Advanced NMR Spectroscopy

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**NMR History**

1937  Rabi predicts and observes nuclear magnetic resonance
1946  Bloch, Purcell first nuclear magnetic resonance of bulk sample
1953  Overhauser NOE (nuclear Overhauser effect)
1966  Ernst, Anderson Fourier transform NMR
1975  Jeener, Ernst 2D NMR
1984  Nicholson NMR metabolomics
1985  Wüthrich first solution structure of a small protein (BPTI) from NOE derived distance restraints
1987  3D NMR + $^{13}$C, $^{15}$N isotope labeling of recombinant proteins (resolution)
1990  pulsed field gradients (artifact suppression)
1996/7  **residual dipolar couplings** (RDC) from partial alignment in liquid crystalline media
        **TROSY** (molecular weight > 100 kDa)
2000s  **Dynamic nuclear polarisation** (DNP) to enhance NMR sensitivity
NMR Nobel Prize Laureates

A brief historical account of the Nobel Prize Laureates clearly shows the track of the discovery, development, and applications of NMR spectroscopy.

- **Otto Stern**, USA: [Nobel Prize in Physics 1943](#), "for his contribution to the development of molecular ray method and his discovery of the magnetic moment of the proton"

- **Isidor I. Rabi**, USA: [Nobel Prize in Physics 1944](#), "for his resonance method for recording the magnetic properties of atomic nuclei"

- **Felix Bloch**, USA and **Edward M. Purcell**, USA: [Nobel Prize in Physics 1952](#), "for their discovery of new methods for nuclear magnetic precision measurements and discoveries in connection therewith"
NMR Nobel Prize Laureates

- **Richard R. Ernst**, Switzerland: **Nobel Prize in Chemistry 1991**, "for his contributions to the development of the methodology of high resolution nuclear magnetic resonance (NMR) spectroscopy"
- **Kurt Wüthrich**, Switzerland: **Nobel Prize in Chemistry 2002**, "for his development of nuclear magnetic resonance spectroscopy for determining the three-dimensional structure of biological macromolecules in solution"
- **Paul C. Lauterbur**, USA and **Peter Mansfield**, United Kingdom: **Nobel Prize in Physiology or Medicine 2003**, "for their discoveries concerning magnetic resonance imaging"
First NMR Spectra on Water

\[ \text{\textsuperscript{1}H NMR spectra of water} \]

Fig. 10. Photographic record of the proton signal in water. The four traces from top to bottom correspond to the times \( t_1, t_2, t_3, t_4 \) of Fig. 9. In the text they are referred to as \( a, b, c, d \), respectively.

NMR History

First Observation of the Chemical Shift

$^1$H NMR spectra ethanol

Modern ethanol spectra

Typical Applications of NMR:

1.) Structural (chemical) elucidation
   - **Natural product chemistry**
   - Synthetic organic chemistry
     - analytical tool of choice of synthetic chemists
     - used in conjunction with MS and IR

2.) Study of dynamic processes
   - reaction kinetics
   - study of equilibrium (chemical or structural)

3.) Structural (three-dimensional) studies
   - Proteins, Protein-ligand complexes
   - DNA, RNA, Protein/DNA complexes
   - Polysaccharides

4.) Metabolomics
5.) Drug Design
   - Structure Activity Relationships by NMR

6.) Medicine - MRI

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**Taxol (natural product)**

**MRI images of the Human Brain**

**NMR Structure of MMP-13 complexed to a ligand**
Classification of Natural Products

1. Alphabetical classification
2. Morphological classification
3. Taxonomic classification
4. Pharmacological classification
5. Chemical classification
6. Chemo - taxonomical classification
1- Alphabetical Classification:

Alphabetical classification is the simplest way of classification of any disconnected items. Crude drugs are arranged in alphabetical order of their Latin and English names (Common names) or sometimes local names. Some of the pharmacopoeias, dictionaries, and reference books which classify crude drugs according to this system are as follows:

1. Indian Pharmacopoeia.
2. British =
3. British Herbal =
4. United States = & National Formulary
5. British pharmaceutical Codex
6. European Pharmacopoeia

Notes: No. (2, 4) these are arranged in English, (6) arranged according to their names in Latin.
2- Morphological Classification :

In this system the drugs are arranged according to the morphological or external characters of the plant parts nor animal parts i.e. ( which part of the plant is used as a drug e.g. ( leaves , roots , stems , …etc ).

Organized Drugs :

**Woods** – Quassia , Sandalwood , Red Sandalwood .

**Leaves** – Digitalis, Eucalyptus, Mint, Senna, Spearmint, Squill, Coca, Hyoscyamus, Belladonna , Tea.

**Barks** – Cascara , Cassia , Cinchona , Wild cherry .

**Flowering parts** – Clove , Pyrethrum , Saffron , Santonica , Chamomile .

**Fruits** – Anise , Bitter orange peel , Capsicum , Caraway , Cardamom , Colocynthis , Coriander , Cumin , Dill , Fennel , Lemon peel , Senna pod , Star anise , Tamarind .

**Seeds** – Bitter almond , Black Mustard , Cardamom , Colchicum , Linseed , Nux vomica , Psyllium , White mustard .

**Roots & Rhizomes** – Aconite , Colchicum corm , Garlic , Gentian , Ginger , Ginsing , Glycyrhrhiza , Podophyllum , Rauwolfia , Rhubarb , Turmeric , Valerian , Squill .

**Plants & Herbs** – Ergot , Ephedra , Yeast , Vinca , Datura .

**Hair & Fibers**- Cotton , Hemp , Jute , Silk , Flax .
Un-Organized Drugs:

Dried latex – Opium, Papain.
Dried Juice – Aloe, Kino.
Dried extracts – Agar, Black catechu, Pale catechu, Pectin.
Waxes – Beeswax, Spermaceti, Carnauba wax.
Gums – Acacia, Guar gum, Indian gum,
Resins – Asafetida, Benzoic, Colophony, Mastic, Coal tar, Tar, Tolo balsam, Storax, sandarac.
Fixed oils & Fats – Arachis, Castor, Coconut, Cotton seed, Linseed, Olive, Sesame, Almond, Theobroma, Cod – liver, Halibut liver, Kokum butter.
Animal Products – Bees wax, Cod – liver oil, Gelatin, Halibot liver oil, Honey, Shark liver oil, Shellac, Spermaceti wax, Wool fat, Musk, Lactose.
Fossil organism & Minerals – Bentonite, Kaolin, Kiesslguhr, Talc.
3- Taxonomical Classification:

Taxonomical classification is purely a botanical classification, its based on principles of natural relationship & evolutionary developments. They are grouped in (Kingdom, Phylum, Order, Family, Genus & Species). As all the entire plants are not used as drugs, parts of plant is used as a drug. For example, Cinnamon bark. This it is of no significance from identification point of view to put plants in a taxonomic order.
4- Pharmacological Classification:

In this system grouping of drug according to their pharmacological action or of most important constituent or their therapeutic use is termed as pharmacological or therapeutic classification of drug. This classification is more relevant and is mostly followed method. Drugs like digitalis, squill and strophanthus having **cardiotonic action** are grouped together irrespective of their parts used or phylogenetic relationship or the nature of phytoconstituents they contain.
5- Chemical classification

The crude drugs are divided into different groups according to the chemical nature of their most important constituent. Since the pharmacological activity and therapeutic significance of crude chemical classification of drugs is dependent upon the grouping of drugs with identical constituents. An out of this classification is as follow:

5-1- Carbohydrates
5-2- Glycosides
5-3- Tannins
5-4- Volatile Oils
5-5- Lipids
5-6- Resins
5-7- Alkaloids
5-8- Protein
5-9- Vitamins
5-10- Triterpines
5-1- **Carbohydrates**– Carbohydrates are polyhydroxy aldehydes or ketones containing an unbroken chain of carbon atoms.

**Gums**– Acacia, Tragacanth

**Mucilages**– Plantago seed

**Others** - Starch, Honey, Agar, Pectin, Cotton.

5-2- **Glycosides**– Glycosides are compounds which upon hydrolysis give rise to one or more sugars (glycone) and non – sugar (aglycone)

**Anthraquinone Glycosides** – Aloe, Cascara, Rhubarb, Senna

**Saponins Glycosides** – Quillaia, Glycyrrhiza

**Cyanophore Glycosides** – Wild cherry bark

**Isothiocyanate Glycosides** – Mustard

**Cardiac Glycosides** – Digitalis, Strophantus

**Bitter Glycosides** – Gentian, Calumba, Quassia
5-3- Tannins – Tannins are complex organic, non-nitrogenous derivatives of polyhydroxy benzoic acids. Ex: Pale catechu, Black catechu, Ashoka bark, Galls, Amla.

5-4- Volatile Oils – Monoterpenes & Sesquiterpenes obtained from plants. Ex: Cinnamon, Fennel, Dill, Caraway, Coriander, Cardamom, Orange peel, Mint, Clove, Valerian.
# Terpene Classification

<table>
<thead>
<tr>
<th>Class Name</th>
<th>Carbon Number</th>
<th>Isoprene Units</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monoterpene</td>
<td>10</td>
<td>2</td>
</tr>
<tr>
<td>Sesquiterpene</td>
<td>15</td>
<td>3</td>
</tr>
<tr>
<td>Diterpene</td>
<td>20</td>
<td>4</td>
</tr>
<tr>
<td>Triterpene</td>
<td>30</td>
<td>6</td>
</tr>
</tbody>
</table>
## Monoterpene Examples

<table>
<thead>
<tr>
<th>Selected Monoterpences</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myrcene</td>
</tr>
<tr>
<td>menthol, a monoterpenoid, not a true terpene.</td>
</tr>
<tr>
<td>limonene</td>
</tr>
<tr>
<td>carvone is a monoterpenoid, a modified monoterpene</td>
</tr>
</tbody>
</table>

![Chemical Structures]

- Myrcene
- menthol, a monoterpenoid, not a true terpene.
- limonene
- carvone is a monoterpenoid, a modified monoterpene
Sesquiterpene Examples

Zingiberene, a sesquiterpene abundant in ginger.

δ-Cadinene, one of a family of sesquiterpene.

Humulones are sesquiterpenoids that give "hoppy" flavor to beer.
Representative Diterpenes

**Taxadiene**: a tricyclic diterpene

**Retinol**: a diterpenoid is one of the animal forms of vitamin A

**Phytol**: a diterpenoid is used in the biosynthesis of vitamin E and vitamin K1
Isoprene Units in Terpenes

Limonene

Camphor

β-Selinene

Guaiol
Non-Isoprenoid Terpenes

Valerane

β-Vetivone

Eremophilone
5-5- Lipids–
   Fixed oils – Castor, Olive, Almond, Shark liver oil.
   Fats – Theobroma, Lanolin.
   Waxes – Beeswax.
5-6- Resins– Complex mixture of compounds like resinols, resin acids, resinotannols, resenes.
Ex: Colophony, Podophyllum, Cannabis, Capsicum, Turmeric, Balsam of Tolu and Peru, Myrrh, Ginger .
5-7- Alkaloids – Nitrogenous substance of plant origin
   Pyridine and Piperidine – Lobelia, Nicotiana
   Tropane – Coca, Belladonna, Datura, Stramonium, Hyoscyamus, Henbane.
   Quinoline - Cinchona
   Isoquinoline – Opium, Ipecac, Calumba .
   Indol – Ergot, Rauwolfia .
   Amines – Ephedra
   Purina – Tea, Coffee.
Alkaloids

Nitrogen containing, basic natural products often have powerful biological activity. Alkaloids are not as conveniently organized by some common structural component.
8- Protein – Gelatin, Ficin, Papain
9- Vitamins – Yeast
10- Triterpines – Rasna, Colocynth

**Squalene:** One of the most important triterpene

**Hopane:**
An example of a pentacyclic triterpene

**Cholesterol:**
one of the simplest but most important triterpenoids
6- Chemotaxonomic Classification:
This system of classification relies on the chemical simillarity of taxon i.e. it is based on the existence of relationship between constituents in various plants. There are certain types of chemical constituents that characterize certain classes of plants. This gives birth to entierly new concept of chemotaxonomy that utilizes chemical facts/characters for understanding the taxonomical status, relationships and the evolution of the plants. For example, tropane alkaloids generally occur among the members of Solanaceae thereby, serving as a chemotaxonomic marker. Similarly plant metabolites can serve as the basis of classification of crude drugs. The berberine alkaloid in Berberis and Argemone; Rutin in Rutaceae members, ranunculaceous alkaloids among its members etc are examples.
It is the latest system of classification and gives more scope for understanding the relationship between chemical constituenets, their biosynthesis and their possible action.
Structure elucidation step by step
# Spectroscopic Techniques and Their Applications in Structure Elucidation of Organic Molecules

<table>
<thead>
<tr>
<th>Spectroscopic Techniques</th>
<th>Radiation Absorbed</th>
<th>Effect on the Molecule</th>
<th>Structural Information Deduced</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ultraviolet/Visible spectrophotometry</td>
<td>Ultraviolet-visible λ, 190–400 nm and 400–800 nm</td>
<td>Changes in electronic energy levels within the molecule</td>
<td>Extent of π-electron systems. Presence of conjugated unsaturation, and conjugation with nonbonding electrons</td>
</tr>
<tr>
<td>Infrared spectrophotometry</td>
<td>Infrared (mid infrared) λ, 2.5–25 mm ν, 400–4000 cm^-1</td>
<td>Changes in the vibrational and rotational movements of the molecule</td>
<td>Detection of functional groups, which have specific vibration frequencies, for example, C=O, NH₂, OH, etc.</td>
</tr>
<tr>
<td>NMR spectroscopy</td>
<td>Radiofrequency λ, 25 cm</td>
<td>Nuclei placed under the static magnetic field change their spins after absorption of radiofrequency radiations</td>
<td>The electronic environment of nuclei, their numbers and number of neighboring atoms</td>
</tr>
<tr>
<td>Circular dichroism (CD)/Optical rotatory dispersion (ORD)</td>
<td>Ultraviolet/visible λ 200–600 nm</td>
<td>Changes in the electronic energy level of a molecule</td>
<td>Identification of absolute configurations of small molecules in solution</td>
</tr>
<tr>
<td>Mass spectrometry</td>
<td>High-speed electron beam and other ionization sources</td>
<td>Effects of ionization and fragmentation of charged particles</td>
<td>Relative masses of molecular ions and fragments</td>
</tr>
</tbody>
</table>
Purification by TLC, HPTLC, GC-MS, HPLC, UPLC, etc.

Pure

Weigh the sample

More than 2 μM

Check EI-MS and $^1$H-NMR spectra and re-isolate, if compound appears to have a complex unknown structure

Not pure

Less than 2 μM

FIGURE 10.1 Preparation of samples for spectroscopic analysis.
FIGURE 10.2 Preparation of samples for structure elucidation. Only pure (>99%) compounds which are in sufficient quantities (2 µM) are processed further.
FIGURE 10.3 Determination of molecular mass and formula by using mass spectrometry.
FIGURE 10.4 Steps in the determination of molecular mass and formula by using mass spectrometry.
Record IR spectrum
1. Nondestructive technique
2. Requires at least 2.0 μmol of sample
3. Record as KBr disk, as solution in CHCl₃ or as nujol mull
4. No alcohol or water should be used

Good IR spectrum
1. Check literature
2. Identify functional groups

Bad IR spectrum
1. Recheck solubility
2. Recheck purity

FIGURE 10.5 Determination of functional groups by using infrared spectrophotometry.
FIGURE 10.6 Determination of unsaturation and conjugated sites (chromophores) by using ultraviolet spectrophotometry.
Submit sample for one-dimensional (1D) $^1$H-NMR spectrum
1. Nondestructive technique.
2. Recheck purity.
3. Filter through small cotton plug to remove solid impurities.
4. Degas if possible to remove dissolved gases (not required for routine $^1$H-NMR analysis).
5. Check solubility at room temperature before measurement.
6. Requires about 1 $\mu$M of sample.

FIGURE 10.7 Quick checks on sample for $^1$H-NMR spectroscopic measurement.
FIGURE 10.8 Steps in $^1$H-NMR spectroscopic measurement of sample.
Submit for broad-band decoupled and DEPT $^{13}$C-NMR experiments
1. Non-destructive technique
2. Requires at least 2–3 mM.
3. Check solubility before submission

Good $^{13}$C-NMR spectra

1. Check well-resolved peaks (signals) in the spectrum
2. Count the number of signals corresponding to various carbons of the molecule, and compare with the molecular formula (HREI-MS based formula)
3. Deduce the methyl, methylene and methine carbons from the DEPT (distortionless enhancement by polarization transfer) experiment recorded at $\theta = 135^\circ$
4. Deduce the methine carbons directly from DEPT experiment $\theta = 90^\circ$
5. Quaternary carbons can be deduced by subtraction of signals which appeared in DEPT spectrum ($\theta = 135^\circ$) from the broad-band decoupled $^{13}$C-NMR spectrum.

Bad $^{13}$C-NMR spectra

1. Recheck solubility
2. Recheck purity
3. Recheck quantity of the sample

Check literature

FIGURE 10.9 $^{13}$C-NMR spectroscopic measurement of sample.
1. Proceed to 2D techniques, such as HMQC, HMBC, etc.
2. Look for compounds of similar carbon skeleton
3. Record INADEQUATE (direct $^{13}$C–$^{13}$C coupling) if enough sample is available (50 mg or more for compounds of molecular weight below 500)

1. Stop
2. Work on other compounds

**FIGURE 10.10** Various steps in NMR spectroscopic measurement of sample.
1. 1D $^1$H-NMR spectrum provides information about chemical shifts, coupling constants, and integrals.

**FIGURE 10.11** Various types of NMR spectroscopic methods.
2. 2D $^1\text{H}$$-$$^1\text{H}$ correlations (COSY) identifies $J$-coupling relationships between protons.
3. 1D $^{13}$C-NMR spectrum, such as broad-band (BB) decoupled $^{13}$C-NMR spectrum, provides the carbon count and chemical shift values, while multiplicity information can be obtained by recording polarization transfer $^{13}$C-NMR experiments, such as DEPT.
4. 2D $^1$H-$^{13}$C one-bond heteronuclear techniques, such as HMQC/HSQC provide information about one-bond carbon/proton correlations.
5. 2D $^1$H-$^{13}$C long-range heteronuclear NMR techniques, i.e., HMBC, provide information about $^{13}$C/$^1$H long-range correlations.

FIGURE 10.11 Various types of NMR spectroscopic methods.
6. 2D NOESY, 1D nOe difference spectra or selective 1D NOESY provide stereochemical information from through-space couplings.
7. 2D $^1$H–$^1$H long-range TOCSY spectrum yields $^1$H–$^1$H correlations within various spin systems.

FIGURE 10.11 Various types of NMR spectroscopic methods.
8. 2D INADEQUATE spectrum provides information about $^{13}$C–$^{13}$C connectivities.

**FIGURE 10.11** Various types of NMR spectroscopic methods.
<table>
<thead>
<tr>
<th>S. No.</th>
<th>Software</th>
<th>Task</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>ACD/Labs (Advanced Chemistry Development Company)</td>
<td>To predict $^1$H- and $^{13}$C-NMR spectra</td>
</tr>
<tr>
<td>2.</td>
<td>CMC-se™ (Bruker)</td>
<td>Perform an automatic analysis of different NMR spectra types and proposes structural solutions</td>
</tr>
<tr>
<td>3.</td>
<td>CMC-assist™ (Bruker)</td>
<td>Providing an automated processing and an automated analysis of the NMR data</td>
</tr>
<tr>
<td>4.</td>
<td>CASE</td>
<td>A computer-assisted structure elucidation (CASE) program to generate chemical structures by utilizing 1D- and 2D-NMR data</td>
</tr>
<tr>
<td>5.</td>
<td>Chemical Concepts</td>
<td>To predict $^1$H- and $^{13}$C-NMR spectra</td>
</tr>
<tr>
<td>6.</td>
<td>Sadtler (University of Texas)</td>
<td>To predict $^1$H- and $^{13}$C-NMR spectra</td>
</tr>
<tr>
<td>7.</td>
<td>Artificial Neural Networks (ANN)</td>
<td>To predict $^1$H- and $^{13}$C-NMR spectra</td>
</tr>
<tr>
<td>8.</td>
<td>PREDICT-IT NMR</td>
<td>To predict $^1$H- and $^{13}$C-NMR spectra</td>
</tr>
<tr>
<td>9.</td>
<td>CS ChemDraw Pro (Cambridge Softwares)</td>
<td>To predict $^1$H- and $^{13}$C-NMR spectra</td>
</tr>
<tr>
<td>10.</td>
<td>SPECTOOL</td>
<td>To predict $^1$H- and $^{13}$C-NMR spectra</td>
</tr>
<tr>
<td>11.</td>
<td>COSMOS (COSMOS software)</td>
<td>To predict $^1$H- and $^{13}$C-NMR spectra</td>
</tr>
<tr>
<td>12.</td>
<td>GAUSSIAN (Gaussian, Inc.)</td>
<td>To predict $^1$H- and $^{13}$C-NMR spectra</td>
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<tr>
<td></td>
<td>Program Details</td>
<td></td>
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<tr>
<td>13</td>
<td>ACD/PNMR (Advanced Chemistry Development Company) To predict $^{31}$P-NMR spectra</td>
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</tr>
<tr>
<td>14</td>
<td>ACD/FNMR (Advanced Chemistry Development Company) To predict $^{19}$F-NMR spectra</td>
<td></td>
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<tr>
<td>15</td>
<td>X-PERT system (Eaton) Structure elucidation of organic molecules containing up to 30 skeletal atoms (C, N, O, P, S, B, Si, and the halogens) using a combination of IR, $^{1}$H- and $^{13}$C-NMR spectral data</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>SpecInfo system (Wiley-VCH) To predict $^{1}$H- and $^{13}$C-NMR spectra and structural interpretation from NMR, IR, and mass spectra</td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>SpecSolv (Wiley-VCH) Structural interpretation from NMR, IR, and mass spectra</td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>SESAMI-H, SESAMI-C Computer-aided structure generator on the basis of 1D- $^{1}$H/$^{13}$C-NMR and 2D-NMR data</td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>HOUDINI (Side Effects Software) Computer-aided structure generator on the basis of 1D- $^{1}$H/$^{13}$C-NMR and 2D-NMR data</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>COCOA (Pascal program) Computer-aided structure generator on the basis of 1D- $^{1}$H/$^{13}$C-NMR and 2D-NMR data</td>
<td></td>
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<tr>
<td>21</td>
<td>CISOC-SES (SGI Indy.) Computer-aided structure generator on the basis of 1D- $^{1}$H/$^{13}$C-NMR and 2D-NMR data</td>
<td></td>
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<tr>
<td>22</td>
<td>NMR-SAM (Spectrum Research, LLC) Computer-aided structure generator on the basis of 1D- $^{1}$H/$^{13}$C-NMR and 2D-NMR data</td>
<td></td>
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<tr>
<td>23</td>
<td>LUCY Uses 2D-NMR data for computer-aided structure elucidation</td>
<td></td>
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<tr>
<td>24</td>
<td>StrucEluc Structure elucidation of organic molecules and biomolecules from 1D- $^{13}$C-NMR data</td>
<td></td>
</tr>
</tbody>
</table>
The following is a summary of the Logical protocol:

1. Obtain a secure **molecular formula**, and calculate the DoU.

   \[ \text{DoU} = \frac{2C + 2 + N - X - H}{2} \]

   “degree of unsaturation” (DoU)

2. Record a **$^1$D 1H-NMR** spectrum, and **assign each proton** a letter of an alphabet.

3. Record the **HSQC (or HMQC)**, and **label each proton** at the respective chemical shifts with the corresponding letters of the alphabet as in the **$^1$D spectrum**.

4. Label the protons along the **diagonal line** of the **COSY-45°** spectrum with the same alphabet letters as in the **$^1$H-NMR spectrum**. Deduce the **vicinal 1H/1H connectivities** from the cross-peaks in the **COSY-45°** spectrum by deleting all the coupling interactions from **geminal protons** (geminal protons are readily identified from the **HMQC/HSQC** or **HETCOR** spectrum).
5. Identify the **various fragments** (spin networks) obtained from the **TOCSY spectra (20, 40, 60, 120 ms)**. Alternatively, deduce them from the **COSY and HMBC spectra**.

6. Using **HMBC and nOe difference spectra**, connect the fragments obtained using this procedure.