

SMASH 2004 NMR Conference

Breckenridge, Colorado

September 12th - 15th, 2004

SMASH 2004 NMR Conference

Dear SMASH 2004 attendees,

Welcome to Breckenridge, Colorado and the 5th SMASH NMR Conference. This year, the program encompasses many divergent areas of small-molecule research and includes the following Sessions: New NMR Experiments, Natural Products and Small Organic Molecules, Dynamic NMR Spectroscopy, Carbohydrates, Small Molecule Solid-State NMR Applications, Small Molecule F-19 NMR Applications, Post-Acquisition Data Processing and Post-Graduate Research. In addition, there is an evening Poster Session with a buffet dinner and Workshops on the following topics: Molecular Modeling, Tips and Techniques, Practical N-15 NMR, and Cryoprobos. Finally, we are honored to have an exceptional NMR spectroscopist and accomplished organic chemist, Frank Anet, as our keynote speaker for the conference banquet.

On behalf of the entire SMASH Organizational Committee, we wish to thank you for your continued interest in, and support of, the SMASH NMR Conferences. We hope that you enjoy the Conference and the beautiful Rocky Mountains. In this regard, please bear in mind that Beaver Run's elevation is nearly 10,000 ft. You can avoid dehydration and minimize altitude problems by drinking plenty of water.

With warmest regards,

Dave Lankin and Gene Mazzola

Co-Chairs, SMASH 2004 NMR Conference

SMASH 2004 NMR Conference Program

Sunday September 12th

5:00 PM - 6:00 PM	Registration
6:00 PM - 8:00 PM	Dinner
8:00 PM - 11:00 PM	Mixer

Monday September 13th

7:00 AM - 8:15 AM	Breakfast
8:15 AM - 8:30 AM	Opening Remarks
8:30 AM - 10:00 AM	New NMR Experiments - Bruce Hilton <ul style="list-style-type: none">• <i>Microflow NMR: Capabilities and Applications of the CapNMR Approach</i> Tim Peck - MRM• <i>Accelerated Structure Elucidation Using FT/MS and CapNMR Spectroscopy</i> Mark O'Neil-Johnson / Sequoia Sciences• <i>Cryo-LC-NMR and Peak Trapping Applications</i> Adrian Davis / Pfizer
10:00 AM - 10:30 AM	Break
10:30 AM - 12:00 PM	Natural Products and Small Organic Molecules - Bill Reynolds <ul style="list-style-type: none">• <i>Small Molecules at Sea - NMR in Studies of Fish Disease, Stress and Nutrition</i> John Walter - NRC Institute of Marine Biosciences• <i>The Terrestrial Environment: The Largest Pool of Natural Products on Earth ?</i> Andre Simpson - University of Toronto• <i>Relative Stereochemical Determination of β-Hydroxy Carbonyl Compounds (Aldol Products) Utilizing the J-Based Configuration Analysis Method</i> Brian Marquez - Pfizer
12:00 PM - 1:30 PM	Lunch
1:30 AM - 3:00 PM	Dynamic NMR Spectroscopy - Alex Bain <ul style="list-style-type: none">• <i>Dynamics of Molecular Reorganization of Some Ion-Pairs and Some Internally Solvated Organolithium Compounds</i> Gideon Fraenkel - The Ohio State University• <i>Conformational Studies of Organic Compounds by Dynamic NMR Spectroscopy and Computational Methods</i> Eric Noe - Jackson State University• <i>Rotational Mechanisms and Rates in Sterically Hindered Molecules</i> Eric Johnston - University of North Carolina at Greensboro
3:00 PM - 3:30 PM	Break
3:30 PM - 5:00 PM	Workshops (Concurrent) <ul style="list-style-type: none">• I. <i>Tips & Techniques</i> - Thomas Williamson• II. <i>Using Modeling with NMR</i> - James P. Snyder
5:00 PM - 6:00 PM	Free Time
6:00 PM - 6:30 PM	Pre-Dinner Social Gathering
6:30 PM - 9:00 PM	Social Dinner / After Dinner Speaker: Frank Anet
9:00 PM - 11:00 PM	Mixer

SMASH 2004 NMR Conference Program

Tuesday September 14th

- 7:00 AM - 8:30 AM **Breakfast**
- 8:30 AM - 10:00 AM **Carbohydrates** - Bruce Coxon
- *Probing Structure and Dynamics in Disaccharides Using Residual Dipolar Couplings*
Daron Freedberg - FDA
 - *NMR Scalar Couplings in Isotopically Labeled Oligosaccharides: New Correlations with Molecular Structure*
Anthony S. Serianni - University of Notre Dame
- 10:00 AM - 10:30 AM **Break**
- 10:30 AM - 12:00 PM **Small Molecule Solid-State NMR Applications** - Fred Vogt
- *Solid-state NMR Spectroscopy of Pharmaceuticals: Challenges and Applications*
Eric J. Munson - University of Kansas
 - *Through-Bond ^{13}C - ^{13}C Correlation at the Natural Abundance Level: Refining Dynamic Regions in the Crystal Structure of Vitamin-D3 with Solid-State NMR*
Jochem Struppe - Bruker BioSpin Corp
 - *MAS NMR of Nanocrystalline Proteins*
Kurt Zilm - Yale University
- 12:00 PM - 1:30 PM **Lunch**
- 1:30 PM - 3:00 PM **Small Molecule Fluorine-19 NMR Applications** - Steve Cheatham
- *^{19}F Filtered NMR Experiments: An Alternative to Chromatography?*
Thomas O'Connell - GlaxoSmithKline
 - *Use of 2D ^{19}F - ^{19}F NOESY for the Assignment of Fluorochemicals*
Richard Newmark and John Battiste - 3M
 - *Two Phases of Fluorine NMR: Gas Phase and Solution Studies*
Chris Roe - DuPont
- 3:00 PM - 3:30 PM **Break**
- 3:30 PM - 5:00 PM **Workshops (Concurrent)**
- I. *Practical ^{15}N NMR* - Gary E. Martin
 - II. *Cryoprobes* - Richard J. Upton
- 5:00 PM - 6:00 PM **Free Time**
- 6:00 PM - 6:30 PM **Pre-Dinner Social Gathering**
- 6:30 PM - 8:00 PM **Dinner**
- 8:00 PM - 10:00 PM **Poster Session with Mixer**
- 10:00 PM - 11:00 PM **Mixer continues**

SMASH 2004 NMR Conference Program

Wednesday September 15th

- 7:00 AM - 8:30 AM **Breakfast**
- 8:30 AM - 10:00 AM **Post - Graduate Session - Laura Lucas**
- *Improved Transient NOE Buildups*
Kristin Cano - UC Irvine
 - *Characterization of the Metabolic Actions of Natural Stresses in the California Red Abalone, *Haliotis rufescens*, Using ¹H NMR Based Metabolomics*
Eric Rosenblum - UC Davis
 - *NMR Studies of the Interactions of Peptides with Heparin*
Jing Wang - UC Riverside
- 10:00 AM - 10:30 AM **Break**
- 10:30 AM - 12:00 PM **Post-Acquisition Data Processing - Eriks Kupce**
- *Protein NMR Without the Protein: Using Filter Diagonalization to Analyze High-Dimensional Small-Molecule NMR Spectra*
A.J. Shaka - University of California at Irvine
 - *Hadamard and Radon Transforms in NMR*
Eriks Kupce – Varian Ltd.
 - *Digital Resolution: Its Determination and Enhancement by Zero Filling and Linear Prediction*
Eugene Mazzola - University of Maryland – FDA Joint Institute
- 12:00 PM - 12:15 PM **Closing Remarks**
- 12:15 PM - **Box Lunch and Departure**

SMASH 2004 NMR Conference Acknowledgements

The SMASH 2004 Conference gratefully acknowledges the support provided by the following companies.

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Poster Session

Gregory Nemeth
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Monday, September 13th 8:30 AM - 10:00 AM

New NMR Experiments

Bruce Hilton, Session Chair

Speakers:

Tim Peck, Protasis/MRM

Mark O'Neil-Johnson, Sequoia Sciences, Inc.

Adrian Davis, Pfizer Global R&D

Microflow NMR: Capabilities and Applications of the CapNMR Approach

T.L. Peck¹, J.A. Norcross¹, D.L. Olson¹, D.J. Detlefsen², D.M. Leatzow³, and R.W. Albrecht⁴

1. Protasis/MRM, 101 Tomaras Avenue, Savoy, IL 61874

2. Novatia, 301A College Road East, Princeton, NJ 08540

3. Protasis/Metaphoresis, 1345 N.E. Terreview Dr., Sect. D, Pullman, WA 99163

4. Protasis Corporation, 734 Forest St., Marlboro, MA 01752

The pressing demand to have NMR meet the analytical challenges of the coming years forces changes to the techniques, hardware, and software employed. In drug discovery, the need to collect greater information earlier in the discovery process requires that instrument designers remain committed to “faster, cheaper, better.” A myriad of complicated and sometimes seemingly contradictory design challenges arise that include integration, reliability, functional complexity, operator simplicity, molecular diversity, economy, automation, efficient packaging, and speed. The advancement over the past decade of high-resolution NMR has involved a technological drive toward high field magnets, high sensitivity detection probes, new pulse sequences, and new methods of spectral interpretation. While continued advancement of these component technologies will result in further gains in NMR performance, these alone will not suffice to maintain NMR’s utility alongside complementary analytical techniques for routine laboratory analysis. Systems must evolve to meet new demands. Already requiring serious consideration prior to instrument purchase are choices that include hyphenated configurations vs. stand-alone platforms; the dedication of a magnet to a single probe vs. accommodation of multiple probes; the means of sample introduction (flow vs. tubes); and accommodation of users (open access vs. dedicated spectroscopist). Several trends seem necessary to meet future demands placed on NMR. These include the analysis of smaller quantities of sample, gathering of greater breadth of information in shorter time, and automated decision-making. Protasis/MRM offers a suite of automated NMR detection and sample management products. The year 2004 represents several significant milestones, including higher sensitivity flow cell offerings for 5 and 10 microliter volume samples, new software for unattended automation, CTC (Leap Technologies) liquid handler support, and development of software to streamline analyses and leverage increased sample throughput (Novatia). This talk describes ongoing and future developments at Protasis and its affiliates in microfluidics and capillary NMR, and illustrates a positioning of this instrumentation strategy toward the themes of technological advancement outlined above.

Accelerated Structure Elucidation Using FT/MS and CapNMR Spectroscopy

Mark O'Neil-Johnson and Eliane Garo

Sequoia Sciences, Inc.
11199 Sorrento Valley Rd.
San Diego, CA 92121

Natural product chemistry has traditionally been a long and time-consuming process for drug discovery research. From extraction, isolation and purification to structure elucidation, active fraction identification is a real challenge in creating value for today's HTS programs in large pharmaceutical companies. Sequoia Sciences has developed rugged HPLC extraction and isolation methodologies for purified natural product chromatographic fractions that fit right into today's HTS screening platforms. Sequoia has also overcome the barrier of structure elucidation on minute quantities, i.e. microgram quantities of active natural product compounds, a necessary requirement in satisfying the "H" criteria in high-throughput screening programs. A successful natural product discovery program in today's drug discovery process must have all of these elements of the isolation process rapidly completed and validated as well as rapid structure elucidation to follow up on leads. This must be done on scales approaching traditional screening quantities.

With the introduction of the CapNMRR probe, new opportunities were created to take the sample requirements down to the microgram scale. Sequoia has previously published papers on its complete process as well as several papers on the use of the CapNMRR probe in working on mass limited samples. In our quest to become better and faster in providing the structure of an active unknown to the drug discovery process, Sequoia has created addition speed and value with the addition of an FT/MS to the process. For full NMR data set acquisitions on compounds from challenging HPLC isolations at the single digit microgram scale, utilizing minimal NMR data with high resolution MS data, Sequoia is able to accelerate the discovery of active and novel compounds from natural product sources.

Data will be presented demonstrating routine acquisition of proton and COSY on 1-10 micrograms of material originating from complex HPLC separations of natural products. With the FT/MS and its SORI technique, Sequoia has created a new way for HPLC chemists and NMR spectroscopists to uncover unknown structures at very low amounts without the need to recollect and scale up. By eliminating this process and using modern instrumentation in an intelligent and creative way, natural product research can realize its full potential in drug discovery.

Cryo-LC-NMR and Peak Trapping Applications

Steve Coombes, **Adrian Davis** and Rosalind Richards

Pfizer Global R&D, Sandwich Laboratories, U.K.

The application of LC-NMR-MS for the identification of impurities has been routinely used in our laboratories for a number of years. Data other than simple ^1H , COSY and TOCSY spectra have been difficult to obtain due to sensitivity limits of conventional flow probes. For the analysis of low-level impurities, excessively long experiment times are required to obtain even a simple ^1H spectrum.

With the advance of cryo-probe technology it has become possible to obtain ^1H data from LC-NMR experiments on limited samples 4-12 times more rapidly than with conventional probes. Structurally useful heteronuclear correlation data can also be obtained by the use of pre-concentration or directly from higher-level impurities. This advance is an important step towards increased productivity in an environment of accelerating drug discovery and development.

Herein we describe our experiences at Pfizer, Sandwich with two systems: a dedicated cryo-flow probe and a cold probe with a removable flow cell.

Monday, September 13th 10:30 AM - 12:00 PM

Natural Products and Small Organic Molecules

Bill Reynolds, Session Chair

Speakers:

John Walter, Institute for Marine Biosciences

Andre Simpson, University of Toronto

Brian L. Marquez, Pfizer Global R&D

Small Molecules at Sea: NMR in Studies of Fish Disease, Stress and Nutrition

John Walter¹, Kirty Solanky¹, Andrew Dacanay¹, Ian Burton¹, Shawna MacKinnon¹, Vanya Ewart and William Driedzic²

1. Institute for Marine Biosciences, 1411 Oxford Street, Halifax NS, Canada B3H 3Z1
2. Ocean Sciences Centre, Memorial University of Newfoundland, St Johns NL A1C 5S7

As part of a program on fish health concentrating on commercially-important cold-water marine species, and problems that arise from aquaculture, we are exploring some possible roles for small molecule NMR-based methods. The problems generally fall into the categories of disease, stress and nutrition, and the methods have received relatively little prior application to fish and other marine systems.

In some of these applications we are taking a metabolomic/ metabonomic approach, exploiting the relatively uniform concentration response of NMR across a variety of small molecules when in free solution, an advantage that offsets the low sensitivity compared to other spectroscopic and spectrometric techniques for which response is highly non-linear. With little prior preparation, NMR may be used to survey the complement of metabolites present in cell cultures, biofluids, tissues, organs, or in some cases intact organisms, and to follow changes in response to many classes of physiological perturbation.

One of our first studies of this type concerns furunculosis in salmonids, a bacterial disease with major consequences in commercial aquaculture. The aim is to gain information about the host-pathogen interaction, and to see if progress of the disease might be assessed from samples that could be obtained without harm to the animal. We have found NMR of salmon plasma samples shows trends indicative of covert infection, and distinct from the effects of anorexia that is a common sign of the disease. Another class of experiment for which NMR offers unique information is the use of stable isotope labeling to follow metabolic processes. When used with multiply labeled precursors it is the only method that enables the integrity of bonds between labeled atoms to be monitored, a property exploited in a wealth of metabolic studies of microorganisms, terrestrial animals and plants, but in relatively few of marine animals. We have used this approach to study an unusual example of cold adaptation: the production of glycerol in very high concentrations as an antifreeze agent by rainbow smelt (a species closely related to salmon) when exposed to seawater temperatures below 0°C. NMR spectra of liver samples show the profound effects of temperature on the distribution of major metabolites, and provide convincing evidence for the processes leading to the extraordinarily high rate of glycerol production.

Other investigations in progress, including further studies of disease, effects of heat shock, nutritional supplements and maturation, are providing opportunities to assess the comparative advantages of NMR, MS and IR methods, and may be also be discussed.

The Terrestrial Environment: The Largest Pool of Natural Products on Earth ?

Andre Simpson¹, Brent Lefebvre², Arvin Moser², Kirill Blinov², and Manfred Spraul³

1. Department of Chemistry, University of Toronto, Canada.
2. Advanced Chemistry Development, Toronto, Canada.
3. Bruker BioSpin, GmbH, Rheinstetten, Germany.

Non-living natural organic matter (NOM) is ubiquitous in the oceans, atmosphere, sediments, and soils. It is generally accepted that NOM is present in the environment as complex organic mixtures which are for the large part “molecularly uncharacterized”. There is a lot of interest in NOM, for environmental reasons, as it is known to play important roles in the transport of xenobiotics, heavy metals, bacteria such as *E. coli*, the reproduction of invertebrates, global warming, and the carbon, nitrogen, and oxygen cycles. Thus it is imperative to understand these fundamental constituents in nature if we are to understand their role in the environment. However, these complex mixtures also represent the most abundant organic carbon reserves on earth, with over 3 times the organic carbon found in all living organisms. These mixtures likely contain many novel structures, resulting from a plethora of microbial and chemical transformations which occur in the environment; many of which could have potential pharmaceutical or other commercial applications.

Unfortunately the complexity of NOM does not facilitate its analysis by conventional analytical techniques. It is insoluble in most organic solvents and novel structures cannot be identified by the limited information that can be obtained from LC-MS methods alone. Thus information obtained from NMR is critical to further our understanding of these mixtures.

This talk will initially consider the intact mixtures and the information which can be gained through a range of conventional NMR approaches and diffusion based methods. Such approaches allow us to assess the major structural classes of components present and their abilities to form stable colloids. Secondly, emphasis will be placed on various hyphenated NMR based methods used in combination with Computer Assisted Structure Elucidation (CASE) with the goal to identify complete unknowns and potentially novel structures.

Relative Stereochemical Determination of β -Hydroxy Carbonyl Compounds (Aldol Products) Utilizing the J-Based Configuration Analysis Method

Brian L. Marquez

Pharm. Sciences NMR Laboratory
Pfizer Global R&D
Groton CT, 06430

Beta-hydroxy carbonyl moieties (Aldol products) are common features in a variety of molecular structures. Whether this structural motif is synthetically prepared or incorporated into a natural product, the determination of the anti or syn (threo and erythro, respectively) relationship between the a and b centers using previously reported NMR methods has, up to this point, been unreliable. Herein, we report a method to determine the relative configuration of Aldol products through the combined use of $^3J_{HH}$ homonuclear and, $^nJ_{CH}$ long-range heteronuclear coupling constants in tandem with dipolar couplings in a manner related to the previously described J-based configuration analysis method.

Monday, September 13th 1:30 PM - 3:00 PM

Dynamic NMR Spectroscopy

Alex Bain, Session Chair

Speakers:

Gideon Fraenkel, Ohio State University

Eric A. Noe, Jackson State University

Eric R. Johnston, University of North Carolina

Dynamics of Molecular Reorganization of Some Ion-Pairs and Some Internally Solvated Organolithium Compounds

Gideon Fraenkel

Ohio State University, Columbus, Ohio

Organolithium compounds assemble into a variety of structures both unsolvated: octahedral hexamers and cubic tetramers and solvated: cubic tetramers, Li bridged dimers and monomers as well as different ion-pairs. Structures have been elucidated from the multiplicities of C-13 resonances due to Li bound carbon together with different NOE procedures. These compounds reorganize rapidly at equilibrium on the NMR time scale via carbanionic inversion, bond rotations, bimolecular C-Li exchange and Li ligand exchange. NMR line shape analysis using our density matrix treatment has been used to determine the dynamics of these processes and in some cases mechanisms have been proposed. For example averaging of the directly bonded Li-6 - C-13 coupling directly provides the rate of bimolecular C-Li exchange. Examples will be given of compounds undergoing some of these processes. In particular we will describe the behavior of selected ion-pairs and some internally solvated organolithium compounds.

Conformational Studies of Organic Compounds by Dynamic NMR Spectroscopy and Computational Methods

Eric A. Noe

Department of Chemistry, Jackson State University, Jackson, MS

Conformational studies of several compounds, including cycloundecane, cyclodecane, and formic acid, will be described. Cycloundecane showed eleven sharp peaks in the low-temperature carbon NMR spectra, corresponding to a C₁ conformation, which was assigned to the [12323] conformation on the basis of these spectra, calculations of ground-state and transition-state energies, and the findings by other workers of this conformation and/or the [335] conformation in the solid state using X-ray crystallography for derivatives of cycloundecane. Additional broad absorption in the low-temperature carbon spectra was attributed to the [335] conformation. This conformation also has C₁ symmetry, but partial averaging of peaks is expected at -182 degrees. MM3 strain energies for cycloundecane, reported earlier by Saunders, were repeated in this study. In addition, MM4 and ab initio calculations were performed in order to obtain geometries, free energies, and NMR chemical shifts for various conformations. Barriers for conformational changes obtained by Kolossvary and Guida with the MM2 force field are compared with those obtained by other methods.

Cyclodecane was shown to exist at low temperatures as a mixture of conformations, consisting mostly of the boat-chair-boat(BCB) with a small amount of the twist-boat-chair-chair. Rate constants for equilibration of carbon sites in BCB cyclodecane by way of the TBC conformation, which predicts that C-1 exchanges with C-4, etc., were calculated by approximating the BCB as a 5 spin system. The calculated spectra reproduced the general features of the experimental BCB spectra, and a free-energy barrier of 5.73 kcal/mol was obtained for -137.4 degrees.

Slow exchange between E and Z conformations of formic acid was found by a low-temperature NMR study in an ether-Freon solvent, although evidence was obtained that exchange of the acidic proton between molecules having the Z conformation is a faster process than E-Z interconversion. Dynamic NMR studies of trifluorothioacetic acid will be described in connection with this observation. Signals for E and Z conformations of propynoic acid were also observed.

Rotational Mechanisms and Rates in Sterically Hindered Molecules

Eric R. Johnston¹, Joseph E. Darty², and James C. Barborak¹

1. The University of North Carolina at Greensboro

2. The University of Texas at Austin

Dynamic NMR studies are presented of correlated bond rotation in some sterically congested molecules. The movement of the rings in these systems is strongly coupled and correlated motion becomes energetically preferred over independent bond rotation. Such interlocked behavior in suitably designed systems has previously led to the synthesis of molecular analogs of brakes, switches, and gears. Triptycene-based three-toothed gears and two-toothed gears based on aromatic tertiary amides are noteworthy examples. The presentation describes the design, synthesis and NMR investigation of several of the latter class of compounds. Ring rotations about the aryl-C=O and amide C-N bonds occur largely or entirely concertedly. We address chirality in some of these molecules which do not formally possess chiral centers, atropisomerism, gear slippage, the experimental demonstration of concerted rotation and the measurement of rates and activation parameters by a variety of NMR methods. A bonus is the indirect quantitative observation (via scalar coupling to carbon-13) of deuterium quadrupolar relaxation in perdeuterated chlorobenzene, a solvent used for some of the high-temperature dynamic NMR experiments.

Tuesday, September 14th 8:30 AM - 10:00 AM

Carbohydrates

Bruce Coxon, Session Chair

Speakers:

Darón I. Freedberg, CBER/FDA

Anthony S. Serianni, University of Notre Dame

Probing Structure and Dynamics in Disaccharides Using Residual Dipolar Couplings

Richard M. Venable, Scott E. Norris, Susan O. Ano, and **Darón I. Freedberg**

CBER/FDA, Bethesda Maryland

Carbohydrate structure-function relationships are not well-developed, because their three-dimensional structures are not easily determined. But carbohydrate three-dimensional structure is difficult to determine in solution because long-range distance or angular data has been sparse. Ideally, one would like to determine the spatial and angular relationships between remote atoms. RDCs (residual dipolar couplings), dipolar coupling measured under partially oriented conditions, allow the relation of all bond vectors to a common alignment frame, thus facilitating three-dimensional structure elucidation. RDCs have been successfully applied to many biomolecules. However, accurate structure determination in carbohydrates is complicated by the fact that a minimum of five independent RDCs are required for the equations used to fit RDCs to structures. In addition to the near parallel orientation of the C-H bonds in pyranose rings, carbohydrates are usually not carbon labeled. Thus, many RDCs are needed in order to ascertain carbohydrate three-dimensional structure.

We have measured 35 RDCs for sucrose, 23 for the glucopyranosyl ring and 12 for the fructofuranosyl ring, including one- and two-bond ^1H - ^{13}C RDCs, three-bond ^1H - ^1H RDCs and one-bond ^{13}C - ^{13}C RDCs. We fit each of the rings separately and find that the alignment tensors for the two rings are different. This suggests that the two rings are moving relative to one another. We also fit single structures of sucrose and find that multiple conformations are indicated. Finally, we use new methods to fit one set of RDCs to two structures. We find that the fits for the two structure models are better than the single structure fits. Further, we find that the two dominant conformations are found in the $\phi = 120$, $\psi = 270$ and the $\phi = 45$, $\psi = 300$ regions. The results will be compared to molecular mechanics calculations.

NMR Scalar Couplings in Isotopically Labeled Oligosaccharides: New Correlations with Molecular Structure

Anthony S. Serianni

Department of Chemistry and Biochemistry
University of Notre Dame
Notre Dame, Indiana 46556

We are interested in determining the conformational and dynamics properties of high-mannose and complex-type N-glycans associated with human CD2 and IgG, respectively, as free oligosaccharides and when bound to protein. These oligosaccharides play critical roles in the biochemistry of these and other human glycoproteins. The role of structural context in determining the conformation and dynamics properties of glycosidic linkages remains an important question to be addressed in glycobiology. Until recently, however, it was difficult to determine experimentally the importance of context because the analytical tools were too coarse to yield the necessary quantitative answers. We will show that scalar trans-glycoside J-couplings (six per phi/psi linkage; $^2J_{COC}$, $^3J_{COCH}$, $^3J_{COCC}$) can be treated quantitatively to yield rotameric populations about each C-O bond. With construction of suitable $^{13}C/^2H$ labeled disaccharides containing all unique linkages found in N-glycans, the complete ensemble of trans-glycoside J-couplings can be measured accurately and as a function of a variable (e.g., temperature). Parameterization of equations pertinent to each coupling can then be accomplished from validated density functional theory (DFT) calculations. The pertinent equations and observed J-couplings are treated by a computer program, GlyFit, to determine rotamer populations for the "isolated" linkage in the disaccharide (reference states). For 1,6-linkages where three bond torsions affect conformation, multiple redundant J-couplings (J_{CH} and J_{CC}) involving the terminal carbons (C4, C5 and C6) on the reducing side of the linkage yield information on omega (C5-C6 torsion) and theta (C6-O6 torsion). Note that psi and theta are the same torsion viewed from a different vantage point. Interestingly, some of these J-couplings, notably $^2J_{CCH}$, are sensitive to both omega and theta, and equations containing both variables can be derived. By solving these multiple equations simultaneously, correlated information about omega and theta can be obtained. This information can be combined with that obtained from J-couplings arising from the non-reducing side of the linkage to give a detailed picture of conformational properties. We will also show that $^2J_{CCH}$ values may serve as constraints on phi in glycosidic linkages, and that $^4J_{CCCCH}/^4J_{COCCH}$ may serve as sensitive constraints on exocyclic hydroxymethyl group conformation (omega). New results on parameterizing $^3J_{HCOH}$ and $^3J_{CCOH}$ scalar couplings, and J-couplings involving N-acetyl side chains, will also be discussed. Armed with these parameters, and with labeled nested fragments of high-mannose and complex-type oligosaccharides, the question of how structural context influences oligosaccharide linkage conformation and dynamics can be addressed.

Tuesday, September 14th 10:30 AM - 12:00 PM

Small Molecule Solid-State NMR Applications

Fred Vogt, Session Chair

Speakers:

Eric J. Munson, University of Kansas

Jochem Struppe, Bruker BioSpin Corp.

Kurt W. Zilm, Yale University

Solid-State NMR Spectroscopy of Pharmaceuticals: Challenges and Applications

Eric J. Munson¹, Dewey H. Barich¹, Joseph W. Lubach¹, Loren J. Schieber¹, Benjamin N. Neson¹, Thomas J. Offerdahl¹, Jonathon S. Salisbury¹, Jacob M. Davis¹, Zedong Dong², and David J. W. Grant²

1. Department of Pharmaceutical Chemistry, University of Kansas, Lawrence, Kansas
2. Department of Pharmaceutics, University of Minnesota, Minneapolis, Minnesota

The ability to effectively deliver solid pharmaceuticals is directly related to the form of the drug in the solid state. This is important because more than 90% of all pharmaceuticals are formulated as solids. Drugs may be formulated in several different states, including amorphous, crystalline, or diluted with excipients. In addition, many drugs exhibit polymorphism, or the ability to exist in two or more crystalline phases that differ in the arrangement or conformation of the molecules in the crystal lattice. We are developing solid-state NMR spectroscopy as a technique for the analysis of pharmaceuticals. We are particularly interested in characterizing the effects of formulation on the properties of pharmaceutical solids.

In this seminar new developments and applications of solid-state ¹³C NMR spectroscopy with cross polarization (CP) and magic-angle spinning (MAS) to study pharmaceuticals will be presented. A new probe design that facilitates high-throughput solid-state NMR will be shown. The ability to quantify the amounts of multiple crystalline and amorphous forms present in formulations of the artificial sweetener neotame will be described. The effects of differences in relaxation parameters and cross polarization efficiencies on characterizing mixtures of forms will be addressed. Finally, correlations of formulation parameters with line widths and relaxation times will be presented.

Through-Bond ^{13}C - ^{13}C Correlation at the Natural Abundance Level: Refining Dynamic Regions in the Crystal Structure of Vitamin-D3 with Solid-State NMR

Ryan A. Olsen¹, Garrett M. Leskowitz¹, Douglas W. Elliott¹, **Jochem Struppe**², and Leonard J. Mueller¹

1. Department of Chemistry, University of California, Riverside, CA 92521

2. Bruker BioSpin Corporation, Billerica, MA 01821

Two-dimensional ^{13}C correlation spectroscopy at the natural abundance isotope level has enabled the application of nuclear magnetic resonance to industrial and academic problems that would be impractical if labeled materials were required. The challenge of natural abundance ^{13}C spectroscopy is the loss in signal intensity due to the 1% isotope concentration of the spins. This loss is particularly acute for correlation spectroscopy, which relies on pairs of nuclei to be spin active, decreasing the sensitivity of the technique by 4 orders of magnitude compared to correlation experiments on uniformly labeled materials. In solids, there are few examples of natural abundance ^{13}C correlation experiments, particularly on molecules with more than a dozen carbon sites. Here we show that ^{13}C natural abundance correlation in solids can be extended to moderately sized molecules, using the uniform-sign cross-peak double-quantum-filtered correlation spectroscopy (UC2QF COSY) to assign the 54 peaks of the solid-state NMR spectrum of microcrystalline vitamin-D3. In this case, comparison between the assigned peaks and ab initio calculations of the chemical shifts based on the crystal coordinates permits a refinement of the average structure in dynamic regions reported as disordered in the crystal structure.

MAS NMR of Nanocrystalline Proteins

Kurt W. Zilm¹, Eric K. Paulson¹, Corey R. Morcombe¹, Lyle A. Crum¹, Van Phan¹,
Vadim Gapanenko² and R. Andrew Byrd²

1. Department of Chemistry, Yale University, New Haven, Connecticut

2. Structural Biophysics Laboratory, National Cancer Institute, Frederick, Maryland

Characterization of lead compounds bound to macromolecular receptors can be of great value in designing better pharmacophores if the resulting structural models are accurate enough. Macromolecular crystallography in many instances has difficulty in determining the state of protonation of either the ligand or receptor binding site, and often does not provide an accurate enough structure to be of use in rational drug design. Solution NMR approaches based on transferred nOes can be much more enlightening, especially if the on/off rates are fast enough and the receptor sufficiently soluble. X-ray diffraction does however have one great advantage in that very weakly binding ligands can be readily complexed by soaking into crystals.

Solid state NMR has great potential for combining the chemical specificity of solution NMR with the advantage of being able to work with much more weakly bound ligands and receptors with insufficient solubility. To realize this potential, new sample preparation protocols, resonance assignment techniques and structure determination methods amenable to small proteins will be required. The use of uniform 2D, ¹³C and ¹⁵N enrichment, 800 MHz operation and MAS rates over 20 kHz makes feasible complete resonance assignment of ¹H, ¹³C and ¹⁵N shifts in small proteins such as ubiquitin. ¹H-¹H structural constraints can be also be obtained in a variety of solid state experiments analogous to ¹H-¹H heteronuclear edited NOESYs. Progress towards using these solid state NMR methods to determine a macromolecular structure will be described. We will also discuss our progress in applying these methods to probing ligand structures in macromolecular complexes. The utility of solid state NMR for characterizing solid protein formulations will also be briefly examined.

Tuesday, September 15th 1:30 PM - 3:00 PM

Small Molecule Fluorine-19 NMR Applications

Steve Cheatham, Session Chair

Speakers:

Thomas O'Connell, GlaxoSmithKline

John Battiste, 3M

D. Christopher Roe, DuPont

^{19}F -NMR Filter Experiments: An Alternative to More Chromatography?

Thomas O'Connell¹, Steve Castellino¹ and V.V. Krishnamurthy²

1. GlaxoSmithKline, Drug Metabolism, RTP NC

2. Varian NMR

The low concentration of analytes and the complexity of the matrix have hampered the application of NMR spectroscopy to the elucidation of drug metabolites from biological fluids. One of the most important advances in this area has been the coupling of HPLC and NMR to provide stop flow analysis. There are still limits to this approach because what may appear to be a single component based on UV may contain a multitude of endogenous compounds that swamp-out detection of the drug metabolite by ^1H NMR. Consequently, we have been interested in experimental NMR methods that will allow us to characterize drug metabolites when the compound of interest is only partially resolved chromatographically.

The use of isotope filters to effectively eliminate the protein background in the NMR studies of biopolymers has been well established. We decided to try a similar approach, taking advantage of the presence of fluorine nuclei in many drugs under development. The idea is to start with a ^1H -pulse sequence which selectively displays all protons having fluorine scalar coupling. Subsequent ^{19}F -filtered experiments build on key resonances through the application of homo- and heteronuclear experiments. In this approach, the metabolite structure is elucidated through a series of experiments, which rely on the effective filtering of endogenous ^1H resonances.

This talk will focus on the advantages and limitations of this approach in metabolite identification from biological matrices.

Use of 2D ^{19}F - ^{19}F NOESY for the Assignment of Fluorochemicals

John Battiste¹ and Richard Newmark²

1. 3M Pharmaceuticals, 3M Center, St. Paul, MN 55144

2. 3M Corporate Research Analytical Laboratories, 3M Center, St. Paul, MN 55144

Two dimensional (2D) NMR is an invaluable technique for the complete analysis and assignment of chemical structures. Although ^{19}F - ^{19}F COSY experiments are routinely used for assignment of fluorochemicals, the ^{19}F - ^{19}F NOESY experiment is rarely described as assignment tool. We will demonstrate that ^{19}F - ^{19}F NOESY experiments can be readily obtained in reasonable time frames on fluorochemical samples and represent an enhancement in the arsenal of 2D ^{19}F NMR experiments. Applications will be presented for assignment of linear perfluorochemical chains and regiospecific assignment of cyclic compounds including a substituted perfluoro tetrahydrofuran and an isomeric mixture of perfluoro 1,3-dimethylcyclohexane.

Two Phases of Fluorine NMR: Gas Phase and Solution Studies

D. Christopher Roe, Paul J. Krusic and Valdimir V. Grushin

Central Research and Development, E. I. Du Pont de Nemours & Company,
Wilmington, Delaware 19880-0328

Although gas-phase NMR is well established for physical studies, it has been generally overlooked as a tool for studying gas-phase reactions. We suggest that gas-phase NMR should be viewed as a powerful tool for quantitative kinetic studies and is particularly useful in organofluorine chemistry. The availability of commercial high-temperature probes with an upper temperature limit of 400°C facilitates in situ studies of both homogeneous and heterogeneously-catalyzed reactions. The experimental details of this technique will be described along with a number of examples, including the pyrolysis of hexafluoropropylene oxide and the thermal decomposition of ammonium perfluorooctanoate. Recent solution NMR studies will be illustrated by the fluorine analog of Wilkinson's catalyst, fluorotris(triphenylphosphine)rhodium.

Wednesday, September 15th 8:30 AM - 10:00 AM

Post-Graduate Session

Laura Lucas, Session Chair

Speakers:

Kristin E. Cano, University of California, Irvine

Eric S. Rosenblum, University of California, Davis

Jing Wang, University of California, Riverside

Improved Transient NOE Buildup

Kristin E. Cano¹, Mari A. Smith¹, Michael J. Thrippleton²,
James Keeler² and A.J. Shaka¹

1. Chemistry Department, University of California, Irvine, CA 92697-2025

2. Department of Chemistry, University of Cambridge, Lensfield Road,
Cambridge CB2 1EW, UK

Zero quantum coherence (ZQC) is known to distort signal line shapes of coupled spins in NMR experiments. For the short mixing times required for distance measurements from the initial linear NOE buildup curve, these distortions can easily overshadow and conceal the appearance of weak nOe signals in a nOe difference experiment. Phase cycling or pulsed field gradients alone cannot remove ZQC signals.

Recently, Thrippleton and Keeler have reported the application of a frequency-swept inversion pulse in conjunction with a strong pulsed field gradient to suppress ZQC signals in one scan. Although the z-filter eliminates an appreciable amount of ZQC, further suppression can be obtained by simply employing two or more in a z-filter cascade. Experimental results clearly show the benefit of using a z-filter in the z-axis followed by another in the x- or y-direction. Combined with improved selection of the target spin, and further improvements that remove multiplet distortions that arise from spin state mixing, we believe we have the current best way to conduct these important experiments.

Characterization of the Metabolic Actions of Natural Stresses in the California Red Abalone, *Haliotis rufescens* Using ¹H NMR Based Metabolomics

E.S. Rosenblum¹, M.R. Viant, M.R.², B.M. Braid³, J.D. Moore³, C.S. Friedman⁴, and R. S. Tjeerdema¹

1. Department of Environmental Toxicology, University of California, One Shields Avenue, Davis, CA 95616, U.S.A.
2. School of Biosciences, The University of Birmingham, Birmingham, B15 2TT, U.K.
3. Bodega Bay Marine Laboratory, PO Box 247, Bodega Bay, CA 94923
4. School of Aquatic and Fishery Sciences, University of Washington, Box 355020, Seattle, WA 98195 USA

Withering syndrome in California red abalone (*Haliotis rufescens*) is caused by the Rickettsiales-like prokaryote (RLP) *Candidatus Xenohaliotis californiensis*. Affected animals undergo metaplastic changes within the digestive gland and it is hypothesized that as digestive gland architecture changes animals lose the ability to digest food. RLP-infection, however, does not necessarily produce signs of withering, and for reasons not yet well understood additional stressors such as elevated seawater temperature appear to influence the development of the disease. Using nuclear magnetic resonance (NMR) based metabolomics we have investigated the effects of bacterial infection, temperature, and food availability, both individually and in combination, on the metabolic status of the red abalone. High-resolution ¹H NMR spectroscopy was particularly appropriate for investigating metabolic status since multiple endogenous metabolites could be quantified rapidly in foot muscle and digestive gland tissues. Food limitation caused dramatic reductions in all classes of foot muscle metabolites while at the same time metabolite levels within the digestive gland were preserved or increased. We also found that food limitation along with the additional stress of elevated seawater temperature led to greater metabolic perturbations in both tissue types than those observed under food limitation alone. RLP infection and food limitation resulted in many of the same metabolic changes within the tissues studied, although the effects of infection were less severe. We observed increased levels of homarine in the digestive gland of both food limited and RLP-infected animals yet only observed increased homarine levels in the foot muscle of infected abalone. These results further support the recently established glucose to homarine ratio for differentiating foot muscle tissue of RLP-infected animals from that of both healthy and starved abalone. Furthermore, we found that the NMR metabolic data correlates well with histological measurements supporting the use of the metabolomics approach for characterizing both normal and pathological events in marine species, particularly during periods of environmentally relevant stress.

NMR Studies of the Interaction of Peptides with Heparin

Jing Wang and Dallas Rabenstein

Chemistry Department, University of California, Riverside

Heparin has been used clinically as a blood anticoagulant for more than half a century. In some situations, it is necessary to neutralize the anticoagulant activity. We are studying several synthetic peptides as potential therapeutic neutralization agents. We report here the results of NMR studies of the interaction of three peptides with heparin.

The three peptides are analogs of heparin interacting peptide (HIP). The amino acid sequences of HIP and the three analog peptides are:

HIP: CRPKAKAKAKDQTK
SGV-HIP: Ac-SRGKAKVKAKVKDQTK-NH₂
SGHV-HIP: Ac-SRGKAHVHAKVKDQTK-NH₂
SGRV-HIP: Ac-SRGKARVRAKVKDQTK-NH₂

The interaction of each analog peptide with heparin was studied by (i) measuring the change in chemical shifts of backbone NH protons, (ii) the intensity of the backbone NH resonances as a function of pH, (iii) the effect of binding on the chemical shifts of heparin and peptide resonances and (iv) the effect of binding on the pK_A values of the lysine side chain ammonium groups.

To assign spectra and to achieve sufficient resolution of resonances to study the binding, 1D and 2D ¹H NMR spectra were measured using BAnd Selective Homonuclear Decoupling (BASHD) experiments. With the 2D BASHD experiments, ¹H-¹H coupled multiplets are collapsed to singlets in the F1 dimension. Examples of 1D and 2D BASHD spectra will be presented. With the increased resolution, it was possible to measure pK_A values from chemical shift-pH titration curves for each of the six lysine ammonium groups of SGV-HIP.

The results indicate that the binding is mostly an electrostatic interaction. Results also indicate that heparin binding protects the peptide NH protons, the ammonium groups of lysine and the NH protons of the guanidinium group of arginine from rapid exchange with solvent.

Upon binding of SGHV-HIP by heparin, the resonance for the A3 proton on the glucosamine residues of heparin is displaced up-field, consistent with a site-specific binding between peptide and heparin in which the imidazolium side chains interact with an imidazolium binding pocket on heparin.

Wednesday, September 15th 10:30 AM - 12:00 PM

Post-Acquisition Data Processing

Eriks Kupce, Session Chair

Speakers:

A.J. Shaka, University of California, Irvine

Eriks Kupce, Varian Ltd.

Eugene P. Mazzola, University of Maryland / FDA

Protein NMR Without the Protein: Using Filter Diagonalization to Analyze High-Dimensional Small-Molecule NMR Spectra

A. J. Shaka¹, Vladimir Mandelshtam¹, Kristin Cano¹, Alpay Dermenci¹, Kathryn Hedges¹, Geoff Armstrong², and Brad Bendiak²

1. Chemistry Dept., University of California, Irvine CA 92697
2. Dept. of Cell and Developmental Biology, UCHSC, Denver, CO 80262

Most of the progress in macromolecular NMR of proteins would not have occurred without the bold step, in the early 1990s, of uniformly enriching the molecule in nitrogen-15, carbon-13, and finally deuterium. Isotopic labeling allows high-sensitivity, high-resolution multidimensional heteronuclear spectra to be obtained, so that overlap in the proton NMR spectrum is removed. Without decent resolution, it is next to impossible to assign the proton resonances, and without a proper assignment a structure is usually not possible to obtain.

Of course, proteins have a number of other advantages: (1) they have a simple repeating motif, with no NMR-inactive nuclei along the important backbone; (2) they can be grown up in a prokaryotic expression system that allows them to be labeled uniformly; (3) the primary structure is usually known beforehand; (4) they have tertiary structure that tends to disperse the chemical shifts of identical amino acids; and (5) there are many bug-free pulse sequences specifically optimized for proteins, including structure calculation software.

Suppose, however, that none of this pertains. Suppose that the sample gives a poorly-resolved proton NMR spectrum, and that the absolute amount of sample makes it essentially impossible to obtain even a 2D natural abundance heteronuclear spectrum within a reasonable time. Suppose that isotopic enrichment of the natural or synthetic product is not possible, and that purification can only be carried out partially. Finally, suppose that essentially no important tertiary structure is present, as in most small-molecule NMR. This puts us back in the "BPTI" protein era. I will try to show that these cases are not hopeless, and that a lot of progress can be made. The focus will be mostly carbohydrate NMR where the spectra are often intractable even at 800 MHz.

Hadamard and Radon Transforms in NMR Spectroscopy

Eriks Kupce¹ and Ray Freeman²

1. Varian Ltd., 28 Manor Rd., Walton-on-Thames, UK

2. Jesus College, Cambridge, UK

Considerable sensitivity gains provided by cryogenic probes coupled with availability of increasingly high magnetic field strength open new possibilities for development of fast methods for data acquisition in NMR spectroscopy. Such methods can speed up multi-dimensional experiments by several orders of magnitude [1]. We shall discuss two new fast multi-dimensional NMR techniques that are based on Hadamard [2] and Radon [3] transforms.

The Hadamard technique employs an extensive array of soft radiofrequency pulses and offers certain practical advantages over the traditional Fourier transform method. The key is the introduction of an encoding scheme based on Hadamard matrices. By eliminating the need for step-by-step exploration of the evolution dimensions in multidimensional spectroscopy, the Hadamard technique can speed up data acquisition by orders of magnitude, paving the way for high-dimensional experiments in a reasonably short time. Practical examples of two- and three-dimensional Hadamard spectroscopy are presented for both small molecules and proteins.

The projection-reconstruction method (PR NMR) [3] is based on the Radon transform and is an alternative scheme for fast multidimensional spectroscopy. This technique is closely related to back-projection methods employed in X-ray tomography where the internal structure of any three-dimensional object is computed by measuring the X-ray absorption in several different directions. In an analogous manner, a three-dimensional NMR spectrum can be reconstructed from projections of the absorption intensity onto planes inclined at different angles. In contrast to the continuous nature of a physiological sample, NMR spectra are discrete and usually well-resolved, presenting a much more favorable case for reconstruction. Only a very small number of different projections suffice to recreate the entire three-dimensional spectrum, speeding up data acquisition by an order of magnitude.

1. Kupce, E. and Freeman, R., *J. Biomol. NMR*, 27, 101–113, (2003).
2. Kupce, E., Nishida, T. and Freeman, R., *Prog. NMR Spectrosc.*, 42, 95-122 (2003).
3. Kupce, E. and Freeman, R., *J. Amer. Chem. Soc.*, 126, 6429–6440, (2004).

Digital Resolution: Its Determination and Enhancement by Zero Filling and Linear Prediction

Eugene P. Mazzola¹ and Que N. Van²

1. University of Maryland-FDA Joint Institute, College Park, MD 20742

2. SAIC-Frederick, NCI, Frederick, MD 21702.

Resolution in NMR, the ability to distinguish two signals, is a source of considerable confusion. It is dependent on magnetic field homogeneity, signal acquisition time, and transverse relaxation times. Information that is lost due to truncation of free induction decays can be restored by zero-filling provided that the minimum acquisition time, for the signals whose resolution is sought, is met or exceeded. In the case of 1D NMR experiments, FID truncation is not usually a serious problem. However, this is not the case for 2D NMR experiments due to the much shorter acquisition times involved. Now one level of zero-filling is especially important in both dimensions. A complicating factor arises from the fact that F1 resolution affects the resolution obtained in F2. A series of DQ-COSY experiments has been carried out to determine the effects of (i) zero-filling in both domains and (ii) linear prediction in the F1 dimension. Both were found to be important, with linear prediction saving a great deal of experimental time. Relatively short T2s and line widths in excess of 3 Hz (due to spin coupling) were found to render unobservable couplings of less than 2 Hz. In addition, apparent active couplings that are erroneously large couplings were also observed in cases where active and passive couplings were very similar in magnitude. Judicious choice and fitting of weighting functions and careful phasing of signals are also important.

Tuesday, September 14th 8:00 PM - 10:00 PM

Poster Session

Sponsored by Varian Inc.

Gregory Nemeth, Session Chair

Index To Posters

1. Through-Bond ^{13}C - ^{13}C Correlation at the Natural Abundance Level: Refining Dynamic Regions in the Crystal Structure of Vitamin-D3 with Solid-State NMR
2. NMR Studies of the Interaction of Peptides with Heparin
3. Probing Structure and Dynamics in Disaccharides Using Residual Dipolar Couplings
4. Small Molecules at Sea: NMR in Studies of Fish Disease, Stress and Nutrition
5. Hadamard and Radon Transforms in NMR Spectroscopy
6. Microflow NMR: Capabilities and Applications of the CapNMR Approach
7. Digital Resolution: Its Determination and Enhancement by Zero Filling and Linear Prediction
8. Characterization of the Metabolic Actions of Natural Stresses in the California Red Abalone, *Haliotis rufescens* Using ^1H NMR Based Metabolomics
9. Solvent Effects on the Chemical Exchange of a Push Pull Ethylene
10. Fully Automatic First-Order Multiplet Analysis (FAFOMA)
11. Improvement of Reliability in NMR Structures of Small Molecules Oriented in Liquid Crystalline Solutions
12. Development and Application of State-of-the-Art NMR Methods to Analytical Challenges Encountered During Drug Discovery and Development
13. HPLC-SPE-NMR, -a New and Efficient Natural Products Research Tool
14. Changes in Spin-Lattice Relaxation Dynamics Upon Processing of Crystalline Organic Solids
15. Some New Aspects of the Out-of-Coil Magnetization Contribution to Signal Detection
16. Using NMR Based Metabonomics to Classify Animals in a Dominant/Submissive Behavioral Model by an Exogenous Biomarker
17. Quality Control in Drug Discovery: Identification and Quantitative Analysis by MicroNMR
18. Multiple-Sample Probe for High-Throughput Solid-State NMR Spectroscopy of Pharmaceutical Solids
19. Chemical Shifts in 4D Structural Analysis of Peptides: Cyclosporin A
20. Identification of an Impurity Observed in a Stability Sample of Naltrexone Base by LC/MS and LC/SPE/NMR.

21. Study of Particle Size of Crystalline Pharmaceutical Solids by Solid-State NMR Spectroscopy
22. Varian's VNMR Chempack in the Open Access GMP Environment
23. Testing NMR Quantitation Parameters
24. Metabolic Profiling of Myocardial Ischemia by Microdialysis and Microcoil NMR
25. Structural Dynamics of a Small, Flexible Model Compound
26. Improved Workflow and Results in the NMR Lab
27. Approaches to the Identification of Impurities in Pharmaceutical Development
28. ^1H NMR Spectra of Renal Carcinomas: Data Mining with Partial Least Squares
29. A Natural Abundance F2 Coupled HSQC (F2HSQC) NMR Experiment for the Measurement of Dipolar Couplings of Chiral Compounds Present in a Chiral Liquid Crystal Solvent and Structural Identification with the use of Molecular Dynamics
30. LC-SPE-NMR: Automated Transfer of SPE-Fractions to 1 mm NMR Tubes
31. Optimization of Analyses: Cross-Divisional Benchmarking Studies
32. Conclusive Determination of Presence of Polymorphic Mixtures by SSNMR
33. Molecular Factor Analysis Applied To Collections of NMR Spectra
34. GraPES: A New Innovation for the Study of Protein Ligand Interactions
35. Rapid Sample Identification: NMRanalyst Dereplication, Verification, and Structure Elucidation
36. A Pure Standard Material for Accurate Quantitative NMR
37. Carbon-Fluorine Correlation Spectra
38. Reaction Monitoring and Optimisation
39. Rapid, High-Resolution 4D NMR Spectroscopy for Structural Determination of Oligosaccharides Derivatized with ^{13}C -Acetyl Groups
40. NMR Characterization of a Glucuronide Metabolite of the Potent, Novel, Non-steroidal Progesterone Receptor Agonist Tanaproget
41. Completely Automated Structure Elucidation
42. Intelligent Bucketing for Metabonomics—Part 1
43. Spectrum-Structure Verification Accuracy
44. Utilizing the Power of ^{31}P NMR Prediction Software for Structural Validation and Data Storage

45. ^{13}C NMR Spectrum of 10 Micrograms of Cholesterol
46. A Test Case for Metabonomics/Metabolomics as a Screening Tool for Neurological Disorders: A Bioinformatics Approach to the Study of Batten Disease
47. A Modified CRISIS-HSQC for Band-Selective IMPRESS
48. Deuterium Isotope Effects in Carbohydrates Revisited. Cryoprobe Studies of the Anomerization and NH to ND Induced ^{13}C Shifts of Acetamido-Deoxy Sugars
49. Structural Studies of Novel Antifungal Lipopeptides from Fungal Culture 38G272
50. Optimization of Sample Parameters for Cryogenic Probes
51. Sample Delivery: From Traditional LC-NMR to Column Trapping Capillary Flow NMR
52. Interfacing NMR with HPLC: The Application of LC-SPE-cryoflow NMR in Pharmaceutical Development
53. Absolute Configuration Assignment of Primary and Secondary Amines by NMR
54. Isolation and Characterization of 16R and 16S Hydroxy-Roquefortine C, Novel Diastereomeric Metabolites from *Penicillium crustosum*
55. Comparison of Stop-Flow LC/NMR and LC/SPE/NMR in the Characterization of Metabolites from *Penicillium roqueforti*
56. Identification of Metabolite Profiles of Canadian Isolates of *Penicillium* by Stop-Flow LC/NMR
57. SepNMR: A System for Isolation, Purification and NMR Data Collection on Trace Components in Mixtures.
58. Application of Metabonomics in Obesity
59. Increasing NMR Sample Availability: Capillary Flow NMR within Lead Discovery
60. Unexpected Chemistry in an NMR Tube: a Caveat Concerning the Use of CDCl_3
61. Study of Acid-Base Interactions in CP-724,714 Salts/Complexes by ^{15}N Solid State NMR
62. Adiabatic Pulse Sequences for Improved Performance in High-Throughput Analysis
63. Weak Alignment of Small Organic Molecules in Small Volume Probes Using a Deuterated Liquid Crystal
64. Combinations of Long-Range HMBC-Type Experiments

65. Automated NMR workflow at UCB Pharma
66. Investigations of Ligand/Peptide Interactions in a Membrane System by NMR
67. The NMR Stereochemical Distinction of Cyclic Sulphites or What to do When Long-Range Correlations are
68. Absent Application of solid state NMR in conjunction with multivariate data calibration to quantify the amount of amorphous Org xxx in crystalline material

1 Through-Bond ^{13}C - ^{13}C Correlation at the Natural Abundance Level: Refining Dynamic Regions in the Crystal Structure of Vitamin-D3 with Solid-State NMR

Ryan A. Olsen¹, Garrett M. Leskowitz¹, Douglas W. Elliott¹, Jochem Struppe², and Leonard J. Mueller¹

1. Department of Chemistry, University of California, Riverside, CA 92521

2. Bruker BioSpin Corporation, Billerica, MA 01821

Two-dimensional ^{13}C correlation spectroscopy at the natural abundance isotope level has enabled the application of nuclear magnetic resonance to industrial and academic problems that would be impractical if labeled materials were required. The challenge of natural abundance ^{13}C spectroscopy is the loss in signal intensity due to the 1% isotope concentration of the spins. This loss is particularly acute for correlation spectroscopy, which relies on pairs of nuclei to be spin active, decreasing the sensitivity of the technique by 4 orders of magnitude compared to correlation experiments on uniformly labeled materials. In solids, there are few examples of natural abundance ^{13}C correlation experiments, particularly on molecules with more than a dozen carbon sites. Here we show that ^{13}C natural abundance correlation in solids can be extended to moderately sized molecules, using the uniform-sign cross-peak double-quantum-filtered correlation spectroscopy (UC2QF COSY) to assign the 54 peaks of the solid-state NMR spectrum of microcrystalline vitamin-D3. In this case, comparison between the assigned peaks and ab initio calculations of the chemical shifts based on the crystal coordinates permits a refinement of the average structure in dynamic regions reported as disordered in the crystal structure.

2 NMR Studies of the Interaction of Peptides with Heparin

Jing Wang and Dallas Rabenstein

Chemistry Department, University of California, Riverside

Heparin has been used clinically as a blood anticoagulant for more than half a century. In some situations, it is necessary to neutralize the anticoagulant activity. We are studying several synthetic peptides as potential therapeutic neutralization agents. We report here the results of NMR studies of the interaction of three peptides with heparin.

The three peptides are analogs of heparin interacting peptide (HIP). The amino acid sequences of HIP and the three analog peptides are:

HIP: CRPKAKAKAKAKDQTK
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The interaction of each analog peptide with heparin was studied by (i) measuring the change in chemical shifts of backbone NH protons, (ii) the intensity of the backbone NH resonances as a function of pH, (iii) the effect of binding on the chemical shifts of heparin and peptide resonances and (iv) the effect of binding on the pK_A values of the lysine side chain ammonium groups.

To assign spectra and to achieve sufficient resolution of resonances to study the binding, 1D and 2D ¹H NMR spectra were measured using Band Selective Homonuclear Decoupling (BASHD) experiments. With the 2D BASHD experiments, ¹H-¹H coupled multiplets are collapsed to singlets in the F1 dimension. Examples of 1D and 2D BASHD spectra will be presented. With the increased resolution, it was possible to measure pK_A values from chemical shift-pH titration curves for each of the six lysine ammonium groups of SGV-HIP.

The results indicate that the binding is mostly an electrostatic interaction. Results also indicate that heparin binding protects the peptide NH protons, the ammonium groups of lysine and the NH protons of the guanidinium group of arginine from rapid exchange with solvent.

Upon binding of SGHV-HIP by heparin, the resonance for the A3 proton on the glucosamine residues of heparin is displaced up-field, consistent with a site-specific binding between peptide and heparin in which the imidazolium side chains interact with an imidazolium binding pocket on heparin.

3 Probing Structure and Dynamics in Disaccharides Using Residual Dipolar Couplings

Richard M. Venable, Scott E. Norris, Susan O. Ano and Darón I. Freedberg

CBER/FDA, Bethesda Maryland

Carbohydrate structure-function relationships are not well-developed, because their three-dimensional structures are not easily determined. But carbohydrate three-dimensional structure is difficult to determine in solution because long-range distance or angular data has been sparse. Ideally, one would like to determine the spatial and angular relationships between remote atoms. RDCs (residual dipolar couplings), dipolar coupling measured under partially oriented conditions, allow the relation of all bond vectors to a common alignment frame, thus facilitating three-dimensional structure elucidation. RDCs have been successfully applied to many biomolecules. However, accurate structure determination in carbohydrates is complicated by the fact that a minimum of five independent RDCs are required for the equations used to fit RDCs to structures. In addition to the near parallel orientation of the C-H bonds in pyranose rings, carbohydrates are usually not carbon labeled. Thus, many RDCs are needed in order to ascertain carbohydrate three-dimensional structure.

We have measured 35 RDCs for sucrose, 23 for the glucopyranosyl ring and 12 for the fructofuranosyl ring, including one- and two-bond ^1H - ^{13}C RDCs, three-bond ^1H - ^1H RDCs and one-bond ^{13}C - ^{13}C RDCs. We fit each of the rings separately and find that the alignment tensors for the two rings are different. This suggests that the two rings are moving relative to one another. We also fit single structures of sucrose and find that multiple conformations are indicated. Finally, we use new methods to fit one set of RDCs to two structures. We find that the fits for the two structure models are better than the single structure fits. Further, we find that the two dominant conformations are found in the $\phi = 120$, $\psi = 270$ and the $\phi = 45$, $\psi = 300$ regions. The results will be compared to molecular mechanics calculations.

4 Small Molecules at Sea: NMR in Studies of Fish Disease, Stress and Nutrition

John Walter¹, Kirty Solanky¹, Andrew Dacanay¹, Ian Burton¹, Shawna MacKinnon¹, Vanya Ewart¹ and William Driedzic².

1. Institute for Marine Biosciences, 1411 Oxford Street, Halifax NS, Canada B3H 3Z1

2. Ocean Sciences Centre, Memorial University of Newfoundland, St Johns NL A1C 5S7

As part of a program on fish health concentrating on commercially-important cold-water marine species, and problems that arise from aquaculture, we are exploring some possible roles for small molecule NMR-based methods. The problems generally fall into the categories of disease, stress and nutrition, and the methods have received relatively little prior application to fish and other marine systems.

In some of these applications we are taking a metabolomic/ metabonomic approach, exploiting the relatively uniform concentration response of NMR across a variety of small molecules when in free solution, an advantage that offsets the low sensitivity compared to other spectroscopic and spectrometric techniques for which response is highly non-linear. With little prior preparation, NMR may be used to survey the complement of metabolites present in cell cultures, biofluids, tissues, organs, or in some cases intact organisms, and to follow changes in response to many classes of physiological perturbation..

One of our first studies of this type concerns furunculosis in salmonids, a bacterial disease with major consequences in commercial aquaculture. The aim is to gain information about the host-pathogen interaction, and to see if progress of the disease might be assessed from samples that could be obtained without harm to the animal. We have found NMR of salmon plasma samples shows trends indicative of covert infection, and distinct from the effects of anorexia that is a common sign of the disease. Another class of experiment for which NMR offers unique information is the use of stable isotope labeling to follow metabolic processes. When used with multiply labeled precursors it is the only method that enables the integrity of bonds between labeled atoms to be monitored, a property exploited in a wealth of metabolic studies of microorganisms, terrestrial animals and plants, but in relatively few of marine animals. We have used this approach to study an unusual example of cold adaptation: the production of glycerol in very high concentrations as an antifreeze agent by rainbow smelt (a species closely related to salmon) when exposed to seawater temperatures below 0°C. NMR spectra of liver samples show the profound effects of temperature on the distribution of major metabolites, and provide convincing evidence for the processes leading to the extraordinarily high rate of glycerol production.

Other investigations in progress, including further studies of disease, effects of heat shock, nutritional supplements and maturation, are providing opportunities to assess the comparative advantages of NMR, MS and IR methods, and may also be discussed.

5 Hadamard and Radon Transforms in NMR Spectroscopy

Eriks Kupce¹ and Ray Freeman²

1. Varian Ltd., 28 Manor Rd., Walton-on-Thames, UK

2. Jesus College, Cambridge, UK

Considerable sensitivity gains provided by cryogenic probes coupled with availability of increasingly high magnetic field strength open new possibilities for development of fast methods for data acquisition in NMR spectroscopy. Such methods can speed up multi-dimensional experiments by several orders of magnitude [1]. We shall discuss two new fast multi-dimensional NMR techniques that are based on Hadamard [2] and Radon [3] transforms.

The Hadamard technique employs an extensive array of soft radiofrequency pulses and offers certain practical advantages over the traditional Fourier transform method. The key is the introduction of an encoding scheme based on Hadamard matrices. By eliminating the need for step-by-step exploration of the evolution dimensions in multidimensional spectroscopy, the Hadamard technique can speed up data acquisition by orders of magnitude, paving the way for high-dimensional experiments in a reasonably short time. Practical examples of two- and three-dimensional Hadamard spectroscopy are presented for both small molecules and proteins.

The projection-reconstruction method (PR NMR) [3] is based on the Radon transform and is an alternative scheme for fast multidimensional spectroscopy. This technique is closely related to back-projection methods employed in X-ray tomography where the internal structure of any three-dimensional object is computed by measuring the X-ray absorption in several different directions. In an analogous manner, a three-dimensional NMR spectrum can be reconstructed from projections of the absorption intensity onto planes inclined at different angles. In contrast to the continuous nature of a physiological sample, NMR spectra are discrete and usually well-resolved, presenting a much more favorable case for reconstruction. Only a very small number of different projections suffice to recreate the entire three-dimensional spectrum, speeding up data acquisition by an order of magnitude.

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6 Microflow NMR: Capabilities and Applications of the CapNMR Approach

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The pressing demand to have NMR meet the analytical challenges of the coming years forces changes to the techniques, hardware, and software employed. In drug discovery, the need to collect greater information earlier in the discovery process requires that instrument designers remain committed to “faster, cheaper, better.” A myriad of complicated and sometimes seemingly contradictory design challenges arise that include integration, reliability, functional complexity, operator simplicity, molecular diversity, economy, automation, efficient packaging, and speed. The advancement over the past decade of high-resolution NMR has involved a technological drive toward high field magnets, high sensitivity detection probes, new pulse sequences, and new methods of spectral interpretation. While continued advancement of these component technologies will result in further gains in NMR performance, these alone will not suffice to maintain NMR’s utility alongside complementary analytical techniques for routine laboratory analysis. Systems must evolve to meet new demands. Already requiring serious consideration prior to instrument purchase are choices that include hyphenated configurations vs. stand-alone platforms; the dedication of a magnet to a single probe vs. accommodation of multiple probes; the means of sample introduction (flow vs. tubes); and accommodation of users (open access vs. dedicated spectroscopist). Several trends seem necessary to meet future demands placed on NMR. These include the analysis of smaller quantities of sample, gathering of greater breadth of information in shorter time, and automated decision-making. Protasis/MRM offers a suite of automated NMR detection and sample management products. The year 2004 represents several significant milestones, including higher sensitivity flowcell offerings for 5 and 10 microliter volume samples, new software for unattended automation, CTC (Leap Technologies) liquid handler support, and development of software to streamline analyses and leverage increased sample throughput (Novatia). This talk describes ongoing and future developments at Protasis and its affiliates in microfluidics and capillary NMR, and illustrates a positioning of this instrumentation strategy toward the themes of technological advancement outlined above.

7 Digital Resolution: Its Determination and Enhancement by Zero Filling and Linear Prediction

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Resolution in NMR, the ability to distinguish two signals, is a source of considerable confusion. It is dependent on magnetic field homogeneity, signal acquisition time, and transverse relaxation times. Information that is lost due to truncation of free induction decays can be restored by zero-filling provided that the minimum acquisition time, for the signals whose resolution is sought, is met or exceeded. In the case of 1D NMR experiments, FID truncation is not usually a serious problem. However, this is not the case for 2D NMR experiments due to the much shorter acquisition times involved. Now one level of zero-filling is especially important in both dimensions. A complicating factor arises from the fact that F1 resolution affects the resolution obtained in F2. A series of DQ-COSY experiments has been carried out to determine the effects of (i) zero-filling in both domains and (ii) linear prediction in the F1 dimension. Both were found to be important, with linear prediction saving a great deal of experimental time. Relatively short T2s and line widths in excess of 3 Hz (due to spin coupling) were found to render unobservable couplings of less than 2 Hz. In addition, apparent active couplings that are erroneously large couplings were also observed in cases where active and passive couplings were very similar in magnitude. Judicious choice and fitting of weighting functions and careful phasing of signals are also important.

8 Characterization of the Metabolic Actions of Natural Stresses in the California Red Abalone, *Haliotis rufescens* Using ^1H NMR Based Metabolomics

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Withering syndrome in California red abalone (*Haliotis rufescens*) is caused by the Rickettsiales-like prokaryote (RLP) “*Candidatus Xenohaliotis californiensis*”. Affected animals undergo metaplastic changes within the digestive gland and it is hypothesized that as digestive gland architecture changes animals lose the ability to digest food. RLP-infection, however, does not necessarily produce signs of withering, and for reasons not yet well understood additional stressors such as elevated seawater temperature appear to influence the development of the disease. Using nuclear magnetic resonance (NMR) based metabolomics we have investigated the effects of bacterial infection, temperature, and food availability, both individually and in combination, on the metabolic status of the red abalone. High-resolution ^1H NMR spectroscopy was particularly appropriate for investigating metabolic status since multiple endogenous metabolites could be quantified rapidly in foot muscle and digestive gland tissues. Food limitation caused dramatic reductions in all classes of foot muscle metabolites while at the same time metabolite levels within the digestive gland were preserved or increased. We also found that food limitation along with the additional stress of elevated seawater temperature led to greater metabolic perturbations in both tissue types than those observed under food limitation alone. RLP infection and food limitation resulted in many of the same metabolic changes within the tissues studied, although the effects of infection were less severe. We observed increased levels of homarine in the digestive gland of both food limited and RLP-infected animals yet only observed increased homarine levels in the foot muscle of infected abalone. These results further support the recently established glucose to homarine ratio for differentiating foot muscle tissue of RLP-infected animals from that of both healthy and starved abalone. Furthermore, we found that the NMR metabolic data correlates well with histological measurements supporting the use of the metabolomics approach for characterizing both normal and pathological events in marine species, particularly during periods of environmentally relevant stress.

9 Solvent Effects on the Chemical Exchange of a Push Pull Ethylene

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Chemical exchange rates provide a sensitive probe of solvent effects in solution. The push –pull ethylene methyl 3-dimethylamino-2-cyanocrotonate (MDACC) has three exchange processes E-Z, E-E', and Z-Z', and we have measured these rates in Acetone-d₆, chloroform-d, tetrahydrofuran-d₈, toluene-d₈, methanol-d₄, acetonitrile-d₃, and methylene chloride-d₂. From this we obtain ΔH , ΔS of activation for each solvent. As the solvent changes, ΔH is approximately constant whereas ΔS follows the solvent polarity.

An interesting point is that the process of going from the major site to the minor site is exothermic, so not only is the rate governed by entropy effects, but also the equilibrium constant between E and Z.

10 Fully Automatic First-Order Multiplet Analysis (FAFOMA)

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The interpretation of every 1D NMR spectrum includes multiplet analysis in order to extract the information about chemical shifts and coupling constants, which is important for both structure elucidation and stereochemical studies. The multiplet analysis is generally not difficult, but rather boring even for simple cases of 1st order spectra, rather time consuming for more complicated cases of 1st order spectra and/or for cases with overlapped multiplets. With the increase of the NMR spectrometers frequency most of the studied ^1H NMR spectra become first order, suitable for First-Order Multiplet Analysis of their peak picking files. This analysis can be applied in the field of combinatory chemistry, multiple parallel synthesis and HPLC-NMR, where a lot of spectra are measured and the automatic assignment is necessary.

The last studies in this area are directed to find simple and effective approach for analysis of 1st order multiplets, but the multiplet overlap makes the known approaches difficult or even not applicable. That is why it is important to develop a new approach, which will be applicable for the analysis of overlapping multiplets.

We developed the program FAFOMA in order to automate the procedure of analysis of peak picking lists utilizing new and more general algorithm. FAFOMA will help either the novice in the analysis of NMR spectra or the experienced user of NMR spectrometer in extracting of as much as possible information from the peak picking lists of 1st order NMR spectra.

The program FAFOMA was implemented in an automatic au program for our BRUKER DRX 250 spectrometer. FAFOMA produces graphical output on screen in X-window and a text output. Now we offer also a free web version of the program FAFOMA for testing the program by the broad NMR community.

11 Improvement of Reliability in NMR Structures of Small Molecules Oriented in Liquid Crystalline Solutions

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One of the main challenges for NMR spectroscopy of oriented molecules is that the structural parameters obtained from experiments in different conditions (solvents, temperature, concentrations) show a large disagreement with one another, as well as with the data obtained by other structural methods. This has been the major obstacle for NMR spectroscopy of oriented molecules to become a well-established structural method. There have been many attempts to understand this large variation of structural data, but none of the existing theories can explain all experimental data.

We analyzed a great number of structural data for small model molecules (benzene, substituted methanes, thiophene, furan, etc.) dissolved in different thermotropic and lyotropic liquid crystals under various experimental conditions, both those compiled from literature and those obtained from our own experiment, and compared their spectral aberrations. In thermotropic liquid crystals, the aberrations are observed not to be random but inversely proportional to the order parameter. Therefore, weakly aligned molecules show maximal aberrations. In contrast, in weakly aligning lyotropic liquid crystals no significant aberrations of structural data have been observed in a wide range of experimental conditions. Thus, the fundamental difference between lyotropic and thermotropic liquid crystals exists regarding the character of averaging the anisotropic interactions of oriented molecules.

In both cases: oriented molecules in thermotropic liquid crystals after appropriate corrections, and those dissolved in weakly aligned lyotropic systems, we may have structural information that is consistent to other structural methods. In addition, these results validate the use of weak-aligning lyotropic liquid crystalline systems for the accurate 3D structure determination of biomolecules by NMR spectroscopy.

12 Development and Application of State-of-the-Art NMR Methods to Analytical Challenges Encountered During Drug Discovery and Development

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Nuclear magnetic resonance (NMR) spectroscopy is unmatched by other analytical techniques in its ability to provide detailed information regarding molecular structure, dynamics, and interactions. The broad applicability of NMR spectroscopy to samples and analytical challenges of pharmaceutical relevance has been exploited in the development and application of several state-of-the-art NMR methods described in this poster.

Mixture analysis is commonly encountered after combinatorial synthesis, a popular approach for drug lead generation. One-dimensional proton NMR spectroscopy is often insufficient for resolving physicochemical information about mixture components due to resonance overlap. The 2D-J-DOSY method resolves mixture components based on differential translational diffusion coefficients and homonuclear coupling constants. This method permitted the unequivocal determination of diffusion coefficients for two sugars whose resonances were overlapped in the 1D proton spectrum and is potentially useful for characterizing larger combinatorial mixtures and for screening assays.

Under the proper experimental conditions, NMR diffusion measurements also provide useful information for characterizing structure-activity relationships during drug discovery. The diffusion NMR method was shown as a viable strategy for epitope mapping of binary and ternary complexes of ligands binding to human serum albumin as well as for characterizing competitive binding interactions.

Finally, drug safety and efficacy studies require reliable analytical methods for determining the biochemical effects of a drug. Metabolic profiling of biofluids and tissues using NMR spectroscopy is a developing approach for these studies. Two NMR experiments were compared to determine a reproducible strategy for automated data processing of serum proton spectra acquired en masse. The sensitivity of high-resolution magic angle spinning (HR-MAS) NMR spectroscopy was also demonstrated for quantitation of a model drug compound in rat skin and muscle tissues.

13 HPLC-SPE-NMR, -a New and Efficient Natural Products Research Tool

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Nature is an important source of bioactive compounds that can be useful as new drugs or lead compounds. Traditionally, structure elucidation of secondary metabolites is performed by spectroscopic analysis of purified compounds. Thus, structural information is obtained at the end of an expensive and laborious process of isolation and purification of trivial or already known compounds, emphasizing the need for new research tools in the field of natural products chemistry. HPLC-NMR has shown to be an advantageous tool, which can reveal structural information about secondary metabolites in complex mixtures at an early stage. However, the use of HPLC-NMR has been suffering from sensitivity constraints and limitations related to solvents. A solution to these problems is provided by HPLC-SPE-NMR, a new development in the field of hyphenated techniques, which uses solid phase extraction as interface between HPLC and NMR. Some major advantages of HPLC-SPE-NMR compared to HPLC-NMR methods are listed below:

HPLC performance can be optimized with a free choice of solvents.

Sensitivity of NMR spectra is improved because peaks are eluted with only 30 μL of deuterated solvent.

A peak can be collected on the same SPE cartridge several times in automation, making a complete structural elucidation possible without preparative-scale purification.

In this study, the major goal has been to develop an efficient protocol for identification of secondary metabolites in plant extracts using HPLC-SPE-NMR. Proof-of-concept studies have been conducted on an extract made from roots of the Iranian plant *Smirnowia iranica*. In summary, it has been shown that HPLC-SPE-NMR enables rapid identification of natural products in plant extracts after separation on an analytical-scale HPLC column. 1D ^1H spectra of excellent quality were obtained of most chromatographic peaks. Besides identifying already known compounds it was evident that several other compounds were present in the extract. Multiple trapping on SPE cartridges was shown to improve the signal to noise ratio in 1D ^1H spectra. Results have shown that the HPLC-SPE-NMR method is an efficient, productivity-increasing tool for analysis of complex mixtures like plant extracts. It has the potential to enable complete structural elucidation of unknown compounds with 2D NMR methods without preparative-scale isolation and purification of compounds.

14 Changes in Spin-Lattice Relaxation Dynamics Upon Processing of Crystalline Organic Solids

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Relaxation dynamics have been used to measure domain sizes in polymers using spin diffusion data (1). Spin diffusion mediates ^1H T1 relaxation in solid samples where parts of the sample are highly mobile and relax quickly (e.g. protons in rotating methyl groups) and others are rigid and relax slowly. Spin diffusion to highly mobile sites results in a common T1 for the entire sample. Here we investigate how the introduction of highly mobile domains (i.e. crystal defect sites, amorphous material) affects the relaxation dynamics of a crystalline organic compound containing no relaxation sinks in the molecular structure.

The ability to predict stability of formulations is critical to the pharmaceutical industry. Crystalline lactose monohydrate was subjected to various forms of physical processing, and ^1H T1 measurements were performed to observe how sample mobility was affected. Measurement of ^1H spin-lattice relaxation times in the solid state may serve as an indicator of which formulations will be most stable. Cryogrinding was found to produce a mixture of amorphous and crystalline lactose, with the amorphous component being relatively mobile, and the crystalline domain presumably containing more defect sites than the bulk material. Tableting bulk crystalline lactose monohydrate at ~5000 psi produced > 3-fold reduction in ^1H T1, without producing any significant changes in the ^{13}C CPMAS NMR spectrum. Cryogrinding for 2 minutes reduced the ^1H T1 by an order of magnitude, also without producing any significant changes in the NMR spectrum. Spray-drying and lyophilization produced completely amorphous lactose, but with ^1H T1 values longer than that of the sample cryoground for 30 minutes. This is likely due to the plasticizing effects of water lost from the monohydrate crystal lattice, which is taken up by the amorphous component. Karl Fischer analysis confirmed that the cryoground samples contained about 3 times as much residual water as the spray-dried samples. We propose that crystal defects and small amounts of amorphous material produced under physical stress serve as relaxation sinks to shorten relaxation times through spin diffusion.

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15

Some New Aspects of the Out-of-Coil Magnetization Contribution to Signal Detection

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In spite of some prior research into the area (1,2) the signal unwittingly but inevitably generated in sample regions falling outside of the RF coil's active region is a notoriously ignored and misunderstood topic in high-resolution NMR. In particular, out-of-coil effects are often confused with lead pick-up, can mimic radiation-damping-like behavior and their role is usually not fully recognized in signal suppression experiments. Moreover, off-coil signals tend to exhibit a rather obstinate and unexpected behavior, in effect serving as a physical "apodization function" with respect to the active-region signal for pulse angles smaller than 180° while pulse angles larger than 180° unveil the active-region component due to the out-of-coil magnetization's overdamped response to the driving field. Following some new research into the topic, we consider some implications of off-coil effects in solvent suppression experiments as well as in the apparently well-established concept of $T2^*$. Demonstrative experimental examples will be shown on some of the smallest "hot" molecules such as CHCl_3 .

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16 **Using NMR Based Metabonomics to Classify Animals in a Dominant/Submissive Behavioral Model by an Exogenous Biomarker**

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The present study investigated the applicability of metabonomics using NMR to classify rats in a dominant-submissive behavioral model. This method was found extremely useful in classifying the animals. A description of the animal model follows. The rats were given access to food and sweetened milk for 1 h daily during the week. Water was always available. The experiments were performed using an apparatus for dominance-reversal test: boxes made of clear plastic and connected by a tube with a feeder placed at the center that is only accessible to one rat at a time. The rats were acclimated to the apparatus. The behavioral measure was sweetened milk drinking, and a point was scored by the rat during each 5s interval unit that it was drinking milk in the test apparatus. The rats were paired according to their scores (a high scoring rat with a low scoring rat) to establish dominance-submissive relationships. The pairs were then observed once daily within the apparatus for 5 min on 5 consecutive days. The average daily drinking score was then calculated from 5 days of measurement for each rat. The dominant rat was identified by the higher daily average drinking score relative to the submissive rat. The level of dominance for each pair was then calculated as the difference between the average daily drinking score for the dominant and submissive rat of each pair. Two weeks are required to score and classify the rats before compound screening can commence. In an effort to expedite the above model or simplify the scoring process urinary metabonomics by NMR was used. Urine was collected from the rats and prepared for NMR analysis using standard protocols. The proton spectra from the urine after appropriate manipulation were subjected to principal component analysis (PCA). The PCA successfully grouped the animals into two groups and the grouping agreed with the dominant or submissive classification based upon the scoring method briefly mentioned above. The metabolite most responsible for differentiating the two groups was galactose derived from the lactose in the milk. This finding highlights the use of exogenous biomarkers in classifying or ranking animals in certain animal models.

17 **Quality Control in Drug Discovery: Identification and Quantitative Analysis by MicroNMR**

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Over the past few years there has been a significantly growing demand for a more accurate characterization of the contents of lead discovery substance libraries of small molecules. In many cases mass spectrometry alone seems not to be sufficient. NMR is an ideal addition to the MS data since intrinsically it yields the most structural information and it can also be used for quantification.

Here we present a completely new and automated NMR setup to measure minute quantities of sample taken from parallel synthesis labs or directly taken from lead discovery depositories with outstanding sensitivity. This extremely time efficient method relies on the use of new spectrometer hardware which consumes only 5 micro liters of the stock solution and therefore sample recovery is obsolete in most cases. NMR experiments can be measured with outstanding sensitivity in these small volumes, which leads to experiment times on 5 micro liters of a 10mM solution of only a few minutes per sample. Since only a very small volume is needed for the structure verification, the use of expensive deuterated solvent is reduced to a minimum or can be omitted completely.

We also present the NMR setup and a new software tool for structure verification and automated yield determination in parallel synthesis as well as for the quality control in aging existing libraries.

18

Multiple-Sample Probe for High-Throughput Solid-State NMR Spectroscopy of Pharmaceutical Solids

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Solid-state NMR spectroscopy is an extremely powerful technique for the analysis of drug compounds and pharmaceutical formulations because of the vast quantity of information that can be obtained from the NMR spectrum. New drug compounds often are poorly crystalline or amorphous, and are present at low levels in a formulation. A larger number of transients needed to acquire an adequate signal to noise ratio (SNR), which could take hours to days for an acceptable SNR.

When a sample is in the spectrometer, the actual data acquisition time is orders of magnitude smaller than the time spent in the magnet. This is due to the fact that magnetization has an extremely short T2 relaxation (milliseconds) and a much longer T1 (seconds to hours). Thus, the sample is idle for a high percentage of the time that it occupies the spectrometer. In an effort to take advantage of the idle time between pulses, a multiple-sample probe is being developed.

The two-module prototype has been constructed which has independent RF circuits and tuning and airlines and angle adjust for magic-angle spinning. A stepper motor rapidly shuttles the spinning modules in and out of the homogeneous region of the magnet while the bulk magnetization of the idle sample regenerates. The probe can simultaneously run two identical samples and reduce the length of an acquisition without sacrificing SNR, or the probe can be set up to run different samples thereby increasing throughput two fold.

Spectra of aspirin and ibuprofen were acquired using this probe. The spectrum of aspirin acquired with sample shuttling was comparable to the spectrum of aspirin acquired without shuttling. A spectrum of ibuprofen was acquired at the same time as a spectrum of aspirin was acquired. In addition, parameters such as shuttling time between samples, decoupling powers, signal to noise ratios, and field homogeneity were found to be comparable to conventional MAS probes.

This probe can increase both the throughput and signal to noise ratio of a sample. The number of modules is only limited by the diameter of the bore. With a smaller module it is conceivable that a probe could consist of 10-20 spinning modules to further increase the efficiency of the spectrometer.

19 Chemical Shifts in 4D Structural Analysis of Peptides: Cyclosporin A

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Use of ^1H NMR chemical shift prediction in peptide conformational analysis was assessed. First, the complete spectral analysis of 800 MHz ^1H NMR high resolution spectrum of cyclosporin A was performed.

Next, the observed chemical shifts and coupling constants were predicted starting from the X-ray structure, using a more complete conformation space analysis with Metropolis Monte Carlo and molecular dynamics simulation. The rms of the chemical shift prediction was 0.25 ppm.

The results propose that the solution structure of CsA differs from the X-ray structure only with respect of the conformational freedom, in agreement with previous studies. The results also propose that the chemical shift prediction forms a potential tool for peptide and protein structural analysis and that the chemical shifts carry valuable information about molecular dynamics.

20 Identification of an Impurity Observed in a Stability Sample of Naltrexone Base by LC/MS and LC/SPE/NMR.

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An HPLC peak of unknown origin, at a 0.15 level by normalized area percent, was observed in stability samples of the drug substance, Naltrexone base. After pre-concentration by preparative HPLC, the unknown's structure was elucidated by a combination of LC/MS and LC/SPE/NMR experiments. The LC/MS analysis, including positive electrospray MS and MS/MS, revealed that the unknown had a molecular weight that was twice that of Naltrexone itself. LC/SPE/NMR analysis, including proton, HSQC, HMBC, Double Quantum Filtered COSY and NOESY experiments, confirmed that the unknown was an aldol dimer of Naltrexone. The NOESY spectral data allowed the determination of the stereochemical configuration of one of the two new chiral centers created by the formation of the dimer.

21

Study of Particle Size of Crystalline Pharmaceutical Solids by Solid-State NMR Spectroscopy

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NMR line widths depend partially upon the magnetic susceptibility of the sample.⁽¹⁾ Ellipsoidal solids have isotropic bulk magnetic susceptibility (IBMS). When particle shapes are non-spherical, the tensor components describing the magnetic susceptibility are no longer symmetric, resulting in anisotropic bulk magnetic susceptibility (ABMS). The degree to which ABMS influences line widths for crystalline organic compounds depends strongly upon particle characteristics including particle size, morphology, and density of material containing ABMS.

Solid-state NMR spectroscopy has been used to study particle size and morphology in pharmaceutical formulations. Particle size and/or morphology were altered in pure ibuprofen samples by recrystallizing from different solvents, melt-quenching, or cryogrinding. The environment of the ibuprofen particles was also altered by preparing physical mixtures of bulk ibuprofen in diluents.

For pure crystalline samples, narrower lines were observed from larger particle sizes and more uniform (ellipsoidal) particle shapes. The line widths in a physical mixture depend on all materials present (i.e., the ibuprofen, the diluents, and air between particles). Line widths can be decreased by mixing with suitable diluents, and particle morphology of diluents can have a dramatic effect on the line width of ibuprofen spectra.

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22 **Varian's VNMR Chempack in the Open Access GMP Environment**

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1. Chemical Process Research and Development

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Varian's Chempack was modified for use in an open access walkup environment which is also used to satisfy GMP compliant (21 CFR part 11) requirements. The system functions as a completely secure log-in instrument with a full complement of 1D and 2D NMR experiments being allowed on a Mercury 300 NMR. Besides walkup experiments, a totally automated robot controlled GMP autosampler can be utilized. The NMR experiments are automatically archived locally and also to a central GMP validated network server with IT oversight. Some examples of the login, NMR experiment menus and archival procedures will be detailed.

23 Testing NMR Quantitation Parameters

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NMR has been used for decades as a quantitative instrument for drug analysis. Since the first commercial 60 MHz proton NMR's, papers showed how NMR could determine the molecular weight of an unknown compound and perform accurate quantitative proton and carbon analyses of pharmaceuticals and drugs of forensic interest. This paper will endeavor to illustrate how to determine and optimize the NMR's uniformity of response throughout the spectral width, determine the delay needed before gradient shimming to optimize peak resolution, and other parameters need to be adjusted in order to produce accurate and precise quantitative spectra.

24 **Metabolic Profiling of Myocardial Ischemia by Microdialysis and Microcoil NMR**

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Small molecule levels in tissues can yield valuable information about the metabolic state of an organism. Studies of changes in the metabolic state, or metabonomics, can reflect disease pathways, drug action, or toxicity. Currently, metabolic profiling experiments typically rely on the analysis of body fluids such as blood and urine or the excision of the tissue of interest. The results of blood and urine analysis typically reflect an average of the metabolic state of the organism and may not reflect the metabolic profiles of affected organs or tissues. Additionally, excision of tissue is an invasive procedure that does not allow for long-term experiments on the same animal. Thus, there is a need in metabonomics research for noninvasive, localized sampling of metabolites.

This research aims to develop this new approach for metabonomics studies. Microdialysis sampling and microcoil NMR analysis using a commercially available MRM probe is employed to compare metabolic states of tissues (blood, liver, brain, muscle, and heart) in Sprague-Dawley rats. Microdialysis sampling is localized, making the metabolic profile tissue specific. It is also minimally invasive, allowing metabolite sampling over the period of hours to days. Coupling microdialysis to NMR analysis is highly advantageous, because a complete metabolic profile is obtained in a single spectrum. However, the small sample volumes and low analyte concentrations make analysis of microdialysis samples challenging. Microcoil NMR is amenable to the analysis of small volume samples and has good mass sensitivity, thus overcoming traditional NMR limitations. The coupling of these techniques is a potentially powerful tool for metabonomic analysis.

These techniques were applied to study effects of myocardial ischemia on rat tissues. Identifying changes in the relative intensities of the metabolites can give important information about potential biomarkers for ischemia, as well as the metabolic pathways inhibited or activated by the condition.

Three systems have been profiled before and after ischemia was induced in rats. These include blood (sampled from the jugular vein), brain (sampled from the cerebral cortex), and heart. Basal and ischemic metabolic profiles were obtained. In all cases, distinct differences in the tissue metabolic profiles were observed. These spectra demonstrate that microdialysis sampling with microcoil NMR detection can distinguish between tissues in basal and ischemic states, as well as between the tissues themselves. Thus, from the data, this new method shows considerable promise for metabonomics studies.

25 Structural Dynamics of a Small, Flexible Model Compound

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A recently introduced model compound(1), exhibits structural dynamics in solution. This thirty-two atom organic molecule, which contains a peptide bond, can be found in two conformational states, as seen both by x-ray crystallography and NMR spectroscopy: open (linear/unfolded/inter-molecularly bonded) and closed (8-membered ring/folded/intra-molecularly bonded).

We have used ^1H and ^{13}C NMR to study the relative populations of the two forms as a function of solvent and temperature. The overall spectral features in chloroform-d, toluene-d₈, and benzene-d₆, are nearly identical. Both conformations are observed in nearly equal populations at room temperature. In aqueous solution however, the room temperature spectrum only exhibits a single, well-defined molecular conformation. Coupling constants were determined using NUMMRIT simulations within Spinworks(2).

Variable temperature ^1H NMR experiments were used to determine the energetics of the conformational change in chloroform-d. In the slow exchange limit the singlet methyl resonances have resolved chemical shifts for the two forms. The dynamic NMR program within Spinworks(2), DNMR(3), was used to determine the rate of change as a function of temperature. Thermodynamic parameters were extracted from an Eyring plot of the data. Additional, internal dynamics of the methyl groups are observable in the open form as the temperature is further reduced.

A correlation exists between the preference for the closed form at low temperatures and deshielding of the proton resonance assigned to the hydroxyl group of the closed form. Future directions include elucidating the relative contributions of the intra- and intermolecular hydrogen bond strengths.

1. Submitted for publication
2. K. Marat, Univeristy of Manitoba,
<http://www.umanitoba.ca/chemistry/nmr/nmrsource2.html>, 2004.
3. DNMR3 program of G. Binch and D. Kleier

26 Improved Workflow and Results in the NMR Lab

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Having the ability to store, organize, search and retrieve spectral and chemical information can be an important component of a company's long term plan to manage and maintain its internal knowledge base. It is often the case that a single sample or compound will be examined by a number of techniques to provide enough analytical information to characterize it properly. Because of the variety of spectral techniques and the variety of spectrometers within a given technique, managing NMR and other data is a challenge for any laboratory.

This poster will examine a number of steps that should be undertaken when developing a resource of informatics tools. This poster also introduces a system that combines tools within a fully integrated environment to include tools for processing, prediction, database building, management, search, analysis, and reporting for HNMR, CNMR, and XNMR. It also demonstrates how working within a single interface can help scientists bring data together to manage workflow, improve productivity, and share information with others in the organization.

27

Approaches to the Identification of Impurities in Pharmaceutical Development

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Identification of impurities in drug development is commonly accomplished by the analysis of NMR data acquired using multidimensional techniques. Isolation of impurities as pure compounds prior to NMR data acquisition appears to be a way to minimize the ambiguities in the final structure of complex molecules. Using ¹H and ¹³C data from 1-D and 2-D NMR experiments, identification of a series of impurities associated with COX-2 inhibitor project will be discussed.

In some cases, ¹H and ¹³C data alone may be insufficient to overcome the challenges in solving structural problems. Inclusion of ¹⁵N, at least through indirect detection can be very helpful in such circumstances. The problems associated with detection of natural abundance ¹⁵N due to its low sensitivity can be circumvented to some extent by exploiting higher sensitivity provided by CryoProbes. This concept will be demonstrated with an example.

28 ¹H NMR Spectra of Renal Carcinomas: Data Mining with Partial Least Squares

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Histopathological examination of renal tumors remains the gold standard for diagnosing the type and grade of cancer present. While this empirical approach serves its medical purpose, it does not provide insight into the biochemical conditions that promote malignant tumor evolution and growth. Also, pathological classification of the tumor grade is subjective due to inter-observer variability.

MAS NMR of tissue samples has recently been gaining in prominence due to the increased resolution it provides for ¹H spectra of biopsies without having to resort to making extracts of the tissue. Working with the intact tissue has proven invaluable since the relative concentrations of fats and small molecule metabolites have been shown to be of diagnostic value.

In our studies, we have collected tissues from a variety of different renal tumor types including benign oncocytomas and angiomyolipomas and malignant tumors including a variety of different grades of renal clear cell and papillary carcinomas. To analyze the ¹H MAS data, we have utilized a peak searching algorithm paired with Partial Least Squares (PLS) analysis to identify peaks that are diagnostic between the different tumor types. This methodology allows us to classify normal tissue versus malignant tumors accurately and has led to a variety of biochemical insights.

29 **A Natural Abundance F2 Coupled HSQC (F2HSQC) NMR Experiment for the Measurement of Dipolar Couplings of Chiral Compounds Present in a Chiral Liquid Crystal Solvent and Structural Identification with the use of Molecular Dynamics**

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A high-resolution, phase-sensitive, natural abundance F2-coupled ^1H - ^{13}C HSQC (F2HSQC) NMR experiment has been developed to simultaneously measure both long and short range ^1H - ^1H and one bond ^{13}C - ^1H residual dipolar couplings (RDCs) of small molecules present in polypeptide chiral liquid crystal solvents (PBLG/ CDCl_3). Because this is an indirect-detection NMR experiment, the relatively low amount of sample (7.5 mg in this study) and short acquisition times (5 hours) required make this HSQC experiment well suited for samples that are limited in either solubility or quantity or short analysis times are necessary. The F2-HSQC experiment can be performed without any specialized equipment or sample modification and can enhance our ability to accurately and rapidly measure RDCs in polypeptide liquid crystal solvents. The RDCs derived from the F2HSQC experiment have been used in calculations to determine the stereochemistry of small organic molecules.

30 LC-SPE-NMR: Automated Transfer of SPE-Fractions to 1 mm NMR Tubes

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The introduction of SPE-NMRTM has greatly facilitated structure elucidation of compounds present in low concentration by LC-NMR, since repeated injection and accumulation of analyte is possible. However, the use of NMR tubes instead of flow-probes may offer some advantages, e.g., sample storage, lack of diffusion, and sample focusing. For 1 mm SPE cartridges, it is possible to manually elute the trapped analyte into 1 mm tubes using a volume of ~10 ml, i.e., near the ideal volume for a Bruker 1 mm TXI probe. In this work we demonstrate the possibility of eluting loaded SPE cartridges to 1 mm tubes in automation by the use of the Spark Prospekt II under HyStarTM control, a Gilson 215 Liquid Handler under Gilson 735 Instrument Control Software, and optionally Icon-NMR for retrieval of sample details and chromatographic data. An auxiliary output signal coordinates the software of the different modules. It has been found that elution with an excess volume of a volatile deuterated solvent followed by evaporation of excess solvent is the most robust method for non-volatile analytes. The evaporation is achieved by blowing nitrogen through a slowly moving capillary in the tube.

31

Optimization of Analyses: Cross-Divisional Benchmarking Studies

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High resolution NMR is an indispensable tool for structural determination, stability, kinetics, time course studies, pharmacokinetics, and metabolism. The often complex matrices and low purity of many samples presented for investigation stretch NMR to the limits of its usefulness. This study was initially undertaken to benchmark our systems so to provide guidelines for intelligent project planning. For example, if the world's supply of a material is represented by a miniscule amount of material in my vial, what is the best way to handle this material, which is the key experiment to perform, and which spectrometer and associated hardware is the best choice for this particular sample? Or on the other hand, what approximate amount of material needs to be provided to allow a reasonable chance that NMR analysis will be successful? Flurbiprofen and quinidine were used as compounds representative of pharmaceutical molecules under development. Although this study initially began as a benchmarking study, we realized as we began to handle such minute amounts of material that special precautions above and beyond those we'd planned were mandatory. Assumptions as to sample stability and reactivity in seemingly inert solvents had to be rethought. We share our learnings in this presentation.

32 Conclusive Determination of Presence of Polymorphic Mixtures by SSNMR

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Each time a new sample of an active pharmaceutical ingredient (API) or a formulation is prepared, it is important to know whether a single crystal form or a mixture of polymorphs was formed. Properties of the sample such as dissolution rate or chemical or physical stability depend on the type of form present. Typically, differential scanning calorimetry (DSC) is a technique of a choice to determine the phase purity. However, in many cases DSC is not sensitive enough, typically due to the similar transition properties of the crystalline forms or due to the not well-defined transitions of amorphous phases. Proton spin diffusion-based carbon and fluorine experiments are presented that conclusively discriminate between the presence of a single form and a mixture of crystal forms. The basic requirement to apply the proposed experiments is the existence of distinct chemical shifts for the components of the mixture. A presence of multiple peaks per any atom in the molecule of interest is then an indication of either a single phase with multiple molecules per asymmetric unit cell or a mixture of polymorphs. Both ^{13}C and ^{19}F afford well-resolved spectral lines, but ^{19}F is preferred due to its exquisite sensitivity and lack of background in pharmaceutical formulations. In the most simple application, it is shown that one can discriminate between the two cases (single crystal phase with multiple sites vs. mixture) by observing different ^1H relaxation rates in carbon or fluorine detected 1D proton relaxation experiments. However, the positive proof of distinction between the two cases can come only from 2D experiments, correlating the peaks of interest by their distance. Proton spin diffusion based experiments are the ideal candidates. The sample is proven to be a single phase if at least some of the multiple peaks per atom show mutual cross peaks in 2D ^{19}F - ^{13}C or ^{19}F - ^{19}F correlation experiments, which use proton spin diffusion mediated coherence transfer steps. In combination with other techniques such as PXRD, such info may enable solving the structure in the absence of a single crystal.

33 Molecular Factor Analysis Applied To Collections of NMR Spectra

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It is often useful to identify and quantify mixture components by analyzing collections of NMR spectra. Such collections arise in metabonomics and many other applications. Many mixtures studied by NMR can contain hundreds of compounds, and it is challenging to analyze the resulting complex spectra. We have approached the problem of separating signals from different molecules in complex mixtures by using self-modeling curve resolution as implemented by the alternating least squares algorithm. Alternating least squares uses non-negativity criteria to generate spectra and concentrations from a collection of mixture spectra. Compared to previous applications of alternating least squares, NMR spectra of complex mixtures possess unique features, such as large numbers of components and sample-to-sample variability in peak positions. To deal with these features, we developed a set of data preprocessing methods, and we made modifications to the alternating least squares algorithm. We use the term “Molecular Factor Analysis” to refer to the preprocessing and modified alternating least squares methods. Molecular factor analysis was tested using an artificial data set and spectra from a metabonomics study. The results show that the tools can extract valuable information on sample composition from sets of NMR spectra.

34 **GraPES: A New Innovation for the Study of Protein Ligand Interactions**

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NMR diffusion experiments have emerged as a powerful tool for examining protein-ligand interactions. The bipolar pulse pair stimulated echo (BPPSTE) experiment is often the diffusion experiment of choice to investigate protein ligand binding interactions. However, it is important to suppress the protein background in protein/small molecule ligand mixtures when measuring the diffusion coefficients of the ligand. Either spectral subtraction or spectral editing can accomplish the required suppression of the protein background. Previously, CPMG pulse trains have been added before or after the BPPSTE pulse sequence to provide T2 spectral editing. Recently it has been shown that when combining the BPPSTE experiment with other experiments such as 2DJ, HMQC and INEPT, applying the diffusion encoding pulse sandwiches during the experiment provides distinct advantages. (Nilsson, et. al. *Anal. Chem.* 2004, accepted for publication., and Stchedroff, et. al. *Phys. Chem. Chem. Phys.* 2004, 6, 3221.) Along similar lines we propose a pulse sequence that applies the gradient encode and decode pulse sandwiches before and after the CPMG pulse train. This new pulse sequence is known as Gradient Phase Encoded Spin lock or GraPES.

The GraPES experiment can be used to both calculate diffusion coefficients as well as suppress the protein background. Results from a simple sample of alpha-cyclodextrin will be presented to demonstrate the ability to obtain diffusion coefficients by the GraPES experiment. The GraPES experiment was also applied to more complex samples, including tryptophan and human serum albumin (HSA), to demonstrate protein spectral background suppression. The advantage of the GraPES experiment is that it is faster and more sensitive than the BPPSTE-CPMG experiment.

35 Rapid Sample Identification: NMRanalyst Dereplication, Verification, and Structure Elucidation

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NMR is a powerful technique to elucidate unknown compounds, to verify proposed structures, and to identify known compounds. This poster presents automated approaches for these tasks, employing sensitive NMR techniques.

Most organic molecules are sufficiently protonated for elucidation by sensitive indirect detection methods. Acquisition of a 1D carbon or nitrogen spectrum on an indirect detection probe remains impractical. The NMRanalyst software can determine such resonances from F1 frequencies of HSQC and HMBC correlations. Carbon-proton bonds and carbon multiplicities are determined from an edited HSQC. Longer-range correlations are determined from HMBC (for molecular nitrogens from ^{15}N -HMBC). Bonds between protonated carbons can be identified from an optional DQF-COSY. With the 1D proton spectrum, this is the standard NMR spectrum set for NMRanalyst structure elucidations.

NMRanalyst automates transform and analysis of NMR data. Its AssembleIt module exhaustively combines detected correlations to derive molecular structures. This generative approach does not require the molecular formula, which is often unknown. Unobserved 2-bond HMBC correlations are automatically added when derivable from observed longer-range correlations. Chemical shift prediction of observed nuclei identifies likely positions and types of unobserved heteroatoms. The poster will present NMRanalyst structure elucidation examples with down to 200 micro gram samples and of molecules up to 30 non-proton atoms.

Sometimes the structure of a molecule is known. But its consistency with NMR data needs to be confirmed. Or from a database of known molecular structures, best matches to available NMR data need to be identified. Both applications require significantly less acquisition time than high resolution 2D spectra used for structure elucidations. NMRanalyst provides VerifyIt and FindIt modules for these applications. FindIt uses a database of over 200,000 biologically active molecular structures provided by the National Cancer Institute. Other databases can be searched and further molecular structures to consider can be added. Examples will also be presented in this poster.

36 A Pure Standard Material for Accurate Quantitative NMR

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Since Fourier transform NMR has taken over the continuous wave NMR, accuracy in quantitative analysis using NMR has not widely been discussed in detail. Recently, the quantitative analysis using NMR has gained an enormous attention in inter comparison studies organized by Comité Consultatif pour la Quantité de Matière, CCQM, under Comité International des Poids et Mesures, CIPM, because the principles of the quantitative analysis by NMR that essentially compares areas of NMR signals is simple, can be expressed with equations, and is an International System of Units (SI) traceable method. Unlike chromatographic techniques, a standard for different material can be used and a calibration curve is not necessary for the quantitative analysis. Therefore, a universal standard can be used for analysis of many materials. Furthermore, the method has an advantage to analyze materials with wide variety of concentration range, from a pure material to a dilute solution. Last few years, we have participated in CCQM inter comparison studies and have demonstrated validity of NMR as a tool of accurate quantitative analysis. However, there is a lack of standard materials that can be used for the quantitative NMR analysis. Currently, we are in process of making a certified reference material that can be utilized as the universal standard in the quantitative NMR measurement. Once this reference material is certified, quantitative analysis using NMR is going to be expanded in its application. We will discuss our new certified reference material under development for quantitative NMR analysis and some applications using the material.

37 Carbon-Fluorine Correlation Spectra

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Direct detection heteronuclear correlation (HETCOR) $^{13}\text{C}\{^{19}\text{F}\}$ and indirect detection gHMQC and gHSQC $^{19}\text{F}\{^{13}\text{C}\}$ two dimensional correlation spectra have been compared for 1,3-perfluoro dimethylcyclohexane. Correlations to the geminally inequivalent fluorines in the CF_2 groups are complex and difficult or impossible to unambiguously observe using HMQC or HETCOR in this typical cyclic fluorochemical in which two bond J(FF) coupling constants are greater than one bond J(CF) coupling constants, but they are readily observed and assigned in the HSQC experiment. However, the HMQC experiment is more forgiving to pulse width calibration and gives better spectra (except for inequivalent CF_2 's) for molecules with short transverse relaxation times. Inverse detection experiments with ^{19}F adiabatic inversion pulses effectively permit observation of the entire ^{19}F chemical shift range in one experiment, but require a probe with ^{19}F pulse widths under about 12 microsec pulse widths at 470 or 564 MHz. Examples of the 2D NMR spectra will be shown.

38 Reaction Monitoring and Optimisation

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We have used NMR to follow the course of reactions that were under development for bulk drug production. The technique augments classical analytical and spectroscopic methods, such as UV and IR/Raman. Solute concentrations were typically high and we were able to record ^1H and ^{13}C NMR spectra with adequate time resolution.

With the simplest experiment an NMR tube was used as the reaction vessel. For more demanding cases, we have been successful in monitoring a reaction in a vessel outside of the magnet. Continuous circulation of a small portion of the reaction medium through an NMR flow cell allowed useful data to be collected.

NMR has a number of advantages and we shall demonstrate that it reports quite subtle effects in solution, such as protonation. We will also illustrate the ease with which high-quality data can be generated for kinetic model generation.

39

Rapid, High-Resolution 4D NMR Spectroscopy for Structural Determination of Oligosaccharides Derivatized with ^{13}C -Acetyl Groups

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Nuclear Magnetic Resonance (NMR) spectroscopy is an effective tool for the study of complex carbohydrates. Unfortunately, even for small carbohydrates the proton spectrum is extremely crowded, limiting the amount of chemical information that can be extracted. However, by peracetylating the oligosaccharide¹ using ^{13}C -labeled acetyl groups (“isotags”), the spectral range of the protons in the carbohydrate is increased, reducing overlap and strong coupling effects. In addition, the uniformly labeled acetyl groups afford a point of entry for magnetization to be transferred into the saccharide units, allowing the analysis of the sugar ring proton spin systems through multi-dimensional correlations to the acetyl group spin-1/2 nuclei.

Multi-dimensional (2D and 3D) NMR experiments have been presented previously¹ as a method for extracting the linkages of isotag-labeled oligosaccharides. The method was effectively limited to 3D experiments, restricting the amount of information that could be extracted in any one experiment. By adding correlations to the frequencies of every NMR active nucleus, the maximum amount of information can be obtained. We present, for the first time, the application of the Filter Diagonalization Method (FDM)² in four dimensions, which markedly improves both the time and resolution requirements for this challenging application. The resolving capabilities of the FDM are based on the total product of the data points, and therefore, a large number of points in each dimension is no longer required to obtain high-resolution spectra. The corresponding reduction in experimental time makes high-dimensional experiments (4D and higher) accessible, allowing the analysis of couplings between sugar ring protons in oligosaccharides to be more readily achieved.

1. Jones, D. N. M. and Bendiak, B., *J. Biomol. NMR* 1999, 15, 157-168.
2. Mandelshtam, V. A., *Prog. NMR. Spec.* 2001, 38, 159-196.

40 NMR Characterization of a Glucuronide Metabolite of the Potent, Novel, Non-steroidal Progesterone Receptor Agonist Tanaproget

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Tanaproget is a potential first-in-class, non-steroidal progesterone receptor agonist which is under development for contraception. A major metabolite of tanaproget formed in humans was initially characterized as a glucuronide of tanaproget. Whether the glucuronide was attached to the nitrogen or sulfur of the benzoxazine-2-thione group in tanaproget could not be determined by LC/MS and LC/MS/MS analysis. To obtain additional structural details for this metabolite, additional quantities were generated from rat liver microsomal incubations and purified by HPLC for NMR analysis. The NMR data for the metabolite confirmed that the glucuronide was covalently bound to either the sulfur or the nitrogen of the benzoxazine-2-thione moiety, but the lack of key scalar and dipolar NMR couplings and correlations in the metabolite spectra (due primarily to low sample concentration) precluded an unambiguous structure elucidation. Subsequent synthesis of the S- and N-glucuronides of tanaproget from tanaproget and a protected form of glucuronic acid facilitated the unambiguous regio- and stereochemical assignment of the metabolite by comparison of 1D NMR chemical shifts and scalar coupling constants, 2D NMR correlations, and HPLC and LC/MS characteristics between the synthetic compounds and the metabolite. From extensive comparison of the spectral and chromatographic data of the microsomally-derived metabolite and the synthetic compounds, the metabolite has been determined to be the S-(beta)-D-glucuronide of tanaproget.

41

Completely Automated Structure Elucidation

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The traditional method for elucidating an unknown structure in solution state is to collect many NMR spectra of a purified sample and then use the different pieces of data from the different experiment types to draw conclusions about the structure. Attachments between atoms can be inferred and the electronic environments of atoms seen in peak shift locations can lead to assignment of peaks to the structure. The process is a tedious one for the most part, but does not need to be so. In the work presented here, a new system for automatically elucidating a chemical structure is shown. The system starts with NMR instrument control software that is capable of completely and accurately peak picking all 1D and 2D spectra for input into the elucidation. The data is then fed into the CASE (Computer Assisted Structure Elucidation) system via a command line interface. This step is key to the complete automation of the process. No data needs to be transferred manually and the execution of the structure elucidation software is completely automatic as well.

The steps in the process of elucidating the structure of *cis*-3-hexenyl cinnamate will be shown in detail. Automated elucidations of many more structures with and without direct detect carbon experiments were performed but will not be examined here.

42 Intelligent Bucketing for Metabonomics— Part 1

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Typical Principal Components Analysis (PCA) of a metabonomics data set starts with the important step of splitting up the spectra into small integral units called bins or buckets. Traditionally, the divisions for these regions were determined arbitrarily—based solely on the width the user had chosen. This method has worked reasonably well, but there was certainly room to improve upon it.

Intelligent bucketing is an algorithm that was designed to make intelligent decisions as to where a bucket division should be. A common shortcoming of traditional bucketing occurs when the edge of a bucket situates itself in the middle of a peak, resulting in a contribution of that peak to two integral regions. The nature of PCA lends itself to correcting this error, by lumping those two regions together into a single principal component vector. An inaccurate result is obtained however, when the peak invariably shifts due to minor pH or salt variations between samples, and now the contribution of the same peak is split asymmetrically across more than one integral region. Intelligent bucketing chooses integral divisions based on local minima and will therefore avoid this common, yet unfortunate scenario.

PCA results from both bucketing techniques will be compared and contrasted with a set of urine samples. The specific advantages of the intelligent bucketing technique will be demonstrated.

43

Spectrum-Structure Verification Accuracy

Brent Lefebvre, Sergey Golotvin and Ryan Sasaki

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The process of matching a chemical structure to an NMR spectrum can be a very time-consuming and challenging task depending on the complexity of the compound of interest. In a research facility, considerable time is spent determining the chemical identity of a compound through an NMR spectrum. This process of checking the consistency of a structure to an experimental spectrum is often referred to as verification. The verification process can be carried out manually, using the professional knowledge of a chemist or an NMR expert to create and evaluate the structure-to-spectrum correspondence. Alternatively, NMR prediction software can aid in the process of verification in two distinct ways. The first involves comparing predicted and experimental spectra side-by-side and evaluating the quality of the match. The second approach involves an automatic verification process where the software evaluates the quality of the structure to spectrum correspondence. Both of these approaches can result in substantial time savings for the user as well as increased confidence in their assignments.

Here we report the results of a validation of our verification process using 47 randomly selected spectra and their corresponding structures. Each spectrum was matched to each structure in the respective dataset and ^1H verification was performed on all combinations. In addition, with the understanding that structural analysis can often be done with compounds similar in structure (within the same chemical family), we conducted a similar study of the ^1H verification of 12 indole derivatives. The results of these studies will investigate how well the software can differentiate false positives from real positives along with the sensitivity and specificity of this technique.

44

Utilizing the Power of ^{31}P NMR Prediction Software for Structural Validation and Data Storage

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Advanced Chemistry Development

^{31}P NMR can be a powerful analytical technique for the verification and elucidation of chemical structures. This technique is common for carbon and hydrogen atoms, but for the phosphorus-31 nucleus, this method of analysis is not nearly as common. The particularly high sensitivity of the phosphorus nucleus entails that small structural changes around the phosphorus atom can be observed in a ^{31}P NMR spectrum. As a result, structure validation is quite easy with this exceptionally diagnostic piece of information. Alternatively, an elucidation of the structure with this information is also much easier because of the unique information of the chemical environment that is obtained in a ^{31}P NMR spectrum.

For the chemist attempting to elucidate a chemical structure, the ^{31}P chemical shift can offer direct information regarding the phosphorus environment. When a database of these structures (either commercially available or produced by the user) and NMR shifts is made available, this can greatly speed up the process of identifying the compound. There is therefore value in predicting the ^{31}P NMR chemical shift for a new structure to aid in structure identification. To this end, Advanced Chemistry Development, Inc., (ACD/Labs) has developed a computer-based shift prediction program, (ACD/PNMR). This program is accompanied by a content database (culled from recent literature), that has been validated by NMR experts and assembled into a structure-based database that currently contains 23,124 structures. Details of the scientific principles and workflow of this system will be presented.

45 ¹³C NMR Spectrum of 10 Micrograms of Cholesterol

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Detection limit of ¹³C NMR spectrum was estimated for a weekend experiment in the field of 11.7T at 298K with cholesterol as a sample. We examined the ¹³C NMR experimental conditions using a Varian ¹³C Nano probe to gain a higher signal to noise ratio (S/N) for a quaternary carbon. We collected a series of spectra on a 600 ug/40 ul CDCl₃ solution with various pulse repetition times from 0.03 to 3 seconds and flip angles from 5 to 70 degrees in a fixed experimental time. The effect of the two parameters on the S/N of a quaternary carbon at C5 position was examined. When the pulse repetition time was shorter than 0.1 seconds, the optimum flip angle was 5 times larger than that predicted by the Ernst equation. With the best experimental conditions, the S/N of the quaternary carbon C5 in the spectrum acquired in 62 hours was 5.9 for 10 ug (26 nmol) of cholesterol, and the detection limit was estimated to be 5 ug for the S/N of 3.

A Test Case for Metabonomics/Metabolomics as a Screening Tool for Neurological Disorders: A Bioinformatics Approach to the Study of Batten Disease

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To understand the increasing sequence data that has characterised the post genomic era requires the integration of an assorted array of functional genomic techniques. The large scale analysis of metabolites by ¹H NMR or mass spectrometry, in conjunction with statistical pattern recognition, is one such technique, referred to as metabolomics/metabonomics. Juvenile neuronal ceroid lipofuscinoses (Batten disease) is the most common progressive neurodegenerative disorder of childhood, and is caused by mutations in the Cln3 gene. In this study, we have investigated a mouse model of Batten disease [1], to examine whether ¹H NMR based metabolomics can be used to identify associated metabolic abnormalities and help determine the mechanism responsible for neurodegeneration. Brains were taken from control and Cln3 mice aged 1 month (n=10 control, n=10 Cln3), 2-6 months (n=5 control, n=5 Cln3), 12-14 months (n=3 control, n=5 Cln3) and 15-20 months (n=12 control, n=17 Cln3), and dissected into the cerebellum, cortex and remaining tissue. Liver and heart tissue from the same mice were also examined. Metabolic profiles of control and Cln3 tissue were prepared using a combination of solution state and high resolution magic angle spinning (HR MAS) ¹H NMR. The resultant spectra were analysed by principal components analysis (PCA) and projections to latent structures by partial least squares – discriminant analysis (PLS-DA). To date, spectral profiles from control and Cln3 mice have been successfully categorised according to disease state and pertinent metabolic changes identified by PLS-DA. Key metabolite changes identified in the brain consisted of relative increases in glutamate, glutamine, myo-inositol, lactate, taurine and choline in Cln3 tissue, alongside decreases in N-acetyl aspartate, N-acetyl glutamate, creatine and gamma-aminobutyric acid. Moreover, HR MAS ¹H NMR analysis revealed differences in the ratio of CH₂CH₂CH₂/CH₂CH₃ lipid moieties, in agreement with the characteristic disruption of lipid metabolism evident in Batten disease. Overall, these changes reflect a perturbation of glutamate-glutamine metabolism and are suggestive of reactive gliosis and neuronal cell loss. This investigation records the first subclinical symptoms of the Cln3 mouse at 1 month of age, preceding histological changes identified from 3 months of age.

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47

A Modified CRISIS-HSQC for Band-Selective IMPRESS

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CRISIS (compensation of refocusing inefficiency with synchronized inversion sweep) is a powerful technique for obtaining multiplicity-edited HSQC spectra without the significant signal loss at the periphery of the spectrum. Unfortunately, the stringent requirement for the duration of the CRISIS waveforms makes them unsuitable for any other function. To this end, we will present a modified CRISIS-gHSQC pulse sequence that is useful for acquiring band-selective spectra individually or in tandem with IMPRESS (improved resolution using symmetrically shifted pulses). Band selection in this sequence, referred to as IMPRESS-CRISIS-bs-gHSQC (IC-bs-gHSQC), is achieved by replacing both C-13 p/2 pulses with time-reversed EBURP-2 waveforms. We will demonstrate that (1) high-resolution, multiplicity-edited spectra can be obtained for several regions in significantly less time by using IMPRESS and (2) IC-bs-gHSQC has a significant sensitivity advantage over the original IMPRESS pulse sequences.

48

Deuterium Isotope Effects in Carbohydrates Revisited. Cryoprobe Studies of the Anomerization and NH to ND Induced ^{13}C Shifts of Acetamido-Deoxy Sugars

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Acetamido-deoxy sugars are a common structural motif in bacterial polysaccharides. As part of a broader study of such structures as they relate to vaccine development, we have determined or confirmed the complete ^1H and ^{13}C NMR assignments of a series of N-acetyl amino sugars and some selected secondary amino sugar derivatives by 2D NMR methods. The work was facilitated by a cryoprobe, which allowed simple 1D and 2D ^1H and ^{13}C NMR spectra to be obtained from solutions of essentially single anomers of the acetamido-deoxy sugars before significant anomerization had occurred. During these studies, we observed that the resonances of ^{13}C nuclei in and around the acetamido group undergo a deuterium induced isotope shift on NH to ND exchange. In contrast to previous measurements of the OH to OD induced, differential deuterium isotope shifts of carbohydrates, which required the use of separate solutions in dual concentric cells, the NH and ND forms of the amino sugar derivatives could be detected directly as separate signals of one solution, because, in these derivatives, NH to ND chemical exchange is slower than OH to OD exchange. Nevertheless, the rates of NH to ND exchange varied widely under different conditions of solvent and D_2O availability. These rates have been monitored by ^1H and ^{13}C NMR. The magnitudes of the NH to ND induced ^{13}C shifts found were beta (CH and C=O) -0.072 to -0.100 ppm, gamma (CH) 0 to -0.043 ppm, gamma (CH₃) -0.045 to -0.053 ppm, and delta 0 to -0.012 ppm. The beta ^{13}C shift for C-1 of an N-(2,4-dinitrophenyl) group was unusually large at -0.138 ppm. Experimentally, these shifts are readily observed as splittings in some of the ^{13}C resonances of single solutions of the amino sugar derivatives, and could be useful for the selective confirmation of ^{13}C NMR assignments in situations where 2D NMR correlation methods yield ambiguous results.

49 **Structural Studies of Novel Antifungal Lipopeptides from Fungal Culture 38G272**

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Human fungal pathogens are eukaryotes and thus show significant biochemical similarity to their hosts. Therefore, a major prerequisite for the discovery of non-toxic anti-fungal therapeutics is the identification of novel fungal-selective biochemical targets and specific compounds that inhibit these targets. Fungal cell wall biosynthesis is such a target. We report here high resolution and hyphenated NMR structural studies of a series of potent, novel lipopeptide inhibitors of fungal cell wall biosynthesis from fungal strain 38G272. The structural and chemical complexity of these compounds, together with their low abundance in the producing organism make these molecules a significant challenge for structure characterization.

50 Optimization of Sample Parameters for Cryogenic Probes

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The introduction of cryogenic NMR probe technology has significantly improved the ability to apply NMR analysis to very small samples. Because the cryogenic NMR probe operates in a regime that is fundamentally different from that of a conventional "warm" probe, some long-standing sample preparation methods need to be revisited. This poster will present various aspects of NMR sample preparation and format, and how those parameters impact instrument performance.

51 **Sample Delivery: From Traditional LC-NMR to Column Trapping Capillary Flow NMR**

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In the pharmaceutical business we encounter a variety of structural problems that can be solved by using several possible NMR configurations. The most favorable setup depends on the nature and amount of sample available, as well as the question(s) being asked. Each configuration contains key common elements such as sample delivery methodology (tube, flow, etc.) and type of probe (flow, cold, capillary) that need to be optimized and appropriately matched. Since NMR spectroscopists and instrument time are always in high demand, it is vital to identify the most efficient path. With that in mind, making use of probe and delivery technology that allows for the rapid analysis of mass limited samples is critical. In the end, obtaining reasonable NMR data and most importantly, obtaining a correct structure from this data, is essential in the drug discovery and development process.

Our focus is on optimizing sample delivery, NMR time, and the spectroscopist's time. We will describe when it is best to use traditional tube NMR, LC-NMR, CapNMR, and/or column-trapping. We will show some improved direct injection and trapping techniques used for flow probes so the maximum amount of sample is delivered and observed in the active volume of the probe. Furthermore, the approximate acquisition time for various experiments vs. amount of sample for a particular delivery technique will be illustrated. From this, it will be shown that differences in 1D proton S/N that one might deem insignificant, in fact has a substantial impact on overall acquisition time when running typical 2D experiments.

52 **Interfacing NMR with HPLC: The Application of LC-SPE-cryoflow NMR in Pharmaceutical Development**

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A relatively new technique is available for interfacing a NMR spectrometer with an HPLC instrument, LC-SPE-cryoflow NMR (Liquid Chromatography-Solid Phase Extraction-cryoflow NMR). There is a general awareness of the sensitivity limitations of the cryo probes, however, often the sample limitation is in the chromatography step as is the case with low level species such as impurities. In order to make the LC-SPE-cryoflow NMR generally more useful and easier to implement, we have explored the sensitivity limitations not only as a function of probe concentration, but as a function of HPLC peak area percent. A mixture of steroids was used that gave a separation that is very close to what one would encounter in actual pharmaceutical development with the advantage of having known concentration HPLC peaks. We will show what type of NMR experiments one could expect to obtain usable information from with respect to the HPLC peak concentration. We will also discuss the realistic limitations in obtaining high quality NMR spectra from low level HPLC peaks.

53 Absolute Configuration Assignment of Primary and Secondary Amines by NMR

Jinhai Gao, Hansjoerg Haas, Yaping Wang, Karl Gunderson, Gregorz Maniara, Serge Moss, Julian Chen, Werner Breitenstein, Srinivasan Rajan

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Quick and convenient methods for the assignment of absolute configuration of chiral compounds are very important and appealing to medicinal chemists in drug discovery. The NMR method based on chiral derivatizing agents (CDAs) has been developed for this purpose. The most recent advance in this area includes the “mix and shake” technique using solid matrix-bound auxiliary reagents for the assignment. The general procedure consists of the derivatization by mixing CDA resin with the chiral substrate directly and the NMR data are acquired without any type of separation, workup or manipulation.

We have applied the “mix and shake” method for the configurational assignment of several chiral primary amines. Modifications of the published procedures have been made to extend the application to amine salts. With the aid of detailed conformational analysis by molecular modeling and dynamic NMR experiments, we have also developed a novel application of this method and successfully determined the absolute configurations of several chiral secondary amines from medicinal chemists.

54 Isolation and Characterization of 16R and 16S Hydroxy-Roquefortine C, novel Diastereomeric Metabolites from *Penicillium crustosum*

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The fungus *Penicillium crustosum* occurs on poorly ensiled grain and can be found in colder dairy production areas including Quebec and the Maritime Provinces of Canada where such contaminated silage has been associated with cattle toxicosis. Small scale growth experiments in Roux bottles has shown that this organism produces primarily roquefortine, cyclopenin and penitrem A, at roughly equal rates. In order to fully characterize the toxic potential, it was necessary to identify the minor metabolites as well. Previous LC/NMR studies have identified several of these metabolites as falling into three categories: biosynthetic precursors or derivatives of cyclopenin, metabolites of the penitrem family and derivatives of Roquefortine C, including two whose ¹H NMR and Mass spectra were indicative of a hydroxy-roquefortine C.

A sub-fraction from a large scale fermentation of *P. crustosum* was analysed by LC/NMR. The chromatogram contained two peaks with different retention times, whose ¹H spectra were indicative of a 16-OH Roquefortine C. The individual peaks were isolated by repeated injection and peak collection followed by complete characterization by NMR and MS. Analysis of the three bond proton-carbon coupling constants allowed the absolute stereochemistry of both compounds to be determined. The possible origin of these metabolites is discussed.

55 Comparison of Stop-Flow LC/NMR and LC/SPE/NMR in the Characterization of Metabolites from *Penicillium roqueforti*

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A crude fungal extract of an isolate of *Penicillium roqueforti* isolated from Quebec silage was analysed by both stop-flow LC/NMR and by LC/SPE/NMR. In the former, metabolites could be identified by both retention time and ¹H NMR spectra without the requirement for isolation or comparison with standards. However, positive identification was often difficult because important resonances were often obscured by residual signals from the deuterated LC solvents or by the applied solvent suppression techniques. Poor S/N was also a factor that made peak identification difficult.

An equivalent injection was made into the LC using non-deuterated solvents, and the main peaks were collected on SPE cartridges, dried and eluted into NMR tubes with a suitable NMR solvent. ¹H NMR spectra obtained under identical conditions were compared.

The advantages of the SPE trapping method are discussed including removing the isotope effect from the LC separation, dispensing with costly deuterated solvents for LC, maintaining metabolite integrity for labile compounds (in this case, light sensitive), and obtaining better S/N with the same amount of sample.

56 Identification of Metabolite Profiles of Canadian Isolates of *Penicillium* by Stop-Flow LC/NMR

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Saprophytic moulds of the genus *Penicillium* occur on a wide variety of both human and animal foodstuffs under poor storage conditions and generate toxic secondary metabolites (mycotoxins) that are associated with both animal and human illness. Several isolates were obtained from corn and grass silage that were associated with incidences of cattle toxicosis in Quebec, Canada. These were initially identified morphologically as belonging to the species *P. paneum*, *P. crustosum* and *P. roqueforti*. Crude fungal extracts of these isolates grown in both still and shake cultures were analysed by stop-flow LC/NMR to further chemotaxonomic identification through the metabolites detected.

While these isolates produce predominately Roquefortine C, various other metabolites were identified by both retention time and ¹H NMR without isolation or purification and often without comparison to a purified standard. Results are compared to those obtained by LC/MS. LC/NMR gives a more accurate portrayal of the relative concentrations of the compounds, which vary widely in structure and therefore in extinction coefficient at 254nm and ionization potential, present in these crude extracts.

57 **SepNMR: A System for Isolation, Purification and NMR Data Collection on Trace Components in Mixtures.**

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Mixture analysis continues to be a persistent challenge in the application of NMR in chemical and biological research. The problem can be addressed using preparation scale chromatography, fraction collection/pooling, sample cleanup and volume reduction. However, these methods require access to equipment (preparation HPLC, fraction collectors, sample drying equipment) and expertise (scale-up chromatography methods development) that are not resident in traditional NMR laboratories. Even if these are available, there is a significant time investment to obtain samples suitable for NMR analysis particularly if the component of interest is present at low levels.

Initial hopes were that LC-NMR, with its advantages of on-line separation and subsequent delivery via a flow probe, would be a powerful new tool for NMR mixture analysis. Time savings result by using chromatographic methods developed by separation science experts that could be easily adapted to an LC-NMR system. LC-NMR is useful where the component of interest is in high abundance but is patently inadequate if the compound is present at trace levels (less than 10%). While advances have been made in NMR Mixture Analysis, clearly significant barriers still exist in terms of obtaining enough pure material in the appropriate solvent and volume.

There appears to be an emerging consensus that the ideal solution is some combination of off-line preparation chromatography, on-line LC-NMR spectroscopy and high sensitivity NMR probes. Put simply, the best approach seems to be coupling a high sensitivity probe (CapNMR, cold/cryo probe) that minimizes the amount of sample required, with an off-line sample preparation system that allows for the rapid isolation, solvent exchange and volume reduction of components from a mixture. Here we describe the development and performance of such a system (SepNMR), that combines novel four pump HPLC methods, plumbing scheme and software for routine isolation and NMR analysis of ug quantities of a component of interest from complex mixtures. In addition, examples of NMR data using a high sensitivity probe on SepNMR derived samples from mixtures will be shown.

58 Application of Metabonomics in Obesity

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Metabonomics is increasingly used in the early phase of drug discovery to examine the response of the whole organism to drug exposure. The mechanism of action of weight loss agents can be defined by measuring biomarkers of efficacy and adverse effects. We have used metabonomics to evaluate the changes in metabolite levels in biofluids from dietary-induced-obese (DIO) mice upon administration of drug candidates of obesity targets. We will show how a new sample preparation technique effectively eliminates the lipoprotein fraction in the serum, and demonstrate dramatic spectral improvements prior to metabonomic analysis. The identification of several markers of different metabolic pathways can be used to classify compounds as fat-reducing or lean (muscle) reducing agents.

59

Increasing NMR Sample Availability: Capillary Flow NMR within Lead Discovery

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The ability to verify high throughput screening (HTS) compounds from the actual HTS plates has been implemented here at the Bayer Research Center. Within the high throughput screening paradigm, compounds are initially placed in a mother plate at high concentration (10mM). Replicate daughter plates are then generated at lower concentration (1mM) that feed into the HTS cascade or are used in secondary analysis. In the past, the ability for NMR to adequately analyze these sets of compounds has been limited to the analysis of the mother plates. This is due to the low concentration and limited sample quantities available from the daughter plates. A capillary flow probe (CapNMR probe) has been installed that now allows the analysis of these plates in an automated flow NMR system. Installation included retrofitting a Gilson 215 liquid handling system to accommodate a capillary flow path and modification of instrument software so that the spectrometer can be converted between BEST-NMR and capillary flow NMR. This CapNMR probe now allows both MS and NMR analysis using only 10ul from a daughter plate (1mM concentration) while conserving repository compound.

60 Unexpected Chemistry in an NMR Tube: a Caveat Concerning the Use of CDCl_3

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During the course of the characterization by NMR of a series of cassane diterpenes isolated from a Barbadian plant, we noticed some unusual reactions of one of the compounds in CDCl_3 . This compound, which had an exocyclic methylene group reversibly isomerized over time to give an intermediate with a methyl group and an endocyclic double bond. Over longer time periods, the intermediate reacted further by formal loss of H_2 to convert what was originally a substituted cyclohexene ring to an aromatic ring. In a second sample of this compound which contained traces of CH_3OH , a second final product was formed competitively, involving both aromatization and formal addition of H_2 to an olefin bond. Further investigations, in which a sample was deliberately spiked with CD_3OD , have provided some mechanistic insights. The first step in particular is almost certainly catalyzed by traces of acid from photochemical decomposition of CDCl_3 . While it is fairly widely recognized that CDCl_3 may sometimes cause sample decomposition, the observation of clear chemistry is unusual. However, a search of the literature reveals that compounds similar to our final product have been reported earlier, in cases where CHCl_3 was used extensively in extraction and/or isolation, suggesting that these compounds may also be artifacts. Treatment of CHCl_3 and CDCl_3 to remove acid impurities is recommended, i.e. use of molecular sieve.

61 Study of Acid-Base Interactions in CP-724,714 Salts/Complexes by ^{15}N Solid State NMR

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CP-724,714 is a ErbB2 inhibitor candidate for treatment of cancer. This compound has five nitrogens and as such shows multiple basic sites. Salt screen was performed based on criteria such as robust physical profiles, adequate exposure, ease of chemical synthesis, physical and chemical stability, tableting properties and compatibility with excipients. Natural abundance ^{15}N solid state NMR study of acid-base interactions in CP-724,714 succinate, malonate and maleate is presented and compared to the free base. Based on single crystal X-ray structures, the acid-base behaviours of these three samples range from a fully hydrogen transferred ionic crystal of maleate to just proton shared succinate complex. ^{15}N solid state NMR chemical shifts were found to be very sensitive indicators of the ionization states of the involved nitrogens. The observed chemical shifts correlate well with the measured pKa of the different acid-base species. In the absence of single crystal X-ray data, ^{15}N SSNMR can be a powerful tool to characterize the acid-based nature of new salts and complexes.

62 **Adiabatic Pulse Sequences for Improved Performance in High-Throughput Analysis**

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Our group has been looking at NMR experiments that meet the requirements of high-throughput analysis. Such experiments must be run quickly and reliably, producing satisfactory results without sample-to-sample calibration. A number of groups have shown the advantages of experiments using adiabatic pulses. This poster describes some new adiabatic pulse sequences based on our previous work with the DEPT-HMQC experiment. The new sequences outperform our earlier experiment, with improved sensitivity and tolerance to miscalibration.

63 Weak Alignment of Small Organic Molecules in Small Volume Probes Using a Deuterated Liquid Crystal

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We have previously reported a deuterated liquid crystal solvent, 4-*n*-pentyl-4'-cyanobiphenyl- d_{19} , that can be used *above its transition temperature* to weakly align small organic-soluble molecules, thereby permitting residual dipolar couplings to be observed¹. As the solvent (available from CDN isotopes) is rather expensive, we have compared the capabilities of the MRM/Protasis probe with the Nalorac 3 mm probe with a Shigemi tube for this specific application.

The solvent in the MRM probe aligns small molecules comparably to the Nalorac probe, and is capable of being used with all higher-dimensional pulse sequences. The probe, of course, has a much smaller internal volume so the cost of the solvent for an analysis is lowered from about \$200/sample in the Nalorac probe to about \$20/sample in the MRM probe. In addition, the MRM probe proved far easier to shim, and, provided appropriate care is taken in flushing the capillary with acetone and drying, can be used for repetitive sample analysis.

One observation of note is that NOESY and ROESY intensities change as a function of alignment. The effects are clearly related to changes in the strengths of dipolar couplings between nuclei, as the NOESY experiment under isotropic conditions is normally performed where dipolar couplings are vanishingly small. However, in this specific system, separating the extraction of detailed distance and orientation information from NOESY cross-peak intensities is rather complex because net orientation, distance, and rate of tumbling all contribute to the effects observed with increased alignment.

1. Bendiak, B., JACS, 124, 14862-14863, 2002

64

Combinations of Long-Range HMBC-Type Experiments

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Recently, numerous variations of long-range HMBC pulse sequence have been reported for the detection of long-range correlations. These include such techniques as adiabatic inversions, 2-bond and n-bond differentiations, band selection via Hadamard phase encoding and accordion optimization. Many of these features/variations are complementary to each other and can be used in conjunction with one another. Results from an investigation of the benefits of these techniques will be presented.

65

Automated NMR workflow at UCB Pharma

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Spectral data management at UCB Pharma has over the last couple of years been optimized in order to accommodate an ever increasing number of samples and the corresponding amount of data. A number of largely Perl and Php based scripts and a custom database have been developed to integrate commercial software (XWINnmr, ICONnmr, ACD Specmanager/1H Predictor) into a consistent workflow.

The system is largely based on free- and open-source software, can be rapidly extended and new functionalities implemented. Initially developed for NMR, it has in the meanwhile been extended to other analytical techniques as well.

The general scheme as well as the details of the individual modules that constitute the system will be discussed :

- web-based sample submission
- automated data processing
- semi-automated structure validation
- custom web interface for database interrogation and data retrieval
- automated backup/archiving on CD/DVD

66 **Investigations of Ligand/Peptide Interactions in a Membrane System by NMR**

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The interactions of small ligand molecules with peptides or proteins have been the focus of numerous NMR studies. The insights provided by such research help elucidate the nature of biological functions and direct the drug discovery process. The interaction between two molecules, however, can vary dramatically as a function of the local environment. NMR spectroscopy offers a unique opportunity to study and understand the relationship between environment and activity.

A number of small ligand molecules under recent investigation have been shown to interact weakly with a target peptide *in vitro*, yet are required for achieving biological activity in model rat systems. Examining this system in a membrane environment has revealed marked differences in the effects that structurally similar ligands impart upon a model membrane. A variety of NMR techniques have been used to probe this tripartite system of ligand, peptide and membrane. The current work will demonstrate how ^{31}P NMR was used to explore the effects of a peptide, in the absence and presence of 'good' and 'bad' ligands, on DPC micelles. Results from diffusion ordered spectroscopy will show the impact on diffusion for the structurally similar ligands, and 1 and 2D NMR spectra will demonstrate the structural changes occurring in the peptide under various conditions.

67 The NMR Stereochemical Distinction of Cyclic Sulphites or What to do When Long-Range Correlations are Absent

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Cyclic sulphites from 1,2-diols commonly known as 1,3-dioxathiane-2-oxide, are widely used in synthetic chemistry. In the last 25 years, many studies were carried out aiming to understand the conformational behavior of cyclic sulphites and often, conflicting conclusions are found in the literature. Calculations using ¹H NMR coupling constants have been used to support the envelope-like conformation and a pseudoaxially oriented S=O group in the case of five membered ring sulphites. Here, the *cis* protons to the S=O group are assigned as the higher frequency signals respect to the *trans* protons. The origin of such effect has been assigned to the so called “*gauche* effect” from the adjacent electronic pairs (-CHR – :O: – S=O) to polar bonds and also from repulsive stereoelectronic and electrostatic repulsive interactions between bulky groups and the S=O bond.

Here we report the synthesis and conformational analysis of the two diastereomeric forms from 4-naphtalen-1-yl-methyl-5-phenyl-[1,3,2]-dioxathiolan-2-oxide (**1** and **2**). The pair of diastereomers was obtained in good yield (> 80 %) via the reaction of *erythro*-3-naphtalen-1-yl-1-phenyl-propane-1,2-diol with thionyl chloride in dichloromethane. The ¹H NMR spectra of **1** and **2** showed significant differences in the chemical shifts of the methyne and methylene diastereotopic protons. Nevertheless it was not possible to correlate this differences with the correspondent structure.

The analysis of the crystalline structure of **1** (obtained from X-ray diffraction), the theoretical conformational analysis and theoretical prediction of nmr chemical shifts [B3LYP/6311G(d,p)//B3LYP/6311G] allowed us to correlate the experimental chemical shifts observed with the molecular interactions present in both diastereomers.

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68

Application of solid state NMR in conjunction with multivariate data calibration to quantify the amount of amorphous Org xxx in crystalline material

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The solid state form of an active pharmaceutical ingredient (API) is important to know because of its influence on dissolution rate, solubility and stability. A solid can be amorphous or it may crystallize in one or more different forms, either in the presence of solvent molecules or not. This is called (pseudo) polymorphism.

Solid state NMR is an established technique to qualitatively investigate the solid state form of an API. However, quantitative information can be obtained as well. Here we present data on compound Org xxx, which show that solid state NMR in conjunction with multivariate calibration, can be used to quantify the amount of amorphous Org xxx in crystalline material.

Spectra of calibration samples with known composition of amorphous and crystalline Org xxx were used to construct a partial least squares calibration model. The multivariate character of the method greatly enhanced precision, enabling the quantification of several percent of amorphous material in a furtherwise crystalline sample.