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Epitope Mapping of AMP-ADH and NAD-ADH with the aid of
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Declaration of Authorship

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- Where I have consulted the published work of others, this is always clearly attributed.
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“Education is the most powerful weapon which you can use to change the world.”

Nelson Mandela

UNIVERSITY OF VIENNA

Abstract

Faculty of Life Sciences

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Master of Science

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A novel assignment method has been employed to determine the binding activity and conformational arrangement of a small-molecule ligand present in incremental excess of a high-molecular-weight receptor. The method applied is an analogon of the classical 1D NOESY experiment, with additional use of an adiabatic inversion pulse. This Adiabatic Fast Passage NOESY (AFP-NOESY) experiment provides an accurate predication about the stereochemical configuration of the ligand molecule inside the binding pocket of the protein. The latter information is based on the NOE build-up curve resulting from the pulse sequence, which is displayed as the sum of two jointly contiguous effects, during the relaxation runtime of the nuclear spins. The two effects involve the direct and indirect NOE magnetization transfer pathway, whose relative contingent for each individual ligand proton holds information about the spherical positioning of the ligand inside the binding cavity of the protein, thus determining its binding epitope. A supplementary experiment introducing the Saturation-Transfer-Difference-Spectroscopy (STD), allows for a means of certification for the newly implemented AFP-NOESY experiment.

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Abbreviations

AFP	Adiabatic fast passage
β	Pulse flip angle
B_0	Strength of the external magnetic field
DD	Dipole-Dipole
CSA	Chemical Shift anisotropy
FID	Free Induction Decay
γ	Gyromagnetic ratio
$\hbar$	Planck's constant divided by 2π
I	Nuclear spin quantum number
$J(\omega)$	Spectral Density Function
μ	Magnetic moment
m	Magnetic quantum number
M_0	Equilibrium net magnetization
$M_{x/y/z}$	Magnetization along the axis $x/y/z$
NMR	Nuclear magnetic Resonance
NOE	Nuclear Overhauser Effect
NOESY	Nuclear Overhauser Effect Spectroscopy
ω_0 , ν_0	Larmor frequency
p	Nuclear angular momentum
ROESY	Rotating Frame Overhauser Effect Spectroscopy
R_1	Longitudinal relaxation rate
R_2	Transverse relaxation rate
$R_{1\rho}$	see Chapter 4
T_1	Longitudinal relaxation time
T_2	Transverse relaxation time
TPPI	Time-proportional phase incrementation
TROSY	transverse rrelaxation-optimized NMR spectroscopy

Dedicated to Michael Gibson.

Part I

Theoretical Background

Chapter 1

Introduction

NMR spectroscopy is arguably the most important analytical method available today. There are manifold reasons for this: it is applied by chemists and physicists to gases, liquids, liquid crystals and solids (including polymers). Biochemists use it routinely for determining the structures of peptides and proteins, and it is also widely used in medicine (where it is often called MRI, Magnetic Resonance Imaging). With the advent of spectrometers operating at very high magnetic fields (up to 21.1 T, i.e. 900 MHz proton resonance frequency), it has become an extremely sensitive technique.

One of the most powerful attributes of NMR spectroscopy is its ability to detect molecular motion in proteins and polymers. Other methods of detecting molecular motion are limited by a small number of sites that can be probed or the narrow time scale over which the motion can be characterized. The ability to observe and characterize resolved NMR resonance lines from individual atoms, provides information on dynamics from a large number of sites within the protein [13].

Simultaneously, NMR is useful in probing molecular interactions, which is the focal point of this scientific work. The most considerable interactions for investigation are protein-protein as well as protein-drug interactions, with the latter one providing the scaffold of structure-based drug design. On the basis of the structures of protein-ligand complexes, new compounds can be designed that optimize intermolecular interactions and improve the physical characteristics of a lead compound without disrupting physical association between the drug and the target. Two of the most successful techniques for obtaining structural information on protein-ligand complexes are X-ray crystallography and NMR. One major disadvantage of X-ray crystallography is the need to obtain suitable crystals of the protein-ligand complex. Although the field of crystallography has advanced over the past decade, the production of crystals continues to be a major hurdle. High-resolution structure determination using NMR spectroscopy also suffers from many disadvantages, the greatest limitation being the need to observe, resolve and assign the many signals that arise from the protein resonances. This limits the application of high-resolution NMR to targets with a molecular weight of less than about 30 KDa. In the

absence of high-resolution structural data on protein-ligand complexes, computational docking strategies can be used to attempt prediction of not only the ligand-binding site, but also the structure of the protein-ligand complex for use in structure-based design. In these cases, the structure of the target biomolecule for use in docking simulations must be obtained either from the experimentally derived structure of the target protein itself or from homology modelling. However, computational algorithms for predicting structures of protein-ligand complexes are still maturing and often give rise to multiple solutions that cannot be reliably discriminated on the basis of the predicted binding energies [3].

Here, a novel NMR-based strategy for deriving structural information on protein-ligand complexes is presented, that provides an alternative to traditional X-ray crystallography and NMR methods for structure-based drug design. The method is a one-dimensional Adiabatic Fast Passage Nuclear Overhauser Effect Spectroscopy (1D AFP-NOESY Spectroscopy). It is based on the dipolar relaxation mechanism of two spins communicating with each other through space, whereas strongly depending on the internuclear distance r , typically with a $\frac{1}{r^6}$ dependence. The dipolar interaction not only constitutes a dominant relaxation mechanism, but also leads to a phenomenon called cross-relaxation. Cross-relaxation causes one of two spins interacting with each other to depart from equilibrium, hence perturbing the equilibrium magnetization of the second spin as well. The amount of perturbation is called the Nuclear Overhauser Enhancement. The aim of my diploma work, was to examine the mechanisms of NOE magnetization transfer, which is, in the case of protein-ligand complexes, the sum of two counteracting NOE transfer methods of dipolar relaxation, namely the direct and indirect pathway. The fractional apportionment between the latter two terms, holds crucial information about the spherical positioning of the ligand inside the binding pocket of the protein, termed epitope mapping.

In the course of this diploma work, a second affirmative experimental method has been chosen to compare the results. The method implemented is called Saturation-Transfer-Difference Spectroscopy (STD), and is based on the Nuclear Overhauser Effect as well. It relies on performing a saturation transfer difference experiment on a ligand complexed to a protein sample, wherein only signals from binding substances are displayed in the spectrum.

Although both methods are a means of investigating binding activities and binding epitopes of ligands, the former one is indubitably the more accurate one in providing accurate conformational arrangements of ligands inside the binding cavity of the protein.

Chapter 2

Nuclear Magnetic Resonance - An Outline

The NMR phenomenon is a consequence of the existence of nuclear spin. Not all nuclei have spin, but those that do also have an associated magnetic field and hence a nuclear magnetic dipole moment, μ [6]. A magnetic dipole can be imagined as a microscopic bar magnet, with separated magnetic north and south poles, aligned with the nuclear spin axis.

When placed in a static magnetic field (B_0), such as that provided by the magnet of an NMR spectrometer, the energy of a nucleus becomes orientation dependent, which leads to the nucleus attempting to align itself with its magnetic dipole parallel with the static field. Twisting it into an antiparallel orientation requires an input of energy.

As the nucleus is a subatomic particle rather than a macroscopic object its behaviour is constrained by the laws of quantum mechanics - where the energy of the nucleus is quantized, the number of energy levels (eigenstates) being $2I + 1$, and I being the nuclear spin quantum number. The most considerable nuclei in NMR research are spin $\frac{1}{2}$, that is nuclei such as 1H , ^{13}C , ^{15}N , ^{31}P and ^{19}F for which $I = \frac{1}{2}$. For such nuclei there are only two possible levels, called α and β . these are the low- and high energy states, respectively, and they correspond to two particular orientations of μ relative to the static magnetic field axis [6].

Since the axes of the nuclear spin and the nuclear magnetic dipole are, in fact, the same, they are reciprocally interconnected by a scalar constant of proportionality, which is called the gyromagnetic ratio, or γ . Quantum mechanics defines the magnitude of the angular momentum due to the nuclear spin as $\hbar\sqrt{I(I+1)}$, so that γ is defined by

$$\mu = \gamma\hbar\sqrt{I(I+1)} \quad (2.1)$$

TABLE 2.1: A selection of isotopes and their properties.

nucleus	spin	natural abundance	$\gamma/\text{rad s}^{-1} \text{T}^{-1}$
^1H	$1/2$	$\approx 100\%$	$267.522 \cdot 10^6$
^2H	1	0.015%	$41.066 \cdot 10^6$
^{12}C	0	98.9%	
^{13}C	$1/2$	1.1%	$67.283 \cdot 10^6$
^{14}N	1	99.6%	$19.338 \cdot 10^6$
^{15}N	$1/2$	0.37%	$-27.126 \cdot 10^6$
^{16}O	0	$\approx 100\%$	

The differences in γ between different nuclear species can be pictured as corresponding to differences in the strength of their associated magnetic dipoles. This in turn implies a difference in their NMR frequencies; the stronger a magnetic dipole is, the more energy is required to change its orientation in a static magnetic field of given strength, and, consequently for spin $\frac{1}{2}$ nuclei, the greater is the energy separation of the α and β states. Another important result from quantum mechanics is that the projection of μ along the static magnetic field axis is $\gamma\hbar m$, where m is the magnetic quantum number, having values $I, I-1, I-2, \dots, -I$. For spin $\frac{1}{2}$ nuclei, these projections, thus, only have magnitudes $(\frac{1}{2})\gamma\hbar$ or $-(\frac{1}{2})\gamma\hbar$, corresponding to the α and β energy states, respectively. Since $m < \sqrt{I(I+1)}$, these projections have magnitudes smaller than μ itself, implying that in either energy state the magnetic dipole is not aligned parallel with the field, but instead makes an angle $\cos^{-1}(m\sqrt{I(I+1)}) = 54.73^\circ$ with the static field axis, as shown in Figure 2.1 [6].

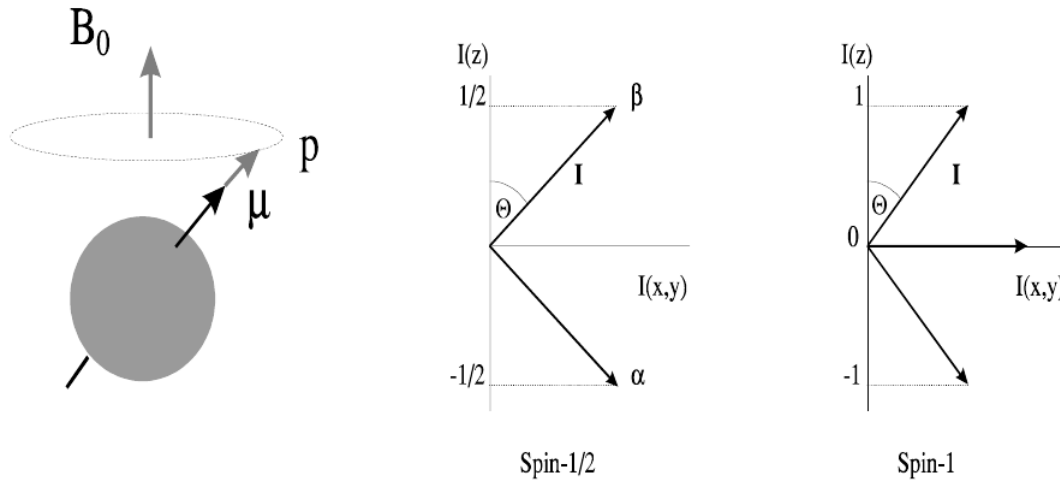


FIGURE 2.1: “Two-cone” representation of the α and β spin states for a single-spin system with $I = \frac{1}{2}$ showing the slightly higher population of the lower energy α state [2].

The energy separation of the α and β states at thermal equilibrium relative to kT at typical room temperatures is minute. The latter statement is a formalization of the Boltzmann distribution law, which says that the populations of the two states at thermal equilibrium will be very similar. This indicates that there will be slightly more spins occupying the α state than the oppositely directioned β state. The ratio is given by the Boltzmann distribution as follows:

$$\frac{N_\alpha}{N_\beta} = e^{\frac{E_\beta - E_\alpha}{kT}}. \quad (2.2)$$

As shown in Figure 2.1 each magnetic dipole μ is depicted as a vector starting at a common origin. Those in the lower-energy α state are evenly distributed around the surface of the upper cone, while the very slightly smaller number in the high-energy β state are distributed around the surface of the lower cone [6].

In terms of this representation, the net nuclear magnetization across the whole sample corresponds to the vector sum of all individual dipoles μ . The result of such a summation is a macroscopic magnetic moment M_z^0 , parallel to the static applied field B_0 and of length proportional to the population difference between the α and β states at thermal equilibrium. This equilibrium magnetization M_z^0 , which is called *longitudinal* because it is aligned along the static field axis B_0 , represents the starting point for all NMR experiments. The role of the RF field is to introduce a transverse (xy) component to the total magnetic field, so that the total field axis is no longer parallel to M_z^0 , and the magnetization precesses about the new field axis. This means that the precession about B_{total} while the RF field is on causes the magnetization to move away from the B_0 axis, and leaves a *transverse* component of magnetization (M_{xy}) when the RF field is switched off. Once created, the transverse magnetization (M_{xy}) precesses about B_0 at a frequency ν (in Hertz) given by the Larmor equation [6]:

$$\nu = \frac{\gamma}{2\pi} |B_0| \quad (2.3)$$

$$= \frac{1}{h} [E_\beta - E_\alpha] \quad (2.4)$$

this equation forms the link between the vector description of NMR and the quantum mechanical description. In the vector description, the rate of precession ν of each nucleus depends on the strengths both of the static field B_0 and the nuclear magnetic dipole μ ; the stronger either of these is, the faster the precession rate will be. The strength of B_0 is a function of the spectrometer, but the strength of the nuclear magnetic dipole depends on the gyromagnetic ratio γ . γ relates the nuclear dipole moment to the nuclear spin angular momentum $p = \sqrt{I(I+1)}\hbar$, but this angular momentum is itself related to the spin energy of the nucleus, in the form of:

$$E = \gamma m \hbar B_0 \quad (2.5)$$

Thus, the precession rate can also be expressed in terms of the nuclear spin energy (2.4), where the spin energy corresponding to resonance is set equal to the energy difference between the α and β nuclear spin states. This is the proof of the Bohr frequency condition familiar from other branches of spectroscopy, where an energy difference between two quantum mechanical states is related, via Planck's constant h , to the frequency of radiation that can interconvert the states ($\Delta E = h\nu$) [6].

It is the Larmor precession of transverse magnetization that NMR spectrometers measure. As it rotates the transverse magnetization induces an alternating current within the receiver coil of the spectrometer which is the NMR signal. This NMR signal depends both on the intrinsic strength of the equilibrium magnetization, given by the length of the vector M_z^0 , and also on the angle through which it is turned by the pulse [6].

In practice it is not only one resonance we detect, but there are usually several present. They result in many contributions to $\vec{M}$. After a pulse, each resonance will precess at its own characteristic Larmor frequency. The sum of these contributing frequencies constitute the free-induction decay (FID), which gives the spectrum on Fourier transformation.

The Radio-Frequency Field and the Free Induction Decay

The effect of a radio-frequency field is as already mentioned in the previous chapter to tip away the magnetization vector $\vec{M}$ from the z axis by introducing a second field $\vec{B}_1$ that is perpendicular to $\vec{B}_0$. This leads to $\vec{M}$ precessing around the resultant field $(\vec{B}_0 + \vec{B}_1)$. When the $\vec{B}_1$ field is switched off, $\vec{M}$ will no longer be aligned with $\vec{B}_0$. However if $\vec{B}_1$ were a static field, it would need to be of similar magnitude as $\vec{B}_0$ in order to influence the orientation of $\vec{M}$. This is practically impossible. Fortunately it is possible to achieve the same result with the aid of a resonant $\vec{B}_1$ field. In the latter one, $\vec{B}_1$ will rotate around $\vec{B}_0$, with the same frequency as $\vec{M}$ does. So, the aim of this model is a conversion from a laboratory reference frame, in which $\vec{M}$ precesses with an angular velocity of $\omega_0 = \gamma B_0$ to a rotating reference frame in which the angular velocity precession of $\vec{M}$ changes to $(\omega_0 - \omega)$ rather than ω_0 . Then, according to this point of view, the field strength cannot be equal to B_0 but must be reduced to $(B_0 - \omega/\gamma)$. And if we are exactly "on resonance" (*i.e.* $\omega = \omega_0$), then the effective field will be shifted entirely onto the x -axis in the transverse plane. Now ω is just the angular velocity with which B_1 is rotating, so that relative to the rotating frame, B_1 appears to be static. In this frame the magnetization precesses around an effective field B_{eff} with a strength [2]:

$$B_{eff} = \sqrt{B_1^2 + (B_0 - \omega/\gamma)^2}. \quad (2.6)$$

The effective precession frequency is then $\omega_{eff} = \gamma B_{eff}$; hence:

$$\omega_{eff} = \sqrt{\omega_1^2 + (\omega_0 - \omega)^2}, \quad (2.7)$$

where:

$$\omega_1 = \gamma B_1. \quad (2.8)$$

The convention is that frequencies indicated by ω are in (rad s^{-1}) and frequencies notated as ν are in Hz (s^{-1}); the conversion is $\omega = 2\pi\nu$. Usually ω is referred to as the "carrier" frequency of the radio-frequency (RF) field, and the quantity $(\omega_0 - \omega)$ is known as the "offset"; that is the difference in frequency between the carrier and the actual Larmor frequency of a spin. The simplest case is obtained when the carrier is on resonance, that is when there is no offset at all and $B_{eff} = B_1$. Then the precession is around B_1 only, which is usually aligned with the x axis of the rotating frame. Then $\vec{M}$ starts its precession along the z axis and rotates in the yz plane. When the B_1 field is on for a duration t_p , and t_p is rather short, typically in the microsecond range, then t_p is denoted as a radio-frequency pulse. The vector $\vec{M}$ has moved during this time by an angle α to the Z axis. This angle α is referred to as the *flip angle* caused by the RF pulse, which is therefore called an α degree pulse. The flip angle is given by [2]:

$$\alpha = \gamma B_1 t_p. \quad (2.9)$$

In order to consider the pulse phase, the right-handed coordinate system is used. For example a pulse along x results in $M_z = M_0 \cos \beta$ and $M_y = -M_0 \sin \beta$. Most NMR experiments are almost exclusively built up from 90° and 180° pulses. The 90° pulse generates the strongest induced current in the detection coil, as the entire magnetization is brought into the transverse plane. On the contrary the 180° pulse has the effect of inverting the magnetization. A 90° pulse with phase x would rotate the magnetization M_z to $-y$ while a 180° pulse would rotate it to $-z$. In practice we want to obtain a complete NMR spectrum, which means that we want to apply an RF pulse that causes a 90° flip angle not only for spins that conform to an on resonance condition but also for spins which have an offset resonance frequency, i.e. a chemical shift difference of $(\omega_0 - \omega)$. This chemical shift comes about because 2 nuclei within a molecule experience a different local magnetic field if their electronic environments are different. Therefore for the largest offset present in the spectrum to be transposed onto the transverse plane it requires that B_{eff} is still very close to B_1 , implying that $B_1 \gg (B_0 - \omega/\gamma)$. For this purpose, pulses were designed to affect in good approximation all nuclei of one isotope in the very same way. Such pulses have a rectangular shape in the time domain while in the frequency domain, gathered by a Fourier transformation, they are characterized by a sinc i.e. $\frac{\sin x}{x}$ profile. These pulses, when narrow enough in the time domain, will cover a frequency band sufficiently broad to be resonant with all chemical shifts. However,

occasionally it is necessary for a pulse to affect one nucleus selectively (e.g. for water suppression). If this is the case, a long weak rectangular pulse can be applied, since a large profile in the time domain is defined by a narrow profile in the corresponding frequency domain. Alternatively a shaped pulse can be used, that has a gaussian or sinc shape instead of a rectangular form in the time domain, resulting in the corresponding gaussian and rectangular profile in the frequency domain [20].

The Free Induction decay (FID) is the information detected and transformed into a spectrum after a basic pulse acquire experiment. If the pulse-acquire experiment has used a 90° pulse about x , $90^\circ(x)$, the generated x- and y-components of the transverse magnetization can be written as [9]:

$$M_y = -M_0 \cos \Omega t \quad M_x = M_0 \sin \Omega t \quad (2.10)$$

where M_0 is the equilibrium magnetization and Ω is the offset (in rad s^{-1}). The precession of the magnetization gives rise to a current in the RF coil which results in a signal voltage that can be digitized and stored away in computer memory. The spectrometer is capable of detecting simultaneously both the x- and y-components of the magnetization, each giving rise to separate signals, S_x and S_y . These signals are proportional to M_x and M_y , but their absolute values aren't of any interest, so the maximum value can be approximated by a constant of proportion S_0 as follows [9]:

$$S_x = S_0 \cos \Omega t \quad S_y = S_0 \sin \Omega t \quad (2.11)$$

However, the magnetization, and hence the signal will decay over time as a result of relaxation processes, which have to be considered in 2.11 to yield:

$$S_x = S_0 \cos \Omega t \exp\left(\frac{-t}{T_2}\right) \quad S_y = S_0 \sin \Omega t \exp\left(\frac{-t}{T_2}\right). \quad (2.12)$$

T_2 is a time constant which characterizes the decay, the shorter T_2 , the more rapid the decay. The simplest case to acquire the x- and y-components of an NMR signal is to detect them in the form of a complex signal, with the x-component being the real part and the y-component the imaginary part. The complex signal $S(t)$ is written as:

$$\begin{aligned} S(t) &= S_x + i S_y \\ &= S_0 \cos \Omega t \exp\left(\frac{-t}{T_2}\right) + i \left[S_0 \sin \Omega t \exp\left(\frac{-t}{T_2}\right) \right] \\ &= S_0 (\cos \Omega t + i \sin \Omega t) \exp\left(\frac{-t}{T_2}\right) \\ &= S_0 \exp(i \Omega t) \exp\left(\frac{-t}{T_2}\right). \end{aligned} \quad (2.13)$$

This complex signal is represented by a vector rotating at a frequency Ω , with a length corresponding to $S_0 e^{\frac{-t}{T_2}}$, where T_2 is the transverse decay constant. We can simplify the division by T_2 by introducing the first-order rate constant for the transverse magnetization decay, defined as:

$$R = \frac{1}{T_2}, \quad R \text{ in units of } s^{-1} \text{ or Hz} \quad (2.14)$$

Thereby equation 2.13 is simplified to:

$$S(t) = S_0 \exp(i\Omega t) \exp(-Rt) \quad (2.15)$$

As R increases the decay becomes more rapid and so the corresponding line in the spectrum becomes broader. If there are several resonances present, the the complex time-domain signal is a sum of the following terms:

$$S(t) = S_0^{(1)} \exp\left(\frac{-t}{T_2^{(1)}}\right) + S_0^{(2)} \exp\left(\frac{-t}{T_2^{(2)}}\right) + \dots$$

where each resonance has its own intensity $S_0^{(i)}$, frequency Ω_i , and decay constant $T_2^{(i)}$. Finally, Fourier transformation of a given complex time-domain signal of Eq. 2.15 yields a complex frequency domain signal or spectrum, $S(\omega)$:

$$S(t) \xrightarrow{\text{FT}} S(\omega)$$

$$S_0 \exp(i\Omega t) \exp(-Rt) \xrightarrow{\text{FT}} \underbrace{\frac{S_0 R}{R^2 + (\omega - \Omega)^2}}_{\text{real}} + i \underbrace{\frac{-S_0 (\omega - \Omega)}{R^2 + (\omega - \Omega)^2}}_{\text{imaginary}}. \quad (2.16)$$

The real part of the spectrum is a peak with the *absorption mode Lorentzian lineshape*, whereas the imaginary part has the *dispersion mode Lorentzian lineshape*, defined as:

$$A(\omega) = \frac{R}{R^2 + (\omega - \Omega)^2} \quad D(\omega) = \frac{-(\omega - \Omega)}{R^2 + (\omega - \Omega)^2}. \quad (2.17)$$

The absorption lineshape is always positive and is centred at Ω ; if $\omega = \Omega$, the height of the peak is $1/R$:

$$\begin{aligned} S(\Omega) &= \frac{R}{R^2 + (\Omega - \Omega)^2} \\ &= \frac{R}{R^2} \\ &= \frac{1}{R} \end{aligned}$$

Using the lineshape functions $A(\omega)$ and $D(\omega)$ from Eq. 2.17, we can write the result of the Fourier transformation as:

$$S(t) \xrightarrow{\text{FT}} S(\omega)$$

$$S_0 \exp(i\Omega t) \exp(-Rt) \xrightarrow{\text{FT}} S_0 [A(\omega) + i D(\omega)]. \quad (2.18)$$

BLOCH-Equations and the Product Operator Formalism

For the description of an NMR experiment there are various possible representations [2]:

1. The energy level diagram, is only for polarisations, not for time-dependent phenomena
2. The classical treatment by BLOCH (BLOCH EQUATIONS) : only for isolated spins - no J coupling
3. the Vector diagram, is a pictorial representation of the classical approach, has the same limitations
4. The quantum mechanical treatment (density matrix), is rather complicated, however using appropriate simplifications and definitions - the product operators - a fairly easy and correct description of most experiments.

BLOCH-Equations

The behaviour of isolated spins can be described by classical differential equations [2]:

$$dM/dt = \gamma M(t) \times B(t) - R \{M(t) - M_0\} \quad (2.19)$$

with M_0 being the Boltzmann magnetization and R the relaxation matrix:

$$R = \begin{bmatrix} 1/T_2 & 0 & 0 \\ 0 & 1/t_2 & 0 \\ 0 & 0 & 1/T_1 \end{bmatrix} \quad (2.20)$$

The external magnetic field consists of a static magnetic field B_0 and the oscillating r.f.field B_{rf} :

$$B(t) = B_0 + B_{rf} \quad (2.21)$$

B_{rf} is linearly oscillating:

$$B_{rf} = B_1 \cos(\omega t + \phi) e_x \quad (2.22)$$

The time-dependent behaviour of the magnetization vector thus corresponds to rotations in space (plus relaxation), with the B_x and B_y components derived from r.f. pulses and B_z from the static field:

$$dM_z/dt = \gamma B_x M_y - \gamma B_y M_x - (M_z - M_0)/T_1 \quad (2.23)$$

$$dM_x/dt = \gamma B_y M_z - \gamma B_z M_y - M_x/T_2 \quad (2.24)$$

$$dM_y/dt = \gamma B_y M_x - \gamma B_x M_z - M_y/T_2 \quad (2.25)$$

Product Operators

To include coupling a special quantum mechanical treatment has to be chosen for description. An operator, called the spin density matrix $\rho(t)$, completely describes the state of a large ensemble of spins. All observable (and non-observable) physical values can be extracted by multiplying the density matrix with the appropriate operator and then calculating the *trace* of the resulting matrix [2]. The time-dependent evolution of the system is calculated by unitary transformations (corresponding to "rotations") of the density matrix operator with the proper Hamiltonian H (including r.f. pulses, chemical shift evolution, J coupling etc.):

$$\rho(t') = \exp\{iHt\} \rho(t) \exp\{-iHt\} \quad (2.26)$$

For calculations these exponential operators have to be expanded into a Taylor series. The density operator can be written as a linear combination of a set of basis operators. Two specific bases turn out to be useful in NMR experiments:

- the real Cartesian product operators I_x, I_y , and I_z . They are useful for the description of observable magnetization and effects of r.f.pulses, J coupling and chemical shift)
- the complex single-element basis set I^+, I^-, I^α and I^β . These are the raising and lowering operators useful for coherence order selection, phase cycling and gradient selection.

Cartesian Product Operators

Single spin operators . Correspond to magnetization of single spins, analogous to the classical macroscopic magnetization M_x, M_y, M_z [2].

I_x, I_y in-phase coherence, observable

I_z z polarization, not observable

Two-spin operators

$2I_{1x}I_{2y}, 2I_{1y}I_{2z}, 2I_{1z}I_{2x}, 2I_{1z}I_{2y}$	anti-phase coherence, not observable
$I_{1z}I_{2z}$	longitudinal two-spin order, not observable
$2I_{1x}I_{2x}, 2I_{1y}I_{2x}, 2I_{1x}I_{2y}, 2I_{1y}I_{2y}$	multiquantum coherence, not observable
$2I_{1x}I_{2x} + 2I_{1y}I_{2y} = I_1^+ I_2^- + I_1^- I_2^+$	zero-quantum coherence
$2I_{1y}I_{2x} + 2I_{1x}I_{2y} = I_1^+ I_2^- - I_1^- I_2^+$	not observable
$2I_{1x}I_{2x} - 2I_{1y}I_{2y} = I_1^+ I_2^+ - I_1^- I_2^-$	double-quantum coherence
$2I_{1x}I_{2y} - 2I_{1y}I_{2x} = I_1^+ I_2^+ + I_1^- I_2^-$	not observable

The single-element operators I^+ and I^- correspond to a transition from the $m_z = -\frac{1}{2}$ to the $m_z = +\frac{1}{2}$ state and back, resp., hence termed as the "raising" and "lowering operator". Products of three and more operators are also possible. Only the operators I_x and I_y represent observable magnetization. However, other terms like antiphase magnetization $2I_{1x}I_{2z}$ can evolve into observable terms during the acquisition period. [2]

Single element operators

In some cases (phase cycling, gradient coherence selection) it is necessary to use operators with a defined order (Eigenstates of coherence order). Coherence order describes the changes in quantum numbers m_z caused by coherence. A *spin* $-\frac{1}{2}$ *system* (no coupling) can assume two coherent states: a transition from $\alpha(m_z = +\frac{1}{2})$ to $\beta(m_z = -\frac{1}{2})$, i.e., a change (coherence order) of -1. [2]

This can be described by the lowering operator $I^- = |\beta\rangle\langle\alpha|$, the coherent transition from the β to α state by the raising operator $I^+ = |\alpha\rangle\langle\beta|$ (coherence order +1).

The real Cartesian operators I_x and I_y correspond to the observable x and y components

of the magnetization. Their relationship with the complex I^{+-} operators is [2]:

$$I^+ = I_x + iI_y \quad \text{raising operator}$$

$$I_x = \frac{1}{2}(I^+ + I^-)$$

$$I^- = I_x - iI_y \quad \text{lowering operator}$$

$$I_y = -i/2(I^+ - I^-)$$

$$I^\alpha = \frac{1}{2}1 + I_z \quad \text{polarisation operator}(\alpha)$$

$$I_z = \frac{1}{2}(I^\alpha + I^\beta)$$

$$I^\beta = \frac{1}{2}1 - I_z \quad \text{polarisation operator}(\beta)$$

$$1 = I^\alpha + I^\beta$$

The effect of r.f. pulses (here: an x pulse with flip angle φ on single-element operators is:

$$I^{+/-} \longrightarrow \varphi_x \quad I^{+/-} \cos^2 \{\varphi/2\} + I^{-/+} \sin^2 \{\varphi/2\} (+/- iI_z \sin \{\varphi\}) \quad (2.27)$$

$$I^\beta \longrightarrow \varphi_x \quad I^\beta \cos^2 \{\varphi/2\} + I^\alpha \sin^2 \{\varphi/2\} + \left(\frac{1}{2}\right) \sin \{\varphi\} [I^+ - I^-] \quad (2.28)$$

$$I^\alpha \longrightarrow \varphi_x \quad I^\alpha \cos^2 \{\varphi/2\} + I^\beta \sin^2 \{\varphi/2\} - \left(\frac{1}{2}\right) \sin \{\varphi\} [I^+ - I^-] \quad (2.29)$$

Generally it is easier to calculate the effects of r.f. pulses on Cartesian operators and then use the conversion rules to get the single-element version.

Phase Cycling

Changing the phase of one or more selected pulses in combination with the phase of the receiver in an orderly manner, signals either add up or cancel each other out at the end of a phase cycle depending on the pathway they went through. Thus unwanted pathways are avoided, and artefacts disappear [20].

Pulsed field gradients

Usually whatever possible is done in order to achieve a homogenous magnetic field. In the case of pulsed field gradients, though, controlled inhomogeneity is introduced by applying an additional magnetic field $G(z)$ that changes linearly along z . The total magnetic field is therefore:

$$B = B_z = B_0 + G(z). \quad (2.30)$$

Since $\omega_0 = -\gamma B$, all nuclei have a different Larmor frequency depending on their position within the sample. Therefore, any transverse magnetization would decay very quickly, but refocussing is possible either by applying the same gradient in the other direction (*i.e.* $G(z) - G(-z)$) or via a simple spin echo experiment (*i.e.* $G(z) - 180^\circ - G(z)$) [20].

Chapter 3

Relaxation Mechanisms in NMR

If a sample is allowed to be undisturbed for a long time in the magnetic field, it reaches a state of *thermal equilibrium*. This state implies that all coherences are absent and that the populations are given by the Boltzmann distribution, at the temperature of the molecular environment. R.f.pulses disturb the equilibrium of the spin system. The populations after a pulse usually deviate from their thermal equilibrium values and, in many cases, coherences are created. For example, a π pulse inverts the population distribution, whereas a $\pi/2$ pulse equalizes spin state population and generates coherences. Relaxation is the process by which equilibrium is regained, through interaction of the spin system with the thermal molecular environment [8].

Relaxation processes may be divided into two types. *Spin-lattice relaxation* is concerned with the movement of spin populations back to their Boltzmann distribution values. *Spin-spin relaxation* is concerned with the decay of coherences. Spin-lattice relaxation is also known as *longitudinal relaxation*, spin-spin-relaxation is also known as *transverse relaxation*.

In an ensemble of isolated spins $-\frac{1}{2}$, there are only two time constants for the relaxation processes: these are the *spin-lattice time constant* T_1 for the equilibration of populations and the *transverse relaxation time constant* T_2 [9].

3.1 Longitudinal Relaxation

When a sample is introduced into a magnetic field, a net magnetization parallel with the surrounding static magnetic field, along the $+z$ -axis (M_o), is generated. Following a radiofrequency pulse, this bulk magnetization starts precessing around the axes of the direction of the pulse. As the bulk magnetization of the spins is rotated away from the z -axis, the energy of the spins is increased, as a consequence of decreasing the number of spins, whose magnetic moments are aligned with the z -axis, the low energy arrangement. For the spins to come back to equilibrium, they therefore need to lose

energy, which involves its flow from the spins to the surroundings. Due to the bias of the spins to occupy the lower energy state, the probability for a certain amount of energy to flow into the surroundings is higher than the other way round. Based on this fact, the z -components of the nuclear magnetic moments will not be distributed randomly, but in such a way as to lead to a rearrangement of a net z -magnetization. As the longitudinal relaxation mechanism involves a flow of energy from the spins to the surroundings mediated by the local fields, we can state from this that longitudinal or more explicitly the spin-lattice relaxation is an enthalpic process. Although the excess energy is transferred as heat, the effect is so small that no increase of temperature can be detected [9]. The relaxation of a perturbed spin $-\frac{1}{2}$ system follows a simple exponential behaviour, described by equations

$$\frac{dM_z}{dt} = \frac{M_0 - M_z}{T_1} \quad (3.1)$$

and

$$M_z = M_0 \left(1 - e^{-\frac{t}{T_1}} \right)$$

M_0 is the equilibrium net magnetization, M_z is the magnetization along the z -axis at every point of time t , and T_1 is referred to as the relaxation time constant that is connected to the relaxation rate constant R_1 in a reciprocal manner ($R_1 = \frac{1}{T_1}$). It depends on the nucleus, on temperature, on viscosity, etc. and usually lies in the order of 10^{-3} to 1 s but can also go up to years in extraordinary cases [20].

The determination of T_1 occurs in its simplest form by measuring a gated inversion recovery experiment. The gated inversion recovery pulse sequence (IR) makes use of a $180^\circ(x)$ -pulse followed by a delay during which time T_1 relaxation occurs, and is followed by a $90^\circ x$ -pulse which allows the recovered magnetization to be measured. The IR experiment is repeated with an array of delays, τ , between the 180° -pulse and the 90° -pulse that cover the range from complete inversion through complete recovery. A shorthand notation that describes the pulse sequence is [20].:

$$T_d - 180_x^\circ - \tau - 90_x^\circ - \text{detect (FID)} \quad (3.2)$$

wherein T_d represents a delay interval during which the spin system returns to (virtually complete) equilibrium. Typically 10 – 20 delays are employed in the IR experiment to support robust fitting of the intensity versus time data. By varying τ , the intensity of the FID is modified, and T_1 can be extracted from a plot $I(t)$ vs (t) using the following relationship

$$\frac{d\Delta I(t)}{dt} = \Delta I(0) e^{-\frac{t}{T_1}}, \quad (3.3)$$

with $\Delta I(t) = I(t) - I_{eq}$, where $I(t)$ is the peak intensity after a delay t , and I_{eq} is the equilibrium magnetization.

3.2 Transverse Relaxation

The process by which transverse magnetization decays away to its equilibrium value of zero is called transverse relaxation or alternatively spin-spin relaxation. It is the result of individual magnetic moments changing orientation while interacting with an oscillating transverse local field. These local fields have transverse components along the x - and y -axis, which drive the transverse magnetization to its equilibrium value. This is termed the non-secular contribution to transverse relaxation. However, there is a second major mechanism for mediating the recurrence of the equilibrium condition. The magnetic moments from individual spins precessing about the applied magnetic field B_0 at the Larmor frequency, do not only experience this B_0 field, but also an additional field originating from the z -component of the local field. The sum of the latter two, generates a local field that slightly varies from spin to spin, leading to a slightly different precession frequency for each spin. Consequently, over time the precession of the individual moments will get out of step with one another, and thus the transverse magnetization will shrink. This is called the secular contribution to transverse relaxation and is an irreversible entropic process [20].

Similarly, to the longitudinal relaxation the transverse relaxation can also be described in the form of an exponential decay of intensity. The measurement of the transverse relaxation can be derived from a simple 90° -detect pulse scheme, however the complication in the measurement of T_2 arises due to Larmor precession and the limited spatial homogeneity of the sample chamber. The reason for this is that the magnetic field varies in different parts of the sample due to the finite field homogeneity-thus spins in the various regions of the sample cell will have different Larmor frequencies [2].

In a standard lock-in detection system, spins with resonance frequencies that are higher than the carrier frequency rotate in one sense, while spins with resonance frequencies lower than the carrier frequency rotate in the opposite sense.

In a simple experiment the decrease in measured magnitude (intensity) will thus be the sum of an artifactual component arising from finite field homogeneity (inhomogeneous broadening) and the actual T_2 relaxation. Distinguishing these effects is critical to accurate measurement of T_2 .

The solution to the problem is what is called the Carr-Purcell-Meiboom-Gill experiment, CPMG, which is based on the original spin-echo technique invented by Erwin Hahn. Hahn discovered that in a system that contains multiple spins with different chemical shifts, in which perhaps none will resonate at the carrier frequency, the direction of the precession can be reversed by applying a 180° -pulse-this experiment is known as the Hahn spin-echo experiment, because the intensity of monitored magnetization appears to first decrease and then increase again [2].

The shorthand notation of the pulse sequence is:

$$90_x^\circ - \tau - 180_y^\circ - \tau - \text{detect}. \quad (3.4)$$

In the CPMG variant, the $(\tau - 180_y^\circ - \tau)$ component of the Hahn echo is repeated, thus allowing an array of time intervals to be produced.

The CPMG sequence may be written as:

$$90_x^\circ - (\tau - 180_y^\circ - \tau)_n - \text{detect}. \quad (3.5)$$

The total length of the transverse relaxation interval will thus be defined by the length of a CPMG-cycle multiplied by the number of cycles.

By varying τ , the intensity of the FID is modified, and T_2 can be extracted from a plot $I(t)$ vs (t) using the following relationship

$$I(t) = I_0 e^{-\frac{t}{T_2}} \quad (3.6)$$

with T_2 as the transverse relaxation constant. Again the transverse relaxation constant R_2 can be gained from the reciprocal value of T_2 . T_2 values cannot be larger than the respective T_1 values, but they can be several orders of magnitude smaller, above all in large molecules that tumble slowly in solution. However, it has to be mentioned that, although the description of both longitudinal and transverse relaxation by a single exponential decay is over-simplified, it is by far acceptable in practical cases [2].

3.3 Dipole-Dipole Relaxation

The relaxation behaviour of any magnetic nuclei in a molecule interacting via the dipolar mechanism can be described as an energetic exchange of two spins or dipoles that is stored in the excited state(s). This interaction comes from one spin generating an instantaneous magnetic dipolar field, which is proportional to the magnetic moment of that nucleus, and a second spin experiencing this local field. As the molecule tumbles in solution, this local field fluctuates and hence causes a mechanism of relaxation of nearby spins. The efficacy of dipolar relaxation depends on a number of parameters. The first to be mentioned is the distance r between the two spins, which falls off rather quickly as $(1/r^3)$. A second parameter is the gyromagnetic ratio of the spin; the larger the gyromagnetic ratio, the larger the magnetic moment and the larger the local field. This means that protons will give rise to larger local fields than ^{13}C nuclei. Finally the orientation of the vector joining the two spins relative to the applied magnetic field (the z -axis is of fundamental importance. Because of the dependence of relaxation on the inverse sixth power of the distance r , nuclear spin relaxation can be used to determine distances between the interacting nuclei, most efficiently by protons, due to their large

gyromagnetic ratios, which constitute a sensitive probe for internuclear distances [9]. Pure dipole-dipole relaxation of spin I elicited by spin S or vice versa leads to the following relaxation rate for longitudinal magnetization.

$$R_{Iz} = d_{DIP} [J(w_I - w_S) + 3J(w_I) + 6J(w_I + w_S)] \quad (3.7)$$

$$d_{DIP} = \frac{\mu_0^2 h^2 \gamma_I^2 \gamma_S^2}{r_{IS}^6} \quad (3.8)$$

$$J(w) = \frac{2}{5} \frac{\tau_c}{1 + \omega^2 \tau_c^2} \quad (3.9)$$

μ_0 is the magnetic susceptibility of the vacuum, h is Planck's constant divided by 2π , γ_I and γ_S are the magnetogyric ratios of the nuclei I and S respectively, r_{IS} is the interatomic distance, and $J(w)$ is the spectral density function. It gives an idea of all frequencies ω involved in the molecular motions of a compound in solution. It depends on the rotational correlation time τ_c that can be approximated for a protein by the Einstein-Stokes formula (M is the molecular mass, V is the volume, η is the viscosity, R the gas constant, and T the temperature):

$$\tau_c = \frac{MV\eta}{RT} \quad (3.10)$$

As can be concluded from these equations, the larger the molecule, the slower is its tumbling motion and the faster the relaxation of an excited spin state. For transverse magnetization we can write the following equation:

$$R_{Ix} = \frac{1}{2} d_{DIP} [4J(0) + J(w_I - w_S) + 3J(W_I) + 6J(w_I + w_S) + 6J(w_S)] \quad (3.11)$$

Are more coupling partners present, the individual contributions from each spin pair can be added up. For double product operators, DD relaxation is given by:

$$R_{IzSz} = d_{DIP} [3J(w_I) + 3J(w_S)] \quad (3.12)$$

If spin I has other coupling partners i , the term $\sum_i d_{DIP}^i [3J(w_i) + 3J(w_I)]$ is added.

$$R_{IzSx} = \sum_i d_{DIP}^i [3J(w_i) + 3J(w_I)] + R_{Sx} \quad (3.13)$$

3.4 Chemical Shift Anisotropy

The usual description given for the chemical shift is to say, that in the presence of a strong applied field, the electrons in the molecule give rise to a small induced (local) field at the nucleus. The nucleus therefore experiences the sum of the applied field and this induced field, thus shifting the Larmor frequency by an amount which depends on the size of the induced field [9].

For most molecules, the size of the induced field, and hence the size of the chemical shift, depends on the orientation of the molecule with respect to the applied magnetic field. This is described by saying that the chemical shift is *anisotropic*. In liquid samples the molecules are tumbling so rapidly that the nuclei see an average local field, and hence have an average chemical shift, called the isotropic shift. Nevertheless, at any instant, the local field is different for molecules at different orientations and thus has to be considered if investigating relaxation [9].

Accordingly, when a molecule is placed in a static magnetic field, in order to work out the size and direction of the local field affecting a particular nucleus, we need to consider the chemical shielding tensor as well as the orientation of the tensor with respect to the applied field. The size of the tensor is specified by its three principle components, denoted $\sigma_{\parallel}$, $\sigma_{\perp}$ and σ_{zz} , which remain fixed as the molecule tumbles. For simplification we assume the tensor to be axially symmetric, with two of its components being equal. Such a tensor can be represented as a three-dimensional ellipsoid i.e. a sphere which has been stretched along one axis [20].

CSA relaxation only involves one spin, so there is no possibility of cross-relaxation, and hence transfer of magnetization between spins. The effect of CSA on longitudinal magnetization of spin I follows the equation

$$R_{Iz} = d_{CSA}^I J(w_I) \quad (3.14)$$

$$d_{CSA}^I = \frac{1}{3} (\sigma_{\parallel}^I - \sigma_{\perp}^I)^2 w_I^2 = \frac{1}{3} (\sigma_{\parallel}^I - \sigma_{\perp}^I)^2 \gamma_I^2 B_0^2. \quad (3.15)$$

$\sigma_{\parallel}$ represents the parallel component, thus the distance along the symmetry axis from the centre of the ellipsoid to the edge. $\sigma_{\perp}$ as the distance from the centre of the ellipsoid to the edge perpendicular to the symmetry axis stands for the second contribution to the chemical shift tensor. For transverse magnetization the subsequent equation holds :

$$R_{Ix} = d_{CSA}^I \left[\frac{2}{3} J(0) + \frac{1}{2} J(w_I) \right]. \quad (3.16)$$

3.5 Cross Correlation

A particular nucleus in a molecule is likely to experience random fields from more than one source, for example by dipolar interaction with several other nuclei, from its CSA or from paramagnetic species. The simplest assumption that can be made, is that these different sources of relaxation are independent, and that the total relaxation rate constant is the sum of the rate constants due to each source of local fields. However this assumption is wrong, as for two sources of relaxation to be independent of one another, the time dependence of the associated random fields must be completely different. This means that there must be no correlation between these random functions. This is certainly not the case for cross-correlation, or in other words relaxation interference, where the time dependence of the local field comes in both cases from the rotational reorientation of the molecule due to thermal motion. Thus the variations of the random fields arising from the two sources must be correlated to some extent as both are modulated by the same motion. Depending on the way in which the fields are correlated, they might reinforce one another to some extent, thus increasing the relaxation rate constant and leading to an undesired effect of line broadening in the spectrum. The other possibility is, that the two fields will cancel one another to some extent, reducing the relaxation rate constant and resulting in sharper linewidths and thus in an improved signal to noise ratio [9].

The presence of such narrow lines for large molecules greatly improves the sensitivity, and therefore makes it much easier to obtain structural information, which is exploited in a method called TROSY (Transverse Relaxation Optimized Spectroscopy).

This line narrowing effect is particularly pronounced at high magnetic fields, where CSA relaxation becomes competitive to dipole-dipole relaxation [20].

Chapter 4

Adiabatic Fast Passage

Adiabatic fast passage (AFP) experiments allow the measurement of heteronuclear self-relaxation. Pulses generating AFP are chirped pulses, i.e. their frequency is a function of time. During the pulse the radiofrequency is swept e.g. linearly. If the magnetization vector follows the effective field $\omega_{eff}(t)$ that is time-dependent in the same way the chirped pulse is, the adiabaticity condition is fulfilled. For this to occur, the pulse needs to exceed a limiting power, and the frequency has to be swept slowly enough imitating a spin-lock. AFP pulses show an efficient inversion potential, and to ensure total inversion, apodization of the pulses at the beginning and the end is usually done. Spin inversion by adiabatic fast passage is typically accomplished using adiabatic frequency sweeps which are very large compared with the radio-frequency field intensity. Hence it is commonly assumed that spin inversion occurs at the point where the adiabatic frequency sweep passes through the chemical shift of a given spin. Relaxation during the adiabatic fast passage is therefore mainly longitudinal. In contrast, if the radio-frequency field is comparable to the frequency sweep range, significant transverse magnetization will be created. At a given sweep rate, different net amounts of transverse magnetization during the time course of the adiabatic inversion pulse can be created by a variation of the rf amplitude [4][20]. This leads to different *effective* relaxation rate (adiabatic spin-lock frame relaxation rate $R_{1\rho}$), which is given as a weighted average of longitudinal and transverse relaxation.

The adiabatic spin-lock frame is shown in Figure 4.1. $\Delta\omega(t)$ is the offset that depends on the time as the pulse is frequency swept. As soon as the frequency reaches a spin chemical shift it is zero. $\omega_1(t)$ is the frequency of the pulse at a given time during the pulse. The sum of these two vectors is the time-dependent effective field $\omega_{eff}(t)$ that starts in the direction of z , goes through zero and ends in $-z$ i.e. inversion. If the frequency sweep occurs slowly enough, the magnetization rotates around ω_{eff} and is therefore in fact spin-locked and inverted as well. During the inversion the magnetization vector can at every time step be split up using trigonometric functions into a longitudinal share that relaxes according to R_1 and a transverse that relaxes according to R_2 . The adiabatic

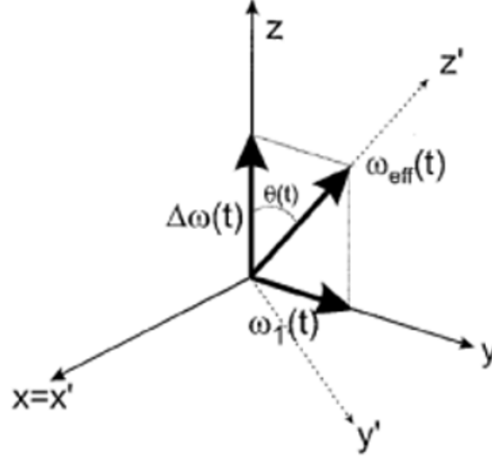


FIGURE 4.1: Adiabatic spin-lock frame [4].

self-relaxation rate in the spin-lock can therefore be written as

$$R_{SL}(t) = \cos^2 \theta(t) R_1 + \sin^2 \theta(t) R_2, \quad (4.1)$$

where $\theta(t)$ is the time-dependent tilt angle represented in Figure 4.1. Using the well known relationship of $\sin^2 \theta + \cos^2 \theta = 1$ the equation above can be written as

$$R_{SL}(t) = R_1 + \sin^2 \theta(t) (R_2 - R_1). \quad (4.2)$$

If the magnetization relaxes purely mono-exponentially, the adiabatic relaxation rate can be written as

$$R_{1\rho} = R_1 + (R_2 - R_1) \frac{1}{\tau_p} \int \sin^2 \theta(t) dt, \quad (4.3)$$

with τ_p being the duration of the adiabatic inversion pulse. Integration results in

$$R_{1\rho} = R_1 + (R_2 - R_1) \sin^2 \theta_{eff} \quad (4.4)$$

where θ_{eff} is the effective tilt angle. The intensity after the adiabatic fast passage of duration τ_p , $I(\tau_p)$, is given by

$$I(\tau_p) = I(0) \exp[-R_{1\rho} * \tau_p] \quad (4.5)$$

The value of $\sin^2 \theta_{eff}$ can be calculated numerically as all the parameters governing the effective tilt angle are defined (e.g. sweep rate and rf amplitude). The exact value of $\sin^2 \theta_{eff}$ can be chosen at will, limited only by the rf amplitude requirement for adiabatic inversion and by hardware parameters.

Chapter 5

NMR techniques in structure-based drug-design

The biological function of a protein generally depends on the interaction of the protein with its ligand substrates. Among the well-established examples are interactions of hormone receptors with the corresponding hormones, that trigger elaborate signalling cascades, the interplay of proteins with oligonucleotides, responsible for the regulation of replicational, transcriptional or translational processes and eventually the highly specific interactions of cell surface antigens with receptors, residing on different cells [1].

For the understanding of biological functions one needs a precise description of protein-ligand interactions on a nuclear level. Of specific interest are protein-ligand interactions involved in the emergence of profound diseases, such as AIDS or cancer. The understanding of the principles underlying the mutual reaction between a protein receptor and the respective biological ligands, approves the development of effective substances that act as agents for the upcoming pharmaceutical products. The identification and optimization of the aforementioned candidates for drug design comprises several steps, a considerable part of which is the recognition of substances with binding activities for the protein receptor of interest in order to generate so called leads (leading substances). The developmental process requires the screening of a large amount of compounds - normally exceeding 10^6 . The classical procedure in the screening process is accomplished by biological or functional assays like the "Enzyme-linked Immunosorbent Assay" (ELISA). Recently though, it has been shown that a number of novel NMR techniques provide us with information about the binding process on atomary level. This allows for the identification of new bioactive compounds [1].

Several NMR techniques have been developed for identifying and characterizing binding activities, for the analysis of bioactive conformations of ligands as well as for the appointment of ligand binding epitopes. The most considerable of them however, are associated with facile determination and a high sensitivity. Examples of the latter are

changes of chemical shifts, relaxation times, diffusion constants, NOEs or saturation exchange. In general 2 approaches are employed: the first one is based on effects, that are measured on NMR signals of the ligands and typically depend on NOE effects between ligand and protein (which is explained in detail in section STD/transfer NOE). The second approach, focusses on the changes of chemical shifts of the target protein upon the binding of a ligand. The two latter methods are complementary and have different benefits. Several additional approaches predicate on changes in the relaxational or diffusional behaviour of the ligand upon binding a protein [1].

5.1 The NOE Effect

The Nuclear Overhauser Effect (NOE) is characterized by resonance line intensity changes caused by dipolar relaxation from neighbouring spins with perturbed energy level populations. To understand the nature of the NOE, the energy level diagram by Solomon can be used to depict the processes for a two spin system I^1 and I^2 . The Solomon diagram exemplifies the nature of possible processes underlying the NOE effect, which are merely polarizations, e.g. population differences between α and β states, excluding coherences which do not evolve [2].

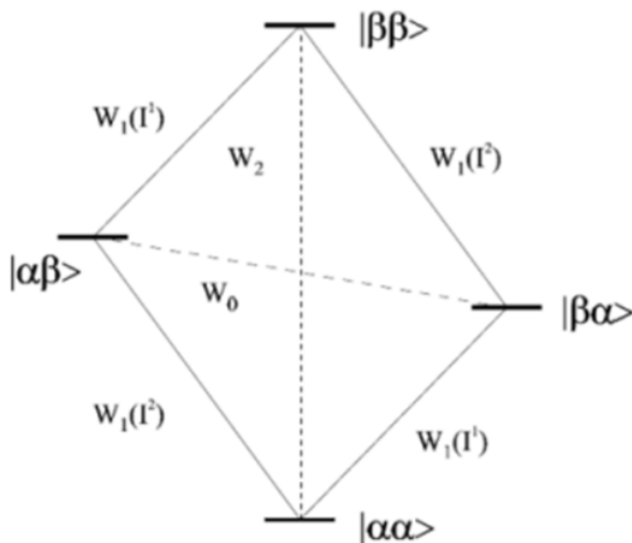
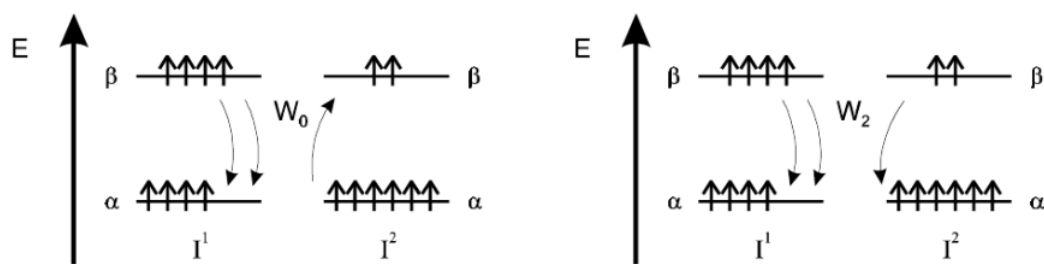


FIGURE 5.1: Solomon diagram for a two spin system composed of two nuclear spins.

Taking into consideration a two spin system, there are several possible transitions that can be classified into three groups:

- W_1 transitions involving a spin flip of only one of the two spins (either I^1 or I^2), corresponding merely to T_1 relaxation of the spin.

FIGURE 5.2: W_0 and W_2 transitions [7].

- a W_0 transition involving a simultaneous spin flip $\alpha \rightarrow \beta$ for one spin and $\alpha \rightarrow \beta$ for the other one (i.e., in summa a zero quantum transition).
- a W_2 transition involving a simultaneous flip of both spins in the same direction, corresponding to a net double quantum transition.

The aforementioned spin state transitions are caused by relaxation and do not involve a coherent process (like, e.g. I_x/I_y coherences, which require a phase coherent transition between two states generated by a radio-frequency pulse).

If one spin is perturbed, e.g. I_1 , i.e., changes its populations of the α and β state (by saturating the resonance = creating equal populations of both states), then relaxation will force I_1 back to the equilibrium Boltzmann distribution. With the W_1 mechanism, spin I_1 will just relax without effecting spin I_2 . However the other two mechanisms, W_0 and W_2 will effect I_2 as shown in Figure 5.2 [2].

With the I_1 polarization going back from saturation to the Boltzmann equilibrium, the W_0 mechanism will cause the neighbouring (so far unperturbed) spin to deviate from its Boltzmann equilibrium towards a decrease in α, β population difference. After a 90° pulse, this will result in a decrease in signal intensity for I^2 - a "negative Noe effect". On the other hand, the W_2 mechanism will cause the population difference of the undisturbed spin I^2 to increase in signal intensity : a "positive NOE effect". These effects can be directly observed in a very simple experiment, the 1D difference NOE sequence [2]:

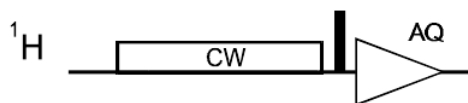


FIGURE 5.3: 1D difference NOE sequence [2].

One spin is selectively saturated by a long, low-power CW (continuous wave) irradiation. As soon as the spin deviates from its Boltzmann population distribution, it starts with T_1 relaxation. Via the W_0 or W_2 mechanisms it causes changes in the population

distribution of neighbouring spins. After a 90° pulse, these show up as an increase or decrease in signal intensity. Usually the experiment is repeated without saturation, giving the normal 1D spectrum. This is then subtracted from the irradiated spectrum, so that the small intensity changes from the NOE effects can be easier distinguished: spins with a positive NOE (i.e., higher intensity in the NOE spectrum than in the reference 1D) show a small positive residual signal, spins with a negative NOE yield a negative signal, spins without an NOE cancel completely [2].

Distinguishing between positive or negative NOEs. First of all, an NOE requires that there is a significant interaction between the magnetic dipoles of the two spins. Dipolar interaction drops off very fast with distance, so a 1H , 1H NOE can only occur with a distance $> 5\text{-}6 \text{ \AA}$ (500-600 ppm) [2].

Due to the very small energy differences between NMR spin states, a spontaneous transition from a higher to a lower energy state is very improbable (with average lifetimes of several years for the spin states). All transitions have to be induced by electromagnetic fields which are on resonance, i.e. have the right frequency corresponding exactly to the energy difference of the two spin states involved in the transition [2].

For the W_1 transitions, this frequency is identical with the Larmor frequency of the spin that is undergoing the spin flip, i.e., the resonance frequencies $\omega(I_1)$ and $\omega(I_2)$. For the W_0 transition, the energy difference is identical to the difference $\omega(I_1) - \omega(I_2)$, and for W_2 it is the sum of the resonance frequencies for the two spins, $\omega(I_1 + \omega(I_2))$.

If we would consider, e.g., 1H in a 500 MHz spectrometer, then the W_1 mechanism would require frequencies of 500 MHz to induce transitions, the W_2 mechanism (corresponding to a simultaneous flip of two 1H spins) requires fields of 1000 MHz, and the W_0 transitions frequencies equal to the resonance frequency difference between the two protons, i.e., a few ppm. (some 100 or 1000 Hz).

Origin of these electromagnetic fields. If two small magnets are close together at a fixed distance (like two 1H spins in a molecule in an external magnetic field, then the interaction between the two depends on the orientation of the molecule in the external field: In position a), there will be a repulsive interaction between the two, in position c), it will be attractive, and somewhere in between (position b), the interaction will be zero. As the molecule is not static, but tumbled around all the time in the sample solution, a rotation of the molecule will change its orientation in the static magnetic field and cause a modulation of the field strength, that a spin actually "experiences" - through the effects of the neighbouring spins. Thus the rotation of the other spins generates an electromagnetic field - but not with a well-defined frequency, but with a wide range of frequency components, due to the random nature of molecular rotation.

The easiest approximation to describe the rotation of a molecule in solution is the assumption of the molecule as a rigid sphere. If we calculate how "much" of a certain frequency is generated in the random rotation, we find the following expression for the

spectral density at a frequency ω :

$$J(\omega) = \frac{2\tau_c}{1 + \omega^2\tau_c^2} \quad (5.1)$$

It means that there is a maximum at $\omega = 0$ and the spectral density then drops off for higher frequencies. How fast it drops off is controlled by the parameter τ_c , the molecular rotational correlation time. A long correlation time means a rather sluggish rotation, and a fast drop off of J (very little high-frequency contributions). A short τ_c corresponds to a fast random rotation, causing a much wider frequency distribution (more high-frequency contributions).

The correlation time is determined by different factors: mainly, the molecular weight. The larger a molecule, the slower its re-orientation, i.e., the longer its τ_c . As a rule of thumb, the correlation time of a molecule can be estimated from the molecular weight MW: $\tau_c [ps] \approx 0.5 MW [Da]$ (e.g., a MW of 1000 Da corresponds to ca. 0.5 ns). But τ_c is also influenced by other parameters: temperature (the higher the temperature, the shorter τ_c), solvent viscosity (the more viscous, the longer τ_c), aggregation in solution.

Detection of NOE. The observable NOE is determined by the cross relaxation rate

$$\sigma = (W_2 - W_0) \quad (5.2)$$

which is a rate constant characterizing the dipolar relaxation between the two spins, that are reasonably close to one another in space. The phenomenon of cross relaxation leads to what is called the Nuclear Overhauser effect.

With the dependence on the spectral densities at the resonance frequencies for W_0 ($\omega = 0$) and W_2 ($\omega = 2\omega_0$) we get:

$$\sigma_{ij} \propto \frac{1}{r_{ij}^6} 6J(2\omega_0) - J(0) \quad (5.3)$$

For $6J(2\omega_0) > J(0)$, we get positive NOEs. This requires a relatively high spectral density at $\omega = 2\omega_0$, i.e., a short τ_c . In the opposite case (long τ_c), W_0 will dominate: negative

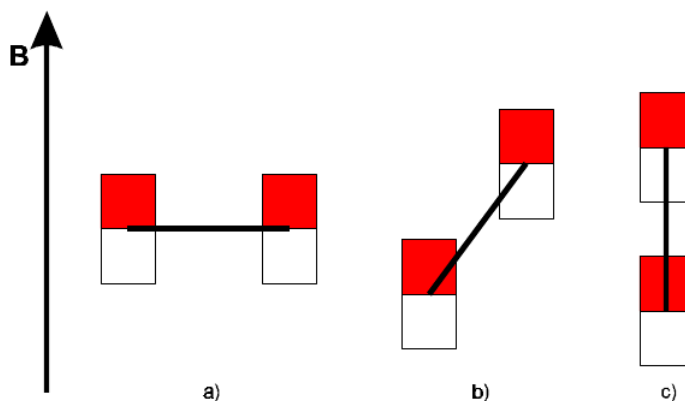


FIGURE 5.4: Orientation of a molecule in the external magnetic field [2].

NOEs. The crossing point is at $\omega_0\tau_c = \frac{\sqrt{5}}{2} \approx 1$, where the effects from W_0 and W_2 cancel each other and there is no NOE to be observed.

5.2 NOESY - The Nuclear Overhauser Enhancement Spectroscopy

A considerable phenomenon employed in NMR to study the spatial structure of a molecule is by means of 2D NOE Spectroscopy. The purpose of the NOESY experiment is to establish connectivities between the spins via cross relaxation [5]. Instead of the transfer of antiphase coherences $I_y S_z \rightarrow I_z S_y$ which is the key process of correlated spectroscopy, NOESY, relies on the transfer of longitudinal magnetization:

$$I_z \rightarrow S_z \quad (5.4)$$

The key features of the NOESY experiment are discerned into four stages: preparation, evolution, mixing and detection. These stages are indicated in the pulse sequence of Figure 5.5.

The experiment starts with a relaxation delay, to allow z magnetization to be restored after a previous scan, followed by a 90° pulse to create transverse magnetization. This concludes the preparation stage. Assuming the radio-frequency pulse to have x phase, the spin density is then given by [5]:

$$\sigma = I_0 I_y + S_0 S_y \quad (5.5)$$

where I_0 and S_0 are the equilibrium z magnetizations.

$$I_0 = \frac{\gamma_I B_0}{4kt} \quad S_0 = \frac{\gamma_S B_0}{4kt} \quad (5.6)$$

Next is the evolution stage, during which the spins are allowed to precess and become labeled with their offset frequencies ω_I and ω_S :

$$I_y \xrightarrow{\omega_I t_1} \begin{Bmatrix} I_y \\ I_x \end{Bmatrix} \quad S_y \xrightarrow{\omega_S t_1} \begin{Bmatrix} S_y \\ S_x \end{Bmatrix} \quad (5.7)$$

The frequency labels are carried over to the z components by the second 90_x° pulse, after which the spin density is given by:

$$\sigma = I_0 [-I_z \cos(\omega_I t_1) + I_x \sin(\omega_I t_1)] + S_0 [-S_z \cos(\omega_S t_1) + S_x \sin(\omega_S t_1)] \quad (5.8)$$

If the second 90° pulse is applied along the $-x$ axis one obtains:

$$\sigma = I_0 [I_z \cos(\omega_I t_1) + I_x \sin(\omega_I t_1)] + S_0 [S_z \cos(\omega_S t_1) + S_x \sin(\omega_S t_1)] \quad (5.9)$$

The x components are affected neither by a 90_x° nor by a 90_{-x}° pulse, while the y components are transferred to $-z$ by a 90_x° and to $+z$ by a 90_{-x}° pulse. Thus if two experiments are carried out, once with x and once with $-x$ phase for the second 90° pulse, and the results subtracted, then whatever signal originates from the I_x and S_x terms is cancelled out.

This phase cycling is indeed part of the NOESY experiment, so at the start of the mixing stage, one only considers the longitudinal magnetization terms:

$$\sigma' = -I_z I_0 \cos(\omega_I t_1) - S_z S_0 \cos(\omega_S t_1) \quad (5.10)$$

Hence at the start of the mixing time, τ_m , the longitudinal components I_z and S_z deviate from their equilibrium values, to which they will relax back. If τ_m is short enough the following approximation holds:

$$\Delta\langle I_z \rangle = -R_{II} [\langle I_z(0) \rangle - I_0] - R_{IS} [\langle S_z(0) \rangle - S_0] \tau_m \quad (5.11)$$

$$\Delta\langle S_z \rangle = -R_{SS} [\langle S_z(0) \rangle - S_0] - R_{SI} [\langle I_z(0) \rangle - I_0] \tau_m \quad (5.12)$$

At the end of the mixing time the longitudinal component of I is then:

$$\langle I_z(\tau_m) \rangle = \langle I_z(0) \rangle + \Delta\langle I_z \rangle \quad (5.13)$$

which after substitution of:

$$\langle I_z(0) \rangle = -I_0 \cos(\omega_I t_1) \quad \langle S_z(0) \rangle = -S_0 \cos(\omega_S t_1) \quad (5.14)$$

yields:

$$\begin{aligned} \langle I_z(\tau_m) \rangle = & \\ & -I_0 \cos(\omega_I t_1) (1 - R_{II} \tau_m) + S_0 R_{IS} \tau_m \cos(\omega_S t_1) + (I_0 R_{II} + S_0 R_{IS}) \tau_m \end{aligned} \quad (5.15)$$

Hence the third term which would have given rise to axial peaks in the 2D NOESY spectrum, is not sign inverted and is cancelled by the phase cycling.

The final 90_x° pulse converts I_z to I_y , the Larmor precession of which is monitored during the detection stage t_2 . The total signal matrix, including the terms originating

from the S spin, is then:

$$\begin{aligned}
 F(t_1 t_2) \propto & \\
 & I_0 [(1 - R_{II}\tau_m) \cos(\omega_I t_1) \cos(\omega_I t_2) - R_{SI}\tau_m \cos(\omega_I t_1) \cos(\omega_S t_2)] \\
 & + S_0 [(1 - R_{SS}\tau_m) \cos(\omega_S t_1) \cos(\omega_S t_2) - R_{IS}\tau_m \cos(\omega_I t_2)] \quad (5.16)
 \end{aligned}$$

The first and the third term give rise to diagonal peaks after 2D FT, they exhibit the same precession frequency during t_1 and t_2 . The second and fourth term yield the NOE cross peaks. For short mixing time τ_m their intensity is directly proportional to the cross relaxation rate $R_{IS} = R_{SI}$.

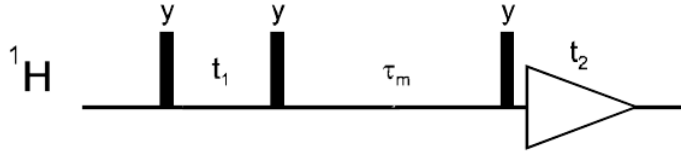


FIGURE 5.5: The basic NOESY pulse sequence [2].

The structural information contained in the 2D NOESY spectrum comes from the fact that the cross relaxation rate R_{IS} is dependent on the internuclear distance between I and S. If the dipolar interaction between the spins dominates the cross relaxation, this is almost always the case for protons, and if the molecule rotates isotropically with a rotational correlation time τ_c , the relaxation rates are given by:

$$R_{II} = \frac{\hbar^2 \gamma_I^2 \gamma_S^2}{10r^6} \left(\frac{6\tau_c}{1 + (\omega_I + \omega_S)^2 \tau_c^2} + \frac{\tau_c}{1 + (\omega_I - \omega_S)^2 \tau_c^2} + \frac{3\tau_c}{1 + \omega_I^2 \tau_c^2} \right) \quad (5.17)$$

$$R_{SS} = \frac{\hbar^2 \gamma_I^2 \gamma_S^2}{10r^6} \left(\frac{6\tau_c}{1 + (\omega_I + \omega_S)^2 \tau_c^2} + \frac{\tau_c}{1 + (\omega_I - \omega_S)^2 \tau_c^2} + \frac{3\tau_c}{1 + \omega_S^2 \tau_c^2} \right) \quad (5.18)$$

$$R_{IS} = \frac{\hbar^2 \gamma_I^2 \gamma_S^2}{10r^6} \left(\frac{6\tau_c}{1 + (\omega_I + \omega_S)^2 \tau_c^2} - \frac{\tau_c}{1 + (\omega_I - \omega_S)^2 \tau_c^2} \right) \quad (5.19)$$

$$R_{SI} = R_{IS} \quad (5.20)$$

where ω_I and ω_S are the Larmor frequencies of the spins. What the above equations 5.17 till 5.20 show is that all four relaxation rates are proportional to the internuclear distance as:

$$R \propto \frac{1}{r^6} \quad (5.21)$$

For the homonuclear case the above equations simplify to:

$$R_{II} = R_{SS} = \frac{\hbar^2 \gamma^4}{10r^6} \left(\frac{6\tau_c}{1 + 4\omega_0^2 + \tau_c^2} + \tau_c + \frac{3\tau_c}{1 + \omega_0^2 \tau_c^2} \right) \quad (5.22)$$

$$R_{IS} = R_{SI} = \frac{\hbar^2 \gamma^4}{10r^6} \left(\frac{6\tau_c}{1 + 4\omega_0^2 + \tau_c^2} - \tau_c \right) \quad (5.23)$$

There are two motional regimes to be distinguished: the fast motional regime with $\omega_0 \ll 1$ prevailing for small molecules and the slow motional regime with $\omega_0 \gg 1$ prevailing for large molecules. From this the auto- and cross relaxational rates for small molecules can be defined as:

$$R_{II} \approx \frac{\hbar^2 \gamma^4}{r^6} \tau_c \quad R_{IS} \approx \frac{\hbar^2 \gamma^4}{2r^6} \tau_c \quad (5.24)$$

For large molecules we get:

$$R_{II} \approx \frac{\hbar^2 \gamma^4}{10r^6} \tau_c \quad R_{IS} \approx -\frac{\hbar^2 \gamma^4}{10r^6} \tau_c \quad (5.25)$$

The above equation 5.24 verifies that R_{IS} is positive for small molecules, and since according to equation 5.16 the NOE cross-peak intensity is proportional to $-R_{IS}$, cross-peaks and diagonal peaks in 2D NOESY then have opposite signs. For large molecules the situation is contrariwise - the cross- and diagonal peaks have the same sign, since R_{IS} is negative here. Molecules with a $MW \approx 1kDa$ typically have τ_c s near the "cross-over point" for which no NOE's are observable:

$$\tau_c(null) = \frac{1}{\omega_0} \sqrt{\frac{5}{4}} \quad (5.26)$$

This category of medium-sized molecules is less amenable to an NMR structural study via NOEs. But one can change the ω_0 by switching to a spectrometer with a different magnetic field, or change τ_c , for example by changing the sample temperature, or record so-called "ROESY" spectra.

5.3 ROESY - The Rotating Frame Nuclear Overhauser Enhancement Spectroscopy

In a ROESY experiment the NOE (or better to say the ROE) is always positive, i.e., cross-peaks always have the opposite sign as the diagonal peaks and never become zero, which is a very important matter when for the structural determination of medium-sized molecules [9].

The pulse sequence for the ROESY experiment is relatively simple. It employs a considerable technique called spin-lock. A spin-lock is a phenomenon used for measuring

transverse cross relaxation for a certain time. The spin-lock sequence suppresses the differential precession of spins with different chemical shifts, by applying a strong radio-frequency field with the same phase as the transverse magnetization. The nutation frequency of the r.f. field has to be strong compared to both chemical shift offset frequencies, so as to allow spin-locked magnetization components to communicate through cross relaxation processes in the transverse plane [8].

After the first 90° pulse and the t_1 evolution time, a spin-lock sequence follows.

The pulse sequence of the ROESY experiment starts with a 90° pulse after which a variable evolution time t_1 and along mixing interval of continuous r.f. irradiation (the spin-lock) follows. During this spin-lock sequence the ROE builds up. The signal is detected in the subsequent t_2 interval.

Thus at the start of the mixing time (i.e. spin-locking), $\tau_m = 0$, the x magnetizations of the two spins are [5]:

$$I_{1x}(0) = \sin(\omega_1 t_1) I_z^0 \quad \text{and} \quad I_{2x}(0) = \sin(\omega_2 t_1) I_z^0 \quad (5.27)$$

At the end of the mixing time the magnetizations are:

$$I_{1x}(\tau) = \underbrace{(1 - R_{xy}^1 \tau) \sin(\omega_1 t_1) I_z^0}_{\text{diagonal peak}} - \underbrace{\sigma_{xy} \tau \sin(\omega_2 t_1) I_z^0}_{\text{cross peak}} \quad (5.28)$$

$$I_{2x}(\tau) = \underbrace{(1 - R_{xy}^2 \tau) \sin(\omega_2 t_1) I_z^0}_{\text{diagonal peak}} - \underbrace{\sigma_{xy} \tau \sin(\omega_1 t_1) I_z^0}_{\text{cross peak}} \quad (5.29)$$

From the above equations 5.28 and 5.29 we see that there are two cross peaks, with the intensity $-\sigma_{xy}\tau$, and two diagonal peaks with the intensity $(1 - R_{xy}^{(1)}\tau)$ and $(1 - R_{xy}^{(2)}\tau)$. In the initial rate limit $R_{xy}\tau \ll 1$, so the diagonal peaks are positive and the cross peaks are negative. The transverse cross relaxation rate constant σ_{xy} is positive for all values of the correlation time, so it is always the case that the cross and diagonal peaks will have opposite sign.

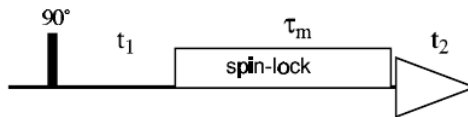


FIGURE 5.6: The basic ROESY pulse sequence.

In the ROESY experiment, relaxation does not occur in the static B_0 field along the z axis but in the transverse field generated by the r.f. irradiation: the non-equilibrium transverse component (e.g. I_{1x} relaxes with $T_{1\rho}$ while "frozen" in the spin-lock field generating a transverse component I_{2x} in the neighbouring spin by cross relaxation.

With the exception of the different dependence on correlation time, everything else works

like for the NOE: the initial ROE build-up rate shows a r^{-6} dependence, but with longer mixing (spin-lock) times, the build-up becomes non-linear and, indirect ROEs start to occur [2].

Due to the very limited power of the r.f. spin-lock fields (a few kHz, compared to several hundred MHz for the static field), an efficient spin-lock in the transverse x, y plane occurs only close to the resonance frequency of the irradiated field, usually in the center of the spectral window. Towards the edges of the spectrum, the spin-lock becomes too weak to force the spins into a precession about the transverse axis. As a result the spins precess about an effective axis that gradually tilts from the x, y plane (on resonance) back to the z axis. of the static field (far off-resonance).

Off-resonance the efficiency of ROE drops off with $(\sin \theta_i \sin \theta_j)$ for the ROE from proton i to proton j , where θ is the angle between the spin's precession axis and the x, y plane. This angle can be calculated for each spin from the ratio between the frequency offset and the spin-lock field :

$$\theta_i \propto \arctan \frac{\gamma B_1}{\omega_i - \Delta} \quad (5.30)$$

where ω_i is the offset of the spin from on-resonance, γB_1 the field strength of the spin-lock field and Δ the offset of the spin-lock field.

Since we go into the spin-lock field with pure transverse magnetization of spin i (from the initial 90° pulse), an additional factor of $(\sin \theta_i)$ has to be added, since only magnetization parallel to the spin-lock axis can be locked. Similarly another $(\sin \theta_j)$ has to be added, when the resulting spin j magnetization is detected during t_2 , since only the x, y component of the spin-locked magnetization is visible [2].

This means that the measured $ROE_{exp.}$ has to be corrected to get the true ROE, correlated with the interproton distance :

$$ROE_{true}^{ij} = ROE_{exp.}^{ij} \cdot \frac{1}{\sin^2 \theta_i \sin^2 \theta_j} \quad (5.31)$$

All these offset corrections are inversely proportional to the spin-lock field strength, increasing as the spin-lock field strength decreases.

5.4 Interference of NOE and ROE

For $\omega_0 \tau_c \approx 1$ there is no significant NOE so we don't need any offset corrections when measuring our ROESY cross-peaks. However if this is not the case, we will simultaneously get NOE contributions from the z component of the spin-locked magnetization (off-resonance, where the spin-lock axis tilts away from the transverse plane) [2]. So the result of cross relaxation during the spin-lock mixing time will be a combination of ROE and NOE:

$$\sigma_{ij} = ROE_{ij} \sin \theta_i \sin \theta_j + NOE \cos \theta_i \cos \theta_j \quad (5.32)$$

The ratio between the NOE and ROE is related to the exact correlation time τ_c of the molecule. Only for extreme cases this correction is relatively easy:

$$\omega_0\tau_c \gg 1 \quad NOE : ROE = 1 \quad (5.33)$$

$$\omega_0\tau_c \ll 1 \quad NOE : ROE = 1 \quad (5.34)$$

5.5 Direct Enhancements and spin-diffusion

Each enhancement in an NOE experiment depends on the relative positions of all nearby spins, not merely those saturated and observed. There are in fact two distinct types of contribution to a given enhancement, the first of which is the *direct contribution* which represents the total relaxation of the observed spin I that occurs by cross relaxation with the saturated spin S . this corresponds to:

$$f_I S_{direct} = \eta_{max} \frac{r_{IS}^{-6}}{r_{IS}^{-6} + \sum_X r_{IX}^{-6}} \quad (5.35)$$

where $f_I S_{direct}$ is the fractional enhancement. At the simplest level the shorter the internuclear distance r_{IS} the larger the corresponding enhancement $f_I S$. However if other spins are neighbouring the observed spin I , and cross-relax with I the term $f_I S$ diminishes the enhancement, as it introduces distance terms that increase the denominator without affecting the numerator. The effect of this dilution increases the closer these other spins are to I .

The latter indirect contribution to the enhancement is defined as:

$$f_I S_{indirect} = -\eta_{max} \sum_X \left[\frac{f_x \{S\} r_{IX}^{-6}}{r_{IS}^{-6} + \sum_X r_{IX}^{-6}} r_{IX}^{-6} \right] \quad (5.36)$$

The above equation 5.36 represents the indirect contribution to $f_I S$. This corresponds to an enhancement at (I) that arrived via cross relaxation of S with some third spin X followed by cross relaxation of (X) with (I). Such an enhancement on X means that the populations on X are not at equilibrium, and therefore cross relaxation of X with its neighbour I is bound to perturb its population in turn.

However these indirect contributions are not limited to pass only through one intermediate spin. Instead the enhancement $f_X S$ will itself contain an indirect term, which in turn will contain other enhancements carrying further indirect terms, so that indirect terms over a number of intermediate spins are presented. This can be pictured as a population disturbance initially present only on spin S , that spreads outward through the molecule by cross relaxation from spin to spin, until at steady-state every spin is affected to a greater or lesser extent. This process of population propagation is referred to as

spin-diffusion. It has very different properties in the positive NOE regime ($\eta_{max} < 0$), than it does for the negative NOE regime ($\eta_{max} > 0$), as it is supposed to only exist in the negative regime. This differing significance of spin-diffusion distinguishes the two NOE regimes [9].

5.6 STD and Transfer-NOE

Saturation-Transfer-Difference-NMR-spectroscopy (STD-NMR) together with transfer NOE is one of the most widespread NMR methods for the study of the interactions between small ligands and macromolecular receptors.

The NOE effect underlying these methods are very advantageous for the determination of three-dimensional structures of molecules in solution, especially for the structural analysis of proteins. NOEs change dramatically upon binding of a ligand to its macromolecular receptor, which leads to the so called transfer-NOE-effect. The observation of trNOEs is based on differential correlation times τ_c for the free and the bound molecule. Molecules with a low or medium molecular weight ($MW < 1000 - 2000$) have short correlation times τ_c . As a result of that they will show in dependence of their shape, molecular weight and of the magnetic field strength, strong positive NOEs, no NOEs or very small negative NOEs. Large molecules however yield strong negative NOEs. Thus, if a small molecule, (ligand) binds a macromolecular receptor (protein) it starts to simulate a large molecule, and resumes its NOE properties, hence exhibiting strong negative NOEs, the so called trNOEs. These trNOEs are being measured in solution and reflect the conformation of the bound ligand. As a consequence the binding of ligand to a receptor can easily be identified when appointing the sign and magnitude of the observed NOEs. A distinguishment between trNOEs, originating from the bound state and those from the free ligand in solution, is accomplished according to the buildup of the maximum intensity of NOE effects. For trNOEs this is in the range of 50-100 ms, whereas for free molecules the buildup is 4-10 times larger. Concluding from this, trNOEs exhibit the maximum amplification at considerably shorter mixing times τ_{mix} than small ligands do.

Generally trNOEs are categorized into inter- and intramolecular trNOEs. Intramolecular trNOEs are employed for characterizing the conformation of bound ligands. In the case of intermolecular trNOEs, these are implemented for defining the orientation of a bound ligand in the binding pocket of a protein. There are several methods based on trNOE effects utilized for detecting binding activities purposes in combinatorial libraries, an important part of which is the Saturation-Transfer-Difference method (STD) [11].

Saturation-Transfer-Difference

Saturation-Transfer-Difference-NMR-Spectroscopy is being applied for several years in order to characterize the binding properties in strongly associated receptor-ligand complexes. If a ligand exhibits, due to a slow exchange, 2 separate signal sets for the free and the bound state, then a transfer of saturation between the free and the bound form is possible. By saturating the signals of the free ligand one can identify the ones from the bound form. Overall this saturation transfer method enables the assignment of signals of the bound ligand as well as the analysis of the binding kinetics of protein-ligand complexes [1].

A new technique based on saturation transfer from the protein to the associated ligand has been developed in order to detect this saturation that is being brought into solution by means of chemical exchange. This method of difference spectroscopy is based upon the subtraction of a spectrum containing the saturated protein nuclei from a spectrum without saturation, yielding a difference spectrum harbouring only signals from the bound ligand. The irradiation frequency for the saturation of the protein nuclei is being set to a value where only resonances from the protein nuclei and none of the ligand are incurred. Hence the spectrum which selectively saturates the protein nuclei is termed the "On-Resonance" experiment. The frequencies used for the selective irradiation of protein resonances appears in the region of about -1ppm where no ligand resonances are to be observed and because on the account of the large linewidth of the protein signals a selective excitation is still ensured. If the ligand shouldn't exhibit resonances in the downfield area of aromatic protons, at $\delta = 11$ or 12 ppm then the saturation frequency can be positioned here as well. To obtain the desired selectivity and simultaneously to avoid an excitation through side bands, so called shaped pulses are employed for the saturation of protein nuclei [1].

The main principle of an STD experiment and the pulse sequence is depicted in Figure 5.7. In a ^1H experiment of a protein solution with a successively added ligand, the protein nuclei are saturated with a radio-frequency that is at least 700 Hz afar from the nearest ligand resonances ("On-Resonance" experiment). These regions can be found easily depending on the nature of the ligand, typically at an irradiation frequency of -1.5 ppm at 500 MHz magnetic field strength. Although the irradiation is highly selective, resembling a bandwidth of just several Hz, a complete saturation of all protein protons in the macromolecule is achieved by efficient spin-diffusion within 50 – 200 ms. Ligands binding to the protein are being saturated as well, whereas the degree of saturation of the ligand protons evidently depends on the dwell time of the ligand in the protein binding pocket. Via the dissociation of the ligand, the saturation is transferred into solution, in which the ligand exhibits narrow linewidths. For ligand protons interacting with protein protons through the mechanism of intermolecular trNOE, the intensity of signals decreases. Usually, however it is not possible to monitor this decrease in intensity

in presence of the protein and other molecules, such as impurities and non-binding components. For this reason the experiment is repeated, this time displacing the irradiation frequency to a region far-off any kind of signal, for instance at 40 ppm ("Off-Resonance" experiment). The recorded spectrum thus complies with a normal NMR spectrum of the compound. Subtracting the "On-Resonance" spectrum from the "Off-Resonance" one, yields a difference spectrum, that merely contains signals from the protons being attenuated by the saturation transfer. Molecules lacking binding activities don't exhibit signals in the spectrum [1].

The saturation of proton nuclei and those of the bound ligand is being accomplished

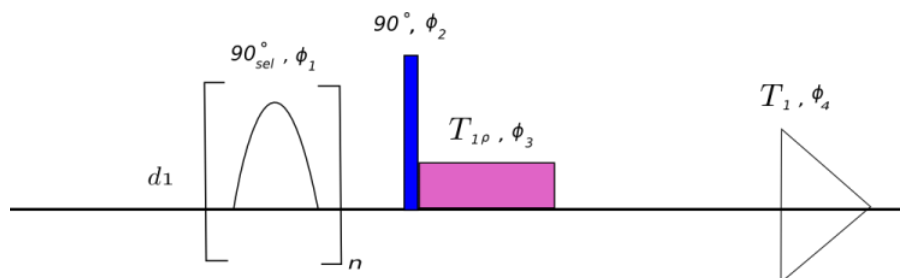


FIGURE 5.7: The basic STD pulse sequence.

quite fast, typically within 100 ms. A high dissociation rate transfers the saturation of the ligand nuclei together with the ligand into solution. If the ligand is present at high excess, a majority of ligand molecules can be saturated within a few seconds via the binding site of the protein. Because the time constant of the T_1/T_2 relaxation is at about 1s for small molecules, the fraction of saturated ligands in solution increases during the saturation time. Via this saturation transfer, from the protein to the ligand, the required amount of protein is being reduced. If, however the binding of the ligand to the receptor is very strong, inferring a low dissociation rate of k_{diss} between 0.1 – 0.01 Hz, the saturation transfer onto the ligand in solution doesn't occur very efficiently. This is estimated for K_D values < 1nM. K_D values in the range of 100 nM or more, correlate with a fast exchange between the free and bound ligand state, which leads to an efficient buildup of ligand saturation in solution.

STD-NMR-effects strongly depend on the dissociation rate, defined by k_{diss} , whereat higher k_{diss} values implicate stronger NMR signals. If the binding is weak, then too will the detention time of the ligand in the binding pocket of the receptor be short, which in turn leads to weak STD intensities. Thus STD can be applied for very strong binding limits of K_D values around 10 mM.

Concerning the pulse sequence shown in Figure xy, a spin-lock filter can be employed shortly after the detection pulse in order to attenuate the background signals from the protein. Values between 10 and 20 ms for the duration of the spin-lock pulse are sufficient for the purpose of signal repression. The intensity of the STD-NMR signals is dependent on the saturation time, on the excess of ligand molecules, whereas the longer the saturation time and the larger the excess of ligand, the stronger the STD-NMR signal

will be. An irradiation time of 2 s and a 100 fold excess of ligand provide good results. The excess of ligand yields a stronger STD signal, although a smaller fraction of ligand is being saturated. Upon the dissociation of the ligand, the saturation is transferred into solution, where it is just merely being degraded by relaxation processes. Before the ligands lose their saturation, a repeated process of association and dissociation with other ligands can occur, which leads to an accumulation of many saturated ligands in solution. The net effect of ligand proton saturation is at most when operating with large ligand excess, as then the newly occurring assignment of the binding pocket with an already saturated ligand would be highly improbable. The necessary amount of protein for the measurement is very small, shifting around 0.3 nM [1].

Part II

Experimental Work

Chapter 6

STD and AFP-NOESY

6.1 Adenosine Monophosphate in complex with Alcohol Dehydrogenase

Adenosine monophosphate (AMP), also known as 5'-adenylic acid is a nucleotide found in RNA. It is an ester of phosphoric acid and the nucleoside adenosine. AMP consists of a phosphate group, the sugar ribose, and the nucleobase adenine. Within the course of this diploma work, adenosine monophosphate has been used as a ligand for the interaction with the enzyme alcohol dehydrogenase (ADH). Alcohol dehydrogenases is a group of 7 dehydrogenase enzymes that occur in many organisms and facilitate the interconversion between the alcohols and aldehydes and ketones with the reduction of the coenzyme nicotinamide adenine dinucleotide (NAD⁺) to NADH. In humans and many other animals they serve to break down alcohols which could otherwise be toxic, in yeast and many bacteria, some ADH catalyze the opposite reaction as part of the fermentation process. From the physical point of view, Alcohol dehydrogenase is a 141k Da tetramer containing 4 equal subunits. The active site of each subunit contains a zinc atom. Each active site also contains 2 reactive sulfhydryl groups and a histidine residue [15][18]. The aim of my diploma work was to ascertain the sterical configuration of the ligand adenosine monophosphate inside the binding pocket of the *Saccharomyces Cerevisiae* alcohol dehydrogenase enzyme. For this purpose, two comparative experiments were implemented, namely the Adiabatic Fast Passage Nuclear Overhauser Effect Spectroscopy (AFP-NOESY), and the Saturation Transfer Difference Spectroscopy (STD). All experiments in the following sections were set up on a Varian NMR 500 spectrometer, at a temperature of 20 ° and with completely deuterized (²H probes).

6.1.1 The AFP-NOESY-Experiment

The first experiment applied, was a NOESY experiment with the aid of an additional adiabatic inversion pulse. The pulse sequence of this AFP-NOESY-Experiment is depicted in Figure 6.2. The experiment starts with a 90° selective inversion pulse irradiating one of the proton resonances of the ribose ring, neighbouring the H1' nucleus of the sugar. Following this selective inversion pulse, is an adiabatic spin inversion pulse, which is accomplished by using adiabatic frequency sweeps, which are very large compared with the radio-frequency field intensity. In this case the sweep ranges from -16 dB up to 35 dB within an array of 13 successive values. Spin inversion then occurs at a point where the adiabatic frequency sweep passes through the chemical shift of a given spin. Relaxation during the adiabatic fast passage is therefore mainly longitudinal, unless the radio-frequency field intensity is comparable in size to the frequency sweep range, then a significant amount of transverse magnetization can be expected. At a given sweep rate, different net amounts of transverse magnetization during the time course of the adiabatic inversion pulse can be created by a variation of the radio-frequency amplitude. This leads to a different *effective* relaxation rate (adiabatic spin-lock frame relaxation rate $R_{1\rho}$), which is given as the weighted average of longitudinal and transverse relaxation. The frequency sweep results from a parabolic phase modulation of the pulse. The B_1 field is kept constant throughout the majority of the pulse with the exception of the edges, where the B_1 field is ramped from zero to w_1^{max} and from w_1^{max} to zero during the first and the last fractions of the pulse, respectively. This ensures that the magnetization is placed along the -z-axis at the end of the inversion [4]. The time-dependent adiabatic spin-lock frame self-relaxation rate ρ^* of the irradiated proton resonance H'_x on the ribose ring of the AMP is given as

$$\rho^*(t) = \rho_l \cos^2 \theta(t) + \rho_t \sin^2 \theta(t) \quad (6.1)$$

Similarly, the adiabatic spin-lock frame cross relaxation rate is given as

$$\sigma^*(t) = \sigma_{NOE} \cos^2 \theta(t) + \sigma_{ROE} \sin^2 \theta(t) \quad (6.2)$$

where ρ^* and σ^* are the overall longitudinal and transverse relaxation rates and $\theta(t)$ is the time-dependent tilt angle. This equation can be linearized in $\sin^2(t)$ to result in

$$\sigma^*(t) = \sigma_{NOE} + \sin^2 \theta (\sigma_{ROE} - \sigma_{NOE}) \quad (6.3)$$

where σ_{NOE} and σ_{ROE} stand for the respective longitudinal and transverse cross relaxational fractions, in dependence of the value of effective field tilt angle $\theta(t)$. When the radio-frequency field intensity B_1 overweighs the adiabatic frequency sweep, then the Rotating Nuclear Overhauser Effect (ROE) will dominate the Nuclear Overhauser Effect (NOE), and the magnetization vector will be aligned predominantly in the transverse

plane. For the opposite case, where the radio-frequency field is weaker than the adiabatic pulse, the NOE effects will dominate and lead to a bestriding longitudinal relaxation. An important difference between the NOE and ROE dipole-dipole cross relaxation, is that with NOE, the cross-peaks are generated due to cross relaxation between the z-magnetization of different spins, whereas ROESY cross peaks arise from cross relaxation of spin-locked magnetization. The cross-peaks in a ROESY experiment always have the same sign, regardless of the value of the correlation time, which is a huge advantage in looking for NOE enhancements in molecules whose correlational times make the conventional NOEs fall to zero or close to zero. In NOESY, the frequency labelled magnetization is rotated onto the z-axis, where cross relaxation takes place. The sign of the NOE enhancement (the cross relaxation rate constant σ_{IS} changes during the course of the NOESY experiment, in dependence of the correlation time. As the correlation time is increased, the cross relaxation rate constant σ_{IS} changes sign from positive to negative, meaning an alteration from a fast motion limit to a slow motion limit on the motional regime scale [9]. This changeover between fast and slow motion can be very informative when performing protein-ligand studies, as is the case for my diploma work. When a ligand, which exerts a fast motion, binds to a high-molecular-weight receptor like a protein, then the correlation time of the ligand is increased, as it mimics the motion of a high-molecular-weight compound, which he is complexed to. High-molecular-weight compounds move in the slow motional regime, and thus have longer correlation times, than the ligand itself. The latter fact, gives fundamental information about binding activities of ligands to, e.g. proteins, changing their characteristic motional traits that can be tracked.

A second very informative event within the pulse sequence of the AFP-NOESY experiment, is the effect of spin-diffusion. Normally, we expect our experiment to show a smooth transition from NOESY to ROESY until there is a cross-over point, where the latter two, σ_{NOE} and σ_{ROE} have opposite signs, and thus cancel one another out, with σ_{eff} becoming zero. However, the phenomenon of spin-diffusion can degrade the accuracy of these σ_{eff} measurements. In spin-diffusion, cross-peak intensity is transferred from spin A, via spin B, to spin C. An A,C cross-peak is observed, although the A,C distance is long. Such spin-diffusion-mediated cross-peaks can be recognized by a characteristic dependence of the cross-peak intensity on θ . Instead of the normal NOE-ROE cross-over, the intensities level off for higher values of θ , or become positive again. This attenuation is nicely depicted in the following Figure 6.1.

The adiabatic fast passage inversion pulse lasts for the period of the mixing time, which is . The pulse sequence follows with an EBURP excitation pulse, and a double watergate sequence for water suppression. The final detection in the transverse plane, is mediated by the 90° detection pulse. The pulse sequence is shown in Figure 6.2 below.

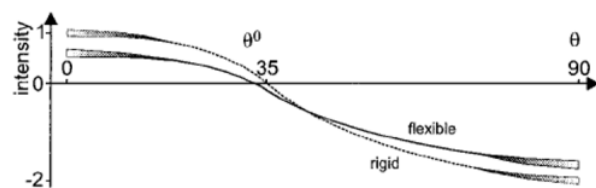


FIGURE 6.1: Ratio of NOESY and ROESY cross-peak intensities influenced by spin-diffusion.

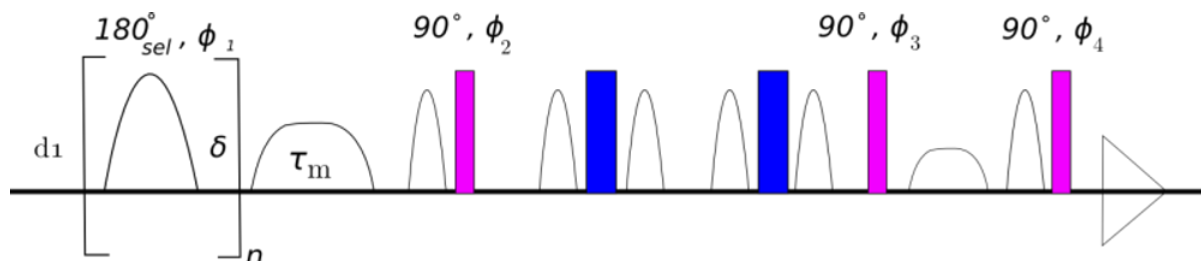


FIGURE 6.2: Pulse sequence of the AFP-NOESY Experiment. The pulse sequence starts with an 180° inversion pulse IBURP for magnetization transfer of M_z to M_{-z} , following an adiabatic frequency sweep lasting for a mixing time of 400 msec, and swept through a range of 13 values. Subsequently comes an EBURP pulse, and a double watergate of 500 s duration is applied. In the final part of the sequence, a 90° pulse is implemented, succeeding in a constant AFP pulse of 30 msec and ending with a 7 msec IBURP pulse with the final 90° detection pulse, bringing the magnetization into the transverse plane.

The experiment was set up to selectively irradiate one of the neighbouring proton resonances (Hx') of the AMP ligand on the ribose ring of the nucleoside, proximally to the $H1'$ nucleus. Following selective inversion by a shaped pulse, intramolecular NOEs are generated within the ligand molecule, generating cross-peaks which come in two different modes. The direct NOEs arise from enhancements between adjacent spins. Such enhancements, reflect the total relaxation of spin 1 that is assigned to the neighbouring spin 2 via cross relaxation. At the simplest level, the shorter the internuclear distance r_{12} , the larger the corresponding enhancement $f_1 S$ is likely to be. The second manifestation of cross-peaks come from indirect contributions, mediated by spin-diffusion. Spin-diffusion mediated cross-peaks originate from an extended spin-system, where NOE enhancements are observed between spins, that are not cross-relaxing one another. These relayed NOEs build up much more slowly than the direct ones. This is because the relayed NOEs require first that a normal direct NOE is generated, and then that this enhancement causes a second NOE to the remote spin. However, these transferred NOEs are dependent on the mixing time of the experiment, during which they have time to build up, which for rather modest mixing times, is often a bottleneck in the experimental setup. Yet, in the slow motion limit, the cross relaxation rate constant depends only on W_0 , which in turn depends on $j(0)$, where $j(0) = 2\tau_c$. As the correlation time gets longer and longer, the cross relaxation rate therefore also increases.

So, for large molecules with long correlation times, such as proteins, cross relaxation can be quite efficient. In such cases, relayed NOE enhancements can build up quickly, and indeed multiple relays along a chain of spins are also possible.

In Figure 6.4 we see the magnitude of the direct intramolecular NOE enhancements of the ligand AMP.

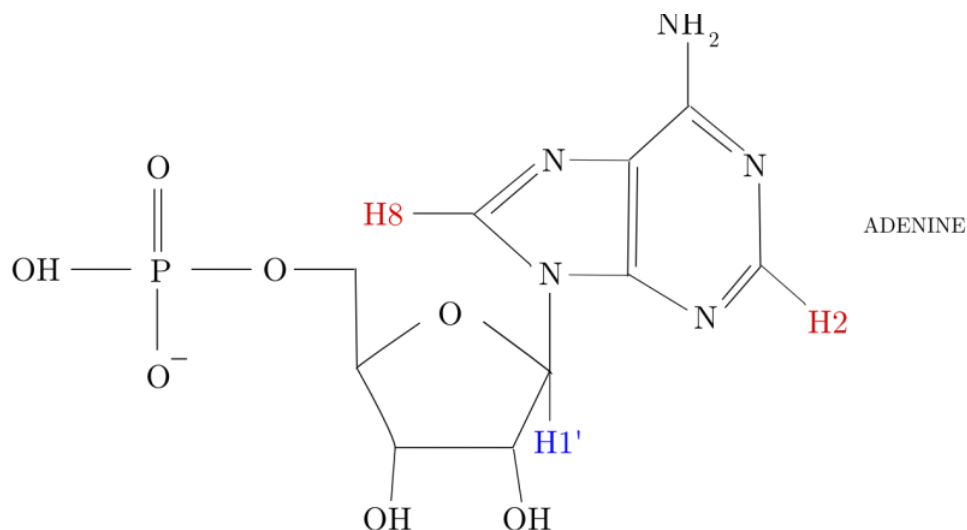


FIGURE 6.3: Adenosine Monophosphate Structure (AMP). The red labelled atoms, on the adenine depict the corresponding cross-peaks of the AFP-NOESY experiment, after irradiation of the Hx' proton resonance on the ribose ring of adenine, highlighted in blue.

The pivotal result gained from this experiment, in reference to the conformational assignment of AMP inside the binding pocket of ADH, was purveyed by a comparison of the different natures of the NOE build up curves of H8 and H2 resonances, shown in Figure 6.5. The H8 build up curve is characterized by the well-known direct NOE contribution via Hx', having a precise value for the zero-crossing θ at lower values of the effective field tilt angle θ , and exhibiting the sign change of the cross-relaxation rate constant as the correlation time increases. The sign changes from negative to positive values, as the ligand binds to the protein and starts to simulate the slow motion properties of high-molecular-weight compound. In comparison to the H8 cross-peak devolution, the H2 build up curve, which is dominated by the indirect spin-diffusion mediated NOE enhancements, does not show a well-defined zero-crossing, which levels off for higher values of the effective field tilt angle θ .

From these informations, we can imply that the H2 proton resonance, exhibiting the spin-diffusion is located on the inner side of the binding pocket, oriented towards the protein surface. This means, the NOE enhancement pathway for the H2 proton resonance starts off with the Hx' inversion, upon which cross-relaxation is propagated via spin-diffusion throughout the protein, finally generating a cross-peak on the H2 proton

resonance. For the second cross-peak on the H8 proton resonance, the NOE enhancement does not propagate throughout the protein, but rather emerges as a direct NOE from the inverted Hx' proton resonance. Thus we can assume, that there is no proximate contact between the surface of the protein and the ligand. The H8 proton is oriented towards the outside of the protein binding cavity, having contact with the surrounding environment. Figure 6.5 particularly exemplifies the difference in the course of the NOE development between the direct NOEs (the H8 proton resonance) and the indirect, spin-diffusion mediated NOE enhancement formation (the proton H2 resonance).

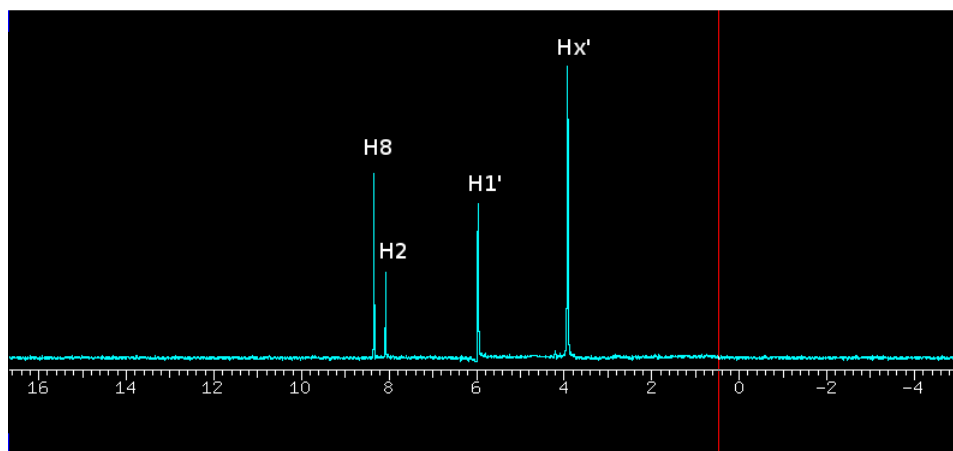


FIGURE 6.4: . AMP-ADH direct NOE enhancements of adenine protons H8, H2, and the H1' ribose proton after cross-relaxation from the Hx' proton on the ribose ring. The chemical shifts of the aforementioned resonances are, proceeding from left: H8 - 8.345 ppm, H2 - 8.083 ppm, H1' - 5.964 ppm and finally the selectively irradiated spin Hx' - 4.206 ppm.

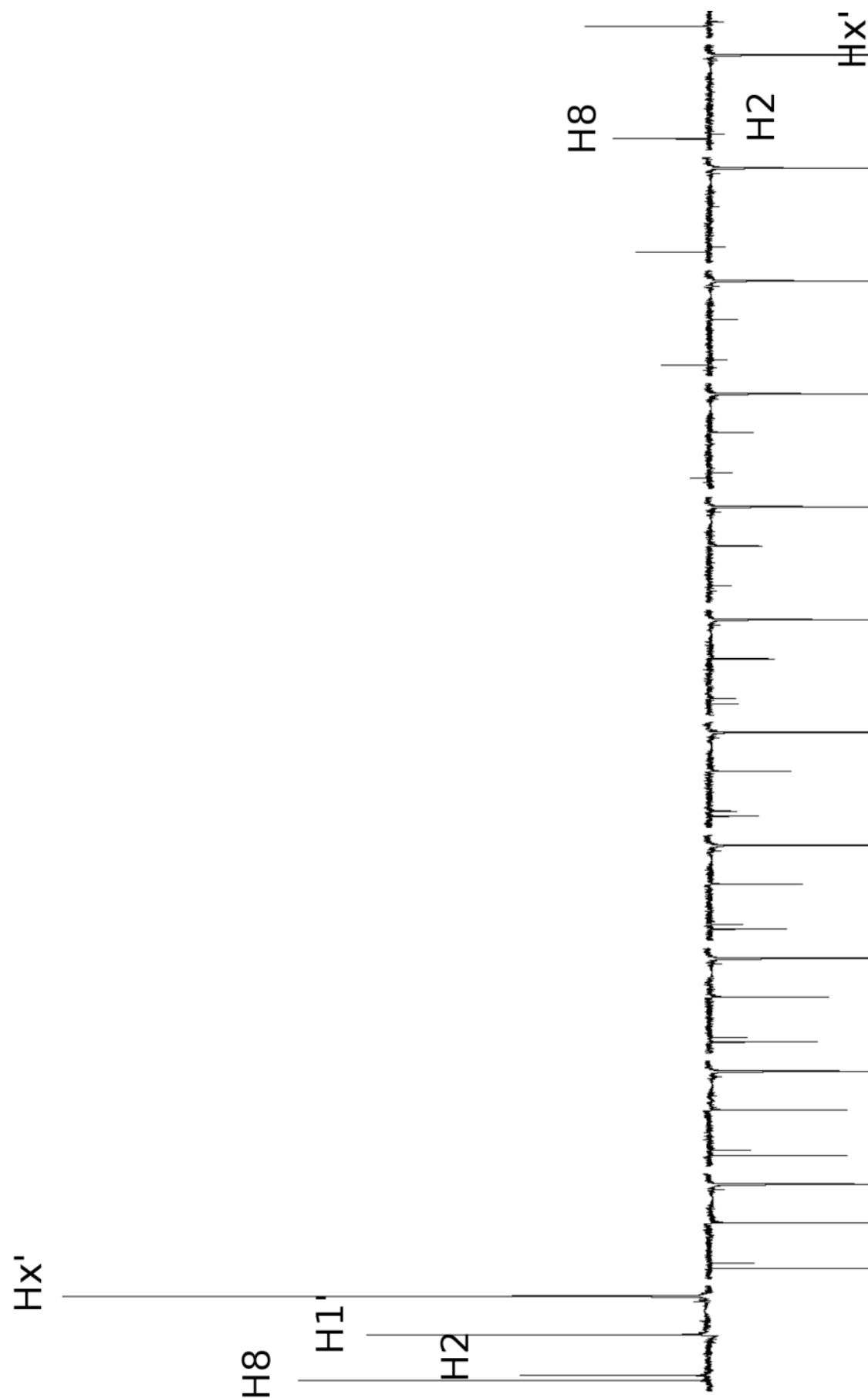
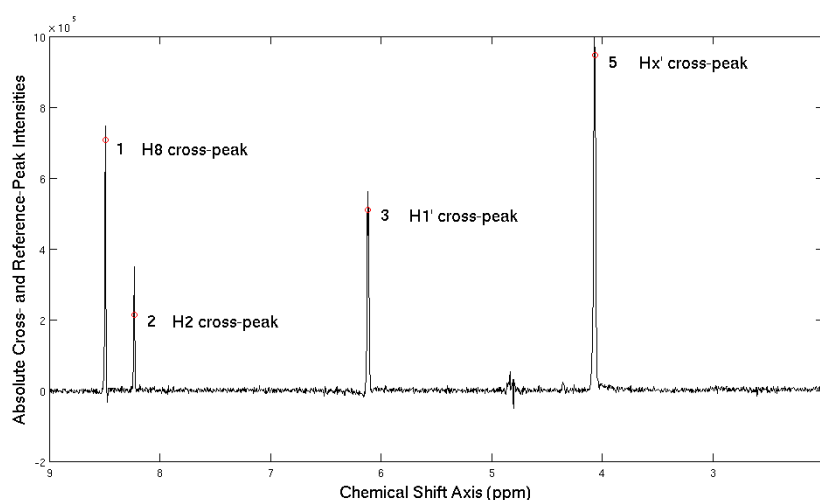
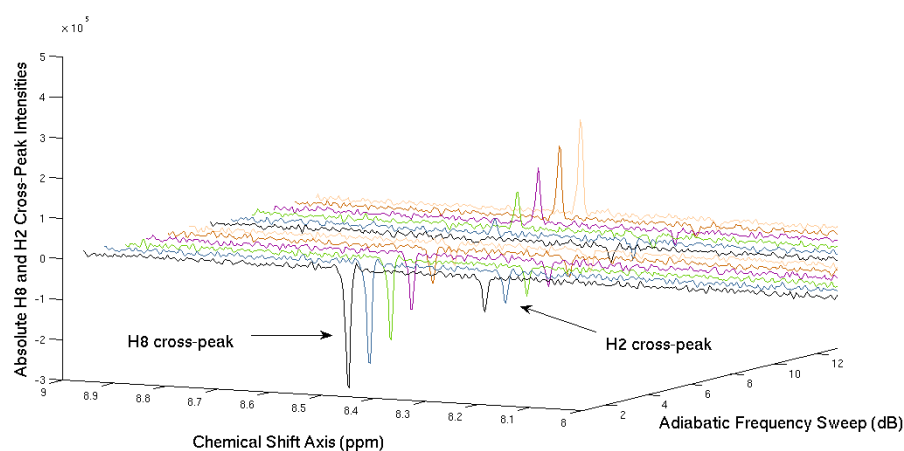


FIGURE 6.5: The H8 and H2 adenine proton resonance build up curves of the ligand AMP when complexed to ADH. The corresponding concentrations used in the experiment are 2 mM for the ligand, 10 μM for the protein.

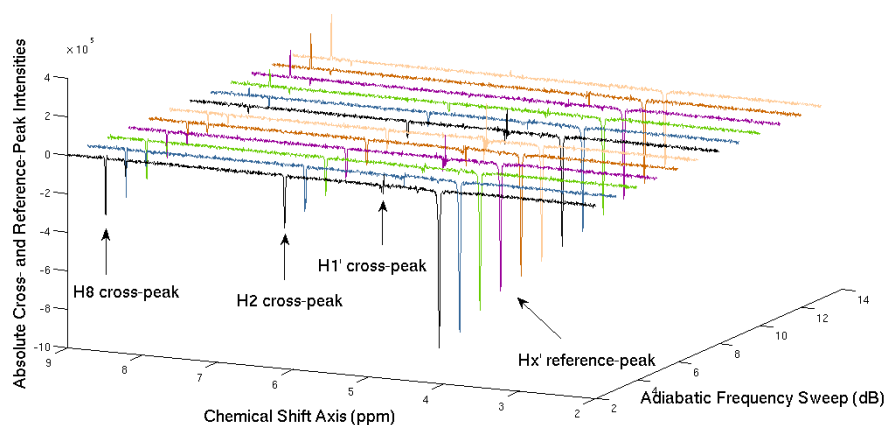
Furthermore, the next section depicts the evaluation of the H8 and H2 adenine NOE enhancements in the time course of the adiabatic frequency sweep, encompassing 13 values from -16 dB up to the eventual 35 dB. The representation in the Figures [6.6](#) and [6.7](#) were accomplished with MATLAB, a numerical calculation and simulation tool [\[10\]](#).



(a) Peak picking of H8 and H2 adenine proton resonances.

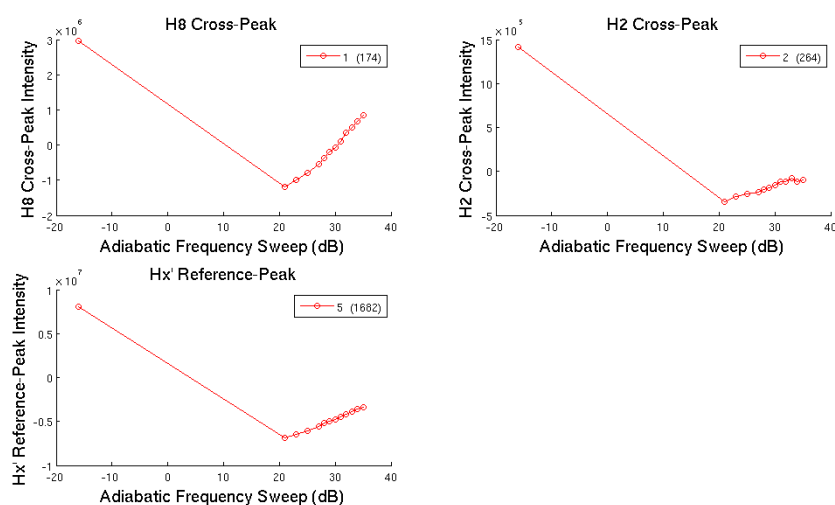


(b) H8 and H2 adenine NOE build up curves during an Adiabatic Frequency Sweep, ranging from -16 up to +35 dB.

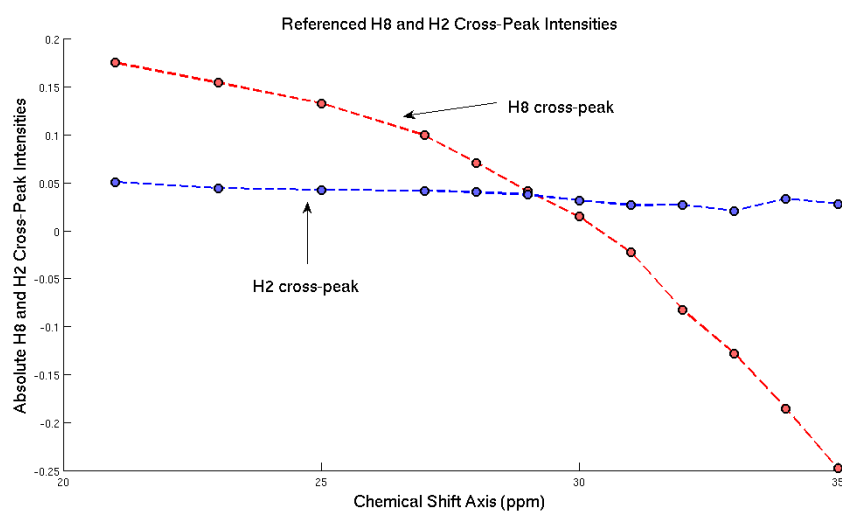


(c) H8, H2 adenine and H1'ribose NOE build up curves during an Adiabatic Frequency Sweep, ranging from -16 up to +35 dB.

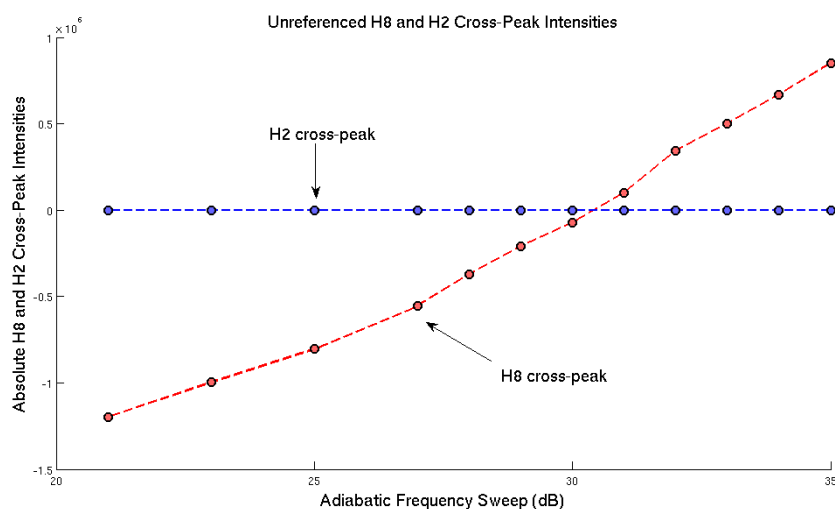
FIGURE 6.6: The H8 and H2 adenine NOE build up curves of 2mM AMP complexed with 10 μ M ADH.



(a) Cross-Peak (H8, H2) and Reference-Peak (Hx') Intensities during the Adiabatic Frequency Sweep.



(b) Referenced Cross-Peak Intensities (H8, H2).



(c) Unreferenced Cross-Peak Intensities (H8, H2).

FIGURE 6.7: The H8 and H2 NOE build up curves of 2mM AMP in complex with 10 μ M ADH.

6.1.2 The STD Experiment

As a comparative experiment, with the objective to confirm the accurateness of the conformational assignment predetermined by the AFP-NOESY experiment, the Saturation-Transfer-Difference method has been made use of. Saturation Transfer Difference (STD) Spectroscopy allows to detect transient binding of small molecule ligands to macromolecular receptors. Receptor species include proteins - free in solution or immobilized, whole virus particles. It is deployed to determine which part of the ligand molecule is responsible for binding, since most strongly interacting groups of the ligand will show a stronger STD effect. The method relies on selectively saturating protons of the macromolecular receptor by irradiating the spectral region containing "wings" of broad resonances of the macromolecule which is also free of any smaller molecule signals, at the chemical shift region around -1 ppm. Due to effective spin diffusion, saturation quickly propagates across the entire receptor. If the smaller molecule ligand binds the receptor, saturation will also spread onto the ligand. The result will be that intensity of the ligand signal will be attenuated. Substraction of the resulting spectrum from the reference spectrum, without saturation, yields the STD spectrum containing only signals of the binding ligands [19]. The pulse sequence of an STD experiment is shown in Figure 6.8.

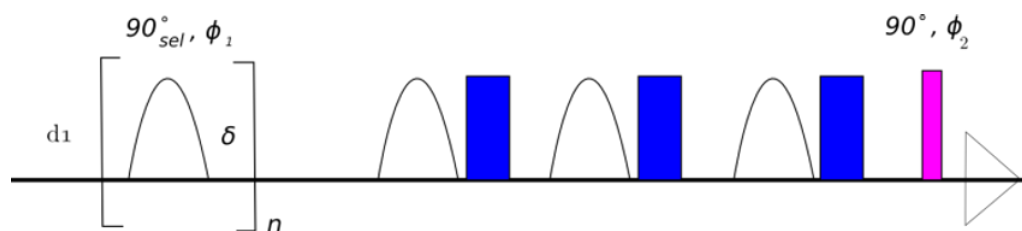


FIGURE 6.8: Pulse sequence of the STD Experiment. The sequence starts with an IBURP inversion pulse of 3.8 msec duration, repeated within 750 cycles. What follows is an EBURP excitation pulse and a double watergate, which lasts 4 msec. The difference experiment is achieved by 2 separate spectra, one at the spectral region of about -1 ppm, where only protein resonances are irradiated, called the "On-Resonance-Spectrum" and a consecutive "Off-Resonance-Spectrum" at an offset jump of 100 ppm in the downfield direction, ending up at +99 ppm. The detection is in the transverse plane, observing the enhancement of the intermolecular NOEs of ligand resonances that are in direct contact with the protein.

The outcome of the STD experiment is illustrated in Figure 6.9 below. It approves AMP as a binding component of ADH, as indicated by the intermolecular spin-diffusion mediated NOEs, measured on the free ligand in solution.

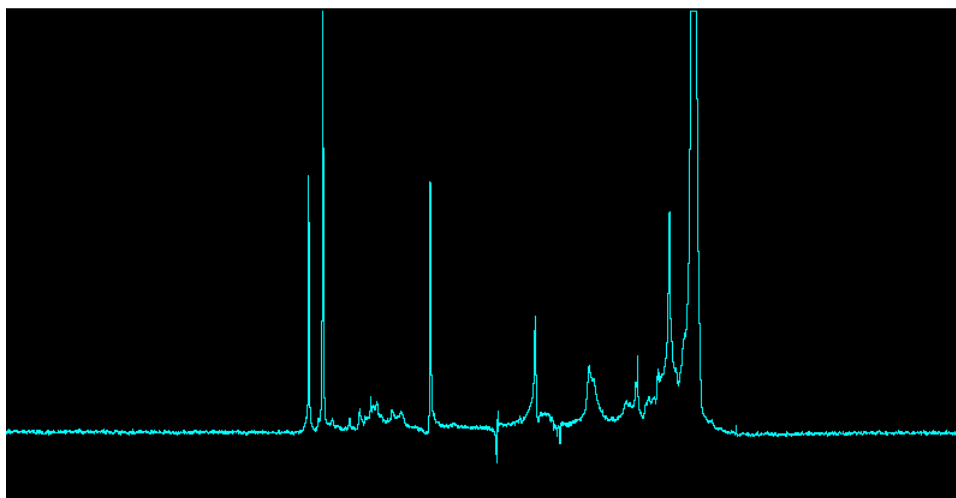


FIGURE 6.9: The STD experiment of AMP bound to ADH. The corresponding concentrations used for the ligand is 2 mM and 10 μ M for the protein.

6.2 Nicotineamide Adenine Dinucleotide in complex with Alcohol Dehydrogenase

Nicotinamide adenine dinucleotide, abbreviated NAD⁺, is a coenzyme found in all living cells. The compound is a dinucleotide, since it consists of two nucleotides joined through their phosphate groups, with one nucleotide containing an adenine base and the other containing nicotinamide. In metabolism, NAD⁺ is involved in redox reactions, carrying electrons from one reaction to another. The coenzyme is therefore found in two forms in cells: NAD⁺ is an oxidizing agent it accepts electrons from other molecules and becomes reduced, this reaction forms NADH, which can then be used as a reducing agent to donate electrons. These electron transfer reactions are the main function of NAD⁺. However, it is also used in other cellular processes, notably as a substrate of enzymes that add or remove chemical groups from proteins, in posttranslational modifications. Due to the importance of these functions, the enzymes involved in NAD⁺ metabolism are targets for drug discovery [16][18]

In this section I investigate a comparative AFP-NOESY and STD experiment to the ones performed on the AMP-ADH complex. The consideration behind this experimental setup, is to compare or more precisely, to affirm a related conformational configuration of the two ligands AMP and NAD inside the binding cavity of one and the same enzyme ADH. To approve this presumption, the very same experiments, namely AFP-NOESY and STD were carried out with the NAD-ADH complex, as described in the next section.

6.2.1 The AFP-NOESY-Experiment

The identical AFP-NOESY experiment has been carried out with the difference of replacing the ligand by Nicotineamide Adenine Dinucleotide (NAD), which shares, from its structural point of view, many similarities with AMP. The only difference is that it interchanges the base of an additional nucleoside for nicotinamide instead of adenine, which is the case for AMP. So, based on this fact, NAD is an outstanding ligand for safeguarding our hypothesis on the epitope mapping sites of AMP when complexed to ADH. What the result of the experiment exemplifies, is a crucial implication, namely that the two ligands AMP and NAD exhibit the same characteristics of the NOE build up curves of H8 and H2 proton resonances on the adenine nucleoside, meaning they share the same conformational orientation of this nucleoside in the binding pocket of ADH. As exemplified in Figures 6.14 and 6.13. In both cases the H8 resonances are represented by a stronger direct NOE, which is apparent from the earlier NOE/ROE cross-over point, and the sign changeover of the NOE intensities. The H2 resonance, is dominated by the phenomenon of spin-diffusion, which as a result leads to the NOE intensities leveling-off for higher values of the effective tilt angle θ , respectively not exhibiting a sign changeover. The direct NOE intensities are depicted in Figure 6.10 below.

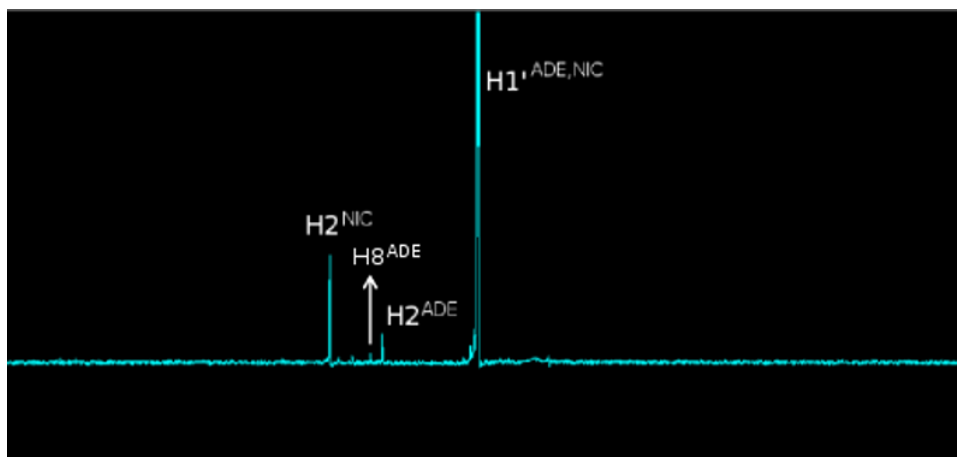


FIGURE 6.10: NAD-ADH direct NOE enhancements of adenine protons H8, H2 and nicotineamide H2 proton after cross-relaxation from the ribose proton $H1'$ of adenine and nicotineamide simultaneously. The chemical shifts of the aforementioned resonances are, proceeding from left: $H2^{NIC}$ - 8.26 ppm, $H8^{ADE}$ - 7.99 ppm, $H2^{ADE}$ - 8.21 ppm, and finally the selectively irradiated spin $H1'^{ADE,NIC}$ - 5.92 ppm.

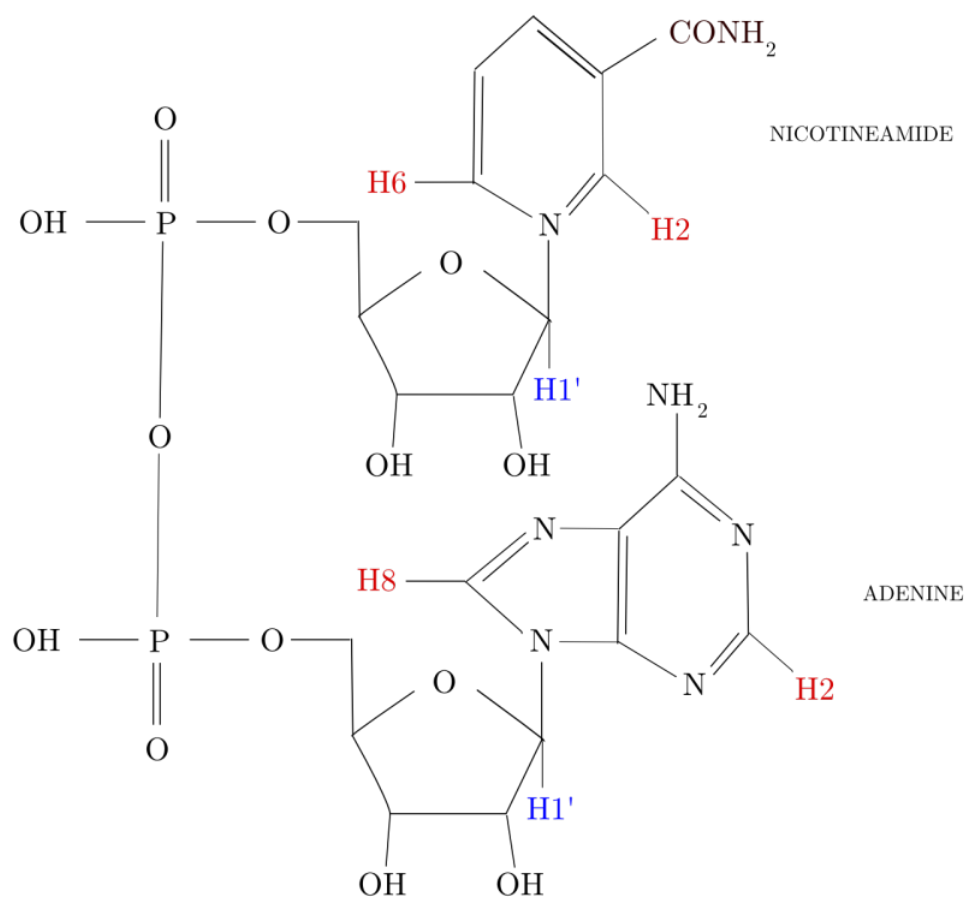


FIGURE 6.11: Nicotinic Acid Adenine Dinucleotide (NAD) Structure. The red labelled atoms on the nicotinic acid - $H2^{NIC}$, $H6^{NIC}$, and $H2^{ADE}$, $H8^{ADE}$ on the adenine, depict the corresponding cross-peaks of the AFP-NOESY experiment, after irradiation of the $H1'^{ADE,NIC}$ proton resonances, highlighted in blue, on the adenine and nicotinic acid, simultaneously.

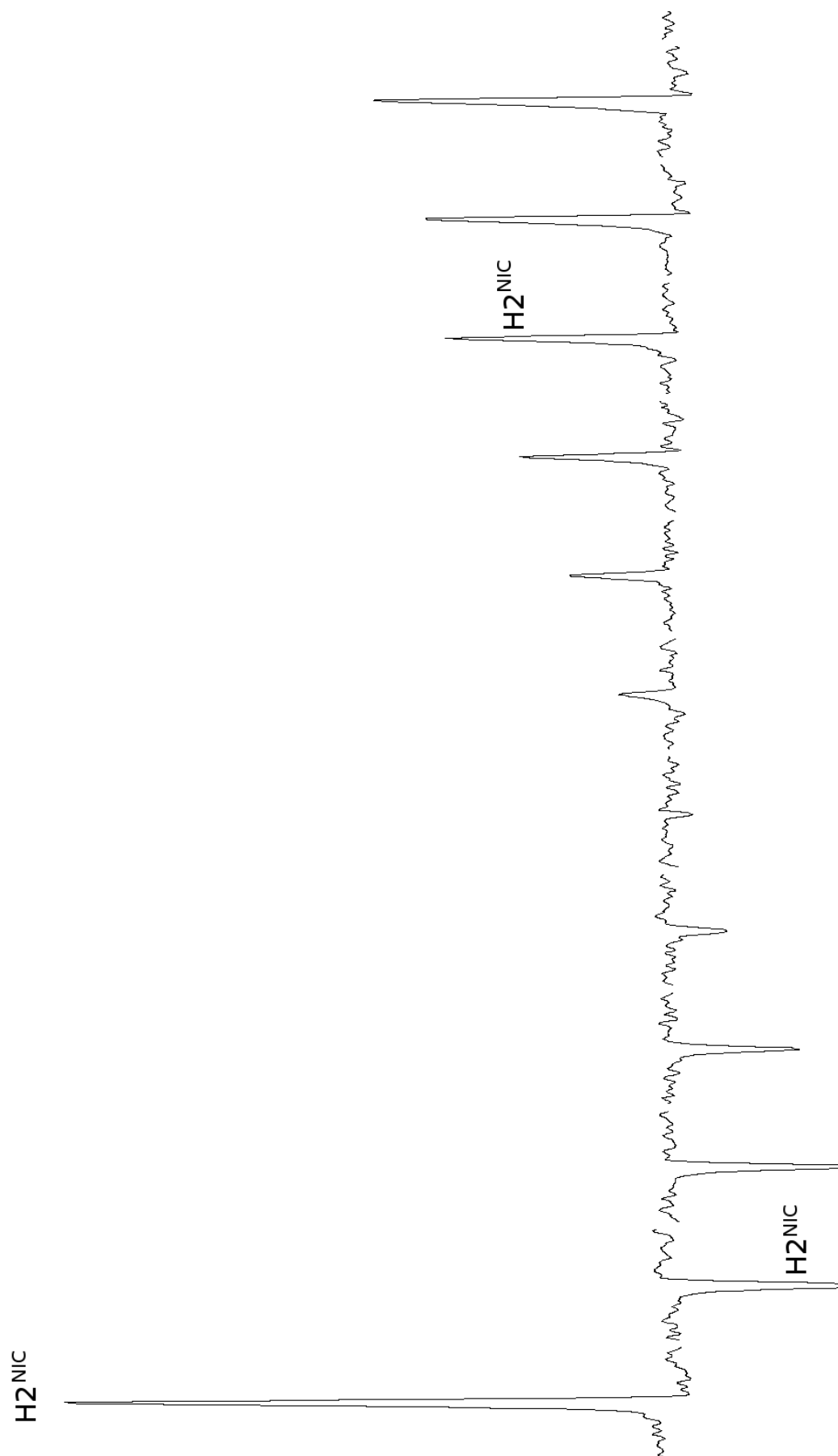


FIGURE 6.12: The H2^{NIC} proton resonance build up curves of the ligand NAD when complexed to ADH. The corresponding concentrations used are 2 mM for the ligand, 10 μM for the protein.

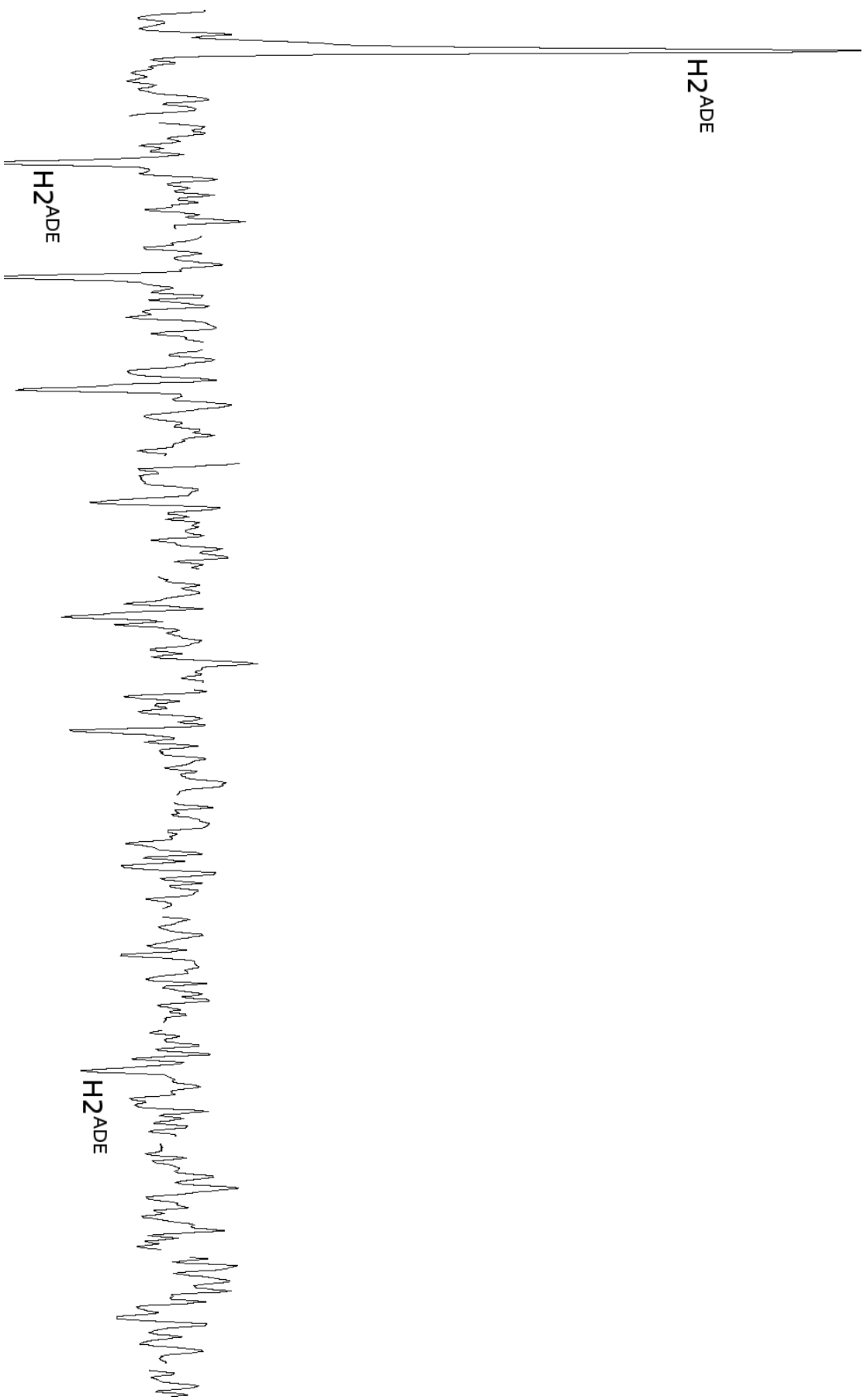


FIGURE 6.13: The H_{2}^{ADE} proton resonance build up curves of the ligand NAD when complexed to ADH. The corresponding concentrations used are 2 mM for the ligand, 10 μ M for the protein.

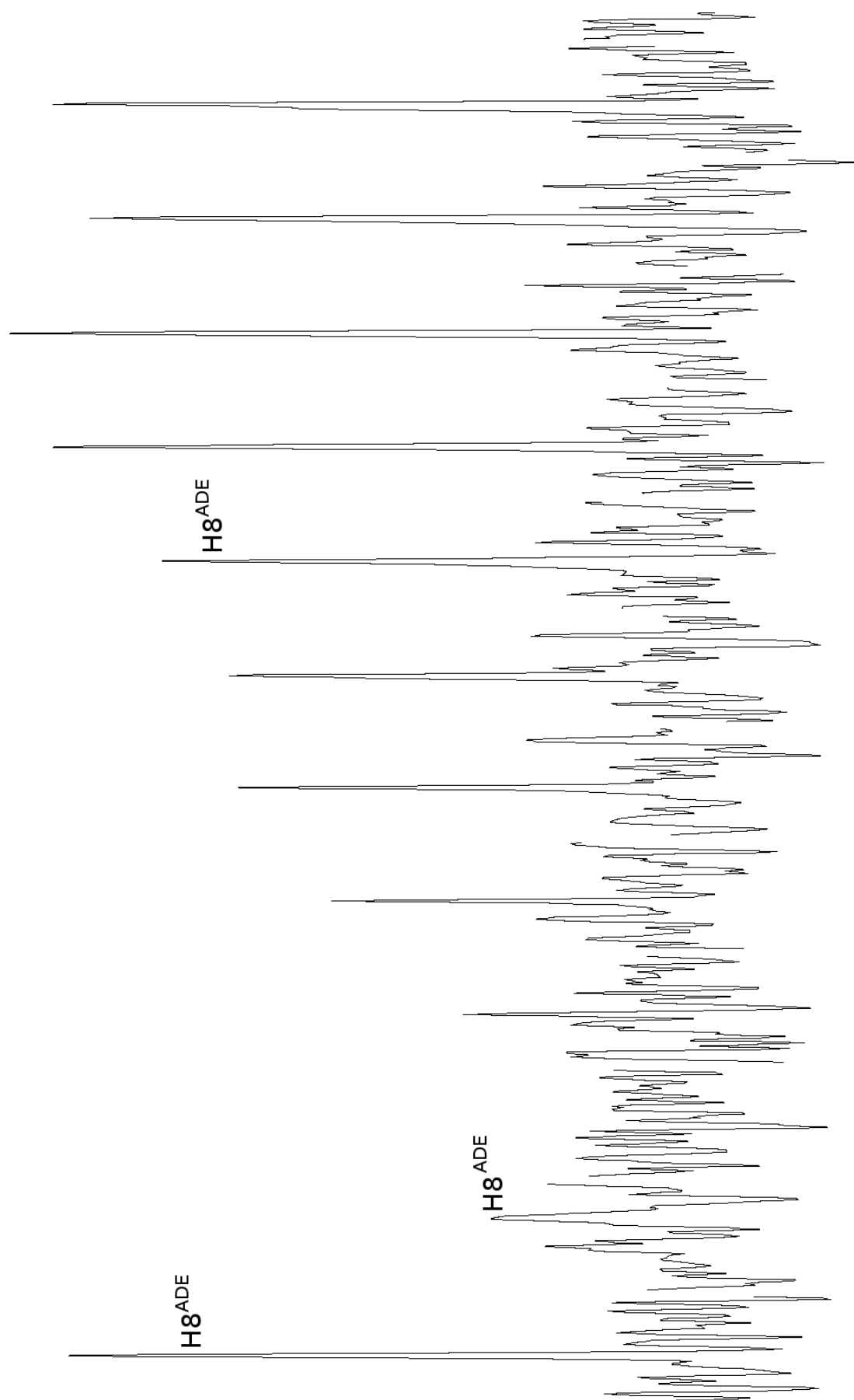


FIGURE 6.14: The $H8^{ADE}$ proton resonance build up curves of the ligand NAD when complexed to ADH. The corresponding concentrations used are 2 mM for the ligand, 10 μ M for the protein.

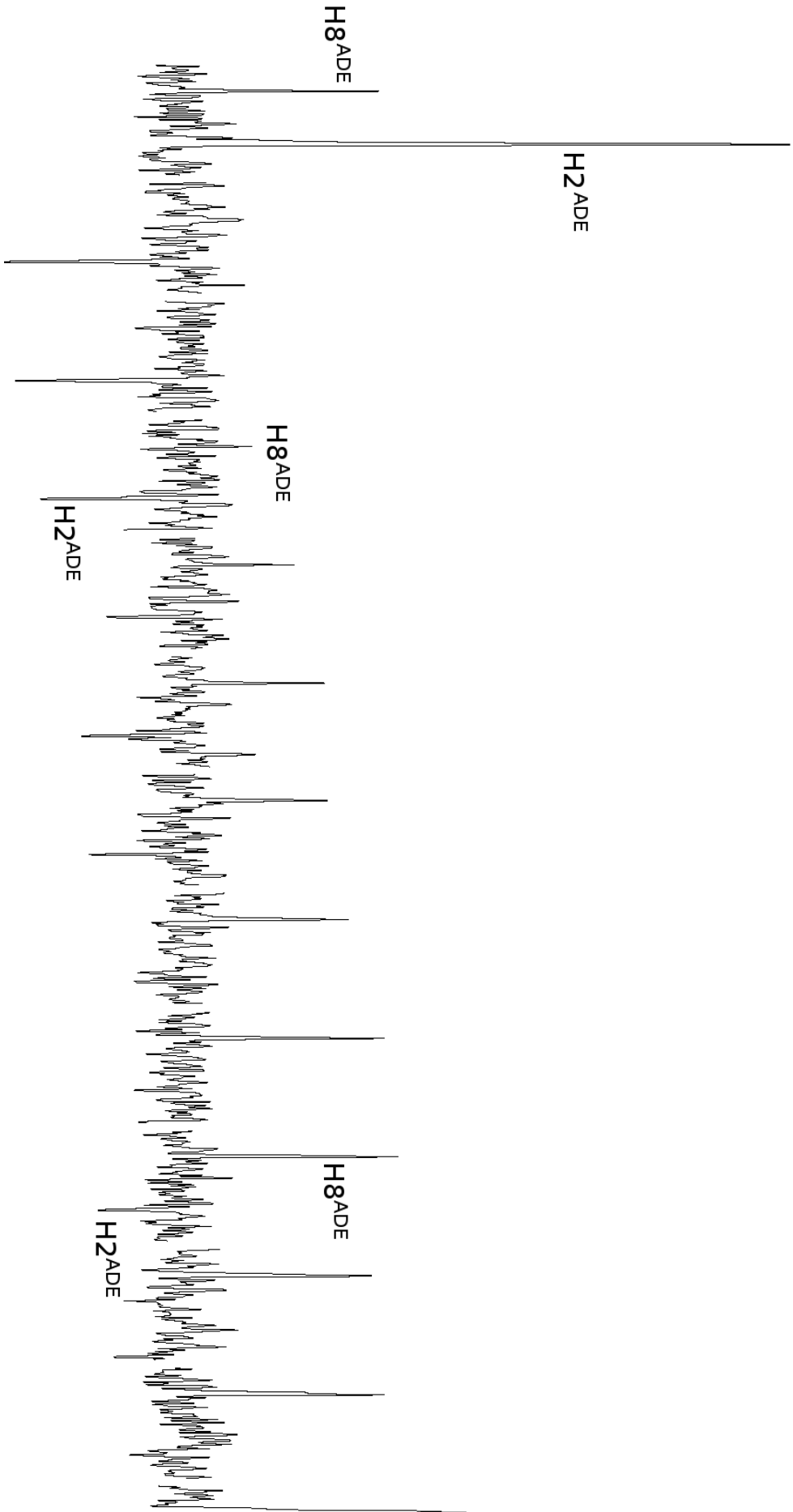
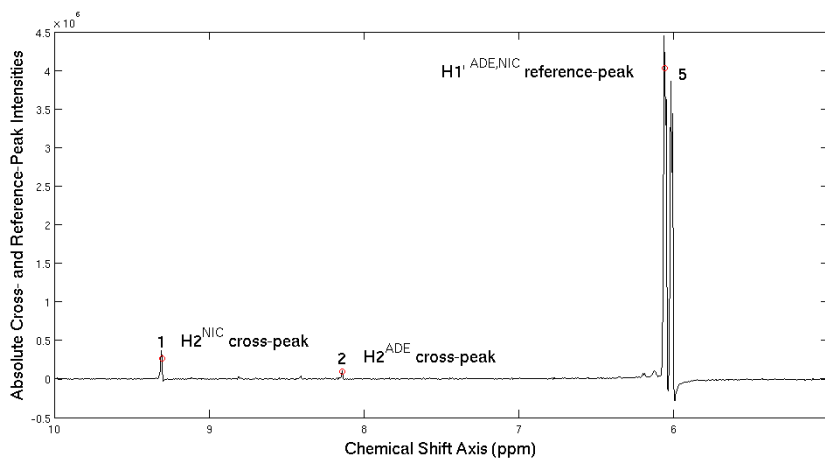
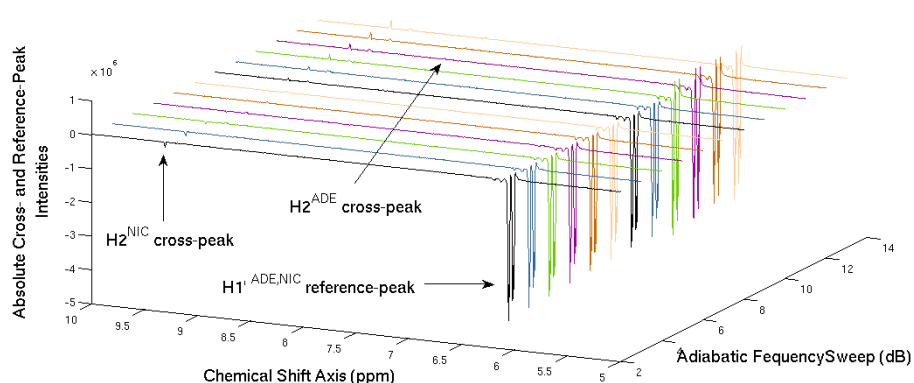


FIGURE 6.15: The H8^{ADE} and H2^{ADE} proton resonance build up curves of the ligand NAD when complexed to ADH. The corresponding concentrations used are 2 mM for the ligand, 10 μM for the protein.

The next section depicts the evaluation of the H2 nicotineamide and H2 adenine NOE enhancements in the time course of the adiabatic frequency sweep, encompassing 13 values from -16 dB up to the eventual 35 dB. The representation in the Figures 6.16 and 6.17 were accomplished with MATLAB, a numerical calculation and simulation tool [10].

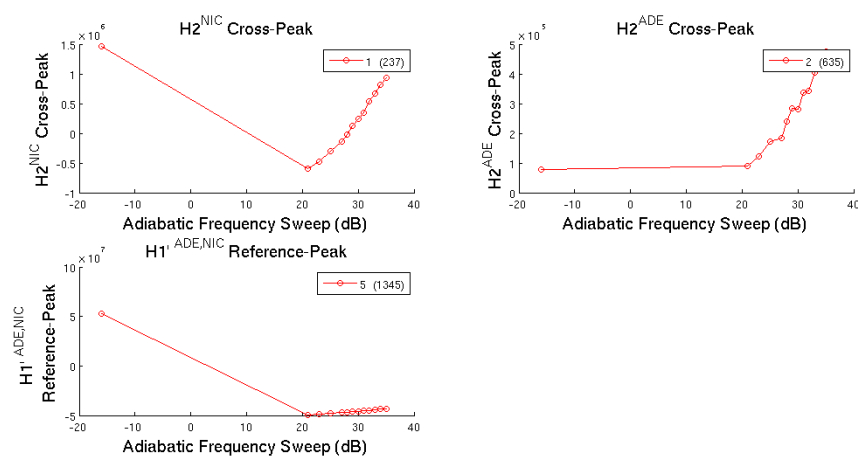


(a) Peak picking of H2 adenine and H2 nicotineamide proton resonances.

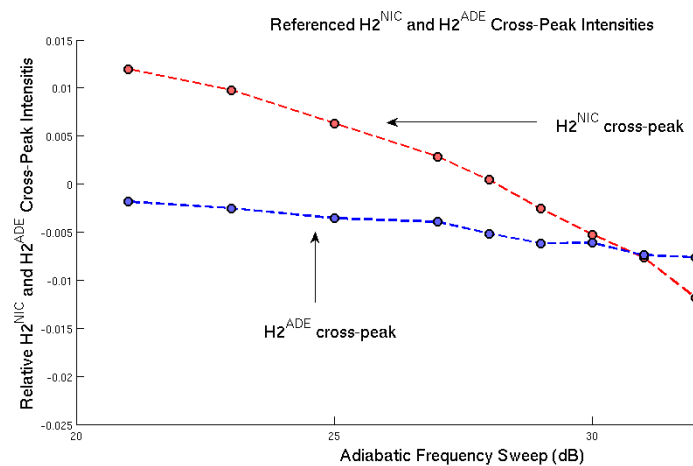


(b) H2 adenine and H2 nicotineamide NOE build up curves during an Adiabatic Frequency Sweep, ranging from -16 up to +35 dB.

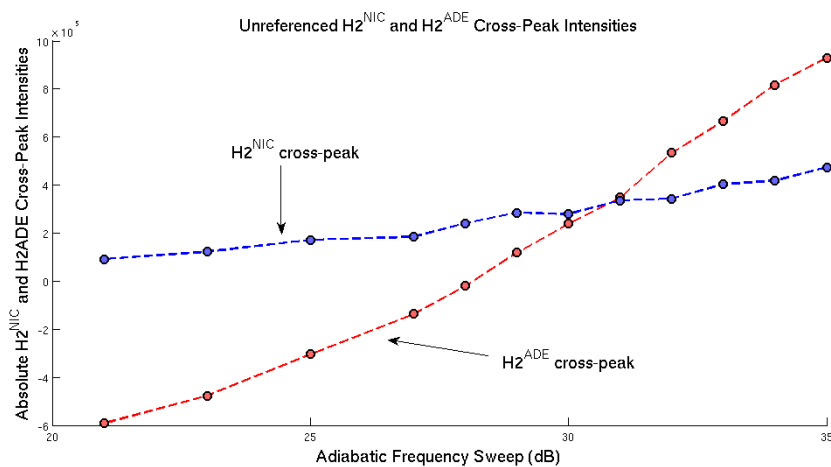
FIGURE 6.16: The $H2^{NIC}$ and $H2^{ADE}$ NOE build up curves of 2mM NAD in complex with 10 uM ADH.



(a) Cross-Peak ($H2^{NIC}$, $H2^{ADE}$) and Reference-Peak ($H1^{ADE,NIC}$) Intensities during Adiabatic Frequency Sweep.



(b) Referenced Cross-Peak Intensities ($H2^{NIC}$, $H2^{ADE}$).



(c) Unreferenced Cross-Peak Intensities ($H2^{NIC}$, $H2^{ADE}$).

FIGURE 6.17: The $H2^{NIC}$ and $H2^{ADE}$ NOE build up curves of 2mM NAD in complex with 10 μ M ADH.

Figure 6.18 was accomplished with a molecular graphics and modelling program, Pymol, and illustrates the situation of NADP(H) bound to *Saccharomyces Cerevisiae* Alcohol Dehydrogenase in the three-dimensional environment. What can be seen is the different surroundings of the H8 and H2 proton resonances from the adenine nucleoside of NADP. H2 is oriented towards the inner surface of the protein ADH, whereby encircled entirely by hydrophobic apolar amino acid residues, like Valine and Isoleucine, as shown in Figure 6.18, which lack the potential of chemical exchange, and thus allow for a spin-diffusion mediated NOE enhancement via protons of the protein. This means, that the H2 proton must be in close contact to the protein surface. On the contrary, the H8 proton resonance is solely enclosed by hydrophilic polar amino acids, like Serines, which are prone to chemical exchange of ^1H protons for deuterized ^2H heavy hydrogens at sites of hydroxyl and amine functional groups and thus prevent the indirect spin-diffusion mediated NOE transfer. Instead, the H8 proton from the adenine exhibits a stronger direct NOE and is oriented away from the protein surface. The H2 proton resonance from the nicotineamide, is located loosely inside a pocket, as the zinc-binding site is empty, most probably occupied by water molecules. Since the H2 proton is motile inside the pocket, it reveals an elevated flexibility.



FIGURE 6.18: . NADP(H)-dependent Cinnamyl Alcohol Dehydrogenase from *Saccharomyces Cerevisiae*. NADP(H) is shown as a stick scaffold, with nitrogen atoms marked blue, oxygen atoms marked red. The regions coulored in magenta display the amino acid residues Valine 186, (VAL186), Valine 249, (VAL249), Valine 208, (VAL208), Isoleucine 209 (ILE209), Isoleucine 257 (ILE257), and Arginine 211 (ARG211). Regions coloured in cyan display the amino acid residues Serine 252, (SER252) and Serine 253 (SER253).

6.3 Vanillic Acid in complex with Lipocalin (Q83)

Vanillic acid, or 4-hydroxy-3-methoxybenzoic acid, is a benzoic acid derivative used as a flavoring agent. It is an oxidized form of vanillin. It is also an intermediate in the production of vanillin from ferulic acid [14].

Lipocaline, or Q83, belongs to a family of proteins which transport small hydrophobic molecules such as steroids, bilins, retinoids, and lipids. They share limited regions of sequence homology and a common tertiary structure architecture. This is an eight stranded antiparallel beta-barrel with a repeated + 1 topology enclosing an internal ligand binding site. These proteins are found in gram negative bacteria, vertebrate cells, and invertebrate cells, and in plants. Lipocalins have been associated with many biological processes, among them immune response, pheromone transport, biological prostaglandin synthesis, retinoid binding, and cancer cell interactions [17]. From a physical point of view, Lipocalin is a low-molecular-weight protein with approximately 160-180 amino acid residues.

6.3.1 The AFP-NOESY-Experiment

In the following section I elucidate the experimental setup of the AFP-NOESY experiment for the complex Vanillic Acid-Lipocalin (Q83). The aim of the experiment was to investigate the sensitivity of the ligand binding when successively increasing the protein concentration. For this purpose we selectively irradiate the 3-methoxy group of the vanillic acid, which builds intramolecular NOE enhancements on the neighbouring 2-H proton resonance. This NOE effect is enhanced when the protein concentration starts to increase. Thus this intramolecular NOE enhancement is directly proportional to the molecular-weight of the protein-ligand complex. A second considerable criterion is the sign changeover, characterizing the motional regime change from fast to slow, thus from a free ligand to the bound form. From the results, in Figures 6.20(b) till Figure 6.22(c) as well as in the array screenshots in Figure 6.24 up to Figure 6.31, one can see that the cross-over point is reached at the protein concentration of 60 μ M.

The next section depicts the evaluation of the 2-H NOE enhancements in the time course of the adiabatic frequency sweep, encompassing 13 values from -16 dB up to the eventual 35 dB. The representation in the Figure 6.32 till Figure 6.47 was accomplished with MATLAB, a numerical calculation and simulation tool [10].

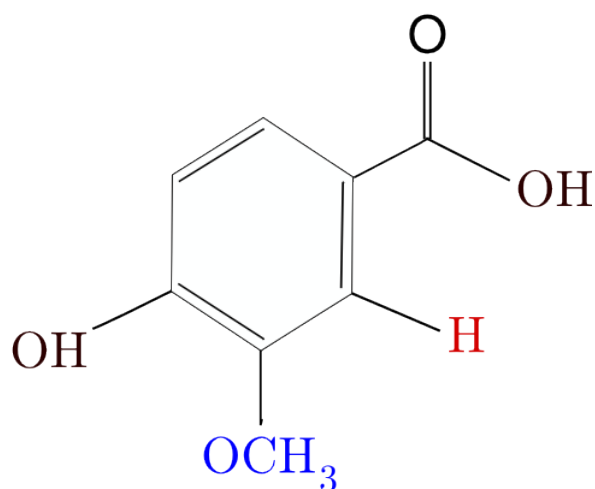
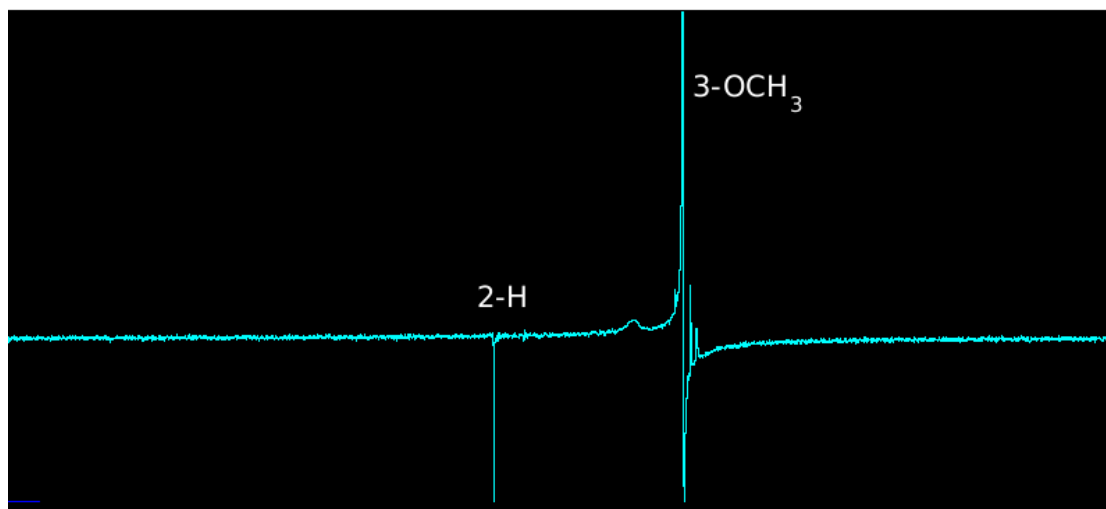
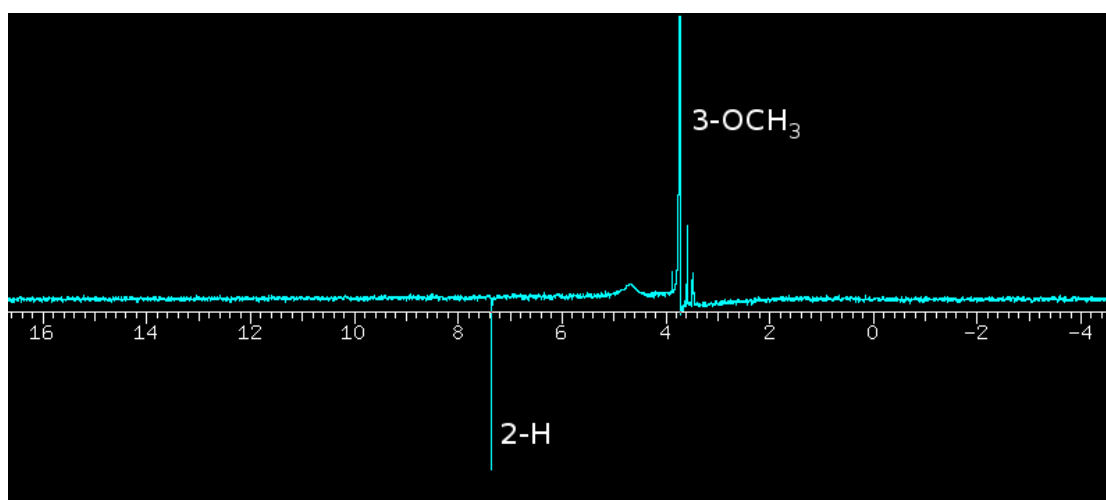


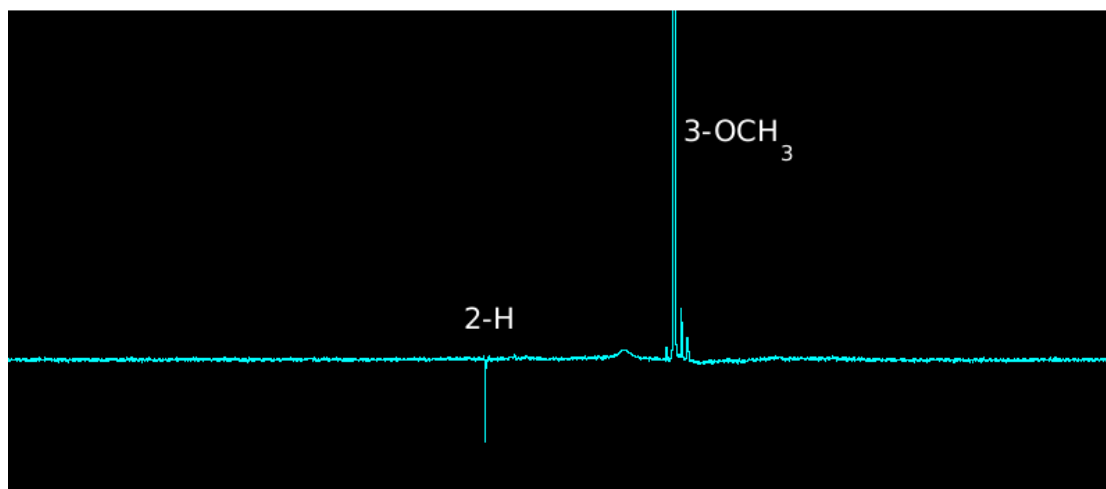
FIGURE 6.19: Vanillic Acid Structure. The red labelled atom, 2-H on the benzene ring, depict the corresponding cross-peaks of the AFP-NOESY experiment, after irradiation of the 3-methoxy (3 – *OCH*3) proton resonance on the benzene ring, highlighted in blue.



(a) Reference spectrum of vanillic acid at a concentration of 2 mM.

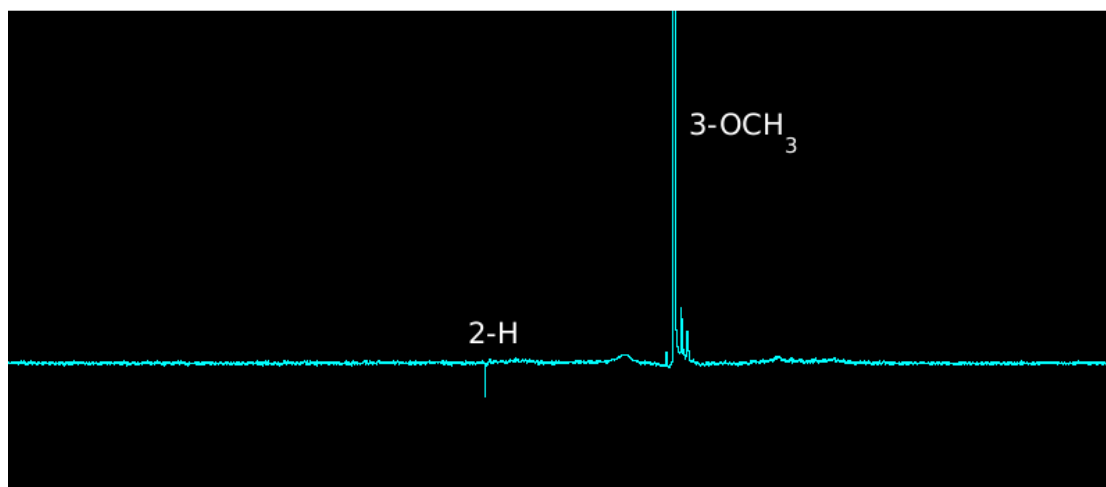


(b) 2-H direct intramolecular vanillic acid NOE enhancements after irradiation of 3-OCH₃, at a Lipocalin concentration of 10 uM.

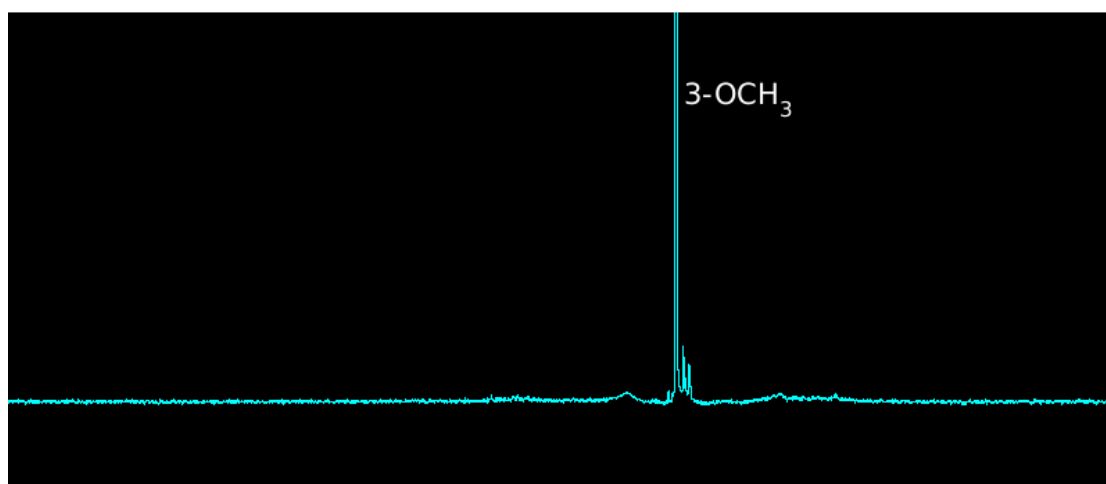


(c) 2-H direct intramolecular vanillic acid NOE enhancements after irradiation of 3-OCH₃, at a Lipocalin concentration of 20 uM.

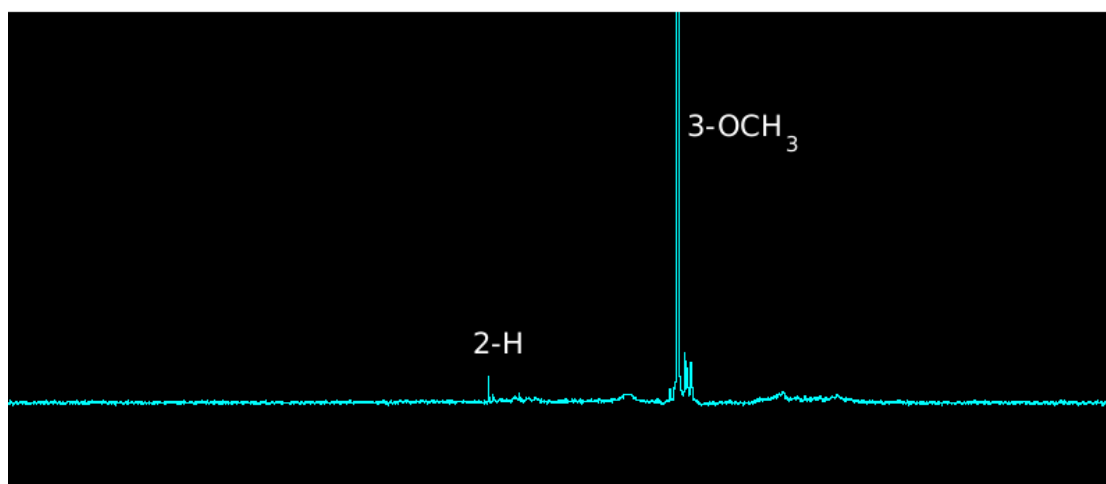
FIGURE 6.20: 2-H direct intramolecular vanillic acid NOE enhancements after irradiation of 3-OCH₃.



(a) 2-H direct intramolecular vanillic acid NOE enhancements after irradiation of 3-OCH₃, at a Lipocalin concentration of 30 uM.

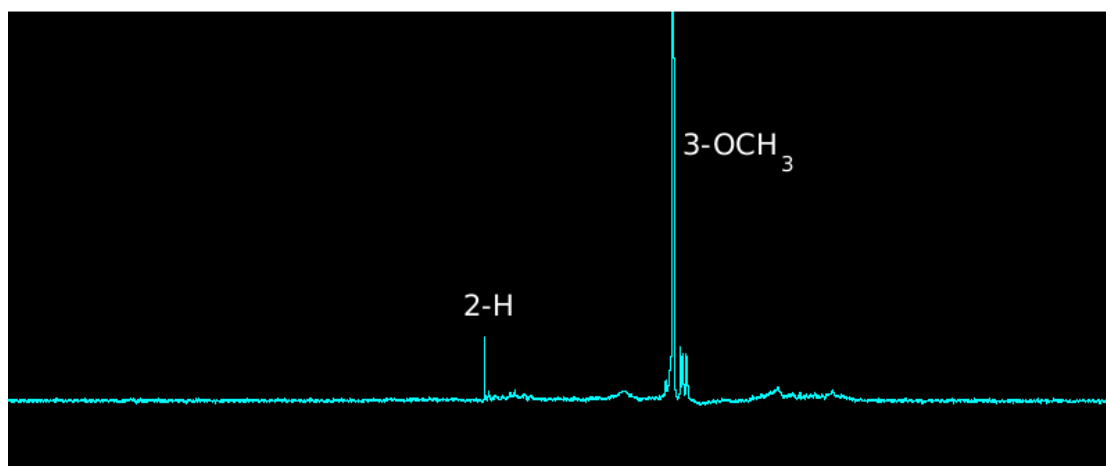


(b) 2-H direct intramolecular vanillic acid NOE enhancements after irradiation of 3-OCH₃, at a Lipocalin concentration of 40 uM.

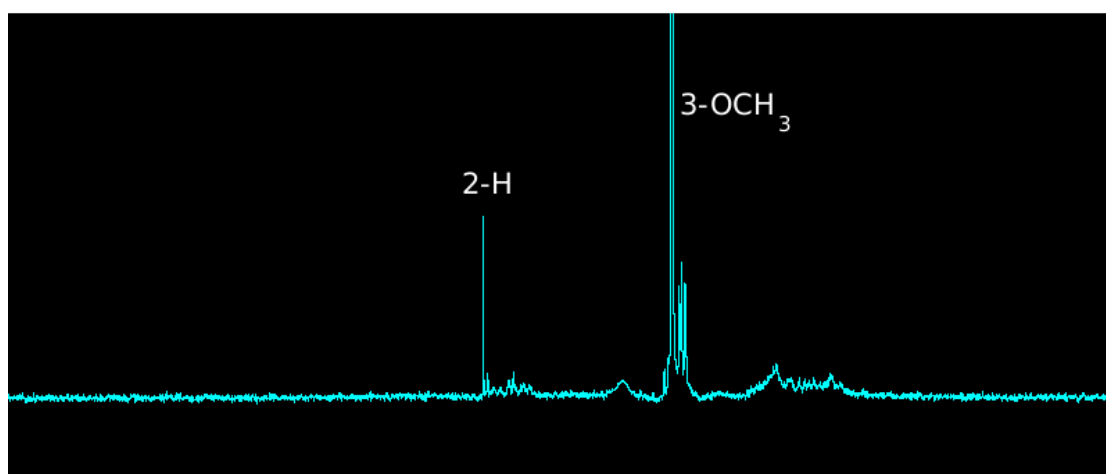


(c) 2-H direct intramolecular vanillic acid NOE enhancements after irradiation of 3-OCH₃, at a Lipocalin concentration of 50 uM.

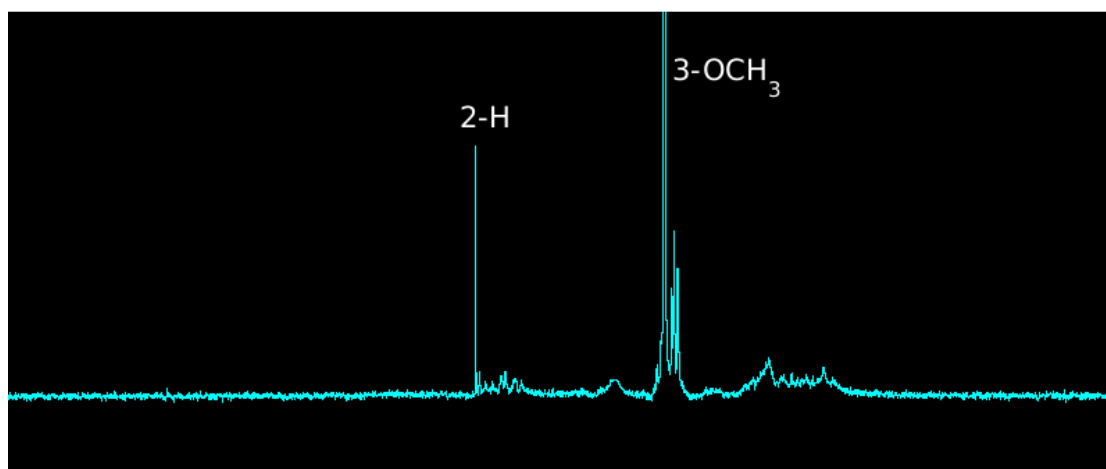
FIGURE 6.21: 2-H direct intramolecular vanillic acid NOE enhancements after irradiation of 3-OCH₃.



(a) 2-H direct intramolecular vanillic acid NOE enhancements after irradiation of 3-OCH₃, at a Lipocalin concentration of 60 μ M.



(b) 2-H direct intramolecular vanillic acid NOE enhancements after irradiation of 3-OCH₃, at a Lipocalin concentration of 70 μ M.



(c) 2-H direct intramolecular vanillic acid NOE enhancements after irradiation of 3-OCH₃, at a Lipocalin concentration of 80 μ M.

FIGURE 6.22: 2-H direct intramolecular vanillic acid NOE enhancements after irradiation of 3-OCH₃.

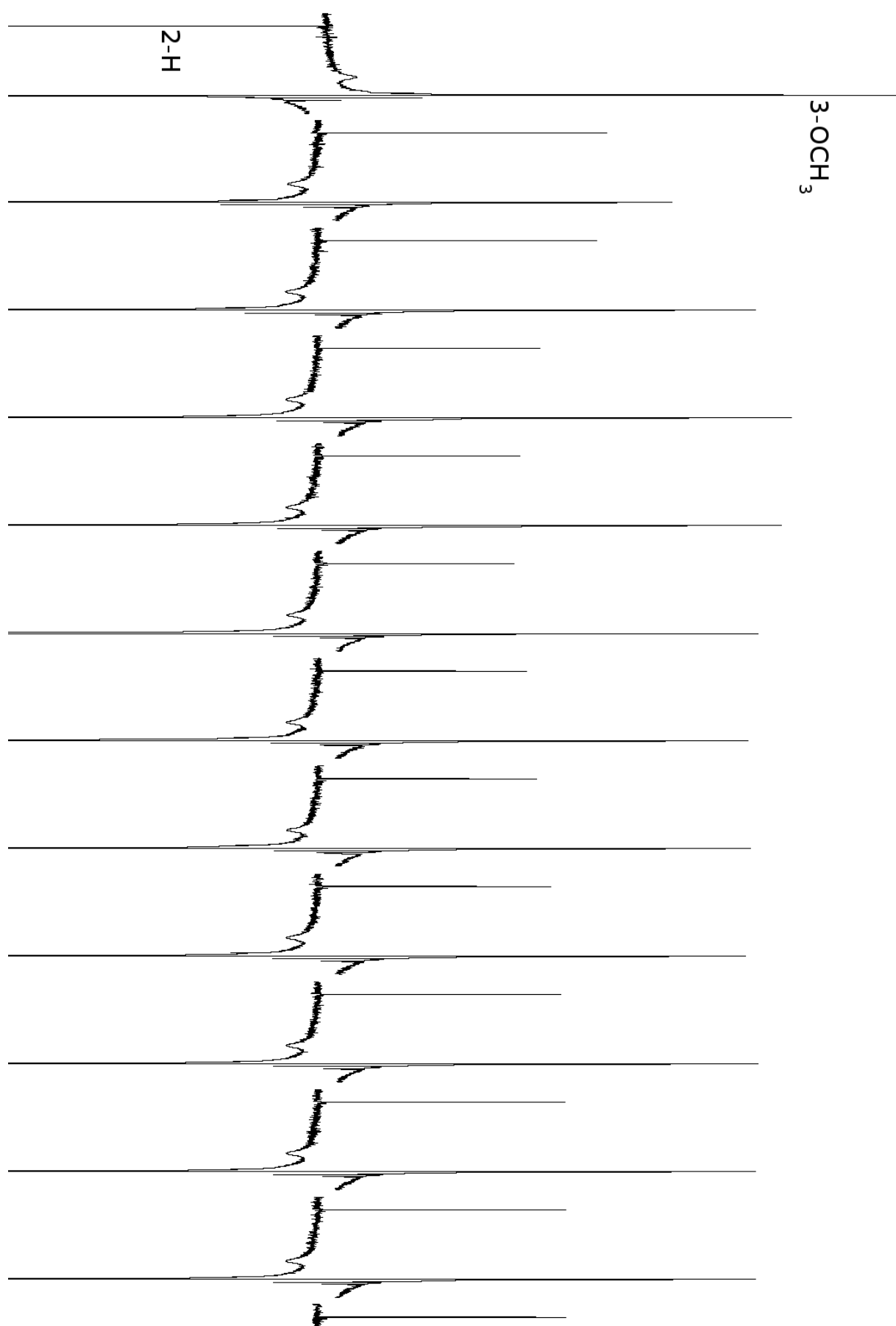


FIGURE 6.23: The 2-H intramolecular NOEbuild up curves of vanillic acid, measured at the corresponding concentrations: 2 mM ligand.

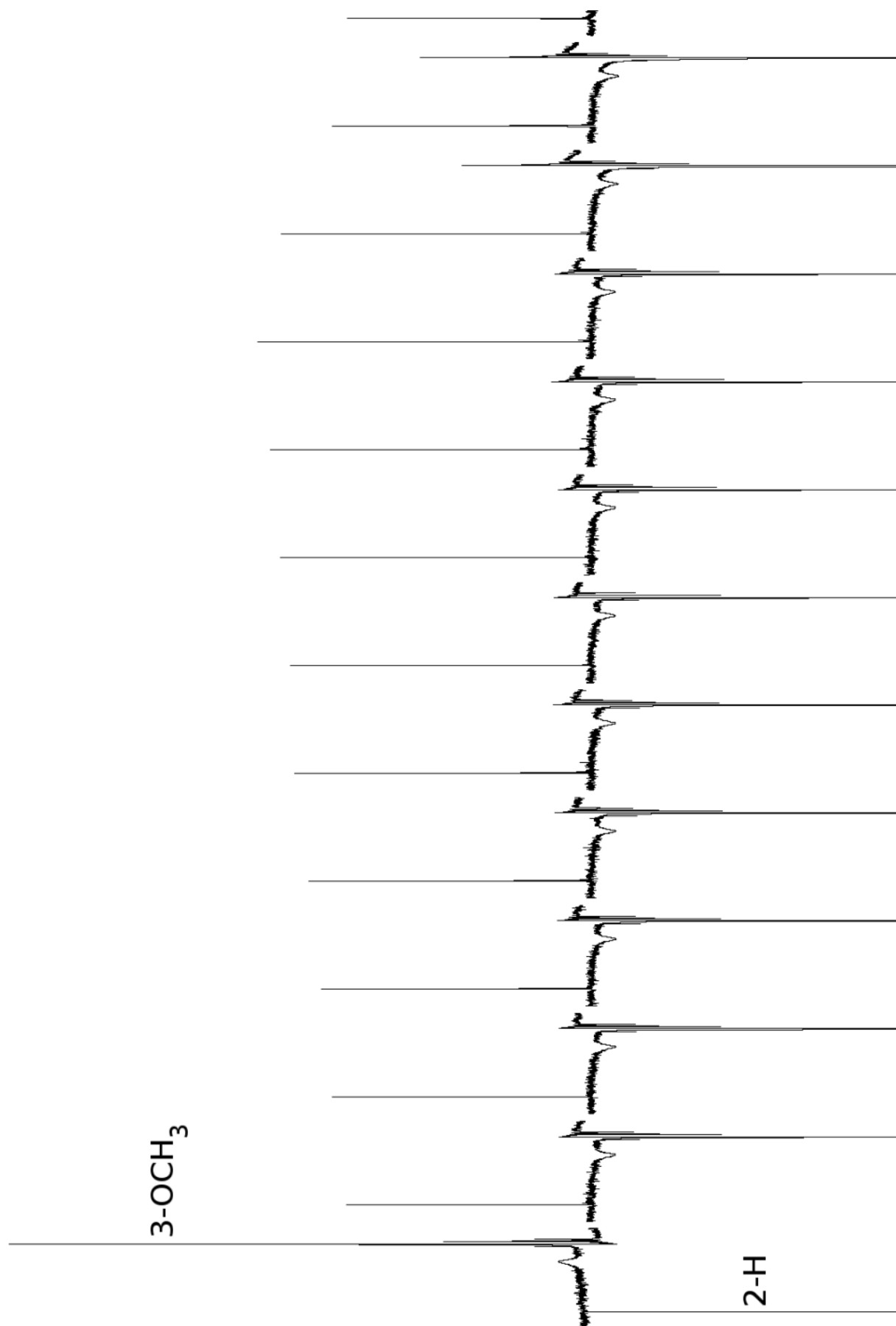


FIGURE 6.24: The 2-H intramolecular NOE build up curves of vanillic acid when complexed to Lipocalin measured at the corresponding concentrations: 2 mM ligand and 10 μ M for the protein.

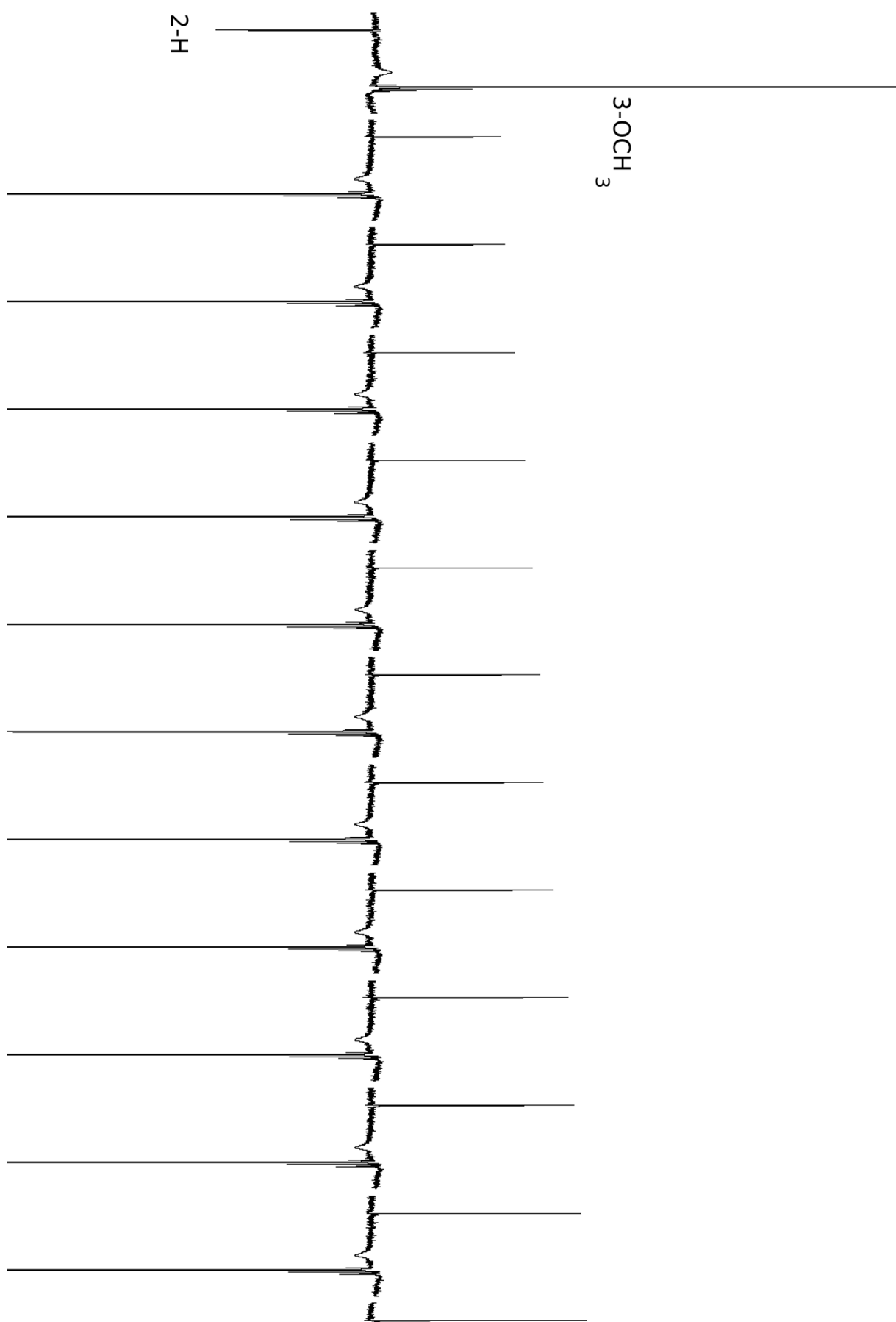


FIGURE 6.25: The 2-H intramolecular NOE build up curves of vanillic acid when complexed to Lipocalin measured at the corresponding concentrations: 2 mM ligand and 20 μ M for the protein.



FIGURE 6.26: The 2-H intramolecular NOE build up curves of vanillic acid when complexed to Lipocalin measured at the corresponding concentrations: 2 mM ligand and 30 μ M for the protein.

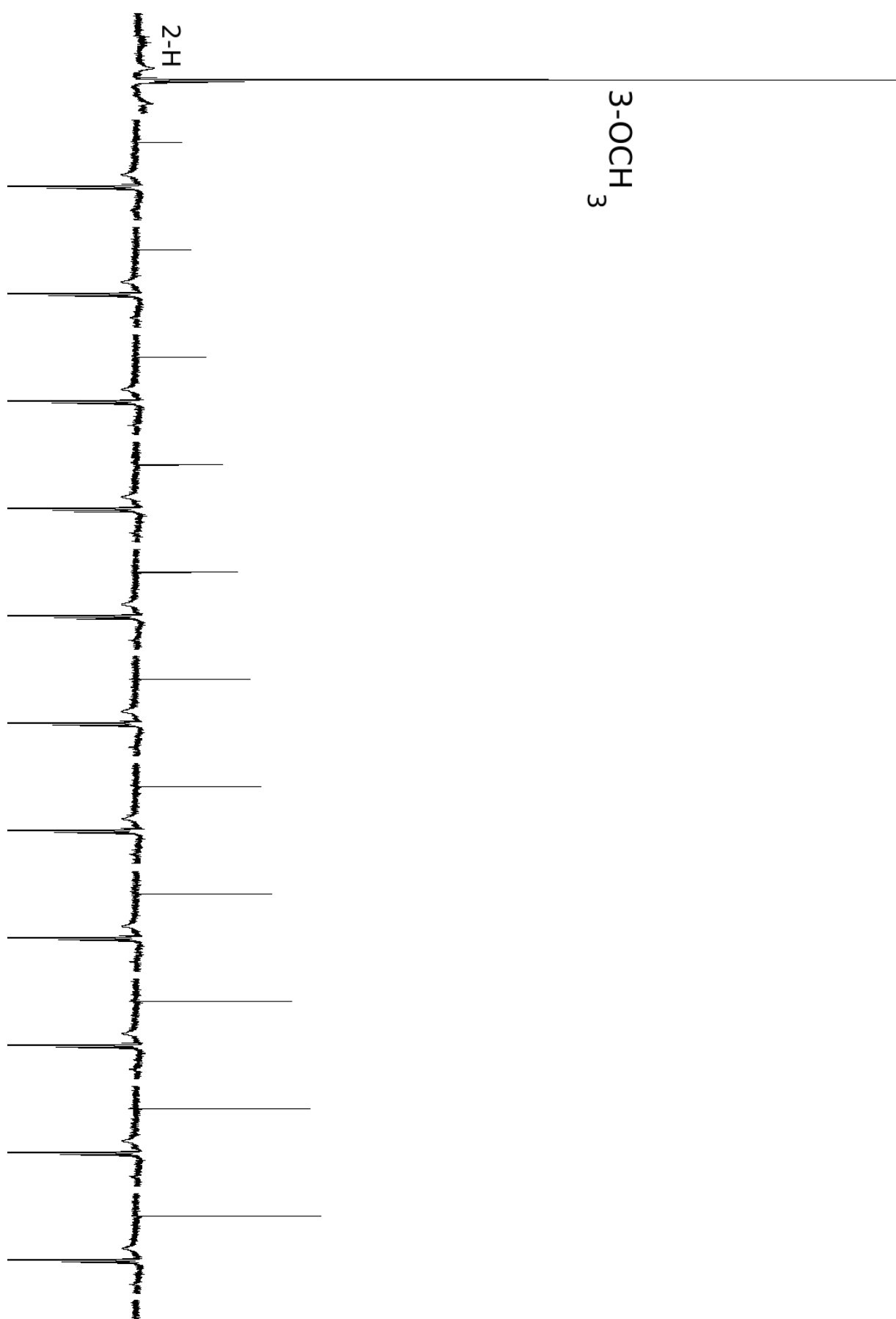


FIGURE 6.27: The 2-H intramolecular NOEbuild up curves of vanillic acid when complexed to Lipocalin measured at the corresponding concentrations: 2 mM ligand and an 40 μ M for the protein.

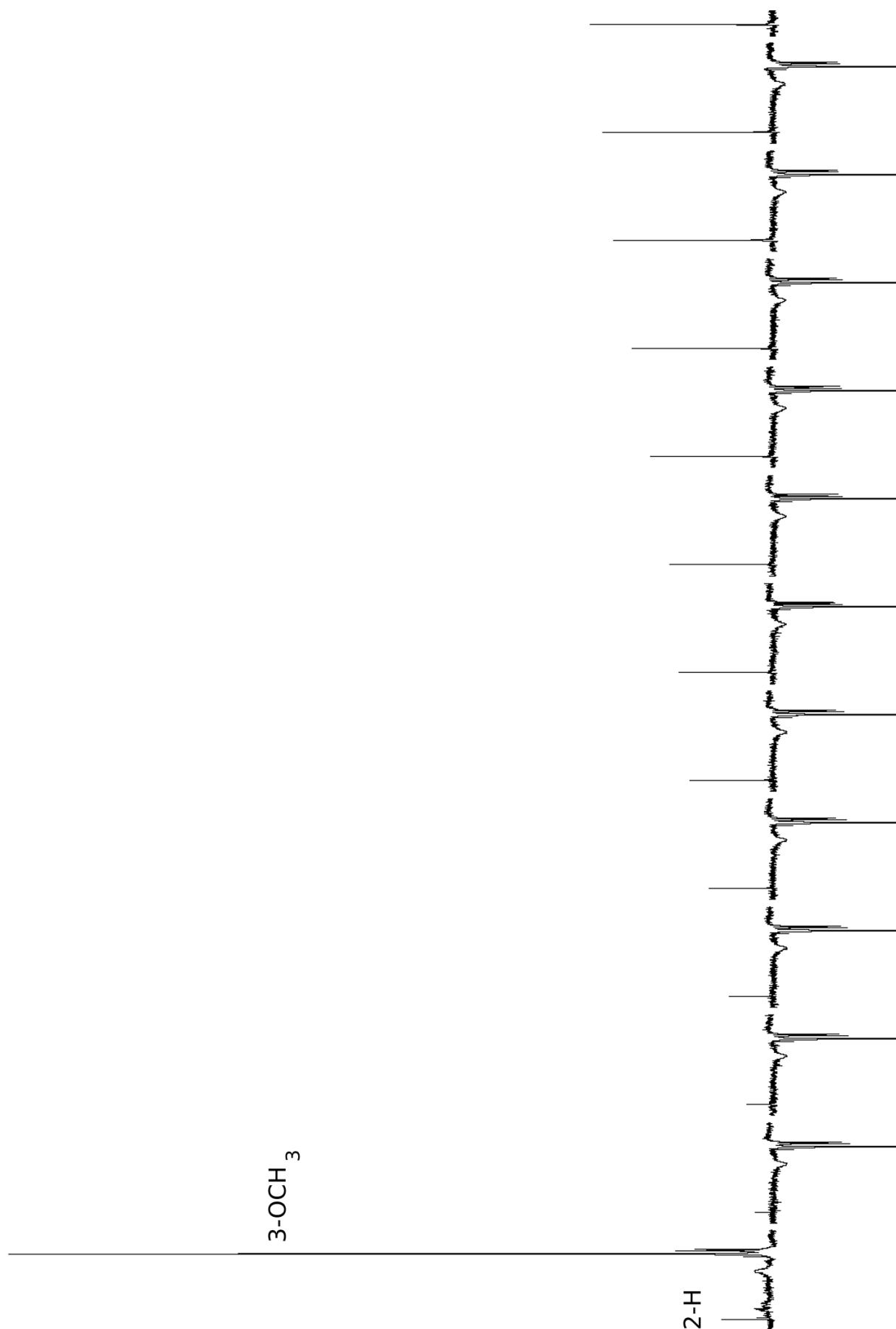


FIGURE 6.28: The 2-H intramolecular NOE build up curves of vanillic acid when complexed to Lipocalin measured at the corresponding concentrations: 2 mM ligand and 50 μM for the protein.

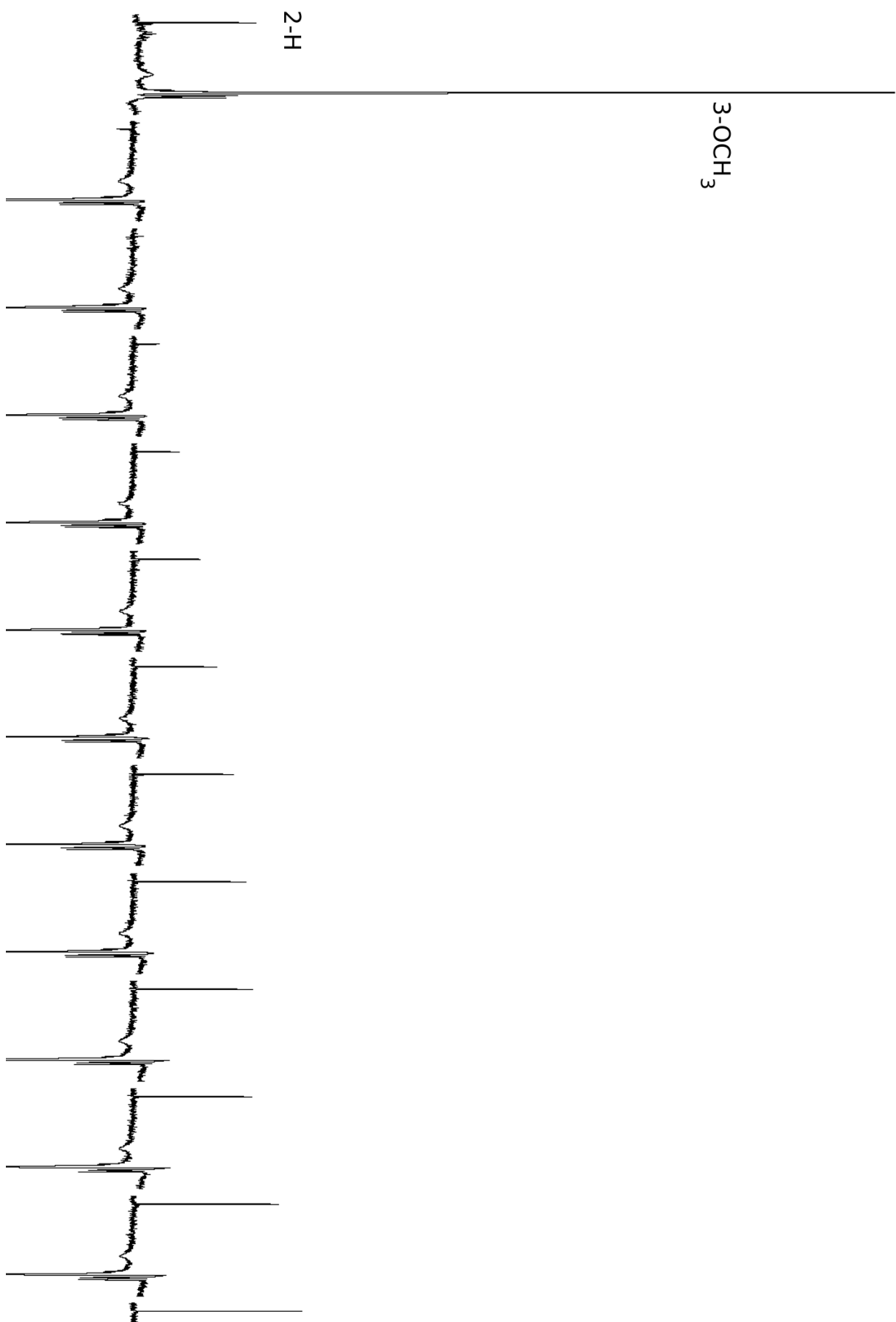


FIGURE 6.29: The 2-H intramolecular NOE build up curves of vanillic acid when complexed to Lipocalin measured at the corresponding concentrations: 2 mM ligand and an 60 μ M for the protein.

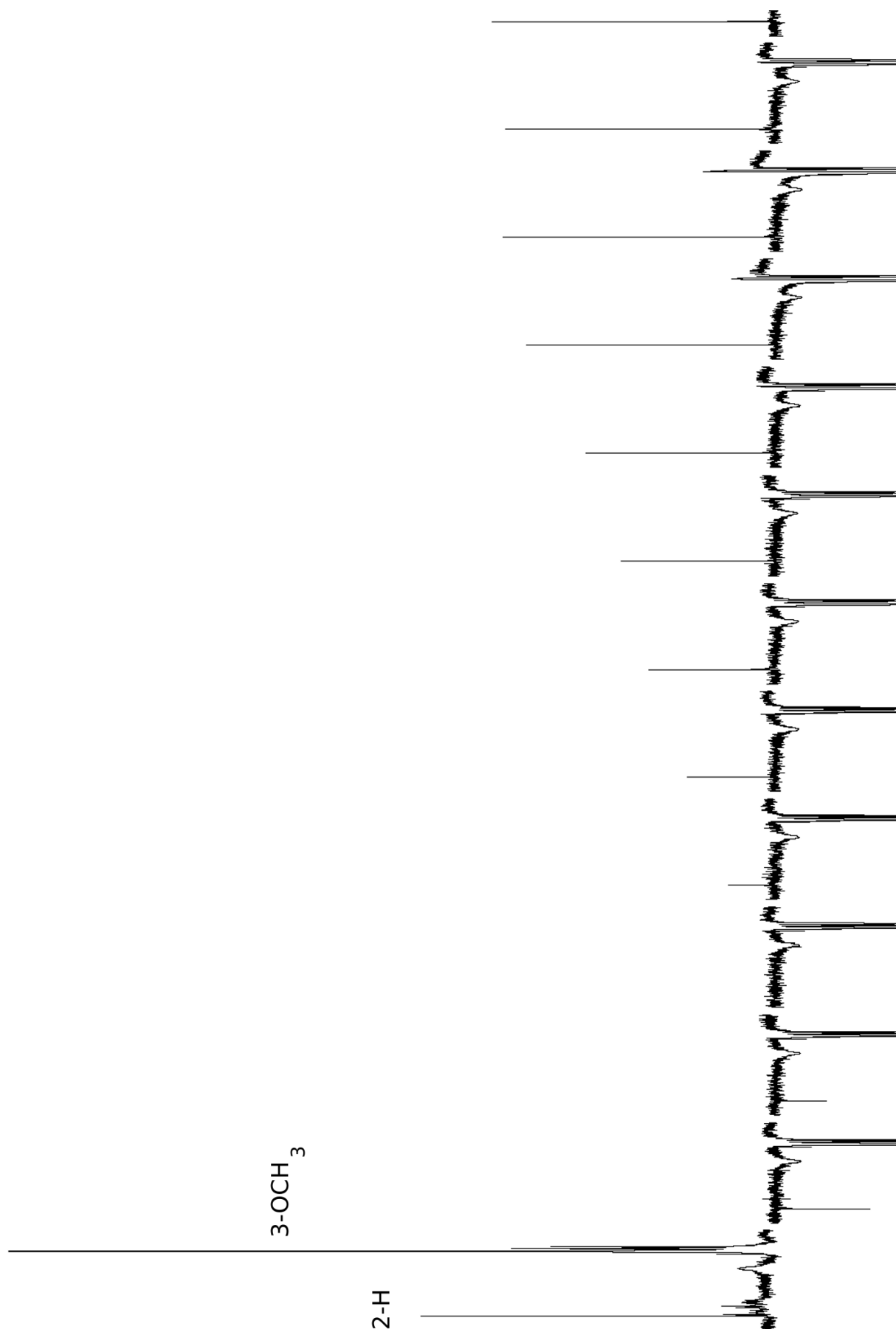


FIGURE 6.30: The 2-H intramolecular NOE build up curves of vanillic acid when complexed to Lipocalin measured at the corresponding concentrations: 2 mM ligand and an 70 uM for the protein.

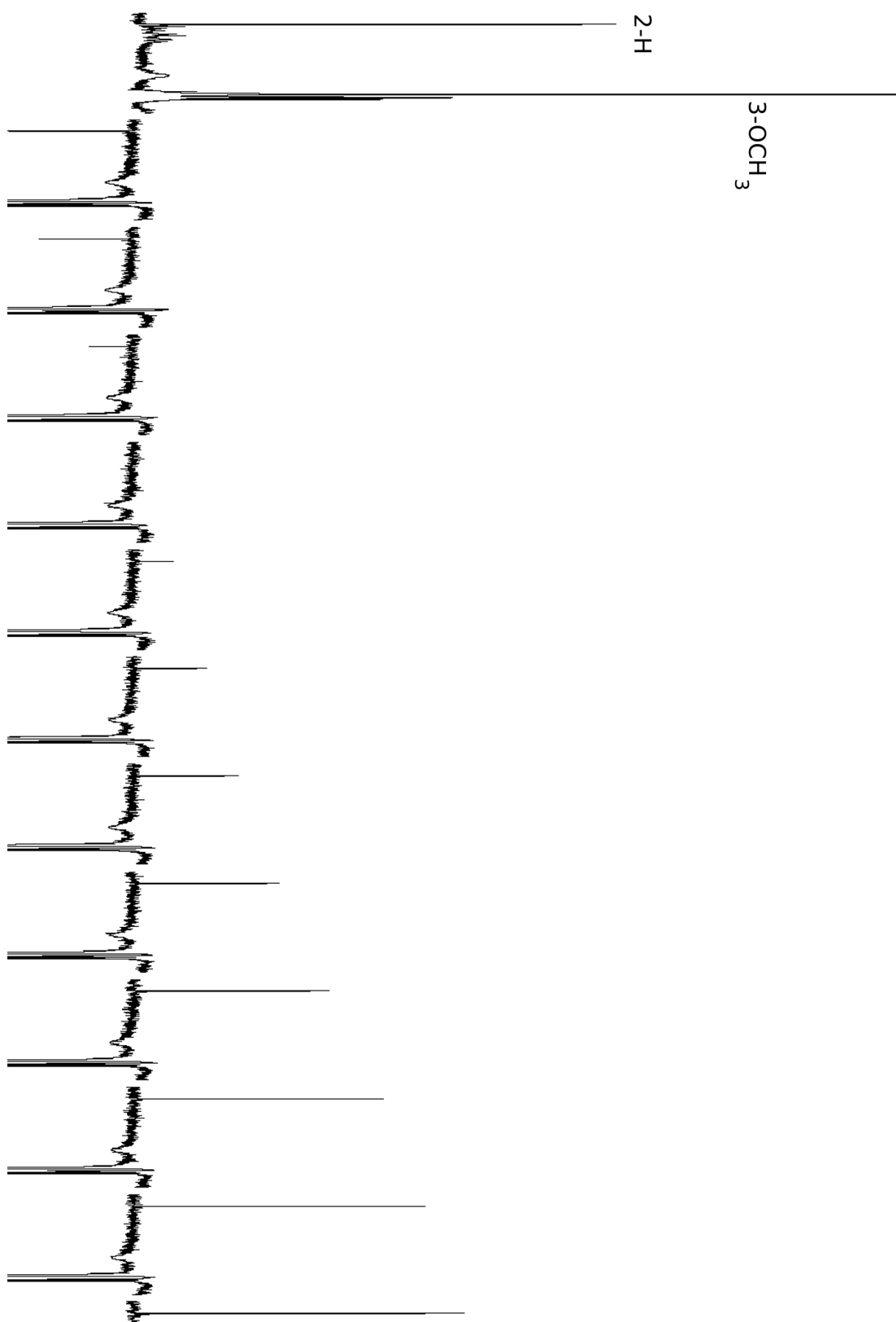
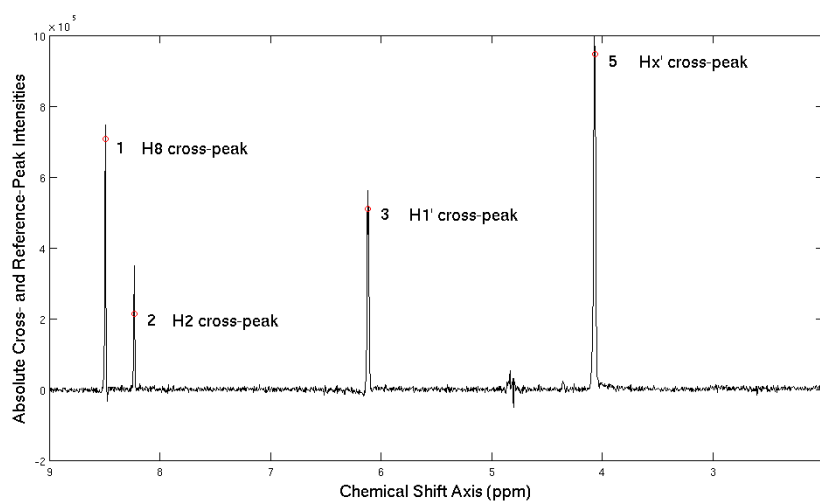
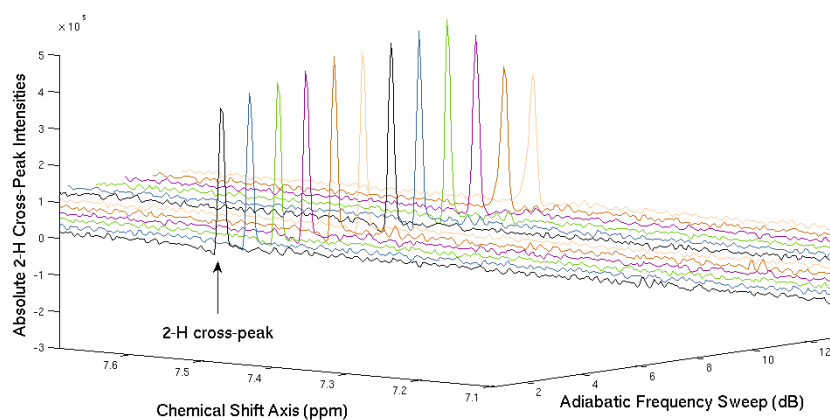


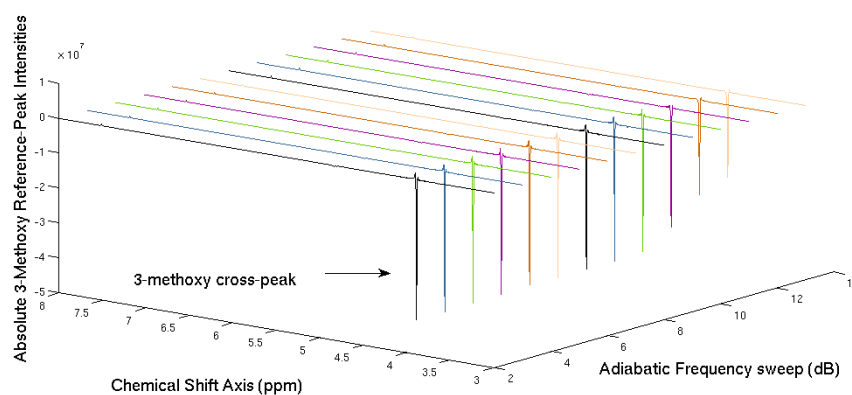
FIGURE 6.31: The 2-H intramolecular NOEbuild up curves of vanillic acid when complexed to Lipocalin measured at the corresponding concentrations: 2 mM ligand and $80\text{ }\mu\text{M}$ for the protein.



(a) Peak picking of 2-H vanillic acid proton resonances.

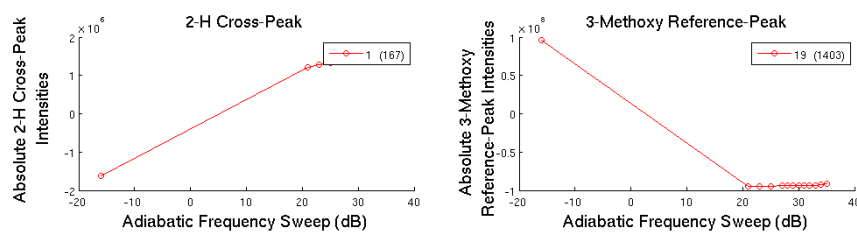


(b) 2-H NOE build up curves during an Adiabatic Frequency Sweep, ranging from -16 up to +35 dB.

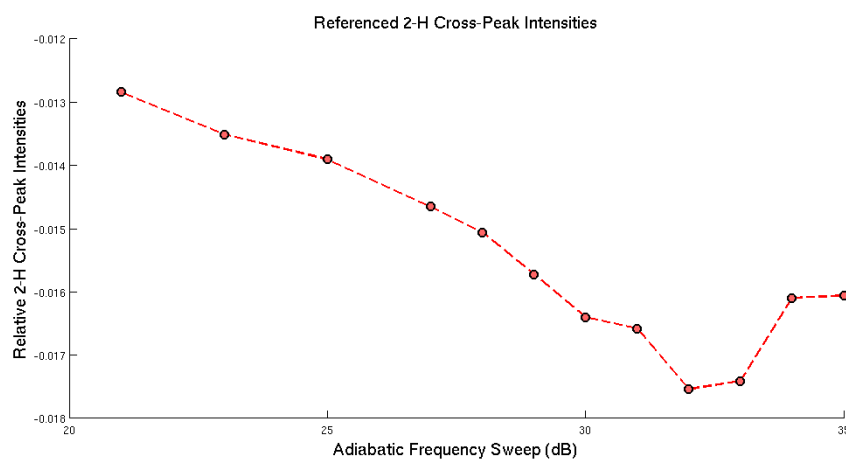


(c) 2-H NOE build up curves during an Adiabatic Frequency Sweep, ranging from -16 up to +35 dB.

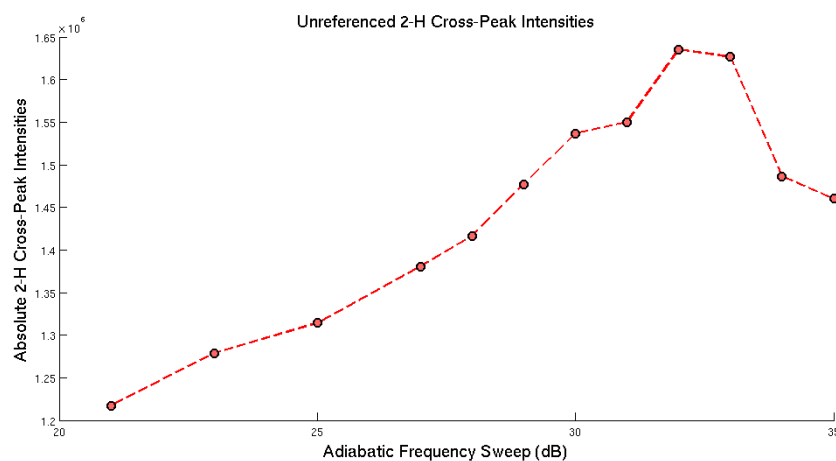
FIGURE 6.32: The 2-H NOE build up curves of 2mM Vanillic Acid in complex with 10 μ M Lipocalin (Q83).



(a) Cross-Peak (2-H) and Reference-Peak (3-OCH₃) Intensities during Adiabatic Frequency Sweep.

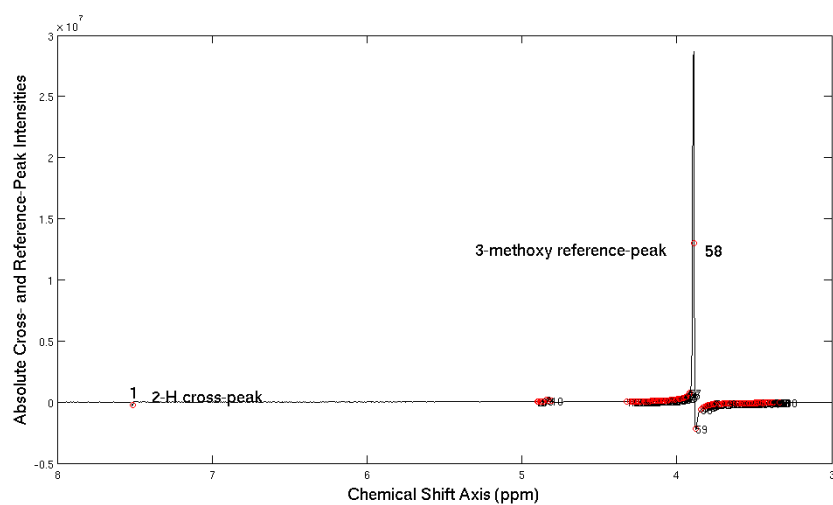


(b) Unreferenced Cross-Peak Intensities (2-H).

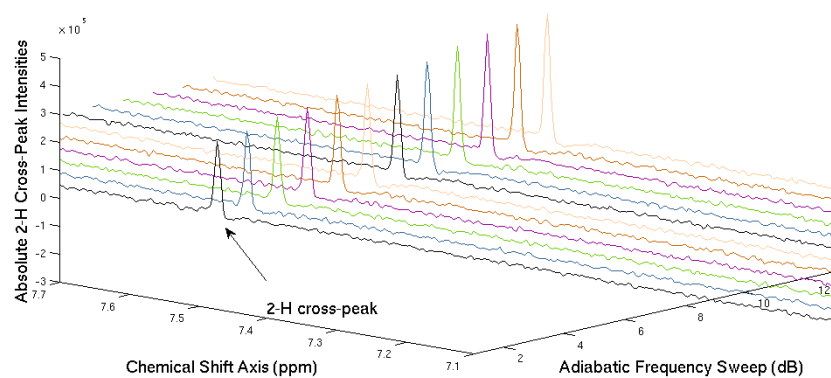


(c) Referenced Cross-Peak Intensities (2-H).

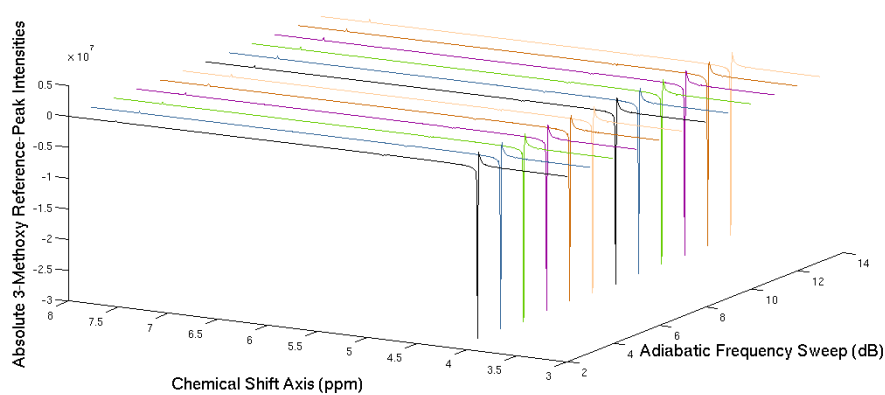
FIGURE 6.33: The 2-H NOE build up curves of 2mM Vanillic Acid in complex with 10 μ M Lipocalin (Q83).



(a) Peak picking of 2-H vanillic acid proton resonances.

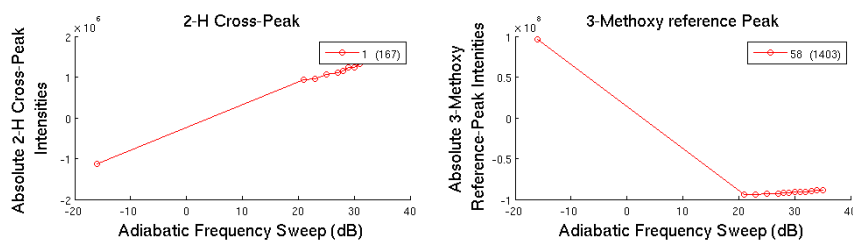


(b) 2-H NOE build up curves during an Adiabatic Frequency Sweep, ranging from -16 up to +35 dB.

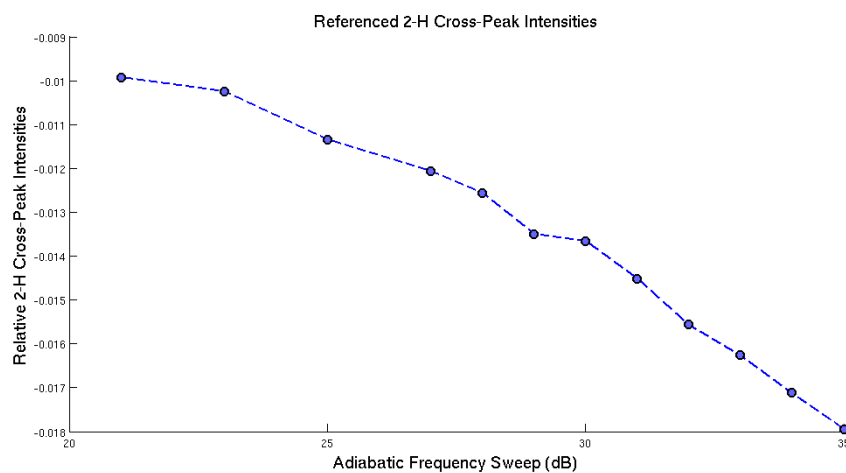


(c) 2-H NOE build up curves during an Adiabatic Frequency Sweep, ranging from -16 up to +35 dB.

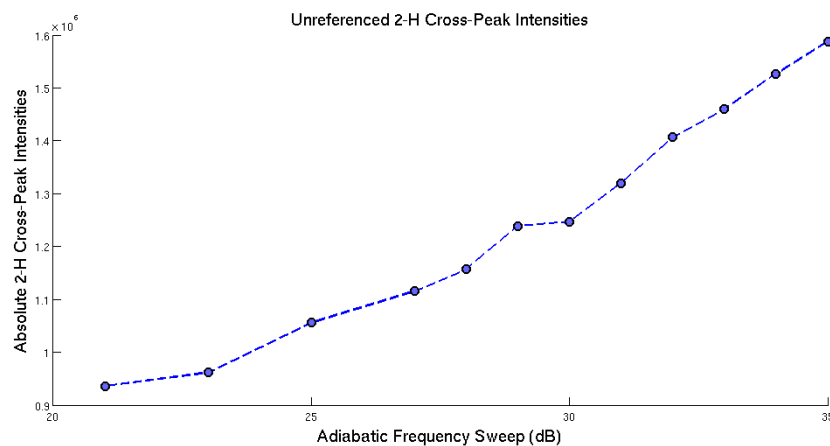
FIGURE 6.34: The 2-H NOE build up curves of 2mM Vanillic Acid in complex with 20 μ M Lipocalin (Q83).



(a) Cross-Peak (2-H) and Reference-Peak (3-OCH₃) Intensities during Adiabatic Frequency Sweep.

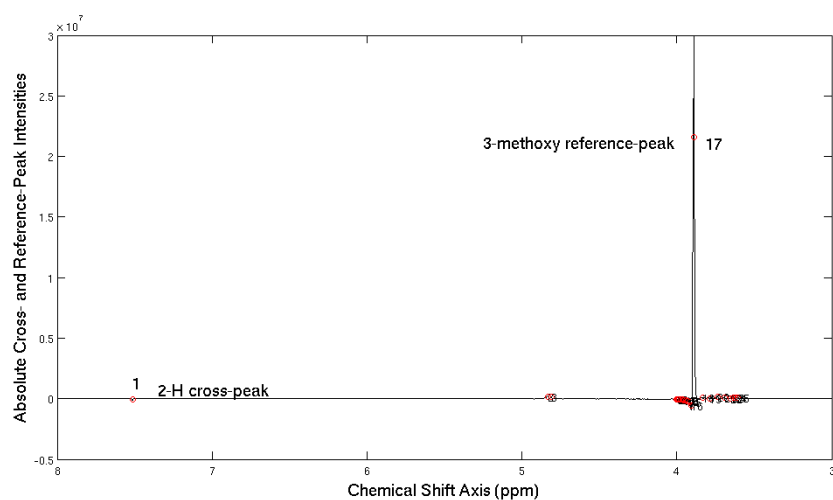


(b) Unreferenced Cross-Peak Intensities (2-H).

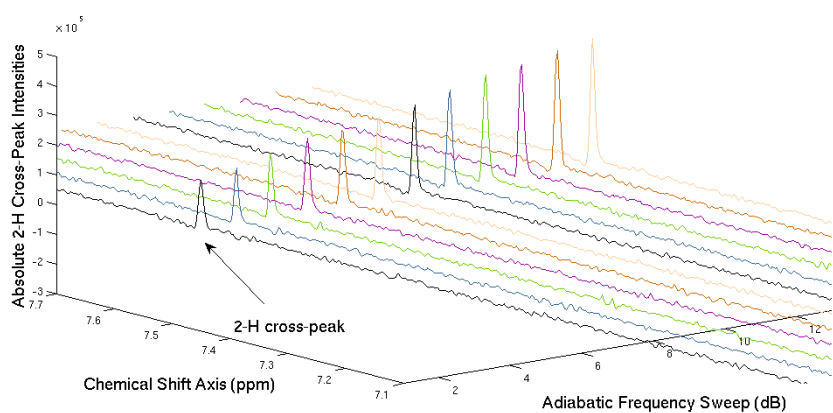


(c) Referenced Cross-Peak Intensities (2-H).

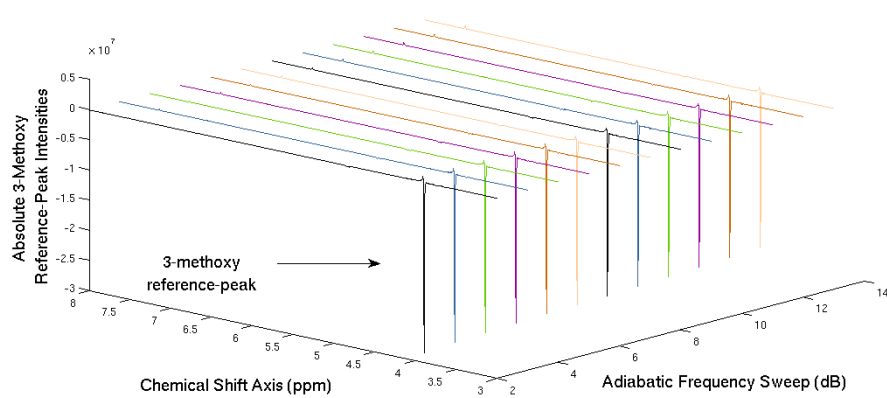
FIGURE 6.35: The 2-H NOE build up curves of 2mM Vanillic Acid in complex with 20 μ M Lipocalin (Q83).



(a) Peak picking of 2-H vanillic acid proton resonances.

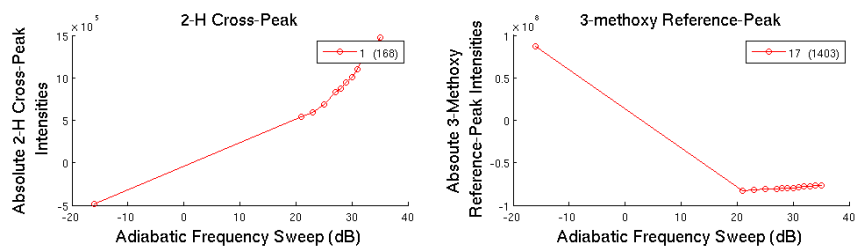


(b) 2-H NOE build up curves during an Adiabatic Frequency Sweep, ranging from -16 up to +35 dB.

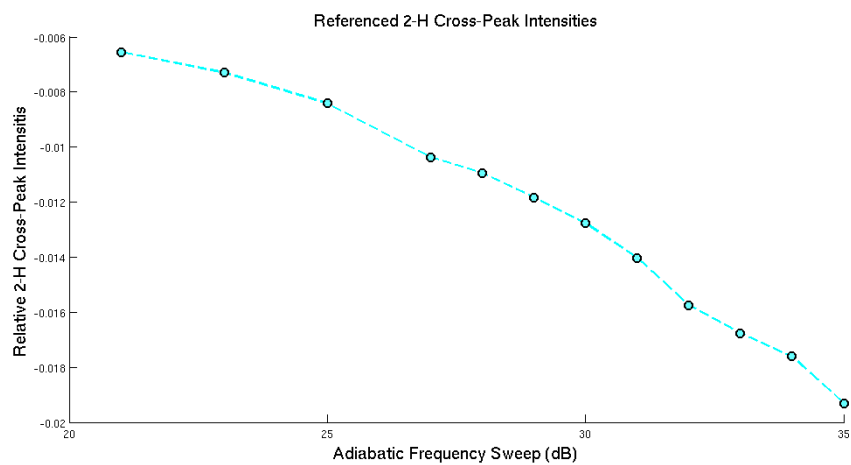


(c) 2-H NOE build up curves during an Adiabatic Frequency Sweep, ranging from -16 up to +35 dB.

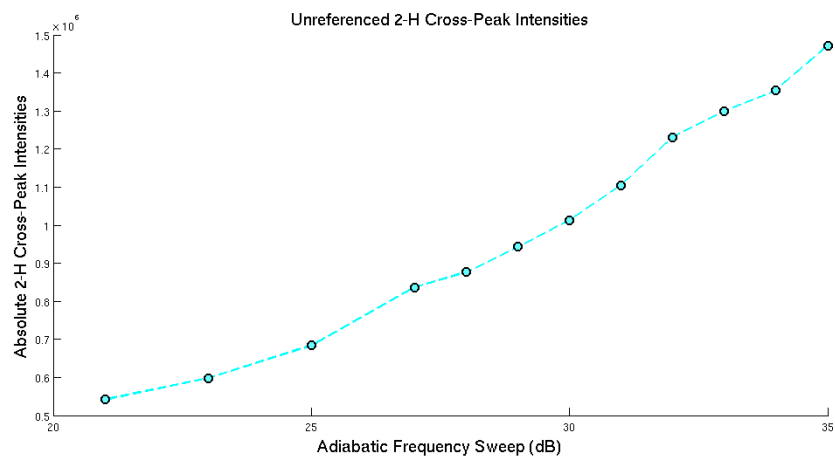
FIGURE 6.36: The 2-H NOE build up curves of 2mM Vanillic Acid in complex with 30 μ M Lipocalin (Q83).



(a) Cross-Peak (2-H) and Reference-Peak (3-OCH₃) Intensities during Adiabatic Frequency Sweep.

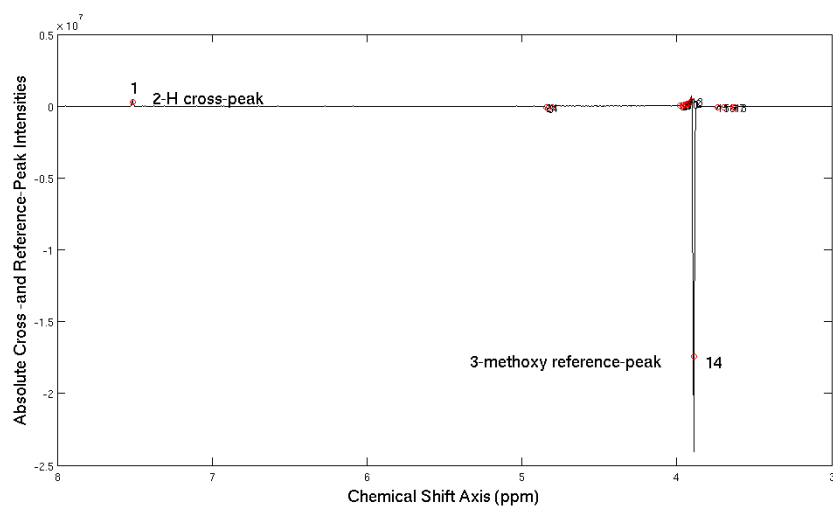


(b) Unreferenced Cross-Peak Intensities (2-H).

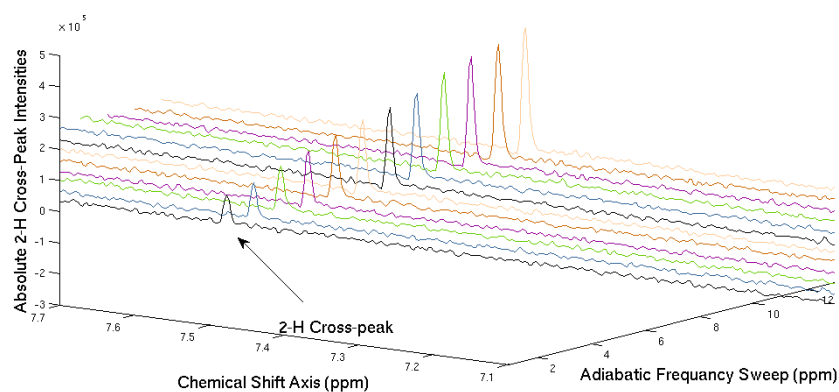


(c) Referenced Cross-Peak Intensities (2-H).

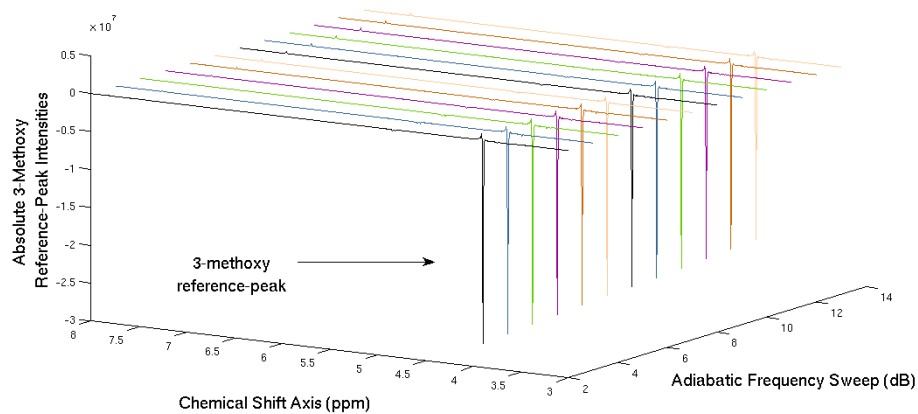
FIGURE 6.37: The 2-H NOE build up curves of 2mM Vanillic Acid in complex with 30 μ M Lipocalin (Q83).



(a) Peak picking of 2-H vanillic acid proton resonances.

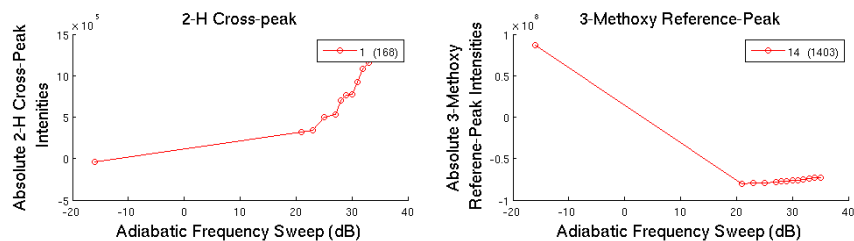


(b) 2-H NOE build up curves during an Adiabatic Frequency Sweep, ranging from -16 up to +35 dB.

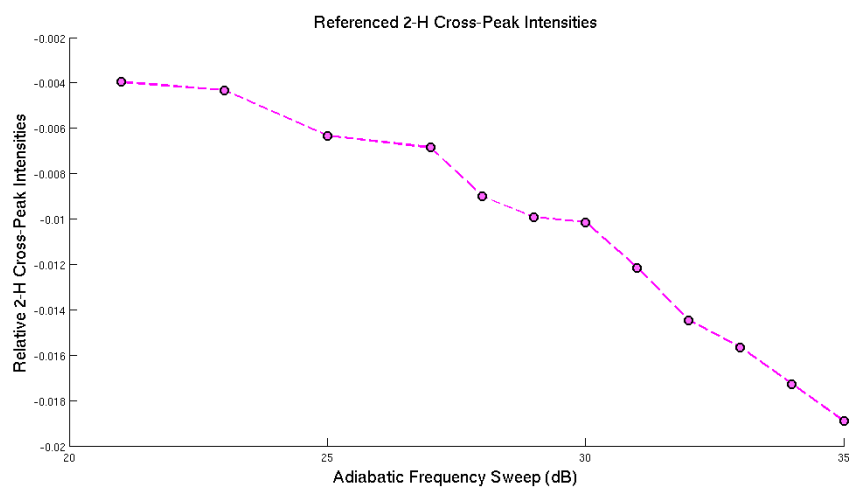


(c) 2-H NOE build up curves during an Adiabatic Frequency Sweep, ranging from -16 up to +35 dB.

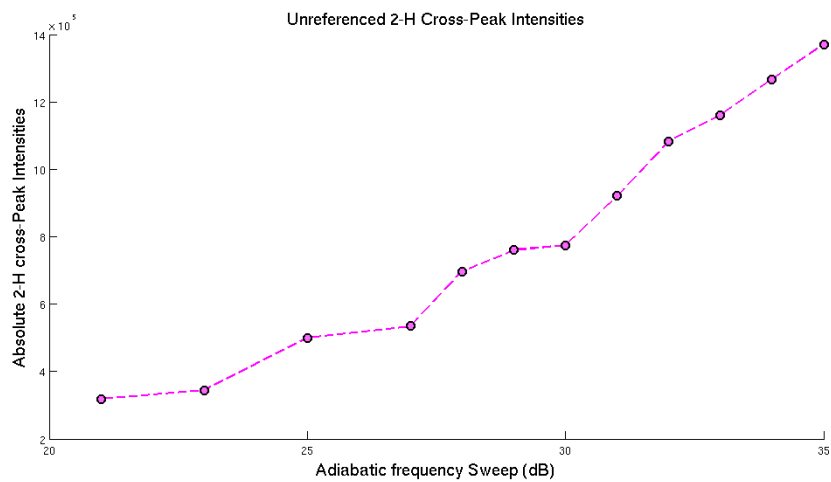
FIGURE 6.38: The 2-H NOE build up curves of 2mM Vanillic Acid in complex with 40 μ M Lipocalin (Q83).



(a) Cross-Peak (2-H) and Reference-Peak (3-OCH₃) Intensities during Adiabatic Frequency Sweep.

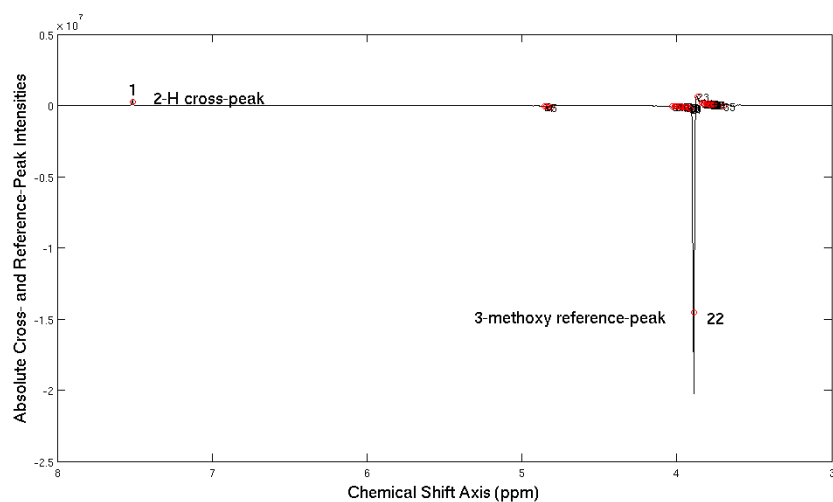


(b) Unreferenced Cross-Peak Intensities (2-H).

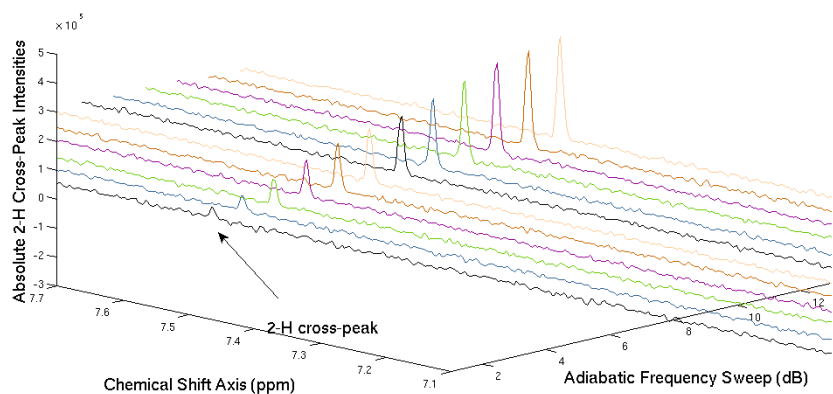


(c) Referenced Cross-Peak Intensities (2-H).

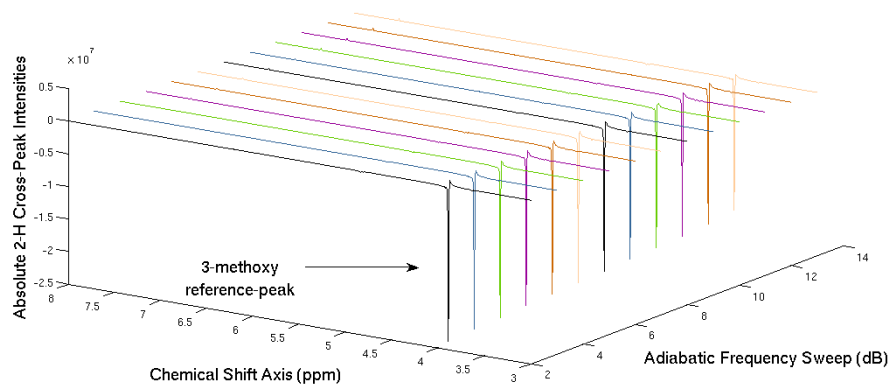
FIGURE 6.39: The 2-H NOE build up curves of 2mM Vanillic Acid in complex with 40 μ M Lipocalin (Q83).



(a) Peak picking of 2-H vanillic acid proton resonances.

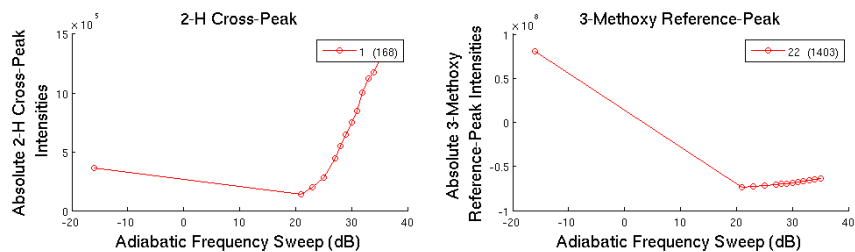


(b) 2-H NOE build up curves during an Adiabatic Frequency Sweep, ranging from -16 up to +35 dB.

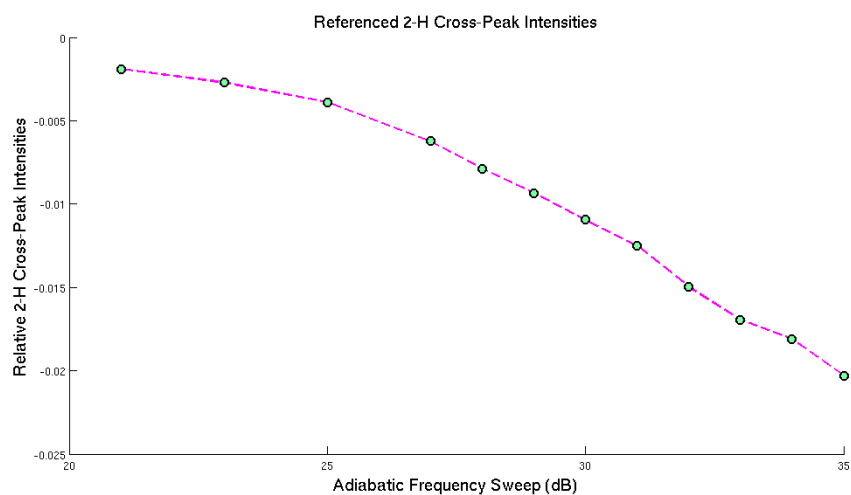


(c) 2-H NOE build up curves during an Adiabatic Frequency Sweep, ranging from -16 up to +35 dB.

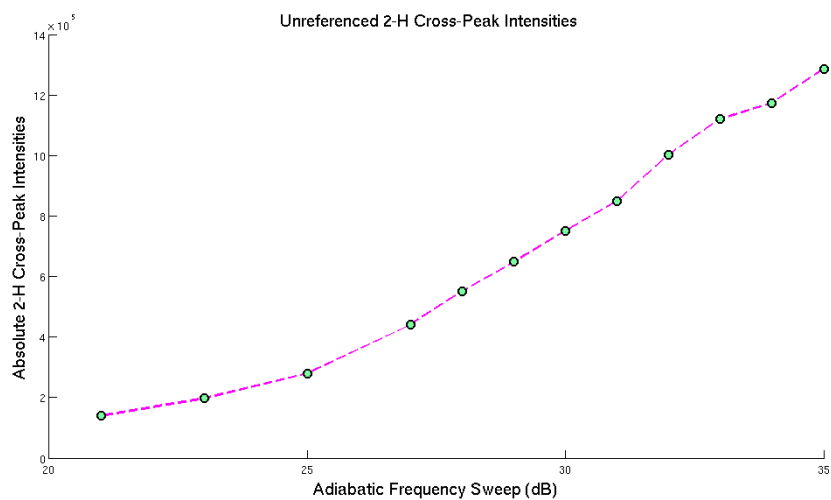
FIGURE 6.40: The 2-H NOE build up curves of 2mM Vanillic Acid in complex with 50 μ M Lipocalin (Q83).



(a) Cross-Peak (2-H) and Reference-Peak (3-OCH₃) Intensities during Adiabatic Frequency Sweep.

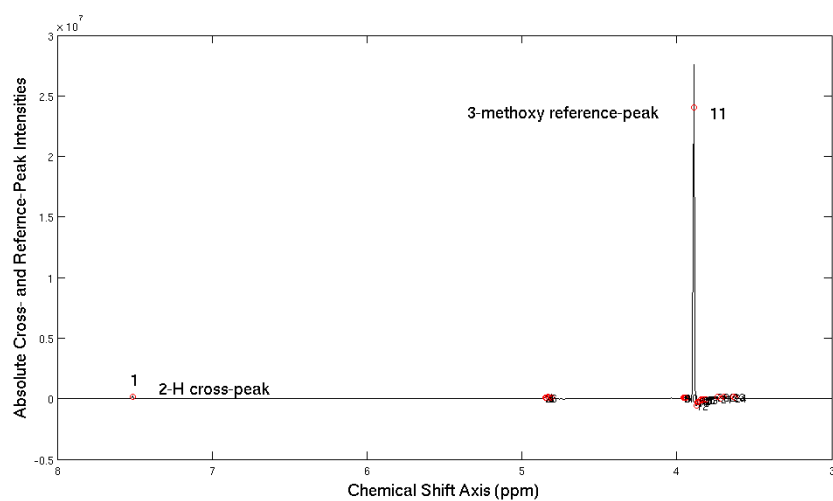


(b) Unreferenced Cross-Peak Intensities (2-H).

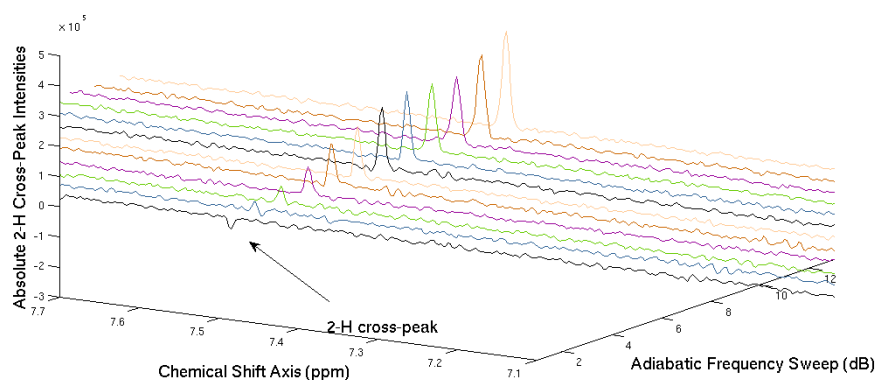


(c) Referenced Cross-Peak Intensities (2-H).

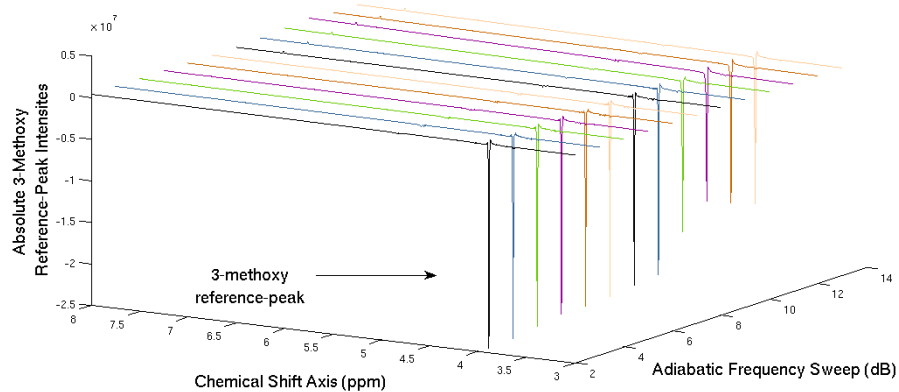
FIGURE 6.41: The 2-H NOE build up curves of 2mM Vanillic Acid in complex with 50 μ M Lipocalin (Q83).



(a) Peak picking of 2-H vanillic acid proton resonances.

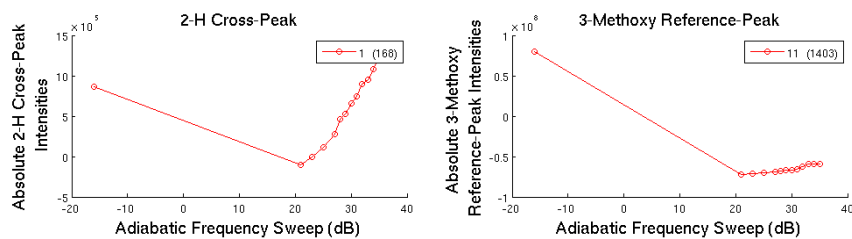


(b) 2-H NOE build up curves during an Adiabatic Frequency Sweep, ranging from -16 up to +35 dB.

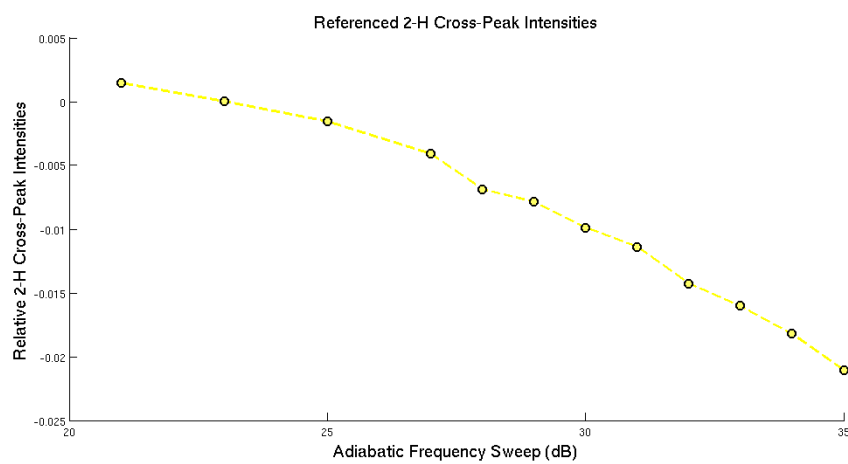


(c) 2-H NOE build up curves during an Adiabatic Frequency Sweep, ranging from -16 up to +35 dB.

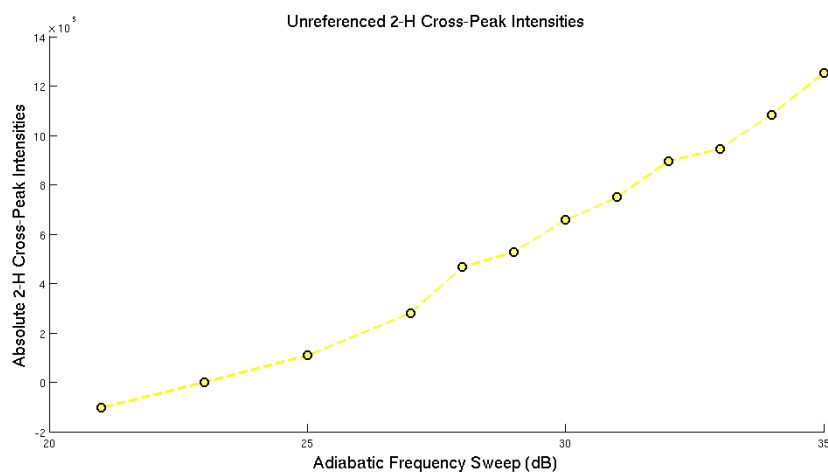
FIGURE 6.42: The 2-H NOE build up curves of 2mM Vanillic Acid in complex with 60 μ M Lipocalin (Q83).



(a) Cross-Peak (2-H) and Reference-Peak (3-OCH₃) Intensities during Adiabatic Frequency Sweep.

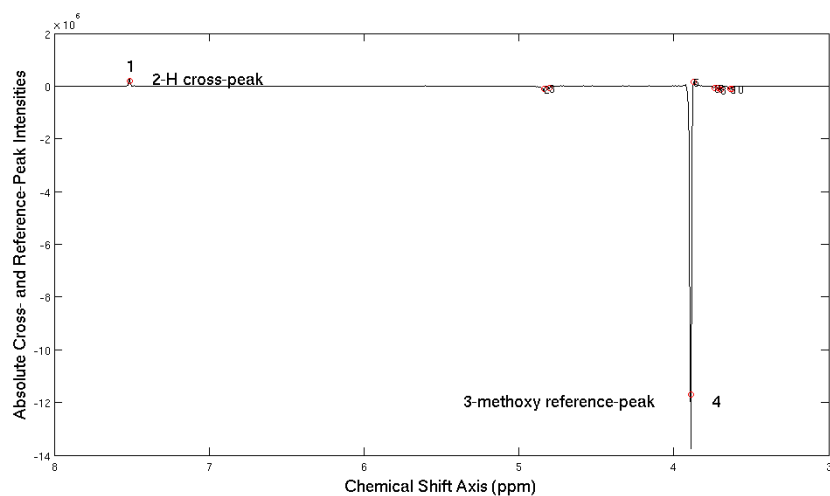


(b) Unreferenced Cross-Peak Intensities (2-H).

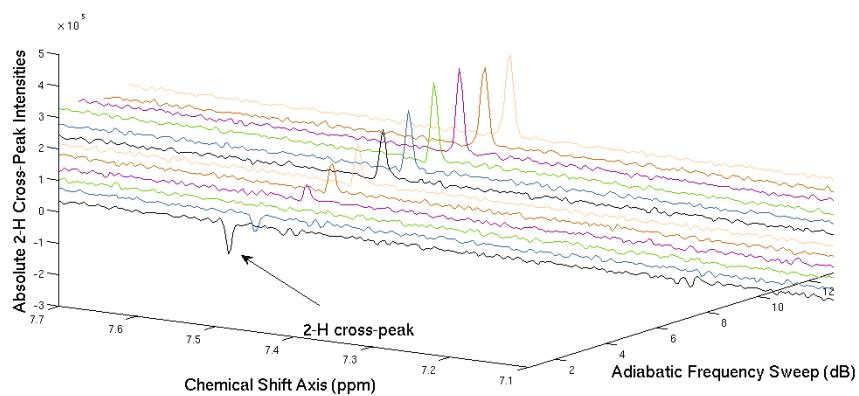


(c) Referenced Cross-Peak Intensities (2-H).

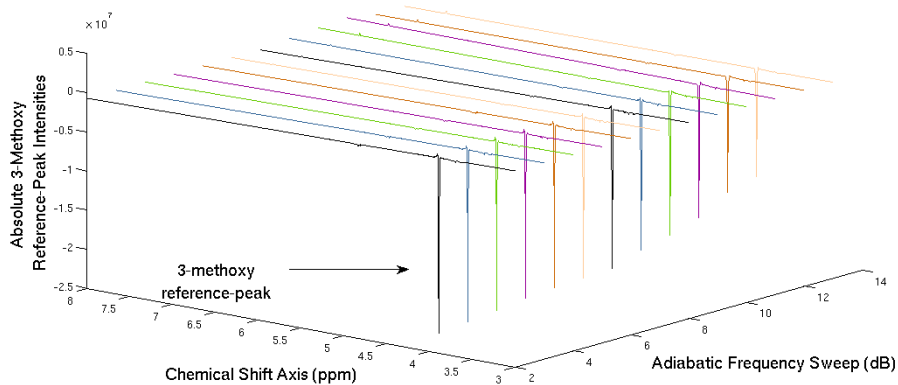
FIGURE 6.43: The 2-H NOE build up curves of 2mM Vanillic Acid in complex with 60 μ M Lipocalin (Q83).



(a) Peak picking of 2-H vanillic acid proton resonances.

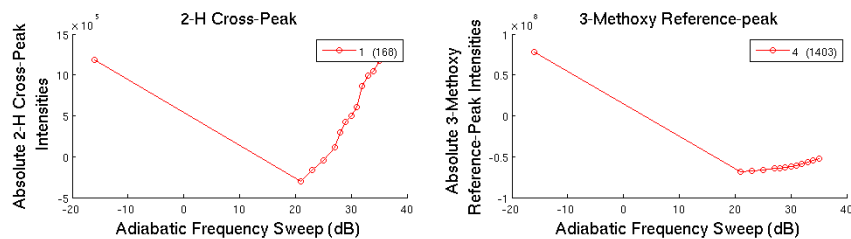


(b) 2-H NOE build up curves during an Adiabatic Frequency Sweep, ranging from -16 up to +35 dB.

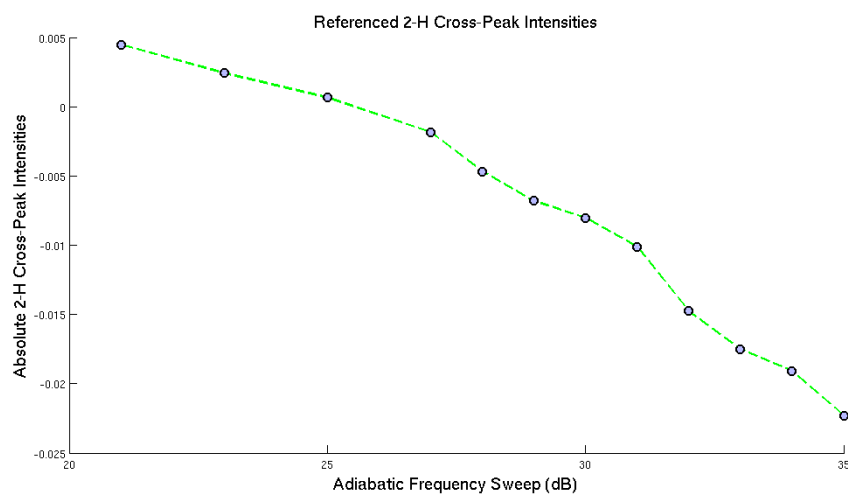


(c) 2-H NOE build up curves during an Adiabatic Frequency Sweep, ranging from -16 up to +35 dB.

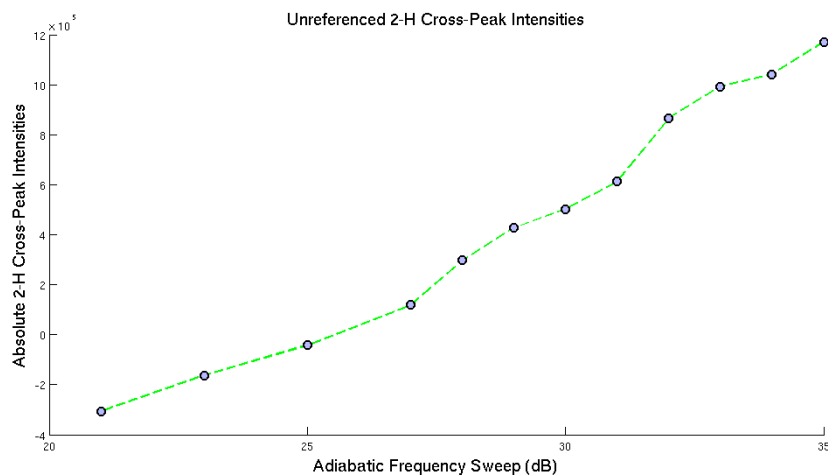
FIGURE 6.44: The 2-H NOE build up curves of 2mM Vanillic Acid in complex with 70 μ M Lipocalin (Q83).



(a) Cross-Peak (2-H) and Reference-Peak (3-OCH₃) Intensities during Adiabatic Frequency Sweep.

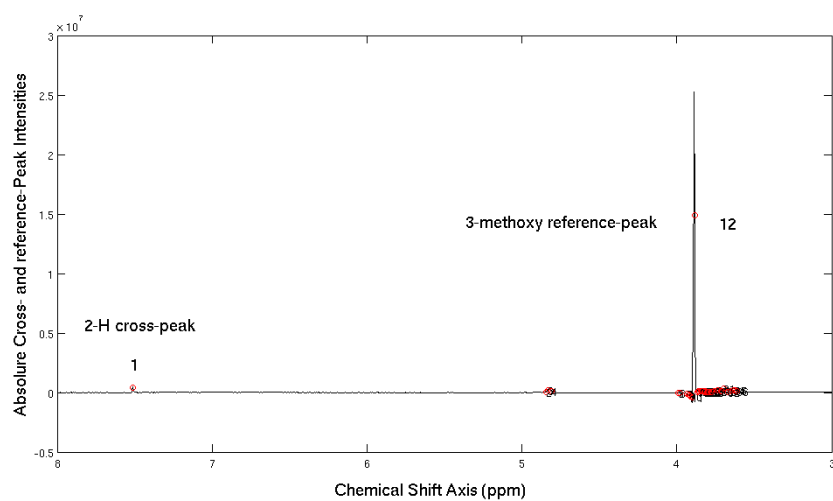


(b) Unreferenced Cross-Peak Intensities (2-H).

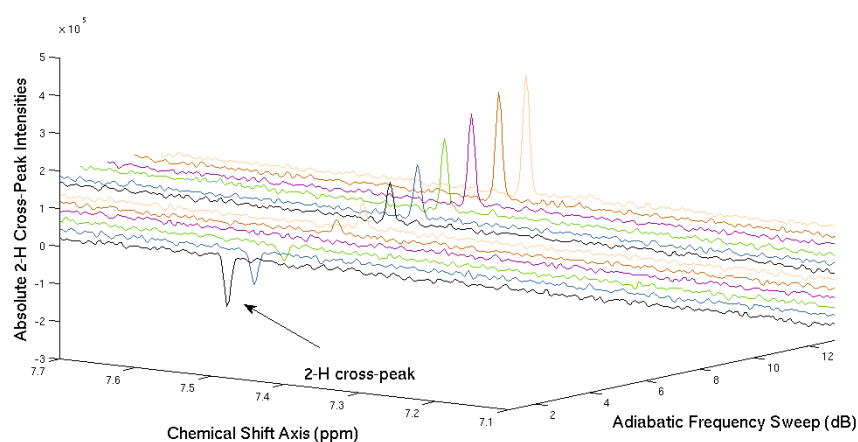


(c) Referenced Cross-Peak Intensities (2-H).

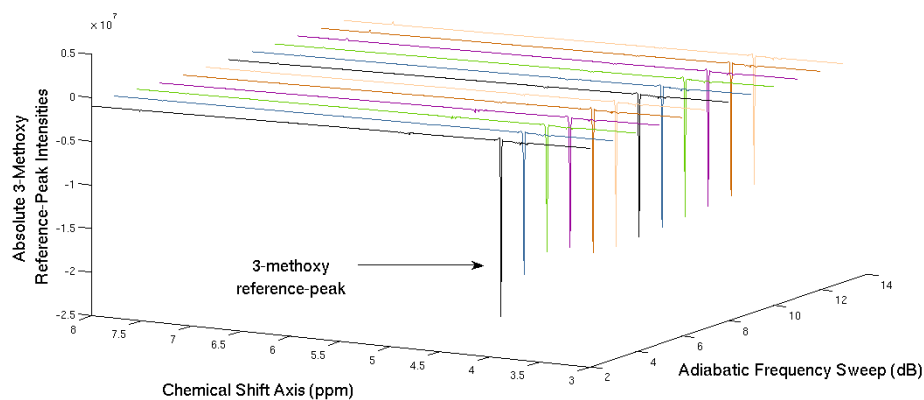
FIGURE 6.45: The 2-H NOE build up curves of 2mM Vanillic Acid in complex with 70 μ M Lipocalin (Q83).



(a) Peak picking of 2-H vanillic acid proton resonances.

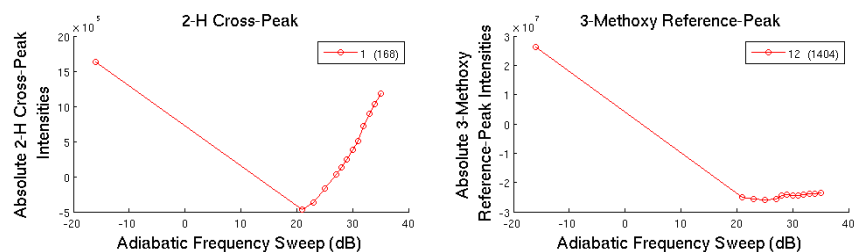


(b) 2-H NOE build up curves during an Adiabatic Frequency Sweep, ranging from -16 up to +35 dB.

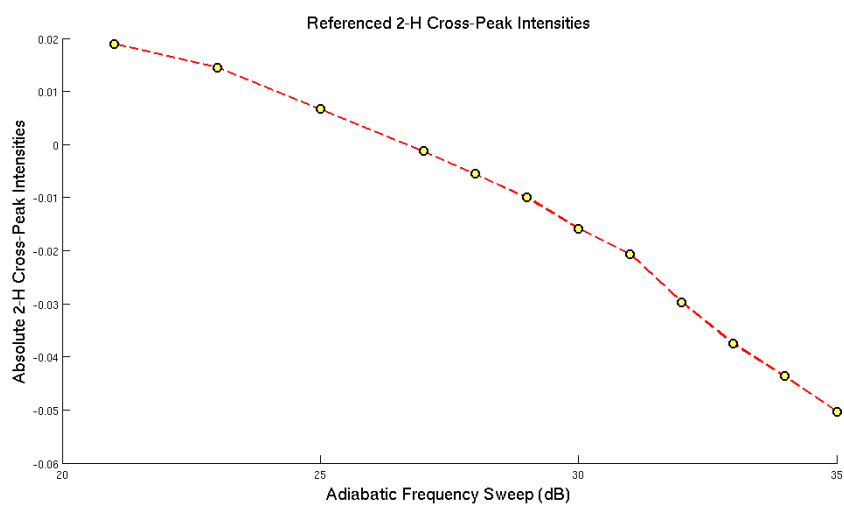


(c) 2-H NOE build up curves during an Adiabatic Frequency Sweep, ranging from -16 up to +35 dB.

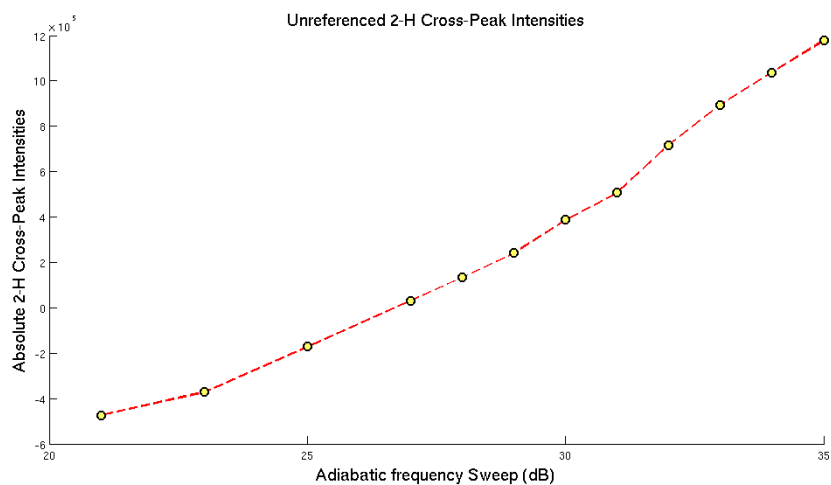
FIGURE 6.46: The 2-H NOE build up curves of 2mM Vanillic Acid in complex with 80 μ M Lipocalin (Q83).



(a) Cross-Peak (2-H) and Reference-Peak (3-OCH₃) Intensities during Adiabatic Frequency Sweep.



(b) Unreferenced Cross-Peak Intensities (2-H).



(c) Referenced Cross-Peak Intensities (2-H).

FIGURE 6.47: The 2-H NOE build up curves of 2mM Vanillic Acid in complex with 80 μ M Lipocalin (Q83).

6.3.2 The STD Experiment

A supporting STD experiment has been carried out with this complex aswell. The outcome is illustrated in Figure 6.48 below. It affirms Vanillic acid as a binding component of Lipocalin, as indicated by the intermolecular spin-diffusion mediated NOEs, measured on the free ligand in solution. Again, one detects two spectra, one "On-Resonance" (at about -1 ppm), and a second "Off-Resonance" spectrum (about +100 ppm downfield), which are then subtracted from one another to yield the netto intermolecular NOE enhancement effect on the ligand.

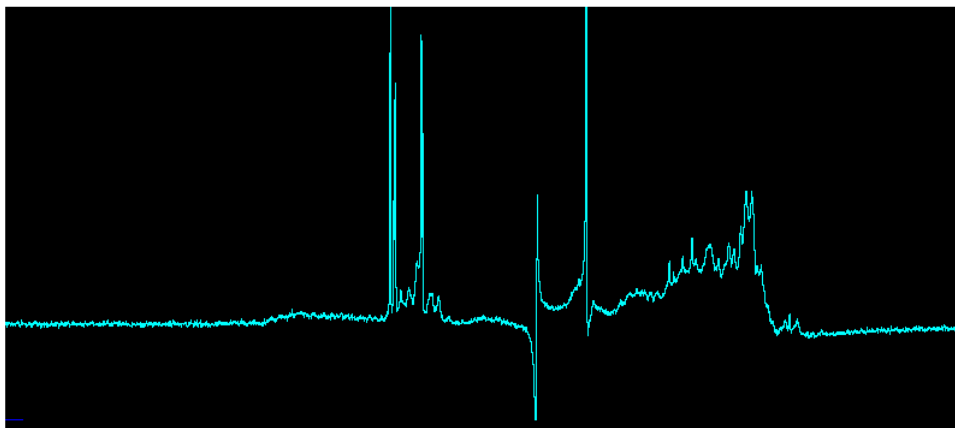


FIGURE 6.48: The STD experiment of Vanillic Acid bound to Lipocalin (Q83). The corresponding concentrations are 2 mM for the ligand and 40 μ M for the protein.

Kapitel 7

Zusammenfassung

Im Kontext dieser Diplomarbeit wurde eine innovative Methode für die Bestimmung der Bindungsaktivität als auch der konformationellen Charakterisierung des Bindungs-epitops von Liganden in der Bindungstasche des Proteins implementiert. Die Methode bedient sich eines Analogons der klassischen 1D-NOESY-Spektroskopie, unter Zuhilfenahme eines adiabatischen Inversionspulses (Adiabatic Fast Passage). Die 1D-AFP-NOESY-Sequenz gewährleistet die Detektion des relativen Anteils an direktem und indirektem NOE Transfers, welches während der Relaxationszeit der Kernspins auftritt und determinierend ist für die Bestimmung der Konfiguration des Liganden innerhalb der Bindungskavität des Proteins. Der Anteil an direktem und indirektem NOE Magnetisierungstransfers wird charakterisiert durch die NOE Aufbaukurve während der Relaxationszeit und ist für jeden individuellen Kern im Ligandenmolekül unterschiedlich. Bei einem eindimensionalen NOESY Experiment werden üblicherweise die Protonen des Liganden detektiert. Ligandenprotonen, die sich durch einen starken indirekten NOE Transfer, dem sog. Phänomen der Spin Diffusion auszeichnen, werden sich in der Bindungstasche des Proteins auf der Berührungsfläche zwischen Protein und Ligand anordnen. Im Gegensatz dazu, werden Ligandenprotonen, die wiederum einen vergleichsweise stärkeren direkten NOE Transfer aufweisen, die Position auf der Außenfläche der Bindungskavität des Proteins einnehmen.

In einem zweiten, zum 1D-AFP-NOESY komplementären Screening-Experiment wurde das Prinzip der Sättigungs-transfer-Differenz-NMR-Spektroskopie (STD) eingesetzt und verglichen. Beide Methoden beruhen auf einer Differenzbildung zwischen Sättigungs-transfer- und normalem NMR-Spektrum, und resultieren in Spektren, die nur Signale bindender Liganden enthalten. Mit diesem Experiment lässt sich das Bindungs-epitop von Liganden leicht bestimmen, da mit dem Protein in direktem Kontakt stehende Ligandenreste intensivere STD-Signale liefern. Das STD-Experiment wurde als 1D-Experiment aufgesetzt, mit Proton als zu detektierenden Kern. Liganden, die reversibel mit der Bindungsdomäne eines Proteins in Wechselwirkung stehen, zeigen charakteristische Relaxations- und Mobilitätsunterschiede, zu den Molekülen, die keine Affinität

zum Protein aufweisen.

Diese zwei ergänzenden Experimente wurden mit drei verschiedenen Protein-Liganden Komplexen durchgeführt: mit Adenosinmonophosphat (AMP) und des aus *Saccharomyces Cerevisiae* stammenden Enzyms, der Alkoholdehydrogenase, mit dem Coenzym Nicotinamidadenindinucleotid (NAD) und der Alkoholdehydrogenase, und schließlich mit der Vanillinsäure und dem Transporterprotein, Lipocalin (Q83).

Das entscheidende Ergebnis lieferte das Vergleichsexperiment zwischen AMP/ADH und dem Komplex NAD/ADH, welches im AFP-NOESY Experiment den eindeutigen Beweis für eine identische Konformation im Bezug auf die syn-/anti Isomerie des N-glykosidischen Bindungswinkels zwischen der Ribose und der Base Adenine, im AMP/-ADH als auch im NAD/ADH Komplex lieferte.

Chapter 8

Summary

In the course of this diploma work a novel assignment method has been employed for the determination of the binding activity and the conformational constitution of the binding epitope of protein-ligand complexes. The method implements an analogon of the classical 1D-NOESY spectroscopy, wherein additionally utilizing an adiabatic inversion pulse, termed as the Adiabatic Fast Passage pulse sequence. The 1D-AFP-NOESY experiment provides for an accurate detection of the fractional participation of direct and indirect NOE magnetization-transfer pathways, characterized by the NOE build-up curve during the runtime of spin relaxation. The NOE build-up curve is different for each individual nuclear spin, which leads to an exact predication of the configuration of the ligand inside the binding pocket of the protein. This difference originates from a differing fractional apportionment of the direct and indirect magnetization transfer pathways between nuclear spins. In a one-dimensional experiment, as applied in this case, protons are being the detected nuclei at the end of the pulse sequence. The results we yield represent two characteristic modes. In the first case, ligand protons exerting a very strong indirect NOE signal, the so called phenomenon of spin diffusion, will reside on the protein-ligand interface, whereas ligand protons excelling a dominating direct NOE effect, will position themselves on the outer surface of the protein binding cavity. In a second, complementary screening experiment, the principle of the Saturation-Transfer-Difference Spectroscopy (STD) has been deployed and compared. Both methods are based on a difference experiment, between a saturation transfer spectrum and a normal NMR spectrum and provide spectra in which only binding substances are displayed. The STD-NMR experiment is a powerful tool for monitoring ligand binding and for mapping the epitopes of ligands that make contact with the target protein. In this work a one-dimensional STD experiment has been applied, with the proton as the nucleus to be detected. Ligands exerting reversible interactions with the binding domain of the protein, show characteristical relaxation- and mobility differences in comparison to molecules lacking affinity for the protein in question.

These two aforementioned assignment methods have been employed to investigate the

docking properties of 3 different protein-ligand complexes, the nucleotide adenosine monophosphate (AMP) with the enzyme alcohol dehydrogenase (ADH), the coenzyme nicotinate dinucleotide (NAD) with ADH and finally vanillic acid with the transporter protein lipocalin (Q83).

The crucial result was provided by the 1D-AFP-NOESY comparison experiment of the two former complexes, AMP/ADH vs. NAD/ADH. It adduced evidence that both complexes have the substantial similarity in the conformational alignment of the N-glycosidic bonding angle between the ribose and the base adenine, as to to their syn-/anti isomerism.

Part III

Appendix

Appendix A

General Information

All NMR spectral data were recorded with a Varian 500 MHz NMR Spectrometer with a ^2H probe. They were transformed using the software package NMRPipe by Delaglio *et al.* [12]. The NMR samples for the spectroscopic section were dissolved in deuterized 1M D_2O potassium phosphate buffer, at a pH of 6.5.

NMR relaxation data were evaluated using MATLAB [10].

In all pulse sequences narrow magenta bars symbolize 90° pulses, while wide blue bars stand for 180° flip angle. Selective pulses are of 90° flip angle. Unless indicated differently all pulses are applied along the x- axis.

Appendix B

Curriculum Vitae

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PERSONAL DATA

★ Born on 24th October 1984 in Bratislava.

★ Nationality: Dutch/Slovak

EDUCATION

October 2008 - June 2009	Diploma thesis supervised by Prof. R. Konrat (NMR)
October 2006 - May 2008	Part-time employment at the Department of Chromosomal Biology Prof. V. Jantsch Laboratory University of Vienna
2002-10	Studies of Molecular Biology at the University of Vienna
1998-2002	Secondary school "Gymnázium Metodova Ulica" in Bratislava, Slovak Republic with focus on natural sciences
1994-1998	Secondary school "Deutsche Schule Den Haag", in The Hague, the Netherlands
1990-1994	Primary school in Bratislava, Slovak Republic

PROFESSIONAL EXPERIENCE

★ Part-time employment at the Department of Chromosomal Biology, University of Vienna, October 2006 - May 2008.

SKILLS

- ★ Languages: Slovak (mother tongue), Dutch (fluent), English (fluent), German (fluent), French (fluent).
- ★ Computational Skills: Windows (ECDL), Linux, L^AT_EX, Matlab, Mathematica, Java Script.

INTERESTS

- ★ Programming
- ★ Travelling
- ★ Sports: tennis, swimming, skiing, endurance sports, athletics
- ★ Languages and literature

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