LABORATORY OF ELEMENTARY BIOPHYSICS

Experimental exercises for III year of the First cycle studies

Field: “Applications of physics in biology and medicine”

Specialization: “Molecular Biophysics”

One- and two-dimensional NMR spectroscopy in organic structure determination (PPB17d)

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The objective

Nuclear magnetic resonance (NMR) spectroscopy is a versatile research technique used in biology, chemistry, physics and medicine. It is one of the widely-applied methods used to study the structure and Dynamics of organic molecules in solution (solution-state NMR). Together with crystallography it is the main method of structural elucidation of molecules – that is of determining their atomic structure.

Even though many specialized NMR experiments have been developed, the methods used to design them, to process the obtained data and present it are usually similar, which is one of the reasons for the methods popularity. The goal of this exercise is to acquaint the students with commonly used one- and two-dimensional NMR spectra in the process of structural elucidation of a simple organic compound using only the most basic external information – such that could have been obtained using elemental analysis and mass spectrometry.

Introduction

Basics

The theory of NMR spectroscopy was presented during the „Molecular spectroscopy" course and can be review in the literature (recommended literature can be found at the end of this handout). A full description of experimental design in NMR spectroscopy would require using the methods of quantum mechanics of complex systems, but for practical applications a simplified description should be sufficient. In this exercise we will limit ourselves to the spectroscopy of spin ½ nuclei in solution state, which is by far the most developed area of NMR spectroscopy.

With each spin ½ nuclei we can associate a parameter called its chemical shift δ (a real number). The chemical shifts of different isotopes (hydrogen-1, carbon-13) have separate scales. The value of the chemical shift can often be used to determine the functional group the nuclei comes from.

The individual spin ½ nuclei in a molecule can interact with each other and most of this interaction is mediated by electrons – which means that it is partly determined by molecular structure. The measure of the strength of this interaction is called the J coupling constant. Due to the importance of bonding electrons the coupling constants are indexed by the number of bonds separating the nuclei in question : ^{1}J is for directly bound nuclei (for example the proton and carbon in a CH group), for geminal protons the coupling constant is ^{2}J and for vicinal protons it is written as ^{3}J. For nuclei that are further away the interaction is usually too weak to be of interest.

On one-dimensional spectra the couplings can be observed as splittings of signals into groups (multiplets) – details can be found in the literature. By using radio pulses of well-defined length and power and separated by exact delays these interactions can be used to perform multidimensional experiments. The simplest example of such experiments are 2D spectra, where the signal intensity is a function of two chemical shifts. Due to practical concerns one of them is almost always the chemical shift of a proton, the second one can a proton one as well (homonuclear experiments) or belong to a different type of nuclei (heteronuclear experiments).

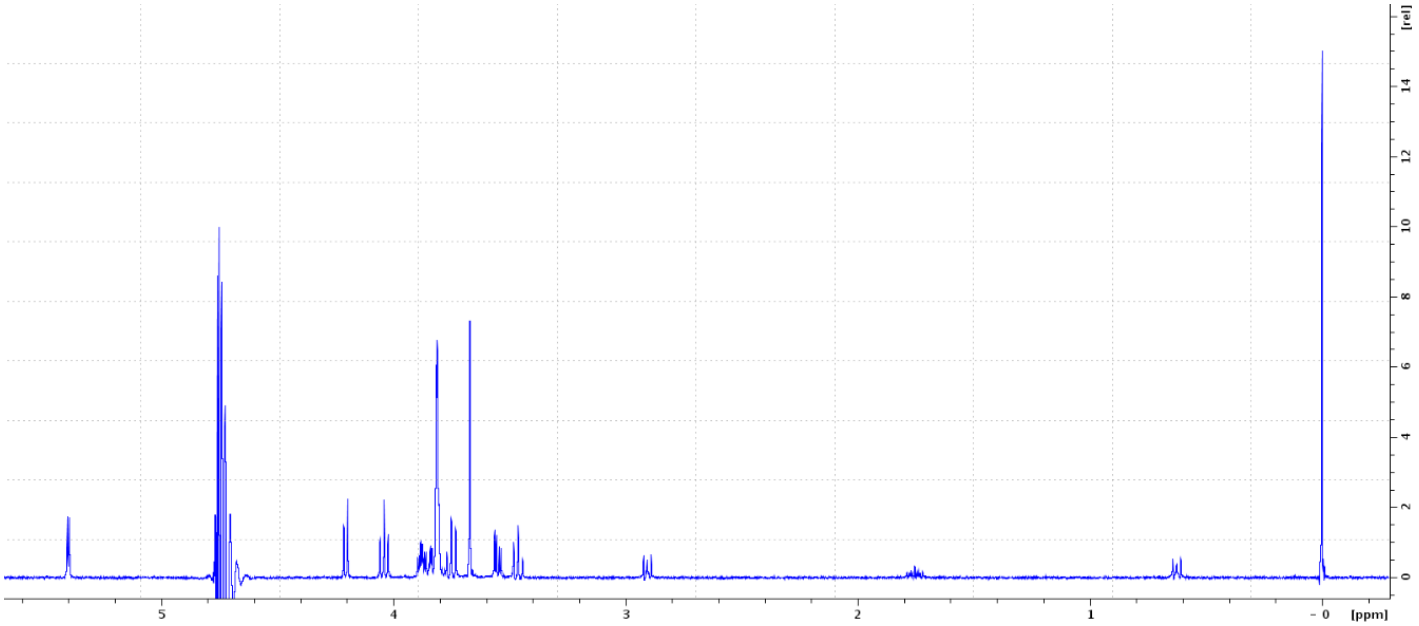

Fig. 1 An example of a one-dimensional proton spectrum recorded for a sample of sucrose in water. The signal near 0 ppm is due to a reference compound (DSS), while the signal near 4.7 ppm is a residual water signal, distorted by a solvent suppression procedure.

The simplest type of a 2D experiment is a homonuclear COSY (correlation spectroscopy) spectrum. Signals are observed on the spectrum for scalar coupled nuclei – mostly for vicinal protons. On HSQC (*heteronuclear single quantum coherence*) spectra the signal appear for pairs of proton and directly coupled heteronuclei (in organic chemistry usually carbons). HMBC (*heteronuclear multiple-bond correlation*) experiments are similar to HSQCs but they are designed in such a way that signals from directly coupled nuclei are suppressed and one can observe weakly interacting pairs. For natural-abundance samples the acquisition of HMBC spectra can take many hours but the information they supply can be of great use in the structure elucidation of complex organic molecules. Further information on all the experiments employed during the exercise will be given by the laboratory assistant.

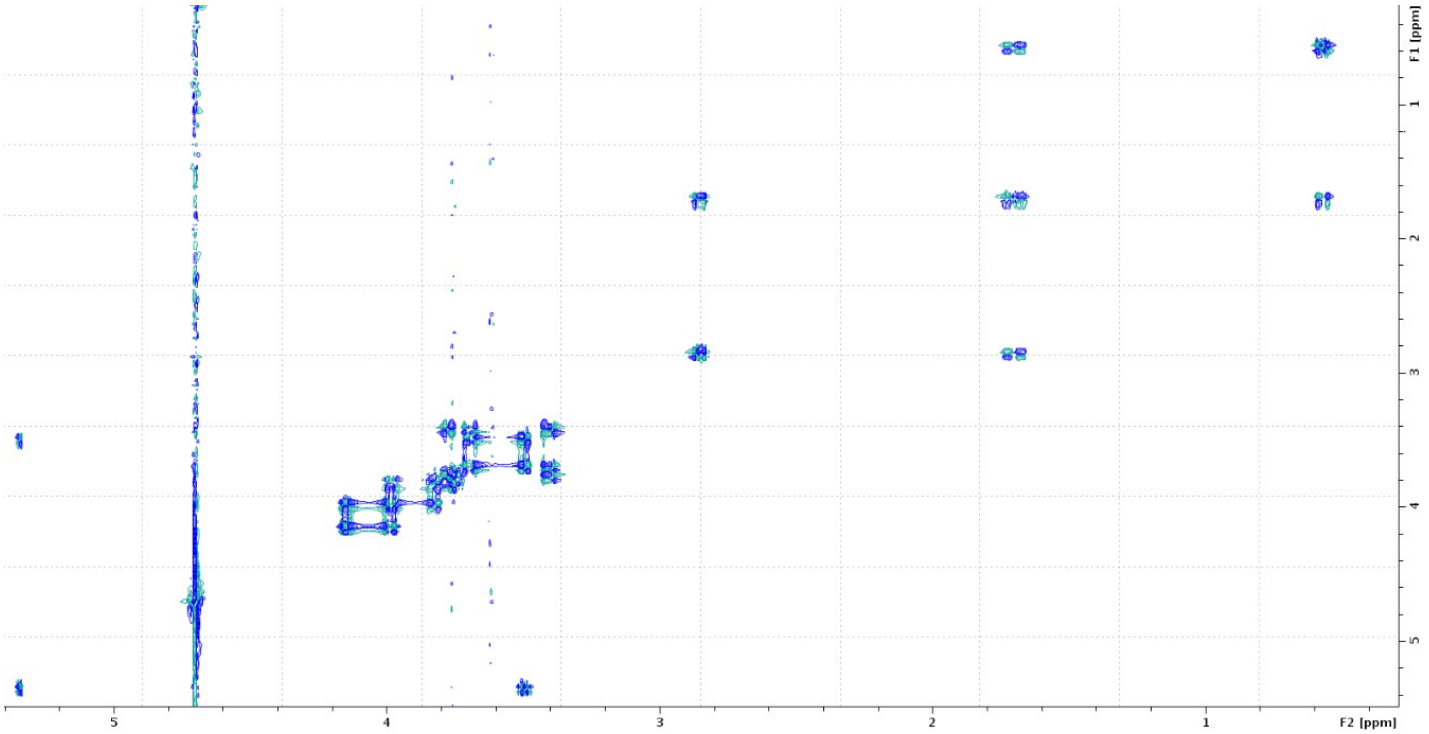

Fig. 2 A 2D NMR spectrum – ^{1}H, ^{1}H DQF COSY of sucrose in water.

Practical aspects of NMR spectroscopy

In order to acquire high-resolution NMR spectra it is important to maintain the stability of the magnetic field during the experiment. There are two main kinds of magnetic field perturbations in NMR spectroscopy. The first one is due to the slow discharge of the superconducting magnet which leads to a weakening of the field and a drift of the resonant frequency of protons on the order of several Hz per hour. The other one is the presence of external magnetic fields of different amplitude and frequency. They can be associated with events such as changing power load on the electric wiring or a tram passing as far away as several dozen meters away. In order to detect on correct (at least in first order) these effects modern NMR spectrometers are equipped with the so called lock system. It is essentially a stand-alone spectrometer registering the signal from deuterium nuclei in the sample and changing the current in a supplementary coil of the magnet (called the z_0 or field coil) in such a way that the signal's resonant frequency stays constant. As the natural abundance of deuterium is very low (under 0.02%) it has to be added to the sample – usually in the form of deuterated solvent – in case of water solutions heavy water (D_2O) is added to a final concentration of 5-10%. In case of organic solvents full deuteration is often employed. This has the additional effect of removing the proton signal of the solvent from the spectra and is often

beneficial as it could overlap with signals from the compound of interest.

The part of the spectrometer inserted into the superconducting magnet is called the probe head. A specially-constructed coil assembly is used to irradiate the sample and record the signal. The properties of this electrical circuit are dependent upon the sample composition (especially on its electric permittivity and magnetic susceptibility) and the material used in the construction of the NMR tube. Before starting an experiment these electric properties have to be optimised in order to maximise the transfer of energy between the coil and the sample and between the coil and other parts of the circuit (like the amplifiers). This is accomplished by moving (using rods) two capacitors, called match and tune. This process is called “tuning the probe” and is analogous to radio tuning. In modern spectrometers the rods are often actuated be electric motors controlled by the acquisition computer.

The homogeneity of the magnetic field of the superconducting magnet itself is usually insufficient for NMR experiments. This situation is exacerbated by the inhomogeneity of the tube holding the sample and by edge effects. In order to increase the homogeneity NMR magnets are equipped with many (usually more than 20) supplementary coils of different geometry. By changing the electric currents in individual coils (called “shims” due to historical reasons) the magnetic field in the sample volume can be changed, which is called “shimming the magnet”.

The length of the radio pulse needed to rotate the magnetisation vector by a given angle has to be determined empirically. As its length is determined by both the geometry and material properties of the NMR tube as well as the sample solutions (especially by the salt concentration) it has to be calibrated for every new sample. This is most often done by finding the length of a pulse that rotates the magnetization vector by 360^{o} (giving close to no signal) starting with known pulse length for a similar sample.

NMR spectra are acquired by subjecting the sample to a train of electromagnetic pulses of well-defined power, length and shape interspersed with exactly-timed delays. Because of that the practical realisation of a NMR experiment is called a pulse sequence or a pulse program. A single train RF pulses ending with the recording of the transverse magnetization-induced signal is called a scan.

The system under investigation returns to thermodynamic equilibrium in finite time and the mechanism (longitudinal and transverse relaxation) is described in the literature. In practice for protons longitudinal relaxation times are between 1 and 10 seconds. The inter-scan delay must be long enough to allow for the recovery for at least a part of the longitudinal magnetization and for proton experiments is often set as 1-2 s. This time is often too short to allow for complete recovery and in order for subsequent scans of an experiment to start with the system in roughly the same state the experiment proper is preceded by “dummy scans”. No data is collected from them but the system can reach a steady-state. Additionally since some experiments can deposits large amounts of energy in both the sample and probe during these scans give the spectrometer time to regulate air flow and heating to maintain constant temperature during the experiment.

Spectra more complex then the most basic one-dimensional experiments require the use of many RF pulses and any miscalibration can lead to the presence of unwanted signals. By repeating the experiment’s scans while changing the phases of some of the pulses these signals can be suppressed by summing the acquired data. The repetitions are called phase programs. A phase cycle of length n increases the duration of the experiment n-fold and the S/N (signal-to-noise ratio) $n^{½}$–fold. Many state-of-the-art experiments do not require any phase cycling but it was once usually for pulse sequences to require them. For that reason before using a pulse sequence one should check the minimal and maximal phase cycles.

Experimental procedure

The aim of the exercise is the structure elucidation of the supplied compound, using NMR spectra. The compound is known to be (at room temperature) a colourless liquid with a gasoline-like odour. From elemental and mass analysis it was determined that its empirical formula is C_8H_{10}. The sample is a mixture of 10% of the compound and 90% deuterated chloroform ($CDCl_3$). Due to the solution's volatility it should not be heated above 40^{o}C.

After acquainting yourself with the spectrometer and operational procedures:

1. Place the sample in the spinner and after centering in the sample gauge inject into the spectrometer.
2. Tune the proton channel and check the tuning for the carbon channel.
3. Lock the sample and set the deuterium lock parameters.
4. Increase the magnetic field homogeneity by shimming the sample.
5. Calibrate the proton pulse by finding the duration of pulse rotating the water signal by 360 degrees.
6. Acquire the following 1D spectra: proton, carbon, carbon with proton decoupling, DEPT-90 and DEPT-135.
7. Fourier transform the acquired spectra. Is the data from these experiments enough to determine the origins of individual signals, magnitudes of coupling constants and/or assign the signals to individual positions in a chemical structure? What is the importance and effect of decoupling and of using the steady-state magnetisation of proton nuclei in transfer experiments?
8. Acquire the ^{1}H, ^{13}C HSQC spectrum (time: up to an hour). Fourier transform and analyse it. What information can be gained from this spectra, does it help in separating the multiplets in 1D spectra?
9. Acquire the ^{1}H, ^{13}C HMBC spectrum (time: up to an hour) optimised for proton-carbon scalar coupling constants of 8 Hz. Analyse the spectrum after a Fourier transform.
10. Depending on the remaining time perform additional experiments (COSY, TOCSY, NOESY). Each should yield good quality spectra in 20-40 minutes. Do they yield any new information about the compound. Discuss their congruence with the spectra recorded earlier.

Laboratory report guidelines

It is expected that the final report will follow the conventional structure for a experimental report, in particular it should contain:

- an abstract,
- a short theoretical introduction,
- descriptions of the equipment used, the sample and experimental details (numbers of points, spectral widths) and data processing (weighting functions)
- data analysis which should include information on the assignment of signals (or groups thereof) on the acquired spectra. A chemical structure should be proposed for the compound under investigation and its compatibility with the experimental results should be explained. The report should contain feature a list of assigned chemical shifts and coupling constants. For individual spectra the question listed in the “Experimantal instructions should be answered.

Introductory test

Commencing with laboratory work is contingent upon passing the pre-laboratory test. The form of the test will be decided by the lab assistant. Participants should familiarize themselves with the literature given at the end of this handout. The exam will cover the following topics:

- Spin ½ isotopes (in the ground state) of biologically import ant elements and their natural abundance.
- Spins in a (external) magnetic field. The magnetic resonance phenomenon, magnetogyric ratio.
- Nuclear shielding and the effective field. Definition of the δ chemical Shift and the reason for introducing this quantity
- Interactions between spins in a molecule. Coupling constant J. Fine structure and multiplet structure of NMR spectra.
- Qualitative description of the factors influencing the appearance of a NMR spectrum using a simple organic molecule (like ethanol) as an example.
- The Fourier transform and its applications in NMR spectroscopy.

Literature

Basic:

- Section 3.7 in
  David Sheehan *Physical Biochemistry. Principles and Applications. Second Edition*. Wiley-Blackwell, 2009

- Chapter J1 in
  Igor N. Serdyuk, Nathan R. Zaccai and Joseph Zaccai, *Methods in Molecular Biophysics. Structure, Dynamics, Function*, Cambridge University Press, 2007

- Description of solution-state NMR in
  Peter Atkins *Physical Chemistry* OUP (any recent edition)

Supplementary:

- Chapters 3-5 in
  Robert M. Silverstein, Francis X. Webster and David J. Kiemle, *Spectrometric identification of organic compounds*, Wiley, 2005
  (A description of many 1 and 2D NMR experiments and their applications in organic chemistry)

- Chapter J2 in
  Igor N. Serdyuk, Nathan R. Zaccai and Joseph Zaccai, *Methods in Molecular Biophysics*. Structure, Dynamics, Function, Cambridge University Press, 2007
  (An extender description of multidimensional NMR spectroscopy)

- Kazimierczuk, K., Misiak, M., Stanek, J., Zawadzka-Kazimierczuk, A. and Koźmiński, W. (2012). *Generalized Fourier transform for non-uniform sampled data*. W *Novel Sampling Approaches in Higher Dimensional NMR* (pages 79-124). Springer Berlin Heidelberg.
  (Mostly signal processing, that is the pathway from the measured signal to spectrum. It also contains a presentation of performing high dimensionality and/or resolution experiments using non-uniform sampling)

- James Keeler, *Understanding NMR Spectroscopy* Wiley, 2013
  (An accessible description of NMR theory on a basic level. Unfortunately there is some terminological confusion in the chapters with regard to quantum mechanics – especially the concepts of a mixed state, density matrix and reduced density matrix.)