Dynamic Stereoselection of Peptide Helicates and Their Selective Labeling of DNA Replication Foci in Cells

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Dedicated to baby Vega

Abstract: Although largely overlooked in peptide engineering, coordination chemistry offers a new set of interactions that opens unexplored design opportunities for developing complex molecular structures. In this context, we report new artificial peptide ligands that fold into chiral helicates in the presence of labile metal ions such as Fe(II) and Co(II). Heterochiral β -turn promoting sequences encode the stereoselective folding of the peptide ligands and define the physicochemical properties of their corresponding metal complexes. CD and NMR spectroscopy in combination with computational methods allowed us to identify and determine the structure of two isochiral $\Delta\Delta$ -helicates, folded as topological isomers. Finally, in addition to the *in vitro* characterization of their selective binding to DNA three-way junctions, cell microscopy experiments demonstrated that a rhodamine-labeled Fe(II) helicate was internalized and selectively stains DNA replication factories in functional cells.

Introduction

Metal ions offer vast opportunities for the structural control at the molecular scale; exploiting their different coordinative properties, researchers can create complex assemblies of great beauty and unique properties,¹ on par with the extraordinary structural and functional complexity displayed by peptides.² Bridging the architectural potential of coordination chemistry with the structural predictability of peptide scaffolds would allow greater complexity in (supra)molecular designs by combining well-established peptide engineering concepts and tools with the supramolecular organization mediated by metal ions.³

Helicates are discrete metal complexes in which one or more organic ligands coil around—and coordinate—two or more metal ions.⁴ Helicates display helical chirality, according to the orientation in which the ligands twist around the axis defined by the metal centers. Besides their fundamental interest in supramolecular chemistry,⁵ helicates have also shown exciting properties as antifreeze agents,⁶ as inhibitors of amyloid aggregation,⁷ as well as G-quadruplex and three-way DNA binding properties that result in promising antimicrobial and antitumoral activities.⁸ Therefore, as a challenging and relevant model system to demonstrate the potential of peptide platforms for engineering metallo-supramolecular entities, we decided to approach the stereoselective synthesis of peptide helicates.

We have previously described the synthesis of peptide ligands containing a O1Pen-2,2'-bipyridine amino acid derivative, which predictably fold into coordination compounds with defined chirality,⁹ including self-assembled helicates,¹⁰ in the presence of metal ions. Unfortunately, the two hydrophobic Pro-Gly β -turns that encoded the folding of these peptide ligands,¹¹ also made our designs poorly soluble and prone to aggregation. Moreover, these helicates, based on the kinetically labile Fe(II)-trisbipyridine coordination, were inherently dynamic and displayed relatively weak metal binding constants. Herein we describe the rational design of alternative oligocationic peptide helicates with improved solubility and thermodynamic stability, and the synthesis of kinetically inert Co(III) helicates through oxidation of their dynamically assembled Co(II) precursors. We also show that these optimized self-assembled Fe(II) helicates bind to three-way DNA junctions *in vitro* and in functional cells, selectively labelling DNA replication foci in the cell nuclei.

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Results and Discussion

Rationale

In order to improve the solubility of the peptide ligands (and that of the final helicates) we decided to replace the original β -turn modules, (D/L)-Pro-Gly, with more polar sequences that were also capable of inducing a reverse β -turn to direct the folding of the peptide chain into the desired three-stranded helicates.¹² Based on the known tendency of heterochiral sequences to promote the formation of type II or type II' β -turns,¹³ we focused our attention on the combination of residues with mixed chirality, D-D-L (or its mirror image L-L-D) which were reported to increase turn formation and stability in short peptides.¹⁴ This pattern was very attractive for our purposes, because it allowed us great freedom in the choice of the residues in the loops, as long as they maintained their relative chirality. Thus, in order to maximize the solubility, and considering the prevalence of Arg residues in protein-DNA complexes,¹⁵ as well as the known beneficial effects of arginine residues in DNA-binding helicates,¹⁶ we selected the sequences L-Arg-L-Pro-D-Arg and D-Arg-D-Pro-L-Arg to define the β -turns and direct the folding of the peptide ligands into two enantiomeric peptide helicates, **LLD** and **DDL** respectively (Scheme 1). Additionally, we replaced the original O1PenBpy building block with a shorter β -Ala derivative (β AlaBpy) to reduce the conformational freedom of the loops and therefore increase the preorganization and thermodynamic stability of the assembled helicates.

Synthesis and characterization of the peptide helicates

The precursor peptide ligands **LLD** and **DDL** were obtained by standard Fmoc solid-phase peptide synthesis protocols.¹⁷ The Bpy ligands were introduced in the form of an Fmoc-protected building block, Fmoc- β AlaBpy-OH (**1**, Scheme 1), which was synthesized with minor modifications of reported procedures reported for the synthesis of Fmoc-O1PenBpy-OH.⁹ The final peptide ligands were purified by HPLC, and their identity confirmed by mass spectrometry (See the Experimental Section and Supporting Information for details). As expected, the four charged arginine residues in the sequence made these new peptide ligands readily soluble in water, thus radically improving the poor solubility of our previous design.¹⁰

The assembly of the Fe(II)₂**LDD** and Co(II)₂**LDD** helicates in solution was characterized by monitoring the quenching in the fluorescence emission intensity of the 5'-amido-[2,2'-bipyridine]-5-carboxamide upon metal coordination (Figure 1a).^{18,19} The resulting titration profile was successfully fitted to a 1:2 binding mode with dissociation constants of about 0.5 and 0.4 μ M for the first, and second metal ion coordination for both Fe(II) and Co(II).²⁰ These values are two orders of magnitude smaller than those of the original O1Pen-based peptide helicates,¹⁰ demonstrating the beneficial effect of the reduced conformational freedom of the β AlaBpy building block leading to helicates high thermodynamic stability. Furthermore, the observed positive cooperativity suggests that the coordination of the first metal ion

further preorganizes the peptide for the binding of the second ion.²¹ MALDI spectra of the solutions at saturating concentrations of metal ions showed peaks at 2555.06 and 2561.02, consistent with the formation of the Fe(II)₂**LDD** and Co(II)₂**LDD** helicates, respectively (See Figure S5 and S7 in the Supporting information). As expected, the fluorescence titrations with the **DDL** ligand reproduced these results.

Scheme 1. Top: Key elements in our first-generation design using (D/L)-Pro-Gly and flexible O1PenBpy building block. Bottom: Solid-phase peptide synthesis of the helicate ligand **LLD** using the Fmoc- β AlaBpy-OH building block. β -turn sequences (RPr) are highlighted in light blue, and one β AlaBpy building block in green. After cleavage and purification, the helicate is stereoselectively folded under thermodynamic control in the presence of coordinating metal ions, Fe(II) or Co(II). The **LDD** ligand contains two loops featuring the heterochiral L-Arg-L-Pro-D-Arg sequence, while the **DDL** contains enantiomeric D-Arg-D-Pro-L-Arg loops ($\text{H}-(\beta\text{AlaBpy})_2\text{-rPr}-(\beta\text{AlaBpy})_2\text{-rPr}-(\beta\text{AlaBpy})_2\text{-NH}_2$). L-amino acids are indicated in upper-case, and D-amino acids in lower case (i.e., R, P for L-Arg and L-Pro, and r, p for D-Arg and D-Pro).

Importantly, the **LLD** and **DDL** peptide ligands displayed mirror image circular dichroism spectra. The CD was dominated by a strong Cotton effect band at c.a. 320 nm, which pointed to a significant preorganization of the peptide chain and the effective chiral induction by the heterochiral β -turn modules in combination with π -stacking interactions between the bipyridine units.^{9a, 22} Addition of Fe(II) ions to a **LLD** solution resulted in a large enhancement of the positive Cotton effect band at c.a. 320 nm, as well as the appearance of a weaker negative Cotton effect band about 525 nm, both bands consistent with the formation of a $\Lambda\Lambda$ - (or *M*) helicate (Figure 1b). Likewise, the enantiomeric peptide ligand **DDL** gave rise to opposite bands at 320 and 525 nm, as expected for the induction of the mirror image $\Delta\Delta$ - (or *P*) helicate (Figure 1b). Taken together, these data are consistent with a dynamic process in which the mixture of the peptide ligands and the Fe(II) ions evolves towards the more stable folded helicates under thermodynamic control.

Figure 1. Titration of the **LLD** peptide ligand with Fe(II) ions. a) Normalized emission spectrum of a 2 μ M solution of **LLD** in 1 mM phosphate buffer NaCl 10 mM, pH 6.5 (thick line), and spectra of the same solution in the presence of increasing concentrations of Fe(II) ions (thin lines); inset showing the titration profile of three independent experiments and best fit to a one to two binding model (same range as main plot); b) circular dichroism spectra of 5 μ M solutions of **LLD** (black dashed line) and **DDL** (red dashed line) in 1 mM phosphate buffer NaCl 10 mM, pH 6.5, and in the presence of 25 μ M Fe(II) ions (same colors, continuous lines).

Locking the dynamic equilibrium by oxidizing Co(II) to Co(III)

The dynamic self-assembly of the helicates limits their potential applications and prevents their detailed structural characterization, because these self-assembled species are intrinsically dynamic and could disassemble, exchange ligands, or modify their structure or association state in response to changes in their environment.²³ To overcome these limitations we tested the *in situ* formation of robust Co(III) helicates by oxidation of the dynamically assembled Co(II) analogs.²⁴ This strategy has been extensively used for the assembly of kinetically-inert metal complexes and described for the immobilization of His-tagged proteins,²⁵ but, to our knowledge, has never been used in the

case of artificial metallopeptides. Thus, preformed solution of the Co(II)₂**LLD** helicate was treated with ceric ammonium nitrate, (NH₄)₂Ce(NO₃)₆, and analyzed by HPLC. Gratifyingly, while the labile helicates Fe(II)₂**LLD** and Co(II)₂**LLD** could never be observed in the acidic RP-HPLC conditions (0.1% TFA H₂O/CH₃CN), after oxidation we could isolate a new peak with mass consistent with the formation of the Co(III)₂**LLD** helicate, and a circular dichroism spectrum indicating the presence of species with the expected $\Lambda\Lambda$ -chirality (Figure S10).²⁶

Structural determination of two helicate topoisomers

The kinetic stability of this new Co(III)₂**LLD** helicate allowed us to obtain 2D NMR spectra with higher quality than those with the complex with Fe(II), and the 1D ¹H NMR spectra confirmed the folding of the peptide ligand into stable and ordered structures (Figure S13, Supporting Information), which encouraged us to pursue their detailed structural characterization in solution. Based on our previous work with metallopeptides and NMR studies of dinuclear ruthenium (II) polypyridyl complex,²⁷ we decided to apply the standard approach for peptide sequence assignment and structural characterization,²⁸ which consists in 2D NMR data acquisition in protonated solvent (90% H₂O/10% D₂O), proton/carbon resonance assignment and the use of the identified NOEs from the amino acid/ β Ala residues to obtain distance restraints for molecular dynamics (See the Supporting Information for a more detailed description of this strategy, 1D/2D spectra and NOEs restraints). Curiously, the isolated fraction (Supporting Information, Figure S8) showed two main components in solution, isomers **A** and **B**, in slow exchange in the NMR chemical shift timescale (Figure 2). The resonances corresponding to 1D aromatic/amide region and 2D ¹H-¹H NOESY correlations for NH- α / β CH₂ (in particular, for β -Ala3/5 at helicate turns) revealed the conformational flexibility of the peptidic turns in the metal complex. In contrast, the region of the Bpy ligands coordinated to the metal ions is more rigid. For this reason, due to ¹⁵N T1 long relaxation times, the ¹⁵N bipyridine resonances could not be observed in the ¹⁵N-¹H HMBC spectrum (data not shown). Based on the NMR experimental data,²⁹ and considering the similar coordination geometries of the Co(III) and Fe(II) trisbipyridyl complexes, we generated two reasonable starting point models of the Fe(II) isomers **A** and **B**, and assessed their stability by Molecular Dynamics (MD) simulations in explicit solvent and periodic boundary conditions (See the Supporting Information for details). During the simulations, the helical twist of the helicates, as well as the octahedral coordination geometry of the Fe(II) metal centers with the Bpy units were conserved. The root-mean square deviation (RMSD) on the heavy atoms (excluding the side chains) was computed during the trajectories using the minimized initial structures as a reference. In both cases the trajectories stabilize after the first 25 ns, reaching relatively stable RMSDs of 1.5 and 2.5 Å in average for isomers **A** and **B**, respectively. A cluster analysis performed on the full-length MDs showed a predominant geometry occupied about 64% and 89% of the total conformation repartition for **A** and **B**. Starting from the representative structures of these clusters, further molecular dynamics refinements where

carried out along 60 additional ns under NOE's distance restraints (Tables S3 and S4) in explicit solvent. The NMR refinement was performed using SANDER including flat-bottom NOE-derived distance potential function.³⁰ Overall, the results show that the two model structures, **A** and **B**, are valid candidates consistent with the experimental observations (see Experimental Section for a detailed analysis). Helicates **A** and **B** are topoisomers, that is, they have the same chirality but different connectivity between the Bpy units around the metal centers. In isomer **A**, the N-terminal (blue, Figure 2) and the C-terminal (red, Figure 2) bipyridine units are placed in the same side of the metalocylinder, whereas in isomer **B**, both terminal Bpy units are disposed in opposite sides of the helicate.³¹

Figure 2. Top: assembly-followed-by-fixing approach for the construction of kinetically-inert $\text{Co}(\text{III})_2\text{LLD}$ peptide helicates. The initial dynamic selection under thermodynamic control is followed by the oxidation of $\text{Co}(\text{II})$ to $\text{Co}(\text{III})$ to yield kinetically-inert complexes **A** and **B**, which have the same chirality, but different topology. Bottom: representative structures for the $\text{Fe}(\text{II})$ helicates of the two most populated clusters of the MD trajectories (stick) together with 100 frames of each trajectory. N-terminal bipyridine in blue, and C-terminal bipyridine in red.

The peptide helicates selectively recognize three-way DNA junctions in vitro

Helicates are known to selectively interact with DNA three-way junctions by insertion into the hydrophobic cavity created by the surface of the base pairs at the center of the DNA junction.^{8d,32} To study the DNA binding properties of these new peptide helicates we prepared a 2 μM solution of a fluorescein-labeled three-way junction DNA (**Flu-twDNA**), and measured its luminescence after the addition of successive aliquots of a solution of the preformed

helicate $\text{Fe}(\text{II})_2\text{LLD}$. The progressive quenching of the fluorescein emission at 515 nm could be fitted to a simple 1:1 binding mode (**Flu-twDNA**/ $\text{Fe}(\text{II})_2\text{LLD}$) with an apparent dissociation constant $K_D \approx 0.45 \mu\text{M}$,³³ which is in line with the data obtained for the original Pro-Gly $\text{Fe}(\text{II})$ peptide helicate (Figure 3). Curiously, in the case of the helicate $\text{Co}(\text{III})_2\text{LLD}$, the titration profile of the emission intensity at 515 nm had to be fitted to a more complex model with a lower-affinity binding site, likely corresponding to the insertion of the $\text{Co}(\text{III})_2\text{LLD}$ metalloprotein in the hydrophobic cavity, with a $K_D \approx 7.9 \mu\text{M}$, and three equivalent higher-affinity sites ($K_D \approx 4.9 \mu\text{M}$), which based on reported X-ray structures of related helicate/DNA complexes could correspond to nonspecific stacking interactions of the helicate with the blunt ends of the twDNA arms.^{8d}

Figure 3. a) Normalized emission spectra of a titration of a 2 μM solution of the fluorescein-labeled three-way junction DNA, **Flu-twDNA**, in 1 mM phosphate buffer, 10 mM NaCl, pH 6.5 (thick line) with increasing concentrations of the $\text{Fe}(\text{II})_2\text{LLD}$ helicate (thin lines); inset: corresponding normalized titration profile of the fluorescein quenching at 515 nm and best fit to a one to one binding model; b) Normalized emission of a 2 μM solution of the fluorescein-labeled three-way junction DNA, **Flu-twDNA**, in 10 mM phosphate buffer, 100 mM NaCl, 10 mM MgCl_2 , pH 6.5 with increasing concentrations of the $\text{Co}(\text{III})_2\text{LLD}$; the best fit to a simple one to one binding model is shown as a dashed line, and the fit to a model including non-specific binding is shown as a continuous line. Titration data are the average of three independent experiments. **Flu-twDNA**, Y1 5'-TTT CAC CGC TCT GGT CCT C-3'; Y2 5'-CAG GCT GTG AGC GGT G-3'; Y3 5'-GAG GAC CAA CAG CCT G-3'.

To better understand the interaction of the $\text{Fe}(\text{II})$ and $\text{Co}(\text{III})$ helicates with the DNA, we carried out electrophoretic mobility shift assays (EMSA) in polyacrylamide gel under non-denaturing conditions and using **SYBRGold** as the DNA stain.³⁴ In agreement with the fluorescence studies, we found that incubation of **twDNA** with increasing concentrations of the preformed $\text{Fe}(\text{II})_2\text{LLD}$ helicate resulted in the appearance of new slow-migrating band, consistent with the formation of the **twDNA**/ $\text{Fe}(\text{II})_2\text{LLD}$ complex (Figure 4a). As expected for a high-affinity interaction, complete band shift is observed already at the lowest concentrations tested, and gives rise to a single new band with no smearing, even at high concentration of the $\text{Fe}(\text{II})_2\text{LLD}$ helicate. On the other hand, no new slow-migrating bands were observed upon incubation of

Fe(II)₂LLD with a double-stranded DNA (dsDNA), which confirms the low affinity of this metalloprotein for regular B-DNA (Figure 4b). In agreement with the fluorescence titrations, the EMSA assays showed reduced affinity of the Co(III)₂LLD helicate for the **twDNA**, so that the saturation of the **twDNA** is only observed at the highest concentrations of Co(III)₂LLD (500 nM, Figure 4c). Remarkably, these assays also confirmed the tendency of the highly charged Co(III)₂LLD helicate to form non-specific complexes, as shown by the disappearance of the **dsDNA** band upon incubation with this helicate (Figure 4d).³⁵

Figure 4. a) EMSA analysis of 200 nM **twDNA** in the presence of 0, 50, 100, 250, 500 nM of Fe(II)₂LLD. The new, slow-migrating band (indicated with an arrow), corresponds to the three-way DNA structure assembled in the presence of the Fe(II) helicate; b) 75 nM **dsDNA** with 0, 1000, and 1500 nM of Fe(II)₂LLD; gels in c) and d) correspond to the same concentrations of **twDNA** and **dsDNA** as a) and b) incubated with the same concentrations of the Co(III)₂LLD helicate (**dsDNA** 5'-AAC ACA TGC AGG ACG GCG CTT-3'; **twDNA**, **Y1** 5'-CAC CGC TCT GGT CCT C-3'; **Y2** 5'-CAG GCT GTG AGC GGT G-3'; **Y3** 5'-GAG GAC CAA CAG CCT G-3').

Based on the previous structural characterization, we used the new *GaudiMM* platform to obtain plausible models for two Fe(II)₂LLD topoisomers threaded into the DNA three-way junction.³⁶ The structures of the Fe(II) helicases extracted from the MD simulation under NOE constraints were docked to the DNA extracted from the X-ray structure of the helicate/three-way DNA junction complex reported by Hannon *et al.* (PDB ID: 2ET0).^{8d} Energetically preferred orientations of the helicases inside the central cavity of the DNA three-way junction were found when considering displacements of up to 2.5 Angstroms along the ten lowest-energy normal mode vectors of the breathing modes of the **twDNA** (see Docking studies in the Experimental section), thus demonstrating that the accommodation of the helicases requires adaptation of the DNA target to the guests and that leads to a slightly different conformation than in the available X-ray structure taken as reference. Calculations with the two different isomers also showed a better accommodation of isomer **B** into the three-way DNA cavity showing higher scoring and lower clashes respect to the isomer **A** (see Supporting Information for further details). No clear penalizing interactions between the three-way DNA junction and the helicate units are observed although the center of mass of **B** aligned better with the **twDNA** and the entire complex fits more deeply into the DNA receptor. This is likely related to the smaller volume of **B** and its slightly more stretched geometry (Figure 5 and S17).

Figure 5. Lowest energy pose of isomer **B** of the Fe(II)₂LLD helicate in the central cavity of the **twDNA**. Left: top view showing the tight fit between the helicate and the **twDNA** hydrophobic site, which appears to be too narrow to comfortably accommodate isomer **A**. Right view of the same complex.

A rhodamine-labelled Fe(II)-helicate selectively labels DNA replication sites in functional cells

Having demonstrated the binding of these helicases to **twDNA** in vitro, we envisioned the application of these helicases to study these DNA structures in cells. Preliminary experiments in which the tetramethylrhodamine- and fluorescein-labeled Fe(II) helicases, Fe(II)₂TAMRA-LLD or Fe(II)₂Flu-LLD,³⁷ were incubated overnight with HeLa cells showed that these complexes remain trapped in endosomes (see Figure S28 in the Supporting information).³⁸ However, if the cells were pretreated with Digitonin,³⁹ then Fe(II)₂TAMRA-LLD was effectively internalized and showed in the red emission channel as a punctuated pattern in the cytoplasm and the nucleus (Figure 6a). Remarkably, the nuclear distribution of the helicate partially matched the localization of the DNA replication sites labeled with the proliferating cell nuclear antigen (PCNA) fused to GFP (Figure 6b),⁴⁰ which demonstrates that Fe(II) helicate is capable of targeting the three-way junctions transiently formed during DNA replication in functional cells (Figure 6c).⁴¹ The staining of what appears to be the cell nucleolus is consistent with Fe(II)₂TAMRA-LLD binding to three-way RNA structures, which are also potential biological targets of helicases.⁴² To our knowledge, this is the first demonstration of *in cellula* **twDNA** staining with designed fluorescent probes. As expected from the in vitro experiments, the Co(III)₂TAMRA-LLD helicate displayed poor specificity, leading to indiscriminate labeling in Digitonin treated cells (data not shown).

Figure 5. $\text{Fe(II)}_2\text{TAMRA-LLD}$ selectively stains DNA replication sites. HeLa cells expressing protein GFP-PCNAL2 were incubated with 25 $\mu\text{g/ml}$ Digitonin for 3 min, then 5 μM of **TAMRA-LLD** peptide ligand and 25 μM of $(\text{NH}_4)_2\text{Fe}(\text{SO}_4)_2$ for 30 min. a) Red channel emission showing the distribution of the rhodamine-labeled helicase; b) green channel, corresponding to the emission of the GFP-PCNAL2 probe labeling the DNA replication foci; c) overlay of the green and red channels of the square region shown in a) Arrows highlight some foci where the staining of GFP-PCNAL2 and $\text{Fe(II)}_2\text{TAMRA-LLD}$ overlap.

Conclusion

We have demonstrated that peptide design rules can be applied to encode both structural and chiral information in *de novo* metallopolypeptides. In particular, we report new oligocationic peptides containing bipyridine ligands that fold into three-stranded helicates in the presence of Fe(II) ions and bind with high affinity and specificity to DNA three-way junctions. For the first time, we have determined the atomic structure of these peptide helicates by a combination of CD, NMR and sophisticated computational methods, and applied a new theoretical approach to explore the nature of their DNA complexes. Finally, we also demonstrate that the $\text{Fe(II)}_2\text{TAMRA-LLD}$ helicate binds to DNA three-way junctions in functional cells, selectively labeling DNA replication foci in the nuclei, thus demonstrating for the first time the selective staining of three-way DNA *in cellula* with designed fluorescent probes.

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Entry for the Table of Contents

Artificial bipyridine-containing peptide ligands encode in their sequence the stereoselective folding into chiral three-stranded Fe(II) or Co(II) peptide helicates, and as kinetically-inert dinuclear Co(III) complexes by in situ oxidation. These peptide helicates selectively label DNA replication foci in functional cells.

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Dynamic Stereoselection of Peptide Helicates and Their Selective Labeling of DNA Replication Foci in Cells

Supporting information

Dynamic Stereoselection of Peptide Helicates and Their Selective Labeling of DNA Replication Foci in Cells

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Author attributions

The following table describes the contribution of each author according to the CRediT taxonomy as described in A. Brand, L. Allen, M. Altman, M. Hlava, J. Scott, *Learn. Publ.* **2015**, 28, 151–155. This taxonomy provides a detailed classification of the various roles performed by each author. The degree of contribution is coded as lead (black), equal (dark grey), or supporting (light grey).

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Electronic spectroscopy and mass spectrometry

UV-Vis Spectroscopy

UV measurements were made in a *Jasco V-630* spectrophotometer coupled to a *Jasco ETC-717* temperature controller, using a standard *Hellma* semi-micro cuvette (108.002-QS) with a light path of 10 mm. Measurements were made at 20 °C.

Acquisition parameters were: 220-700 nm range, scan speed of 200 nm/min, resolution of 0.2 nm.

Fluorescence spectroscopy

Coordination experiments and direct quenching of fluorescein-labelled DNA's

Luminescence experiments were made with a Varian Cary Eclipse Fluorescence Spectrophotometer coupled to a Cary Single Cell Peltier accessory (Agilent Technologies) temperature controller. All measurements were made with a *Hellma* semi-micro cuvette (108F-QS) at 20 °C. The settings for these measurements were adapted depending on the studied system.

Single point anisotropy titrations

Single point anisotropy was carried out in a *Jobin-Yvon Fluoromax-3 (DataMax 2.20)* coupled to a temperature controller *Wavelength Electronics LFI-3751*. All measurements were made with a *Hellma* semi-micro cuvette (108F-QS) at 20 °C. The values are an accumulation of three measurements.

Circular Dichroism

Circular dichroism measurements were made with a *Jasco J-715* coupled to a *Neslab RTE-111* thermostatic water bath, using a *Hellma* 100-QS cuvette (2 mm light pass). Scan speed was 200 nm/min and the obtained spectra are the mean of three accumulations.

DNA hybridization

Oligonucleotides were obtained from *Biomers*. Concentration of the oligonucleotides was measured by UV-VIS by using the extinction coefficient given by the Comercial House Biomers.

For the hybridization process, a stoichiometric mixture of the DNA strands was prepared in PBS buffer (10 mM, 100 mM NaCl, pH 7.0) and heated at 90 °C for 10 minutes. After this time the mixture was slowly cooled down until room temperature, obtaining the resulting 3w/ds hybridized DNA.

Mass spectrometry

Matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) was performed with a *Bruker Autoflex* MALDI-TOF model in positive scan mode by direct irradiation of the matrix-adsorbed peptide. 4-HCCA (α -cyano-4-hydroxycinnamic acid) was the selected matrix for all these experiments.

Synthesis of Fmoc-βAlaBpy-OH

All the solvents employed for the synthesis of the Fmoc-βAlaBpy-OH were obtained from different commercial sources: *Fisher Chemical* (acetonitrile for HPLC, chloroform, toluene, DMF, methanol, NaOH), *Scharlau* (absolute ethanol), *Sigma Aldrich* (5,5'-dimethyl-2,2'-bipyridine, KMnO₄, SOCl₂, hydrazine monohydrate, xylene, DIPEA), *Panreac* (celite and NaNO₂).

The synthesis of this chelating residue was carried out following the methodology recently published by our group with minor modifications.¹ All the obtained products were analyzed by HPLC-MS to test their purity.

Synthesis of 2,2'-bipyridine-5,5'-dicarboxylic acid (2)

A mixture of 39 g of potassium permanganate and 7 g of 5,5'-dimethyl-2,2'-bipyridine in 250 mL of H₂O was heated for 2 h (115 °C), cooled at rt and filtrated through celite. The filtrate was cooled to 4 °C and acidified with HCl until precipitation of a white solid, which was filtrated, washed with water, and lyophilized to afford the desired product in a 93% yield (8.7 g)

¹H-NMR (500 MHz, DMSO-*d*₆, δ): 13.50 (br); 9.18 (dd, ⁴*J* = 2.15, ⁵*J* = 0.8 Hz 2H); 8.55 (dd, ³*J* = 8.3; ⁵*J* = 0.8 Hz, 2H); 8.44 (dd, ³*J* = 8.3; ⁴*J* = 2.15 Hz, 2H).

¹³C-NMR (125 MHz, DMSO-*d*₆, δ): 165.49, 156.84, 149.82, 137.97, 126.65, 120.62.

MALDI-TOF (m/z) [M+H]⁺ Calcd. for [C₁₂H₈N₂O₄] 245.0; found 245.0.

Synthesis of diethyl [2,2'-bipyridine]-5,5'-dicarboxylate (3)

2,2'-bipyridine-5,5'-dicarboxylic acid (10.0 g, 41 mmol) was suspended in 150 mL of absolute ethanol. Concentrated sulfuric acid (20.0 mL) was slowly added and the resulting mixture was refluxed for 18 h. The solution was cooled to rt and added over 400 mL of water at 4 °C causing the precipitation of a white solid, which was filtered, washed with water and freeze dried. 11.5 g (93.0 %)

¹H-NMR (500 MHz, DMSO-*d*₆, δ): 9.20 (dd, ⁴*J* = 2.15, ⁵*J* = 0.8 Hz 2H); 8.57 (dd, ³*J* = 8.3; ⁵*J* = 0.8 Hz, 2H); 8.46 (dd, ³*J* = 8.3; ⁴*J* = 2.15 Hz, 2H); 4.0 (q, ³*J* = 7.1 Hz, 4H); 1.37 (t, ³*J* = 7.1 Hz, 6H).

¹³C-NMR (125 MHz, DMSO-*d*₆, δ): 164.2, 157.2, 149.8, 138.0, 126.2, 121.0, 61.1, 13.9.

MALDI-TOF (m/z) [M+H]⁺ Calcd. for [C₁₆H₁₆N₂O₄] 301.1; found 301.1.

Synthesis of Ethyl 5'-Carbohydrazido-2,2'-bipyridine-5-carboxylate (4)

Diethyl [2,2'-bipyridine]-5,5'-dicarboxylate (15.00 g, 50 mmol) and hydrazine hydrate (3.75 mL, 55 mmol) are dissolved in a mixture of EtOH (42 mL) and toluene (128 mL). The resulting solution was heated at 80 °C for 48 h. The precipitate was filtered, washed with chloroform and dried under vacuum to afford the desired product in an initial 80% yield; the unreacted diethyl ester was concentrated and reacted again following the same procedure to obtain a global yield of (90%).

¹H-NMR (500 MHz, DMSO-*d*₆, δ): 1.35 (t, 3H), 4.37 (q, 2H), 8.35 (d, ³*J* = 8.4 Hz, 1H), 8.45 (dd, ³*J* = 8.4 Hz, ⁴*J* = 2 Hz, 1H), 8.52 (d, ³*J* = 8.4 Hz, 1H), 8.57 (d, ³*J* = 8.4 Hz, 1H), 9.1 (d, ⁴*J* = 2 Hz, 1H), 9.2 (d, ⁴*J* = 2 Hz, 1H), 10.1 (br, 1H).

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¹³C-NMR (125 MHz, DMSO-*d*₆, δ): 14.1, 61.3, 120.9, 120.9, 126.1, 129.6, 136.2, 138.2, 148.1, 150.0, 155.8, 157.8, 163.8, 164.5.

MALDI-TOF (m/z) [M+H]⁺ Calcd. for [C₁₄H₁₄N₄O₃] 287.1; found 287.1.

Synthesis of Ethyl 5'-(Carbohydrazido)-2,2'-bipyridine-5-carboxylate (5)

To a solution of ethyl 5'-(hydrazinecarboxyl)-[2,2'-bipyridine]-5-carboxylate (5.7 g, 20 mmol) in concentrated HCl (100 mL) was cooled to 0 °C. Aqueous NaNO₂ (1.73 g, 25 mmol; 15 mL) was added dropwise, maintaining the temperature at 0 °C. After 60 min, the resulting yellow solution was diluted with water (300 mL) to precipitate the monoester **5** as a white powder, which was filtered, washed with water, and lyophilized to afford the desired product (5.5 g, 92 %)

¹H-NMR (500 MHz, Acetone-*d*₆, δ): 9.13 (d, ⁴*J* = 1.91 Hz, 1H), 9.10 (d, ⁴*J* = 1.91 Hz, 1H), 8.71 (d, ³*J* = 12.5 Hz, 1H), 8.69 (d, ³*J* = 12.5 Hz, 1H), 8.517 (m, 2H); 4.31 (q, ³*J* = 7.15 Hz, 2H); 1.29 (t, ³*J* = 7.15, 3H).

¹³C-NMR (125 MHz, Acetone-*d*₆, δ): 170.8, 164.5, 159.2, 157.7, 150.3, 150.0, 138.0, 137.9, 127.0, 121.3, 61.2, 13.6.

Synthesis of Ethyl 5'-(ethoxycarbonyl)amino-2,2'-bipyridine-5-carboxylate (6)

A solution of ethyl 5'-(azidocarbonyl)-[2,2'-bipyridine]-5-carboxylate (8.6 g, 29 mmol) in EtOH (100 mL) and xylene (100 mL) was heated at 90 °C for 4 h. The solvent was evaporated under reduced pressure and the yellow residue was washed with EtOH and dried in vacuo to give the desired product (8.21 g, 90%).

¹H-NMR (500 MHz, DMSO-*d*₆, δ): 1.27 (t, *J* = 7.2 Hz, 3H), 1.35 (t, *J* = 7.2 Hz, 3H), 4.17 (q, *J* = 7.2 Hz, 2H), 4.36 (q, *J* = 7.2 Hz, 2H), 8.08 (dd, ³*J* = 8 Hz, ⁴*J* = 2 Hz, 1H), 8.35 (dd, ³*J* = 8.3 Hz, ⁴*J* = 2 Hz, 1H), 8.40 (dd, ³*J* = 8.3 Hz, ⁵*J* = 1 Hz, 1H), 8.45 (d, ³*J* = 8 Hz, 1H), 8.70 (d, ⁴*J* = 2 Hz, 1H), 9.12 (d, ⁴*J* = 2 Hz, ⁵*J* = 1 Hz, 1H), 10.13 (s, 1H).

¹³C-NMR (125 MHz, DMSO-*d*₆, δ): 13.6, 13.9, 60.3, 60.6, 119.1, 121.2, 124.46, 124.9, 136.7, 137.4, 139.0, 147.4, 149.4, 153.1, 158.1, 164.2.

MALDI-TOF (m/z) [M+H]⁺ Calcd. for [C₁₆H₁₇N₃O₄] 316.1; found 316.1.

Synthesis of 5'-Amino-2,2'-bipyridine-5-carboxylic acid hydrochloride (7)

A solution of ethyl 5'-(ethoxycarbonyl)amino-2,2'-bipyridine-5-carboxylate (14.55 g, 45.9 mmol) in a mixture of EtOH (50 mL) and 2.5 M aqueous NaOH (50 mL) was heated at 75 °C for 14 h. The EtOH was concentrated in vacuum, and the aqueous solution was acidified with HCl to afford a bright yellow precipitate, which was filtered, washed with cold water, and lyophilized to afford the desired bipyridine amino acid (9.80 g, 85%).

¹H-NMR (500 MHz, D₂O, δ): 7.00 (dd, ³*J* = 8.5 Hz, ⁴*J* = 2.8 Hz, 1H), 7.55, 7.61 (d, ³*J* = 8.5 Hz, 2H), 7.81 (d, ⁴*J* = 2.9 Hz, 1H), 7.98 (dd, ³*J* = 8.5 Hz, ⁴*J* = 2.9 Hz, 1H), 8.65 (d, ⁴*J* = 2.8 Hz, 1H)

¹³C-NMR (125 MHz, D₂O, δ): 123.3d, 126.2, 126.7, 133.8, 139.8, 141.34, 147.5, 147.4, 152.4, 159.6, 176.04

MALDI-TOF (m/z) [M+H]⁺ Calcd. for [C₁₁H₉N₃O₂] 216.1; found 216.1.

Synthesis of Fmoc-βAlaBpy-OH

10 mL of SOCl₂ were slowly added over 4.58 g (14.72 mmol) of Fmoc-βAla-OH 10 mL at rt. The resulting mixture was stirred for 30 min; the thionyl chloride was evaporated under reduced pressure and the yellow residue was washed with dichloromethane (10 mL, three times) and dried under reduced pressure.

The bipyridine amino acid **7** (3.7 g, 14.8 mmol) was dissolved in 20 mL of dichloromethane and 5 mL of DIEA and the mixture was added onto the Fmoc-Ala-Cl. The suspension was stirred at rt overnight. The solvent was evaporated, the solid was suspended in acetonitrile 0.1 %TFA/H₂O (2:1), centrifuged and washed with acetonitrile and H₂O and lyophilized to give the desired product (6.70 g, 89 %).

¹H-RMN (300 MHz, DMSO-*d*₆, δ): 10.44 (s, 1H); 9.13 (d, ⁴*J* = 1.36 Hz, 1H); 8.89 (d, ⁴*J* = 2.12 Hz, 1H); 8.42 (m, 3H); 8.25(dd, ³*J* = 8.48 Hz, ⁴*J* = 2.12 Hz, 1H); 7.88 (d, ³*J* = 7.46 Hz, 2H); 7.67 (d, ³*J* = 7.38 Hz, 2H); 7.49 (t, ³*J* = 5.34 Hz, NH); 7.40 (t, ³*J* = 7.21 Hz, 2H); 7.30 (t, ³*J* = 7.38 Hz, 2H); 4.30 (d, ³*J* = 6.95 Hz, 2H); 4.21 (t, ³*J* = 6.95 Hz, 1H) 3.32 (t, ³*J* = 6.70 Hz, 2H); 2.60 (t, ³*J* = 6.70 Hz, 2H).

¹³C-NMR (75 MHz, DMSO-*d*₆, δ): 169.76 (C), 168.62 (AlaC), 157.23 (C), 150.94 (C), 149.21 (C), 144.74 (C), 143.47 (C), 142.27 (C), 141.59 (C), 140.28 (CH), 138.29 (CH), 138.08 (CH), 129.73 (CH), 128.09 (CH), 125.96 (CH), 122.14 (CH), 120.87 (CH), 120.79 (CH), 118.73 (CH), 110.34 (CH), 67.38 (CH₂), 63.77 (CH₂), 47.62 (CH), 41.71 (CH₂).

MALDI-TOF (m/z) [M+H]⁺ Calcd. for [C₂₉H₂₅N₄O₅] 509.1; found 509.1.

Solid phase peptide synthesis

Fmoc- amino acids for peptide synthesis were from *Sigma Aldrich*. The resin employed for the SPPS was H-Rink-Amide *ChemMatrix* 35-100 mesh particle size. 6-Carboxytetramethylrhodamine succinimidyl ester (TMRA) was provided by *Carbosynth* while Fluorescein isothiocyanate was provided by *Sigma Aldrich*.

Peptides were synthesized following standard solid-phase peptide synthesis protocols. Natural residues were coupled using HBTU/HOBt (5 equiv) as coupling mixture and DIEA 0.2M in DMF (4 mL) as solvent. Non-natural β AlaBpy residues were coupled using HATU (3.5 equiv) as coupling reagent. Deprotection of the temporal Fmoc protecting group was performed with 20% piperidine in DMF for 15 min. Cleavage/deprotection of the final ligands was performed by treatment of the resin-bound peptides for 2–2.5 h with 900 μ L TFA, 50 μ L CH_2Cl_2 , 25 μ L H_2O and 25 μ L TIS (~1 mL of cocktail/40 mg resin). The crude products were purified by reverse-phase HPLC. HPLC was done using an *Agilent 1100* series Liquid Chromatograph Mass Spectrometer system. Analytical HPLC was carried out using an *Eclipse XDB-C18* analytical column (4.6 $\times$ 150 mm, 5 μ m), 1 mL/min, gradient 5 to 75% B over 30 min (A: H_2O 0.1% TFA, B: CH_3CN 0.1% TFA). Purification of the peptides was performed on a semipreparative *Phenomenex Luna-C18* (250 $\times$ 10 mm) reverse-phase column, 3 mL/min, gradient 10 to 50% B over 30 min. ESI/MS was performed with an *Agilent 1100* LC/MSD VL G1956A in positive mode.

N-capping with TAMRA and Flu were performed by reacting the N-terminal deprotected amine with the appropriate activated fluorophore—6-Carboxytetramethylrhodamine succinimidyl ester (TAMRA-OSu), or Fluorescein isothiocyanate, respectively—in 1 mL DIEA/DMF 0.2 M during 2 h (approximately 7.5 mg of the reactive fluorophore/40 mg of resin).

Cleavage from the resin and final deprotection

Cleavage and deprotection of the peptide were simultaneously performed using standard conditions by incubating the resin for 2.5 h with an acidic mixture containing 50 μ L CH_2Cl_2 , 25 μ L of H_2O , 25 μ L of TIS (triisopropylsilane), and 900 μ L of TFA. The resin was filtered, and the TFA filtrate was concentrated under a nitrogen stream to an approximate volume of 1 mL, then added onto ice-cold diethyl ether (20 mL). After 10–30 min, the precipitate was centrifuged and washed again with 5 mL of ice-cold ether. The residue was dissolved in a mixture of H_2O and MeCN and purified by reverse-phase preparative HPLC.

HPLC and UHPLC

UHPLC-MS

Peptide analysis was performed by analytical UHPLC-MS with an *Agilent 1200* series LC/MS using a SB C18 (1.8 μ m, 2.1 $\times$ 50mm) analytical column from Phenomenex. Standard conditions for analytical UHPLC consisted on a linear gradient from 5% to 95% of solvent B in 12 min at a flow rate of 0.350 mL/min (A: water 0.1% TFA, B: acetonitrile 0.1% TFA). Compounds were detected by UV absorption at 222, 270 and 330 nm. Electrospray Ionization Mass Spectrometry (ESI/MS) was performed with an *Agilent 6120* Quadrupole LC/MS model in positive scan mode using direct injection of the purified peptide solution into the MS detector.

Preparative HPLC

Peptide purification was performed by preparative RP- HPLC with a *Waters 1500* series Liquid Chromatograph using a Sunfire Prep C₁₈ OBD (5 μ m, 19 $\times$ 150 mm) reverse-phase column from Waters. Standard conditions for preparative RP- HPLC consisted on an isocratic regime during the first 5 min, followed by different linear gradients of solvent B during 30 min (A: water 0.1% TFA, B: acetonitrile 0.1% TFA). The gradients were adjusted for each peptide.

UHPLC-MS of the purified peptide ligands

DDL: H-(β AlaBpy)₂rpR(β AlaBpy)₂rpR(β AlaBpy)₂-NH₂

Figure S1. Left: chromatogram of the purified free ligand DDL. Right: mass spectra corresponding to the peak with t_R of 5.4 min.

HPLC-MS (ESI): (5-95% B, $t_R = 5.4$ min) Calcd. for C₁₁₈H₁₃₇N₄₃O₁₈ = 2444.11; found [M+2H+TFAH]²⁺ = 1289.7; [M+3H]³⁺ = 816.0; [M+4H]⁴⁺ = 612.3; [M+5H]⁵⁺ = 490.0; [M+6H]⁶⁺ = 408.8.

MS (MALDI-TOF): Calcd. for C₁₁₈H₁₃₇N₄₃O₁₈ = 2444.11; found: [M+H]⁺ = 2446.19.

LLD: H-(β AlaBpy)₂RPr(β AlaBpy)₂RPr(β AlaBpy)₂-NH₂

Figure S2. Left: chromatogram of the purified free ligand LLD. Right: mass spectra corresponding to the peak with t_R of 5.4 min.

HPLC-MS (ESI): (5-95% B, $t_R = 5.4$ min) Calcd. for C₁₁₈H₁₃₇N₄₃O₁₈ = 2444.11; found [M+2H+TFAH]²⁺ = 1289.7; [M+3H]³⁺ = 816.0; [M+3H+TFAH]³⁺ = 854.1; [M+4H]⁴⁺ = 612.3; [M+5H]⁵⁺ = 490.0; [M+6H]⁶⁺ = 408.8.

MS (MALDI-TOF): Calcd. for C₁₁₈H₁₃₇N₄₃O₁₈ = 2444.11; found: [M+H]⁺ = 2446.14.

Flu-LLD: Flu-Amhx(β AlaBpy)₂RPr(β AlaBpy)₂RPr(β AlaBpy)₂-NH₂

Figure S3. Left: chromatogram of the purified free ligand **Flu-LLD**. Right: mass spectra corresponding to the peak with t_R of 6.2 min.

HPLC-MS (ESI): (5-95% B, t_R = 6.2 min) Calcd. for $C_{145}H_{159}N_{45}O_{24}S$ = 2946.23; found $[M+2H]^{2+}$ = 1474.8; $[M+3H]^{3+}$ = 983.3; $[M+4H]^{4+}$ = 738.0; $[M+5H]^{5+}$ = 590.4; $[M+6H]^{6+}$ = 492.4; $[M+7H]^{7+}$ = 422.0.

MS (MALDI-TOF): Calcd. for $C_{145}H_{159}N_{45}O_{24}S$ = 2946.23; found: $[M+H]^+$ = 2948.51.

TAMRA-LLD: TAMRA-O1Pen(β AlaBpy) $_2$ RPr(β AlaBpy) $_2$ RPr(β AlaBpy) $_2$ -NH $_2$

Figure S4. Left: chromatogram of the purified free ligand **TAMRA-LLD**. Right: mass spectra corresponding to the peak with t_R of 5.9 min.

HPLC-MS (ESI): (5-95% B, t_R = 5.9 min) Calcd. for $C_{147}H_{166}N_{46}O_{24}$ = 2959.31; found $[M+3H+TFAH]^{3+}$ = 1025.0; $[M+3H]^{3+}$ = 987.1; $[M+4H]^{4+}$ = 740.5; $[M+5H]^{5+}$ = 592.7; $[M+6H]^{6+}$ = 494.1; $[M+7H]^{7+}$ = 423.9.

MS (MALDI-TOF): Calcd. for $C_{147}H_{166}N_{46}O_{24}$ = 2959.31; found: $[M+H]^+$ = 2960.43.

Synthesis of the Fe(II) peptide helicates

To a 2 μM solution of any of the peptide ligands (**LLD** or **DDL**) in phosphate buffer (1 mM, 10 mM NaCl, pH 6.5), 5 eq. of Mohr's salt $(\text{NH}_4)_2\text{Fe}(\text{SO}_4)_2 \cdot 6 \text{H}_2\text{O}$ (Mohr's salt) were added as source of Fe(II) ions.

Figure S5. Top: UV-VIS spectra of a 4 μM solution of **LLD** ligand before (black solid line) and after the addition of 5 eq equivalents of $(\text{NH}_4)_2\text{Fe}(\text{SO}_4)_2 \cdot 6 \text{H}_2\text{O}$ (black dashed line). Formation of the corresponding Fe(II) complex can be confirmed with the appearance of a MLCT band centered at 535 nm. Bottom: MALDI-TOF of $\text{Fe(II)}_2\text{LLD}$. Calculated for $[\text{Fe(II)}_2\text{LLD}]^{4+}$ $\text{C}_{118}\text{Fe}_2\text{H}_{137}\text{N}_{43}\text{O}_{18} = 2555.98$; found: $[\text{M}-2\text{Fe}+\text{H}]^+ = 2446.4$; $[\text{M}+\text{Fe}-\text{H}]^+ = 2500.4$; $[\text{M}+2\text{Fe}-3\text{H}]^+ = 2555.3$.

MS (MALDI-TOF): Calcd. for $\text{Fe(II)}_2\text{DDL}$ $\text{C}_{118}\text{Fe}_2\text{H}_{137}\text{N}_{43}\text{O}_{18} = 2555.97$; found: $[\text{M}+2\text{Fe}-3\text{H}]^+ = 2555.31$.

MS (MALDI-TOF): Calcd. for $\text{Fe(II)}_2\text{LLD}$ $\text{C}_{118}\text{Fe}_2\text{H}_{137}\text{N}_{43}\text{O}_{18} = 2555.97$; found: $[\text{M}+2\text{Fe}-3\text{H}]^+ = 2554.99$.

MS (MALDI-TOF): Calcd. for $\text{Fe(II)}_2\text{Flu-LLD}$ $\text{C}_{145}\text{Fe}_2\text{H}_{159}\text{N}_{45}\text{O}_{24}\text{S} = 3058.09$; found: $[\text{M}+2\text{Fe}-3\text{H}]^+ = 3056.24$.

MS (MALDI-TOF): Calcd. for $\text{Fe(II)}_2\text{TAMRA-LLD}$ $\text{C}_{147}\text{Fe}_2\text{H}_{166}\text{N}_{46}\text{O}_{24} = 3071.17$; found: $[\text{M}+2\text{Fe}-3\text{H}]^+ = 3068.26$.

Synthesis of the Co(II) peptide helicate

A fluorescence titration of a 2 μM solution of the **DDL** peptide in phosphate buffer (1 mM, 10 mM NaCl, pH 6.5) with increasing concentrations of $\text{Co}(\text{ClO}_4)_2 \cdot 6 \text{H}_2\text{O}$ resulted in a series of emission spectra with decreasing emission intensity due to the quenching of the 5'-amido-[2,2'-bipyridine]-5-carboxamide units upon coordination of the Co(II) ions. The resulting titration profile at 420 nm could be fitted to a 1:2 binding mode using *DynaFit* software with dissociation constants for the first, and second iron coordination of 0.54 and 0.44 μM .

Figure S6. Left: emission spectra of a 2 μM **LLD** peptide solution (1 mM PBS buffer, 10 mM NaCl, pH 6.5) with increasing concentrations of $\text{Co}(\text{ClO}_4)_2 \cdot 6 \text{H}_2\text{O}$. Right: Titration profile of the maximum emission wavelength at 410 nm with increasing concentrations of Co(II) ions. The best fit according to the 1:2 model in *DynaFit* is also shown. Experimental data points correspond to the average of three independent titrations.

Figure S7. Top: UV-VIS spectra of a 4 μM solution of **LLD** ligand before (black solid line) and after the addition of 5 eq equivalents of $\text{Co}(\text{ClO}_4)_2 \cdot 6 \text{H}_2\text{O}$ (black dashed line). Bottom: MALDI-TOF of $\text{Co}(\text{II})_2\text{LLD}$. Calculated for $[\text{Co}(\text{II})_2\text{LLD}]^{4+}$ $\text{C}_{118}\text{Co}_2\text{H}_{137}\text{N}_{43}\text{O}_{18} = 2561.97$; found: $[\text{M}-2\text{Co}+\text{H}]^+ = 2445.1$; $[\text{M}+\text{Co}-\text{H}]^+ = 2504.1$; $[\text{M}+2\text{Co}-3\text{H}]^+ = 2561.0$.

MS (MALDI-TOF): Calcd. for $\text{Co}(\text{II})_2\text{LLD}$ $\text{C}_{172}\text{Co}_2\text{H}_{241}\text{N}_{45}\text{O}_{45} = 2561.97$; found: $[\text{M}-3\text{H}]^+ = 2561.02$.

Synthesis of the Co(III) peptide helicate

Over a 600 μM solution of **LLD** in PBS 1 mM, 10 mM NaCl, pH 6.5, 5 equivalents of $\text{Co}(\text{ClO}_4)_2 \cdot 6 \text{H}_2\text{O}$ (3 mM) were added from a 100 $\times$ stock solution. After 30 min of stabilization, 5.5 equivalents of $(\text{NH}_4)_2\text{Ce}(\text{NO}_3)_6$ (3.3 mM) were added, and the resulting mixture was incubated for 30 min. HPLC analysis of the solution showed two major peaks, which were purified by semipreparative HPLC in reverse phase (0-40% MeCH/H₂O, 0.1% TFA in 30 min), and freeze dried.

Figure S8. Left: chromatogram of the isolated peak 1 of $\text{Co(III)}_2\text{LLD}$. Right: mass spectra corresponding to the peak with t_R of 5.3 min.

HPLC-MS (ESI): (5-95% B, $t_R = 5.3$ min) Calcd. for $\text{C}_{172}\text{Co}_2\text{H}_{241}\text{N}_{45}\text{O}_{45} = 2561.97$; found $[\text{M}+3\text{H}+\text{TFAH}]^{3+} = 891.5$; $[\text{M}+3\text{H}+2\text{TFAH}]^{3+} = 929.2$; $[\text{M}+3\text{H}+3\text{TFAH}]^{3+} = 966.9$; $[\text{M}+4\text{H}]^{4+} = 640.5$; $[\text{M}+4\text{H}+\text{TFAH}]^{4+} = 669.0$.

MS (MALDI-TOF): Calcd. for $\text{C}_{172}\text{Co}_2\text{H}_{241}\text{N}_{45}\text{O}_{45} = 2561.97$; found: $[\text{M}+\text{H}]^+ = 2561.11$.

Figure S9. Left: chromatogram of the purified isomer 2 of $\text{Co(III)}_2\text{LLD}$. Right: mass spectra corresponding to the peak with t_R of 5.3 min.

HPLC-MS (ESI): (5-95% B, $t_R = 5.3$ min) Calcd. for $\text{C}_{172}\text{Co}_2\text{H}_{241}\text{N}_{45}\text{O}_{45} = 2561.97$; found $[\text{M}+3\text{H}+\text{TFAH}]^{3+} = 891.5$; $[\text{M}+3\text{H}+2\text{TFAH}]^{3+} = 929.2$; $[\text{M}+3\text{H}+3\text{TFAH}]^{3+} = 967.1$; $[\text{M}+4\text{H}]^{4+} = 640.2$; $[\text{M}+4\text{H}+\text{TFAH}]^{4+} = 668.8$.

MS (MALDI-TOF): Calcd. for $\text{C}_{172}\text{Co}_2\text{H}_{241}\text{N}_{45}\text{O}_{45} = 2561.97$; found: $[\text{M}+\text{H}]^+ = 2561.13$.

Circular Dichroism studies of the Co(III) peptide helicates

CD measurements of the two isolated HPLC peaks (1 and 2) were made in phosphate buffer (1 mM, 10 mM NaCl, pH 6.5) in order to analyze their chirality after the oxidation step to afford the helicate Co(III)₂**LLD**. As expected, both spectra are superimposable and display a positive Cotton effect c.a. 350 nm that is consistent with a left-handed helicate.

Figure S10. CD spectra of the two Co(III)₂-**LLD** HPLC peaks isolated (1 and 2).

Nuclear Magnetic Resonance Experiments

NMR samples containing the peptides helicates were prepared weighting the lyophilized molecule and dissolving it in HEPES-d18 buffer in 90% H₂O/10 % D₂O. After dissolution, we measured the pH for all NMR samples and the value was 4.9-5.0 (uncorrected for the deuterium isotope effect). All data were acquired using a Bruker Avance-III 500 MHz spectrometer equipped with a z-axis pulsed field gradient triple resonance (¹H, ¹³C, ¹⁵N) TCI cryoprobe. Two-dimensional (2D) ¹H-¹H experiments (NOESY, ROESY, TOCSY and clean-TOCSY) spectra were acquired at 298 K in the phase-sensitive mode using the States-TPPI method and using a time domain data size of 512-600 t₁ and 4096 t₂ complex points and 24 transients per complex t₁ increments. The conventional TOCSY spectra contained both positive peaks associated to magnetization transfer through coupled spins (*J* transfer) and negative peaks due to cross relaxation. To avoid missing cross peaks due the interference of cross relaxation effects overlapping with TOCSY transfer and signal cancellation, we also acquired the “clean” TOCSY version, modifying Bruker TOCSY pulse sequence by the introduction of delays into an MLEV-17 pulse. The water resonance was suppressed by “3-9-19” pulse sequence with gradients using flip-back pulse in NOESY experiments and only 3-9-19 sequence in ROESY experiments, using excitation sculpting with gradients in TOCSY experiments and watergate W5 pulse sequence with gradients in clean TOCSY experiments. The pulses sequences used were *noesyfpgpphwg*, *roesygpph19* from Bruker library and modified *mlevgpphw5* with additional delay for the “clean” TOCSY version.² TOCSY spectra were obtained with a mixing time of 75 ms, NOESY spectra with a mixing time of 150 ms and ROESY with a mixing time of 75 ms. The comparison of NOESY and ROESY cross peaks allowed to detect those cross peaks in NOESY spectra originated by spin-diffusion. 2D ¹H-¹³C HSQC (Bruker pulse sequence *hsqcetgpprpsisp2.2*) and ¹H-¹³C HSQC-TOCSY (Bruker pulse sequence *hsqcdiedetgpprpsisp2.2*) spectra were recorded with 2048 (¹H, t₂) and 512 (¹³C, t₁) complex points, 30-60 transients per complex t₁ increments and spectral widths of 16 (¹H) and 230 ppm (¹³C), respectively. 2D experiments were processed with the standard TOPSPIN program (Bruker Biospin, Karlsruhe, Germany). The 2D data matrices were multiplied by square-sine bell window function with the corresponding shift optimized for every spectrum and zero-filled to 2x1 K complex matrices prior to Fourier transformation. Baseline correction was applied in both dimensions. Helicates 2D NMR spectra were analyzed using MestreNova software (MestreLab Research S.L.). NOE cross-peaks were integrated in the 150 ms NOESY spectra and the NOE volumes were converted to distances, which were calibrated using the average NOE volume of resolved geminal b-methylene proton cross-peaks from βAla3 (see Figure S12 for numbering).

Assignment strategy

There are few NMR structural studies on helicates in the literature. Some of them are ligand-binding studies (NMR assignment of ligand-DNA complex and MD calculation of the complex structure using previously known ligand-free helicate crystal structure),³ or using selective deuteration of the 2,2'-bipyridyl rings to obtain sufficient spectral resolution to characterize the corresponding metal-complex and ligand-complex structures.⁴

For helicates **LLD**-turn regions (L-Arg-L-Pro-D-Arg-βAla sequences), homonuclear 2D ¹H-¹H NMR methods (methodology established by Wüthrich and coworkers for peptides sequential assignment) were used.⁴ 2D ¹H-¹H COSY and TOCSY spectra allowed to identify all the resonances within the individual spin system, followed by acquiring 2D ¹H-¹H NOESY and ¹H-¹H ROESY spectra to link the amino acids (Arg, Pro or βAla) of the same side (turn). We also acquired the 2D ¹H-¹⁵N HSQC spectrum (Figure S18) to identify the corresponding NH chemical shifts, which could help in the assignment of crowded ¹H-¹H 2D NMR spectra. We detected three (x 2) NH β-Ala3/5 resonances, two of them corresponding to the main two species observed for HPLC-peak 1 (the main two sets of NMR peaks are also observed and assigned in 2D ¹H, ¹H NOESY spectrum; labeled isomers **A** and **B**). In Figure S22, we show the resonance assignment for the L-Arg-D-Pro-L-Arg NH-αH correlations in 2D ¹H-¹H TOCSY spectrum. Also, the 2D spectra helped to confirm the presence of four prolines isomers, two with *trans* and two with *cis* conformation (specific chemical shifts/NOEs in Figure S19, Figure S20, Figure S21, and Table S1, Table S2), that together with the differences observed for HPLC-peaks 1 and 2 after time (commented below, Figure S16), confirmed the presence of two main species in solution for HPLC-peak 1 (isomers **A** and **B**). NOE peaks between proline and its preceding residue Xaa can be utilized to distinguish between *cis* and *trans* Xaa-Pro peptide bonds: *trans* Xaa-Pro shows strong NOEs/ROEs between the two δH(Pro) and αH(Xaa) and between the two

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$\delta\text{H}(\text{Pro})$ and $\text{NH}(\text{Xaa})$, while *cis* Xaa-Pro between $\alpha\text{H}(\text{Pro})$ and $\alpha\text{H}(\text{Xaa})$ and between $\alpha\text{H}(\text{Pro})$ and $\text{NH}(\text{Xaa})$. Due to the partial overlapping between water resonance and Pro H α and H γ relevant cross peaks in the 2D ^1H - ^1H NOESY spectrum, and lower sensitivity of 2D ^1H - ^1H ROESY spectrum, not all the typical cross-peaks for *trans/cis* Pro bonds could be assigned unambiguously (Figure S19 and Figure S20, Figure S11 and Table S2), in particular for isomer **B** (HPLC-peak 1) and HPLC-peak 2.

Figure S11. Cross peaks for *trans/cis* Pro bonds observed in the corresponding 2D ^1H - ^1H NOESY/ROESY spectra.

To confirm the proline isomers, carbon chemical shifts were also measured by acquisition of 2D ^1H - ^{13}C heteronuclear correlation spectra. In these spectra, the signals of proline ring carbons are well resolved for *cis* and *trans* isomers. The pattern of the chemical shifts compared to the random coil values for individual amino acids were used to identify the amino acid type (Arg or Pro). Heteronuclear 2D ^1H - ^{13}C HSQC and HSQC-TOCSY experiments allowed us the characterization of ^{13}C NMR chemical shifts for each of the two prolines of the two main species of $\text{Co}(\text{III})_2\text{LLD}$ (isomers **A** and **B**, Figure S22, Figure S25 and Figure S26) and the confirmation of the 2D ^1H - ^1H proton spectra observation of two Prolines in *cis* and two prolines in *trans*. The chemical shift assignments for the Arg-Pro-Arg and β -Ala proton and carbon resonances are given in Table S1. It is known that the chemical shift difference between C_β and C_γ ($\Delta\delta_{\beta\gamma}$) in proline rings could help to identify *trans/cis* Pro conformations.⁵ This difference for the prolines residues of the two main components (isomers **A** and **B**) of $\text{Co}(\text{III})_2\text{LLD}$ HPLC-peak 1 (last column, Table S1) are the expected for *cis/trans* prolines and confirmed the chemical shift assignment and the 2D ^1H - ^1H NOE/ROE correlations used for the identification of *cis/trans* prolines in the helicate (listed at Table S2). The observed trend for ^{13}C - C_β values of L-Arg/D-Arg in isomer **A** helicate (Table S1) must be pointed out, since their respective chemical shift values are exchanged when comparing the peptidic loops: L-Arg1 (25.8 ppm) and D-Arg1 (27.2 ppm) for the first loop and L-Arg2 (27.5 ppm) and D-Arg2 (25.2 ppm). This trend is related to the differences in the proximity of the L-Arg/D-Arg amino acid nuclei to the shielding cone of the heterocycles depending on the loop where they are located, as observed in the MD optimized structures. For the isomer **B** (HPLC-peak 1), we found a higher ^{13}C C_β chemical shift (lower shielding) than for isomer **A** and we did not observe differences in L-Arg/D-Arg ^{13}C C_β chemical shifts between the two loops (Table S1). This can be related to the more stretched shape of isomer **B**, setting the corresponding loops at longer distance from the bipyridine rings.

The expected structure for $\text{Co}(\text{III})_2\text{LLD}$ is an octahedral complex in which the 2,2'-bipyridyl groups are fixed in the *cis*-planar conformation. The ^1H chemical shifts of 3 and 3' bipyridyl protons in the metal complex are the most deshielded, 4 and 4' protons are the most spread and 6 and 6' are the most shielded, following the trends observed previously in the literature for bipyridyl-metal complexes and the magnetic anisotropy effects known for aromatic rings (Figure S23).⁶ Protons 6 and 6' are shielded because they are lying very closely above the plane of neighbor pyridine rings, protons 3 and 3' are deshielded because they are on the side of their pyridyl rings and outside the effects of neighboring aromatic rings. We observed a high number of resonances for bipyridyl protons due to differences in bipyridyl environments and lack of structural symmetry, so the specific assignment of aromatic protons is not completely accessible. Aromatic

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proton/carbon chemical shift could be affected by different substituents (R-C(=O)-NH-bipy for 3,4 and 6 protons or bipy-C(=O)-NH-R for 3',4' and 6' protons), pyridine rings pointing inwards the structure interior or towards the external peptidic turns, differences in peptidic turn conformation (for example, *trans/cis* Pro isomerism), the coexistence of diverse helicate isomers in slow exchange, etc. Based on ^1H and ^{13}C chemical shifts alone, we have not enough resolution to assign specific bipyridyl protons, but ^1H - ^1H NOEs/ROEs with the amino acid protons near them helped to assign some aromatic protons at short-medium distance to the protons in the loops (Figure S25).

Metal/Dynamics effects

a) Metal effects

Comparison of 1D- ^1H and 2D ^1H - ^1H spectra of Fe(II) and Co(III) **LLD** helicates (Figure S14 and Figure S15) showed that:

- There are spectral consequences of the increase of the ionic radii from the Co(III) to the Fe(II) (from 0.55 pm to 0.75 pm; Low Spin states).⁷ This can be translated for Fe(II) helicates in less steric strain, faster proline isomerization and could be related to the fluxionality of the Fe(II) complexes that will show faster chemical exchange in the NMR tube in solution
- Due to its characteristic chemical shift (the most downfield resonances), we could detect an upfield shift of NHbipy2/4/6 resonances when going from the Co(III) to the Fe(II) complexes.
- The spectra in the aliphatic region is very similar for both metal complexes, the aromatic region has more relevant differences in chemical shifts and resonances intensities.
- Broadened resonances in the 2D ^1H - ^1H NOESY spectrum of Fe(II) complex and fewer NOE cross peaks.

b) Co(III) helicate dynamics

We observed spectral changes over time for HPLC-peak 2 in solution. After 72 h, its NMR spectra have the spectral features of HPLC-peak 1, with two main components identified in solution, isomers **A** and **B**, in slow equilibria in the NMR chemical shift timescale. Figure S16 and Figure S17 show these changes for the resonances corresponding to 1D aromatic/amide region and 2D ^1H - ^1H NOESY correlations for NH- $\alpha/\beta\text{CH}_2$ (in particular, for β -Ala3/5, the b-Ala at helicate turns), thus revealing the conformational flexibility of the peptidic turns in the metal complex.

Our hypothesis for the origin of the two HPLC fractions considering the experimental data (HPLC/NMR) is the incomplete helicate folding with the final topology due to slow dynamics. This leads to the isolation of two peaks after the oxidation process, HPLC-peak 1 (stable, with two isomers **A** and **B** in slow exchange) and peak-2 (Figure S16 and Figure S17 show the progressive formation of the two isomers **A** and **B** over time). See more details in synthesis of the Co(III) peptide helicate subparagraph. The different media for the HPLC purification (acidic with acetonitrile) and the NMR experiments (buffered water at neutral pH) could account for these observations.

We also suggest that proline isomerization could introduce an additional conformational variable in the metal complex structure in solution and to give rise to the observed NMR spectra changes in the isomers and the changes over time, as observed in Figure S20 where helicate HPLC peak-2 ROEs are missing for *cis*-Pro conformations.

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NMR Structural analysis

For peptides with *trans* X-Pro bonds and Pro at position 2 of the turn, short interproton distances are described for $d_{NN}(3,4)$ (type I and II), $d_{\delta N}(2,3)$ (type I) and $d_{\alpha N}(2,3)$ (type II) and long for $d_{\alpha N}(2,4)$ for type I and type II β -turns.⁸

For linear peptides, these short distances give characteristic ^1H - ^1H NOE/ROE connectivity and could help to identify and detect peptides populations with β -turns depending of population levels and the averaging of conformations in solution. Pro-D-Xaa sequences are known to strongly promote type II β -turns and to reduce the population of the *cis*-Pro isomer.⁹

For the helicates, we have the advantage that the presence of metal and bipyridine “locks” the turn structure, so we decrease the number of conformations available and we slow the conformational averaging in solution, so we could detect specific NOEs and conformations for isomer **A** (HPLC-peak 1):

- We observed NOEs between $d_{NN}(3,4)$ for both sides of the helicate (Table S2).
- Due to resonances overlap, for *t*Pro1-D-Arg1 pair we could not unambiguously discard $d_{\alpha N}(2,3)$, but we observed $d_{\delta N}(2,3)$.
- We also observed $d_{\alpha N}(1,3)$ NOEs for *c*Pro2-D-Arg2 pair.
- We observed NOEs between $d_{NN}(1,3)$ for both sides of the helicate (Table S2).¹⁰
- Other criterion for β -turn formation is the observation of weak $d_{\alpha N}(i,i+2)$ connectivity, which unambiguously establishes the bend of the peptide chain (Table S2).¹¹

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Figure S12. Free **LLD**, **Fe(II)₂LLD** and **Co(III)₂LLD** helicates nomenclature used in this paper (labeling 1 to 6 from N- to C-terminal end).

Figure S13. Aromatic (top) and aliphatic (bottom) region of the ^1H 1D NMR spectra of the $\text{Fe(II)}_2\text{LLD}$ and the $\text{Co(III)}_2\text{LLD}$ HPLC-peak1 helicates. The spectra were acquired at 298K in 15 mM HEPES buffer (90% $\text{H}_2\text{O}/10\%$ D_2O , pH 5).

Figure S14. 2D ^1H - ^1H NOESY NMR spectra of the $\text{Fe(II)}_2\text{LLD}$ (top) and $\text{Co(III)}_2\text{LLD}$ HPLC-peak 1 (bottom) helicates. The spectra were acquired at 298K in 15 mM HEPES buffer (90% H_2O /10% D_2O , pH 5).

Figure S15. Top: aromatic region of the ¹H 1D NMR spectra of the Co(III)₂LLD HPLC-peak 1 helicite isolated by reverse-phase HPLC at different temperatures. The spectra were acquired in 15 mM HEPES buffer (90% H₂O/10% D₂O, pH 5). Bottom: comparison between the free ligand **LLD** and the Co(III)₂LLD HPLC-peak 1 helicite spectra.

Figure S16. Aromatic region of the ^1H 1D NMR spectra of the two $\text{Co(III)}_2\text{LLD}$ HPLC-peaks isolated (1 and 2) by reverse-phase HPLC (HPLC-peak 1-2 mM and HPLC-peak 2-1 mM, the spectra in the figure are scaled taking into account the concentration difference). The spectra were acquired at 298 K in 15 mM HEPES buffer (90% H_2O /10% D_2O , pH 5). We observed interconversion of the HPLC-peak 2 towards the HPLC-peak 1 after 72 hours in buffer solution, also confirmed by 2D NMR (see below).

Figure S17. Top: 2D correlations for NH- $\alpha/\beta\text{CH}_2$ $\beta\text{-Ala3/5}$ (2D ^1H - ^1H NOESY), showing the interconversion of the $\text{Co(III)}_2\text{LLD}$ HPLC-peak 2 towards the HPLC-peak 1 after 72 h in buffer solution. Bottom: comparison of αC - αH Arg/Pro (2D ^1H - ^{13}C HSQC) and αH - δH Pro (2D ^1H - ^1H TOCSY) correlations for both isomers.

Figure S18. 2D ^1H - ^{15}N HSQC spectrum of $\text{Co(III)}_2\text{LLD}$ HPLC-peak 1 (2 mM in 15 mM HEPES buffer, 90% $\text{H}_2\text{O}/10\%$ D_2O , pH 5). The spectrum had three (x2) NH β -Ala3/5 peaks, two of them corresponding to the main two species observed for $\text{Co(III)}_2\text{LLD}$ HPLC-peak 2 (the main two sets of NMR peaks are also observed in 2D ^1H - ^1H NOESY spectrum), consistent with the presence of two major conformations for HPLC-peak 1. The NH β -Ala3/5 HPLC-peak 1 minor species proton resonance correspond to the proton resonances from main species in the 2D ^1H - ^1H NOESY spectrum of HPLC-peak 2 (see below).

Figure S19. 2D ^1H - ^1H ROESY spectrum of $\text{Co(III)}_2\text{LLD}$ HPLC-peak 1. The characteristic ROEs used to assign the *cis* and *trans* conformations of the isomer **A** (HPLC-peak 1) are labeled. We used some of the observed NOEs peaks between (*cis*/*trans*) Pro and L-Arg/D-Arg amides NHs to confirm the *trans*/*cis* conformation of Pro1/Pro2 for isomer **B** (HPLC-peak 1) (see NOE's list in tables S3 and S4). For spectra peak labels, the same conventions that in the main text and chemical shift tables were used (figure S1), but Greek letters (α , β , ...) were replaced for Latin letters (a, b, ...).

Figure S20. 2D ^1H - ^1H ROESY spectrum of $\text{Co(III)}_2\text{LLD}$ helicate-peak 2 after 48 hr in solution. Some of the characteristic ROEs used to assign the *trans*-Pro1 conformation of peak 1 major species are observed, but the expected ROEs for *cis*-Pro2 are missing (empty box).

Figure S21. (Top) 2D ^1H - ^{13}C HSQC-TOCSY aC-NH region for helicate turn amino acids; confirmation of the presence of two main species in $\text{Co(III)}_2\text{LLD}$ HPLC-peak 1 sample (labeled isomers **A** and **B**). (Bottom) 2D ^1H - ^{13}C HSQC aC-aH region for helicate turn amino acids; confirmation of the presence of two main species in HPLC-peak 1 sample (labeled isomers **A** and **B**).

Figure S22. 2D ^1H - ^1H TOCSY NH-aH region for helicate turn Arg-Pro-Arg amino acids; confirmation of the presence of two main species in $\text{Co(III)}_2\text{LLD}$ HPLC-peak 1 sample (labeled isomers **A** and **B**).

Figure S23. 2D ^1H - ^{13}C HSQC-TOCSY $\text{Co(III)}_2\text{LLD}$ HPLC-peak 1 helicate bipyridine region overview.

Figure S24. $\text{Co(III)}_2\text{LLD}$ HPLC-peak 1 2D ^1H - ^{13}C HSQC-TOCSY bipyridine region with some specific assignments (with the help from 2D ^1H - ^1H NOESY through space correlations).

Figure S25. $\text{Co(III)}_2\text{LLD}$ HPLC-peak 1 2D ^1H - ^{13}C HSQC-TOCSY of aC amino acids region with corresponding assignments.

Table S1. ^1H and ^{13}C chemical shifts assignment (in ppm) for the most characteristic resonances (amino acids and β -Ala at the turns) of $\text{Co(III)}_2\text{LLD}$ HPLC-peak 1 two main species (isomers **A** and **B**) in 15 mM HEPES buffer, 90% $\text{H}_2\text{O}/10\%$ D_2O , pH 5.

Residue	Component	NH	α -CH		β -CH		γ -CH		δ -CH		$\Delta\delta_{py}$
t Pro1	A		4.07	61.80	2.12, 1.72	28.53	2.02, 1.85	25.36	3.73, 3.44	47.60	3.17
c Pro2	A		4.45	59.98	2.20, 1.91	31.56	1.85	22.10	3.46, 3.32	47.60	9.46
L-Arg1	A	9.06	4.56	53.05	1.81, 1.68	25.80	1.52, 1.27	24.18	3.14	40.60	
L-Arg2	A	9.02	4.22	53.00	1.70, 1.65	27.58	1.68, 1.48	24.43	3.14	40.60	
D-Arg1	A	8.66	4.03	53.62	1.89	27.12	1.54	24.47	3.09	40.60	
D-Arg2	A	8.58	4.07	53.28	1.95	25.21	1.48	24.47	3.09	40.60	
t Pro1	B		4.21	62.00	2.19, 1.68	29.19	1.85, 1.68	25.07	3.69, 3.38	47.90	4.12
c Pro2	B		4.17	61.13	2.22, 1.92	31.60	1.94	21.90	3.69, 3.44	47.90	9.70
L-Arg1	B	8.58	4.17/4.30	53.28/53.00	1.62, 1.54	29.68	1.41	24.40	3.05	40.60	
L-Arg2	B	8.52	4.17/4.30	53.28/53.00							
D-Arg1	B	8.63/8.66	4.24	52.00	1.74	28.60	1.42	24.40	3.07	40.60	
D-Arg2	B	8.63/8.66	4.24	52.00							
β -Ala3	A	8.09	2.43	36.67	3.14, 3.43	35.1-35.7					
β -Ala5	A	8.12	2.44	36.67	3.18, 3.43	35.1-35.7					
β -Ala3	B	7.71/7.65	2.59	35.24	3.07, 3.48	35.1-35.7					
β -Ala5	B	7.71/7.65	2.57	35.24	3.00, 3.51	35.1-35.7					

Table S2. NMR cross-peaks features as indicators of turn-like structures (top) and trans/cis Pro peptide bond (bottom) obtained from 2D ^1H - ^1H NOE and ROESY spectra of $\text{Co(III)}_2\text{LLD}$ (for HPLC-peak 1 isomer **A**). Nomenclature: distances (1 to 4) from L-Arg to β -Ala.

f1 (ppm)	f2 (ppm)	Assignment	Comments
4.23	8.58	$\alpha\text{HArg2-NHDAArg2}$	ROE; $d_{\alpha\text{N}}(1,3)$
4.56	8.66	$\alpha\text{HArg1-NHDAArg1}$	ROE; $d_{\alpha\text{N}}(1,3)$
8.66	8.08	$\text{NH}\beta\text{Ala3-NHDAArg1}$	NOE; $d_{\text{NN}}(3,4)$
8.58	8.13	$\text{NH}\beta\text{Ala5-NHDAArg2}$	NOE; $d_{\text{NN}}(3,4)$
3.70	8.66	$\text{NHDAArg1-}\delta^2\text{HtPro1}$	NOE; $d_{\delta\text{N}}(2,3)$
9.07	8.66	NHArg1-NHDAArg1	NOE; $d_{\text{NN}}(1,3)$
9.02	8.59	NHArg2-NHDAArg2	NOE; $d_{\text{NN}}(1,3)$

f1 (ppm)	f2 (ppm)	Assignment	Comments
4.46	4.22	$\alpha\text{HcPro2-}\alpha\text{HArg2}$	species A, ROE
4.55	3.44	$\alpha\text{HArg1-}\delta^1\text{HtPro1}$	species A, ROE
4.56	3.73	$\alpha\text{HArg1-}\delta^2\text{HtPro1}$	species A, ROE
4.46	1.68	$\alpha\text{HcPro2-}\beta^1\text{HArg2}$	species A, ROE
4.45	8.58	$\alpha\text{HcPro2-NHDAArg2}$	species A, ROE
3.73	8.66	$\delta^2\text{HtPro1-NHDAArg1}$	species A, NOE
1.92	4.08	$\beta^1\text{HcPro2-}\alpha\text{HDAArg2}$	species A, ROE

Table S3. Inter-residual and intra-residual NOEs for Co(III)₂LLD (isomer **A** of HPLC-peak 1).

f1 (ppm)	f2 (ppm)	Assignment (f2-f1)	Species	Protons number	Distances (NOEs)
3.12	1.67	bHLArg2-dHLArg2	A	4	3.01
4.44	1.68	bHLArg2-aHcPro2	A	2	n.m.
4.06	1.72	aHtPro1-bHtPro1	A	1	2.72
4.06	2.12	aHtPro1-bHtPro1	A	1	2.39
3.32	2.22	bHcPro2-dHcPro2	A	1	3.00
3.42	2.22	bHcPro2-dHcPro2	A	1	3.13
2.42	3.41	bHbAla3/5-aHbAla3/5	A	4	3.06
3.15	3.43	bHbAla3/5-bHbAla3/5	A	2	2.35
2.02	3.42	gHtPro1-dHtPro1	A	1	2.45
3.72	3.43	dHtPro1-dHtPro1	A	1	2.27
2.02	3.73	gHtPro1-dHtPro1	A	1	2.52
1.85	3.72	gHtPro1-dHtPro1	A	1	2.29
9.03	7.47	6Hbipy4-NHLArg2	A	1	3.74
7.30	7.47	6Hbipy-6Hbipy	A		n.m.
7.36	7.47	6Hbipy-6Hbipy	A		n.m.
3.40	7.54	6Hbipy3/5-bHbAla3/5	A	1	4.03
3.39	7.59	6Hbipy3/5-bHbAla3/5	A	1	4.06
1.53	8.08	gHDArg1-NHbAla3	A	2	4.07
1.88	8.08	bHDArg1-NHbAla3	A	2	4.88
8.66	8.08	NHbAla3-NHDArg1	A	1	3.03
1.49	8.12	gHDArg2-NHbAla5	A	2	4.41
4.06	8.12	aHDArg2-NHbAla5	A	1	2.98
1.93	8.12	bHDArg2-NHbAla5	A	2	4.88
8.58	8.13	NHbAla5-NHDArg2	A	1	3.13
1.69	8.12	bHLArg2-NHbAla5	A	2	4.43
2.43	8.29	4Hbipy5-aHbAla5	A	1	4.09
3.42	8.29	4Hbipy5-bHbAla5	A	1	4.06
3.15	8.29	4Hbipy5-bHbAla5	A	1	3.88
2.42	8.41	4Hbipy3-aHbAla3	A	1	3.99
3.13	8.41	4Hbipy3-bHbAla3	A	1	3.90
3.41	8.42	4Hbipy3-bHbAla3	A	1	4.70
1.92	8.57	NHDArg2-bHcPro2	A	1	3.43
4.43	8.57	NHDArg2-aHcPro2	A	1	2.79
2.22	8.57	NHDArg2-bHcPro2	A	1	4.23
3.13	8.57	NHDArg2-bHbAla5	A	1	4.00
3.41	9.09	NHbipy3/5-bHbAla3/5	A	1	2.15
3.40	9.11	NHbipy3/5-bHbAla3/5	A	1	2.55
3.08	8.65	NHDArg1-bHbAla3	A	1	3.58
1.72	8.66	NHDArg1-bHtPro1	A	1	3.57
2.12	8.66	NHDArg1-bHtPro1	A	1	3.60
3.70	8.66	NHDArg1-dHtPro1	A	1	4.33
9.07	8.69	NHLArg1-NHDArg1	A	1	3.25
1.81	8.74	4Hbipy-bHLArg1	A	1	4.15
1.68	8.74	4Hbipy-bHLArg1	A	1	3.00
7.42	8.85	NHbAla2/4/6-6Hbipy1/3/5	A	1	3.59
7.62	8.90	NHbAla2/4/6-6Hbipy1/3/5	A	1	3.43
7.52	8.91	NHbAla2/4/6-6Hbipy1/3/5	A	1	3.34
7.39	10.61	NHbipy2/4/6-4Hbipy2/4/6	A	1	2.88
7.30	10.65	NHbipy2/4/6-4Hbipy2/4/6	A	1	2.77
7.59	10.66	NHbipy2/4/6-4Hbipy2/4/6	A	1	3.17
2.41	10.61	NHbipy2/4/6-aHbAla2/4/6	A	2	3.08
2.53	10.61	NHbipy2/4/6-aHbAla2/4/6	A	2	2.62
2.54	10.65	NHbipy2/4/6-aHbAla2/4/6	A	2	2.48
9.10	7.45	NHbipy3/5-6Hbipy3/5	A	1	3.50
2.54	7.30/7.32	aHbAla2/4/6-4Hbipy2/4/6	A	2	2.67
2.54	7.37/7.39	aHbAla2/4/6-4Hbipy2/4/6	A	2	2.71
3.39	10.65	bHbAla2/4/6-NHbipy2/4/6	A	2	3.70
3.44	10.66	bHbAla2/4/6-NHbipy2/4/6	A	2	4.25

Table S4. Inter-residual and intra-residual NOEs for Co(III)₂**LLD** (isomer **B** of HPLC-peak 1).

f1 (ppm)	f2 (ppm)	Assignment (f2-f1)	Species	Protons number	Distances (NOEs)
4.22	1.68	bHtPro1-aHtPro1	B	1	2.66
4.22	2.20	bHtPro1-aHtPro1	B	1	2.47
3.00	2.57	aHbAla3/5-bHAla3/5	B	2	2.24
8.64	9.11	NHbipy3/5-4Hbipy3/5	B	1	3.50
8.74	9.05	NHbipy3/5-4Hbipy3/5	B	1	3.42
2.55	9.08	aHbAla3/5-NHbipy3/5	B	1	2.36
2.54	9.11	aHbAla3/5-NHbipy3/5	B	1	2.05
9.12	7.32/7.35	6Hbipy3/5-NHbipy3/5	B	1	2.08
9.08	7.51/7.54	6Hbipy3/5-NHbipy3/5	B	1	2.36
2.56	7.52	6Hbipy3/5-aHbAla3/5	B	1	3.15
2.55	7.58	6Hbipy3/5-aHbAla3/5	B	1	2.88
1.62	7.64	bHLArg2-NHbAla5	B	2	2.82
1.41	7.64	gHLArg2-NHbAla5	B	2	3.72
4.32	7.64	NHbAla5-aHLArg2	B	1	2.58
1.62	7.71	bHLArg1-NHbAla3	B	1	2.65
1.54	7.71	bHLArg1-NHbAla3	B	1	3.10
1.42	7.71	gHLArg1-NHbAla3	B	2	3.83
4.32	7.71	NHbAla3-aHLArg1	B	1	2.56
8.47	7.72	NHLArg2-6H'bipy4	B	1	2.15
1.94	8.51	NHLArg2-dHcPro2	B	1	3.08
1.68	8.58	NHLArg1-dHtPro1	B	1	2.28
2.54	8.59	NHLArg1-aHbAla3	B	2	3.07
1.68	8.63	NHDArg1-bHtPro1	B	1	2.54
2.54	8.65	NHDArg1/2-aHbAla3/5	B	4	2.94
2.56	8.86	NHbAla2-aHbAla4	B	2	3.20
2.56	10.41	NHbipy2/4/6-aHbAla2/4/6	B	1	2.85
7.87	10.41	NHbipy2/4/6-4Hbipy2/4/6	B	1	2.81
2.41	10.42	NHbipy2/4/6-aHbAla2/4/6	B	1	2.75
2.63	10.43	NHbipy2/4/6-aHbAla2/4/6	B	1	2.69
2.41	10.46	NHbipy2/4/6-aHbAla2/4/6	B	1	3.10
2.55	10.47	NHbipy2/4/6-aHbAla2/4/6	B	1	2.67
7.90	10.48	NHbipy2/4/6-4Hbipy2/4/6	B	1	2.62
7.92	10.50	NHbipy2/4/6-4Hbipy2/4/6	B	1	2.74

Molecular Modeling

Structural determination by NMR and MDs. Molecular dynamics (MD) simulations were set up with the xleap program solvating the helicates structures with a box of pre-equilibrated TIP3P water molecules, the total charge was balanced with chlorine ions (ions94.lib library). For the standard residues the amber99sb force field was used while for the Fe-bipyridines centers, the Fe-bonding force constants and equilibrium parameters, were obtained through the *Seminario* method,¹² the GAFF force field was adopted for the remaining atoms. The point charges were derived using the RESP (Restrained ElectroStatic Potential) model.¹³ The force fields building operations were carried out using the MCPB.py.¹⁴ The solvent first and the whole system next, were submitted to 3000 energy minimization steps to relax possible steric clashes then, thermalization of water molecules and side chains was achieved by increasing the temperature from 100 K up to 300 K. MD simulations under periodic boundary conditions were carried out during 100 ns with *OpenMM* engine.¹⁵ Restrained molecular dynamics were carried out with AMBER using SANDER Engine including flat-bottom NOE-derived distance potential function. The weight of the restrains strength has been increased gradually in 2 ns steps. Clustering analysis was performed with *cptraj* along all the trajectories considering all the heavy atoms.

Docking Studies. Docking studies were carried out with the *GaudiMM* platform, using the two Fe(II) helicate structures obtained from the clustering analysis as initial conformations, together with the unique crystallographic structure of a helicate bound to a DNA three way junction (PDB: 2ET0). The original helicate was removed previous to docking. Global motions of the DNA strands were allowed through Normal Modes Analysis (NMA) setting a maximum displacement of 2.5 Å along the 11 lowest energy modes, while the rotamers and torsions of the helicate were also considered. The latter ensures a correct degree of flexibility that could be achieved by the helicate during the binding process. In fact, from the unrestrained MDs: i) no substantial backbone motion is observed, displaying RMSDs of 1.5 and 2.5 Å in average for isomers **A** and **B**, respectively; and ii) a predominant conformation occupying about 64% and 89% of the whole trajectory are observed for **A** and **B**, respectively. The *GaudiMM* genes used for the exploration included the finding of all the possible rotamers and torsions of the helicate. Moreover, the helicates were allowed full rotational in an evaluation sphere of 26.2 Å from the centroid of the triplex core. Finally, the *GaudiMM* results were evaluated considering those displacements in term of genes (conformational exploration) and clashes as well as vina scores for the objectives.¹⁶

Molecular Dynamics simulations

Molecular dynamics (MD) simulations were set up with the xleap program solvating the proteins structures with a box of pre-equilibrated TIP3P water molecules, the total charge was balanced with chlorine ions (ions94.lib library). For the standard residues the amber99sb force field was used while for the Fe-bipyridines centers, the Fe-bonding force constants and equilibrium parameters, were obtained through the *Seminario* method,¹⁷ the GAFF force field was adopted for the remaining atoms.

Geometry and harmonic frequencies were computed through *Gaussian 09* (revision D.01),¹⁸ using the B3LYP functional including Grimme' empirical correction for dispersion.¹⁹ Fe atoms were described using

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the scalar-relativistic Stuttgart–Dresden SDD double- ζ basis set complemented with a set of f polarization functions and its associated pseudopotential.²⁰ The 6-31g(d,p) basis set was used for the oxygen atoms

The point charges were derived using the RESP²¹ (Restrained ElectroStatic Potential) model. The force fields building operations were carried out using the MCPB.py.²² The solvent first and the whole system next, were submitted to 3000 energy minimization steps to relax possible steric clashes. Then, thermalization of water molecules and side chains was achieved by increasing the temperature from 100 K up to 300 K. MD simulations under periodic boundary conditions were carried out during 100 ns with *OpenMM* engine.²³

Restrained molecular dynamics were carried out with AMBER using SANDER Engine including flat-bottom NOE-derived distance potential function. The weight of the restrains strength has been increased gradually in 2 ns steps.

Clustering analysis was performed with *cptraj* along all the trajectories considering all the heavy atoms.

GaudiMM calculations

The structural simulations of the helicates into the **twDNA** were performed with GaudiMM platform.²⁴ The structure of DNA junction was obtained from the Protein Data bank (pdb code 2ET0), in the unique crystallographic entry of the entire databank that presents both triplex DNA and an helicate complex.²⁵ The original helicate was removed previous to docking. For the helicates tested in this study, two representative structures of the two most populated clusters (from section 1) of both isomers of the helicate were selected to perform the calculations. Hence calculations account for four systems, IsoA (fatty) and IsoB (skinny), each of them with two different conformations (A1 and A2 for IsoA; B2 and B3 for IsoB). For the DNA, global motions of the strands were allowed throughout normal modes analysis (NMA). In order to assess the maximum possible conformational variability of the strand without losing the coherence of the triplex structure, different magnitudes were tested (from 2 to 3 Å) for the maximum displacement along the 11 lowest energy modes. The *GaudiMM* genes used for the exploration included the finding of all the possible rotamers and torsions of the helicate, but the first coordination sphere of the metal remained fixed. The exploration was performed along 100 generations with a population of 100 individuals in three replicas for each isoform, using a local evaluation sphere of 26.2 Å from the centroid of the triplex core. Finally, the results were evaluated considering those displacements in term of genes (conformational exploration) and clashes as well as vina scores²⁶ for the objectives.

Table S5. GaudiMM exploration results (Clashes and Vina Score) for the binding of both isomers (**A** and **B**) of the Fe(II)₂LLD helicate. The radius of the artificial part of each helicate is also reported.

Cluster	Isomer A		Isomer B	
	1 st	2 nd	1 st	2 nd
Clashes	91.508	28.161	15.12	23.722
Vina Score	10.677	-4.276	-11.231	-5.319
Helicate radius	7.5 Å	7 Å	6.3 Å	6.4 Å

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Figure S27. A and B pictures show the front and lateral views of the complex formed by isomer **A** (2nd cluster) with twDNA with a RMSD margin for the normal modes of 2.5 Å. C and D pictures correspond to isomer **B** (2nd cluster) with a RMSD margin for the normal modes of 2.5 Å.

Cell Internalization studies

HeLa cells were grown in monolayers in medium D-MEM supplemented with 5% fetal bovine serum (FBS), 2% L-glutamine and 1% antibiotic mix (penicillin and streptomycin) on glass bottom 35 mm dishes. Cell monolayers were transfected with plasmid pc653 using TransIT®-LT1 (Mirus),²⁷ according to the manufacturer's instructions and incubated at 37 °C. 24 h post-transfection, the cells were washed twice with cold transfer buffer (25 mM HEPES, 125 mM potassium acetate, 2 mM magnesium acetate, 5 mM sodium acetate, 1 mM EDTA, pH 7.3), and permeabilized with 25 mg/mL Digitonin (Sigma) in the same buffer for 3 min on ice. Then, the cells were washed twice with transfer buffer containing 10 mg/mL of BSA and incubated in the same buffer with the preformed helicate (5 μ M **TAMRA-LLD** peptide ligand and 25 μ M $(\text{NH}_4)_2\text{Fe}(\text{SO}_4)_2$) for 30 min, then washed again three times with the BSA-containing buffer, overlaid with D-MEM containing 10% FBS, and observed directly with no further processing.²⁸ Confocal images were captured on an Andor Dragonfly spinning disk confocal system mounted on a Nikon TiE microscope equipped with a Zyla 4.2 PLUS camera (Andor). Images were processed either with Imaris (Oxford Instruments), or Adobe Photoshop CC (Adobe System, California, USA).

Figure S28. HeLa cells were grown in DMEM (Dulbecco modified Eagle Medium) with 10% of fetal bovine serum in a 12-plate box with a glass coverslip of 15 mm. Before carrying out the experiments, cells were washed three times with PBS and finally 0.5 mL of DMEM was added. A determined volume of stock solutions of $\text{Fe}(\text{II})_2\text{Flu-LLD}$ or $\text{Fe}(\text{II})_2\text{TAMRA-LLD}$ were added to each plate in order to reach a final concentration of 5 μ M of the compound. The resulting mixture was incubated overnight under an atmosphere of 5% of CO_2 at 37 °C. After this time the cells were washed three times with PBS and visualized with DAPI staining to label the cell nuclei. Images were alternatively obtained with an Olympus DP-71 digital camera mounted on an Olympus BX51 fluorescence microscope, or with an Andor Dragonfly spinning disk confocal system mounted on a Nikon TiE microscope equipped with a Zyla 4.2 PLUS camera (Andor).

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EMSA experiments

EMSAs were performed with a *BioRad* Mini Protean gel system, powered by an electrophoresis power supplies Power Pac Basic model, maximum power 150 V, frequency 50-60 Hz at 140 V (constant V). Binding reactions were performed over 35 min in 18 mM Tris-HCl buffer (pH 7.5), 90 mM KCl, 1.8 mM MgCl₂, 9% glycerol, 0.11 mg/mL BSA, 2.2% NP-40 and 0.02 mM of ZnCl₂. In the experiments we used 75 nM of the dsDNAs and 200 nM of the twDNAs in a total incubation volume of 20 μ L. twDNA was previously hybridized by mixing equimolar quantities of the different three strands (**Y1**, **Y2**, **Y3**) and heating the mixture at 90 °C during 10 min, followed by slow cooling until rt. dsDNA was also hybridized following the same procedure. Stock solutions of the Fe(II)₂**LLD** helicate were prepared by mixing the ligand (0.5, 1 and 2 μ M) and 5 eq of (NH₄)₂Fe(SO₄)₂ in water followed by 15 min of stabilization. For the Co(III)₂**LLD** helicate, different stock solutions (0.5, 1 and 2 μ M) were prepared in water. After this, aliquots corresponding to the different lanes were prepared by mixing the corresponding DNA (**twDNA** or **dsDNA**) and different concentrations of the preformed helicates (Fe(II)₂**LLD** or Co(III)₂**LLD**). After incubation for 30 min products were resolved by PAGE using a 10% non-denaturing polyacrylamide gel and 0.5 $\times$ TBE buffer for 35 min at 20 °C and analyzed by staining with SyBrGold (Molecular Probes: 5 μ L in 50 mL of 1 $\times$ TBE) for 10 min and visualized by fluorescence. 5 $\times$ TBE buffer: 0.445M Tris, 0.445 M Boric acid.

Control binding experiment with Flu-LLD and twDNA

An additional fluorescence anisotropy titration of a 2 μ M solution of the fluorescein labelled **LLD** derivative (**Flu-LLD**) with increasing concentrations of **twDNA**, demonstrated the key role of metal coordination in **twDNA** recognition. In this case the descending and oscillating pattern observed in the titration showed the lack of interaction between the ligand and the **twDNA**.

Figure S29. anisotropy control of a 2 μ M solution of **Flu-LLD** with increasing concentrations of **twDNA**, where no interaction is appreciated in the decreasing and oscillating pattern of the data.

Curve fitting

Experimental data were fitted with the *DynaFit 4.0* software, which performs a numerical treatment of the system.²⁹ The program is available free of charge for academia at <http://www.biokin.com/dynafit/>.

Dynafit requires plain text files called *scripts* that contains information about the chemical model underlying the experimental data, the values of model parameters, such as starting concentrations of reactants, as well as information about location of the files. A typical script used in the analysis titrations is included below. The file has been commented to indicate the purpose of the keywords and sections, but the reader is recommended to review the *DynaFit* scripting manual distributed along the program or available at the *DynaFit* website.

```
[task]                                ;semicolons indicate comments from actual instructions
task = fit                            ;nature of the calculation to be performed
data = equilibria

[mechanism]                           ;Free-form 1:1 binding model with Kd
R + L <=> RL : Kd dissociation        ;to be calculated as dissociation constant

[constants]                           ;Initial Kd value for iteration
Kd = 1.0 ?                            ;the "?" indicates that this will be optimized

[concentrations]                      ;Fixed conc. of the DNA during the peptide titration
R = 2.0

[responses]                           ;contribution to the spectroscopic signal of each
R = 0.1 ?                             ;of the different components of the equilibrium
RL = 1.5 ?                            ;these will be optimized ("?" after the values).

[data]                                ;location of files and information about the data
variable L                            ;the species that changes conc. during the titration
offset auto ?
directory ./exp/brHis                 ;file path (relative to DynaFit program location)
extension txt
file fl                               ;name of the experimental data file

[output]
directory ./exp/hel/out               ;path indicating location of DynaFit output files
```

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