20th Annual Biocatalysis and Bioprocessing Conference

“Current Topics in Industrial Biotechnology”

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October 17-18, 2011
20th Annual
Biocatalysis and Bioprocessing Conference

“Current Topics in Industrial Biotechnology”

Sponsored by:

The University of Iowa
Center for Biocatalysis and Bioprocessing

October 17-18, 2011

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Mr. Mitchell Rotman

Director
Dr. Mani Subramanian
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# 20th Annual Center for Biocatalysis and Bioprocessing Conference

“Current Topics in Industrial Biotechnology”

Iowa Memorial Union, Iowa City, IA

## Monday, October 17, 2011

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<td>4:00 pm</td>
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<td>4:00–6:00</td>
<td>Poster set up – Main Lounge, 1st floor IMU</td>
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<td>4:30</td>
<td>Tour of CBB – please register for tour on-line</td>
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<td>6:00–7:00</td>
<td>Welcome Dinner/Buffet – Main Lounge, 1st floor IMU</td>
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<td>7:00–8:00</td>
<td><strong>Ganesh Kishore, Ph.D., CEO, Burrill &amp; Company</strong>&lt;br&gt;“The Convergence of Green and White Biotechnology”</td>
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## Tuesday, October 18, 2011

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<tr>
<td>7:00–8:00 am</td>
<td>Registration – outside Main Lounge, 1st floor IMU</td>
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<td>7:30</td>
<td>Continental Breakfast – Main Lounge, 1st floor IMU</td>
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<td>8:30</td>
<td><strong>Program</strong> – Bijou, 1st floor IMU</td>
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<td>8:30</td>
<td><strong>Introduction and Welcome</strong></td>
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<td>9:00</td>
<td><strong>Mani Subramanian, Ph.D., DEO, Center for Biocatalysis and Bioprocessing</strong>&lt;br&gt;Professor, Department of Chemical and Biochemical Engineering, The University of Iowa</td>
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<td>9:00</td>
<td><strong>Simon Charnock, Ph.D., Manager &amp; Technical Director, Prozomix Ltd.</strong>&lt;br&gt;“Perpetual Impact of Genomics on Biocatalyst Discovery and Development”</td>
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<td>9:45</td>
<td><strong>Tom Beardslee, Ph.D., Senior Director, Renewable Chemicals, Verdezyne, Inc.</strong>&lt;br&gt;“Renewable Nylon: Engineering Yeast for the Production of Adipic Acid”</td>
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<td>10:30</td>
<td><strong>Break</strong> – Main Lounge, 1st floor IMU</td>
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<td>10:45</td>
<td><strong>Charles Brenner, Ph.D., Professor, Department of Biochemistry, The University of Iowa</strong>&lt;br&gt;“New Steps in NAD Metabolism: Biology, Biotechnology and Potentials for Further Development”</td>
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<td>11:30</td>
<td><strong>Lunch</strong> – Main Lounge, 1st floor IMU</td>
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Afternoon Session – Bijou, 1st floor IMU

1:15 pm  Yoshimi Kikuchi, Ph.D., Associate General Manager, Ajinomoto  
"Recombinant Protein Expression System of Corynebacterium"

2:00–3:00  CBB/NIH Fellow Presentations  
Four 10 minute talks plus 5 minutes for questions by selected graduate students

Cynthia Darnell, Kirby Laboratory, Department of Microbiology, The University of Iowa  
"Multicellular Development in Myxococcus Xanthus Requires the Putative Fatty Acid Desaturase Des7"

Mark Ericson, Rice Laboratory, Division of Medicinal and Natural Products Chemistry, The University of Iowa  
"The Solid Phase Synthesis of Reducible Polypeptides"

Alexander Lodge, Quinn and MacGillivray Laboratories, Department of Chemistry, The University of Iowa  
"Kinetic Evaluation of Dual Binding Human Acetylcholinesterase Inhibitors"

Heidi Schwanz, Kerns Laboratory, Division of Medicinal and Natural Products Chemistry, The University of Iowa  
"Differential Binding of DNA with Fluoroquinolones Affects the Ability of the Fluoroquinolone to Kill Microorganisms"

3:00–5:00  Poster Session – Main Lounge, 1st floor IMU  
Wine/hors d’oeuvres  
Announcement of the Usha Best Poster Prize Award winner

5:00  Adjourn
LIST OF ORAL PRESENTATIONS

1. THE CONVERGENCE OF GREEN AND WHITE BIOTECHNOLOGY
   Ganesh M Kishore, Ph.D.
   CEO, Burrill & Company, San Francisco, CA 94111

2. PERPETUAL IMPACT OF GENOMICS ON BIOCATALYST
   DISCOVERY AND DEVELOPMENT
   Simon Charnock, Ph.D.
   Managing & Technical Director, Pozomoix Limited, Haltwhistle,
   Northumberland, NE 49 9HN UK

3. RENEWABLE NYLON: ENGINEERING YEAST FOR THE
   PRODUCTION OF ADIPIC ACID
   Thomas A. Beardslee, Ph.D.
   Senior Director, Renewable Chemicals, Verdezyne, Inc., Carlsbad, CA 92010

4. NEW STEPS IN NAD METABOLISM: BIOLOGY,
   BIOTECHNOLOGY AND POTENTIALS FOR FURTHER
   DEVELOPMENT
   Charles Brenner, Ph.D.
   Professor, Biochemistry Department, The University of Iowa, Iowa City, IA
   52242

5. RECOMBINANT PROTEIN EXPRESSION SYSTEM OF
   CORYNEBACTERIUM
   Yoshimi Kikuchi, Ph.D.
   Associate General Manager, Institute for Innovation, Ajinomoto Co. Inc.,
   Kawasaki-ku, Kawasaki 210-8681 Japan

6. MULTICELLULAR DEVELOPMENT IN MYXOCOCCUS XANTHUS
   REQUIRES THE PUTATIVE FATTY ACID DESATURASE DES7
   Cynthia Darnell
   Department of Microbiology, Roy J. and Lucille A. Carver College of
   Medicine, The University of Iowa, Iowa City, IA 52242

7. THE SOLID PHASE SYNTHESIS OF REDUCIBLE POLYPEPTIDES
   Mark D. Ericson
   Division of Medicinal and Natural Products Chemistry, College of Pharmacy,
   The University of Iowa, Iowa City, IA 52242
8. KINETIC EVALUATION OF DUAL BINDING HUMAN ACETYLCHOLINESTERASE INHIBITORS
Alexander M. Lodge
Department of Chemistry, College of Liberal Arts and Sciences, The University of Iowa, Iowa City, IA 52242

9. DIFFERENTIAL BINDING OF DNA WITH FLUOROQUINOLONES AFFECTS THE ABILITY OF THE FLUOROQUINOLONE TO KILL MICROORGANISMS
Heidi A. Schwanz
Division of Medicinal and Natural Products Chemistry, College of Pharmacy, The University of Iowa, Iowa City, IA 52242
Abstracts of Oral Presentations
Since the dawn of agriculture, humans have largely depended upon agricultural resources for a vast array of their existential needs. While food and feed have been at the very core of societal needs, fuel, fiber and medicinal needs of humans were also met by agriculture and plant resources for a very long time. The discovery of abundant energy resources in the form of coal, natural gas and petroleum ushered an agriculture independent energy sector and significant fuel and fiber needs are now being met by these non-renewable resources. This migration was accelerated by the inconsistency of agricultural production, concerns about meeting human food needs and the high energy density of fossil energy. Recent concerns about energy security, greenhouse gas concentrations in our environment and overdependence on non-renewable resources - especially the notion that these are finite, have invigorated fresh thinking and leveraging plant and agricultural resources for energy as well as material production. Biorenewables is now on the agenda of policy makers of multiple nations at this time.

The biochemical machinery of plants has vast potential to harvest the abundant yet dilute solar energy and transforming that energy into chemical energy. There is global recognition that solar energy is one the most viable of the energy options. Unlike the human designed solar cells that transform solar energy to electricity, carbohydrates, the products of biological solar harvesting that capture carbon dioxide, can be transformed to a diverse array of bio-renewables. Carbohydrates and other metabolic products have significant potential for not only for addressing human caloric and nutritional needs but also fiber and fuel needs. For this potential to be transformed into reality, it is important that reliability and productivity of agriculture are both substantially improved. Significant opportunities exist to improve every crop cultivated today as well as, new crops on the horizon; both breeding and biotechnology based crop improvement can play a major role in realizing these improvements. Thus Green Biotechnology is focused on improving productivity, consistency and quality of the photosynthesis metabolic products across diverse production systems.

White biotechnology has emerged as an important aspect of biotechnology over the last thirty years. Initially directed towards production of specialty chemicals – both complex organic molecules and bio molecules, this technology is now being directed towards the production of commodity and bulk chemicals. While earlier biocatalysts used glucose and/or sucrose as the feedstock, newer biocatalysts are capable of using a diverse array of bio molecules as substrates. The products of white biotechnology have applications in fuel, fiber, nutrition and pharmaceutical industries. For white biotechnology to become a big industry, it is important that the issue of feedstock is addressed. While the option of using photosynthetic microbes to bypass agriculture exists, the reality is that we are several years away from making this an economic reality and for the foreseeable future, agriculture based feedstocks are the more likely substrates for the white biotech industry.

Deployment of green biotech has been significantly hampered by an uncertain regulatory and public acceptance climate. Three decades of experience in discovering, developing and deploying Green Biotech products have illustrated that much of the concern expressed about this technology is neither about technology nor its safety but much more rooted in business and control issues. It is important that these issues are addressed with a sense of urgency if we have to manage the rising cost of food and feed. While one could argue whether using food and feed products for fuel and industrial purposes is ethical, one must also realize that under deployment of technology especially safe products of technology that have potential to increase production and productivity of agriculture on a global basis is at least in part, responsible for the lack of supply of adequate grains and other products. When coupled with issues of climate change, tribal warfare, lack of storage and infrastructure for grain movement – it is clear that much of food security and access is a “man made” issue and solutions are already available to address concerns regarding food vs. non food uses of grains and land for cultivation of the same.
PERPETUAL IMPACT OF GENOMICS ON BIOCATALYST DISCOVERY AND DEVELOPMENT

Simon Charnock, Ph.D.
Prozomix Limited, Station Court, Haltwhistle, Northumberland, NE49 9HN UK

The impact of Genomics on biocatalysis began with the sequencing of the first whole bacterial genome, that of Haemophilus influenza, in mid1995. The significance of this was not that this organism was found to be particularly rich in useful enzymes, but because this achievement heralded the start of an explosion in DNA sequence information that has since resulted in the nucleotide database doubling in size approximately every 18 months. Keeping pace with this exponential expansion of genomic data has been the development of ever more rapid, cost-effective and sophisticated molecular biology techniques, enabling greater exploitation of the ever increasing number of putative enzyme encoding genes. An acute need to compile and annotate the expanding database, along with an increasing desire to mine the new data, coincided with greater computer processing and storage capacity, leading to the rapid development of the powerful Bioinformatics applications in use today.

Historical dependence on laborious practical methods such as the preparation and screening of gene libraries for new recombinant biocatalyst development quickly diminished as putative enzyme encoding genes became increasingly easy to simply “cherry-pick” from the expanding nucleotide database by way of multiple sequence alignment algorithms such as BLAST. A significant increase in the number of biocatalysts studied academically and also exploited commercially thus resulted. A notable advantage of the recombinant biocatalysis enzyme technology over traditional microbial screening was that a simple standard expression system such as Escherichia coli could be employed, which was both easy to establish and scale in most biological laboratories. In addition, advances in enzyme evolution techniques such as DNA shuffling, error-prone PCR and site-directed mutagenesis enabled the biochemical and biophysical characteristics of essentially any recombinant enzyme discovered to be optimized towards a specific application(s), with increasing ease and reliability.

In today’s “Post-Genomics Era” with approximately 2000 completed genomes to mine, and falling gene synthesis prices enabling increased exploitation of both rapidly expanding metagenomic data and intron-containing genes, there is currently a developing debate with respect to what will become the most effective / practical approach for the discovery and development of new biocatalysts; (1) to screen a relatively small panel of well studied enzymes exhibiting biophysical / biochemical characteristics proven amenable historically to biocatalysis application, and then use enzyme evolution methods to perfect the desired final biocatalyst out of the best hit, or (2) to screen a significantly larger panel of putative biocatalysts rationally selected from the database with the expectation of either finding a suitable candidate in the first instance, or at least finding a significantly “better” starting enzyme for subsequent more efficient evolution experiments.

This question is certainly very situational at the moment, depending largely on the class of enzyme in question, desired time-scales and budget. However, the recent proliferation of next generation DNA sequencing services brings with it the promise of a second renaissance with respect to the impact genomic data has on biocatalysis. Indeed, a step-change is guaranteed over the coming 1-3 years, resulting from a precipitous fall in sequencing costs in conjunction with the sheer quantity of new genomic data generated. Among others, likely developments will feature the renewed utility of microbial screening (now with associated genome sequencing), and significantly larger off-the-shelf biocatalyst panels, with advanced enzyme optimization services being widely offered commercially.
RENEWABLE NYLON: ENGINEERING YEAST FOR THE PRODUCTION OF ADIPIC ACID

Thomas A. Beardslee, Michael Walbridge, Jian Yi and Stephen Picataggio*
Verdezyne, Inc., Carlsbad, CA 92010

Adipic acid is an important industrial chemical used to make Nylon 6,6 and polyurethane resins for consumer products ranging from fibers for carpets and apparel, to shoes and engineering plastics. With an estimated global market of 5 billion pounds per year, it is currently produced from petrochemical sources by nitric acid catalyzed oxidation of cyclohexane. The production of adipic acid from renewable sources would allow the production of completely bio-based nylons and polyurethanes. Towards this goal, we have been engineering the diploid yeast *Candida tropicalis* to produce bio-based adipic acid from renewable feedstocks. This yeast can normally utilize alkanes or fatty acids as the sole carbon source for growth via cyclic degradation through the \( \beta \)-oxidation pathway. However when \( \beta \)-oxidation is blocked, *C. tropicalis* can convert these substrates at high yield and selectivity to the corresponding dicarboxylic acids via the \( \beta \)-oxidation pathway. Thus, a completely \( \beta \)-oxidation blocked strain would convert the mixed fatty acids from plant-based oils to a mixed dicarboxylic product with the same chain-length distribution as the feedstock. We have engineered both the \( \beta \)-oxidation and \( \omega \)-oxidation pathways to enable selective production of adipic acid from any plant-based oil, regardless of its fatty acid composition.
NEW STEPS IN NAD METABOLISM: BIOLOGY, BIOTECHNOLOGY AND POTENTIALS FOR FURTHER DEVELOPMENT

Szu-Chieh Mei, Samuel Trammell, Allyson Mayer, Denise Lieuson and Charles Brenner*
Department of Biochemistry, Roy J. and Lucille A. Carver College of Medicine, The University of Iowa, Iowa City, IA 52242

The activity of nicotinamide adenine dinucleotide (NAD), the major co-enzyme for hydride transfer reactions in biology, was first measured by Arthur Harden in 1905 in his reconstitution of cell-free alcohol fermentation. Nicotinic acid and nicotinamide, collectively “niacin,” the pellagra-preventing NAD precursor vitamins, were discovered by Conrad Elvehjem in 1938. The enzymic basis for NAD biosynthesis from amino acids and from nicotinic acid were largely solved by 1958, based on major contributions from Arthur Kornberg, Jack Preiss, Philip Handler and others. After being a leading molecule in early 20th century biology, NAD seemed to retreat to the relative obscurity of a “housekeeping molecule,” thought to be too ubiquitous and abundant to serve regulatory functions. In 2000, the yeast silent information regulator 2 gene product, Sir2, was shown to deacetylate acetyllysine protein modifications with an absolute requirement for NAD. However, unlike NAD-dependent hydride transfer enzymes, Sir2 is an NAD-consuming enzyme, producing nicotinamide plus acetylated ADPribose, and thereby necessitating salvage biosynthesis. Other NAD-consuming enzymes are poly(ADPribose) polymerases, ADPribose transferases, cADPribose synthetases. The signaling functions mediated by these reactions are manifold. Among the many 21st century problems being addressed that interface with NAD metabolism are how calorie restriction modulates the activity of Sir2 in order to extend lifespan. Whereas two major research groups used the available information about NAD biosynthesis to predict how the ratios of key metabolites might be modulated by glucose availability to affect Sir2 function, our group considered the gene-enzyme assignments and pathway connectivity to be untested with respect to completeness. Since 2004, we have described and characterized 8 new genes and two metabolites involved in NAD biosynthesis. The most striking of the discoveries, that nicotinamide riboside is a previously unappreciated vitamin with unique properties that provide added value over niacin, will be discussed.
RECOMBINANT PROTEIN EXPRESSION SYSTEM OF CORYNEBACTERIUM

Yoshimi Kikuchi
Institute for Innovation, Ajinomoto Co., Inc., Japan

*Corynebacterium glutamicum* discovered in 1957 as a natural producer of glutamate is a gram-positive, non-sporulating bacterium with approximately 54% GC content of DNA. For several decades, this strain has been heavily used in industrial production of amino acids such as glutamate, lysine, and others, which is used in human food additive, animal feed additive, or pharmaceutical product. Compared to its ability to produce amino acids, little is known about protein production in *C. glutamicum*. However, we have successfully developed a novel recombinant protein expression system by using *C. glutamicum* [1-5], and named this protein expression system CORYNEX™.

The CORYNEX™ is a protein secretion system, and it has mainly three advantages compared with other protein secretion systems. First, *C. glutamicum* naturally little amount of the secreted protein in the culture medium. Therefore, the purity of the secreted-target protein in this strain is very high. Second, little degradation of the secreted-target protein occurs in the culture supernatant, since there are no major extracellular proteases in *C. glutamicum*. The third advantage is on correct folding of the secreted-target protein. A protein which has complex structure such as including disulfide bonds, homo-dimer, and hetero-dimer can be secreted in *C. glutamicum* as active form.

Furthermore, we identified the *C. glutamicum* Tat-pathway, which is a novel protein secretion pathway that differs from the general protein-secretion pathway well known as Sec-pathway. We demonstrated that a protein which could not be secreted in the Sec-pathway could be efficiently secreted in the *C. glutamicum* Tat pathway, and that this pathway would be capable of industrial protein production [6-8].

We has launched this protein expression service for customers who have had troubles on protein expression (www.corynex.com). As a result, the CORYNEX™ has succeeded to produce many proteins which have not been produced in many other protein production systems so far.

In this conference, we'd like to introduce a summary of the CORYNEX™ for recombinant protein production.

MULTICELLULAR DEVELOPMENT IN MYXOCOCCUS XANTHUS REQUIRES THE PUTATIVE FATTY ACID DESATURASE DES7

Cynthia Darnell and John Kirby*
Department of Microbiology, Roy J. and Lucille A. Carver College of Medicine, The University of Iowa, Iowa City, IA 52242

*Presenting author

Myxococcus xanthus is a gram-negative soil bacterium with a complex life cycle involving multiple multicellular behaviors. During vegetative growth, M. xanthus preys upon other microbes in the environment, assembling into an efficient and coordinated motility pattern termed rippling. This behavior allows M. xanthus to lyse prey cells, releasing macromolecular nutrients into the environment, and to maximize uptake of these molecules. When nutrients become scarce, M. xanthus begins a highly regulated developmental program resulting in large, highly ordered structures known as fruiting bodies. Within the fruiting bodies, a percentage of the cells convert to resistant spores; these spores germinate when nutrients become available to begin the cycle anew.

The ability to recognize both neighbor cells (self) and prey cells (non-self) is essential throughout this life cycle. While many bacteria have been shown to communicate using quorum sensing molecules such as homoserine lactones and small peptides, these highly diffusible molecules are less useful in a terrestrial environment. Instead, M. xanthus has the ability to recognize and respond to specific lipids. Recently, we have uncovered a chemosensory signal transduction system, Che7, which appears to post-translationally regulate the fatty acid desaturase Des7. Through genetic analysis, we have shown that Che7, and in particular Des7, is important for organization into fruiting bodies during development. We hypothesize Des7 is producing a specific lipid signal to properly aggregate and subsequently sporulate. Mutations in che7 genes result in improper aggregation. cheA7 and mcp7 strains form fruiting bodies prematurely and produce 10-fold more viable spores than the wildtype parent. The des7 strain is delayed in aggregation and produces less spores compared to the parent. This defect appears to be specific to starvation induced sporulation. Characterization of regulation of Des7 through the Che7 pathway will further our knowledge of intercellular communication and multicellular development.
THE SOLID PHASE SYNTHESIS OF REDUCIBLE POLYPEPTIDES

Mark D. Ericson and Kevin Rice*
Division of Medicinal and Natural Products Chemistry, College of Pharmacy,
The University of Iowa, Iowa City, IA 52242

Since the first dipeptide synthesized in 1901 by Fischer, peptides and proteins have been synthetic targets due to their myriad of biological properties. The advent of solid phase peptide synthesis in 1963 by Merrifield has allowed innumerable short length peptides and proteins to be synthesized on a solid support. The ease and automation of peptide synthesis has greatly simplified the investigation of biologically active peptides and proteins, leading to many important advances. Here we present our work in developing a new methodology of solid phase peptide synthesis. Unlike traditional peptide synthesis, where individual amino acids are sequentially added, we propose to add full length cysteine flanked peptides in each coupling cycle. A polypeptide synthesized in this manner would be composed of many peptide subunits individually constructed of amide bonds and subunits linked together through disulfide bonds. Such a monodisperse reducible polypeptide would have many applications in gene and drug delivery. For example, a gene therapy vector could be assembled as a homogeneous polymer with a DNA binding domain, a targeting ligand, a PEG stealthing portion, and a fusogenic peptide that readily disassembles upon cellular entry.

We first developed a synthetic strategy that could form a reducible polypeptide in solution. The selection of the terminal cysteine protecting group was critical, and a thiazolidine protected cysteine was found to be stable to purification and coupling conditions, while the deprotection conditions (aqueous methoxyamine-HCl) did not disrupt previously formed disulfide bonds. The conversion of the thiazolidine to free cysteine requires careful manipulation of the reaction conditions: suboptimal pH, concentration, peptide length, and reaction time result in the formation of peptide dimers or disulfide scrambling, where the liberated cysteine reacts internally with a previously formed disulfide bond and cleaves the polypeptide. Using optimized conditions, we have been able to synthesize many reducible polypeptides, though are limited in yields to about 20\% per coupling due to multiple purifications of the intermediates per cycle.

To overcome the low yields, we have begun exploring a solid phase approach. An activated cysteine flanked peptide can react with an immobilized peptide on resin, and all excess peptide and byproducts can be washed away. A simple cleavage step would then yield the desired polypeptide, improving yields and purity. While many water soluble resins are commercially available, we found the ethylene glycol backbone NovaPEG resin to achieve the highest coupling. Conjugation of a second peptide to the resin via a disulfide bond was found to be highly dependent on reaction volume and peptide equivalents, and can now be reliably coupled in excellent purity. The addition of a third peptide to the growing polypeptide has been observed, but in small yields due to the growing peptide cleaving itself from resin. We are currently exploring a variety of reaction conditions to overcome this autocleavage, so as to be able to make reducible polypeptides consisting of three or more peptide subunits.
KINETIC EVALUATION OF DUAL BINDING HUMAN ACETYLCHOLINESTERASE INHIBITORS

Alexander M. Lodge, Manza B. Atkinson, Elizabeth Elacqua, Daniel M. Quinn* and Leonard R. MacGillivray*
Department of Chemistry, College of Liberal Arts and Sciences, The University of Iowa, Iowa City, IA 52242

Synthesis and kinetic evaluation of dual binding acetylcholinesterase (AChE) inhibitors targets both the cholinergic and β-amyloid plaque pathways of Alzheimer’s disease (AD) treatment. Aryl-trifluoroketones, ladderane natural product derivatives, and paracylcophane moieties have been evaluated as potential human AChE inhibitors. Dose response assays, using the Ellman¹ method, of quinoline and N-methylquinolinium aryl-trifluoroketones showed IC50 values in the 10⁻⁹ M range. Additionally, both aryl-trifluoroketone moities were observed to be tight binding while only the N-methylquinolinium showed time dependent inhibition. Dose response assays of chiral and achiral tetrapyridyl-5-ladderane (TPL5) showed IC50 values in the 10⁻⁶ M range. Similarly, the tetrapyridyl-paracylcophane (TPPCyc) and N-methyl tetrapyridyl-paracylcophane (Me-TPPCyc) showed IC50 values in the 10⁻⁵ M and 10⁻⁶ M range respectively. Lineweaver-Burk analysis of Me-TPPCyc showed its mode of inhibition to be noncompetitive.
DIFFERENTIAL BINDING OF DNA WITH FLUOROQUINOLONES AFFECTS THE ABILITY OF THE FLUOROQUINOLONE TO KILL MICROORGANISMS

Heidi A. Schwanz and Robert J. Kerns* Division of Medicinal and Natural Products Chemistry, College of Pharmacy, The University of Iowa, Iowa City, IA 52242

Drug resistant bacteria are responsible for increasing incidences of Staphylococcus aureus, Pseudomonas aeruginosa, Streptococcus pneumonia, and Mycobacterium tuberculosis infections in humans. Fluoroquinolones (FQs), broad-spectrum bactericidal antibiotics, exert their effects by inhibiting type II topoisomerases through the formation of a ternary complex with the enzyme and DNA. Recently, it has been shown that newer, structurally unique FQs rapidly kill cells by promoting chromosomal fragmentation. Because the new FQs are able to rapidly kill in the presence and absence of protein synthesis, they have potential to be used in the treatment of microorganisms that go into a dormant state, as well as microorganisms that produce persister cells.

Previous studies with older generation FQs have demonstrated that the ability of a FQ to bind single-stranded (SS) and double-stranded (DS) DNA does not correlate with activity. Newer generation FQs have not been examined for DNA binding. Recent work suggests that there is a unique mechanism of action by which these newer FQs rapidly kill microorganisms, both in the presence and absence of the known protein synthesis inhibitor chloramphenicol. The goal of the studies presented here is to determine if FQs with distinct modes of killing bind different types of DNA in unique ways. Binding of FQs to SS, DS, and nicked DNA, all mimics of possible DNA types present in the active ternary complex, will be discussed. The ability of the different FQs to destabilize or stabilize DNA will also be presented, as we attempt to gain further insights into the unique mechanism of action observed with the newer FQs.
1. EFFECT OF A MUTANT WITH ALTERED DYNAMICS ON HYDRIDE TRANSFERS CATALYZED BY THYMIDYLATE SYNTHASE
   Thelma Abeysinghe, Zhen Wang and Amnon Kohen*
   Department of Chemistry, College of Liberal Arts and Sciences, The University of Iowa, Iowa City, IA 52242

2. ROLE OF HOMOLOG CuZnSOD IN VIRAL PROPAGATION
   Bhakti Bapat and David Murhammer*
   Department of Chemical and Biochemical Engineering, College of Engineering, The University of Iowa, Iowa City, IA 52242

3. MATERIALS-BASED APPROACH TO IMPLANT INFECTION MITIGATION
   Ann Baumhoer and Eric Nuxoll*
   Department of Chemical and Biochemical Engineering, College of Engineering, The University of Iowa, Iowa City, IA 52242

4. SYNTHESIS OF N-ARYLACYL O-SULFONATED AMINOGLYCOSIDES THAT BIND HEPARAN SULFATE BINDING PROTEINS
   Ioana Craciun, Amanda M. Fenner and Robert J. Kerns*
   Division of Medicinal and Natural Products Chemistry, College of Pharmacy, The University of Iowa, Iowa City, IA 52242

5. ENHANCING GENE DELIVERY THROUGH NUCLEAR TARGETED PLA2
   Samuel T. Crowley, Nick Baumhover and Kevin G. Rice*
   Division of Medicinal and Natural Products Chemistry, College of Pharmacy, The University of Iowa, Iowa City, IA 52242

6. MULTICELLULAR DEVELOPMENT IN MYXOCOCCUS XANTHUS REQUIRES THE PUTATIVE FATTY ACID DESATURASE DES7
   Cynthia Darnell and John Kirby*
   Department of Microbiology, Roy J. and Lucille A. Carver College of Medicine, The University of Iowa, Iowa City, IA 52242

7. BIOCATAYTIC PRODUCTION OF XYLITOL FROM CORN STOVER HEMICELLULOSE HYDROLYSATE AND PURIFICATION OF THE PRODUCT BY SMB CHROMATOGRAPHY
   Shuvendu Das¹, Michael Louie¹, Naveen Sudharsan², Anil Oroskar² and Mani Subramanian¹,*
   ¹Center for Biocatalysis and Bioprocessing, The University of Iowa Research Park, Coralville, IA 52241
   ²Orochem Technologies Inc., Lombard, IL 60148

8. EVALUATION OF qPCR PRIMERS TO DETECT METHANOTROPHS IN CONTAMINATED GROUNDWATER
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EFFECT OF A MUTANT WITH ALTERED DYNAMICS ON HYDRIDE TRANSFERS CATALYZED BY THYMIDYLATE SYNTHASE

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Thymidylate synthase (TSase EC 2.1.1.45) catalyzes the final step in the de novo biosynthesis of 2’-deoxythymidine 5’-monophosphate (dTMP), where the cofactor (6 R)-N^5, N^10-methylene-5,6,7,8-tetrahydrofolate (CH_2_H_4_F) serves both as the methylene donor and as the hydride (H^-) donor (from its C6 position). The essential role of TSase makes it a common target for chemotherapeutic and antibiotic drugs. Recent focuses on the mutants of highly conserved residue Tyr-209 (in ecTSase) have shown a key role of stabilizing the closed enzyme conformation.

According to the crystal structures of of wt ec TSase and its Y209W mutant at a 1.3 Å resolution, the structures are nearly identical. The distinct feature of Y209W is the anisotropic B factors of the phosphate-binding loop, which are not uniformly oriented as in the wt. To assess whether this dynamic effect altered on the hydride transfer step (the rate determining step at the physiological temperatures) of the Y209W TSase mutant, KIEs (kinetic Isotope effects) and other kinetic features were examined.

Competitive KIEs on the second- order rate constant (V/K) were measured over a temperature range of 5-35°C. The observed H/T (1/V/K_H) and D/T (1/V/K_D) KIEs were used to calculate the intrinsic KIEs throughout the temperature range. The outcome indicated that the hydride transfer was not altered significantly, but that other kinetic and dynamic steps were dramatically affected. While the Tyr-209 is involved mostly with binding of dUMP, Y209W has little effect on dUMP but allows for greater mobility of 5,6,7,8-tetrahydrofolate (THF) prior to the hydride transfer step. This finding emphasizes the importance of long range protein effects in catalysis.

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ROLE OF HOMOLOG CuZnSOD IN VIRAL PROPAGATION

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Baculoviruses are widely used with insect cells to express recombinant proteins. While the rate of recombinant protein expression can be very high in this system, the protein expression ceases within 2-3 days following baculovirus infection due to production of excess reactive oxygen species (O$_2^-$) that result in cell death. The baculovirus contains a Copper-Zinc Superoxide Dismutate (CuZnSOD) homolog gene that has a significant effect on the baculovirus infection process in insect cells. Specifically, the CuZnSOD homolog inactivates the CuZnSOD enzyme by removing the copper, thereby leading to increased oxidative stress and presumably more rapid cell death. Note that CuZnSOD and other SOD’s convert O$_2^-$ to H$_2$O$_2$, which is subsequently converted to H$_2$O by other enzymes. Cell longevity potentially can be extended by increasing cellular antioxidant defenses, e.g., by removing the CuZnSOD homolog. This presentation tests this idea through studies with a modified baculovirus in which the CuZnSOD homolog gene has been removed.
MATERIALS-BASED APPROACH TO IMPLANT INFECTION MITIGATION

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Bacterial biofilm infection is a common (~ 2 to 4%) complication for recipients of surgically implanted medical devices. Due to the tremendous chemical resistance of these biofilms, present treatment is a combination of antibiotics and replacement of the device, often requiring multiple surgeries. The objective of the current study is to determine the feasibility of using heat to deactivate biofilm as an alternative therapy. It is hypothesized that heat can be used to thermally eradicate the biofilm. In this study, Psuedomonas aeruginosa biofilm was cultured (37°C, 5% CO2 for 72 hours using glucose-enhanced media) and subjected to heat shocks. The live cell count per area was quantified for both control and heat shocked samples using confocal fluorescent microscopy. Decreased live cell count per area correlated to biofilm deactivation due to the heat shock and established the feasibility of thermal deactivation of infectious biofilms.

Keywords: Biofilm, thermal, resistance, confocal fluorescent microscopy, Pseudomonas aeruginosa
SYNTHESIS OF N-ARYLACYL O-SULFONATED AMINOGLYCOSIDES THAT BIND HEPARAN SULFATE BINDING PROTEINS

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Heparan sulfate is a cell surface glycosaminoglycan that plays a profound role in numerous physiological processes. HS chains are comprised of repeating disaccharide units with regions of high anionic content which selectively and/or nonselectively bind diverse proteins. Over 200 proteins have been identified to bind HS and the structurally similar glycosaminoglycan heparin. Over twenty therapeutic applications have been proposed for molecules to bind specific HS-binding proteins and block or modulate HS-mediated biological activities. In our work, synthetic modifications of glycosaminoglycans has been undertaken toward identifying molecules that selectively, if not specifically bind HS-binding site of individual HS-binding proteins.

Aminoglycosides are small, naturally occurring saccharides originally used as antibiotics. This class of compounds provides an untapped resource as a carbohydrate scaffold for designing new therapeutic agents. The hydroxyl and amino functional groups are synthetically useful handles for designing compound libraries, and the saccharides core provides structural support for positioning structural moieties to enhance binding contacts with specific proteins.

The Kerns lab has previously identified certain N-arylacylated and carboxamide derivatives of glycosaminoglycans that selectively bind HS-binding proteins. In the studies presented here, this work was furthered by synthesizing a panel of N-arylacyl O-sulfonated aminoglycosides to identify structural derivatives that selectively bind HS-binding proteins. Based on this approach, progress toward developing methods for the synthesis of multiple N-arylacyl O-sulfonated aminoglycosides to create a compound library will be described.
ENHANCING GENE DELIVERY THROUGH NUCLEAR TARGETED PLA2

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Gene therapy has been a goal of medicine for nearly 30 years. Current gene therapy research delivers DNA to tissues through the use of viruses, which have evolved their DNA delivery capabilities over millions of years. Viral gene delivery carries several risks, including immune response. These risks create an interest in nonviral gene delivery methods. However, nonviral techniques have long suffered from low efficiency in vivo compared to viral methods. It is believed that borrowing features from viruses may help to improve gene transfer efficiency. One such feature is phospholipase A2 activity seen in the capsid proteins of several viruses. Phospholipases hydrolyze ester bonds in phospholipids, which disrupts membrane bilayers. When the PLA2 activity is removed, the virus becomes far less infective, implying PLA2 activity is important for delivering the viral genome into the host cell.

It is hypothesized that PLA2 activity will increase transgene expression in vivo by creating pores in membrane bilayers that plasmid DNA can pass through. The effect of Bee venom phospholipase A2 on hydrodynamic gene delivery was tested in mice. PLA2 and plasmid DNA encoding firefly luciferase was injected into the tail vein of mice and luciferase activity was measured through bioluminescent imaging. PLA2 was modified with a nuclear localization sequence by treating the enzyme with 2-iminothiolane to create free thiols, which are then used to attach the NLS peptide through a maleimide linker. This directs the enzyme to the nucleus, where it would be more likely to create pores in the nuclear envelope, allowing plasmid DNA to enter and be expressed. This ability could allow for efficient nonviral DNA delivery and gene therapy for the treatment of hereditary disorders, cancers, and other medical issues.

In step 1, DNA and nuclear targeted PLA2 are injected into the cell. PLA2 then enters the nucleus in step 2. In step 3, PLA2 creates a pore in the nuclear envelope by hydrolyzing the phospholipids there. In step 4, the plasmid DNA enters the nucleus where it can be expressed.
MULTICELLULAR DEVELOPMENT IN MYXOCOCCUS XANTHUS REQUIRES THE PUTATIVE FATTY ACID DESATURASE DES7

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Myxococcus xanthus is a gram-negative soil bacterium with a complex life cycle involving multiple multicellular behaviors. During vegetative growth, *M. xanthus* preys upon other microbes in the environment, assembling into an efficient and coordinated motility pattern termed rippling. This behavior allows *M. xanthus* to lyse prey cells, releasing macromolecular nutrients into the environment, and to maximize uptake of these molecules. When nutrients become scarce, *M. xanthus* begins a highly regulated developmental program resulting in large, highly ordered structures known as fruiting bodies. Within the fruiting bodies, a percentage of the cells convert to resistant spores; these spores germinate when nutrients become available to begin the cycle anew.

The ability to recognize both neighbor cells (self) and prey cells (non-self) is essential throughout this life cycle. While many bacteria have been shown to communicate using quorum sensing molecules such as homoserine lactones and small peptides, these highly diffusible molecules are less useful in a terrestrial environment. Instead, *M. xanthus* has the ability to recognize and respond to specific lipids. Recently, we have uncovered a chemosensory signal transduction system, Che7, which appears to post-translationally regulate the fatty acid desaturase Des7. Through genetic analysis, we have shown that Che7, and in particular Des7, is important for organization into fruiting bodies during development. We hypothesize Des7 is producing a specific lipid signal to properly aggregate and subsequently sporulate. Mutations in *che7* genes result in improper aggregation. *cheA7* and *mcp7* strains form fruiting bodies prematurely and produce 10-fold more viable spores than the wildtype parent. The *des7* strain is delayed in aggregation and produces less spores compared to the parent. This defect appears to be specific to starvation induced sporulation. Characterization of regulation of Des7 through the Che7 pathway will further our knowledge of intercellular communication and multicellular development.
BIOCATAYTIC PRODUCTION OF XYLITOL FROM CORN STOVER HEMICELLULOSE HYDROLYSATE AND PURIFICATION OF THE PRODUCT BY SMB CHROMATOGRAPHY

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Xylitol is a five-carbon sugar alcohol that has numerous beneficial health properties. It has almost the same sweetness as sucrose, but with an energy value of 2.4 cal/g compared to the 4 cal/g of sucrose. Metabolism of xylitol is insulin-independent; thus it is an ideal sweetener for diabetics. It also prevents dental caries and ear infection in small children. Therefore, xylitol has found many applications in food products, oral and personal care, and animal nutrition (1, 2). In addition, U.S. Department of Energy has listed xylitol as one of the twelve biobased platform chemicals that can subsequently be used for the synthesis of other high-value chemicals and materials (3).

Xylose reductase from \textit{Pichia stipitis} (PsXR) was expressed in \textit{Pichia pastoris} GS115 (4) for this study. This recombinant organism, PG4000-4, was spray-dried to permeabilize the cells. Spray-dried PG4000-4 cells were used as biocatalyst for conversion of D-xylose in crude hemicellulose hydrolysate into xylitol. Three hundred fifteen mM of xylitol was produced from 414 mM of D-xylose in the hemicellulose hydrolysate within 1 hour, which represents a 76% conversion. Exogenous addition of expensive enzyme cofactors such as NAD(P)⁺/NAD(P)H or auxiliary substrates such as glucose and formate were not required. Xylitol from post reaction mixture was purified to 99.35% in a single step by using simulated moving bed chromatography (SMBC). The purified xylitol crystallized at 4°C from the purified concentrate.

References:
EVALUATION OF qPCR PRIMERS TO DETECT METHANOTROPHS IN CONTAMINATED GROUNDWATER

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The extensive use of tetrachloroethene (PCE) and trichloroethene (TCE) as cleaning solvents has resulted in widespread contamination of groundwater systems with vinyl chloride (VC). VC, a known human carcinogen, is primarily formed in groundwater via incomplete anaerobic reductive dechlorination of PCE and TCE. Aerobic, methane-degrading bacteria (methanotrophs), which are capable of VC cometabolism while growing on methane, could be important in natural attenuation of VC plumes that escape anaerobic treatment. Real-time PCR (qPCR) represents an innovative approach for detecting and quantifying the presence and activity of these VC-degrading microbes. This technique could provide lines of evidence for natural attenuation of VC and thus support existing anaerobic bioremediation technologies that generate VC as a metabolic intermediate.

In this work, we evaluated several PCR primer sets from the literature for use in methanotroph qPCR assays of groundwater samples. PCR primers targeting two functional genes involved in VC cometabolism, \textit{pmoA} (sub-unit of particulate methane monoxygenase (pMMO)) and \textit{mmoX} (sub-unit of soluble MMO (sMMO)), as well as 16S rRNA gene primers that targeted Bacteria, and Type I and Type II methanotrophs were tested. These assays were made quantitative by constructing standard curves with DNA from \textit{Methylococcus capsulatus} (Type I) and \textit{Methylocystis} sp. strain Rockwell (Type II). Primer sets were evaluated by comparing gene abundance estimated against known amounts of Type I and Type II methanotroph DNA. After primer validation, qPCR was used to investigate methanotroph populations in groundwater samples taken from VC-contaminated sites. Some samples studied were also subjected to 16S rRNA gene pyrosequencing, allowing for relative abundance comparisons with qPCR analyses.

Following our primer assessment experiments, effective primer sets were used to estimate the relative abundance of methanotrophs in environmental samples by comparing the Type I and Type II primer estimates to those of the 16S universal primers. Methanotrophs in these groundwater samples ranged from 0.2% to 6.6% of the total bacterial population. Pyrosequencing analysis of the same samples showed methanotroph relative abundances that ranged from 1.7% to 54%. In groundwater samples where both DNA and RNA was extracted, the quantities of functional gene transcripts per gene copy was compared, revealing that the transcripts/gene ratio for both \textit{pmoA} and \textit{mmoX} was less than one, implying relatively low methanotroph activity.

Our data suggest: 1) these primers adequately detect methanotrophs and will be useful in bioremediation strategies; 2) the pyrosequencing analysis should be used in conjunction with other evaluation tools such as qPCR, and TRFLP; 3) methanotrophs at these VC-contaminated sites are present, but not actively expressing pMMO and sMMO genes above basal levels.
MICROGLIA AND A DOPAMINE-DERIVED NEUROTOXIN, 3,4-DIHYDROXYPHENYLACETALDEHYDE: TOXICITY, METABOLISM, ACTIVATION, AND IMPLICATIONS FOR PARKINSON’S DISEASE

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The cause of dopaminergic cell death in Parkinson’s Disease (PD) is unknown, but recent research demonstrates oxidative stress, inflammation, and the endogenous neurotoxin, 3,4-dihydroxyphenylacetaldehyde (DOPAL), are factors in PD pathogenesis. DOPAL is generated from dopamine (DA) by monoamine oxidase and oxidized to DOPAC, the acid metabolite, by aldehyde dehydrogenase (ALDH). DOPAL is highly toxic to dopaminergic cells and must be rapidly metabolized to avoid adverse effects. Interaction of DOPAL with non-neuronal cells (e.g., microglia), including metabolism, activation, and toxicity is unknown. Activated microglia, found in PD-affected areas of the brain, can damage dopaminergic cells through phagocytosis, ROS and proinflammatory cytokine production/release. The ability of DA, DOPAL and DOPAC to activate BV-2 microglia was shown in this work by TNF-α release, and DOPAL but not DA or DOPAC exhibited a significant activational response. Metabolism and toxicity of DA and DOPAL were determined for BV-2 cells, and it was found microglia metabolize DA to DOPAC via DOPAL. Altered metabolism of DOPAL was measured after treatment with malondialdehyde (MDA), an endogenous product of lipid peroxidation, known to inhibit ALDH activity. Also, MDA was found to be toxic to the BV-2 microglia. Aggregation of the PD-relevant protein, α-synuclein, by physiologically relevant levels of DOPAL was also demonstrated in this work. At physiologic levels, neither DA nor DOPAL was toxic to BV-2 cells; however, DOPAL-treated cells exhibited activation. DOPAL-mediated microglial activation as shown in this study represents a viable mechanism for inflammation and dopaminergic cell death seen in PD patients.
INVESTIGATING THE IMPACT OF METHYL – HYDROGEN EXCHANGE ON SUPRAMOLECULAR SOLID-STATE SELF-ASSEMBLY AND REACTIVITY: DIRECTED STEREOSELECTIVE PHOTOCYCLOADDITIONS OF TRISUBSTITUTED OLEFINIC ESTERS

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Supramolecular chemistry focuses on the design of molecular architectures by relying on the complementary recognition, and subsequent assembly, of well-defined subunits. The idea of molecular recognition stems from biological systems. By utilizing noncovalent forces to dictate structural and morphological properties, biological systems have been able to produce single desired products. In an effort to mimic nature, chemists have employed noncovalent interactions to direct supramolecular self-assembly. Specifically, small molecule ‘templates’ have been designed to influence the packing of olefin-containing molecules within the organic solid state, so as to facilitate a [2+2] photocycloaddition reaction. This approach has led to the construction of various pyridine-functionalized frameworks with unique properties (e.g. fluorescence). To expand the applicability of our approach, we pursued the photoreaction of isoprene-like trisubstituted difunctional olefins. Trisubstituted olefins are the building blocks of several natural products, and are well-studied in the context of biosynthetic pathways. Trisubstituted olefin frameworks remain a large focus of solution-phase organic synthesis, wherein the inherent bioactivity of a compound can be rendered inactive with subtle changes in molecular structure (e.g. methyl – hydrogen or methyl – ethyl substitution). Whereas trisubstituted olefins are widely studied in biochemistry owing to their prevalence in bioactive compounds, these olefins remain mostly dormant in terms of deliberate installation of solid-state reactivity.

In this contribution, we will present a series of pyridine-based trisubstituted olefinic esters, and discuss the impact of the methyl group on the photoreactive properties of the series. We will also compare the solid-state reactivity properties of the trisubstituted olefins to the disubstituted analogues. Lastly, we will also present the difficulties encountered in achieving predictable supramolecular assemblies in more complex systems.
THE SOLID PHASE SYNTHESIS OF REDUCIBLE POLYPEPTIDES

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Since the first dipeptide synthesized in 1901 by Fischer, peptides and proteins have been synthetic targets due to their myriad of biological properties. The advent of solid phase peptide synthesis in 1963 by Merrifield has allowed innumerable short length peptides and proteins to be synthesized on a solid support. The ease and automation of peptide synthesis has greatly simplified the investigation of biologically active peptides and proteins, leading to many important advances. Here we present our work in developing a new methodology of solid phase peptide synthesis. Unlike traditional peptide synthesis, where individual amino acids are sequentially added, we propose to add full length cysteine flanked peptides in each coupling cycle. A polypeptide synthesized in this manner would be composed of many peptide subunits individually constructed of amide bonds and subunits linked together through disulfide bonds. Such a monodisperse reducible polypeptide would have many applications in gene and drug delivery. For example, a gene therapy vector could be assembled as a homogeneous polymer with a DNA binding domain, a targeting ligand, a PEG stealling portion, and a fusogenic peptide that readily disassembles upon cellular entry.

We first developed a synthetic strategy that could form a reducible polypeptide in solution. The selection of the terminal cysteine protecting group was critical, and a thiazolidine protected cysteine was found to be stable to purification and coupling conditions, while the deprotection conditions (aqueous methoxyamine-HCl) did not disrupt previously formed disulfide bonds. The conversion of the thiazolidine to free cysteine requires careful manipulation of the reaction conditions: suboptimal pH, concentration, peptide length, and reaction time result in the formation of peptide dimers or disulfide scrambling, where the liberated cysteine reacts internally with a previously formed disulfide bond and cleaves the polypeptide. Using optimized conditions, we have been able to synthesize many reducible polypeptides, though are limited in yields to about 20% per coupling due to multiple purifications of the intermediates per cycle.

To overcome the low yields, we have begun exploring a solid phase approach. An activated cysteine flanked peptide can react with an immobilized peptide on resin, and all excess peptide and byproducts can be washed away. A simple cleavage step would then yield the desired polypeptide, improving yields and purity. While many water soluble resins are commercially available, we found the ethylene glycol backbone NovaPEG resin to achieve the highest coupling. Conjugation of a second peptide to the resin via a disulfide bond was found to be highly dependent on reaction volume and peptide equivalents, and can now be reliably coupled in excellent purity. The addition of a third peptide to the growing polypeptide has been observed, but in small yields due to the growing peptide cleaving itself from resin. We are currently exploring a variety of reaction conditions to overcome this autocleavage, so as to be able to make reducible polypeptides consisting of three or more peptide subunits.
ENGRIERING OF CHLORELLA VULGARIS FOR BIOCATALYSIS TO GENERATE HIGH VALUE PRODUCTS

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CO₂ emission is a global problem with respect to climate change and sustainability (http://www.climatetechnology.gov). Recent emphasis on shifting energy and chemical production via bio-refineries, as opposed to oil-refineries, does not diminish CO₂ issues. For example, ethanol production via fermentation generates considerable CO₂. Several solutions have been proposed to deal with CO₂ such as, capturing it underground, capture by algae for production of biodiesel, etc. While these are interesting approaches, it does not create “value” and solution at the same time. The larger vision of the present work is to use algae to capture CO₂ from ethanol plants, and use the ‘generated biomass’ as biocatalyst for production of high value chemicals such as xylitol, from agricultural residues. The first step towards achieving this vision is to demonstrate that algal biomass can be used as efficient biocatalyst.

Towards this, our aim is to (i) develop a procedure for stable nuclear and chloroplast transformation of Chlorella vulgaris. C. vulgaris was chosen because of its robust autotrophic growth, and (ii) to perform nuclear and chloroplast expression of foreign proteins of industrial importance such as xylose reductase (XR) and glycolate oxidase (GO). XR has been shown in our lab for conversion of corn stover hemicellulose extract (xylose) to xylitol, a non-caloric sweetener. Likewise, GO has been scaled up in our lab for production of pyruvic acid from lactic acid.

For optimization of nuclear transformation, a genetic construct composed of bacterial aadA gene under the control of CaMV35S promoter and 3’UTR of opine synthase was designed. Agrobacterium tumefaciens was used to introduce the transgene into Chlorella. Additional constructs for electro transformation of C. vulgaris were created to express aadA under the control of the ribulose 1,5-bisphosphate carboxylase small subunit (rbcS) promoter and 3’UTR of rbcS. In both cases C. vulgaris resistant colonies on selective medium of spectinomycin was obtained, indicative of successful nuclear transformation.

To our knowledge, chloroplast transformation has not been successfully demonstrated in C. vulgaris. Testing of several vectors for expression of bacterial aadA under control of either the small subunit ribosomal RNA with 3’ UTR of psaB gene or ATP synthase β-subunit promoter with 3’ UTR of psbA gene is currently in progress. Each of these constructs is carried in a fragment which has homology with a fragment at the 3’UTR of ribulose 1,5-bisphosphate carboxylase (rbcL). Alternative construct using the rbcL promoter and 3’UTR of psaB gene was cloned into fragment which will introduce the aadA cassette at the 5’UTR of the psbB gene. Appearance of resistant colonies and PCR of bacterial aadA have confirmed the presence of the transgene in the chloroplast of C. vulgaris. Growth for homoplasmicity is in progress for visualization of an additional phenotype, i.e., lack of photosynthesis due to introduction of a point mutation in the rbcL and psbB genes. Due to presence of multiple gene copies in the chloroplast genome, this marker is not visible before the strains become homoplasmic.

Once the transformation protocols are established, XR and GO will be introduced into C. vulgaris to test its potential for biocatalysis.
COMPARISON OF PURIFICATION OF RECOMBINANT N-DEMETHYLASE B (NdmB) USING SIMULATED MOVING BED AFFINITY CHROMATOGRAPHY (SMB) AND FPLC

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Simulated moving bed (SMB) is a well-established technology based on simulating a countercurrent contact between the solid (stationary) phase and liquid phase. It is widely used in separation of sugars, racemic drugs and drug intermediates at commercial scale. SMB has been shown to be more beneficial in terms of productivity; such as product concentration as well reduced use of solvents/buffers, while retaining product purity. Use of SMB in protein purification in biopharma industry is not common. This is in spite of the fact that most of the work reported in the area of FPLC-based protein purification is on separation of binary mixtures. Protein purification is ideally suited for SMB where two components are separated as raffinate and extract. There are a couple of reports on the protein purification of complex mixtures using SMB. We have developed a four-zone SMB method (Bind, Wash, Elute and Regenerate) using Nickel (HIS-Select) affinity chromatography to purify recombinant N-demethylase B (NdmB). NdmB (Mol.wt: 35 kDa) from Pseudomonas putida CBB5 catalyzes the N-3-demethylation of caffeine. This enzyme has been cloned and expressed E. coli as his-tag protein. We carried out 30-L fermentation of E. coli expressing NdmB and the cell paste obtained from this fermentation was used for purification of NdmB using SMB (Octave 100, Semba Biosciences) and FPLC (Akta). About 24 grams of cells paste was used for each method of purification. For SMB we used 8 columns of 5-mL size and for FPLC, one 40-mL (XK26) column. Equal amount of protein was loaded on to both systems. After washing the column with Buffer (25mM KPi, 300mM NaCl, 10mM Imidazole, pH 7.5), NdmB was eluted with Elution Buffer (25mM KPi, 300mM NaCl, 250mM Imidazole, pH 7.5). The recovery of NdmB from SMB and FPLC was 30% and 23% respectively. Based on band intensity on SDS-PAGE of NdmB, it was assessed that SMB achieved 95 % purity whereas from FPLC it was 91%. Measuring the overall productivity in terms of (a) time taken for purification, 150 minutes vs. 350 minutes (SMB vs FPLC), (ii) buffer use, 1 vs 1.5 times (SMB vs FPLC), and (iii) and column regeneration (no additional unit operation for SMB), SMB method was better than the FPLC (batch chromatography). SMB being a continuous process is more suitable for large scale manufacturing of proteins/therapeutics. CBB will continue to explore SMB application for protein purification and for chiral molecule separations with the intent of developing this technology for industrial applications.
SYNTHESIS OF PICOLYL AZIDE ANALOGUES OF NICOTINAMIDE AS POTENTIAL TWO-DIMENSIONAL INFRARED PROBES OF NAD(P)-DEPENDENT ENZYME DYNAMICS

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Characterizing protein motions and understanding their functional relevance are of significant interest, but the role of dynamics at various time scales especially the fast subnanosecond protein structural fluctuations in enzyme-catalyzed reactions is an emerging topic in enzymology. Two-dimensional infrared (2D IR) spectroscopy is one of the few methods that can examine chemical motions at the femtosecond (fs) to picosecond (ps) time scale, but all the IR probes used so far were specific to only a few unique enzymes. The lack of general IR probes limits the studies of enzyme dynamics using 2D IR. Here we describe the synthesis of two picolyl azide analogs of nicotinamide: picolyl azide adenine dinucleotide (PAAD+) and picolyl azide adenine dinucleotide 2’-phosphate (PAADP+), which are derived of ubiquitous redox cofactors in biology – nicotinamide adenine dinucleotide (NAD+) and nicotinamide adenine dinucleotide 2’-phosphate (NADP+), respectively. Both new analogues of nicotinamide cofactors serve as chromophores in the mid-IR range because of the azido stretch. Independent 2D IR studies reported for PAAD+ and azide anion at the active site of formate dehydrogenase (FDH) showed consistent dynamics between the ternary complexes of FDH-PAAD+-azide and FDH-NAD+-azide. Additionally, isothermal titration calorimetry of enzyme complexes of PAAD+ to FDH and PAADP+ to dihydrofolate reductase (DHFR) show dissociation constants similar to those of the native cofactors, indicating PAAD and PAADP have significant potential as 2D IR probes to investigate femtosecond to picosecond dynamics of nicotinamide-dependent enzymes.
IDENTIFYING OPTIMAL CONDITIONS FOR DETECTING VARYING CONCENTRATIONS OF ATRAZINE USING RAMAN SPECTROSCOPY

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Atrazine, an endocrine disrupter compound and group C-possible carcinogen, is the second most widely used herbicide in the United States. An estimated 70 million pounds per year is applied to desired crops and recreational landscapes, resulting in ground water, precipitation, and surface water runoff contamination. Raman spectroscopy has been shown to detect atrazine and will be used to follow its biodegradation by Pseudomonas ADP (P.ADP) in future experiments. Therefore, the purpose of this research is to optimize spectral conditions required to detect atrazine at its solubility limit using deionized water, methanol, and chloroform as solvents. The Raman effect was induced using 532 or 785 nm laser at varying exposure times in the probehead or microscope configuration. Results obtained from this research will aid in detecting atrazine degradation in P.ADP biofilms. Even though Raman spectra of biofilm matrices consist of complex superpositions of different components, it is possible to identify changes in structure and chemistry. Applying Raman spectroscopy to examine the chemical changes occurring within the system is a promising approach to probe biotransformation activity. Success of this research will lead to a firmer grasp of scientific fundamentals associated with biofilm degradation, which will ultimately lead to improved remediation applications and to reduced pollutant-associated illness.
Regulators of G-protein signaling (RGS) proteins temporally regulate G-protein coupled receptor (GPCR) signal transduction cascades by acting as GTPase accelerating proteins (GAPs) upon binding activated $\alpha$ subunits. In previous studies, we investigated the mechanism of the small-molecule RGS inhibitor CCG-4986 [methyl-$N$-[(4-chlorophenyl)sulfonyl]-4-nitro-benzenesulfinimidoate] and found it covalently modified key cysteine residues on RGS4 both at the RGS/$\alpha$ interface, Cys132, and at a distal, allosteric site, Cys148. The allosteric inhibition at Cys148 ablated $\alpha$-RGS protein-protein interaction much more (75%) than modification of Cys132 (25%), and this effect was selective for RGS4 and not RGS8 or RGS16, other members of the R4 family that contain an equivalent of Cys148. Additional work in our lab has shown that Cys148 is also sensitive to modification by the endogenous lipid peroxidation product 4-hydroxynonenal (4-HNE). In this current study, we investigate the biophysical, allosteric changes that occur in RGS4 upon CCG-4986 and 4-HNE binding that efficiently displace $\alpha$. To ensure structure homogeneity, we expressed recombinant RGS4 with all cysteines removed, except Cys148, in Escherichia coli, and following purification, performed structural analysis using high-field biological NMR for free RGS4 and modified RGS4. Overlaid $^{15}$N-$^1$H HSQC spectra indicate significant changes in RGS4 structure upon CCG-4986 binding. Our interest in the structural allosteric changes in RGS4 upon modification of Cys148 is twofold: first, it will provide structural insights into the unique allostery present in RGS4 that may be targeted for modulation and second, we have identified the presence of cysteine adducts on RGS4 in the presence of pro-oxidants in a cellular context. These modifications, as observed in cells, suggest a role for regulation of RGS4 in the pathogenesis of diseases characterized by oxidative stress such as Parkinson’s disease, Alzheimer’s disease, schizophrenia and others.
BUILDING CHEMICAL LIBRARIES OF SPATIALLY COMPLEX TRICYCLIC MOLECULES USING A CASCADE REACTION

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High-throughput screening (HTS) can be a valuable tool for finding chemical compounds with determined biological activity when chemical libraries are structurally diverse. To that end, we have developed a strategy to create high spatial complexity in tricyclic molecules, starting from a highly versatile heterocyclic compound. Cascade reactions are very desirable to create these compounds because tandem reactions occur in one flask without the need of isolating each reaction intermediate.

We have developed a green chemo-enzymatic oxidation of alkenes utilizing a lipase and hydrogen peroxide as the oxidant. We have also developed a new thiazolidinethione from indene oxide and shown the chemical versatility of this heterocycle. In this presentation, we will show details on the cascade reaction of \( N \)-enoyl thiazolidinethiones with aldehydes in the presence of a Lewis acid. We are employing this strategy to build a small library of highly complex tricyclic compounds possessing a quaternary carbon attached to four heteroatoms.

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DEVELOPMENT OF MOLECULAR BIOLOGY TOOL FOR MONITORING BIOREMEDIATION OF VINYL CHLORIDE IN GROUNDWATER

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Vinyl chloride (VC), a known human carcinogen and common groundwater (GW) contaminant, often occurs in GW as result of incomplete reductive dechlorination of the more highly chlorinated ethenes and ethanes. At some sites, the VC formed has migrated away from anaerobic GW, forming a dilute plume. Ethene may also form and comigrate along with the VC. Under this scenario, aerobic, ethene-assimilating bacteria (i.e. etheneotrophs) could either cometabolize the VC in the presence of ethene or possibly adapt to VC as a growth substrate\(^1\). Here, we aim to extend a newly described quantitative, real-time PCR (qPCR) method for estimating the abundance of functional genes associated with etheneotrophs and VC-assimilators in GW to also include quantification of mRNA transcript abundance.

Nocardioides strain JS614 was grown on ethene (0.25 mM), VC (1.6 mM), or acetate (20 mM) as a sole carbon and energy source, sampled at different time points, and collected with Sterivex filters. For RNA preserve, RNAlater was applied. GW samples from a VC-contaminated site were collected with Sterivex filters and preserved with RNAlater as appropriate. DNA (or RNA) extraction was performed with the MOBIO PowerWater DNA (or RNA) Isolation Kit. Luciferase mRNA (or DNA) was incorporated immediately after the cell lysis step to provide internal controls for mRNA or DNA loss. Previously described degenerate qPCR primers were used along with SYBR green qPCR chemistry. These primers target \(etnC\), which encodes the alkene monooxygenase (AkMO) alpha and \(etnE\), which encodes the epoxyalkane:coenzyme M transferase (EaCoMT). These functional genes are known to be involved in both the aerobic VC and ethene biodegradation pathways.

The data from pure cultures indicated that acetate grown culture showed 0.5 \((etnC)\) and 0.6 \((etnE)\) transcripts/gene. When the cultures were at starvation condition, transcripts/gene ranged 0.1-0.2. While the cultures were metabolizing ethene or VC, transcriptase/gene had higher than 2. Analysis of corrected qPCR data revealed 1,300-22,000 \(etnC\) and 2,500-16,000 \(etnE\) genes per liter (L) of GW. In contrast, there were 6,500-16,000 \(etnC\) and 3,500-13,000 \(etnE\) transcripts were present per L of GW. This corresponded to DNA and RNA recovery efficiencies of 16-68% and 1-4%, respectively. Normalizing gene expression to gene abundance showed that there were 0.4-5.7 \(etnC\) and 0.4-2.2 \(etnE\) transcripts per gene. Especially, RB64I had 5.7 \(etnC\) and 2.2 \(etnE\) transcripts/gene, which suggests that etheneotrophic gene expression in this well was active.

This novel RT-qPCR method shows promise for quantifying the abundance of \(etnC\) and \(etnE\) transcripts in GW and therefore represents an approach for demonstrating metabolic functionality of etheneotrophs therein.
MITOCHONDRIAL TARGETED TRIPHENYLPHOSPHONIUM DERIVATIVES FOR TREATMENT OF METASTATIC MELANOMA

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Background: An emerging class of cancer therapeutics comprises mitochondrial-targeted molecules that exploit cancer cell-specific differences in oxidative metabolism to selectively kill cancer cells relative to healthy cells. It has been suggested that dysfunctional electron transport chain (ETC) activity during aerobic respiration results in both heightened hydroperoxide production and an increased voltage potential across the mitochondrial membrane, which represent potential targets for mitochondrial targeted therapies. One class of mitochondrial targeted reagents that have been used extensively to study the biophysical properties of mitochondrial membranes is derivatives of triphenylphosphonium (TPP). TPP variants localize preferentially to the mitochondrial membrane by virtue of a positive charge distributed over a large lipophilic surface, which facilitates transport across cell and mitochondrial membranes. Preferential accumulation of TPP derivatives in cancer cells is thought to be mediated by high mitochondrial density and heightened mitochondrial membrane potential of cancer cells relative to surrounding tissue.

Hypothesis: We hypothesized that TPP molecules modified with aliphatic alkyl “tails” would localize to the mitochondrial membrane and disrupt the ETC to drive over-production of cytotoxic hydroperoxides. Furthermore, we hypothesized the length and composition of the molecular “tail” could be manipulated synthetically to optimize electron transport disruption and preferential cancer cell cytotoxicity.

Methods: In this study, we examined TPP derivatives modified with 5, 10, 15, and 20-atom length alkyl chains to test our working hypothesis. High throughput MTT assays were performed to measure the cytotoxic effects of test compounds by colorimetric assay. A375 human melanoma cells were incubated with TPP variants in the presence and absence of buthionine sulfoximine (BSO), an inhibitor of the hydroperoxide metabolizer glutathione to support the hypothesis that cytotoxicity is driven by overproduction of hydroperoxide species.

Results and Conclusions: No apparent cytotoxicity to A375 cells was observed for the 5-TPP compound (with or without BSO), while 10 and 15-TPP compounds exhibited cytotoxicity that was enhanced by BSO administration. The 20-TPP compound demonstrated increased cytotoxicity compared to TPP variants with shorter alkyl chains, regardless of BSO administration. These results indicate that TPP derivatives exhibit anti-cancer activity and the length of the alkyl chain can have a significant impact on melanoma cell cytotoxicity. These results demonstrate the promising potential of the combination of mitochondrial targeted TPP variants, used together with hydroperoxide metabolism suppressor BSO for therapy of metastatic melanoma.
VC DEGRADATION BY METHANE-OXIDIZING AND ETHENE-OXIDIZING BACTERIA IN THE PRESENCE OF METHANE AND ETHENE MIXTURES

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Vinyl chloride (VC) is a known human carcinogen that is primarily formed in anaerobic groundwater zones by incomplete reductive dechlorination of chloroethenes, where significant amount of methane and ethene may also generated. When a VC plume intersects with groundwater containing molecular oxygen, natural attenuation of VC could occur via oxidation by aerobic microorganisms. Under aerobic conditions, ethene-oxidizing bacteria (etheneotrophs) are able to perform cometabolic VC oxidation, where the enzyme Alkene monooxygenase (AkMO) fortuitously oxidizes VC while utilizing ethene as primary growth substrate. Methane-oxidizing bacteria (methanotrophs) utilize methane as their sole carbon and energy source through the enzyme methane monooxygenase (MMO), which can also cometabolize VC.

We are studying how the combined metabolic activities of the two organisms affect the VC biodegradation process. Also, VC inhibitory activity to primary substrates utilization and which monooxygenase is more compatible with VC cometabolism are of particular interest. In this work, pure culture mixtures of ethene-oxidizing Mycobacterium strain JS622 and methane-oxidizing Alphaproteobacterium, Methylocystis sp. strain Rockwell (ATCC 49242) were used to investigate the degradation activity of ethene, methane, and vinyl chloride mixtures in serum bottles. The following treatments are evaluated in individual bottle: (1) methane alone; (2) ethene alone; (3) methane+VC; (4) ethene +VC ; (5) VC alone; (6) methane+ethene; (7) methane+ethene+VC; (8) culture medium control containing methane+ethene+VC. Masses of ethene, methane, and vinyl chloride added to each bottle were 400 µmoles, 400 µmoles, and 20 µmoles, respectively. The total mass of a compound remaining in a serum bottle was determined by gas chromatographic analysis of headspace samples (88 ml from the 160 ml serum bottles). In culture mixtures with 50% methanotrophs and 50% etheneotrophs, the bottles containing methane only, VC only, and methane + VC showed smooth decreasing degradation curves, but bottles containing ethene only, and ethene+VC are degraded at slower rate at the beginning and higher rate after two, or three days. The presence of ethene inhibited methane biodegradation, thereby inhibiting VC consumption. Results have shown that the order of monooxygenases compatibility to the three compounds is ethene,VC, and methane. By comparing different concentration ratios of methanotrophs and etheneotrophs in the presence of the environmentally relevant ratios of VC, ethene, and methane, we aim to gain a better understanding of how the interactions of the two microbial groups affect VC degradation in a scenario where methane, ethene, VC, and oxygen are all present in a groundwater system.
BACTERIAL COMMUNITY STRUCTURES AND POLYCHLORINATED BIPHENYL BIODEGRADATION POTENTIAL IN SEDIMENTS FROM INDIANA HARBOR SHIPPING CANAL

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Polychlorinated biphenyls (PCBs) are a group of 209 synthetic chemicals that were widely used in industry during the last century. They pose a possible threat to both human and environmental health, because certain PCB congeners are toxic, carcinogenic and recalcitrant. Microorganisms could play an important role in removing and detoxifying PCBs in the environment. Microbial PCB biodegradation is known to proceed via anaerobic dechlorination catalyzed by reductive dehalogenases, and by aerobic biphenyl ring cleavage, catalyzed by a series of enzymes (e.g. biphenyl dioxygenase). In this study, we are characterizing microbial community structures and searching for evidence of PCB-degrading microbes in contaminated sediments from Indiana Harbor Shipping Canal (IHSC), IN. Microbial community structures at different sediment depths and locations were characterized by terminal restriction fragment length polymorphism (T-RFLP) and 16S rRNA gene pyrosequencing approaches. At least twelve bacterial phyla were detected by pyrosequencing in five sediment samples, among which Proteobacteria was found to be the most abundant taxa. Changes in the T-RFLP profiles were correlated with changes in PCB congener profiles along sediment depth of core samples, as determined by a multivariate statistical analysis, Mantel test (p<0.05). A PCR-based approach was performed to search for the presence of genes related to PCB degraders. Eighteen partial biphenyl dioxygenase gene sequences were successfully retrieved from surficial and core sediment samples, some of which were found identical to sequences from known PCB degraders such as Acidovorax sp. KKS102 and Comamonas testosteroni TK102, indicating the potential for aerobic, in situ microbial PCB degradation at IHSC. Five Dehalococcoides-specific 16S rRNA gene sequences were found in deep sediment samples; however, they were not similar to sequences from known anaerobic PCB degraders. Future experiments will also be conducted to identify reductive dehalogenase genes in the sediment samples, which would provide stronger evidence for anaerobic in situ PCB degradation potential.
Synthesis and kinetic evaluation of dual binding acetylcholinesterase (AChE) inhibitors targets both the cholinergic and β-amyloid plaque pathways of Alzheimer’s disease (AD) treatment. Aryl-trifluoroketones, ladderane natural product derivatives, and paracylcophane moieties have been evaluated as potential human AChE inhibitors. Dose response assays, using the Ellman¹ method, of quinoline and N-methylquinolinium aryl-trifluoroketones showed IC₅₀ values in the 10⁻⁹ M range. Additionally, both aryl-trifluoroketone moieties were observed to be tight binding while only the N-methylquinolinium showed time dependent inhibition. Dose response assays of chiral and achiral tetrapyridyl-5-ladderane (TPL5) showed IC₅₀ values in the 10⁻⁶ M range. Similarly, the tetrapyridyl-paracylcophane (TPPCyc) and N-methyl tetrapyridyl-paracylcophane (Me-TPPCyc) showed IC₅₀ values in the 10⁻⁵ M and 10⁻⁶ M range respectively. Lineweaver-Burk analysis of Me-TPPCyc showed its mode of inhibition to be noncompetitive.

HIGH-THROUGHPUT SCREENING AND CHARACTERIZATION OF SMALL MOLECULE INHIBITORS OF THE Gα:RGS17 PROTEIN: PROTEIN INTERACTION FOR TREATMENT OF LUNG AND PROSTATE CANCERS

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G-Protein Coupled Receptors are one of the most important targets in drug development, making up over 60% of drug targets. Recent studies have implicated a role of Regulator of G-Protein Signaling (RGS) proteins in the development and progression of pathologies, including some cancers. RGS17, the most-recently identified family member of the RZ family of RGS proteins, has been implicated in the growth, proliferation, metastasis and migration of prostate tumors as well as small-cell and non-small cell lung cancers. In neoplastic tumor tissues RGS17 is up-regulated 13 fold over patient-matched normal tissues. Studies have shown that RGS17 knockdown inhibits colony formation and decreases tumorigenesis in nude mice. In this study, we implemented AlphaScreen® technology to develop a high-throughput screening method for interrogating small molecule libraries for inhibitors of Gα:RGS17 interaction as well as preliminary characterization of confirmed hits. We screened the NCI Diversity Set II and determined 35 initial hits of which 16 were confirmed after screening against controls. The 16 compounds exhibited IC50 <10μM in dose-response experiments for inhibiting the Gα:RGS17 interaction. Four exhibited IC50 values <6μM while inhibiting the Gα:RGS17 interaction >50% when compared to a biotinylated GST control. Compounds RL-1 and RL-2 were confirmed by flow cytometry protein interaction assay (FCPIA) while RL-3 and RL-4 were unable to disrupt this PPI in FCPIA. All four compounds were tested using the differential scanning fluorimetry (DSF) method, which is based on energetic coupling between ligand binding and protein unfolding and found compounds RL-1 to RL-4 all increased protein stability upon ligand binding. Our future approach will involve miniaturization of our assay into 1536 well plates, functional exploration of compounds in GTPase steady-state assays and the screening of the in-house 50,000 diverse compound library in the new high-throughput screening center. This screen has established lead pharmacophores for further optimization of structure with the focused on activity in enzymatic, whole cell, xenograft and whole animal models as well as potential new avenues for anticancer therapies.
Several human pathogens rely on the activity of flavin-dependent thymidylate synthase (FDTS) for the production of thymidylate, a DNA nucleotide. The chemical mechanism of this enzyme is different from that of its human analog and not entirely delineated, thus impeding the development of mechanism-based inhibitors. Although several catalytic mechanisms have been proposed for FDTS, no experimental evidence supporting existence of the proposed intermediates is available to this date. In this work, we report the chemical trapping and identification of such an intermediate in the FDTS-catalyzed reaction. The identity of the trapped intermediate sheds light on the nature and timing of chemical steps during flavin-dependent catalysis. The presented methodology provides an important experimental tool for further studies of FDTS chemistry, knowledge of which may assist the efforts to rationally design inhibitors as leads for future antibiotics.
NEW PATHWAY FOR CAFFEINE DEGRADATION IN *PSEUDOMONAS* sp. CBB1:
NOVEL ENZYMES, GENES, AND METABOLITES

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Soil bacteria *Pseudomonas* sp. CBB1 grows on caffeine as sole source of carbon and
nitrogen. LC-MS analysis of spent media of CBB1 grown on caffeine suggested transient
accumulation of 1,3,7-trimethyluric acid (TMU, m/z 211.0) and 3,6,8-trimethylallantoin
(TMA, m/z 201.0). CBB1 initiates caffeine metabolism by a quinone-dependent caffeine
dehydrogenase (Cdh) by direct oxidation at the ‘C-8’ position to form TMU\(^1\). Subsequently
TMU gets converted to TMA by a NAD(P)H dependent trimethyluric acid monooxygenase
(TMU-MO). This enzyme has been purified from CBB1; besides TMU, the enzyme also
oxidizes di- and monomethyluric acids at lower rates, but had no activity on uric acid. The
gene for TMU-MO (designated as *tmuO*) was isolated from a genomic Fosmid library by
using degenerate primers designed from the N-terminal sequence of the protein, and
conserved FAD-binding domain of monooxygenases. *tmuO* is composed of 1191 nt,
encoding a 396-amino-acid protein with a theoretical \(M_t\) of 42,619 and pI of 6.12. The gene
was cloned into the pET32a plasmid with C-terminal His\(_6\)-tag, over expressed in soluble
form, and purified to homogeneity. The purified protein was active and displayed same
substrate specificity as that of purified enzyme from strain CBB1. The apparent \(M_t\) of the
protein was estimated to be 43 KDa. The enzyme was yellow in color, showed UV-visible
absorption maxima at 271, 380 and 456 nm indicative of a flavoprotein. Product analysis of
the reaction using the purified enzyme showed transient accumulation of 1,3,7-trimethyl-5-
hydroxyisourate (TM-HIU, m/z 227.0). TM-HIU was found to be unstable, resulting in the
formation of racemic TMA, which was confirmed by high-resolution mass spectrum and
NMR. TMA produced enzymatically was further analyzed using Chiralpak IA column in
HPLC and found to be racemic. Based on (i) these results (ii) analysis of ORFs flanking
*tmuO*, and (iii) its homology with HIU-hydrolases, allantoinases, and other enzymes of uric
acid pathway, a new pathway has been proposed for caffeine metabolism. CBB1
metabolizes caffeine via TMU and TM-HIU to one of the enantiomeric forms of TMA,
which gets further degraded to TMAA.

**Figure 1:** Proposed C-8 oxidation pathway for caffeine degradation by CBB1.
MODIFICATION AND FUNCTIONAL INHIBITION OF REGULATOR OF G-PROTEIN SIGNALING 4 BY 4-HYDROXY-2-NONENAL

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Regulators of G-protein signaling (RGS) proteins are a family of proteins responsible for regulating G-protein coupled receptors (GPCRs). RGS proteins inhibit signal transduction from GPCRs by acting as GTPase accelerating proteins (GAPs), increasing the rate of hydrolysis of GTP to GDP in the Ga subunit. RGS proteins inactivate Go, Gi, and Gq but not Gs. One of these proteins, RGS4, has been implicated in several neurological diseases. In this study, we analyze the effect of 4-hydroxy-nonenal (4HNE) on purified RGS4, lacking the first 51 amino acids. 4HNE forms covalent adducts on reactive amino acids such as cysteine. Purified RGS4 was analyzed by western blot to detect for 4HNE adducts. Purified RGS4 was also analyzed by LC-MS after tryptic digest to detect 4HNE adducts. Purified RGS4 exposed to 4HNE revealed that modification only occurs at select cysteine residues on RGS4. Several mutants were made containing only one of the modified cysteines, either C71, C148, or C183. These constructs were treated with 4HNE at ratios of 1:1, 1:10, and 1:100 and then assayed for coupling to the native binding partner Go, by ALPHA-Screen. The wild type construct showed sensitivity to 4HNE but the cysteine null mutant did not. Of the single cysteine containing mutants, only C148 conferred sensitivity to RGS4 while the others behaved similarly to the cysteine null mutant. Research support provided by the Pharmacological Sciences Training Grant (NIH/NIGMS T32GM067795).
**BEAUVARIA BASSIANA OXIDATIVE PERFORMANCE**

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The filamentous fungus *Beauveria bassiana* have the capacity to enhance the yield and selectivity of oxidative biotransformations. Organic solvents serve as carbon sources during growth of *B. bassiana* and have been shown to induce the expression of oxidative enzymes. Expression varies with the alkane compound. Current research efforts apply organic solvents to adapt cell growth under these minimum conditions. In addition the oxidative performance is evaluated by conversion of androst-4-ene-3, 17-dione to desired steroid products and sulfoxidation of 2-[(diphenylmethyl)thio]acetic acid. The long-term goal is to standardize and control the biocatalysis capacity of *B. bassiana* in the biotransformation of a variety of oxidizable substrates under different operational parameters. The selection of suitable n-alkane solvent for optimal yield and selectivity, and the enhancement of oxidative enzymes expression. This research will provide new insight on the *B. bassiana*’s oxidative performance. It will also offer opportunities for industrial applications utilizing steroid or sulfide molecules.
We explore this system by means of single molecule spectroscopy, an extremely sensitive confocal microscopic technique in which bursts of photons are detected as a fluorophore travels through the laser probe volume. Using this method, we can observe both diffusion of a fluorophore through the nanopore – which may be considerably different than diffusion in the bulk solution – and adsorption of a fluorophore to the nanopore wall – which elucidates the inherent heterogeneity of the surfaces of these particles. Because methods of chemical modification of silica surfaces are extremely diverse, well documented, and relatively simple, a wide variety of closely related particles with different surface functionalization are available for study. Understanding the behavior of small molecules in nanoporous silica can provide the necessary information to tailor these nanomaterials to a wide variety of beneficial uses.
ROLE OF THE DNA-BINDING PROTEIN IsaB IN \textit{STAPHYLOCOCCUS AUREUS} BIOFILMS

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\textit{Staphylococcus aureus} is a gram-positive bacterial pathogen, and the cause of a wide variety of both acute and chronic infections. The chronic infections include osteomyelitis, endocarditis, and implant-associated infections, and the ability of \textit{S. aureus} to form a biofilm and persist is a conserved feature of chronic disease. Bacterial biofilms are surface-associated communities composed of cells and encased in an extracellular matrix. The extracellular matrix is composed of polysaccharide, extracellular DNA (eDNA), and protein. Recent studies have uncovered important roles for eDNA and protein in the matrix, but the mechanisms that might enable DNA-protein interactions are not known. The secreted \textit{S. aureus} protein, IsaB, was recently reported to bind extracellular DNA. A chromosomal knockout of IsaB was constructed in the wild-type \textit{S. aureus} strain, HG001, a strong biofilm former. HG001 has also been reported to form large amounts of extracellular IsaB. Here we show, that the HG001\textit{Δ}isaB strain produces significantly less biofilm than its parent in a microtiter plate biofilm assay. Additionally, we show this phenotype can be complemented in trans on a plasmid with an IPTG inducible promoter. This finding could provide insight into the interaction between eDNA and protein in a biofilm.
Kinetic isotope effects (KIEs) have been used to probe the nature of the electrostatic and
dynamic interactions that enzymes use to catalyze H-transfers. In recent years, though, it has
become apparent that the molecular interpretation of $2^\circ$ KIEs and the temperature
dependence of $1^\circ$ KIEs is either qualitative or requires expensive and specialized QM/MM
calculations to provide a more quantitative molecular understanding. We recently used $2^\circ$
KIEs to quantitatively model the transition state structure of an enzyme in the context of
Marcus-like models of hydrogen tunneling\(^1\). Here we continue to narrow the gap between
observation and interpretation of KIEs by developing a simple method to quantitatively link
the size and temperature dependency of $1^\circ$ KIEs to a conformational distribution that reflects
the dynamic nature of the catalyzed reaction. The present model assumes fully adiabatic
hydrogen tunneling, and by fitting experimental KIE data, the model yields a population
distribution for fluctuations of the distance between donor and acceptor atoms. Fits to data
from a variety of proton or hydride transfers catalyzed by enzymes and their mutants,
revealed that steeply temperature dependent KIEs indicate the presence of at least two
distinct conformational populations, each with different kinetic behaviors. We will present
the results of these calculations for several published cases and discuss how the predictions
of the calculations might be experimentally tested.

\(^1\) Roston, D. and A. Kohen, 2010, "Elusive Transition State of Alcohol Dehydrogenase
ORGANIC NANOCRYSTALS OF SUPRAMOLECULAR MATERIALS

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The size-dependent properties related to nanocrystals make such solids of great interest. Whereas inorganic nanocrystals have experienced immense utility in a variety of areas, nanocrystals comprised of purely organic components have remained relatively unexplored. With this in mind, we initially targeted the synthesis of purely organic, multi-component, nanocrystals to exploit the physiochemical properties (e.g. molecular recognition) of organic solids. In recent years cocrystals (i.e. multi-component molecular crystals) have garnered immense interest in the field of pharmaceutics owing to an ability to improve the physiochemical properties of pharmaceutical agents (PAs). Coupling the benefits of pharmaceutical cocrystals with a decrease in particle size to the nanoscale can be expected to further improve the properties of PAs. To achieve pharmaceutical nano-cocrystals of our model system, caffeine 2,4-dihydroxybenzoic acid monohydrate (caff)- (dhba)·(H\textsubscript{2}O), we turned to sonochemical approach which employed multiple-solvents and surfactant. The result was an average particle size of 136.4 nm ± 65.05. The technique has been expanded to include additional pharmaceutical cocrystals and the host-guest system C- methylcalix[4]resorcinarene. The resorcinarene crystallizes in a cubic space group and self-assembles to form a chiral sphere composed of six resorcinarene molecules and eight water molecules. Originally the hexamer was prepared via slow-solvent evaporation to afford crystals ranging from 5 μm to 1 mm. Sonochemistry reduced the average size of the crystals to 630 nm (s.d. 315 nm).

\textbf{Figure 1.} Scanning electron microscopy micrograph of the resorcinarene hexamer.
DIFFERENTIAL BINDING OF DNA WITH FLUOROQUINOLONES AFFECTS THE ABILITY OF THE FLUOROQUINOLONE TO KILL MICROORGANISMS

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Drug resistant bacteria are responsible for increasing incidences of Staphylococcus aureus, Pseudomonas aeruginosa, Streptococcus pneumonia, and Mycobacterium tuberculosis infections in humans. Fluoroquinolones (FQs), broad-spectrum bactericidal antibiotics, exert their effects by inhibiting type II topoisomerases through the formation of a ternary complex with the enzyme and DNA. Recently, it has been shown that newer, structurally unique FQs rapidly kill cells by promoting chromosomal fragmentation. Because the new FQs are able to rapidly kill in the presence and absence of protein synthesis, they have potential to be used in the treatment of microorganisms that go into a dormant state, as well as microorganisms that produce persister cells.

Previous studies with older generation FQs have demonstrated that the ability of a FQ to bind single-stranded (SS) and double-stranded (DS) DNA does not correlate with activity. Newer generation FQs have not been examined for DNA binding. Recent work suggests that there is a unique mechanism of action by which these newer FQs rapidly kill microorganisms, both in the presence and absence of the known protein synthesis inhibitor chloramphenicol. The goal of the studies presented here is to determine if FQs with distinct modes of killing bind different types of DNA in unique ways. Binding of FQs to SS, DS, and nicked DNA, all mimics of possible DNA types present in the active ternary complex, will be discussed. The ability of the different FQs to destabilize or stabilize DNA will also be presented, as we attempt to gain further insights into the unique mechanism of action observed with the newer FQs.
HIGHLY EFFICIENT SYNTHESIS OF NOVEL OSW-1 ANALOGS

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OSW-1 is an extremely potent and highly selective anticancer natural product isolated from the bulbs of *Ornithogalum saundersiae*. It exhibits anticancer activities that are about 10-100 times more potent than many well-known anticancer agents in clinical use such as etoposide, methotrexate, mitomycin C, adriamycin, cisplatin, camptothecin, 5-Fu, and paclitaxel. In addition, it shows equal potency against cancer cells resistant to a number of anticancer agents in clinical use. Whereas non-malignant cells are significantly less sensitive to OSW-1, with the IC$_{50}$ values 40-150 fold greater than those observed in cancer cells. Furthermore, OSW-1 doesn’t show any hemolytic toxicity. However, OSW-1 showed weak *in vivo* activity. After eight years of extensive pharmacological studies it has been speculated that the weak *in vivo* activity is primarily due to the metabolic deactivation of the drug. The specific aim of my research project is to synthesize designed analogs of OSW-1 with improved *in vivo* activity. The completion of this project will lead to the discovery and development of potentially highly effective novel anticancer drugs. The analogs have been designed to circumvent the metabolism, which leads to the deactivation of OSW-1. These designed analogs are expected to significantly improve its *in vivo* anticancer activity. The novel OSW-1 analogs contain one steroidal fragment, a modified arabinose fragment and a modified xylose fragment. Presented here is the efficient route for the synthesis of the steroidal fragment. Upon completing the synthesis of OSW-1 analogs, they will undergo extensive *in vitro* and *in vivo* evaluation by which we will be able to develop highly efficacious drugs.
NANOSTRUCTURED PHOTO CROSS-LINKED BIOPOLYMERS IN WOUND-HEALING AND DRUG DELIVERY APPLICATIONS

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Chitosan materials have attracted significant attention in the past decade due to their inherent antimicrobial and haemostatic properties, biocompatibility, and renewable nature. Wound dressings containing these cationic polysaccharide fibers have been shown to promote wound healing and prevent infection while also slowing blood loss. In addition to the already-promising potential of chitosan, further improvement can be accessed by more closely mimicking the structure of the extra cellular matrix. Increased cell survival and attachment have been demonstrated on nanostructured synthetic polymers relative to their isotropic counterparts. The overall goal of this work is to determine if sub-micron order, as caused by surfactant templating of the solution prior to cross-linking, can improve the transport properties and wound-healing capabilities of cross-linked chitosan materials. Low-molecular weight chitosan was functionalized with photopolymerizable groups via Michael-addition of 3-(acryloyloxy)-2-hydroxypropyl methacrylate (AOHPMA). Various surfactants were added to induce nanostructure in the solution and subsequent hydrogel polymers. Nanostructure was characterized using small-angle x-ray scattering and polarized light microscopy. The transport properties of the hydrogels, including water uptake and drug release rates, were measured gravimetrically and spectroscopically, respectively. Although only small indications of nanostructure presence were observed, very significant differences in transport properties resulted from the use of varying types and concentrations of surfactant. The results thus indicate that the sub-micron order of these chitosan materials can be manipulated and tailored to specific applications using surfactant templating. The cytotoxicity of the templated hydrogels and non-cross-linked solutions was evaluated using a colorimetric cell viability assay with human fibroblast cells. The cytotoxicity results further enforced the potential of the material as biocompatible. Future studies will investigate the mechanical properties of chitosan hydrogels as well as their effect on the wound-healing process in vivo. Better control of polymer nanostructure and further understanding of its role in cell-material interactions are crucial to the advancement of chitosan biomaterials for use in many applications, including wound-healing, drug delivery, and tissue scaffolding applications.
SUPRAMOLECULAR CATALYSIS IN THE ORGANIC SOLID STATE

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The main task of synthetic chemistry is to design new molecules and materials and synthesize natural products. However, when synthesizing complex molecules, chemists often face almost insuperable limitations largely due to issues of low reaction yields and byproduct formations. On the other hand, the same natural products are synthesized in biological systems with high accuracy, fidelity and almost defect-free. The key of such efficiency of biological systems is hidden in principles of supramolecular chemistry. Supramolecular chemistry employs non-covalent interactions and self-assembly to direct reactivity, exponentially increase yields and ensure strict regio- and stereocontrol. We developed a reliable approach based on the principles of supramolecular chemistry to control reactivity in the organic solid state. In our approach, small-molecule linear templates direct covalent-bond formations via hydrogen bonding.

Catalysis is essential for efficient and sustainable organic syntheses. However, catalysis in the organic solid state is expected to be difficult to achieve due to low diffusion rates of molecules in the crystals. A recently reported example of supramolecular catalysis in the organic solid state will be presented, as well as results of expanding the scope to new substrates. The first reported small-molecule supramolecular catalyst, 4,6-dichloro-resorcinol (4,6-diCl-res), catalyzes a [2+2] photodimerization of trans-1,2-bis-(4-pyridyl) ethylene (4,4’-bpe) to give rctt-tetrakis-(4-pyridyl)cyclobutane (4,4’-tpcb) via hydrogen bonding. To achieve turnover, dry mortar-and-pestle grinding was utilized. A reaction of 2,2’-bpe, catalyzed by a similar ditopic receptor res, is now reported wherein the turnover proceeds spontaneously. The catalyses were investigated using 1H NMR spectroscopy, XRPD analysis and single-crystal X-ray diffraction.
GENETIC CHARACTERIZATION OF CAFFEINE DEGRADATION via N-DEMETHYLATION BY PSEUDOMONAS PUTIDA CBB5

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Pseudomonas putida CBB5 is capable of utilizing caffeine (1,3,7-trimethylxanthine) as the sole source of carbon and nitrogen. Caffeine is metabolized by three sequential N-demethylation reactions to xanthine. The first two steps, N1-demethylation of caffeine to theobromine and N3-demethylation of theobromine to 7-methylxanthine, are catalyzed by two Rieske [2Fe-2S] N-demethylases, NdmA and NdmB, in conjunction with a specific reductase, NdmD. An N7-specific N-demethylase activity, NdmC, co-purified with NdmD, but the two activities could not be resolved by traditional biochemical techniques.

The fraction containing NdmC/D activity was purified to three major bands, as observed by SDS-PAGE, which were submitted for N-terminal sequencing. Simultaneously, a 13.2-kb region of genomic DNA from CBB5 containing ndmA and ndmB was sequenced in order to identify other genes involved in caffeine degradation. This DNA fragment contained one partial and ten complete ORFs. The N-terminal sequences of the three major bands in the NdmC/D fraction matched those of the theoretical proteins encoded in orf9, orf10, and orf11 of the 13.2-kb fragment. The deduced orf10 protein sequence contained a Rieske-type [2Fe-2S] domain at its N-terminal half. Additionally, a flavin binding domain, an NADH-binding domain, and a plant-type [2Fe-2S] domain were identified at its C-terminal half, similar to FNR-type reductases of Rieske oxygenases (ROs). This domain arrangement could represent a unique gene fusion of a ferredoxin gene and a RO reductase gene into a single ORF.

orf10 was cloned and expressed as an N-terminal His-tagged fusion protein and purified with a Ni-NTA column. His6-orf10 oxidized NADH and reduced cytochrome c concomitantly, similar to several RO reductases. Furthermore, His6-orf10 was capable of passing reducing equivalents to NdmA-His6 and NdmB-His6 to catalyze N1- and N3-demethylation of caffeine, respectively. Thus, orf10 was confirmed as ndmD. orf11 is similar in sequence to many glutathione S-transferases. We hypothesize that orf9 encodes NdmC because its deduced protein sequence contains a deep hydrophobic ligand binding pocket domain plus a catalytic triad motif for non-heme iron, similar to ndmA and ndmB. Interestingly, a Rieske [2Fe-2S] domain was not present in the deduced protein sequence of orf9. Expression of orf9 as recombinant protein for functional characterization is in progress. The deduced functions of the remaining ORFs include two transcriptional regulators, two outer membrane proteins, and a glutathione-dependent formaldehyde dehydrogenase.

N-Demethylation of many xenobiotics and naturally occurring purine alkaloids, such as caffeine and theophylline, are primarily catalyzed in higher organisms, ranging from fungi to mammals, by the well-studied membrane-associated cytochrome P450s. This work represents the first report of soluble Rieske enzymes genotypes for N-demethylation of purine alkaloids from bacteria that enable them to live on caffeine.
INVESTIGATING THE MECHANISM OF HEPATOCYTE INVASION BY MALARIA-CAUSING \textit{Plasmodia} BY DESIGNING AND SYNTHESIZING CHEMICAL PROBES

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The disease malaria is caused by a number of members of the protozoan genus \textit{Plasmodium}. The infection is initiated by a bite from a \textit{Plasmodium} carrying mosquito, which releases the sporozoite form of the parasite into the bloodstream. The parasite must first infect hepatocytes, where it undergoes transformation into merozoites and replicates. This is the liver stage of the parasite. Prevention of hepatocyte infection represents an attractive goal for treatment of malaria.

It has been shown that the trisubstituted pyrrole (TSP) 4-[2-(4-fluorophenyl)-5-(1-methylpiperidine-4-yl)-1H-pyrrol-3-yl] pyridine has activity against erythrocytic stages of the parasite both \textit{in vitro} and \textit{in vivo}. This activity has been shown to be due to the inhibition of cGMP-dependent protein kinase (PKG) in the parasite. Additionally, this TSP has been shown to inhibit sporozoite infection of hepatocytes. Interestingly, a conditional PKG knockout of the sporozoite is still able to infect hepatocytes and the TSP retains the ability to prevent sporozoite invasion of hepatocytes. This suggests that there is another protein target of the TSP that is essential for sporozoites to infect the liver. Identification of this protein would give insight into the mechanism by which sporozoites invade hepatocytes as well as represent a potential new target for the treatment or prevention of malaria.

Described here is the synthesis of molecular probes for the pursuit of identifying the protein targets of TSPs in \textit{Plasmodium} spp. Two different biotinylated TSP derivatives and control analogs, including parent drug and linker-only derivative, have been synthesized. These compounds have been tested against sporozoite infection to evaluate their activity and determine their suitability for use in a protein pull-down to identify the molecular target.
INHIBITION AND PROTEIN MODIFICATION OF GAPDH BY THE ENDOGENOUS NEUROTOXIN-3,4-DIHYDROXYPHENYLACETALDEHYDE

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Parkinson’s disease (PD) is a neurological disorder that affects 1% of the population over age 55. The physiological symptoms include resting tremor, muscle rigidity, bradykinesia and posture instability. However, the pathological mechanism of selective dopaminergic neuronal loss is unknown. Investigating neuronal protein targets is essential in determining the cause of toxicity. Dopamine is oxidatively deaminated and catalyzed by monoamine oxidase to form the endogenous neurotoxin 3,4-dihydroxyphenylacetaldehyde (DOPAL). A reduction in levels of DOPAL is biologically critical as this aldehyde has been shown to be toxic to dopaminergic cells and is a highly reactive electrophile. An essential protein-GAPDH (e.g., glyceraldehyde-3-phosphate dehydrogenase) is an abundantly expressed enzyme known for its glycolytic activity and recent research has implicated its role in oxidative stress-mediated neuronal death. GAPDH has been shown to be highly susceptible to covalent modification and inactivation by DOPAL. Upon treatment of DOPAL (5-25 µM), enzyme activity was significantly inhibited compared to control. Extensive protein crosslinking by DOPAL was observed and is augmented in the presence of pro-oxidant metals such as Cu^{2+}. Given GAPDH’s intracellular abundance and its pivotal role in multiple metabolic/apoptotic pathways, compromise on enzymatic activity may have devastating effects on cellular homeostasis. Thus, GAPDH is a viable and possible target of modification by DOPAL. In addition, the intracellular formation of GAPDH may serve as a cellular phenotype in neurodegenerative disorders.
THE ROLE OF ENZYME DYNAMICS IN THYMIDYLATE SYNTHASE CATALYZED REACTION: A COMBINED EXPERIMENTAL - THEORETICAL APPROACH

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How enzyme dynamics contributes to their functions has been a hotly debated topic in recent years. An interactive approach where experimental and theoretical studies examine the same chemical transformations during enzyme catalysis is instrumental in addressing this issue. Thymidylate synthase (TSase) catalyzes the biosynthesis of 2’-deoxythymidine-5’-monophosphate (dTMP, one of the four DNA bases) in most organisms including humans. The mechanism of TSase comprises a series of bond cleavages and formations including activation of two different C-H bonds: a rate-limiting C-H-C hydride transfer and a non-rate-limiting C-H-O proton transfer. This provides an excellent model system to examine the nature of different bond activations along the same catalytic cascade. We have developed both experimental and theoretical methods to investigate the nature of both C-H cleavages, primarily using temperature dependence of kinetic isotope effects (KIEs), site-directed mutagenesis, and quantum mechanical/molecular mechanical (QM/MM) calculations. Our experimental studies with the wild type E. coli TSase have revealed temperature-independent KIEs for the hydride transfer step and temperature-dependent KIEs for the proton transfer step. The QM/MM calculations on the hydride transfer step have suggested significant coupling between protein dynamics and the reaction coordinate, which limits the sampling of reaction paths to increase the H-transfer probability, leading to temperature-independent KIEs. The theoretical studies on the proton transfer step have suggested a mechanism different from the conventionally proposed one, and the role of enzyme dynamics in this step is being investigated. The synergistic enhancement of the experimental and theoretical efforts in this project will not only provide a deeper understanding of the TSase mechanism, but also illustrate the role of enzyme dynamics in different chemical steps during catalysis.
NOVEL ANTIMUTANT C-7 ARYL FLUOROQUINOLONES

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Antibiotic resistance eventually emerges in the treatment of most bacterial infections because current pharmaceutical practices place antibiotic dosing levels directly within the mutant selection window (MSW). The MSW is a dosing range between the minimum inhibitory concentration (MIC) of wild-type bacteria and the mutant prevention concentration (MPC) of the least susceptible mutant populations. Dose levels below the MIC do not effectively lower bacterial populations to combat disease, and dose levels above the MPC are possibly toxic. Though doses within the MSW effectively treat infection, the resultant selection of mutant strains has given rise to highly resistant bacteria such as multi-drug resistant (MDR) and extensive drug resistant (XDR) *M. tuberculosis*. The programmatic goal of the work presented here is to develop novel approaches for the development of new antibiotics that restrict the emergence of resistant strains of *M. tuberculosis* by having a very narrow MSW. In the studies presented here, computer modeling was employed to generate further understanding of the drug binding sites in topoisomerase IV and DNA gyrase A, the protein targets of ciprofloxacin and other antibiotics, to design new antimutant fluoroquinolones. The synthesis of aryl-substituted C-7 fluoroquinolone analogues will be described and their activity with wild-type and fluoroquinolone-resistant bacteria will be presented.
APPROACHES TO SYNTHESIS OF THE STELLETTINS

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The stellettins (e.g. 1–4), a small family of natural products isolated from marine sponges, have been of interest because they exhibit potent and differential cytotoxicity in the National Cancer Institute’s (NCI) 60-cell line assay. Furthermore their pattern of activity does not correlate to any known mechanism of action. For some years our group has pursued synthesis of the schweinfurthins, and many of the goals of that program have been met. While there is little similarity of structure between the schweinfurthins and the stellettins, there is a strong correlation between their profiles of biological activity, with the most prominent activity against CNS, leukemia, and renal cancer cell lines. This strong correlation suggests that both families of compounds affect the same target or at least the same biological pathway, but their target remains unknown. Furthermore, the stellettins are both more potent and more selective than the schweinfurthins. Because the stellettins are not readily available from their natural source, we have decided to pursue a chemical synthesis of these complex natural products.

Synthesis of stellettin A has been divided into a left and right half, and poses two main challenges: stereochemistry of the left half ring system and the polyene tail in the right half. The stellettins feature a trans-syn-trans tricyclic core that causes the B ring to be in a strained twisted boat conformation. Therefore, care must be taken to control the stereochemistry around this ring so that the trans-anti-trans system is not formed. Installation of the polyene tail late in the synthesis of stellettin A is imperative because it isomerizes and decomposes when exposed to light, so our initial efforts have focused on preparation of the tricyclic core. Key steps in the formation of the left half include oxidation of farnesol, Wittig olefination, hydroboration-oxidation to provide homofarnesol, bromination of the alcohol, and a Shi epoxidation to introduce a stereogenic center. The Shi epoxidation will be followed by a cascade cyclization initiated by a Lewis acid, which opens the epoxide to afford the desired carbocycle. Our synthesis of the stellettin family will allow a more in-depth exploration of the biological activity of these rare but very potent compounds.

1 R=R’=O, 13-E = stellettin A
2 R=R’=O, 13-Z = stellettin B
3 R=H, R’=OAc, 13-E = stellettin C
4 R=H, R’=OAc, 13-Z= stellettin D
MODULAR AND EXPANDABLE DETECTION PLATFORM FOR CURRENT AND POTENTIAL FOOD TOXINS AND ADULTERANTS: SCREENING AND IDENTIFICATION OF MICROORGANISMS CONTAINING RICININE NITRILASE ACTIVITY

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Background: Food security is susceptible to real or perceived danger of chemical contamination to the food supply. The establishment of a rapid, inexpensive detection system/technique to specifically detect harmful chemicals in food will greatly alleviate the potentially damaging impact on food security in case of food supply contamination. The Department of Homeland Security has identified the need to detect Ricinine/Ricin as high priority. Ricinine itself is a toxic compound that is also a marker for ricin, a highly toxic protein. Ricinine is also representative of a number of known organo-nitrogen toxins, such as cyanide, strychnine (rat poison), botulism toxin, etc. The presence of a nitrile group in all of them provides a target for detection by nitrilase. In this study, identification of microorganisms containing ricinine nitrilase activity was (i) screened from CBB/UI Culture Collection, and (ii) isolated via soil-enrichment.

Methods: Both ricinine and related analogs 2-Hydroxy-3-cyanopyridine (HCP) and 3-Cyano-2-hydroxy-6-methylpyridine (CHMP) were tested as substrates or used as enriching agents to select organisms from soil samples. A rapid colorimetric assay, indophenol blue method (Berthelot assay) was used for screening nitrilase activity and quantitative detection of ammonia/ammonium formation.

Results: Screening for ricinine nitrilase activity from (i) UI Culture Collection and (ii) isolation of ricinine utilizers via soil enrichment were conducted in three phases. In Initial Screening (Phase 1), 54 microorganisms selected from the CBB/UI Culture Collection and 34 morphologically different colonies isolated via soil enrichment, were screened for their ability to produce nitrilases specific for ricinine analogs, HCP and CHMP. Seventeen bacterial strains from CBB/UI Culture Collection and 14 strains isolated from soil enrichment were found have ammonia-formation activity. Quantitative detection of ammonia/ammonium formation from Phase 1 positives were conducted in Phase 2 screening. Six bacterial strains from CBB/UI Culture Collection and 9 strains from soil enrichment exhibiting activity on HCP and CHMP were selected to test ammonia formation with ricinine in Phase 3. Three Strains, Acinetobacter AP1200, Pseudomonas sp.4NT and Burkholderia cepacia R34 from CBB/UI culture collection, and three soil enrichment strains, SE No.13, SE No.21 and SE No.28 showed activity on ricinine. Nitrilase activity on ricinine, HCP and CHMP of these 6 strains was further confirmed in the crude cell extract of the selected strains. All six strains have been submitted for sequencing in order to annotate nitrilases and clone them for activity confirmation.
CONTINUOUS NEAR INFRARED MONITOR FOR PICHIA PASTORIS BIOREACTORS

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ASL Analytical is currently developing a real-time monitor for controlling and optimizing the production of bio-therapeutics and industrial enzymes by protein expression in *Pichia pastoris*. The ASL *Pichia* monitor continuously and noninvasively follows the concentrations of glycerol and methanol and the accumulation of cell biomass density during the fermentation process. Glycerol and methanol are the principal carbon sources for *Pichia pastoris* and the ability to monitor, control, and optimize the concentrations of these substrates is critical for efficient production of the targeted protein product. Currently, key analytes are monitored using off-line analyses that are time consuming and labor intensive, which makes optimization of reactor productivity difficult. The ASL *Pichia* monitor operates by passing specific wavelengths of near infrared light through a sample of the fermentation broth. The concentrations of glycerol and methanol, and the biomass level, are determined from a real-time analysis of the resulting near infrared spectrum. Successful concentration measurements rely on the concept of “Constrained Variance”, an ASL innovation that reduces sources of spectral variance and focuses the measurement on the molecular structure of the targeted analyte. Additionally, the ASL *Pichia* monitor can be used to provide closed-loop feedback control of both glycerol and methanol concentrations during fermentations.
CONTINUOUS PROTEIN PURIFICATION USING SIMULATED MOVING BED: TAKING THE CHROMATOGRAPHY PLATFORM TO THE NEXT LEVEL

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Chromatography as a bioprocessing platform has not kept pace with the demand for lower costs, higher flexibility, and higher productivity in the manufacture of protein-based pharmaceuticals. Simulated moving bed chromatography (SMBC) elevates the chromatographic platform by conversion of the conventional batch process to a continuous process. SMBC has been successfully applied to small and large-scale binary separations of hydrocarbons, sugars, and enantiomers but has rarely been used for protein purifications. The highly efficient zones of separation created by SMBC enable continuous bind-wash-elute chromatography and peak shaving of high purity target protein. A bench-scale SMBC instrument was employed to investigate several purification methods including Protein A, Protein G, immobilized metal affinity and size exclusion chromatography. The results demonstrate that the dramatic increases in productivity and purity SMBC offers for small molecule separations can also be realized for recombinant proteins and monoclonal antibodies. These advantages can help alleviate the bioprocessing bottleneck created by increased protein production upstream making SMBC an improved process alternative to single column methods.
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