Small Molecule NMR Research
(and a little Protein NMR)

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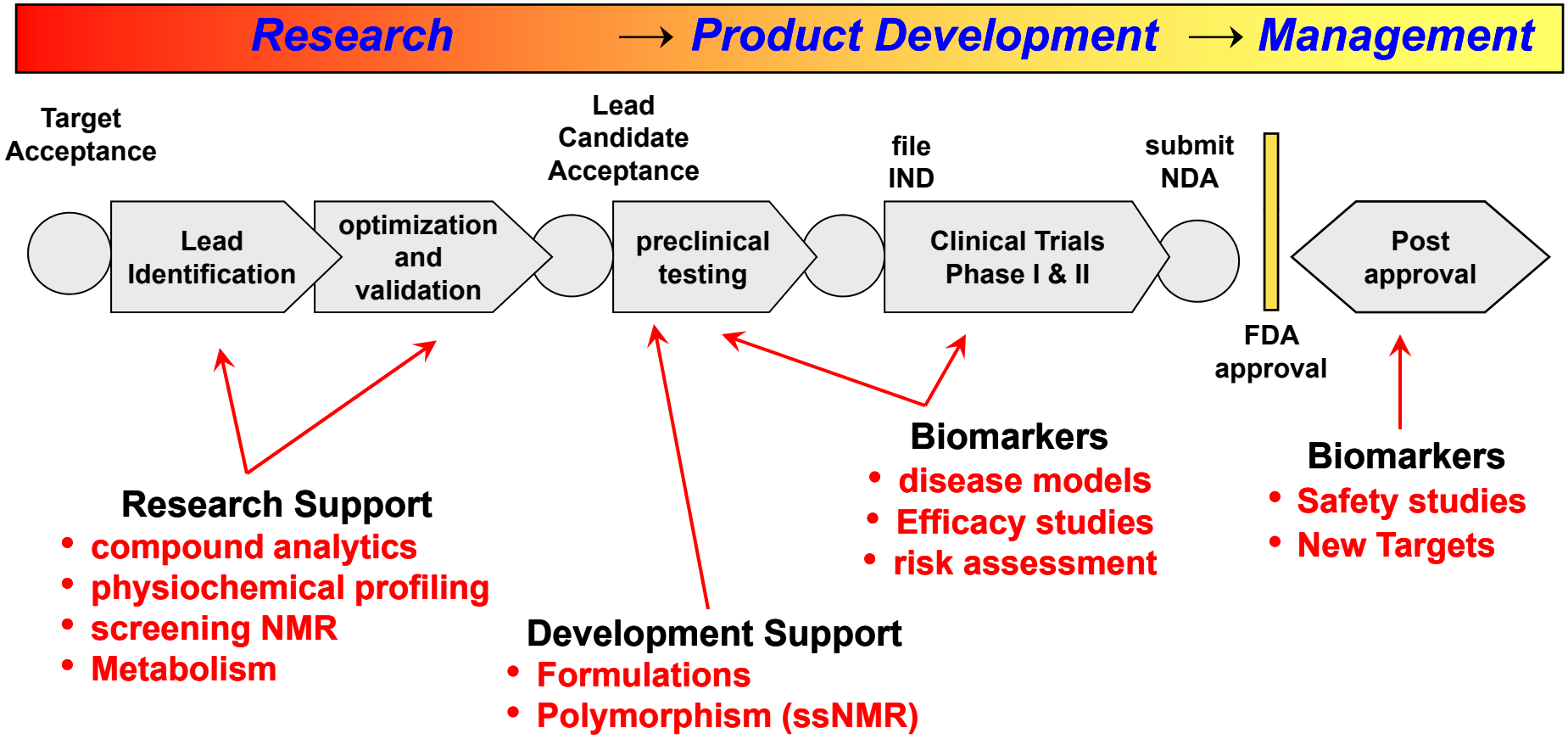
LDPCM

Bristol-Myers Squibb

Outline

- Utility of NMR throughout the Drug Discovery Process
- NMR basics (relevant to the chemist)
- Drug Metabolism Support
- Screening methods
- Metabonomics
- Quantitative NMR

NMR Opportunities*



*Generalized timeline

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NMR – Chemistry support

Provide NMR solutions to improve their chemistry

- Maintain open access/instruments
- Desktop data manipulations
- Structure elucidation
  - Confirm reaction products
  - Determine Stereochemistry
  - Identify unknowns

NMR – Biology Support

Biology

- Protein NMR
- Fragment screening

HTS

- NMR screening
- Confirm dosing plates

What else ?

Drug Metabolism & Pharmacokinetics (DMPK)

- metabolite ID
- excretion mass balance

Metabonomics/metabolomics

- urine, tissue analysis
- metabolic flux analysis

Structure Elucidation

Chemists have a general idea of the products

^{1}H, ^{13}C information is needed

^{1}H, TOCSY, NOESY, ^{13}C, HSQC, HMBC standard suite of experiments

Proton Experiments

COSY/TOCSY

- 2D experiments
- correlations show protons that are connected

give backbone information

NOESY/ROESY

- 2D experiments
- correlations show protons that are close in space

gives conformation information

Proton -Carbon Experiments

HSQC

- Inverse heteronuclear (^{1}H - ^{13}C) correlated expt
- Better sensitivity then HetCor (^{13}C detected)

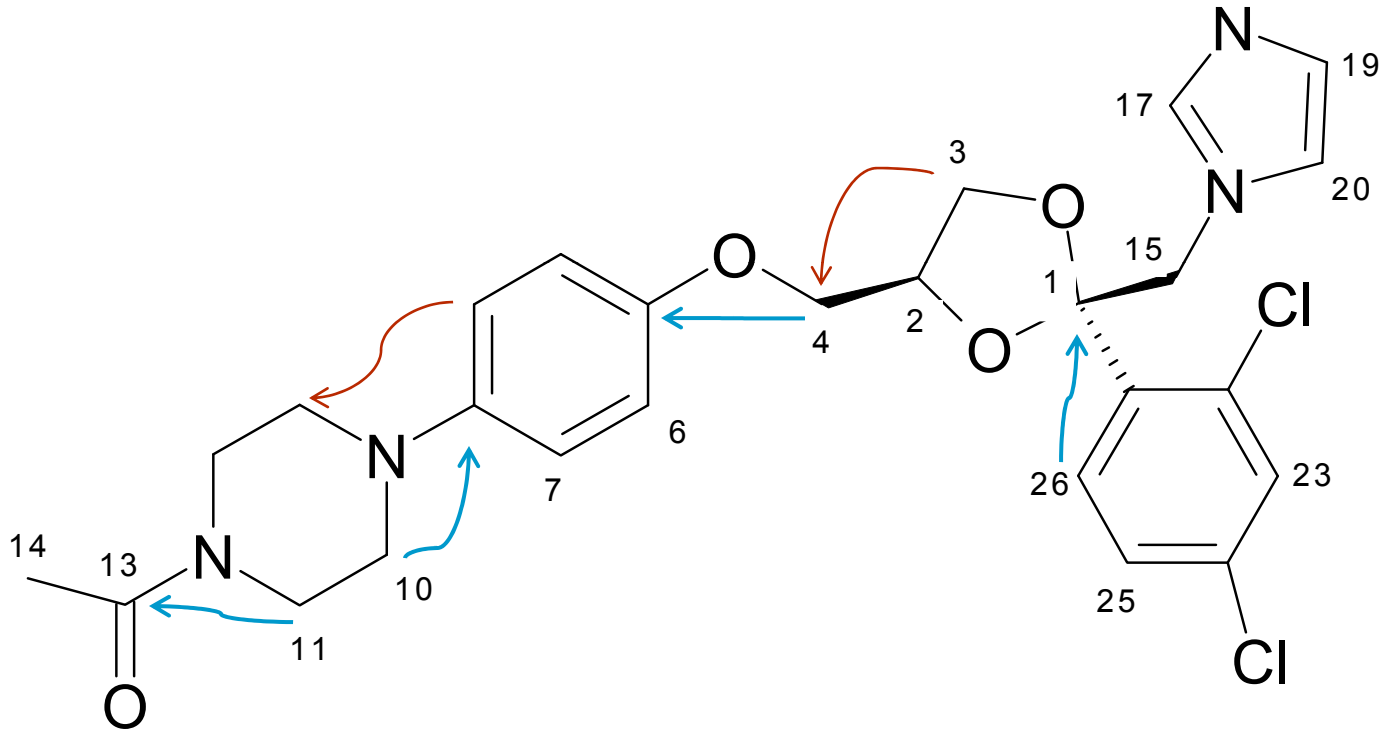
Allows proton-carbon assignments

HMBC

- Inverse Heteronuclear experiment (^{1}H-^{13}C)
- Long range $^{2\text{-}3}J_{CH}$ correlations
- Allows correlations through heteronuclei (N, O) or quat. Carbons

Connects groups together

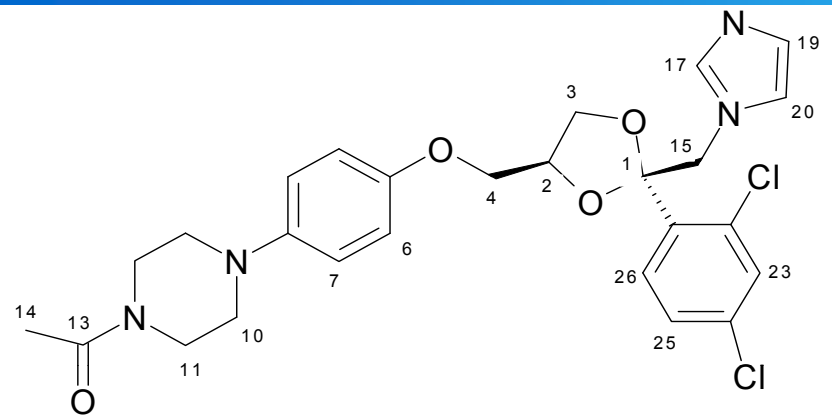
Ketoconazole – NMR correlations



HMBC correlations

NOE correlation

Ketoprofen



	^{1}H	^{13}C	
1	X	107.69	
2	4.33	74.53	
3	3.85	66.68	
4	3.65, 3.52*	67.72	
5	X	152.04	H4/C5, H7/C5
6	6.79	115.07	H6/H4
7	6.9	117.83	
8	X	145.5	H6/C8
9	X	X	
10	3.01, 2.94	49.61, 50.04	H7/H10
11	3.63, 3.55*	45.59	
12	X	X	
13	X	168.16	H11/C13, H14/C13
14	2.03	21.16	
15	4.54,4.5	50.52	H15/C17
16	X	X	
17	7.47	138.51	
18	X	X	
19	6.81	127.59	
20	7	121.07	H15/C20
21	X	135.91	
22	X	134.44*	
23	7.67	130.59	
24	X	132.35*	
25	7.44	127.25	
26	7.56	130.03	

Whitehouse *et. al, J. Pharm. Biomed. Anal., 12(11)pp 1425 -1441.*

Data generated yield

^{1}H & ^{13}C assignments and J Couplings both of which are needed for major publications

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Quality vs. Quantity

How much noise is acceptable?

- High quality ^{1}H data takes more time
  - the more time used per sample => lower throughput

How much sample?

- How much is the chemists willing to sacrifice?

How much solvent?

Metabolism

Drug Metabolism

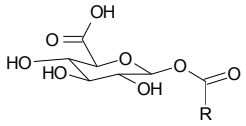
FDA would like to know the fate of a drug compound

Drug metabolism groups typically rely on LC-MS and radioisotopes

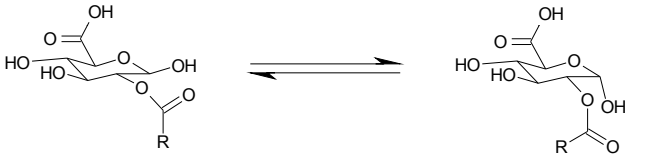
- **regioisomers can be problematic**

LCNMR is most advantageous within this area

Drug Metabolism

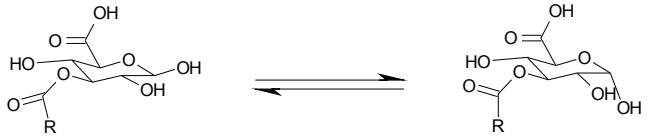


1-O-Acyl (β) glucuronide



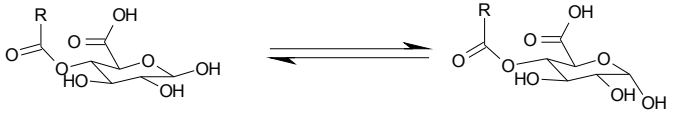
2-O-Acyl (β) glucuronide

2-O-Acyl (α) glucuronide



3-O-Acyl (β) glucuronide

3-O-Acyl (α) glucuronide



4-O-Acyl (β) glucuronide

4-O-Acyl (α) glucuronide

J. Nicholson et al. Anal.Chem (1995), 67, 4441-4445

Glucuronides can undergo acyl migration

Utilized LC-NMR for the identification of the glucuronides

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ADME Study

Excretion Mass Balance

- How long does the drug stay in the body?
- Where does it come out?
  - Urine, plasma, Feces
- What are the major metabolites?

Traditional methods

- Radioisotope (^{3}H or ^{14}C)

LCNMR can add valuable information

ADME Study

Compound contained fluorine

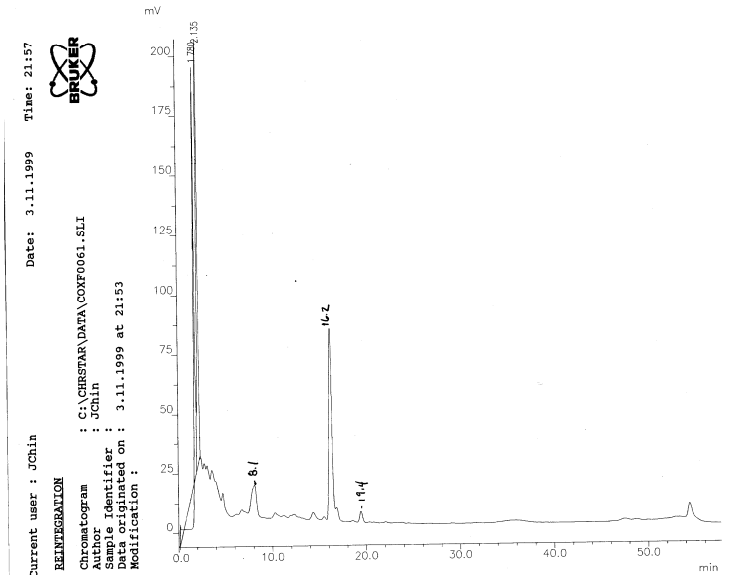
^{19}F NMR - Nearly as sensitive as ^{1}H

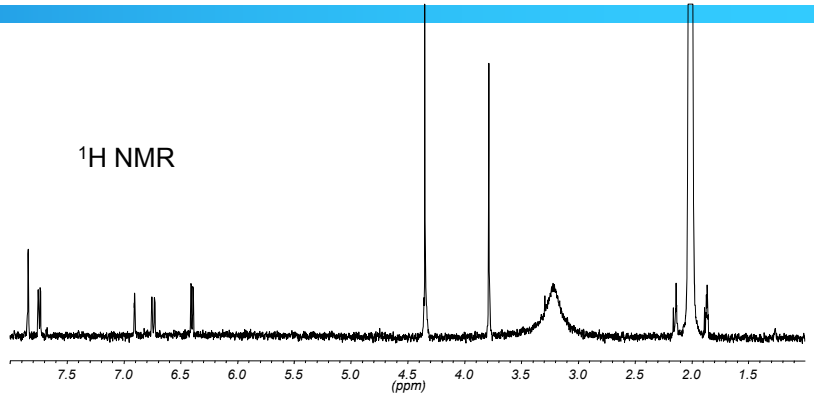
- No radiolabel – no extra synthesis required

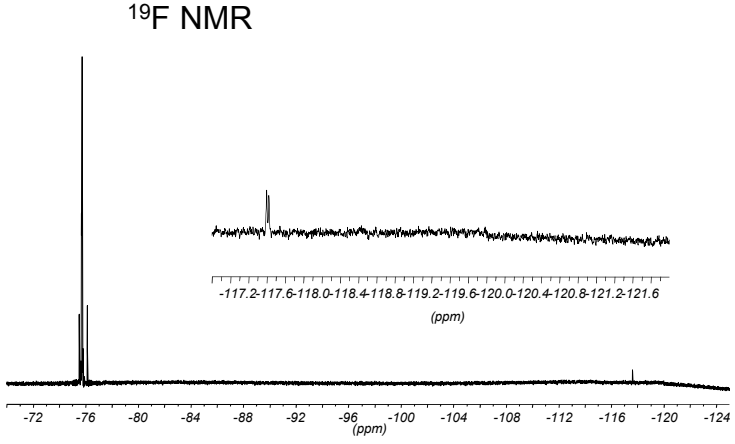
Utilize LCNMR

- Use HPLC UV & ^{19}F for estimation of metabolite elution
- ^{1}H for structural identification

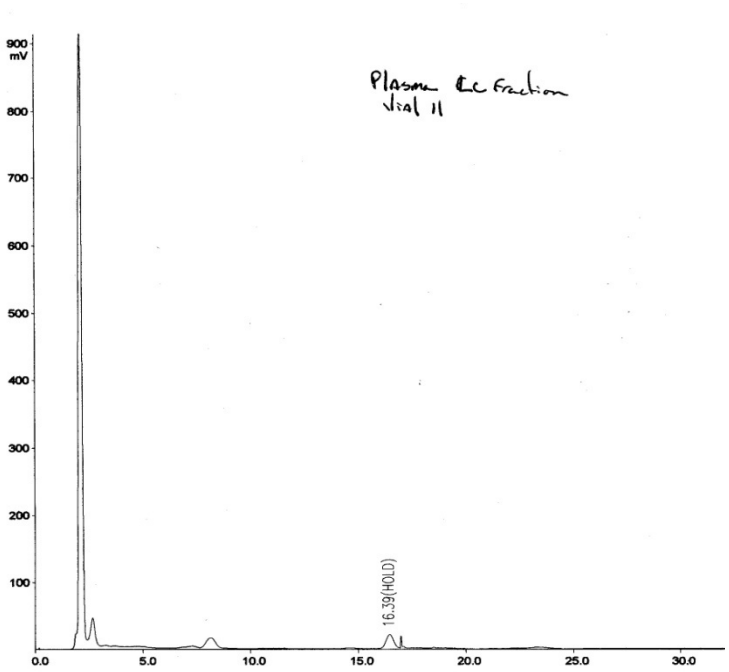
LCNMR - Stop Flow Urine

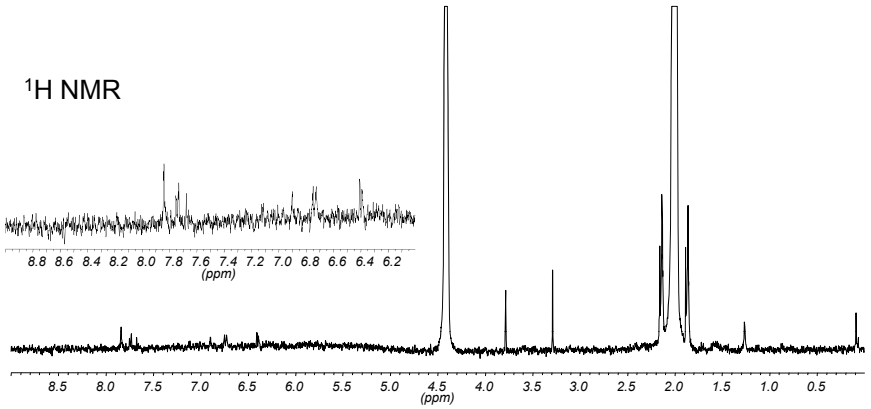


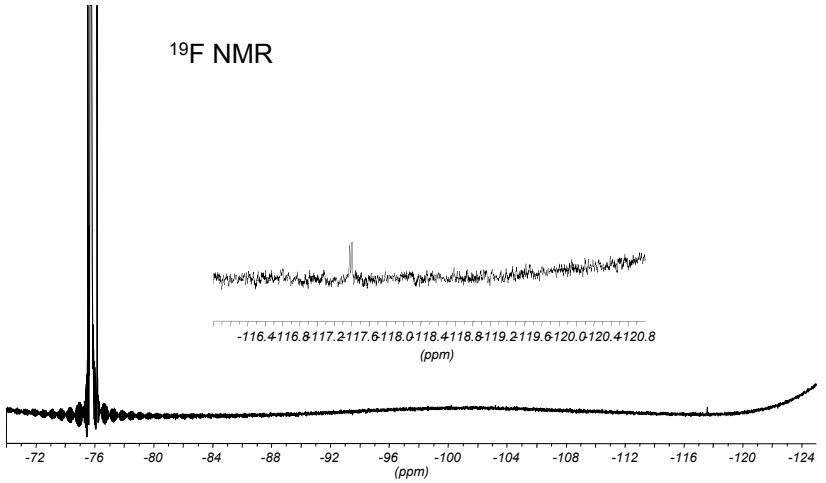




LCNMR - Stop Flow Plasma







^{1}H acquisition - 5hrs

^{19}F acquisition - 6hrs

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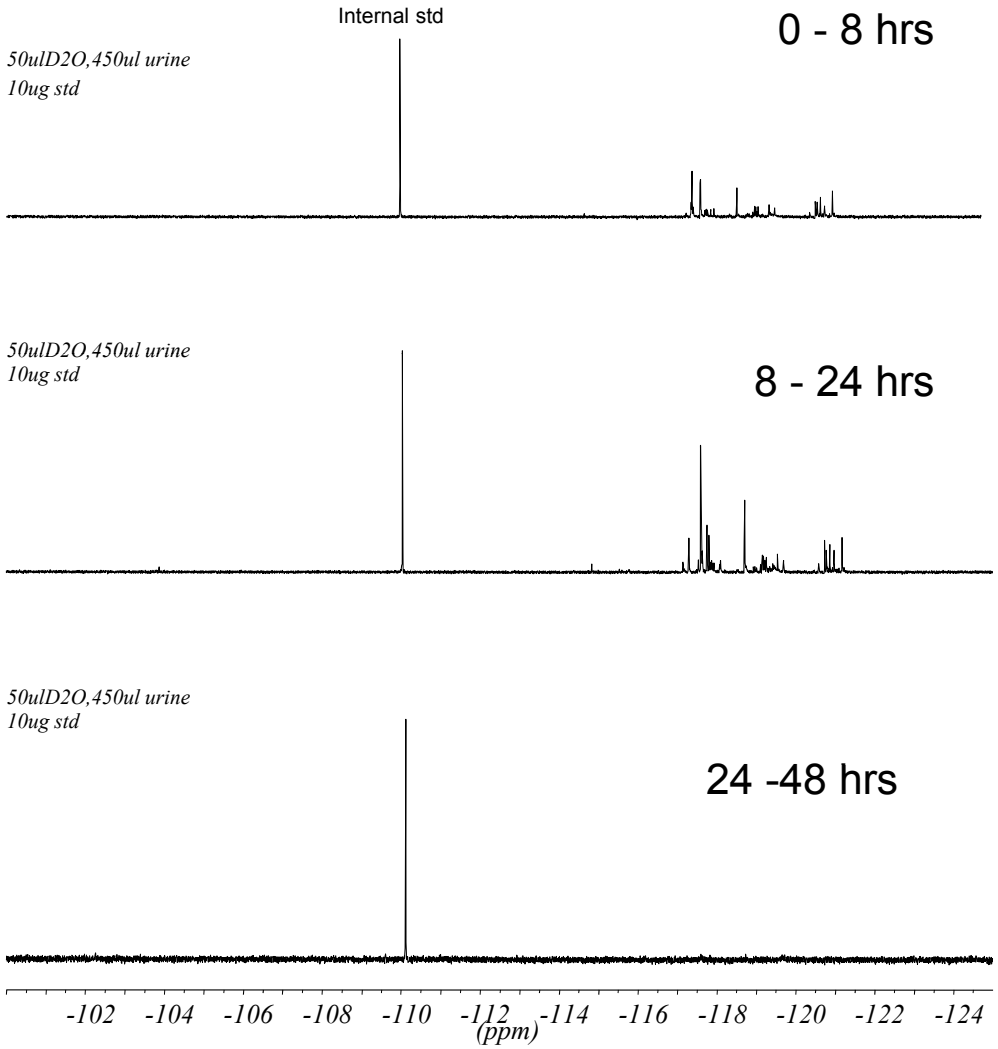
Urine - ^{19}F NMR

Urine samples

- **4 subjects - 4 time points/subject**

Observed the excretion of parent drug and its metabolites



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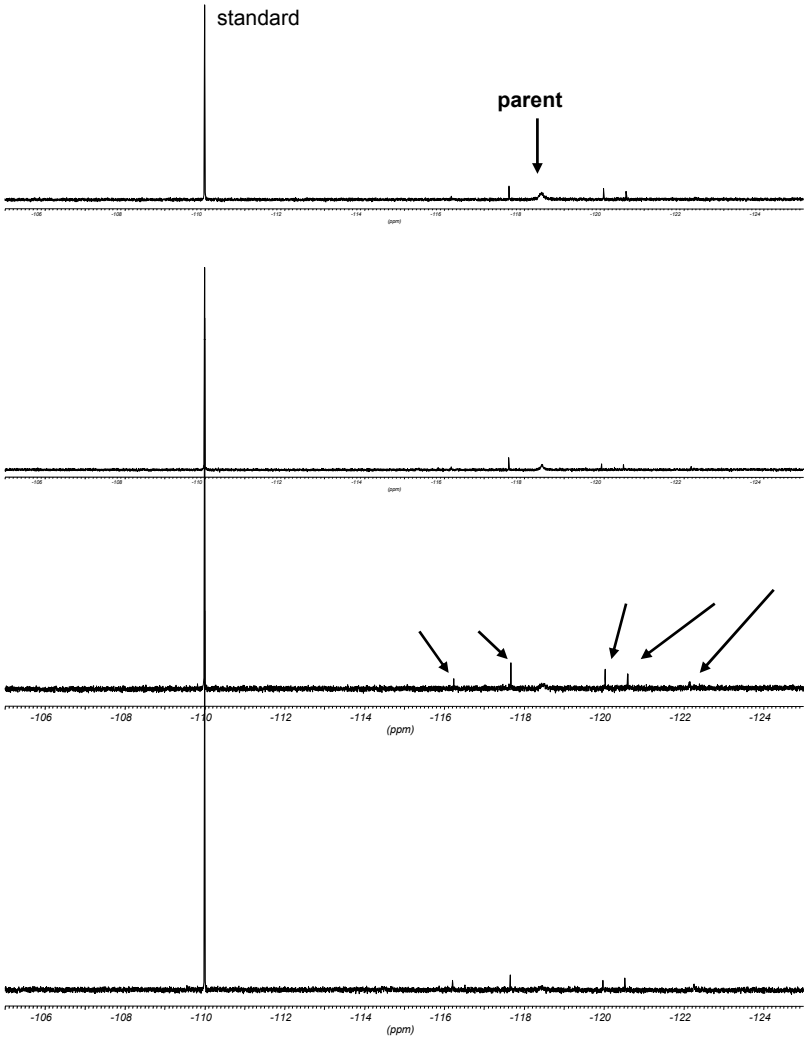
Plasma - ^{19}F NMR

Pooled plasma samples across 5 subjects at specific time points

Observed 5 metabolites and parent



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Feces - ^{19}F NMR

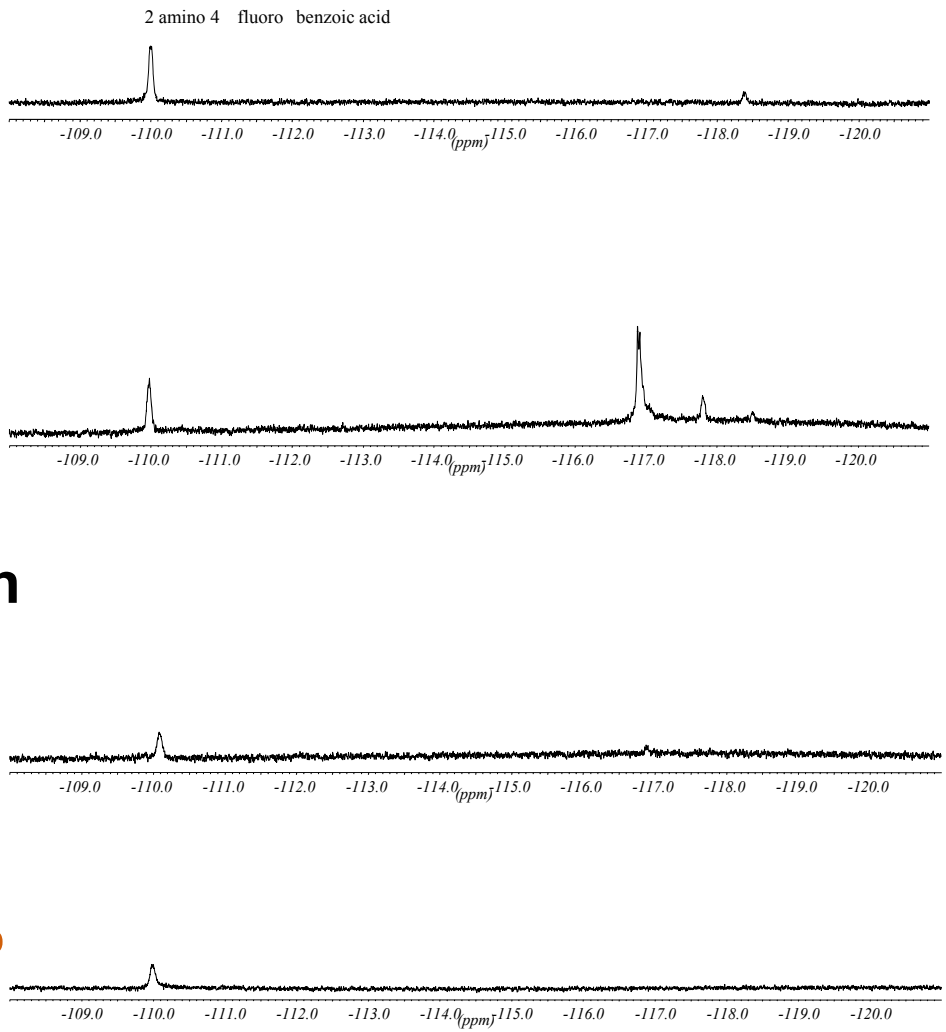
Feces sample

- 4 subjects - 4 time points/subject

^{19}F hr MAS NMR

- lyophilized samples in duplicate
- 19hr acquisitions

Quantitation results ~ 80% recovery



Metabolite Identification

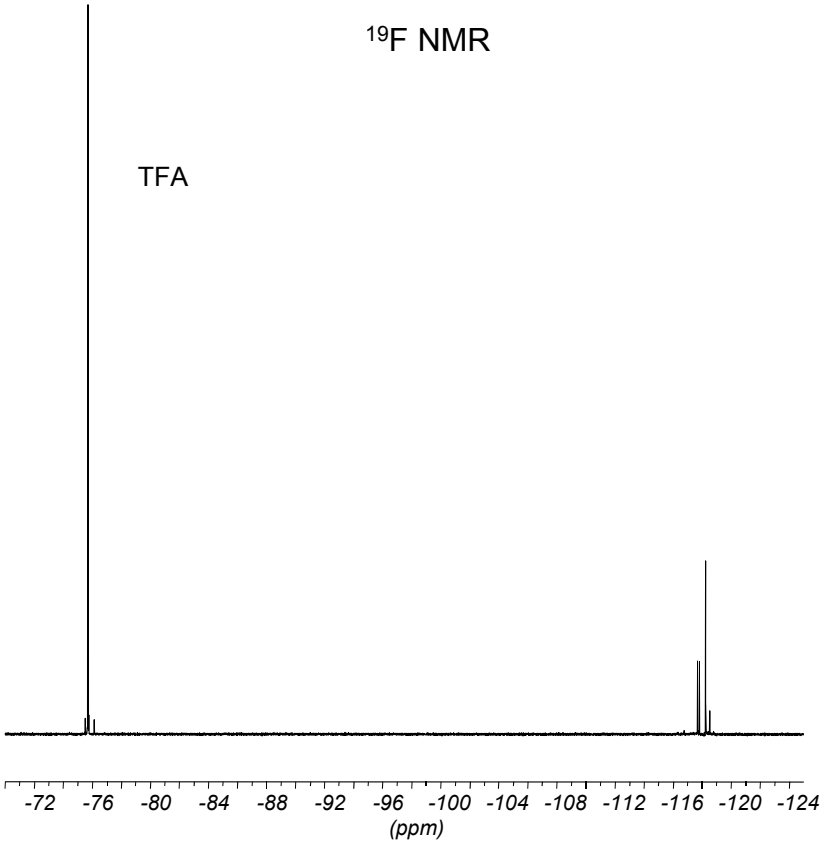
Concentrated urine was fractionated (DMPK)

Fractions still contained multiple ^{19}F resonances

LCNMR on the fractions

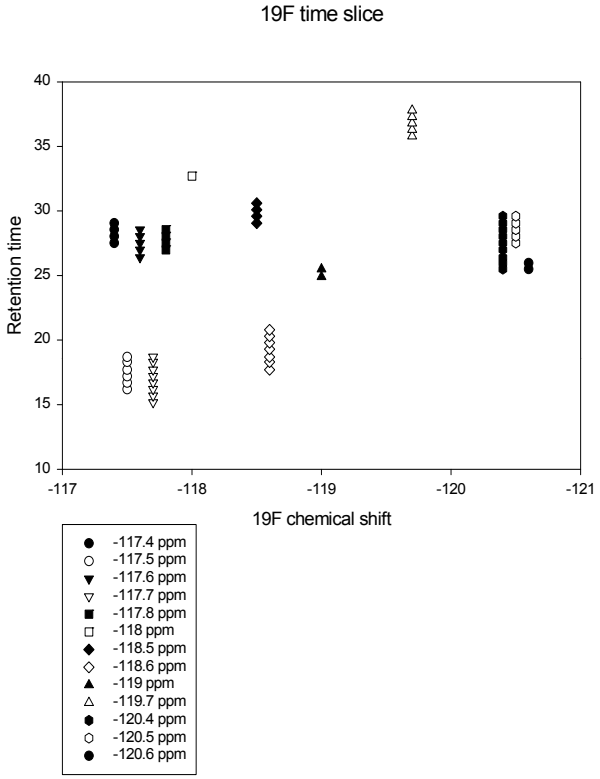
Stop Flow

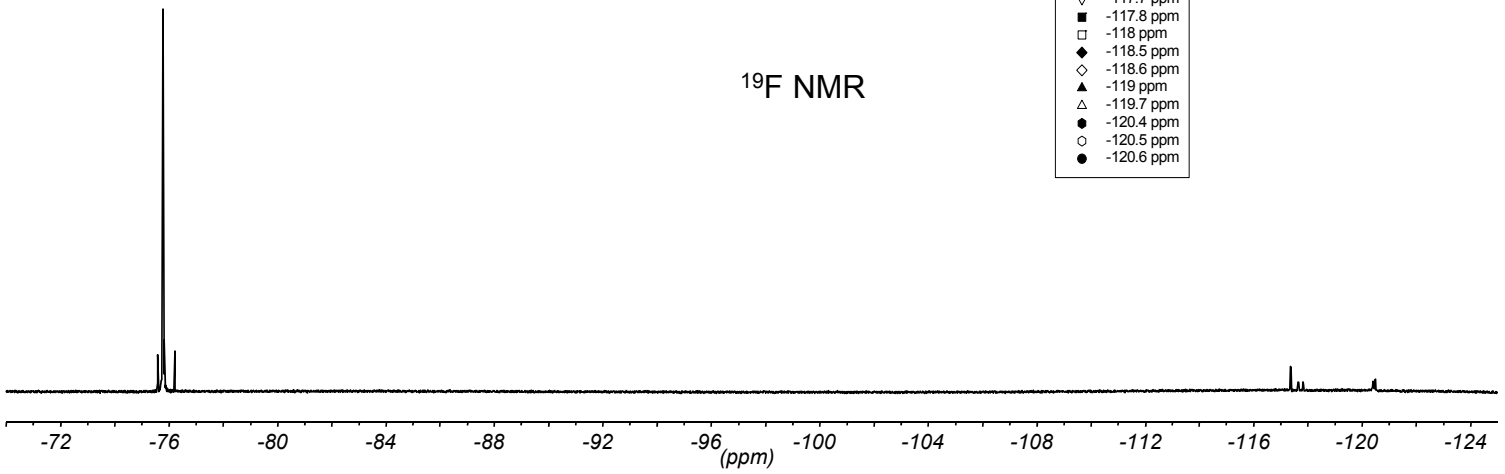
- **^{19}F NMR first**
- **^{1}H NMR for structures**



LCNMR - Time Slicing

- ^{19}F NMR acquisition
- Each slice 15 minute acquisition
- Approximate idea of where the metabolites are in separation





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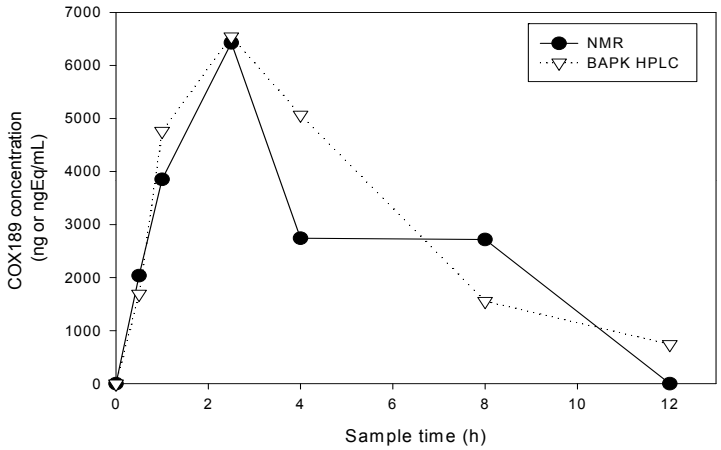
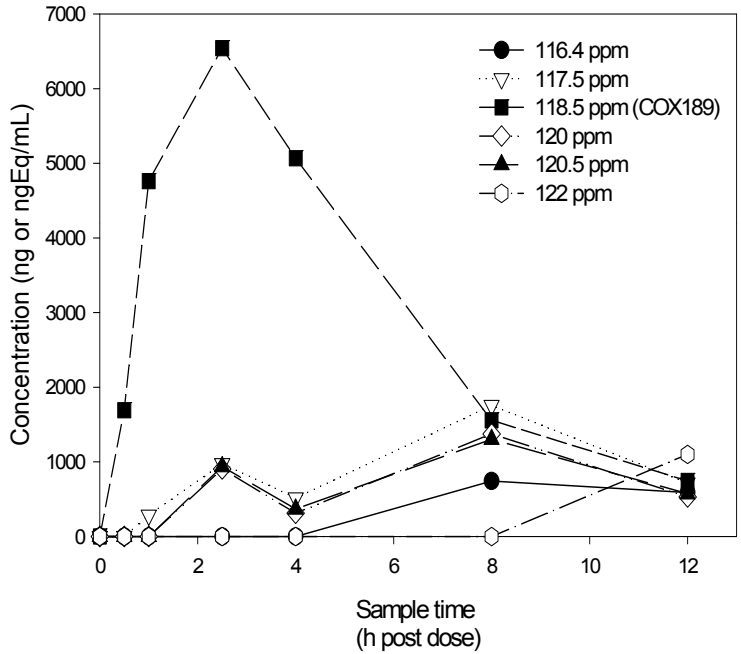
^{19}F NMR vs HPLC Plasma samples





Standard methodology for excretion mass balance is with a radioisotope and HPLC

19F -NMR is comparable

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Summary

Mass balance - Urine, feces, plasma recoveries approaching that observed by radiolabel

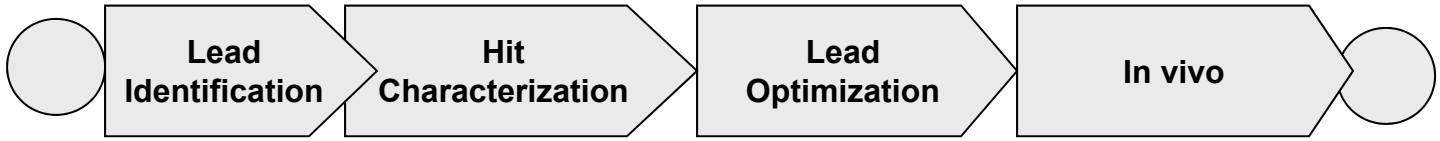
- + **Sample work-up minimized - lyophilization**
- – **Quantitation may takes longer (data acquisition)**

LCNMR was used to identify metabolites

- **Analyzed urine and plasma, used ^{19}F and ^{1}H NMR**

MAS NMR provides information with minimal sample manipulation

NMR Screening



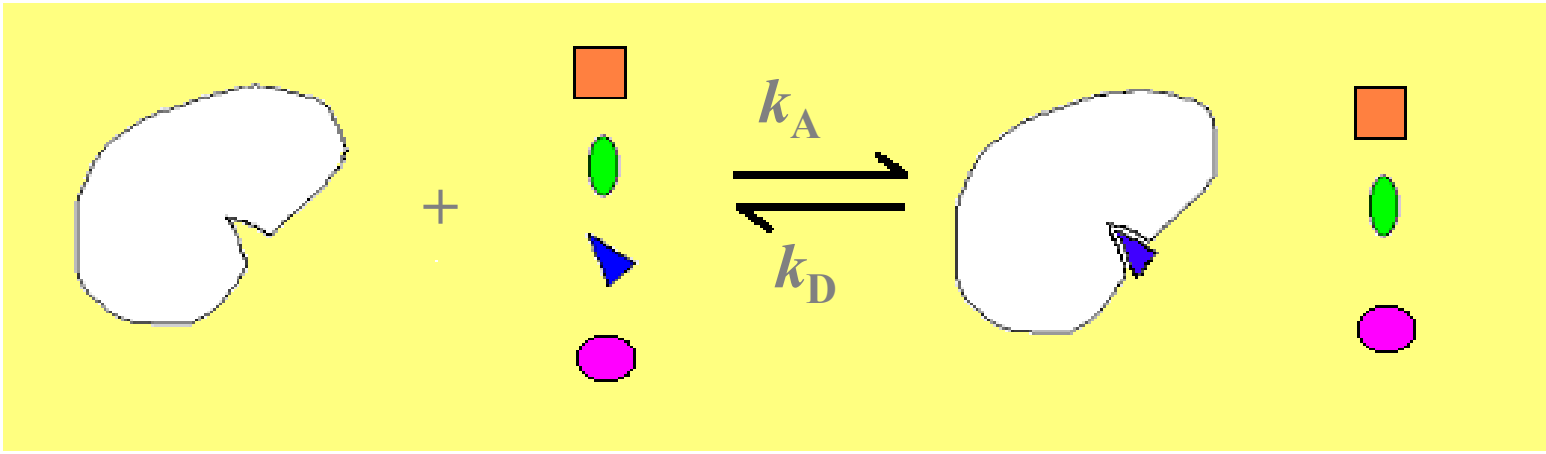
- Fragment Based DD
- Isolated target
- Cellular Assay

- SAR by NMR
- Fragment screening

Biomarkers

- disease models
- Efficacy studies
- risk assessment

Protein-Ligand Screening



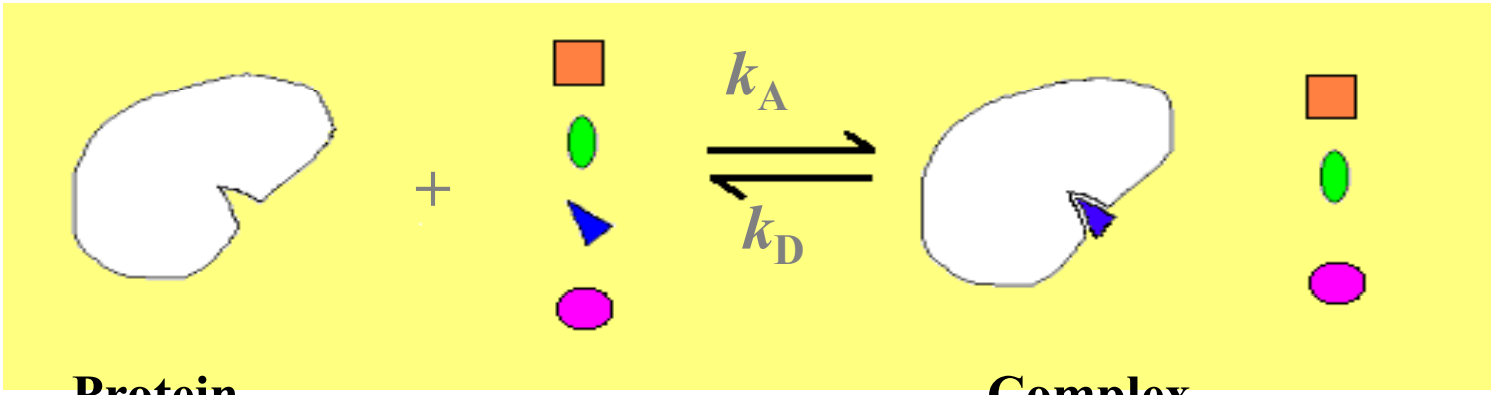
Looking for a small molecule (drug) to interact with a protein, enzyme….

Variety of ways to look for ligands

- **Fluorescence , Radiolabels, Competition assays**
- **High-throughput formats - 96, 384, 1536 plates**

Protein or ligand observe experiments

Protein Observe



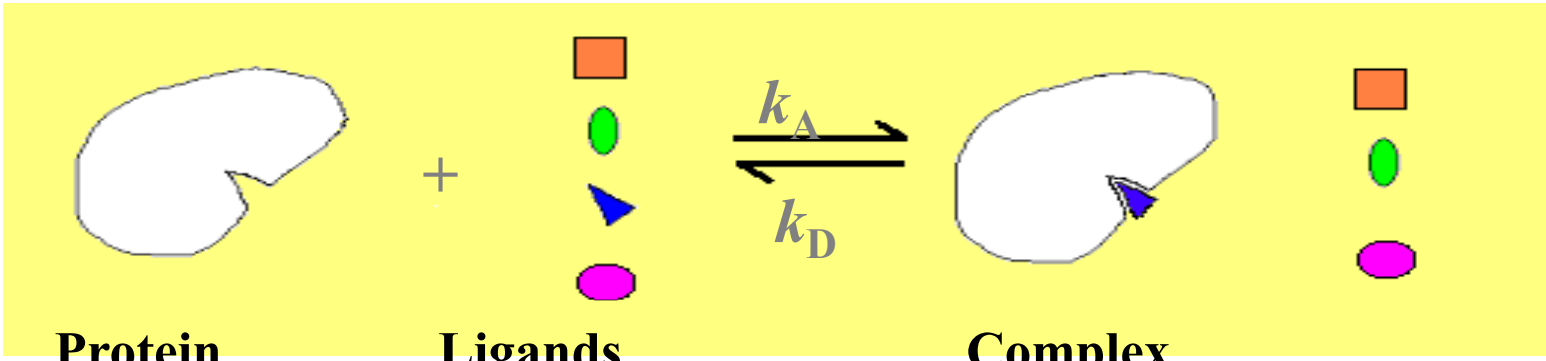
Protein
fast relaxation
slow diffusion
negative NOE

Complex
similar properties
to protein

Observe the binding site of the protein

Need knowledge of the binding site

Ligand Observe



Protein	**Ligands**	**Complex**
fast relaxation	slow relaxation	similar properties
slow diffusion	fast diffusion	to protein
negative NOE	positive NOE	

Observation of ligands compared to protein

NMR properties will change for ligands that bind to the protein

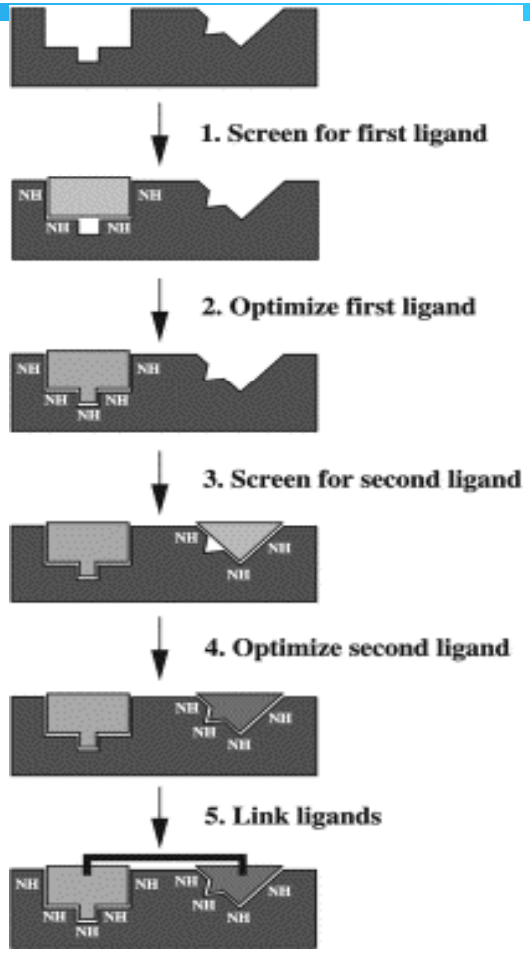
Ligand Fishing by NMR Spectroscopy

- **Protein Signal Detection**
  - chemical shift changes in 2D spectra
  - isotope labels
  - size limitations
  - structural assignments
- **Ligand Signal Detection**
  - simple 1D experiments
  - unlabeled protein
  - no size limit
  - smaller amount s of protein
  - no deconvolution required

SAR by NMR

Lead generation or optimization

A larger, better binding compound can be built from smaller compounds with lower affinity



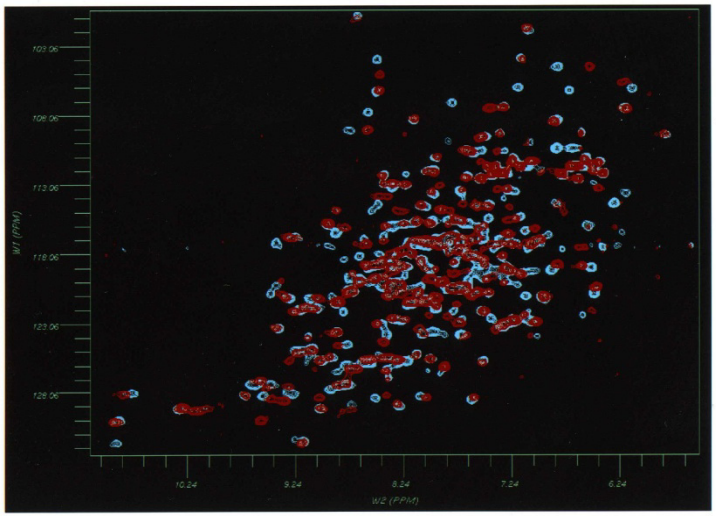
Shuker, SB;Hadjuk, PJ; Meadows, RP; Fesik, SW, *Science*, **274**, 1996, 1531

SAR by NMR

Protein observe expts

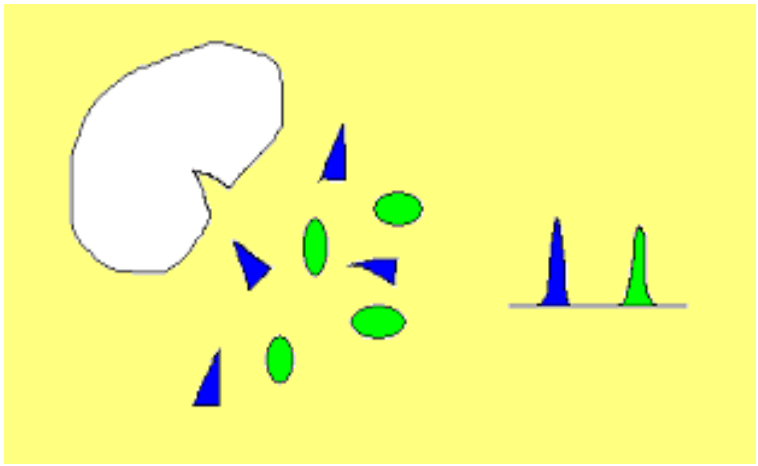
Chemical shifts are influenced by the environment

^{15}N chemical shifts of the binding site should change when there is a substrate

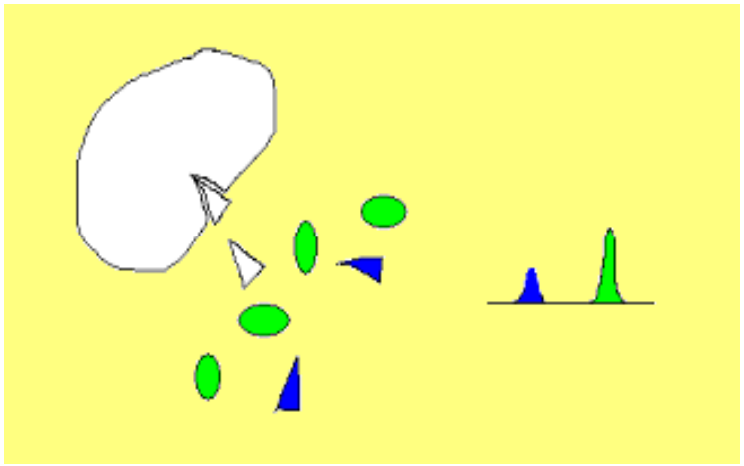


Shuker SB, Hadjuk PJ, Meadows RP, Fesik SW, *Science*, 1996, **274**, 1531-1534

Relaxation Based Experiments



No Binding

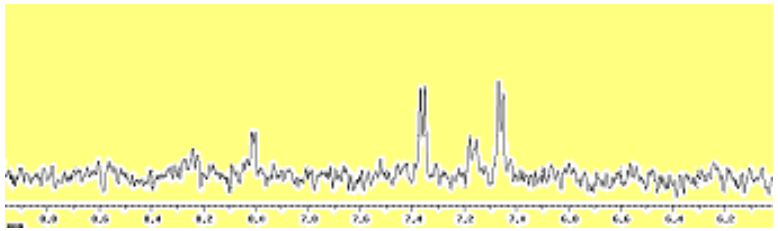


Blue Compound is Binding

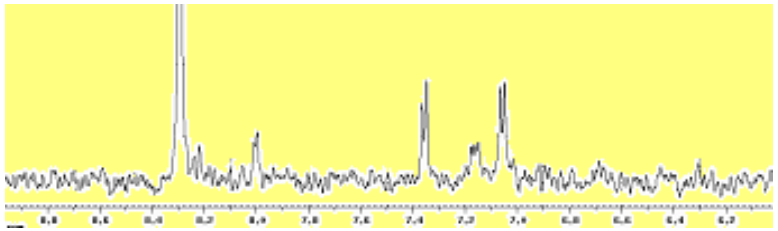
Ligand observe experiments

Relaxation Based Experiment

No Binding

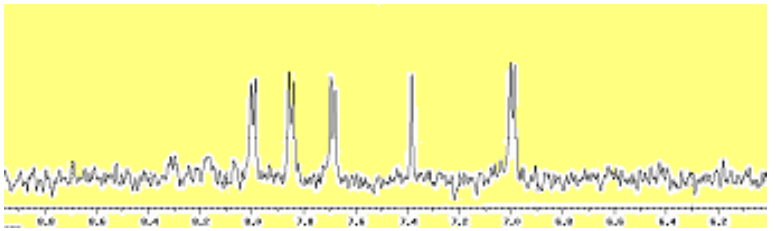


Control Spectrum

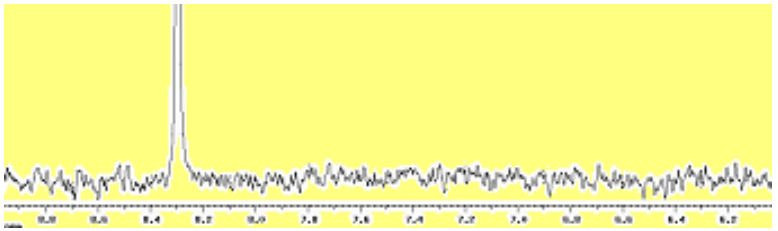


Relaxation Based Spectrum

Binding

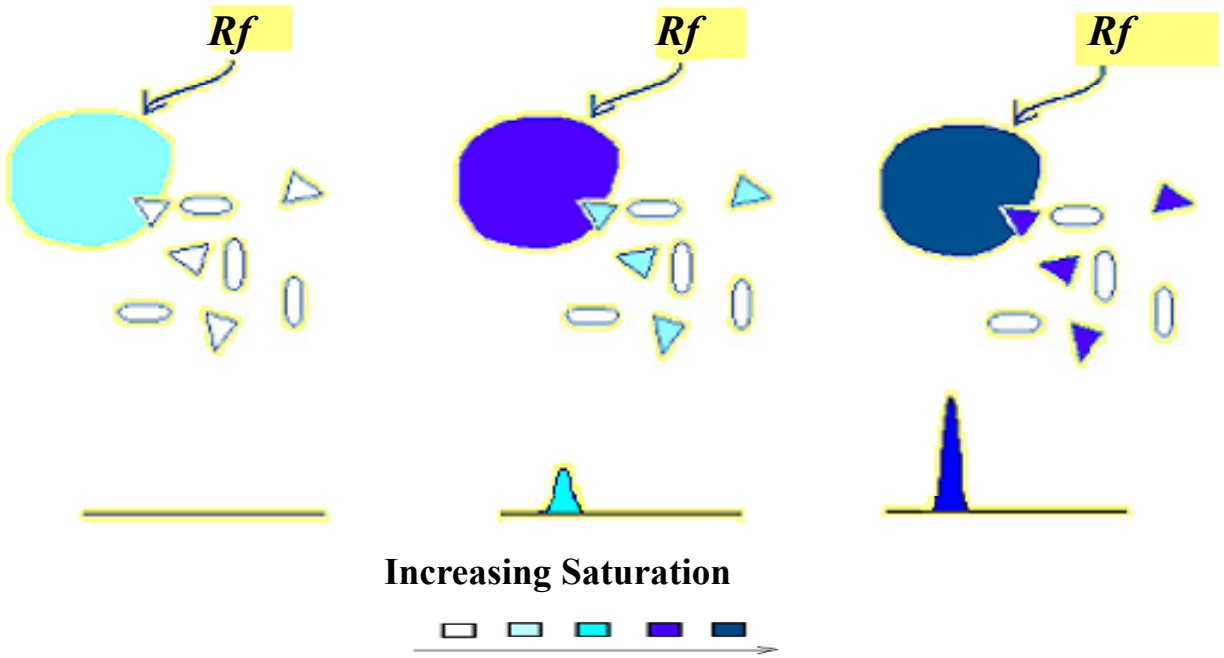


Control Spectrum



Relaxation Based Spectrum

Magnetization Transfer Experiment



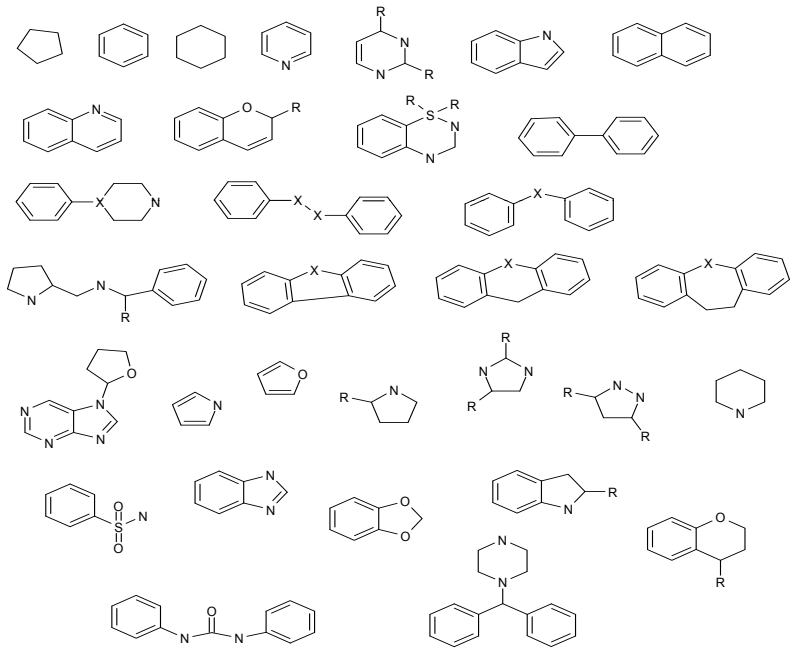
Ligand observe experiments

SHAPES

Library Design

Ligand Observe experiments

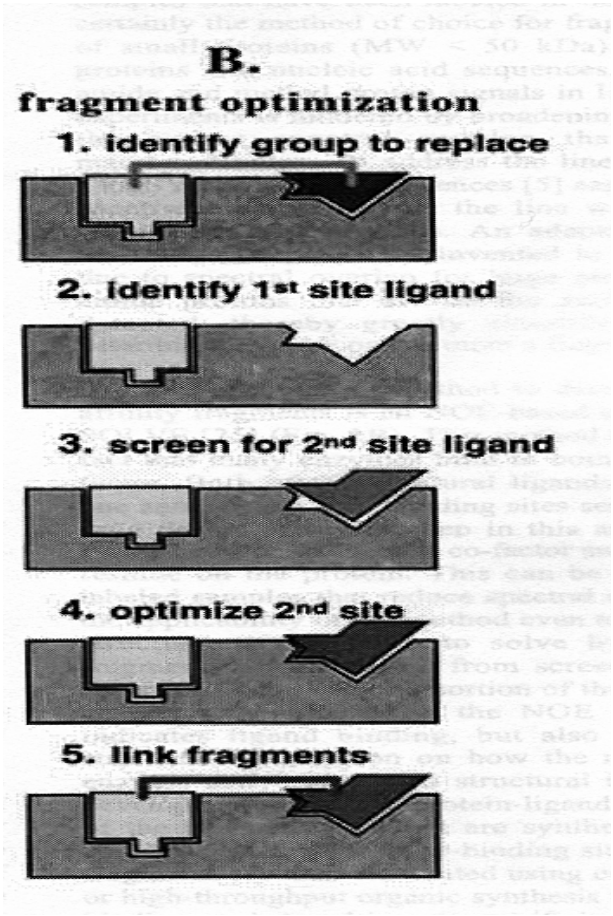
Use these core groups to screen and find a starting block



J. Fejzo,C.A Lepre, J.W. Peng, G.W. Bemis, Ajay, M.A. Murcko, J.M. Moore, Chem. Biol, 6 (1999), 755.

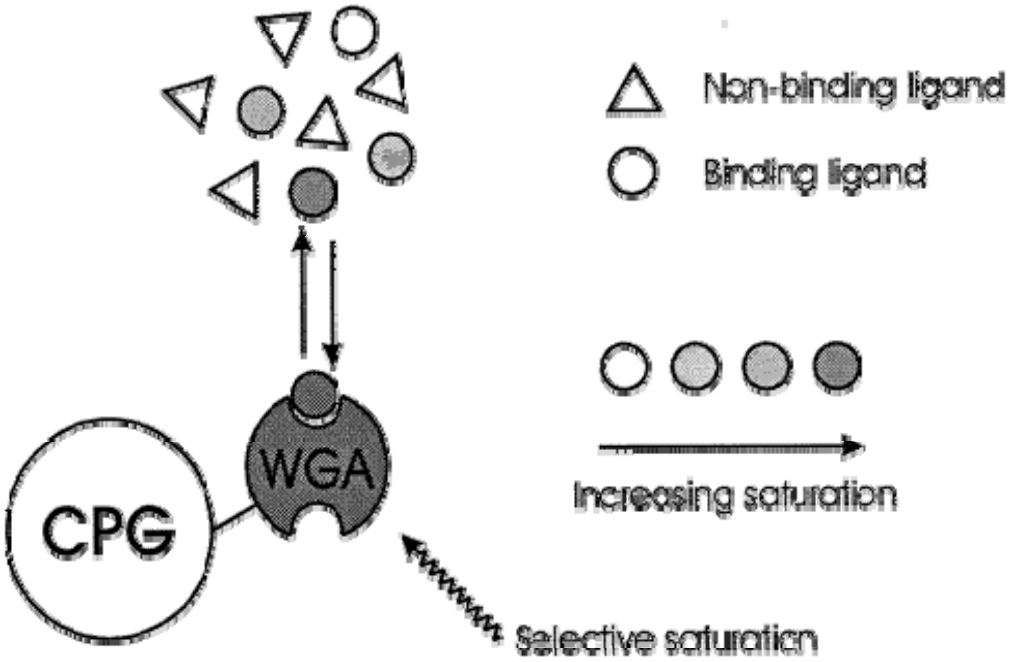
Fragment based NMR

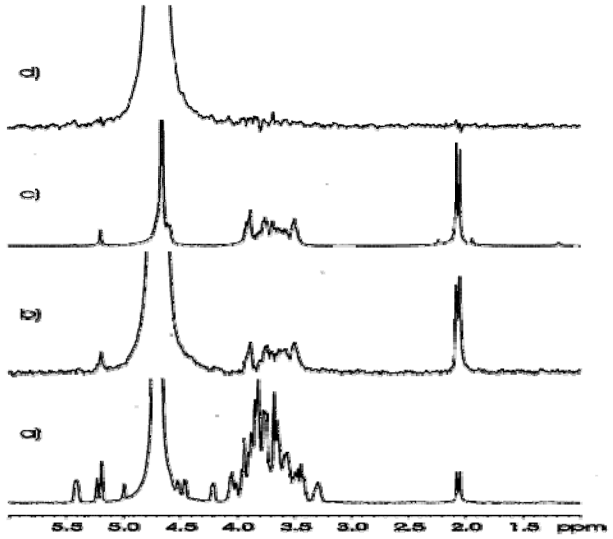
- Extension of SAR by NMR
- Lead Optimization
- Start with the lead compound
- Cleave off the offending group(s)
- Rescreen by SAR by NMR for better ligands



Huth JR; Sun C,
Comb.Chem. & High Throughput Screening, **5**, 2002, 631-643

STD hr MAS





Klein J, Meinecke R, Mayer M, Meyer B,
J. Am. Chem. Soc.,, **121**, 1999, 5336-5337

Other NMR Screening methods

Diffusion NMR

- relaxation based experiments

WaterLogsy

- magnetization based experiments

Spin label Enhanced NMR screening

HSA binding

Focussed Libraries

Virtual screening

Solubility of compounds

Tox. properties

Functional Libraries

Functional Libraries

Genomics era - providing new targets where their functions may not be known

Libraries can be built with compounds that have known targets

Metabonomics

The body is a machine constantly working to maintain status quo

Perturbations within the body, forces the system to try and re-equilibrate

The body will consume energy and produce byproducts to carry out this re-equilibration

Biofluids will contain these byproducts

- urine, plasma, feces

Metabonomics

NMR is a universal analyzer

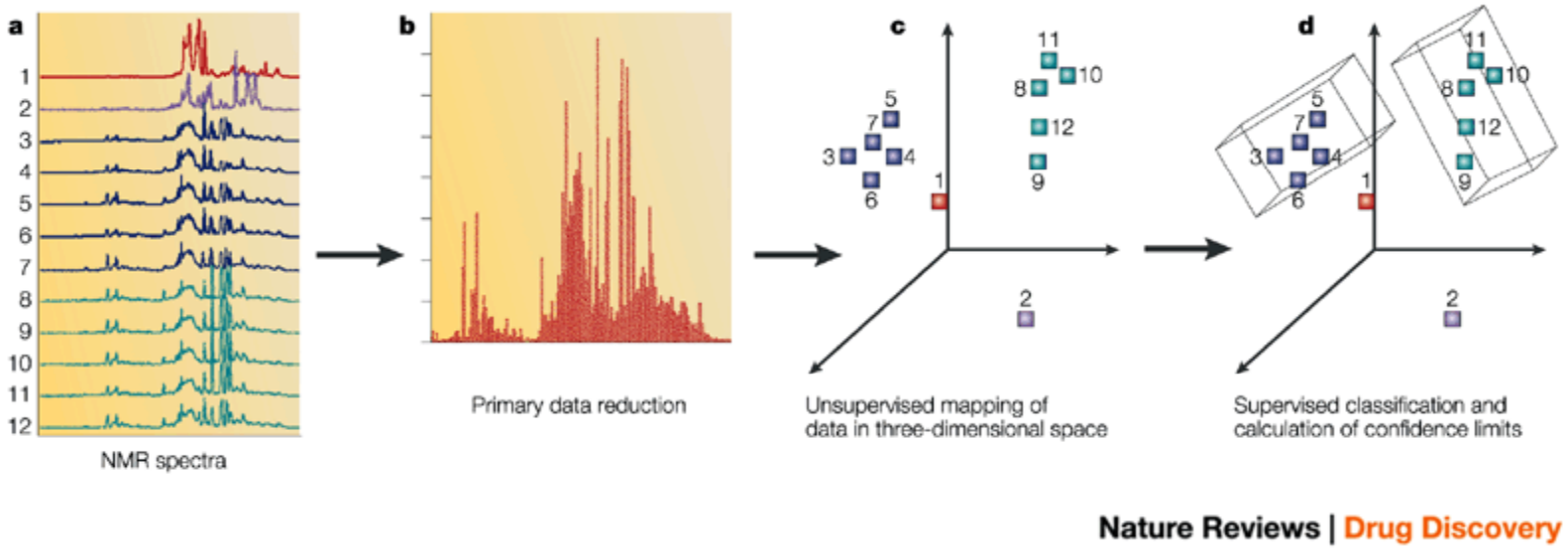
- do not need to know the byproduct first

NMR of biofluids

- Solution NMR : urine, plasma
- MAS NMR : feces

Results - indicate what is happening

Metabonomics



Nicholson JK, Wilson ID; Nat Rev Drug Discov. 2003 Aug 2(8): 668-676

Lindon JC. Holmes E, Nicholson JK; FEBS J 2007 Mar, 27 4(5): 1140-1150

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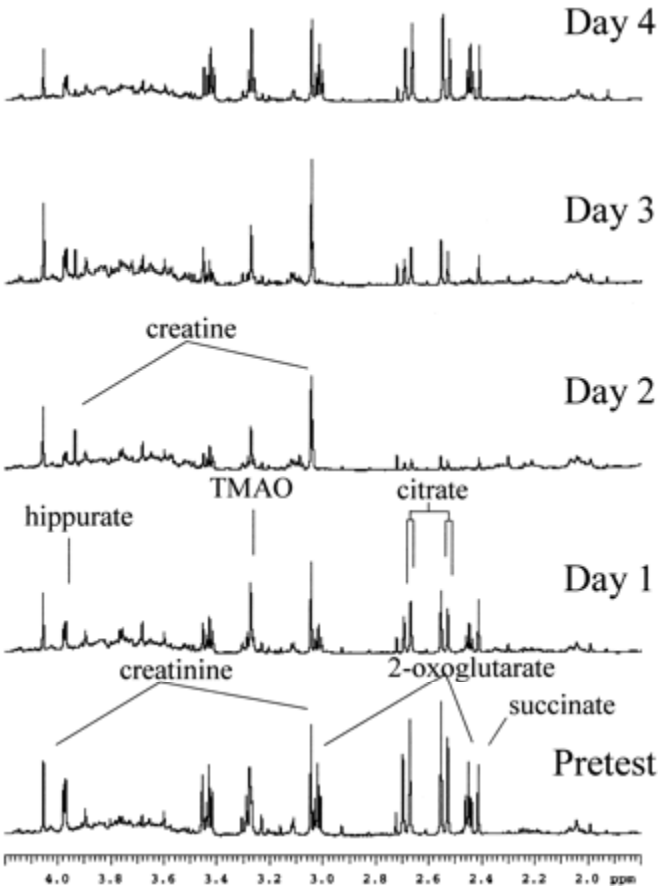
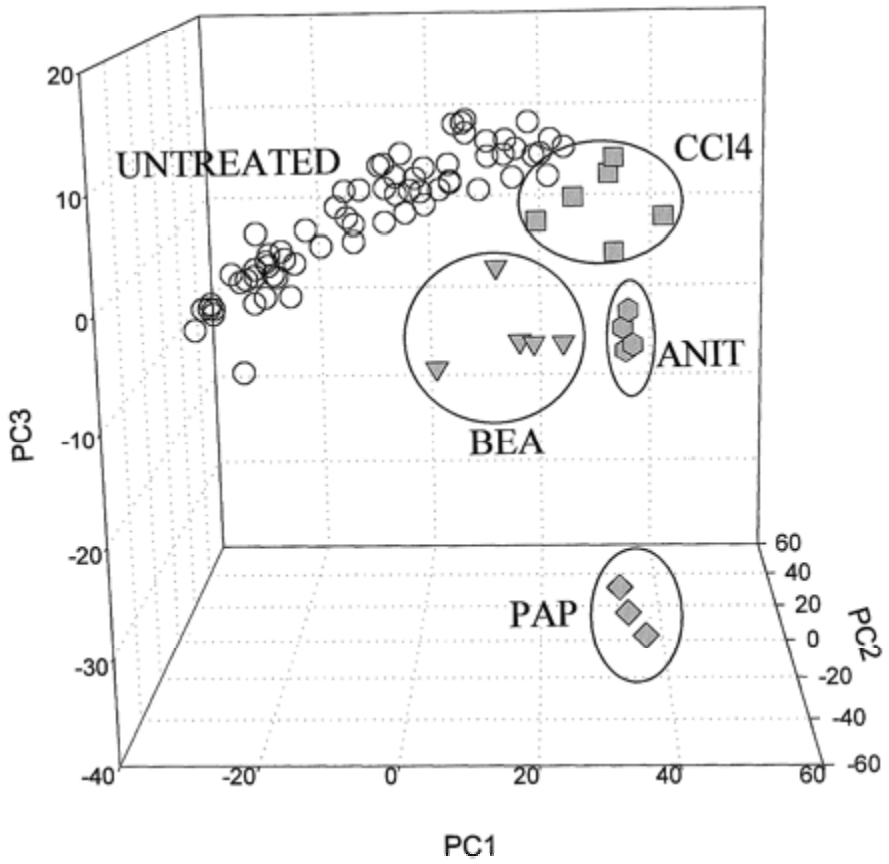
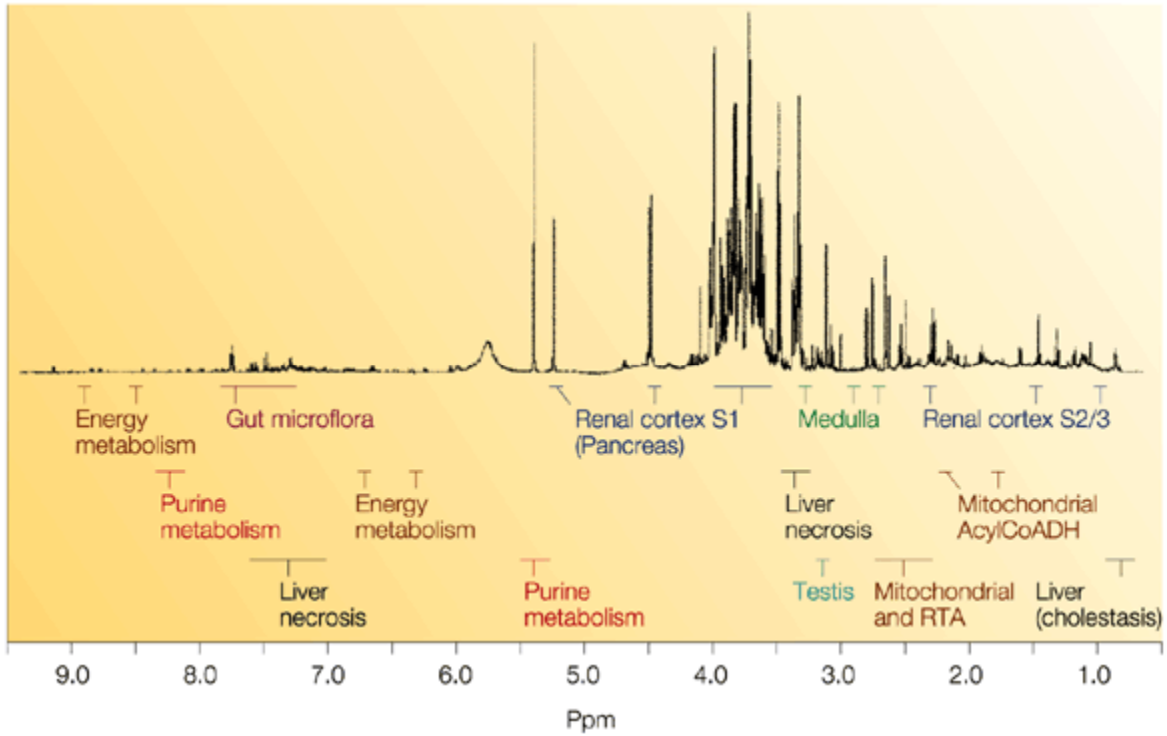
Metabonomics





Metabonomics



Nature Reviews | Drug Discovery

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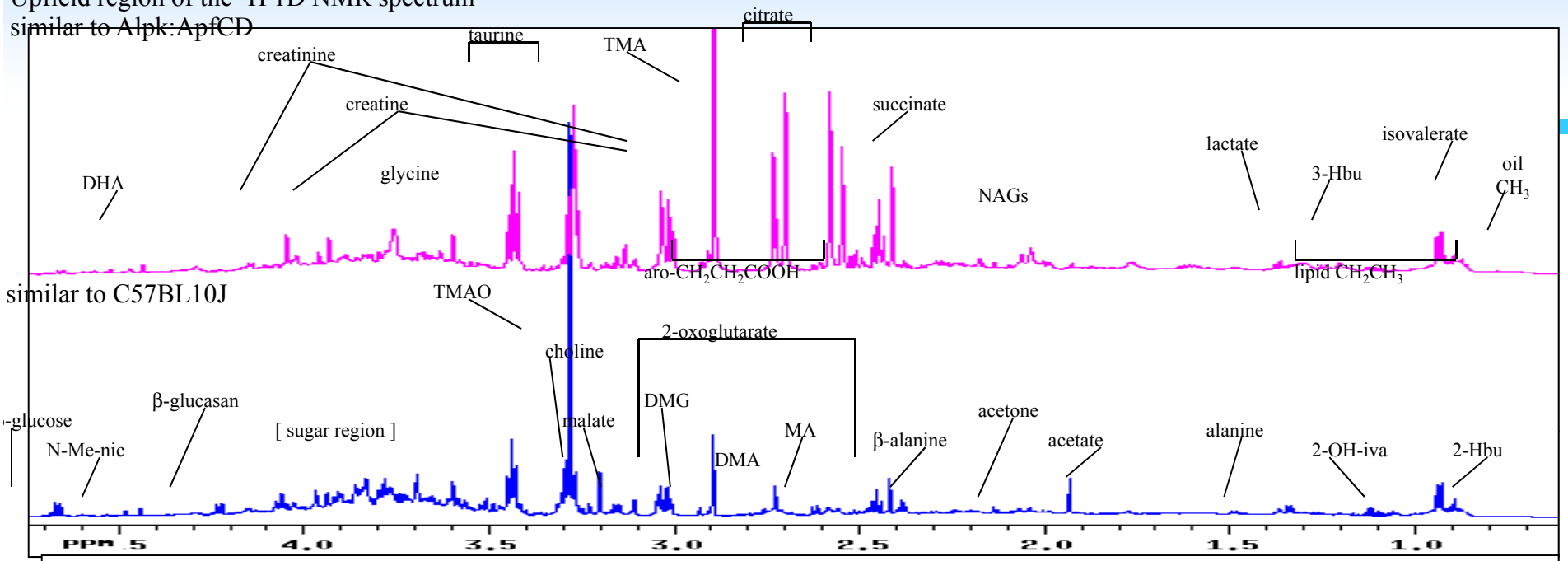
Metabonomics

- Toxic insults to rodents can be observed by NMR analysis of their urine
- Extended to tissue analysis
- Experiments are reproducible
- QC of animals before dosing
- Efficacy assay?

In-House controls: PPARα lean vs NCR nude {*see*: FEBS Lett 484: 169-174}

Upfield region of the ^{1}H 1D NMR spectrum

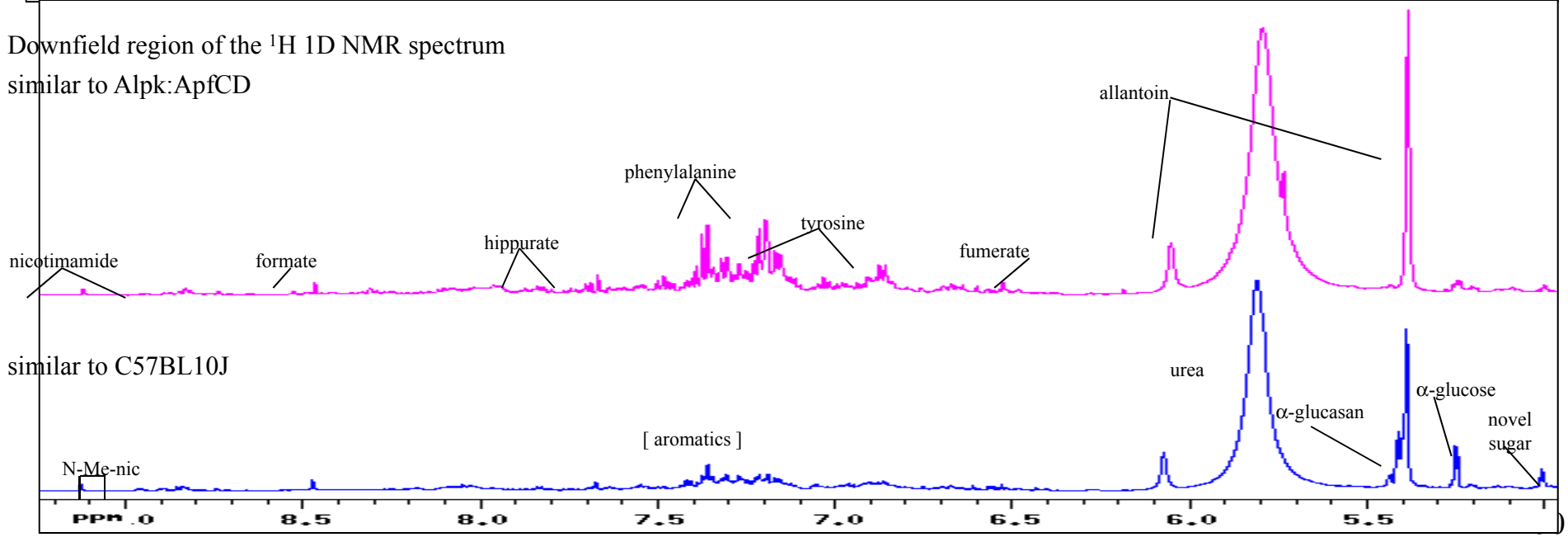
similar to Alpk:ApfCD



similar to C57BL10J

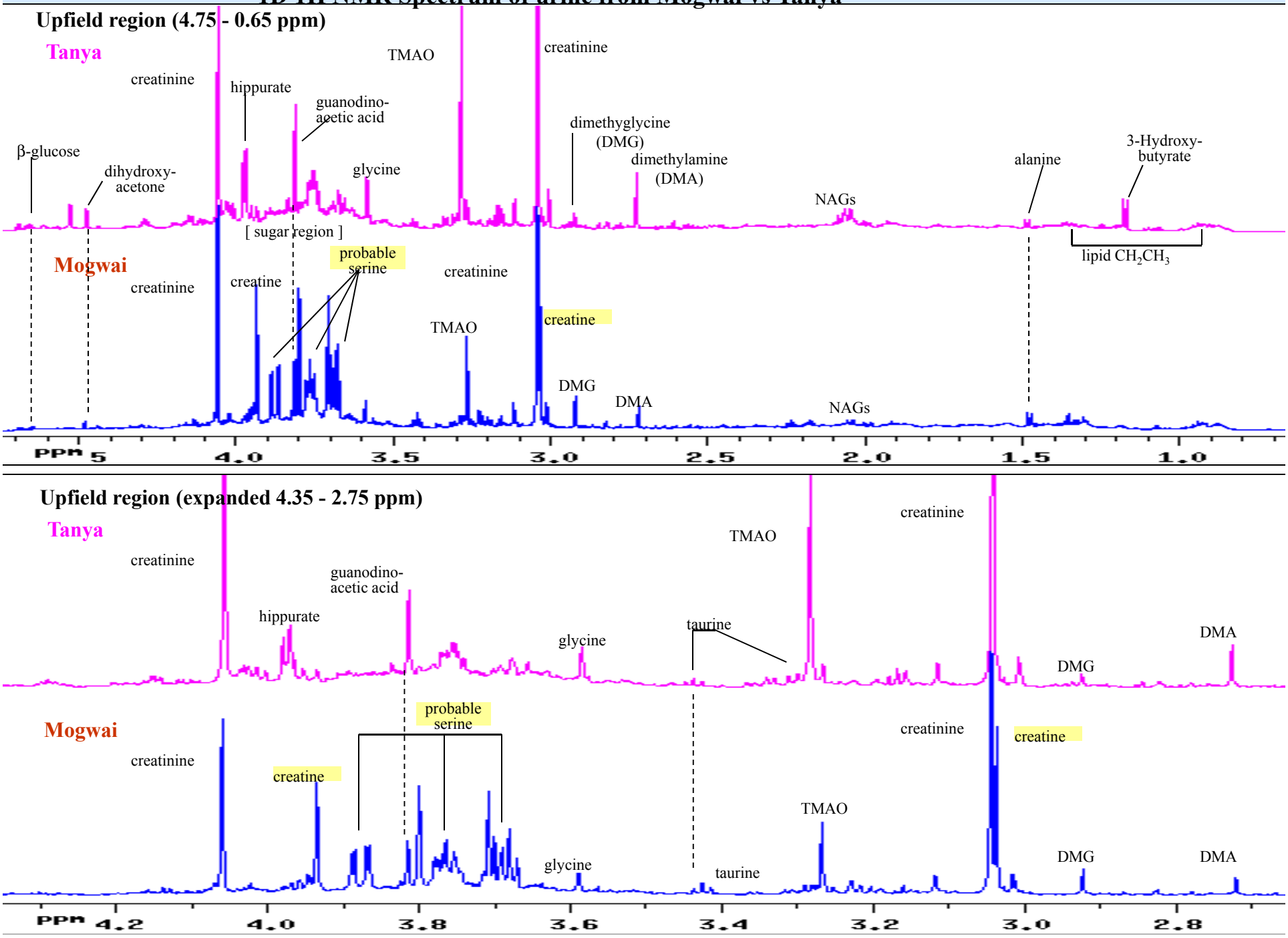
Downfield region of the ^{1}H 1D NMR spectrum

similar to Alpk:ApfCD

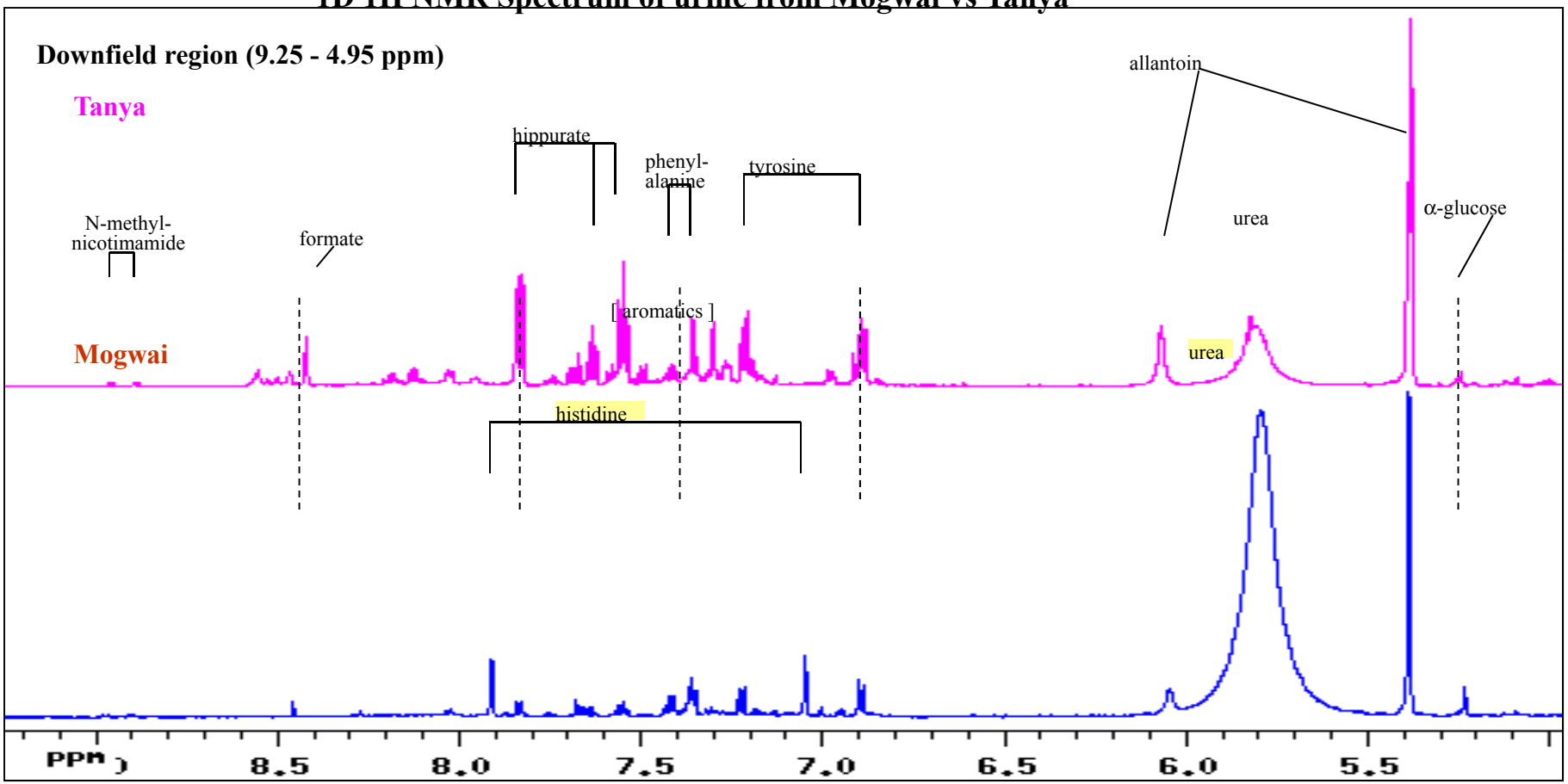


similar to C57BL10J

1D 1H NMR Spectrum of urine from Mogwai vs Tanya



1D 1H NMR Spectrum of urine from Mogwai vs Tanya



Cellular NMR (Metabolomics)

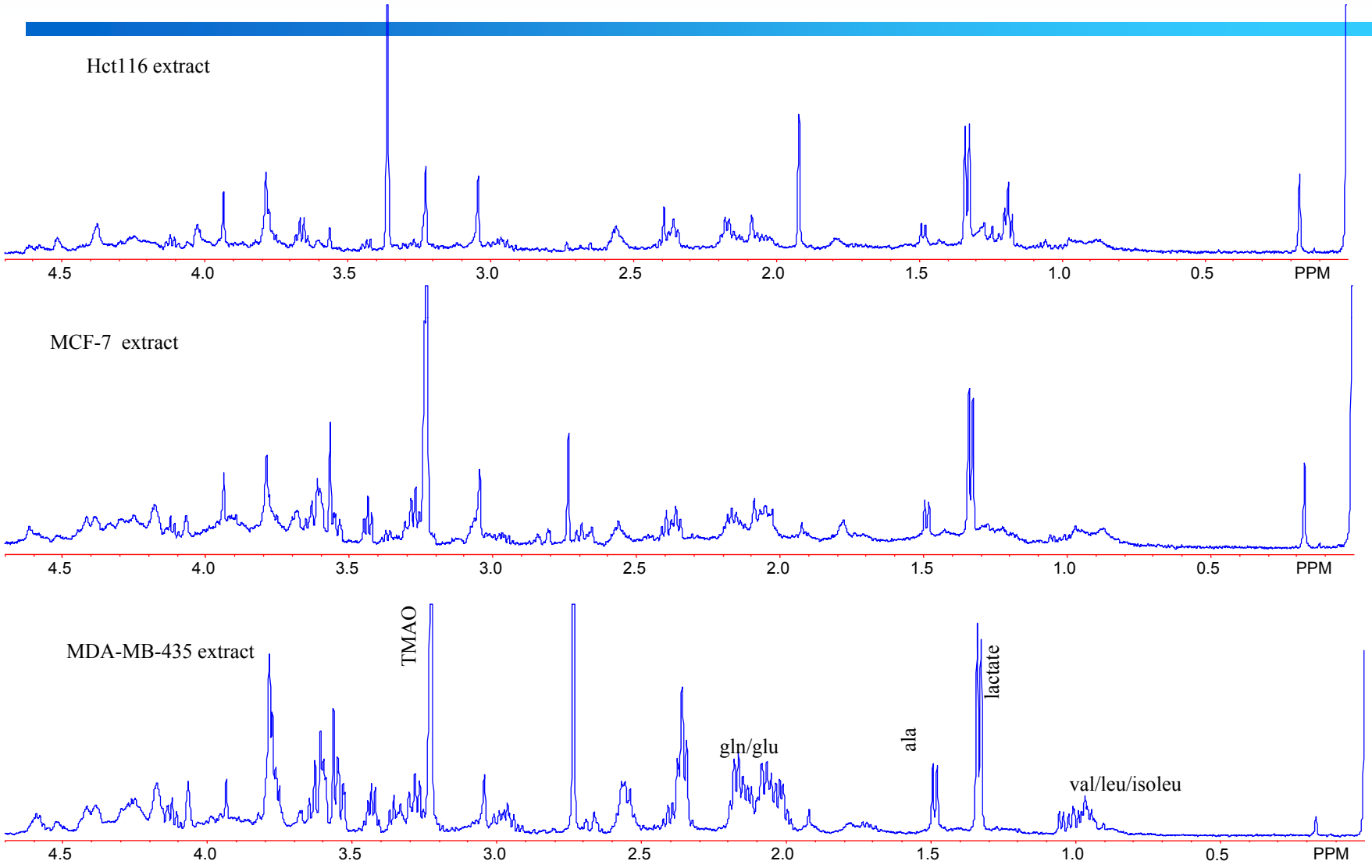
Metabolomics similar concept with Metabonomics except on the cellular level

Mode of action

- **perturbation of a single protein may cause a cascade effect**

Applicable to functional genomics?

Aqueous extracts

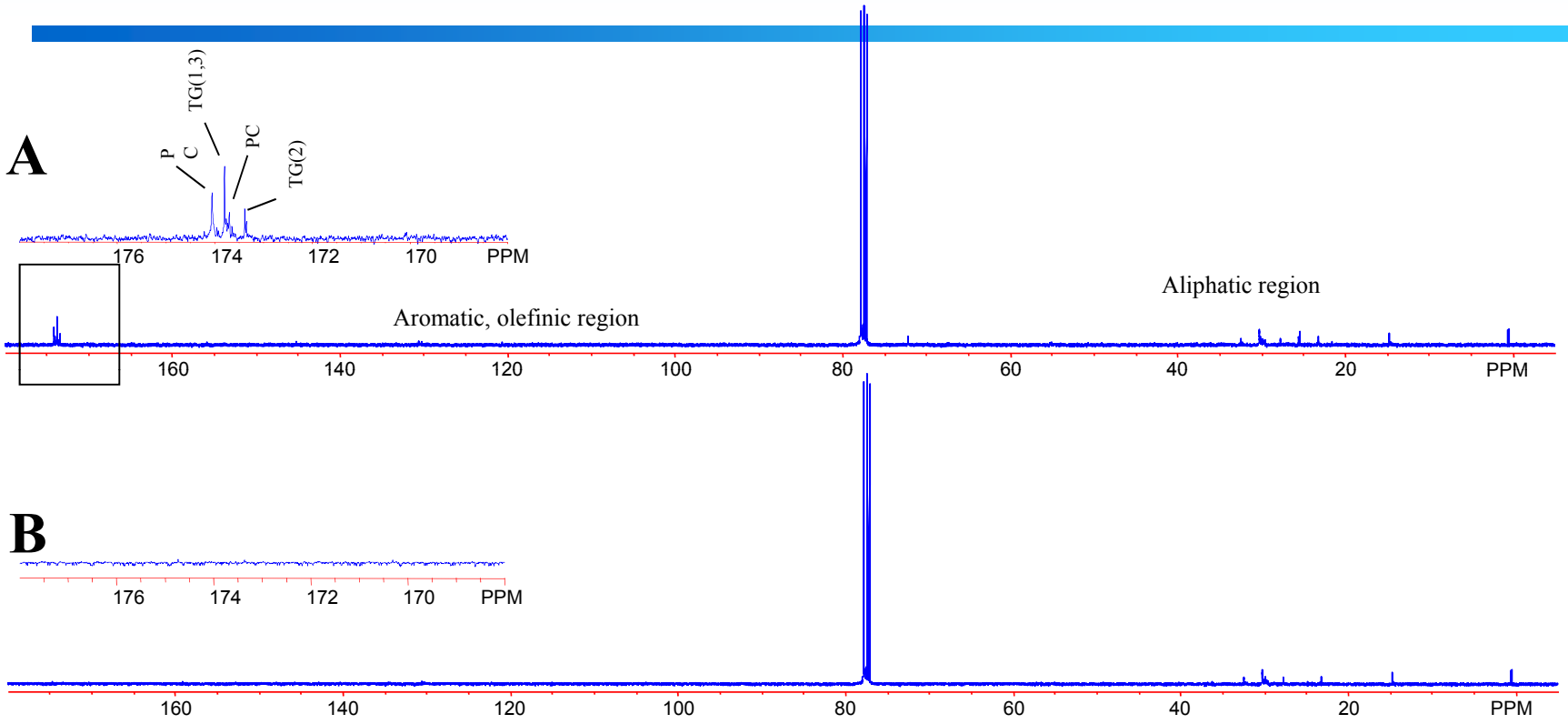


Metabolic pathways

Heteronuclei NMR is used extensively to elucidate metabolic pathways.

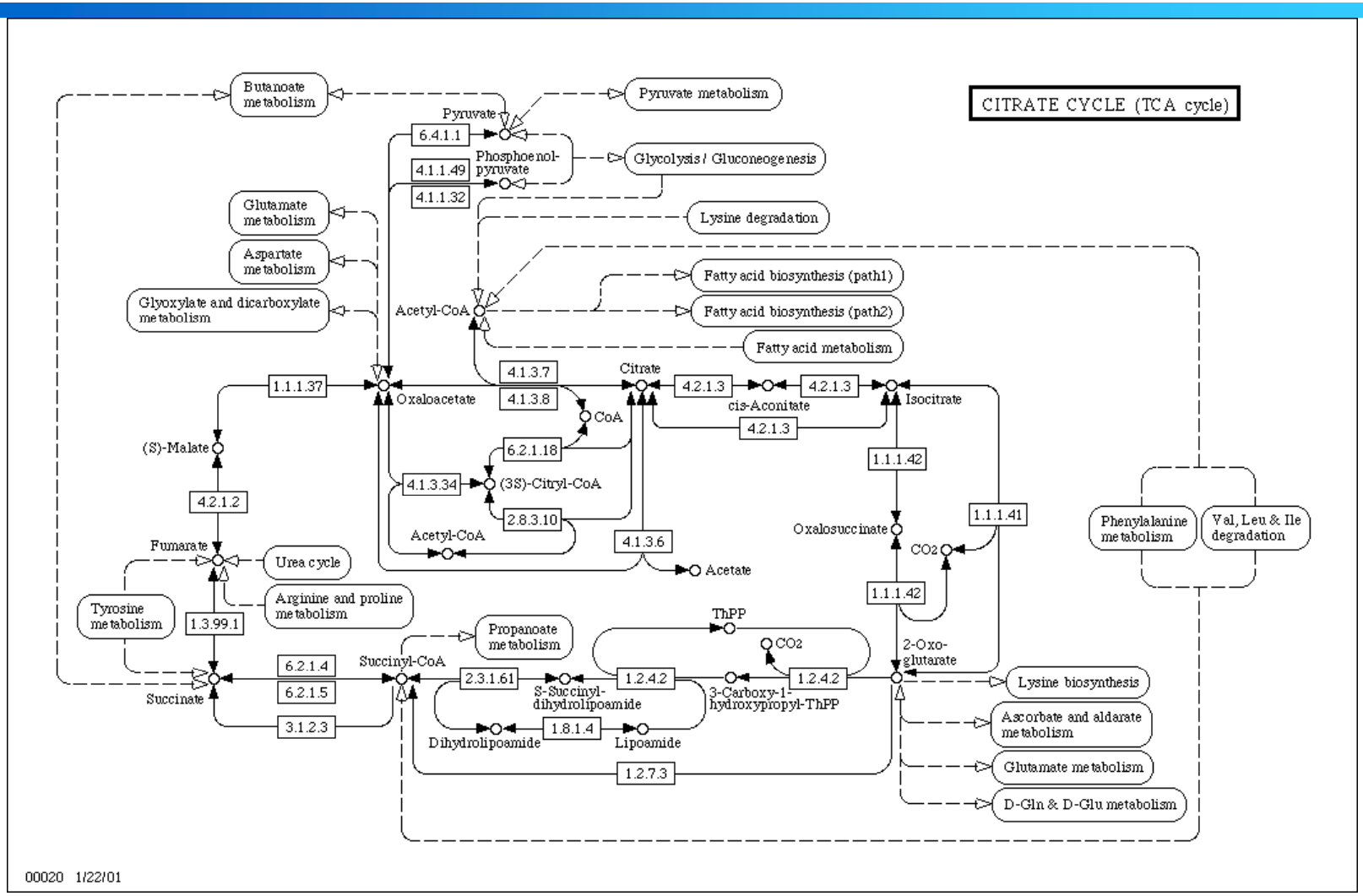
^{13}C or ^{2}H labeled compounds can be utilized to monitor the fate of the compounds or alterations within the cell's metabolism

^{13}C incubated cells



^{13}C NMR spectra of cell extractions. Carbon spectra were acquired with identical NMR conditions
Top - Cell were treated with 13C labeled palmitic acid.
Bottom - Non treated cells

TCA Cycle


CITRATE CYCLE (TCA cycle)
Butanoate metabolism
Pyruvate metabolism
Pyruvate
6.4.1.1
Phosphoenol-pyruvate
4.1.1.49
4.1.1.32
Glycolysis / Gluconeogenesis
Glutamate metabolism
Aspartate metabolism
Glyoxylate and dicarboxylate metabolism
Lysine degradation
Fatty acid biosynthesis (path1)
Fatty acid biosynthesis (path2)
Fatty acid metabolism
Acetyl-CoA
4.1.3.7
4.1.3.8
Citrate
4.2.1.3
cis-Aconitate
Isocitrate
1.1.1.37
Oxaloacetate
CoA
(S)-Malate
6.2.1.18
(3S)-Citryl-CoA
4.1.3.34
2.8.3.10
1.1.1.42
4.2.1.2
Fumarate
Urea cycle
Acetyl-CoA
4.1.3.6
Acetate
Oxalosuccinate
CO2
1.1.1.41
Phenylalanine metabolism
Val, Leu & Ile degradation
Tyrosine metabolism
1.3.99.1
Arginine and proline metabolism
ThPP
Propanoate metabolism
CO2
2-Oxo-glutarate
6.2.1.4
6.2.1.5
Succinyl-CoA
2.3.1.61
1.2.4.2
1.2.4.2
Lysine biosynthesis
Succinate
S-Succinyl-dihydrolipoamide
3-Carboxy-1-hydroxypropyl-ThPP
Ascorbate and aldarate metabolism
3.1.2.3
1.8.1.4
Dihydrolipoamide
Lipoamide
Glutamate metabolism
1.2.7.3
D-Gln & D-Glu metabolism
00020 1/22/01

Quantitative NMR

Concentration & CM

The ability for CM to quantitate compound solutions would be a quality improvement. Why?

Our tightest controls still can not prevent

- **Precipitation, crystallization in DMSO**
- **Precipitation from DMSO to another solvent (H2O)**
- **Instrument errors**

The ability to evaluate the concentration whenever would be beneficial

Improved concentration number may improve the assay's results

Concentration Verification Using NMR

The ability for Compound Management to quantitate compound concentrations in DMSO solutions would provide improvement in quality for determining accurate potency in biological assays.

Even with best practices in process, challenges to accurate concentration determination include:

- **Weight discrepancies**
- **Precipitation, crystallization in DMSO**
- **Precipitation during solvent changes**
  - **i.e dilution into aqueous solvent**
- **Instrument errors**

NMR's usefulness

Non-destructive – can recover the sample if needed

Can also obtain structural integrity and purity assessment – depending on conc. and time (99.5% purity)

Regio isomer and enantiomers can be determined

Quantitate the concentration of the NMR (qNMR) sample using NMR (^{1}H, ^{13}C, ^{31}P, ^{19}F)

Nuclear Magnetic Resonance (NMR)

Nuclear Magnetic Resonance is an analytical technique that is inherently quantitative. NMR detects protons (or other NMR active nuclei) based upon its magnetic environment and its detection relies directly upon the number of protons observed.

Benefits of NMR

- **Non-destructive – can recover the sample if needed**
- **Can also obtain structural integrity and purity assessment – depending on conc. and time (99.5% purity)**
- **Isomeric structure can be determined**
- **Sample concentration can be quantified by using NMR (^{1}H, ^{13}C, ^{31}P, ^{19}F)**

Quantitation methods

Internal

Spike the sample with the known compound

Integrals of the spectrum are relative to concentration

External

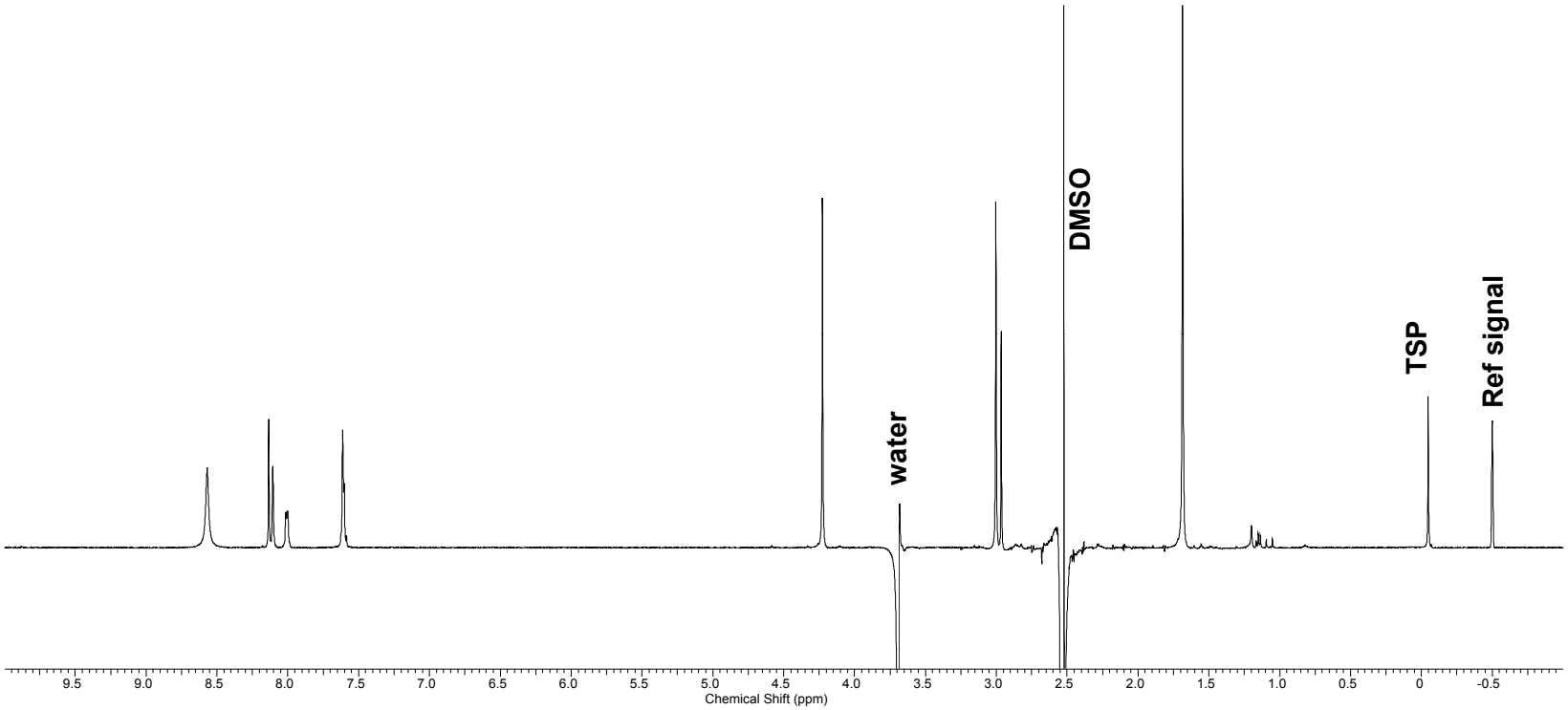
Acquire calibration curve

Integration of the unknown should fall within the calibration curve

Digital NMR – absolute integration values can be used as the reference

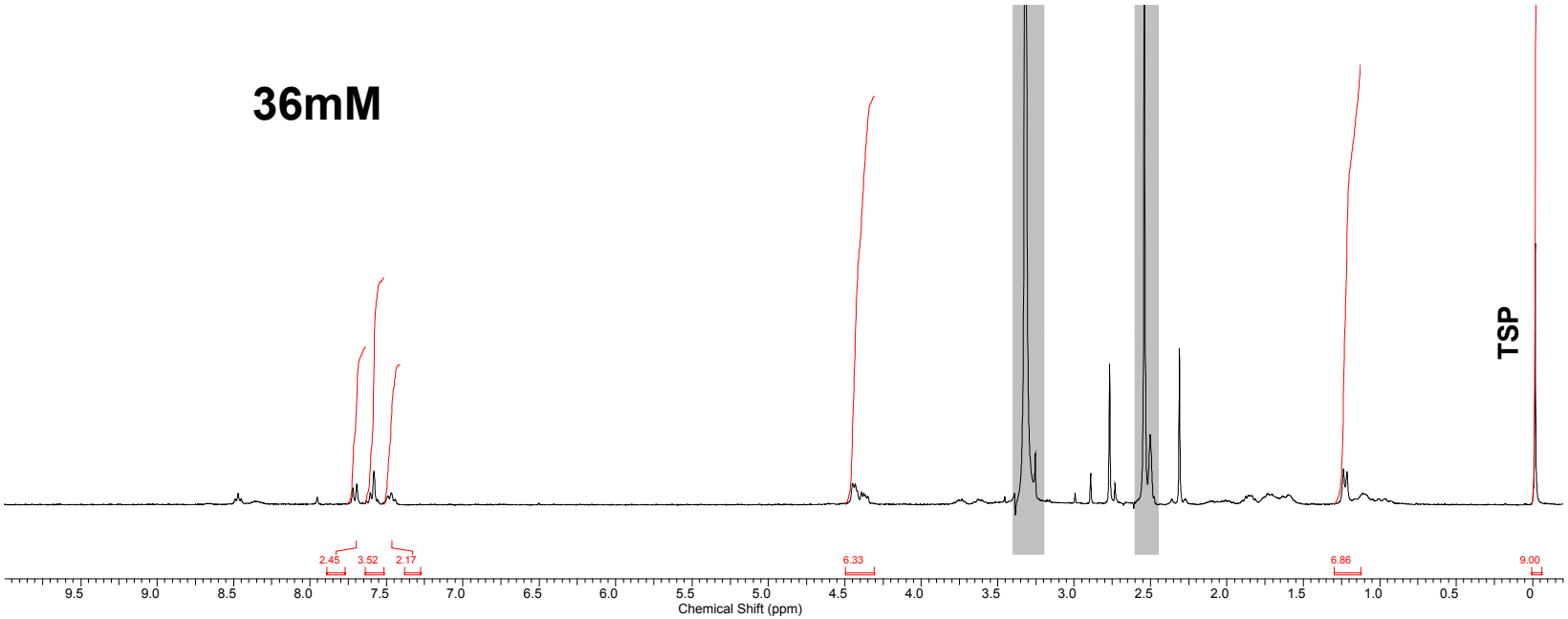
$$Conc_{cpd} = conc_{std} * (H_{std}/H_{cpd}) * (Int_{cpd}/Int_{std}) * \text{dil. factor}$$

Electronic Reference Standard



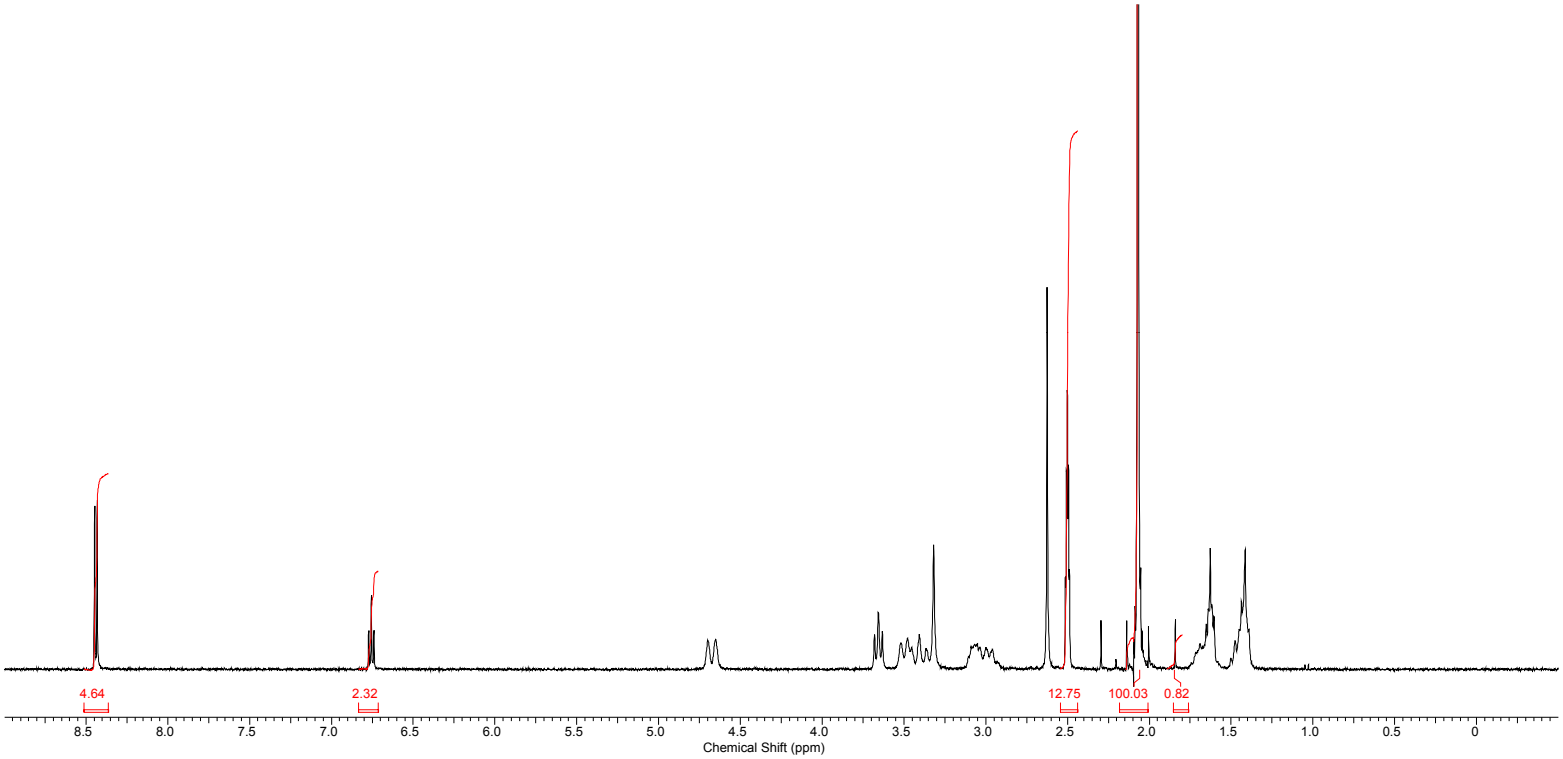
concentration is determined relative to the electronic reference signal

Internal Reference Standard



concentration is determined relative to known amount of TSP

“Traceless” Internal standard



concentration is determined relative to known amount of acetonitrile

Purity of the compound is not compromised

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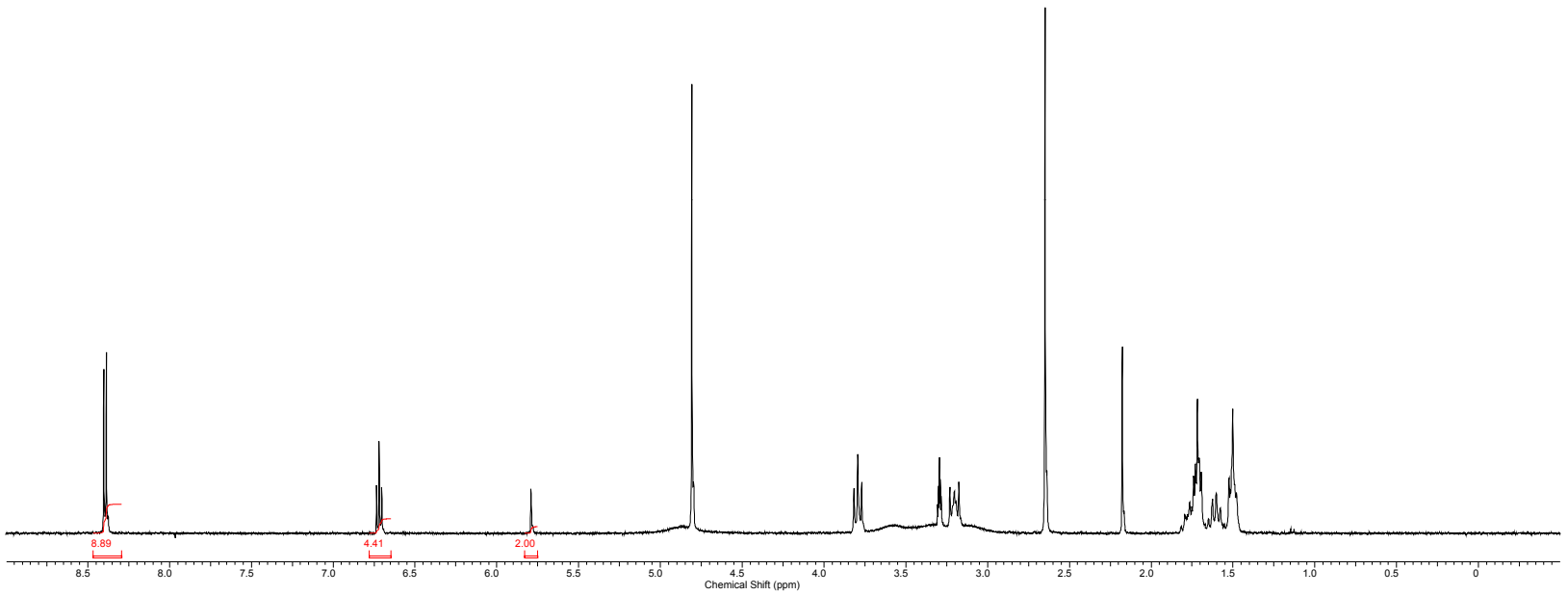
“Traceless” Internal standard



concentration is determined relative to known amount of dimethyl furan internal standard

Purity of the compound is not compromised

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Future

The way research is being performed is constantly changing

- **Combinatorial chemistry**
- **HTS**

NMR Research has ample opportunity to add value is just one analytical tool that complements many

Acknowledgements

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