Chapter 4

Dinuclear iron complexes for direct double strand DNA cleavage

Achieving direct double strand DNA cleavage continues to be a major challenge to the field of oxidative DNA cleavage. To date, double strand DNA cleavage is observed only with natural compounds, such as bleomycin. Although many researchers have claimed that their particular synthetic system displays direct double strand DNA cleavage, careful evaluation of the data often reveals that such claims have to be taken with caution. In this chapter, a new approach to obtain double strand cleavage agents is introduced and discussed. Rather than screening for active DNA cleavage agents, a rational design is applied in linking of two single strand cleavage agents. This approach enables the development of the first synthetic system, which can function as a direct double strand cleavage agent. An attempt was made to establish a link between the topology of the dinuclear complexes and the cleavage activity, but the activity seems to be unaffected by changes in size, rigidity and relative orientation between two active metal centers.*

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Chapter 4

4.1 Introduction

The cleavage chemistry of the mononuclear non-heme iron complex Fe$^{II}$(N4Py) was discussed in Chapter 3.\textsuperscript{1} It has been demonstrated that DNA is cut solely via a single strand cleavage pathway, and that, in contrast to Fe(BLM), no direct double strand cleavage was observed. The covalent attachment of a DNA intercalator resulted in an increase in the DNA cleavage rate, albeit without a change in the cleavage pathway. To access the direct double strand cleavage pathway, two DNA strand cuts have to be introduced in close proximity in opposite strand in rapid succession. The ensemble of the metal binding domain, the spacer, and the DNA intercalator of Fe(BLM) makes that this requirement for double strand cleavage can be met within one turnover of the catalyst (see Chapter 1). The design and synthesis of a Fe(BLM) model, which has all these particular features required for direct double strand cleavage, would be a time consuming and tedious process. Therefore, another approach will have to be followed to meet the requirements for the direct double strand cleavage of DNA, which is more accessible and straightforward.

4.1.1 Dinuclear complexes for DNA oxidation

As mentioned, to mimic the double strand cleavage activity of bleomycin, the most important challenge is to introduce two single strand cuts in both DNA strands in rapid succession. The approach taken here will be the covalent linking of two single strand cleavage agents to each other, to result in a net double strand cleavage agent. The idea of tethering two metal complexes together is not unprecedented. In recent years, considerable attention has been given to multinuclear copper systems in the cleavage of DNA. Karlin and Rokita found that dinuclear copper tripyridyl systems (Figure 4.1, top) have an increased activity when compared to the monotopic complex based on an equimolar amount of copper.\textsuperscript{2} Moreover, selective DNA cleavage was observed at junctions between single and double stranded DNA (see Chapter 1, section 1.4.3.2).\textsuperscript{3}

![Figure 4.1 Dicopper complexes used by Karlin and Rokita in the oxidation of DNA.](image)

This increased activity and selectivity has been observed for another dinuclear complex also (Figure 4.1, bottom), although these complexes are engaged in oxidation of guanine base pairs rather than DNA backbone oxidation.\textsuperscript{4,5} It is proposed that the increase in activity of the dinuclear copper complexes is the result of more efficient oxygen activation,\textsuperscript{5-8} due to a synergistic effect of both Cu$^{2+}$ centers.\textsuperscript{9}

Related complexes were investigated by Guo and co-workers (Figure 4.2).\textsuperscript{10} They confirmed the increased DNA cleavage activity of dinuclear copper complexes, compared to the corresponding mononuclear complex. Moreover, a structural relationship between the relative orientation of the two copper centers and the DNA cleavage activity was found.
The complex with the 1,3-structural motif (Figure 4.2, center) has a higher activity in oxidative DNA cleavage than the corresponding complex with the 1,4-structural motif (Figure 4.2, right). Also here it is proposed that the difference in reactivity is the result of a synergistic effect of the two copper centers in the activation of dioxygen.

Figure 4.2 Complexes studied by Guo in oxidative DNA cleavage.

It is somewhat peculiar that with the latter formation of linear DNA is not observed when supercoiled DNA is used as a benchmark substrate. Rather, immediate fragmentation of the formed nicked DNA into smaller DNA fragments (smear formation) was observed. Unfortunately, the authors do not provide any explanation for these strange findings. Moreover, this interesting discrepancy with all combined results in the field of DNA oxidation is being ignored in the remainder of the article.

Interestingly, the covalent attachment of a third copper center did not increase the activity further, but rather displayed a reduced activity when compared to the 1,3-substituted dinuclear complex (trinuclear complexes will be discussed in more detail in Chapter 5). Several other dinuclear copper systems have been reported for the oxidation of DNA, however, no examples of dinuclear iron complexes for oxidative DNA cleavage are known. Related examples include dinuclear iron complexes, which are used as synthetic nucleases and phosphatases, which are engaged in Lewis acid activation mechanisms rather than in oxidative mechanisms.

4.1.2 Target complexes for direct double strand oxidative DNA cleavage

The approach presented in this chapter will be the covalent linking of two single strand cleavage agents to result in a net double strand cleavage agent (Figure 4.3). The single strand cleavage agent of choice is the FeII(N4Py) complex (1), due to its high activity in the oxidation of DNA. Moreover, the synthesis of the N4Py ligand allows for facile derivatization steps to introduce additional functionalities, which are required to obtain the target complexes.

It should be noted that the target dinuclear iron complexes are inherently different than the known dinuclear copper complexes from a mechanistic point of view. The approach taken in this chapter is to have two independently operating cleavage agents in proximity to each other, which can lead to increased activity and double strand DNA cleavage. In contrast, the increased activity from the dinuclear copper complexes stems from a cooperative interaction between the two copper centers to activate dioxygen or hydrogen peroxide.

Figure 4.3 FeIII(N4Py) (1) (left) and the target dinuclear FeIII(N4Py)2 complex (right).
4.2 Synthesis of the dinuclear complexes

4.2.1 Synthesis of the ligands

Over the years, many derivates of N4Py have been synthesized and much knowledge is gathered about the synthesis of this class of ligands. Examples include N3Py systems (ligands similar to N4Py, but lacking one pyridyl moiety),20-23 monosubstituted N4Py derivatives,18,19,21 disubstituted N4Py derivatives,24 and even tetrasubstituted N4Py complexes have been reported.25-27 Due to this in-house knowledge,28 the synthetic limits and opportunities of N4Py type ligands are quite well known.

The synthesis of these ligands via a common intermediate is useful to allow for rapid access to a whole range of ditopic ligands. A precursor in the synthesis of the N4Py derivative used in the synthesis of the acridine system ($\text{2}$) was considered to be useful in the construction of ditopic ligands. The last step in the synthesis of $\text{2}$ is the reaction of 9-chloroacridine with N4Py ligand $\text{3}$, which was derivatized with an aminopropyl functionality (Scheme 4.1).18,19

![Scheme 4.1 Synthesis of the N4Py acridine derivative 2 from N4Py-C3-NH$_2$ (3) and chloroacridine in phenol.](image)

This amino derivative $\text{3}$ can be used to construct symmetric dimers by reaction with an appropriate difunctionalized linker molecule. Importantly, the linker functionality is introduced in the last step of the synthesis, which allows for a straightforward synthesis of different ditopic ligand derivatives. The amine functionality of $\text{3}$ allows the possibility of employing a straightforward peptide coupling strategy, of which several methods are available.29-32 By taking this approach, two equivalents of $\text{3}$ can be reacted with one equivalent of a diacid (or activated diacid) to furnish the desired ditopic ligands $\text{4}$, $\text{5}$ or $\text{6}$ in one step (Scheme 4.2).

![Scheme 4.2 Synthesis of the ditopic ligands 4, 5 or 6 via reaction of 3 with a diacid.](image)

To investigate a possible effect of the rigidity of the spacer backbone, a 1,2-disubstituted phenyl ring can be used ($\text{4}$). A possible effect of the spacer length can be investigated by comparison of the results obtained from the iron complexes of $\text{5}$ and $\text{6}$. An additional benefit is that these diacids are commercially available starting materials.
The first attempt in the coupling of 3 was a reaction with commercially available diacid chlorides. Indeed, when two equivalents of 3 were reacted with either glutaryl dichloride or ortho-phthaloyl dichloride, 4 and 5 were obtained, respectively. However, 1H NMR spectroscopy showed considerable amounts of unreacted starting material, together with side products. This is probably due to partially hydrolysis of the acyl chloride. A coupling between 3 and a diacid in the presence of N,N’-dicyclohexylcarbodiimide (DCC) resulted in even more complex mixtures, due to considerable contamination of the product mixture by N,N’-dicyclohexylurea (DCU), complicating purification.

To improve the reaction it is essential that the diacid reacts twice and thus must be doubly activated. For this reason, it would be useful to follow a synthetic strategy in which independently synthesized and purified activated diacids are employed. Therefore, it was decided to synthesize the corresponding N-hydroxysuccinimide esters from the acids, which can react efficiently with a free amine and are air and moisture stable.30

The required diesters were synthesized according to different routes. The activated diacids 7 and 8 were obtained by reacting either glutaryl dichloride or ortho-phthaloyl dichloride, respectively, in tetrahydrofuran (THF) with N-hydroxysuccinimide (NHS) (Scheme 4.3). The products obtained could be purified by recrystallization from isopropanol (IPA). Diester 9 was obtained via a DCC-coupling in THF of suberic acid and NHS. This product could also be purified by recrystallization from IPA, although multiple crystallization steps were required due to the presence of DCU in the product.

Scheme 4.3 Synthesis of the NHS-esters 7, 8 and 9.

Reaction of N4Py-amine 3 and the NHS-esters 7, 8 and 9 in dichloromethane (DCM) afforded a clean conversion to ligands 4, 5 and 6, respectively. A final purification was performed by size exclusion chromatography (Sephadex® LH-20) with methanol as eluent, to remove the remaining traces of low molecular weight impurities. The final ligands were obtained in moderate to high yields (60-90%) and were characterized by 1H and 13C NMR spectroscopy and electron spray mass spectral analysis.

4.2.2 Complexation of the ditopic ligands to iron(II)

Several attempts to crystallize the corresponding iron complexes of 4, 5 and 6 were unsuccessful. Despite varying solvents, iron(II) salts or temperature, only oils were obtained. Therefore, the complexes were prepared in situ prior to DNA oxidation experiments (Scheme 4.4). Titration experiments were performed and followed with several techniques to ensure that two iron(II) ions are bound per ligand.
Scheme 4.4 Synthesis of the dinuclear complexes 10, 11, and 12 from the corresponding ligands 4, 5, and 6, respectively.

The titration of 6 with (NH₄)₂Fe²⁺(SO₄)₂•6H₂O was followed with ¹H NMR spectroscopy (Figure 4.4) and UV/Vis spectroscopy (Figure 4.5). The corresponding iron(II) complex of 6 with (NH₄)₂Fe²⁺(SO₄)₂ in D₂O is paramagnetic (Figure 4.4a), due to the formation of a high spin iron(II) complex. The integrations of the characteristic absorptions between δ 25 and 28 with respect to an internal standard (acetone) can be plotted against the number of equivalents of (NH₄)₂Fe²⁺(SO₄)₂ added (Figure 4.4). This plot clearly shows the apparent stoichiometry of two equivalents of iron(II) per ligand.

Figure 4.4 a) ¹H NMR of 6 with two equivalents of (NH₄)₂Fe²⁺(SO₄)₂ in D₂O. Inset: expansion of the region between δ 25 and 28, with 0, 1, or 2 equivalents of (NH₄)₂Fe²⁺(SO₄)₂ added to 6 in D₂O. b) Plot of the relative area of the two absorptions between δ 25 and 28 with respect to the internal standard (acetone) as a function of the number of equivalents of (NH₄)₂Fe²⁺(SO₄)₂.

The same experiment in H₂O was followed with UV/Vis spectroscopy. (NH₄)₂Fe²⁺(SO₄)₂ was added to ligand 6 and the absorption at 355 nm is measured (Figure 4.5a). From these data, the relative molar absorptivity at 355 nm can be calculated, and plotted against the number of equivalents of iron(II) (Figure 4.5b). Again, the data are in agreement with a stoichiometry of two iron(II) ions per ligand.
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Figure 4.5 a) UV/Vis spectrum of 6 with zero (solid line) and two equivalents of (NH₄)₂Fe⁶⁺(SO₄)₂ (dashed line) in H₂O. b) Plot of the relative molar absorptivity at λ 355 nm as a function of the number of equivalents of (NH₄)₂Fe⁶⁺(SO₄)₂.

4.3 DNA oxidation experiments

4.3.1 Oxidation of supercoiled pUC18 plasmid DNA

The freshly prepared ditopic iron(II) complexes 10 (from 4), 11 (from 5) and 12 (from 6) were used in the oxidation of supercoiled DNA and compared to the mononuclear complex Fe⁶⁺(N4Py) (1). Figure 4.6 compares the cleavage of supercoiled pUC18 plasmid DNA (0.1 μg/μL, 150 μM in bp) by (NH₄)₂Fe⁶⁺(SO₄)₂, Fe⁶⁺(N4Py) (1, see Chapter 3), [Fe⁶⁺(4)] (10), [Fe⁶⁺(5)] (11), and [Fe⁶⁺(6)] (12) at a 1.0 μM concentration (based on Fe) under air in the presence of DTT (1.0 mM) as reductant after 30 min.

![Figure 4.6](image-url)

It is apparent from Figure 4.6 that the double N4Py complexes 10, 11 and 12 form significant amounts of linear DNA in addition to nicked DNA after 30 min (lanes 5, 6, and 7, respectively), whereas the monotopic complex Fe⁶⁺(N4Py) (1) has formed nicked DNA only within the same period (lane 4). Moreover, the remaining levels of supercoiled DNA is nearly identical for all complexes. This suggests that the activity per iron center for the
dinuclear complexes is significantly higher than the activity of the mononuclear complex [Fe$^{II}$(N4Py)] (1).

The oxidation of supercoiled plasmid DNA was followed in time for Fe$^{II}$(N4Py) (1) and the dinuclear complexes 10, 11 and 12 (Figure 4.7). Initially (i.e. for approximately the first 10 min), the consumption of supercoiled DNA is accompanied by a steady increase in the fraction of nicked DNA, with approximately similar rates ($t_{1/2}$ ~ 10 min) for all complexes.

After this initial 10 min period, significant differences are observed in the product distribution between the mononuclear complex and the dinuclear complexes. Employing [Fe$^{III}$(N4Py)] (1) as catalyst, relatively little linear DNA is formed within a 60 min time period, which is the result of extensive single strand cleavage activity (Figure 4.7a). This reaction pattern is indicative of a single strand cleavage agent (see Chapter 3). In contrast, a significantly higher amount of linear DNA is formed in the same time period, using the dinuclear complexes 10, 11 or 12 as catalysts (Figure 4.7b, c and d, respectively). This suggests that other mechanisms in addition to single strand cleavage are responsible for the formation of linear DNA, such as direct double strand cleavage.

![Figure 4.7](image)

**Figure 4.7** Aerobic oxidation of supercoiled plasmid DNA (■) into nicked DNA (●) and linear DNA (▲) followed in time catalyzed by a) [Fe$^{II}$(N4Py)(CH$_3$CN)](ClO$_4$)$_2$ (1), b) [Fe$^{II}$(4)] (10), c) [Fe$^{II}$(5)] (11), and d) [Fe$^{II}$(6)] (12). Errors bars represent the root mean square (rms) error based on three runs. A correction factor of 1.31 was used to compensate for the reduced ethidium bromide uptake capacity of supercoiled DNA (see Chapter 2).

A statistical analysis was conducted to further substantiate the hypothesis that a direct double strand cleavage process can be responsible for the high amount of linear DNA formed. From the fractions of supercoiled, nicked and linear DNA, the average number of
single and double strand cuts per DNA molecule can be calculated, using Equation 4.1 and Equation 4.2 (see Chapter 2).

\[ f_{III} = m \times e^{-m} \]

**Equation 4.1** Average number of double strand cuts in one DNA molecule \((m)\), calculated from the fraction of linear DNA \((f_{III})\).

\[ f_I = e^{-(m+n)} \]

**Equation 4.2** Total number of strand cuts (both single \((n)\) and double \((m)\)) in one DNA molecule calculated from the remaining fraction of DNA without any cuts \((f_I)\).

The average number of double strand cuts per DNA molecule \(m\) can be plotted against the average number of single strand cuts per DNA molecule \(n\) (Figure 4.8). As was discussed in Chapter 3, Fe\(^{II}\)(N4Py) (1) displays single strand cleavage activity only, with the data being comparable to that expected from a theoretical single strand cleavage pathway (Freifelder-Trumbo relation).33

![Figure 4.8](image)

**Figure 4.8** Number of double strand cuts \((m)\) plotted as a function of the number of single strand cuts \((n)\) for a) [Fe\(^{II}\)\(\cdot\)(4)] (10), b) [Fe\(^{II}\)\(\cdot\)(5)] (11), c) [Fe\(^{II}\)\(\cdot\)(6)] (12), d) results of 10 (■), 11 (□), and 12 (▲) superimposed. The dotted line is the Freifelder-Trumbo relation.\(^{33}\) Error bars represent the uncertainty of the data, based on a Monte Carlo simulation, taking into account a standard deviation \(\sigma = 0.03\).

A different pattern is observed with the dinuclear complexes 10, 11 and 12 (Figure 4.8). Initially, only single strand cleavage activity is observed with all three complexes, as the \(m/n\) values are comparable to the expected theoretical value. Over the course of the reaction, i.e. when significant amounts of nicked DNA are present (around \(n \sim 1\)), the slope
of the graphs increases steadily and deviate significantly from the Freifelder-Trumbo relation. This indicates that the double strand cuts in the DNA are not the result of extensive single strand cleavage, but rather a different process, such as direct double strand cleavage, is responsible for this observation. This behavior is significantly different to that of the mononuclear Fe(N4Py) complex 1 (see Chapter 3) and in this respect also from other published oxidative DNA cleavage systems, which display single strand cleavage only.

The results in the n/n analysis are identical between the three ditopic systems. Superposition of the data obtained shows no significant differences between the three individual complexes (Figure 4.8d). This means that the spacer used to couple the two DNA cleaving moieties (the Fe(N4Py) moieties) has little influence on the reaction pathway observed.

4.3.2 Oxidation of nicked DNA

It is apparent that the dinuclear complexes display double strand cleavage only after ~10 min, when already a significant amount of the supercoiled DNA is converted into nicked DNA. This observation can be rationalized by considering the structure of the DNA, which is used in these studies. A key observation is that the rate of conversion of supercoiled DNA into nicked DNA is similar for the mononuclear complex 1 and the dinuclear complexes 10, 11 and 12. Because of the compact folded structure of the supercoiled DNA, two oxidation events that are in proximity of each other (i.e. close in space) are not necessarily close in terms of DNA sequence. When the resulting two strand breaks are too distant in the DNA sequence (outside the 16 base pair region), the hydrogen bonding network will prevent linearization of the DNA, and, hence, only the formation of nicked DNA is observed. In contrast, nicked DNA has a relaxed open circular structure, in which there is a much greater probability that the two cleavage events take place on opposite strands, in contrast to supercoiled DNA. In other words, nicked DNA might be more susceptible to double strand cleavage than supercoiled DNA.

This hypothesis was examined further by using nicked DNA as a substrate in the reaction. The nicked pUC18 plasmid DNA was prepared by digestion of supercoiled pUC18 plasmid DNA with the nicking restriction enzyme Nt.BstNBI, and was isolated and purified subsequently (see Chapter 2). The isolated nicked pUC18 plasmid DNA was used as a substrate in the reaction in place of the supercoiled DNA under otherwise identical conditions. The formation of linear DNA was followed in time for 1 and 12 (Figure 4.9).

The rate of linear DNA formation is significantly higher when the dinuclear complex 12 was employed than when the mononuclear complex 1 was used. Already after 25 min, around 30% of linear DNA was formed (Figure 4.9c), which is the maximum, which can be quantified reliably (see Chapter 2 for details). In contrast, approximately 10% of linear DNA is formed in the same period when 1 was employed as catalyst. Since the production of double strand breaks in the DNA are the result of two processes, namely single strand cleavage and direct double strand cleavage, it is difficult to determine a rate constant for the dinuclear complexes. For this reason, the difference in activity between the mononuclear complex 1 and the dinuclear complex 12 cannot be quantified.

The restriction enzyme Nt.BstNBI, which is used for the synthesis of the nicked plasmid DNA has four unique restriction sites. This means that the nicked DNA, which is used as substrate already has four single strand cuts. The rate constant in which 1 induced single strand cuts in the DNA was determined earlier (0.1 min⁻¹, see Chapter 3). Taken together, this means that after 25 min, around 6.5 single strand cuts are expected to be present in the DNA when 1 was used. By using Equation 4.3 (see Chapter 2) the theoretical sum of fractions of supercoiled DNA together with nicked DNA can be calculated, when the
number single strand cuts \((n)\) is known. Together with the fact that the sum of all fractions is always equal to 1 (Equation 4.4), the theoretical fraction size of linear DNA can be calculated for a pure single strand cleavage process. So when \(n = 6.5\), the theoretical fraction of linear DNA is equal to 0.12, which is 12%. This value corresponds well to the percentage of linear DNA found experimentally with 1 (Figure 4.9c).

\[
\frac{1}{6} \approx 0.12
\]

**Figure 4.9** Conversion of nicked pUC18 plasmid DNA into linear pUC18 plasmid DNA in time. a) Agarose gel slab of \([\text{Fe}^{II}(\text{N4Py})(\text{CH}_3\text{CN})](\text{ClO}_4)_2\) (1) (lanes 1-15 correspond to the datapoints in c)), b) Agarose gel slab of \([\text{Fe}^{II}_2(6)]\) (12). (lanes 1-15 correspond to the datapoints in c)), c) Time profile for 1 and 12. Errors bars represent the root mean square (rms) error based on three runs.

\[
f_I + f_{II} = \left[1 - n(2h + 1)/2L\right]^{1/2}
\]

**Equation 4.3** Average number of single strand cuts \((n)\) in a DNA molecule, calculated from the fractions supercoiled \((f_I)\) and nicked \((f_{II})\) DNA. The term \(h\) is the maximum distance in base pairs between nicks on opposite strands to generate a double strand cut (16 base pairs) and \(L\) is the total number of base pairs of the DNA used (2686 base pairs for pUC18 plasmid DNA).

\[
f_I + f_{II} + f_{III} = 1
\]

**Equation 4.4** The total sum of the fractions supercoiled \((f_I)\), nicked \((f_{II})\) and linear \((f_{III})\) DNA are equal to 1.

The fact that the value found experimentally is in agreement with the theoretical value for linear DNA is a clear indication that all linear DNA, which is formed using 1 as catalyst, is the result of random single strand cleavage. However, the fraction of linear DNA is significantly higher when the dinuclear complex 12 was employed as catalyst, demonstrating that another pathway is responsible for the formation of linear DNA, besides single strand cleavage.

There are two possibilities to explain the higher amount of linear DNA formed by 12 from nicked DNA: i) the complex is selective in terms of having a preference for nicked sites or ii) direct double strand cleavage is observed. The observation that 1 displays random single strand cleavage exclusively (see Chapter 3) would argue against the first possibility. The observation that 1 does not display selectivity in the strand cleavage of nicked DNA, which would result in the appearance of defined DNA bands on the agarose gel slab, further
supports this argument. It is rather unlikely that the chemistry displayed by 12 is inherently different from that of 1, which strongly suggests that the dinuclear complex indeed displays direct double strand cleavage.

In conclusion, the results obtained for the experiments using nicked DNA indeed point towards the possibility that the two oxidation equivalents from the dinuclear iron complexes are delivered to the DNA in close proximity. However, in the compact folded structure of supercoiled DNA, two oxidation events that are close in space are not necessarily close in terms of DNA sequence. Therefore, nicked DNA is formed rather than linear DNA. Indeed, when nicked DNA was employed as substrate, the formation of linear DNA is higher than can be accounted for on a statistical basis. This study holds the first examples of synthetic complexes, which mimic the unique direct double strand cleavage activity of bleomycin. Therefore, the dinuclear iron N4Py complexes are functional models for bleomycin.

In this respect, the behavior of the dinuclear iron complexes differs from that of Fe(BLM), which effects double strand cleavage of supercoiled DNA, by direct formation of linear DNA. In Fe(BLM), the two oxidizing equivalents are delivered by a single iron complex, which is proposed to hinge around the intercalating bisthiazole moiety to cleave both DNA strands sequentially.

### 4.4 Topology of dinuclear complexes

Although the DNA cleavage results do not show a significant difference between the cleavage activity of the dinuclear iron complexes, a possible influence of the orientation of the active centers (i.e. the Fe(N4Py) moieties) with respect of each other was investigated in more detail, by analogy to dicopper complexes.10

#### 4.4.1 Ligand synthesis

A series of ligands was prepared with different substitution patterns on a central phenyl ring, allowing a variation of the relative orientation of the metal centers (ligands 4, 15 and 16, Scheme 4.5), to investigate a possible structural relationship between activity and relative orientation of the iron centers.

![Scheme 4.5 Synthesis of ditopic N4Py ligands 4, 13, and 14 with different substitution patterns in the linker moiety, resulting in a difference in the relative orientation of the iron centers in the corresponding complexes.](image)

Ligand 4 with a 1,2-substitution pattern was synthesized earlier (vide supra). The corresponding ligands 15 and 16, with a 1,3- and 1,4-substitution pattern, respectively, can be synthesized analogously. Firstly, the required NHS-activated acids 17 and 18 were synthesized from the corresponding acids and NHS via a DCC coupling (vide supra).
Subsequent reaction of these activated acids 17 and 18 with the N4Py-amine 3 furnished the desired ligands 15 and 16, respectively. The ligands could be purified over a Sephadex® LH-20 size-exclusion column, using methanol as eluent, and were obtained in moderate to excellent yields varying from around 56 to 94%. Ligand purity was determined by $^1$H and $^{13}$C NMR spectroscopy, electrospray mass spectral analysis and RP-HPLC analysis.

### 4.4.2 DNA oxidation experiments

As for the dinuclear complexes prepared discussed above, the ligands 4, 15, and 16 were coordinated to $(\text{NH}_3)_2\text{Fe}^{II}(\text{SO}_4)_2$ to obtain the corresponding diiron complexes 11, 19, and 20, respectively, prior to the DNA oxidation reactions. Titration experiments of 16 and $(\text{NH}_3)_2\text{Fe}^{II}(\text{SO}_4)_2$ followed with $^1$H and UV/Vis spectroscopy showed the uptake of two iron ions per ligand, similar to related dinuclear complexes \textit{(vide supra)}. The experiments using supercoiled plasmid DNA were performed as described earlier. The samples were analyzed by gel electrophoresis and the results of 19 and 20 are displayed in Figure 4.10.

![Figure 4.10](image.png)

**Figure 4.10** Aerobic oxidation of supercoiled pUC18 plasmid DNA (●) into nicked DNA (■) and linear DNA (▲) followed in time catalyzed by a) [Fe$^{III}_2$(15)] (19) and b) [Fe$^{II}_2$(16)] (20). Errors bars represent the root mean square (rms) error based on three runs. A correction factor of 1.31 was used to compensate for the reduced ethidium bromide uptake capacity of supercoiled DNA.

The data obtained for 19 and 20 do not differ significantly from the results obtained with 11 (Figure 4.7). Indeed, a similar rate of supercoiled DNA consumption was observed ($t_{1/2}$ ~ 10 min), together with a comparable increase in nicked DNA and linear DNA over time. Moreover, a similar trend is observed, as linear DNA is formed only when significant amounts of nicked DNA are present. To further substantiate the similarities between the ditopic complexes, the average numbers of single- and double strand cuts per DNA molecule were calculated (Figure 4.11).

Indeed, nearly identical values were calculated for the $m/n$ for 10, 19, and 20. It seems that the complex with the 1,3-structural motif (19) displays a slightly higher degree of double strand cleavage activity than the other two complexes in the end of the reaction. However, a more precise method of determining the $m/n$ ratio are required to substantiate this finding. The uncertainty limits of the method used here are too large to discriminate between the individual complexes in terms of cleavage behavior.
Figure 4.11 Number of double strand cuts (dsc, \(m\)) per DNA molecule plotted as a function of the number of single strand cuts (ssc, \(n\)) per DNA molecule for \([\text{Fe}^2\{4\}]\) (10) (■), \([\text{Fe}^2\{15\}]\) (19) (□), \([\text{Fe}^2\{16\}]\) (20) (▲). The dotted line is the Freifelder-Trumbo relation.33 Error bars represent the uncertainty of the data, based on a Monte Carlo simulation, taking into account a standard deviation \(\sigma\) of 0.03.

In this respect, the newly developed dinuclear iron complexes behave differently to the dinuclear copper complexes. Guo and co-workers have found clear differences in the DNA oxidation activity between a 1,3-substituted dicopper complex and a 1,4-substituted dicopper complex (Figure 4.2).10 The differences between these complexes can be rationalized by a cooperative interaction between the two copper centers to activate dioxygen (or hydrogen peroxide), which is favorable to generate reactive intermediates. The structural limitations of the 1,4-substituted dicopper complex prevents such a favorable interaction, leading to a lower activity. Moreover, evidence was presented that dinuclear copper complexes are engaged in a selective DNA cleavage pathway only (preferences for junctions between double and single stranded DNA), rather than direct double strand cleavage.3-5

It seems that the dinuclear iron N4Py complexes are not engaged in a cooperative mechanism of dioxygen activation. No significant differences are observed between the individual dinuclear complexes 10, 19, and 20 in terms of cleavage pathway. It is more likely that both iron centers in a dinuclear complex activate dioxygen independently. The absence of selective DNA cleavage of nicked DNA together with the observation that linear DNA is produced in higher amounts can be accounted for on a statistical basis, suggests direct double strand cleavage of DNA with dinuclear iron N4Py complexes.

4.5 Summary and conclusions

In this chapter, the oxidative cleavage of supercoiled DNA with dinuclear complexes is discussed. It was envisioned that the covalent tethering of two typical single strand cleavage agents, namely Fe(N4Py), results in a complex, which can induce two single strand cuts in opposite DNA strands and as a consequence in a net direct double strand cut.

The ligands, that were required, were synthesized from a known N4Py derivative, which has an aminopropyl functionality on one of its pyridine rings. Reaction of this amine derivative with activated acids furnished the desired ditopic ligands in one step. Purification of the ligands was performed by size exclusion chromatography. Since attempts to crystallize the corresponding iron(II) complexes failed, the uptake of two iron(II) per ligand was confirmed using titration experiments.
The DNA oxidation experiments with supercoiled pUC18 plasmid DNA as substrate employing the new dinuclear complexes as catalysts showed some peculiar features. Initially, only nicked DNA is formed, which is typical for a single strand cleavage agent, such as Fe^{II}(N4Py) (see Chapter 3). However, after 10 min significant amounts of linear DNA are produced, which are higher than can be accounted for on a statistical basis for extensive single strand cleavage. This observation could be further substantiated by calculating the number of single and double strand cuts in the DNA. Indeed, initially the reaction proceeds according to the Freifelder-Trumbo relationship, and thus displays typical single strand cleavage behavior. However, after a significant amount of nicked DNA was produced (i.e. when on average one or more single strand cuts per DNA molecule are present) the ratio between \( m \) and \( n \) deviates significantly from this Freifelder-Trumbo relationship, and thus another cleavage pathway is involved.

The observation that the rate of decrease in supercoiled DNA is similar between the dinuclear complexes and the mononuclear Fe^{II}(N4Py) complex together with the observation that more linear DNA is produced with the dinuclear complexes when significant amounts of nicked DNA are present, suggests that nicked DNA could be a more appropriate substrate than the supercoiled DNA. Indeed, when nicked DNA was used as a substrate in the reaction, a significantly higher amount of linear DNA was produced over the same time period with a dinuclear complex compared to the mononuclear complex. Furthermore, the linear DNA produced by the latter is the result of extensive single strand DNA cleavage as was confirmed by statistical analysis. Again, these experiments demonstrate that the dinuclear complexes can be involved in an alternative pathway than single strand cleavage, such as direct double strand cleavage. The proposed reason that the nicked DNA is a better substrate than the supercoiled DNA is that the two active centers are indeed involved in two cleavage events in proximity. However, the superhelicity of the supercoiled DNA might result in these two events occurring in close proximity spatially, but not necessarily close in terms of DNA sequence.

The dinuclear complexes behave differently from Fe(BLM). In Fe(BLM) only one iron center is responsible for the two DNA cuts in opposite strands. In practice, this means that the direct double strand cleavage activity can be observed when supercoiled DNA is used as a substrate, whereas with the dinuclear complexes this process is not observed. A variation of the spacer unit between the two iron centers, in terms of length, rigidity or relative orientation, did give rise to significant differences. The complexes retain enough flexibility to induce the two DNA strand cuts in close proximity. Moreover, these results indicate that the two iron centers function as independent strand cleavage agents in contrast to the dinuclear copper complexes.

In conclusion, the ditopic N4Py complexes are capable of inducing direct double strand cleavage of DNA. Both iron centers independently activate dioxygen inducing a single strand cut in the DNA; the observed double strand cleavage is the result of two of these events in close proximity. In this respect, these dinuclear complexes are good functional models for bleomycin, although with the natural system Fe(BLM) only one iron center is responsible for the observed double strand cleavage activity. The dinuclear FeN4Py complexes are the first examples of synthetic DNA cleavage agents, which can induce double strand cleavage catalytically. Moreover, these dinuclear iron complexes are first examples, in which double strand cleavage activity is unequivocally established and quantified.
4.6 Experimental section

Instrumentation

NMR spectra were recorded on a Varian Mercury Plus 200 (1H NMR at 200 MHz, 13C NMR at 50.3 MHz), a Varian VXR-300 (1H NMR at 300 MHz, 13C NMR at 75.5 MHz), or on a Varian Mercury Plus 400 (1H NMR at 400 MHz, 13C NMR at 100 MHz). Chemical shifts (δ) are denoted in ppm and referenced to the residual solvent peak unless stated otherwise (CDCl3, 1H δ = 7.24, 13C δ = 77.0; CD3OD, 1H δ = 3.30, 13C δ = 49.0; CD3CN, 1H δ = 1.93, D2O 1H δ = 4.79). The splitting patterns are designated as follows: s (singlet), d (doublet), dd (double doublet), t (triplet), q (quartet), m (multiplet), and br (broad). Coupling constants (J) between two nuclei separated by n chemical bonds are denoted in Hertz (Hz).

Chemical ionisation mass spectra (MS-Cl+), electron impact (MS-EI+), and exact mass determination (HRMS) were recorded on an AEI MS-902. Electrospray ionisation mass spectrometry (MS-ESI+) was performed a Triple Quadrupole LC/MS/MS mass spectrometer (API 3000, Perkin-Elmer Sciex Instruments).

Elemental analysis was performed on a EuroVector CHNS-O Elemental Analyzer Euro EA 3000. Melting points were recorded on a Büchi B-545 melting point apparatus. UV-Vis spectra were recorded on a Hewlett-Packard 8453 diode array or Jasco V-570 spectrophotometer.

HPLC separations

Analytical reversed-phase high-performance liquid chromatography (RP-HPLC) was performed on a Shimadzu LC-10AD vp at 35°C, which was equipped with a photodiode array detector (SPD M10A) and a fraction collector (Shimadzu FRC 10A). A Waters 2690 Separations Module equipped with a photodiode array detector (Waters 996) and a Micromass ZMD Quadrupole mass spectrometer at room temperature was used for LC-MS analysis. General separation method: A Waters XTerra MS C18 analytical column (3.5 μm, 3.0 x 150 mm) was used with a flow rate of 0.5 mL/min of water (containing 0.05 % acetic acid) and MeOH (containing 0.05 % acetic acid) as mobile phase. A linear gradient of 10% to 70% methanol over a period of 60 min was employed.

Chemicals and methods

For general remarks, see Chapter 3. All reactions were performed under an inert atmosphere (N2 or Ar). The N4Py derivative 3 was synthesized according to literature procedures and all data were in agreement with the published data. Names of new compounds were generated using the software program ACD/IUPAC Name Pro version 3.50.

For general remarks on biochemical studies, see Chapter 2 and 3. The pictures and subsequent analysis data from Figure 4.7a, Figure 4.7b, and Figure 4.7d were obtained using the method described in Chapter 2. All other pictures and subsequent analysis was performed using the method described in Chapter 3. The uncertainty limits of the values of n and m were calculated with a Monte Carlo simulation using the software program Mathematica version 5.2.0.0. as was described in Chapter 3.
Dinuclear iron complexes for direct double strand DNA cleavage

1-[(2-[(2,5-Dioxo-1-pyrrolidinyl)oxy]carbonyl]benzoyl)oxy]-2,5-pyrrolinedione (7)

To a cooled (0°C) solution of NHS (1.27 g; 11.0 mmol) in THF (30 mL) was added triethyl amine (1.11 g; 1.52 mL; 11.0 mmol) and freshly distilled ortho-phthaloyl dichloride (1.02 g; 0.73 mL; 5.00 mmol). The resulting white suspension was stirred for 2 hours at room temperature. The solvent was removed and the residue was taken up in DCM (100 mL), washed with water (3 x 50 mL) and dried over Na₂SO₄. Filtration and evaporation of the solvent yielded an off-white solid, which was recrystallized from IPA to yield a white solid (1.40 g; 3.90 mmol; 78 %). M.p. 161.9-162.0°C.

1H NMR (300 MHz, CDCl₃): δ 8.01-8.06 (m, 2H), 7.71-7.78 (m, 2H), 2.85 (s, 8H).

13C NMR (50.3 MHz, CDCl₃): δ 168.7, 161.5, 133.2, 130.9, 127.3, 25.7.

MS-CI+: m/z 378.1 [M + NH₄⁺].

Anal. calcd (%) for C₁₆H₁₂N₂O₈: C 53.34, H 3.36, N 7.78; found: C 53.0, H 3.34, N 7.68.

1-[(5-[(2,5-Dioxo-1-pyrrolidinyl)oxy]-5-oxopentanoyl)oxy]-2,5-pyrrolinedione (8)

Following the procedure as for 7, starting from freshly distilled glutaryl dichloride (0.845 g; 0.64 mL; 5.00 mmol). A white solid (1.61 g; 4.93 mmol; 99 %) was obtained after recrystallization from IPA. M.p. 129.1-130.7°C.

1H NMR (300 MHz, CDCl₃): δ 2.81 (s, 8H), 2.77 (t, 3J = 7.3 Hz, 4H), 2.16 (quintet, 3J = 7.3 Hz, 2H).

13C NMR (50.3 MHz, CDCl₃): δ 169.0, 167.7, 29.5, 25.5, 19.5.

MS-CI+: m/z 344.1 [M + NH₄⁺].

Anal. calcd. (%) for C₁₃H₁₄N₂O₈: C 47.86, H 4.33, N 8.59; found: C 47.8, H 4.30, N 8.45.

1-[(8-[(2,5-Dioxo-1-pyrrolidinyl)oxy]-8-oxooctanoyl)oxy]-2,5-pyrrolinedione (9)

A solution of suberic acid (1.19 g; 6.84 mmol), DCC (3.10 g; 15.0 mmol) and NHS (1.73 g; 15.0 mmol) in THF (250 mL) was stirred overnight. The white suspension was filtered and the filtrate was concentrated. DCM (150 mL) was added and the solution was washed with water (2 x 50 mL) and aq. NaHCO₃ (50 mL). After drying (Na₂SO₄) and filtration, the solvent was evaporated. Recrystallization from IPA afforded a white solid (1.99 g; 5.40 mmol; 79 %). M.p. 166.2-167.0°C.

1H NMR (400 MHz, CDCl₃): δ 2.80 (s, 4H), 2.58 (t, 3J = 7.3 Hz, 4H), 1.74 (m, 4H), 1.43 (m, 4H).

13C NMR (50.3 MHz, CDCl₃): δ 169.1, 168.5, 30.7, 28.1, 25.6, 24.2.

MS-CI+: m/z 386.2 [M + NH₄⁺].

Anal. calcd (%) for C₁₆H₂₀N₂O₈: C 52.17, H 5.47, N 7.61; found: C 52.3, H 5.54, 7.50.
1-(3-[[2,5-Dioxo-1-pyrrolidinyl]oxy]carbonyl]benzoyl]oxy]-2,5-pyrrolinedione (17)

Following the procedure as for 9, starting from isophthalic acid (2.00 g; 12.0 mmol), DCC (5.52 g; 26.5 mmol) and NHS (3.05 g; 26.5 mmol) in THF (300 mL). A white solid (3.78 g; 10.5 mmol; 86 %) was obtained after recrystallization from IPA. M.p. 255°C (dec.).

$^1$H NMR (400 MHz, CDCl₃): $\delta$ 8.90 (t, $^1J = 1.4$ Hz, 1H), 8.44 (dd, $^3J = 7.9$ Hz, $^4J = 1.4$ Hz, 2H), 7.72 (t, $^3J = 7.9$ Hz, 1H), 2.93 (s, 8H).

$^{13}$C NMR (50.3 MHz, CDCl₃): δ 168.8, 160.6, 136.3, 132.4, 129.7, 126.3, 25.6.

MS-Cl$: m/z 378.1 [M + NH₄]^+$.

Anal. calcd (%) for C₁₆H₁₂N₂O₈: C 53.34, H 3.36, N 7.78; found: C 53.5, H 3.35, N 7.75.

1-(4-[[2,5-Dioxo-1-pyrrolidinyl]oxy]carbonyl]benzoyl]oxy]-2,5-pyrrolinedione (18)

Following the procedure as for 9, starting from terephthalic acid (2.01 g; 12.1 mmol), DCC (5.49 g; 26.6 mmol) and NHS (3.06 g; 26.6 mmol) in THF (300 mL). A white solid (3.74 g; 10.4 mmol; 86 %) was obtained after recrystallization from IPA. M.p. 260°C (dec.).

$^1$H NMR (400 MHz, CDCl₃): $\delta$ 8.26 (s, 4H), 2.92 (s, 8H).

$^{13}$C NMR (100 MHz, APT, CDCl₃): δ 168.8 (C), 160.8 (C), 130.8 (CH), 130.7 (C), 25.7 (CH₂).

MS-Cl$: m/z 378.1 [M + NH₄]^+$.

Anal. calcd (%) for C₁₆H₁₂N₂O₈: C 53.34, H 3.36, N 7.78; found: C 53.4, H 3.44, N 7.73.

Synthesis of ligands 4, 5, 6, 15 and 16, general procedure

Two equivalents of the aminopropyl substituted N₄Py 3 were added to a solution (~ 0.1 mM in DCM) of one equivalent of NHS-ester 7, 8, 9, 17, or 18. The mixture was stirred overnight at room temperature. The mixture was washed with water (3 x 10 mL) and dried over Na₂SO₄. After filtration the solution was concentrated and the product was precipitated with Et₂O. Further purification was achieved by size exclusion chromatography over a Sephadex LH20 column with MeOH as the mobile phase. The fractions were analyzed using RP-HPLC (vide supra). After evaporation of the MeOH, the oily product was taken up in a minimum amount of DCM and the product was precipitated with Et₂O.

$N^1,N^2$-Bis(3-[[6-[[di(2-pyridinyl)methyl]2-pyridinylmethyl]amino]methyl]-3-pyridinyl]carbonyl]amino]propyl)phthalamide (5)

Starting from 7 (39.3 mg; 0.109 mmol) and 3 (0.102 g; 0.218 mmol), 4 was obtained as a pale light brown solid (0.101 g; 95 μmol; 94%).

$^1$H NMR (400 MHz, CD₂OD): $\delta$ 8.78 (d, $^1J = 2.2$ Hz, 2H), 8.45 (m, 4H), 8.35 (m, 2H), 8.04 (dd, $^3J = 8.1$ Hz, $^4J = 2.2$ Hz, 2H), 7.79-7.64 (m, 14H), 7.55 (m, 2H), 7.49 (m, 2H), 7.25 (m, 4H), 7.18 (m, 2H), 5.33 (s, 2H), 3.99 (s, 4H), 3.95 (s, 4H), 3.44 (m, 8H), 1.85 (m, 4H).
Dinuclear iron complexes for direct double strand DNA cleavage

$^{13}$C NMR (50.3 MHz, CD$_3$OD): δ 171.8, 167.7, 163.8, 160.7, 160.3, 150.0, 149.5, 148.7, 138.5, 138.4, 137.2, 136.9, 131.3, 129.9, 128.8, 125.6, 124.8, 124.1, 124.0, 123.7, 74.2, 58.7, 58.3, 38.3, 38.2, 29.8.


$N_1,N_5$-Bis(3-[(6-[di(2-pyridinyl)methyl](2-pyridinylmethyl)amino)methyl]-3-pyridinyl)carbonyl]amino]propyl]pentanediamide (4)

Starting from 8 (33 mg; 0.102 mmol) and 3 (95 mg; 0.203 mmol), 5 was obtained as a pale light brown solid (92 mg; 89 μmol; 87%).

$^1$H NMR (300 MHz, CD$_3$OD): δ 8.79 (d, 4 $^J$ = 2.0 Hz, 2H), 8.46 (dd, 3 $^J$ = 4.8 Hz, 4 $^J$ = 0.7 Hz, 4H), 8.35 (dd, 3 $^J$ = 4.8 Hz, 4 $^J$ = 0.7 Hz, 2H), 8.08 (dd, 3 $^J$ = 8.1 Hz, 4 $^J$ = 2.0 Hz, 2H), 7.67-7.80 (m, 14H), 7.26 (m, 4H), 7.19 (m, 2H), 5.34 (s, 2H), 4.01 (s, 4H), 3.96 (s, 4H), 3.40 (dd, 3 $^J$ = 6.6 Hz, 4 $^J$ = 7.0 Hz, 4H), 3.25 (t, 3 $^J$ = 6.6 Hz, 4H), 2.24 (t, 3 $^J$ = 7.3 Hz, 4H), 1.91 (m, 2H), 1.78 (m, 4H).

$^{13}$C NMR (50.3 MHz, CD$_3$OD): δ 175.5, 167.8, 163.8, 160.8, 160.3, 150.0, 149.5, 148.5, 138.5, 138.4, 136.9, 130.0, 125.6, 124.8, 124.2, 124.0, 123.7, 74.4, 58.8, 58.3, 38.7, 37.7, 36.2, 30.2, 23.1.

MS-ESI$: m/z$ 1031.7 [M + H$^+$]+, 861.6 [M – Py-CH$_2$-Py + H$^+$]+, 516.6 [M + 2H$^+$]$_2^+$, 344.7 [M + 3H$^+$]$_3^+$.

$N_1,N_8$-Bis(3-[(6-[di(2-pyridinyl)methyl](2-pyridinylmethyl)amino)methyl]-3-pyridinyl)carbonyl]amino]propyl]octanediamide (6)

Starting from 9 (0.206 g; 0.558 mmol) and 3 (0.522 g; 1.12 mmol), 6 was obtained as a pale light brown solid (0.338 g; 0.315 mmol; 56%).

$^1$H NMR (300 MHz, CD$_3$OD): δ 8.79 (d, 4 $^J$ = 2.2, 2H), 8.45 (d, 3 $^J$ = 5.1 Hz, 4H), 8.34 (d, 3 $^J$ = 4.8 Hz, 2H), 8.08 (dd, 3 $^J$ = 8.1 Hz, 4 $^J$ = 2.2 Hz, 2H), 7.80-7.65 (m, 14H), 7.26 (m, 4H), 7.19 (m, 2H), 5.35 (s, 2H), 4.02 (s, 4H), 3.97 (s, 4H), 3.39 (dd, 3 $^J$ = 7.0 Hz, 4 $^J$ = 6.6 Hz, 4H) 3.24 (dd, 3 $^J$ = 7.0 Hz, 4 $^J$ = 6.6 Hz, 4H), 2.18 (t, 3 $^J$ = 7.3 Hz, 4H), 1.77 (m, 4H), 1.60 (m, 4H), 1.33 (m, 4H).

$^{13}$C NMR (75.5 MHz, CD$_3$OD): δ 176.3, 167.8, 163.9, 160.8, 160.3, 149.9, 149.5, 148.6, 138.4, 138.3, 136.8, 129.9, 125.6, 124.8, 124.2, 124.0, 123.6, 74.4, 58.8, 58.3, 38.3, 37.8, 37.1, 30.2, 29.9, 26.8.

Chapter 4

$N_1,N_3$-bis($\{6-[\{di(2-pyridinyl)methyl]\{2-pyridinylmethyl\}amino\}methyl\}-3-pyridinyl\}carbonyl\}amino\}propyl\}isophthalamide (15)

Starting from 17 (38 mg; 0.11 mmol) and 3 (0.10 g; 0.21 mmol), 15 was obtained as a pale light brown oil (65 mg; 0.06 mmol; 56%).

$^1$H-NMR (400 MHz, CD$_3$OD) $\delta$ 8.83 (d, $^4J = 1.5$ Hz, 2H), 8.47 (d, $^5J = 4.7$ Hz, 4H), 8.37 (d, $^6J = 4.8$ Hz, 2H), 8.33 (m, 1H), 8.10 (dd, $^7J = 8.1$ Hz, $^8J = 2.2$ Hz, 2H), 7.98 (dd, $^9J = 7.7$ Hz, $^{10}J = 1.8$ Hz, 2H) 7.81-7.66 (m, 15H), 7.54 (m, 2H), 7.27 (m, 4H), 7.21 (t, $^{11}J = 5.9$ Hz, 2H), 5.36 (s, 2H), 4.01 (s, 4H), 3.98 (s, 4H), 3.49 (m, 8H), 1.93 (m, 4H).

$^{13}$C-NMR (50.3 MHz, CD$_3$OD) $\delta$ 169.42, 167.89, 163.86, 160.78, 149.95, 149.50, 148.58, 138.42, 138.35, 136.87, 136.14, 131.16, 129.93, 127.30, 125.62, 124.81, 123.64, 124.16, 124.00, 74.40, 58.77, 58.37, 54.80, 38.43, 30.22.

MS-ESI+: m/z 1065.5 ([M + H$^+$]+, 895.6 [M – Py-CH$_2$-Py + H$^+$]+, 533.5 [M + 2H$^+$]$^{2+}$, 356.1 [M + 3H$^+$]$^{3+}$.

RP-HPLC: $R_f = 33.4$ min.

$N_1,N_4$-Bis($\{6-[\{di(2-pyridinyl)methyl]\{2-pyridinylmethyl\}amino\}methyl\}-3-pyridinyl\}carbonyl\}amino\}propyl\}terephthalamide (16)

Starting from 18 (38 mg 0.11 mmol) and 3 (0.10 g; 0.21 mmol), 16 was obtained as a pale light brown oil (80 mg; 7.5 μmol; 71%).

$^1$H NMR (400 MHz, CD$_3$OD) $\delta$ 8.83 (d, $^4J = 1.5$ Hz, 2H), 8.47 (d, $^5J = 4.8$ Hz, 4H), 8.36 (d, $^6J = 4.8$ Hz, 2H), 8.19 (dd, $^7J = 8.1$ Hz, $^8J = 1.8$ Hz, 2H), 7.91 (s, 4H), 7.81-7.66 (m, 14H), 7.35 (m, 4H), 7.28 (t, $^9J = 5.5$ Hz, 2H), 5.36 (s, 2H), 4.03 (s, 4H), 3.98 (s, 4H), 3.48 (dd, $^{10}J = 6.6$ Hz, $^{11}J = 6.2$ Hz, 8H), 1.92 (t, $^{12}J = 6.6$ Hz, 4H).

$^{13}$C NMR (50.3 MHz, CD$_3$OD): $\delta$ 169.3, 167.9, 163.9, 160.8, 160.3, 145.0, 149.5, 148.6, 138.4, 136.9, 129.9, 128.5, 125.6, 124.8, 124.2, 124.01, 123.7, 74.8, 58.8, 58.4, 38.4, 30.2.

MS-ESI+: m/z 1065.5 ([M + H$^+$]+, 895.5 [M – Py-CH$_2$-Py + H$^+$]+, 533.5 [M + 2H$^+$]$^{2+}$, 356.1 [M + 3H$^+$]$^{3+}$.

RP-HPLC: $R_f = 30.9$ min.

Titration study of (NH$_4$)$_2$Fe$^{II}$(SO$_4$)$_2$ and 6

$^1$H NMR: To a solution of 6 in D$_2$O (with acetone as internal standard) was added 0, 1, 2 or 3 equivalents of [(NH$_4$)$_2$Fe$^{II}$(SO$_4$)$_2$] (Figure 4.4a). The $^1$H NMR spectra were referenced with respect to the acetone absorption ($\delta$ 2.22 ppm), since the solvent residual peak of D$_2$O tends to shift after the addition of the [(NH$_4$)$_2$Fe$^{II}$(SO$_4$)$_2$]. The relative area of the two peaks between $\delta$ 25 and 28 compared to the acetone absorption was plotted against the amount of equivalents of [(NH$_4$)$_2$Fe$^{II}$(SO$_4$)$_2$] (Figure 4.4b).

UV/Vis measurement: To a solution of 6 in H$_2$O was added 0, 1, 2 or 3 equivalents of [(NH$_4$)$_2$Fe$^{II}$(SO$_4$)$_2$] (Figure 4.5a). The relative molar absorptivity ($\varepsilon/\varepsilon_{\text{max}}$) at $\lambda$ 355 nm was plotted against the amount of equivalents of [(NH$_4$)$_2$Fe$^{II}$(SO$_4$)$_2$] (Figure 4.5b).
4.7 References and notes

1. See Appendix for abbreviations used.
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