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Copper complexes as biomimetic models of catechol oxidase: mechanistic studies

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9 Summary, general discussion and outlook

9.1 General

The research described in this thesis deals with the synthesis of copper(II) complexes which can be regarded as model compounds of the active site of catechol oxidase, and with the mechanism of the catalytic oxidation of catechol mediated by these compounds. Catechol oxidase is a type-3 copper enzyme usually encountered in plants and in some crustaceans, which catalyzes a conversion of a wide range of *o*-diphenols (catechols) to the respective *o*-benzoquinones. These highly reactive compounds subsequently auto-polymerize, resulting in the formation of a dark pigment melanin, which is thought to protect a damaged tissue from pathogens.

In Chapter 1 a general overview of the model compounds of catechol oxidase, reported in the literature, is given, and the different approaches used by various authors to study the mechanism of the catalytic reaction are discussed. The general overview of the recognized types of copper proteins and the detailed description of the crystal structure of catechol oxidase, as well as the proposed mechanisms of the enzymatic cycle are also presented in this chapter.

Chapters 2, 4 and 5 deal with the preparation of novel dinucleating phenol-based ligands, designed to mimic certain peculiar features of the active site of the enzyme, *e.g.* the asymmetric surroundings of the two copper ions and the unusual thioether bond in a close proximity to one of the metal centers. The structures and properties of the Cu^{II} complexes with these ligands are also reported in these chapters. In Chapter 3 the structures, spectroscopic and magnetic properties of several Cu^{II}, Mn^{II} and Co^{II} complexes with the phenol-based ligand Hpy2ald, containing pyridine and formyl functions, which has been prepared as an intermediate synthon in the preparation of asymmetric phenol-based ligands, are presented.

In Chapters 6-8 the structures of Cu^{II} and Cu^{I} complexes with two N-donor macrocyclic ligands [22]py4pz and [22]pr4pz, which provide respectively two N_4 and two N_3 donor sets for the metal coordination are presented, and the mechanisms of the catechol oxidation by the copper(II) complexes with these ligands are discussed. Chapter 6 also describes detailed paramagnetic ^1H NMR spectroscopic studies on the hydroxo-bridged dicopper(II) complex with the macrocyclic ligand [22]py4pz.

9.2 Cu^{II}, Co^{II} and Mn^{II} complexes with the phenol-based ligands

The ability of dinucleating phenol-based ligands to bind simultaneously two metal centers, keeping them in a close proximity thanks to the presence of a bridging phenolate group, gave rise to an extensive utilization of in this class of ligands to model bimetallic biosites.[1-7] In this thesis, the syntheses of four novel phenol-based ligands (Figure 9.1) are reported, and the structures and properties of seven Cu^{II}, two Co^{II} and two Mn^{II} complexes with these ligands are discussed.

In Chapter 2 the synthesis of the dinucleating phenol-based ligand Hpy3asym (Figure 9.1, a), which was designed to mimic the asymmetric surrounding of the two copper ions in the active site of catechol oxidase, are reported. This ligand contains one tridentate and one didentate arm attached to the 2 and 6 positions of the phenolic ring in order to achieve a coordination number asymmetry in its dicopper(II) complexes. The developed strategy for the synthesis of this ligand can also be successfully applied to generate other asymmetric phenol-based ligands, as discussed elsewhere.[8]

A dinuclear copper(II) nitrate complex with Hpy3asym $[Cu_2(py3asym)(H_2O)_{1.5}(NO_3)_{2.5}](NO_3)_{0.5}$ shows a donor-atom asymmetry that consists of a N_3O_3 donor set for the Cu1 ion and a N_2O_4 donor set for the Cu2 ion. Both Cu^{II} ions adopt a distorted octahedral surrounding, completed by the donor atoms of the solvent molecules and the nitrate counter ions, besides the donor atoms provided by the ligand. The absence of an exogenous bridging ligand in this complex results in a relatively long metal-metal separation of 3.9003 Å.

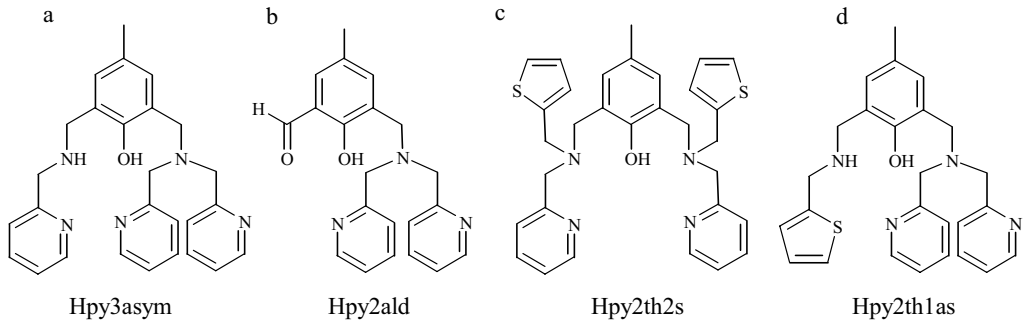

Figure 9.1. Schematic representation of the prepared phenol-based ligands.

Chapter 3 reports the structures and properties of six novel complexes of Cu^{II}, Co^{II} and Mn^{II} with the phenol-based ligand Hpy2ald (Figure 9.1, b), containing a tridentate pyridine-containing arm and a formyl group in two *ortho* positions with respect to the phenol group of the aromatic ring. Due to the presence of a weak donor formyl group in the ligand, the structures of the resulting complexes were found to depend strongly on the donor properties of the counter ions. Thus, the use of metal salts with weakly coordinating anions like perchlorate and tetrafluoroborate (*i.e.* $Co(ClO_4)_2{\cdot}6H_2O$, $Co(BF_4)_2{\cdot}6H_2O$ and $Mn(ClO_4)_2{\cdot}6H_2O$) leads to the formation of

dinuclear complexes with a metal to ligand ratio of 2:2, in which two metal ions are doubly bridged by two oxygen atoms of the deprotonated phenol groups of two different ligands. The metal ions have a distorted octahedral (Co^{II}) or trigonal prismatic (Mn^{II}) surrounding, with the remaining positions in the coordination sphere being occupied by three nitrogen donor atoms of one ligand and the oxygen atom of the formyl group of another ligand. The obtained complexes are thus structurally related to the nickel(II) complexes earlier reported by Adams[9] resulting from the hydrolysis of the imine arm of the original dinucleating phenol-based ligands during their reaction with Ni^{II} salts.

The presence of stronger donors, such as nitrate, bromide or chloride, prevents the coordination of the weaker donor formyl group, resulting in metal complexes of quite different structures. Thus, the reaction of manganese(II) chloride and copper(II) bromide with Hpy2ald leads to the isolation of the complexes $[Mn(Hpy2ald)Cl_2]$ and $[Cu(Hpy2ald)Br_2]\cdot 0.5H_2O$. In these complexes, the phenol group of the ligand remains protonated, failing to bridge two metal ions and instead being semi-coordinated to only one of them. The coordination environment around the metal center in both complexes is a distorted octahedron, constituted by three nitrogen donor atoms from the pendant arm of the ligand, two halogen anions and a loosely bound oxygen atom of the phenol group. The reaction of copper(II) nitrate with the ligand results in the formation of the complex $[Cu_2(py2ald)(\mu\text{-}NO_3)(NO_3)_2]\cdot CH_3CN$ with a metal to ligand ratio of 2:1. Two copper ions in the complex are bridged by the oxygen atom of the deprotonated phenol group and one of the nitrate counter ions. One of the two copper(II) ions has an almost ideal square pyramidal N_3O_2 surrounding, whereas the second shows a distorted octahedral O_6 surrounding, in which only one of the oxygen atoms is provided by the ligand, whereas the other five originate from three nitrate counter ions. Similarly to the copper(II) bromide and manganese(II) chloride complexes, the formyl group of the ligand remains uncoordinated to the metal centers.

In Chapter 4 the preparation of the symmetric phenol-based ligand Hpy2th2s (Figure 9.1, c) containing pyridine and thiophene functions is described. This ligand was designed to mimic the presence of the unusual thioether bond at the active site of catechol oxidase, resulting in the presence of a non-coordinated sulfur atom in the close proximity of one of the metal centers. In the case of Hpy2th2s, the very weak donor properties of the thiophene sulfur atoms prevent their coordination to the copper ions. Two copper(II) complexes with this ligand have been prepared and structurally, spectroscopically and magnetically characterized, *viz.* $[Cu_2(py2th2s)(\mu\text{-}Cl)Cl_2]$ and $[Cu_2(py2th2s)(\mu\text{-}Br)Br_2]$. In these complexes, both copper(II) ions are pentacoordinated and doubly bridged by the oxygen atom of the deprotonated phenol group and a halogen anion. The remaining positions in the metal coordination sphere are occupied by two nitrogen atoms provided by the pendant arms of the ligand and the remaining halogen atom. The magnetic susceptibility studies indicate a moderate antiferromagnetic coupling between the copper(II) ions in both complexes. The complexes do not exhibit

catecholase activity, most likely owing to the presence of the strongly coordinated halogen anions as bridging ligands between the metal centers. The studies on the interaction of these complexes with the model substrate tetrachlorocatechol (TCC) indicate that the bridging ligands indeed cannot be replaced by the incoming catecholate, although in the case of the chloride complex, one of the apical halogen anions undergoes a substitution with TCC. These results emphasize the importance of the bridging ligands on the catecholase activity of the dicopper(II) complexes, as the ability of these ligands to be displaced by the substrate is one of the crucial demands for the catalytic activity.

In Chapter 5, the synthesis of the asymmetric phenol-based ligand Hpy2th1as (Figure 9.1, d), containing a thiophene ring on one of the pendant arms, is reported. Two copper(II) complexes with this ligand, *viz.* $[Cu_2(H_2py2th1as)Cl_2](CuCl_4)_2$ and $[Cu_2(H_2py2th1as)Cl_2](ClO_4)_4 \cdot 6CH_3OH$, have been isolated and structurally characterized. The structures of the complex cations in the two complexes are very similar. Surprisingly, the secondary amine atom of one of the pendant arms undergoes a protonation during the crystallization with copper(II) salts, which prevents it from binding to the metal centers. Furthermore, the phenol group of the ligand remains protonated, similarly to the copper(II) bromide and manganese(II) chloride complexes with the ligand Hpy2ald. As a result, the metal to ligand ratio in the complexes is 2:2, with two metal ions being doubly bridged by two chloride anions. The thiophene rings remain uncoordinated; however, in the tetrachlorocuprate(II) complex they are involved in unusual stabilizing π-stacking interactions with the bridging chloride anions. Despite the large similarities between the two compounds, the major difference being different counter ions, the two coordination compounds exhibit significantly different structural arrangements, showing the important influence of the anion on the crystal packing.

The dimeric core in both complexes dissociates in solution, resulting in the mononuclear $[Cu(H_2py2th1as)Cl]^{2+}$ species. The complexes do not exhibit catecholase activity, although one equivalent of quinone is formed as a result of a stoichiometric reaction between 3,5-di-*tert*-butylcatechol ($DTBCH_2$) and the copper(II) tetrachlorocuprate complex. The absence of catalytic activity is, however, not surprising, since the complexes are essentially mononuclear in solution, which makes them less suitable models of the dinuclear active site of catechol oxidase.

9.3 Cu^{II} and Cu^{I} complexes with dinucleating macrocyclic ligands: structures and properties

Chapters 2, 4 and 5 of this thesis are devoted to the design of new phenol-based ligands in order to mimic the coordination surroundings of the metal centers in the active site of catechol oxidase. Nevertheless, these ligands have a few shortcomings, such as the presence of a phenol group, which, although beneficial in keeping two metal

ions at a short distance from each other, provides an additional oxygen donor atom for the metal coordination spheres. Because the presence of a single hydroxide ligand between the copper(II) centers was proposed for the natural enzyme,[10] the presence of an additional bridging group is therefore superfluous. Furthermore, the poor ability of the phenolate group to bridge two copper(I) centers makes the isolation of the reduced dicopper(I) complexes with such ligands nearly impossible, and it appears that dinuclear Cu^{I} complexes containing bridging phenolate groups have never been structurally characterized.

In addition, the preparation of metal complexes with phenol-based ligands requires a careful control of the pH of the solution, as an unexpected protonation of the pendant arms of the ligands may possibly occur, yielding complexes with unpredictable structural features.

Therefore in Chapters 6-8 the attention has been turned to the synthesis and characterization of dicopper(II) and dicopper(I) complexes with dinucleating macrocyclic ligands [22]py4pz and [22]pr4pz, which are shown in Figure 9.2. These ligands have been designed earlier to mimic the coordination surroundings of the copper centers in the active site of the structurally related type-3 copper protein hemocyanin.[11,12]

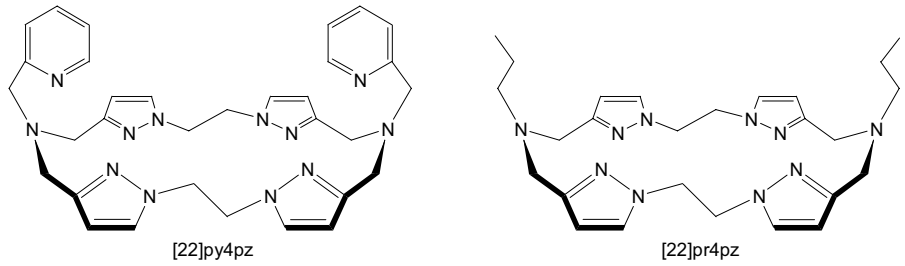

Figure 9.2. Schematic representation of the macrocyclic ligands [22]py4pz and [22]pr4pz.

In Chapter 6 the synthesis, spectroscopic and magnetic properties of the hydroxo-bridged dicopper(II) complex $[Cu_2([22]py4pz)(\mu\text{-}OH)](ClO_4)_3 \cdot H_2O$, as well as the paramagnetic ^1H NMR studies on this complex, are presented. The copper(II) ions in the complex are in a distorted trigonal bipyramidal surrounding, constituted by four nitrogen atoms of the ligand and the oxygen atom of the bridging hydroxide anion, with the oxygen atom and the nitrogen atom of the tertiary amine group of the ligand occupying the apical positions. A large Cu-O-Cu angle of 156.0(3)° results in a very strong antiferromagnetic coupling ($2J$ = -691(35) cm^{-1}) between the two metal ions, allowing the solution study of the complex by ^1H NMR spectroscopy. The complex shows an anti-Curie type behavior, with resonances in the spectrum shifting downfield when increasing the temperature. A complete assignment of the hyperfine-shifted signals was achieved by applying ^1H 1D NOE difference and 2D COSY NMR spectroscopy, through the determination of the relaxation times and by a selective chemical substitution. Temperature-dependent NMR studies were also used for an independent determination of the antiferromagnetic coupling constant $2J$ in solution,

and the obtained value was found to be in a very good agreement with the one obtained from the solid-state magnetic susceptibility measurements.

In Chapter 7 the 3D structure and properties of the dicopper(I) complex with [22]py4pz, *viz.* $[Cu_2([22]py4pz)](ClO_4)_2 \cdot 2CH_3OH$ are reported. The Cu^I ions in this complex are tricoordinated with a distorted trigonal surrounding, the nitrogen atoms of the tertiary amine groups remaining uncoordinated. At low temperature (-40 °C) in acetonitrile, the complex reacts with dioxygen, leading to the formation of a trans-μ-1,2-peroxo-dicopper(II) complex, which has been characterized by UV-Vis and resonance Raman spectroscopic techniques.

In Chapter 8 the structures and properties of the copper(II) and copper(I) complexes with the macrocyclic ligand [22]pr4pz are presented. The copper(II) complex exhibits a tetranuclear structure in the solid state, with one macrocyclic unit accommodating two metal ions, bridged by a carbonate anion. Two oxygen atoms of two carbonate anions then doubly bridge two copper centers of two different macrocyclic units, resulting in the complex $[Cu_2([22]pr4pz)(CO_3)(H_2O)]_2(CF_3SO_3)_4 \cdot 2CH_2CN \cdot 4H_2O$. The copper(II) ions have a distorted square-pyramidal environment, with a long intra-macrocyclic copper-copper distance of 4.5427(18) Å. In a diluted methanol solution, the tetranuclear structure dissociates into two dinuclear units, although at high concentrations it is preserved in solution. The magnetic susceptibility studies indicate that a ferromagnetic coupling is realized between the two Cu^{II} ions of one macrocyclic unit through the *syn, syn*-carbonato bridge, while a very weak antiferromagnetic coupling occurs between two copper(II) ions of two different macrocyclic units through the asymmetric di-μ-$O_{carbonate}$ bridge. The dicopper(I) complex $[Cu_2([22]pr4pz)(CH_3CN)_2](ClO_4)_2$ contains two tetracoordinated Cu^I ions, coordinated by three nitrogen donor atoms from the ligand and a nitrogen atom of a coordinated acetonitrile molecule.

9.4 Catecholase activity of Cu^{II} complexes with macrocyclic ligands: mechanisms of the catalytic reaction

The dinuclear complex $[Cu_2([22]py4pz)(\mu\text{-}OH)](ClO_4)_3 \cdot H_2O$ can be regarded as a satisfactory structural model of the active site of catechol oxidase. In particular, the presence of a single hydroxide bridge between the metal centers is an important structural feature of the natural enzyme. As hydroxo-bridged compounds have been previously reported as successful functional models of catechol oxidase, the catecholase activity of the complex has been investigated. The complex indeed catalytically oxidizes $DTBCH_2$ in acetonitrile, the catalytic reaction showing a Michaelis-Menten behavior with $V_{max} = 1.3 \times 10^{-6}$ M·s^{-1} and $K_M = 4.9$ mM.

It is even more important to appraise the mechanism of the substrate oxidation by model complexes in order to understand the mechanism of action of the natural enzyme. Consequently, the mechanism of $DTBCH_2$ oxidation by this complex was investigated by studying separately every step of the catalytic cycle. On the first stage, the stoichiometric reaction of the complex and the substrate in anaerobic conditions leads to the release of one molar equivalent of quinone and the generation of reduced dicopper(I) species. Dioxygen binding to the latter complex results in the formation of a trans-μ-1,2-peroxo-dicopper(II) adduct, which oxidizes a second molecule of catechol, leading to the restoration of the original hydroxo-bridged dicopper(II) core. This reaction was found to proceed in two successive steps: initially, a proton transfer from the substrate to the peroxo core leads to the formation of a highly reactive intermediate, which was assumed to be a hydroperoxo-dicopper(II) species on the basis of the resonance Raman, UV-Vis and EPR spectroscopy. Secondly, the bound catecholate is oxidized by the hydroperoxo moiety, which is accompanied by the reduction of the peroxide to water. The general mechanism of the catalytic reaction closely resembles the mechanism for catechol oxidase earlier proposed by Krebs;[10] however, the structure of the peroxo-dicopper(II) intermediate species most likely differs, as a μ-η^2:η^2 peroxo-dicopper intermediate has been proposed for the natural enzyme. This is also the first example of catechol oxidation by trans-μ-1,2-peroxo-dicopper(II) species.

It is also interesting to note that while trans-μ-1,2-peroxo-dicopper(II) species oxidize catechol to the corresponding quinone (catecholase activity), they do not perform the hydroxylation of the *o*-position of a phenol ring (monophenolase activity), in contrast to μ-η^2:η^2 peroxo-dicopper(II) species. The ability to hydroxylate the *o*-position of phenolic substrates prior to the oxidation of the resulting catechols into quinones is also a major difference between catechol oxidase and a structurally related type-3 copper enzyme tyrosinase. As the exact structure of tyrosinase remains unknown, no acceptable explanation for this difference in behavior of the two enzymes is available; however, it is tempting to speculate that this difference can be caused by the different structures of the peroxo-dicopper(II) intermediates formed in the catalytic reaction.

In Chapter 8 the mechanism of the catecholase activity of the dinuclear copper(II) species $[Cu_2([22]pr4pz)(CO_3)(H_2O)]^{2+}$in methanol is discussed. During the first few minutes of the catalytic reaction, the substrate oxidation is accompanied by the formation of dihydrogen peroxide, which, however, quickly cedes. The anaerobic interaction of the complex with catechol indicates that instead of a two-electron reduction of the dicopper(II) core, leading to the quinone and the dicopper(I) species formation, only one electron is transferred in the stoichiometric reaction, resulting in the formation of the mixed-valence $Cu^{II}Cu^{I}$- semiquinone species. The oxidation of the latter species by dioxygen leads to the formation of one molar equivalent of quinone and one molar equivalent of dihydrogen peroxide. Thus, the complex initially behaves

as a mononuclear copper(II) species, with only one copper ion participating in the redox process, whereas another plays solely a structural role. This is likely to be caused by the long copper-copper separation within a macrocyclic unit, which makes the simultaneous binding of catechol in a didentate bridging fashion to both copper(II) ions impossible.

However, the dihydrogen peroxide formation stops after a few minutes, suggesting that a different mechanism takes place at later stages of the catalytic reaction, most likely similar to the mechanism proposed by Krebs and co-workers for the natural enzyme.[10] This assumption is also confirmed by the inhibiting influence of the oxidation product DTBQ on the catalytic reaction, indicating that it does not simply accumulate in the reaction mixture, but also participates in the catalytic process. As DTBQ is able to react very quickly with the reduced dicopper(I) species, reoxidizing it to the mixed-valence $Cu^{II}Cu^{I}$-semiquinone species, this is a probable cause of its inhibiting behavior. The formation of dicopper(I) species can in turn be only explained by the mechanistic pathway proposed by Krebs and co-workers.[10] Thus, two different mechanistic pathways are realized in this case, proving that the catechol oxidation by model copper(II) complexes can be a very intricate process. The findings also explain the mechanism of dihydrogen peroxide formation as a by-product of catechol oxidation and indicate that the copper-copper distance plays a substantial role in the catalytic mechanism: a short distance enables a binding of catechol in a didentate bridging fashion, resulting in its further oxidation by the mechanism, similar to that observed for $[Cu_2([22]py4pz)(\mu\text{-}OH)](ClO_4)_3 \cdot H_2O$, whereas a long distance results in the binding of catechol to only one of the copper(II) centers with the formation of the semiquinone intermediate and subsequent reduction of dioxygen to dihydrogen peroxide.

9.5 Future outlook

In conclusion, the mechanism of catechol oxidation by model compounds appears to be very intricate, which obviously explains often contradictory literature reports on the catecholase activity of copper complexes. It should also be added that the investigations carried out by different research groups on the structure and function of catechol oxidase is a perfect example of the essential strategy adopted by the chemist of the 21st century. Indeed, such studies inevitably bring in distinct but complementary disciplines of contemporary chemistry, *i.e.* biochemistry, synthetic and inorganic chemistry, and spectroscopy. It is also evident that research on model compounds of natural enzymes is very inspiring for the development of novel bio-inspired efficient catalysts for oxidation reactions. The design of environmentally benign and clear processes for industrial applications is essential for a sustainable development of industrial chemistry. Therefore one should look at how Nature performs bio-transformations in order to find alternatives to the current environmentally unfriendly procedures. In this context, studies of enzymatic syntheses like the one achieved by

catechol oxidase are crucial since effective, selective, and ecologically friendly catalysts may be produced via a biomimetic approach.

As very promising results have been achieved with copper complexes with macrocyclic ligands, future investigations with these compounds are highly desired. In particular, further studies on the influence of the metal-metal distance on the catalytic behavior would be very beneficial to confirm the hypothesis about it being a crucial factor defining the whole catalytic mechanism. It should also be noted that very recently,[13] the monohydroxo-bridged dicopper(II) complex with the macrocyclic ligand [22]pr4pz has been isolated, the structure of which is shown in Figure 9.3. In this complex, two copper(II) ions are kept at a shorter distance of 3.8207(15) Å, comparable with the copper-copper distance in the complex $[Cu_2([22]py4pz)(\mu\text{-}OH)](ClO_4)_3 \cdot H_2O$. The coordination spheres of the metal ions in the complex also strongly resemble the active site of catechol oxidase, as each copper(II) ion is coordinated by three nitrogen donor atoms of the ligand; the loosely bound perchlorate anions are expected to dissociate in solution and be replaced by solvent molecules. It is very interesting to study the mechanism of the catecholase activity of this complex and to compare it with the mechanism of the catalytic reaction for its carbonate-bridged analogue, as well with the mechanism found for the hydroxo-bridged complex with the related macrocyclic ligand [22]pr4pz.

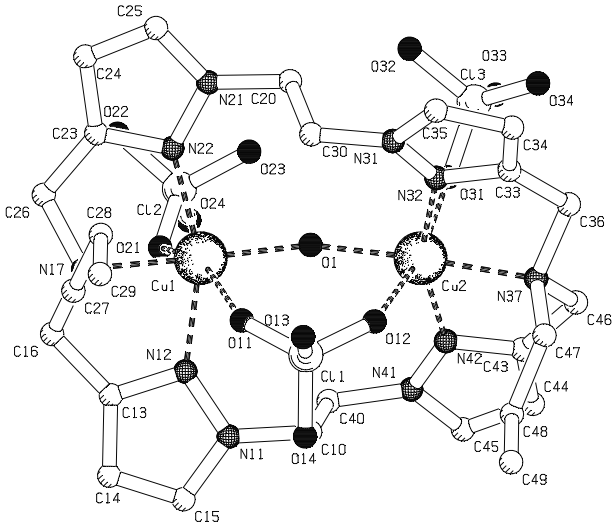

Figure 9.3. Platon[14] projection of the crystal structure of the complex $[Cu_2([22]pr4pz)(\mu\text{-}OH)(ClO_4)_3]$. Hydrogen atoms are omitted for clarity.

Furthermore, it is very appealing to study dioxygen binding by the dicopper(I) complex with [22]pr4pz, since it has been shown that the utilization of ligands with N_3 donor sets generally leads to the formation of $\mu\text{-}\eta^2\text{:}\eta^2$ peroxo-dicopper species, while trans-μ-1,2-peroxo-dicopper(II) complexes are usually obtained upon dioxygen binding to copper(I) complexes with N_4 ligands.[15] As $\mu\text{-}\eta^2\text{:}\eta^2$ peroxo-dicopper complexes are generally known to be able to perform the hydroxylation of the *o*-position of phenolic

substrates, it would be beneficial to study the reactivity of such species with respect to the modeling of the catalytic activities of both tyrosinase and catechol oxidase.

The oxidation of the mixed-valence $Cu^{II}Cu^{I}$-semiquinone species by dioxygen also deserves additional attention. As discussed in Chapter 8, this mechanism implies the formation of intermediate Cu^{II}-superoxide species, the spectroscopic characterization of which would undoubtedly be a highly interesting and challenging task.

The synthesis of copper(II) complexes with phenol-based ligands also has a significant potential interest. Despite certain shortcomings of these ligands, as outlined above, they are very easy to prepare, in contrast to the macrocyclic ligands. It is also relatively easy to synthesize structurally related ligands with only small variations, which would allow to study in detail the influence of certain factors (electron-donating or electron-withdrawing properties, steric hindrance etc.) on the catalytic behavior of the respective copper complexes. It is also very interesting to isolate dicopper(II) complexes, holding an additional exogenous hydroxide bridge between the metal centers, with such ligands. Unfortunately, the attempts to prepare such complexes with the ligands reported in Chapters 2-5 were futile; therefore new ligands can be designed for this purpose. One of such ligands, designed to provide both a coordination number asymmetry and the presence of non-coordinated sulfur atom in a close proximity of one of the copper centers, is depicted in Figure 9.4.

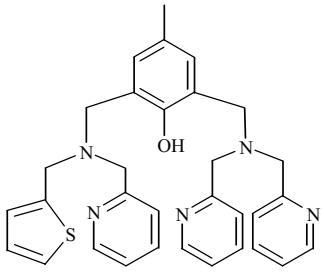

Figure 9.4. Schematic representation of the phenol-based ligand designed to mimic two peculiar features of catechol oxidase: the presence of non-coordinating sulfur atom in a close proximity of one of the copper(II) ions and a coordination number asymmetry of two metal ions.

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