

## **Conference Program**

September 26<sup>th</sup>-29<sup>th</sup>, 2010  
Portland, Oregon

# SMASH 2010 NMR Conference

Dear SMASH 2010 Attendees,

We want to welcome you to the 2010 **S**mall **M**olecules **A**re **S**till **H**ot NMR conference in Portland, Oregon. The conference site is located on the East side of the Willamette River which runs directly through the middle of the city. Aside from a great location to hold the conference, the city of Portland provides many interesting opportunities to enjoy some free time. In particular there is the Oregon Trail, Portland Rose garden, local breweries/wineries, etc... If time allows, there are also many outdoor adventures within a short distance. These include the nearby Mt. Hood, Mt. St. Helens and Multnomah Falls to name a few. The weather is well suited for the outdoors, typically dry and sunny with high temperatures of approximately 70°F (21°C).

We are excited to announce that we will be co-hosting the conference with CoSMoS, the **C**onference on **S**mall **M**olecule **S**cience. Each conference will have their own oral sessions and workshops on Monday and Tuesday, and then combine for joint sessions on Wednesday. Since we wanted to facilitate interaction between the two groups, we will share all meals, breaks, poster sessions, and our after dinner speaker. More importantly, your SMASH registration will allow you to attend any CoSMoS session/workshop, and you'll also see CoSMoS attendees in our sessions. This conference combination highlights the interdisciplinary nature of the work that many of us are doing. To accommodate the joint sessions, we have broken from tradition of ending mid-day on Wednesday and will instead have a full day.

This year's program has oral sessions titled, "*Latest and Greatest in NMR*", "*Solids NMR*", "*Real-Time Reaction Monitoring*", "*Graduate and Postdoctoral Students*", "*How Low Do You 'Need' To Go? – Techniques, Applications & Considerations of Trace Analyses*", and "*NMR & MS Imaging*". We have five excellent workshops lined up, "*Residual Dipolar Coupling, From Sample Preparation to Stereochemistry*", "*So, You Want to Quantitate?*", "*Setting Out on Your Own: Entrepreneurship in a Niche Environment*", "*NMR, It's Not just for Structures: Determination of Physicochemical Properties*", and finally "*Bringing It All Together: Multi-Disciplinary Structure Elucidation*". While posters will be up the entire conference, those with even numbers will present on Monday and odd numbers on Tuesday. After dinner mixers on both evenings in the poster area will allow for discussions to continue.

The Monday keynote after dinner speaker is Bill Fenical, Distinguished Professor of Oceanography and Pharmaceutical Science from the Scripps Institution of Oceanography at the University of California. He will be talking about "*Exploring the Deep Oceans, A Source of Intriguing Small Molecules*". If you ever wondered what's in the depths, this is a presentation you don't want to miss.

On behalf of the SMASH Organizing Committee, we want to extend a warm welcome and thank you for attending SMASH 2010.

Sincerely,

Brian Marquez and Andreas Kaerner  
Co-Chairs, SMASH 2010 NMR Conference

# SMASH 2010 NMR Conference Program

The poster sessions, meals and breaks will be in the Exhibit Hall unless otherwise specified.  
All sessions will be in the Holladay/Broadway/Weidler/Halsey Rooms of the Lloyd Center Ballroom.

## Sunday September 26<sup>th</sup>

**4:30 PM - 6:00 PM Registration** (In front of Holladay Room)

**6:00 PM - 8:00 PM Dinner** (Lloyd Center Ballroom)

**8:00 PM - 11:00 PM Mixer** (Lloyd Center Ballroom)

## Monday September 27<sup>th</sup>

**7:30 AM - 8:30 AM Breakfast**

**8:45 AM - 9:00 AM Opening Remarks**

**9:00 AM - 10:30 AM Latest and Greatest in NMR**

Tim Spitzer, GlaxoSmithKline

*2D NMR in the Gas Phase: Correlation and Diffusion Experiments*

Alexander Marchione, DuPont Central Research and Development

*Enhancing Small Molecular NMR and Molecular Imaging using New Spin Physics*

Warren S. Warren, Duke University

*Denosing One-Dimensional NMR Solution-Phase Spectra*

Howard Taylor, University of Southern California

**10:30 AM - 11:00 AM Break**

**11:00 AM - 12:30 PM Solids NMR**

Ales Medek, Vertex

*Advanced <sup>19</sup>F SSNMR Techniques in Pharmaceutical and Chemical Product Development*

Mark Strohmeier, GlaxoSmithKline

*NMR Crystallography*

James Harper, Department of Chemistry, University of Utah

*Structure and Dynamics of N-terminal Sequences of Dermorphin Neuropeptide in the Solid State-NMR Spectroscopy Versus X-ray Crystallography*

Marek Potrzebowski, Polish Academy of Sciences

*Dynamic Nuclear Polarization at 263 GHz: Experimental Methods and Applications*

Shane Pawsey, Bruker BioSpin Corp.

**12:30 PM - 2:30 PM Lunch, Free Time & Vendor Discussions**

**2:30 PM - 4:00 PM Workshops (concurrent)**

*Residual Dipolar Couplings: From Sample Preparation to Stereochemistry* (Holladay Room)

Roberto Gil, Carnegie Mellon University

Burkhard Luy, Technische Universität München

*So, You Want to Quantitate?* (Broadway/Weidler/Halsey Rooms)

Dave Lankin, University of Illinois-Chicago

Patrick Hays, DEA Special Testing and Research Laboratory

**4:30 PM - 6:00 PM Poster session with CoSMoS (Even numbered posters)**

Gene Mazzola, University of Maryland, FDA Joint Institute

Laurie Galya, Incyte Corp.

**6:30 PM - 9:00 PM Dinner**

**After Dinner Speaker: Professor Bill Fenical**, University of California

*Exploring the Deep Oceans, A Source of Intriguing Small Molecules*

**9:00 PM - 11:00 PM Mixer & Continuation of Poster Discussion**

## Tuesday September 28<sup>th</sup>

**7:30 AM - 8:30 AM Breakfast**

**8:00 AM - 9:00 AM Student/Industry Breakfast Roundtable**

**9:00 AM - 10:30 AM Real-Time Reaction Monitoring**

Mike Bernstein, AstraZeneca

*Practical Applications of Compact High Resolution 60 MHz Permanent Magnet NMR Systems for Reaction Monitoring and Online Process Control*

John Edwards, Process NMR Associates

*Monitoring Organic Reactions: What can UF-NMR Spectroscopy Offer*

Encarnación Fernández Valle/Dolores Molero, Madrid University

*New Chemometric Methods for Alignment (icoshift), Classification (ECVA) and Information Extraction of NMR Data*

Søren Engelsen, Copenhagen University

**10:30 AM - 11:00 AM Break**

**11:00 AM - 12:30 PM Graduate Students & Postdoctoral Students Session**

Xiaohong Li, University of Akron

*New Applications of Filter Diagonalization Method to Small Molecules*

Hasan Celik, University of California, Irvine

*Use of Multiple Homonuclear Decoupling for Small Molecule Structural Elucidation*

Ana Paula Espindola, University of Texas Southwestern Medical Center

*Versatile <sup>1</sup>H-<sup>31</sup>P-<sup>31</sup>P COSY 2D NMR Techniques for the Characterization of Polyphosphorylated Small Molecules*

Yan Sun, Johns Hopkins School of Medicine

*Advanced 2D NMR Techniques for Fluoropolymer Model Compounds*

Xiaohong Li, University of Akron

**12:30 PM - 2:30 PM Lunch, Free Time & Vendor Discussions**

**2:30 PM - 4:00 PM Workshops (concurrent)**

*Setting Out on Your Own: Entrepreneurship in a Niche Environment* (Holladay Room)

Tim Peck, Protasis/MRM Corp.

*NMR, It's Not Just For Structures: Determination of Physicochemical Properties*

(Broadway/Weidler/Halsey Rooms)

Manuel Perez, Pfizer

**4:30 PM - 6:00 PM Poster session with CoSMoS (Odd numbered posters)**

Gene Mazzola, University of Maryland, FDA Joint Institute

Laurie Galya, Incyte Corp.

**5:30 PM - 7:00 PM Wine and Cheese Party - At the CortecNet Exhibitor Table**

**6:30 PM - 8:30 PM Dinner & Best Poster Award**

**8:30 PM - 11:00 PM Mixer & Continuation of Poster Discussion**

## Wednesday September 29<sup>th</sup> (Joint with CoSMoS)

**7:30 AM - 8:30 AM Breakfast**

**8:30 AM - 10:30 AM How Low Do You 'Need' To Go? - Techniques, Applications and Considerations of Trace Analyses**

Charlotte Corbett, US Department of Justice

Elaine Ricicki, Agilent

*Trace Analysis of Seized Drugs using Ultra High Performance Liquid Chromatography Tandem Mass Spectrometry*

Ira S. Lurie, Drug Enforcement Administration

*An Investigation of Experimental Limits of Small Sample Heteronuclear 2D NMR*

Gary Martin, Merck

*Trace Level Detection of Compounds Related to the Chemical Weapons Convention by <sup>1</sup>H-Detected <sup>13</sup>C NMR Spectroscopy Executed with a Sensitivity-Enhanced, Cryogenic Probehead*

Terry J. Henderson, US Army, Edgewood Chemical Biological Center

*Forensic Drug Identification in Blood, Urine and Unconventional Matrices such as Hair, Saliva, Liver, Brain, Vitreous Humor*

Ashraf Mozayani, Harris County Institute of Forensic Sciences, Houston, Texas

**10:30 AM - 11:00 AM Break**

**11:00 AM - 12:30 PM Workshop** (Broadway/Weidler/Halsey Rooms)

*Bringing it All Together: Multi-Disciplinary Structure Elucidation*

David Russell, Agilent Technologies

Jonathan Josephs, Bristol Myers Squibb

**12:30 PM - 2:30 PM Lunch, Free Time & Vendor Discussions**

**2:30 PM - 4:30 PM NMR and MS Imaging**

Andreas Kaerner, Eli Lilly & Co

YingYing Huang, ThermoFisher

*NMR Microscopy of Formulated Pharmaceutical Products*

Andrew Phillips, AstraZeneca, UK

*MS Imaging: Snapshots of Tissue Distribution*

Stephen Castellino, GlaxoSmithKline

*Imaging Mass Spectrometry: A New Tool for Small Molecule Analysis*

Michelle L. Reyzer, Vanderbilt University

**4:30 PM - 4:45 PM Closing Remarks**



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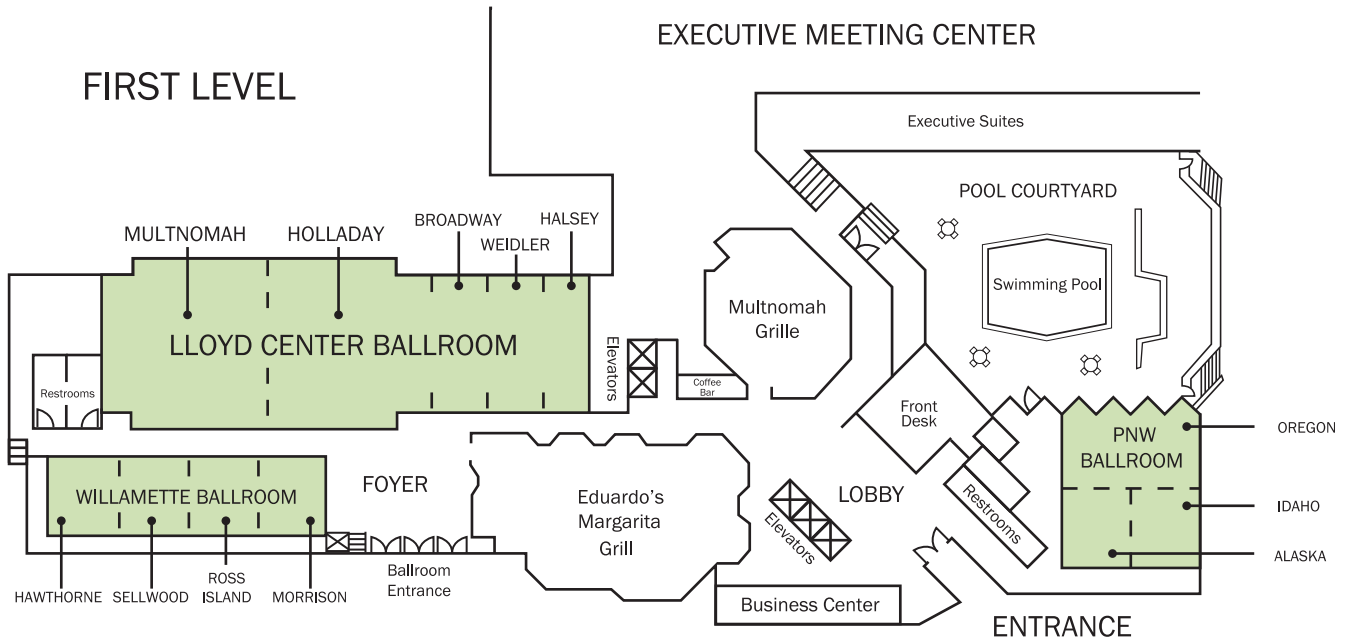


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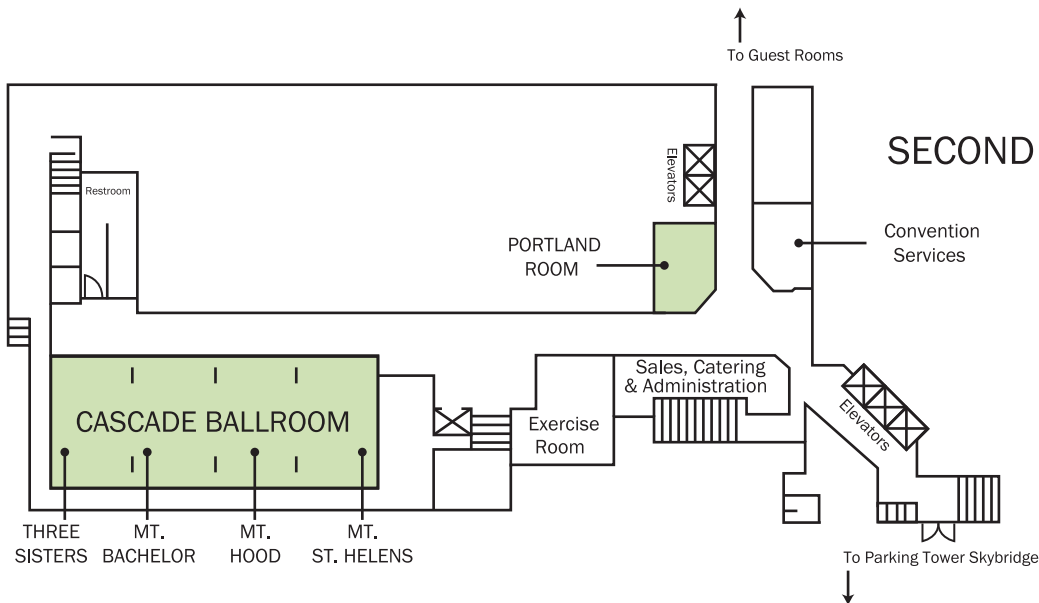
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# **SMASH 2010 NMR Conference Committee**

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## **Poster Session**

Gene Mazzola  
University of Maryland

Laurie Galya  
Incyte Corp.

Monday, September 27<sup>th</sup>  
9:00 AM - 10:30 AM

**Latest and Greatest in NMR**  
Chair: Tim Spitzer

*2D NMR in the Gas Phase: Correlation and Diffusion  
Experiments*

Alexander Marchione, DuPont Central Research and  
Development

*Enhancing Small Molecular NMR and Molecular Imaging using  
New Spin Physics*

Warren S. Warren, Duke University

*Denoising One-Dimensional NMR Solution-Phase Spectra*  
Howard Taylor, University of Southern California

# 2D NMR in the Gas Phase: Correlation and Diffusion Experiments

Alexander A. Marchione

DuPont Central Research and Development, Wilmington, DE, USA

Despite the increasing application of gas phase NMR to the study of chemical reactions in the last 15 years, the application of common two dimensional solution-phase experiments to gaseous analytes has not been reported. Common 2D correlation experiments, including COSY, HMQC, HSQC, and HMBC, are readily applicable to typical hydrocarbons and fluorocarbons at ordinary pressures. The only significant limitation is in the use of pulsed field gradients, where rapid diffusional motion in the gas phase results in significant loss of signal intensity in sequences requiring a relatively long period ( $> 1$  ms) for gradient coherence selection. Experiments using  $^{19}\text{F}$  and  $^{13}\text{C}$  as the observed nucleus benefit, relative to solution phase work, from rapid spin-rotation relaxation, which enhances their effective sensitivity and renders HETCOR and even INADEQUATE experiments possible in the gas phase. Gas phase NMR is thus rendered a useful complement to ubiquitous GC/MS analyses for characterization of gaseous analytes.

Of particular interest to gaseous systems are DOSY-type experiments. The spectral separation of mixtures of fluorocarbons can be readily achieved with standard pulse sequences and instrumentation. Also of interest is the derivation of absolute diffusion rates of gases, which is relevant to industrial process modeling. The diffusion rates of a set of gaseous systems obtained from DOSY experiments matched predicted rates to within 20%.

# Enhancing Small Molecule NMR and Molecular Imaging using New Spin Physics

Warren S. Warren

Department of Chemistry, Duke University, Durham, NC

NMR is by far the most powerful form of spectroscopy to the practicing chemist, and MRI has become a very powerful clinical imaging modality, for two fundamental reasons. First, the hardware is mature: modern NMR spectrometers and MRI machines routinely give complex sequences of arbitrarily shaped radiofrequency pulses to create precise excitation, and give magnetic field gradient pulses to suppress magnetization or obtain spatial resolution. More importantly, however, the theoretical framework is mature. No other modern spectroscopy has such a strong theoretical basis, which of course is used to understand the structures of molecules as complicated as proteins in solution. This maturity is even more important in MRI: complications associated with imaging *in vivo* can often be reduced or eliminated by clever pulse sequence design.

However, the sophistication of this framework carries with it an inherent danger. The magnetic resonance community has long since beatified Bloch, Bloembergen, Hahn, Purcell and many of the other brilliant physicists who pioneered the field, at a time when it was at the very cutting edge of “modern physics.” But none of these men claimed omniscience, and all of their work relied on underlying assumptions. When experimental conditions change (for example, as the community gradually accessed higher fields, or looked at more complex samples such as tissue) it is easy to forget to check if the changes challenge these assumptions, or if the result changes the basic predictions of magnetic resonance. I will discuss several projects in my laboratory which, in fact, contradict what seems “obvious” in the standard picture of magnetic resonance; and I will discuss a variety of application to signal and contrast enhancement in molecular imaging.

1. Over the last decade, our group and others have shown that intermolecular multiple-quantum coherences (iMQCs)-simultaneously flipping multiple spins, separated by a macroscopic distance-can give readily detectable signals that provide different contrast in magnetic resonance imaging with a wide range of applications, including enhanced tumor detection and functional imaging. For example, we have used measurement of fat-water crosspeaks to measure absolute temperature deep in tissue, with far greater accuracy than conventional methods. Temperature, one of the most fundamental intrinsic quantities of matter, is very difficult to measure noninvasively beneath the surface. One pressing need for this technology is in the real-time monitoring of hyperthermic cancer treatments. These adjunctive therapies deliver localized heat to enhance many traditional treatments and have already seen positive Phase III clinical trials. However, delivering a clinically relevant dose requires accurate temperature imaging *in vivo*, and this has proven very challenging. The best endogenous approach to traditional MR thermometry uses the change in chemical shift of water protons (0.01 ppm/°C, or 3Hz/°C in a 7 Tesla magnet) to monitor temperature changes, but lineshapes *in vivo* are broad, and the topography of the local magnetic field (which generates the inhomogeneous lineshape) changes dramatically with motion, heating, drift, and

susceptibility gradients. Our method, which measures the simultaneous spin flips of fat (which has no temperature dependence) and water, removes these complications and thus permits absolute temperature measurement. We have also shown that these methods can be used to do localized spectroscopy of fatty tissues, resolving resonances that are inaccessible by conventional imaging, such as the difference between brown adipose tissue (the “good fat” which burns calories) and white adipose tissue.

2. Magnetic resonance has always suffered from the relative insensitivity, due to the small energy spacing compared to  $kT$ . In recent years, hyperpolarization methods have dramatically improved the range of potential applications; most notably, dynamic nuclear polarization (DNP) coupled with rapid dissolution has led to signal enhancements of  $10^4$  or greater, and is commercially available. However, the major limitation is the  $T_1$  relaxation time, which restricts the application of this method to very rapid processes. I will discuss a new approach to hyperpolarized agent generation using true singlet states, which surmount the previous limitations from short relaxation times. We present specific molecular systems where it is possible to store nonequilibrium magnetization in the singlet, without moving the spins out of the magnetic field or actively decoupling them. The consequence is that it is possible to envision an entirely new class of hyperpolarized reagents with spin lifetimes extending to many minutes, or even longer, and this holds the potential for transforming hyperpolarized NMR from a laboratory curiosity into a genuine molecular imaging method.

3. A recent quantum computing paper made an astonishing prediction that multiple echo sequences can be improved by using a specific, unequal spacing, as opposed to the approach universally used in MR since the 1950s (Carr-Purcell-Meiboom-Gill (CPMG) with equally spaced echo pulses). This was demonstrated to be correct for reducing dephasing of optically trapped ions. We have shown that, in fact, these optimal “UDD sequences” produce different  $T_2$ -weighted contrast in magnetic resonance than do CPMG sequences with substantial gains in most regions. The key is understanding that in MRI of structured materials such as tissue, diffusion in compartmentalized and microstructured environments with susceptibility gradients leads to fluctuating fields on a range of different timescales. The UDD sequence can be shown to do the best possible job of suppressing effects of very low frequency fluctuations, and thus can outperform the conventional multiple echo sequences. We have shown that, both in excised tissue and in a live mouse tumor model, optimal UDD sequences produce different contrast than do CPMG sequences with the same number of pulses and total delay, with substantial enhancements in most regions (signal gains typically were 20-70%). This new source of endogenous contrast has potential applications in human MR (particularly at lower fields, where power dissipation is not an issue), because varying the length of the UDD sequence effectively maps out the spectral density function.

# **Denoising One-Dimensional NMR Solution and Solid Phase Spectra**

**H.S. Taylor** and A. Kershaw

Department of Chemistry, University of Southern California, Los Angeles, California, 90089

An Algorithm and its associated user-friendly software is presented for increasing sensitivity in NMR experiments that are expected to yield Lorentzian Spectra. The Algorithm requires so many less transients than the usual Fourier analysis that  $^{13}\text{C}$ (quaternary),  $^{15}\text{N}$  and  $^{17}\text{O}$  experiments can usually be accelerated and enrichment avoided. The algorithm and method for given signal length yields significantly higher resolution than the Fourier signal processing method. Examples are presented. The disadvantage of the Fourier Method is that it needs many transients ( $S/N$  proportional to  $\sqrt{Ntr}$ ) to remove noise (grass). Its advantage, once the noise is reduced, is its robustness and the fact that it can process any type of signal. The new algorithm overcomes the Fourier disadvantages but requires the introduction of a model for the noiseless signal that here is the weighted sum of damped harmonics which in turn limits it to Lorentzian spectra. The new model shows no "grass" but instead fits noise to Lorentzian looking peaks that could, and in the past, have been mistaken for signal. The main advance in the new algorithm is that it shows how to recognize and discard noise peaks to obtain only signal features. The latter emerge when, locally about each feature of interest, the  $S/N$  ratio in the standard Fourier spectrum is roughly 1.1.

For associated publications and for further explanation and examples go to:

<http://www-rcf.usc.edu/~taylor/>

Monday, September 27<sup>th</sup>  
11:00 AM - 12:30 PM

**Solids NMR**  
Chair: Ales Medek

*Advanced <sup>19</sup>F SSNMR Techniques in Pharmaceutical and  
Chemical Product Development*

Mark Strohmeier, GlaxoSmithKline

*NMR Crystallography*

James Harper, Department of Chemistry, University of Utah

*Structure and Dynamics of N-terminal Sequences of  
Dermorphin Neuropeptide in the Solid State-NMR Spectroscopy  
Versus X-ray Crystallography*

Marek Potrzebowski, Polish Academy of Sciences

*Dynamic Nuclear Polarization at 263 GHz: Experimental  
Methods and Applications*

Shane Pawsey, Bruker BioSpin Corp.

# Advanced $^{19}\text{F}$ SSNMR Techniques in Pharmaceutical and Chemical Product Development

**Mark Strohmeier**

GlaxoSmithKline  
Analytical Sciences  
709 Swedeland Rd  
King of Prussia, PA 19406

The physical and chemical properties of organic molecular crystals greatly depend on the salt and polymorphic form that significantly affects the manufacturability, stability and bioavailability of the drug. Determining the phase purity of drug substances is one of the main challenges in the early development stages of pharmaceuticals since often the knowledge of the material's phase diagram and polymorphic forms is incomplete, crystal structures are elusive and only a few different batches of material are available.

Assessing the phase purity with solid-state NMR relies on the assignment of the observed resonances in a given spectrum to magnetically inequivalent positions in the crystal structure. This is rather straight forward for known crystal structures with only one molecule per asymmetric unit ( $Z'=1$ ) and in the absence of site disorder. However, often crystal structures are unknown and additional resonances are observed and need to be explained in terms of site disorder, multiple molecules in the asymmetric unit ( $Z'>1$ ), the presence of a second molecular entity in the lattice (inclusion, cocrystal, solid-solution etc) or a phase impurity. This assignment is critical as inclusion of unwanted molecules and phase impurities pose a risk for the process and product performance and need to be identified with high confidence.

We have applied long ranging magnetization transfer experiments such as  $^{19}\text{F}$  2D DARR in conjunction with  $^{19}\text{F}$ - $^{13}\text{C}$  HETCOR experiments to prove phase association of additional unassigned  $^{19}\text{F}$  and  $^{13}\text{C}$  resonances. Several commercially available pharmaceuticals, phase mixtures, molecular cocrystals and solid solutions were investigated to prove the concept and to provide an estimate for the achievable range of  $^{19}\text{F}$ - $^{19}\text{F}$  and  $^{13}\text{C}$ - $^{19}\text{F}$  magnetization transfers.

# NMR Crystallography

James K. Harper and David M. Grant

Department of Chemistry, University of Utah, 315 S. 1400 E. Salt Lake City, UT 84112.

For decades solid-state NMR revealed structural information on fragments of molecules, but failed to provide independent structural characterizations. In 2002, these structural hints were finally combined for the first time to provide complete molecular structures. Crystallographically, these NMR determined structures represent the point group. Since NMR parameters usually monitor short-range interactions ( $\sim 4\text{\AA}$ ), extension of these data to predict space group is challenging and is only now being pursued by two different approaches. Usually, the NMR determined point group is combined with x-ray powder diffraction (XRD) data to establish the space group. Alternatively, crystal structure prediction software allows trial space groups to be generated computationally using a chosen point group and NMR data can then be used to select from among these structures. Work emphasizing the chemical shift tensor will be described including the role of computation of shifts. Recent work demonstrates that structures obtained from combined XRD/NMR analyses can be further refined using NMR tensor data. This refinement converts XRD/NMR structures from relatively low quality structures into geometries rivaling those obtained from single crystal diffraction data.

# Structure and Dynamics of N-terminal Sequences of Dermorphin Neuropeptide in the Solid State - NMR Spectroscopy Versus X-ray Crystallography

Marek J. Potrzebowski<sup>1</sup>, Katarzyna Trzeciak-Karlikowska<sup>1</sup>, Hassan Kassasir<sup>1</sup>, Włodzimierz Ciesielski<sup>1</sup>, Grzegorz Bujacz<sup>2</sup> and Anna Bujacz<sup>2</sup>

1. Centre of Molecular and Macromolecular Studies, Polish Academy of Sciences, Sienkiewicza 112, 90-363 Lodz, Poland
2. Technical University of Lodz, Institute of Technical Biochemistry, Stefanowskiego 4/10, 90-924 Lodz, Poland

In the 1970's the first reports showing that peptides have similar biological properties and functions as opiates were reported. The enkephalins (Tyr-Gly-Gly-Phe-Leu (Lenk) and Tyr-Gly-Gly-Phe-Met (Menk)), the first endogenous opioid peptides, were isolated and identified from pig brain in 1975. Other opioid peptides coming from natural sources, e.g. deltorphin I (Tyr-D-Ala-Phe-Asp-Val-Val-Gly-NH<sub>2</sub>) and dermorphin (Tyr-D-Ala-Phe-Gly-Tyr-Pro-Ser-NH<sub>2</sub>) were found in the skin of South American frogs. The presence of D amino acid is crucial for biological activity. The synthetic analogs of given supra heptapeptides consisting of L-alanine are not biologically active.

Our interest is focused on understanding of influence of the alanine stereochemistry on crystal molecular packing and internal dynamics in the solid state of amino acids in sequences Tyr-Ala-Phe and Tyr-Ala-Phe-Gly. In the first part of the talk the power of NMR spectroscopy as tool for searching of different polymorphs of tri- and tetrapeptides containing L-alanine in sequence will be shown. NMR results will be compared with X-ray data of single crystals. The influence of stereochemistry of alanine on molecular packing in term of weak C-H...π interactions will be presented. In the second part of the talk distinct molecular dynamics of polymorphs of tripeptides and tetrapeptides containing L- and D-alanine will be discussed. Applicability of <sup>1</sup>H -<sup>13</sup>C PILGRIM, <sup>1</sup>H-<sup>13</sup>C PISEMAMAS and <sup>2</sup>H NMR static measurement for analysis of molecular motion will be shown. Finally problem of bioactive conformation of native and fully <sup>13</sup>C labeled tetrapeptide containing D-Ala embedded to phospholipid DMPC/DMPG layers, usefulness of <sup>1</sup>H RF Driven NOESY and variable temperature <sup>13</sup>C NMR measurements, relation of X-ray structure to structure found in physiological environment will be discussed.

# Dynamic Nuclear Polarization at 263 GHz: Experimental Methods and Applications

Shane Pawsey<sup>1</sup>, Melanie Rosay<sup>1</sup>, Ralph T. Weber<sup>1</sup>, Richard J. Temkin<sup>2</sup>, Robert G. Griffin<sup>2</sup>  
and Werner E. Maas<sup>1</sup>

1. Bruker BioSpin Corporation, Billerica, MA
2. Massachusetts Institute of Technology, Cambridge, MA

Dynamic Nuclear Polarization (DNP) can be used to substantially increase the sensitivity of NMR by transferring higher Boltzmann polarization of unpaired electron spins to nuclear spins. To accomplish the polarization transfer unpaired electrons are irradiated with microwaves at or near the electron Larmor frequency. We have developed a spectrometer for DNP experiments of solids at 263 GHz microwave frequency, 400 MHz <sup>1</sup>H frequency, and have recorded signal enhancements up to a factor of 80 at 100 K using the biradical TOTAPOL.<sup>1</sup> A high power gyrotron is used to generate the microwaves, which are transmitted to the NMR probe via a corrugated waveguide, to irradiate the sample in a 3.2 mm rotor for magic angle spinning DNP-NMR experiments. A variety of samples have been successfully polarized ranging from small molecules to proteins. This contribution will focus on the factors influencing DNP transfer efficiency, sample preparation, and potential applications in the area of small molecules.

1. Song, C., et al. *J. Am. Chem. Soc.*, 128, 11385-11390, 2006

Monday, September 27<sup>th</sup>  
2:30 PM - 4:00 PM

## **Workshops**

*Residual Dipolar Couplings: From Sample Preparation to Stereochemistry*

Roberto Gil, Carnegie Mellon University  
Burkhard Luy, Technische Universität München

*So, You Want to Quantitate*

Dave Lankin, University of Illinois-Chicago  
Patrick Hays, DEA Special Testing and Research Laboratory

# Residual Dipolar Couplings: From Sample Preparation to Stereochemistry

Roberto R. Gil<sup>1</sup> and Burkhard Luy<sup>2</sup>

1. Department of Chemistry, Carnegie Mellon University, Pittsburgh, PA, USA

2. Institute of Organic Chemistry, Karlsruhe Institute of Technology, Karlsruhe, Germany

This workshop will illustrate how Residual Dipolar Couplings (RDCs) can provide extremely powerful structural information in the analysis of small molecules, particularly when traditional NMR experiments, such as 3J coupling constant analysis and Nuclear Overhauser Enhancement (NOE) fail to provide a unique configuration and/or conformation. The information obtained by the later methods is of local character and is restricted to structural information in the local environment of a molecule. However, RDCs provide information of non-local character and it is possible to determine the relative configuration of stereocenters no matter how far they are located one from each other. These NMR parameters help to lift the local information limitations provided by traditional methods.

Since RDCs are not directly observed in conventional liquid state NMR experiment (isotropic conditions), the sample needs to be exposed to an anisotropic medium to reveal their values. Anisotropy can be induced in the NMR sample either by using stretched or compressed cross-linked polymers (gels) or liquid crystal solutions of homopolypeptides such as PBLG or PELG in CDCl<sub>3</sub>.

In the present workshop we will cover the features and limitation of the use of RDCs for the stereochemical analysis of small molecules in organic solvents. We will cover the topics of sample preparation, NMR experiments for the acquisition of RDCs in aligned media, as well as computationally based RDC data analysis procedures to determine relative configuration and conformation of small molecules.

We have selected few real problems as examples to demonstrate how to determine the configuration and/or conformation of small molecules.

Note: apart from the speakers, the following scientists have participated in the organization of this workshop:

Christian C. Griesinger, NMR-Based Structural Biology Department, Max Planck Institute for Biophysical Chemistry, Göttingen, Germany.

Armando Navarro-Vázquez, Department of Chemistry, Universidade de Vigo, Vigo, Spain.

Christina M. Thiele, Department of Chemistry, Technical University of Darmstadt, Darmstadt, Germany.

1. G. Kummerlöwe, B. Luy, "Residual dipolar couplings for the configurational and conformational analysis of organic molecules", Annual Reports in NMR Spectroscopy 68,

- 193-232 (2009).
2. C.M. Thiele, "Residual dipolar couplings (RDCs) in organic structure determination", *European Journal of Organic Chemistry* 34, 5673-5685 (2008).
  3. F. Kramer, M.V. Deshmukh, H. Kessler, S.J. Glaser. "Residual dipolar coupling constants: An elementary derivation of key equations". *Concepts in Magnetic Resonance Part A* 21A, 10-21 (2004).
  4. J. A. Losonczi, M. Andrec, M.W.F. Fischer, J.H. Prestegard. "Order matrix analysis of residual dipolar couplings using singular value decomposition". *Journal of Magnetic Resonance* 138, 334-342 (1999).

# So, You Want to Quantitate?

David C. Lankin<sup>1</sup> and Patrick A. Hays<sup>2</sup>

1. UIC College of Pharmacy, Dept. of Medicinal Chemistry & Pharmacognosy, Chicago, Illinois 60612-7231
2. DEA Special Testing and Research Laboratory, Dulles, Virginia 20166

Contemporary NMR spectroscopy constitutes a marvelous qualitative tool for use in complete structure elucidation of small molecule organic compounds. However, the use of NMR as a quantitative tool is becoming equally important. Recently, there has been renewed interest in the quantitative aspects of NMR (qNMR) as a primary analytical tool for the characterization of small organic molecules<sup>1-3</sup> which includes applications for: 1) assessing absolute purity of materials, 2) assaying the quality of primary standards and organic chemicals of all kinds, 3) checking the purity of drug preparations and dietary supplements, 4) quantifying complex mixtures, and 5) quantitatively assessing the content of active species in natural product extracts and fractions, and 6) quantifying metabolite content. The emphasis of the present workshop will be to 1) summarize all of the basic fundamental experimental details of how to establish and optimize NMR data acquisition parameters for acquiring good quantitative NMR data. Currently available methodologies, and their caveats, for extracting the quantitative information from the resultant qNMR spectra will also be presented and discussed. While the focus will largely relate to the use of quantitative proton NMR (qHNMR), the use of other NMR active nuclei for quantifying applications will be elaborated. Handouts of the lecture material together with key references / bibliography will be provided to the workshop attendees.

1. Pauli, Guido F., Jaki, Birgit U., Lankin, David C., Quantitative <sup>1</sup>H NMR: Development and Potential of a Method for Natural Product Analysis, *J. Nat. Prod.*, 68, 133-149 (2005).
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3. Holzgrabe, Ulrike, Quantitative NMR Spectroscopy in Pharmaceutical Applications, *Progress in NMR Spectroscopy*, 57, 229-240 (2010).

Monday, September 27<sup>th</sup>

**After Dinner Speaker**

*Exploring the Deep Oceans, A Source of Intriguing Small  
Molecules*

Professor Bill Fenical, University of California

# Exploring The Deep Oceans, A Source of Intriguing Small Molecules

**William Fenical**

Center for Marine Biotechnology and Biomedicine., Scripps Institution of Oceanography,  
UCSD, La Jolla, CA 92093-0204

and

Skaggs School of Pharmacy and Pharmaceutical Sciences, UCSD, La Jolla, CA 92093-0204

For 50 or more years, ending in the mid 1990s, soil-derived actinomycete bacteria provided a major pharmaceutical resource for the discovery of antibiotics and related bioactive compounds. During this time, a major effort was undertaken to examine virtually every terrestrial habitat. The oceans, representing >70% of the Earth's surface were, however, never seriously considered as a source for bacterial diversity. This is because the prevailing view was that the most important bacterial class, the actinomycetes were exclusively terrestrial. In addition, the marine environment was recognized as being far more difficult to sample. Compounding these views was that little information was available to insure the successful cultivation of "marine bacteria." During the last 15 years, we have examined tropical marine environments and undertaken a systematic approach to cultivate and identify "marine actinomycetes", i.e. those uniquely adapted to growth in the sea. Our studies have revealed that taxonomically-unprecedented representatives of the major actinomycete families can readily be isolated and cultivated. More than 15 new actinomycete taxa have been isolated and described.

In culture, we are now observing the production of a wide variety of bioactive secondary metabolites. One early study (ca. 1998) identified Salinosporamide A, a potent proteasome inhibitor from the new genus *Salinispora*, which is currently in phase II clinical trials for the treatment of cancer. From a member of the new genus "*Marinispora*", we have identified new macrolide antitumor-antibiotics of an unprecedented class, with specificities toward drug resistant bacteria and melanoma. Studies of the deep ocean have significantly added to these discoveries. Small molecules recently discovered that are structurally-unique have posed serious challenges to the classic process of structure elucidation by NMR and other spectroscopic methods. These issues, which include metabolites with low proton to carbon ratios, will be discussed along with the novel bioactivities and biosyntheses of these molecules.

Tuesday, September 28<sup>th</sup>  
9:00 AM - 10:30 AM

**Real-Time Reaction Monitoring**  
Chair: Mike Bernstein

*Practical Applications of Compact High Resolution 60 MHz  
Permanent Magnet NMR Systems for Reaction Monitoring and  
Online Process Control*

John Edwards, Process NMR Associates

*Monitoring Organic Reactions: What can UF-NMR  
Spectroscopy Offer*

Encarnación Fernández Valle/Dolores Molero, Madrid  
University

*New Chemometric Methods for Alignment (icoshift),  
Classification (ECVA) and Information Extraction of NMR Data*  
Søren Engelsen, Copenhagen University

# **Practical Applications of Compact High-Resolution 60 MHz Permanent Magnet NMR Systems for Reaction Monitoring and Online Process Control**

**John C. Edwards** and Paul J. Giammatteo

Process NMR Associates, LLC  
87A Sand Pit Road, Danbury, Connecticut 06810

For the past two decades high resolution  $^1\text{H}$  NMR at 60 MHz has been utilized to monitor the chemical physical properties of refinery and petrochemical feedstreams and products. These approaches involve the use of partial least squares regression modelling to correlate NMR spectral variability with ASTM and other official test methods, allowing the NMR to predict results of physical property tests or GC analysis. The analysis is performed in a stop flow environment where solenoid valves are closed at the beginning of the NMR experiment. This approach allows up to 5 or 6 different sample streams to be sent to the sample in order to maximize the impact of the instrument. The current work with these permanent magnet NMR systems is to utilize them as chemistry detectors for bench-top reaction monitoring, mixing monitoring, dilution monitoring, or conversion monitoring. In the past use of NMR for these applications has been limited by the need to bring the "reaction" to the typical "superconducting" NMR lab. A compact high resolution NMR system will be described that can be situated on the bench-top or in the fume hood to be used as a continuous or stop-flow detector and/or an "in-situ" reaction monitoring system. The system uses a unique 1.5 Tesla permanent magnet with a simple flow cell and total system volumes of 2 to 5 mL depending on the length and diameter of the transfer tubing. Further, detection limits of analytes in the 200+ ppm range are possible without the use of typical deuterated NMR solvents. Analysis times of 5 to 20 seconds are also possible at flow rates of 5 to 20+ ml/minute. Reaction monitoring directly in standard 5 mm NMR tubes again using conventional (non-deuterated) reactants, solvents and analytes will also be described.

# Monitoring Organic Reactions: What can UF-NMR Spectroscopy Offer?

Dolores Molero<sup>1</sup>, Encarnación Fernández-Valle<sup>1</sup>, Antonio Herrera<sup>2</sup>,  
Roberto Martínez-Álvarez<sup>2</sup>, Zulay D. Pardo<sup>2</sup>, Elena Sáez<sup>1</sup>, Maayan Gal<sup>3</sup> and  
Eva M. Gutiérrez<sup>2</sup>

1. CAI de R.M.N. y R.S.E., UCM, Madrid, Spain
2. Departamento de Química Orgánica, UCM, Madrid, Spain
3. Dep. Chemical Physics, Weizmann Institute of Science, Rehovot, Israel

Multidimensional Nuclear Magnetic Resonance serves as a basic tool in the structural elucidation of chemical structures or complex biological systems and offers different possibilities toward the monitoring of chemical and biochemical processes. Traditional multidimensional experiments are intrinsically time consuming, due to the fact that many  $t_1$  increments have to be acquired in order to achieve  $nD$  spectra with adequate digital resolution in the indirect dimension [1]. To overcome this limitation, different proposals for accelerating  $nD$  NMR have been introduced [2]. Among them, ultrafast NMR (UF-NMR) inspired by echo planar imaging (EPI) and developed by Lucio Frydman, permits the acquisition of complete  $nD$  NMR data sets within a single continuous acquisition [3]. If the analyte's signal is sufficiently strong the acquisition time of multidimensional NMR experiments can be shortened by several orders of magnitude. This fascinating possibility enables ultrafast NMR as a key technique for the monitoring of dynamic processes as they happen, in real time.

We have applied UF-NMR TOCSY to monitor the reaction between aliphatic ketones and triflic anhydride in the presence of nitriles. This leads to pyrimidines as main products. The evolution of the reactants from early stages of the reaction, the presence of different nitrilium-type intermediates, and the generation of reaction products has been monitored [4]. Despite these important findings with UF-TOCSY, no direct information regarding structural changes in the carbonyl carbon atom can be obtained. Since it is a quaternary carbon, its evolution cannot be detected using 2D homonuclear sequences such as COSY or TOCSY or heteronuclear such as HSQC. However, UF-HMBC pulse sequence can offer direct and more precise information about the structural changes that take place on the carbon core and protons attached at neighbor positions during the reaction.

In order to complete the information regarding the previous mentioned reaction we have applied this sequence (UF-HMBC) to monitor how the carbonyl carbon atom evolves during the process. Our target was to identify possible intermediates formed. We choose a simple aliphatic-aromatic ketone, (acetophenone), as the model compound and studied its reaction with triflic anhydride in the presence of an excess of acetonitrile. Labeled  $^{13}\text{C}$ -carbonyl-acetophenone and  $[\text{D}_3]$ acetonitrile were used as reactants. In the present case UF-HMBC has permitted the detection of a new intermediate (trifluoromethanesulfonyl)carbenium ion and has confirmed the presence of nitrilium salt intermediates. The use of  $^{13}\text{C}$ -Carbonyl-labeled ketone ensures the detection of short life intermediates and overcomes sensitivity problems [5]. In spite of this we are working now on new sequences which would allow studying reactions with unlabeled compounds.

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5. Herrera, A., Fernández-Valle, E., Gutiérrez, E. M., Martínez-Álvarez, R., Molero, D., Pardo, Z. D., Sáez, E., *Org. Lett.*, 12(1), 144-147, 2010

# New Chemometric Methods for Alignment (icoshift), Classification (ECVA) and Information Extraction of NMR Data

S.B. Engelsen, F. Savorani and R. Bro

Department of Food Science, Quality & Technology, Faculty of Life Sciences – University of Copenhagen, Rolighedsvej 30, 1958 Frederiksberg C, DK

The increasing interest towards metabolomics and metabonomics takes advantage from the Nuclear Magnetic Resonance (NMR) spectroscopy that in many cases is able to replace laborious and time consuming chemical analysis, providing an overwhelming quantity of chemical information. This paper presents some new chemometric tools for data alignment, classification and data extraction.

**Data alignment:** Though a large number of algorithms able to deal with misalignment of NMR signals have been recently published, an open source tool able to handle, in a simple but customizable way, the different cases that can occur, has been missing. Thanks to FFT-CC (Fast Fourier Transform Cross-Correlation) these inherently computational intensive methods are now capable of handling huge NMR datasets in a reasonable time. To this end we present the icoshift algorithm [1] intended for dealing with all kind of signal aligning problems.

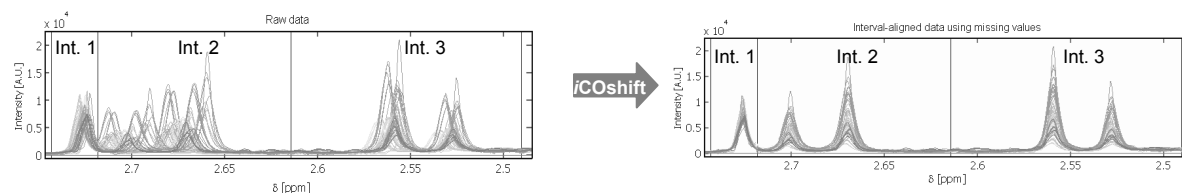


Fig. 1. An example of using icoshift for coping with a case of multiple misalignments.

**Data classification:** For multivariate classification of multiple classes we have developed a new algorithm based on canonical variates analysis (CVA). The new method is called extended canonical variates analysis (ECVA) [2] and its use will be demonstrated in a case study.

**Data extraction:** Finally the application of Multivariate Curve Resolution and Parallel Factor Analysis will be demonstrated for solving the cocktail party effect in complex NMR data ensembles of biological samples [3].

The Matlab codes are made freely available from the website: [www.models.life.ku.dk](http://www.models.life.ku.dk)

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Tuesday, September 28<sup>th</sup>  
11:00 AM - 12:30 PM

**Graduate Students & Postdoctoral Students Session**  
Chair: Xiaohong Li

*New Applications of Filter Diagonalization Method to Small Molecules*

Hasan Celik, University of California, Irvine

*Use of Multiple Homonuclear Decoupling for Small Molecule Structural Elucidation*

Ana Paula Espindola, University of Texas Southwestern Medical Center

*Versatile  $^1\text{H}-^{31}\text{P}-^{31}\text{P}$  COSY 2D NMR Techniques for the Characterization of Polyphosphorylated Small Molecules*

Yan Sun, Johns Hopkins School of Medicine

*Advanced 2D NMR Techniques for Fluoropolymer Model Compounds*

Xiaohong Li, University of Akron

# New Applications of Filter Diagonalization Method to Small Molecules

Hasan Celik and A. J. Shaka

Chemistry Department, University of California, Irvine, CA, USA

Fourier transform nuclear magnetic resonance (FT-NMR) spectroscopy has long been the identification method of choice in small molecule research. It is reliable, robust, and offers a vast library of multidimensional experiments with rich structural, stereochemical and dynamical information content that is hardly rivaled by any other methodology. However, the Fourier time-frequency uncertainty principle imposes a transform-limited lineshape on the indirectly-detected frequencies of multidimensional experiments. This can result in experiments on the order of days or even weeks for NMR beyond 1D in order to achieve the high-resolution desired.

The Filter Diagonalization Method (FDM) [1] has been used to process NMR data in liquids [2-4] in a way that bypasses the Fourier time-frequency uncertainty. Use of FDM over Discrete Fourier Transform (DFT) is particularly advantageous when three specific conditions are met: sparsity of the spectrum, Lorentzian lineshapes and sufficiently high signal-to-noise. While the theory of FDM as a parametric method is well established, there is still much room for improvement in NMR spectral estimation from FDM parameters especially in cases where the necessary conditions are not satisfied. Previous notable advances include the introduction of regularization to the FDM algorithm for the case of noisy data [3], a hybrid method in which FDM is used to pick out peaks that match the theoretical model and DFT is used for the residual [5], and a sensitivity enhanced basis that increases the stability of the spectral estimate in the presence of noise [6].

Here we will present a true phase-sensitive treatment of FDM spectral representation for small molecule NMR experiments, such as HSQC. Previous FDM work on NMR signals employed an "aggressive" method [3] that involved an artificial phasing of the spectrum in order to obtain a pure absorption-mode spectrum and came with serious drawbacks: an uncorrected linear phase in the dataset could lead to disappearance of peaks and a falsified estimate, and a priori knowledge of the absolute phase of each NMR dataset was required. An alternative approach to creating the NMR spectral estimate that bypasses the aforementioned problems associated with the aggressive method will be demonstrated.

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6. Celik, H., Shaka A. J., in revision, *J. Magn. Reson.*

# Use of Multiple Homonuclear Decoupling for Small Molecule Structure Elucidation

Ana Paula D. M. Espindola<sup>1</sup>, Ronald Crouch<sup>2</sup> and John B. MacMillan<sup>1</sup>

1. UT Southwestern Medical Center
2. Applications Laboratory, Varian, Inc

Natural products chemistries rely heavily on NMR experiments for the structure determination of new compounds.[1] Commonly used experiments (like COSY, HSQC and HMBC) are extensively applied on the planar structure elucidation with excellent results. However, one of the remaining limitations of natural products NMR is the ability to obtain relative configuration of small molecules. Recently has grown the use of J-based configuration analysis to determine the relative stereochemistry of complex acyclic and macrocyclic small molecules and natural products.[2] The usefulness of J-based methods relies on the ability to measure coupling constants between protons ( $J_{HH}$ ),[3] a difficult task in molecules with complex multiplets and significant signal overlap. Methods, such as E.COSY[4] and 2D J-resolved suffer from lack of sensitivity and complex data analysis. Alternatively, classic homonuclear decoupling can be used to simplify a complex multiplet, but is limited to irradiating a single proton.[5] We utilized shaped selective pulses to simultaneously decouple multiple protons to deconvolute complex <sup>1</sup>H NMR spectra of small molecules. With the multiple homonuclear decoupling experiment (MDEC) we obtain specific coupling values from a complex spectra in a fast and reliable way.[6]

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# Versatile $^1\text{H}$ - $^{31}\text{P}$ - $^{31}\text{P}$ COSY 2D NMR Techniques for the Characterization of Polyphosphorylated Small Molecules

Yan Sun<sup>1</sup>, Ananya Majumdar<sup>2</sup>, Meha H. Shah<sup>1</sup>, J. Kipchirchir Bitok<sup>1</sup>, Maria E. Hassis-LeBeaub<sup>1</sup> and Caren L. Freel Meyers<sup>1</sup>

1. Department of Pharmacology and Molecular Sciences,  
Johns Hopkins School of Medicine
2. The Johns Hopkins University Biomolecular NMR Center, Baltimore, MD

Poly-phosphorylated compounds play essential roles in numerous chemical and biological processes. Structural characterization of this compound class at natural abundance (lacking isotopic enrichment) is a challenging task, especially at sub-millimolar concentrations. In our work, we have utilized two-dimensional HPP-COSY spectroscopy techniques for the detection and characterization of poly-phosphorylated compounds at low concentration. Results for a diverse group of phosphorylated organic molecules will be presented, including molecules bearing P-O-P or P-C-P connectivity (exhibiting different dihedral angles resulting in different coupling constants), molecules bearing multivalent P(III)-O-P(V) connectivity with large chemical shift anisotropy effects, as well as compounds with virtually overlapping  $^{31}\text{P}$  resonances exhibiting strong coupling effects. With this technique, mono- and diphosphates are quickly distinguished, and distinct P-O-P connectivities are easily detected to provide valuable structural information. Application of these HPP-COSY techniques to characterize intermediates in the bacterial methylerythritol phosphate isoprenoid biosynthetic pathway will also be presented to demonstrate these techniques.

# Advanced 2D NMR Techniques for Studying Fluoropolymer Model Compounds

Xiaohong Li<sup>1</sup>, Elizabeth F. McCord<sup>2</sup>, Peter A. Fox<sup>2</sup>, Jon L. Howell<sup>2</sup>,  
and Peter L. Rinaldi<sup>1</sup>

1. University of Akron, Dept. of Chemistry, Akron, OH
2. EI DuPont de Nemours and Co, Experimental Station, Wilmington, DE

There is considerable interest in commercial applications of fluoropolymers, resulting from their unique physical properties and stability in extreme environments. This interest has created a great need for a family of robust NMR techniques to characterize complex new fluorinated materials. While 2D-NMR methods have been enormously useful for studying hydrocarbon-based structures, the unique NMR characteristics of <sup>19</sup>F have complicated efforts to use similar methods for characterizing fluoropolymers and highly fluorinated materials in general.

In this presentation we describe the use of selective <sup>19</sup>F{<sup>13</sup>C}-HSQC and ECOSY [1] 2D-NMR methods in combination with pulsed <sup>19</sup>F-<sup>19</sup>F homonuclear decoupling techniques [2] to produce ultra-high resolution 2D-NMR spectra with simplified cross-peak patterns. The methods permit the separation and identification of the signals from the many stereo-sequence structures present in the backbone of PPFPO (Polyperfluoropropyleneoxide). Similarly, these methods were used to identify perfluorinated chain-end structures. A small molecule perfluoro-2,5,8-trimethyl-3,6,9-trioxadodecanoyl fluoride was studied as model compound. Full assignments of its <sup>19</sup>F and <sup>13</sup>C chemical shifts are established. The resonances from initiation and termination chain ends structures are resolved and identified. The attachment between monomer units reveals details of reaction parameters that are critical to understanding the polymerization process.

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Tuesday, September 28<sup>th</sup>  
2:30 PM - 4:00 PM

## **Workshops**

*Setting Out on Your Own: Entrepreneurship in a Niche  
Environment*

Tim Peck, Protasis/MRM Corp.

*NMR, It's Not Just For Structures: Determination of  
Physicochemical Properties*

Manuel Perez, Pfizer

# Setting Out on Your Own: Entrepreneurship in a Niche Environment

D. Detlefsen<sup>1</sup>, S. Dominguez<sup>2</sup>, and T. Peck<sup>3</sup>

1. Novatia LLC, Monmouth Jct., NJ
2. MestreLab Research SL, Spain
3. Protasis Corp., Savoy, IL

Small business plays a vital role in small molecules research by addressing demands for new capabilities, dynamically changing/evolving needs in throughput and capacity, and technological innovation. Small businesses generally have greater flexibility and faster response time to adjust to changing market demands. A vital partnership exists between the small business and the end user. Advancement requires that the small business and its customers take measured risks toward the achievement of a desired outcome. Risks include: 1) scientific risk (do the laws of nature and physics allow what is being attempted?), 2) engineering risk (are the available materials, tools, and expertise sufficient to accomplish the task?), 3) commercial risk (will the market endorse the proposed plan or solution?) and financial risk (is the financial opportunity sufficient to pay back the debt incurred in the development, and lead eventually to profits?). Personal and professional risk components are also present.

This workshop will include overviews from three representatives of small businesses that serve the analytical measurements community, and which aim to provide researchers with new and modern tools and services to advance science. Each representative will provide a thumbnail sketch of the histories of their company from the formative concept to present day, citing some of the difficulties and pitfalls encountered along the way. The critical role of the customer and stakeholders will be highlighted. The majority of time allocated for the workshop will be dedicated to a roundtable discussion, intended to facilitate informal exchange between these representatives and the audience. Envisioned discussion topics include logistical matters of business formation, practical day-to-day operational challenges, modes in which customers can participate in product design/development, and challenges facing the pharmaceutical and research communities for which small business needs to respond. In this manner it is the goal of the workshop to provide useful and timely information to not only those who may be considering a small business career or small business startup, but also to those who are already involved in small business and to those in larger corporations or universities where support of small business initiatives may hold competitive advantage for their organization and/or for collaborative scientific advance.

# **NMR, It's Not Just For Structures: Determination of Physicochemical Properties**

Kathleen Farley<sup>1</sup>, William Farrell<sup>2</sup>, Richard Lewis<sup>3</sup>, Manuel Perez<sup>4</sup> and Wei Wang<sup>2</sup>

1. Pfizer, Groton, US
2. Pfizer, La Jolla, US
3. Astra-Zeneca, Charnwood, UK
4. Pfizer, Sandwich UK

Over the last fifteen years there has been a slow but constant change in the focus of many analytical groups. Advances in magnet and console technologies have allowed specialists to move from workflows purely based around structure and purity determination towards studies concerned with measuring physicochemical properties, as well as the rationalisation of the behaviour of molecules in solution.

A wide range of subjects will be detailed in this workshop, to cite a few: how to detect and measure intramolecular and intermolecular interactions in solution, determination of pKa values in non aqueous systems, estimation of the shape of molecules in solution, reaction kinetics or how to measure exposed polar surface area using SFC.

Emphasis will be placed on the need to approach all of these questions with a multidisciplinary mind, the desirable outcomes and the impact on medicinal chemistry projects.

Wednesday, September 29<sup>th</sup>  
8:30 AM - 10:30 AM

**How Low Do You 'Need' To Go? - Techniques,  
Applications and Considerations of Trace Analyses**  
Chairs: Charlotte Corbett and Elaine Ricicki

*Trace Analysis of Seized Drugs using Ultra High Performance  
Liquid Chromatography Tandem Mass Spectrometry*

Ira S. Lurie, Drug Enforcement Administration

*An Investigation of Experimental Limits of Small Sample  
Heteronuclear 2D NMR*

Gary Martin, Merck

*Trace Level Detection of Compounds Related to the Chemical  
Weapons Convention by <sup>1</sup>H-Detected <sup>13</sup>C NMR Spectroscopy  
Executed with a Sensitivity-Enhanced, Cryogenic Probehead*  
Terry J. Henderson, US Army, Edgewood Chemical Biological  
Center

*Forensic Drug Identification in Blood, Urine and  
Unconventional Matrices such as Hair, Saliva, Liver, Brain,  
Vitreous Humor*

Ashraf Mozayani, Harris County Institute of Forensic Sciences,  
Houston, Texas

# **Trace Analysis of Seized Drugs using Ultra High Performance Liquid Chromatography Tandem Mass Spectrometry**

**Ira S. Lurie**

Special Testing and Research Laboratory,  
Drug Enforcement AdministrationDulles, VA

The ultra trace analysis of seized drugs requires highly sensitive and specific methodology. In this vein, hyphenated techniques such as GC-MS or HPLC-MS have been used. Liquid phase separation techniques are advantageous for solutes that are thermally degradable, highly polar or non-volatile. Ultra high performance liquid chromatography (UHPLC), which utilizes sub 2  $\mu\text{m}$  particle column operating at elevated pressure, offers significantly higher peak capacity and lower limits of MS detection than HPLC. MS/MS detection via multiple reaction monitoring (MRM) provides high selectivity and sensitivity. In this presentation, the use of UHPLC-MS/MS for the ultra trace analysis of seized drugs is presented. This lecture will discuss applications which include opium, heroin, methamphetamine, and fentanyl profiling, and the analysis of dietary supplements and candies.

# **An Investigation of Experimental Limits of Small Sample Heteronuclear 2D NMR**

**G. E. Martin** and B. D. Hilton

Merck Research Laboratories  
Discovery and Preclinical Sciences  
Rapid Structure Characterization Laboratory, Summit, NJ 07901

Strategic approaches to many structure characterization problems are defined by the amount of sample available. Examples include natural products, impurities and degradants isolated from pharmaceuticals, metabolites, and forensic samples among others. Often the key experiments for the characterization of a total unknown are a variety of heteronuclear 2D NMR experiments. Small volume NMR probes have been developed and the current state-of-the-art is well represented by a 1.7 mm gradient inverse triple resonance cryoprobe capable of studying samples in 1.7 or 1.0 mm tubes. We report the results obtained using very small samples of strychnine as a model compound to define the practical performance limits of variety of heteronuclear 2D NMR experiments, which include multiplicity-edited  $^1\text{H}$ - $^{13}\text{C}$  GHSQC,  $^1\text{H}$ - $^{13}\text{C}$  and  $^1\text{H}$ - $^{15}\text{N}$  GHMBC, H2BC, IDR-(Inverted-Direct-Response)-GHSQC-TOCSY, and 1,1-ADEQUATE.

# **Trace Level Detection of Compounds Related to the Chemical Weapons Convention by $^1\text{H}$ -Detected $^{13}\text{C}$ NMR Spectroscopy Executed with a Sensitivity-Enhanced, Cryogenic Probehead**

David B. Cullinan, George Hondrogiannis and **Terry J. Henderson**

US Army Edgewood Chemical Biological Center, Aberdeen Proving Ground, MD 21010

Two-dimensional  $^1\text{H}$ - $^{13}\text{C}$  HSQC and fast-HMQC pulse sequences were implemented using a sensitivity-enhanced, cryogenic probehead for detecting compounds relevant to the Chemical Weapons Convention present in complex mixtures. The resulting methods demonstrated exceptional sensitivity for detecting the analytes at trace level concentrations.  $^1\text{H}$ - $^{13}\text{C}$  correlations of target at  $\leq 25$   $\mu\text{g/mL}$  were easily detected in a sample where the  $^1\text{H}$  solvent signal was  $\sim 58,000$ -fold more intense than the analyte  $^1\text{H}$  signals. The problem of overlapping signals typically observed in conventional  $^1\text{H}$  spectroscopy was essentially eliminated, while  $^1\text{H}$  and  $^{13}\text{C}$  chemical shift information could be derived quickly and simultaneously from the resulting spectra. The fast-HMQC pulse sequences generated magnitude mode spectra suitable for detailed analysis in  $\sim 4.5$  h, and can be used in experiments to efficiently screen a large number of samples. The HSQC pulse sequences, on the other hand, require roughly twice the data acquisition time to produce suitable spectra. These spectra, however, were phase-sensitive, contained considerably more resolution in both dimensions, and proved to be superior for detecting analyte  $^1\text{H}$ - $^{13}\text{C}$  correlations. Furthermore, a HSQC spectrum collected with a multiplicity-edited pulse sequence provided additional structural information valuable for identifying target analytes. The HSQC pulse sequences are ideal for collecting high-quality data sets with overnight acquisitions, and logically follow the use of fast-HMQC pulse sequences to rapidly screen samples for potential target analytes. Use of the pulse sequences considerably improves the performance of NMR spectroscopy as a complimentary technique for the screening, identification, and validation of chemical warfare agents and other small-molecule analytes present in complex mixtures and environmental samples.

# **Forensic Drug Identification in Blood, Urine and Unconventional Matrices such as Hair, Saliva, Liver, Brain, Vitreous Humor**

**Ashraf Mozayani**

Harris County Institute of Forensic Sciences, 1885 Old Spanish Trail, Houston, TX 77054

The most common biological specimens for testing drugs and their metabolites in forensic cases are blood and urine. . However, with recent advances in extraction technology and instrumentation it has become more practical to explore unconventional biological matrices such as saliva, and hair. Like any other scientific procedure, the testing of these matrices can be appropriate in some instances and inappropriate in other instances. This presentation is a general review to offer a balanced view of the pros and cons of drug testing in these unconventional matrices.

Wednesday, September 29<sup>th</sup>  
11:00 AM - 12:30 PM

**Workshop**

*Bringing it All Together: Multi-Disciplinary Structure  
Elucidation*

David Russell, Agilent Technologies  
Jonathan Josephs, Bristol Myers Squibb

# Bringing it All Together: Multi-Disciplinary Structure Elucidation

David J. Russell<sup>1</sup>, Jonathan Josephs<sup>2</sup>, and R. Thomas Williamson<sup>3</sup>.

1. NMR Applications Scientist, Agilent Technologies, Walnut Creek, CA.
2. Bristol Myers Squibb.
3. NMR Spectroscopy and PAT, Roche, Florence, SC.

NMR and Mass Spectrometry are two of the most useful tools available for structure elucidation. These complementary techniques are most powerful when used together. In fact, many structure problems cannot be solved using either technique as a standalone approach. This workshop will touch on the comparative strengths and weaknesses of each technique from the pharmaceutical perspective, then move to a general discussion around topics such as:

- How should a structure group be organized?
- How do “proof of structure” and “unknown identification” differ?
- What is required to reach 100% confidence in a structural assignment?
- Does chemical shift prediction help, or can it send you down the garden path?
- What good does a standard do scientifically?
- What good does a standard do vis-à-vis regulatory requirements?
- What to do when it won't ionize, it won't fragment, and it has no protons?
- Vibrational spectroscopy, anyone?
- How many techniques can one hyphenate and, does it help to do so?
- Hyphenated NMR techniques for routine/non-routine structure determination?
- Are isolates really that unstable?
- How much of this work can really be outsourced?
- Is the chemistry behind the sample really that important?

Wednesday, September 29<sup>th</sup>  
2:30 PM - 4:30 PM

**NMR and MS Imaging**

Chairs: Andreas Kaerner and YingYing Huang

*NMR Microscopy of Formulated Pharmaceutical Products*

Andrew Phillips, AstraZeneca, UK

*MS Imaging: Snapshots of Tissue Distribution*

Stephen Castellino, GlaxoSmithKline

*Imaging Mass Spectrometry: A New Tool for Small Molecule  
Analysis*

Michelle L. Reyzer, Vanderbilt University

# NMR Microscopy of Formulated Pharmaceutical Products

Andrew Phillips, Les Hughes and Stephen Wren

AstraZeneca, Pharmaceutical Development, Macclesfield, UK

Pharmacopoeial dissolution methods are the main routine test of the pharmaceutical performance of solid dosage forms. However whilst the pharmacopoeial methods may identify performance differences between formulation variants or batches, they are of limited use in identifying root causes. Therefore techniques which can give a deeper insight into the underlying mechanisms that control pharmaceutical performance are required.

The process known as drug product “dissolution” is complex and involves more fundamental steps such as hydration, gelation, erosion, disintegration, de-agglomeration, substance dissolution and diffusion. Thus macroscopic differences in drug product “dissolution” could arise as a result of differences in any one or more of these fundamental steps.

In this presentation we will show how traditional spin-echo Magnetic Resonance images can be used to probe dissolution. The images can of course be used qualitatively but it is also possible and important to extract quantitative data such as maps of absolute water concentration and spin-spin relaxation times. It is the differences in relaxation time, a measure of water mobility that usually provides image contrast.

The drawback of these traditional sequences is the time it takes to get the required data - often a few minutes per two-dimensional image. However many pharmaceutical products have already released in this timescale. The use of ultra-fast quantitative MRI will also be explored allowing the same information to be obtained but in just a few seconds<sup>1</sup>.

The application of both techniques will be illustrated with examples of both solid dispersion and HPMC matrix tablets, using a flow cell that mimics traditional dissolution methods.

1. Y.Y. Chen, L. P. Hughes, L.F. Gladden and M.D Mantle, Quantitative Ultra-Fast MRI of HPMC Swelling and Dissolution. *J. Pharm. Sci.* In Press. DOI: 10.1002/jps.22110

# **MS Imaging: Snapshots of Tissue Distribution**

**Stephen Castellino**

US Structure ID, GlaxoSmithKline

Determining the distribution of drugs and metabolites in tissue is a critical component of evaluating drug safety, pharmacology, and pharmacokinetics. Historically, tissue distributions in preclinical settings have been determined using autoradiography or LC-MS analysis of tissue homogenates. Autoradiography has been extensively employed because it provides both the spatial distribution as well as analyte quantification. However, this methodology is not able to distinguish between the parent drug and metabolites. Furthermore, radio-labeled drug is required, adding to the cost and time requirements of obtaining distribution data. LC-MS analysis of homogenates can resolve parent drug from metabolites, but spatial information is lost. Imaging Mass Spectrometry (IMS) is an emerging technique which has the promise of providing tissue distributions for both parent and metabolites without the need for a radiolabel.

This seminar will highlight our efforts to implement IMS in a preclinical environment to address issues associated with drug safety, pharmacology, and pharmacokinetics as it relates to tissue distribution.

# **Imaging Mass Spectrometry: A New Tool for Small Molecule Analysis**

**Michelle L. Reyzer**

Vanderbilt University

Over the past decade, MALDI imaging mass spectrometry (IMS) has been utilized for the in situ detection of proteins, peptides, lipids, and small molecules. Matrix is applied to thin sections of tissue, typically fresh frozen, and individual mass spectra are obtained from discrete spots across the tissue surface. The mass spectra contain molecular signatures of the compounds present in the tissue section and two-dimensional ion density maps or images may be created for any signal detected. Recent advances in the technology include the ability to examine formalin fixed paraffin embedded tissues through on-tissue enzymatic digestion and the expansion of the technology to 3-dimensional imaging and integration with other 3-D techniques (including PET and MRI).

The analysis of several drugs by IMS, including rifampicin, isoniazid, and olanzapine will be presented. A few examples of endogenous small molecule and protein imaging will also be discussed to illustrate how tissue microenvironments may be interrogated with this technology. Additionally, key points relating to sample preparation, matrix application, and general imaging technology will be addressed.

Monday, September 28<sup>th</sup> 4:30 PM - 6:00 PM  
Even Numbered Posters

Tuesday, September 29<sup>th</sup> 4:30 PM - 6:00 PM  
Odd Numbered Posters

### Poster Sessions

Chairs: Gene Mazzola & Laurie Galya

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13. Microcoil NMR for the Characterization of Heparin-Derived Oligosaccharides
14. DENA and RASA-HSQC, Two Approaches to Decipher or Select Ambiguous Chemical Shifts in Aliased Spectra
15. RevealX<sup>TM</sup> Technology Improves Isolation and Purification of Natural Products by Flash Chromatography
16. Automated Raw Material Screening Using NMR: Application to a Production Lab Operating Under GMP
17. Reaction Monitoring Using  $^{31}\text{P}$  NMR
18. Automating Gradient Method Development in Flash Chromatography for Greater Productivity and Minimizing Solvent Use
19. A New Tropane Alkaloid from the Roots of *Erythroxylum Pungens* O.E. Schulz
20. Structural Characterization of Synthetic Galactosyl Diglyceride Antigens for Lyme Disease
21. RevealX<sup>TM</sup> Technology Improves Purification of Lead Generation Compounds by Flash Chromatography

22. An Appreciation of Laurie Hall, a Pioneer in Small Molecule NMR
23. pKa-measurements and Conformational Studies of Amine Linked Pseudodisaccharides
24. Method Optimization for Packing Various Polymer and Silica Chromatographic Media with Dynamic Axial Compression Columns
25. Solving the Bloch Equations: Application to the Hahn Echo
26. Binding Studies of Lysozyme with Small Glycans by NMR Spectroscopy and Weak Affinity Chromatography
27. Unique Selectivity Improves Separation of Polar Compounds by HPLC and UHPLC
28. Metabonomic Analysis of Tomato Fruit Ripening Inhibition Phenotypes by NMR
29. Are Pitfalls Unavoidable During the Structure Elucidation of New Organic Compounds?
30. An Open Access Setup to Measure Lipophilicity in Crude Mixtures Using Reversed-Phase HPLC-MS
31. How Many Structures Can Correspond to a Specific Molecular Mass?
32. Probing the Biliary Metabolism of Drugs by the Use of Diffusion-edited NMR Spectroscopy
33. The Use of Multiple Fragmentation Methods for Small Molecule Characterization on an LTQ Orbitrap Velos
34. Structure Determination of Designer Drugs by NMR and Confirmatory Methods: Forensic Case Studies
35. Isolation and Structure Elucidation of In-Process Impurities During Tetrazole Ring Formation of Compound I
36. Solvent Selection for Single-Wall Carbon Nanotubes: New Best Solvents and Acids/Superacids
37. Parahydrogen-induced Polarization Detected by Zero-field NMR
38. Use of Solid State NMR to Aid the Salt-Selection Process for Pharmaceutical Products
39. A Metabolomic Assessment of Cobia Health in Response to Dietary Manipulation
40. Automated Raw Material Screening by NMR: Analysis Methods Used for Automated Report Generation
41. Insights into the RPIP-HPLC Separation of Heparin and Heparan Sulfate Oligosaccharides
42. Application of NMR Spectroscopy to Forensic Characterization of Diastereomeric Superwarfarins

43.  $^{15}\text{N}$  Indirect Detection of N-sulfo Glucosamine Residues in Glycosaminoglycans
44. Continuing Optimization of a Large-scale NMR Facility
45. Quantitative Analysis of Trace Level Impurities by NMR in both Drug Substance and Formulated Products
46. Mandelalides A-D, Cytotoxic Macrolides from Endemic South African Lissoclinum Species
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57. Monitoring Chemical Reactions in Real Time with NMR Spectroscopy
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60. Automated Quantification and Structure Verification in Capillary Flow NMR

# 1 Hybrid Helices: New Motifs for Secondary Structure Scaffolds in Foldamers

Madavi Choudhary<sup>1</sup>, Gangavaram V. M. Sharma<sup>1</sup>, Nagula Chandramouli<sup>1</sup>, Pendem Nagendar<sup>1</sup>, Kallaganti V. S. Ramakrishna<sup>1</sup>, Peter Schramm<sup>2</sup>, and Hans-Jorg Hofmann<sup>2</sup> and Ajit C. Kunwar<sup>1</sup>

1. Indian Institute of Chemical Technology (CSIR), Hyderabad, Andhra Pradesh, India
2. Institute of Biochemistry, Faculty of Biosciences, University of Leipzig, Leipzig, Germany

The concept of “hybrid helices” as a new motif for foldamers is presented. Foldamers are discrete artificial oligomers with well-defined and predictable folding patterns akin to naturally occurring helices, turns and linear strands [1]. Among the non-natural polymers with the propensity to form well-defined secondary structures, the peptidomimetic foldamers are attracting increasing attention. The major advantage of this family of foldamers is that the amide groups, that combine monomers into the chain, also act as cross-linking points via hydrogen bonding between the amide proton and the carbonyl oxygen to fold the chain into a regular structure. Therefore these structures are able to easily form secondary structures (various helix-types, strand-like conformations, and turns) and are capable of forming higher-order self assemblies too.

Earlier in our group a large number of foldameric peptides were designed, based on homologues of  $\alpha$ -peptides and with different sugar substitutions. Various combinations of amino acids in 1:1 alternation with proper chirality provided different mixed helices like 12/10- (in  $\beta$ - peptides), 11/9- (in  $\alpha/\beta$ -hybrid peptides), 12/10- (in  $\alpha/\gamma$ -hybrid peptides), 13/11- (in  $\beta/\gamma$ -hybrid peptides), 13/11- (in  $\alpha/\delta$ -hybrid peptides), and 14/12-helices (in  $\alpha/\epsilon$  -hybrid peptides). In continuation of our interest in accessing new classes of scaffolds having homogeneous or heterogeneous backbones with characteristic folding patterns, similar to the designs on chimeric ( $\alpha/\beta+\alpha$ ) peptides for protein surface recognition by Gellman et al [2], we developed the concept of “hybrid helices”, using a variety of helices obtained by us in different peptide foldamer families as building blocks. In this report, we present several examples of hybrid helices derived from two or more different backbone types of  $\beta$ -peptides [3] and  $\alpha/\beta$ -[4] and  $\alpha/\gamma$ -[5] hybrid peptides sequences within a single oligomer. The structures of the peptides were investigated by extensive NMR studies [6].

Hybrid helices enrich the arsenal of defined foldamer structures for a structural and functional mimicry of native peptides and proteins. The transition from one helix type to another was found to be rather smooth with high compatibility of the different helix types. The high degree of regularity of the hybrid helix along the sequence of the oligomer accommodating the two different helix types without problems can well be seen. Such hybrid helices represent novel motifs of secondary structure scaffolds and open up the possibility to change the direction of helix propagation in a subtle manner, enabling to control the orientation of the side chains.

Combining different foldamer scaffolds will be an effective and perhaps general strategy for protein ligand design as suggested by the studies on binding of chimeric ( $\alpha/\beta+\alpha$ )-peptides to

Bcl-xL by Gellman et al. The path from  $\alpha$ - to  $\beta$ - to  $\alpha/\beta$ - to  $(\alpha/\beta+\alpha)$ -peptide ligands lead to conclude that foldamer-based strategies for disrupting protein-protein interactions will grow in scope and efficacy as the number of foldamer scaffolds with distinct shapes increases. The availability of such new motifs, in combination with other peptide foldamers, may help in attaining a functional mimicry of natural proteins.

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# 2 Conformational Studies on $\beta$ -L-Fucp-(1 $\rightarrow$ 6)- $\alpha$ -D-Glcp-OMe Using MD Simulations and NMR Spectroscopy

Robert Pendrill, Elin Säwén, and Göran Widmalm

Department of Organic Chemistry, Stockholm University, Stockholm, Sweden

In order to exploit the interactions between carbohydrates and proteins in the pursuit of novel treatments for various diseases, a deeper knowledge of the conformational behavior of carbohydrates is needed. In this study, an approach based on a combination of molecular dynamics (MD) simulations and NMR spectroscopy is used to describe the solution conformation of a  $\beta$ -(1 $\rightarrow$ 6)-linked disaccharide,  $\beta$ -L-Fucp-(1 $\rightarrow$ 6)- $\alpha$ -D-Glcp-OMe. The (1 $\rightarrow$ 6)-linkage in carbohydrates presents a larger challenge compared to other linkages due to its inherent larger flexibility [1]. In order to describe the conformation of a (1 $\rightarrow$ 6)-linked oligosaccharide, three torsion angles need to be addressed:  $\phi$ ,  $\psi$  and  $\omega$ .

1D<sup>1</sup>H, <sup>1</sup>H-NOESY and T-ROESY NMR spectra were collected, selectively inverting the resonances at the fucosyl H1' as well as glucoside H6pro-R and H6pro-S and from these experiments effective interproton distances were calculated. Effective rotational correlation time was estimated from the ratio of NOESY to T-ROESY build-up rates as well as calculated from the translational diffusion constant as measured by the BPPLIED [2] experiment.

Carbon longitudinal (T1) relaxation times were measured using an inversion-recovery sequence and from these, additional spectral density related parameters were extracted.

Long-range heteronuclear coupling constants (<sup>3</sup>J<sub>C,H</sub>) related to the glycosidic linkage were measured using the J-HMBC [3] experiment.

From the <sup>3</sup>J<sub>H5,H6</sub> coupling constants related to the glycosidic linkage, the ratios between the accessible states for the  $\omega$  torsion (gt, gg and tg) were estimated using literature values [4] for each conformation.

A 500 ns MD simulation in explicit water using the force field PARM22/SU01 [5] was performed. Effective proton-proton distances for the relevant interactions were extracted, as well as heteronuclear coupling constants through the use of reported Karplus-type relationships. The translational diffusion constant was extracted from the mean square displacement.

The NMR parameters measured are compared to those extracted from the MD simulation and found to be in reasonable agreement. Deviations are brought within uncertainty limits when the ratios between the  $\omega$  torsion states from <sup>3</sup>J<sub>H5,H6</sub> coupling constants are used in the extraction of parameters from the simulation.

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# **3 A Robust Methodology for Rapid Structure Determination of Microgram-level Drug Metabolites by NMR Spectroscopy**

Kim A. Johnson, Xiaohong Liu, Stella Huang, Vikram Roongta, W. Griffith Humphreys and Yue-Zhong Shu

Bristol-Myers Squibb

A robust method for in vitro metabolite generation and facile sample preparation on analytical HPLC was established for rapid structure determination of microgram-level drug metabolites by using high field NMR equipped with a cryoprobe. A single 2-5 mL incubation of drug candidate (10-30  $\mu$ M) in microsomes, hepatocytes, or recombinant drug metabolising enzymes, typically cytochrome P450s and UDP-glucuronosyltransferases, was used for metabolite formation. Following precipitation of proteins and solvent removal, metabolite mixtures were chromatographed with 5-10 injections onto an HPLC/MS system. Metabolites were collected into a 96-well plate, dried, and reconstituted in deuterated NMR solvents. NMR spectra of isolated metabolites were acquired on a 500 MHz spectrometer equipped with a 5 mm cryogenic probe. The methodology has been successfully employed as an extension of HPLC-MS/MS-based metabolite identification and applied frequently to 0.5-10 microgram quantities of metabolite. Most structure determinations were achieved rapidly by 1D  $^1\text{H}$ -NMR with satisfactory signal-to-noise ratio, whereas some required 2D NMR data analysis. This report describes the method development and metabolite structure determination using the model compound trazodone. In addition to trazodone, a large number of examples from our laboratories have proven that the microgram-level NMR method avoids time consuming preparative-scale metabolite generation and purification and circumvents technical complications associated with online LC-NMR. Most importantly, the turnaround time of metabolite structure determination for metabolically unstable compounds using the present methodology is more in sync with the cycle time during which medicinal chemists modify the identified metabolic softspot while performing other iterative lead optimisation activities, demonstrating a real impact to the drug discovery process.

# 4 NMR Study on Conformational Preferences of Diindolylurea and Diindolylthiourea Anion Receptors

Damjan Makuc<sup>1,2</sup> and Janez Plavec<sup>1,2</sup>

1. National Institute of Chemistry, Slovenian NMR Centre, Ljubljana, Slovenia
2. EN→FIST Centre of Excellence, Ljubljana, Slovenia

We have recently analyzed conformational preferences of several 2,7-disubstituted indoles with amide substituents at C2 and urea substituents at C7, which showed presence of distinct conformers in the presence and in the absence of anions [1,2]. In addition, indole and urea groups were strongly involved in hydrogen-bonding interactions with bound anionic guest, whilst the amide group interacted only weakly with the bound anion. These observations led to design of diindolylureas and diindolylthioureas [3,4]. In the current work the conformational preorganization of receptors 1-4 (below) as well as conformational changes upon binding of chloride and several oxoanions were studied by the means of NMR spectroscopy and augmented with energetic preferences established by ab initio calculations.

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## Acknowledgements:

We would like to thank Professor Philip A. Gale and Jennifer R. Hiscock from School of Chemistry, University of Southampton, for providing anion receptors.

# 5 Adiabatic $^{13}\text{C}$ NMR Pulse Sequences for High Precision Quantitative Measurement

Serge Akoka, Christophe Thibaudeau, Virginie Silvestre and Gérald Remaud<sup>1</sup>

1. CEISAM, UMR 6230 CNRS/Université de Nantes, Nantes, France.

Accurate measurement for  $^{13}\text{C}$  levels close to or at natural abundance by NMR is desirable in several domains [1]. A high degree of accuracy (1 %) is therefore needed. The single-pulse  $^{13}\text{C}$  NMR sequence optimised with adiabatic decoupling [2] was used successfully to precise, accurate and reproducible measurements [3]. However, measuring times of several hours associated with the use of this sequence constitute a serious drawback. The alternative is to use multi-impulsional NMR sequences. In this work, several pulse sequences based on polarization transfer were evaluated and optimised for precise quantitative  $^{13}\text{C}$  NMR within a short time. Although being more sensitive than the one-pulse technique, DEPT, INEPT and HCP are intrinsically less precise: the intensity of the NMR signals is influenced by several  $^1\text{H}$  and  $^{13}\text{C}$  flip angles and evolution delays which cannot be set simultaneously to optimal values for all resonances. Signal distortions may be explained by either slightly inaccurate pulse widths or by RF field inhomogeneity. Moreover, hard pulses are also known to be highly sensitive to off-resonance effects which induce frequency dependent modulations of signal intensities and therefore poor precision and inaccuracy.

Adiabatic  $180^\circ$   $^1\text{H}$  and  $^{13}\text{C}$  pulses were incorporated into DEPT and refocused INEPT to minimize the influence of  $180^\circ$  pulse imperfections on the precision of measured  $^{13}\text{C}$  peak areas. A modified HCP experiment featuring  $^1\text{H}$  and  $^{13}\text{C}$  spin-locks with adiabatic  $180^\circ$  pulses is also introduced. Inaccurate  $180^\circ$   $^1\text{H}$  and  $^{13}\text{C}$  pulses lead to incomplete refocusing of chemical shifts and induce a modulation of NMR peak intensities by resonance frequencies. The influence of  $^{13}\text{C}$  and  $^1\text{H}$   $180^\circ$  RF pulse imperfections was studied for each pulse sequence by performing series of experiments where either  $^{13}\text{C}$  or  $^1\text{H}$  offset was varied throughout a narrow range. Incorporation of  $180^\circ$   $^{13}\text{C}$  adiabatic pulses dramatically reduces the intensity modulation. To evaluate the influence of  $^{13}\text{C}$  and  $^1\text{H}$  off-resonance on the precision of  $^{13}\text{C}$  peak surfaces, additional series of experiments were performed on a much wider range of values. In spectra recorded using the standard sequences, the precision of peak surfaces was rather poor (c.v.  $\sim$  4 to 6 %) whereas a minimum contribution of  $^{13}\text{C}$  off-resonance was observed for the adiabatic sequences (c.v.  $\sim$  0.2 to 0.6 %). The adiabatic HCP sequence provides a rather precise (within several %) measurement of  $^{13}\text{C}$  peak surfaces only within a rather limited range of offset values, and may therefore not be applied as such for high precision quantitative  $^{13}\text{C}$  NMR, but might be useful for other NMR applications where other spin-lock patterns are inefficient. Further work would be needed to improve the offset dependence properties of the sequence.

Our results clearly show that adiabatic  $^1\text{H}$  and  $^{13}\text{C}$   $180^\circ$  pulses significantly improve the repeatability of the measurements with DEPT, INEPT and HCP by minimizing the influence of  $180^\circ$  pulse imperfections. Among all sequences tested, the adiabatic DEPT and INEPT sequences both have the potential to be used in high precision quantitative  $^{13}\text{C}$  NMR. Further

work needs however to be done to systematically test whether either or both these sequences will provide reproducible data for measurements performed over a longer period of time on a wide range of experimental and environmental set-ups.

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# **6 Implementation of an Open Access SFC/MS in the Medicinal Chemistry Laboratory**

Christine M. Aurigemma and William P. Farrell

Pfizer Global Research and Development

Medicinal chemists often depend on analytical instrumentation for reaction monitoring and product confirmation at all stages of pharmaceutical discovery and development. To obtain pure compounds for biological assays, the removal of side products and final compounds through purification is often necessary. Prior to purification, chemists often utilize open access analytical LC/MS instruments for fast and reliable mass confirmation. Supercritical fluid chromatography (SFC) is typically utilized to obtain excellent separations and isolation of the parent compound in a salt-free format, and it is an orthogonal technique to LC. In laboratories where SFC is the predominant technique for analysis and purification of compounds, a reasonable approach for quickly determining optimal chromatographic conditions is to screen the sample against different columns. This can be a bottleneck to the purification process. To commission SFC for open access use, a walk-up analytical SFC/MS screening system was implemented in the medicinal chemistry laboratory. Each sample is automatically screened through six column/method conditions, and on-demand data processing occurs for the chromatographers after each screening method is complete. This paper highlights the “FastTrack” approach to expedite samples through purification.

# 7 Reaction Monitoring and Mixture Analysis by NMR

Alexander Idström, Tineke Papavoine and Clas Landersjö

AstraZeneca R&D Mölndal, Mölndal, Sweden

NMR is a versatile technique that can give insight in many aspects of chemical reactions, like physical processes, quantities and structures. The use of NMR as a technique to investigate reaction mechanisms is of increasing interest within the pharmaceutical industry. By understanding the chemical reaction in the early stage of the project, an efficient and cost effective synthetic route can be designed which is optimized for larger scale usage.

While monitoring reaction, the number of compounds might increase to the extent that structural determination of an individual compound is impossible. This can be overcome by using Diffusion Ordered Spectroscopy (DOSY) that will 'separate' the spectra in the diffusion dimension, e.g. it differentiates compounds with different molecular sizes.

If molecules have a very similar size, a variation of the method, MAD, Matrix Assisted DOSY can be used. Compounds of similar size but different functional groups can be separated in the diffusion dimension due to the addition of a polymer. The separation of the analytes resembles TLC [1].

On this poster examples will be described on how chemical reactions can be studied. A comparison will be made between a reaction performed directly in the NMR tube and the reaction performed in a reaction vessel coupled to a NMR flow tube [2]. Furthermore an example will be given of the MAD method and its usage.

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# 8 Selective Labeling in Large Proteins as a Tool for Monitoring Ligand-protein Contacts by NMR. Proof of Concept Demonstrated on dUTPase Complexes

Tatiana Agback<sup>1</sup>, Esmeralda Woestenenka<sup>1</sup>, Christer Sahlberg<sup>1</sup> and Johan Isaksson<sup>2</sup>

1. Medivir AB, Lunastigen 7 SE-14144 Huddinge, Sweden,

2. Department of Chemistry, Tromsø University, N-9037 Tromsø, Norway

Based on the example of a large protein-ligand complex, dUTPase-dUpNHpp (MW~ 50kD, symmetrical trimer), the following has been shown: (1) It was possible to distinguish and assign resonances of amino acids located in the active site near the inhibitor through comparison of the chemical shifts between dUTPase and dUTPase-dUpNHpp, where the protein is selectively labelled with selected  $^{13}\text{C}$ ,  $^{15}\text{N}$ ,  $^1\text{H}$  amino acids, while keeping all other background almost fully deuterated (more than 80%). (2) Assignment has been confirmed through conventional procedures in uniformly labelled  $^{13}\text{C}$ ,  $^{15}\text{N}$ ,  $^2\text{H}$ (70%) dUTPase. (3) Using the proposed labelling scheme it was possible to observe in  $^{15}\text{N}$  ( $^{13}\text{C}$ )-edited NOESY(ROESY) experiment nOe (or rOe) contacts between protons of labelled amino acids and ligand dUpNHpp. (4) In the same sample using a  $^{15}\text{N}$ ,  $^{13}\text{C}$ -filtered NOESY experiment, only the nOe contacts of the ligand are detected. (5) We expect this method to be applicable on non-symmetric proteins of the same size where conventional assignment starts to become ambiguous due to spectral overlap as a way to quickly extract key contacts between inhibitor and a few selected amino acids in the active site. (6) The possibility to explore this approach to detect hetero nOe between labelled amino acids and hetero atoms of the ligand ( $^{31}\text{P}$ ,  $^{19}\text{F}$ ,  $^{15}\text{N}$ ) is discussed and in progress.

# 9 An Integrated LC-MS + Offline Microcoil NMR Platform for Identification of Drug Metabolites

Rose Gathungu<sup>1</sup>; Craig Masse<sup>2</sup>; Roger Kautz<sup>1</sup> and Paul Vouros<sup>1</sup>

1. Barnett Institute and Department of Chemistry and Chemical Biology, Northeastern University, Boston, MA
2. CoNCERT Pharmaceuticals, Lexington, MA

Our LC-MS NMR approach combines two innovations for MS and NMR. It integrates a post-column nanosplitter, for online LC-MS, and offline NMR analysis with a microcoil probe, to utilize each technique at its optimal sensitivity. The nanosplitter provides nanoelectrospray from normal bore LC columns with a higher loading capacity, whilst 99% of the eluant is directed to a fraction collector for NMR. Nanoelectrospray reduces signal suppression and increases sensitivity when compared to conventional electrospray. A microcoil probe provides 10x-increased sensitivity over conventional 5 mm and LC-NMR probes – the limit of detection for a microcoil probe is 50 ng for overnight <sup>1</sup>H spectrum acquisition. Additionally, performing NMR offline prioritizes goals for NMR after analysis of MS results, and reconciles the differences in sample mass and analysis time requirements between NMR and MS. The platform, which was previously demonstrated in natural product discovery, is now being applied to the analysis of drug metabolites in the early stages of drug development.

Drug metabolite identification is routinely done by LC-MS due to its high sensitivity. The addition of NMR as a complementary technique provides more definitive structural identification, thereby reducing the number of metabolites to be confirmed by laborious chemical synthesis. A pilot study to evaluate the platform's applicability to metabolite identification has been done with a mixture of metabolite standards of the anti-cancer drug Lapatinib. The metabolites were first separated and collected by LC-MS, peak fractions were then pooled, dried down, and re-suspended in deuterated solvents, and finally NMR spectra acquired in the microcoil probe. NMR spectra were readily obtained from sub-microgram amounts of metabolites; the combination of LC-MS and NMR provided for unambiguous identification of the metabolites.

# 10 From Chromatogram to Response Factors – an LC-QNMR Approach

Ruth Boetzel<sup>1</sup>, Rosalind Richards<sup>1</sup> and Rebecca E. Joyce<sup>2</sup>

1. Structure Elucidation Group, Pfizer Global R&D, Sandwich, UK

2. University of Sussex, Brighton, UK

Quantitation of pharmaceutically active compounds, impurities or reaction intermediates is a critical step in the drug development and manufacturing process. Current ICH guidelines require the identification of impurities observed at or above 0.1%. Usually impurity levels are estimated by their UV responses; however, depending on the relative UV responses of the parent compound and the impurity this can give misleading results. In the case of overestimation of the level of impurity considerable amounts of time can be consumed trying to identify very low level impurities unnecessarily. The ability to accurately measure the amount of impurity present would enable structure elucidation efforts to be focused where most needed. Furthermore, determining the purity of reaction intermediates or active species that cannot be isolated due to their instability in the absence of solvents is critical for manufacturing to be able to adhere to the required stoichiometry for subsequent steps. Relative response factors were obtained by LC-QNMR without isolating unstable compounds. The results have led to a method that can be transferred to production to perform the charging calculations that are vital to the control of both yield and purity profile.

Quantitative NMR (1) (QNMR) relies on the proportionality between signal integration and number of protons and is commonly used for potency calculations in the later stages of drug development. However, there is scope for the application of qNMR in establishing impurity levels prior to full structure elucidation. Once the stoichiometry of an impurity NMR signal has been understood, the molarity, in relation to an external standard, can be determined using QNMR techniques. Using the molecular weight obtained from MS this can then be converted to a weight percentage. This may be applied to isolated samples run in NMR tubes or to LC-NMR experiments.

This poster will demonstrate quantitation by LC-NMR for impurities and an unstable reaction intermediate by employing a software-generated reference signal (pseudo-ERETIC) as well as the PULCON (2) method.

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# 11 Ultrafast 2D NMR Optimized for Routine Analysis of Small Organic Molecules

Patrick Giraudeau and Serge Akoka

CEISAM UMR 6230, Université de Nantes, Nantes, France

2D NMR is a powerful tool for structural and quantitative analysis of small organic molecules. However, the main drawback affecting 2D NMR experiments is their experimental duration, due to the time incrementation necessary to sample the indirect dimension. This limitation generates time constraints which are particularly problematic for overbooked spectrometers dedicated to structural elucidation, but a more fundamental limitation is the incompatibility with kinetic or dynamic studies of short timescale phenomena. Very recently, L. Frydman and co-workers have suggested a so-called “ultrafast 2D NMR” approach [1], allowing the acquisition of a 2D spectrum in a single scan, and thus in a fraction of a second. However, its generalization has been quite limited so far, due to the limitations of this method in terms of spectral width, sensitivity and resolution [2].

We have developed a number of strategies to make ultrafast 2D NMR applicable to a wide range of small molecule samples. We have carefully studied the factors limiting resolution and sensitivity, and we have proposed a multi-echo excitation scheme [3] to limit the impact of molecular diffusion effects, leading to an optimum compromise between resolution and sensitivity. Moreover, an important limitation affecting ultrafast 2D NMR experiment is the accessible spectral width for a given resolution. We have developed a simple and robust approach [4] leading to a twofold increase in spectral width while preserving the original resolution. This method does not require any additional selective pulse, contrary to other recently proposed methods [5,6].

Thanks to these improvements, ultrafast 2D NMR spectra have been obtained in a fraction of a second on a variety of small molecules. Several examples will be presented, such as the DQF-COSY spectrum of ibuprofen or the TOCSY spectrum of a mixture of citrals. The application of ultrafast 2D HSQC for the kinetic study of the mutarotation of glucose in water will also be described. All spectra were acquired on a 400 MHz spectrometer dedicated to routine structural analysis with no special hardware requirements. These examples highlight the efficiency and the robustness of our optimized ultrafast methods.

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# **12 Evaluation of Large Scale Data Processing for LC/UV & MS Based Compound QC in Support of Medicinal Chemistry and Library Compound Management**

Mark Bayliss and Joseph Simpkins

Virscidian Inc.

In our experiences, we have found a significant number of situations that force us to have to QC a much greater percentage of our LC/MS UV, ELSD compound QC results than we feel should be really necessary. This oftentimes means a 100% QC. Some of the reasons are summarized as: Target(s) Found (Green) but the purity or concentration of the sample being too low to be of practical usage. Targets found but eluting in a region of significant level impurities and therefore more challenging for auto-purification. Targets eluting within the solvent front or end of the chromatographic run typically with poor integration. Targets being poorly classified as found, maybe or not found due to challenges in the signal processing, baselining, peak integration, MS peak classification, poor assignment of adducts and so on. The major issue of course, was that we were not really sure to what level these issues were prevalent or were causing us to over QC results. To better understand these effects we have undertaken a relatively large scale review of our results to determine where most of the problem situation occurs and to remedy as many as possible. We were also looking to increase the trust we have our processing and to be able to trap those situations where an analyst needs to make an informed decision and communicate this effectively. This presentation summarizes some of our finding and how we have attempted to solve these issues.

# 13 Microcoil NMR for the Characterization of Heparin-Derived Oligosaccharides

John F. K. Limtiaco, Szabolcs Beni and Cynthia K. Larive

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

Heparin and its related glycosaminoglycan (GAG) heparan sulfate (HS) are polydisperse linear chain polysaccharides that are involved in numerous biological processes. This involvement is attributed to their interaction with a variety of proteins most of which are located within the extracellular matrix. A wide variety of biological processes are regulated by these glycosaminoglycans including cellular growth, tumor growth and metastasis, as well as viral invasion [1]. The diversity of its biological function is a direct consequence of its microheterogeneous structure. Heparin consists of repeating of uronic acid-(1-4)-D-glucosamine disaccharide subunits with variable substitutions at the 2-O position of the iduronic acid and the 6-O and N- position of the glucosamine residue [1]. Solution state  $^1\text{H-NMR}$ , along with complementary analytical techniques, is commonly used to characterize heparinase derived heparin oligosaccharides.

Because of the structural complexity and heterogeneity of heparin and HS, chemical or enzymatic digestion of the full length polysaccharides is a common practice especially when investigating sequences that bind to proteins. Enzymatic cleavage converts the iduronic acid at the cleavage position into an unsaturated uronate ( $\Delta\text{UA}$ ) residue which serves as a chromophore that can be detected at 232 nm and is utilized in subsequent separations; the unsaturated uronate also introduces a characteristic H-4 NMR signal (5.95-6.01 ppm) [2,3]. Following depolymerization, individual GAG oligosaccharides from the complex solution are detected and isolated with a suite of separation steps that utilizes their differing sizes and charges [4]. Size-exclusion chromatography (SEC) was used to separate digested heparin. Using SEC, we isolated oligosaccharide fractions from disaccharides (dp2) to hexadecasaccharides (dp16) into 4.5 mL aliquots which were further purified using HPLC. Individual tetra- and hexasaccharides were isolated using strong-anion exchange (SAX)-HPLC on a Carbopac PA1 column. Peaks eluting at similar retention times were combined and desalted. Desalted peaks were then lyophilized and stored at  $-20^\circ\text{C}$  until further analysis. A major challenge in the characterization of heparin derived oligosaccharides is obtaining sufficient amounts of pure oligosaccharides for NMR analysis, this is especially difficult for rare heparin sequences. We therefore turned to a commercially available Prostasis micro-coil NMR probe for the characterization of heparin-derived tetra- and hexasaccharides. Microcoil NMR allows the investigator to interrogate smaller amounts of materials by reducing the active volume of the detection coil. The Prostasis probe has a sample cell volume of 3  $\mu\text{L}$  and an active sample volume of 1.5  $\mu\text{L}$ .

Lyophilized oligosaccharide peaks ( $\sim 10\text{-}30\ \mu\text{g}$ ) were reconstituted into 3.5  $\mu\text{L}$  of a sodium phosphate in  $\text{D}_2\text{O}$  buffer (pD = 7.4) for NMR analysis. EDTA-d16 was added to the solution to improve spectral quality as it binds to trace paramagnetic impurities that affect the line width of heparin resonances [5]. Analyte solutions were sandwiched between an immiscible solvent, deuterated- $\text{CDCl}_3$ , and positioned within the active volume of the micro-coil probe. Focusing

the analyte between plugs of CDCl<sub>3</sub> improved the S/N of our measurements. By confining the analyte solution to the region in and around the coil active volume, we obtained a significant improvement in S/N when compared with the same mass analyzed in a conventional 5 mm or even a Shigemi tube.

1D and 2D NMR techniques were used to identify and characterize the isolated oligosaccharides. TOCSY and COSY spectra identified resonances and corresponding functional groups that are present on the individual saccharide units and serve as the building blocks for the total oligosaccharide structure. ROESY spectra provide through-space correlation and allow the assignment of the oligosaccharide sequence as well as conclusive determination of the orientation of the glycosidic linkage.

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# 14 DENA and RASA-HSQC, Two Approaches to Decipher or Select Ambiguous Chemical Shifts in Aliased Spectra

Mohammadali Foroozandeh and Damien Jeannerat

Department of Organic Chemistry, University of Geneva, Geneva, Switzerland

Among the numerous techniques aiming at increasing the resolution of indirect dimensions of 2D NMR spectra, spectral aliasing plays a unique role for its simplicity of application and efficiency [1,2]. As a result of the violation of the Nyquist Theorem, aliasing of signals to ambiguous frequencies is the main drawback of aliasing techniques. We introduced the DENA (Differential Evolution for Non-ambiguous Aliasing) sequence [3] and the RASA (Region Accentuation by Selective Aliasing) technique to either overcome the ambiguity of chemical shifts in indirect dimension, or select a region of interest in heteronuclear spectra. The DENA sequence takes advantage of the relation between aliasing order and spectral width and the two separate coherence pathways of the Sensitivity-Enhanced HSQC to record spectra where the split of signals codes the high-order chemical shifts. The RASA technique represents an alternative to semi-selective experiments. This pseudo-3D experiment favors signals that are non-folded in a very clean manner without the need of selective pulses suffering from imperfect transition regions. Applications to Cyclosporin A will be presented.

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# **15** **RevealX™ Technology Improves Isolation and Purification of Natural Products by Flash Chromatography**

Melissa Wilcox, Scott Anderson and Rakesh Bose

Grace Davison Discovery Sciences

Natural products can play a dominant role in the development of new drugs for medicinal purposes. A crude extract typically contains lead compounds requiring multiple steps to isolate and purify. Conventional flash purification technique used for isolating such compounds either fail to detect those compounds that are non-chromophoric or lack the necessary sensitivity often required for detection during separation.

This work investigates the purification of certain class of natural products using the RevealX™ detection technology of the Reveleris™ flash chromatography system. Equipped with integrated multiple detectors, one can separate, detect, and isolate complex matrix of compounds with higher sensitivity and speed. Natural product extracts that are both chromophoric and non-chromophoric are purified satisfactorily in a single step without the use of additional techniques such as preparative HPLC. Such a novel flash chromatography makes the exploration of nature's therapeutic agents less time consuming and tedious.

# **16 Automated Raw Material Screening Using NMR: Application to a Production Lab Operating Under GMP**

Kimberly L. Colson<sup>1</sup>, Joshua M. Hicks<sup>1</sup>, Mark Garvey<sup>2</sup> and Christian Fischer<sup>2</sup>

1. Bruker BioSpin Corp., Billerica, MA, USA
2. Bruker BioSpin GmbH, Rheinstetten, Germany

Since its discovery, NMR spectroscopy has been known for its capability to provide information on the purity of a compound and the quantity of the material present. With recent adulteration of raw materials for economic gains in the pharmaceutical, food and chemical industry there is increased need to utilize NMR for these strengths to enhance product safety for consumers and limit product liability for manufacturers. Here we present the development of a fully automated screening method for raw materials that (1) evaluates instrument performance, (2) performs NMR acquisition on raw materials, (3) analyzes spectra and (4) issues a raw material quality report that may be used as GMP validation of the raw material. Optimizations for automation to apply NMR Raw Material Screening in a production facility operating under GMP where technical expertise in NMR is not readily available will be discussed.

# 17

## Reaction Monitoring Using $^{31}\text{P}$ NMR

Charles Pathirana<sup>1</sup>, Richard Fox<sup>2</sup> and Mark Bolgar<sup>1</sup>

1. Analytical R&D,
2. Process R&D, Bristol-Myers Squibb, New Brunswick, NJ

Proton NMR is by far the most commonly used NMR technique, primarily due to the relatively high sensitivity and abundance of the proton as a nucleus. However, when dealing with a mixture of organic compounds, and when quantitation is required, overlapping resonances often complicate proton NMR interpretation. These challenges can be overcome by resorting to other less commonly observed nuclei. In this study, we were able to use  $^{31}\text{P}$  NMR to detect the end point of an intermediate reaction that produces a phosphate. Lack of a chromophore in the product made HPLC with UV detection unsuitable as a detection technique.  $^{31}\text{P}$ NMR allowed for undisturbed detection of individual components of the reaction mixture. Trimethyl phosphate was used as an internal standard. The objective was to monitor the reaction to minimize the formation of a possible decomposition product that is formed if the reaction is run too long.  $^{31}\text{P}$ NMR spectra demonstrated that this unwanted product could be easily controlled to below the specified level.

# 18 Automating Gradient Method Development in Flash Chromatography for Greater Productivity and Minimizing Solvent Use

Melissa Wilcox, Scott Anderson and Rakesh Bose

Grace Davison Discovery Sciences

Productivity demands in today's laboratories require the chemist to minimize time spent purifying intermediates and target new molecular entities. Faster, 'greener' flash chromatography methods increase throughput, improve productivity, save solvent, and reduce operating costs. Meeting these goals requires gradient methods that deliver the required resolution in the fastest possible time. The RevealX™ Operating System of the Reveleris® flash chromatography system automatically generates these gradient profiles without the iterative TLC and HPLC screening separations typically used today. Instead, the software features the ability to deliver optimal gradient profiles from a minimal amount of user prescreening to successfully isolate target components of interest quickly and easily.

The RevealX™ Operating System uses only two chromatographic separations (TLC plates or HPLC chromatograms) when generating gradient profiles and can provide methods for both normal phase and reversed phase systems. The synthetic chemist can choose a gradient profile based on either highest purity or fastest speed.

In this work both gradient profiles were compared with traditional gradient development routes for normal and reversed phase chromatography examples. This work shows a productivity gain, when using the RevealX™ Operating System for gradient method development, by increasing chromatographic resolution, reducing time spent optimizing the separation and reducing solvent used compared to traditional methods.

# 19 A New Tropane Alkaloid from the Roots of *Erythroxylum Pungens* O.E. Schulz

José G. Sena Filho<sup>1</sup>, Haroudo S. Xavier<sup>3</sup>, José M. Barbosa Filho<sup>2</sup> and Raimundo Braz Filho<sup>4</sup>

1. EMBRAPA Tabuleiros Costeiros, Av. Beira Mar 3250, Aracaju-SE, Brasil
2. Universidade Federal da Paraíba, 58051-970, João Pessoa, PB, Brazil
3. Universidade Federal de Pernambuco- Laboratório de Farmacognosia, 50740-521, Recife-PE, Brasil
4. Pesquisador Visitante Emérito - FAPERJ/UENF/UFRRJ, 28013-602 Campos, RJ, Brazil

The constant researches in drug discovery from different sources, led our research group study species from caatinga region of Brazil, besides the enormous variety of *Erythroxylum* species in this ecoregion, it has many endemic species with a potential medical purpose, in which could be further investigated. This worked focuses in the alkaloids isolation from the roots of *Erythroxylum pungens* for which no prior investigation has been reported. The methodology was carried out using chromatography methods and spectral analyses, including <sup>1</sup>H-NMR, <sup>13</sup>C-NMR, including 2D-NMR techniques spectra. Tertiary total fraction of alkaloids were used to isolate the compound by CC and HPLC resulting in a new tropane alkaloid as well as a new substitution group, (1R,3R)-8-methyl-8-azabicyclo [3.2.1] octan-3-yl-4-hidroxy-3,5-dimethoxyl called Pungencine.

# 20 Structural Characterization of Synthetic Galactosyl Diglyceride Antigens for Lyme Disease

Bruce Coxon and Vince Pozsgay

Eunice Kennedy Shriver National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, Maryland, USA

In previous studies, we isolated and identified two types of glycolipid antigens in *Borrelia burgdorferi*, the tick-borne bacterium that causes Lyme disease [1]. These are cholesteryl 6-O-acyl- $\beta$ -D-galactopyranoside (BBGL-1) and 1,2-di-O-acyl-3-O- $\alpha$ -D-galactopyranosyl-sn-glycerol (BBGL-2), in which the predominant acyl groups are palmitoyl (16:0) and oleoyl (18:1), but which also include minor amounts of such other fatty acids as myristic (14:0), stearic (18:0), and linoleic (18:2). These simple glycolipids are expressed in the surface membrane of the bacterium, and in the absence of detectable lipopolysaccharide, are thought to assume its function. In on-going vaccine development studies, our lab has synthesized compounds of the BBGL-1 type [2], and has prepared their BSA protein conjugates for immunochemical testing [3]. Compounds of the BBGL-2 type have been synthesized in several labs [4-6], including ours, but usually without detailed NMR assignments.

The apparent antigenicity of BBGL-2 analogs has previously been assessed by measurement of the levels of the anti-inflammatory cytokine, interleukin-2 produced by stimulation of natural killer T-cell hybridomas [4]. Such antigenicity was found to depend significantly on acyl chain length and degree of saturation [4]. In the course of validating the structures of synthetic BBGL-2 variants by 1D and 2D NMR methods, we have made detailed  $^1\text{H}$  and  $^{13}\text{C}$  NMR assignments for four of these structures; namely, 1,2-di-O-oleoyl-, 1-O-oleoyl-2-O-palmitoyl-, 1-O-palmitoyl-2-O-oleoyl-, and 1,2-O-palmitoyl-3-O- $\alpha$ -D-galactopyranosyl-sn-glycerol, the positions of the various substituents being confirmed by HMBC experiments. Interpretation of the  $^{13}\text{C}=\text{O}$  chemical shifts has led to a set of rules that describe the dependence of these  $^{13}\text{C}$  shifts on fatty acid type, and substituent position on the glycerol moiety. The rules are useful for NMR analysis of the mixtures of products that result from acylation reactions of imperfect regiospecificity.

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# 21

## **RevealX™ Technology Improves Purification of Lead Generation Compounds by Flash Chromatography**

Scott Anderson

Grace Davison Discovery Sciences

During pharmaceutical development of biologically active compounds, the identification and purification of unknown impurities is a key requirement for the successful registration of a new molecular entity. Traditional flash chromatography as preferred by synthetic chemists is equipped with Ultraviolet (UV) detection that fails to detect targets and impurities that are either present at low levels or lack chromophores. This may result in producing impure targets and lead to false hits during biotesting.

This study investigates the detection and quantification of an impurity in the presence of the lead compound during purification using multiple signal-processing from UV and ELSD (Evaporative Light Scattering Detector). Using the RevealX™ detection technology in the Reveleris™ flash chromatography system, chemists can detect both chromophoric and non-chromophoric compounds present in the sample matrix. A system comparison to a preparative liquid chromatography with an ELSD shows that Reveleris™ can be more productive during purification for lead generation.

# 22 An Appreciation of Laurie Hall, a Pioneer in Small Molecule NMR

Bruce Coxon

Eunice Kennedy Shriver National Institute of Child Health and Human Development, National  
Institutes of Health, Bethesda, Maryland, USA

Laurance David Hall (1938-2009) made significant contributions to small molecule NMR in its early days, including extensive investigations of the  $^1\text{H}$  and  $^{19}\text{F}$  NMR of deoxyfluoro sugars, studies of a number of other heteronuclei, determination of the signs of coupling constants by double resonance methods, and measurements of the spin-lattice relaxation times of a variety of organic molecules. In this poster, I will give a brief exploration of his life and work, which included the publication of 585 papers.

The aspects addressed will include:

- Early History and Education
- The Vancouver Period (1963-1984)
- The Cambridge Period (1984-2005)
- Honors and Awards (1971-2000)
- Some Key References
- Epilog

# 23 **pKa-measurements and Conformational Studies of Amine Linked Pseudodisaccharides**

Jerk Rönnols, Anja Burkhardt, Ian Cumpstey and Göran Widmalm

Department of Organic chemistry, Arrhenius laboratory, Stockholm University, Stockholm, Sweden

Glycosidase inhibitors tend to be positively charged, either due to their ability to mimic a positive charge build-up at the transition state of glycosidic cleavage, or because of interactions with carboxylates flanking the anomeric binding position, or both.[1]

Some 3-aminoaltropyranosides have been shown to act as  $\beta$ -glucosidase inhibitors. The proposed binding mode has Alt-C-3 in the position occupied by C-1 of the glycoside in the cleavage transition state, with the altropyranosides residing in <sup>1</sup>C<sub>4</sub>-conformation.[2] Amine-linked pseudodisaccharides could behave in a similar way to such aminosugars, hypothetically with enhanced inhibition properties due to binding interactions between the glycosidase and the other carbohydrate moiety.

The acid dissociation and conformational properties of such pseudodisaccharides are apparently of great importance for inhibition to occur.

In the presented work the pKa-values of six recently synthesized amine linked pseudodisaccharides[3] have been determined by the use of NMR-measurements together with internal pH indicators, taking advantage of a procedure developed by Sykes et. al.[4] The ring conformations, as well as their pH-dependence, of amine linked altrosides has also been investigated through NMR-measurements.

The discussed molecules are divided into two groups: primary-primary and primary-sec N-linked. The former consists of one or two glucose units and/or one mannose unit linked together via C6, while the latter consists of one 2- or 3-linked altroside unit connected to C6 of a glucose or mannose unit.

In the described work the current pH in the NMR-tube is determined by measurements of chemical shifts of the pH indicator molecules and simple calculations. The chemical shifts of protons adjacent to the amine functionalities vary a lot with the degree of protonation. Titration curves are obtained by plotting chemical shifts against pH, thus pKa-values can be determined. The pKa-values of the studied molecules turned out to vary in a range from 5.6 to 8.0 depending on linkage site.

The conformational properties of the discussed molecules were determined by analysis of <sup>1</sup>H, <sup>1</sup>H coupling constant patterns at pD 3 and 10, and comparison with calculated values for different conformers. Coupling constants were calculated with the software Janocchio,[5] from energy minimized 3D-structures. Altrose is, due to its stereochemistry, known to be conformationally

labile in solution. In our study the ring conformations of the altrose moieties appear to fluctuate, occupying chair conformations as well as skew conformations. There is an apparent dependence of protonation on the population distribution of the different ring conformations. The  $\omega$ -torsions were also examined, but showed little pH-dependence.

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# 24 **Method Optimization for Packing Various Polymer and Silica Chromatographic Media with Dynamic Axial Compression Columns**

Reno Nguyen, Jochen Saar, Michael Early and Melissa Wilcox

Grace Davison Discovery Sciences

One of the challenges in scaling up chromatography processes from analytical method development to pilot or production scale is optimizing the column packing procedure. Early method development work is typically done on pre-packed columns supplied from HPLC stationary phase manufacturers. However, for performance, economics, availability, and simple logistics issues most users migrate to dynamic axial compression (DAC) columns that are packed in-house once processes require large ID columns, >100mm ID. Chromatographers experience numerous difficulties packing stable beds in process/production columns. These problems include non-uniform compression, bed deformation, channeling, void formation and crushing under improper packing. The use of true dynamic axial compression can overcome these issues and produce high quality beds that will exhibit high performance over a broad range of flow rates as evidenced by HETP (height per each theoretical plate) test. Placing constant pressure on a moveable piston gives it the dynamic capability of maintaining a consistent homogeneous bed. The resulting elimination of voids and channeling provides ultimate chromatographic performance. While stationary phase manufacturers may give guidance on packing procedures for their phases, end users often must engage in a trial and error process to determine how best to pack a HPLC phase using their individual DAC system, often sacrificing valuable product in the process. Here we outline a method for optimizing DAC packing using the MODcol® MultiPacker® and SPRING® Columns for various HPLC phases including polymer resins, 300Å silica phases, 120Å silica phases, and irregular 60Å silica. The availability of smaller ID DAC columns with the MODcol® system allows users to lock-in scalable packing processes early in development to further minimize risk involved in scale-up. The SPRING® Column's main feature is the presence of a set of Belleville washers which provides constant pressure on the resin bed. Examples are given for DAC columns ranging from 25mm ID to 100mm ID.

# 25 Solving the Bloch Equations: Application to the Hahn Echo

Alex D. Bain<sup>1</sup>, Christopher K. Anand<sup>2</sup> and Zhenghua Nie<sup>3</sup>

1. Department of Chemistry and Chemical Biology,
2. Department of Computing and Software,
3. School of Computational Engineering and Science, McMaster University, Hamilton, Ontario  
Canada

Not as easy as you think. The full Bloch equations contain terms dealing with evolution of the spins, the effects of radiofrequency (rf) irradiation and relaxation. Dealing with any pair of these is straightforward and familiar, but the full solution, containing all three effects, is more difficult. For example, we must account for both evolution and relaxation during a pulse. It was a few years after Bloch's original work that Torrey [1-3] published a full solution, obtained with Laplace transform methods. We have recently obtained a symbolic solution of these equations using more familiar methods involving density matrices and propagators [4]. This method includes Torrey's solution, but can easily be extended to systems larger than an isolated spin-1/2.

We have tested this solution by applying it to T2 relaxation measurements using the Hahn echo. In this method, a single refocusing pulse is used and the delay time to the echo is varied, whereas in the CPMG method, the delay is constant and the number of echoes is varied. The CPMG method is the most widely used, since it suppresses diffusion and coupling effects, but it can suffer from problems with the accumulation of pulse imperfections. In a modern spectrometer, diffusion effects without gradients are negligible, and here we deal with a single-line spectrum. We use the proton spectrum of cyclohexane at above the coalescence temperature, since this gives us an easily-varied value of T2. The two methods give values that are well within experimental error.

The main effect we investigate is pulses with weak rf power – in our case, a  $\gamma B_1$  of around 500 Hz, corresponding to a 500  $\mu$ s 90 degree pulse. In this case, even small offsets from resonance will cause substantial evolution during the pulse, and so the concept of a hard 180 reversing all the spins can no longer be used. On resonance, the Hahn echo gives a smooth single-exponential decay, as it should, which matches the CPMG result obtained under ideal conditions. Off-resonance, there is an oscillation introduced in the decay, with a frequency corresponding to the offset from resonance.

Since we have an exact solution, we get an excellent fit to the on-resonance and off-resonance data, even out to offsets about  $0.5 * \gamma B_1$ . After that, the oscillations get larger and faster, so other effects (probably static field inhomogeneities) introduce errors. Because we have a more sophisticated model of the experiment, we can tolerate offset effects much more than with standard methods.

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# 26 Binding Studies of Lysozyme with Small Glycans by NMR Spectroscopy and Weak Affinity Chromatography

Christoffer Hamark<sup>1</sup>, Jens Landström<sup>1</sup>, Maria Bergström<sup>2</sup>, Sten Ohlsson<sup>2</sup>  
and Göran Widmalm<sup>1</sup>

1. Department of Organic Chemistry, Arrhenius Laboratory, Stockholm University, Sweden
2. Department of Natural Sciences, Linneaus University, Kalmar, Sweden

Studies of the interactions between HEWL (hen egg-white lysozyme) and small N-acetyl glucosamine (GlcNAc) based glycans have been performed. Bioactivity was tested in dynamic conditions with ligand-based NMR experiments and weak affinity chromatography (WAC) [1]. The techniques were combined with computational methods to gain quantitative and qualitative insights about the system. The HEWL interaction was studied with synthesized carbohydrates as well as commercially obtained analogues.

Protein – carbohydrate interactions are observed in many different forms in biological systems such as in recognition of pathogens of the human cell or the lysis of bacterial cells from hydrolysis of oligosaccharides by lysozymes. An extended knowledge about these systems is vital in biomimetic applications like drug discovery [2]. More specifically, the interaction between carbohydrates and carbohydrate binding domains of proteins is the central point in studies of such molecular recognition. Model systems with proteins and smaller glycans are often the starting point and suitable combinations of biophysical and computational techniques are essential in the process [3]. HEWL is a well-studied model protein known to catalyze  $\beta$ -(1 $\rightarrow$ 4) linkages of the polysaccharides murein and chitin. The enzyme has a large binding cleft that can be divided into six subsites A-F, that each can contain one monosaccharide unit and the hydrolysis is known to take place between subsites D and E [4].

Here we show the use of ligand detected NMR experiments that exploit transferred NOE effects. Saturation Transfer Difference (STD) and transferred NOESY (trNOESY) experiments were chosen to investigate qualitative molecular recognition. In STD NMR saturation is transferred from the receptor protein to ligand molecules and leads to specific signal attenuation of resonance signals of ligands that bind to the receptor. The attenuation is made visible by difference spectroscopy and allows identification and characterization of affinity ligands. Mapping of binding epitopes was performed with the method. TrNOESY is partially based on the same principles as STD and NOE cross-peak signs reveal binding events. The technique was used to determine ligand conformations in the bound states. For NMR determinations of KD, titrations of ligand molecules into a solution of HEWL were performed while detecting T2 relaxation rates using the CPMG pulse sequence. The affinity constants were confirmed using the high throughput technique WAC.

A set of approximately 20 monomeric and dimeric GlcNAc derivatives was studied. This included inter alia 1 $\rightarrow$ 3, 1 $\rightarrow$ 4 and 1 $\rightarrow$ 6 linked dimers but also more uncommon structures such as the seleno- and thiazoline derivatives. The latter is a known inhibitor of N-acetyl

hexosaminidases and hence a potential inhibitor of HEWL [5]. For two of the disaccharides, namely the methyl glycosides of N,N-diacetyl chitobiose and N-acetyl lactosamine, solution structures in complexes with HEWL were deduced from NMR spectroscopy together with in silico molecular docking and molecular dynamics simulations. In solution the disaccharides bind in syn-conformation to B and C sites of HEWL, a result that is at variance from previously published crystal structures [6], which indicate binding in anti-conformation in the C and D sites. This finding highlights that protein-ligand complexes may differ notably between the solution and solid states. Furthermore, dissociation constants  $K_D$ , were determined for the carbohydrate ligands using both NMR and WAC and the two techniques are in very good agreement. Monomers with  $K_D \approx 40$  mM differ in binding affinity by two orders of magnitude from dimers,  $K_D \approx 0.4$  mM. However, the affinity for the dimers shows a larger variation and a prerequisite for a successful binding is a  $\beta$ -(1 $\rightarrow$ 4) linkage.

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# 27 Unique Selectivity Improves Separation of Polar Compounds by HPLC and UHPLC

Melissa Wilcox, Laura Kaepplinger and Neil Herbert

Grace Davison Discovery Sciences

Over the last 10 years, polar pharmaceutical compounds have come to the forefront and can pose severe problems for standard C18 materials best suited to the separation of non- to moderately polar compounds. Shorter chain RP phases can be used but very short chain materials (<C4) suffer stability problems at low and high pH (<pH2 and >pH8). Polar embedded phases (such as carbamate and amide) and AQ phases have been introduced which give improved polar retention and are stable in 100% aqueous mobile phases. Base-deactivated phases and high coverage inert materials typically give good chromatography with most analytes, including bases, but also give similar selectivity. The Grace® Vision HT™ column phases give markedly different selectivity with polar analytes and therefore can give orthogonal selectivity to a standard high coverage, end-capped C18 or C8. In addition, because of the pure nature of the surface and the uniform covering of inert vicinal silanols, peak shape with chelators and bases is very good.

Here we discuss a new group of rugged sub 2µm phases with complementary selectivities that proves ideal for separating everything from small polar molecules to larger protein/peptides. These phases, combined with unique hardware demonstrate the separation benefits of this universal VisionHT™ column platform.

# 28 Metabonomic Analysis of Tomato Fruit Ripening Inhibition Phenotypes by NMR

Gary D. Strahan<sup>1</sup>, Matthew V. DiLeo<sup>2</sup>, Meghan den Bakker<sup>3</sup> and Owen A. Hoekenga<sup>2</sup>

1. USDA, ARS, Eastern Regional Research Center, Wyndmoor, PA
2. USDA, ARS, Robert W. Holley Center for Agriculture and Health, Ithaca, NY
3. Boyce Thompson Institute for Plant Research, Ithaca, NY

Although transgenic crops comprise the majority of acreage planted in the U.S.A., there remains significant debate about their safety as food and the potential for unintended effects on plant composition, food quality, and the environment. In this work, transgenic and wild-type tomato varieties were examined by NMR-based, non-targeted metabonomics to examine the metabolic changes induced when different mechanisms are employed to inhibit the fruit ripening gene, *LeMADS-RIN*. It is well-known that multiple processes are involved in fruit ripening, and that significant metabolic reorganizations occur as the fruit progresses from mature green through to over-ripe. Many studies have characterized these dramatic changes in pigmentation, flavor, aroma and nutritional quality, as well as in the accumulation and dissolution of major and secondary metabolites (1-8).

The gene, *LeMADS-RIN*, produces a transcription factor that enhances the ethylene-dependent fruit ripening process. If *LeMADS-RIN* is absent or diminished, fruit development does not progress fully or at the same rate. There is also natural, spontaneous mutant form of *LeMADS-RIN*: in the heterozygous state, it slows fruit ripening, while in the homozygous mutant, ripening arrests at the mature green stage. Many commercial fresh market tomatoes are heterozygous mutants, with extended shelf life. More recently, transgenic tomato variants have been produced that also inhibit ripening via this gene. Given the concerns in the general public about genetically modified foods, it is important to better understand the metabolic consequences of these mutations.

Using NMR spectroscopy, the metabolic profiles of six tomato variants (including wild-type and transgenic varieties) were studied to model the impact of this *rin* mutation on the tomato fruit metabolome. Multivariate statistical methods have provided insight into many of the most important metabolic components that distinguish these varieties and their significance is being evaluated.

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# 29

## Are Pitfalls Unavoidable During the Structure Elucidation of New Organic Compounds?

Mikhail E. Elyashberg<sup>1</sup>, Kirill A. Blinov<sup>1</sup>, Antony J. Williams<sup>2</sup> and Ryan Sasaki<sup>3</sup>

1. Advanced Chemistry Development, Ltd., Moscow Department, Moscow, Russian Federation
2. Royal Society of Chemistry, US Office, Wake Forest, USA
3. Advanced Chemistry Development, Inc., Toronto, Canada

This presentation was initiated by the review of Nicolaou and Snider [1] entitled “Chasing molecules that were never there: misassigned natural products and the role of chemical synthesis in modern structure elucidation” published in 2005. The review posits that both imaginative detective work and chemical synthesis still have important roles to play in the process of solving nature's most intriguing molecular puzzles.

According to Nicolaou and Snider, around 1000 articles were published between 1990 and 2004 where the originally determined structures needed to be revised. Figuratively speaking, it means that 40–45 issues of the imaginary “Journal of Erroneous Chemistry” were published where all articles contained only incorrectly elucidated structures and, consequently, at least the same number of articles were necessary to describe the revision of these structures. The associated labor costs necessary to correct structural misassignments and subsequent reassignments are very significant and, generally, are much higher than those associated with obtaining the initial solution. From these data it is evident that the number of publications in which the structures of new natural products are incorrectly determined is quite large, and reducing this stream of errors is clearly a valid challenge. Nicolaou and Snider commented that “there is a long way to go before natural product characterization can be considered a process devoid of adventure, discovery, and, yes, even unavoidable pitfalls.”

We believe that the application of modern CASE systems can frequently help the chemist to avoid pitfalls, or, in those cases when the researcher is challenged, then the expert system can at least provide a cautionary warning. Our belief is based on the fact that molecular structure elucidation can be formally described as deducing all logical corollaries from a system of statements which ultimately form a partial axiomatic theory. These corollaries are all conceivable structures that meet the initial set of axioms [2, 3]. The great potential of expert systems is due to the fact that these systems can be considered as inference engines applicable to the knowledge presented within the set of axioms. Particularly, the expert system ACD/Structure Elucidator [3-5] developed by our group is based on the presentation of all initial knowledge in the form of a partial axiomatic theory. The system is capable of inferring all plausible structures from a combination of a molecular formula and 1D and 2D NMR data even in those cases when the spectrum-structural information is very fuzzy.

This system was used in our investigation for the following reasons; Structure Elucidator has been demonstrated [3] to be the most advanced system containing all intrinsic features contained within other systems, but also has a series of additional features which make it

capable of solving very complex real problems. The system is already installed in many structure elucidation laboratories around the world, and has proven itself on many hundreds of both proprietary and non-proprietary structural problems.

Nicolaou and Snider noted that the development of spectroscopic methods in the second half of the 20th century resulted in a revolution in the methodology of structure elucidation. We believe that the continued development of algorithms, and accompanying software platforms and expert systems, will further revolutionize structure elucidation, and will lead to significant acceleration in the progress of organic chemistry and natural products specifically as a result of reduced errors and increased efficiencies.

This presentation contains the main results reported in our article recently published in Nat. Prod. Rep. [6] which considers the application of the Structure Elucidator system to a series of real world examples in which the original structures were later revised. All examples and spectral data of new compounds were borrowed from the articles published in leading chemical journals. We demonstrate how the chemical structure could be correctly elucidated if 2D NMR data were available, and the expert system Structure Elucidator was employed. We also show that if only 1D NMR spectra were originally used for the purpose of structure elucidation, then just the empirical calculation of  $^{13}\text{C}$  chemical shifts for the hypothetical structures will frequently enable a researcher to realize that a structural hypothesis is likely incorrect. We analyze a number of erroneous structural suggestions made by highly qualified and skilled chemists. The investigation of these mistakes is very instructive and has facilitated a deeper understanding of the complicated logical-combinatorial process for deducing chemical structures.

The examples of the application of Structure Elucidator for resolving misassigned structures has shown that the program can serve as a flexible scientific tool which assists chemists in avoiding pitfalls and obtaining the correct solution to a structural problem in an efficient manner.

As shown in [1], chemical synthesis clearly still plays an important role in molecular structure elucidation. However, the multi-step process requires the structure elucidation of all intermediate structures at each step, for which spectroscopic methods are commonly used. Consequently, the application of a CASE system would be very helpful even in those cases when chemical synthesis is the crucial evidence to identify the correct structure. We believe that the utilization of CASE systems will frequently reduce the number of compounds requiring synthesis.

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# 30

## An Open Access Setup to Measure Lipophilicity in Crude Mixtures Using Reversed-Phase HPLC-MS

Muhammad Alimuddin, Angie Li, Wei Wang, Jason Ewanicki and Jennifer Lafontaine

Pfizer, 10770 Science Center Drive, San Diego, CA 92121

A simple and easy to use open access setup for medicinal chemists to measure a lipophilicity index, analogous to the widely used  $\log D(\text{pH}=7.4)$  parameter, of compounds in crude mixtures was developed using reversed-phase HPLC coupled with a mass spectrometer. The  $\log D$  values (derived from the shake-flask method) of 107 compounds, ranging in lipophilicity (0.50 – 6.10) and  $\text{pK}_a$  (1.0 – 12.8), were compared to data obtained using the Open Access Lipophilicity Index method (also loosely referred to as OA  $\log D$  method in conversation) and showed a correlation coefficient ( $r^2$ ) of 0.92, as shown in the figure below. We found the method to be insensitive toward sample matrix (i.e. changes in pH, sample concentration or solvent type). The reproducibility of the method was investigated via repeated injections over a 4-day period and revealed an  $r^2 = 0.96$  and a relative standard deviation (%RSD)  $< 2.1\%$ , suggesting the method is rugged and accurate enough for a variable, high-use, open access environment.

# 31 How Many Structures Can Correspond to a Specific Molecular Mass?

Kirill A. Blinov<sup>1</sup>, Sergey G. Molodtsov<sup>2</sup>, Mikhail E. Elyashberg<sup>1</sup>, Tatiana S. Churanova<sup>1</sup>  
and Antony J. Williams<sup>3</sup>

1. Advanced Chemistry Development, Moscow Department, Moscow, Russian Federation
2. Novosibirsk Institute of Organic Chemistry, Siberian Division, Russian Academy of Sciences, Novosibirsk, Russian Federation
3. Royal Society of Chemistry, US Office, Wake Forest, NC, USA

The number of structures corresponding to a particular molecular formula or molecular weight is one of the most important criteria to consider in Structure Elucidation theory. This knowledge specifically allows us to estimate the complexity of a particular structure elucidation problem. No mathematical formula at present can calculate the number of structures which can be generated from a given molecular formula. Attempts to determine the number of structures that can be generated from formulae corresponding to the molecular weights up to 150 Da[1] or up to 11 heavy atoms[2] have been conducted previously. In this work we have determined an empirical dependence of the number of structures on molecular weight.

We have conducted experiments in which all molecular formulae for molecular weights up to 180.5 Da and with elemental compositions made up of C, H, N and O have been generated. The total number of possible molecular formulae is 2993. All chemical structures corresponding to standard valence rules have been generated[3-5] for each molecular formula and the dependence of the number of structures on molecular weight has been examined. An analysis of the dependence of the number of structures on molecular weight is non-linear. The dependence shows a linear trend with well-defined oscillations with a periodicity of around 14 Da. The oscillations decay with increasing molecular weight and are weak above 100 Da. A linear trend has been identified for data between 140-180 Da. The following equation has been obtained:

$$\lg N = 0.09(MW) - 4.80 \quad (\text{Equation 1})$$

This dependence can be summarized in the following way: The number of possible chemical structures increases by a factor of 10 when the molecular weight increases by 11 Da.

This observation has been confirmed by the generation of structures for the nominal molecular weights of 190 and 200 Da (67 and 87 molecular formulae respectively). The number of structures obtained shows a good correlation with those expected from the derived equation. This simple empirical equation can therefore be used to quickly estimate the number of possible compounds, and therefore the complexity of a structure elucidation task associated with a given molecular weight.

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# 32 Probing the Biliary Metabolism of Drugs by the Use of Diffusion-edited NMR Spectroscopy

Smriti Khera<sup>1</sup>, Mark P. Grillo<sup>2</sup> and Steven L. Hollis<sup>1</sup>

1. Molecular Structure, CR&D, Amgen Inc.
2. PKDM, Translational Sciences, Amgen Inc.

Unambiguous structural characterization of chemically reactive and potentially toxic metabolites is on the critical path to any successful lead optimization program. To enable characterization, metabolites are usually isolated in small quantities ( $\mu\text{g}$ ) from in vivo biological matrices and can contain endogenous impurities which often co-elute by HPLC along with metabolites of interest. These endogenous impurities (such as bile acids) can complicate if not prevent NMR spectral assignments and may require re-purification resulting in further loss of material.

Here we examine the use of Diffusion-edited NMR spectroscopy in order to save purification and re-purification effort, and simplify the analytical workflow leading up to full characterization of drug metabolites. To this end, we collected bile from a bile duct cannulated rat that was fed acetaminophen (APAP) and aimed to purify the two main metabolites of APAP observed by LC-MS. We carried out protein precipitation of the bile sample followed by solid-phase extraction (SPE) of the supernatant, followed by semi-preparative HPLC purification of the SPE fractions to isolate the two major APAP metabolites. Using DOSY, we were able to identify the glucuronide conjugate of APAP (APAP-O-Glu) from one of the SPE fractions of bile eluted with 10% MeOH in H<sub>2</sub>O and fully assign all the <sup>1</sup>H signals of this metabolite, eliminating the need for any further purification of the metabolite. The glutathione conjugate of APAP (APAP-SG) was purified by HPLC along with a methylated glucuronide of APAP (APAP-O-Glu-Me) from a second SPE fraction. Diffusion-editing was useful in the full spectral assignment of both APAP-SG and APAP-O-Glu-Me by suppressing the solvent signals from the residual HPLC mobile phase as well as residual non-deuterated solvent as previously described [1]. DOSY was also useful in making complete spectral assignments for APAP-SG by ‘separating’ the relevant metabolite signals from those of a co-eluting impurity, thus eliminating the need for any further re-purification of this sample.

Results from this study of diffusion-ordered spectroscopy aided characterization of APAP metabolites from a complex sample of rat bile will be presented along with the advantages and limitations of using diffusion-based experiments for the purpose of drug metabolite characterization.

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# 33

## The Use of Multiple Fragmentation Methods for Small Molecule Characterization on an LTQ Orbitrap Velos

Kate Comstock and Yingying Huang

Thermo Fisher Scientific, 355 River Oaks Parkway, San Jose, CA 95127

Tandem mass spectrometry is a valuable tool for small molecule characterization due to its capability to yield a fragmentation fingerprint which reveals the structure information. The advanced MS/MS and MS<sup>n</sup> capabilities of Collision Induced Dissociation (CID) and Higher-Energy Collision Dissociation (HCD) provide different ways to access fragmentation fingerprints. Such fragmentation capabilities can be combined with high resolution accurate mass to offer better data accuracy and confidence.

In this study, eleven small molecule standards were chosen with a variety of structure features, polarities, and molecular weights. These compounds were mixed and then spiked into biological matrices. An LTQ Orbitrap Velos mass spectrometer coupled to an Accela UHPLC system was used to compare the fragmentation efficiency of CID vs HCD. The sensitivity, spectral quality, and speed of the CID MS<sup>n</sup> vs the HCD MS<sup>2</sup> for small molecule structural elucidation was compared and contrasted. Mass Frontier software was used for spectral annotation.

The results indicate that the sensitivity of CID and HCD MS/MS spectra are comparable. In general, 35% normalized collision energy for CID is efficient for fragmenting the majority of small molecule compounds, while the optimal collision energy for HCD varies depending on the structure features and molecular weight of the compounds. HCD is useful for the determination of low mass product ions and records ions resulting from multiple steps of collision, while the CID MS<sup>n</sup> preserves the structural linkage between fragments. About half of the compounds tested, under different energy level, a significant difference in fragmentation pattern was observed between CID vs HCD MS/MS spectra.

In conclusion, CID and HCD complement each other in providing diversified fragmentation pathways, generating information-rich, structurally diagnostic product ions. Combining both dissociation techniques together facilitates optimal, confident small molecule structure characterization, which is critical for metabolite identification, impurity and degradation product structure elucidation.

# 34 Structure Determination of Designer Drugs by NMR and Confirmatory Methods: Forensic Case Studies

Jonathan J. Litzau and John B. Crowe

U.S. Food & Drug Administration, Forensic Chemistry Center, Cincinnati, OH

Over the last decade there has been a significant increase in the appearance of designer drugs—synthetic versions of controlled substances that are produced with slightly altered molecular structures to avoid classification as illicit drugs. The detection and identification of designer drugs is particularly challenging to the forensic scientist due to the constantly changing chemical landscape and countless possibilities for alteration of the original compounds. The internet provides a huge marketplace for designer drugs, ranging from initial inquiries into synthetic routes and material sources to final sale as a “legal high” or “research chemical” [1]. Established screening methods are often inadequate because recently discovered or yet unknown substances are not included in the libraries used for initial characterization. Molecular structure determination of these compounds is further hindered by the absence of standard reference materials for structural confirmation.

NMR spectroscopy is an essential tool to combat the growing number of cases involving the production, sale, and abuse of these substances. Designer drugs that have been characterized at the Forensic Chemistry Center (FCC) include psychedelic phenethylamines, substituted cathinones, and designer anabolic steroids. Selected case studies are presented where 1D and 2D NMR experiments were employed for molecular structure elucidation. Typical sample submissions from bulk powders to finished dosage forms present additional analytical challenges. Supplementary techniques such as FT-IR, exact mass determination, and GC-MS are used as confirmatory methods.

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# 35 Isolation and Structure Elucidation of In-Process Impurities During Tetrazole Ring Formation of Compound I

Maria Silva Elipe, Chul Yoo, Fang Xia, Jason Simiens, Kevin Crossley, John Huckins, Gary Guo, Jason Tedrow and Kirby Wong-Moon

Amgen, Inc., Thousand Oaks, CA, 91320

**Purpose** Two in-process impurities of identical mass observed at levels of 10-12 LCA% during the formation of the tetrazole ring in Compound I were isolated to determine their structures and apply this knowledge to the process development.

**Methods** The impurities of interest were isolated from the mother liquor under normal phase conditions followed by reversed phase conditions; they were then neutralized, dried down, and de-salted. Isolated peaks of interest were analyzed using high resolution LC/MS and 1D and 2D NMR experiments.

**Results** A multi-step isolation procedure was necessary to obtain isolates of sufficient purity and quantity for structural elucidation studies. High resolution MS analysis suggested that the two isolates had the same protonated masses of ca. 577.32 Da and also displayed identical fragmentation patterns of limited structural information. NMR studies indicated the presence of an additional NH functional group in both isolates with similar spatial and bond correlations to one of the dimethylcarbamoyl moieties and the corresponding aromatic ring. A benzodimethylcarbamoylamino moiety formed as a result can be explained based on the Schmidt reaction mechanism, through the nucleophilic attack of the azide group to the carbonyl of the carbamoyl group, the migration of the phenyl group and the release of N<sub>2</sub> followed by tautomerization.

**Conclusions** Multi-step isolation and structure elucidation of two in-process impurities formed during tetrazole ring synthesis suggested an unexpected reaction pathway. Since the reaction conditions were fixed due to safety concerns, the crystallization protocol was designed to significantly reduce the levels of these impurities.

# 36 Solvent Selection for Single-Wall Carbon Nanotubes: New Best Solvents and Acids/Superacids

Francisco Torrens and Gloria Castellano

Institut Universitari de Ciència Molecular, Universitat de València, Edifici d'Instituts de Paterna

The analysis of 1 octanol-, cyclohexane- and chloroform ( $\text{CHCl}_3$ )-water partition coefficients  $P_{o-ch-cf}$  allows calculating molecular lipophilicity patterns (MLPs), which show that for a given atom  $\log P$  is sensitive to functional groups. Program CDHI cannot properly differentiate between non equivalent atoms. The most abundant single-wall carbon nanotube (SWNT), (10,10), presents consistency between a relatively small aqueous solubility and large elementary polarizability,  $P_{o-ch-cf}$  and kinetic stability. A class of non hydrogen-bonding Lewis bases with good solubility is found. The SWNTs in some organic solvents are cationic while in water/Triton X 100 (TX) are anionic. A group of eight new solvents, two superacids and nitric acid ( $\text{HNO}_3$ ) behaves as best solvents. Categorized solubility is semiquantitatively correlated with solvent parameters. Solvchromic term  $\beta$  correlates positively while permittivity and volume correlate negatively. The electron affinity of D glucopyranoses (D Glc<sub>pn</sub>) suggests aqueous colloids of anionic SWNTs. Dipole moment for D Glc<sub>pn</sub>-linear increases until  $n = 4$  in agreement with an 18 fold helix. The Inz- and SWNT- form inclusion complexes with cyclodextrin (CD) and amylose (Amy). Starch, D Glc<sub>p</sub>, CD and Amy are co solvents of SWNTs. Unperturbed guests-hosts expand the central channel. Composites and conducting/antistatic coatings are some of the most sought-after applications of nanotubes. Homogeneous, stable and spontaneously formed solutions of unmodified nanotubes should be a welcome starting material. Poly(vinyl alcohol) composite films present improved mechanical properties when compared to the pristine polymer. Another development is the possibility to functionalize selective and stoichiometrically the nanotube walls, in the same way as in the early days of C<sub>60</sub> chemistry where C<sub>60</sub>R<sub>n</sub> molecules could be selectively obtained from C<sub>60</sub>n- solutions. Adjusting the charge on the nanotubes one could precisely monitor the amount of attached functional groups on them. The results place nanotube processing onto a solid scientific ground and encourage the engineering of macroscopic nanotube materials.

# 37

## Parahydrogen-induced Polarization Detected by Zero-field NMR

Thomas Theis<sup>1</sup>, Micah. P. Ledbetter<sup>2</sup>, Gwendal Kervern<sup>1</sup>, Paul Ganssle<sup>1</sup>, Dmitry Budker<sup>2,3</sup>  
and Alex Pines<sup>1,4</sup>

1. Department of Chemistry, University of California Berkeley, Berkeley, CA 94720
2. Department of Physics, University of California Berkeley, Berkeley, CA 94720
3. Nuclear Science Division, Lawrence Berkeley Laboratory, Berkeley CA 94720
4. Materials Science Division, Lawrence Berkeley Laboratory, Berkeley CA 94720

Nuclear magnetic resonance (NMR) at high magnetic fields (2-20T) has become a powerful and widely used analytical tool for the determination of molecular structure and biological function. For mainstream NMR applications, large applied magnetic fields are needed in order to polarize spins, resolve peaks, and detect with high sensitivity. Immobile and expensive superconducting magnets are used to attain the necessary magnetic fields. We demonstrate that microfabricated atomic magnetometers enable cost-effective and mobile NMR at zero field. To polarize the spins at zero field, hyperpolarization methods can be used. In the last few decades hyperpolarization techniques, such as dynamic nuclear polarization (DNP), laser-polarized noble gases, and parahydrogen-induced polarization (PHIP) have become well-established methods applicable to a vast range of analytes. Here we show that PHIP can be used to obtain high-resolution J-coupling spectra of molecules containing  $^{13}\text{C}$ - $^1\text{H}$  bonds at natural  $^{13}\text{C}$  abundance. An additional benefit of zero-field is extremely high field homogeneity, both spatially and temporally, allowing narrow lines (100 mHz)[1]. This enables very accurate determination of J-coupling parameters, which contain important structural information. In order to observe the low frequency spin dynamics (0-400Hz) at zero field with high sensitivity ( $\sim 30\text{fT}/\text{Hz}^{1/2}$ ) a Rubidium vapor cell is used in an optical pump probe configuration. We show the experimental setup as well as several zero-field spectra, obtained using two different methods for transferring polarization from parahydrogen to analyte (hydrogenation, SABRE). The structural information encoded in the spectra, together with the obtained signal-to-noise and resolution, demonstrate the strength of this new technique.

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# 38

## Use of Solid State NMR to Aid the Salt-Selection Process for Pharmaceutical Products

Richard J Lewis, David Martin, Anke Fiebig, Talbir K Austin, Steve Connolly  
and Austen D Pimm

AstraZeneca R&D Charnwood, Bakewell Road, Loughborough, Leics. LE11 5RH. UK.

Selection of a suitable drug compound for development involves not only selecting the right compound, but also the correct physical form. Many candidate drugs are hard to crystallise and have poor physical properties and very often addition of a salt results in the drug molecule having more desirable properties than the free acid or base itself. For example, the salt form may be more physically and chemically stable, may be easier to manufacture, may exhibit only a single stable polymorph or have better bioavailability. This work describes how we have used solid state NMR to understand and troubleshoot issues occurring at the salt selection stage when the physical properties of the candidate drugs are poorly understood. The work falls into three main areas:

**Variable Stoichiometry:** Issues can arise when different batches of salt prepared under supposedly the same conditions have different stoichiometries. Two examples are shown exhibiting different causes. In one example, attempts to make a mono carboxylate salt of an amine gave stoichiometries below and up to 1:1. Stoichiometries below 1:1 showed loss of intensity in the salt peaks and some disorder in the molecule, but no new species present. The most likely explanation is that this compound is not a true salt, but rather forms a co-crystal or solvate.

**Protonation State:** It can be useful to determine the protonation state of a salt [1] – in other words whether the proton is transferred in the solid state. Such knowledge enables the “salt” to be described correctly as a salt or a co-crystal and may indicate possible problems (such as that of variable stoichiometry above). We will describe an example where knowledge of protonation state helped assess the risk of taking the compound forward.

**Chemical Stability:** In this example, chemical stability of solid samples was found to vary with salt form. For an unstable salt, mobility resolved ssNMR spectra showed a high degree of mobility around the degradation site. Mobility was estimated from a simple high-powered decoupled carbon experiment in which the relaxation time ( $d_1$ ) was varied. In this experiment, only carbon atoms experiencing motion in the solid state will appear in the spectrum at short relaxation times [2]. A range of alternative salt forms were screened and we found a good qualitative correlation between the mobility of the atoms around the degradation site and the chemical stability measured over a 12 week period. A correlation between mobility and stability has been suggested previously, but we believe that for this compound, the correlation is much stronger than those reported in the literature [3]. We hope to develop this further to enable fast prediction of stability on long term storage.

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# 39 A Metabolomic Assessment of Cobia Health in Response to Dietary Manipulation

Tracey B. Schock<sup>1</sup>, Sarah E. Newton\*<sup>2</sup>, Arezue F. B. Boroujerdi<sup>1</sup> and Daniel W. Bearden<sup>1</sup>

1. National Institute of Standards and Technology, Analytical Chemistry Division, Hollings Marine Laboratory, Charleston, South Carolina
2. University of Arkansas Pine Bluff, NOAA EPP Scholar

Aquaculture produces a significant source of protein for global human consumption. In February 2008, U.S. government agencies, the National Oceanic and Atmospheric Administration (NOAA) and the National Institute of Standards and Technology (NIST), sponsored a workshop to identify issues and technologies that are deemed necessary to enhance the U.S. aquaculture industry's competitiveness. A priority objective resulting from the workshop was to evaluate the ability to improve human health attributes of farmed fish products through dietary manipulation. Commercial fish diets rely heavily on fish meal and fish oil, which can be costly. Alternative protein and lipid sources may provide nutritionally improved high performance growth and may be more cost effective.

A dietary study was conducted by the South Carolina Department of Natural Resources (SCDNR) on the finfish cobia, *Rachycentron canadum*. Juvenile cobia were raised on four different diets: a commercial diet (control), a diet with conventional levels of fish meal (FM100), a diet with a 50% reduction in fish meal (FM50), and a diet with 25% reduced fish meal (FM25). The experimental diets substituted varying amounts of soybean meal and poultry meal for fish meal. A total of 432 fish were stocked in 24 tanks (6 tanks/diet) in three recirculating aquaculture systems. Seventy-two fish (3 fish/tank) were sampled on Day 0, Day 66, and Day 98. Physical growth measurements were taken and serum was sampled prior to fillet and organ collection for metabolomic analysis. In the current study, we examined cobia sera with NMR-based metabolomic techniques to assess the affect of decreasing dietary fish meal on the health of the cobia. Filtered sera <sup>1</sup>H NMR spectra were analyzed by principal components analysis (PCA). Cobia fed reduced fish meal diets were metabolically different than control and FM100 diets in PC1. Betaine increased in cobia fed diets with less fish protein while lactate decreased. Lactate also decreased significantly as cobia matured (PC2). This data suggests that the cobia on reduced fish meal diets were in a state of malnutrition. Growth performance measurements on Day 98 corroborate this data with control and FM100 fed cobia weighing significantly more than the FM50 and FM25 fed cobia. The results show that metabolomic analysis is useful for understanding the effects of alternative diets in aquaculture studies.

\* Visiting Undergraduate Student

# 40

## **Automated Raw Material Screening by NMR: Analysis Methods Used for Automated Report Generation**

Christian Fischer<sup>2</sup>, Joshua M. Hicks<sup>1</sup>, Mark Garvey<sup>2</sup> and Kimberly L. Colson<sup>1</sup>

1. Bruker BioSpin Corp., Billerica, MA, USA
2. Bruker BioSpin GmbH, Rheinstetten, Germany(2)

Since its discovery, NMR spectroscopy has been known for its capability to provide information on the purity of a compound and the quantity of the material present. With recent adulteration of raw materials for economic gains in the pharmaceutical, food and chemical industry there is increased need to utilize NMR for these strengths to enhance product safety for consumers and limit product liability for manufacturers. Here we present the analysis part of a fully automated screening method for raw materials that (1) identifies expected compounds, (2) quantifies, (3) detect unexpected signals, (4) writes a QC and expert report. Automated analysis involves the use of a NMR Spectral Base (SBASE) and Knowledgebase (KBASE) to identify main components, common impurities and adulterants. The identification and quantification of the compounds present in the sample is discussed in detail.

# 41 Insights into the RPIP-HPLC Separation of Heparin and Heparan Sulfate Oligosaccharides

Christopher Jones<sup>1</sup>, Szabolcs Beni<sup>1,2</sup> and Cynthia Larive<sup>1</sup>

1. University of California, Department of Chemistry, Riverside, CA, 92521, USA
2. Semmelweis University, Department of Pharmaceutical Chemistry, Hőgyes Endre u. 9

Reverse-phase ion-pairing HPLC (RPIP-HPLC) is an increasingly popular chromatographic technique for the separation of charged compounds. RPIP separations are useful for the separation of oligosaccharides derived from heparin and heparan sulfate (HS), highly anionic members of the glycosaminoglycan family. Heparin and HS are important pharmaceutical targets due to their involvement in diverse biological processes including angiogenesis, tumor metastasis, and viral invasion. Heparin oligosaccharides are useful compounds to probe the details of the RPIP separation mechanism, aspects of which are still being debated. The affects of IPR structure, concentration, and mobile phase pH on the quality of the RPIP-UPLC separation of heparin oligosaccharides will be presented with particular emphasis on how these factors affect the separation of the anomeric forms of heparin and heparan sulfate derived disaccharides. The results of RPIP-UPLC experiments that vary mobile phase pH demonstrate that separation of anomeric forms of these disaccharides can be eliminated, simplifying chromatographic analysis. <sup>1</sup>H NMR experiments were crucial in assigning the anomeric forms of each disaccharide. In the case of N-acetylated disaccharides, anomeric assignments were not feasible using carbon bound protons alone and relied upon detection of the amide proton resonances. We are also interested in the extent to which changing the mobile phase conditions, in this case pH, affects the electrospray ionization efficiency for heparin disaccharides. The knowledge gained from the presented work could provide significant insights leading to the development of a more sensitive and efficient separation of larger heparin oligosaccharides.

# 42

## Application of NMR Spectroscopy to Forensic Characterization of Diastereomeric Superwarfarins

Paul Alperin, Akim Faisal, and Danylle M. Kightlinger, Kajal Nandy, Aaron T. Wright, Herman Cho and John R. Cort

Fundamental and Computational Sciences Directorate  
Pacific Northwest National Laboratory  
Richland WA 99354

The formation of unequal mixtures of diastereomeric products due to stereoselectivity in organic reactions that introduce a second stereocenter into a racemic starting material is a fundamental principle in organic chemistry. The ratio of diastereomers in such product mixtures commonly exhibits dependence on reaction conditions such as temperature, time, reagents, and other parameters. We are investigating whether these dependencies can be used as a basis for source attribution and sample matching of diastereomeric compounds of forensic interest. Comparison of diastereomer ratios in two samples may indicate or rule out a common origin in the same production batch. As an example, we describe our findings that the diastereomer ratio in the highly toxic anticoagulant (superwarfarin) brodifacoum, which exists as a mixture of two diastereomers, exhibits significant variation from one batch to another. The diastereomer ratio was easily determined by integration of isolated resonance peaks for each diastereomer in NMR spectra of brodifacoum [1]. HPLC was also used to measure diastereomer ratios in brodifacoum samples and corroborated the NMR measurements. In addition, line broadening and secondary chemical shifts in the NMR spectra suggest that different conformational preferences in each brodifacoum diastereomer dictate diastereomer-specific changes in dynamics which depend on the ionization state of a titratable enol group whose enol/enolate proportion varies from sample to sample. These characteristics of the NMR spectra can be used in conjunction with diastereomer ratio measurements as a chemical forensic signature for source attribution and sample matching of brodifacoum and related superwarfarins.

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# <sup>15</sup>N Indirect Detection of N-sulfo Glucosamine Residues in Glycosaminoglycans

Derek J. Langeslay<sup>1</sup>, Szabolcs Beni<sup>1,2</sup> and Cynthia K. Larive<sup>1</sup>

1. Chemistry Department, University of California, Riverside, Riverside, California
2. Department of Pharmaceutical Chemistry, Semmelweis University, Budapest, Hungary

Heparin and heparan sulfate are members of a class of biologically and pharmaceutically significant molecules known as glycosaminoglycans (GAGs). GAGs are complex linear polysaccharides that are both polydisperse in size and microheterogeneous in composition. Their study is of great interest, but it is complicated by the diverse structure of these molecules. Work done by Pomin et al. [1] has shown that the analysis of N-acetylated GAGs can be simplified through acquisition of <sup>1</sup>H-<sup>15</sup>N Heteronuclear Single Quantum Coherence (HSQC) spectra. This approach takes advantage of the slow exchange on the NMR timescale of the nitrogen-bound protons of the N-acetylated glucosamine residues of <sup>15</sup>N-labeled heparan sulfate from cultured mammalian cells. This method is not able to observe more rapidly exchanging nitrogen-bound protons like those found in N-sulfo glucosamine residues, which comprise many protein binding motifs in heparin and heparan sulfate. The goal of the work outlined in this poster is to optimize detection of both the N-acetylated and N-sulfonated nitrogens found in the glucosamine residues typical of heparin and heparan sulfate. This work will lay the groundwork for a comprehensive <sup>1</sup>H-<sup>15</sup>N HSQC-based method that can be used for both structure elucidation and binding studies involving <sup>15</sup>N-labeled heparan sulfate derived oligosaccharides. This approach is common in protein analysis where it is used to identify the amino acids involved in binding events. When applied to heparan sulfate derived oligosaccharides we envision that it will be able to provide information regarding which saccharides interact with a protein, aiding in the elucidation of the glycan sequences responsible for binding to various targets.

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# 44 **Continuing Optimization of a Large-scale NMR Facility**

Dennis P. Anderson, Wei Wang, Andrew P. Butler, Kathleen A. Farley, Geeta Yalamanchi,  
George Perkins and Stephen C. Maginess

Analytical Chemistry, Pfizer Global Research and Development, Groton, CT 06340

The operation of a large scale open-access NMR facility places large demands on an NMR administrator for upkeep of the instrumentation, training of users in the acquisition and processing of data, and the optimization of software, including the implementation of updates. While instrument upkeep is necessarily handled on an instrument by instrument basis, efforts for user training and software optimization can be mitigated somewhat.

The MyNMR interface provides several enhancements over the standard interfaces, including streamlining the data entry process in a “top down” manner, the use of LDAP lookup of user information without the need for separate database software, and the enforcement of time limitations according to local policies for instrument usage. The VOICE application was developed to store the customization files in a central location and quickly deploy changes for any or all instruments when needed.

As instrumentation ages, updates and upgrades are necessary to continue using vendor supported hardware and software. With updated software, a newer customized interface is needed to maintain facile sample entry, while maintaining full functionality of previous versions. For this purpose, the MyNMRj interface is shown, utilizing special macros and xml pages.

# 45 Quantitative Analysis of Trace Level Impurities by NMR in both Drug Substance and Formulated Products

Andrew Phillips, Ian Jones and Steve Coombes

AstraZeneca, Pharmaceutical Development, Macclesfield, UK

NMR is a well-established quantitative technique for looking at relatively small amounts of material – for example residual solvents within an active pharmaceutical ingredient down to levels of 0.1% w/w.

Recently there has been considerable concern from pharmaceutical regulatory agencies over the control of potential genotoxic impurities (PGI) in medicinal products. The Threshold of Toxicological Concern (TTC) for PGIs in commercial products is 1.5  $\mu\text{g}/\text{day}$ , or single-figure ppm with respect to a typical drug substance. Consequently, methods for the measurement of impurities in the single-ppm range are required – presenting a significant analytical challenge. We have recently shown that NMR can detect down to these low level often with significant advantages over other more traditional techniques in terms of method development, sample preparation and experiment time<sup>1,2</sup>.

The key to the success of NMR is overcoming the inherent lack of sensitivity so this poster will focus on some of the important factors that determine the level of detection, such as the performance of the NMR system, substrate concentration, linewidth, resolution and dynamic range.

In addition a number of new genotoxic impurities examples analysing both pure compounds and formulated products will be presented. Furthermore it will shown that the use of NMR can be expanded to other trace analysis problems such as cleaning validation: confirming vessels used for chemical reactions are not contaminated with material from previous experiments.

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# 46 Mandelalides A-D, Cytotoxic Macrolides from Endemic South African Lissoclinum Species

Justyna Sikorska<sup>1</sup>, Michael T. Davies-Coleman<sup>2</sup>, Shirley Parker-Nance<sup>2</sup>, Clemens Anklin<sup>3</sup> and Kerry L. McPhail<sup>1</sup>

1. Department of Pharmaceutical Sciences, College of Pharmacy, Oregon State University, Corvallis, OR, U.S.A.
2. Department of Chemistry, Rhodes University, Grahamstown, South Africa
3. Bruker BioSpin, Billerica, MA, U.S.A.

There are as many as 50 marine-derived natural products or derivatives in clinical and preclinical trials. The most successful of these are Ecteinascidin 743 (Yondelis®) from the tunicate *Ecteinascidia turbinata*, Dehydrodidemnin B (Aplidin®) from the tunicate *Aplidium albicans*, and the protein Neovastat (AE-941) from a shark, all of which are in late clinical trials or approved for specific cancers, and are being used to treat larger numbers of people.[1] That Ecteinascidin-743 and Aplidin are the most clinically advanced marine natural products for cancer treatment is a testament to the potential of tunicates and their symbionts as a prolific source of unique and potently bioactive metabolites. The natural products chemistry of marine tunicates has been studied relatively little compared to that of other marine invertebrates such as sponges and soft corals. This may in part be due to limited availability of biomass, especially of encrusting forms, and also the fact that biologically active compounds are often only minor constituents of the organisms.[2] Enormous improvements in the sensitivity of spectroscopic methods make feasible the structure elucidation of compounds in microgram or even nanogram quantities.[3]

The crude extract of a new species of *Lissoclinum* tunicate collected from Nelson Mandela Bay, South Africa, showed good cytotoxicity to neuro 2a mouse blastoma cells (98% lethality at 30 µg/mL). Bioassay-guided fractionation yielded a series of new macrolides, named mandelalides A-D, in microgram amounts. Structure elucidation by 1D and 2D NMR spectroscopy was greatly facilitated by folded HSQC and HMBC and semi-phase sensitive HMBC experiments acquired at 700 MHz on an inverse cryoprobe. While relative configuration of the compounds can be assigned from analysis of NOE data, the measurement of homonuclear coupling constants ( $^3J_{HH}$ ), heteronuclear coupling couplings ( $^{2,3}J_{CH}$ ) and residual dipolar couplings (RDC) in orienting media is being investigated for the determination of absolute configuration of mandelalides A, B, C and D.

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# 47

## **Adulteration Testing In The Pharmaceutical Industry By Complimentary NMR and DESI-MS**

Ian Marsden<sup>1</sup>, Nari N Talaty<sup>2</sup>, Stephen G Spanton<sup>2</sup>, Michelle A Long<sup>3</sup> and Steven P. Cepa<sup>2</sup>

1. Abbott Laboratories, Structural Chemistry, North Chicago, Illinois
2. Abbott Laboratories, Structural Chemistry, Abbott Park, Illinois
3. Abbott Laboratories, Global Pharmaceutical Operations, North Chicago, Illinois

There are many recent examples of product adulteration. For example, the presence of oversulfated chondroitin sulfate in heparin and melamine in pet food/feedstock and milk/infant formula. Testing of such materials in many cases use simple tests which may easily be deceived by specific adulteration with less expensive ingredients. For heparin, oversulfated chondroitin sulfate is known to have some anticoagulant properties. Whereas, the addition of melamine elevates the total nitrogen content detected by simple protein tests thus inflating the apparent protein content.

Pharmaceutical companies globally are seeking more thorough testing of incoming raw materials based on risk to the final product. Herein the use of NMR and DESI-MS will be described to evaluate the potency and possibility of adulteration of a variety of materials.

# 48

## Hadamard NMR in Automation

Ron Crouch<sup>1</sup>, Bert Heise<sup>2</sup>, Ēriks Kupče<sup>2</sup> and Eugene Mazzola<sup>3</sup>

1. Agilent/Varian NMR, Cary, NC, USA
2. Agilent/Varian NMR, Oxford, UK
3. Univ. of Maryland-FDA Joint inst., College Park, MD, USA

A set of basic NMR experiments that are typically used for structure determination in small organic molecules - COSY, DQ-COSY, TOCSY, NOESY, ROESY, multiplicity edited HSQC, HSQC-TOCSY, HMBC and HETCOR - have been modified to incorporate Hadamard encoding [1, 2] that replaces the conventional systematic exploration of the evolution dimension. Provided the sensitivity is sufficient this speeds up the measurements by a factor  $2M/N$ , where  $N$  is the order of the Hadamard matrix and  $M$  is the number of evolution increments in the conventional, phase sensitive implementation of the experiment. If  $k$  chemical sites are selected,  $k$  must be less than or equal to  $N$ .

A suite of setup and processing macros has been developed that allow fully automatic setup, processing and plotting of a user-selected series of the Hadamard experiments. A 1D proton spectrum is used as a starting point and all of the following Hadamard experiments are conducted in full automation. Depending on the sequence of the chosen Hadamard experiments, the C-13 frequencies of the protonated carbon sites are generated using the refocused INEPT experiment. The signal-to-noise ratio is constantly monitored during recording of the C-13 spectra, and the line list is created automatically when the required S/N threshold is reached. The phase and threshold for the 2D plots are also determined automatically. The construct is then placed into the Study Queue - a standard protocol for automation on Varian NMR systems. This allows investigating multiple samples unattended and in full automation. The spectra are produced either as hard copies or as electronic copies that can be automatically e-mailed to the users as soon as the experiment is finished.

All Hadamard fast methods supported in automation are shown using the dermal toxin, T-2 Toxin, as an example. Some comparisons are made with traditional 2D methods.

### Acknowledgements.

We greatly appreciate the involvement of K. Krishnamurthy in this project.

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# 49 Utility of F2-Coupled-HSQC Experiments in the Intact Structural Elucidation of Complex Saponins

Eugene P. Mazzola<sup>1</sup>, Bruce Coxon<sup>2</sup>, Ainsley Parkinson<sup>3</sup>, Edward J. Kennelly<sup>3</sup>  
and Daron I. Freedberg<sup>4</sup>

1. University of Maryland-FDA Joint Institute, College Park, MD, USA
2. The Eunice Kennedy Shiver National Institute of Child Health and Human Development, NIH, Bethesda, MD, USA
3. Lehman College, CUNY, Bronx, NY, USA
4. Center for Biologics Evaluation and Research, FDA, Bethesda, MD, USA

In the structural elucidation of saponins (triterpenes, steroids and their glycosides), high-resolution F2-coupled-HSQC spectra can be crucial for determining both the identities of intact monosaccharide units [1] and coupling constants in strongly coupled proton spin systems. Identification of the orientation (axial or equatorial) of anomeric hydrogens is frequently trivial, but that of the remaining, non-anomeric, ring hydrogens in each sugar unit, based on the relative magnitudes and multiplicities of coupling constants, can be difficult due to (i) severe signal overlap in the congested carbohydrate region of the <sup>1</sup>H NMR spectra and the occurrence of vicinal hydrogens that have nearly identical chemical shifts and (ii) insufficient resolution in the HSQC spectra. In addition, determination of precise coupling constants of adjacent hydrogens that have nearly identical chemical shifts and thus comprise strongly coupled, second-order spin systems [2] can be similarly arduous. The resulting signals for such hydrogens are frequently uninterpretable in 1-dimensional NMR spectra. However, in both of these cases, F2-coupled-HSQC experiments not only relieve spectral congestion but also transform second-order spectra into easily interpretable pseudo-first-order spectra.

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Matthias Niemitz<sup>1</sup>, Samuli-Petrus Korhonen<sup>1</sup> and Manfred Spraul<sup>2</sup>

1. PERCH Solutions Ltd, Kuopio Finland
2. Bruker BioSpin GmbH, Rheinstetten, Germany

To provide a safe assessment of the consistency between a given structure and its <sup>1</sup>H NMR spectrum we present a process for complete NMR spectral analysis in a fully automated fashion performing a prediction of NMR parameters (chemical shifts, J-couplings) and optimizing them to match the experimental data using iterative quantum mechanical spectral analysis [1] with the optional use of HSQC information.

The process returns the completely assigned spectrum and accurate spectral parameters (chemical shifts, J-couplings, line-shapes) extracted from the data even for overlapping signals as well as for higher order spectra and evaluates the consistency between structure and spectral data upon the quality of the fit and the similarity between predicted and actual spectral parameters.

Extremely high specificity is guaranteed, because the complete spectral analysis assures, that all NMR-parameters are self-consistent. Thus the “false positive rate” is remarkably small (well below 3% even for very similar structures). High throughput with respect to the extracted information and the NMR data acquisition with report generation within an average of 5 min per analysis can be accomplished on standard computer equipment.

A safe assessment of the consistency between structures with MW up to 500 and their good quality spectral data with no major spectral artefacts is given for cases an NMR-experienced user is able to completely assign manually as well. Depending on the complexity and ambiguity of the data high verification rates of up to 80% are achievable.

Supported solvents are DMSO, CDCl<sub>3</sub>, CH<sub>3</sub>OD, D<sub>2</sub>O and Acetone fully included in the iterative spectral analysis as separate spin-particles. Thus exclusions or “dark regions”, which could adversely affect the complete analysis, are avoided and also signals overlapping with solvent signals can be assigned.

A purity estimate is given by the percentage the solute, solvent and impurities contribute to the total spectral intensity.

The poster describes the overall process showing several examples.

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# 51 NMR Studies of AMG221 Salt Formation Using Flow and Solid-State NMR Techniques

L. Steven Hollis<sup>1</sup>, Liane Klingensmith<sup>2</sup> and Michael Bartberger<sup>3</sup>

1. Molecular Structure, Amgen Inc., 360 Binney Street, Cambridge, MA 02142
2. Chemical Process Research and Development, Amgen Inc., 360 Binney Street, Cambridge, MA 02142
3. Molecular Structure, Amgen Inc., One Amgen Center Drive, Mail Stop 29-1-B, Thousand Oaks, CA 91320

11 $\beta$ -Hydroxysteroid dehydrogenase type 1 (11 $\beta$ -HSD1) is known to be an important biological target for the treatment of diseases associated with the metabolic syndrome, including type 2 diabetes. The 11 $\beta$ -HSD1 enzyme catalyzes the conversion of cortisone to cortisol, which is involved in regulating the activation of glucocorticosteroid receptors in humans. Since glucocorticoids are known to regulate glucose and lipid homeostasis, it is believed that selective inhibition of 11 $\beta$ -HSD1 could play an important role in the treatment of metabolic syndrome. As part of our drug discovery efforts, we have developed a series of 11 $\beta$ -HSD1 inhibitors based on *exo*-norbornyl-2-amino-1,3-thiazol-4(5H)-ones, where the lead compound in this series, AMG221, has been chosen for advancement to clinical trials. Initial NMR studies of AMG221 showed that this compound exists as a complex mixture of rotomers and tautomers in solution and that the distribution of components was found to be solvent and pH dependent. A variety of NMR techniques, including VT NMR studies, <sup>1</sup>H-<sup>15</sup>N HSQC/HMBC and ROESY experiments, were employed to identify the exchange products in a variety of solvents. Structural information gathered from these studies also provided useful information on the mechanistic aspects of salt formation, which is important in the development process as the protonated form of AMG221 was found to be a key intermediate in scale-up. The results of our studies of the protonation process using flow NMR, single crystal x-ray diffraction and solid-state NMR techniques, will be presented.

## The Use of Human and Bacterial P450 Libraries for the Synthesis of Drug Metabolites

Martin A. Hayes<sup>1</sup>, Elizabeth M.J. Gillam<sup>3</sup>, James J. de Voss<sup>4</sup>, Dominic J.B. Hunter<sup>3</sup>, Wayne A. Johnston<sup>4</sup>, Gunnar Grönberg<sup>2</sup>, Tineke Papavoine<sup>2</sup> and Eva-Lotte Lindstedt<sup>2</sup>

1. Discovery DMPK, AstraZeneca R&D Mölndal, Sweden
2. Medicinal Chemistry, AstraZeneca R&D Mölndal, Sweden
3. School of Biomedical Sciences, The University of Queensland, Australia
4. School of Chemistry and Molecular Biosciences, The University of Queensland. Australia

The synthesis of mg scale quantities of metabolites remains a challenge particularly in the context of the tight timelines imposed in a drug discovery. The use of P450s for performing both regio- and stereoselective hydroxylation is well documented. Both the physchem properties and biological activity of hydroxylated leads are of interest in drug hunting projects to probe SAR, to increase solubility and to lower logD relative to the parent compound. The introduction of hydroxy group ‘handles’ may facilitate clearance by conjugating enzymes which may then mitigate clinical DDIs should the parent molecule be cleared primarily by a single hP450 isoform. Two approaches are highlighted which lead to the synthesis of metabolites of interest using a) a P450 screening library of BM3 mutants (Codexis) and b) using a library of human P450 3A mutants available to AZ through a collaboration with University of Queensland.

# 53 Controlling the Output of Automated NMR Systems

Anitha S. Gowda<sup>1</sup>, W. John Layton<sup>1</sup>, Anne-Frances Miller<sup>1</sup> and David J. Russell<sup>2</sup>

1. Department of Chemistry, University of Kentucky, Lexington, KY

2. Agilent Technologies

The default output formats supplied by the instrument vendors are not typically very useful when applied to large laboratory courses. Incorporation of NMR experiments in such applications requires flexible control of the data output. We are developing a suite of macros that permit a user to choose criteria for peak selection (such as signal-to-noise) that controls automated data output, resulting in reliable and useful reporting of the important spectral features without user intervention. The goal is for the instrument to automatically adjust parameters to produce usefully annotated spectra for each sample type, without the need for a human to modify detection thresholds or peak separations on a spectrum-to-spectrum basis. Thus, even end-users with little familiarity with NMR spectra can make use of the output. Our macros can be used in conjunction with Chempack 4 tools that allow the user to customize the plotted output

# **54 Efficient Quantitation and Characterization of Process Impurities and Extractables in Antibody-Containing Solutions, Using Surrogate Standards and $^1\text{H}$ NMR**

Ken Skidmore, Chris Cornell, Kathryn Robinson and Yung-Hsiang Kao

Genentech, Protein Analytical Chemistry Process R&D, South San Francisco, CA

A series of studies were carried out which demonstrate the general applicability of NMR spectroscopy to the process validation and characterization of the manufacturing process of protein-based drugs. Properly applied, this technique allows for the characterization and quantitation of excipients, leachables, and impurities in process pools, even in the presence of large amount of protein drug. Several conclusions were drawn from these studies:

- The use of a cpmg pulse sequence (with carefully selected acquisition parameters) allows for accurate quantitation of small molecules, leachables, and unknowns in the presence of protein drug substance.
- Standards of the molecules being quantitated are not needed in order to in order to detect and quantitate these molecules.
- Quantitation by NMR using a surrogate spike as a standard is reliable and accurate enough for process validation, even for higher molecular weight extracts whose identities and structures are not precisely known.
- Spectra of extracts from filters and resins are not required for assessing whether pools are free of leachables or unknowns.

Ron Crouch<sup>1</sup> and David Russell<sup>2</sup>

1. Agilent/Varian NMR, Cary, NC, USA
2. Agilent/Varian NMR, Walnut Creek, CA

Quantification using NMR Spectroscopy is a key technique in Analytical Chemistry. The level of precision afforded by qNMR can be impressive with the use of internal chemical reference standard. Recent advances in methodology allow fair precision with absolutely no reference sample (2 to 5%) with a one time calibration irrespective of the console receiver gain used for subsequent analyses over a time scale of many weeks. Such precision is sufficient to allow decisions to be made about parameterization of any experiment; however, this is not good enough to be called truly Quantified NMR. If a calibration is done on a known sample at the same receiver gain for subsequent analysis and that calibration used for a batch of samples run on a timescale appropriate for the temperature stability of the room, the level of accuracy can improve to the 0.3 to 0.5% level.

It is possible to automate the calibration step for any NMR sample so it is now possible to achieve true quantification on demand in a high-throughput NMR facility with no reference material or signal.

#### Acknowledgements.

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# 56 NMR-Based Quantification of Ligustilide in Dang Gui Botanicals

Tanja Gödecke, Jose G. Napolitano, Shao-Nong Chen, David C. Lankin,  
Norman R. Farnsworth and Guido F. Pauli

Department of Medicinal Chemistry & Pharmacognosy and UIC/NIH Botanical Center, College  
of Pharmacy, University of Illinois at Chicago, Chicago (IL)

*Angelica sinensis* (AS, “Dang Gui”, Apiaceae) is a widely used botanical for Women’s Health. Despite numerous in vitro/vivo and phytochemical studies, the active constituent(s) have not been identified conclusively to enable chemical standardization to bioactive markers [1,2]. Ongoing phytochemical analyses of AS extracts and fractions with activity in a panel of in vitro bioassays have repeatedly pointed to ligustilide as being (associated with) the active principle. Due to instability and related issues in LC-based analysis, there is a demand for new methods capable of quantitating ligustilide without relying on an identical primary reference standard for calibration [3,4].

In order to make the bioactive AS fraction amenable to NMR analysis, a method has been developed that employs primary RP-18 SPE fractionation (H<sub>2</sub>O-MeOH step gradient) of 75% EtOH extracts of *A. sinensis*. Quantification of ligustilide without its primary reference material was achieved by quantitative <sup>1</sup>H NMR (qHNMR, 600 MHz) [5]. The qHNMR method development involved comparing several approaches to quantification in qNMR and provides initial validation data for the measurement of ligustilide within AS preparations. The two-step SPE-qHNMR procedure might serve as an example for the targeted quantification of bioactive secondary metabolites in herbal preparations.

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# 57

## Monitoring Chemical Reactions in Real Time with NMR Spectroscopy

Mark Zell<sup>1</sup>, Brian Marquez<sup>1</sup>, Dave am Ende<sup>1</sup>, Pascal Dube<sup>1</sup>, Eric Gorman<sup>2</sup>, Robert Krull<sup>3</sup>, Don Piroli<sup>3</sup>, Kimberly Colson<sup>3</sup> and Michael Fey<sup>3</sup>

1. Pfizer Global Research and Development, 445 Eastern Point Road, Groton, CT 06340
2. Department of Pharmaceutical Chemistry, University of Kansas, 2085 Constant Drive, Lawrence, KS 66047
3. Bruker Biospin, 15 Fortune Drive, Billerica, MA 01821

Monitoring chemical reactions using a flow system coupled to a chemical reactor is becoming increasingly popular, as it utilizes the power of NMR spectroscopy to understand the complex interplay between molecules during a chemical reaction, making it particularly well suited for interrogating the presence of transient intermediates, providing detailed kinetic information for all peaks in the spectrum that can be resolved via multiple nuclei (<sup>1</sup>H, <sup>13</sup>C, <sup>19</sup>F, <sup>31</sup>P), as well as providing quantitative information for monitoring mass balance throughout the reaction. This poster will present highlights of our work in the area of directly monitoring chemical reactions by continuously flowing the contents of a chemical reactor through a newly developed NMR flow cell. A brief overview of the design as well quantitative kinetic results from chemical reaction steps in pharmaceutical process development will be presented.

Robert Evans<sup>1</sup>, Juan Aguilar<sup>1</sup>, Stefan Haiber<sup>2</sup>, Mathias Nilsson<sup>1</sup> and Gareth Morris<sup>1</sup>

1. School of Chemistry, University of Manchester, Manchester, UK

2. Givaudan, Dept. for Analytical Research, Naarden, Netherlands

Diffusion-ordered NMR spectroscopy (DOSY) allows the spectrum of a mixture to be resolved into individual components on the basis of their diffusion coefficients. Good results require well-resolved spectra; peak overlap in the frequency dimension, almost unavoidable in <sup>1</sup>H NMR, leads to artefacts such as peaks appearing in compromise positions in the diffusion dimension. Signal overlap and its attendant problems can be greatly reduced by simplifying the proton spectrum to give a homodecoupled or 'pure shift' spectrum.

Pure shift techniques based on homonuclear 2D J spectroscopy [1] have been long available but are all more or less unsatisfactory. The properties of the phase twist lineshape that is inherent to the technique [2] necessitate the use of severe weighting functions and absolute value display, so that 45° projection of absolute value 2D J spectra yields pure shift spectra with broad lines and distorted intensities. The introduction of the Zangger-Sterk pulse sequence element [3] has led to significant improvements. This combination of selective pulse and magnetic field gradient, simultaneously slice- and shift-selective, allows a subset of the spins to be treated as heteronuclei which can then be manipulated independently of the rest of the sample. A number of pure shift experiments [4, 5, 6] have been developed that show resolution of complicated multiplets and pure absorption mode pure shift spectra. The extension of such sequences to produce pure shift DOSY experiments will be demonstrated.

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## Dual Sample NMR Probe

James Norcross, Craig Milling, Anthony Audrieth, Robert Albrecht, Dean Olson  
and Timothy Peck

Protasis Corporation, MRM Division, Savoy, IL, USA

A new generation of microscale, CapNMR™ probe technology employs two independent detection elements to accommodate two samples simultaneously. Each detection element in the Dual Sample Probe (DSP) delivers the same spectral resolution and S/N as in a CapNMR probe configured to accommodate one sample at a time. A high degree of electrical isolation allows the DSP to be used in a variety of data acquisition modes. Both samples are shimmed simultaneously to achieve high spectral resolution for simultaneous data acquisition, or alternatively, a flowcell-specific shim set is readily called via spectrometer subroutines to enable acquisition from one sample while the other sample is being loaded. Protasis One-Minute™ NMR automation accommodates loading of two samples via dual injection ports on a CTC/Leap autosampler into two completely independent flowpaths leading to dedicated flowcells in the DSP probe.

J. Norcross, C. Milling, D. Olson, D. Xu, A. Audrieth, R. Albrecht, K. Ruan, J. Likos, C. Jones, and T. Peck, *J. Anal. Chem.*, in press.

# 60 Automated Quantification and Structure Verification in Capillary Flow NMR

Ke Ruan<sup>1</sup> and Wei Wang<sup>2</sup>

1. CRUK - Beatson Institute for Cancer Research, Switchback Rd., Glasgow, G61 1BD, UK
2. Pfizer, 10770 Science Center Drive, San Diego, CA 92121

Knowing the identity and concentration of a discovery compound that entering screening library with confidence is essential to make informed project decision. In this presentation, we will describe an easy-to-implement method, using residual solvent which in turn calibrated by external concentration standards, to determine unknown sample concentration in a fully automated fashion on a capillary flow NMR system.

The even higher throughput achieved with a novel dual sample probe<sup>1</sup> afforded a more robust structure verification using 1-D proton, sensitivity enhance HSQC and Hadamard TOCSY at a maximum capacity of 100 samples/day. The quantitative NMR is first employed to determine the integrals accurately, followed by the conventional combined verification via proton and HSQC. A Hadamard version of TOCSY is then used to cross validate the combined verification results to filter out the most undesirable false positive results. Such modularized protocol could be adapted in a variety of NMR systems.

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