultant adduct dimerizes to give an intermediate similar to 3a (i.e., 5, Scheme 3.7), which then contracts to a six-member ring similar to 3b (i.e., 7). Scheme 3.6 shows the reaction mechanism proposed by Binder et al. for the formation of the bridged complex. We believe the reaction of AMB works in a similar manner once eight-member intermediate 5 is formed. Scheme 3.7 shows the postulated mechanism for formation of BOBF.

The ORTEP diagram of BOBF is shown in Figure 3.6. To avoid formation of BF₃ based complexes we repeated reaction of BCMA with AMB without the addition of AgBF₄, with and without applying heat. However, we were not able to isolate any crystalline products form these reaction mixtures.

BOBF (Figure 3.6) has not been described in the literature. Compounds of this type have been reported to have applications in antibiotic drug synthesis [208] and materials chemistry as luminescent device coatings [209]. For these reasons, we decided to attempt another synthesis of this molecule.
Scheme 3.6. Proposed mechanism for formation of bridged oxygen boron fluoride complex [207].
Scheme 3.7. Proposed scheme for formation of complex BOBF.

Based on our initial hypothesis (Scheme 3.7), we set forth to synthesize BOBF using AMB and AgBF$_4$ as the only reagents in DCM. Unfortunately, monitoring the $^{19}$F NMR showed that AgBF$_4$ was not consumed, suggesting the reaction does not proceed unless an activating reagent is added to generate BF$_3$ from AgBF$_4$.

In order to generate reactive BF$_3$ in situ we added Me$_3$SnCl to the above mix of AMB and AgBF$_4$. These reagents were stirred together in DCM under conditions described in Table 3.3, entries 3–5. The reactions were stirred overnight and the crude mixture was sampled using $^{11}$B and $^{19}$F NMR to compare the results with existing NMR data reported
Figure 3.6. ORTEP diagram of BOBF.
Table 3.3. Attempted reactions for synthesis of BOBF.

<table>
<thead>
<tr>
<th>No.</th>
<th>Reagents (equivalents)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AMB</td>
</tr>
<tr>
<td>1</td>
<td>1.0</td>
</tr>
<tr>
<td>2</td>
<td>1.5</td>
</tr>
<tr>
<td>3</td>
<td>1.0</td>
</tr>
<tr>
<td>4</td>
<td>1.5</td>
</tr>
<tr>
<td>5</td>
<td>1.0</td>
</tr>
<tr>
<td>6</td>
<td>1.0</td>
</tr>
<tr>
<td>7</td>
<td>1.0</td>
</tr>
<tr>
<td>8</td>
<td>1.5</td>
</tr>
<tr>
<td>9</td>
<td>1.0</td>
</tr>
<tr>
<td>10</td>
<td>1.5</td>
</tr>
<tr>
<td>11</td>
<td>1.0</td>
</tr>
<tr>
<td>12</td>
<td>1.5</td>
</tr>
<tr>
<td>13</td>
<td>1.0</td>
</tr>
<tr>
<td>14</td>
<td>1.5</td>
</tr>
<tr>
<td>15</td>
<td>-</td>
</tr>
<tr>
<td>16</td>
<td>-</td>
</tr>
</tbody>
</table>

All reactions were carried out in DCM at room temperature and stirred overnight under argon atmosphere. NaMB = Sodium methoxybenzoate generated in situ via reaction of p-methoxybenzoic acid and NaH in THF.

by the Binder group. We found that the $^{11}$B NMR signals were too close to discriminate the reagents from the products. However, the $^{19}$F NMR signals shifted significantly for reagent / product, and thus we considered $^{19}$F NMR as a diagnostic tool to determine product formation.

We found that none of the crude mixtures showed a fluorine signal for the product (estimated at $\delta = -81$ ppm) in the NMR. We attributed this to a possible slow reaction of tin chloride with AgBF₄ to produce BF₃ as was evident from the mostly unchanged BF₄⁻ signal at $\delta = -150$ ppm. Knowing that silyl compounds have a greater tenacity for fluorine
than do tin compounds, we replaced Me₃SnCl with Me₃SiCl to speed up the formation of reactive BF₃ and thus push the reaction towards the formation of carboxylate-BF₂ dimer intermediate 5 (Scheme 3.7). In the reactions we examined, we did not see any signal for the product near the expected δ –81 ppm (entries 6–8, Table 3.3).

Consequently, after the above attempts at synthesis of BOBF using AgBF₄, we decided to use BF₃•Et₂O (entries 9, 10, 15, 16). Unfortunately, once again the NMR data revealed that no product was formed using these reaction conditions; however, the signals at δ –139 and –143 ppm were again observed in these reactions.

After a slew of unsuccessful attempts and a reconsideration of all parameters from the original synthesis which resulted in formation of BOBF, we decided to test BCl₃ as a possible reagent to catalyze the process (entries 11–14, Table 3.3). These attempts gave us similar disappointing results. To exhaust all options for the synthesis of the boron complex we decided to combine all reagents in various combinations – entries 13, 14 Table 3.3.

None of the reactions listed in Table 3.3 showed ¹⁹F signal downfield of –130 ppm and none of the reactions showed the ¹⁹F NMR signal at –81 ppm (as has been reported by Binder et al. for BOBF-type complex). This led us to conclude that either (1) our X-ray crystallographic data or (2) the reported ¹⁹F NMR values for BOBF complex is incorrect. A report published by Doris et al. in 2014 analyzed the reaction of BF₃•Et₂O with Pb(OAc)₂ [210]. The ¹⁹F NMR signal of products formed in this reaction resonated at –153 ppm to –139 ppm, indicating the formation of an eight-membered intermediate similar to the one proposed by the Binder group, such as intermediate 3a, Scheme 3.6. The reaction
Scheme 3.8. Reaction of acetate in presence of BF$_3$\cdot$Et$_2$O ($R = CH$_3$).

The scheme proposed by the Doris group is shown in Scheme 3.8. Given that we have repeatedly observed a signal at $\sim$139 ppm in several of our reactions, including the original reaction to synthesize BCMA-AMB aromatic molecule, we surmise the intermediate 12 is a likely precursor of BOBF. $^1$H NMR of our reaction mixtures showed a downfield shift of the ortho-Hs of the benzoate from $\delta$ 7.9 ppm for $p$-anisic acid to $\delta$ 8.0 ppm in the product, suggesting the presence of a carboxylate-BF$_2$ complex similar to 12. Moreover, the $^{19}$F NMR data and the reported structures by the Doris group, suggest that BOBF is formed via structure 13 and that the $^{19}$F NMR signal at $\sim$139 ppm is that of a benzoate.
intermediate similar to that structure. Potentially, the hydroxyl group in structure 13 can be deprotonated by a base to facilitate cyclization and thus formation of the oxo-bridge. Moreover, the corresponding $^{11}$B NMR signals observed in our experiments (between −2 ppm and 0 ppm) also fit the reported values for RO−BF$_3$ in the literature (≈0 ppm). This reconciles our X-ray data with the observed NMR signals and suggests that it is possible to access BOBF type complexes via simpler routes than those proposed by Binder et al.

3.3. CONCLUSIONS AND FUTURE DIRECTIONS

In this study, we pursued synthesis of a boron-based probe that would chelate a R−COOH group to form a cationic heteroaromatic structure. The synthesis of such a reagent in pure form proved to be a challenge – isolation and use of BCMA proved too difficult to be of practical value as a reagent for metabolomic analyses. Reaction of the crude reagent with a Ag-benzoate, however, did yield an interesting, previously unreported product. A new oxygen-bridged diboron species was characterized by X-ray crystallography. Similar bridged complexes, such as benzodiazaborines, have been recently reported to have antibacterial properties [208]. Similar complexes are also used in synthesis of organic electroluminescent devices. The ability of boron-based compounds to form better covalent bonds with pyridyl ligands as compared to metal organic frameworks of Al, Zn or Be have generated interest in bridged boron complexes. A study published by Wu et al. have described the use of bridged oxygen based boron complexes in synthesis of blue electroluminescent devices [209]. Thus a simpler synthetic route to BOBF type molecules would be of great value. Further route optimization and characterization of this molecule is needed for its applicability in the said fields.
4.1. INTRODUCTION

4.1.1. Significance of C5 Sugar Isolation from Biomass

Ever increasing energy demands coupled with depletion of fossil fuels and regiopolitical strife in mideastern countries have compelled energy self-sufficiency onto the US and encouraged development of alternate sources of energy in recent years. Lignocellulosic biomass is widely recognized as a possible alternative to supplant the traditional energy sources [211,212]. Lignocellulosic biomass is renewable, inexpensive, abundant, carbon neutral and the processes to generate energy can be readily economized [213]. Hydrolysis of the interlinked glycosidic bonds [214] affords solutions of monomeric saccharides (e.g., glucose, xylose, arabinose) which can be transformed, chemically or enzymatically, into biofuels or can be modified for use in polymer industry. Several hydrolytic techniques, such as treatment with acid [215], steam explosion [216], or various biological, enzyme-mediated conversion processes [217], have been extensively studied to obtain hydrolyzates rich in monosaccharides. Subsequent
conversion to value added chemicals [218] or to biofuels by fermentation [219] constitute principal next-steps for processing the hydrolyzates.

Given that the University of Louisville is in the heart of Bourbon country, one of the readily available lignocellulosic biomass is dried distillers’ grains (DDG). Makers Mark Distillery, a bourbon whisky making company in Kentucky currently disposes off a grain/water mix byproduct that contains considerable amount of sugars [220]. The growth of such companies further exacerbates the challenges involved with waste disposal. The high concentration of hemicellulose presents in DDG hydrolyzate makes it attractive source of pentose sugars [221]. This thesis work aims to utilize this waste product as a resource to isolate C5 sugars from DDG hydrolyzate using sustainable techniques.

4.1.2. Methods for Xylose Isolation from DDG Hydrolyzate

DDG hydrolyzate mixtures are composed of a milieu of chemicals along with C5 and C6 sugars. Harsh hydrolysis conditions and subsequent concentration steps give rise to degradation byproducts that adversely affect sugar composition of the hydrolyzate [222]. The process byproducts are also toxic for enzymatic transformation of sugars to biofuels and severely limit the yields and scope of biofuel production [223]. Techniques, such as over-liming [224], addition of nutrients to offset toxic inhibition [225] and ion-exchange [226], have been employed to overcome these issues. However, several of these methods become the source of even more byproducts [227]. Similar problems are faced when using enzymatic techniques for biofuel conversion.
Figure 4.1. Boronic acids as sugar carriers in reactive solvent extraction.

To avoid poisoning enzymatic transformations of sugars by degradation byproducts, the sugars can be chemically “transported” to a clean aqueous solution via reversible boronic ester formation [228]. Boronic acids can selectively chelate diols on sugar molecules and transfer the bound sugars to into an organic phase via ion matching and then release them in a “clean” aqueous phase prior to subsequent fermentation or other enzymatic processes [229]. This approach employs molecular recognition of cis-diols whereby the sugar diols are deprotonated by alkaline hydrolyzate and chelate with boronic acids across immiscible layers forming anionic tetrahedral boron centers. Lipophilic cationic salts, such as ammonium salts, in organic phase pair with the boronate anions and pull them into the organic solution via a reactive solvent extraction process [229,230]. The resulting salts are readily hydrolyzed in a clean, aqueous acidic solution to regenerate the sugars, which then are employed for either of the techniques mentioned above (Figure 4.1.). The extractions and kinetics of these processes have been greatly
optimized for dynamic transportation of sugars [231,232]. These techniques do provide adequate solutions to the above mentioned challenges but limit the utility of the extracted sugars as chemical modifications are constrained for aqueous solutions. A method that would deliver dry sugars can be expected to enable a greater set of chemical reactions for production of semi-synthetic chemicals in addition to biofuels.

4.1.3. Boron Carbohydrate Interactions

The affinity of boric acid and phenylboronic acids for simple diols and monosaccharides was first reported by Lorand and Edwards [233] in 1959. Since then the reversible reaction between boronic acids and divalent ligands has been extensively documented. Since boronate esters are generally more Lewis acidic than the parent boronic acids, the reaction between a boronic acid and cis-diols in aqueous solutions varies depending on the pH. For example, phenylboronic acid reacts sluggishly with diols to form a neutral, trigonal boronate ester. However, at pH 10 or higher the boronic acid itself exists as an anionic, tetrahedral molecule and chelates rapidly with the diols to form anionic, tetrahedral boronate ester (Figure 4.2). At higher hydroxide concentrations the boronate ester is in its more stable tetrahedral form thus favoring ester formation [234]. A study by Ishihara et al. proved that it is indeed the trigonal boronic acid that exchanges most rapidly with diols regardless of pH [235]. The formation of boronates are most favored in case of cis-1,2-diols, followed by 1,3-diols. The sluggish reaction of trans-1,2-diols as compared to the above mentioned diols renders some selectivity to the boronic acid for chelation to monosaccharides.
A boronate ester is also susceptible to trans-esterification via another diol provided the former boronate ester is thermodynamically less stable than the latter [236]. This remarkable property of esters allows easy recovery of chelated diols in one of the simplest, convenient and mild procedures. Figure 4.3 demonstrates a generic Scheme for transesterification of diols.

**Figure 4.3. Generic transesterification of boronate esters.**

Elegant studies by Matteson and Man [237] reported that ligand exchange reactions are greatly influenced by a number of factors, such as, conformation, entropy, catalyst, solvent polarity, concentration, the entropies of internal rotations of free diols and the steric repulsions among others.

With this background information in mind we hypothesized that if biomass hydrolyzate was treated with a boronic acid, we could chelate sugars as boronate esters.
These esters could be continuously extracted in an organic solvent to concentrate the sugars in the form of their boronate esters. Consequently, the esters could be subjected to transesterification using a diol that would form more thermodynamically stable boronate esters to release sugars in organic solvent. Since the sugar release would occur in an organic solvent, they would precipitate out of the reaction mixture and could be easily collected via filtration. Moreover, we also hypothesize that the resulting boronate esters from the transesterification process could be hydrolyzed for regeneration of the phenylboronic acid as well as the diol. Thus, with this hypothesis in mind we explored the following aims for xylose isolation.

Thus we aim to test our hypothesis using DDG hydrolyzate as pentose source and phenylboronic acid (PBA) as the chelating reagent. Our primary aims were: a) to optimize pH for chelation of sugars using phenylboronic acid and easy extraction in toluene; b) to optimize PBA stoichiometry with respect to pentose concentration in DDG hydrolyzate; c) to identify a ligand for facile transesterification of sugar-boronates in order to precipitate sugars and optimize its use; d) to regenerate and recover PBA via hydrolysis and e) to demonstrate a sub-pilot scale isolation of pentoses using the optimized techniques and to analyze cost of operation.

4.2. RESULTS AND DISCUSSION

Using the 2-stage acid hydrolysis process described by Fonseca et al. [238], our collaborator Dr. Eurick Kim obtained DDG hydrolyzate rich in d-xylose and l-arabinose. The chemical components of this DDG hydrolyzate are shown in Table 4.1.
Table 4.1. Extraction of individual pentoses using 2-stage hydrolysis.

<table>
<thead>
<tr>
<th>Compound</th>
<th>First Stage (kg/L)</th>
<th>Second Stage (kg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arabinose</td>
<td>5.41</td>
<td>2.52</td>
</tr>
<tr>
<td>Xylose</td>
<td>1.23</td>
<td>14.46</td>
</tr>
<tr>
<td>Glucose</td>
<td>0.26</td>
<td>2.22</td>
</tr>
<tr>
<td>Glycerol</td>
<td>1.16</td>
<td>0.88</td>
</tr>
<tr>
<td>Acetic acid</td>
<td>0.53</td>
<td>1.84</td>
</tr>
<tr>
<td>5-Hydroxymethylfurfural</td>
<td>0.01</td>
<td>0.16</td>
</tr>
<tr>
<td>Furfural</td>
<td>0.02</td>
<td>1.07</td>
</tr>
</tbody>
</table>

First Stage: 110 °C and 0.2% mass fraction of H₂SO₄  
Second Stage: 140 °C and 0.4% mass fraction of H₂SO₄

We used phenylboronic acid to test the chelation of these C5 sugars directly from the neutralized hydrolyzate solution. The chelated diol-boronates were extracted into toluene layer as formed and then transesterification of the boronates released sugars as crystalline solids. Once this sugar precipitation route was established we optimized each step with regards to isolation yields, cost of operation, recycling and sustainability.

4.2.1. Determination of Optimal pH for Boronic Ester Formation

To optimize conditions for esterification of C5 sugars, we investigated the ideal pH conditions for boronate formation and extraction. As discussed earlier in this chapter, acidic conditions are not conducive for boronic ester formation while neutral to basic pH conditions promote boronate ester formation. However, at higher pH values trigonal boronate centers tend to become tetrahedral anions and thus become difficult to extract in an organic solvent.

Another aspect to consider for this reaction is that xylose exists predominantly in its pyranose form in aqueous solution [239]. However, PBA reacts predominantly with
Scheme 4.1. Conformers of xylose, PBA chelation and effect of pH on extraction.

the furanose conformer. As the reaction progresses, the chelation of xylo-furanose constantly shifts the equilibrium in favor of the furanose form. This reaction is carried out in presence of toluene which dissolves the boronates as formed, thus removing the product and driving the equilibrium towards more ester formation (Scheme 4.1).

Considering these criteria, we tested esterification of xylose to xylose diesters (XDE) in the pH range 7.0 to 9.0 with intervals of 0.5 units (Figure 4.4.). DDG hydrolyzate was treated with a methanolic mixture of 6 equivalents PBA/xylose (xylose concentration was calculated using HPLC data, Table 4.2.) and toluene at a given pH value. As the reaction progressed XDE formed in these reactions was extracted into toluene along with unreacted PBA. The efficiency of XDE formation at a certain pH and extraction was assessed by integration of the $^1$H NMR signals at $\delta$ 8.25 ppm (boroxine ortho Hs) and $\delta$ 6.23 ppm (XDE C(1)-H) in the spectrum of the crude extract obtained for each condition.
Figure 4.4. XDE extraction as a function of pH.

Plotted according to pH condition (axes) are the extracted weight in grams (x10) of XDE (red pentagon), the percent unreacted xylose (x100) remaining in the hydrolyzate determined using HPLC (green), and the ratio of extracted XDE:PBA determined using $^1$H NMR (blue).

The aqueous layers of DDG hydrolyzate were analyzed using HPLC before and after PBA treatment to determine change in sugar concentration (Figure 4.4.). The extraction efficiency of XDE was slightly affected by pH, with the pH 7.5 condition providing an extract most enriched in XDE and highest extracted weight of XDE. The results noted in Table 4.2. agree with previously reported observations that at neutral pH fewer boronate esters have tetrahedral geometry whereas at higher pH charged, tetrahedral forms (e.g., [XDE•OH]$^-$, Scheme 4.2.) predominate [240] and these are less likely to be extracted into toluene. Consequently, for all subsequent studies we adjusted the hydrolyzate pH to 7.5 for XDE formation and extraction.
Table 4.2. Determination of optimal pH for boronic ester formation.

<table>
<thead>
<tr>
<th>pH</th>
<th>Extracted PBA:XDE Ratios ( \left( ^1 \text{H} \text{NMR} \right) )</th>
<th>Unreacted Xylose in Hydrolyzate (HPLC)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>XDE/PBA</td>
<td>Extracted XDE (g)</td>
</tr>
<tr>
<td>9.0</td>
<td>0.25</td>
<td>1.89</td>
</tr>
<tr>
<td>8.5</td>
<td>0.34</td>
<td>1.89</td>
</tr>
<tr>
<td>8.0</td>
<td>0.33</td>
<td>1.92</td>
</tr>
<tr>
<td>7.5</td>
<td>0.39</td>
<td>2.09</td>
</tr>
<tr>
<td>7.0</td>
<td>0.31</td>
<td>1.91</td>
</tr>
</tbody>
</table>

Scheme 4.2. Trigonal and tetrahedral boronate esters.

4.2.2. Optimization of PBA Stoichiometry

As discussed earlier, DDG hydrolyzate solution consists of a number of chemical compounds that interfere with PBA esterification of sugars and hence, excess PBA is required to effectively chelate sugars for extraction. However, using a large excess of PBA would require also raising the amount of reagents used in the subsequent steps and thus
significantly raise the cost of xylose isolation per cycle. To determine the optimal amount of PBA needed for efficient complexation of the C5-sugars xylose and arabinose in the hydrolyzate milieu, pH 7.5 hydrolyzate (11.4 mg xylose/mL; 10.6 mg arabinose/mL; 0.9 mg glucose mL) was treated with incremental amounts of PBA (2, 4, 6, 8, 10 and 12 eq./xylose) followed by toluene extraction and extract analysis using HPLC. The concentration of sugars in untreated hydrolyzate was measured after neutralization of the hydrolyzate, using HPLC. Then the hydrolyzate was stirred with methanolic solution of PBA in presence of toluene for 16 h. After the reaction, the biphasic layers of reaction mixture were separated and residual methanol in the aqueous layer was evaporated using rota-evaporator. This solution was then analyzed using HPLC to calculate the amount of sugars consumed via PBA chelation. We observed that at 2 equivalent PBA/xylose only ca. 42% of the xylose reacted (unreacted xylose in hydrolyzate measured using HPLC and XDE/PBA measured using NMR, Table 4.3., Figure 4.5.). On addition of another 2 equivalents PBA, more than half the xylose had reacted; but only 40% of the available arabinose had reacted. On examination of even higher PBA:xylose ratios, we noted that 8 eq. of PBA/xylose appeared to provide a good balance for C5-sugar extraction — using 10 or even 12 equivalents of PBA became unwieldy and provided little increase in xylose or arabinose consumption (<15% average C5-sugar reacted per 4 added equivalents of PBA). D-Glucose was not appreciably consumed until more than 8 equivalents of PBA had been used, thus reinforcing the use of 8 equivalents PBA/xylose for C5-sugar extraction.
Table 4.3. Determination of optimal PBA stoichiometry.

<table>
<thead>
<tr>
<th>Eq. PBA/Xylose</th>
<th>D-Xylose</th>
<th>D-Glucose</th>
<th>L-Arabinose</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Calcd. Conc. (g/L)</td>
<td>% Xylose Consumed (measured using HPLC after 16 h)</td>
</tr>
<tr>
<td>12</td>
<td>1.05</td>
<td>91</td>
<td>0.31</td>
</tr>
<tr>
<td>10</td>
<td>1.58</td>
<td>86</td>
<td>0.43</td>
</tr>
<tr>
<td>8</td>
<td>2.22</td>
<td>80</td>
<td>0.59</td>
</tr>
<tr>
<td>6</td>
<td>3.13</td>
<td>72</td>
<td>0.67</td>
</tr>
<tr>
<td>4</td>
<td>4.96</td>
<td>56</td>
<td>0.80</td>
</tr>
<tr>
<td>2</td>
<td>6.58</td>
<td>42</td>
<td>0.71</td>
</tr>
<tr>
<td>Original Concentration of sugars</td>
<td>11.36</td>
<td>0</td>
<td>0.88</td>
</tr>
</tbody>
</table>

Figure 4.5. Influence of PBA Stoichiometry on sugar isolation.

Sugar concentration in the treated hydrolyzate (pH 7.5) as a function of PBA equivalents used in the reaction.
4.2.3. Extraction Efficiency and PBA Recovery

As esterification of xylose progresses, XDE is simultaneously extracted into the toluene layer. This acts as an enriching step for XDE as its solubility in toluene is higher as compared to PBA. Any PBA that gets extracted in toluene is easily dehydrated to form boroxine. This extracted boroxine may interfere with the transesterification step. Hence, the concentration of free PBA should be minimized in the reaction. For this purpose, we determined the efficiency of XDE extraction using toluene and exploited the high solubility of XDE in toluene vs. PBA (Figure 4.6.). We limited the first toluene extraction to half the volume of subsequent toluene extractions. This saturated the first extract with XDE (XDE:PBA ratio of 1:1.5) containing >80% of the sugars while the second and third extracts contained mainly boroxines. Thus, the first extract was suitable for direct use in the subsequent transesterification step. The second and third toluene extracts were then used to recover unreacted PBA from the hydrolyzate. To improve recovery of PBA, the hydrolyzate mixture was acidified to pH ~2 prior to second and third extractions to hydrolyze extraneous PBA adducts and drive the free PBA into toluene.
4.2.4. Transesterification of XDE to Precipitate Xylose

Given our aim to precipitate crystalline sugars from toluene solution we tried various techniques to release sugars from their respective boronic esters. Initially we tried passing HCl gas through toluene solution containing XDE. This did not hydrolyze the boronate esters due to low solubility of HCl gas in toluene solution. Another method to release a diol from a boronate ester was reported by Roy and Brown [241] in 2007 (Figure 4.7.). They exploited the ability of a sterically hindered 1,2-diol (smaller dihedral angle) to displace a less hindered diol chelated to boronic acids to form a more thermodynamically favored boronate ester. For example, chiral (+)-2-phenyl[1,3,2]-dioxaborolane-4,5-dicarboxylic acid diisopropyl ester was easily transesterified using chiral 2,3 butanediol. This facile ligand exchange is attributed to the minimal steric repulsions in the new boronic esters.
Dr. Mandapti Raju, a collaborator and postdoctoral researcher in Nantz group, explored the transesterification of XDE using different diols (Figure 4.8.) in toluene (Table 4.4.) since this solvent was used for extraction of XDE from the DDG hydrolyzate. In most cases, xylose precipitated from the toluene solution as the diol-mediated boronate ester transesterification proceeded. The solid xylose was readily collected by filtration after the reaction was complete. In the cases involving simple 1,2-diols (entries 1-4, Table 4.4.), xylose was isolated in good to moderate yield as a mixture of d-xylopyranose anomers. Although norbornene diol and pinacol diol gave good transesterification yields, the stability of resulting boronate esters made their recycling via acidic hydrolysis sluggish. Pentaerythritol (5) and Rochelle’s salt (7) failed to induce xylose precipitation even after prolonged reaction time (entries 5, 8). In the cases of diethanolamines 6a and 6b (entries 6, 7), precipitation was noted almost immediately after addition of the aminodiol. \(^1\)H NMR analyses confirmed the precipitates were adducts resulting from nitrogen–boron complexation rather than xylose liberated on transesterification. To our delight, treatment of XDE with propylene glycol (PG) (3), among the least expensive of the 1,2-
Figure 4.8. Transesterification of XDE using diols explored in this study.

Table 4.4. Transesterification of XDE by reaction with various diols.\(^a\)

<table>
<thead>
<tr>
<th>Entry</th>
<th>Diol</th>
<th>Time (h)</th>
<th>D-Xylose (% yield)(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>exo-2,3-norbornanediol (1)</td>
<td>18</td>
<td>90</td>
</tr>
<tr>
<td>2</td>
<td>pinacol (2)</td>
<td>32</td>
<td>80</td>
</tr>
<tr>
<td>3</td>
<td>propylene glycol (3)</td>
<td>24</td>
<td>97</td>
</tr>
<tr>
<td>4</td>
<td>ethylene glycol (4)</td>
<td>24</td>
<td>71</td>
</tr>
<tr>
<td>5</td>
<td>pentaerythritol (5)</td>
<td>48</td>
<td>no xylose ppt</td>
</tr>
<tr>
<td>6</td>
<td>diethanolamine (6a)</td>
<td>8</td>
<td>XDE–aminodiol complex(^c)</td>
</tr>
<tr>
<td>7</td>
<td>N-methyldiethanolamine (6b)</td>
<td>8</td>
<td>XDE–aminodiol complex(^c)</td>
</tr>
<tr>
<td>8</td>
<td>Rochelle’s salt (7)</td>
<td>48</td>
<td>no xylose ppt</td>
</tr>
</tbody>
</table>

\(^a\)Conditions: XDE (1 eq.) and diol (2.0 eq., entry 1; 5.0 eq., entries 2-8) reacted in toluene at room temperature for the indicated time; \(^b\)yield of recovered xylose precipitate; \(^c\)addition of the aminodiol causes precipitation of the corresponding XDE-ate complex — no free xylose isolated.
diols we examined and considered non-toxic for food and additives purposes (ingestion of under 25 mg/kg/day) by U.S. Food and Drug Administration [242], produced xylose in excellent yield as well as delivered the corresponding boronate complex, 4-methyl-2-phenyl-1,3,2-dioxaborolane (PGE), in near quantitative yield. The facile hydrolysis of propylene glycol ester to recycle PG after the transesterification made it an ideal choice for this step.

4.2.5. PBA Recovery on PGE Hydrolysis

With the goal of isolating PBA for recycling purposes, we next sought to develop a precipitation protocol devoid of solvent extraction steps for more convenient PBA isolation. Mild-acid induced hydrolysis of PGE has been reported previously [243].

![Scheme 4.3. Hydrolysis of PGE.](image)

We found that direct treatment of PGE with water at room temperature resulted in clean precipitation of PBA and boroxine within 18 h in 62% yield (Scheme 4.3.). The boroxine readily converts to PBA in aqueous solutions.
4.2.6. Preparative Scale Isolation of Pentoses and Cost Analysis

After optimizing all the variables for sugar isolation from DDG hydrolysate we tested the protocol at a semi-pilot scale using 3.5 L of hydrolysate (Figure 4.9.). The hydrolysate was basified to pH 7.5, filtered and then treated with 8 equivalents of PBA/xylose. XDE, the corresponding arabinose diester and unreacted PBA were extracted into toluene. On cooling this toluene extract to −20 °C, some PBA precipitated due to its low solubility in toluene and was readily collected via filtration. The extracted hydrolysate was acidified to hydrolyze any partial boronate esters and re-extracted with toluene to recover and recycle additional PBA. When PG was added to the toluene extract containing XDE and ADE, a mixture of C5-sugars precipitated as a gum. Trituration with ethanol transformed the gum into crystalline solids that were readily collected by filtration. Analysis of the isolated solid sugar mixture indicated a ca. 5:1 ratio of D-xylose to L-arabinose. $^1$H NMR spectral characterization of the precipitated sugar mixture indicated a high level of purity — no contamination from other sugars or organic by-products from the DDG hydrolysate were noted (Figure 4.10.). The $^1$H NMR signal at ~6.3 and ~ 6.0 ppm was characteristic for anomeric –OHs of D-xylose and L-arabinose, respectively. The overall percentages of xylose and arabinose isolation were calculated, based on concentrations measured in the neutralized hydrolysate at the onset of the process, and found to be 48% and 11%, respectively. HPLC analysis of the extracted hydrolysate revealed a xylose depletion of 93% and an arabinose depletion of 60%, thus confirming that pentose degradation also occurs during the overall isolation process (Figure 4.11.). The efficiency of PBA recovery on this preparative scale was 79%, and the
Figure 4.9. Sub-pilot scale demonstration and summary of pentose isolation and PBA recovery cycle.

Solvent recoveries were excellent (95% toluene recovery, 99% EtOH recovery). We also did a cost analysis of the large scale reaction to get a better idea for economic feasibility of this project. This cost analysis is shown in Table 4.5.
Figure 4.10. $^1$H NMR spectra of reference sugars vs. isolated sugars (DMSO-d6).

(top) a reference mixture comprised of 5:1 d-xylose:L-arabinose and (bottom) the pentose mixture isolate. The anomeric-OHs of d-xylose and l-arabinose are marked.
Figure 4.11. Xylose extraction efficiency as determined using HPLC.

Standard xylose concentration curve as determined by HPLC analysis. Red box shows concentration of xylose in hydrolyzate before PBA treatment (11.4 g/L) and green circle shows concentration of xylose post PBA (8 eq./xylose) treatment.
Table 4.5. Cost analysis of xylose isolation from DDG hydrolyzate.

### Xylose Extraction from DDG Hydrolyzate

#### Step 1 - XDE Formation

<table>
<thead>
<tr>
<th>Chemicals</th>
<th>M.W (kg/kmol)</th>
<th>Conc. (kg/l)</th>
<th>Density (kg/l)</th>
<th>Mole</th>
<th>Mole ratio</th>
<th>Volume ratio</th>
<th>Actual Wt (kg)</th>
<th>Volume (L)</th>
<th>Accumulated volume (L)</th>
<th>Wt Ratio</th>
<th>Abs. cost $</th>
<th>Recovery %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Xylose (in DDG Hydrolyzate - Conc 18.6 mg/mL xylose)</td>
<td>150.13</td>
<td>0.0186</td>
<td>0.003031</td>
<td>1.00</td>
<td>0.0465</td>
<td>2.80</td>
<td>2.50</td>
<td>1.00</td>
<td>0.70</td>
<td>65.0%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neutralize hydrolyzate using NaOH pellets to get pH = 7.5</td>
<td>40</td>
<td>0.003031</td>
<td>1.00</td>
<td>0.0124</td>
<td>0.00</td>
<td>2.50</td>
<td>0.27</td>
<td>0.009</td>
<td>0.00</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phenylboronic acid - PBA</td>
<td>121.93</td>
<td>1</td>
<td>0.00048</td>
<td>8.00</td>
<td>0.3051</td>
<td>0.50</td>
<td>3.00</td>
<td>0.00</td>
<td>2.00</td>
<td>27.0%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Add PBA to Hydrolyzate at 25°C</td>
<td>32.04</td>
<td>0.791</td>
<td>0.20</td>
<td>0.3955</td>
<td>0.50</td>
<td>4.55</td>
<td>0.20</td>
<td>0.1</td>
<td>0.08</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Toluene</td>
<td>92.14</td>
<td>0.865</td>
<td>0.50</td>
<td>1.0813</td>
<td>1.25</td>
<td>4.05</td>
<td>13.00</td>
<td>28.1</td>
<td>100.0</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Methanol</td>
<td>32.04</td>
<td>0.865</td>
<td>0.50</td>
<td>2.1625</td>
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<td>5.33</td>
<td>0.00</td>
<td>0.0</td>
<td>0.00</td>
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<td></td>
<td></td>
</tr>
</tbody>
</table>

#### Step 2 - Transesterification / Recycling

<table>
<thead>
<tr>
<th>Chemicals</th>
<th>M.W (kg/kmol)</th>
<th>Conc. (kg/l)</th>
<th>Density (kg/l)</th>
<th>Mole</th>
<th>Mole ratio</th>
<th>Volume ratio</th>
<th>Actual Wt (kg)</th>
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<tbody>
<tr>
<td>Xylose diester (XDE) in toluene</td>
<td>321.93</td>
<td>0.000</td>
<td>1</td>
<td>0.00025</td>
<td>1.00</td>
<td>0.08</td>
<td>0.00</td>
<td>1.00</td>
<td>7</td>
<td>0.859893</td>
<td>100.0</td>
<td></td>
</tr>
<tr>
<td>Add propylene glycol and stir for 24h</td>
<td>78.09</td>
<td>1.04</td>
<td>0.00124</td>
<td>3</td>
<td>0.09</td>
<td>0.09</td>
<td>0.09</td>
<td>1.00</td>
<td>7</td>
<td>0.859893</td>
<td>100.0</td>
<td></td>
</tr>
<tr>
<td>Distill ether</td>
<td>74.12</td>
<td>0.713</td>
<td>37.15</td>
<td>2.11</td>
<td>2.96</td>
<td>3.05</td>
<td>17.00</td>
<td>35.9</td>
<td>100.0</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Theoretical Factor =** 2.1443

**Theoretical Yield =** 0.099712 kg

**Expected Yield =** 0.07977 kg

**% Yield =** 80.0%

**% Purity =** 100.0%

## Cost Analysis

### Stage 1 - XDE Formation

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<td>0.0</td>
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<td></td>
<td></td>
</tr>
</tbody>
</table>

**Stage 1 variable costs:** 701.97 $

**Stage 1 theoretical costs:** 56.00 $

**Stage 1 total variable costs:** 5.64 $

**Theoretical Factor =** 2.1443

**Theoretical Yield =** 0.099712 kg

**Expected Yield =** 0.07977 kg

**% Yield =** 80.0%

**% Purity =** 100.0%

### Stage 2 - Transesterification / Recycling

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<th>Chemicals</th>
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**Theoretical Factor =** 2.1443

**Theoretical Yield =** 0.099712 kg

**Expected Yield =** 0.07977 kg

**% Yield =** 80.0%

**% Purity =** 100.0%

**Stage 2 total variable costs:** 5.64 $

**Total $/kg of xylose**

### Conclusion

- **M.W. of XDE** = 321.9300 kg/kmol
- **Theoretical Factor** = 2.1443
- **Theoretical Yield** = 0.099712 kg
- **Expected Yield** = 0.07977 kg
- **% Yield** = 80.0%
- **% Purity** = 100.0%
4.3. CONCLUSIONS

A three-stage process for isolation of C5-sugars in dry form from DDG hydrolyzate is described. The salient features include extraction of bis(boronic ester) adducts into toluene on treatment of neutralized hydrolyzate with PBA and a subsequent transesterification procedure that results in pentose precipitation. We found that the addition of propylene glycol to the extract is particularly well suited to induce sugar precipitation for ready collection. To our knowledge there is no other process that delivers sugars in crystalline form. Our method delivers dry, crystalline sugars while regenerating the reagents and solvents used in the process. A preparative scale demonstration delivered 48% of the xylose content available in the hydrolyzate while nearly 80% PBA is recovered using a simple hydrolysis procedure. The low yield can be explained by possible degradation of the sugars in hydrolyzate and also some isomerization during PBA reaction. We also did a cost analysis for the isolation of sugars and found that PBA was the most expensive component in the reaction. Hence, we checked for its availability and found that this is a cheap and readily available reagent available in bulk. Given that pentoses can serve as synthetic precursors of numerous value-added materials, these results suggest a feasible means of DDG processing, although additional optimizations still need to be developed.
4.5. FUTURE DIRECTIONS

Dr. Satyavolu’s lab is also exploring enrichment of DDG hydrolyzate with respect to separation of xylose and arabinose streams in the hydrolysis stage. Separation of C5 streams will allow collection of individual sugars rather than a mixture. With the aim to further streamline the xylose isolation process and considering the possible degradation of sugars due to long reaction times, we hope to shorten the XDE formation and extraction time to ~4 hours. This could be accomplished by tuning the electron density of the boronic acid. An electron deficient boronic acid would be expected to chelate the sugars more efficiently while the transesterification of such boronates would be sluggish. Hence, the right balance of electron density should allow for better and faster chelation, thereby reducing the time and cost of XDE formation. Cost analysis of the sugar isolation procedure revealed PBA to be the most expensive component, hence a better or even
100% recycling of PBA would significantly reduce the cost of operation. To accomplish this, we are exploring a polymeric PBA approach. 3 amino phenylboronic acid can be mounted on a carboxylate polymer via amidation reaction. This reactive polymer would selectively bind sugars from the hydrolyzate and can be separated via solid filtration. Transesterification of XDE can also be easily accomplished by treating the polymer with PG in an organic solvent. Lastly, we hope to replace toluene with greener solvents such as vegetable oil or Aromatics 100 in order to make the process sustainable and cheap for sugar isolation.

In order to make the C5 sugar isolation procedure more efficient and cost effective we hope to utilize mathematical modelling techniques by systematically incorporating changes in time, solvents, boronic acids, transesterification diols and hydrolyzate concentration. One such modelling technique, Design of Experiments, developed by the Sigman group at University of Utah allows the flexibility and variability of reaction parameters to mathematically compute the best combination of reagents and reaction conditions for optimal yields [244]. We hope to use the same techniques to explore the economic feasibility of our methods for sugar isolation.
CHAPTER 5

EXPERIMENTAL PROCEDURES

5.1. GENERAL EXPERIMENTAL PROCEDURES

All reagents were purchased from Aldrich Chemical Company (Milwaukee, WI) and used without further purification unless otherwise specified. Phenylboronic acid was purchased from Oakwood Chemicals, West Columbia, SC. Dried distillers’ grains (DDG) hydrolyzate was prepared in-house at the Conn Center for Renewable Energy Research using the reported procedure by Fonseca et al.[245] Xylose diboronic ester (PhB)\(_2\)(α-D-XylfH\(_4\)) was prepared using method described by Reichvilser et al.[246] Reagent grade ACS dichloromethane (DCM), tetrahydrofuran (THF), acetone, hexanes, and ethyl acetate were purchased from Pharmco-AAPER (Shelbyville, KY). THF and DCM were dried using a solvent purification system by LC Technology Solutions, Inc. (Salisbury, MA). CDCl\(_3\) (99.8%) was purchased from Cambridge Isotope Laboratories (Andover, Ma.).

NMR spectra were acquired on a Varian Inova 400 MHz spectrometer. FTIR spectra were acquired on a Mattson galaxy series 5000 DRIFT spectrometer using 64 scans, at a scan speed of 2.1 kHz and a resolution of 4ν. Centrifugation of cell extracts was performed on a Thermo Scientific Sorvall Legend X1R centrifuge; centrifugation of
$n$-BuOH extracts was carried out on Eppendorf centrifuge 5414 R; vacuum centrifugation was performed on an Eppendorf AG 22331 Hamburg vacufuge. Melting points were acquired on Fisher-Johns melting point apparatus.

Positive ion CID mass spectra were obtained using LTQ-IT mass spectrometer. Standard QDE adducts were prepared and MS$^n$ analyses were done independently on standards and cell extracts. Nitrogen was used as the collision gas and the collision induced dissociation energies were between 16-25 eV. All identified fragment ions were compared with ion peaks in corresponding reference spectrum.

All HPLC analyses for the pentose isolation project were performed on a Waters 600E HPLC system (Waters Corporation, Milford, MA) fitted with an Agilent 1260 Infinity refractive index detector and an Agilent Hi-Plex H column (300 mm x 7.7 mm, 8 μm). The column temperature was set to 60 °C with a refractive index detector temperature of 55 °C. The mobile phase consisted of aq. sulfuric acid at a concentration of 0.005 mole/L. The flow rate was set to 0.7 mL/min. Samples were filtered through a 25 mm syringe filter with a 0.45 μm polyether-sulfone membrane prior to analysis. The hydrolyzate samples were analyzed for monomeric sugars (glucose, xylose, arabinose) according to the published method [221].
5.2. CHAPTER 2 EXPERIMENTAL PROCEDURES

5.2.1. Synthesis of QDE

\[
\begin{align*}
\text{Me}_2\text{N} & \quad \text{N} \quad \text{N} \\
\text{O} & \quad \text{O} \\
\text{1} & \quad \text{2}
\end{align*}
\]

\[
\begin{align*}
\text{I}^- & \quad \text{N} \quad \text{N} \quad \text{CH} & \quad \text{Cl} & \quad \text{O} \\
\text{10} & \quad \text{10} & \quad \text{10} & \quad \text{10}
\end{align*}
\]

\[
\begin{align*}
\text{chloroacetamide-QDE} & \quad \text{QDE}
\end{align*}
\]

**Scheme 2.1. Synthesis of QDE.**

Reagents and conditions: a. 1-iodododecane, CH\(_3\)CN, 100 °C, 20 h; b. H\(_2\)NNH\(_2\)•H\(_2\)O, EtOH, 40 °C, 12 h, 83% (2 steps); c. chloroacetyl chloride, K\(_2\)CO\(_3\), CH\(_2\)Cl\(_2\), rt, 12 h; d. NaI, acetone, 65 °C (sealed tube), 20 h, 78% (2 steps).

**N-(2-Aminoethyl)-N,N-dimethyldodecylammonium iodide (2).** A sealed tube was flushed with N\(_2\) and then charged with 1-iodododecane (10.0 mL, 40.7 mmol), aminoimide 1 (8.80 g, 40.3 mmol) and CH\(_3\)CN (150 mL). The tube was sealed and the reaction mixture was heated to 100 °C for 20h. After cooling to room temperature, the tube was opened and the reaction solution was concentrated by rotary evaporation. The remaining solid was washed with hexanes using a Buchner funnel to remove any remaining 1-iodododecane. The crude salt, obtained as a white solid (17.3 g), was used in the next step.
without further purification; mp, 182-184 °C; $^1$H NMR (CDCl$_3$) δ 7.87-7.84 (m, 2H), 7.77-7.74 (m, 2H), 4.21 (t, $J$ = 6.0 Hz, 2H), 3.99 (t, $J$ = 6.4 Hz, 2H), 3.69-3.65 (m, 2H), 3.56 (s, 6H), 1.75 (m, 2H), 1.29-1.18 (m, 18H), 0.84 (t, $J$ = 6.8 Hz, 3H); $^{13}$C NMR (CDCl$_3$) δ 167.6, 134.7, 131.4, 123.9, 64.9, 60.6, 52.1, 32.5, 31.9, 29.5, 29.4, 29.3, 29.3, 29.2, 26.1, 22.9, 22.6, 14.1; FTIR 3015, 2952, 2850, 1782, 1711 cm$^{-1}$.

To a solution of the crude ammonium iodide (17.3 g, 33.4 mmol) in ethanol (200 mL) at room temperature was added hydrazine monohydrate (6.69 mL, 134 mmol). A white precipitate formed within 30 minutes at 40 °C. The reaction suspension was stirred a total of 12 h at 40 °C before cooling to room temperature. The ethanol then was evaporated by rotary evaporation. CH$_2$Cl$_2$ was added and the undissolved solids (e.g., phthalhydrazide) were filtered. The filtrate was concentrated by rotary evaporation to give ammonium iodide 2 (12.7 g) as a pale yellow solid; mp, 70-72 °C; $^1$H NMR (D$_2$O) δ 3.56-3.53 (m, 4H), 3.26 (s, 6H), 3.18-3.14 (m, 2H), 1.88 (m, 2H), 1.46-1.35 (m, 18H), 0.94 (t, $J$ = 6.4 Hz, 3H); $^{13}$C NMR (D$_2$O) δ 65.2, 65.0, 51.3, 34.5, 32.0, 30.0, 29.9, 29.9, 29.8, 29.6, 29.3, 26.2, 22.8, 22.7, 13.9; FTIR 3460, 3190, 2918, 2851, 1615 cm$^{-1}$; HRMS for C$_{16}$H$_{37}$N$_2$I$^+$, calcd, 257.2951; found, 257.2954.

**Dodecyl-$N$-[2-(2-iodo-acetylamino)-ethyl]-$N,N$-dimethylammonium iodide (QDE).** To a solution of amine 2 (12.6 g, 32.8 mmol) in CH$_2$Cl$_2$ at 0 °C was added K$_2$CO$_3$ (27.2 g, 197 mmol). Chloroacetyl chloride (5.20 mL, 65.6 mmol) then was added to the stirred suspension dropwise via syringe. The reaction was stirred 12 h at room temperature and then filtered. The filtrate was dried over Na$_2$SO$_4$ and concentrated by rotary evaporation to give the crude chloroacetamide-QDE (7.69 g). This was confirmed
by the $^{1}$H NMR signal for $\alpha$–methylene protons at $\sim$4.1 ppm as compared to $\sim$3.8 ppm for the same protons in the iodoacetamide version of the molecule. The crude amide (7.69 g, 16.7 mmol) was dissolved in dry acetone (50 mL) and transferred to a sealed tube. To the solution was added NaI (7.50 g, 50.0 mmol) and a magnetic spin bar. The tube was flushed with N$_2$, sealed, and then heated to 65 °C. After stirring 20 h, the reaction was cooled to room temperature, the tube was opened and the precipitated solids were filtered and washed with CH$_2$Cl$_2$. The combined filtrate was concentrated by rotary evaporation and the crude residue was dissolved in CH$_2$Cl$_2$ (150 mL). The resultant solution was washed successively with aqueous NaHCO$_3$ solution (2 x 150 mL), aqueous NaHSO$_3$ solution (20 mL) and then brine (2 x 150 mL). The organic layer was dried (Na$_2$SO$_4$) and concentrated by rotary evaporation. The crude product obtained was purified by SiO$_2$ column chromatography, eluting with 9% MeOH in CH$_2$Cl$_2$, to obtain iodoacetamide QDE (7.18 g, 78% yield) as a pale yellow solid; $R_f$ = 0.22 (12% MeOH in CH$_2$Cl$_2$); mp, 81-82 °C; $^1$H NMR (CDCl$_3$) $\delta$ 8.44 (m, 1H), 3.81-3.77 (m, 6H), 3.55-3.51 (m, 2H), 3.37 (s, 6H), 1.77 (m, 2H), 1.37-1.24 (m, 18H), 0.86 (t, $J$ = 6.8 Hz, 3H); $^{13}$C NMR (CDCl$_3$) $\delta$ 169.3, 66.0, 62.6, 52.0, 34.6, 31.9, 29.5, 29.4, 29.4, 29.3, 29.2, 26.2, 22.9, 22.6, 14.1; FTIR 3230, 2853, 1664, 1533 cm$^{-1}$; HRMS for C$_{18}$H$_{36}$INO$_2^+$, calcd, 425.2023; found, 425.2025.
5.2.2. Synthesis of *QDE

**Scheme 2.2. Synthesis of *QDE.**

Reagents and conditions:  

a. 1-bromododecane, Na₂CO₃, EtOH, reflux, 24 h;  
b. phthalimide, PPh₃, DIAD, THF, 0 °C to rt, 12 h, 29% (2 steps);  
c. ¹³CD₃I, 50 °C (sealed tube), 3 h;  
d. H₂NNH₂•H₂O, EtOH, 40 °C, 12 h, 93% (2 steps);  
e. chloroacetyl chloride, K₂CO₃, CH₂Cl₂, rt, 12 h;  
f. NaI, acetone, 65 °C (sealed tube), 20 h, 32% (2 steps).

**2-[2-(N-Dodecyl-N-methylamino)-ethyl]-isoindole-1,3-dione (3, Scheme 5.2.).**

To a suspension of Na₂CO₃ (4.79 g, 45.2mmol) in EtOH (200 mL) was added 2-(methylamino)ethanol (16.6 mL, 205 mmol) and dodecylbromide (9.87 mL, 41.1 mmol). The reaction mixture was heated to reflux for 24 h. On cooling to room temperature, the precipitated solids were filtered. The filtrate was then concentrated using rotary evaporation and centrifuged to remove any remaining solids. The resulting viscous liquid was collected and placed under high vacuum for 12 h prior to a bulb to bulb distillation (Kügelrohr) to obtain the corresponding alkylated amino alcohol (8.08 g, 81% yield) as a
clear viscous liquid; $^1$H NMR (CDCl$_3$) δ 3.58 (t, $J = 5.2$ Hz, 2H), 2.53 (t, $J = 5.2$ Hz, 2H), 2.42-2.38 (m, 2H), 2.25 (s, 3H), 1.47-1.45 (m, 2H), 1.28-1.26 (m, 18H), 0.88 (t, $J = 6.8$ Hz, 3H).

To a solution of the amino alcohol (8.00 g, 32.9 mmol), phthalimide (5.32 g, 36.2 mmol), and triphenylphosphine (9.48 g, 36.2 mmol) in dry THF (150 mL) at 0 °C was added dropwise, with stirring, diisopropyl azodicarboxylate (DIAD) (7.10 mL, 36.2 mmol) via syringe. The reaction mixture was stirred an additional 30 min at 0 °C and then allowed to warm to room temperature. After 12 h, the reaction mixture was concentrated by rotary evaporation. EtOAc (200 mL) was added to dissolve the residue followed by successive washings with saturated aq. NaHCO$_3$ solution (3 X 150mL), water (70 mL) and brine (3 X 150mL). The organic layer was dried over Na$_2$SO$_4$, filtered and concentrated to 50 mL. The organic layer was cooled using an ice bath and cold 5% HCl solution (50 mL) was added. The reaction mixture was allowed to warm to room temperature and stirred for another 20 minutes. The organic layer was separated and the aqueous layer was extracted with Et$_2$O (3 X 150 mL). The aqueous layer then was cooled to 0 °C and neutralized by gradual addition of saturated aqueous NaHCO$_3$ solution. The neutral aqueous layer was extracted with CHCl$_3$ (3 X 70 mL). The combined CHCl$_3$ extract was dried (Na$_2$SO$_4$), filtered and then concentrated by rotary evaporation. The crude product was purified by silica gel column chromatography using with 2:1 CH$_2$Cl$_2$:EtOAc as the solvent system, to afford phthalimide 3 (4.45 g, 36% yield) as a white waxy solid; mp 27-28 °C; R$_f$ 0.46 (2:1 CH$_2$Cl$_2$:EtOAc); $^1$H NMR (CDCl$_3$) δ 7.84-7.82 (m, 2H), 7.7-7.68 (m, 2H), 3.79 (t, $J = 6.8$ Hz, 2H), 2.63 (t, $J = 6.6$ Hz, 2H), 2.35 (t, $J = 7.2$ Hz, 2H), 2.28 (s, 3H), 1.37-1.16 (m, 20H), 0.87 (t, $J = 6.4$ Hz, 3H); $^{13}$C NMR (CDCl$_3$) δ 168.5, 133.9, 132.4, 123.3, 58.0,
55.0, 42.3, 36.1, 32.1, 29.8, 29.8, 29.7, 29.5, 27.5, 27.4, 22.8, 14.3; FTIR 2914, 2848, 1775, 1714, 1613 cm$^{-1}$.

$[N\{-^{13}C_1,^{2}H_3\}]\cdot N\{-2\text{-Aminoethyl}\}-N\{-dodecyl\}-N,N\{-dimethylammonium\}$ iodide (4, Scheme 5.2). Phthalimide 3 (2.53 g, 6.78 mmol) was dissolved in CH$_2$Cl$_2$ (50 mL) and placed in a sealed tube under a N$_2$ atmosphere. The reaction mixture was then cooled to 0 °C and $^{13}$CD$_3$I (1.00 g, 6.85 mmol) was added in one portion. The tube was sealed and the reaction mixture was warmed to 50 °C and stirred 3 h. The reaction then was allowed to cool to room temperature, the tube was opened and the solvent was removed by rotary evaporation to give the corresponding ammonium iodide salt (3.52 g) as a white solid; mp, 180-182 °C; $^1$H NMR (CDCl$_3$) $\delta$ 7.89-7.86 (m, 2H), 7.80-7.77 (m, 2H), 4.21 (t, $J = 6.2$ Hz, 2H), 4.02-4.00 (m, 2H), 3.67-3.64 (m, 2H), 3.57 (d, $J = 2.8$ Hz, 3H), 1.75-1.69 (m, 2H), 1.31-1.20 (m, 18H), 0.88 (t, $J = 6.8$ Hz, 3H); $^{13}$C NMR (CDCl$_3$) $\delta$ 167.8, 134.9, 131.6, 124.1, 64.9, 60.6, 51.4 ($^{13}$C, m), 32.5, 32.0, 29.7, 29.6, 29.5, 29.3, 26.3, 23.0, 22.8, 14.3; FTIR 2916, 2851, 1710 cm$^{-1}$.

To a solution of the ammonium iodide prepared above (3.50 g, 6.75 mmol) in ethanol (50 mL) at room temperature was added hydrazine monohydrate (1.40 mL, 27.0 mmol). The reaction was warmed to 40 °C whereupon a white precipitate formed within 30 minutes. The reaction suspension was stirred an additional 11.5 h at 40 °C before cooling to room temperature. The ethanol then was evaporated by rotary evaporation. CH$_2$Cl$_2$ was added and the undissolved solids (phthalhydrazide) were filtered. The filtrate was concentrated by rotary evaporation to give product 4 as a pale yellow waxy solid (2.40 g, 93% yield); mp 69–71 °C; $^1$H NMR (D$_2$O) $\delta$ 3.46-3.44 (m, 4H), 3.18 (d, $J = 3.2$ Hz,
3H), 3.13-3.09 (m, 2H), 1.83 (m, 2H), 1.42-1.27 (m, 18H), 0.91 (t, J = 6.4 Hz, 3H); $^{13}$C NMR (D$_2$O) δ 65.0, 64.8, 50.3 ($^{13}$C, m), 34.3, 31.9, 29.8, 29.8, 29.7, 29.6, 29.5, 29.1, 26.1, 22.6, 22.6, 13.9; FTIR 3460, 3006, 2851, 1615, 1469 cm$^{-1}$.

$[N-(^{13}$C$_1,^{2}$H$_3)]-N$-(dodecyl-[2-(2-iodo-acetylamino)-ethyl]-$N,N$-dimethylammonium iodide (*QDE). To a solution of amino ammonium salt 4 (2.20 g, 5.66 mmol) in CH$_2$Cl$_2$ at 0 °C was added K$_2$CO$_3$ (4.70 g, 34.0 mmol). Chloroacetyl chloride (0.90 mL, 11.3 mmol) then was added to the stirred suspension dropwise via syringe. The reaction was stirred 12 hours at room temperature and then filtered and concentrated by rotary evaporation. The crude amide was dissolved in dry acetone (50 mL) and transferred to a sealed tube. To the solution was added NaI (2.50 g, 17 mmol) and a magnetic spin bar. The tube was flushed using N$_2$, sealed, and then heated to 65 °C. After stirring 20 h, the reaction was cooled to room temperature, the tube was opened and the precipitated solids were filtered and washed with CH$_2$Cl$_2$. The combined filtrate was concentrated by rotary evaporation and the crude residue was dissolved in CH$_2$Cl$_2$ (50 mL). The resultant solution was washed successively with aq. NaHCO$_3$ solution (2 x 50 mL), aq. NaHSO$_3$ solution (5 mL) and then brine (2 x 50 mL). The organic layer was dried (Na$_2$SO$_4$) and concentrated by rotary evaporation. The crude product obtained was purified by SiO$_2$ column chromatography, eluting with 9% MeOH in CH$_2$Cl$_2$, to obtain iodoamide *QDE (0.99 g 32% yield) as a pale yellow solid; R$_f$ 0.22, (12% MeOH in CH$_2$Cl$_2$); mp, 79-81 °C; $^1$H NMR (CDCl$_3$) δ 8.43 (m, 1H), 3.81-3.79 (m, 6H), 3.54-3.51 (m, 2H), 3.37 (d, J = 3.6 Hz, 3H), 1.77 (m, 2H), 1.37-1.25 (m, 18H), 0.87 (t, J = 6.6 Hz, 3H); $^{13}$C NMR (CDCl$_3$) δ 169.3, 65.9, 62.5, 51.2 ($^{13}$C, m), 34.6, 31.9, 29.6, 29.4, 29.3, 29.2, 26.2, 22.9, 22.6,
14.1; FTIR 2919, 2850, 1661, 1531 cm⁻¹; HRMS for C₁₇(¹³C)H₃₅D₃INO₂⁺, calcd, 429.2242, found, 429.2246.

5.2.3. Optimization of Thiol-QDE Adduct Formation & Extraction

**QDE:thiol ratios tested for adduct formation.** A 1:1 mixture of QDE (200 μL of a 100 μM solution in 1:1 H₂O:CH₃CN) and L-cysteine (200 μL of a 100 μM solution in H₂O) was prepared in a microfuge vial and agitated for 12 h in the dark at room temperature. The reaction mixture was dried using a vacuum centrifuge and reconstituted in 1 mL MeOH. Aliquots of this solution (15 μL) were then analyzed by FT-ICR-MS. The [QDE-Cys]⁺ adduct was confirmed at m/z = 418.30981. The above experiment was repeated using 3 equivalents of QDE per cysteine (this ratio was sufficient to completely consume all cysteine, as confirmed by negative mode FT-ICR-MS). Also, unreacted QDE was observed in the spectrum.

**Solvent optimization for adduct extraction.** A solution of L-cysteine (200 μL of 20 mM in H₂O) was combined with a solution of QDE (200 μL of 48 mM solution in 1:1 H₂O:CH₃CN) in a microfuge vial and the resultant solution was agitated on a shaker for 12 h in the dark at room temperature. The reaction mixture was then dried using a vacuum centrifuge and multiply extracted (0.5 mL X 5) with EtOAc. The organic layer from each individual extraction was isolated in a separate microfuge vial and dried using a vacuum centrifuge. Each dried extract was then reconstituted in 1 mL MeOH and vortex mixed. One hundred μL of each extract solution was further diluted to 1 mL with MeOH, vortex mixed, centrifuged at 13000 rpm for 10 min at 4 °C. Aliquots (15 μL) of the solutions were analyzed by FT-ICR-MS. This process was repeated using the following solvents:
methylene chloride, \textit{n}-butanol, toluene, chloroform, and diethyl ether. FT-ICR-MS spectra showed signals of the \textit{QDE} adduct only in case of \textit{n}-butanol and chloroform. The peak intensity for the signal in extract of \textit{n}-butanol was much higher as compared to that in chloroform.

\textbf{Examination of alkylation pH.} A solution of \textit{QDE} (150 \(\mu\)L of a 10 \(\mu\)M solution in 1:1 \(\text{H}_2\text{O}:\text{CH}_3\text{CN}\)) and a solution of L-cysteine (50 \(\mu\)L of 10 \(\mu\)M solution in \(\text{H}_2\text{O}\)) were added to pH 5.8 K-phosphate buffer (100 \(\mu\)L) in a microfuge vial. The mixture was agitated for 12 h at room temperature in the dark. The reaction solution was then dried using a vacuum centrifuge and extracted with \textit{n-BuOH} (0.2 mL X 3). The combined extract was dried using a vacuum centrifuge and then reconstituted in 1 mL MeOH followed by vortex mixing and centrifugation (10 mins, 13000 rpm) at 4 \(^\circ\)C. Aliquots (15 \(\mu\)L) of this solution were analyzed by FT-ICR-MS. In similar fashion, solutions of \textit{QDE} (100 \(\mu\)L of a 10 \(\mu\)M solution in 1:1 \(\text{H}_2\text{O}:\text{CH}_3\text{CN}\)) and L-glutathione (50 \(\mu\)L of a 10 \(\mu\)M solution in \(\text{H}_2\text{O}\)) were added to pH 8 K-phosphate buffer (100 \(\mu\)L) in a microfuge vial. Work-up and analysis of the reaction were performed as described above. The reaction of L-cysteine at pH 5.8 showed that the alkylation is possible at lower pH whereas the reaction of GSH at pH 8.0 was carried out to evaluate multiple alkylations by \textit{QDE}.

\textbf{5.2.4. Cell Culture, Formation & Extraction of Thiol-\textit{QDE} Adducts}

Dr. Pawel Lorkiewicz, Dr. Alex Belshoff and Ms. Sathyaramya Balasubramanium carried out the cell culture work in the labs of Dr. Teresa Fan and Dr. Richard Higashi. Human lung adenocarcinoma A549 cells were grown on 4 \(\times\) 10-cm plates with 8 mL DMEM 1640 medium supplemented with 4 mM glutamine, 0.2% glucose, 10% fetal bovine
serum, 100 U/mL penicillin, and 0.1 mg/mL streptomycin at 37 °C, 5% CO₂ and 95% humidity. When cells became 90% confluent, the medium was removed, and the cells were washed quickly three times on ice each with 5 mL cold PBS (phosphate buffered saline) to remove medium components. After PBS removal via vacuum suction, a cold solution of 1:1 QDE:*QDE (300 nmol) in acetonitrile (1 mL) at −20 °C was added immediately to quench metabolism within seconds by precipitating proteins. Then, for the pH 7.4 study, nanopure water (0.75 mL) was added or, for the pH 4 study, aq. HCl (600 μL of a 0.01M HCl solution) was added. The cells were scraped and collected into a 15 mL polypropylene conical centrifuge tube (Sarstedt, Newton, NC) containing three 3 mm diameter glass beads. The pH was measured and adjusted if necessary by addition of additional 0.01M aq. HCl. These steps were then repeated to collect any remaining cells. The centrifuge tubes were covered with aluminum foil, to ensure QDE and *QDE remain in the dark, and agitated on a nutator.

After agitation for 12 h, the tubes were centrifuged at 4.6k x g for 20 min at 4 °C. The polar fraction was carefully transferred to pre-weighed 7 mL PE screw cap vials using a fine tip transfer pipet without disturbing the pellet. The pellet was suspended in 200 μL Millipore water and transferred to pre-weighed 1.5 mL microfuge vials. The 15 mL conical tubes were washed with 2 x 0.25 mL CHCl₃:CH₃OH:BHT (2:1:1 mM) solution into the microfuge tubes. The microfuge vials were shaken vigorously to extract lipids from the pellets. These microfuge vials were then centrifuged at 14000 rpm for 20 min at 4 °C to yield two phases with a solid interphase. The upper aqueous layer (polar extract) was transferred carefully to the 7 mL PE vials and the lower organic layer (lipids) was
transferred to 1.5 mL screw cap glass vials. The organic layer was dried in a vacuum centrifuge, reconstituted in 0.3 mL CHCl₃:CH₃OH:BHT (2:1:1 mM), capped and stored at −80 °C. The solid middle layer was reconstituted in 0.5 mL MeOH, shaken and centrifuged at 14000 rpm for 20 min at 4 °C. The MeOH was decanted and the pellet was dried in vacuum centrifuge before measuring its mass and storing at −80 °C.

The polar extract (7 mL PE vials) was weighed and 1/8ᵗʰ (12.5%) was transferred to pre-weighed, uncapped GC-MS glass vials. Also, 2 X 1/16ᵗʰ (12.5%) of the polar extract was transferred to pre-weighed capped 0.5 mL FT-MS vials. The caps of the 7 mL PE vials containing the remaining 75% of the polar extract and the FT-MS vials containing 12.5% of the polar extract were replaced with punctured caps and the samples were frozen using liquid nitrogen. The samples were lyophilized overnight (−87 °C/vacuum 21 mT). After lyophilization the FT-MS vials (0.5 mL), as well as the GC-MS vials (1.5 mL), were capped and stored at −80 °C.

The dried polar extracts in the 7 mL PE vials were reconstituted in n-BuOH (200 μL) and transferred to microfuge vials. The microfuge vials were shaken vigorously and vortex mixed occasionally to extract the QDE/*QDE adducts of metabolites, and then centrifuged at 13000 rpm for 20 minutes at 4 °C. The n-BuOH supernatant was transferred to another microfuge vial without disturbing the pellet. The n-BuOH extraction procedure was repeated 5 more times to ensure complete extraction of adducts to give a total n-BuOH supernatant volume of 1.2 mL. The leftover pellet was dried in a vacuum centrifuge and stored at −80 °C. The combined n-BuOH extract was dried on a vacuum centrifuge (~3 h), reconstituted in 1 mL MeOH, shaken, vortex mixed
and centrifuged at 13000 rpm for 10 mins. Aliquots (15 μL) of these MeOH solutions (one from each of the four plates) were analyzed by FT-ICR-MS to identify thiol metabolites.

5.2.5. FT-ICR-MS Analyses & Informatics

**FT-ICR-MS analyses.** A hybrid linear ion trap (LIT) FT-ICR mass spectrometer (LTQ-FT, Thermo Electron, Bremen, Germany) equipped with a TriVersa NanoMate ion source (Advion BioSciences, Ithaca, NY) with an “A” electrospray chip (nozzle inner diameter 5.5 μm) was used for mass spectral analysis (Figure 5.1).

![FT-ICR-MS instrument](image)

**Figure 5.1.** FT-ICR-MS instrument used for thiol analyses.

The TriVersa NanoMate was operated in positive ion mode by applying 1.40 kV with 0.3 psi head pressure. MS runs were recorded over a m/z range from 305 to 1,000 Da using optimized ion abundance targets enabled for the selected mass range. Initially,
low resolution LIT-MS scans were acquired for 0.50 min to track the stability of the ion spray, after which high mass accuracy data were collected using the FT-ICR-MS analyzer where MS scans were acquired for 14 min at a target resolving power of \( m/\Delta m = 200,000 \) at \( m/z = 400 \) (10% valley). Five “microscans” (ICR-MS transients) were accumulated before Fourier transformation to produce each saved spectrum; thus the cycle time for each transformed, saved spectrum was about 5 s. The LTQ-FT was tuned and calibrated according to the manufacturer’s default standard recommendations, which typically achieved better than 0.5 ppm mass accuracy at a resolving power of 200,000 at \( m/z = 400 \). FT-ICR-MS mass spectra were centroided by Xcalibur and exported as exact mass lists into an Excel file using QualBrowser 2.0 SR2 (all software from Thermo Electron, “Bremen” version for the LTQ-FT).

**Mass corrections and global assignments of peak pairs.** Peaks were exported as numerical values of exact masses and ion counts into MS Excel 2010 file and those with ion counts less than 150 were excluded as noise. All peak pairs \( (m/z; m/z + 4.02188) \) representing QDE and *QDE adducts of given metabolites were selected using an in-house program (PREMISE - PREcalculated Exact Mass Isotopologue Search Engine) [247]. Only peak pairs with intensity ratios from 0.7-1.3 were used for further analyses. This list was manually checked to ensure the accuracy of the PREMISE program. The masses were compared across 4 experiments performed at both pH 4 and pH 7.4, and those persistently present in at least 50% of the experiments were retained. In the final step (QDE—I) was subtracted from all monoisotopic peaks to obtain molecular formulae of metabolites and compared against HMDB [248,249].
5.2.6. Synthesis of Standards for MS Analysis

**QDE-GSH**

To a solution of *QDE (0.20 g, 0.36 mmol) in EtOH (30 mL) was added a solution of L-glutathione (GSH) (0.09 g, 0.30 mmol) in pH 7.4 K-phosphate buffer (10 mL of a 0.1 M solution). After stirring at room temperature for 12 h in the dark, the reaction mixture was concentrated to ~10 mL by rotary evaporation. The concentrated mixture then was washed with CHCl₃ (3 X 10 mL). The aqueous layer was concentrated and the residue purified using reverse phase flash chromatography (C18 Aq), eluting with 1:1 CH₃CN:H₂O. The fractions containing the adduct (UV abs. at 214 nm) were lyophilized to afford *QDE-GSH (0.21 g, 94 % yield) as a white solid; mp 163-165 °C; ¹H NMR (D₂O) δ 4.61 (m, 1H), 3.81 (s, 2H), 3.71 (t, J = 6.4 Hz, 2H), 3.49 (m, 2H), 3.36 (m, 4H), 3.15 (s, 3H), 3.09 (m, 1H), 2.94 (m, 1H), 2.56 (m, 2H), 2.18 (m, 2H), 1.79 (m, 2H), 1.38-1.30 (m, 18H), 0.90 (t, J = 7.0 Hz, 3H); ¹³C NMR (D₂O) δ 175.8, 174.8, 173.8, 172.3, 171.5, 64.5, 61.1, 54.0, 52.8, 50.2 (¹³C, m), 43.3, 35.0, 33.6, 33.5, 31.4, 29.0, 28.9, 28.8, 28.7, 28.4, 26.2, 25.6, 22.2, 21.9, 13.6 ppm; FTIR 3256, 2924, 2854, 1645, 1594, 1467 cm⁻¹; HRMS for C₂₇(¹³C)H₅₁D₃N₅O₇S⁻, calcd, 608.3957, observed, 608.3969.
QDE-Cys

To a solution of QDE (0.26 g, 0.47 mmol) in EtOH (10 mL) was added a solution of L-cysteine (Cys) (0.04 g, 0.31 mmol) in pH 7.4 K-phosphate buffer (10 mL of a 0.1 M solution). After stirring at room temperature for 12 h in the dark, 5 μL of this solution was diluted to 1 mL using MeOH, vortexed, centrifuged for 10 min at 13000 rpm and used as standard for MS/MS analysis; HRMS for C_{21}H_{44}N_{3}O_{3}S^{+}, calcd, 418.3098, observed, 418.3098.

QDE-Hcy (racemic)

To a solution of QDE (0.12 g, 0.22 mmol) in EtOH (10 mL) was added a solution of DL-homocysteine (Hcy) (0.02 g, 0.15 mmol) in pH 7.4 K-phosphate buffer (10 mL of a 0.1 M solution). After stirring at room temperature for 12 h in the dark, 5 μL of this solution was diluted to 1 mL using MeOH, vortexed, centrifuged for 10 min at 13000 rpm and used as standard for MS/MS analysis; HRMS for C_{22}H_{46}N_{3}O_{3}S^{+}, calcd, 432.3254, observed, 432.3255.
QDE-CysGly

To a solution of QDE (0.05 g, 0.08 mmol) in EtOH (5 mL) was added a solution of cysteinylglycine (CysGly) (0.01 g, 0.06 mmol) in pH 7.4 K-phosphate buffer (5 mL of a 0.1 M solution). After stirring at room temperature for 12 h in the dark, 10 μL of this solution was diluted to 1 mL using MeOH, vortexed, centrifuged for 10 min at 13000 rpm and used as standard for MS/MS analysis; HRMS for C_{23}H_{47}N_{4}O_{4}S^{+}, calcd, 475.3313, observed, 475.3314.

QDE-HT

To a solution of QDE (0.15 g, 0.27 mmol) in EtOH (10 mL) was added a solution of hypotaurine (HT) (0.02 g, 0.18 mmol) in pH 7.4 K-phosphate buffer (10 mL of a 0.1 M solution). After stirring at room temperature for 12 h in the dark, 10 μL of this solution was diluted to 1 mL using MeOH, vortexed, centrifuged for 10 min at 13000 rpm and used as standard for MS/MS analysis; HRMS for C_{20}H_{44}N_{3}O_{3}S^{+}, calcd, 406.3098, observed, 406.3108.
5.2.7. Quantification of Glutathione

Human lung adenocarcinoma A549 cells were grown on 3 x 10-cm plates with 8 mL DMEM 1640 medium supplemented with 4 mM glutamine, 0.2% glucose, 10% fetal bovine serum, 100 U/mL penicillin, and 0.1 mg/mL streptomycin at 37 °C, 5% CO₂ and 95% humidity. The cells were harvested when ~90% confluent (ca. 2 days). The medium was removed, and the cells were washed quickly three times on ice, each with 5 mL cold PBS (phosphate buffered saline) to remove medium components. After PBS removal via vacuum suction, cold solutions of QDE (360 and 220 nmol) in acetonitrile (1 mL) at –20 °C were added to the control plates, to quench the metabolism. Then, nanopure water (0.75 mL) was added and the cells were scraped and collected into a 15 mL polypropylene conical centrifuge tube (Sarstedt, Newton, NC) containing three 3 mm diameter glass beads. These steps were then repeated to collect any remaining cells. The centrifuge tubes were covered with aluminum foil, to ensure QDE remained in the dark, and agitated on a nutator.

After agitation for 12 h, the tubes were centrifuged at 4.6k x g for 20 min at 4 °C. The polar fraction was carefully transferred to pre-weighed 7 mL PE screw cap vials using a fine tip transfer pipet without disturbing the pellet. The pellet was suspended in 200 µL Millipore water and transferred to pre-weighed 1.5 mL microfuge vials. The 15 mL conical tubes were washed with 2 x 0.25 mL CHCl₃:CH₃OH:BHT (2:1:1 mM) solution into the microfuge tubes. The microfuge vials were agitated for 5 mins to extract lipids from the pellets. These microfuge vials were then centrifuged at 14000 rpm for 20 min at 4 °C to yield two phases with a solid interphase. The upper aqueous layer (polar extract) was
transferred to the 7 mL PE vials and the lower organic layer (lipids) was transferred to 1.5 mL screw cap glass vials. The organic layer was dried in a vacuum centrifuge, reconstituted in 0.3 mL CHCl₃:CH₃OH:BHT (2:1:1 mM), capped and stored at –80 °C. The solid middle layer was reconstituted in 0.5 mL MeOH, agitated and centrifuged at 14000 rpm for 20 min at 4 °C. The MeOH was decanted and the pellet was dried in vacuum centrifuge before measuring its mass and storing at –80 °C.

The polar extract (7 mL PE vials) was weighed and 1/8<sup>th</sup> (12.5%) was transferred to pre-weighed, uncapped GC-MS glass vials. Also, 2 X 1/16<sup>th</sup> (12.5%) of the polar extract was transferred to pre-weighed capped 0.5 mL FT-MS vials. The caps of the 7 mL PE vials containing the remaining 75% of the polar extract and the FT-MS vials containing 12.5% of the polar extract were replaced with punctured caps and the samples were frozen using liquid nitrogen. The samples were lyophilized overnight (–87 °C/vacuum 21 mT). After lyophilization the FT-MS (0.5 mL) vials, as well as the GC-MS (1.5 mL) vials, were capped and stored at –80 °C.

The dried polar extracts in the 7 mL PE vials were reconstituted in n-BuOH (200 μL) and transferred to microfuge vials. The microfuge vials were agitated and vortex mixed occasionally to extract the [QDE-GSH]<sup>+</sup> adduct, and then centrifuged at 13000 rpm for 20 minutes at 4 °C. The n-BuOH supernatant was transferred to another microfuge vial without disturbing the pellet. This procedure was repeated 5 more times to ensure complete extraction of adducts to give a total n-BuOH supernatant volume of 1.2 mL. The n-BuOH supernatant was divided into seven equal fractions (7 x 145 μL) and increasing concentrations of the [*QDE-GSH]<sup>+</sup> standard (4 μL, 10 μL, 20 μL, 60 μL, 100 μL, 300 μL or
500 μL of 10 μM solution in MeOH) were added to these fractions. The resultant solutions were dried using a vacuum centrifuge and then reconstituted in 1 mL MeOH, vortex mixed and centrifuged at 13000 rpm at 4 °C for 10 min. Aliquots (15 μL) of these MeOH solutions (seven solutions from each plate) were analyzed by FT-ICR-MS to quantify reduced glutathione (GSH).

The pellet remaining after n-BuOH extraction was dried in a vacuum centrifuge and suspended in nanopure water (100 μL). To the suspension was added a freshly prepared aq. solution of tris(2-carboxyethyl)phosphine hydrochloride (TCEP•HCl) (20 μL of 10 mM solution in water) to reduce GSSG. After agitating on a nutator for 12 h, QDE (12.5 μL of a 20 mM solution in acetonitrile) was added and the mixture was agitated for 12 h. The mixture then was dried on a vacuum centrifuge and the [QDE-GSH]^+ adduct was extracted with n-BuOH (6 x 200 μL) as described above. The n-BuOH extracts were combined to give a total volume of 1.2 mL. This n-BuOH phase then was divided into seven equal fractions (7 x 145 μL) and increasing concentrations of the [*QDE-GSH]^+ standard (4 μL, 10 μL, 20 μL, 60 μL, 100 μL, 300 μL or 500 μL of 10 μM solution in MeOH) were added to these fractions. The resultant solutions were dried using a vacuum centrifuge and then reconstituted in 1 mL MeOH, vortex mixed and centrifuged at 13000 rpm at 4 °C for 10 min. Aliquots (15 μL) of these MeOH solutions (seven solutions from each plate) were analyzed by FT-ICR-MS to quantify GSSG.
5.3. CHAPTER 3 EXPERIMENTAL PROCEDURES

5.3.1. Synthesis of BCMA

Scheme 3.3. Synthesis of BCMA

**Bis(trimethylstannyl)mesitylamine 4.** A 250 mL two-necked round-bottomed flask equipped with a dropping funnel with a pressure equalizing side arm, a reflux condenser, a thermometer and a magnetic stirrer was thoroughly flushed with argon. Argon atmosphere was maintained throughout the reaction.

A 1.6 M solution of n-BuLi in hexanes (40 mL, 63.6 mmol) was introduced into the flask using a syringe. This was cooled to −78 °C using a dry ice/acetone bath and a solution of diethylamine in diethyl ether (6.6 mL, 63.6 mmol) was then added dropwise, with stirring over a period of 15 mins. This reaction mixture was allowed to warm to room
temperature and then a solution of trimethyltin chloride in diethyl ether (12.6 g, 63.6 mmol) was added at such a rate as to cause slow reflux. After the addition was complete, the reaction mixture was heated to reflux and stirred for another 2 hours. After completion of reaction, the mixture was cooled to room temperature and then centrifuged at 1500 rpm for 20 mins to separate the precipitated solids. The solids were washed with diethyl ether and the organic layers containing crude (diethylamino)trimethylstannane were combined.

The crude was introduced into a two-neck, 250 mL round-bottomed flask equipped with a magnetic stirrer and a short path distillation unit. 2,4,6-trimethylanilne (4.1 mL, 29 mmol) was added to this solution at room temperature using a syringe. The solution was heated to 80 °C for 1 hour to remove the volatile solvents and then the temperature was increased to 150 °C and the reaction mixture was stirred for 5 days. As the reaction progressed, the generated diethylamine was distilled via short path distillation. After 5 days the reaction was cooled to room temperature and then subjected to fractional distillation using a 12 cm Vigreaux column at 200 °C and 30 torr pressure. However, no product distilled into the receiving flask. Subsequently, a short path distillation of the remaining reaction mixture at 210 °C (oil-bath temperature) and 30 torr successfully distilled the product between 170–176 °C (distilling temperature) at 30 torr pressure. Pale yellow, viscous liquid bis(trimethylstannyI)mesitylamine, 4 was collected in 98 % yield; bp, 170-176 °C at 30 torr; \textsuperscript{1}H NMR (CDCl\textsubscript{3}) \( \delta \) 6.77 (s, 2H), 2.20 (s, 3H), 2.14 (s, 6H), 0.13 (s, 18H); \textsuperscript{13}C NMR (CDCl\textsubscript{3}) \( \delta \) 151.0, 135.9, 129.2, 128.2, 20.7, 20.1, −4.4.
**Bis(chlorophenylboryl)mesitylamine (BCMA).** MesN(SnMe$_3$)$_2$ 4, (3.0 g, 6.45 mmol) was dissolved in DCM and added dropwise to a solution of PhBCl$_2$ (0.9 mL, 13.2 mmol) in DCM over a period of 15 mins while stirring. The reaction mixture was stirred for 16 h and then sampled for $^{11}$B NMR analysis to check for completion of reaction. We observed 3 major signals in $^{11}$B NMR at $\delta$ 60, 47, and 37 ppm; the signals at 47 and 37 ppm have been reported for BCMA and MesN(SnMe$_3$)(PhBCl) respectively, however the signal at 60 has not been reported in the literature. We spiked the NMR samples with PhBCl$_2$ and observed that this resulted in an increase in the intensity of the signal at 60 ppm. As the reaction progressed, the intensity of this signal decreased while a simultaneous increase was observed for the BCMA boron signal. Hence, we hypothesized this to be an intermediate-complex leading to the formation of the product.

Once the maximum conversion of the $^{11}$B was observed for BCMA production (after 12 days, the signal intensities did not change), the volatiles were removed under reduced pressure. This reaction mixture was then subjected to high vacuum (~30 torr) for 8 hours to remove the generated Me$_3$SnCl. However, $^1$H NMR of the sample revealed Me$_3$SnCl to be present in the reaction mixture. Successive attempts to remove Me$_3$SnCl via heating the reaction mixture upto 35 °C while under 30 torr vacuum caused the production of a byproduct Mes–N=B–Ph which was observed in $^{11}$B NMR at 34 ppm. Hence crude BCMA was used to test reaction with silver-p-methoxycarboxylate.

5.3.2. Synthesis of Silver-p-methoxybenzoate (AMB)

$p$-Methoxybenzoic acid (1 g, 6.6 mmol) was dissolved in 10 mL THF and mixed with a solution of NaOH (0.26 g, 6.6 mmol) in 5 mL water and stirred for 10 mins. The solvents
were evaporated and the residual solids were suspended in 10 mL THF. A solution of AgNO3 (1 g, 5.9 mmol) in 10 mL water was added to this suspension and stirred for 15 mins. The product was filtered, washed with water and dried at 70 °C for 8 h to remove the solvents. The product was collected as an off-white solid in 92 % yield; 1H NMR (CDCl₃) δ 7.88 (d, 2H), 6.89 (d, 2H), 3.78 (s, 3H).

5.3.3. Attempted Synthesis of BCMA-AMB Adduct.

A suspension of AMB (1.3 g, 5.0 mmol) in DCM was added to solution of BCMA (1.5 g, 4.0mmol) in DCM in a 50 mL round bottomed flask equipped with a magnetic stir bar. The solution was stirred for 30 mins and then a suspension of AgBF₄ (1.0 g, 5.1 mmol) in DCM was added to this mixture with stirring. Vigorous precipitation was observed upon addition of AgBF₄. The solution was stirred for 16 hours at room temperature and then the solids were allowed to settle and the liquids were cannulated into another flask. This solution was analyzed using ¹¹B NMR spectroscopy. The boron signals showed a downfield shift from 47 ppm to –8 ppm and –7 ppm. This proved that the BCMA had reacted, however, it did not give any information as to the identity of the product. To get a better idea about product/s formed during the reaction, we crystallized the formed products via liquid-liquid diffusion technique.
Variations in synthesis of BCMA-AMB adduct. The above synthesis was repeated with chlorobenzene as the solvent instead of DCM. The reactions were sampled for NMR analysis after 16 h and 5 days for NMR as well as crystallographic analysis.

The reactions that were carried out in DCM were repeated at reflux temperature (~40 °C). However, this resulted in the reaction mixture turning dark brown color and precipitation of black colored solids. An NMR analysis of these solids could not be carried out due to their insolubility in CDCl₃. Similarly, the reactions carried out in chlorobenzene were repeated at 80 °C and also resulted in precipitation of black solids (could not be analyzed using NMR).

5.3.4. Crystallizations

The crude products from the above reactions were crystallized using a biphasic solvent diffusion technique using a combination of solvents. All crystallizations were carried out under argon atmosphere. The crude was diluted in DCM to give a concentration of ~200 mg of the product per mL of the solvent. ~2mL of this was transferred to a cylindrical crystallization tube. Freshly distilled hexanes was then carefully layered on top of the DCM solution. This tube was allowed to sit on a stable surface for 5 days to allow slow diffusion of hexanes into the DCM layer. Fine, colorless needles were obtained on the 5th day and isolated from the crystallization milieu for X ray analysis. Identically treated samples were allowed to stand for longer periods of time (7 and 10 days). It was observed that on allowing the diffusion to proceed beyond 7 days another set of yellow prismatic crystals were obtained. These crystals, however,
degraded as soon as isolated due to their instability to air and moisture. X-ray analysis of these yellow colored crystals did not provide any meaningful data.

Other solvents tried for crystallization include chlorobenzene for dissolving the product and hexanes, cyclohexane and n-heptane as the diffusing solvents. Chlorobenzene/hexanes was found to be the best binary solvent combinations for the crystallization of yellow crystals and DCM/heptanes were ideal for crystallization of colorless crystals. The first set of colorless crystals yielded the mesitylene aniline•BF3 adduct. The second set of colorless crystals obtained were found to be BOBF complex. This compound was analyzed using X-ray crystallography (Figure 3.6).

5.4. CHAPTER 4 EXPERIMENTAL PROCEDURES

5.4.1. Calibration Data for Pentose Quantification

Glycerol (200 mg), acetic acid (300 mg), ethanol (250 mg), hydroxymethylfurfural (HMF, 125 mg), furfural (125 mg), D-glucose (600 mg), D-xylose (600 mg) and L-arabinose (600 mg) were mixed in a 25 mL volumetric flask and diluted to 25 mL by addition of deionized water. The resultant stock solution, containing 24.0 mg/mL each of D-xylose, D-glucose and L-arabinose, was diluted with water successively to obtain 19.1, 14.4, 9.6, 4.7, 2.9 and 1.0 mg/mL solutions. These solutions were passed through a 25 mm syringe filter with a 0.45 μm polyethersulfone membrane prior to HPLC analysis. The standards were plotted as concentration (g/L) vs. peak area and were analyzed 3 times to obtain a standard deviation. The regression values for inter-day triplicate analysis was found to be greater than 0.999.
5.4.2. pH Optimization for Chelation and Extraction of Pentoses

DDG hydrolyzate was prepared by Dr. Robert Lupitsky, Dr. Mayank Gupta and Dr. Eurik Kim at Conn Center, University of Louisville. The hydrolyzate was prepared using a 2 stage hydrolysis procedure, first by heating DDGs at 110 °C and 0.2% mass fraction of H$_2$SO$_4$ and then increasing to 140 °C and 0.4% mass fraction of H$_2$SO$_4$ [238].

Sodium hydroxide (2.18 g, 54.5 mmol) was added to the DDG hydrolyzate (400 mL) in 3-neck, round bottom flask at room temperature and stirred for 1 h. The pH of the solution was measured to be 9.3. An aliquot (60 mL) of the pH 9.3 hydrolyzate was filtered and analyzed using HPLC. By comparing the areas under curve (HPLC signal) of standard sugar solution with the test hydrolyzate solution we determined the concentration of xylose in this solution to be determined to be 15.6 mg/mL. The remaining pH 9.3 hydrolyzate (ca. 340 mL) was then acidified by addition of untreated hydrolyzate in an amount sufficient to decrease the pH to 9.0. An aliquot (60 mL) of the pH 9.0 hydrolyzate was filtered and stored. This acidification approach was repeated to generate 4 additional 60 mL filtered samples of pH 8.5, 8.0, 7.5 and 7.0 hydrolyzate.

To each individual 60 mL aliquot was added phenylboronic acid (PBA; 4.59 g; 6 eq. PBA/xylose), toluene (60 mL) and MeOH (30 mL). The reaction mixture was vigorously stirred at room temperature for 16 h. The phases were then allowed to separate and organic layer was isolated. The aqueous layer was extracted with toluene (2 x 60 mL) and the organic layers were combined, dried over Na$_2$SO$_4$ (~20 g) and then concentrated by rotary evaporation. The solids were analyzed using $^1$H NMR to determine the ratio of
XDE:PBA. In addition, the aqueous layer for each pH examined was analyzed by HPLC for unreacted xylose.

5.4.3. Determination of Optimal PBA Stoichiometry

A 250 mL sample of pH 7.5 hydrolyzate, prepared according to the method described above, was divided into five 50 mL aliquots, each containing 11.4 mg xylose/mL. To each aliquot then was added PBA to generate PBA:xylose molar ratios of 2, 4, 6, 8, 10 and 12 by addition of 1.03, 2.07, 3.10, 4.14, 5.17 and 6.21 g of PBA, respectively. Then toluene (50 mL) and MeOH (25 mL) were added to each aliquot and the mixtures were stirred at room temperature for 16 h. The phases were allowed to separate and the organic layers of each mixture were removed. The aqueous layers were extracted with additional toluene (2 x 50 mL) and then each aqueous layer was concentrated to ca. 40 mL followed by dilution to 50 mL by addition of water. Analysis by HPLC enabled the measurement of unreacted xylose, glucose and arabinose in treated hydrolyzate.

5.4.4. Evaluation of XDE & PBA Extraction Efficiency

To a basified hydrolyzate solution (50 mL, pH 7.5) containing 15.6 mg xylose/mL was added PBA (5.1 g, 8.0 eq./xylose), toluene (50 mL) and MeOH (25 mL) at room temperature. This mixture was stirred 20 h whereupon the organic layer was isolated and dried over Na$_2$SO$_4$. The solvents were removed by rotary evaporation, and the residue was weighed and analyzed using $^1$H NMR to determine the ratio of XDE:PBA. The aqueous layer was then extracted using a second volume of toluene (50 mL) followed by extraction with a third volume of toluene (50 mL). Both organic extracts were treated as above to
determine the XDE:PBA ratio. The extraction protocol was repeated as described above but using reduced volumes of toluene (25 mL) and MeOH (10 mL).

5.4.5. Transesterification of XDE & D-Xylose Precipitation

To a stirred solution of XDE (9.00 g, 28.0 mmol) in toluene (180 mL) at room temperature was added propylene glycol (PG) (10.3 mL, 140 mmol). After 24 h, stirring was ceased and the precipitated solids were allowed to settle. The toluene layer was decanted and to the remaining humectant precipitate was added diethyl ether (200 mL). After stirring 30 min, the resulting fine solids were collected by filtration to obtain D-xylopyranose (4.07 g, 97 %) as a 1:1.5 mixture of β:α anomers that was spectroscopically identical to previously published data [250]. The decanted toluene layer was concentrated by rotary evaporation to obtain the corresponding boronate ester 4-methyl-2-phenyl-1,3,2-dioxaborolane (PGE) (8.52 g, 94 %) as an oil; 1H NMR (CDCl₃): δ 7.81 (d, J = 6.8 Hz, 2H), 7.49-7.45 (m, 1H), 7.38 (t, J = 7.6 Hz, 2H), 4.75-4.70 (m, 1H), 4.46 (t, J = 16 Hz, 1H), 3.89 (t, J = 16 Hz, 1H), 1.42 (d, J = 2.0 Hz, 3H) ppm; 13C NMR (100 MHz, CDCl₃): δ 135.1, 131.7, 128.1, 74.1, 72.9, 22.1 ppm.

5.4.6. Propylene Glycol Ester Hydrolysis

Water (20 mL) was added to PGE (5.10 g, 31.5 mmol) at room temperature and the mixture was stirred. After 18 h, the precipitated solids were collected by filtration to obtain the PBA anhydride, phenylboroxine (2.40 g, 62 %).
5.4.7. Preparative Scale Xylose Extraction Using DDG Hydrolyzate

**Pretreatment.** DDG hydrolyzate (5.0 L) stirred in a 12 L round bottom flask at room temperature was basified to pH 7.5 by slow addition of NaOH (21.4 g). The reaction solution then was centrifuged (IEC International Centrifuge - model CS at 3/4th speed setting for 20 min) followed by filtration of the collected supernatant using a Büchner funnel. Analysis of the filtrate determined the concentration of xylose in the basified, filtered hydrolyzate to be 11.4 mg/mL.

**C5-Sugar and PBA Extraction.** A 22 L two-neck, round bottom flask connected to a mechanical, overhead stirrer was charged with the basified, filtered DDG hydrolyzate (3.5 L). Toluene (1.75 L), MeOH (0.7 L) and PBA (260 g, 8 eq./xylose) were added to the hydrolyzate and the reaction mixture was vigorously stirred at room temperature. After 15 h, the organic layer was separated using a separatory funnel, dried over Na$_2$SO$_4$ and filtered. The filtrate was concentrated to 820 mL by rotary evaporation and then cooled to $-20$ °C for 5 hours. The precipitated PBA (10.0 g) was collected using a Büchner funnel. The filtrate was analyzed using $^1$H NMR prior to the PG-mediated transesterification protocol to reveal an XDE:PBA ratio of 1:1.1. The extracted hydrolyzate was acidified to ca. pH 2 by addition of conc. HCl (36%, 25 mL) and then extracted using toluene (2 x 3.5 L). The organic layers were combined, dried over Na$_2$SO$_4$ and filtered. The volatiles were distilled (and recovered) by rotary evaporation to afford a second batch of PBA (93.8 g).

**Transesterification.** To the toluene filtrate ( ~800 mL) containing the bis(boronate ester) adducts and unreacted PBA in a 2 L two-neck round bottom flask at room temperature was added PG (170 mL). Additional toluene (200 mL) was added and the
mixture was stirred. After 24 h, stirring was ceased and the precipitated viscous solids were allowed to settle. The toluene layer was decanted and precipitated solids were rinsed with toluene (140 mL). EtOH (300 mL) then was added to the solids and the resultant suspension was stirred 30 min before filtering. The solids were collected and dried under vacuum at ambient temperature to afford a 1:0.2 mixture of D-xylopyranose:L-arabinopyranose as a crystalline solid (23.0 g). The decanted toluene layer and toluene rinse solution were combined and concentrated by rotary evaporation to obtain PGE (166 g).

**PGE Hydrolysis.** To PGE (166 g, 1.02 mol) was added water (664 mL) at room temperature. After stirring 18 h, the precipitated solids were collected by filtration to obtain the third batch of PBA (101 g), bringing the total quantity of recovered PBA to 205.1 g (79% recovery).
REFERENCES


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82. Lawrence Livermore National Laboratory and the Department of Energy. DOE/EIA MER, 2015.


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156. Glowacki, R.; Bald, E. Fully Automated Method For Simultaneous Determination Of Total Cysteine, Cysteinylglycine, Glutathione And Homocysteine In Plasma By


# APPENDIX – A

## LIST OF ACRONYMS AND ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>ADB</td>
<td>Azadiboriridine</td>
</tr>
<tr>
<td>AIDS</td>
<td>Acquired Immune Deficiency Syndrome</td>
</tr>
<tr>
<td>AMB</td>
<td>Silver Methoxy Benzoate</td>
</tr>
<tr>
<td>BCMA</td>
<td>Bis(chlorophenylboryl)mesitylene Amine</td>
</tr>
<tr>
<td>BOBF</td>
<td>Bridged Oxygen Boron Fluoride Complex (Figure 3.5)</td>
</tr>
<tr>
<td>BODIPY</td>
<td>Boron-Dipyrrromethene</td>
</tr>
<tr>
<td>CA</td>
<td>Carboxylic Acid</td>
</tr>
<tr>
<td>CID</td>
<td>Collision Induced Dissociation</td>
</tr>
<tr>
<td>CNS</td>
<td>Central Nervous System</td>
</tr>
<tr>
<td>CS</td>
<td>Chemoselective</td>
</tr>
<tr>
<td>Cys</td>
<td>Cysteine</td>
</tr>
<tr>
<td>DBD-PZ-NH₂</td>
<td>7-(N,N-dimethylaminosulfonyl)-4-(aminoethyl)piperazino-2,1,3-benzoxadiazole</td>
</tr>
<tr>
<td>DCM</td>
<td>Dichloromethane</td>
</tr>
<tr>
<td>DDG</td>
<td>Distillers’ Dried Grains</td>
</tr>
<tr>
<td>DIAD</td>
<td>Diisopropyl Azodicarboxylate</td>
</tr>
<tr>
<td>DmAPBr</td>
<td>p-Dimethylaminophenacyl Bromide</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl Sulfoxide</td>
</tr>
<tr>
<td>DNBS</td>
<td>2,4-Dinitrobenzene Sulfonyl</td>
</tr>
<tr>
<td>DPDS</td>
<td>2,2’-Dipyridyl Disulfide</td>
</tr>
<tr>
<td>DTNB</td>
<td>5,5’-Dithiobis-2-Nitrobenzoic Acid</td>
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ESI-MS  Electrospray Ionization Mass Spectrometry
FRET  Förster Resonance Energy Transfer
FT-ICR-MS  Fourier Transform Ion Cyclotron Resonance Mass Spectrometry
GC-MS  Gas Chromatography Mass Spectrometry
GSH  Glutathione
GSHPx  Glutathione Peroxidase
GSSG  Glutathione Disulfide
HBTU  2-(1H-Benzotriazol-1-yl)-1,1,3,3-Tetramethyluronium Hexafluorophosphate
HMDB  Human Metabolome Database
HPLC  High Performance Liquid Chromatography
HRMS  High Resolution Mass Spectroscopy
LC  Liquid Chromatography
LC-MS  Liquid Chromatography Mass Spectrometry
Mes  2,4,6-Trimethylaniline
MS  Mass Spectrometry
Mtb  Metabolite
NADP  Nicotinamide Adenine Dinucleotide Phosphate
NEM  N-Ethyl Maleimide
NIR  Near Infrared
NMR  Nucelar Magnetic Resonance Spectroscopy
PBA  Phenylboronic Acid
PBS  Phosphate Buffer Solution
PD  Parkinson's Disease
PET  Photoinduced Electron Transfer
PG  Propylene Glycol
PGE  Propylene Glycol Phenylboronic Acid Ester
PREMISE  Precaculated Exact Mass Isotopologue Search Engine
QDA  Quaternary Ammonium Dodecyl Dimethyl Ethyl Aminoxyl
<table>
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<tr>
<th>Acronym</th>
<th>Full Description</th>
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<tr>
<td>QDE</td>
<td>Quaternary Ammonium Dodecyl Dimethyl Ethyl Iodoacetamide</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive Oxygen Species</td>
</tr>
<tr>
<td>S&lt;sub&gt;N&lt;/sub&gt;2</td>
<td>Substitution Nucleophilic Bimolecular</td>
</tr>
<tr>
<td>TCA</td>
<td>Tricarboxylic Acid</td>
</tr>
<tr>
<td>TCEP•HCl</td>
<td>Tris(2-Carboxyethyl)phosphine Hydrochloride</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>XDE</td>
<td>Xylose Phenylboronic Acid Diester</td>
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</table>
APPENDIX – B

SPECTRA

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B.3. SPECTRA FROM CHAPTER 3
B.4. SPECTRA FROM CHAPTER 4
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B.2. SPECTRA FROM CHAPTER 2

Figure B.2.1. 400 MHz $^1$H NMR Spectrum of Compound 2 in D$_2$O

The NMR confirms alkylation of the amine with C$_{12}$ chain as well as the hydrazinolysis of phthalamide.
Figure B.2.2. 100 MHz $^{13}$C NMR Spectrum of Compound 2 in D$_2$O
This NMR confirms the amidation of compound 2 and also the halide exchange via Finkelstein reaction.
Figure B.2.4. 400 MHz 2D COSY $^1$H NMR Spectrum of QDE in CDCl$_3$
Figure B.2.5. 100 MHz $^{13}$C NMR Spectrum of QDE in CDCl$_3$
Figure B.2.6. 400 MHz $^1$H NMR Spectrum of Compound 3 in CDCl$_3$

This NMR confirms the alkylation of ethanolamine as well as the phthalimidation of the alcohol.
Figure B.2.7. 100 MHz $^{13}$C NMR Spectrum of Compound 3 in CDCl$_3$
Figure B.2.8. 400 MHz $^1$H NMR Spectrum of Compound 4 in D$_2$O
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Figure B.2.10. 400 MHz $^1$H NMR Spectrum of *QDE in CDCl$_3$
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Figure B.2.12. 400 MHz $^1$H NMR spectrum of [*QDE-GSH] in D$_2$O
Figure B.2.13. 100 MHz $^{13}$C NMR spectrum of [$^{*}$QDE-GSH]$^+$ in D$_2$O
Figure B.2.14. FT-MS spectrum of QDE

QDE

$C_{18}H_{38}IN_2O^+$

Exact Mass: 425.20234
Figure B.2.15. FT-MS spectrum of *QDE

*CDE

C_{17}^{13}CH_{35}D_3IN^2O^+

Exact Mass: 429.22422
Figure B.2.16. FT-MS spectrum of \([^{*}\text{QDE-GSH}]^+\)

\[\text{C}_{27}^{13}\text{CH}_5\text{D}_3\text{N}_8\text{O}_7\text{S}^+\]

Exact Mass: 608.39573
Figure B.2.17. CID MS/MS/MS spectrum of QDE-Cys

Top: QDE-Cys in derivatized cell extracts; Bottom: QDE-Cys Standard
Figure B.2.18. CID MS/MS spectrum of QDE-HT

Top: QDE-HT in derivatized cell extracts; Bottom: QDE-HT Standard
Figure B.2.19. CID MS/MS/MS spectrum of QDE-Hcy

**Top:** QDE-Hcy in derivatized cell extracts; **Bottom:** QDE-Hcy Standard
Figure B.2.20. CID MS/MS/MS spectrum of QDE-CysGly

Top: QDE-CysGly in derivatized cell extracts; Bottom: QDE-CysGly Standard
Figure B.3.1. 400 MHz $^1$H NMR spectrum of bis(trimethyltin)mesitylamine in CDCl$_3$

The NMR confirms the transmetallation of mesitylene aniline.
Figure B.3.2. 100 MHz $^{13}$C NMR spectrum of bis(trimethyltin)mesitylamine in CDCl$_3$
Figure B.3.3. 400 MHz $^1$H NMR spectrum of AMB in DMSO
Figure B.3.4. 128 MHz $^{11}$B NMR spectrum of BCMA in DCM
Figure B.3.5. 128 MHz $^{11}$B NMR spectrum of BCMA / AMB reaction in DCM.

The NMR shows the downfield shift of the B signals after reaction.
Figure B.4.1. 400 MHz $^1$H NMR spectrum of XDE in CDCl$_3$
Figure B.4.2. 400 MHz $^1$H NMR spectrum of extracted solids (XDE + PBA-anhydride) in CDCl$_3$
Figure B.4.3. 400 MHz $^1$H NMR spectrum of PGE in CDCl$_3$
### Identification code
mn06ltc

### Empirical formula
$C_{16}H_{14}B_{2}F_{2}O_{7}$

### Formula weight
377.89

### Temperature
100.05(10) K

### Wavelength
0.71073 Å

### Crystal system
Monoclinic

### Space group
$C 2/c$

### Unit cell dimensions

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<td>b</td>
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<tr>
<td>β</td>
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### Volume
1542.08(12) Å³

### Z
4

### Density (calculated)
1.628 Mg/m³

### Absorption coefficient
0.139 mm⁻¹

### F(000)
776

### Crystal color, habit
colorless plate

### Crystal size
0.35 x 0.18 x 0.01 mm³

### Theta range for data collection
3.48 to 28.16°.

### Index ranges
$-22 \leq h \leq 22$, $-5 \leq k \leq 5$, $-28 \leq l \leq 27$

### Reflections collected
8328

### Independent reflections
1895 [R(int) = 0.0378]

### Completeness to theta = 28.16°
99.7%

### Absorption correction
Semi-empirical from equivalents

### Max. and min. transmission
1.000 and 0.983

### Refinement method
Full-matrix least-squares on $F^2$

### Data / restraints / parameters
1895 / 0 / 151

### Goodness-of-fit on $F^2$
1.075

### Final R indices [I>2σ (I)]
R1 = 0.0390, wR2 = 0.0841
R indices (all data) \[ R1 = 0.0547, \; wR2 = 0.0914 \]

Largest diff. peak and hole \[ 0.330 \text{ and } -0.258 \text{ e.Å}^{-3} \]

Table C1. Atomic coordinates (x 10^4) and equivalent isotropic displacement parameters (Å^2 x 10^3) for mn08ltc. U(eq) is defined as one third of the trace of the orthogonalized U^ij tensor.

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Table C2. Bond lengths [Å] and angles [°] for mn08ltc.

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O(2)-C(7)-C(4) 119.41(12)
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H(8A)-C(8)-H(8C) 108.8(14)
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H(8A)-C(8)-H(8B) 113.1(14)
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F(1)-B(1)-O(4) 117.60(13)
F(1)-B(1)-O(3)#1 107.39(12)
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F(1)-B(1)-O(2) 107.53(11)
O(4)-B(1)-O(2) 110.04(11)
O(3)#1-B(1)-O(2) 103.74(11)

Symmetry transformations used to generate equivalent atoms: #1 -x+1,y,-z+3/2
Table C3. Anisotropic displacement parameters (Å² x 10³) for mn08ltc. The anisotropic displacement factor exponent takes the form: –2\pi² [ h² a*² U¹¹ + ... + 2 h k a* b* U¹² ].

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Table C4. Hydrogen coordinates (x 10⁴) and isotropic displacement parameters (Å² x 10³) for mn08ltc.

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### Table C5. Torsion angles [°] for mn08ltc.

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Symmetry transformations used to generate equivalent atoms: #1 -x+1,y,-z+3/2
APPENDIX – D

LIST OF PUBLICATIONS

D.1. List of Publications

D.2. Copyright Permissions

D.3. Manuscript Title Pages
D.1. List of Publications

The work presented in this dissertation was published in various peer-reviewed journals and/or patented. The following list summarizes the publications and patents that arose from my research.

Publications:


Patent:

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A Chemoselective, One-Pot Transformation of Aldehydes to Nitriles

Sébastien Laulhé, Sadakatali S. Gori, and Michael H. Nantz*

Department of Chemistry, University of Louisville, Louisville, Kentucky 40292, United States

Supporting Information

ABSTRACT: This paper describes a procedure for direct conversion of aldehydes to nitriles using O-(diphenylphosphoryl)hydroxylamine (DPPH). Aldehydes are smoothly transformed to their corresponding nitriles by heating with DPPH in toluene. The reaction can be accomplished in the presence of alcohol, ketone, ester, or amine functionality.

The preparation of nitriles by transformation of carbon-equivalent functional groups is an important synthetic route to these versatile intermediates and valued target pharmacophores. Among the most popular approaches is the oxidation-state neutral conversion of carboxylic acids to nitriles via dehydration of intermediate primary amides (Figure 1) either using traditional dehydrating reagents8 or recently developed metal-mediated procedures.9 An efficient complement to amide dehydration is the oxidative transformation of aldehydes to nitriles. Isolation of aldoxime intermediates (Figure 1) generally is followed by activation of the oxime hydroxyl group (e.g., as a sulfonate ester derivative) and then its elimination to afford the nitrile.10 The appeal of this approach has led to several one-pot methods for direct synthesis of nitriles from aldehydes using either hydroxylamine or ammonia in combination with a variety of activating reagents.10

Unfortunately, the accompanying reagents for these one-pot approaches, such as CaCl2/N2O4/MeOH/O2,11 Pb(OAc)4,12 OsO4,13 H2O2,14 I2,15 NBS,15 IBX,16 and NaI,17 often are not tolerant of other functional groups or require somewhat harsh conditions to effect the transformation. Ideally, an aldehyde-selective reagent that would facilitate the conversion to the nitrile under neutral conditions would greatly expand the utility of this direct approach. We report here the use of O-(diphenylphosphoryl)hydroxylamine (DPPH) as such a reagent.

Our interest in aminoxy chemistry led us to consider the use of DPPH (Ph2P(O)ONH2) as a possible chemoselective alternative to hydroxylamine or ammonia for introduction of nitrogen onto the carbonyl carbon of aldehydes. Since the reaction of this reagent with an aldehyde would directly form an activated oxime ester as an intermediate (e.g., 1, Scheme 1), we reasoned that it should be possible to thermally induce an electrocyclic rearrangement resulting in the elimination of diphenylphosphonic acid. A similar mechanism involving elimination of methanesulfonic acid has been proposed for formation of nitriles from intermediate sulfonlated aldoximes.18 Although DPPH is well appreciated as an electrophilic reagent for the annihilation of a variety of nucleophiles, including Grignard reagents,19 enoles,20 or for annihilation of tertiary amines in syntheses of aziridines from α,β-unsaturated carbonyl substrates,21 as use as a nucleophilic counterpart, especially in chemoselective “click” transformations, has received limited attention.22 To test the action of DPPH as a suitable reagent for oxime ester formation as well as the subsequent elimination to the nitrile, we examined the reaction between DPPH and α-naphthaldehyde (Scheme 1).

While presently not commercially available, DPPH can be readily prepared in good yield in one step from commercially available diphenylphosphinic chloride and hydroxylamine hydrochloride.23 Reaction of DPPH with naphthaldehyde in THF gave oxime ester 1 in 79% yield. Subsequent heating in toluene revealed that the elimination of diphenylphosphonic acid from 1 required warming to above 80 °C to achieve a significant rate of formation of naphthonitrile 2. Of particular note is that no

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Published: August 28, 2012
Profilng thiol metabolites and quantification of cellular glutathione using FT-ICR-MS spectrometry

Sadakatafi S. Gori · Pawel Lorkiewicz · Daniel S. Ehringer · Alex C. Belshoff · Richard M. Higashi · Teresa W.-M. Fan · Michael H. Nantz

Received: 8 January 2014 / Revised: 31 March 2014 / Accepted: 3 April 2014 / Published online: 25 May 2014
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Abstract We describe preparation and use of the quaternary ammonium-based α-iodoacetamide QDE and its isotopologue *QDE as reagents for chemoselective derivatization of cellular thiols. Direct addition of the reagents to live cells followed by adduct extraction into n-butanol and analysis by FT-ICR-MS provided a registry of matched isotope peaks from which molecular formulae of thiol metabolites were derived. Acidification to pH 4 during cell lysis and adduct formation further improves the chemoselectivity for thiol derivatization. Examination of A549 human lung adenocarcinoma cells using this approach revealed cysteine, cysteinylglycine, glutathione, and homocysteine as principal thiol metabolites as well as the sulfenic acid hypotaurine. The method is also readily applied to quantify the thiol metabolites, as demonstrated here by the quantification of both glutathione and glutathione disulfide in A549 cells at concentrations of 34.4 ± 11.5 and 10.1 ± 4.0 mmol/mg protein, respectively.

Keywords Chemoselective · Metabolomics · Iodoacetamide · Hypotaurine · Oxidative stress · Cysteine

Introduction

Cellular thiol-containing metabolites, such as cysteine and glutathione, are crucially important for maintaining and regulating redox homeostasis in addition to protein functions [1, 2]. For example, the reduced form of cellular glutathione (GSH) is oxidized to its dimer glutathione disulfide (GSSG) in response to oxidative stress as a means to minimize cellular damage from reactive oxygen species [3, 4]. Since many diseases are linked to high levels of oxidative stress, the intracellular levels of GSH and GSSG can be indicative of cell pathogenesis [5–7]. Indeed, studying the ratio of GSH to GSSG and quantifying the dysregulation of GSH and GSSG levels are keys to understanding oxidative stress and the etiology of many important human diseases including cancer and type 2 diabetes [8–10]. To profile cellular thiols, chemoselective (CS) reagents for reaction with the sulphydryl (–SH) moiety have been developed [11, 12]. These CS approaches can enable faster as well as more sensitive and selective analyses of thiol metabolites directly in crude extracts, and this, in turn, can improve the understanding of metabolite roles in human disease development.

Several methods, such as fluorescent detection [13, 14], colorimetric assays [15–17], capillary electrophoresis [18], electrochemical detection [19], SALDI-MS [20], and chromatographic methods coupled with mass spectrometry (GC-MS [21], LC-MS [22]) have been reported for the quantification of glutathione. The reported colorimetric and fluorometric probes are all designed to covalently modify (i.e., tag) glutathione for detection and quantification, and these assays also are useful for quantification of known metabolites using reference standards. However, they are unsuited for quantifying
Isolation of C5-Sugars from the Hemicellulose-Rich Hydrolyzate of Distillers Dried Grains

Sadakatuli S. Giri,‡ Mandapati V. Ramakrishnam Raju,§ Dania A. Fonseca,∥ Jagannadh Satyavolu,‡ Christopher T. Burns,† and Michael H. Nantz*†

‡Department of Chemistry, 2320 South Brook Street, University of Louisville, Louisville, Kentucky 40292, United States
§Conn Center for Renewable Energy Research, University of Louisville, Louisville, Kentucky 40292, United States

Supporting Information

ABSTRACT: A three-stage process for isolation and separation of C5-sugars in dry form from the hydrolyzate of distillers dried grains (DDG) is described. The salient features include extraction of bis(boronic ester) adducts of xylose and arabinose into toluene on treatment of neutralized hydrolyzate with phenylboronic acid (PBA) and subsequent addition of propylene glycol to the organic phase to induce sugar precipitation for ready collection. The PBA used in the process is largely reclaimed on hydrolysis of the propylene glycol boronic ester formed during the process. A preparative scale example afforded 48% of the xylose content in DDG as a crystalline solid also containing an additional 11% of the arabinose content.

KEYWORDS: Boronate, Transesterification, Xylose, Arabinose, Extraction, Precipitation, Dioxane

INTRODUCTION

The depletion of fossil fuels coupled with increasing global energy demands has prompted searches for sustainable sources of energy. One widely investigated renewable energy source is lignocellulosic biomass.1,2 Because lignocellulosic biomass is renewable, inexpensive, and abundant, economization of processes for its conversion to biofuels is postulated to be one possible solution to the ever-increasing demand for energy.3 Its degradation by hydrolysis of the interlinked glycosidic bonds affords solutions of monomeric saccharides (e.g., glucose, xylose, arabinose). Several hydrolytic techniques, such as treatment with acid,4 steam explosion,5 or various biological, enzyme-mediated conversion processes,6 have been extensively studied to obtain hydrolyzates rich in monosaccharides. Subsequent conversion to value-added chemicals7 or to biofuels by fermentation8 constitute principal next-steps for processing the hydrolyzates.

The sugar concentrations in hydrolyzates are typically lower than desired for downstream processing steps; consequently, hydrolyzate concentration is often required.9 These concentration steps can deteriorate the sugars. Many of the sugar degradation compounds are toxic to the fermentation process and severely limit yields and effectiveness of overall processes.10 Techniques, such as overliming,11 addition of nutrients to offset toxic inhibition12 and ion-exchange,13 have been employed to overcome these issues. However, several of these methods become the source of even more byproducts.14 Similar problems are faced when using enzymatic techniques for biofuel conversion.

One hydrolyzate treatment approach developed in the past decade relies on the chemical affinity of sugars toward boronic acids to form reversible boronic esters.15 Lipophilic boronic acids selectively extract sugars from the hydrolyzate into an organic phase and then release them in a "clean" aqueous phase prior to subsequent fermentation or other enzymatic processes.16 This approach employs a reactive solvent extraction method wherein lipophilic boronic acids in organic solution are stirred with basic aqueous hydrolyzate solution. The aqueous base causes interphase deprotonation of the boronic acids across the immiscible layers. The ionized boronic acids then form boronate complexes with cis-diol moieties of sugars. Extraction into organic phase can be promoted by ion pairing with lipophilic quarternary ammonium cations.17,18 The resulting salts are readily hydrolyzed in a clean, aqueous acidic solution to regenerate the sugars, which are then employed for either of the techniques mentioned above. Extraction capabilities and kinetics of these processes have been well studied and documented in recent years.16,19,20 Although these processes do extract sugars from hydrolyzates, their utility is chemically limited because the sugars are obtained as aqueous solutions. Indeed, a method that would deliver dry sugars can

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CURRICULUM VITAE

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RESEARCH EXPERIENCE

University of Louisville, Louisville, KY, USA 2011–2016
Department of Chemistry

Doctoral Candidate (with Prof. Michael Nantz)

• Established a protocol for high-throughput analysis of thiol metabolites in cancer cells using a novel chemoselective reagent and FT-ICR mass spectrometry. This protocol is currently being used to identify and quantify thiols in human tissue samples at University of Kentucky.

• Led the development of a sustainable method to isolate pentose sugars from biomass. This process allows isolation of crystalline pentoses at ambient conditions while recycling the reagents used. The method is now being scaled up at Conn Center, Louisville to provide pentoses for further biofuel research.

• Developed a facile, one-pot transformation of aldehydes to nitriles using an aminooxy reagent with a colleague. The reagent was found to be robust and easy to use across a wide spectrum of aldehydes.

Evonik Industries AG, Hanau, Germany July–Dec 2014
Innovation Management: Process, Research & Development

Research Associate - Intern

• Lead a team of chemists to establish and optimize multistep synthesis of a drug intermediate (undisclosed molecule) with specific substitution pattern. These synthetic procedures were employed to fulfill an order of 150 kg of the intermediate. Conducted independent literature search, supervised research/analyses and reported results to the director.

• Optimized heterogeneous Suzuki coupling for synthesis of an API-intermediate using Design of Experiments (DOE) technique. A 50 gm representative sample was synthesized after screening eight catalysts in four solvents with five bases at two different temperatures.
National Institute of Pharmaceutical Education and Research, India 2009–2010
Department of Natural Products
Technical Assistant

- Supervised synthetic/semi-synthetic reactions for preparation of natural product analogues and their analyses using HPLC, GC and MS. Responsible for oversight and maintenance of Natural Products synthesis and analytical labs.
- Developed and optimized bio-activity guided fractionation of plant extracts using extractions and chromatography for pharmacology and toxicology labs. The extracts were evaluated for their pharmacological and pharmacokinetic activities.

Unimark Remedies Ltd., Vapi, Gujarat, India 2008–2009
Quality Control
Chemist

- Characterized active pharmaceutical ingredients and intermediates using wet analysis and analytical instruments such as HPLC, MS, FTIR, UV, polarimeter, Karl Fisher, etc.
- Specialized in method development and method validation of intermediates and final products of Lisinopril, Montelukast Sodium, Albendazole, Ramipril, Ganciclovir, etc.

Publications

(4) SS Gori, M Mashuta and MH Nantz
Synthesis of a Bicyclic Boron Fluoride Complex With a Bridged Oxygen.
*Manuscript in Preparation.*

(3) SS Gori, MVR Raju, DA Fonseca, J Satyavolu, CT Burns and MH Nantz
Isolation of C5-Sugars from the Hemicellulose-Rich Hydrolyzate of Distillers Dried Grains
*ACS Sustainable Chem. Eng.* 2015, 3, 2452–2457

(2) SS Gori, P Lorkiewicz, DS Ehringer, AC Belshoff, RM Higashi, TW-M Fan and MH Nantz
Profiling Thiol Metabolites and Quantification of Cellular Glutathione using FT-ICR-MS Spectrometry

(1) S Laulhé, SS Gori and MH Nantz
A Chemoselective, One-Pot Transformation of Aldehydes to Nitriles

Patent

Presentations

(8) **SS Gori**, P Lorkiewicz, DS Ehringer, AC Belshoff, RM Higashi, TW-M Fan and MH Nantz

Metabolomic Analysis of Thiols Using Chemoselective Derivatization Approach

**Oral**, Institute For Molecular Diversity and Drug Design (IMD) Fall Fest, Louisville, KY, US

10 Nov, 2015

(7) **SS Gori**, MVR Raju, DA Fonseca, J Satyavolu, CT Burns and MH Nantz

Isolation of C5 Sugars from Distillers Dried Grains Hydrolyzate Using Sustainable Processes

**Poster**, WasteStock Challenge 2015, Louisville, KY, USA


(6) **SS Gori**, P Lorkiewicz, DS Ehringer, AC Belshoff, RM Higashi, TW-M Fan and MH Nantz

Chemoselective Analysis of Thiol Metabolites in A549 Cells Using Mass Spectrometry

**Poster**, 2015 RC-SIRM Symposium and Workshop, Univ. of Kentucky, Lexington, KY, USA

26 Jul, 2015

(5) **SS Gori**, MVR Raju, DA Fonseca, J Satyavolu, CT Burns and MH Nantz

D-Xylose Isolation from the Hemicellulose-Rich Hydrolyzate of Dried Distillers Grains

**Poster**, 2015–Renewable Energy & Energy Efficiency Workshop, Louisville, KY, USA

22–24 Mar, 2015

(4) **SS Gori**, P Lorkiewicz, DS Ehringer, AC Belshoff, RM Higashi, TW-M Fan and MH Nantz

Profiling Thiol Metabolites and Quantification of Cellular Glutathione using FT-ICR-MS

**Poster**, Metabolomics 2015, 11th Annual Intl. Conf. of the Metabolomics Soc., Burlingame, CA, USA

29 Jun–2 Jul, 2015

(3) **SS Gori**, P Lorkiewicz, DS Ehringer, AC Belshoff, RM Higashi, TW-M Fan and MH Nantz

Profiling Thiol Metabolites and Quantification of Cellular Glutathione using FT-ICR-MS

**Oral**, Mass Spectrometry: Applications to Clinical Lab 2014 EU (MSACL 2014 EU), The First Annual European Congress, Salzburg Congress Center, Austria

2–5 Sep, 2014

(2) **SS Gori**, M Rajani and S Anandjiwala

HPTLC Densitometric Quantification of Berberine, Curcumin, Piperine, Gallic Acid and Ellagic Acid from *Eladya Modaka*

**Oral**, National Symposium on Medicinal Plants, Surat, Gujarat, India

9 Jun, 2010

(1) **SS Gori**

Green Chemistry

**Oral**, University Grants Commission State Level Seminar, Kadi, Gujarat, India

27 Feb, 2008
Invited Talks

(2) Protecting Groups and Utility
University of Louisville – Dept. of Chemistry, KY, USA
19 Jan 2016

(1) Chemoselective Reagents for Metabolite Identification and Analysis
Berea College, 101 Chestnut St., Berea, KY, USA
9 Jul, 2015

EDUCATION

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FELLOWSHIPS AND AWARDS

• Doctoral Dissertation Completion Award, School of Interdisciplinary and Graduate Studies, University of Louisville, 2015
• Institute for Molecular Diversity and Drug Design (IMD³) – Travel Award, University of Louisville, 2015
• Deutscher Akademischer Austauschdienst (DAAD–RISE) Scholarship, Bonn, 2014
• Young Investigator Award, MSACL EU, Salzburg, 2014
• Graduate Research Assistantship, University of Louisville, 2013
• Graduate Scholarship Award, Maa Foundation, 2008
• Undergraduate Research Scholarship, Sardar Patel University, 2005–2006
TEACHING EXPERIENCE

University of Louisville, Department of Chemistry 2011–2015

• Lab: Organic Chemistry Lab I & II (CHEM 343 & 344) – Fall 2015, Spring 2014, Spring 2012, Fall 2011

• Lab: Synthesis and Analysis I (CHEM 528) – Spring 2015

• Lab: Separations and Spectroscopy (CHEM 527) – Fall 2012

• Recitation: General Chemistry (CHEM 201) – Spring 2011

National Institute of Pharmaceutical Education and Research, Dept. of Natural Products 2009–2010

• Chromatographic Separations Lab (NP 510)

• Oversight, operation and handling of HPTLC, HPLC and MS

• Supervise MS pharmacy students in synthetic labs and dissertation work

COMPUTER SKILLS

• Operating Systems: Mac OS, Microsoft Windows

• Applications: MS Office, ChemBioDraw, Xcalibur, MODDE, MestReNova, Photoshop, etc.

• Databases and Resource Websites: SciFinder, Reaxys, ChemSpider, SDBS, KEGG, HMDB, etc.

• Nantz Lab Webmaster 2013–2016

PERSONAL DETAILS

• Date of Birth: 15th January 1985

• Gender: Male

• Marital Status: Married

• Nationality: Indian

• Languages (fluent): English, Hindi, Gujarati
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