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Lanthanide Tag in Search for Paramagnetically Induced Residual Dipolar Couplings in Small Molecules

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ABSTRACT

The structure elucidation of molecules and determination of their characteristic interactions and thermodynamic processes have become routine measurements while using Nuclear Magnetic Resonance (NMR) spectroscopy as an indispensable technique. The theoretical description of NMR spectroscopy and treatment of the dynamics of nuclear spin systems is highly dependent on quantum mechanics. More precisely, Residual Dipolar Coupling (RDC) provides global orientation information of magnetic dipole-dipole interaction in a reference frame and distance-independent angular insights to local relaxation. RDC measurements necessitates the use of an anisotropic orientation medium for the partial alignment of the target molecule with respect to the magnetic field. Such media include liquid crystals or paramagnetic centers with a lanthanide ion. Anisotropic paramagnetic tags have been successful in biomolecular NMR applications of large proteins but their use in small organic molecules remains imperfect due to challenges in designing functional lanthanide-complexes with varying degrees of nondirectional bonding in the Ln(III) inner coordination sphere. In this study, we propose a strategy for the synthesis of a lanthanide tag and the measurement of RDCs in a target organic molecule using several paramagnetic lanthanide complexes.

1. INTRODUCTION

Residual dipolar couplings (RDCs) have become a fundamental approach in structural determination of proteins, nucleic acids, and carbohydrates. RDCs are spectroscopic interactions that appear in high resolution Nuclear Magnetic Resonance (NMR) spectroscopy. Although it was originally discovered for small molecules in liquid crystal solvents,¹ the RDC spectra were too complex for practical use in structure determination. However, the discovery of weak orienting media in aqueous solutions led to a boost in their application in biomolecular analysis. RDCs can provide long-range angular and distance restraints to solve structural ambiguities, an advantage over the conventional Nuclear Overhauser Effect (NOE) analysis, which is limited to 5Å radius.² RDCs are orientation-dependent interactions that are observed in an anisotropic environment provided by suitable alignment media. This anisotropy allows extraction of angular information relative to an external reference, determination of conformations and configurations of molecules and the distinguish of enantiomers.³ While dipolar coupling interactions are dominant in solid-state NMR, they are averaged to zero in liquid-state NMR due to isotropic uniform distribution of the orientations based on the *Rotational Brownian Diffusion*. Consequently, a wealth of structural insights is lost once the dipolar couplings vanish. Nevertheless, once displayed in a homogenous magnetic field together with an alignment media, the molecule adapts a preferred orientation that favors anisotropy. This allows the anisotropic magnetic interactions to become observable.⁴

Using quantum mechanics, the dipolar interaction of nuclear spins rotating at the *Larmor frequency* is described in a simplified two-spin system. Spins I and S with a fixed distance \mathbf{R}_{IS} are oriented at an internuclear angle Θ_{IS} in a static magnetic field \mathbf{B}_0 (**Figures 1a and 1b**). Spin I

induces a magnetic field added to the static magnetic field felt by spin S and causes a shift in its resonance frequency, the dipolar coupling.² Since spins parallel and anti-parallel to \mathbf{B}_0 are equally populated, splitting into the dipolar coupling $2D_{IS}$ is observed and is directly proportional to $3\cos^2\Theta_{IS} - 1$ (**Figure 1c**). This RDC contains valuable structural information and covers a large portion of the NMR timescale allowing its application in small organic molecules⁵⁻⁶, in biomolecules⁷⁻⁸ and in newly discovered types of materials.⁹⁻¹⁰

The behavior of a molecule with \mathbf{B}_0 differs largely if it is in crystal form, in isotropic solution or in anisotropic solution with a partially oriented medium (**Figure 1d**). In the latter case, the quantum mechanical approach of RDCs is based on the averaging process of the anisotropic tumbling motion, which occurs between the molecule being subjected to a magnetic field, and is described by the alignment tensor A .¹¹ The dipolar coupling Hamiltonian H_D depicting the two spins I and S has the form:

$$\overline{H_D} = 2\pi D \left\{ I_{z^L} S_{z^L} - \frac{1}{2} I_{x^L} S_{x^L} - \frac{1}{2} I_{y^L} S_{y^L} \right\} \quad (1)$$

If the spins I and S are heteronuclear, H_D is simplified to:

$$\overline{H_D} = 2\pi D I_{z^L} S_{z^L} \quad (2)$$

and the dipolar coupling constant D is represented by the equation:

$$D = \frac{K}{R^3} \left(\cos^2\Theta_{IS} - \frac{1}{3} \right) \quad (3)$$

K is a term that gathers the physical constants of the spins:

$$K = -\frac{3}{8\pi^2} (\gamma_I \gamma_S \mu_0 \hbar) \quad (4)$$

where $\overline{\gamma_I}$ and $\overline{\gamma_S}$ are the gyromagnetic ratios of spins I and S, respectively, $\overline{\mu_0}$ is the vacuum permeability, and $\overline{\hbar}$ is Planck's constant. It is worth mentioning that D and H_D are both time-

dependent, and therefore, the time-averaged *RDC constant* \overline{D} is represented as:

$$\overline{D} = \frac{K}{R^3} \left(\overline{\cos^2 \Theta_{IS}} - \frac{1}{3} \right) \quad (5)$$

To calculate \overline{D} for any pair of spins, the alignment properties of a molecule in the molecular frame approach have to be first described. In this reference system, the orientation of the magnetic field is time-dependent, the inter-nuclear vector is set constant, and the *probability tensor* \mathbf{P} is introduced. \mathbf{P} is a second order approximation of the orientation probability distribution for the direction of the external magnetic field¹² and is represented by an ellipsoid with fixed orientation in the (x, y, z) molecular frame (**Figure 1e**). The three principal values of \mathbf{P} are the probabilities of finding the magnetic field along the principal axes of the ellipsoid such that

$$\overline{P_x + P_y + P_z} = 1 \quad (6)$$

This simplifies the calculation of the RDC constants and requires only knowledge of the three Cartesian components $\overline{r_x}$, $\overline{r_y}$ and $\overline{r_z}$ of a given inter-nuclear unit vector $\overline{\mathbf{r}}$:

$$\overline{\cos^2 \Theta_{IS}} = \overline{P_x r_x^2 + P_y r_y^2 + P_z r_z^2} \quad (7)$$

The alignment tensor \mathbf{A} of the anisotropic tumbling motion is then extracted from \mathbf{P} and fitted with the principal components:

$$\overline{A} = \overline{P} - \frac{1}{3} \quad (8)$$

$$\overline{A_x + A_y + A_z} = 0 \quad (9)$$

$$\overline{\left(\overline{\cos^2 \Theta_{IS}} - \frac{1}{3} \right)} = \overline{A_x r_x^2 + A_y r_y^2 + A_z r_z^2} \quad (10)$$

The time-averaged RDC representing the direct dipole-dipole interaction for spins \mathbf{I} and \mathbf{S} is represented by the equation:

$$\overline{D}_{IS} = -\frac{\gamma_I \gamma_S \mu_0 \hbar}{16\pi^2} \left\langle \frac{1}{R_{IS}^3} \left(3\overline{\cos^2 \Theta_{IS}} - 1 \right) \right\rangle \quad (11)$$

The observed anisotropic dipolar coupling D_{IS} adds to the scalar J coupling, and both contribute to the total coupling constant T :

$$\underline{|T| = |J + D|} \quad (12)$$

Therefore, two measurement series are required to determine the dipolar coupling D for a spin system; an isotropic spectrum that determines J scalar couplings, and another anisotropic spectrum to measure T total couplings. The difference between the two spectra determines D .

The advancement of RDCs in protein NMR spectroscopy has become a favorable and routine tool for accurate protein solution structure determination.¹³⁻¹⁵ RDCs provide orientation information of magnetic dipole-dipole interaction vectors and its measurement requires a nonisotropic orientation, through a direct or indirect magnetic field alignment, of the protein in solution. The conventional pulse sequences used for coupling measurements, *HMQC and HSQC* experiments, can be recorded without heteronuclear decoupling during acquisition. To decipher RDCs in protein NMR spectroscopy, 2D ^1H - ^{15}N HSQC spectra of isotropic or anisotropic solution decoupled in ^{15}N dimension are usually measured and analyzed (**Figure 1f**). A regular HSQC spectrum of an isotropic solution decoupled in both dimensions shows no observed ^1H - ^{15}N splitting. On the contrary, when the HSQC spectrum of the same isotropic solution is recorded without decoupling in ^{15}N dimension, the splitting becomes observable and is equal to a one-bond scalar coupling $^1J_{\text{NH}}$, bearing a negative value between 92 and 95 Hz. The same ^{15}N decoupled HSQC spectrum but of a partially oriented sample shows positive and negative *RDC* values in addition to the observable ^1H - ^{15}N splitting of the one-bond scalar coupling. This observation demonstrates the benefits and advantages of using an orienting medium to induce partial molecular alignment for target molecules as a method to permit accurate molecular conformation and configuration information in solution NMR via the measurement of dipolar couplings, and consequently internuclear distances.

The most common orienting media used for partially aligning a molecule are liquid crystals, strain-induced alignment gels (SAGs), and anisotropic paramagnetic tags. The application of liquid crystals is limited to small symmetric molecules since they induce high degree of order and consequently large dipolar couplings.⁴ On the other hand, the alignment stretch of SAG is solely determined by mechanical stretching, and its use is limited due to the

lengthy preparation time for solutes to diffuse into the polymer gel.¹⁶ An alternative alignment method, the paramagnetic lanthanide tag, has implemented considerable advances in the field of biomolecular NMR of large proteins, oligonucleotides and drug discovery.¹⁷⁻²⁰ Mainly, this involves a lanthanide ion that orients molecules by the anisotropy of the paramagnetic susceptibility.²¹ Paramagnetic centers have the advantage of providing long-range structural restraints and accordingly ingenious applications in structure determination of proteins, ligand-protein interactions and complex dynamics.^{8, 22} A largely unexplored method in small molecule structural determination is the application of internal alignment using non-covalent paramagnetic tags. So far, the use of lanthanide tags has not been applied to small organic molecules due to the broadening effects in spectral lines of neighboring nuclei covalently attached to the paramagnetic ions.² If the intrinsic binding affinity of a paramagnetic tag is high enough, paramagnetically induced RDCs and pseudo-contact shifts (PCS) will be observed due to the alignment of the molecule with the magnetic field. Importantly, non-covalent tags, also called chiral lanthanide shift reagents, have been widely used to measure relative concentrations of enantiomers. By direct attachment of the lanthanide, the relative mobility of the tag versus the molecule will be minimized, whereas the internal mobility of the small molecule will still exist. Starting from *ab initio* calculation from the structure of the local complex, the alignment tensor will determine the induced RDCs and PCSs from both a paramagnetic and diamagnetic contribution to the magnetic susceptibility anisotropy.

Once the alignment tensor, which is attached to the moiety of the molecule that binds the tag has been determined, mobility can be considered much simpler than in case of external alignment, since the induced alignment tensor is fixed. Several of the chiral lanthanide tags are commercially available or can be easily synthesized in large quantities. In this study, we describe the strategy of synthesizing and applying a dipicolinic acid known for its lanthanide tagging of proteins using a single cysteine residue.²³ The tag 4-mercaptomethyl-dipicolinic acid (4-MMDPA) coordinates metal ions in a non-chiral fashion and can be readily attached to a cysteine thiol group via disulfide bridging. Sulfur chemistry has proven to be a powerful tool in synthetic strategies due to its divergent functions and potencies in different oxidative states which have led to a rich chemistry in organic synthesis.²⁴⁻²⁶ Sulfide bonds have been reported to create molecular junctions in thiophene-based molecules for example,²⁷ while thiols were used to functionalize gold nanoparticles with electro-polymerizable thiophene precursors.²⁸

Using such thiol chemistry, we synthesized the 4-MMDPA tag and covalently attached it to naphthalene-2-thiol through disulfide bonding which is seen as a requirement for the synthesis of many biologically active compounds involved in chemical and biological processes.²⁹ Diamagnetic and paramagnetic lanthanide ions were then applied to compound (**10**) to induce RDC measurements in organic molecules.

2. MATERIALS AND METHODS

Benzotriazole, Dimethyl 2,6-pyridinedicarboxylate, 5,5'-dithiobis(2-nitrobenzoic acid), Naphthalene-2-thiol and pyridine dicarboxylic acid were purchased from Sigma Aldrich Chemie GmbH (Schnelldorf, Germany). All lanthanide compounds were purchased from Merck Chemicals GmbH (Darmstadt, Germany). Deuterated solvents were purchased from Deutero GmbH (Kastellaun, Germany). All NMR measurements were recorded at 25 °C on a BRUKER DRX 400 spectrometer and were referenced internally to TMS. The measured RDCs were obtained from the ¹³C dimension of the 2D ¹H-¹³C-HSQC spectra recorded without carbon decoupling and with a digital resolution of 0.4 Hz/data point. Electron Ionization Mode (EI-HRMS) analysis was conducted on Thermo Fischer Scientific 8230 Finnigan MAT Double Focusing Mass spectrometer with BE Geometry and EI mode at 70 eV (Bremen, Germany).

Mono-Hydroxymethylation: Synthesis of 2,6-dimethoxy carbonyl -4-hydroxyl methylpyridine (**2**) – Compound (**1**) was first identified by ¹H NMR (400 MHz, CDCl₃) δ 8.32 (d, *J* = 7.87 Hz, 2H); δ 8.05 (t, *J* = 7.84 Hz, 1H); δ 4.01 (s, 6H), melting point 121 °C. (**1**) (2.226 g, 11.4 mmol) was added to sulfuric acid (30% v/v). A saturated aqueous solution of FeSO₄·7H₂O was added dropwise followed by H₂O₂ solution (30% v/v, 131 mmol) with temperature control between 42–45 °C. The reaction was stirred for an additional hour at room temperature and the solution was pH 6.4. The suspension was filtered and extracted with ethyl acetate. The organic phase was dried, filtered, and evaporated to obtain product (**2**). After column chromatography (SiO₂, 100% ethyl acetate), product (**2**) (0.665 g, yield: 26%) was identified by ¹H NMR (400 MHz, CDCl₃): δ 8.31 (s, 2H); δ 4.91 (s, 2H); δ 4.01 (s, 6H). This reaction was repeated while varying the concentrations of H₂O₂ and H₂SO₄ and at different temperatures. The optimal conditions are the ones mentioned above.

Tosylation: Synthesis of 2,6-dimethoxy carbonyl -4-tosyl oxymethylpyridine (**3**) – A solution

of *p*-toluene sulfonyl chloride (0.4 g, 1.51 mmol) and dichloromethane was added dropwise to a cold solution containing product **(2)** (0.665 g, 2.96 mmol) in dichloromethane. Under stirring at 0 °C, triethylamine (0.8 ml) was added dropwise in three portions at 30 minutes interval. The mixture was diluted with ethyl acetate and the organic phase was extracted with water and 3M hydrochloric acid. The tosylated product **(3)** (0.576 g, yield: 86.5%) was identified by ¹H NMR (400 MHz, CDCl₃): δ 8.17 (s, 2H); δ 7.81 (d, *J* = 8.31 Hz, 2H); δ 7.36 (d, *J* = 8.24 Hz, 2H); δ 5.19 (s, 2H); δ 4.01 (s, 6H); δ 2.45 (s, 3H).

Bromination: Synthesis of 2,6-Dimethoxy carbonyl –4-bromo methylpyridine **(4)** – Lithium bromide (0.400 g, 4.60 mmol) was added to a solution containing product **(3)** (0.576 g, 1.524 mmol). The solution was stirred at room temperature for 3 hours and the suspension filtered, concentrated under vacuum and the residues triturated with chloroform. The product was dissolved in ethyl acetate and purified by preparative thin layer chromatography to obtain the brominated compound **(4)** (0.374 g, yield: 65%). ¹H NMR (400 MHz, CDCl₃): δ 8.32 (s, 2H); δ 4.50 (s, 2H); δ 4.03 (s, 6H), melting point 114 °C.

Thiolation: Synthesis of 4-Mercaptomethyl–2,6–pyridinecarboxylic acid **(6)** – Under nitrogen atmosphere, a solution of **(4)** (0.120 g, 0.418 mmol) and thiourea (0.060 g, 0.788 mmol) in methanol was refluxed overnight. The exchange of the bromine by the thiol functional group yielded product **(5)**, which was then dissolved in deoxygenated water and sodium hydroxide. The mixture was refluxed overnight under nitrogen atmosphere. Product **(6)** was obtained (0.07 g, 58.3%) ¹H NMR (400 MHz, D₂O): δ 8.28 (s, 2H); δ 3.86 (s, 2H), melting point 140 °C, EI–HRMS C₈H₇NO₄S M–H 212.0016.

Disulfide Bridging: Synthesis of 4-(Naphtalen-2-yl-disulfanyl)-pyridine-2,6,-dicarboxylic acid **(10)** – 5,5'-Dithio-Bis-(2-Nitrobenzoic acid) (DTNB) **(8)** was identified by ¹H NMR (400 MHz, D₂O) δ 7.97 (d, *J* = 8.76 Hz, 2H); δ 7.59 (d, *J* = 8.92 Hz, 2H); δ 7.53 (m, 2H). **(8)** (1 g, 2.53 mmol) was dissolved in 1:1 methanol/H₂O solution. Naphtalene-2-thiol **(7)** (0.4 g, 2.49 mmol) was added dropwise into DTNB solution. **(6)** (0.02 g, 0.094 mmol) was poured over a solution containing **(7)** and **(8)**. The resulting compound (0.3 g, 76.2%) was concentrated, extracted with diethyl ether and identified by ¹H NMR spectroscopy as compound **(10)**.

Incorporation of Lanthanide Ion: A solution of the tagged molecule **(10)** (1.67 mg) was dissolved in DMSO and different lanthanide ions were added as follows: LaCl₃·7H₂O (3.7 mg) in

1:1 ratio (10 mM); Eu(FOD)₃ (2.068 mg) in 0.2:1 ratio (2 mM); Eu₂(SO₄)₃ (1.184 mg) in 0.2:1 ratio (2 mM); Eu(FOD)₃ (5.17 mg) in 0.5:1 ratio (5 mM); Eu₂(SO₄)₃ (2.959 mg) in 0.5:1 ratio (5 mM); Eu₂(SO₄)₃ (5.918 mg) in 1:1 ratio (10 mM); Tb(NO₃)₃ (3.809 mg) in 0.5:1 ratio (10 mM).

3. RESULTS AND DISCUSSION

The design of functional lanthanide-containing coordination compounds requires the precise control of the lanthanide inner coordination sphere.³⁰ We propose a strategy for measuring RDCs in organic compounds using paramagnetic lanthanide tags. The synthesis of 4-MMDPA (**6**) is outlined in **Figure 2** based on modified literature methods.^{23, 31} The starting material Dimethyl 2,6-pyridinedicarboxylate (**1**) undergoes a direct nucleophilic aromatic substitution to incorporate a mono-hydroxymethyl group (**2**), which is then protected by tosylation (**3**). The tosyl group is by nature electron-withdrawing hence readily substituted by bromine. The resulting bromomethyl pyridine (**4**) undergoes thiolation and generates product (**5**) which upon deprotection of its carboxylic moieties yields the desired product 4-MMDPA (**6**). All intermediate products were identified by 1D ¹H NMR spectroscopy (**Figure 2 and Supplementary Figure 1**) and chemical shift values were accordingly assigned (**Table 1**).

The above reactions were successful after optimizing different conditions. For instance, the temperature control of the mono hydroxymethylation of the starting material (**1**) to yield intermediate (**2**) was most challenging. At 10 °C, no appreciable conversion of (**1**) to (**2**) was detected, but after increasing the reaction temperature up to 48 °C, a peak rise was observed (singlet at 8.32 ppm), between the doublet of the starting material, indicating production of the desired intermediate (**2**) (**Supplementary Figure 2**). At 50 °C, this desirable intermediate is the major product of the incomplete reaction and shows a sharp resolved singlet between the doublet of the residual starting material. However, the appearance of a new peak on the right side of the doublet (8.29 ppm) indicates the production of an undesirable by-product; which indeed is pronounced in higher yields as the temperature is beyond 50 °C; accompanied by the disappearance of the desired product and its singlet peak at 8.32. (**Supplementary Figure 2**). Most of the methods developed for the synthesis of unsymmetrical disulfides involve nucleophilic substitution of a sulfonyl derivative by a thiol or its derivative. However, the preparation of the sulfonyl intermediate requires several steps and the use of toxic chlorinating agents such as SOCl₂ and Cl₂. Our attempt to prepare the unsymmetrical disulfide compound

from the corresponding thiols, 4-MMDPA (**6**) and naphthalene-2-thiol (**7**), followed a modified approach with milder conditions that was adopted from disulfide bridging methods of protein chemistry. 5,5'-Dithiobis (2-nitrobenzoic acid), **DTNB**, also referred to as Ellman's reagent, is a symmetrical disulfide commonly used in analytical biochemistry, particularly in labeling cysteine residues. 4-MMDPA can be readily attached to a cysteine thiol group of a protein via a disulfide bridge using established DTNB chemistry.³² DTNB breaks its disulfide bridge to form the thiol nitrobenzoic acid, which forms a disulfide bond to a thiol-containing compound. We have therefore depicted this method to couple naphthalene-2-thiol (**7**) with 4-MMDPA (**6**) (**synthesis scheme reported in Figure 3** and ¹H NMR spectra reported in **Supplementary Figure 3**). DTNB (**8**) reacts with naphthalene-2-thiol (**7**) to generate the disulfide intermediate product (**9**). 4-MMDPA (**6**) readily exchanges the TNB moiety of (**9**) to yield the desired asymmetrical disulfide product (**10**).

Paramagnetic lanthanides are capable of orienting the target molecule in high magnetic fields where each lanthanide orients according to its anisotropic magnetic susceptibility tensor.³³⁻³⁴ The principle states that when a paramagnetic lanthanide ion is introduced to a solution, a series of shifted resonances appear in the ¹H spectrum far outside the spectral range. But with an appropriate order of alignment, RDC measurements are obtained within spectral range from the differences of the paramagnetic and diamagnetic states. The paramagnetic equivalents of scalar couplings, *the contact shifts*, are restricted to nuclei in the immediate vicinity of lanthanides. This induces pseudocontact shifts (PCSs), paramagnetic relaxation enhancements (PREs), and RDCs as the three major effects caused by paramagnetic lanthanide ions in the NMR spectra. Paramagnetic lanthanides provide long-range structural restraints where *PCS* can reach 40 Å from the metal ion and *RDCs* pertain to the entire molecular system due to paramagnetically induced alignment in the magnetic field.^{21, 35-36} Lanthanide ions behave differently when placed in the same chemical environment. This results in different magnitudes of the magnetic susceptibility tensor and its associated anisotropy.³⁷⁻³⁸ Consequently, the lanthanides are ranked according to the magnitude of this anisotropic component as highly paramagnetic (Dy³⁺, Tb³⁺ and Tm³⁺), moderately paramagnetic (Er³⁺ and Yb³⁺), and little paramagnetic (Eu³⁺, Ce³⁺ and Sm³⁺). Importantly, there are also diamagnetic lanthanides such as La³⁺, Y³⁺, Lu³⁺ and Sc³⁺ that serve as diamagnetic references. A diamagnetic reference presents the target molecule-metal complex in its diamagnetic state and allows *RDCs* measurements from the differences of the

paramagnetic and diamagnetic states. The diamagnetic properties and chemical shifts in particular are sensitive to metal binding,³⁵ nevertheless, the molecule of interest must be site-specifically labeled with a lanthanide in order to fully exploit the lanthanide paramagnetism.

We have investigated the effect of paramagnetic lanthanides by selecting four lanthanide reagents: (a) Lanthanum chloride heptahydrate ($\text{LaCl}_3 \cdot 7\text{H}_2\text{O}$) as a diamagnetic reference; (b) Europium as a moderately paramagnetic lanthanide ion in its organic state, $\text{Eu}(\text{FOD})_3$ (tris (6,6,7,7,8,8,8-heptafluoro-2,2-dimethyl-3,5-octanedionato) europium consisting of three bidentate acetylacetonato ligands bound to Eu^{3+} center, and inorganic state ($\text{Eu}_2(\text{SO}_4)_3$) and (c) Terbium (III) nitrate pentahydrate ($\text{Tb}(\text{NO}_3)_3 \cdot 5\text{H}_2\text{O}$) for its highly paramagnetic effect. ^1H NMR and ^{13}C HSQC experiments were first recorded for the diamagnetic reference (1:1 Tag- $\text{LaCl}_3 \cdot 7\text{H}_2\text{O}$ solution, 10 mM) The ^1H NMR spectra (**Figure 4**) showed chemical shifts and peak broadening. A difference in the J scalar coupling of the C-H bond of the naphthalene moiety of 3.53 Hz was observed in the ^1H - ^{13}C HSQC set as J_{dia} , the scalar coupling from the diamagnetic reference. It is worth mentioning a diamagnetic reference, in this case La^{3+} , represents the target molecule-metal complex in its diamagnetic state and allows accurate $RDCs$ measurements from the differences of the paramagnetic and diamagnetic states. The diamagnetic properties and chemical shifts are sensitive to metal binding, nevertheless, the molecule of interest must be site-specifically labeled with a lanthanide to fully exploit the lanthanide paramagnetism. The splitting of 3.5 Hz is a clear indication to why it is important to use a diamagnetic reference to report the total coupling rather than using the free tag as a reference. The paramagnetic compound induces shifts in the protons near the Lewis basic sites of the molecule. We measured 1D ^1H and 2D ^{13}C HSQC experiments on three samples of the lanthanide tag (10 mM) while varying $\text{Eu}(\text{FOD})_3$ concentrations (2 mM, 5 mM and 10 mM). The proton ^1H spectra (**Figure 4**) showed broadening of the peaks and loss of resolution with increasing concentration accompanied with changes in the value of the *total coupling* T . The differences between T and J_{dia} correlate directly to the $RDCs$ values. Indeed, the differences were significant (between 0.76 and 2.21 Hz). Similarly, we measured 1D ^1H and 2D ^{13}C HSQC experiments of the lanthanide tag (10 mM) while varying concentrations of $\text{Eu}_2(\text{SO}_4)_3$ (2 mM, 5 mM, and 10 mM). The proton ^1H spectra (**Figure 4**) again showed dramatic peak broadening and loss of resolution as a function of lanthanide concentration. However, the broadening is more significant than in the $\text{Eu}(\text{FOD})_3$ measurements since the sulfate ions behave differently in solution than the organic ligand, and the Eu ions are

more readily available to induce paramagnetism. Similarly, ^{13}C HSQC experiments (**Figure 4**) showed again significant changes in the value of the total coupling T . The differences between T and J_{dia} in the case of $\text{Eu}_2(\text{SO}_4)_3$ (3.93 and 5.37 Hz) were larger than the values obtained with $\text{Eu}(\text{FOD})_3$. All RDCs extracted from the 2D ^1H - ^{13}C HSQC spectra of the free tag, and in complex with the diamagnetic lanthanide LaCl_3 , or the paramagnetic lanthanides $\text{Eu}(\text{FOD})_3$, $\text{Eu}_2(\text{SO}_4)_3$, $\text{Tb}(\text{NO}_3)_3$ are summarized in **Supplementary Figure 4**. RDCs are based on J coupling constants and total coupling constants T for all complexes and include the analysis of errors.

Moreover, the highest lanthanide concentration induced a strong paramagnetic interaction which resulted in peak broadening beyond detection. The ^1H NMR spectrum of the lanthanide tag in presence of $\text{Tb}(\text{NO}_3)_3$ (10mM) proved the strong paramagnetic effect on peak broadening and dramatic loss in resolution. Upon inspection of 1D ^1H NMR spectra of the 4-MMDPA-naphthalene 2-thiol with different lanthanide concentrations (**Figure 4**), the change in intensity demonstrated that there was partial binding of the lanthanide by the time one molar equivalent had been reached in the titration. This made it possible to confer partial alignment and measure small RDCs. The choice of a paramagnetic ion with a higher magnetic moment (Tb^{3+}) induced a higher degree of alignment upon binding as reflected by signal broadening. It is important to mention that the extraction of accurate and small proton-carbon coupling constants is often complicated and made more difficult by the low natural abundance of ^{13}C and spectral overlap. While large one bond proton-carbon coupling constants ($^1J_{\text{HC}}$) can be determined easily from ^{13}C satellites, J values between 0 and 15 Hz are typically much more difficult to be measured and complicated by the similar magnitude of proton-proton and proton-carbon coupling constants.³⁹⁻
⁴⁰ To overcome this, the introduction of H-C coupling in the directly detected dimension by excluding carbon decoupling is a valid approach, rather than in the indirect dimension and omitting the ^1H refocusing pi-pulse, might solve spectral overlap but at a cost of decreasing the overall signal to noise ratio.

4-MMDPA coordinates metal ions in a nonchiral fashion and is readily attached to a cysteine thiol group via a disulfide bridge. Lanthanides bind DPA with nanomolar affinity for the first DPA ligand with decreasing affinities for the additional ligands up to the complex $[\text{Ln}(\text{DPA})_3]^{3-}$.⁴¹⁻⁴² In presence of the lanthanide ion, a target molecule tagged to one unit of 4-MMDPA can further strengthen its coordination using carboxyl groups, if available, and act as

additional anchors for tethering the lanthanide ion. Simultaneous coordination of the lanthanide ion by 4-MMDPA and one or several carboxyl groups provides high-affinity lanthanide binding sites that immobilize the lanthanide ion with respect to the target molecule. Importantly, the lanthanide ion must be rigidly attached to the target molecule to avoid averaging due to tag mobility since RDCs are greatly reduced when lanthanide tag reorientates with respect to the protein.⁴³⁻⁴⁴ This rigidity attachment is accomplished either by tethering the lanthanide tag simultaneously to two different attachment sites of the target molecule⁴⁵⁻⁴⁷ or by using a bulky lanthanide tag for which reorientation is prevented by steric hindrance.^{44, 48} However, lanthanide tags that are anchored to two different sites, such as the case of a protein via two disulfide bonds, immobilize the tag effectively with respect to the protein. Yet, this requires two cysteine residues with thiol groups positioned at the correct distance to react with a single tag molecule.⁴⁹⁻⁵⁰

Our attempt to determine the alignment tensor of the 4-MMDPA naphthalene-2-thiol system was unsuccessful due to signal overlap and broadening of all spin pairs other than 1H-C. It is accepted that the size of the observed *RDCs* is directly proportional to the degree of order induced by the anisotropic medium. This degree of orientation should be small otherwise the spectra become too complex to be interpreted. It is indeed essential to maintain both the weak alignment region, where D is smaller than J , and the quality of the high-resolution NMR spectra so that dipolar coupling constants can be measured easily by comparing line splitting in isotropic and in the aligned samples. However, due to signals overlap of H2, H3, H4, H5 and H6 in the spectrum measured at 400 MHz and signal broadening after the addition of a lanthanide ion, we were limited in using H7 for unambiguous determination of the RDCs. Although this approach proves the concept, a higher magnetic field spectrometer will enhance the quality of the spectra and allow the use of more H-C spin pairs in the analysis.

On the other hand, the single covalent bond between the tag and the thiol group of the target molecule may trigger some flexibility. The presence of such conformational flexibility develops serious consequences in data interpretation and with the structure determination approaches used for rigid compounds as they are inapplicable to flexible compounds. This is due to the simultaneous observation of two averaging processes one from the tumbling motion of the compound and the second due to conformational flexibility. The outcome is an averaging of distances and angles, thus making their use much more complicated. So far, few applications of *RDCs* in flexible molecules have been published; assuming a restricted number of conformers to

be present.^{5, 51}

Metal ions behave as chiral centers such that enantiomers of the metal chelate result in diastereomeric protein-tag complexes, thereby doubling the number of NMR peaks.⁵² As a result, the tags' enantiomeric purity prevents peak doubling caused by metal coordination.⁵³ Moreover, the proximity of the metal ion to the protein is also significant, because accurate measurement of the alignment tensor A necessitates appropriate sampling of the region around the metal ion.⁵⁴ Interactions with the alignment media change the structure of the macromolecule and, as a result, affect the RDCs, therefore alignment by paramagnetic lanthanides rather than alignment media is potentially appealing. Furthermore, lanthanide tags placed at different places on the molecule, or several tags placed at the same region, conveniently offer varied alignment orientations, which are crucial for thorough structural and dynamics research.

Paramagnetism causes line broadening for nuclear spins close to the metal ion, making it difficult to measure small RDCs for such nuclei. However, the alignment is independent of the molecule's molecular weight, implying that a single lanthanide ion might be capable of aligning massive particles. Even though diamagnetic susceptibility grows with molecular size and may exceed paramagnetic susceptibility, an experiment omitting a diamagnetic reference effects would reflect the paramagnetic component of molecular alignment.

4. CONCLUSION

A largely unexplored method in small molecule structural determination is the use of internal alignment through non-covalent paramagnetic tags. If the intrinsic binding affinity of a paramagnetic tag is high enough, paramagnetically induced RDCs and PCS will be observed due to the alignment of the molecule with the magnetic field. The non-covalent tag $[\text{Ln}(\text{pyridine-2,6-dicarboxylic acid})_3]^{3-}$, $(\text{Ln}(\text{DPA})_3)^{3-}$ is highly efficient in inducing RDCs and PCSs in biomolecules despite the large number of possible attachment sites. In case of small molecules only a limited number of attachment sites are available suggesting that non-covalent tagging might be even more applicable. Importantly, non-covalent tags are used since decades as chiral lanthanide shift reagents to measure relative concentrations of enantiomers.⁵⁵ However, lanthanide induced RDCs for determination of relative and absolute configurations of small

molecules are very rarely used due to the fact that lanthanide shift reagents were introduced well before the tools to determine conformations accurately enough were available. Also, the computational tools to use PCSs as restraints were not available. By direct attachment of the lanthanide, the relative mobility of the tag versus the molecule will be minimized, whereas the internal mobility of the small molecule will still exist. Calculation of the local complex *ab initio* alignment tensor from the structure that determines the induced RDCs and PCSs will then be possible. Once the alignment tensor, which is attached to the moiety of the molecule that binds the tag has been determined, mobility can be considered much simpler than in case of external alignment, since the induced alignment tensor is fixed.

In this study, we propose the application of a chiral paramagnetic lanthanide tag (4-MMDPA) to induce partial alignment in a target molecule (naphthalene-2-thiol) and measure residual dipolar couplings RDCs. A diamagnetic reference (LaCl_3) is compared with different paramagnetic lanthanides ($\text{Eu}(\text{FOD})_3$, $\text{Eu}_2(\text{SO}_4)_3$ and $\text{Tb}(\text{NO}_3)_3$). Once a proper alignment within the magnetic field is achieved, the paramagnetic solution shows total coupling constants, T , and the differences with the scalar couplings from the diamagnetic solution, J , would mark the RDC constants, D . Paramagnetic broadening yields interactions between the NMR-active nuclei and the paramagnetic electrons. Consequently, this shortens the spin-lattice relaxation times of the nuclei and causes uncertainty broadening and loss of resolution. The NMR signals of nuclei near the paramagnetic center are easily broadened beyond detection depending on the magnitude of the paramagnetic dipole moment of the metal ion. This method allows structural determination of small molecules with accurate molecular conformation and configuration information. The lanthanide tag method is widely used in protein structural determination, while no or little research has been done with small molecules due to the broadening effect. The best lanthanide tag is not the one that binds lanthanides very tight, but the one that binds lanthanides with sufficiently rapid exchange kinetics. Moreover, the best metal ions for RDC measurements are those with a highly anisotropic tensor. This strategy broadens the application of paramagnetic lanthanide tags for the measurement of RDCs in organic molecules.

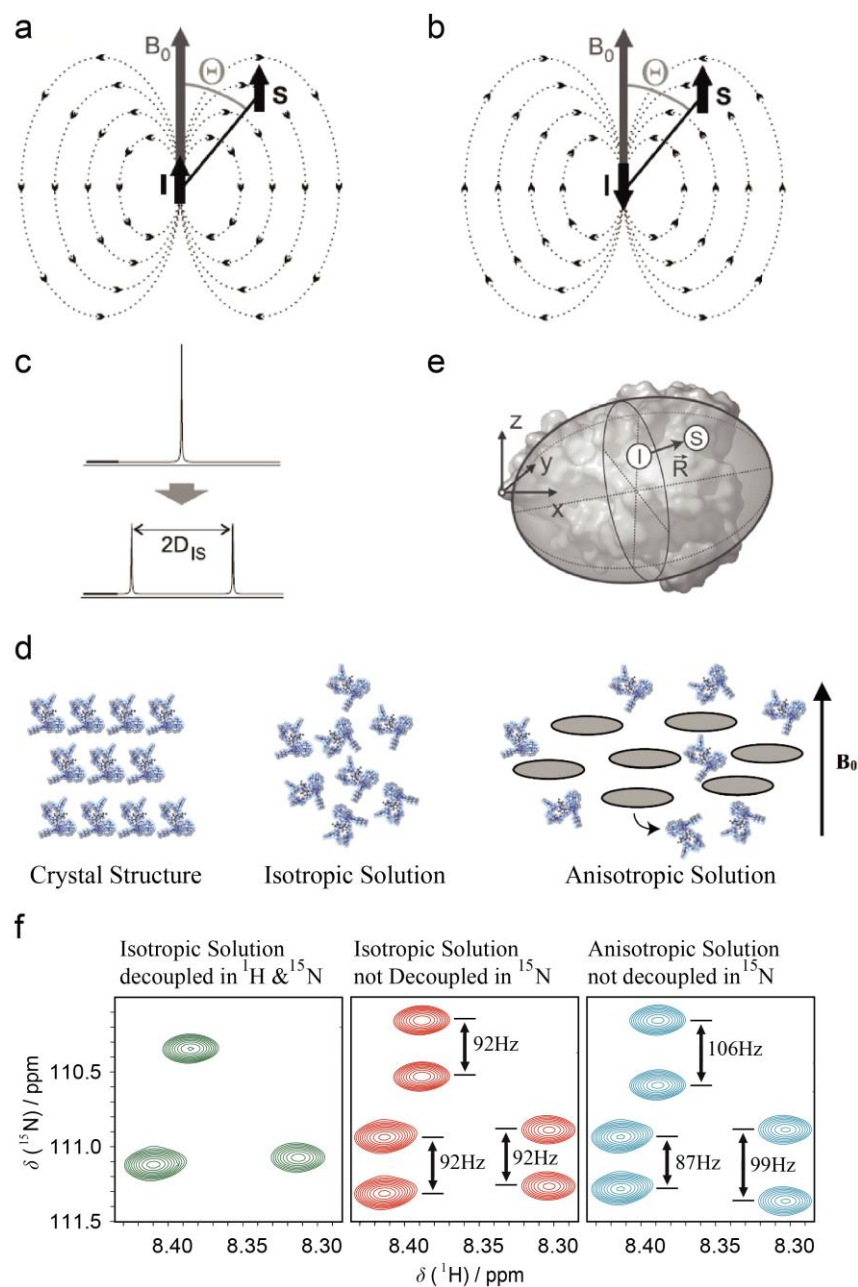


Figure 1. Residual Dipolar Couplings in NMR Spectroscopy. Dipolar interaction between spins I and S (a) parallel and (b) anti-parallel to the magnetic field B_0 . (c) Dipolar coupling splitting $2D_{IS}$ relative to B_0 . (d) The behavior of a molecule with the magnetic field B_0 depends on physical properties. In its crystal form, the dipolar couplings are too large, in an isotropic solution the dipolar interaction average to zero due to tumbling and no coupling is observed, whereas in an anisotropic solution with a partially oriented medium, small residual dipolar couplings are visible. (e) Representation of the probability tensor P in the molecular frame reference system.¹¹ (f) 2D ^1H - ^{15}N HSQC spectra of a protein where each peak corresponds to the N-H resonance of an amino acid of the backbone. The spectra in green and red correspond to an isotropic solution decoupled in both ^1H and ^{15}N dimensions or only ^1H , respectively. The latter spectrum shows the same ^{15}N - ^1H splitting for every amino acid peak and represents ^{15}N - ^1H one-bond scalar coupling. The blue spectrum corresponds to the anisotropic solution without decoupling in ^{15}N dimension. The partial orientation allows observation of ^{15}N - ^1H splitting and represents ^{15}N - ^1H one-bond scalar coupling in addition to the residual dipolar coupling RDC, the total coupling.

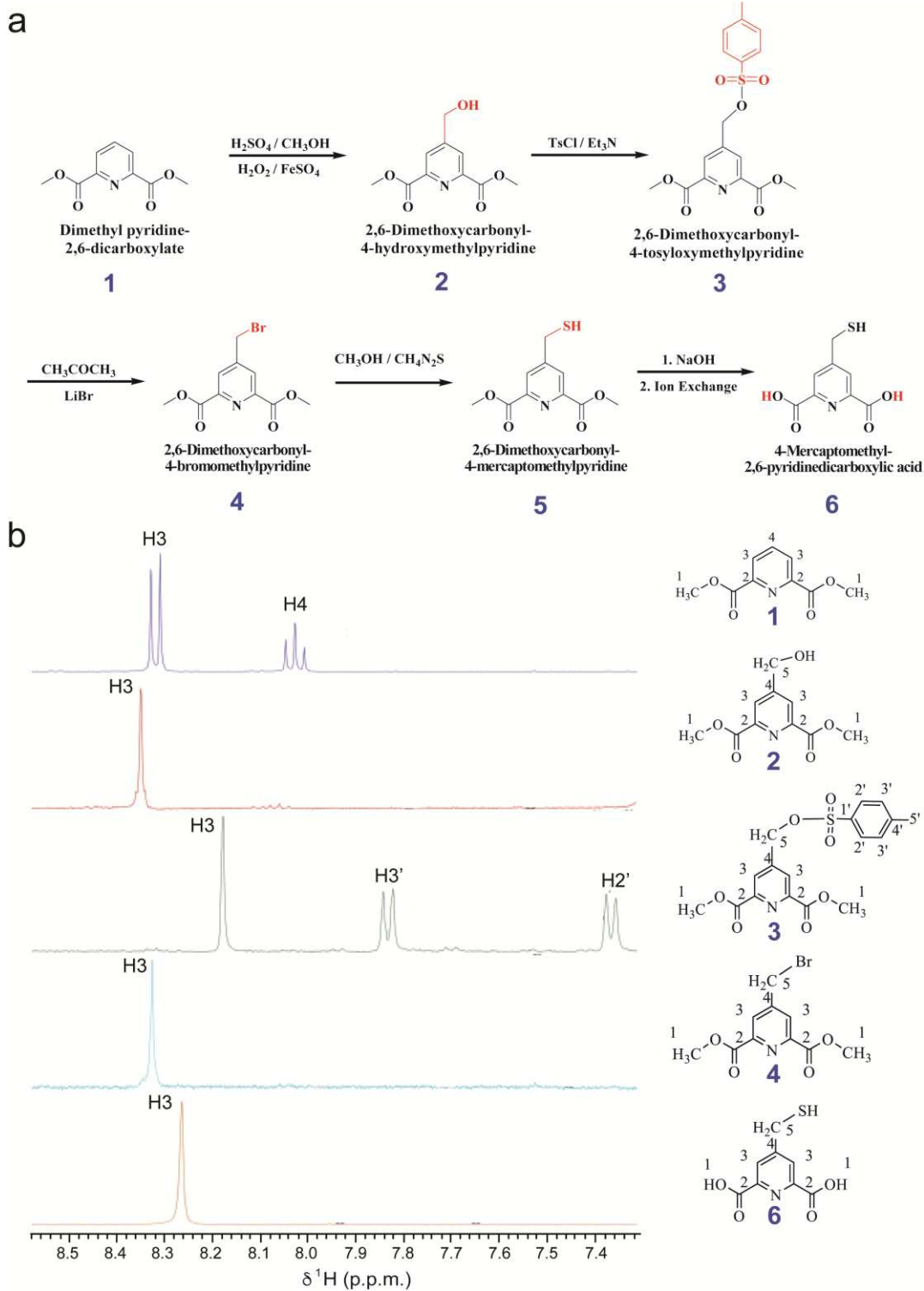


Figure 2. Strategy for the synthesis of 4-MMDPA. (a) synthesis of 4-MMDPA (b) 1D ^1H NMR spectra showing the aromatic region of the intermediates generated along the pathway of 4-MMDPA synthesis. The full spectra of all intermediates is reported in Supplementary Figure 1. The color labeling of atoms highlights the substitution of certain functional groups in every step.

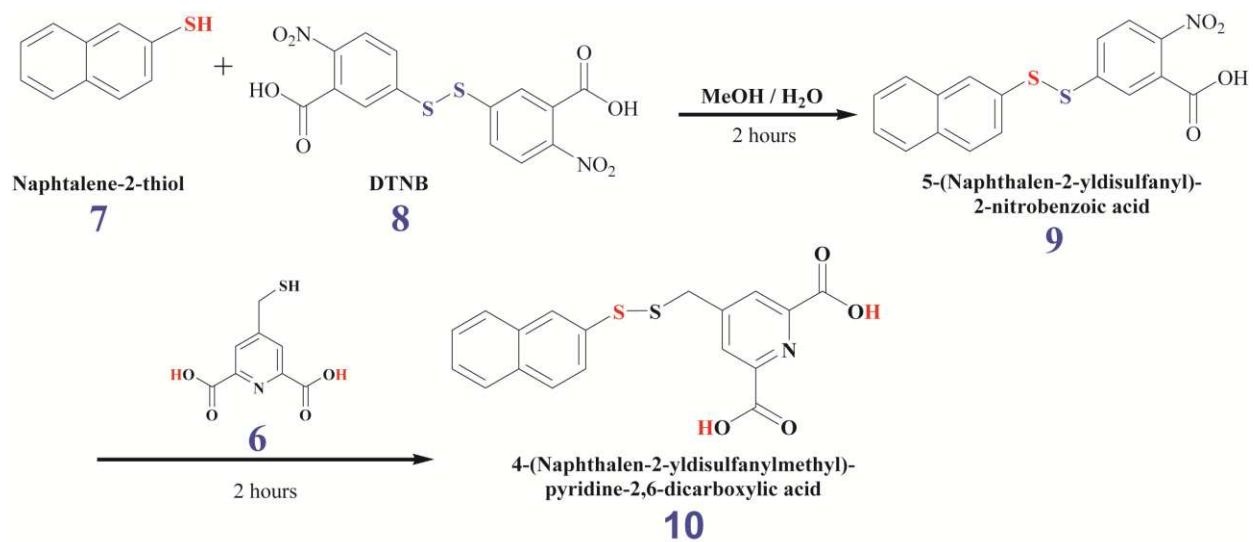


Figure 3. Synthesis of the unsymmetrical disulfide between 4-MMDPA and naphthalene-2-thiol using DTNB chemistry. The color labeling of atoms highlights the substitution of certain functional groups in every step.

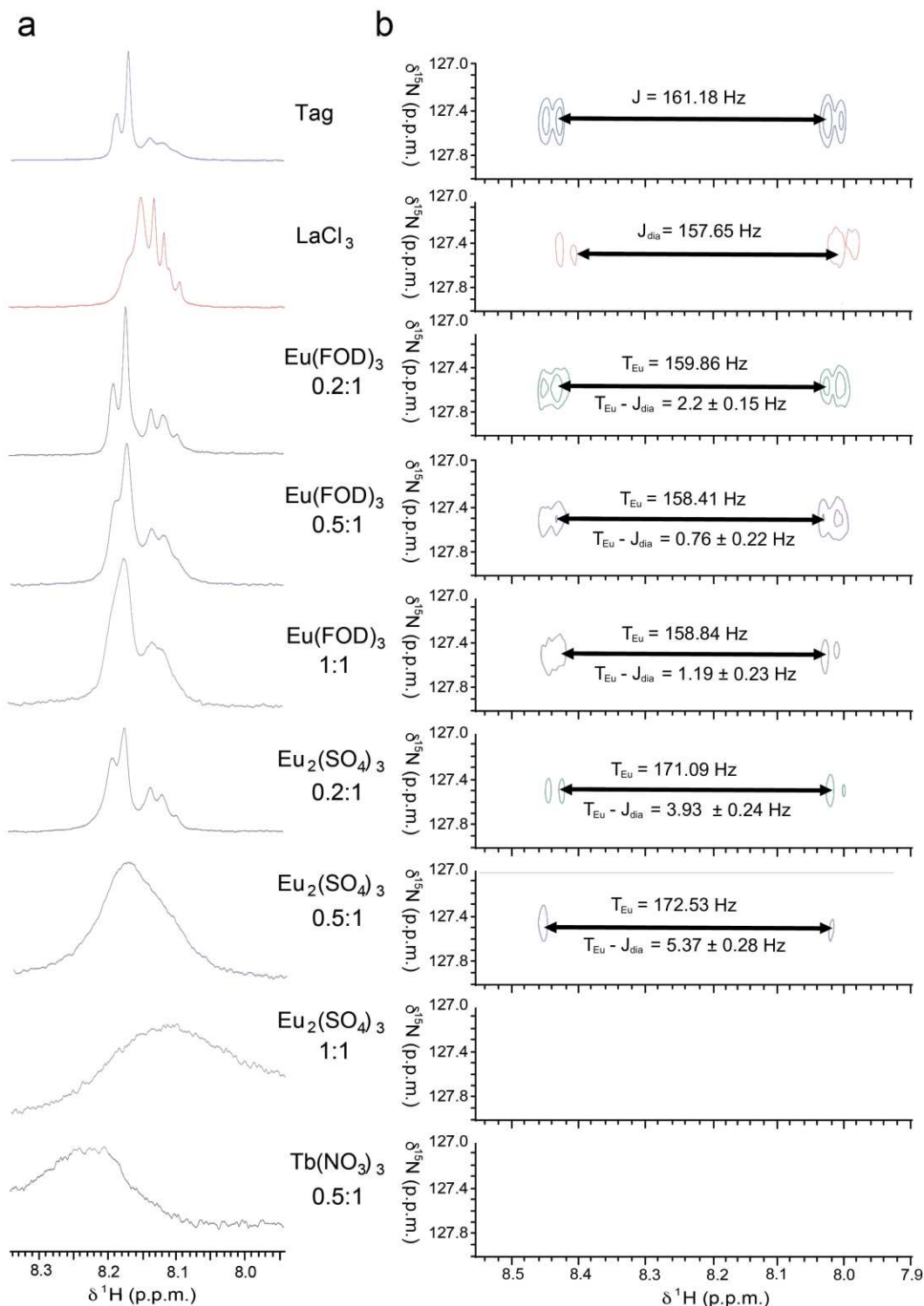
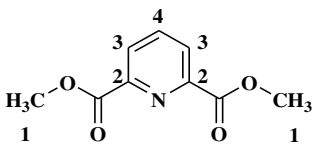
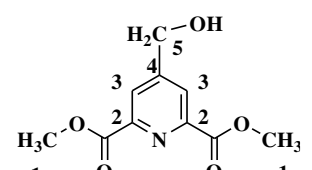
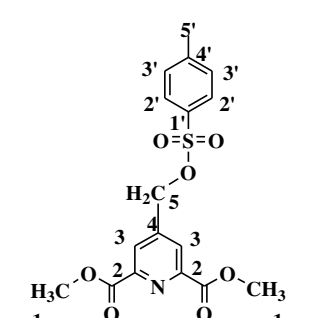
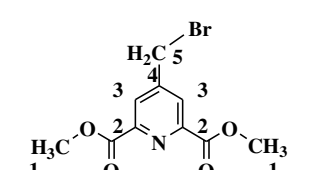
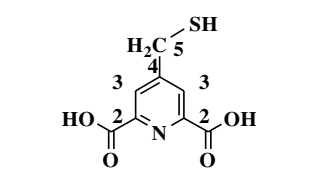


Figure 4. RDC Measurements in Lanthanide tagged 4-MMDPA naphthalene-2-thiol. (a) 1D ¹H NMR and (b) 2D ¹H-¹³C HSQC spectra of the free tag, and in complex with the diamagnetic lanthanide LaCl₃, or the paramagnetic lanthanides Eu(FOD)₃, Eu₂(SO₄)₃, Tb(NO₃)₃. The J coupling constants and total coupling constants T for all complexes are shown on the 2D spectra. The error ranges in RDC measurements are evaluated from the contour levels and the peak intensity. H7 of Naphthalene thiol is reported in the spectra.

Table 1. ^1H NMR Chemical Shifts of the Intermediate Products in the synthesis of 4-MMDPA.

Product	Structure	^1H NMR
<i>Dimethyl pyridine-2,6-dicarboxylate</i>		8.32 (d, 2H, H ₃) 8.05 (t, 1H, H ₄) 4.01 (s, 6H, H ₁)
<i>2,6-Dimethoxycarbonyl-4-hydroxymethylpyridine</i>		8.31 (s, 2H, H ₃) 4.91 (s, 2H, H ₅) 4.01 (s, 6H, H ₁)
<i>2,6-Dimethoxycarbonyl-4-tosyloxymethylpyridine</i>		8.17 (s, 2H, H ₃) 7.81 (d, 2H, H _{3'}) 7.36 (d, 2H, H _{2'}) 5.19 (s, 2H, H ₅) 4.01 (s, 6H, H ₁) 2.45 (s, 3H, H _{5'})
<i>2,6-Dimethoxycarbonyl-4-bromomethylpyridine</i>		8.32 (s, 2H, H ₃) 4.50 (s, 2H, H ₅) 4.03 (s, 6H, H ₁)
<i>4-Mercaptomethyl-2,6-pyridinedicarboxylic acid</i>		8.28 (s, 2H, H ₃) 3.86 (s, 2H, H ₅)

ASSOCIATED CONTENT

Supporting Information.

The following files are available free of charge.

Synthesis of 4-MMDPA followed by NMR spectroscopy (PDF)

Optimization of the monohydroxy methylation step (PDF)

NMR spectra of DPA and Naphthalene Thiol (PDF)

Representation of RDCs extracted from 2D NMR spectra (PDF)

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Author Contributions

A. Yassin and S. El Kantar performed organic synthesis and analyzed the data, S. Obeid analyzed the data, E. Akoury designed the study, performed organic synthesis, conducted NMR experiments, analyzed the data, and wrote the manuscript.

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ABBREVIATIONS

NMR, nuclear magnetic resonance, MMDPA, 4-mercaptomethyl-dipicolinic acid, RDC, residual dipolar coupling

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