FOOD stars — Innovative Food Product Development Cycle: Frame for Stepping Up Research Excellence of FINS

Basics of Primary and Secondary Metabolites

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Difficulty of Nat. Prod. Res.: Diversity of metabolites

- Metabolites in organisms

- Constitutive metabolites : primary and secondary metabolites

- Non constitutive metabolites : phytoalexin

- Exogenous metabolites : by other organism or chemicals

- about 250,000 natural products are known
- more than 3,500 metabolites in single plant
- about 4000 new ones are reported every year
- about 15% of all plants have been studied to some extend for their constituents

NADES Workshop

- **The 1st day (Introduction)**
  - **Natural Products Research: What to do and Where to go?**
  - **Green Technology: DES, ILs and SFE**
  - **Basic Phytochemistry**
- **The 2nd day (Application of NADES)**
  - **Sample Preparation and Analysis**
  - **Application of NADES to Natural Products Extraction**
- **The 3rd day (Practice)**
  - **NADES extraction of Flavonoid from *Sophora japonica* flowers**

The 1st day

- **Natural Products Research**
- **Green Technology**
- **Basic Phytochemistry**

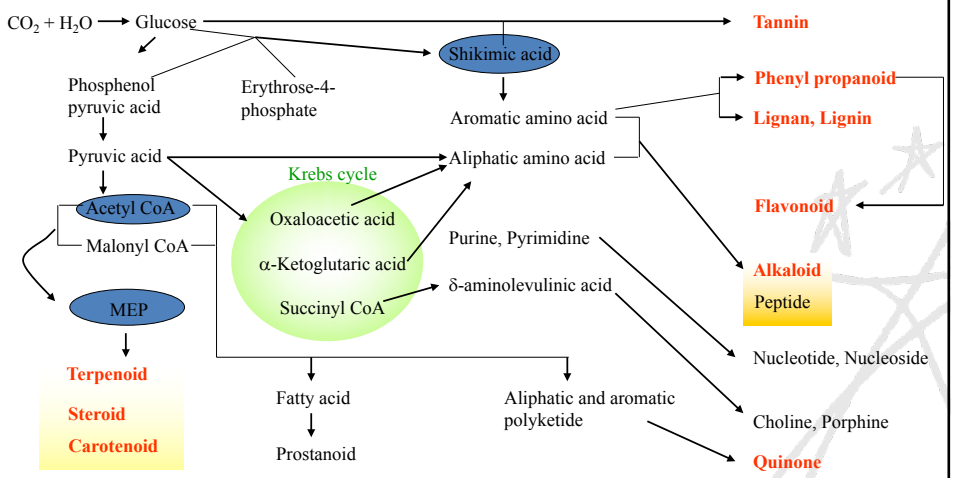
Now, you have samples for metabolomics!

Basics of plant metabolites

- **The 1st step of research to know about metabolites present in living organisms**
- **Chemical characteristics of group of metabolites**
  (carbohydrates, amino acids, flavonoids, terpenoids, alkaloids…)
  detected by diverse analytical methods (UV, MS, NMR)
- **Common metabolites (usual suspect) detected in plants**
- **Exceptional metabolites which show different analytical features from others**

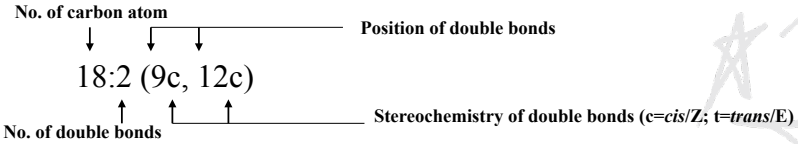
Are you afraid of huge number of metabolites?

Plant Metabolites



Fatty Acid and Lipid (1)

- Simple and complex
- Simple lipid: fatty acid, glyceride (ester of fatty acid and glycerol), wax (ester of higher fatty acid and higher alcohol)
- Complexed: glycolipid (sphingo, glycero), phospholipid (sphingo, glycero)



Saturated

butyric	$CH_3(CH_2)_2COOH$ (4:0)	stearic	$CH_3(CH_2)_{16}COOH$ (18:0)
caproic	$CH_3(CH_2)_4COOH$ (6:0)	arachidic	$CH_3(CH_2)_{18}COOH$ (20:0)
caprylic	$CH_3(CH_2)_6COOH$ (8:0)	behenic	$CH_3(CH_2)_{20}COOH$ (22:0)
capric	$CH_3(CH_2)_8COOH$ (10:0)	lignoceric	$CH_3(CH_2)_{22}COOH$ (24:0)
lauric	$CH_3(CH_2)_{10}COOH$ (12:0)	cerotic	$CH_3(CH_2)_{24}COOH$ (26:0)
myristic	$CH_3(CH_2)_{12}COOH$ (14:0)	montanic	$CH_3(CH_2)_{26}COOH$ (28:0)
palmitic	$CH_3(CH_2)_{14}COOH$ (16:0)	melissic	$CH_3(CH_2)_{28}COOH$ (30:0)

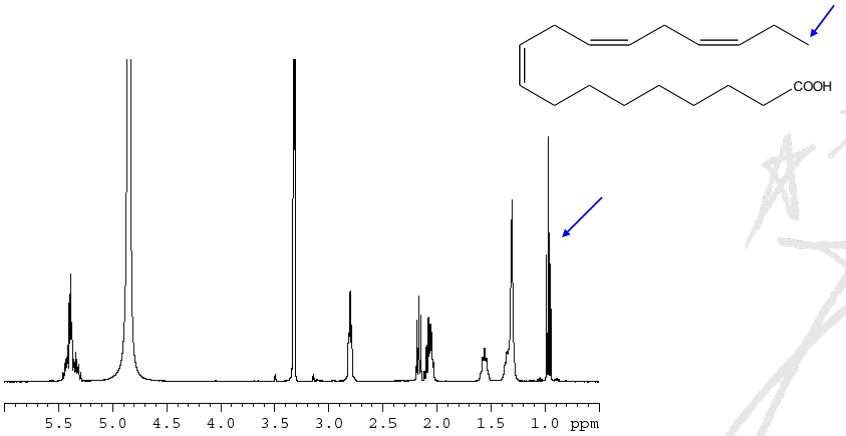
Fatty Acid and Lipid (2)

Unsaturated

- **Abundant in the seeds**
- **Choline, α-linolenic acid**
- **Difficult identify each lipid by NMR -> GC-MS**

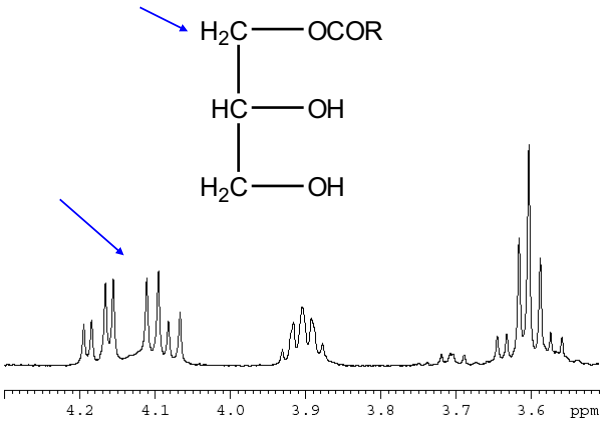
palmitoleic	$CH_3(CH_2)_5CH=CH(CH_2)_7COOH$
oleic	$CH_3(CH_2)_7CH=CH(CH_2)_7COOH$
cis-vaccenic	$CH_3(CH_2)_5CH=CH(CH_2)_9COOH$
linoleic	$CH_3(CH_2)_4CH=CHCH_2CH=CH(CH_2)_7COOH$
α-linolenic	$CH_3CH_2CH=CHCH_2CH=CHCH_2CH=CH\ (CH_2)_7COOH$
γ-linolenic	$CH_3(CH_2)_4CH=\ CHCH_2CH=\ CHCH_2CH=CH\ (CH_2)_4COOH$
gadoleic	$CH_3(CH_2)_9CH=CH(CH_2)_7COOH$
arachidonic	$CH_3(CH_2)_4CH=CHCH_2CH=\ CHCH_2CH=CHCH_2CH=CH(CH_2)_3COOH$
eicosapentanoic (EPA)	$CH_3CH_2CH=\ CHCH_2CH=\ CHCH_2CH=CHCH_2CH=CHCH_2CH=CH(CH_2)_3COOH$
erucic	$CH_3(CH_2)_7CH=CH(CH_2)_{11}COOH$
docosapentaenoic (DPA)	$CH_3CH_2CH=\ CHCH_2CH=\ CHCH_2CH=CHCH_2CH=CHCH_2CH=CH(CH_2)_5COOH$
docosahexaenoic (DHA)	$CH_3CH_2CH=CHCH_2CH=CHCH_2CH=CHCH_2CH=CHCH_2CH=\ CHCH_2CH=CH(CH_2)_2COOH$
nervonic	$CH_3(CH_2)_7CH=CH(CH_2)_{13}COOH$

^{1}H-NMR spectrum of α-linolenic acid



Terminal CH_3 might be a characteristic in NMR

^{1}H-NMR spectrum of glycerol moiety of lipid



Most fatty acids exist as conjugated form with glycerol

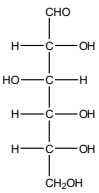
GC analysis of fatty acids (methylation): lipidomics

1. Add 1 mL of 0.5 M NaOH solution (10 g/500 mL in MeOH) to extract
2. React for 30 min at 75 °C
3. Transfer to 10 mL test tube
4. Extract with *n*-hexane (2 mL x 3) and transfer to 25 mL evaporating flask
5. Evaporate *n*-hexane extract
6. Add 0.5 mL of BF_3 in MeOH and transfer to 1.5 mL-glass vial
7. React for 20 min at 75 °C
8. Add 0.5 mL of *n*-hexane
9. Injection of 1 μL of *n*-hexane solution to GC
10. GC condition is the same to nonpolar metabolites analysis

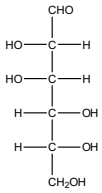
Carbohydrate (1)

- Polyhydroxy aldehyde or polyhydroxy ketone Stereoisomer having $C_n(H_2O)_n$
- Aldose (Aldehyde functional group), Ketose (Ketone functional group)
- Pentose: C5, Hexose: C6
- Sugars
  - Monosaccharides (aldoses and ketoses)
    Trioses, Tetroses, Hexoses
  - Oligosaccharides
    Disaccharides, Trisaccharides
  - Sugar derivatives
    Alcohols, Acids, Esters, Glycosides
  - Polysaccharides (glycans)
    Hexosans (Glucans, Fructans, Galactans, Mannans, Glucomannans, Galactomannans)
    Pentosans (Xylans, Arabans)
    Glycouronans (Glucouranans, Galactouronans)

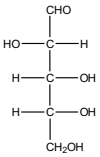
Carbohydrate (2)



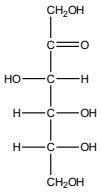
D-Glucose



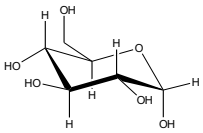
D-Mannose



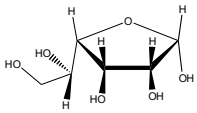
D-Arabinose



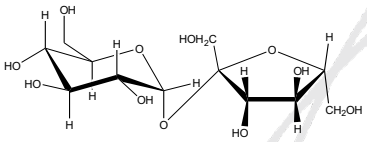
D-fructose



α-D-Glucopyranose



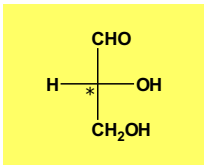
α-D-Glucofuranose



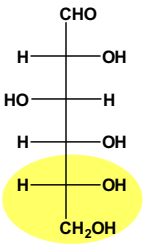
β-D-Fructofuranosyl-(2→1)-α-D-glucopyroanoside
(Sucrose)

Carbohydrate (3)

D- and L- form: Fischer-Rosanoff's rule



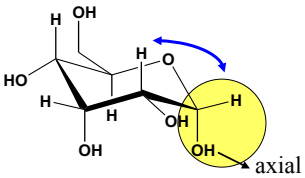
(+)-D-glyceraldehyde



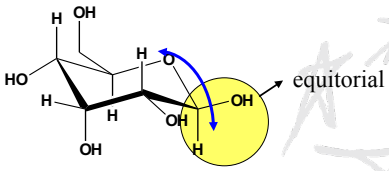
D-glucose : natural form

Carbohydrate (4)

Anomeric configuration : α- and β form



α-D-Glucopyranose

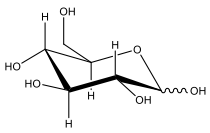


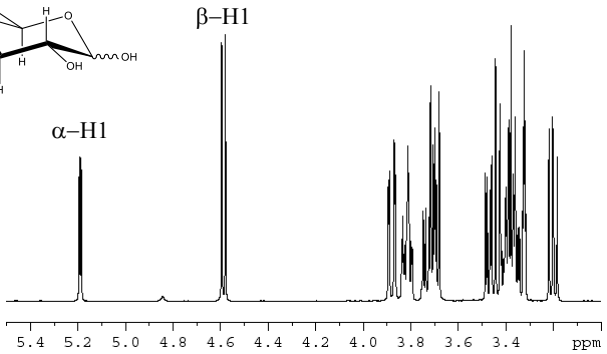
β-D-Glucopyranose

α form- 60° : J=2-3 Hz
β form- 180° : J=7-8 Hz

Ratio of glucose (α:β)= 36:64

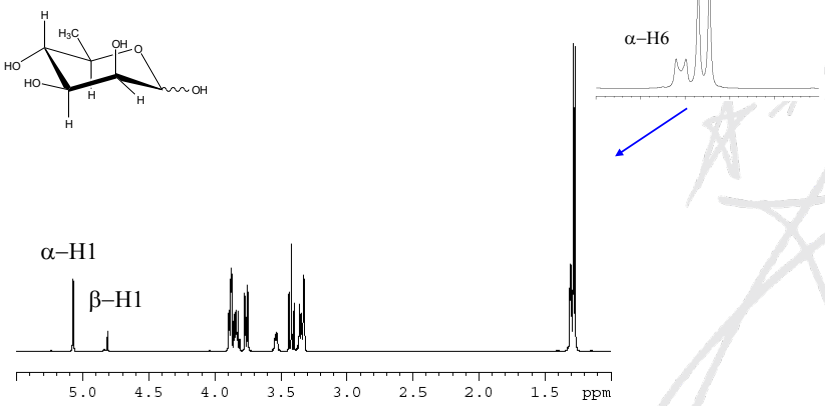
^{1}H-NMR spectrum of glucose



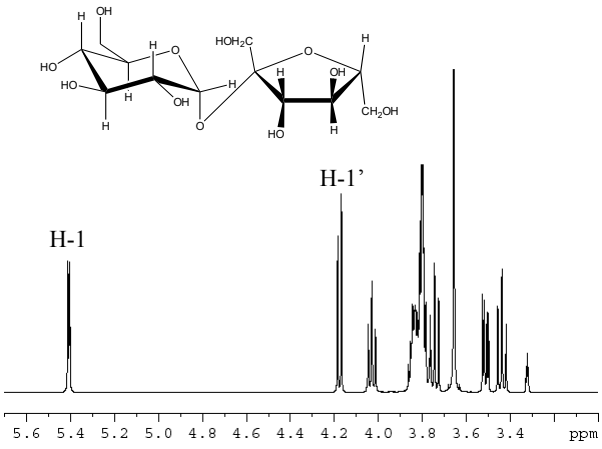


Monosaccharide has two forms in water solution

^{1}H-NMR spectrum of rhamnose



^{1}H-NMR spectrum of sucrose



If OH at C-1 position attach with other molecules there is only one form

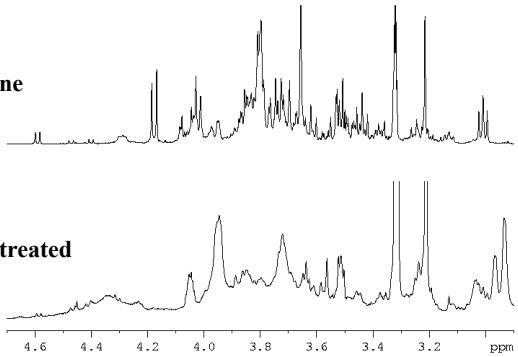
Carbohydrate (5)

- Low sensitivity in LC-MS (derivatization needed for increasing sensitivity)
- Good sensitivity and resolution in GC-MS after TMS-derivatization
- Several signals in GC-MS analysis
- Oligosaccharides make NMR signals broadening
- In NMR analysis CPMG pulse needed

^{1}H-NMR spectrum of cannabis cell line

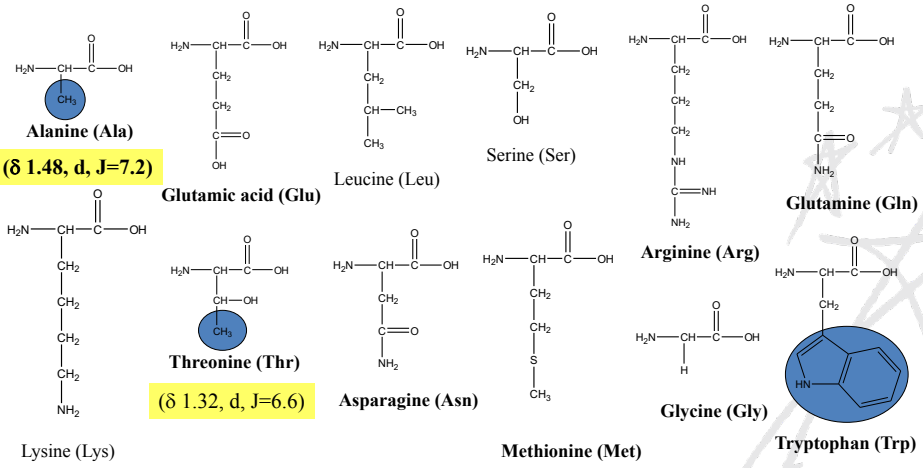
Normal cell line

Polysaccharide-treated



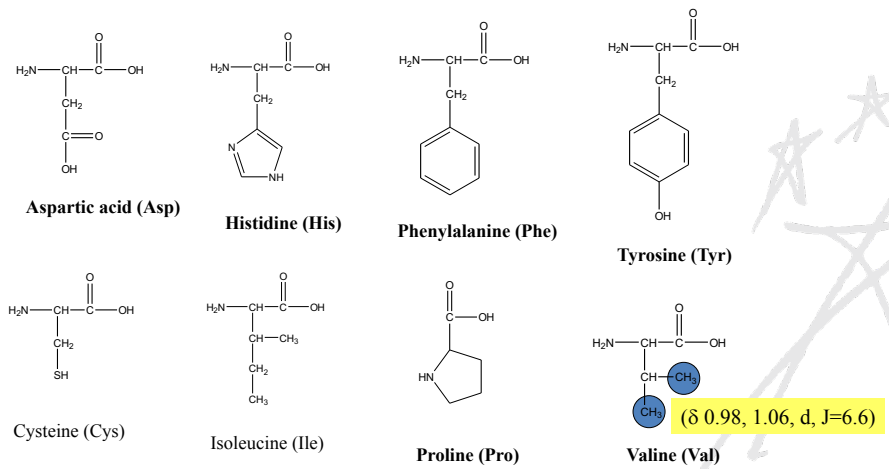
Oligo- or polysaccharide-rich sample cause signal broadening

Amino acid (1)

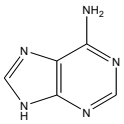


Progress in Nuclear Magnetic Resonance Spectroscopy, 1996, 28: 161-219.

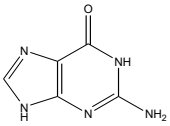
Amino acid (2)



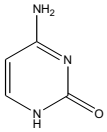
Nucleic acid



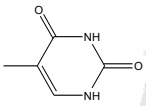
Adenine



Guanine

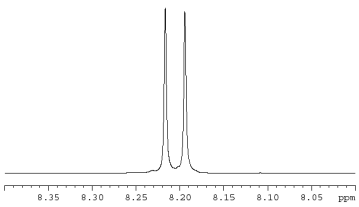


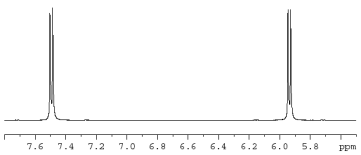
Cytosine

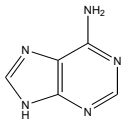


Thymine

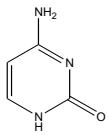
^{1}H-NMR spectrum of adenine and cytosine







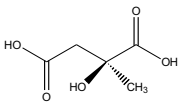
Adenine



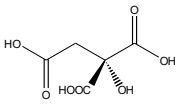
Cytosine

Signals are very pH sensitive

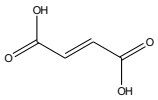
Organic acid



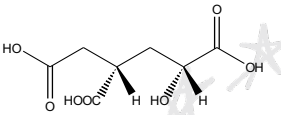
Citramalic acid



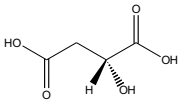
Citric acid



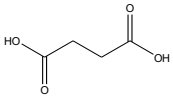
Fumaric acid



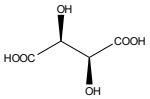
Isocitric acid



Malic acid

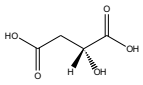


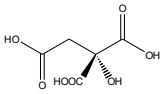
Succinic acid



Tartaric acid

^{1}H-NMR spectrum of citric acid and malic acid

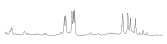
Malic acid 

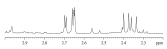
Citric acid 

pH effect: nalic acid in plants

pH 6 (acetate) 

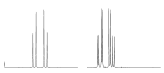
pH 6 (phosphate) 

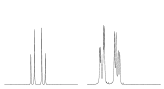
pH 7 (phosphate) 

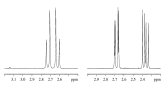
pH 8 (phosphate) 

^{1}H-NMR spectra of *Senecio jacobea* (δ 2.5 – δ 3.0)

Concentration effect: citric acid and malic acid

10 mg/mL 

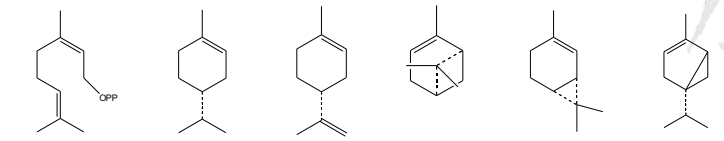
5 mg/mL 

1 mg/mL 

^{1}H-NMR spectra of *malic- and citric acid* (δ 2.5 – δ 3.0)

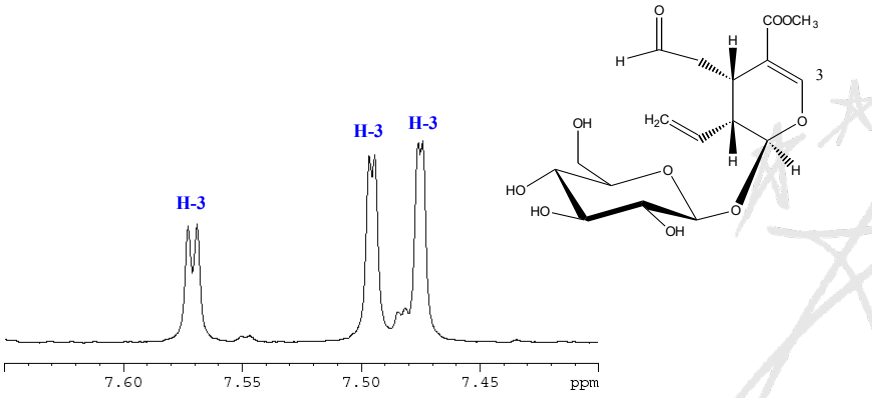
Monoterpenoid (C10)

- Component of plant essential oil
- GC-MS targeted analysis



nerylprophosphate menthane limonene α-pinene carnene thujene

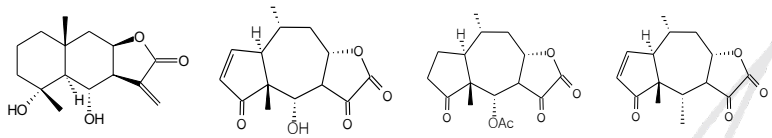
^{1}H-NMR spectrum of secologanin



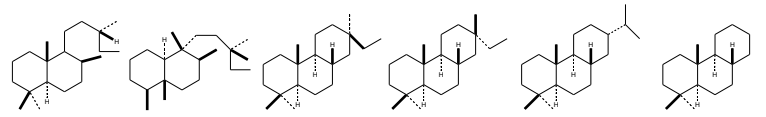
H-3 has 1-3 signals depending pH (one signal in strong acid condition)

Sesquiterpenoid (C15)

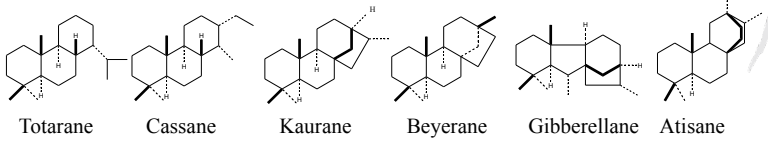
- **Most diverse group of compounds (more than 100 skeletons)**
- **Cytotoxic: sesquiterpene lactone**
- **Phytoalexins in Solanaceae**



Diterpenoid (C20)

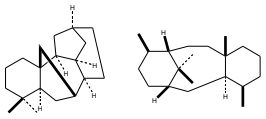


Labdane Cleordane Pimarane Isopimarane Abietane Podocarpane



Totarane Cassane Kaurane Beyerane Gibberellane Atisane

stevioside



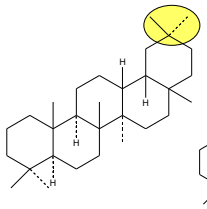
Aconane Taxane

taxol

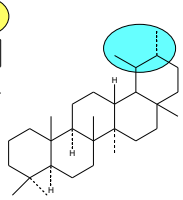
Specific in some plant family (Abietane diterpene: Labiate)

Triterpenoid (C30)

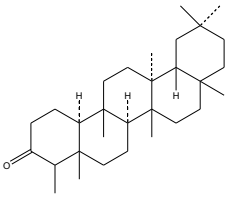
Generally present in plant/Tetracyclic, pentacyclic
Oleanane: oleanolic acid, β-amyrin
Ursolic acid: ursolic acid, α-amyrin
Glycoside (C3, C28); saponin



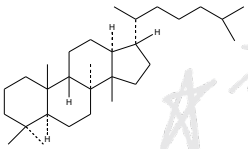
Oleanane



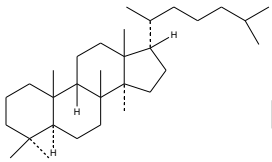
Ursane



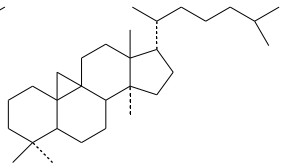
Friedelane



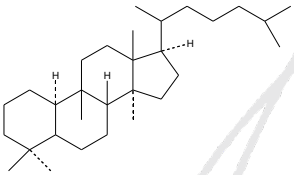
Prostane



Lanostane



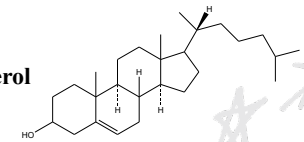
Cycloartane



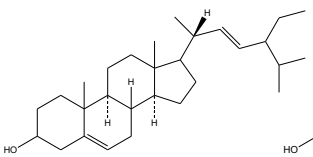
Curcurbitane

Steroid (C27)

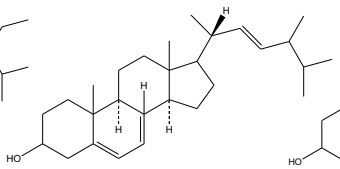
- **Sterol: steroid alcohol (3-OH)**
- **β-sitosterol: most abundant phytosterol**
  mostly present with stigmasterol, campesterol
- **steroid glycoside: saponin**



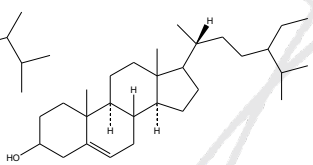
Cholesterol



Stigmasterol



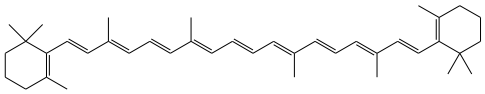
Ergosterol



β-sitosterol

Carotenoid (C40)

- 8 isoprenoid
- color (yellow, red)
- trans (natural form)/cis (light)
- abundant in carrot



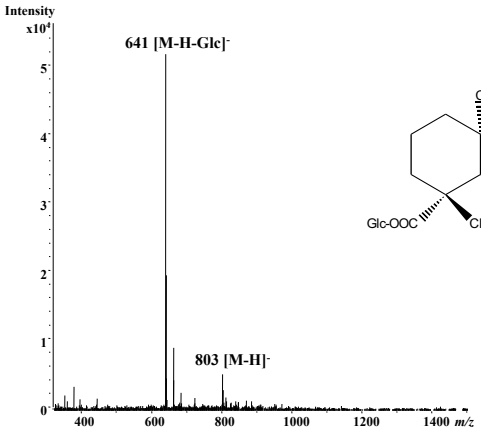
β-carotene

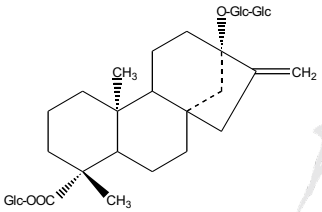
Analysis of terpenoid and steroid

- MS-based method is more powerful than NMR for individual terpenoid or steroid
- GC-MS for aglycones and LC-MS for glycosides
- Methyl and anomeric proton of sugar are characteristic features in NMR spectra
- Many terpenoids exist as glycosides

  triterpenoids and steroids glycosides : saponin

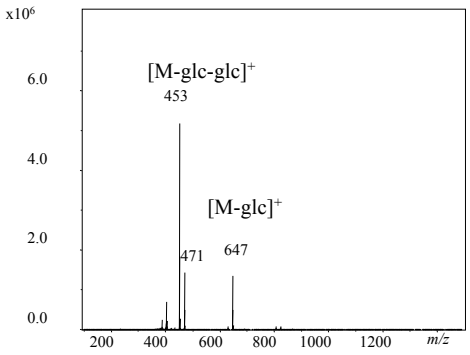
ESI-MS spectrum of stevioside

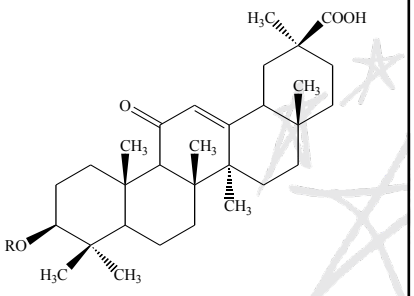




MW peak of glycoside is not always base (the highest) signal in ESI or APCI

APCI-MS spectrum of glycyrrhizin

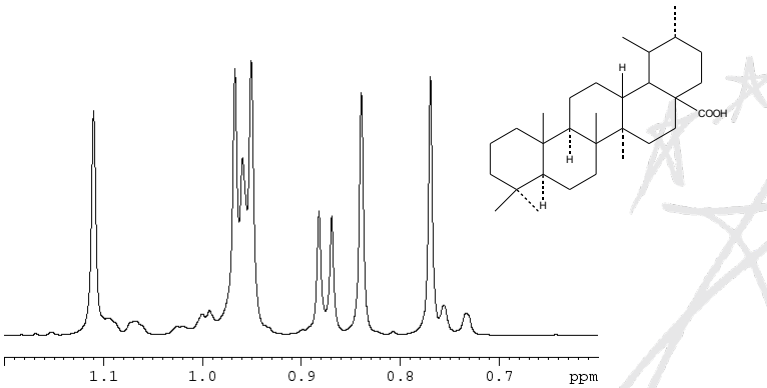




R= Glucose-Glucose

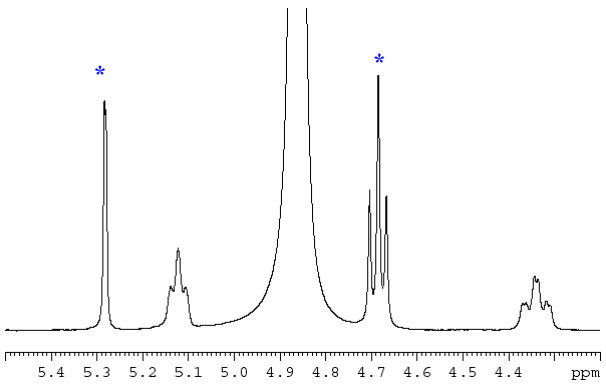
Sometimes MW peak of glycosides can not be detected

^{1}H-NMR spectrum of ursolic acid



Methyl signals are characteristics for terpenoids in NMR

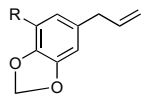
^{1}H-NMR spectrum of a saponin



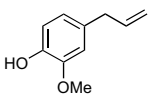
** Anomeric proton of sugars in saponin*

Phenylpropanoid

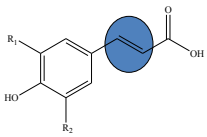
- C_6-C_3 (phenyl + propane)
- C_3 : carboxyl acid, aldehyde, alcohol or olefin
- High vapor pressure: Essential oil
- Chlorogenic acid : Ester of quinic acid and caffeic acid
- Cinnamic acid : Cinnamonum, volitile
- Coniferin, syringin, anethole, eugenol, safrole, myristicin



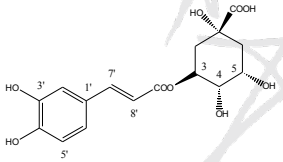
R=H, safrole
R=ome, myristicin



eugenol



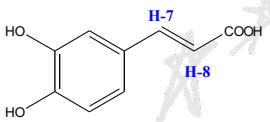
$R_1 = OCH_3$, R_2 = H, Ferulic acid
R_1 = OH, R_2 = H, Caffeic acid
R_1 = H, R_2 = H, Coumaric acid
$R_1 = OCH_3$, $R_2 = OCH_3$, Sinapic acid

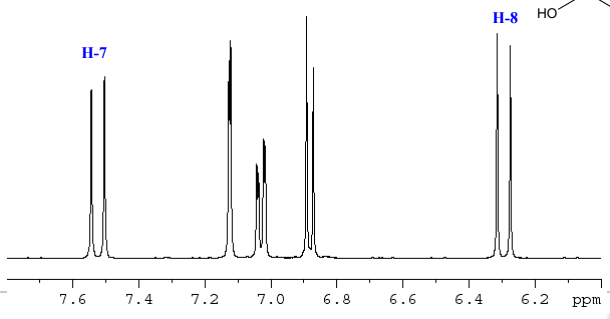


chlorogenic acid

^{1}H-NMR spectrum of caffeic acid

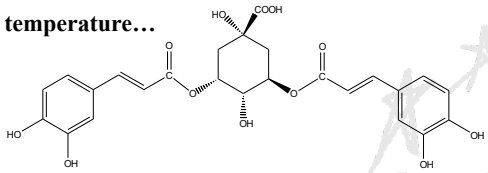
- NMR: most powerful method for structure elucidation
- Large coupling constant (16Hz): *trans* form
- Mostly conjugated with organic acid or sugars

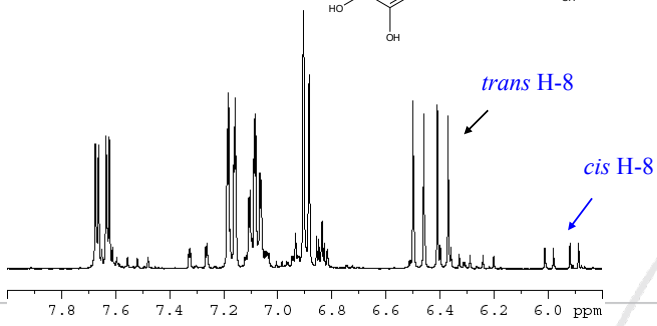




^{1}H-NMR spectrum of dicaffeoyl quinic acid

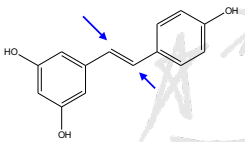
- **isomerization from *trans* forms to *cis* forms**
- **artifact by light, solvent, temperature…**

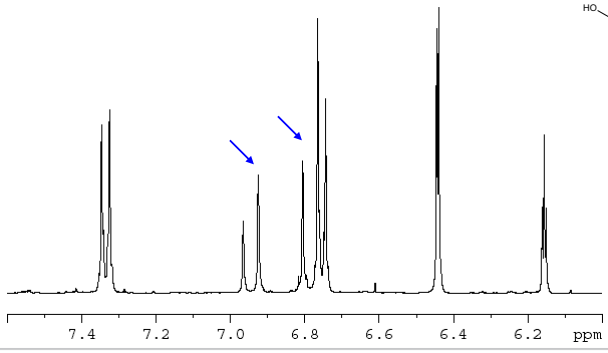




^{1}H-NMR spectrum of a stilbenoid (resveratol)

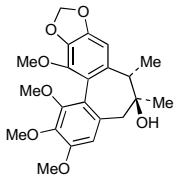
- **Stilbenoid: C6-C2**
- **The same coupling constant but higher chemical shift compared to phenylpropanoid**



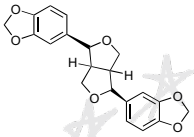


Lignan

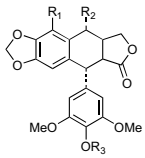
- Oxidative coupling of C6-C3 : C_{18}
- Generally β coupling
- podophyllotoxin : *Podophyllum, Juniperus* species
  Precursor of etoposide, tenuposide
- gomisin: Schisandra species, hepatoprotective
- enterodiol, enterolactone: from human and animal,1980
- pinoresinol, sesamin: polarity (+) (*Sesamum indicum*)
- neolignan: not β coupling, magnol, honokiol
- norlignan: C_{17}



gomisin A



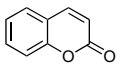
sesamin



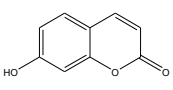
R_1=H, R_2=OH, R_3=Me, podphyllotoxin
R_1=OH, R_2=R_3=H, α-peltatin
R_1=OH, R_2=OH, R_3=Me, β=peltatin

Coumarin

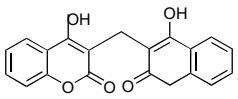
- 2*H*-1-Benzopyran-2-one
- Umbeliferae, Ranunculaceae, Leguminosae, Compositae
- simple coumarin, furanocoumarin, pyroanocoumarin, biscoumarin
- Open cycle by alkali
- dicoumarol: *Medicago sativa*, *Melilotus officinalis*
  inhibition vitamin K inhibition
- novobiocin (gram negative infection, *Streptomyces niveus*), umbelliferone, scopoletin



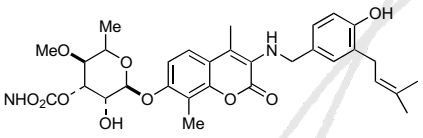
coumarin



Umbeliferone

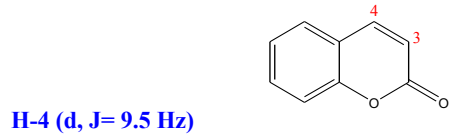


dicoumarol

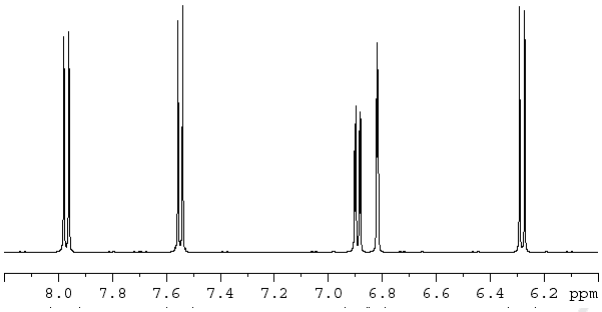


novobiocin

^{1}H-NMR spectrum of umbelliferone

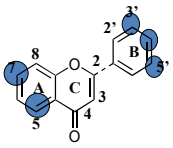


H-4 (d, J= 9.5 Hz) **H-3 (d, J= 9.5 Hz)**

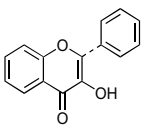


H-3 and H-4 are characteristics in NMR

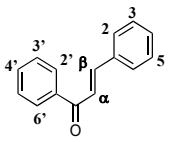
Flavonoid (C6-C3-C6)



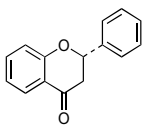
flavone



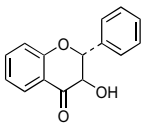
flavonol



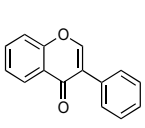
chalcone



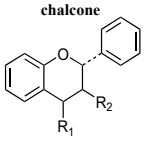
flavanone



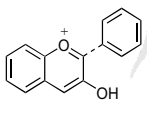
dihydroflavonol



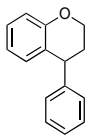
isoflavone



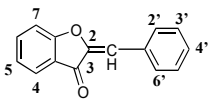
R_1=H, R_2=OH, catechin
R_1=R_2=OH, leucoanthocyanidin



anthocyanidin

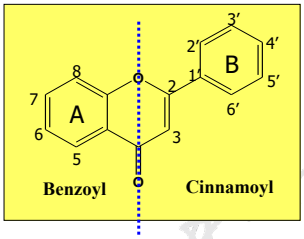


neoflavonoid



aurone

UV spectrum of flavone & flavonol



- 240-400nm region
- Band I: 300-380 nm (B ring)
- Band II: 240-280 nm (A ring)
  1. A ring: more OH group, Band II bathochromic shift
  2. B ring: more OH group, Band I bathochromic shift

Flavone	A-ring pattern	Band II
flavone	-	250
5-OH flavone	5	268
7-OH	7	252
5,7-OH flavone	5,7	268
baicalein	5,6,7	274
norwogonin	5,7,8	281

Flavonol	B-ring pattern	Band I
galangin	-	359
kaempferol	4′	367
quercetin	3′,4′	370
myricetin	3′,4′,5′	374

Flavonoid type	Band I
Flavone	304-350 nm
Flavonol (3-OH)	352-385 nm
Flavonol (sub. 3-OH)	328-357 nm

UV shift reagent for flavonoid

1. NaOMe: 3-OH (band I 40-65 nm bathochromic shift)
   4'-OH (band I 40-65 nm shift, intensity decrease)

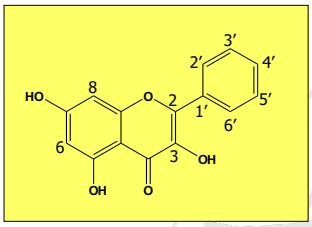
2. NaOAc: 7-OH (band II 5-20 bathochromic shift)
   4'-OH, no 3,7-OH (band I shoulder)

3. NaOAc/H_3BO_3: B ring ortho diOH (band I 12-30nm bathochromic shift)

4. $AlCl_3$ & $AlCl_3$/HCl :
   3 or 5-OH (acid stable complex), ortho-diOH (acid unstable complex)
   -> B ring diOH: $AlCl_3$ & $AlCl_3$/HCl 30-40 nm shift
   triOH 20 nm shift
   5-OH: 35-55 nm
   3-OH: 60 nm
   3,5-diOH: 50-60 nm

(Ref: Systemic identification of Flavonoids, T.J.Mabry, K.R.Markham & M.B.Thomas, Springer-Verlag, 1970)

^{1}H-NMR spectrum of flavonoid



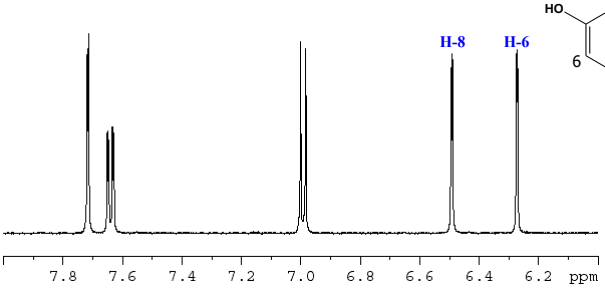
A-ring proton: H-6, H-8
5,7-diOH: 6.0-6.5 ppm, d, J=2.5 Hz

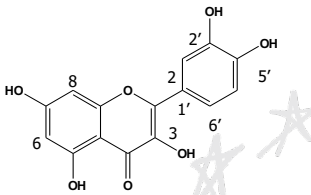
compound	H-6	H-8
5,7-diOH flavone, isoflavone	6.0-6.2	6.3-6.5
5-OH, 7-glycosyl flavone, isoflavone	6.2-6.4	6.5-6.9
5,7-diOH flavanone, dihydroflavonol	5.75-5.95	5.9-6.1
5-OH, 7-glycosyl flavanone, dihydroflavonol	5.9-6.1	6.1-6.4

B-ring proton: downfield than A-ring, 6.7-7.9 ppm

C-ring proton: H-3 6.3 ppm

^{1}H-NMR spectrum of quercetin

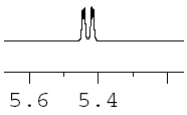




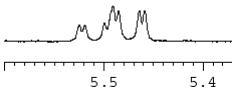
H-6 and H-8 are characteristics in NMR

^{1}H-NMR spectrum of naringenin and naringin

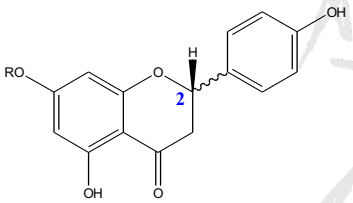
- **Diasteromeric exchange in naringin**
- **Naringenin might have two enantiomers which are not separated by NMR**
- **Sugar attachment change enantiomers to diastereomers separated by NMR (Naringin)**



H-2 of naringenin



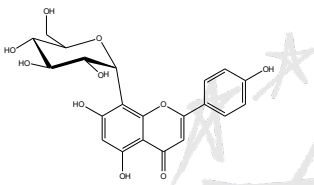
H-2 of naringin

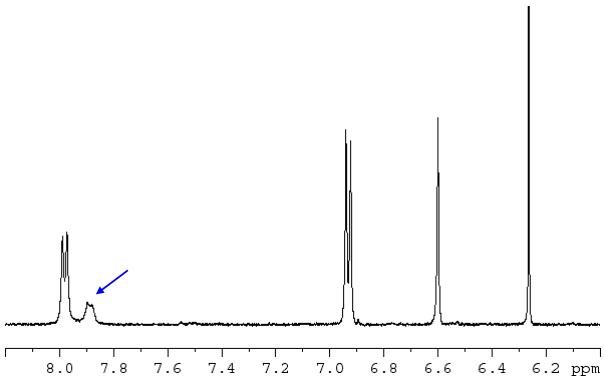


R=H, naringenin
R=rhamnoglucose (neohesperidose), naringin

^{1}H-NMR spectrum of vitexin

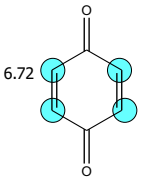
- **Rotamers in C-glycoside**
- **Sugar attachment on C-8 makes rotation of B-ring slower**



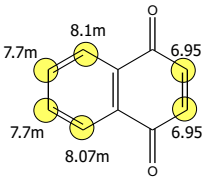


Quinone

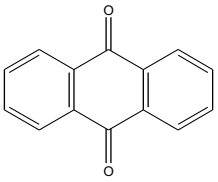
- Animal, plant, microrganismn
- Anthraquinone, naphthoquinone, benzoquinone
- Ubiquinone, vitamin K, mitomycin, sennoside, alkannin, alizarin
- Diverse color: 1,4-quinone (yellow), 1,2-quinone (red), OH makes color darker



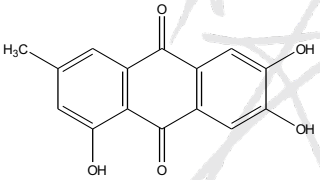
Benzoquinone



Naphtoquinone

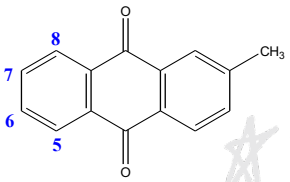


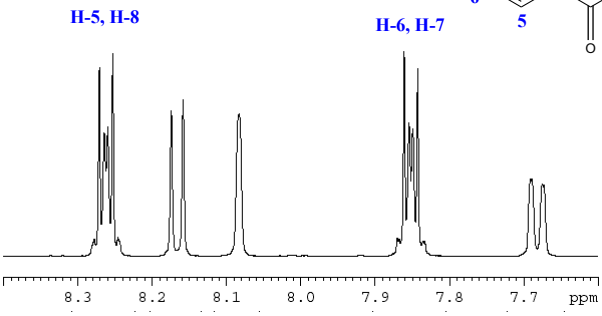
Anthraquinone



Emodin

^{1}H-NMR spectrum of tectoquinone



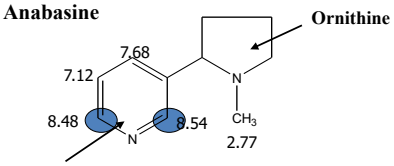


Alkaloid (1)

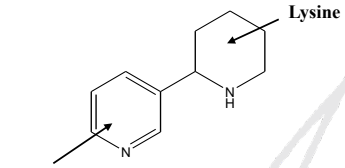
- Heterocyclic Nitrogen containing compound from plant, Basic, Intensive biological activity
- There is no exact definition
- Now, just nitrogen containing natural products
- Very plant-specific

Pyridine alkaloid

- Nicotine
- 5% in dried leaves of *Nicotiana tabacum*
- Insectcidal
- Anabasine



Nicotine

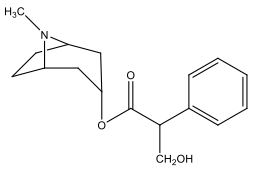


Anabasine

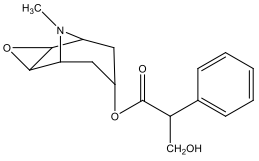
Alkaloid (2)

Tropane alkaloid

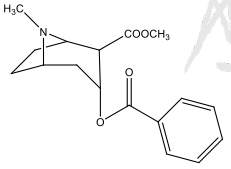
- Atropine: 1833, Solanaceane *Atropa belladonna,* isolated by Mein
- Cocaine: from Coca leaves
- Scopolamine



Atropine



Scopolamine

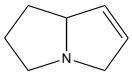


Cocaine

Alkaloid (3)

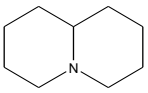
Pyrrolizidine alkaloid

- Cytotoxic, carcinogenic
- Compositae senecio species
- Senecionine, monocrotaline



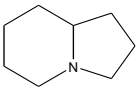
Quinolizidine alkaloid

- Lupinane group alkaloid
- Matrine, nuphridine, sparteine



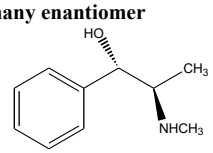
Indolizidine alkaloid

- minor
- Elaeocarpine, tylophorine



Benzylamine alkaloid

- Ephedrine group alkaloid
- 1887, Nagai isolated from Ephedra species
- many enantiomer

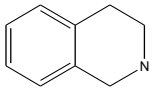


Ephedrine

Alkaloid (4)

Isoquinoline alkaloid

- Morphine, berberine
- Mostly benzylisoquinoline

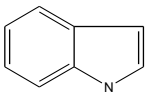


Piperidine alkaloids

- Arecoline
- *Areca catechu*

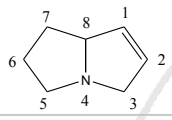
Indole alkaloid

- Biosynthesized from Tryptophan, secologanin
- Reserpine (*Rauwolfia serpentiana*)
- Yohimbine, vincristine, vinblastine

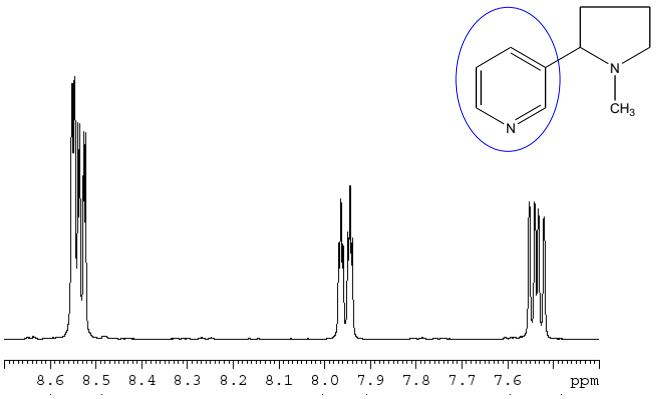


Pyrrolizidine Alkaloid

- Widespread occurrence
- Diverse range of biological activities
- Hepatotoxic

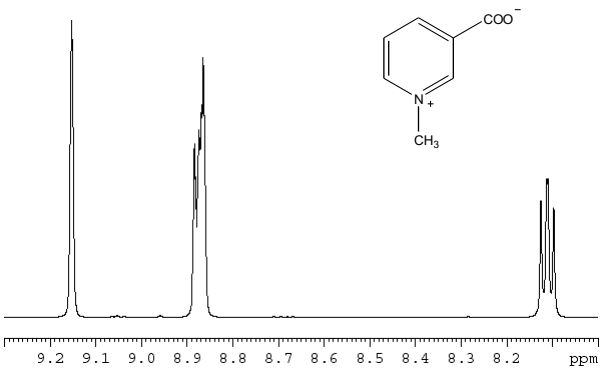


^{1}H-NMR spectrum of nicotine



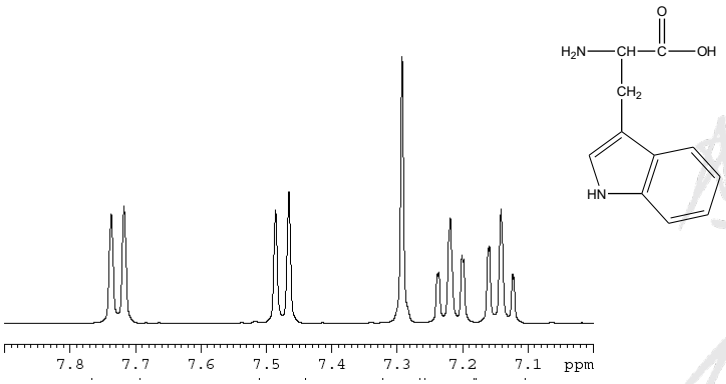
Signals are very pH sensitive

^{1}H-NMR spectrum of trigonelline



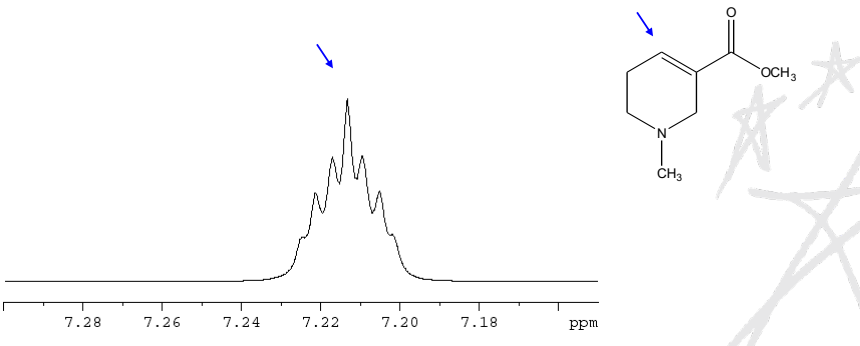
Common in Solanaceae plants

^{1}H-NMR spectrum of tryptophan (for indole alkaloids)



Typical pattern of indole moiety

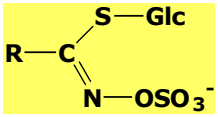
^{1}H-NMR spectrum of arecoline



Higher chemical shift because of ketone group

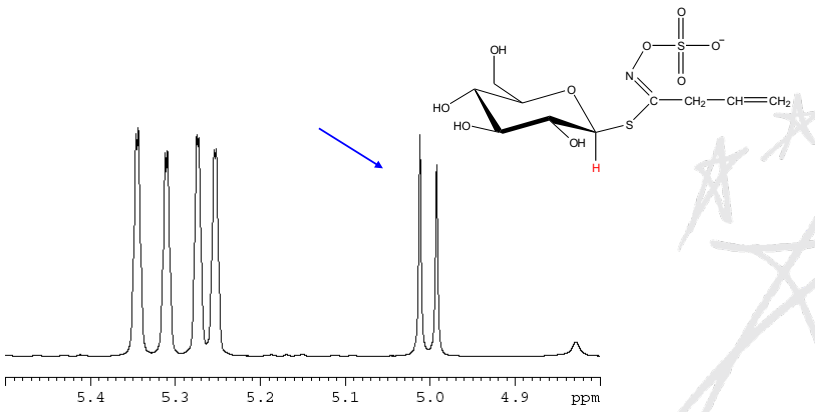
Glucosinolates

- Mostly occur in Cruciferae (Arabidopsis, Brassica)
- β-thioglucoside-N-hydroxysulfates



Sulfur-linked b-*D*-glucopyranose
R = side chain
- ¤ aliphatic (ω-methylthioalkyl) – most abundant
- ¤ aromatic (benzyl)
- ¤ heterocyclic (indole)

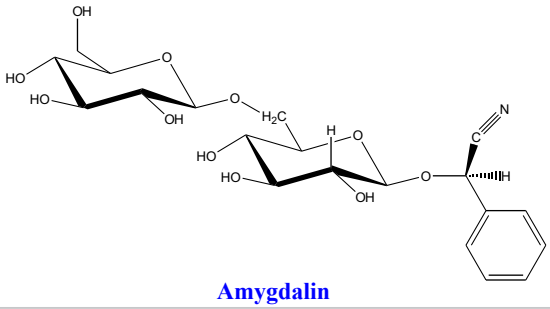
^{1}H-NMR spectrum of sinigrin



H-1 of glucosinolate has big coupling constants (10 Hz)

Cyanogenic glycoside

- **Producing HCN gas by treatment of dil. acid or alkali**
- **Mostly monoglucoside**
- **Diglycoside: amygdalin, vicianin, lucumin, linustatin, neolinustatin containing gentiobiose [glucose-glucose (1→6)]**



Amygdalin

Tomorrow

- **Sample Preparation and Analysis**
- **Application of NADES to Natural Products Research**
- **Preparation of NADES**