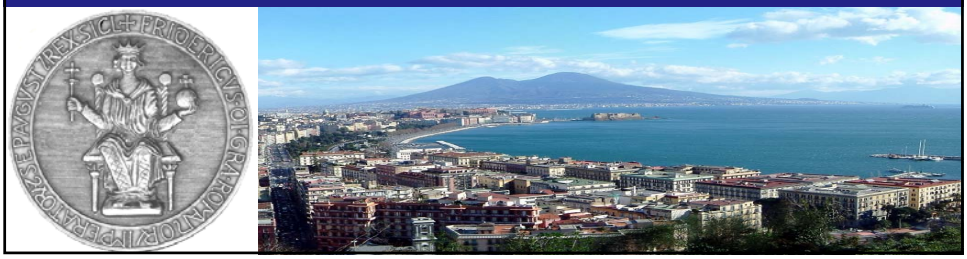
NMR of oligosaccharides and protein oligosaccharide complex

Antonio Molinaro

molinaro@unina.it

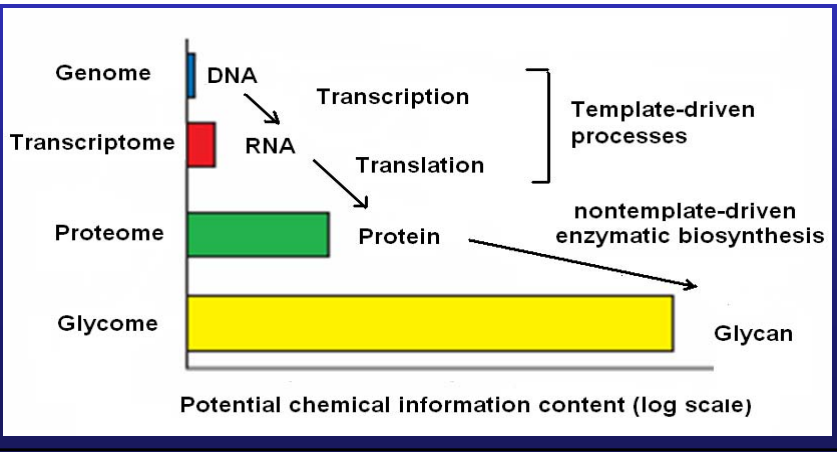
Department of Chemical Sciences
University of Naples Federico II



Glycomics Hits the Big Time

Cells run on carbohydrates. Glycans, sequences of carbohydrates conjugated to proteins and lipids, are arguably the most abundant and structurally diverse class of molecules in nature. Recent advances in glycomics reveal the scope and scale of their functional roles and their impact on human disease

Cell, 2010



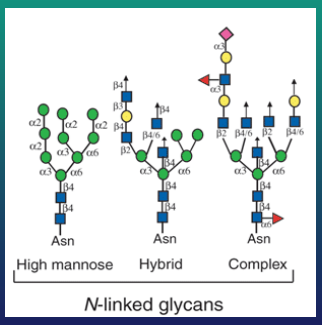
Carbohydrate complexity

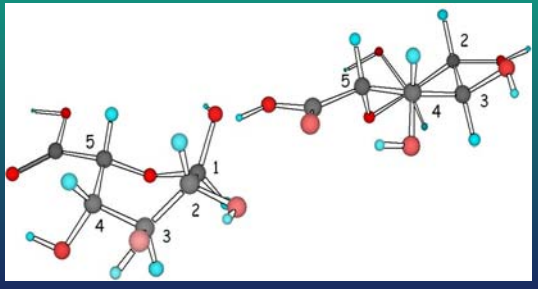
4 L-aminoacids → 256 tetra-peptides

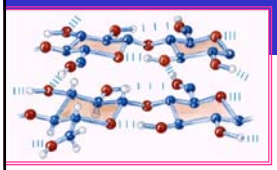
4 D-aldohexoses → 7.602.176 tetrasaccharides

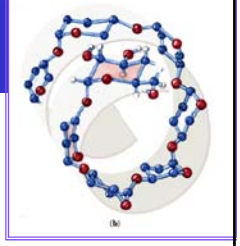
Not always linear polymers but frequently branched

Not to speak about conformation !

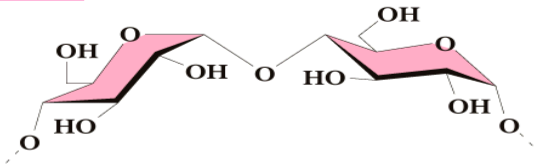








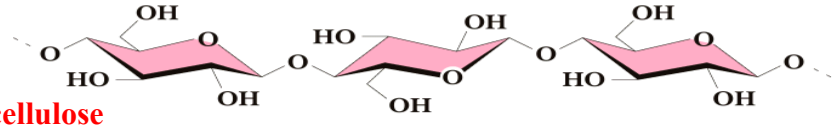
amylose



α-1,4-Linked D-glucose units

(a)

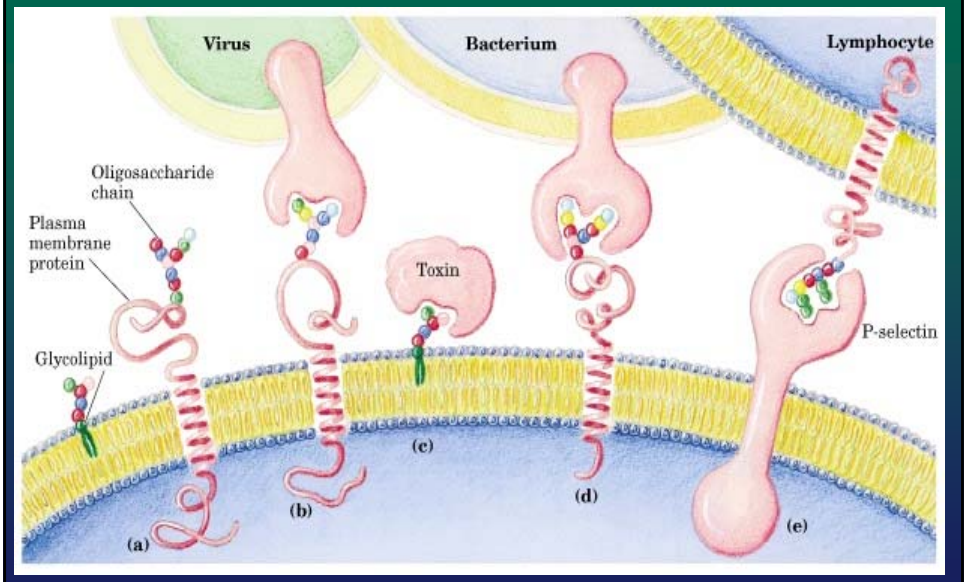
cellulose



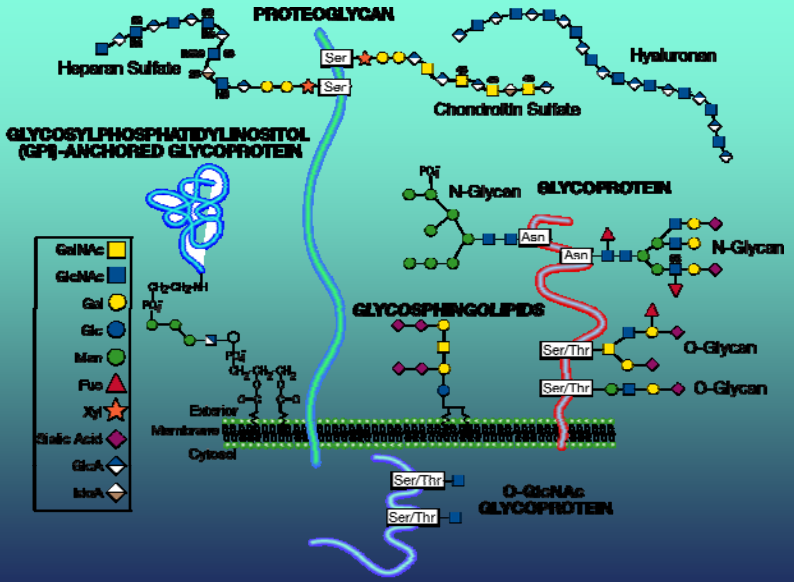
β-1,4-Linked D-glucose units

(b)

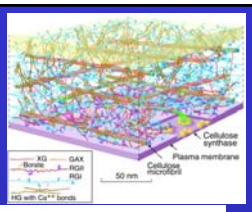
Mediators of microbial social life

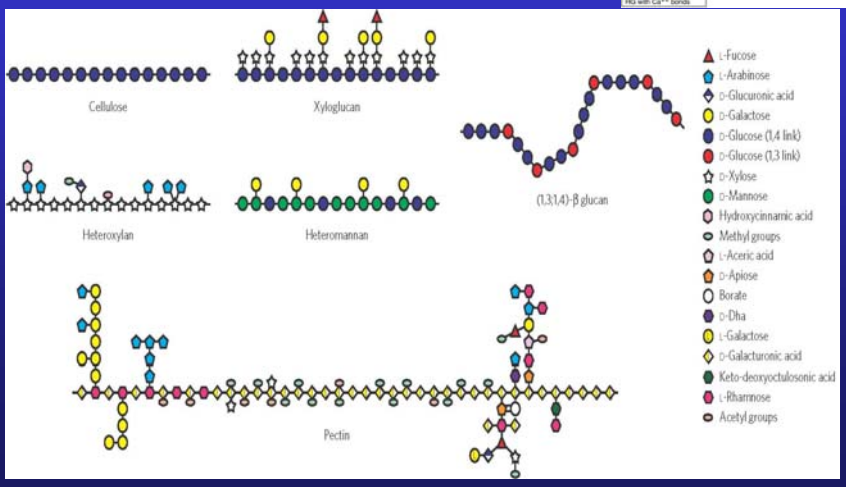


Major Glycan Classes in Vertebrate Cells



The plant cell wall





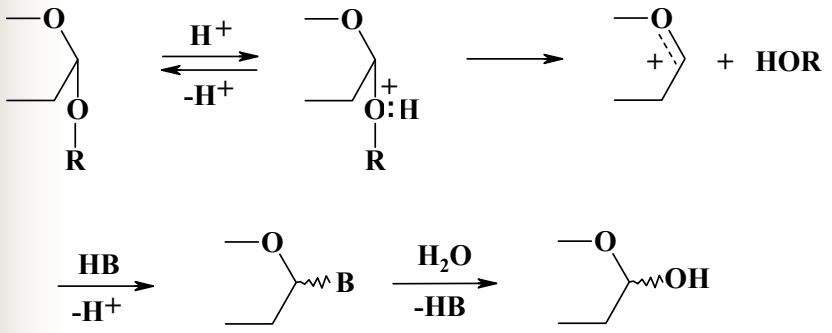
Structure determination of a glycan chain: the steps

De Castro et al., Meth. Enzymol., 2010; glycopedia.eu

- Quali-quantitative analysis (GC-MS, NMR)
- Absolute configuration (GC-MS, NMR)
- Size of the ring (GC-MS, NMR)
- Anomeric configuration (NMR)
- Linkage analysis (GC-MS, NMR)
- Monosaccharides sequence (MALDI-MS,2D NMR)
- Determination of non-carbohydrate appendages (GC-MS,MALDI-MS, 2D NMR)

Quali-quantitative analysis (GC-MS)

The major approach to the determination of chemical composition is full solvolytic depolymerization of polysaccharides followed by identification of monomers



Advantages of acid hydrolysis:

- hydrolysis is simple to handle
- it is easy to vary conditions of hydrolysis

Disadvantages of acid hydrolysis:

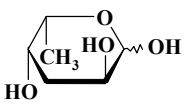
- some monosaccharides are too unstable
- some glycosidic linkages are too stable
- many non-sugar substituents are eliminated

Different conditions for the hydrolysis may be used for analysis of different monosaccharides

O-polysaccharide of *Pseudoalteromonas tetraodonis*

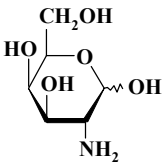
→2)-α-Col*p*-(1→4)-β-Glc*p*NAc-(1→4)-β-Glc*p*A-(1→3)-β-Gal*p*NAc-(1→
α-Col -(1→2)-β-Gal-(1→2)┘

0.5 M CF_3COOH, 100°, 1 h



Col (colitose)

2 M CF_3COOH, 120°, 3 h

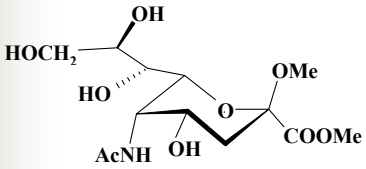


GalN

Acid-labile monosaccharides may be stabilized using methanolysis, giving methyl derivatives

O-polysaccharide of *Salmonella arizonae*

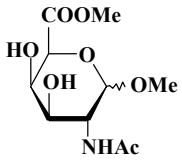
↓ 0.5 M HCl/MeOH, 85°



Neu5Ac

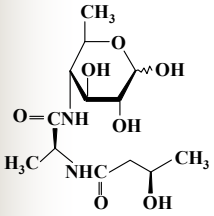
O-polysaccharide of *Shigella dysenteriae*

↓ 1. 1 M HCl/MeOH, 100°
2. Ac_2O

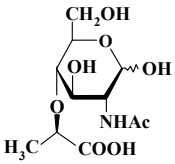


GalNAcA

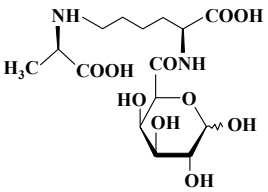
Solvolysis with anhydrous hydrogen fluoride or trifluoromethanesulfonic acid enable isolation of complex monosaccharide amide derivatives



serogroup O4



serogroup O15

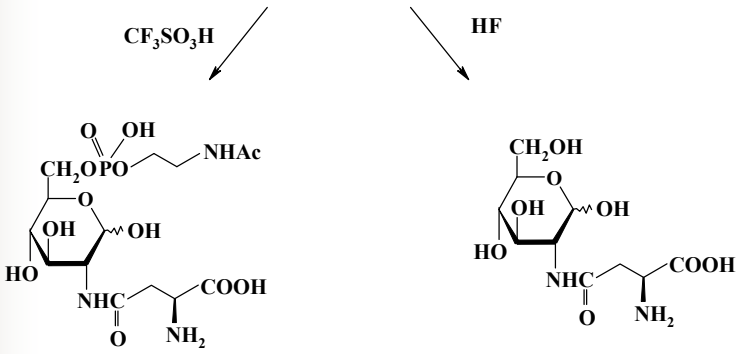


serogroup O13

Isolated components of *Proteus* O-polysaccharides

Solvolysis with trifluoromethanesulfonic acid, but not with hydrogen fluoride, enables isolation of phosphorylated monosaccharide derivatives

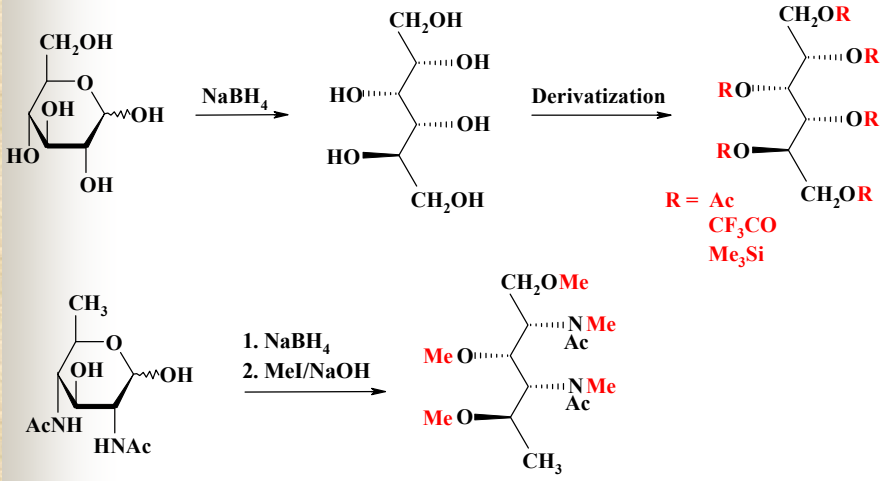
O-polysaccharide of *Proteus mirabilis* O38



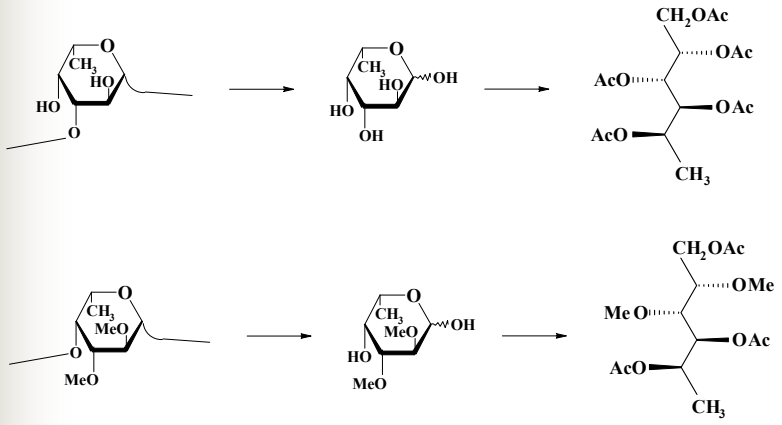
Identification of monosaccharides

- Chromatography, including liquid chromatography and gas-liquid chromatography.
- Determination of optical rotation and circular dichroism for enantiomeric differentiation.
- Mass spectrometry, including combined gas-liquid chromatography/mass spectrometry.
- NMR spectroscopy.

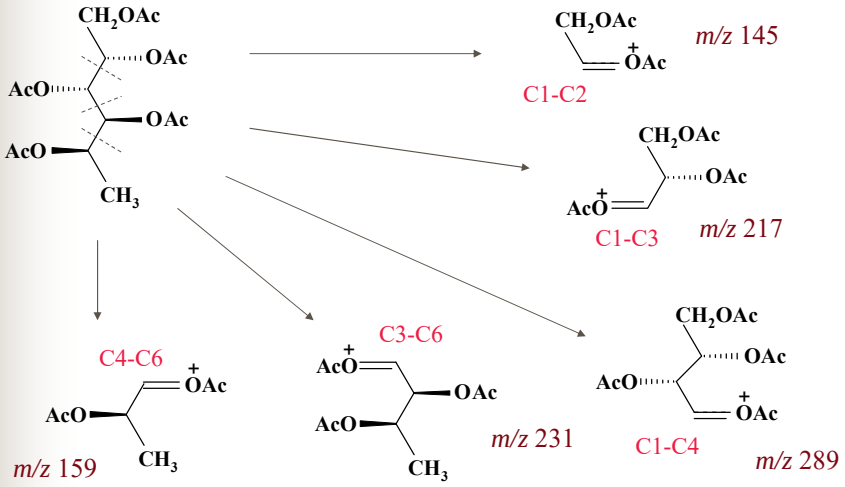
Gas-liquid chromatography requires derivatization of monosaccharides, usually to alditol derivatives



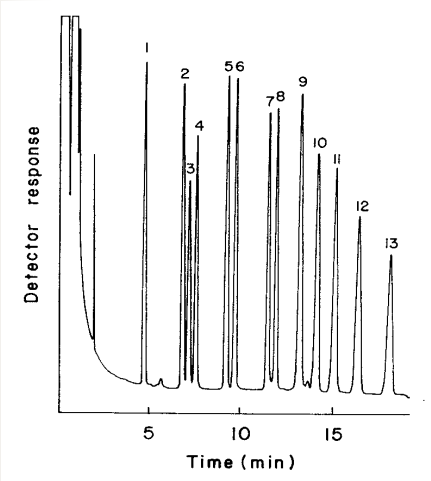
EI MS is commonly used for identification of alditol derivatives in sugar and methylation analyses



EI MS fragmentation of the fully acetylated 6-deoxy alditol in sugar analysis



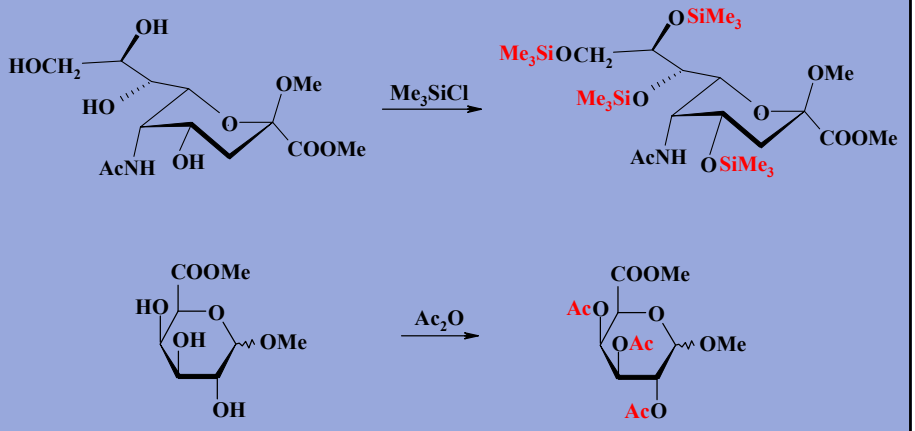
GLC separation of sugar alditol acetates



Column: Silar 10C
Detection: Flame ionization

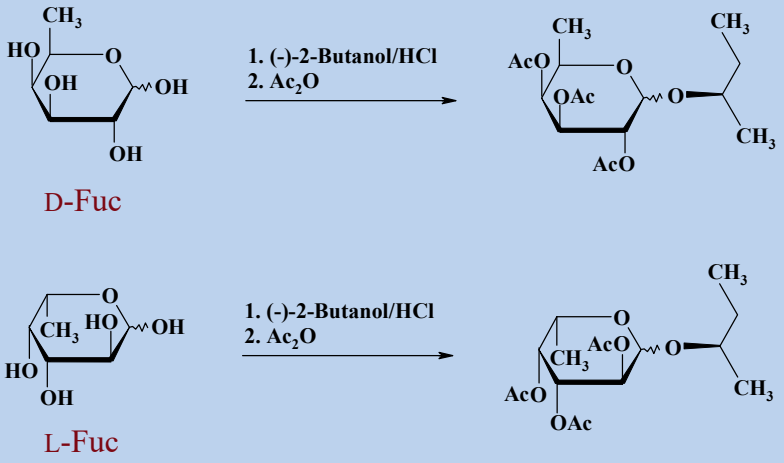
Peaks:
1. Tetritol
2. 2-Deoxypentitol
3. Rhamnitol
4. Fucitol
5. Ribitol
6. Arabinitol
7. Xylitol
8. 2-Deoxyhexitol
9. Allitol
10. Mannitol
11. Galactitol
12. Glucitol
13. *myo*-Inositol

Glycosides obtained by methanolysis are useful for identification of sialic and uronic acids



- Absolute configuration determination (GC-MS)

Glycosides with chiral alcohols are used for enantiomeric differentiation of sugars



D-Fuc

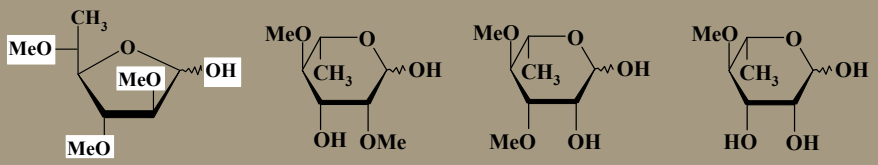
L-Fuc

- Linkage analysis, size of ring (methylation analysis) (GC-MS)

Methylation is the most widely used chemical approach to linkage analysis

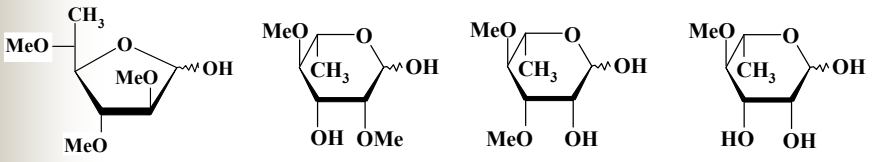
→3)-α-Rha*p*-(1→3)-α-Rha*p*-(1→2)-α-Rha*p*-(1→
α-Fuc*f*-(1→4)┘

↓ 1. MeI/NaOH
2. CF_3COOH

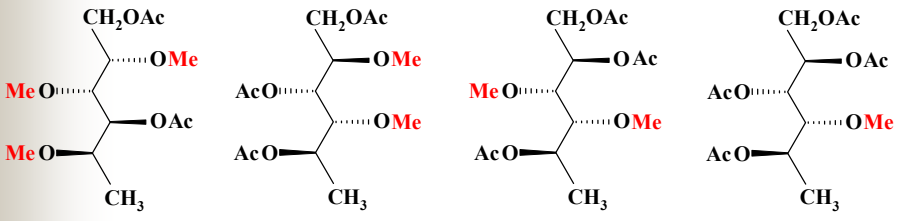


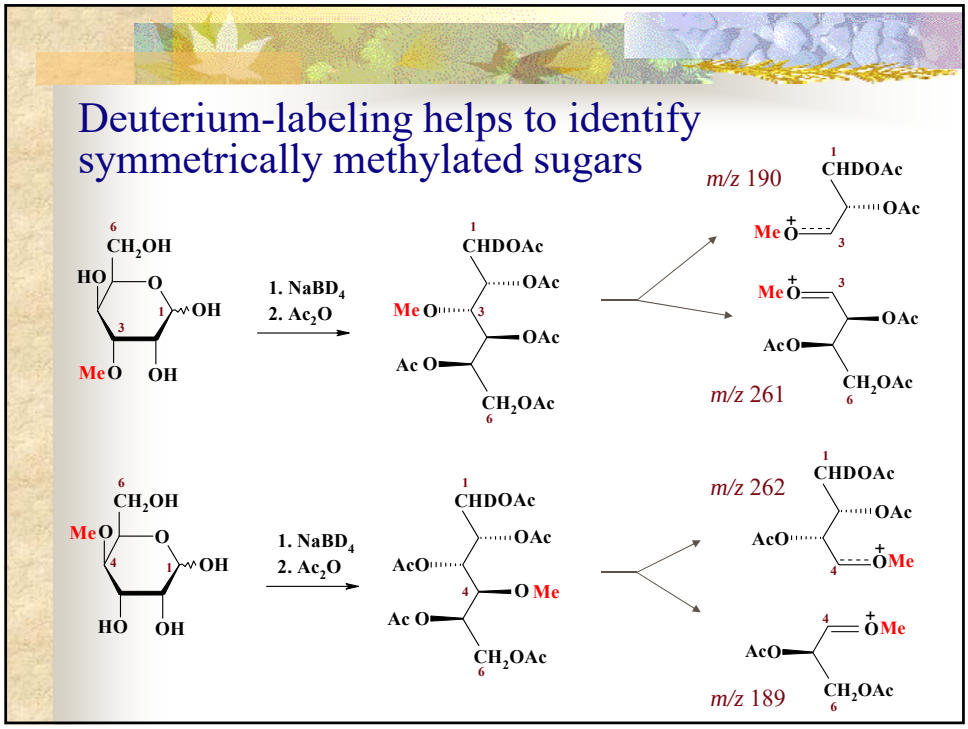
Terminal fucofuranose | 3-Substituted rhamnopyranose | 2-Substituted rhamnopyranose | 3,4-Disubstituted rhamnopyranose

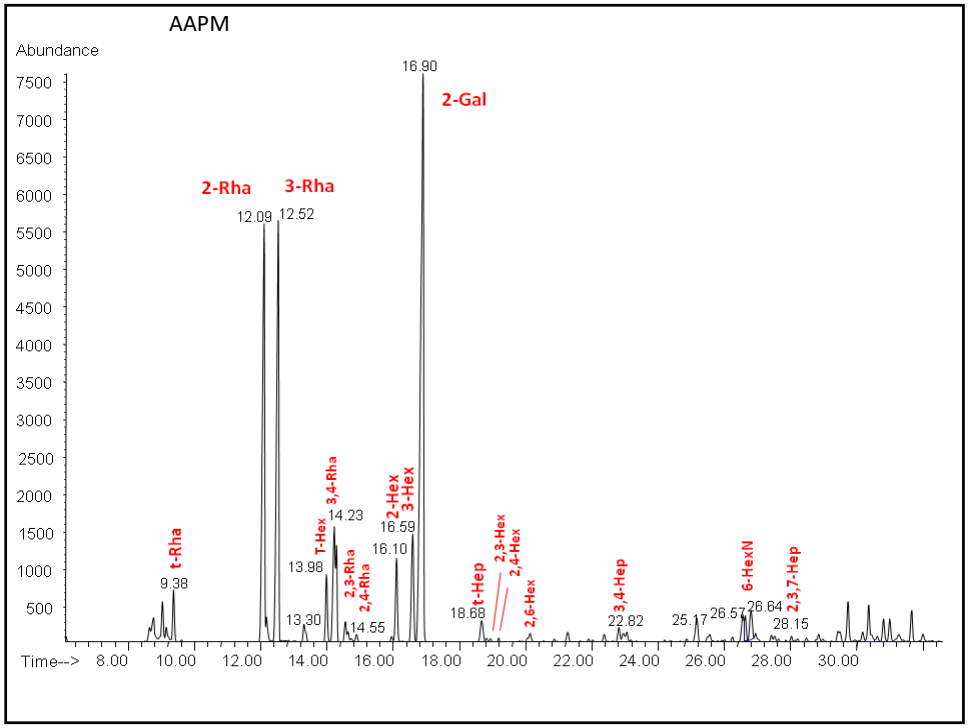
Partially methylated monosaccharides are identified by GLC/MS of the acetylated alditols



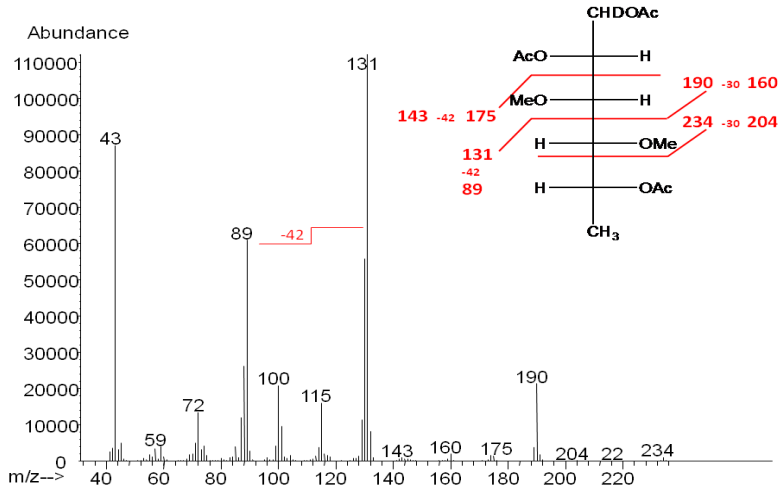
↓ 1. $NaBH_4$
2. Ac_2O




Deuterium-labeling helps to identify symmetrically methylated sugars
1. NaBD4
2. Ac2O
m/z 190
m/z 261
1. NaBD4
2. Ac2O
m/z 262
m/z 189


AAPM
Abundance
Time-->
2-Rha
3-Rha
2-Gal
t-Rha
T-Hex
3,4-Rha
2,3-Rha
2,4-Rha
2-Hex
3-Hex
t-Hep
2,3-Hex
2,4-Hex
2,6-Hex
3,4-Hep
6-HexN
2,3,7-Hep

2-Rha Fragmentation pattern



Nuclear Magnetic Resonance:
key sequences to structure/sequence determination of carbohydrate containing molecules

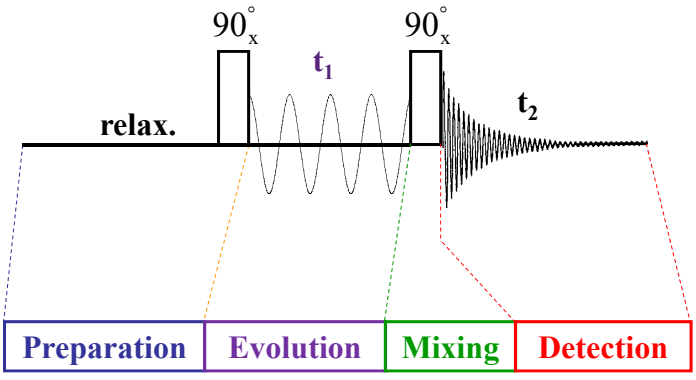
Nuclear Magnetic Resonance - NMR

- **Measures the absorption of electromagnetic radiation in the radio-frequency region (~4-900 MHz)**

- sample needs to be placed in magnetic field to cause different energy states

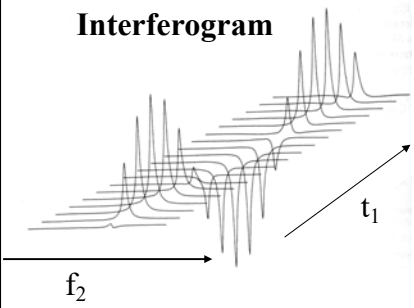
NMR is routinely and widely used as the preferred technique to rapidly elucidate the chemical structure of most organic compounds.

Anatomy of a 2D NMR Experiment



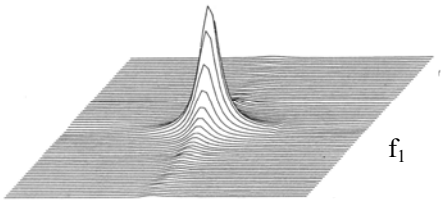
2D NMR - The Interferogram

Interferogram

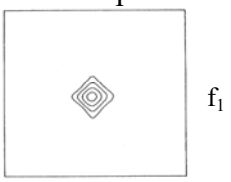


A 2D data set can be thought of as a series of 1D .
Each 1D file is different from the next by a change in t_1.
Fourier transformation of each 1D in the t2 domain creates an interferogram.

2D plot of data



Contour plot.



Two Dimensional NMR

✓A 2D data set can be thought of as a series of 1D experiments collected with different timing.

✓Fourier transformation of each 1D in the t2 domain creates an interferogram.

✓The t1 domain is then Fourier transformed resulting in a 2D file with the frequency in each dimension.

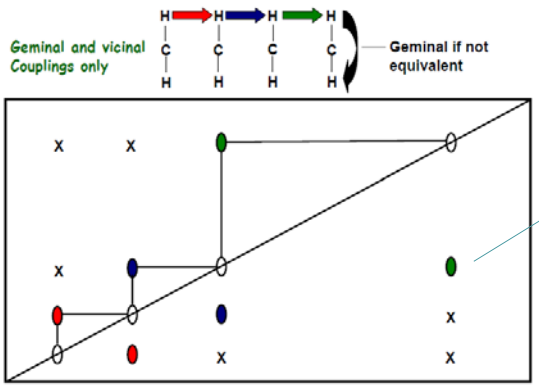
✓This 2D file will provide a map of all spin-to-spin correlations

✓Each 2D experiment can provide either through bond (COSY type) or through space (NOESY type) correlation

COrrelation SpectroscopY (COSY)

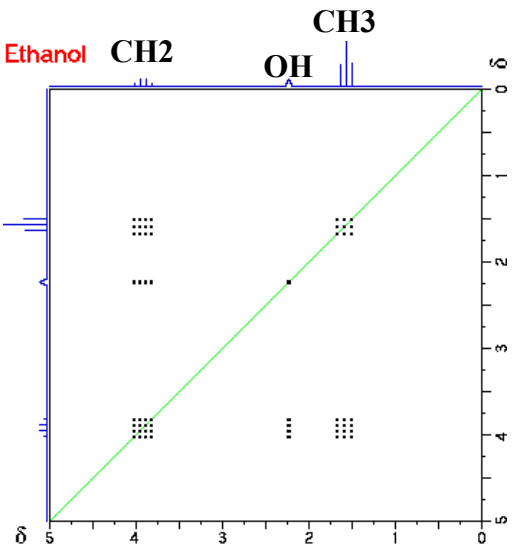
In a 2D COSY spectrum, cross-peaks will exist where there is spin-spin coupling between nuclei.

^{1}H-^{1}H COSY and DQFCOSY Experiments



Used to identify spins which are coupled to each other.

2D Experiments – COSY

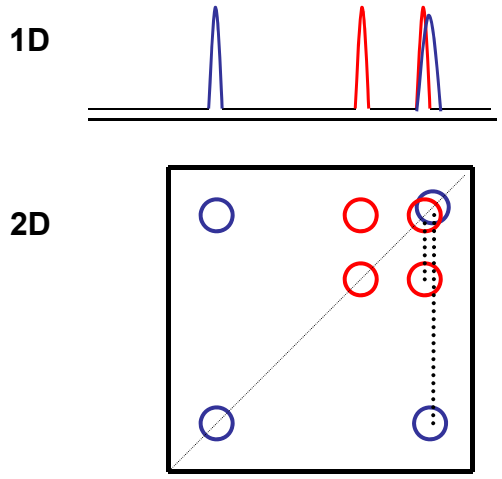


Cross peaks due to geminal and vicinal coupling

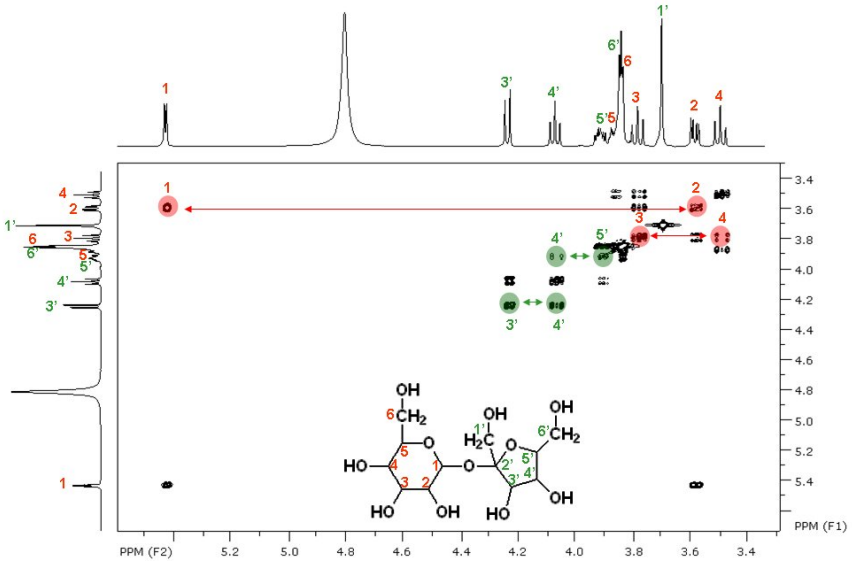
CH3-CH2-OH

2D Experiments – COSY

The Power of 2D NMR:
Resolving Overlapping Signals



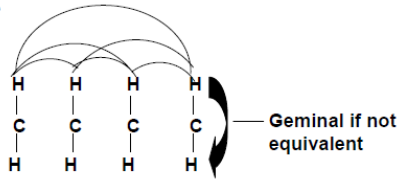
COSY of sucrose

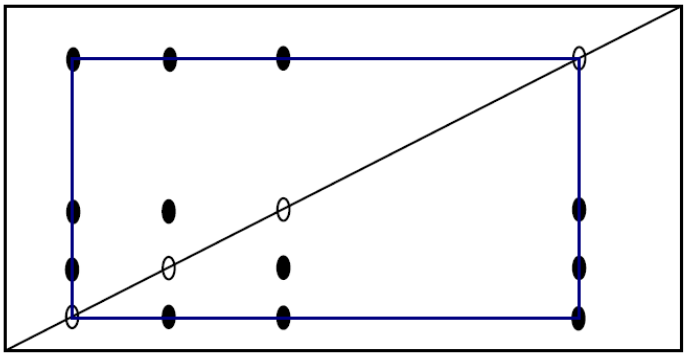


TOtal Correlation SpectroscopY (TOCSY) experiment

TOCSY Experiment

In general, the TOCSY mixing time determines the number of bonds over which signal can be Transferred, assuming that none of the coupling Constants = 0

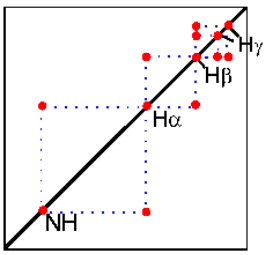




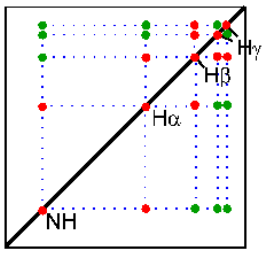
TOCSY

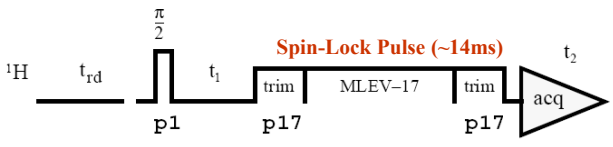
•cross peaks are generated between all members of a coupled spin network
• NMR resonances for the complete side-chain spin systems is obtained
• coherence transfer period occurs during a multi-pulse spin-lock period;
•length of spin-lock determines how far the spin coupling network will be probed

COSY

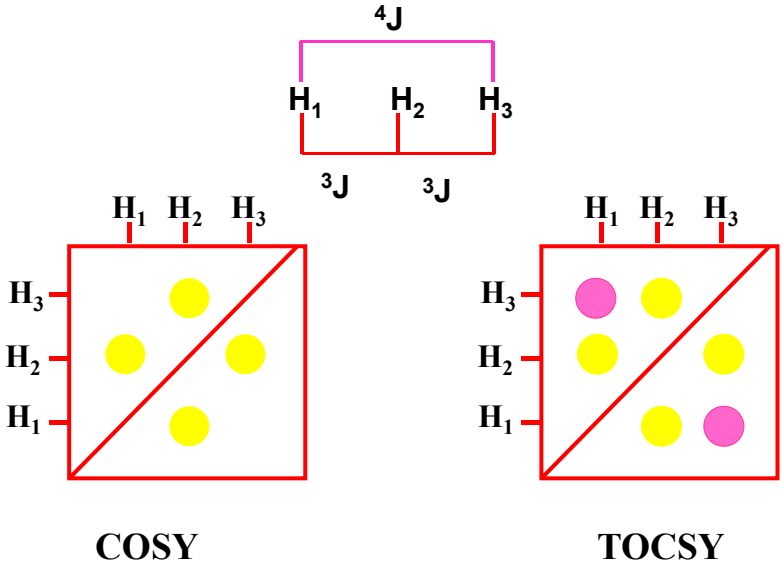


TOCSY

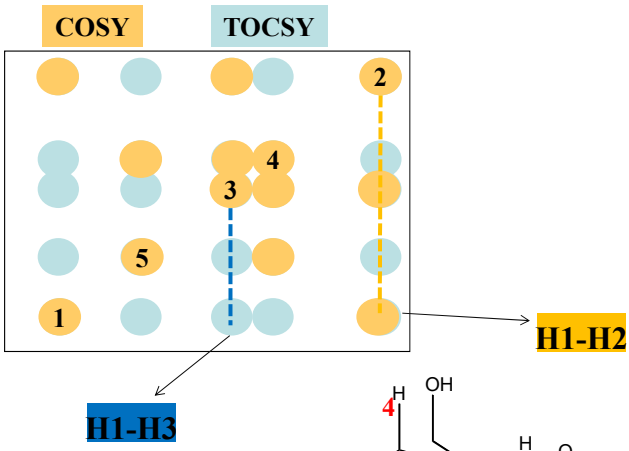


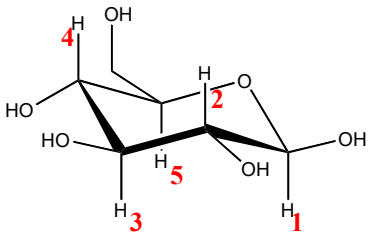


COSY and TOCSY



COSY and TOCSY

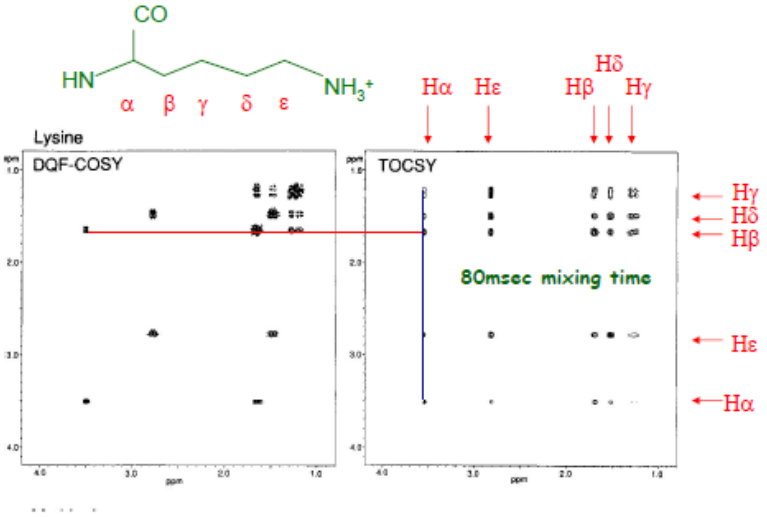




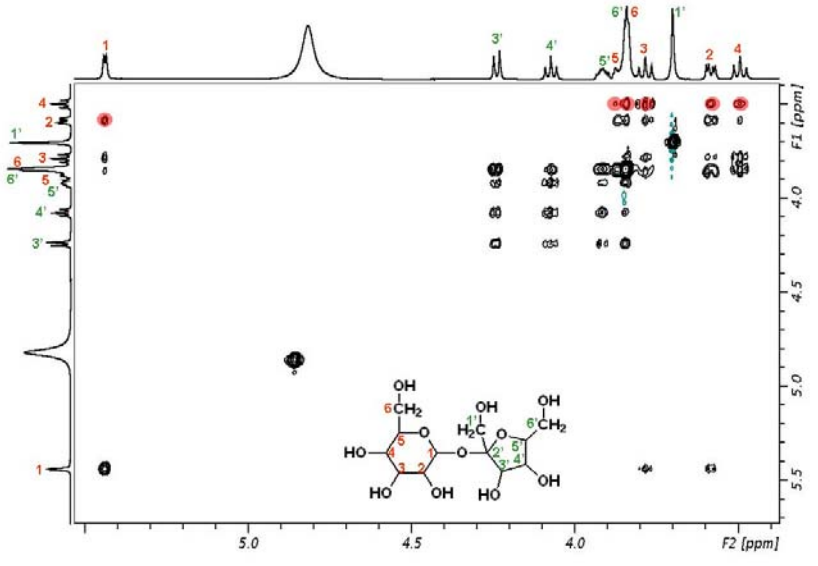
In Glucose, H1 and H2 protons are scalarly coupled, H2 and H3 not, Through COSY, H1 andH2 correlations are observed ; Thought TOCSY, correlation between H1 and H3 are observed

TOCSY

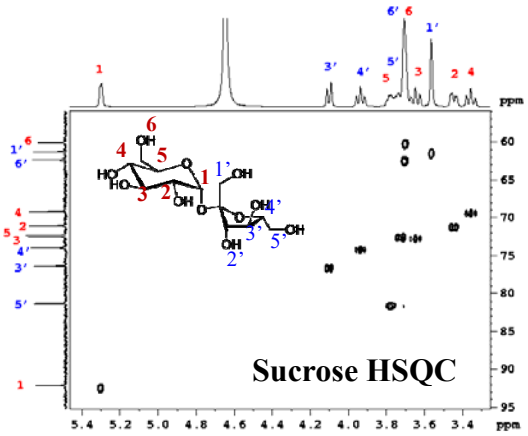
Example of lysine spin system



TOCSY of sucrose



HSQC: Heteronuclear Single-Quantum Correlation



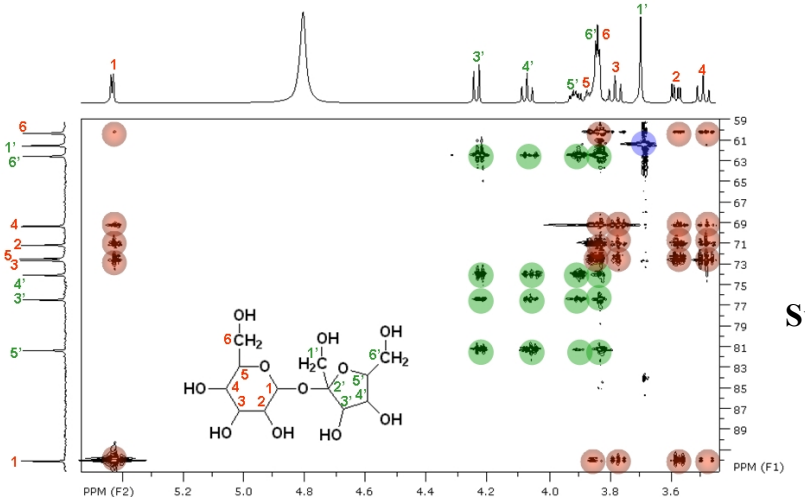
HSQC experiment:
one axis for ^{1}H and the other for a *heteronucleus*

The spectrum contains a peak for each unique proton attached to the heteronucleus being considered.

The **2D HSQC experiment** permits to obtain a 2D heteronuclear chemical shift correlation map between directly-bonded 1H and X-heteronuclei (an atomic nucleus other than a proton), often ^{13}C or ^{15}N.

HSQC-TOCSY

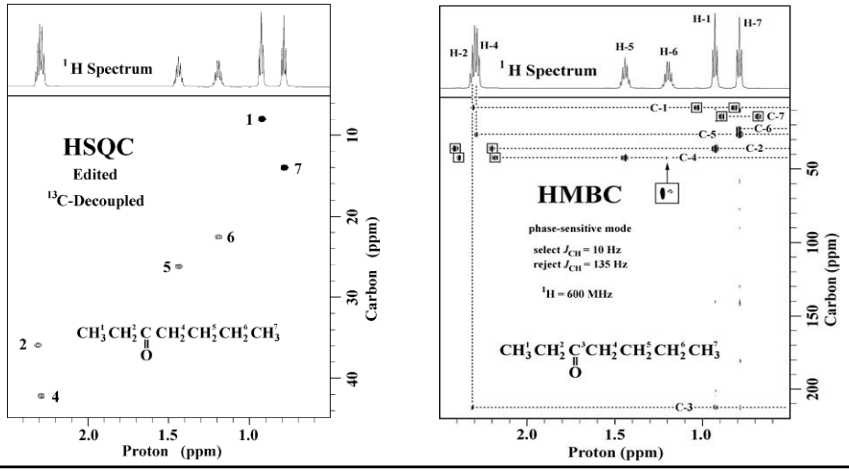
The HSQC-TOCSY is a 2D TOCSY that has been resolved into the carbon dimension.
Especially useful in case of huge overlap in the proton spectrum



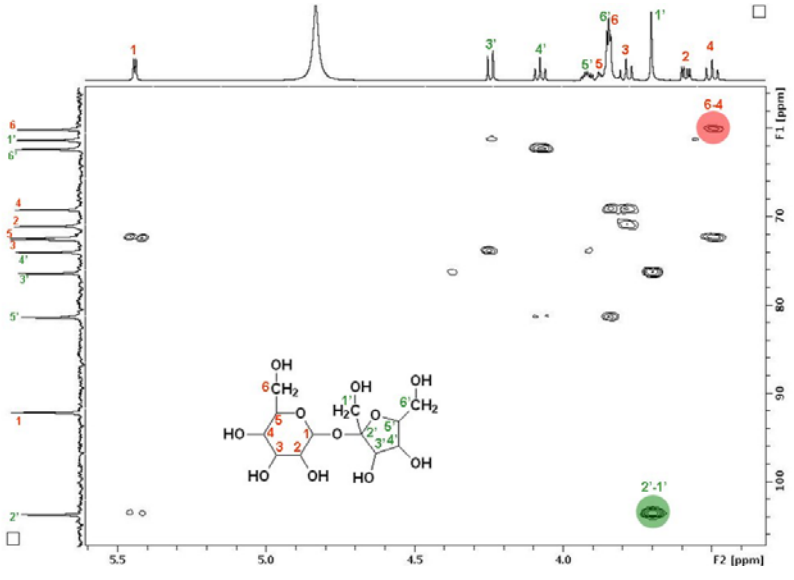
Sucrose

HMBC (Heteronuclear Multiple Bond Correlation)

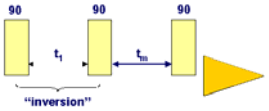
2D HMBC (Heteronuclear Multiple Bond Correlation) experiment correlates chemical shifts of two types of nuclei **separated from each other with two or more chemical bonds.**



HMBC of Sucrose

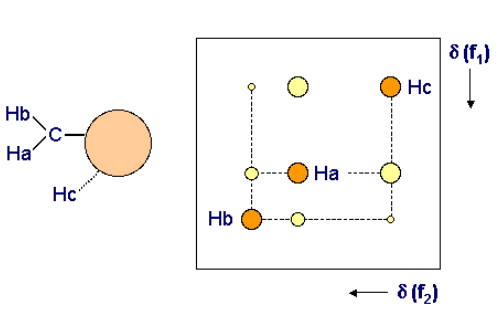


Nuclear Overhauser Effect (NOE) Spectroscopy



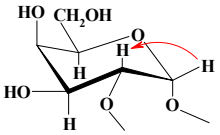
The 2D spectrum will have chemical shifts in f1 and f2.

The cross peaks are for nuclei that are dipolar coupled.

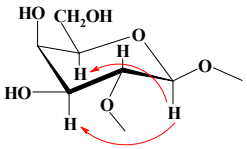


Intra-residue NOE contacts in monosaccharides: relative configuration of sugar residues

***gluco, galacto* configuration**

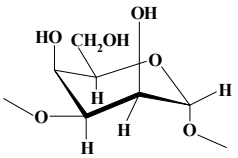


α-linkage: H1/H2

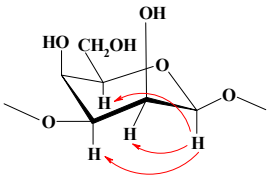


β-linkage: H1/H3, H1/H5

***manno* configuration**

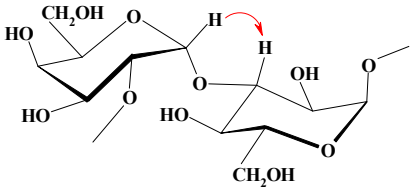


α-linkage: no contact



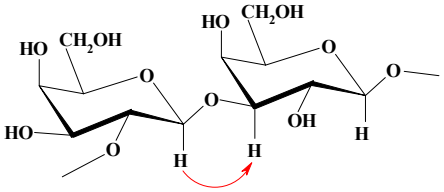
β-linkage: H1/H2, H1/H3, H1/H5

Inter-residue NOE contacts in saccharides

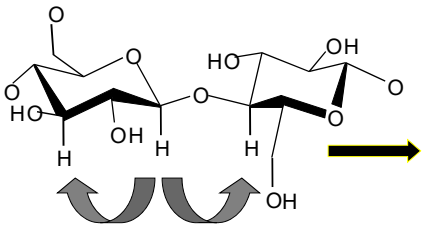


α-(1-3)linkage

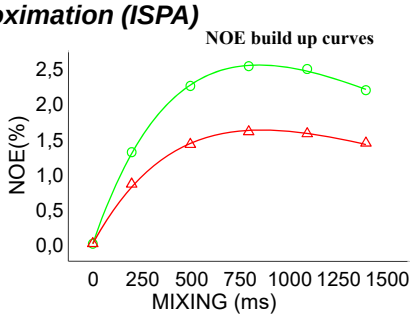
β-(1-3) linkage



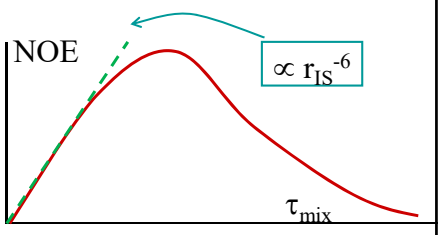
NOE and Distances
Isolated spin pair aproximation (ISPA)



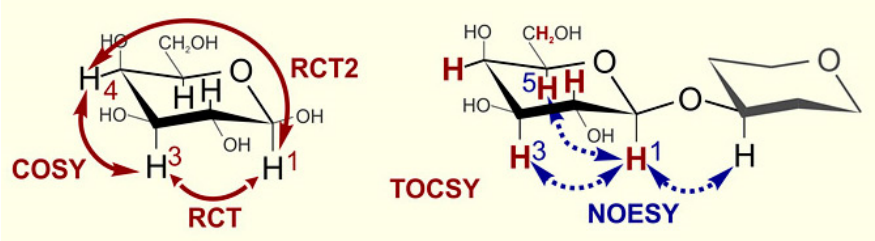
NOE H1'-H3' NOE H1'-H4

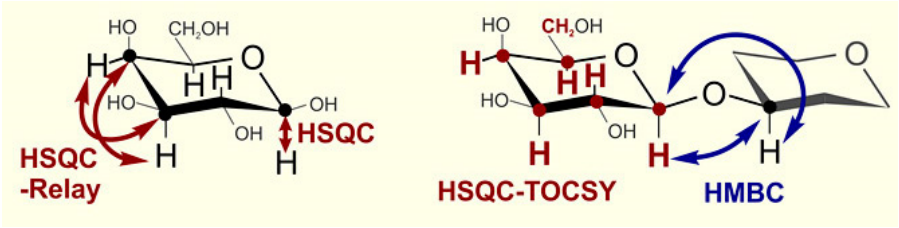


$\eta_{ab} \propto r_{ab}^{-6}$
$r_{ac} = r_{ab} * (\eta_{ab} / \eta_{ac})^{-1/6}$
$\eta_{ac} \propto r_{ac}^{-6}$



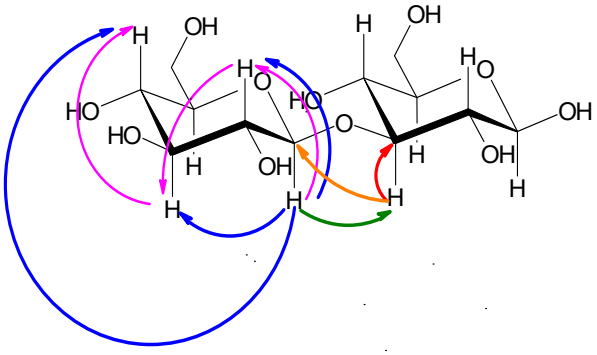
Application of various NMR techniques to carbohydrates



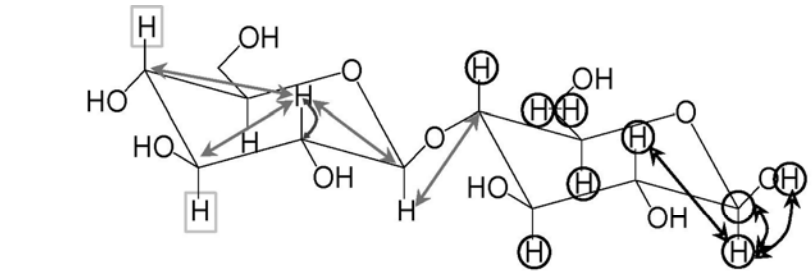


Application of various NMR techniques to carbohydrates

- HOMONUCLEAR (^{1}H-^{1}H)
- HETERONUCLEAR (^{1}H-^{13}C)



HOMONUCLEAR ^{1}H-$^{1}H
COSY
TOCSY
NOESY/ROESY
HETERONUCLEAR ^{1}H-$^{13}C
HMQC/HSQC
HMBC



COSY: neighboring ^{1}Hs ($^{3}J_{HH}$)

HMBC: typically $^{2}J_{HC}$ and $^{3}J_{HC}$

HSQC, HMQC: $^{1}J_{HC}$

TOCSY and HSQC-TOCSY: $^{n}J_{HH}$, and $^{1}J_{HC}$ and $^{n}J_{HH}$ and respectively. Where n is the number of bonds separating coupled nuclei. Different mixing time is used to detect different sized *J* value.

Fig. 1 Flowchart comparing a generalized approach for solution structural determination of biomolecules. Dashed squares identify aspects in the structural determination of glycans that need improvement or are underutilized.

^{1}H and ^{13}C typical regions of carbohydrates:

The ^{1}H NMR Spectra can be roughly divided into the following regions:

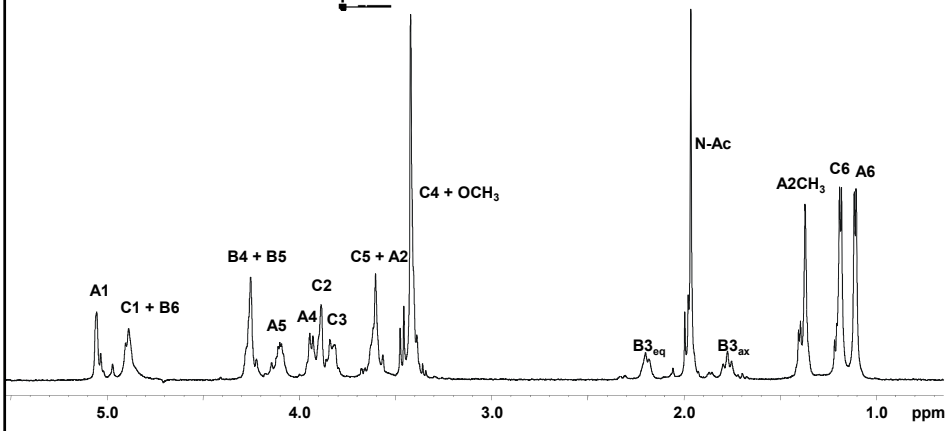
Anomeric and Acylated Protons : 5.5-4.5 ppm.
Ring Protons : 4.5-3 ppm
Acetyl Groups, Methylene Protons: 3-2 ppm
Methyl Groups: 0.8-2.0 ppm

The ^{13}C NMR Spectra can be roughly divided into the following regions :

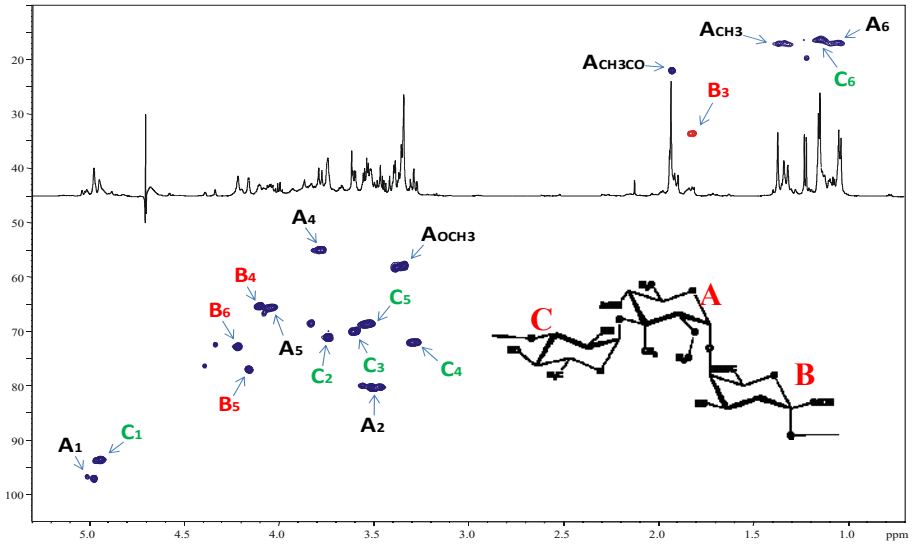
Anomeric Carbons Resonate Between 90-105 ppm
Ring Carbons Between 52-78 ppm
Nitrogen Bearing Carbons (In Amino Sugar) 50-60 ppm
Acetyl Groups XXX ppm
Methylene Protons: XXX ppm
Methyl Groups: XXX ppm

O-chain isolated from *Rhodopseudomonas palustris* sp. BIS A53

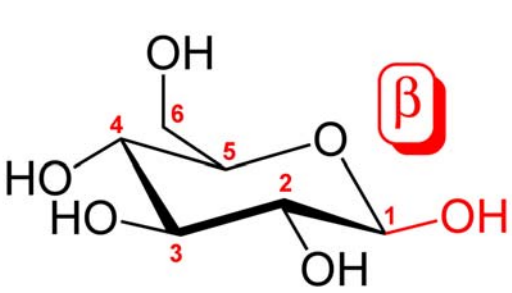
A: 2-*O*-Metil-3-deossi-3-Metil-4-ammino-4-deossi-chinovosio
B: 3-deoxy-D-lyxo-2-heptulosaric acid (DHA)
C: Rhamnose (Rha)

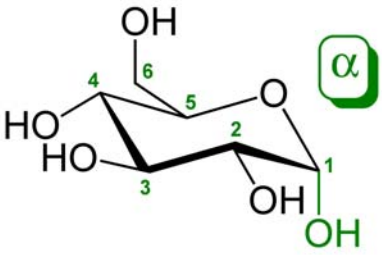


HSQC spectrum of the *O*-chain isolated from *Rhodopseudomonas palustris* sp. BIS A53

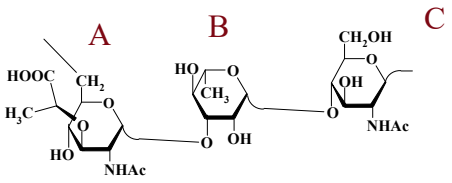


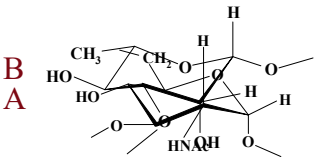
❖Anomeric configuration (NMR)



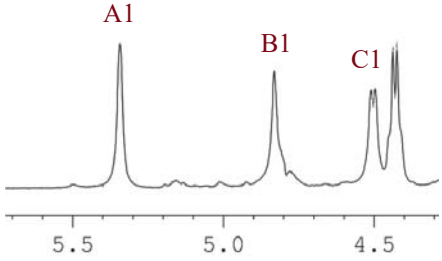


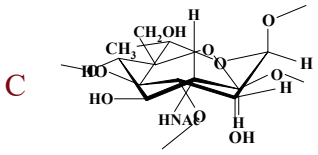
^{1}H NMR spectrum contains information on the configuration of glycosidic linkages





β-linkage: $J_{1,2} < 2$ Hz
α-linkage: $J_{1,2} < 4$ Hz



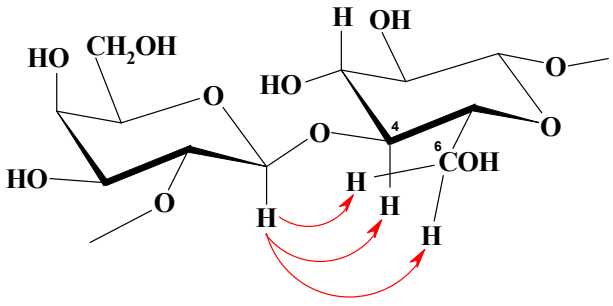


β-linkage: $J_{1,2} > 6$ Hz
α-linkage: $J_{1,2} < 2$ Hz

Monosaccharide Sequence

- NOE contact
- Glycosylation shift (HSQC spectrum)
- Inter-residual long range correlation (HMBC spectrum)

NOE in disaccharides may occur not only at the linkage protons but also at the neighbouring protons

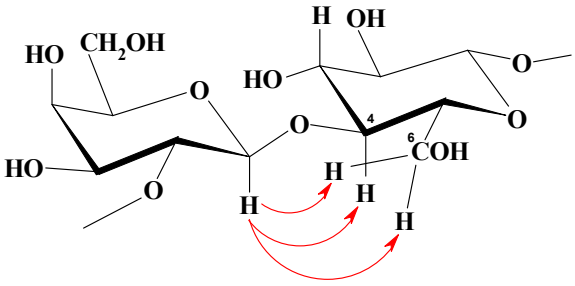


….Saccharide conformation…

Characteristic chemical shifts in ^{1}H and ^{13}C NMR spectra of polysaccharides

Component	Group	δ_H	δ_C
3-Deoxy sugar	CH_2	1.9-2.6	30-42
6-Deoxy sugar	CH_3	1.1-1.4	15-21
Uronic acid	COOH		173-178
Amino sugar	CHN		44-59
O-acetyl	CH_3	2.1-2.3	21-22
	CO		174-176
N-acetyl	CH_3	1.8-2.1	23-24
	CO		174-176
N-formyl	HCO	8.0-8.1	164.5-165.5
1-carboxyethyl	CH_3	1.4-1.6	18-20
	COOH		175-179
ethanolamine	CH_2N	3.25-3.30	40-42
	CH_2O	4.0-4.2	62-64

NOE in disaccharides may occur not only at the linkage protons but also at the neighbouring protons



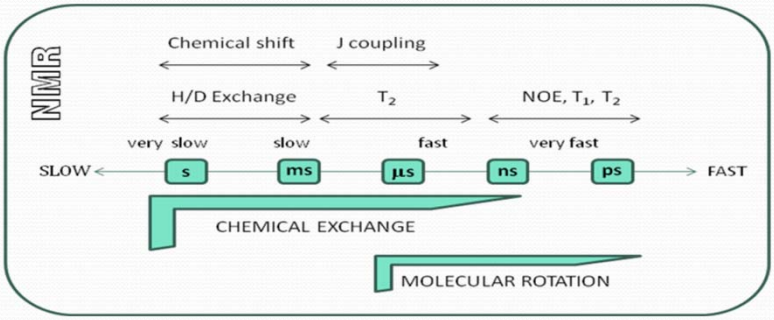
NMR as a tool for studying protein-ligand interactions"

Which NMR methods are useful to look at the interaction between a small thing and a large entity?

Rules of Engagement" of Protein–Glycoconjugate Interactions: A Molecular View Achievable by using NMR Spectroscopy and Molecular Modeling

Roberta Marchetti, Serge Perez, Ana Arda, Anne Imberty, Jesus Jimenez-Barbero, Alba Silipo, Antonio Molinaro

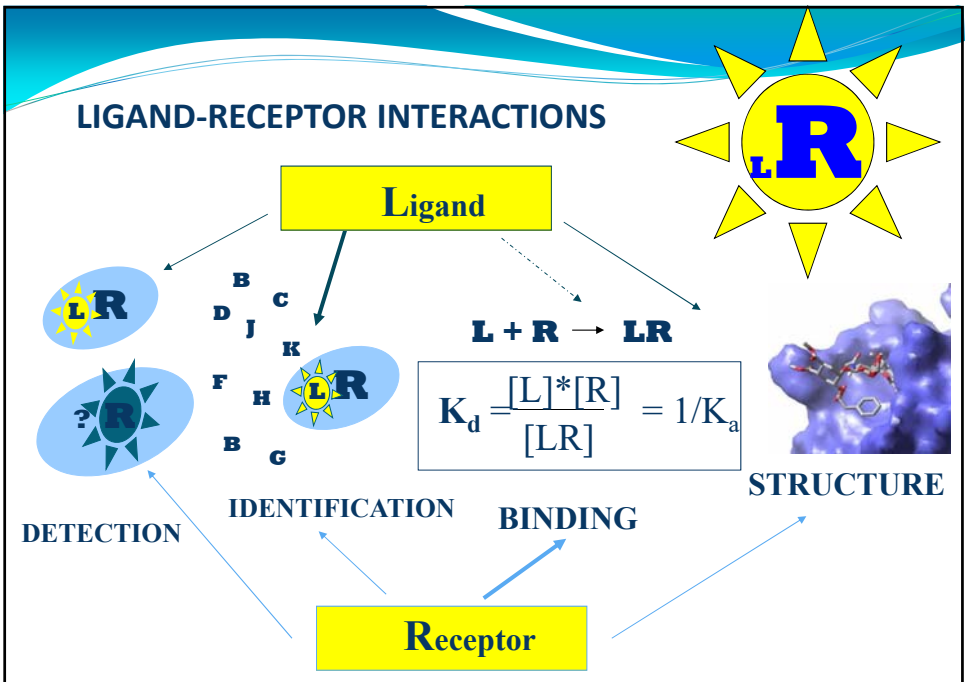
Time scale in NMR Dynamics



Protein – carbohydrate interactions exhibit low-medium affinity

Systems in fast exchange

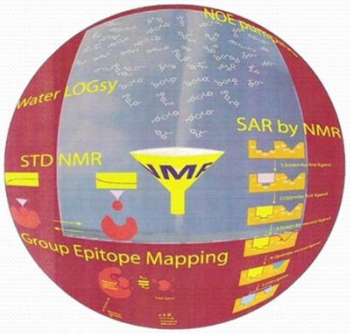
LIGAND-RECEPTOR INTERACTIONS



MOLECULAR INTERACTIONS BY NMR

Ligand- based NMR techniques

- **Transferred Noe (trNOE)**
- **Saturation Transfer Difference (STD NMR)**



- Small amount of protein
- Study of the interaction in solution
- Non destructive technique
- ^{15}N, ^{13}C labeling not required

Angewandte Chemie International Edition ,2003, vol. 8, pages 864-89

Ligand observation

$$L + R \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} LR$$

Two states equilibrium

L_{free} L_{bound} Molar fractions

FAST EXCHANGE

$$K_d = \frac{k_{off}}{k_{on\ diffusion\ controled}}$$

k_{off} = $>10^2$ (s^{-1})

k_{on} = $>10^7$ ($s^{-1}M^{-1}$)

$$R_{Lobs} = L_f * R_{Lf} + L_b * R_{Lb}$$

$$\Delta R = L_b * (R_{Lb} - R_{Lf})$$

Experimental procedure: $L_0 >> R_0$;

$L_0/R_0 > 10 - 100....$ $L_f >>> L_b$

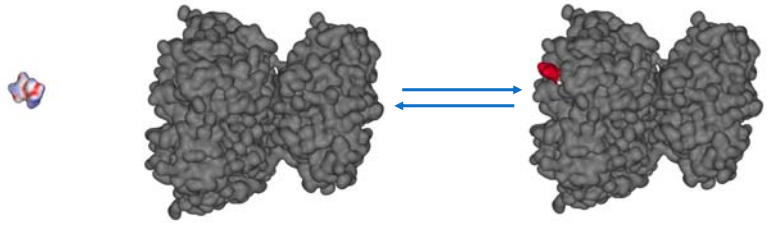
Necessary condition: $|(R_{Lb} - R_{Lf})| >> 0$

R_{Lb} Strong dependency on molecular size

NMR observable parameter R : NOE; Diffusion; Line Shape

LIGAND OBSERVED

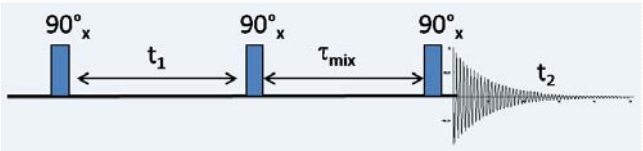
-RELAXATION IS PERTURBED



SMALL **LARGE**

TRANSFERRED NOE

Information on the ligand bioactive conformation



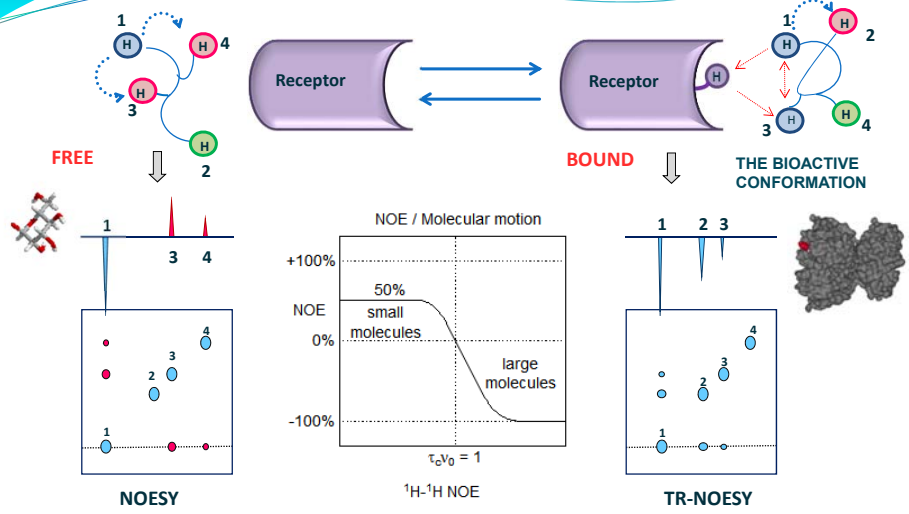
Ligand-protein 1:5

❖ **During the mixing time *inter* and *intra*-molecular NOE effects build up**
❖ ***Inter*-molecular tr-NOE effects are visible, intermolecular trNOEs are usually much larger than intramolecular effects**

Important notes

- ❖The mixing time must be short enough so that the contribution of the free ligand is negligible and long enough to allow visualization of the signal in the spectrum.
- ❖The molar ratio of ligand to receptor. It should be emphasized that the trNOESY experiment works well for ligands that have K_D in the range 10^{-3} – 10^{-6} M / mM- mM range
- ❖Small amount of purified receptor
- ❖Routinely used to probe ligand-receptor interaction

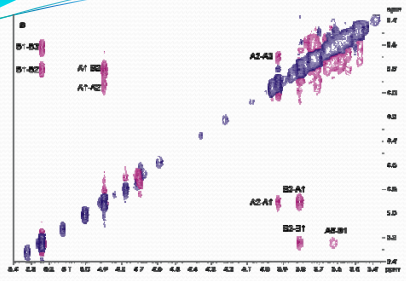
The bioactive conformation: Transfer NOESY

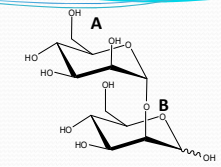


- Is There Any Binding?
- Which Is The Ligand Bioactive Conformation?

Chem Soc Rev 27 (1998) 133; Methods Enzymol (2003) 417; Curr Opin Struct Biol (1999) 549 , ibid (2003) 646

MBL - DISACCHARIDE





NOESY on free state ligand

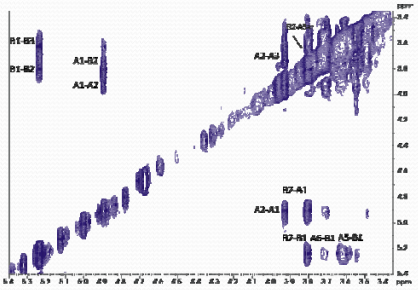
↓

Crosspeaks with opposite sign of the diagonal

tr-NOESY on bound state ligand

↓

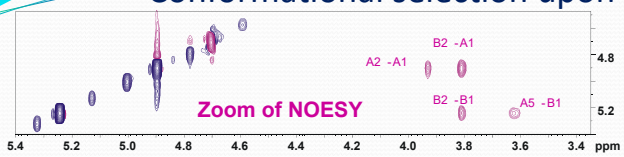
Crosspeaks with same sign of the diagonal

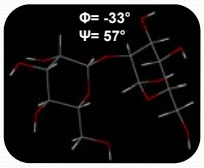


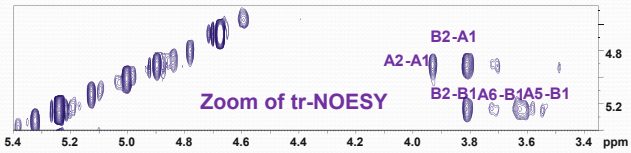
R. Marchetti, R. Lanzetta, I.C. Michelow, A. Molinaro, A. Silipo, Eur. J. Org. Chem., 2012, 27, 5275–5281

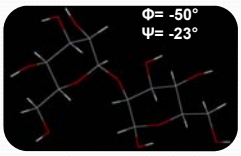
MBL - DISACCHARIDE

Conformational selection upon binding

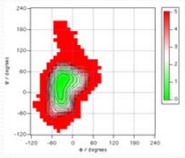








Distance	Experimental Free state	Experimental Bound state	Calculated Φ= -33° Ψ0 57°	Calculated Φ= -50° Ψ= -23°
A1-B2	2.3	2.35	2.4	2.5
A1-B1	nd	nd	2.7	4.3
B1-A5	2.6	2.5	2.7	2.4
B1-A6	nd	3.3	3.4	4.9

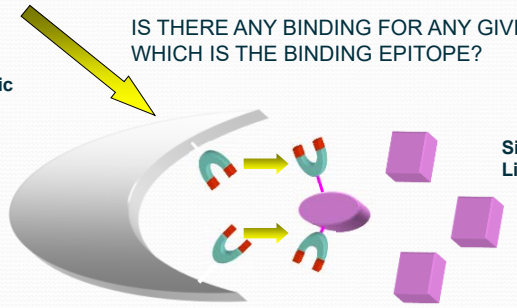


R. Marchetti, R. Lanzetta, I.C. Michelow, A. Molinaro, A. Silipo, Eur. J. Org. Chem., 2012, 27, 5275–5281

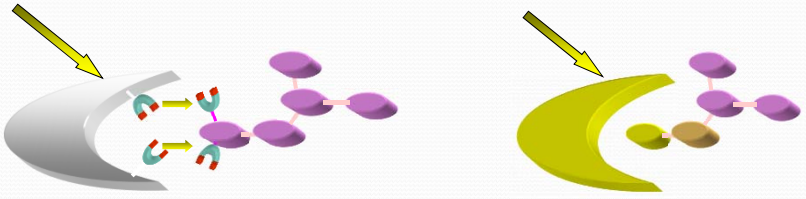
Saturation Transfer Difference NMR Spectroscopy – STD NMR

IS THERE ANY BINDING FOR ANY GIVEN COMPOUND?
WHICH IS THE BINDING EPITOPE?

IRRADIATION at the aromatic or aliphatic NMR regions

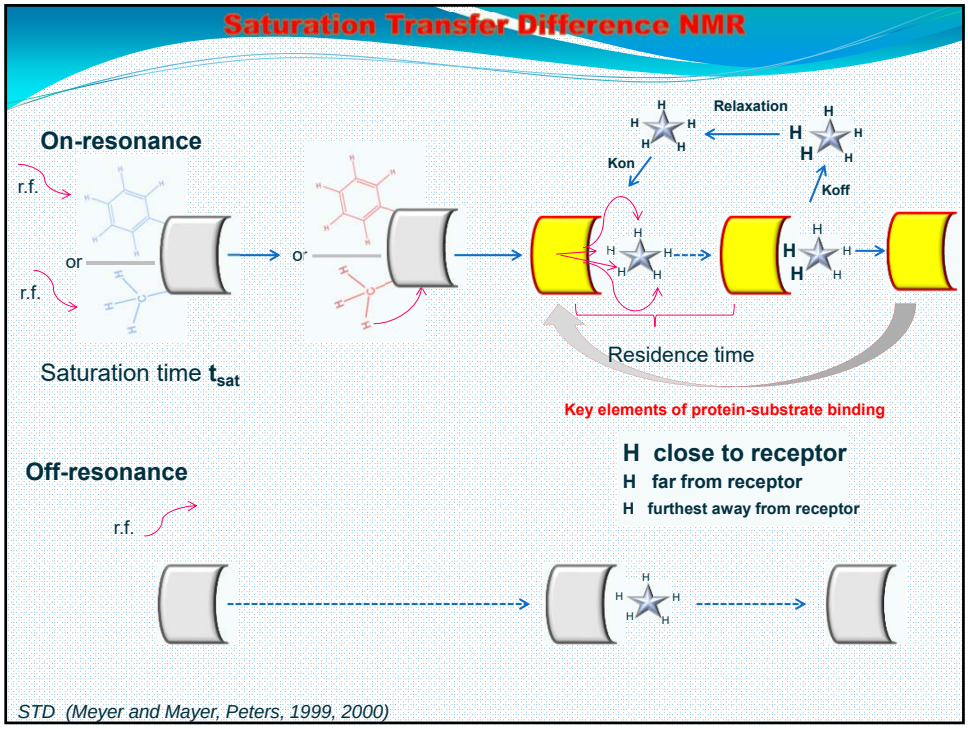


Single Compound or Library

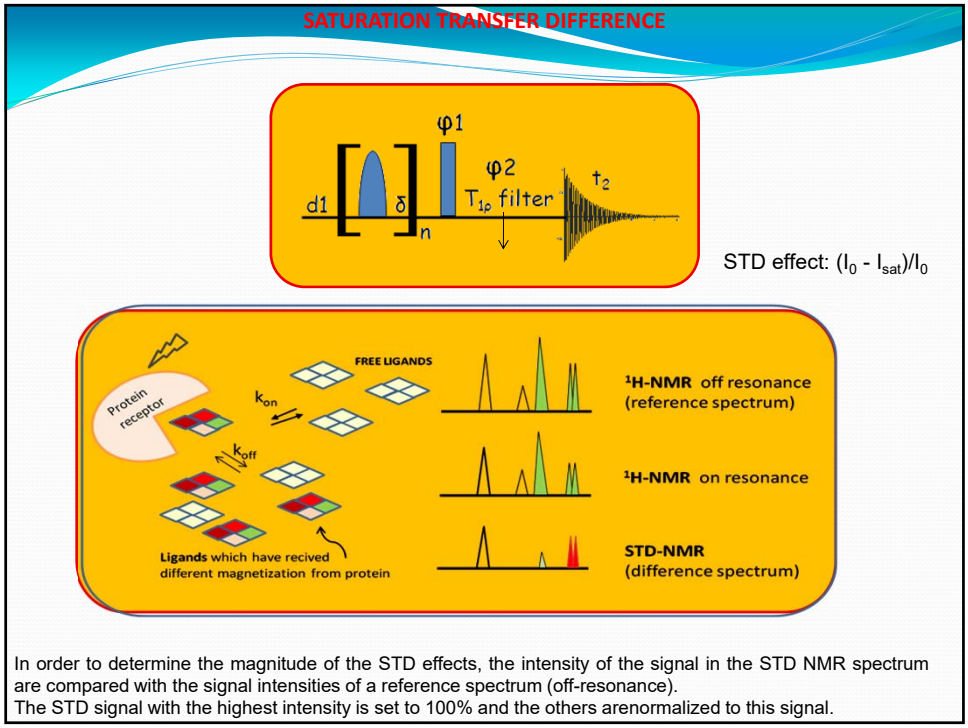


At long irradiation times, the saturation is transferred to the bound ligand, first to the protons belonging to the ligand epitope, then to the rest of the ligand

(Meyer and Mayer, Peters, 1999, 2000)



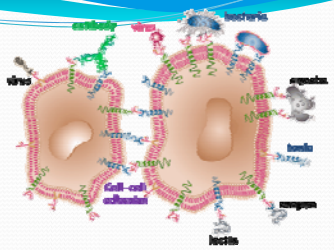
STD (Meyer and Mayer, Peters, 1999, 2000)



In order to determine the magnitude of the STD effects, the intensity of the signal in the STD NMR spectrum are compared with the signal intensities of a reference spectrum (off-resonance).
The STD signal with the highest intensity is set to 100% and the others arenormalized to this signal.

Host-microbe interactions

symbiotic *commensal* *pathogenic*



human-pathogen interactions

- mAb 5D8 – *B. anthina* LPS (*ChemBiochem* , **2013**)
- rhMBL – mannosides (*Eur. J. Org. Chem.*, **2012**)

microbe-microbe interactions

- BclA lectin – heptoses (*Glycobiology*, **2012**)
- *B. subtilis* PrkC – PGN (*Biochem. J.*, **2011**; *JACS*, **2011**)

plant-microbe interactions

- *C. japonicus* Xyl31A – xyloglucans (*Chemistry*, **2012**)
- *Plant LysM* – chitooligosaccharides (PNAS, under second revision, 2013)

GLYCOSIDE HYDROLASE (GH) – XYLOGLUCAN

Xyloglucan

ubiquitous plant polysaccharides

cell strength and shape

defensive barrier

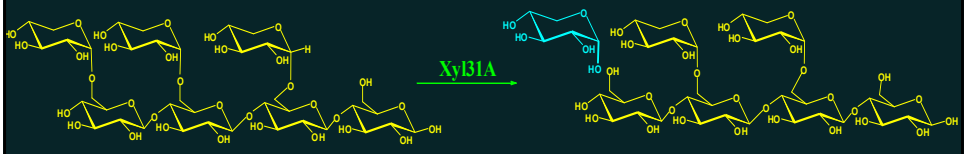
carbohydrate store

Xyl31A: α-xylosidase from *Cellvibrio japonicus*

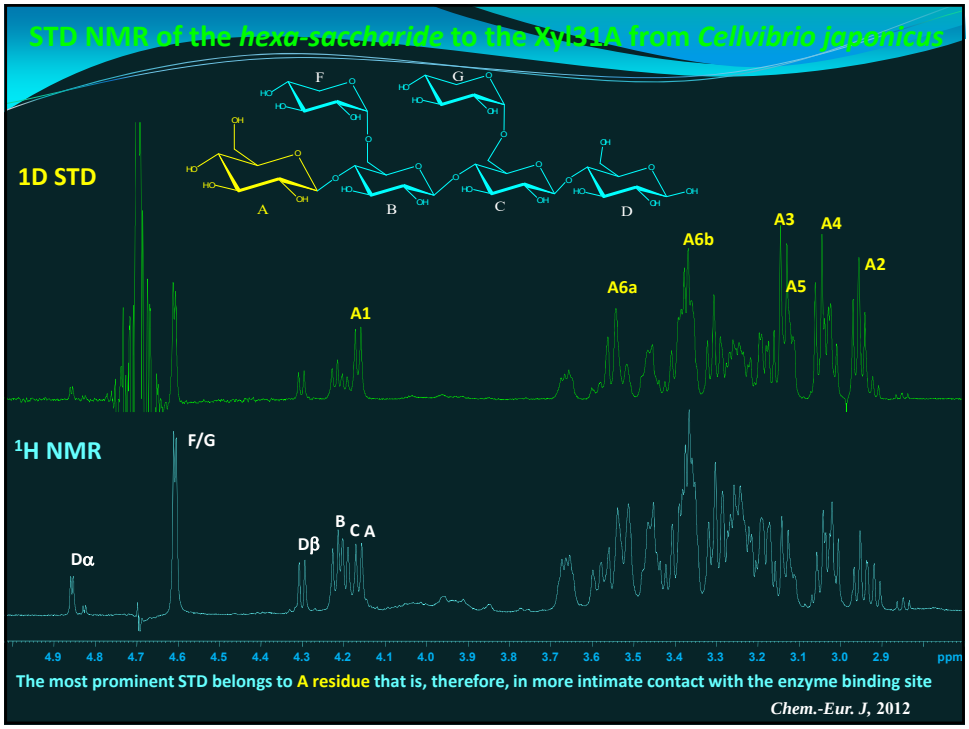
member of glycoside hydrolase family 31

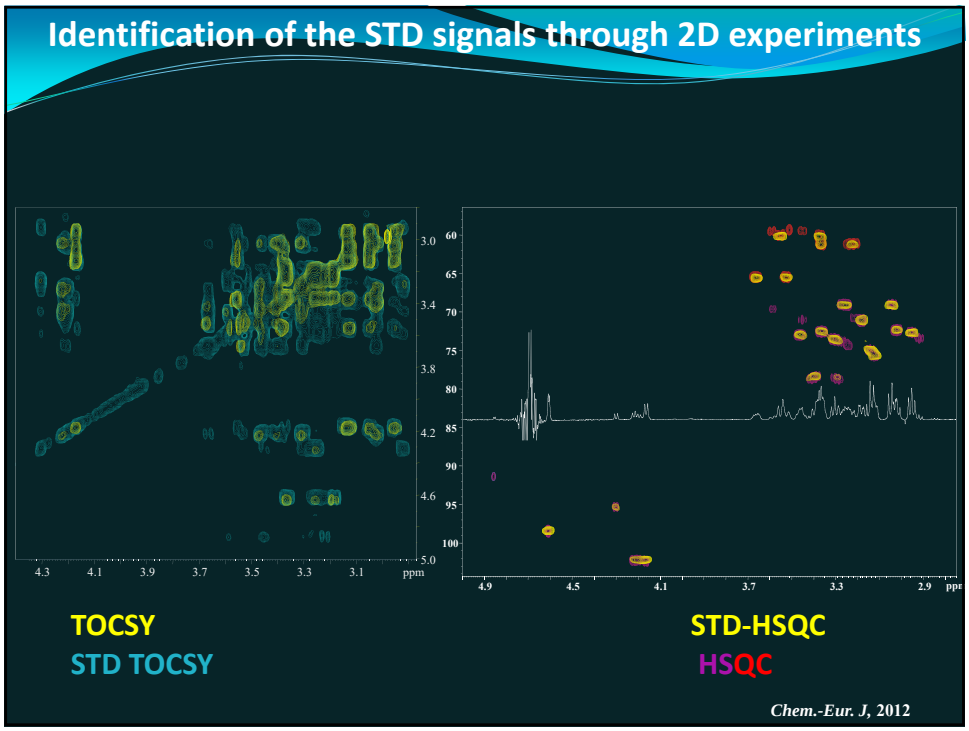
exo-active enzyme

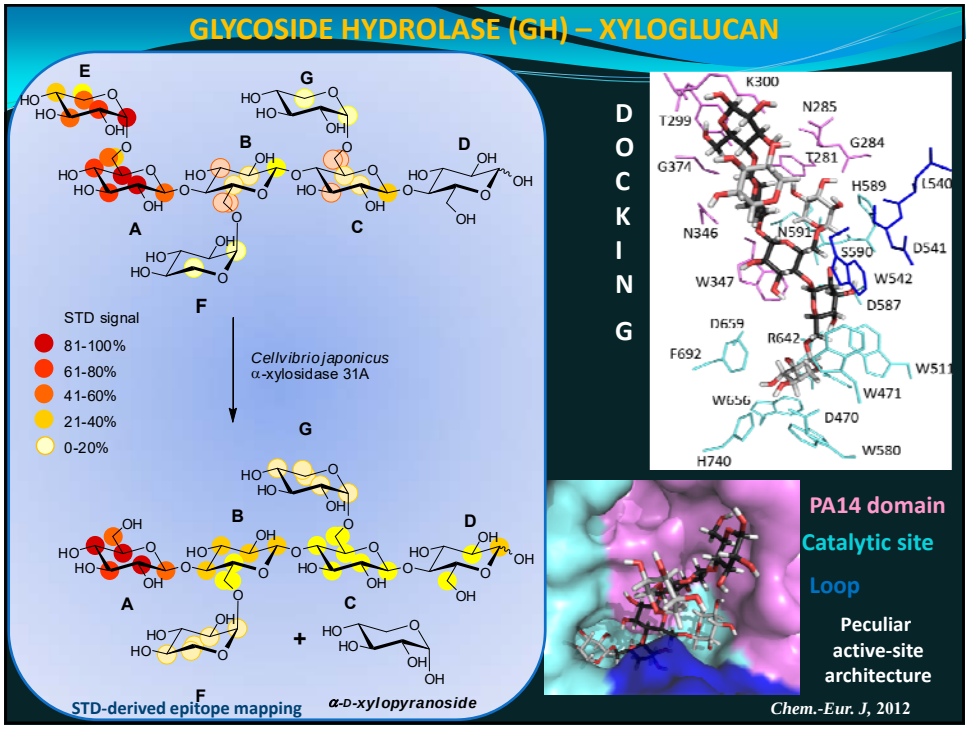
Preference for long substrates (appended PA14 domain)



J. Larsbrink, A. Izumi, F. Ibatullin, A. Nakhai, H.J. Gilbert, G.J. Davies, H. Brumer, *Biochem. J.*, **2011**


STD NMR of the hexa-saccharide to the Xyl31A from Cellvibrio japonicus
1D STD
F
G
A
B
C
D
A1
A6a
A6b
A3
A4
A5
A2
1H NMR
F/G
Dα
Dβ
B
C A
4.9 4.8 4.7 4.6 4.5 4.4 4.3 4.2 4.1 4.0 3.9 3.8 3.7 3.6 3.5 3.4 3.3 3.2 3.1 3.0 2.9 ppm
The most prominent STD belongs to A residue that is, therefore, in more intimate contact with the enzyme binding site
Chem.-Eur. J, 2012


Identification of the STD signals through 2D experiments
3.0
3.4
3.8
4.2
4.6
5.0
4.3 4.1 3.9 3.7 3.5 3.3 3.1 ppm
60
65
70
75
80
85
90
95
100
4.9 4.5 4.1 3.7 3.3 2.9 ppm
TOCSY
STD TOCSY
STD-HSQC
HSQC
Chem.-Eur. J, 2012


GLYCOSIDE HYDROLASE (GH) – XYLOGLUCAN
STD signal
81-100%
61-80%
41-60%
21-40%
0-20%
Cellvibrio japonicus
α-xylosidase 31A
STD-derived epitope mapping
α-D-xylopyranoside
DOCKING
K300
N285
T299
G284
T281
G374
H589
L540
N346
N591
S590
D541
W347
W542
D587
D659
R642
F692
W511
W471
W656
D470
W580
H740
PA14 domain
Catalytic site
Loop
Peculiar active-site architecture
Chem.-Eur. J, 2012


Questions?

Optional material

Gas Chromatography-
Mass Spectrometry (GC-MS)
power and limits
(for carbohydrates)

Gas Chromatography – Mass Spectrometry

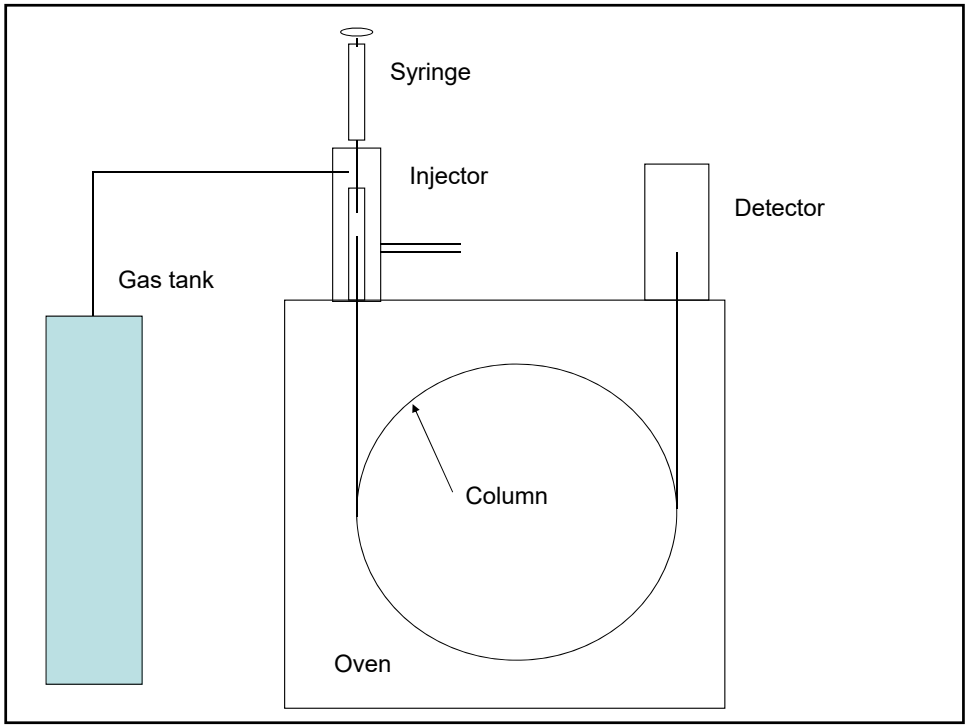
- Components (EI GC-MS)
- Monosaccharide analysis as:
  - Acetylated Alditols
  - Partially Methylated Acetylated Alditols
  - Acetylated Methylglycosides
  - Acetylated Octyl Glycosides

Function

- Separation of volatile organic compounds
- Volatile – when heated, VOCs undergo a phase transition into intact gas-phase species
- Separation occurs as a result of unique equilibria established between the solutes and the stationary phase (the GC column)
- An inert carrier gas carries the solutes through the column

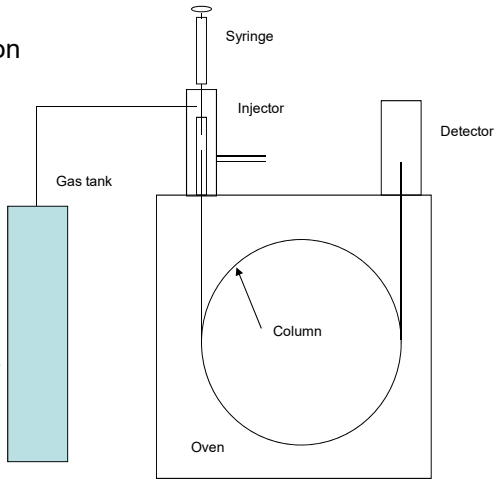
Components

- Carrier Gas, N_2 or He, 1-2 mL/min
- Injector
- Oven
- Column
- Detector

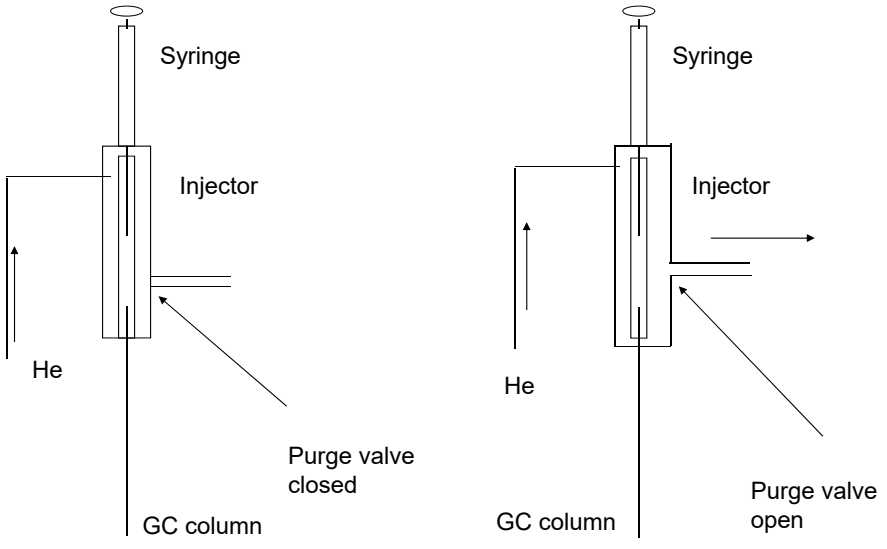


Injector

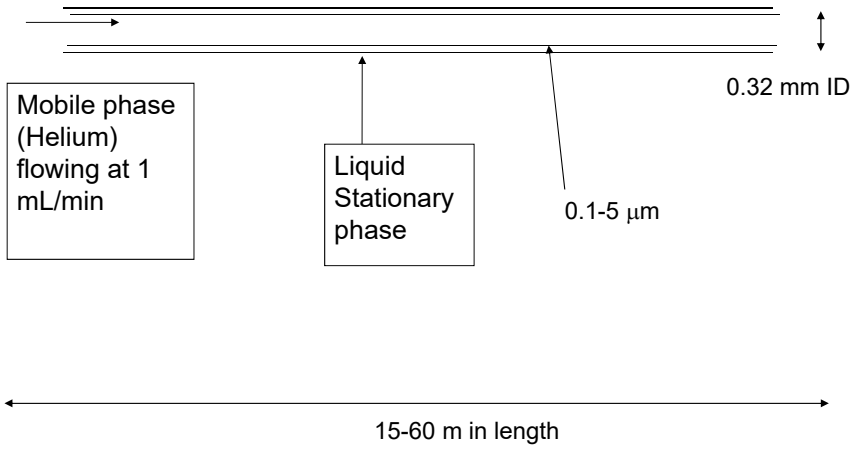
- A GC syringe penetrates a septum to inject sample into the vaporization camber
- Instant vaporization of the sample, 280 °C
- Carrier gas transports the sample into the head of the column
- Purge valve controls the fraction of sample that enters the column



Splitless (100:90) vs. Split (100:1)



Open Tubular Capillary Column

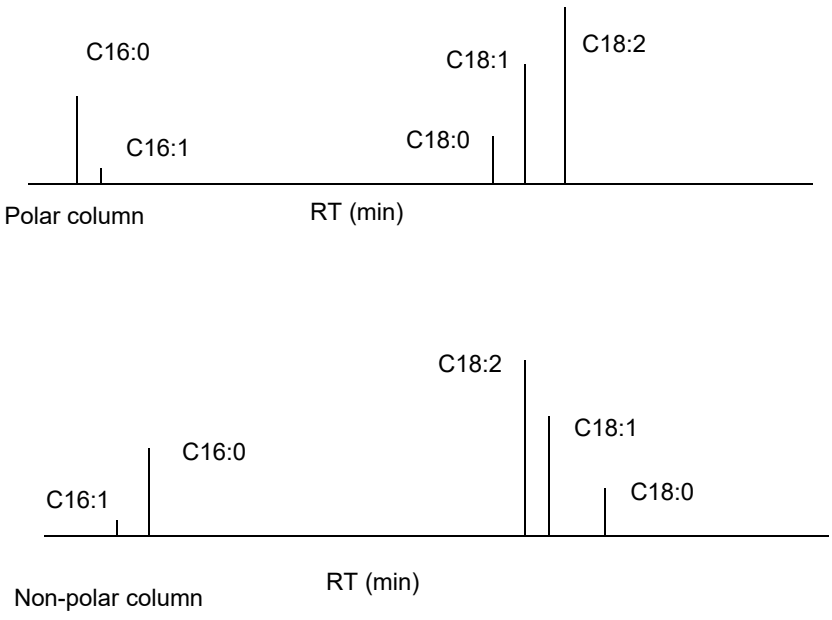


Fused Silica Open Tubular (FSOT) columns

- Coated with polymer, crosslinked
  - Polydimethyl siloxane (non-polar)
  - Poly(phenylmethyldimethyl) siloxane (10% phenyl)
  - Poly(phenylmethyl) siloxane (50% phenyl)
  - Polyethylene glycol (polar)
  - Poly(dicyanoallyldimethyl) siloxane
  - Ploy(trifluoropropyldimethyl) siloxane

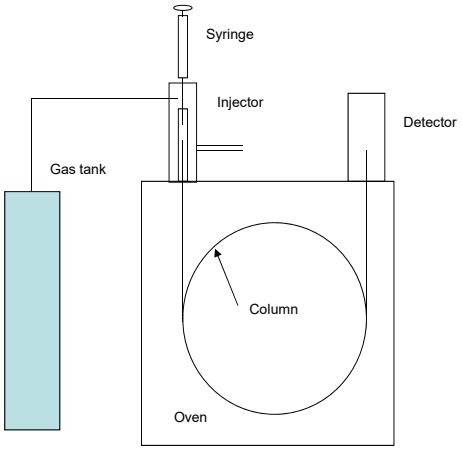
Polar vs. nonpolar

- Separation is based on the vapor pressure and polarity of the components.
- Within a homologous series (alkanes, alcohol, olefins, fatty acids) retention time increases with chain length (or molecular weight)
- Polar columns retain polar compounds to a greater extent than non-polar
  - C18 saturated vs. C18 saturated methyl ester

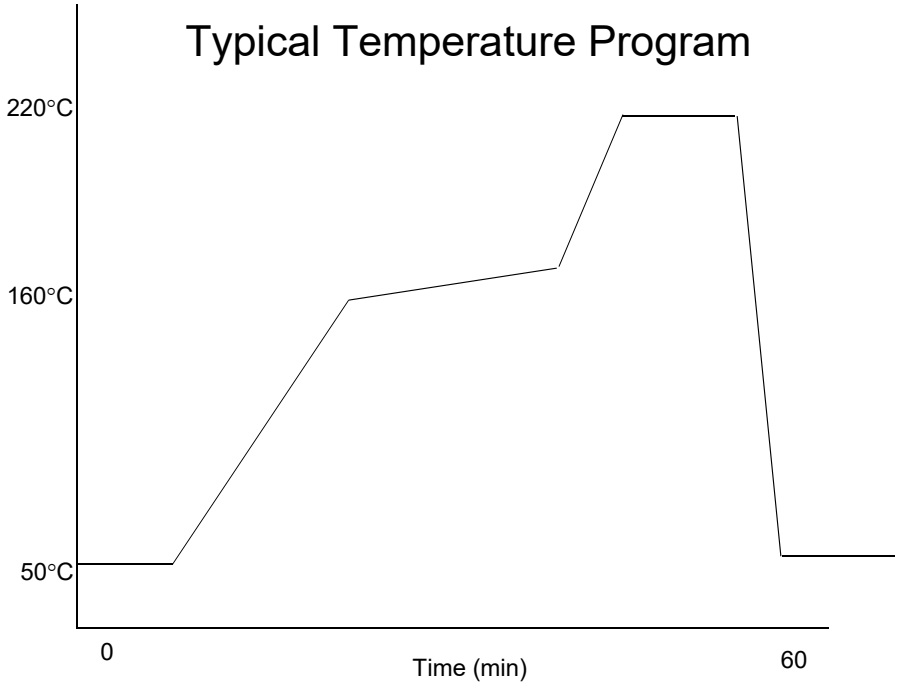


Oven

- Programmable
- Isothermal- run at one constant temperature
- Temperature programming - Start at low temperature and gradually ramp to higher temperature
  - More constant peak width
  - Better sensitivity for components that are retained longer
  - Much better chromatographic resolution
  - Peak refocusing at head of column



Typical Temperature Program



Detectors

- Flame Ionization Detectors (FID)
- Electron Capture Detectors (ECD)
- Electron impact/chemical ionization (EI/CI) Mass spectrometry

What kind of info can mass spec give you?

- Molecular weight
- Elemental composition (low MW with high resolution instrument)
- Structural info (hard ionization or CID)

How does it work?

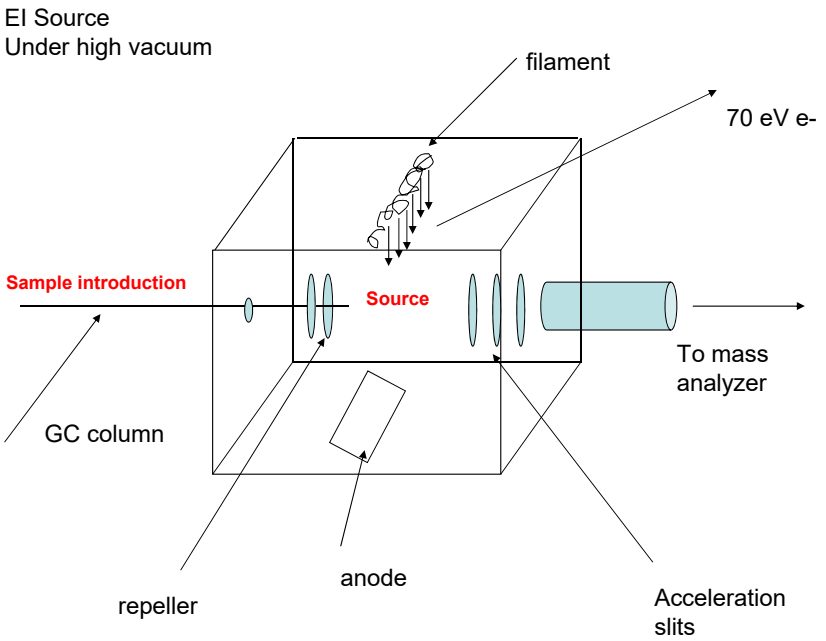
- Gas-phase ions are separated according to mass/charge ratio and sequentially detected

Parts of a Mass Spec

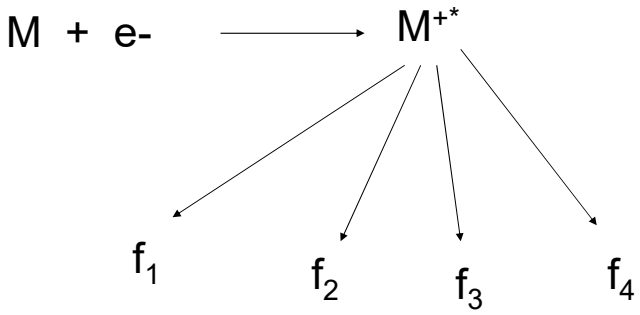
- Sample introduction
- Source (ion formation)
- Mass analyzer (ion sep.) - high vac
- Detector (electron multiplier tube)

EI, CI

- EI (hard ionization)
  - Gas-phase molecules enter source through heated probe or GC column
  - 70 eV electrons bombard molecules forming M+* ions that fragment in unique reproducible way to form a collection of fragment ions
  - EI spectra can be matched to library stds
- CI (soft ionization)
  - Higher pressure of methane leaked into the source (mtorr)
  - Reagent ions transfer proton to analyte



EI process

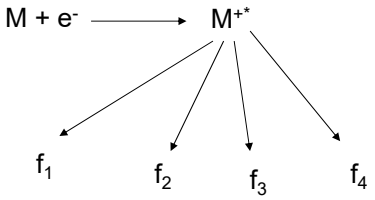


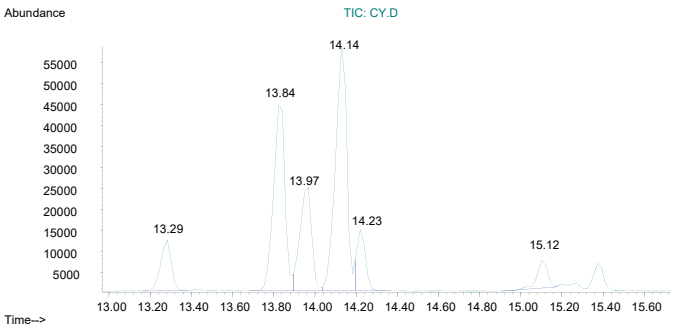
This is a remarkably reproducible process. M will fragment in the same pattern every time using a 70 eV electron beam

GC-MS chromatogram

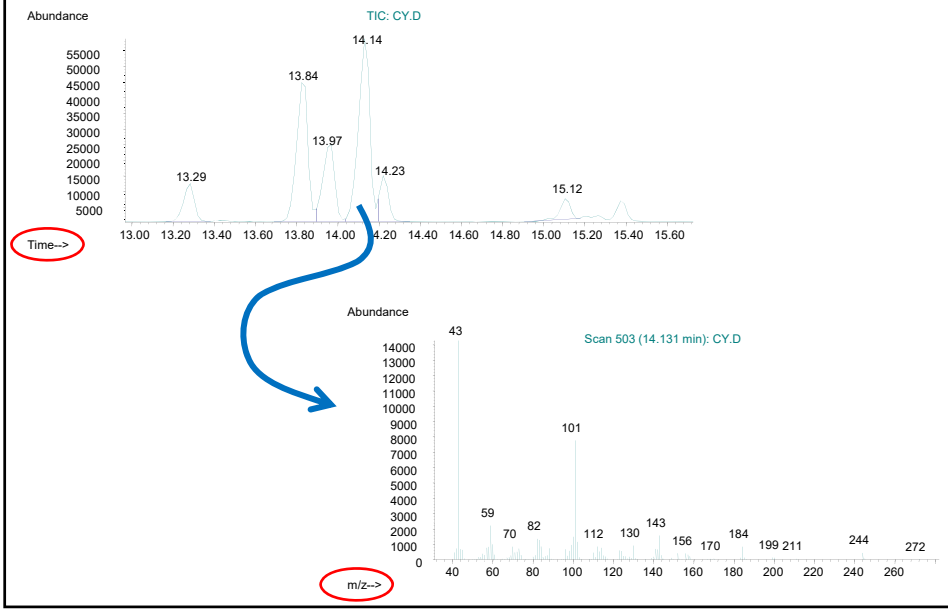
TIC: Total Ion Chromatography

Abundance = Σf_i^+





GC-MS chromatogram



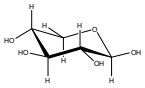
Mass Analyzers

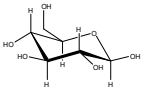
- Low resolution
  - Quadrupole
  - Ion trap
- High resolution
  - TOF time of flight
  - Sector instruments (magnet)
- Ultra high resolution
  - ICR ion cyclotron resonance

Gas Chromatography – Mass Spectrometry

- Monosaccharide composition as:
  - Acetylated Alditols
  - Acetylated Methyl glycosides
- Additional info
  - Partially Methylated Acetylated Alditols
  - Acetylated Octyl Glycosides
  - N.B.: amount of sample required ~ 0.2 mg

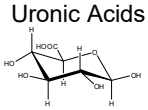
Monosaccharide diversity

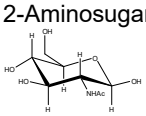
Pentose  8

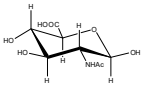
Hexose  16

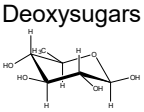
Ulosonic acids

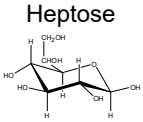
Kdo
Sialic acid
Legionamminic acid
….

Uronic Acids  16

2-Aminosugars  16

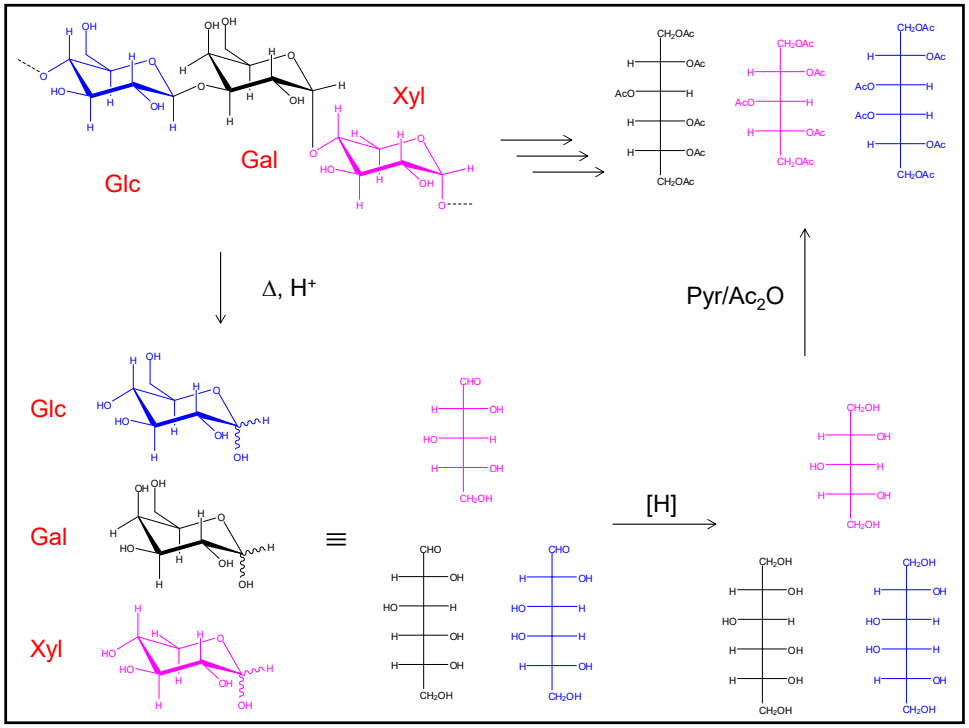
2-Aminuronic acids  16

Deoxysugars  16

Heptose  32

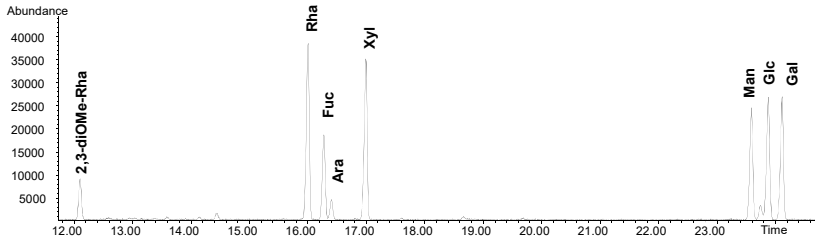
Dideoxysugars
Aminodeoxysugars
Branched sugars
…..
∞

Acetylated Alditols



Acetylated Alditols: advantages and limits

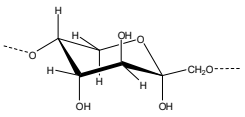
- Advantages:
  - one residue ⇒ one peak … except for ketoses
  - No special reaction conditions setup required
  - Suitable for neutral sugars (aldose and ketoses) and aminosugars



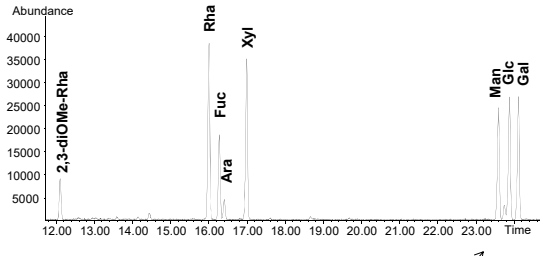
Acetylated alditols, example …

1 sugar ⇒ 1 peak,…

except for ketoses

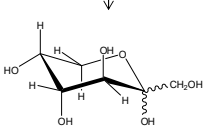


Fructose

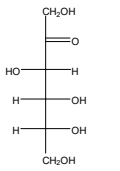


Ac_2O, Pyr

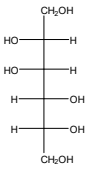
H^+



≡

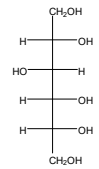


[H]



Man-ol

+



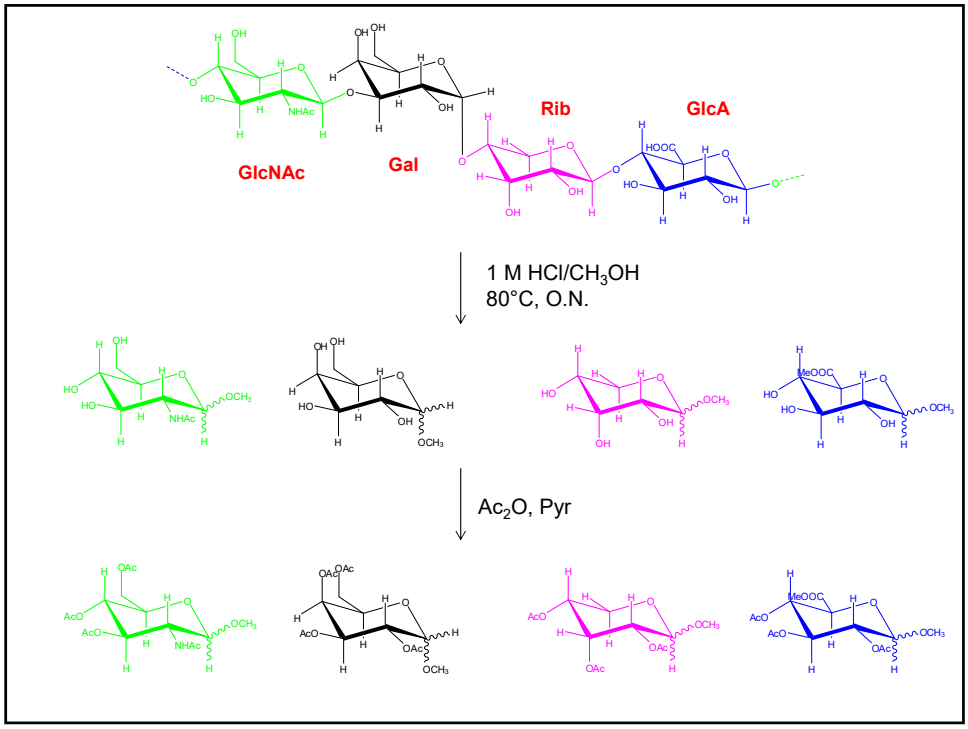
Glc-ol

Acetylated Alditols: advantages and limits

- Limits:
  - Free emiacetals degrade during hydrolysis
  - Care needed for hydrolysis conditions selection
    - Ideal conditions: 100% hydrolysis – 0% degradation
      - Ketoses linkages are more labile than those of hexoses
      - Aminosugars linkages are very strong
      - Sugars carrying an aminosugar or an uronic acid are understimated
  - Acidic monosaccharides are not detected even if their hydrolysis occurs

Solution: other types of derivatives

Acetylated Methyl glycosides

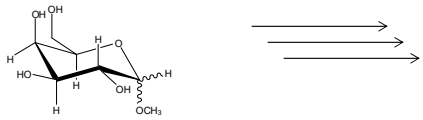


Acetylated Methyl glycosides

- Advantages:
  - Less reactions' step compared to Acetylated Alditols
  - O.N. reaction yields to almost complete methanolysis of the product
  - No free aldehyde group is produced during methanolysis ⇒ monosaccharide degradation is minimized
  - Suitable for most type of sugars
    - Hexoses
    - Aminosugars
    - Uronic acid
    - Ulosonic acids
    - ……

Acetylated Methyl glycosides

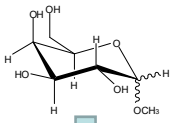
- Limits:
  - One sugar ⇒ more peaks
  - Respect anhydrous conditions during methanolysis
  - Ketose residues are lost



Galactose

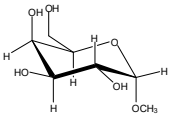
Acetylated Methyl glycosides

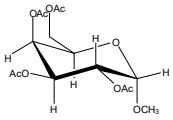
Galactose



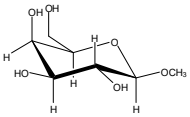
One sugar …
4 signals!!

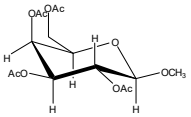
α-Gal*p*



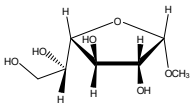


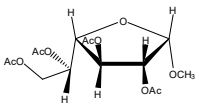
β-Gal*p*



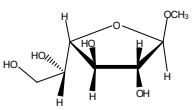


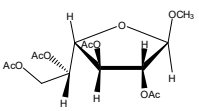
α-Gal*f*



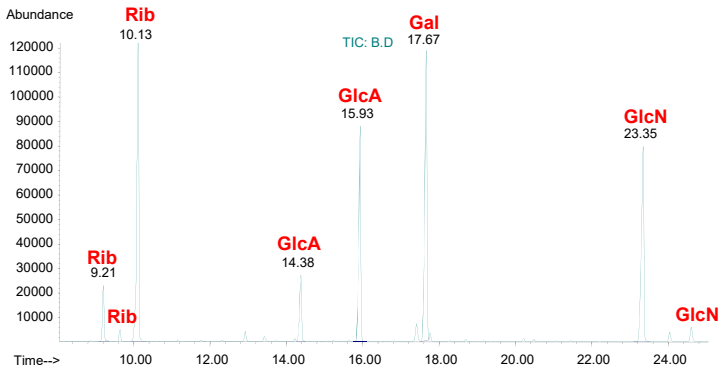


β-Gal*f*





Acetylated Methyl glycosides



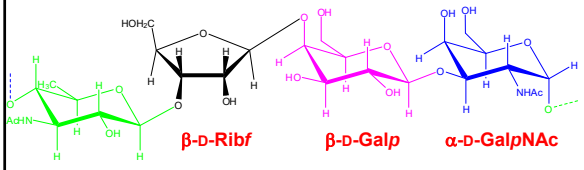
1 sugar ⇒ more peaks

An advantage or a limit?

GC-MS
not only composition but also for other info

- Partially Methylated Acetylated Alditols ⇒ Substitution Pattern
- Acetylated Octyl Glycosides ⇒ Absolute Configuration

Partially Methylated Acetylated Alditols

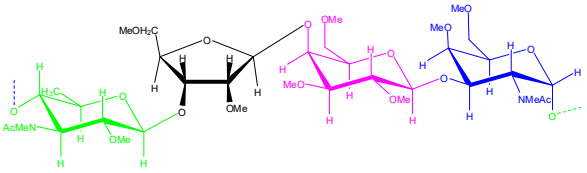


β-D-Rib*f* β-D-Gal*p* α-D-Gal*p*NAc

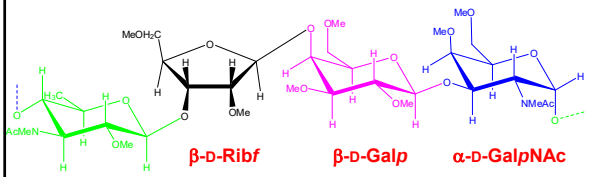
β-D-Qui*p*3NAc

O-antigen from *E. coli* O5:K4:H4

Exhaustive polysaccharide methylation



Partially Methylated Acetylated Alditols

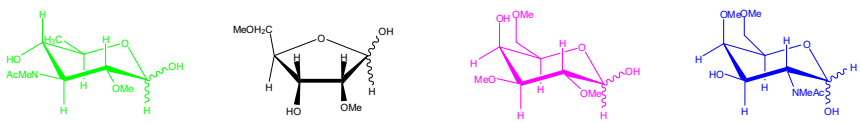


β-D-Rib*f* β-D-Gal*p* α-D-Gal*p*NAc

β-D-Qui*p*3NAc

All free –OH are transformed in methyl-ethers

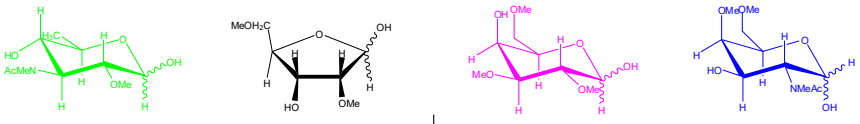
[H⁺, Δ]



Hydrolysis frees those –OH groups previously engaged in a linkage

Partially Methylated Acetylated Alditols

D-Qui3NAc D-Rib D-Gal D-GalNAc

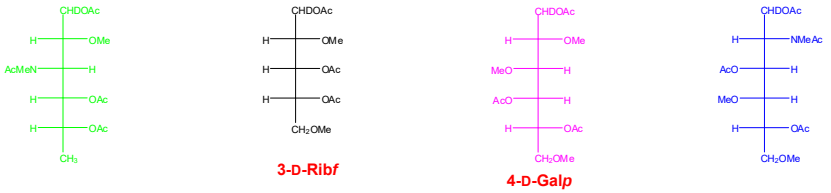


[H]
Reduction usually performed with deuterated hydride

Anomeric position is marked with a deuterium

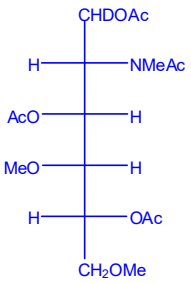
Ac_2O, Pyr

Partially Methylated Acetylated Alditols



4-D-Qui*p*3NAc 3-D-Rib*f* 4-D-Gal*p* 3-D-GalNAc

Features of each sugar derivative: 3-linked GalNAc as example



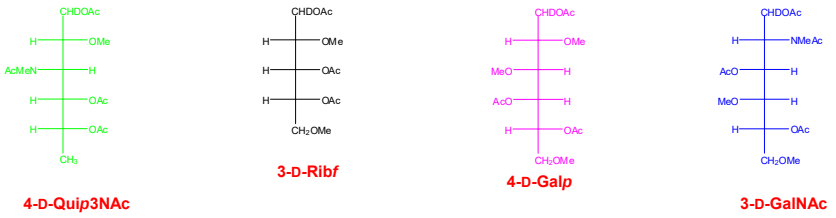
Each PMAA must have:

- One CHDOAc group deriving from the anomeric carbon
- One H-C-Oac deriving from OH- group involved in sugar cyclization

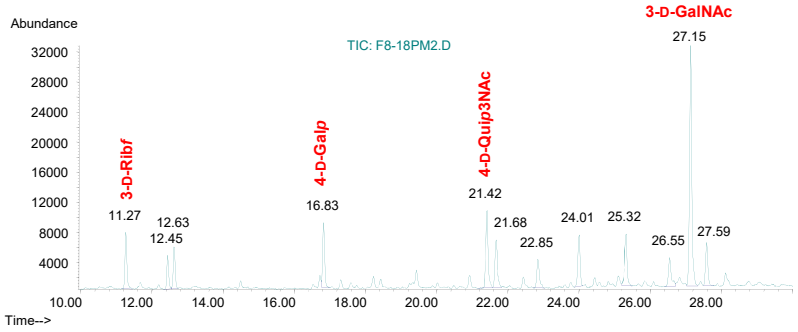
Aside from the two "musts"

- Each H-C-OMe indicates a free OH- group in the polysaccharide
- Each H-C-OAc (if present) indicates a substituted OH- group

Partially Methylated Acetylated Alditols



4-D-Quip3NAc **3-D-Ribf** **4-D-Galp** **3-D-GalNAc**



Partially Methylated Acetylated Alditols

Advantages:

•Interpretation rules easy and clear

•One analysis determines the substitution pattern of the residues in the polysaccharides

•Analysis almost mandatory to understand complex poly/oligosaccharide

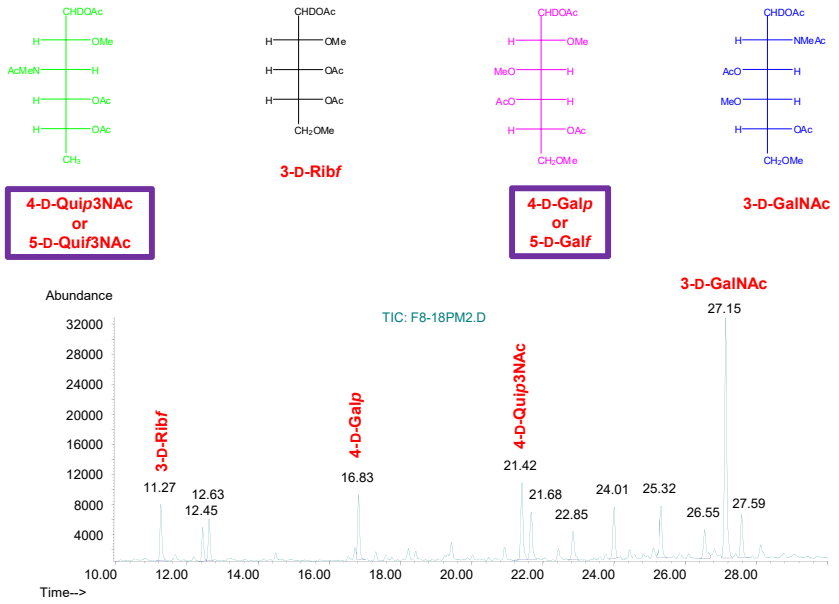
Limits:

•Polysaccharide undermethylation yields to false results

•Procedure needs to be adapted for uronic acids detection

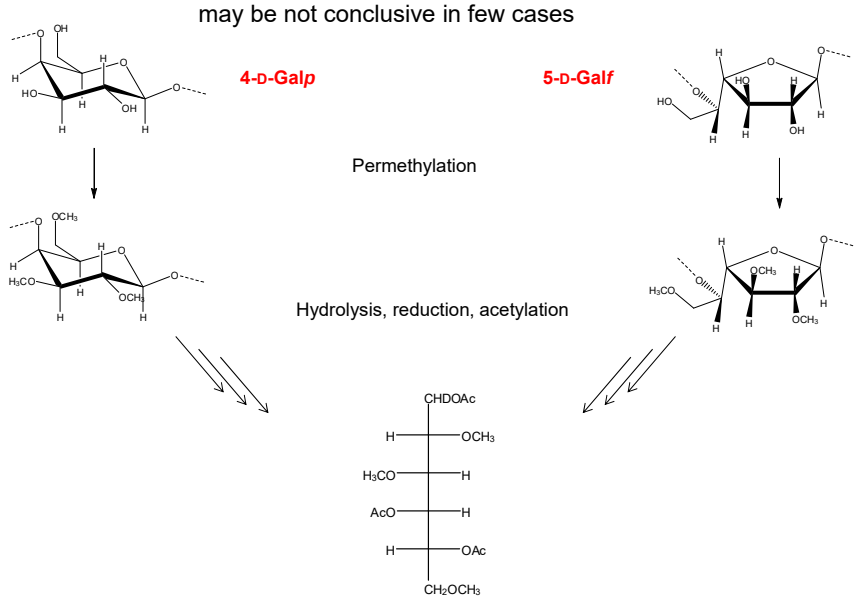
•Even if interpretation of PMAA is clear, it may be not conclusive in few cases

Partially Methylated Acetylated Alditols



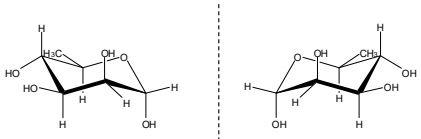
Partially Methylated Acetylated Alditols

•Even if interpretation of PMAA is clear, it may be not conclusive in few cases



Acetylated Octyl Glycosides
(Absolute configuration determination)

Many sugars exist in both configuration: es. D-Rha and L-Rha

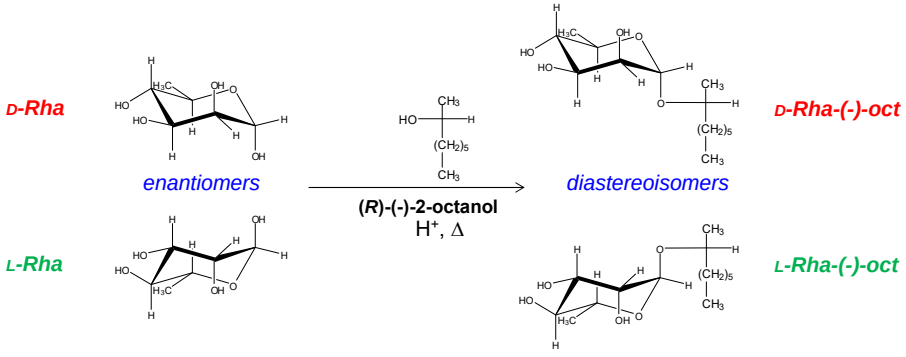


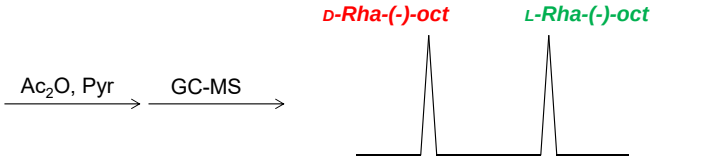
enantiomers

Problem: acetylated methyl glycosides or acetylated alditols do not discriminate among the two forms; the MGA (or AA) are still enantiomers

Solution: derivation of enantiomeric sugars in diastereoisomers, as 2-octylglycosides

Acetylated Octyl Glycosides

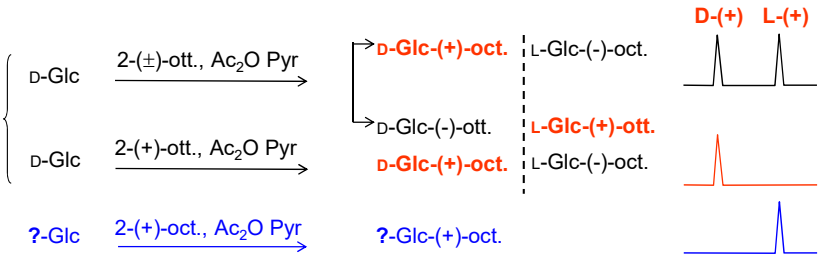




Acetylated Octyl Glycosides

Standard Preparation

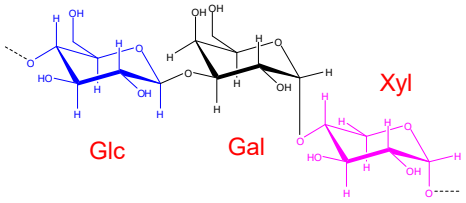
- Many sugar configurations are rare and the corresponding monosaccharide are not commercially available
- Standards are prepared using one sugar configuration and a combination of enantiomeric pure and racemic 2-octanol



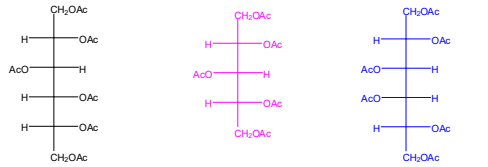
Interpretation of GC-MS carbohydrate spectra

Refer to:
Lonngren, J. and S. Svensson, *MASS SPECTROMETRY IN STRUCTURAL ANALYSIS OF NATURAL CARBOHYDRATES. Advances in Carbohydrate Chemistry and Biochemistry, 1974.* ***29: p. 41-106.***

Acetylated Alditols



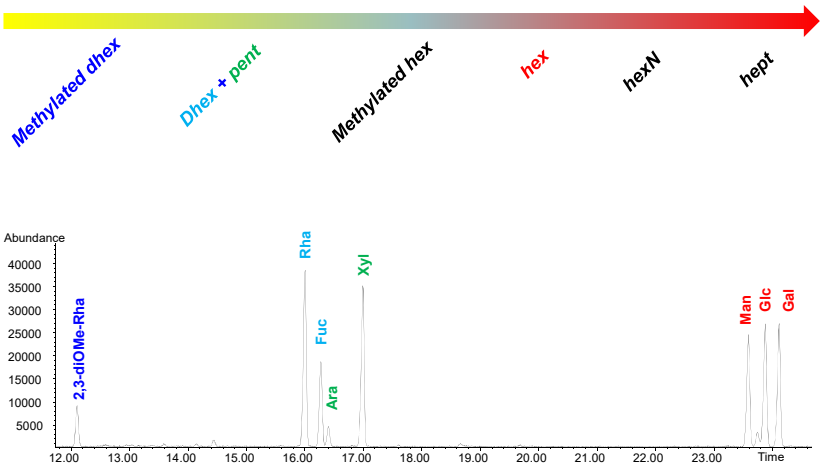
- Acid hydrolysis
- Reduction
- Acetylation



Peracetylated glucitol | Peracetylated xylitol | Peracetylated galactitol

Acetylated Alditols:

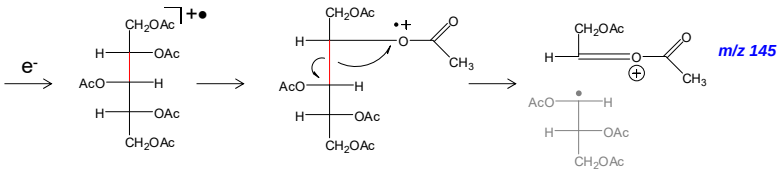
Elution time is important



Acetylated Alditols:

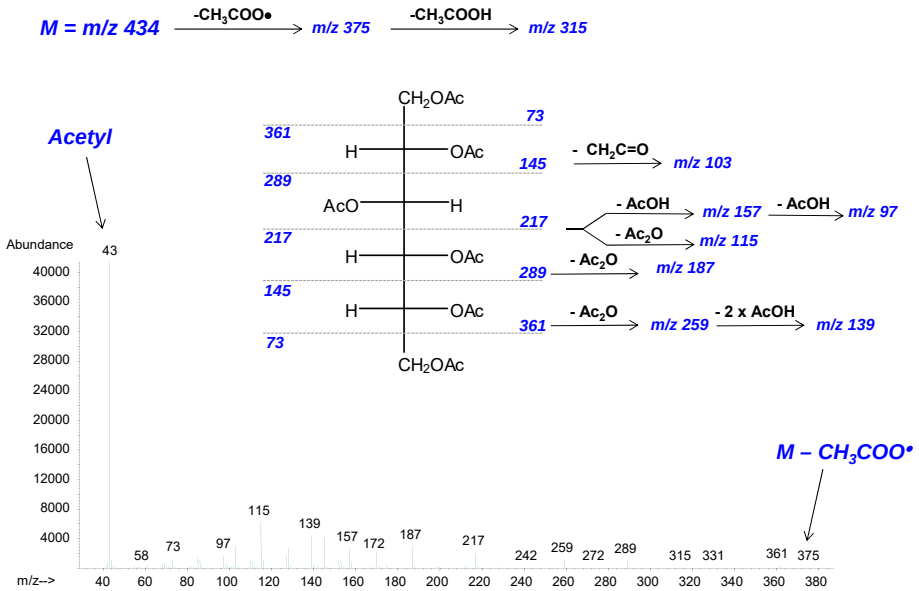
Fragmentation pattern, few rules to understand part/most of the fragments:

- Mass spectra of stereoisomers (as Glc and Gal) are very similar
- Molecular ion is never detected
- Primary ions are formed by
  - Elimination of an acetoxyl group ($CH_3COO^{\bullet}$)
  - from alditol backbone rupture

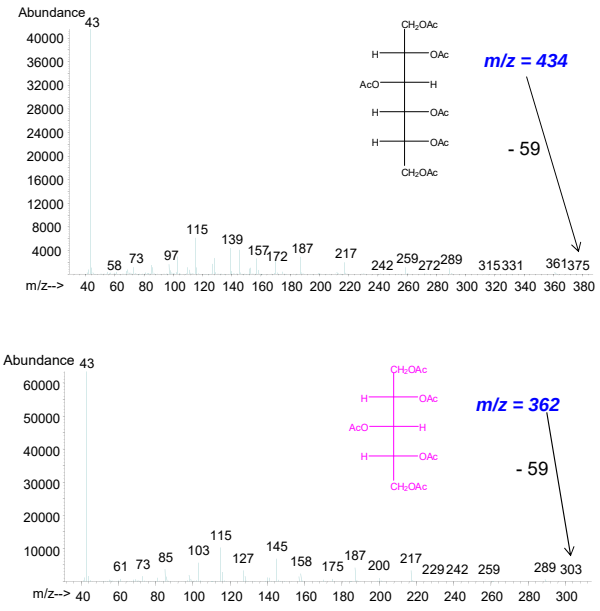


- Intensity of the primary fragments decreases with increasing molecular weight
- Primary ion further loose neutral molecules:
  - AcOH (m/z 60), Ac_2O (m/z 102) or CH_2=C=O (m/z 42)

Acetylated Alditols: hexitol

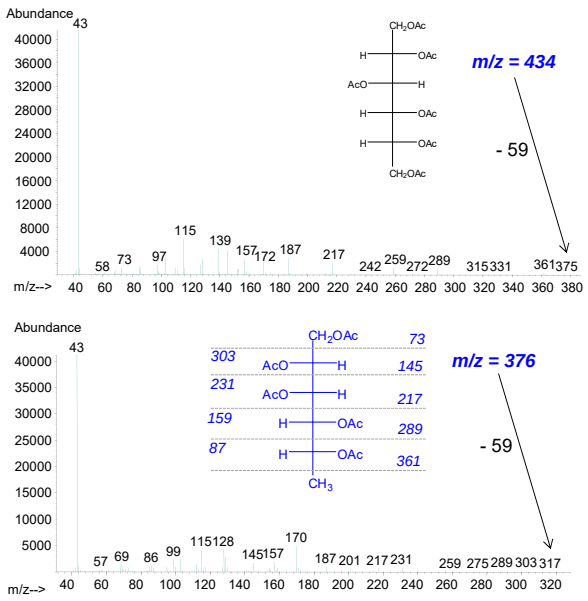


Acetylated Alditols: hexitol vs pentitol



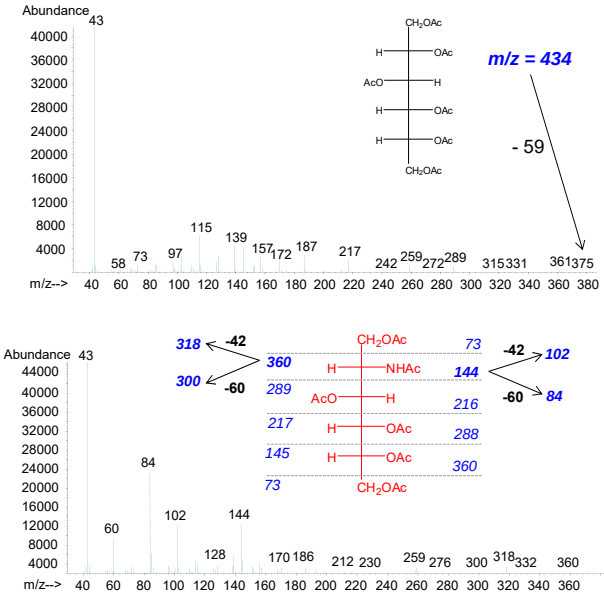
- Very similar EI-MS spectra
- Low m/z almost identical
- High m/z values discriminate among the two alditols but …
- High m/z values are less abundant fragment and might be undetected

Acetylated Alditols: hex vs d-hex

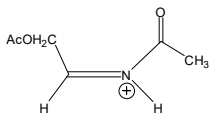


- Different fragment pattern of the EI-MS spectra
- Differences related to the occurrence of the methyl group of the 6-deoxysugar (rhamnose)

Acetylated Alditols: hex-ol vs HexN-ol

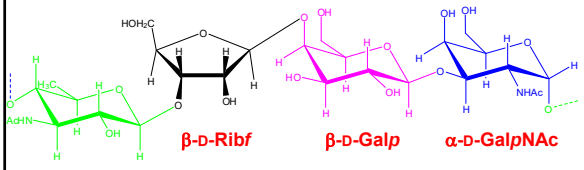


- Very different EI-MS spectra
- Fragmentation containig NHAc are more important than others
- Superior ability of nitrogen with respect to oxygen to stabilize the intermediate cation



Partially Methylated and Acetylated Alditols: PMAA

Partially Methylated Acetylated Alditols



β-D-Rib*f* β-D-Gal*p* α-D-Gal*p*NAc

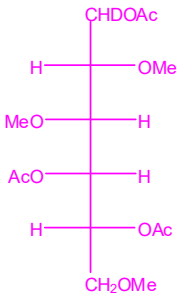
β-D-Qui*p*3NAc

O-antigen from *E. coli* O5:K4:H4

Methylation
Hydrolysis
Reduction
acetylation

Partially Methylated Acetylated Alditols

Features of each sugar derivative: 4-linked Gal as example



Each PMAA must have:

- One CHDOAc group deriving from the anomeric carbon
- One H-C-OAc deriving from OH- group involved in sugar cyclization

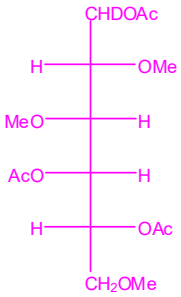
Aside from the two "musts"

- Each H-C-OMe indicates a free OH- group in the polysaccharide
- Each H-C-OAc (if present) indicates a substituted OH- group

Interpretation rules follow those from Acetylated Alditols

Some few integration are necessary

Partially Methylated Acetylated Alditols



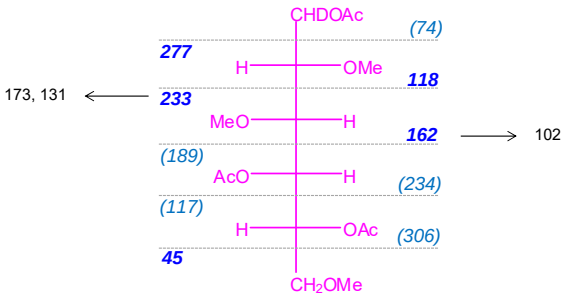
PMAA fragmentation pattern, extension of AA rules

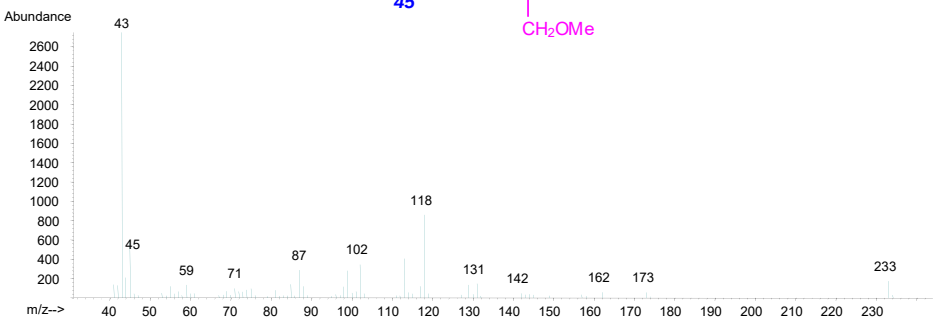
- Mass spectra of stereoisomers (as Glc and Gal) are very similar
- Molecular ion is never detected
- Primary ions are formed by from alditol backbone rupture
- Backbone rupture is governed by the stability of the fragment formed
  - **Fission among two methoxyl-bearing carbons is preferred with respect that among one methoxyl and one acetoxyl.**
  - **Fission among two acetoxyl bearing carbons is neglectable**
- **The charged fragment is always that with the methoxy group**
- Intensity of the primary fragments decreases with increasing molecular weight
- Primary ion further loose neutral molecules:
  - AcOH (m/z 60), Ac_2O (m/z 102) or CH_2=C=O (m/z 42)
  - **But also CH_3OH (m/z 32), CH_2O (m/z 30)**

Partially Methylated Acetylated Alditols

Remember:

CHDOAc	= 74
H-C-OMe	= 44
H-C-OAc	= 72
CH_2OAc	= 73
CH_2OMe	= 45
H-C-NMeAc	= 85
CH_3	= 15



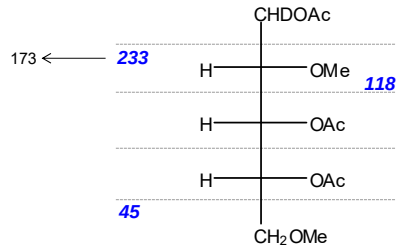


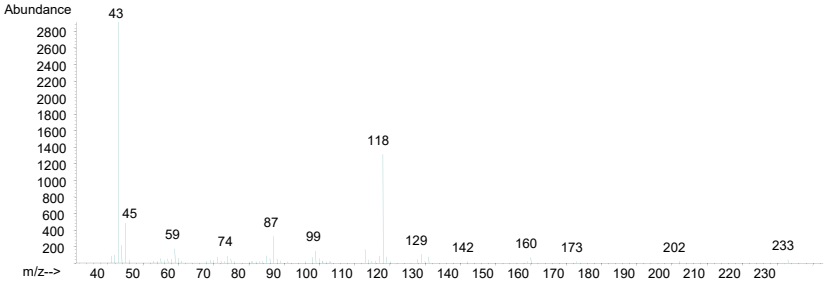
N.B.: fragmentation containing C-1 are even, fragmentation from the "tail" are odd

Partially Methylated Acetylated Alditols

Remember:

CHDOAc = 74
H-C-OMe = 44
H-C-OAc = 72
CH_2OAc = 73
CH_2OMe = 45
H-C-NMeAc = 85
CH_3 = 15

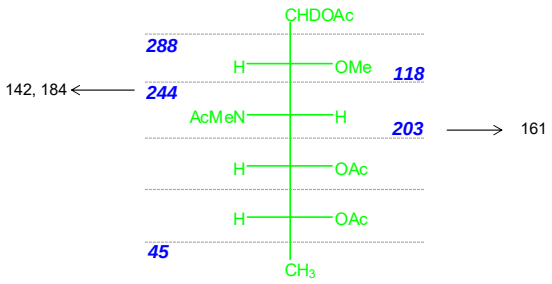


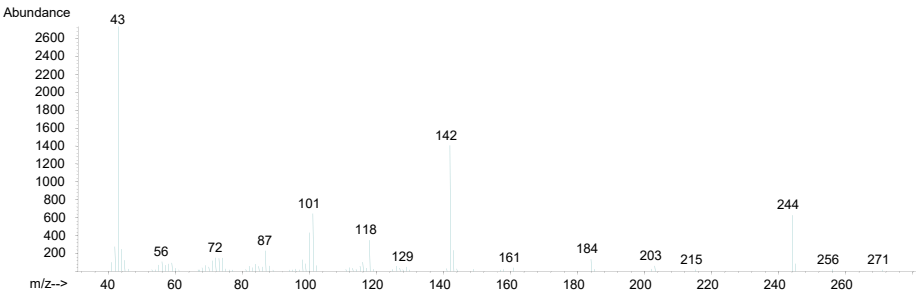


Partially Methylated Acetylated Alditols

Remember:

CHDOAc = 74
H-C-OMe = 44
H-C-OAc = 72
CH_2OAc = 73
CH_2OMe = 45
H-C-NMeAc = 85
CH_3 = 15

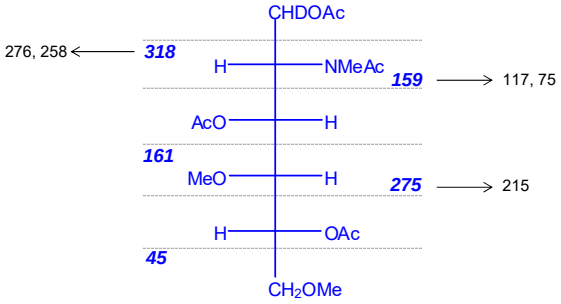


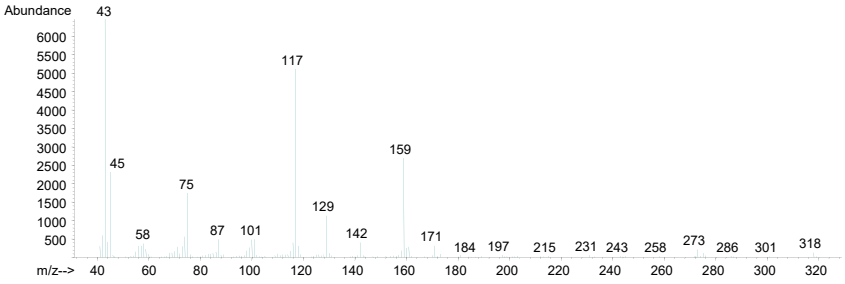


Partially Methylated Acetylated Alditols

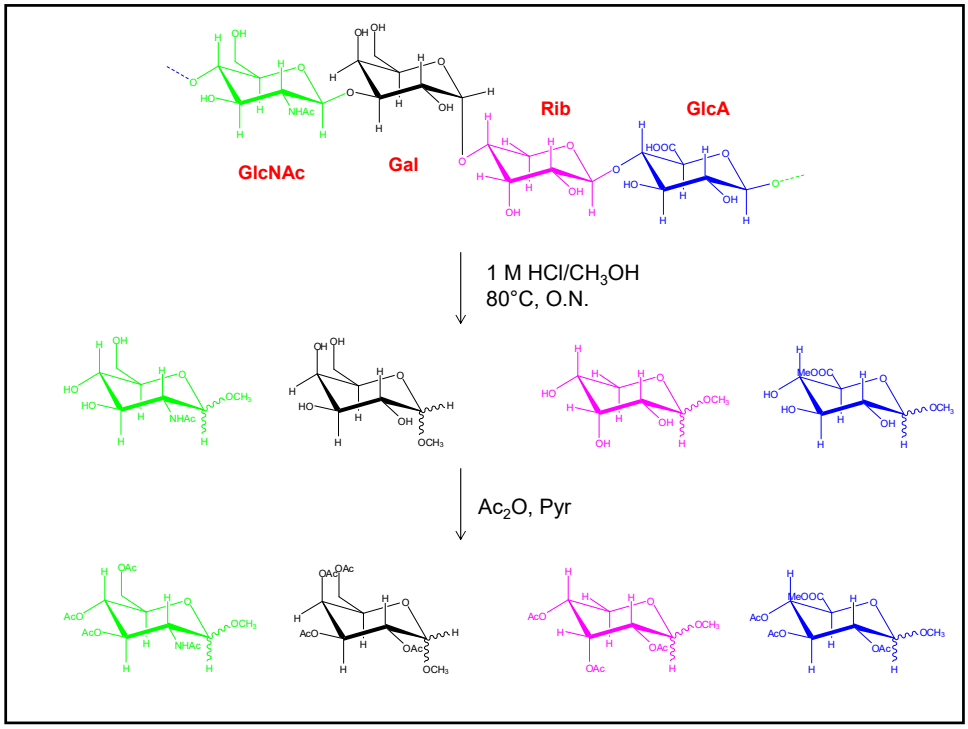
Remember:

CHDOAc = 74
H-C-OMe = 44
H-C-OAc = 72
CH_2OAc = 73
CH_2OMe = 45
H-C-NMeAc = 85
CH_3 = 15





Acetylated Methyl glycosides



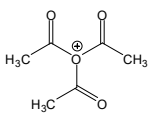
Acetylated Methyl glycosides

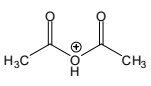
Fragmentation rules

Fragmentation rules:

- The most stable ions will be observed in the EI-MS spectrum
- Isomeric sugars (as Glc and Gal) give the same EI-MS spectrum
- The radical cation of the methylglycosides undergoes several pathways:
- A, B, C, D, E, F, H, J, and K (example given for an hexose)
- Fragments gives a series of daughter ions by loss of neutral molecules (AcOH, Ac_2O, AcO•, CH_2=C=O)
- Occurrence of acetamido, or deoxy groups, change the preferential fragmentation pathway
- Along with the ions from the fragmentation pathways, triacetoxonium and diacetoxonium ions maybe observed.

m/z 145

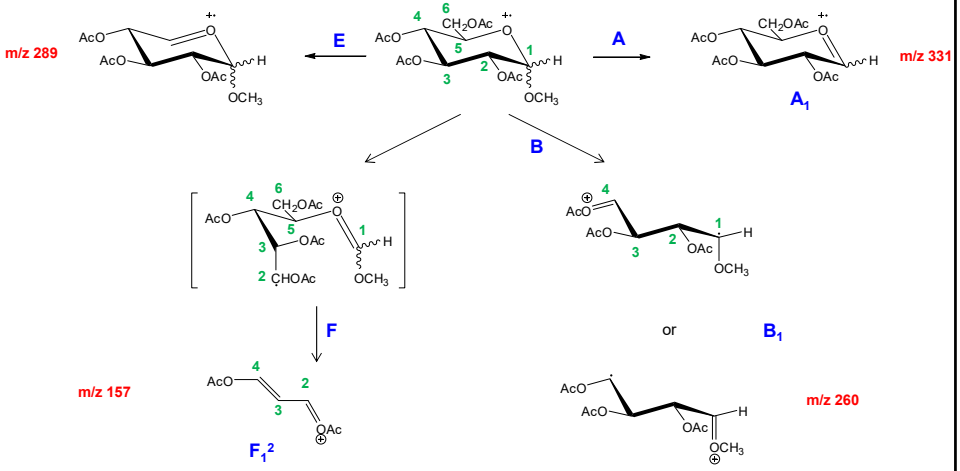




m/z 103

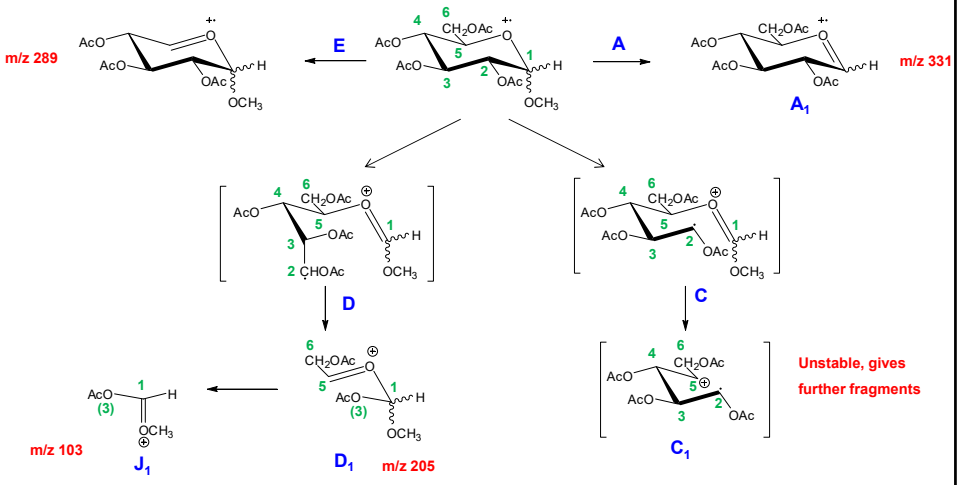
Acetylated Methyl glycosides

Fragmentation pathway (hexose)



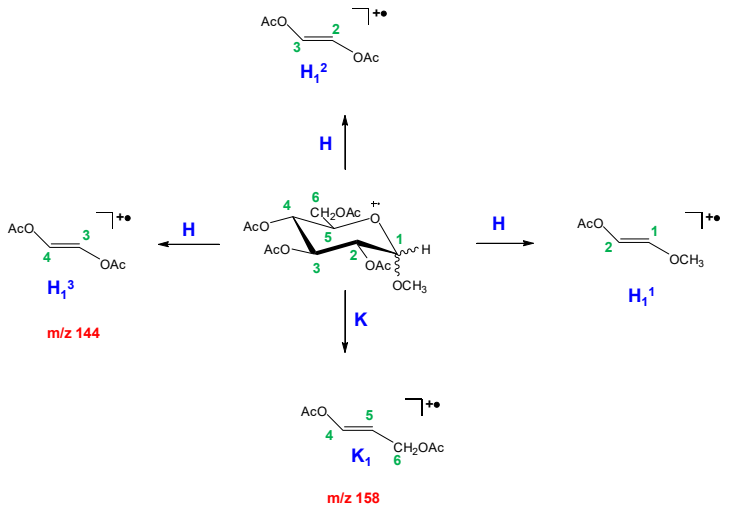
Acetylated Methyl glycosides

Fragmentation pathway (hexose)

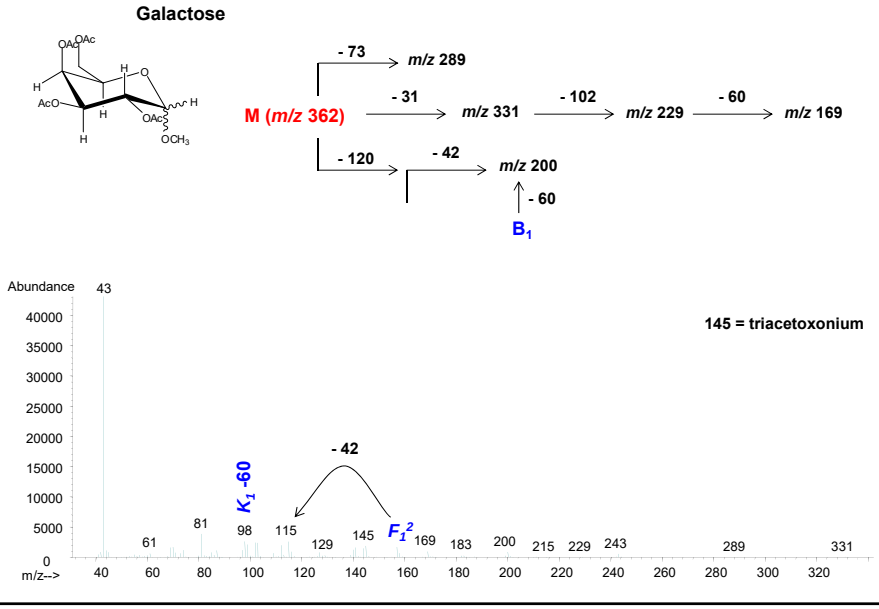


Acetylated Methyl glycosides

Fragmentation pathway (hexose)

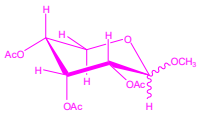


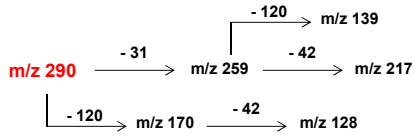
Acetylated Methyl glycosides

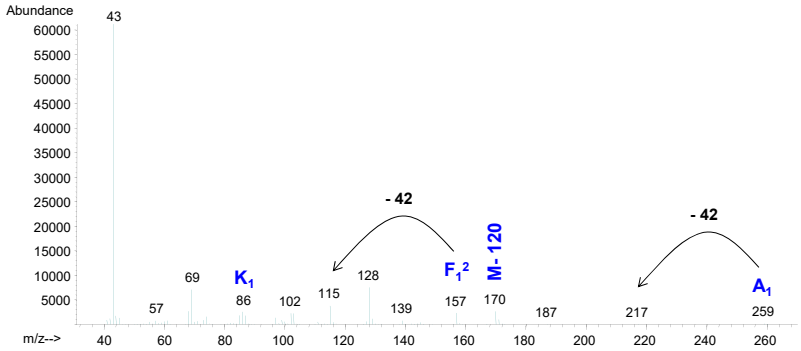


Acetylated Methyl glycosides

Ribose

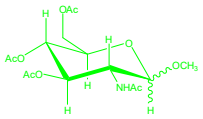


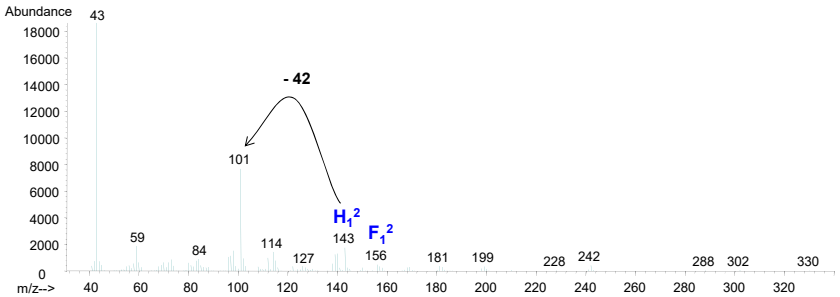




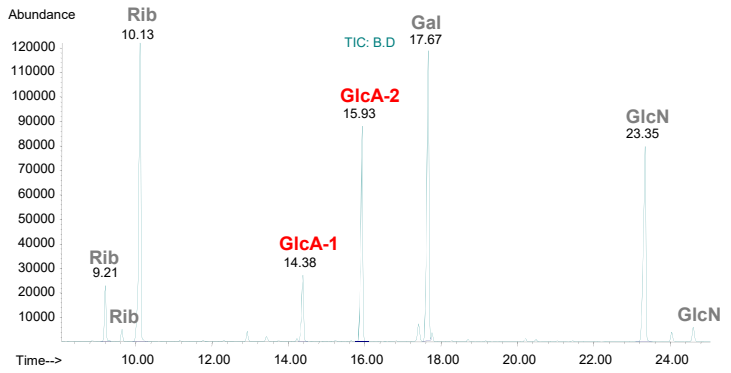
Acetylated Methyl glycosides

Glucosamine



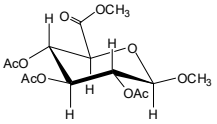


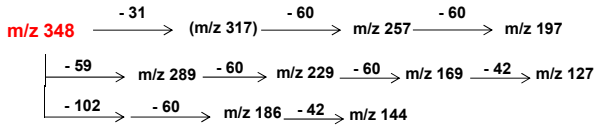
Acetylated Methyl glycosides

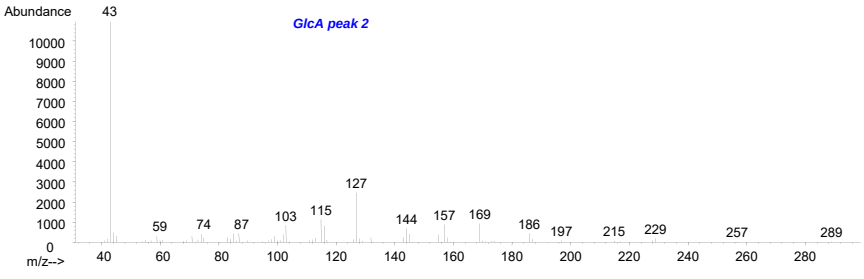


Glucuronic acid: sometimes knowledge of sugar chemistry is important to understand their fragmentation

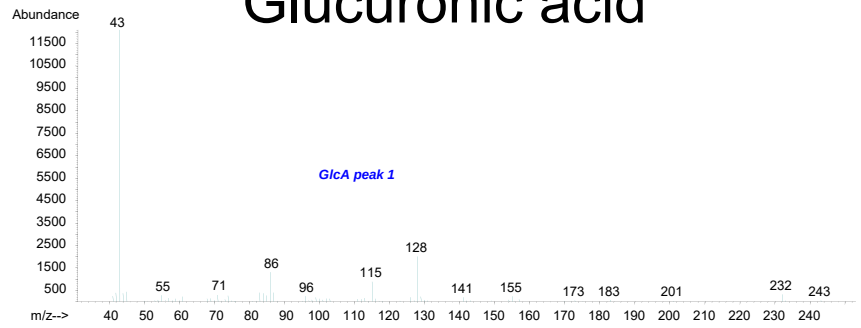
Glucuronic acid

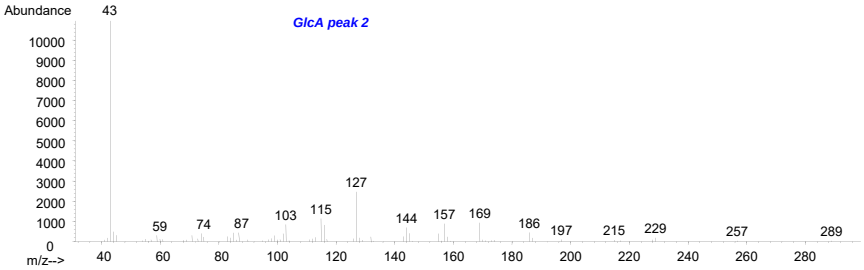






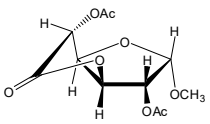
Glucuronic acid

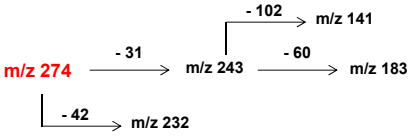


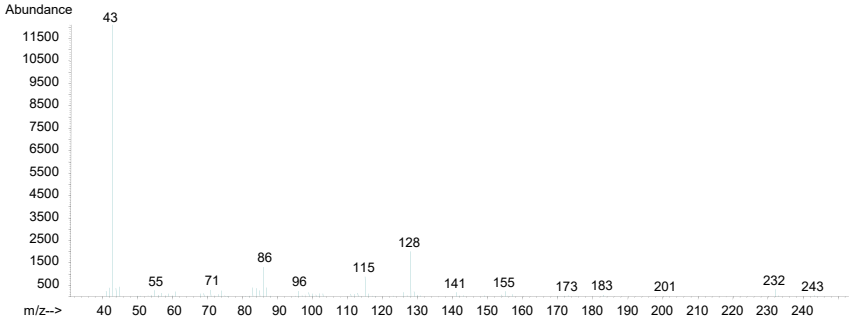


Acetylated Methyl glycosides

Glucuronolacton

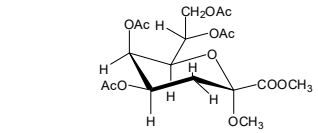




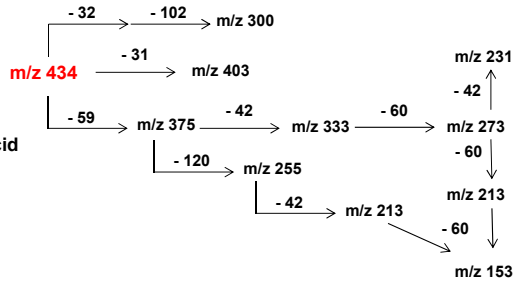


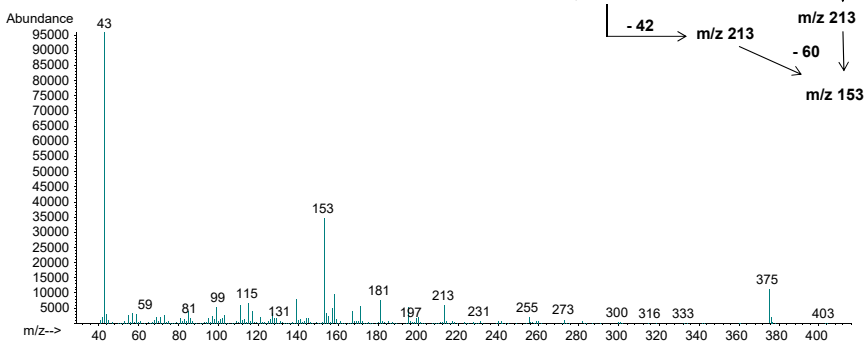
Acetylated Methyl glycosides

Ulosonic acids are detected, as Kdo



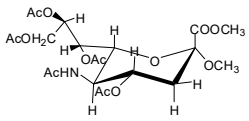
3-keto-2-deoxy-D-*manno*-octulosonic acid



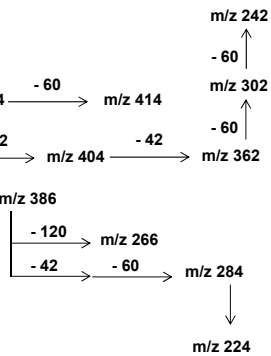


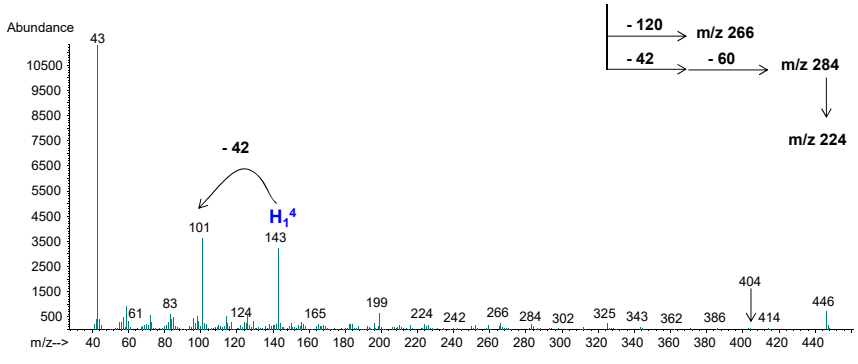
Acetylated Methyl glycosides

Ulosonic acids are detected, as Sialic acid



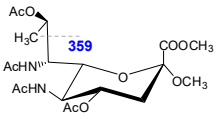
Sialic or neuraminic acid



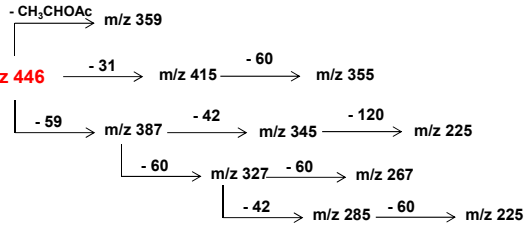


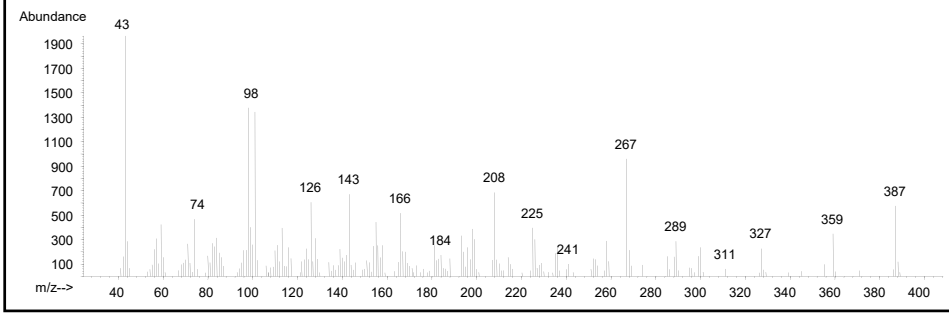
Acetylated Methyl glycosides

Ulosonic acids are detected, as Legionaminic acid



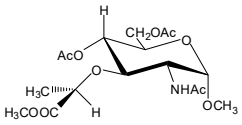
Legionaminic acid



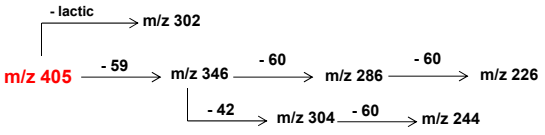


Acetylated Methyl glycosides

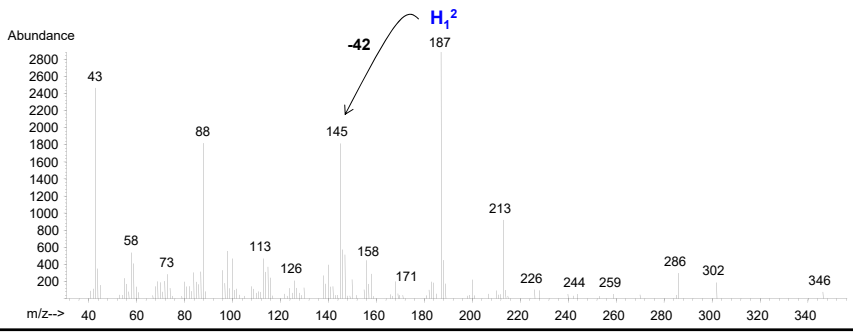
Other rare sugars



Muramic acid

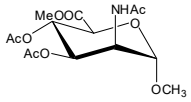


m/z 88 = McLafferty rearrangement from lactic residue

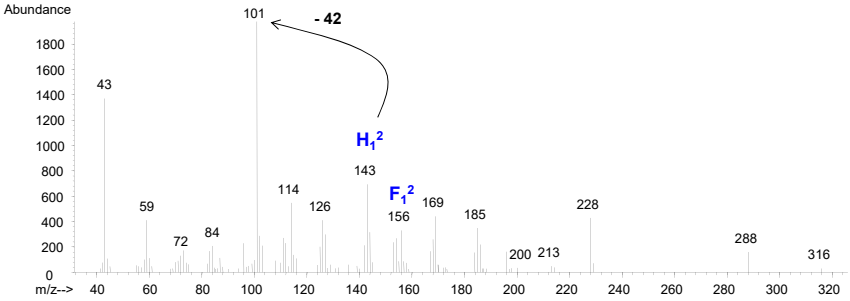


Acetylated Methyl glycosides

Other rare sugars

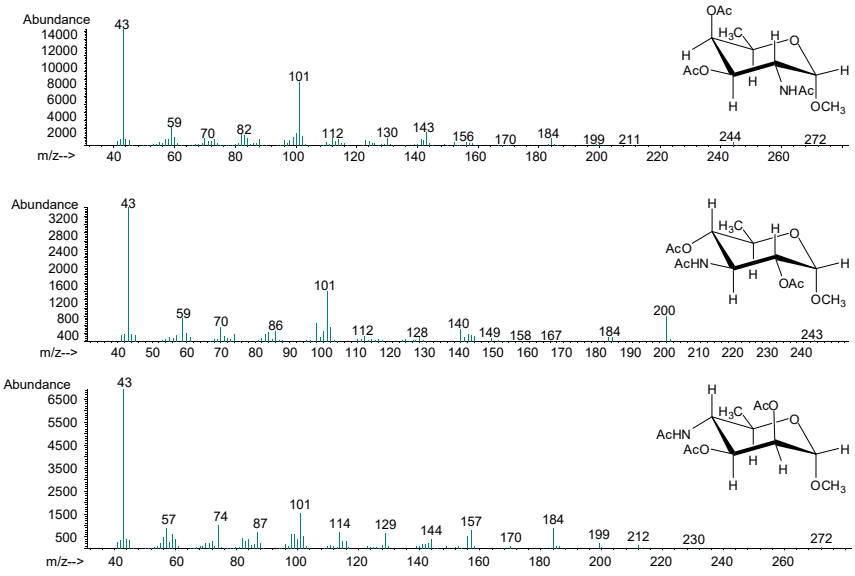


Aminouronic acid



Acetylated Methyl glycosides

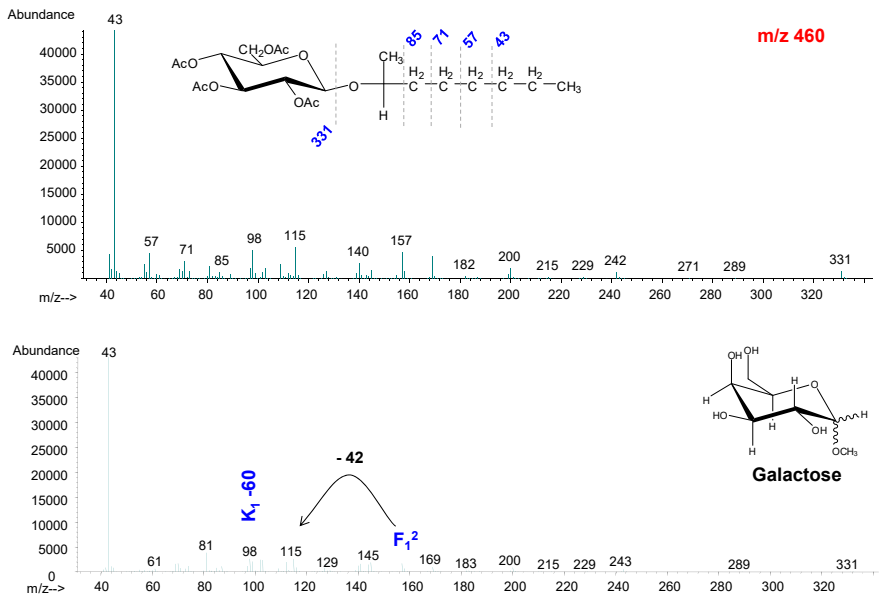
Fragmentation pattern diagnostic for related isomeric sugars



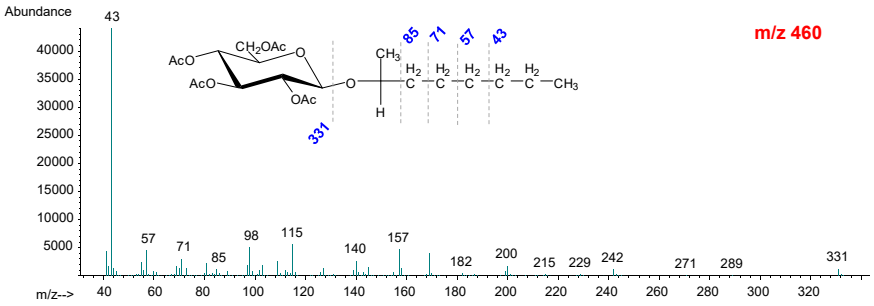
Acetylated Octylglycosides …

an extension of the previous rules.

Acetylated Octyl Glycosides



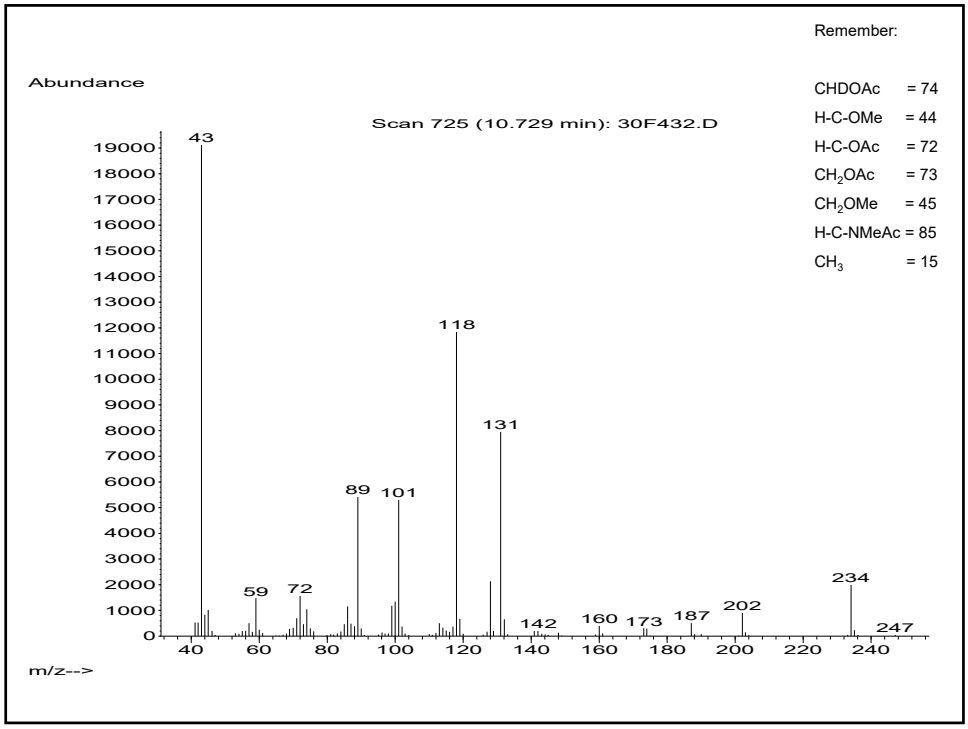
Acetylated Octyl Glycosides

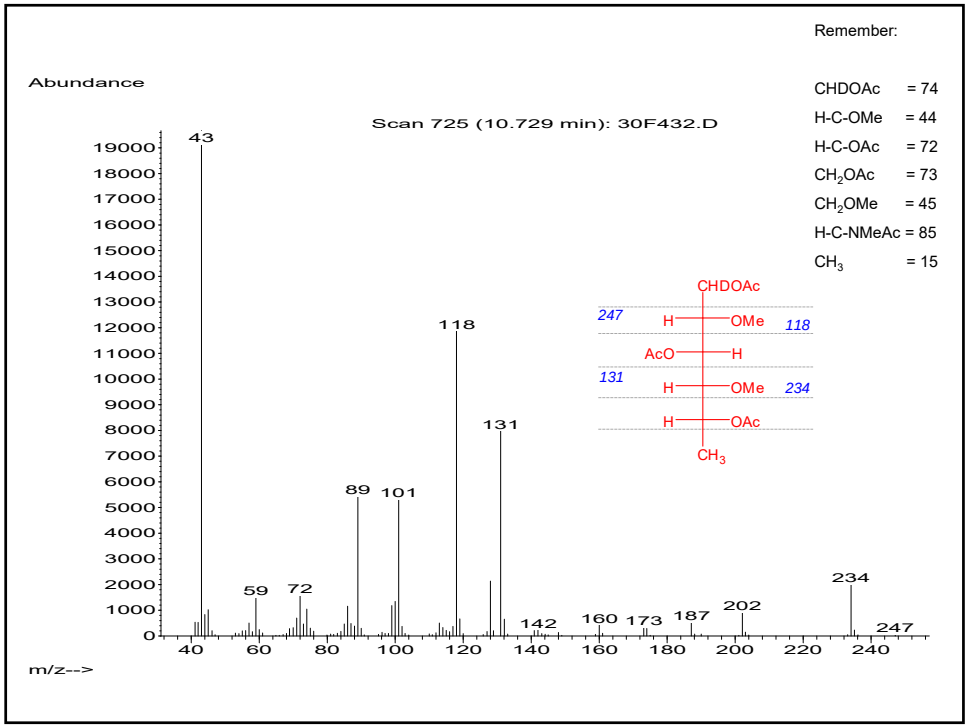


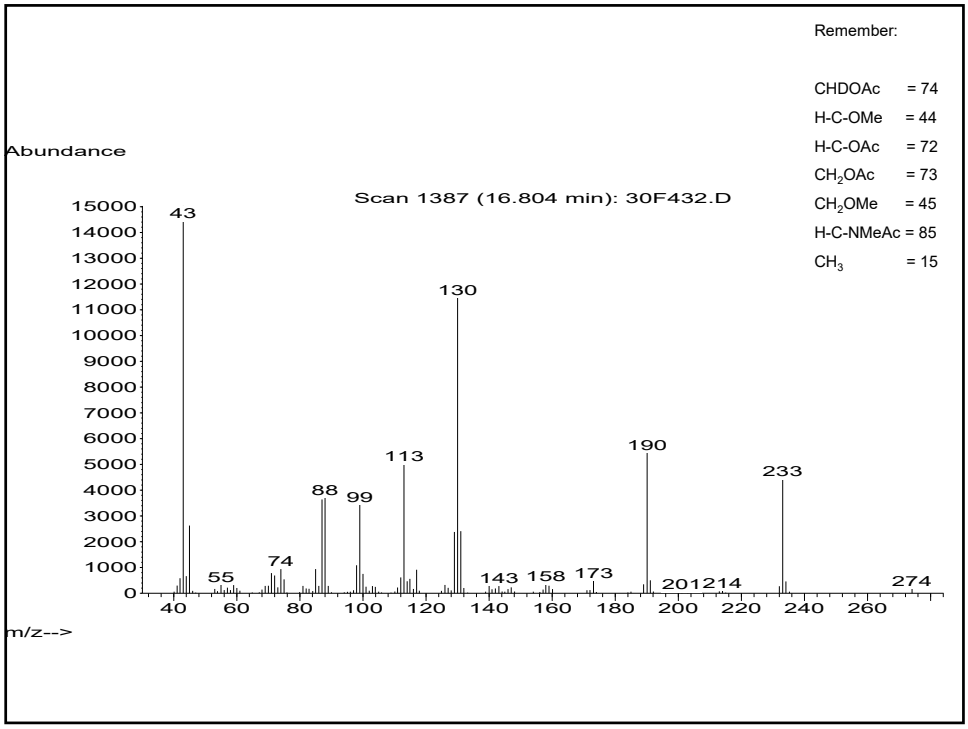
- Octyl or methylglycosides give almost the same EI-MS spectra
- Small contribute from the lipophilic tail of octanol
- Main diffence among these derivatives is their column retention time

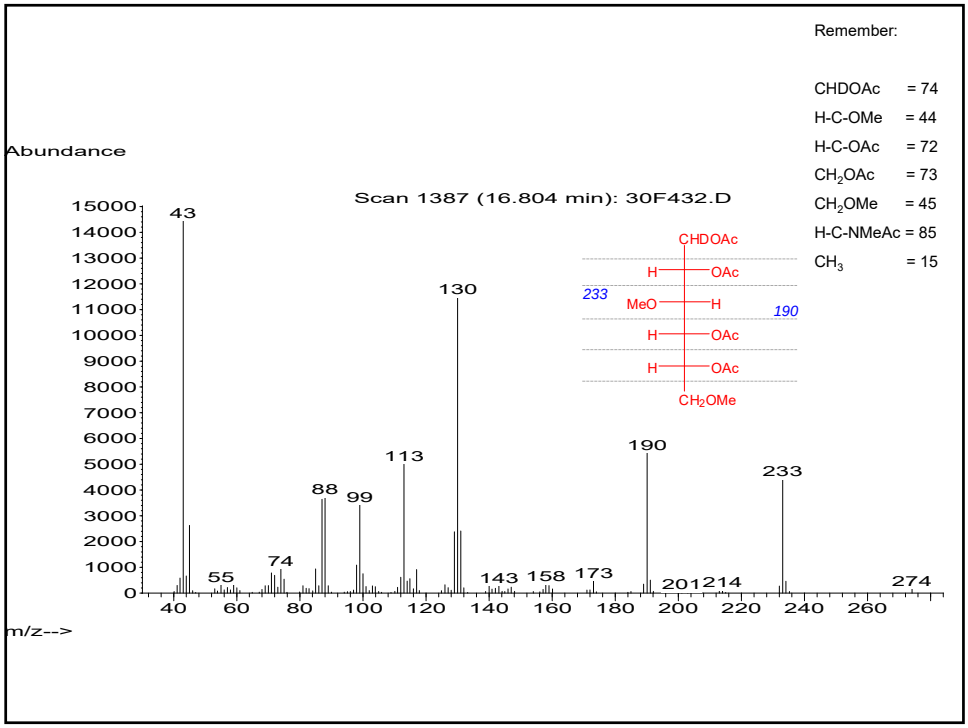
HANDS ON …..

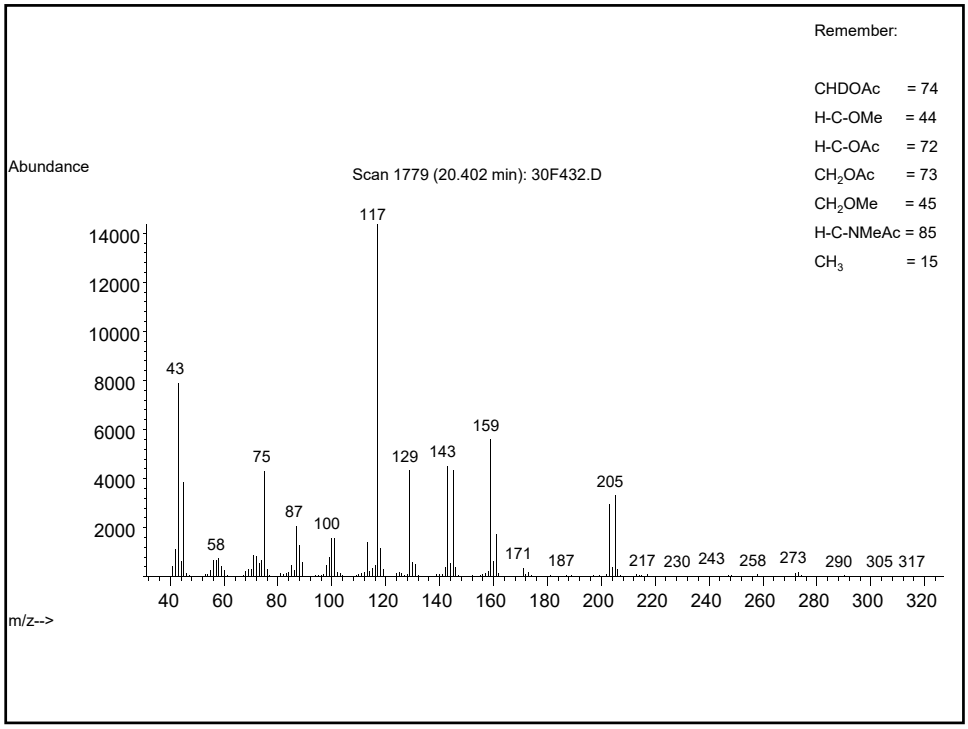
Partially Methylated Acetylated Alditols

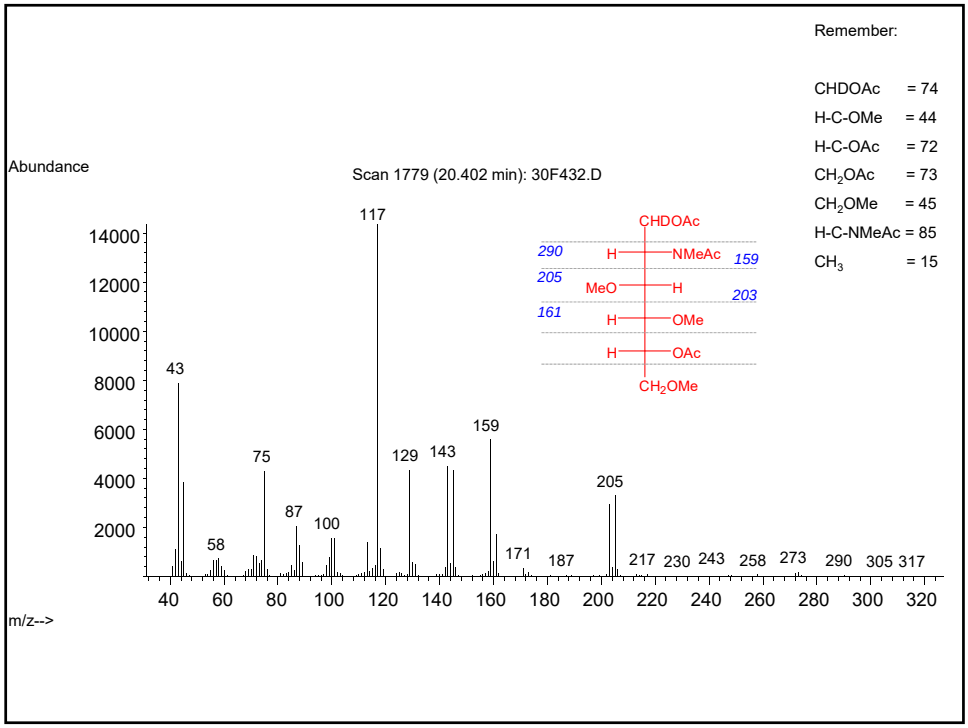

Remember:
CHDOAc = 74
H-C-OMe = 44
H-C-OAc = 72
CH2OAc = 73
CH2OMe = 45
H-C-NMeAc = 85
CH3 = 15
Abundance
Scan 725 (10.729 min): 30F432.D
m/z-->

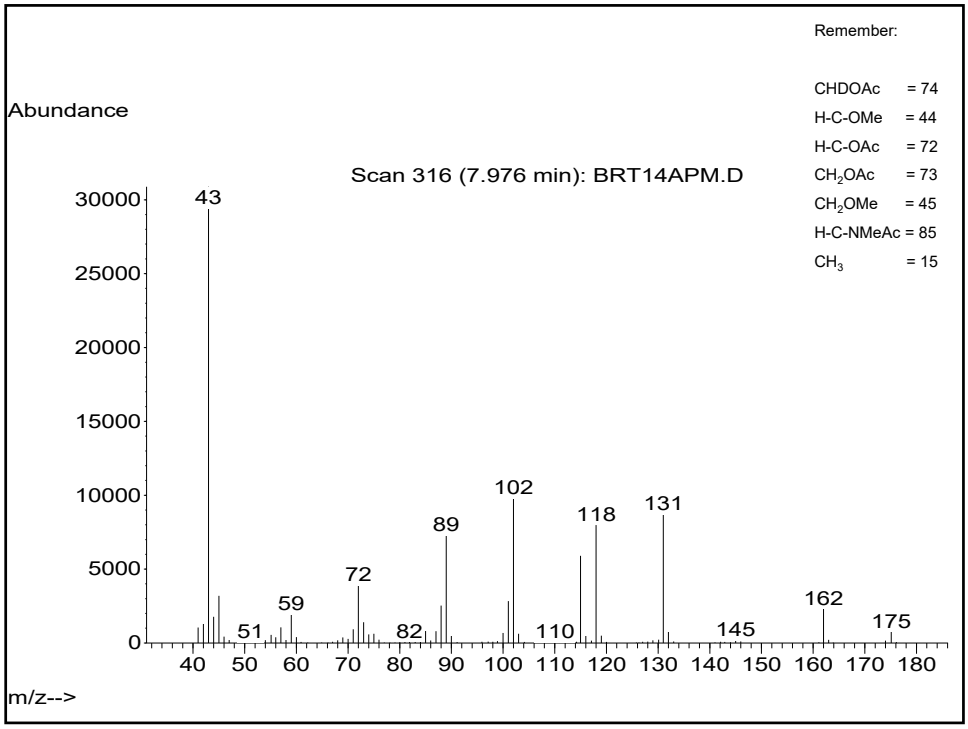

Remember:
CHDOAc = 74
H-C-OMe = 44
H-C-OAc = 72
CH2OAc = 73
CH2OMe = 45
H-C-NMeAc = 85
CH3 = 15
Abundance
Scan 725 (10.729 min): 30F432.D
CHDOAc
247
H
OMe
118
AcO
H
131
H
OMe
234
H
OAc
CH3
m/z-->

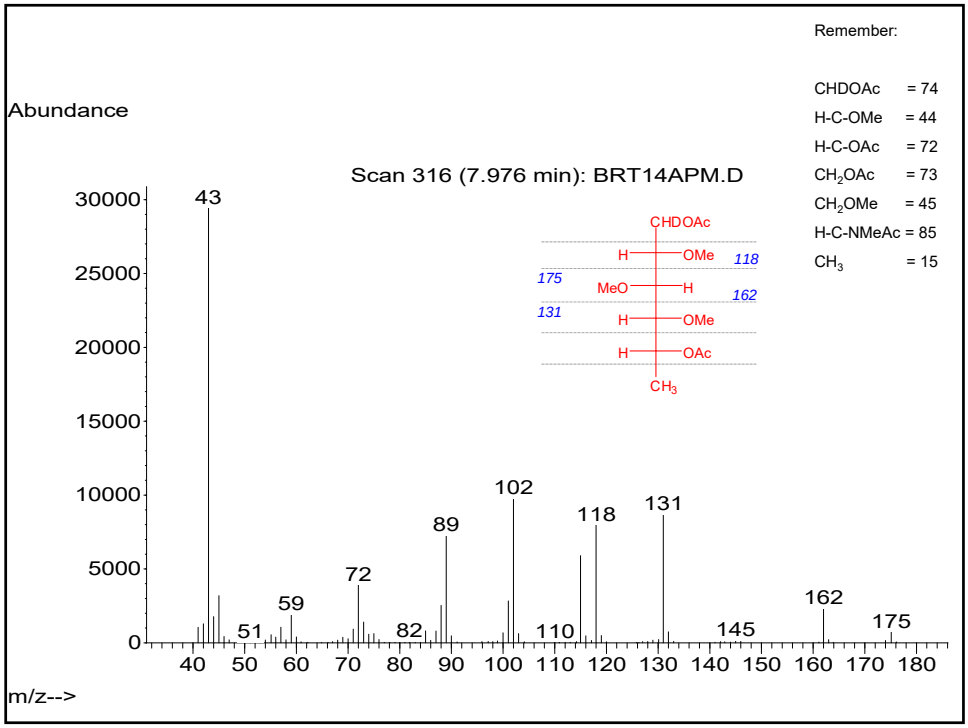

Remember:
CHDOAc = 74
H-C-OMe = 44
H-C-OAc = 72
CH2OAc = 73
CH2OMe = 45
H-C-NMeAc = 85
CH3 = 15
Abundance
Scan 1387 (16.804 min): 30F432.D
m/z-->

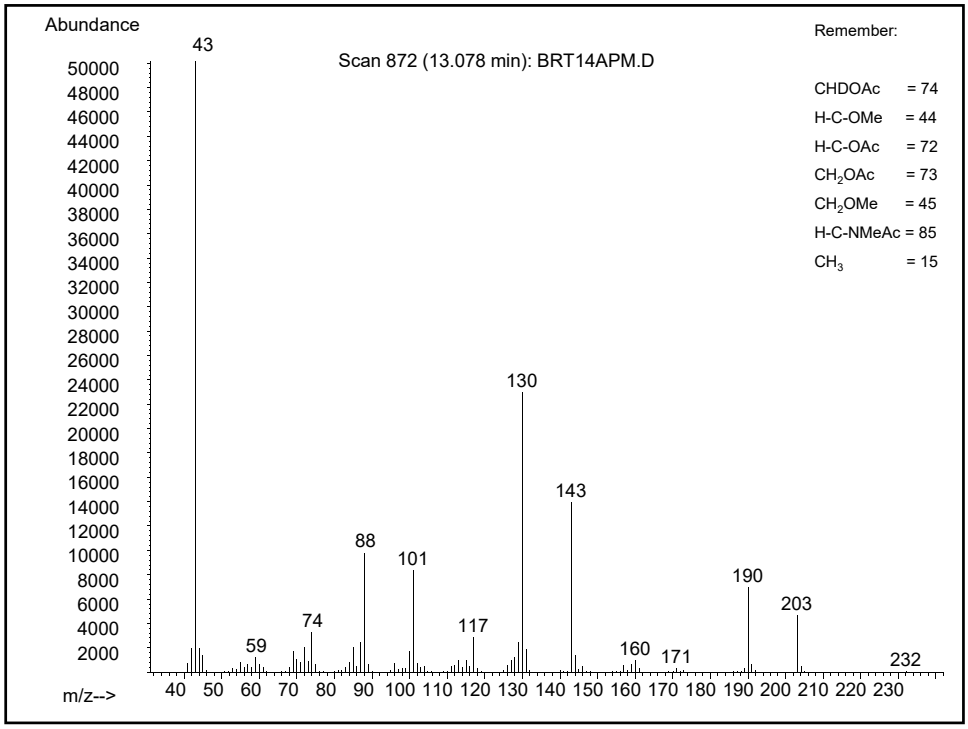

Remember:
CHDOAc = 74
H-C-OMe = 44
H-C-OAc = 72
CH2OAc = 73
CH2OMe = 45
H-C-NMeAc = 85
CH3 = 15
Abundance
Scan 1387 (16.804 min): 30F432.D
CHDOAc
H
OAc
233
MeO
H
190
H
OAc
H
OAc
CH2OMe
m/z-->

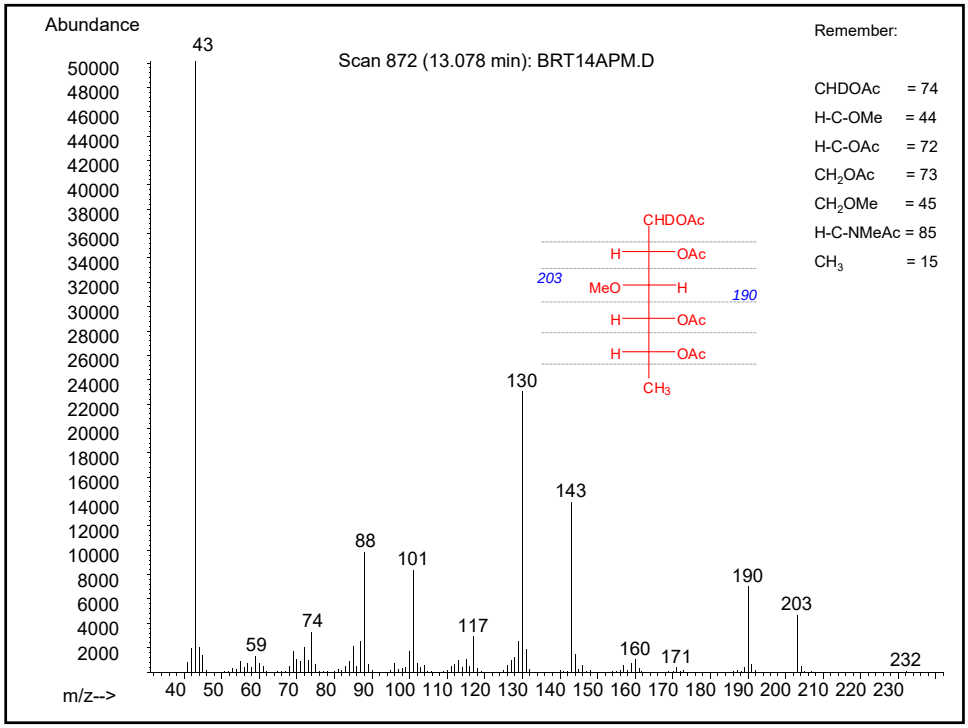

Remember:
CHDOAc = 74
H-C-OMe = 44
H-C-OAc = 72
CH2OAc = 73
CH2OMe = 45
H-C-NMeAc = 85
CH3 = 15
Abundance
Scan 1779 (20.402 min): 30F432.D
m/z-->

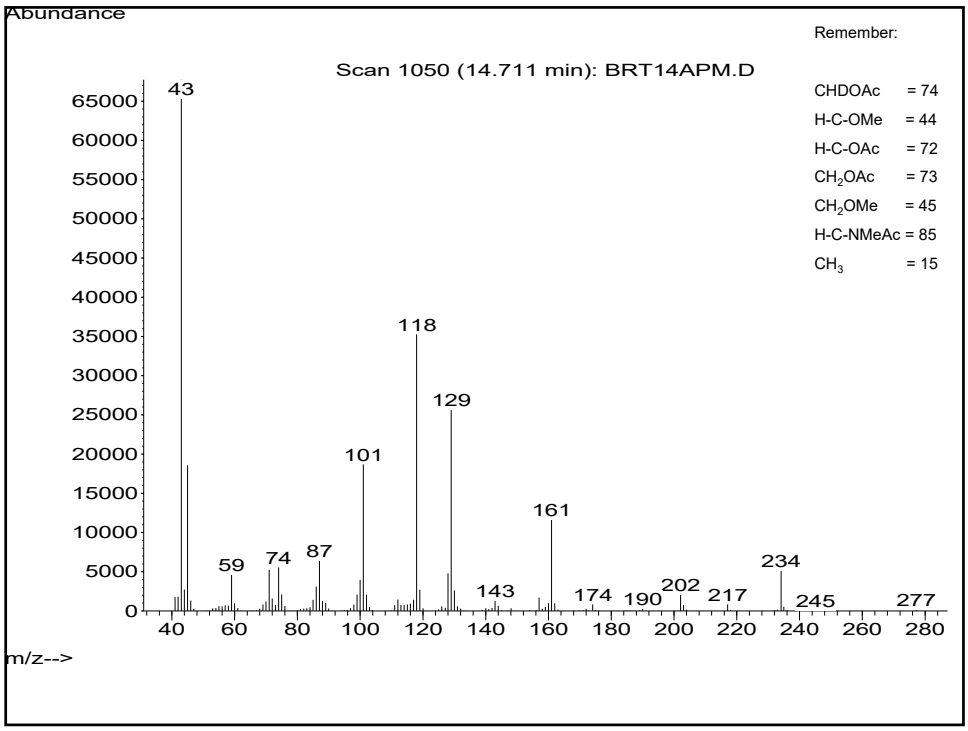

Remember:
CHDOAc = 74
H-C-OMe = 44
H-C-OAc = 72
CH2OAc = 73
CH2OMe = 45
H-C-NMeAc = 85
CH3 = 15
Abundance
Scan 1779 (20.402 min): 30F432.D
CHDOAc
290
H
NMeAc
159
205
MeO
H
203
161
H
OMe
H
OAc
CH2OMe
m/z-->

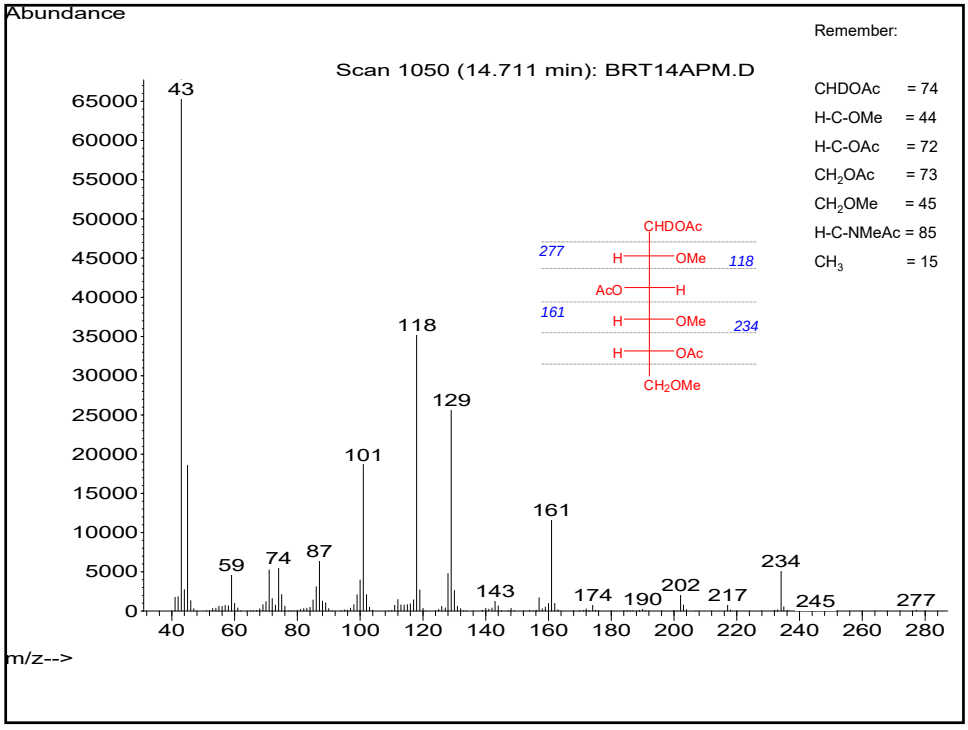

Remember:
CHDOAc = 74
H-C-OMe = 44
H-C-OAc = 72
CH2OAc = 73
CH2OMe = 45
H-C-NMeAc = 85
CH3 = 15
Abundance
Scan 316 (7.976 min): BRT14APM.D
m/z-->

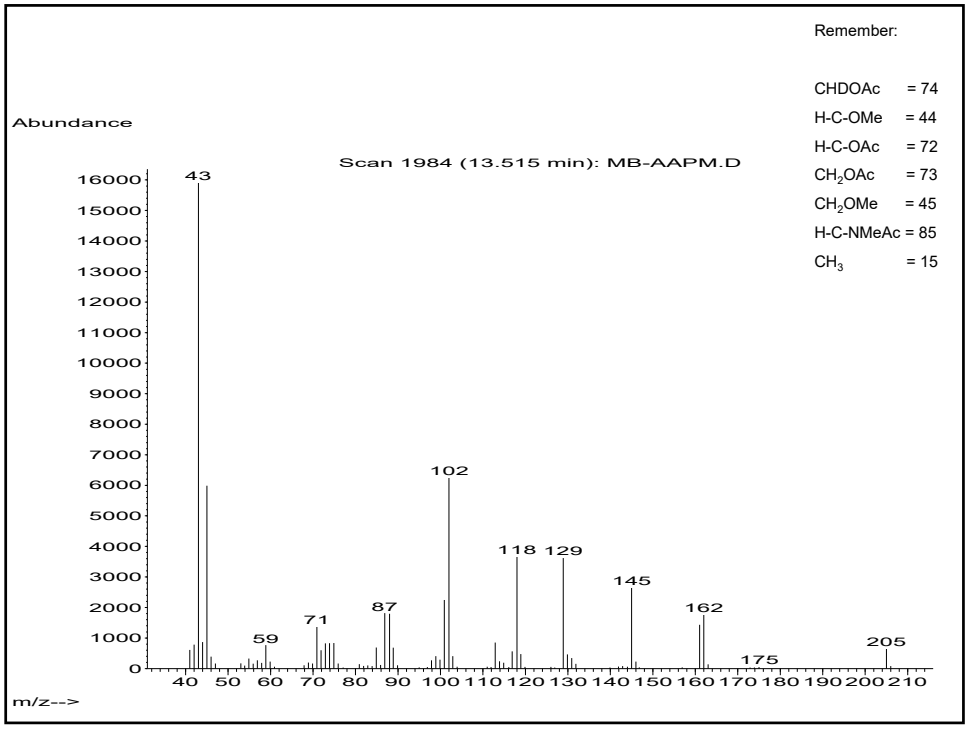

Remember:
CHDOAc = 74
H-C-OMe = 44
H-C-OAc = 72
CH2OAc = 73
CH2OMe = 45
H-C-NMeAc = 85
CH3 = 15
Abundance
Scan 316 (7.976 min): BRT14APM.D
CHDOAc
H OMe 118
175 MeO H 162
131 H OMe
H OAc
CH3
m/z-->

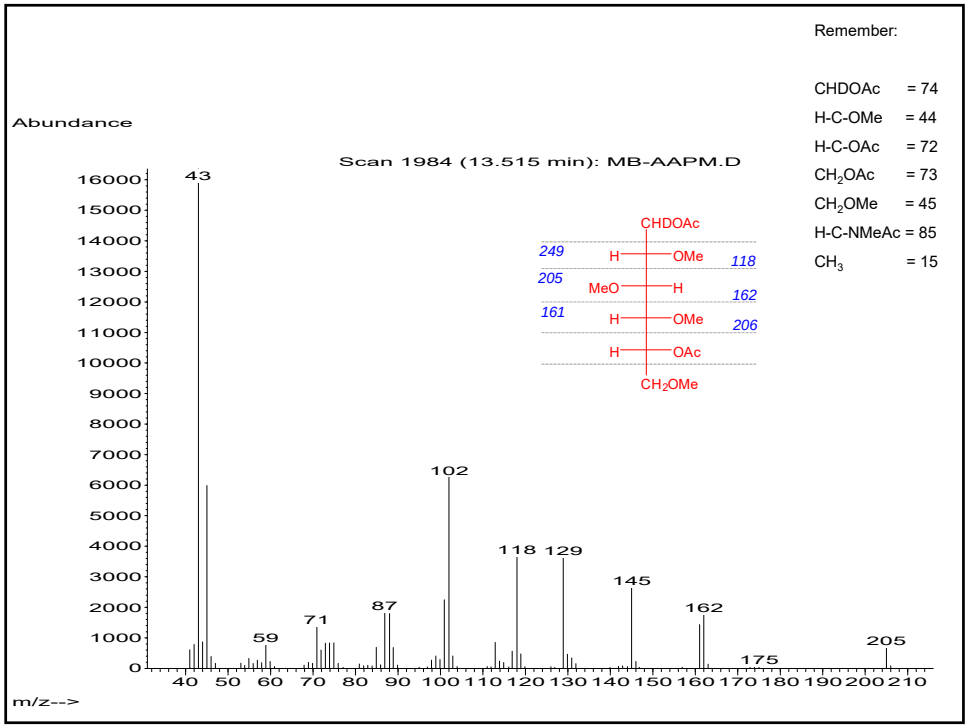

Abundance
Scan 872 (13.078 min): BRT14APM.D
Remember:
CHDOAc = 74
H-C-OMe = 44
H-C-OAc = 72
CH2OAc = 73
CH2OMe = 45
H-C-NMeAc = 85
CH3 = 15
m/z-->

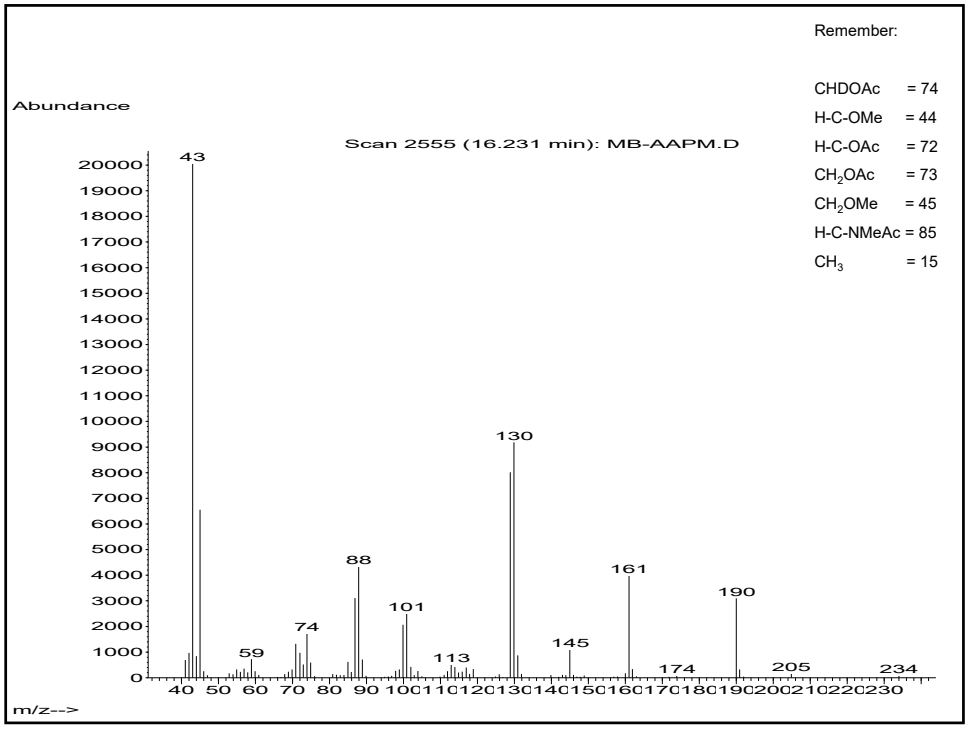

Abundance
Scan 872 (13.078 min): BRT14APM.D
Remember:
CHDOAc = 74
H-C-OMe = 44
H-C-OAc = 72
CH2OAc = 73
CH2OMe = 45
H-C-NMeAc = 85
CH3 = 15
CHDOAc
H
OAc
203
MeO
H
190
H
OAc
H
OAc
CH3
m/z-->

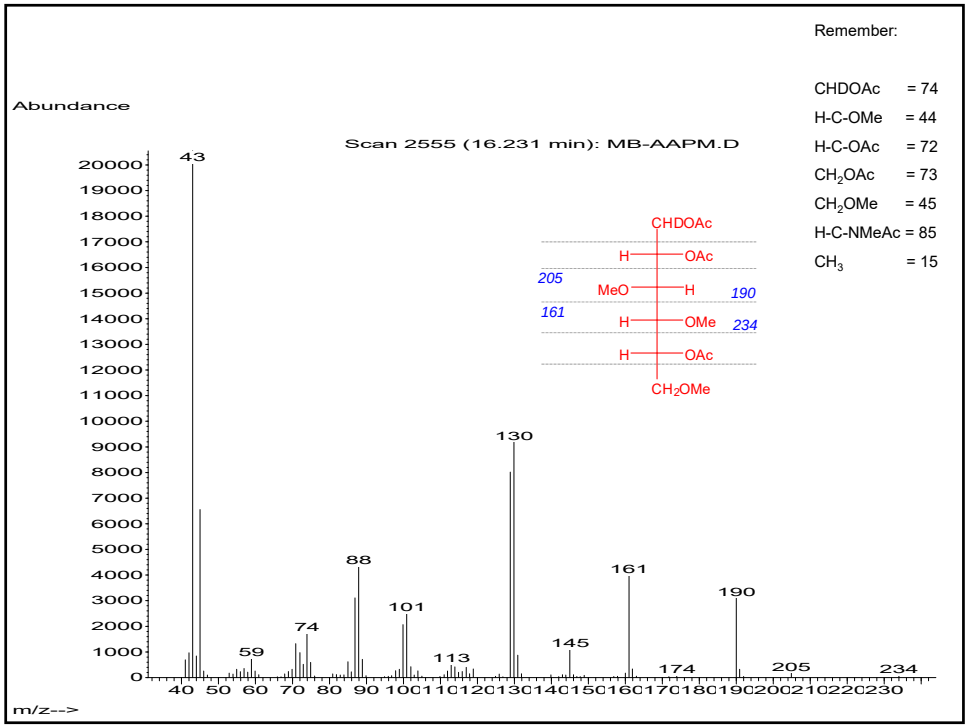

Abundance
Scan 1050 (14.711 min): BRT14APM.D
Remember:
CHDOAc = 74
H-C-OMe = 44
H-C-OAc = 72
CH2OAc = 73
CH2OMe = 45
H-C-NMeAc = 85
CH3 = 15
m/z-->

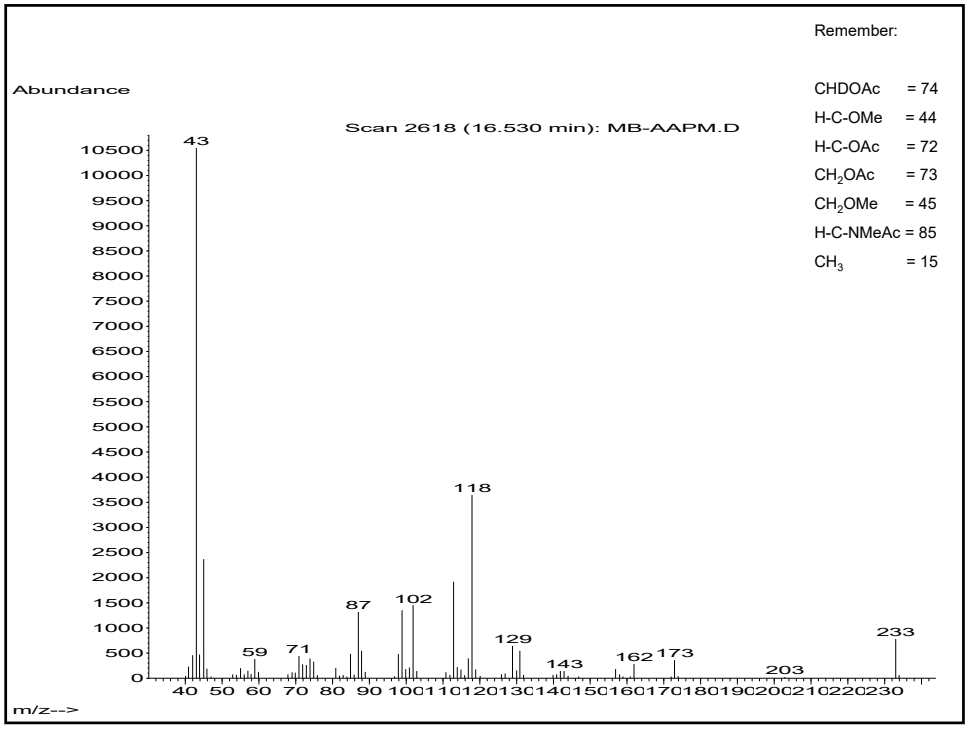

Abundance
Scan 1050 (14.711 min): BRT14APM.D
Remember:
CHDOAc = 74
H-C-OMe = 44
H-C-OAc = 72
CH2OAc = 73
CH2OMe = 45
H-C-NMeAc = 85
CH3 = 15
CHDOAc
277
H
OMe
118
AcO
H
161
H
OMe
234
H
OAc
CH2OMe
m/z-->

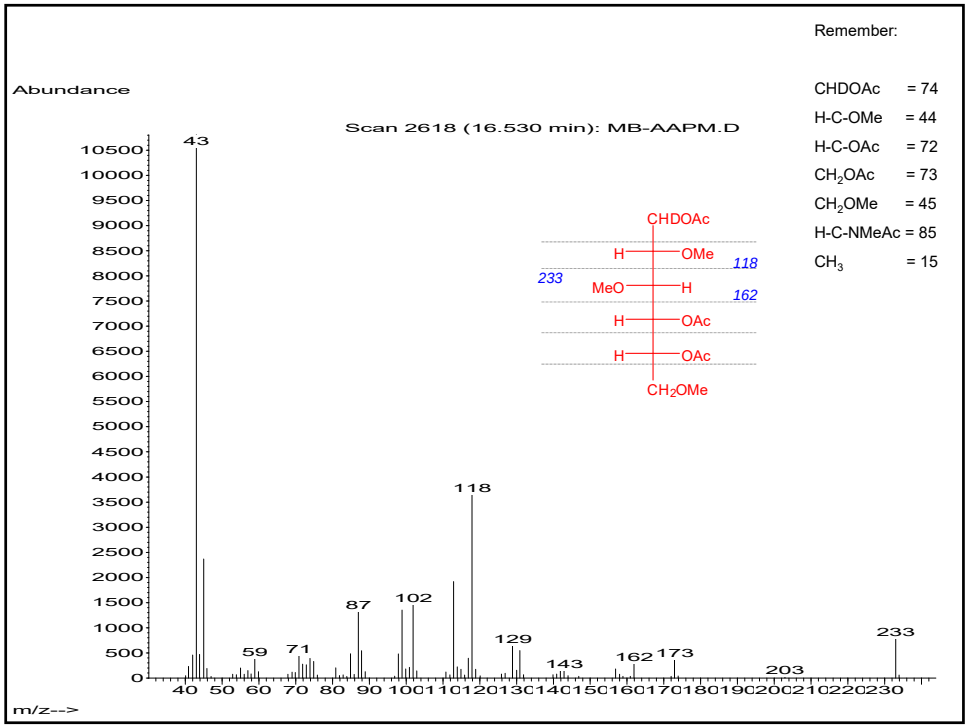

Remember:
CHDOAc = 74
H-C-OMe = 44
H-C-OAc = 72
CH2OAc = 73
CH2OMe = 45
H-C-NMeAc = 85
CH3 = 15
Abundance
Scan 1984 (13.515 min): MB-AAPM.D
m/z-->

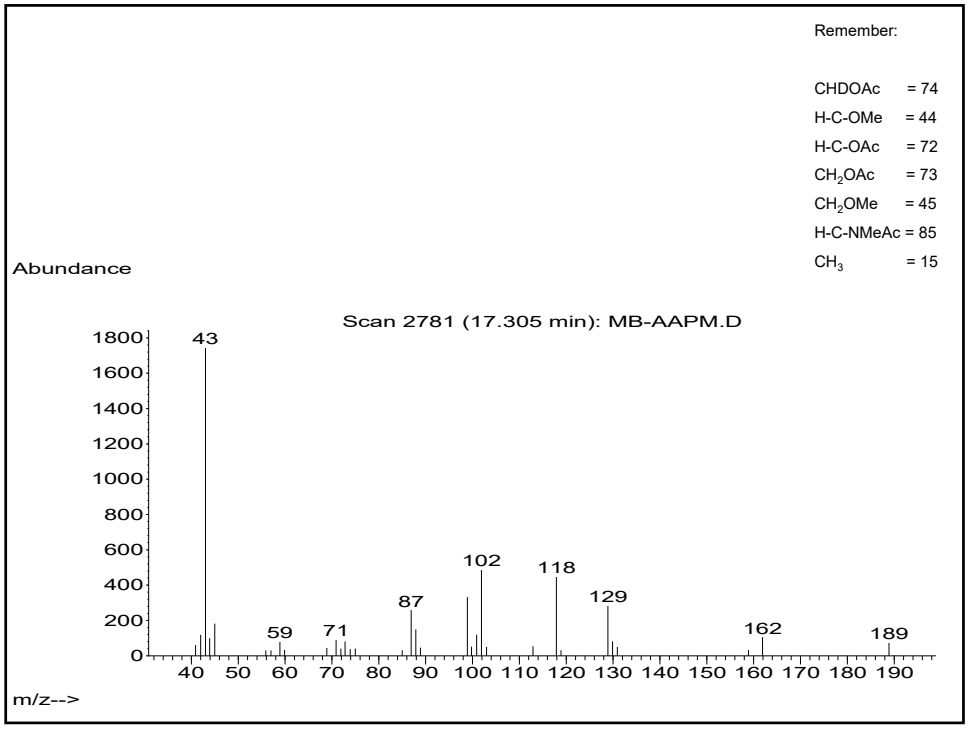

Remember:
CHDOAc = 74
H-C-OMe = 44
H-C-OAc = 72
CH2OAc = 73
CH2OMe = 45
H-C-NMeAc = 85
CH3 = 15
Abundance
Scan 1984 (13.515 min): MB-AAPM.D
CHDOAc
249
H
OMe
118
205
MeO
H
162
161
H
OMe
206
H
OAc
CH2OMe
m/z-->

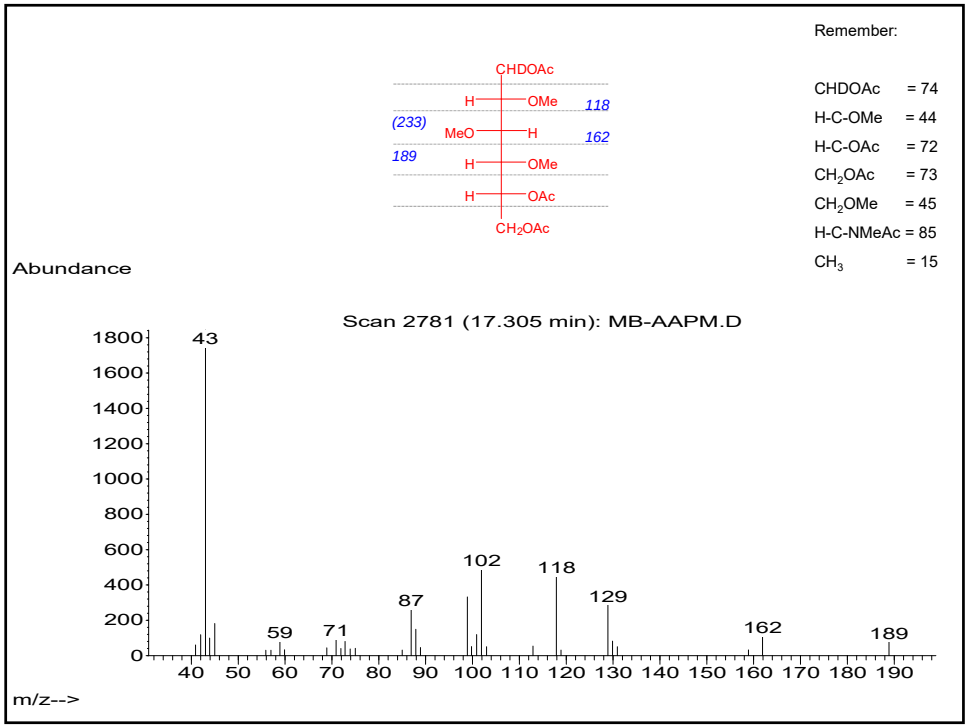

Remember:
CHDOAc = 74
H-C-OMe = 44
H-C-OAc = 72
CH2OAc = 73
CH2OMe = 45
H-C-NMeAc = 85
CH3 = 15
Abundance
Scan 2555 (16.231 min): MB-AAPM.D
m/z-->

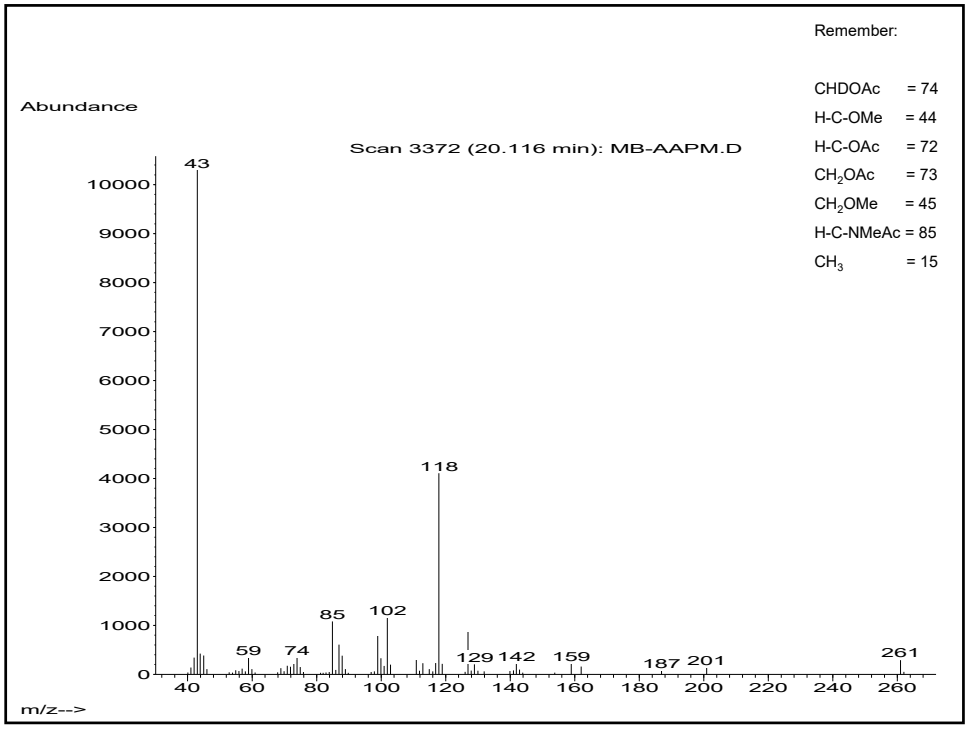

Remember:
CHDOAc = 74
H-C-OMe = 44
H-C-OAc = 72
CH2OAc = 73
CH2OMe = 45
H-C-NMeAc = 85
CH3 = 15
Abundance
Scan 2555 (16.231 min): MB-AAPM.D
CHDOAc
H
OAc
205
MeO
H
190
161
H
OMe
234
H
OAc
CH2OMe
m/z-->

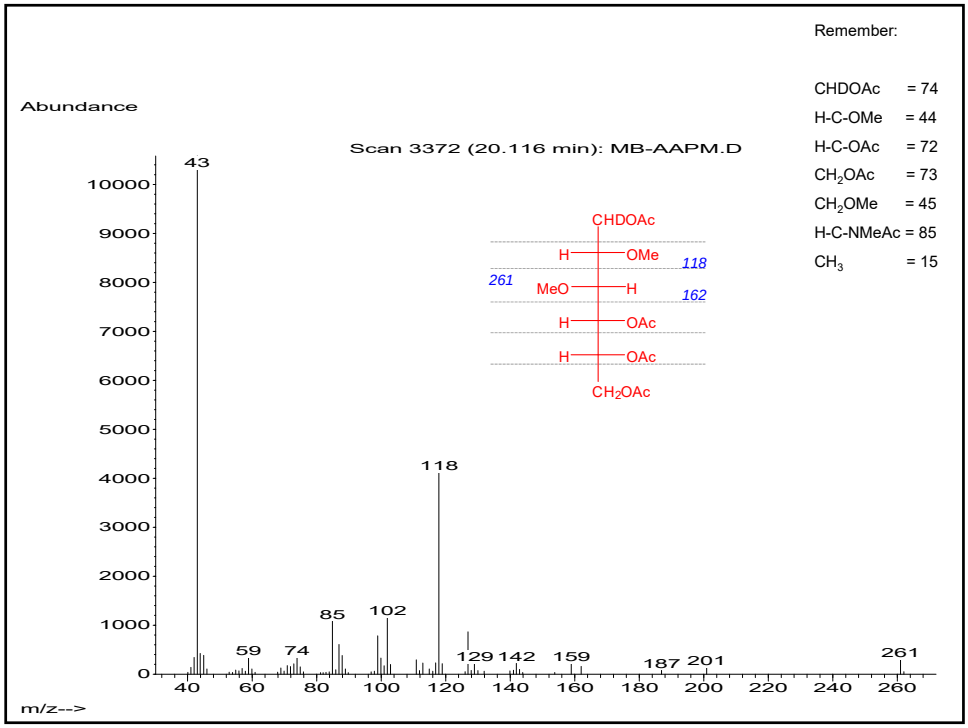

Remember:
CHDOAc = 74
H-C-OMe = 44
H-C-OAc = 72
CH2OAc = 73
CH2OMe = 45
H-C-NMeAc = 85
CH3 = 15
Abundance
Scan 2618 (16.530 min): MB-AAPM.D
m/z-->


Remember:
CHDOAc = 74
H-C-OMe = 44
H-C-OAc = 72
CH2OAc = 73
CH2OMe = 45
H-C-NMeAc = 85
CH3 = 15
Abundance
Scan 2618 (16.530 min): MB-AAPM.D
CHDOAc
H
OMe
118
233
MeO
H
162
H
OAc
H
OAc
CH2OMe
m/z-->


Remember:
CHDOAc = 74
H-C-OMe = 44
H-C-OAc = 72
CH2OAc = 73
CH2OMe = 45
H-C-NMeAc = 85
CH3 = 15
Abundance
Scan 2781 (17.305 min): MB-AAPM.D
m/z-->

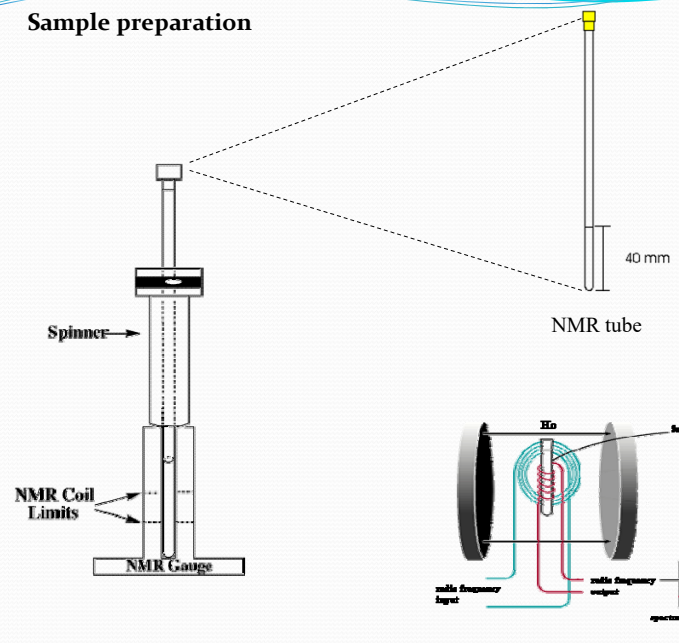

Remember:
CHDOAc
H
OMe
118
(233)
MeO
H
162
189
H
OMe
H
OAc
CH2OAc
CHDOAc = 74
H-C-OMe = 44
H-C-OAc = 72
CH2OAc = 73
CH2OMe = 45
H-C-NMeAc = 85
CH3 = 15
Abundance
Scan 2781 (17.305 min): MB-AAPM.D
m/z-->


Remember:
CHDOAc = 74
H-C-OMe = 44
H-C-OAc = 72
CH2OAc = 73
CH2OMe = 45
H-C-NMeAc = 85
CH3 = 15
Abundance
Scan 3372 (20.116 min): MB-AAPM.D
m/z-->

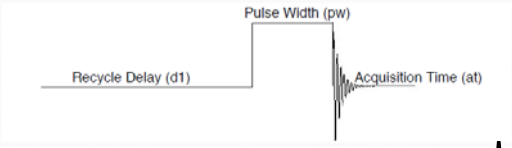

Remember:
CHDOAc = 74
H-C-OMe = 44
H-C-OAc = 72
CH2OAc = 73
CH2OMe = 45
H-C-NMeAc = 85
CH3 = 15
Abundance
Scan 3372 (20.116 min): MB-AAPM.D
CHDOAc
H
OMe
118
261
MeO
H
162
H
OAc
H
OAc
CH2OAc
m/z-->

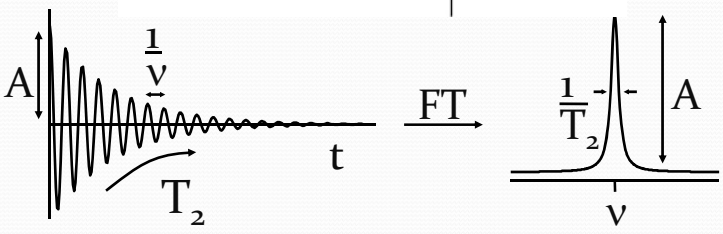
NMR spectra assignment of carbohydrate containing molecules

Sample preparation



Data analysis - Analysis of FIDs





time domain (FID)

frequency domain (spectrum)

The ADC (analog-to-digital converter) converts the FID into a series of points

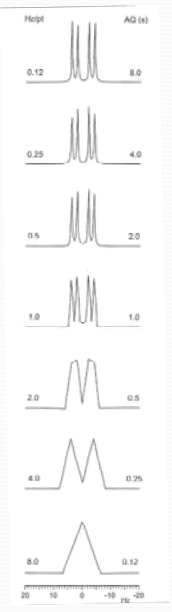
Digital Resolution: is equal to (acquisition time)$^{-1}$

$$DW = \frac{1}{2SW}$$

$$AQ = DW \bullet TD = \frac{TD}{2SW}$$

$$DR = \frac{SW}{SI} = \frac{2SW}{TD} = \frac{1}{AQ}$$

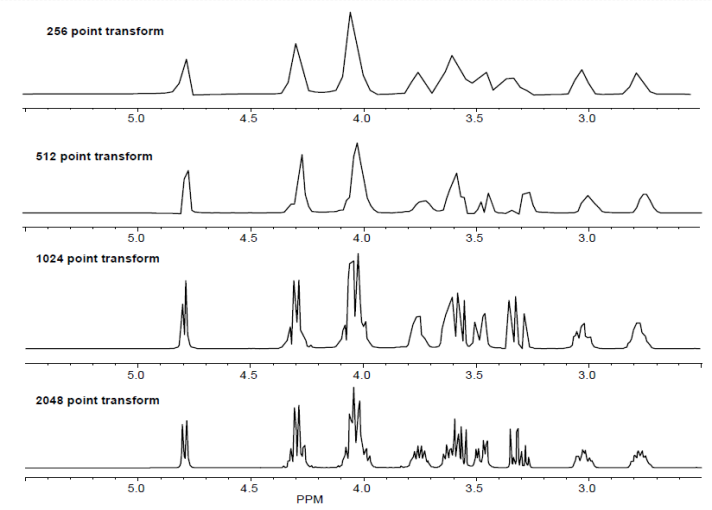
DW is dwell (time between digital points)
AQ is the acquisition time (time the FID is sampled)
TD is the number of points collected in the FID
DR is the digital resolution
SI is the number of points in the frequency spectrum
TD and SI are base 2 numbers (2, 4, 8, …,1024, 2048,…) to make FFT work



NMR Data Processing

Digitalization: Convert FID (Volt/Time) in Digital form

Want to maximize digital resolution, number of data points in each dimension



NMR Data Processing

Data Manipulations

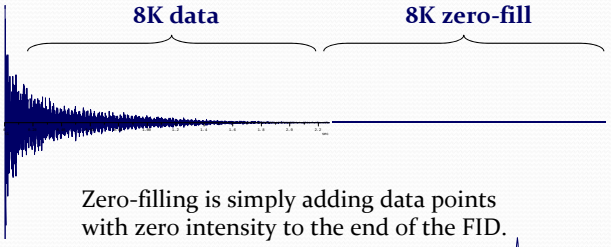
The optimal spectrum can almost never be obtained by Fourier transformation of the data directly from the spectrometer.

At least three manipulations are generally required:

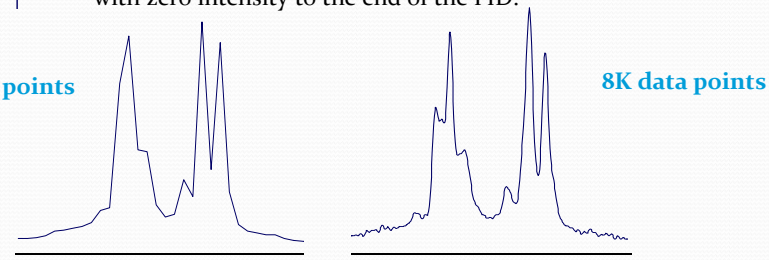
- Zero Filling
- Apodization
- Phasing

NMR Data Processing

Zero filling



Zero-filling is simply adding data points with zero intensity to the end of the FID.

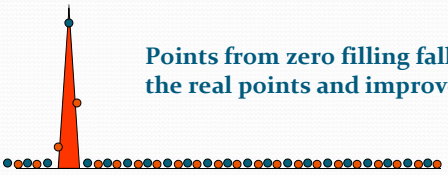


Collect data until FID goes to zero. However, you still may not be able to define the top of the peak. Then zero fill.

NMR Data Processing

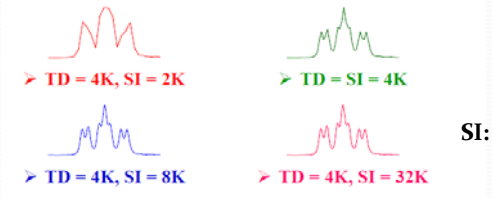
Zero filling

frequency



Points from zero filling fall between the real points and improve *digital* resolution.

frequency

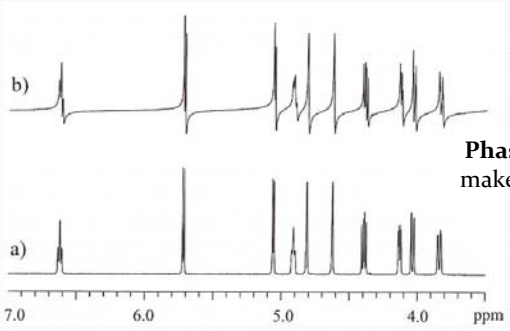


TD: Size of FID
SI: Size of real spectrum (after zero filling

NMR Data Processing

Phase correction

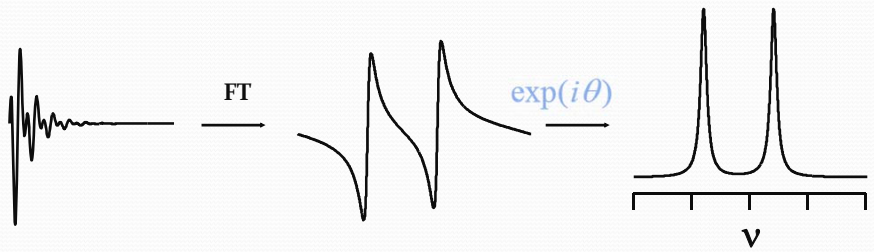
Phase errors arise because detection of an NMR signal (FID) can begin at any point in an oscillation, depending on the instrumental and experimental parameters. Phase of the peak is determined by the relative phase of the pulse and the receiver
They appear as twists in the baseline of the Fourier-transformed spectrum, and are corrected automatically by the spectrometer's computer or manually by trial-and-error.



Phasing: pho is constant rotation to make the real signal absorptive and the imaginary signal dispersive

NMR Data Processing

Processing of NMR signals



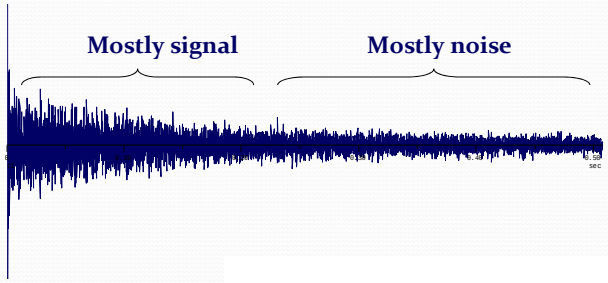
FID (time domain)

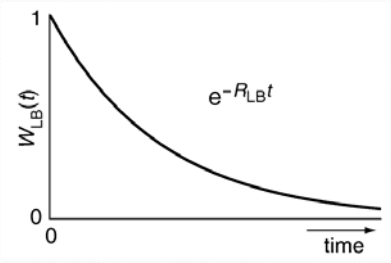
spectrum - unphased (frequency domain)

spectrum - phased (frequency domain)

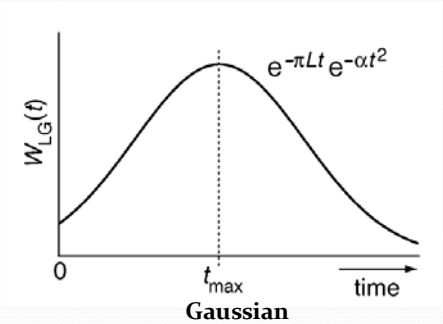
NMR Data Processing

Apodization - Window functions



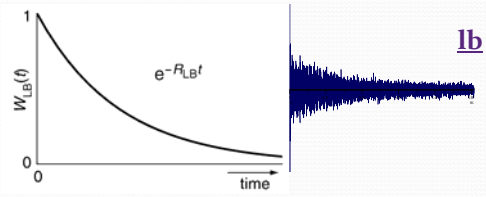


Exponential



Gaussian

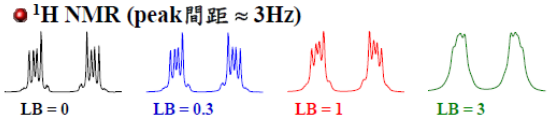
Exponential functions and Line broadening



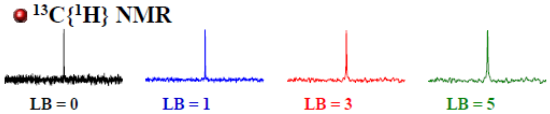
lb (line broadening factor) > 0

Multiplying the FID by an exponential curve should result in improved S/N.

- ^{1}H NMR (peak間距 ≈ 3Hz)

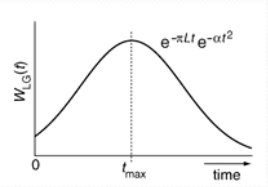


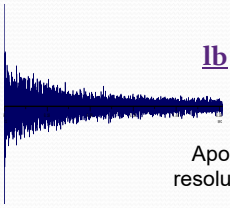
- ^{13}C{^{1}H} NMR



Improved S/N, worst resolution

Gaussian functions and resolution

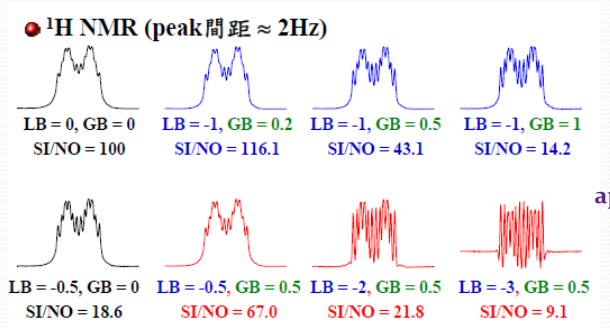




lb (line broadening factor) < 0

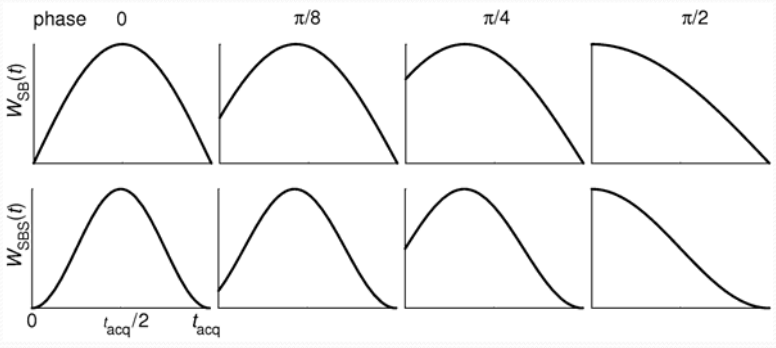
Apodization can also be used to improve resolution by emphasizing the tail of the FID.

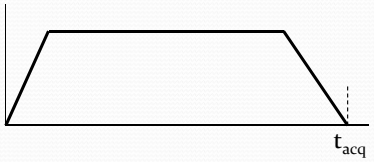
This function emphasizes the middle and end of the FID



The price to pay for this apodization is a significant decrease in S/N;

Other windows functions





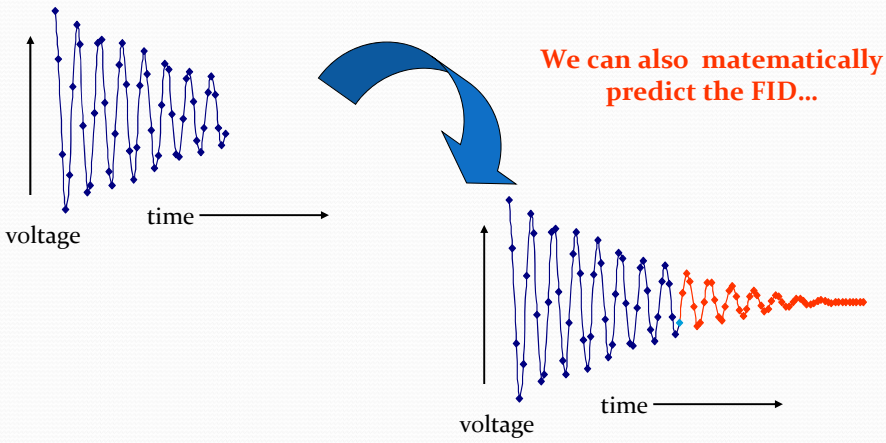
.....Bruker command:

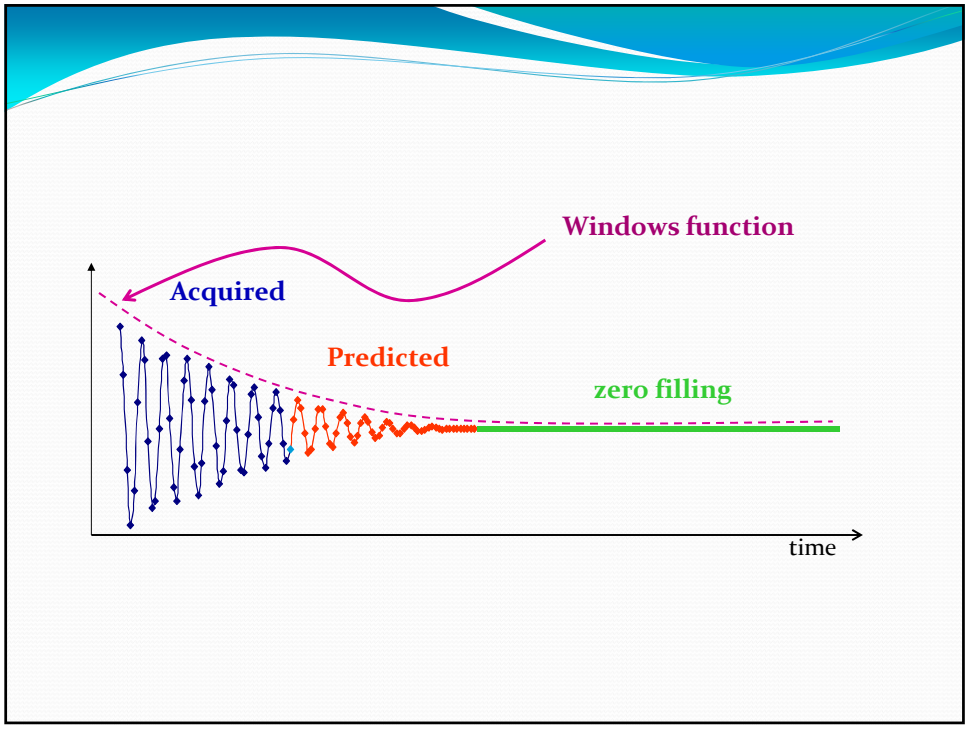
- em: Exponential window multiplication of the FID
- gm: Gaussian window multiplication of the FID
- pk: Phase correction according to PHC0/PHC1
- ft: Fourier transform

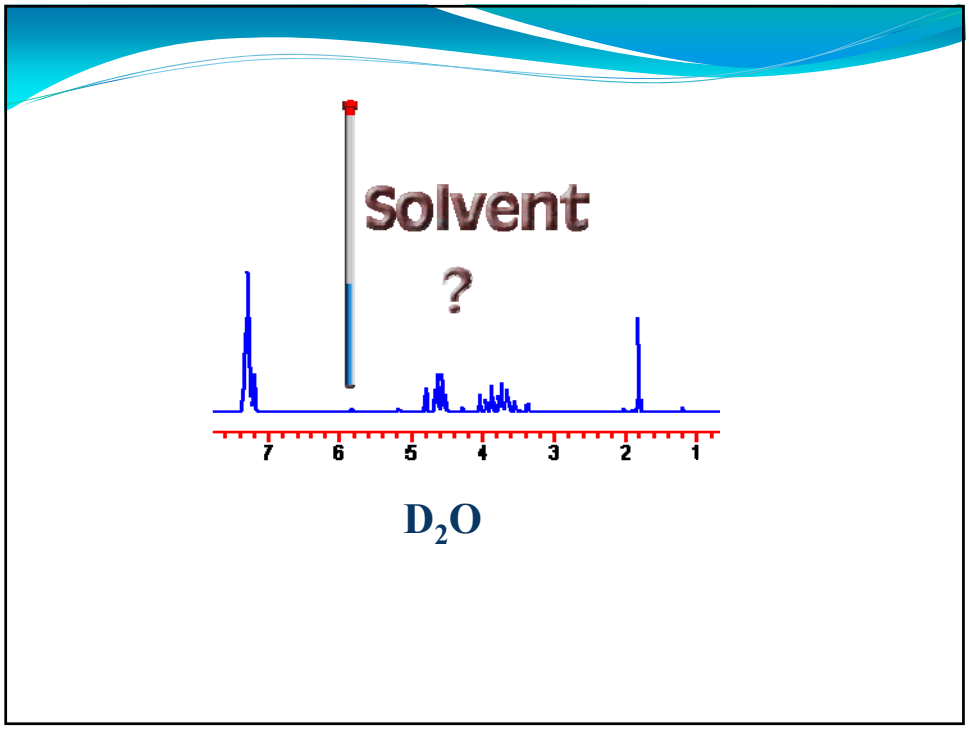
組合指令

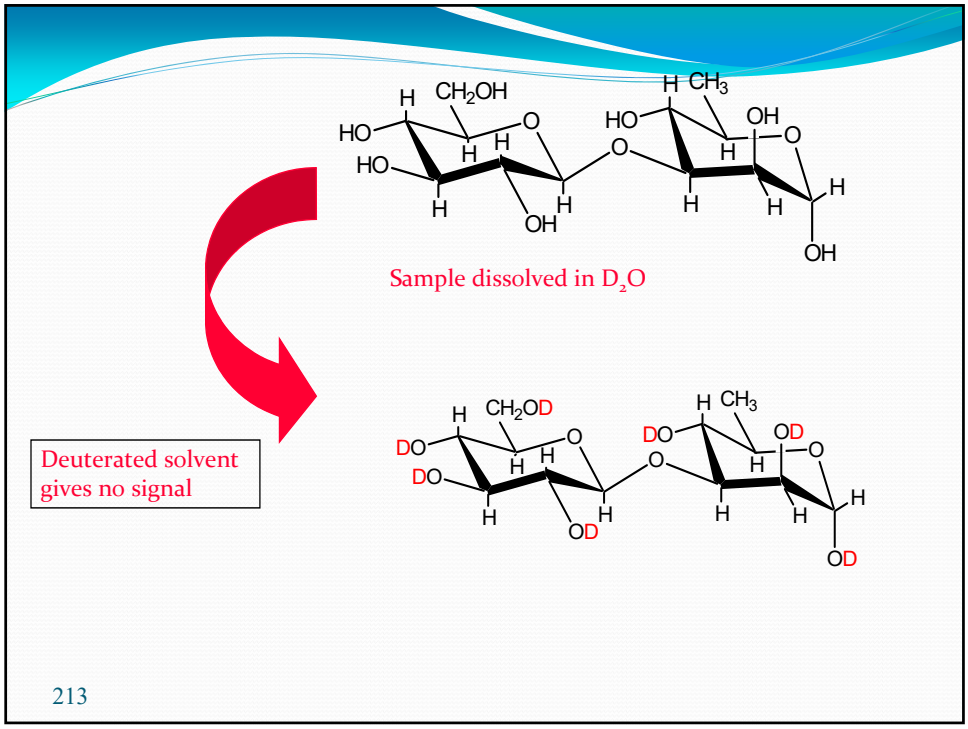
- em + ft = ef
- gm + ft = gf
- em + ft + pk = efp
- gm + ft + pk = gfp
- ft + pk = fp

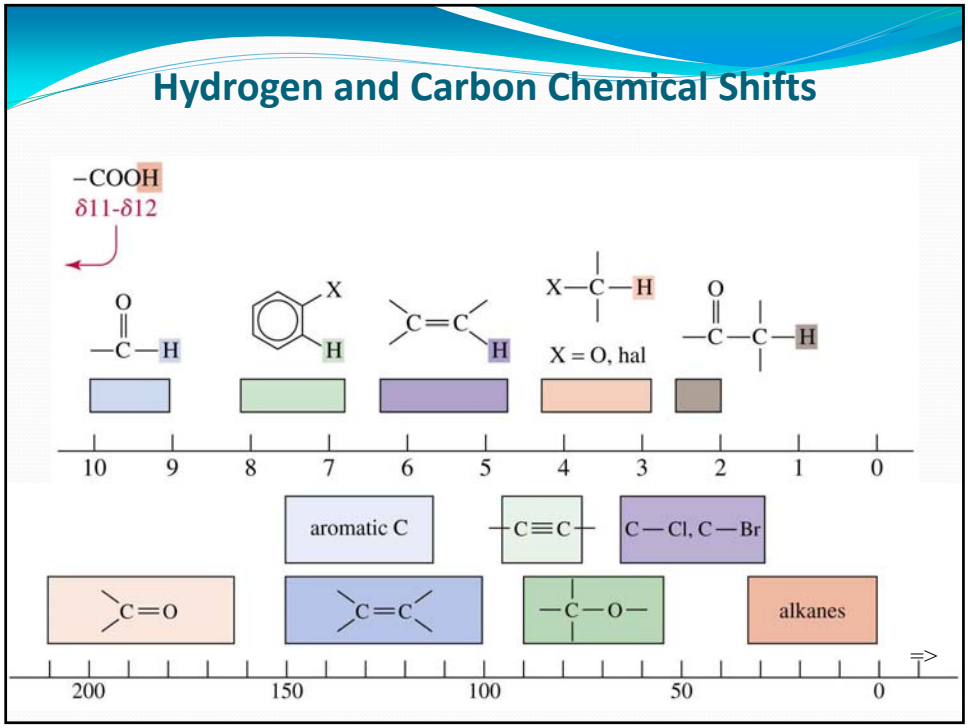
Linear prediction




Windows function
Acquired
Predicted
zero filling
time


Solvent
?
7
6
5
4
3
2
1
D2O


Sample dissolved in D_2O
Deuterated solvent gives no signal
213


Hydrogen and Carbon Chemical Shifts
–COOH
δ11-δ12
X = O, hal
10 9 8 7 6 5 4 3 2 1 0
aromatic C
C—Cl, C—Br
alkanes
200 150 100 50 0

Parameters in NMR

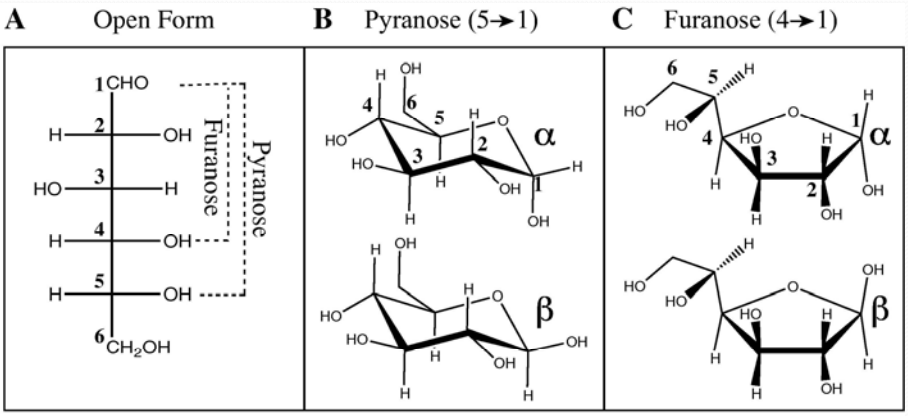
2. **Coupling constant (J) → Hz**
   Structural and
   Conformational information
3. **Area of peaks**
   Relative proportion of nuclei
4. **Distance between nuclei**
   Information is contained in relaxation and NOE
5. **Molecular Motion**
   Information is contained in relaxation, NOE and Variable Temperature

NMR Assignments

What is the NMR Assignment Issue?

• **Each observable NMR resonance needs to be assigned or associated with the atom in the protein structure.**

Sugar complexity



Open, pyranose and furanose forms of an aldose, showing the many equivalent OH groups. The OH groups can be modified by $-PO_4^{-3}$, $-SO_4^{-2}$, $-NH_2$, $-CO-CH_3$, $-NH-CO-CH_3$, -H.

^{1}H and ^{13}C typical regions of carbohydrates:

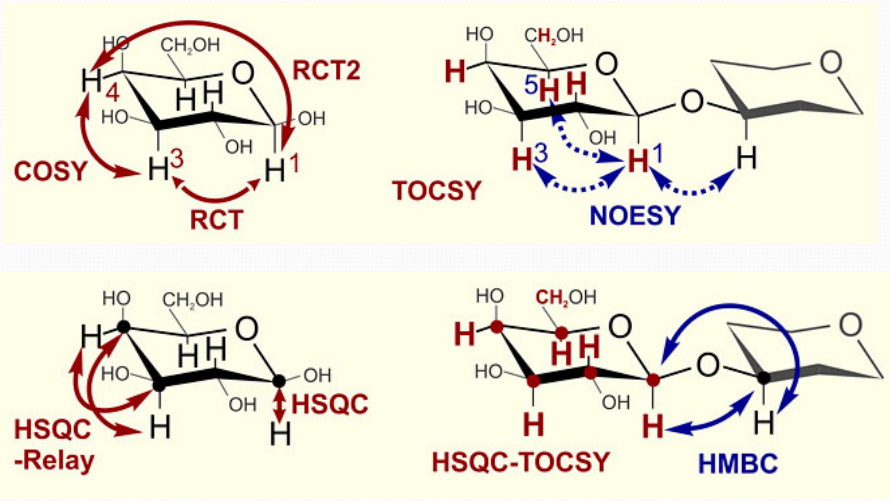
The ^{1}H NMR Spectra can be roughly divided into the following regions:

Anomeric and Acylated Protons : 5.5-4.5 ppm.
Ring Protons : 4.5-3 ppm
Acetyl Groups, Methylene Protons: 3-2 ppm
Methyl Groups: 0.8-2.0 ppm

The ^{13}C NMR Spectra can be roughly divided into the following regions :

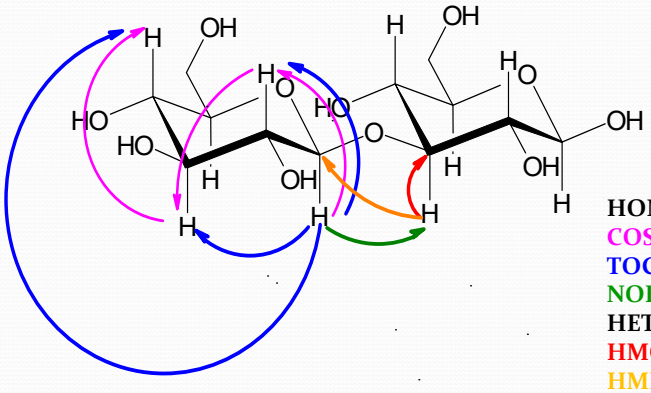
Anomeric Carbons Resonate Between 90-105 ppm
Ring Carbons Between 52-78 ppm
Nitrogen Bearing Carbons (In Amino Sugar) 50-60 ppm
Acetyl Groups XXX ppm
Methylene Protons: XXX ppm
Methyl Groups: XXX ppm

Application of various NMR techniques to carbohydrates

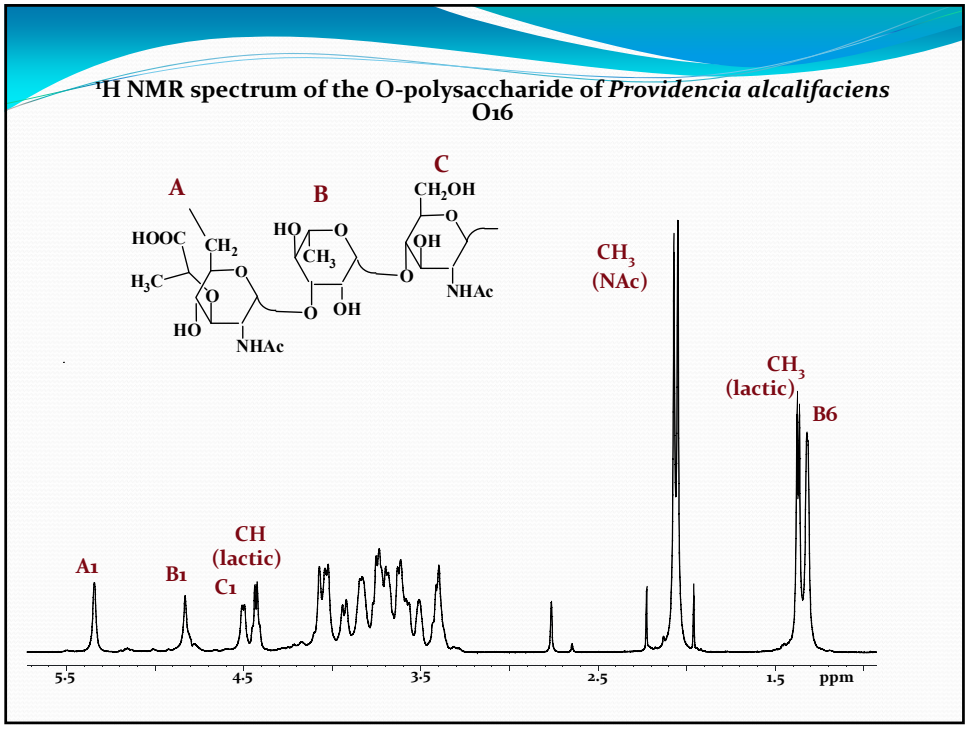


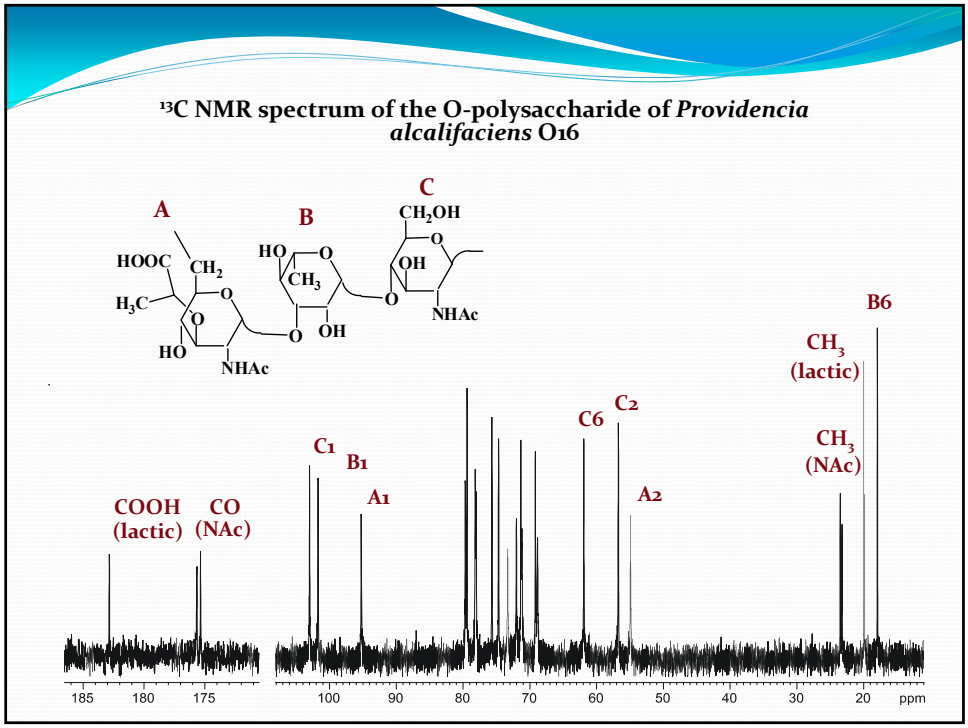
Assignment of sugar residues

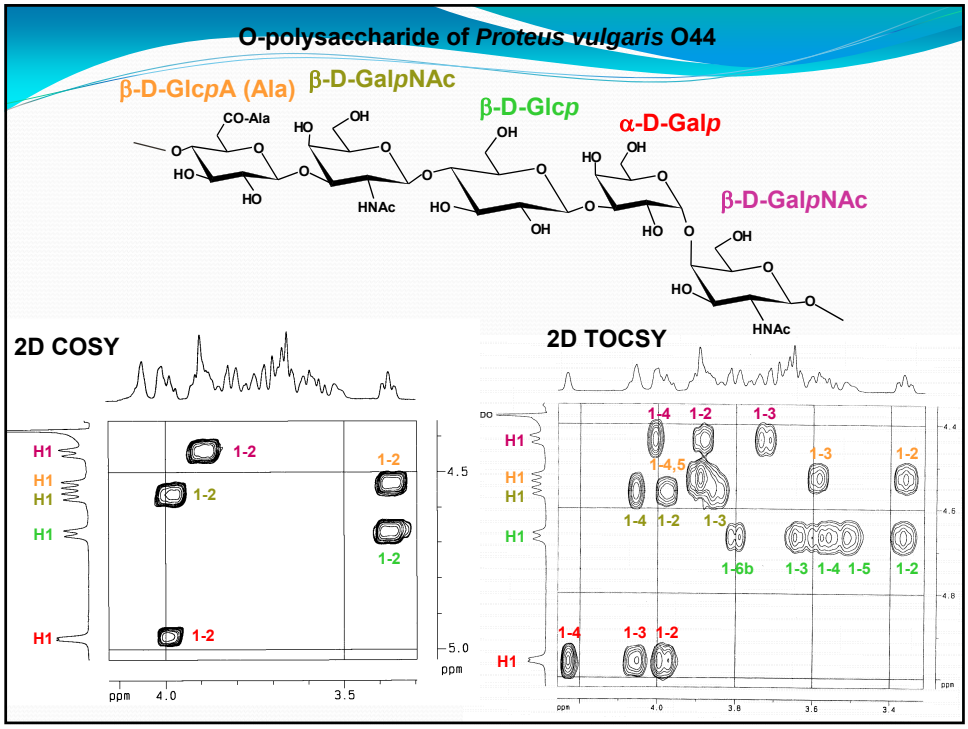
- HOMONUCLEAR (^{1}H-^{1}H)
- HETERONUCLEAR (^{1}H-^{13}C)

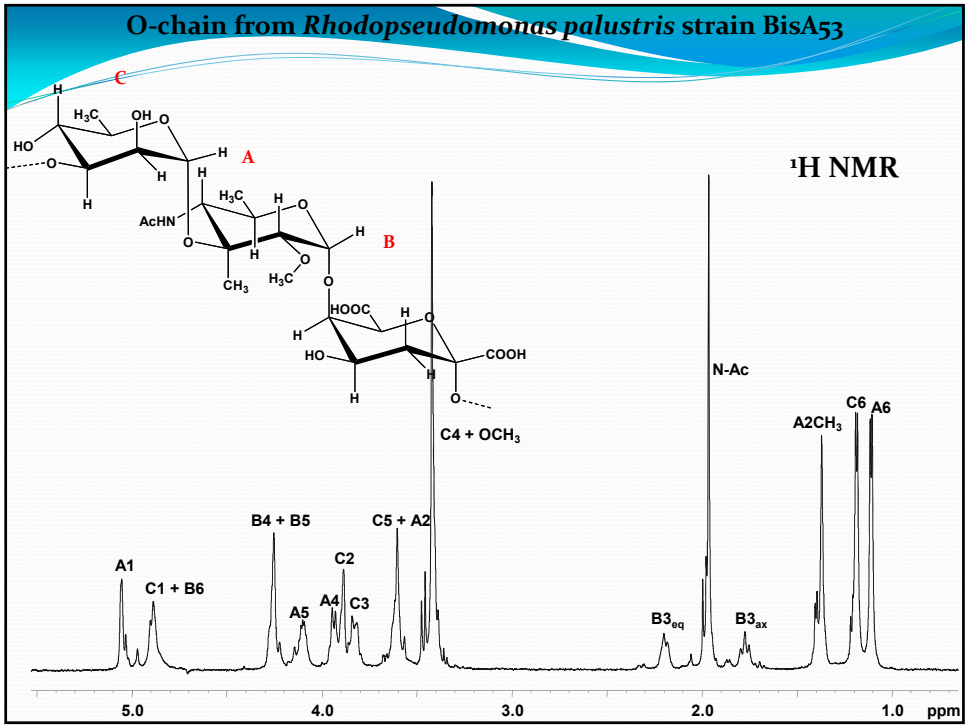


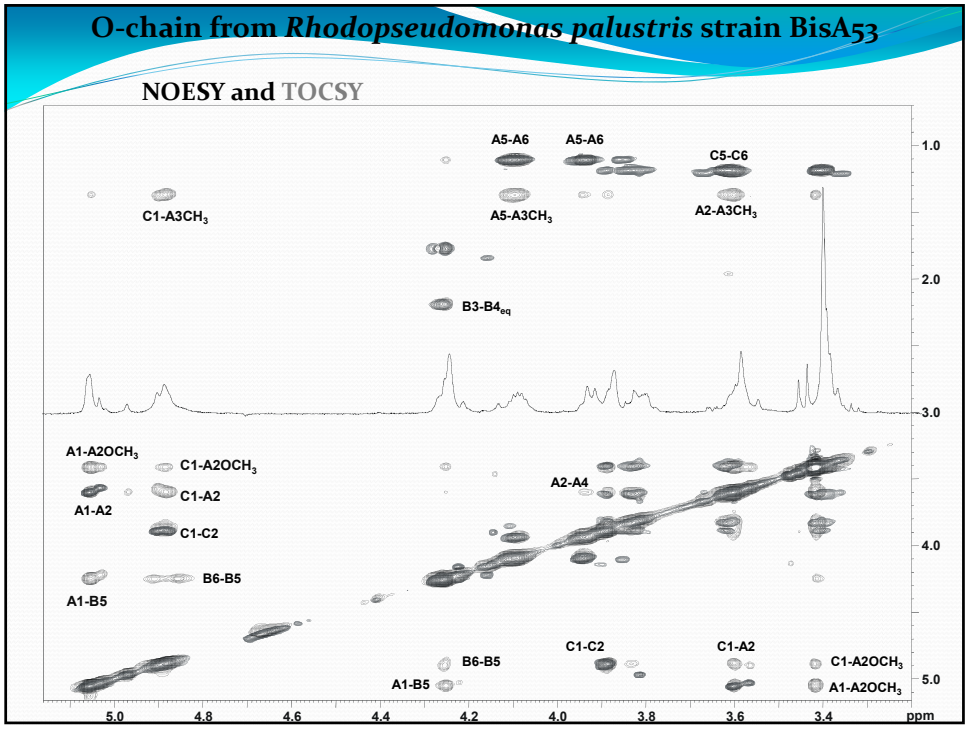
HOMONUCLEAR ^{1}H-^{1}H
COSY
TOCSY
NOESY/ROESY
HETERONUCLEAR ^{1}H-^{13}C
HMQC/HSQC
HMBC

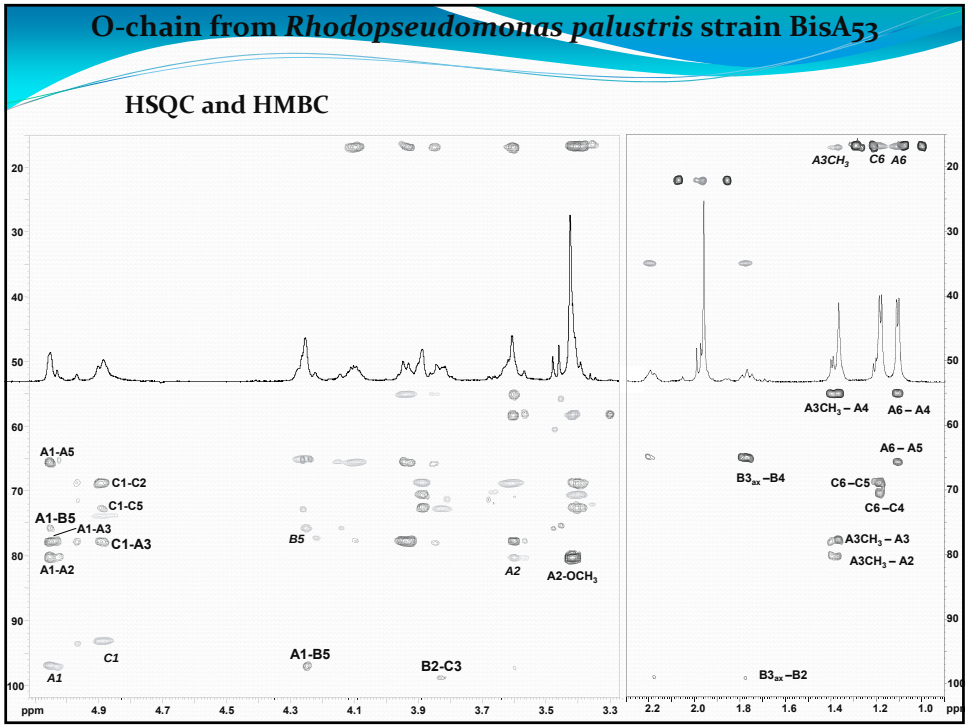

¹H NMR spectrum of the O-polysaccharide of *Providencia alcalifaciens* O16
A
B
C
CH₂OH
HOOC
CH₂
H₃C
HO
CH₃
OH
NHAc
CH₃ (NAc)
CH₃ (lactic)
B6
A1
B1
CH (lactic)
C1
5·5
4·5
3·5
2·5
1·5
ppm


¹³C NMR spectrum of the O-polysaccharide of *Providencia alcalifaciens* O16
A
B
C
CH₂OH
HOOC
CH₂
H₃C
HO
CH₃
OH
NHAc
B6
CH₃ (lactic)
C6
C2
C1
B1
A1
CH₃ (NAc)
A2
COOH (lactic)
CO (NAc)
185
180
175
100
90
80
70
60
50
40
30
20
ppm

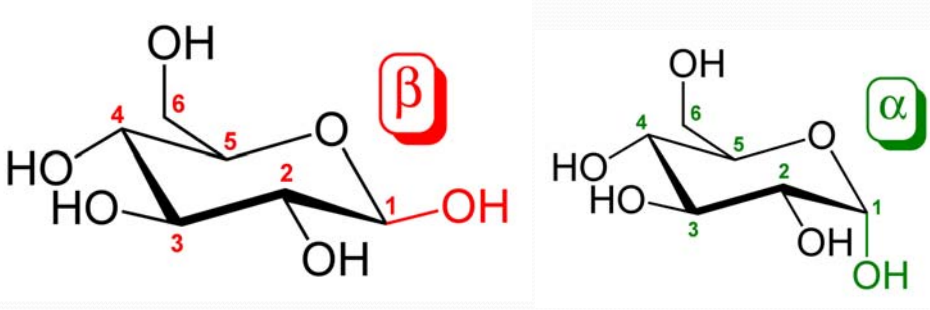

O-polysaccharide of *Proteus vulgaris* O44
β-D-Glc*p*A (Ala)
β-D-Gal*p*NAc
β-D-Glc*p*
α-D-Gal*p*
β-D-Gal*p*NAc
CO-Ala
HNAc
2D COSY
2D TOCSY
H1
1-2
1-3
1-4
1-4,5
1-6b
1-5
ppm


O-chain from *Rhodopseudomonas palustris* strain BisA53
A
B
C
AcHN
HOOC
COOH
C4 + OCH3
¹H NMR
N-Ac
A2CH3
C6
A6
B4 + B5
C5 + A2
C2
A1
C1 + B6
A5
A4
C3
B3eq
B3ax
5.0
4.0
3.0
2.0
1.0
ppm

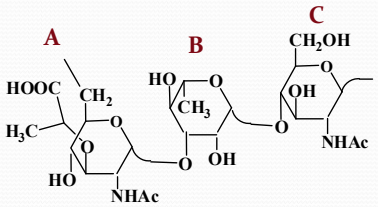

O-chain from *Rhodopseudomonas palustris* strain BisA53
NOESY and TOCSY
A5-A6
A5-A6
C5-C6
C1-A3CH3
A5-A3CH3
A2-A3CH3
B3-B4eq
A1-A2OCH3
C1-A2OCH3
A2-A4
C1-A2
A1-A2
C1-C2
B6-B5
A1-B5
C1-C2
C1-A2
B6-B5
C1-A2OCH3
A1-B5
A1-A2OCH3
1.0
2.0
3.0
4.0
5.0
5.0
4.8
4.6
4.4
4.2
4.0
3.8
3.6
3.4
ppm

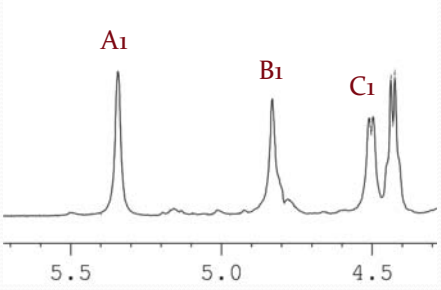

O-chain from *Rhodopseudomonas palustris* strain BisA53
HSQC and HMBC
A3CH3
C6
A6
A3CH3 – A4
A6 – A4
A6 – A5
B3ax –B4
C6 –C5
C6 –C4
A3CH3 – A3
A3CH3 – A2
B3ax –B2
A1-A5
C1-C2
C1-C5
A1-B5
A1-A3
C1-A3
A1-A2
B5
A2
A2-OCH3
C1
A1
A1-B5
B2-C3
20
30
40
50
60
70
80
90
100
ppm
4.9
4.7
4.5
4.3
4.1
3.9
3.7
3.5
3.3
2.2
2.0
1.8
1.6
1.4
1.2
1.0

•Anomeric configuration

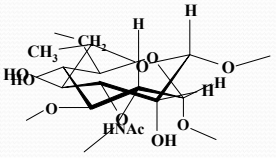


¹H NMR spectrum contains information on the configuration of glycosidic linkages





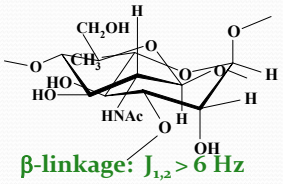
A B



α-linkage: $J_{1,2} \leq 4$ Hz

β-linkage: $J_{1,2} \leq 2$ Hz

C

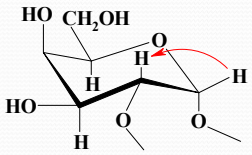


β-linkage: $J_{1,2} > 6$ Hz

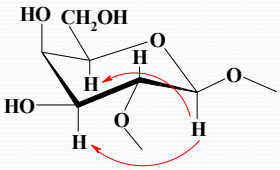
α-linkage: $J_{1,2} < 2$ Hz

Intra-residue NOE contacts in monosaccharides

gluco, galacto configuration

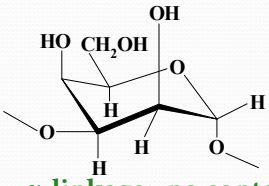


α-linkage: H1-H2

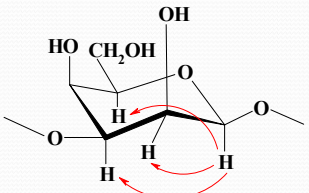


β-linkage: H1-H3, H1-H5

manno configuration



α-linkage: no contact



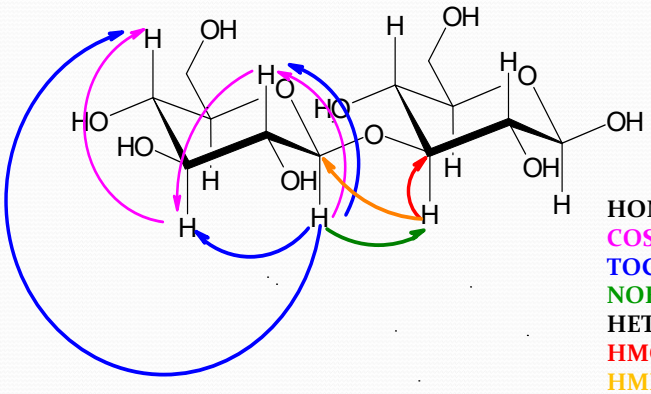
β-linkage: H1-H2, H1-H3, H1-H5

❖Monosaccharide Sequence

- *Inter*-residual NOE contact
- Glycosylation shift
- Long range inter-residual correlation in the HMBC spectra

Monosaccharide Sequence

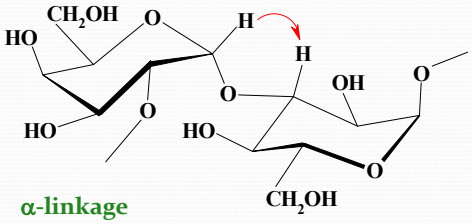
•*Inter*-residual NOE contact



HOMONUCLEAR ^{1}H-^{1}H
COSY
TOCSY
NOESY/ROESY
HETERONUCLEAR ^{1}H-^{13}C
HMQC/HSQC
HMBC

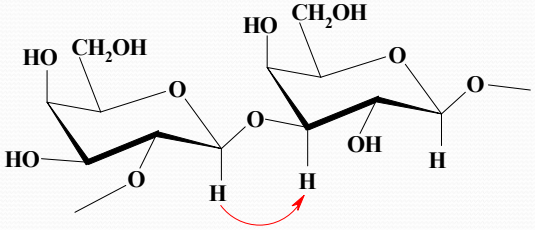
Monosaccharide Sequence

***Inter*-residue NOE contacts in saccharides**



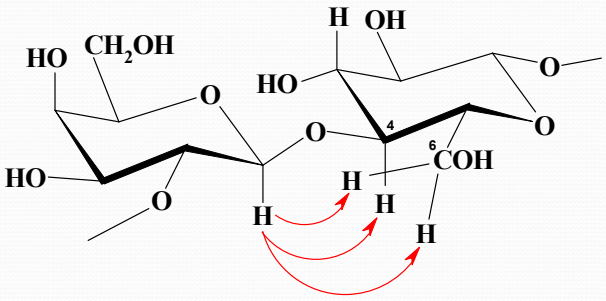
α-linkage

β-linkage

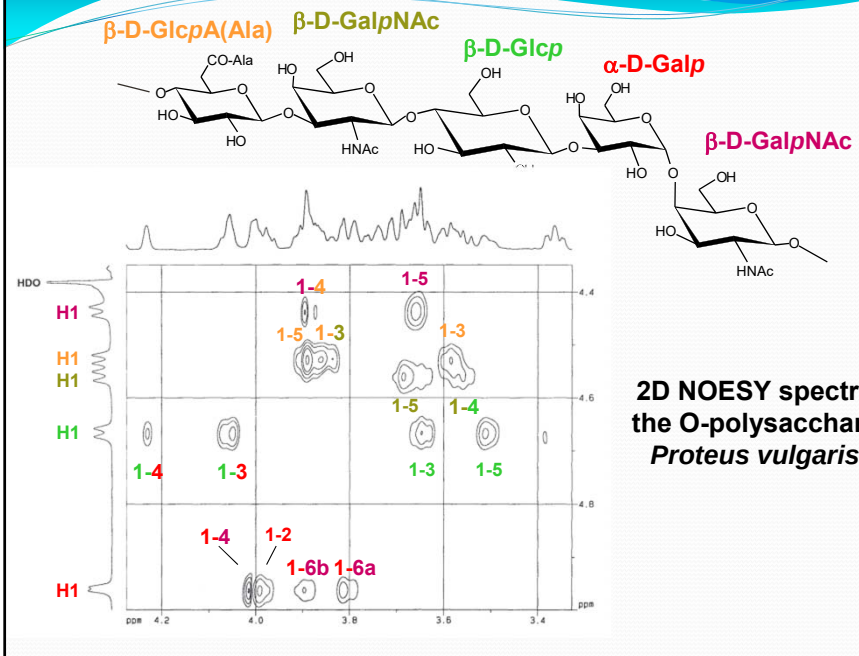


❖Monosaccharide Sequence

NOE in disaccharides may occur not only at the linkage protons but also at the neighbouring protons



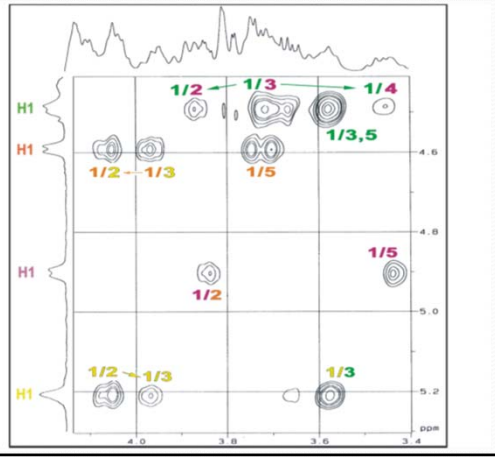
Monosaccharide Sequence



2D NOESY spectrum of the O-polysaccharide of *Proteus vulgaris* O44

Monosaccharide Sequence

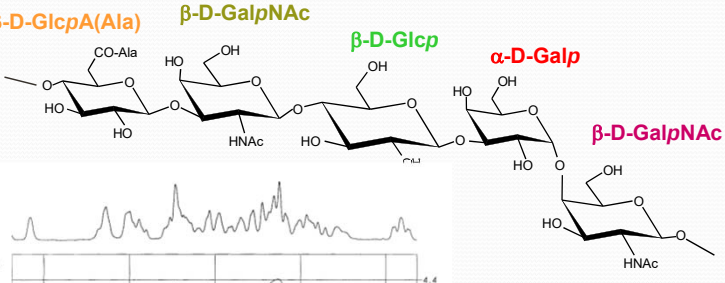
β-D-Gal*p* α-D-Glc*p*NAc β-D-Glc*p* β-D-Glc*p*NAc

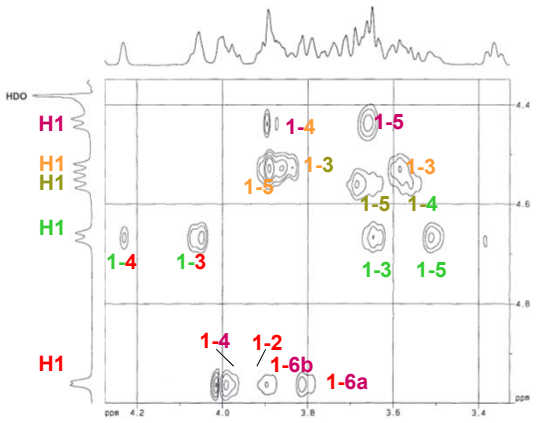


2D NOESY spectrum of the dephosphorylated O-polysaccharide of *Proteus mirabilis* O33

Monosaccharide Sequence

β-D-Glc*p*A(Ala) β-D-Gal*p*NAc β-D-Glc*p* α-D-Gal*p* β-D-Gal*p*NAc

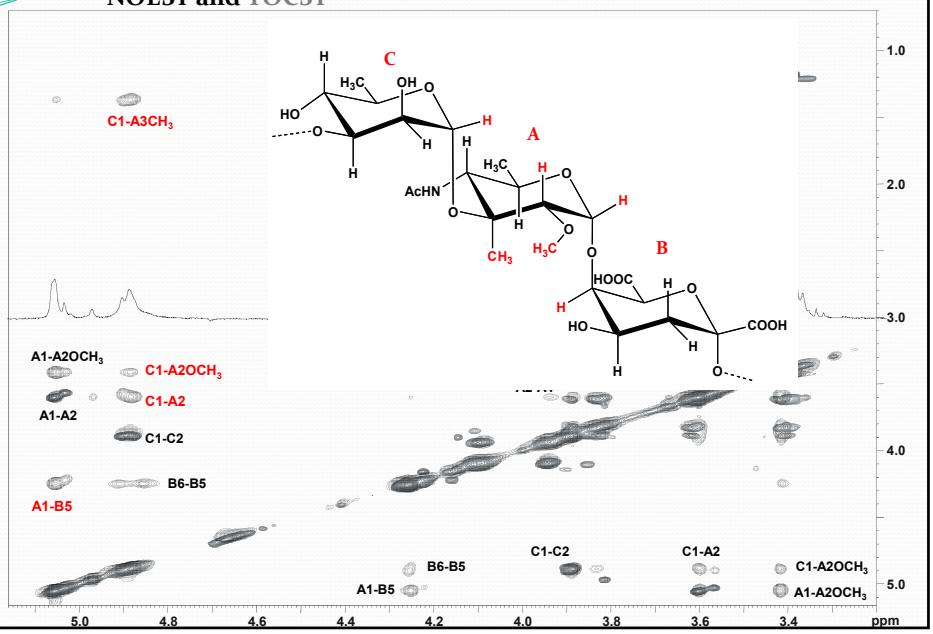




2D ROESY spectrum of the O-polysaccharide of *Proteus vulgaris* O44

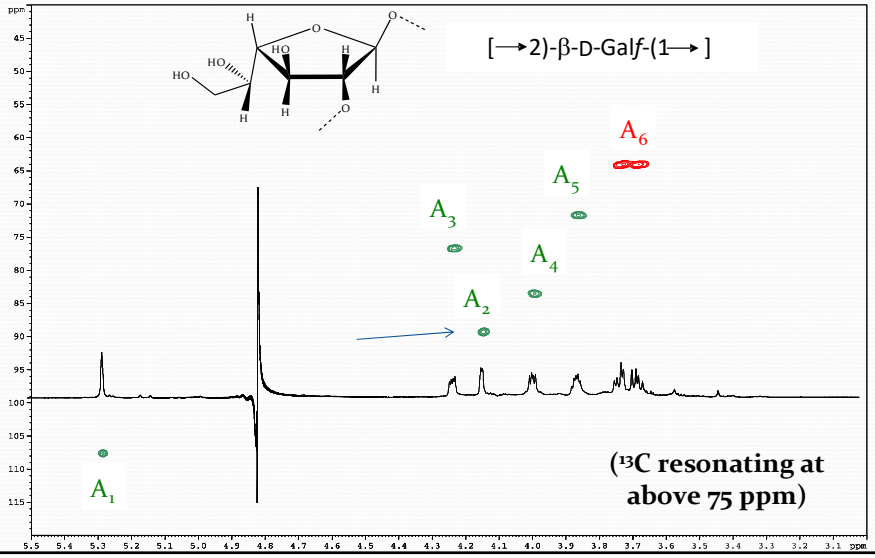
O-chain from *Rhodopseudomonas palustris* strain BisA53

NOESY and TOCSY



Monosaccharide Sequence

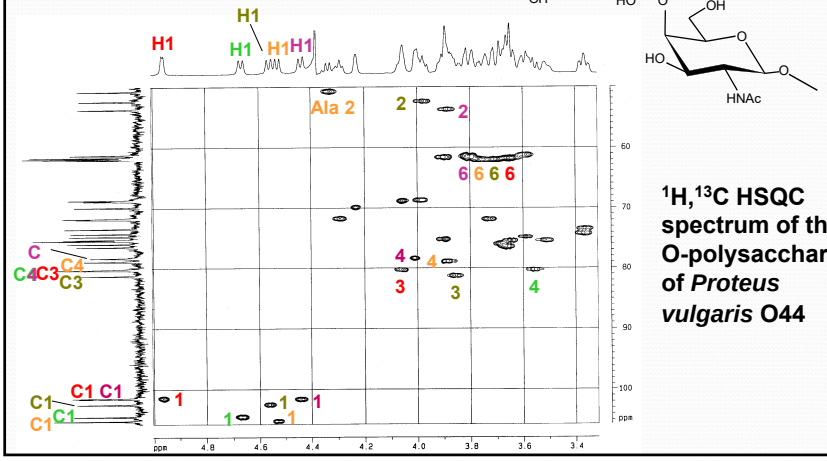
Glycosylation shift: downfield shift of carbon resonances at glycosilated position present in the HSQC spectrum



Monosaccharide Sequence

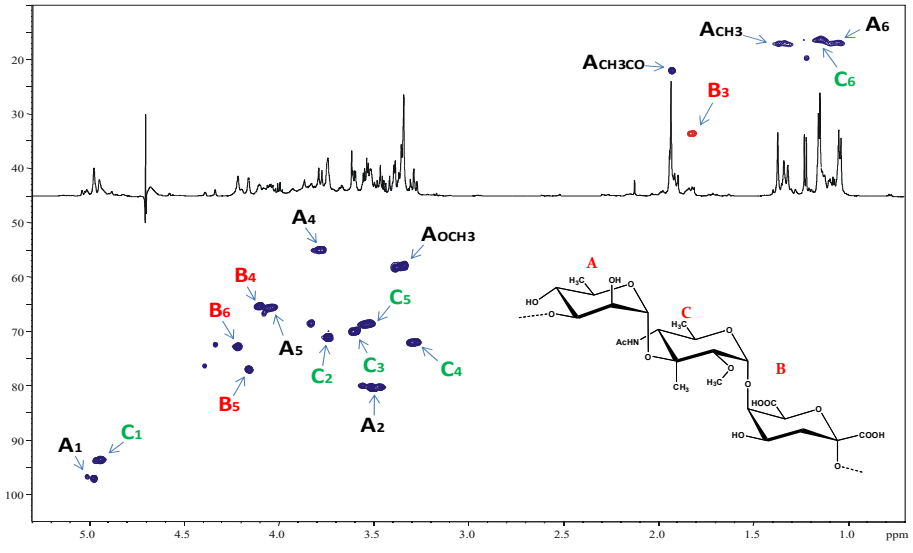
Glycosylation shift

β-D-Glc*p*A(Ala) β-D-Gal*p*NAc β-D-Glc*p* α-D-Gal*p* β-D-Gal*p*NAc



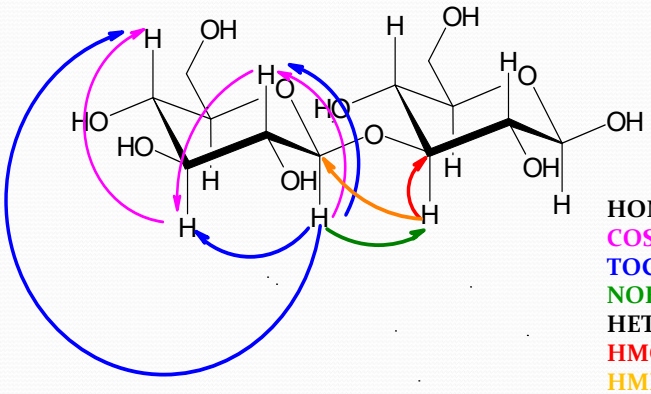
$^{1}H,^{13}C$ HSQC spectrum of the O-polysaccharide of *Proteus vulgaris* O44

HSQC spectrum of the *O*-chain isolated from *Rhodopseudomonas palustris* sp. BIS A53



Monosaccharide Sequence

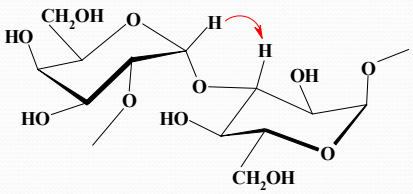
Long range *inter*-residual correlations in the HMBC spectrum



HOMONUCLEAR ^{1}H-^{1}H
COSY
TOCSY
NOESY/ROESY
HETERONUCLEAR ^{1}H-^{13}C
HMQC/HSQC
HMBC

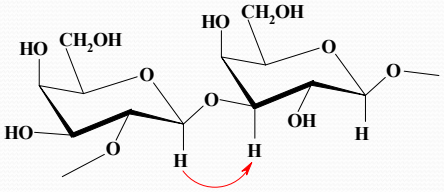
❖Monosaccharide Sequence

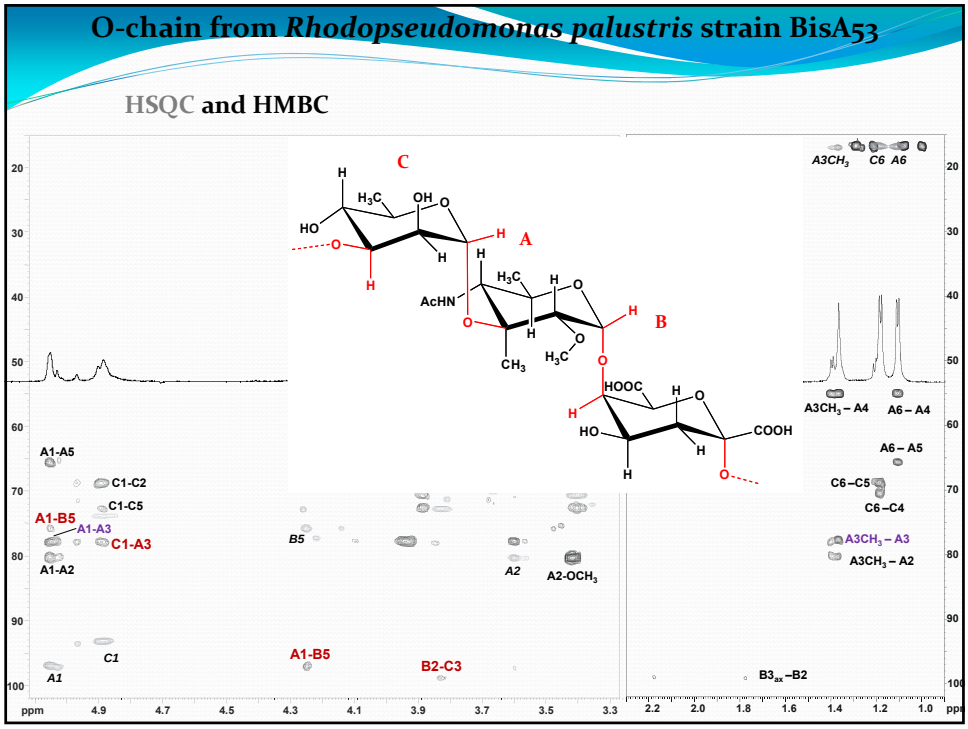
Long range *inter*-residual correlations in the HMBC spectrum



α-linkage

β-linkage




O-chain from *Rhodopseudomonas palustris* strain BisA53
HSQC and HMBC
C
A
B
A1-A5
C1-C2
C1-C5
A1-B5
A1-A3
C1-A3
A1-A2
C1
A1
B5
A1-B5
B2-C3
A2
A2-OCH3
A3CH3
C6
A6
A3CH3 – A4
A6 – A4
A6 – A5
C6–C5
C6–C4
A3CH3 – A3
A3CH3 – A2
B3ax–B2
ppm