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Novel NO Donating Iron-Sulfur Nitrosyl Complex with High Signaling and Antioxidant Reactivity

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Abstract: The rates of NO-releasing, signaling and antioxidant functions of complex $[Fe(SC(NH_2)_2)_2(NO)_2]_2[Fe_2(S_2O_3)_2(NO)_4]$ (**1**) in comparison with parent compound $Na_2[Fe_2(S_2O_3)_2(NO)_4]4{\cdot}H_2O$ (**2**) have been studied for the first time by amperometry, EPR-spectroscopy and molecular genetics with mutant *E. coli* cells bearing fusion promoters in the tester genes: lacZ in the SOS and SoxRS regulons and biofilm processing with *Ps.aeruginosa*. The most important finding of our work was the significant increase in expression levels of the *E. coli* soxS and sfiA genes in complex 1-treated cells versus untreated controls, and, in contrast, after combined cell treatment with a solution mixture of complex **2** and thiourea. In frozen, *E. coli* cells **1** and **2** produced EPR signals of the DNICs family with the same characteristics (g=2.03). The intensities of the signals correlated with the rates of gene expression. Antioxidant functions of the complex **1** were most pronounced in *Pseudomonas sp.* biofilm production and dispersal. Our data indicate that compound 1, with strong signaling and antioxidant activities, is promising for further development as a novel pharmaceutically and clinically important drug.

Keywords: NO donor; signaling; antioxidant; biofilm; Ps.aeruginosa; E.coli.

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1. Introduction

The unique properties of nitric oxide (NO) ensure the high reactivity of its endogenously formed intermediates and their role in cell signaling [1-4]. The biological functions of NO and other gasotransmitters are numerous and well established, especially those related to medicine. NO is a physiological regulator and a signal transducer in biological systems, a chain-breaking antioxidant that mediates cellular immunity, neurotransmission, angiogenesis, platelet aggregation, etc. [2,5,6]. The introduction of NO into known drugs is widely used as a modern approach to the design of hybrid NO-releasing compounds that directly interact with cellular targets [7,8]. These dual-mechanism hybrids provide combination therapies that are more effective than the sum of their parts. NO-aspirin, NO-ketoprofen, NO-indomethacin, etc., contain fragments of non-steroidal anti-inflammatory drugs (NSAIDs) and NO_2 groups with nitrate functions [9]. NSAIDs, being promising drugs, obtained negative statistics of gastrointestinal bleeding and ulceration due to problems with cyclooxygenase

enzyme inhibition and stimulating inflammation. Linking NSAIDs to free NO-donating groups became an excellent decision to avoid gastric lesions [9, 10,11]. A powerful protective action of NO in cardiovascular disorders and oncology is due to its antioxidant, anti-inflammatory, and antiradical properties, as well as its unique role in cell signaling and the regulation of nuclear proteins, such as NF-kappa B and AP-1 [3,12].

In vivo, NO can be stored as nitrosothiols (RSNOs) bound with low-molecular thiols (RSH) existing in the cells, as well as dinitrosyl iron complexes DNICs [13-15]. DNICs possess NO mimetic characteristics with regard to phenotypic response [3,4, 13,14], but unlike RSNOs and other NO-derived cellular adducts, the fate of DNIC in cells is not known. DNICs are the largest intracellular pool of chelated iron. In the EPR spectra, the paramagnetic DNICs form the unique anisotropic signal with g_{aver}=2.03 with $g_{\perp}$=2.04, $g_{\|}$ = 2.014; the sizes of the signals are absolutely dependent on the cellular index of DNICs and the iron availability. In this regard, synthetic analogs of DNICs have attracted special attention in recent years. They have several advantages over other classes of exogenous NO donors, including the presence of NO groups and functional thiolate ligands in their structure, effective action at low therapeutic doses, and no need for activation to generate NO. DNICs exhibit a broad range of pharmacological properties, depending on the structure of the thiolate ligands [16]. They have shown high potential for the treatment of oncological [17-26], cardiovascular [27-29], endocrine [30], and infectious [31-37] diseases.

Recently, synthetic nitrosothiols, as nitric oxide donors, have been shown to have intraocular pressure-lowering activity in glaucoma by increasing aqueous humor outflow and relaxing the trabecular meshwork [38]. Glaucomatous pathology is associated with decreased antioxidant enzyme levels in ocular tissues, leading to increased reactive oxygen species (ROS) production and reduced NO bioavailability. Another example of a hybrid molecule is 4-(5-Amino-1,2,3-oxadiazol-3-yl)-2,2,6,6-tetramethyl-1-piperidinol (SA-2), which contains both antioxidant and NO-donor functionalities that provide a therapeutic level of NO necessary to promote angiogenesis and protect endothelial cells against hydrogen peroxide-induced oxidative stress [39].

This work aimed to study NO-donating, signal transduction, and functional activity of the octanitrosyl iron complex with formula $[Fe(SC(NH_2)_2)_2(NO)_2]_2[Fe_2(S_2O_3)_2(NO)_4]$ (**1**). This molecule simultaneously contains two different structural types of iron-sulfur nitrosyl fragments (see Fig.S1 in Supplementary file), namely, viz. two DNIC cations with thiourea ligands and the dianion of the DNIC dimer with thiosulfate ligands [40]. The structure of the dianion in **1** is similar to the previously studied dianion in the salt $Na_2[Fe_2(S_2O_3)_2(NO)_4]\cdot 4H_2O$ (**2**) (see Figure S2 in Supplementary file). Antioxidant and antiradical properties have been demonstrated for salt **2** *in vitro* [41].

Particular attention was paid to the dose-dependence between the NO-generation (nM) and signaling activity of the SOS and the SoxRS-DNA repair pathways in *E.Coli* cells. We focused on identifying doses and mechanisms of antioxidant function, namely the inhibition of ROS accumulation by NO-mediated reversible effects on biofilm production in *Ps. aeruginosa*. We suppose that data obtained from amperometry, EPR spectroscopy, and molecular-genetic experiments will help establish the objective regularities and the potential of a new hybrid NO molecule in studying mechanisms of its signal transduction and antioxidant reactivity.

2. Materials and Methods

2.1. Synthesis of NO-donors.

Complexes **1** and **2** with functional sulfur-containing ligands were synthesized as described in protocols [40] and [42]. The structures of the synthesized complexes have been confirmed by IR spectroscopy.

2.2. Reagents.

In the work, commercial chemicals $Fe_2SO_4{\cdot}7H_2O$, $Na_2S_2O_3{\cdot}H_2O$, hydrochloric acid (37%), HEPES, a chromogen o-nitrophenyl-β-D-galactopyranoside (ONPG); menadione; 4-nitroquinoline-1-oxide (4NQO); a chelator of bivalent iron o-phenanthroline (OF, 0.5 mM), and thiourea were used (Sigma-Aldrich, USA). Some study solvents were purified according to the procedures described in [43]. Fluoroquinolone antibiotic ciprofloxacin (CF, Promed Exports, India, 0.1μM) was used as the positive control biofilm inhibitor of cell growth.

2.3. Electrochemical determination of NO donor activity.

The measurements were performed with an inNO Nitric Oxide Measuring System, "amiNO-700" (Innovative Instruments, USA). Amounts of NO generated by complexes 1 and 2 were recorded for ~500 s with 0.2 s intervals in 1% aqueous solution of dimethylsulfoxide (DMSO) with the concentration of complexes of $0{,}4{\cdot}10^{-5}$ M. DMSO was purified according to the procedure [43]. Standard aqueous $NaNO_2$ solution (100 μmol) was added to the mixture of 0.12 M KI and 2 mL of 1 M H_2SO_4 in 18 mL of water to calibrate the electrochemical sensor. All experiments were performed in aerobic solutions in a cell with temperature controlled using a circulating thermostat LT-324 (LOIP, Russia) at 25°C, in phosphate buffer solution pH 7.0 (Fisher Chemical) controlled using a membrane pH-meter HI 8314 (HANNA instruments, Germany). Data for determining NO amounts by amperometry were obtained for each kinetic curve several times for five repetitions.

2.4. EPR measurements.

Our work used the EPR methodology to characterize **1** in frozen *E. coli* cells, in powder, and in water solution *in vitro* at room temperature. *E. coli* cells harvested from 48 ml of standard suspension by centrifugation (10 min at 4000 rpm) were suspended to 0.5 ml and frozen in liquid nitrogen. The EPR spectra were recorded at 77K with an X-range radio spectrometer assembled of Radiopan modules (Poland) at HF modulation amplitude 0.2 mT and microwave power 5 mW. In the EPR study in vitro, we used the ELEXSYS-II E500 radiospectrometer ("Bruker") at room temperature.

2.5. Bacterial strains.

In the experiments, we used *Escherichia coli* strains with fusion promoters in tester genes and lacZ gene: *E. coli* PQ37 (sfiA::Mu d(AP lac) cts lac ΔU169mal+ uvrA galE galY PhoC rfa F- thr leu his pyrD thi trp::Muc+ srl300::Tn10] a gift from Prof. Hofnung, Pasteur Inst., Paris [44] and *E. coli* TN530 (F2 Δ(lacZYA-argF)U169 rpsL λ(soxS'-lacZ) soxRS+ a gift from Prof. Nunoshiba, Japan [45].

The opportunistic pathogen *Pseudomonas aeruginosa* PA103 (reference strain, laboratory collection) was used in the biofilm experiments.

2.5.1. β- galactosidase assay.

In the test system with *E. coli* PQ37 [sfiA::lacZ], a level of sfiA- gene expression (the SOS-regulon) was studied non directly –on the basis of enzymatic activity of β-galactosidase in a colorimetric test at λ= 420 nм with ONPG as the chromogen. A PD-303UV digital spectrophotometer (Apel Co. Ltd., Japan) was applied [46]. β – gal activity in Miller units [47] was calculated according to E=1000xOD420 /t, where OD- is the optical density at λ= 420 nм, t – time of incubation with ONPG. A similar methodology was used at work with the *E. coli* TN530 [soxS::lacZ] in studying the *E. coli* SoxRS regulon induction.

2.5.2. Biofilm processing.

The detailed basic protocol is described in [48]. The level of *Pseudomonas aeruginosa* plankton cell growth at 25°C (OD600) and mass of mature biofilm (OD570) production in the LB medium in single-use polypropylene cuvettes (Kartell, Italy) were assessed after 24 h incubation with PD-303UV digital spectrophotometer (Apel Co. Ltd., Japan) in the control and in NO-treated cells.

To evaluate the efficacy of short NO-donor-mediated dispersal of mature biofilm, we used samples from untreated cells incubated for 21 h in LB medium, then treated them with NO donors.

2.5.3. Statistical analysis.

The gene expression results are present in Miller Units as the mean values of 3 experiments and a 95% confidence interval.

3. Results and Discussion

NO functions as a unique signaling molecule, but its diverse chemistry and biological activity are contradictory. NO is also a pro-oxidant and a potential antioxidant. It acts as a pro-oxidant at high concentrations or when it reacts with superoxide O_2, giving rise to highly toxic peroxynitrite. At the same time, NO is an effective chain-breaking antioxidant in free-radical-mediated lipid oxidation, and only low doses of NO are required to inhibit cellular lipid oxidation [50].

Complexes **1** and **2,** as nitric oxide donors, generate NO without additional activation during hydrolysis under aerobic conditions. Maximum amounts of NO generation for both compounds were approximately equal to 25-35 nM; it was stated that there was an equal sum of NO molecule generation for 500 s in the compounds, but their kinetics were different. In the case of **1,** it reaches a “plateau” at 300 s after the start of the experiment. The kinetic curve of NO-donation by 2 does not go to a “plateau”; moreover, there are two stages in NO-releasing: at the first 50 seconds, NO generation kinetics is observed as “the bell” (Figure 1).

The mixture of solutions of complex **2** and thiourea (0,3 M and 0,7 M) has demonstrated prolonged kinetics in NO-releasing, like complex **1** (up to 1.5 h), though the level of NO-releasing was inhibited a little (23,0 nM and 18,0 nM, respectively). Early on, we noted that the rate of NO donating in vitro did not correlate directly with the signaling activity of NO donors; it proved to depend on intracellular iron.

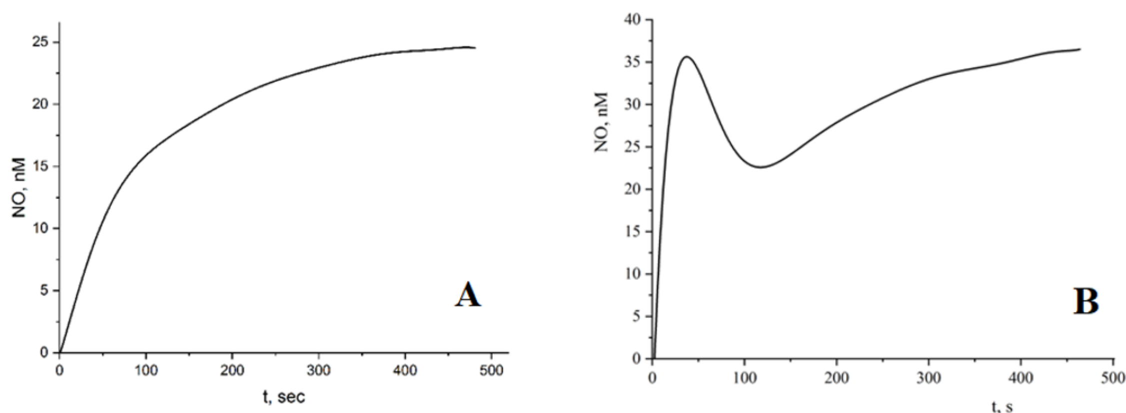

Figure 1. Time dependences of NO amounts generated by complexes **(A)** 1; **(B)** 2 (the concentration of complexes was 0.4·10-5 M) in 1% aqueous solution of DMSO under aerobic conditions (T =25°C).

A complex study of signaling and antioxidant functions of complex **1**, in comparison with its parent compound, viz complex **2,** and of a mixture of complex **2** and thiourea solutions, was fulfilled for the first time in the bacterial cells.

E. coli PQ37 is the basic tester strain for expressing quantitative study and prediction of the genotoxic/carcinogenic/anticancer activity of chemicals and their mixtures, with a special accent on the DNA repair inducible error-prone (mutagenic) SOS process [44]. In 1999, Vasilieva and coauthors were the first to substantiate and experimentally confirm the DNA SOS-inducing activity of NO-donors in *E. coli* cells [46]. Molecular-genetic mechanisms of the multifunctional DNA-repair SOS pathway have been studied for many years, and most details are now clear [50,51]. *E. coli* TN530, a multifunctional SoxRS [2Fe-2S] regulon member, consists of nearly 10 promoters and is activated by the coordinated action of the SoxR and the SoxS genes. SoxR is a transcription factor that induces the expression of SoxS to initiate the production of enzymes in response to oxidative stress. In addition to superoxide, SoxR, sensitive to NO, produces a protein-bound dinitrosyl-iron complex (DNIC) with a characteristic EPR signal at g_{aver} =2.03. SoxR molecular contains [2Fe-2S] cluster, the preferred target for NO, so the *E. coli* SoxRS DNA repair system demonstrates dynamic modulation of cell resistance to oxidative agents [47].

In present EPR studies of *E. coli* TN530 treated with complex **2** or with the mixture of **2** and thiourea solutions, we observed the traditional anisotropic broad EPR signals of g=2.03 family of mononuclear dinitrosyl iron complexes (DNICs) (Figure 2). Fe^{2+} chelator o-phenanthroline (OF) leads to the disappearance of g=2.03 signals and the appearance of the red color complex in the cell suspension. The last one was a new complex with Fe^{2+} as a result of NO-donor decay [Figure 2]. The complex **2** EPR spectra with g=2.03 signals resembled complex 1 and were responsible for activating the SOS- and SoxRS DNA repair systems in *E. coli* cells [15,23]. Two washes of the cells or a temperature shift to 24°C didn’t change the typical EPR characteristics of the signals; all of these are evidence of their protein nature. In NO donors' EPR spectra, we observed typical anisotropic signals of the g=2.03 family of mononuclear dinitrosyl iron complexes (DNICs). The nature of paramagnetic centers is universal, so the appearance of the g=2.03 signal means the production of the DNICs with thiol-containing ligands in NO-treated cells. EPR spectra with g=2.03 signals resembled for the SOS and the SoxRS DNA repair systems in *E. coli* cells; the EPR signals' size was correlated with corresponding gene expression levels.

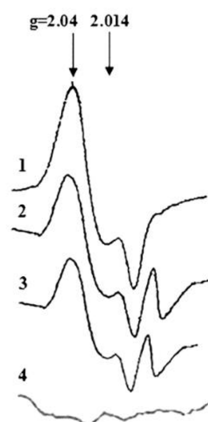

Figure 2. EPR spectra of *E. coli* cells TN530 incubated with complex **1** (1), the mixture of complex **2** and thiourea (1:10) (2), complex **2** (3), and thiourea (4). Experimental conditions: 77 K, microwave power 5 mW, modulation amplitude 0.5 mT, spectrometer amplification $1 \cdot 10^6$.

These NO donors' high signaling and functional activity were established for the first time in the alternative *E. coli* DNA–repair pathways. Figures 3A and B show dose-dependent levels of both *E. coli* SoxS and sfiA gene expression. At the same effective dose of 0.05 mM, complex 1 causes expression of soxS and sfiA genes 22 and 5.2 fold (resp.) higher versus untreated control cells, while it was 14 and 4.5 fold higher after combined cell treatment with complex **2** and thiourea (1:10). The complex **2** was the least effective in these experiments (8 fold at soxS versus 2.5 fold at sfiA.). The mixture of complex **2** and thiourea (1:10) demonstrated *in vitro* more dilatory prolonged kinetics in NO-releasing up to 1.5 hours, as noted above, which correlated with its prolonged DNA signaling activity (Figures 3 A, B). Pretreatment of the cells with Fe^{2+} chelating agent o-phenanthroline (OF) prevented the expression of both genes by NO-donors (Figures 3 A, B, the lower lines).

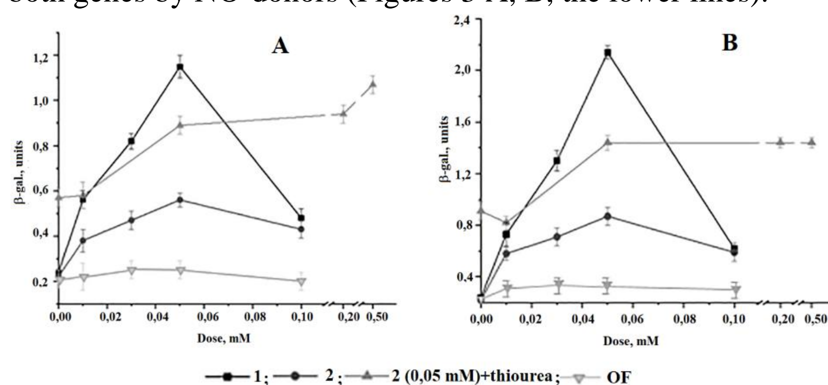

Figure 3. Dose dependences of sfiA gene expression in *E. coli* PQ37 (**A**) and SoxS gene in *E. coli* TN530 (**B**) cells, incubated in LB medium after treatment with complexes **1**, **2**, the mixture of complex 2 and thiourea (1:10), and the iron (2+) chelator, viz 0.05mM o-phenanthrolin (OF).

Thus, in genetic experiments with complex **1**, we described a high-level increase in the expression of *E. coli* DNA-repair genes of the SoxRS and the SOS DNA-repair systems relative to the controls. Complex **2** turned out to be an effective signaling agent, such as alternative NO donors with "physiological ligands" - DNICcys, DNICglu, or GSNO [46, 48, 49]. We also observed an interesting phenomenon, viz in contrast to complex **2**, the mixture of complex **2** and thiourea (as an organic compound) solutions demonstrated many-fold prolonged kinetics in NO-releasing in aqueous solution (up to 1.5 hours), though the rate of the process was significantly lower. At the same time, this mixture's signaling activity level was higher than that of complex **2** (Figures 3, 4). A similar correlation was first observed in slow-releasing

organic H_2S-donor GYY4137 in the proliferation effect in human breast adenocarcinoma MCF-7 cells. Inorganic fast donors NaHS/Na_2S did not influence the process of cell proliferation of all previously studied cancer cells. Of course, increasing up to several days of medical use of H_2S donor with a low rate of donation, like GYY4137 (it was proposed as a prospective model to study acute joint inflammation in human cartilage cells), is much more promising than applying high doses of H_2S inorganic doses - NaHS and Na_2S [52]. Understanding the mechanisms of “organic influence” on H_2S- and NO-slow-donating agents is a key challenge for further designing and developing these compounds as novel pharmaceutically and clinically important drugs.

Bacterial biofilm processing is now a worldwide medical task. In our study, low doses of complex **1** were most “efficient” in *Pseudomonas aeruginosa* biofilm production and dispersal. Biofilm suppressive levels of 0.05-0.07 mM of **1** were as potent as the antibiotic ciprofloxacin. *Ps. aeruginosa* biofilm suppressive levels of 0.01-0.07mM complex **1** were more efficient than those for complex **2** at the higher doses (Fig 4). Application of pure thiourea water solutions, as well as other antioxidants, was not effective in the NO biofilm processing, but in the combined cell treatment with a mixture of complex 2 and thiourea, the lowest level of biofilm production was observed, comparable with the antibiotic ciprofloxacin (CF).

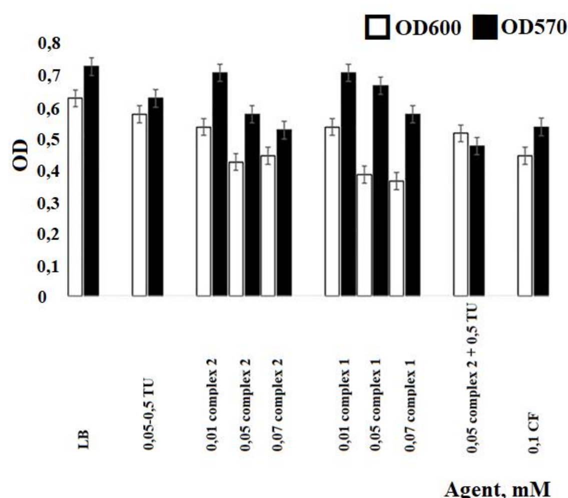

Figure 4. Optical density (OD600) of *Ps. aeruginosa* plankton cells (dark) and mass of mature biofilms (OD570) (light) incubated in LB medium and treated with complexes **1**, **2**, the mixture of **2** and thiourea (TU) in the ratio 1:10 and antibiotic ciprofloxacin (CF).

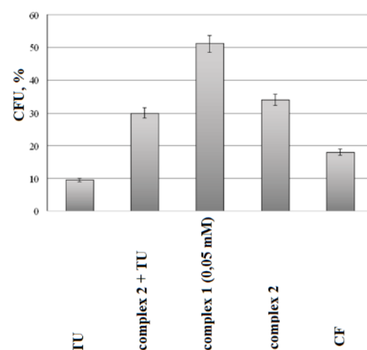

Figure 5. Dispersal of daily *Pseudomonas sp.* biofilm (%) in the cells, treated with 0.05 mM NO-donors, 0.5 mM thiourea (TU) and 0,1 μM ciprofloxacin (CF).

Using the alternative methodology, we observed an influence of NO donors on *Ps. aeruginosa* biofilm production within 21 hours. After cell treatment with 0.05 mM complex **1,**

the highest index of *Pseudomonas* dispersal was estimated at 51.16 + 0.14%, versus pure thiourea (8.5%), complex 2 (34%), the mixture of complex 2 and thiourea (30%), and 0.1 μM CF (18%) (Fig.5).

Hence, in our experiments, the new hybrid NO donor with thiourea was the most effective in Pseudomonas biofilm production and dispersal.

4. Conclusions

Signaling and functional activities of complex **1** with thiourea were first studied in *E. coli* mutants with particular attention on (1) dose-dependence between NO-generation and signaling at the SOS and SoxRS-DNA-repair pathways and (2) identifying its antioxidant activity, connected with bacterial biofilm production and dispersal at *Pseudomonas aeruginosa*.

The data obtained from amperometric, EPR spectroscopy, and molecular-genetics experiments established the objective regularities and preferences of **1** as a signaling molecule over complex **2**. The data obtained from amperometric, EPR spectroscopy, and molecular-genetics experiments established the objective regularities and preferences of 1 as a signaling molecule over complex 2. This is an example of a promising approach to designing new NO-donating hybrid molecules for the current therapy of socially significant diseases.

Authorship contributions

Conceptualization: S.V.V.; Investigation: S.V.V., N.A.S.; Writing – original draft: S.V.V.; Writing – review & editing: N.A.S. All authors have read and agreed to the published version of the manuscript.

Data Availability Statement

Data supporting the findings of this study are available upon reasonable request from the corresponding author.

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Conflicts of Interest

The authors declare no conflict of interest. The funders had no role in the design of the study, in the collection, analyses, or interpretation of data, in the writing of the manuscript, or in the decision to publish the results.

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Supplementary materials

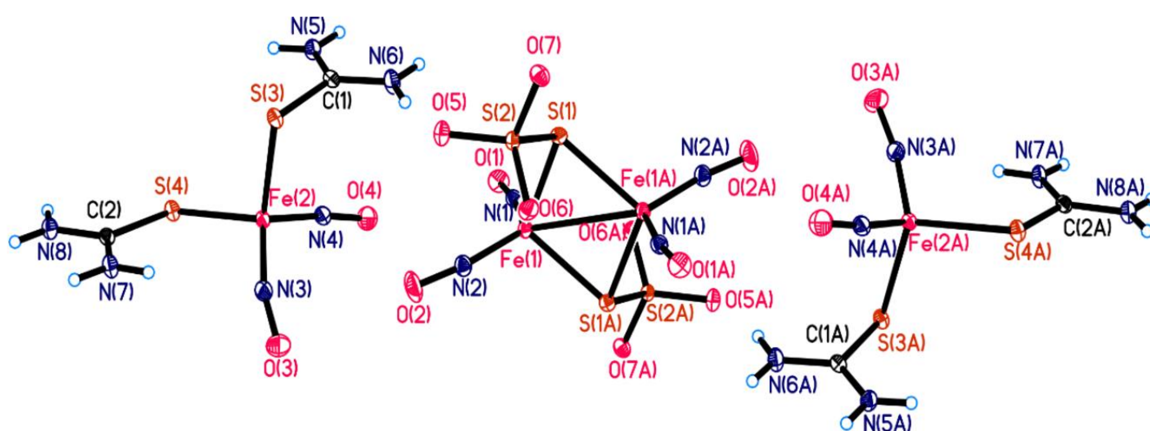

Figure S1. Molecular structure of complex 1 (according to ref.[40]).

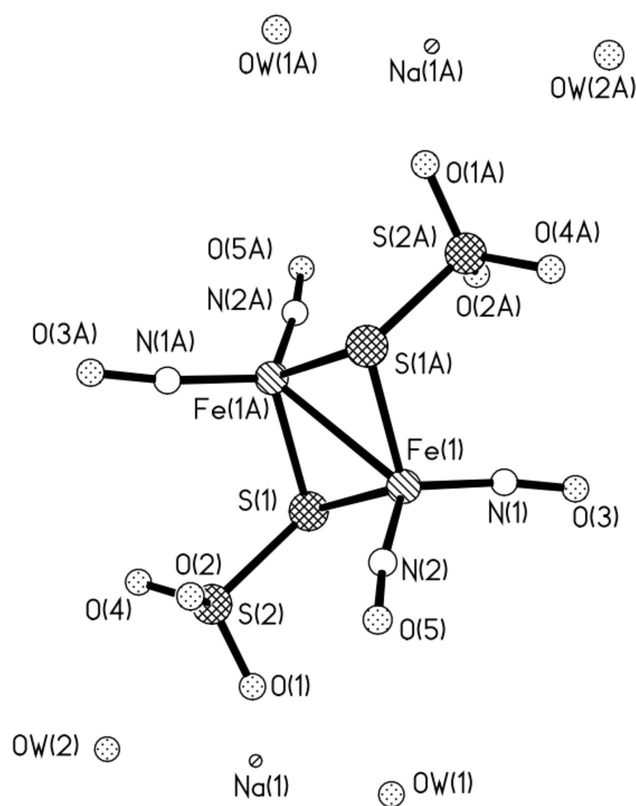

Figure S2. Molecular structure of complex 2 (according to ref.[42]).