Short thesis for the degree of doctor of philosophy (PhD)

Enhancement of selectivity and efficiency of ligands and their derivates having medicinal significance by using their Co(III) complexes

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I. INTRODUCTION AND OBJECTIVES

Limiting antibacterial resistance and cancer treatment are among the most serious duties for medicine in recent days.

Despite several attempts in the last century antibacterial resistance is still an unsolved problem. Resistant bacteria appeared in a short period of time after the release of a new type of antibiotic. Since the increasing level of infections casued by resistant bacteria this problem might became one of the relevant medicinal threats in the near future. Lack of selectivity in cancer therapy leads to many unwanted side effects of the used anticancer agents making cancer treatment another important problem nowadays.

To increase the efficency and selectivity of the drug and to overcome the problem of resistance the use of metal complexes as carriers of the drug molecules can be a solution for both antibacterial and anticancer agents. The use of Co(III) as a central metal ion in the complexes can be an option to transport the biologically active molecules selectively to the tumor tissues. Since the Co(III) complexes are kinetically inert their reduction to the corresponding Co(II) complexes might happen only in the reducing environment of the tumor. Co(II) complexes are much more labile therefore the dissociation of the complex can occur shortly after the reduction. In order to avoid the reduction of the Co(III) complexes in the healthy cells before reaching the tumor, tetradentate ligands with N donor atoms (4N) and capable of forming stable fused chelates are coordinated to the metal ion. The remaining two coordination sites of the Co(III) ion are occupied by the drug molecule having O donor atoms in chelating position (O,O) that can selectively be released in the hypoxic cancer cells. Beside antitumor agents the formation of Co(III) complexes can also be useful for the antibacterial agents to enhance their efficiency. Since patients after cancer treatment have

weakened ability to fight against infections delivering the antibacterial agent directly to the treated area might be more effective; furthermore, complexation of the drug molecule may also solve the problem of resistance.

The aim of my work was to synthesize $[Co(4N)(O,O)]^{n+}$ type complexes. Tren and tpa tripodal amines were used as 4N donor ligands and several quinolones and retrohydroxamic acid based molecules were selected for (O,O) type ligands with antibacterial activity. Differently substituted flavonols and quinizarin compounds were used as anticancer agents having O donor atoms (***Figure 1***). Characterization of the obtained complexes with NMR, IR, MS, elemental anlysis and X-Ray diffraction analytical methods was also purposed. Furthermore, investigation of the redox properties of the products was planned alongside the study of the effects of the differently substituted functional groups on the redox properties of the complexes. Biological screening of the complexes and ligands in cooperation with other research groups was purposed too and we planned to investigate the relationship between the biological activity and the structural and redox properties of the compounds.

II. EXPERIMENTAL METHODS

Characterization of the obtained ligands and complexes was carried out by different analytical techniques.

NMR measurements were carried out on Bruker WP 360 SY and Bruker Avance 400 type instruments using D_2O or d^6-DMSO solvents and the spectra were analysed by MestReNova program. Chemical shift values were referenced to the residual solvent peaks for the ^1H NMR studies (DMSO: 2.50 ppm, D_2O: 4.79 ppm), and a 10 mM NaF solution was used as reference for ^{19}F NMR measurements. Data were given in ppm values.

Infrared spectroscopic (IR) measurements were carried out at the Department of Organic Chemistry, University of Debrecen on a Perkin Elmer FTIR Paragon 1000 PC device using KBr pellets. IR measurements were perfomed by Dr. László Tóth and **elemental analysis** data were measured on an Elementar Vario MICRO CUBE device by Dr. Attila Kiss.

Mass spectrometric (MS) experiments were performed at the Department of Applied Chemistry, University of Debrecen on a Bruker micrOTOF-Q ESI-TOF device and carried out by Dr. Tibor Nagy and at the Department of Inorganic and Analytical Chemistry by Dr. Attila Gáspár and Dr. Gizella Csire using Bruker MaXis II. uhr ESI-TOF device. Spectra were registered in the positive mode and analysed with DataAnalysis 3.4. program. Water or methanol were used as solvents for sample preparation.

Single crystal analysis was carried out on a Bruker-Nonius MACH3 or Bruker-D8 Venture type X-Ray diffraktometer, at the Department of Physical Chemistry, University of Debrecen and performed by Dr. Attila Bényei. For HFA and GSK compounds Bruker-D8 Quest ECO device was used at the Trinity College of Dublin and the studies were performed by Dr. Brendan Twamley.

Characteristic K_α radiation of Cu and Mo (Cu: λ = 1.54184 Å, Mo: λ = 0.71073 Å) were used for the measurements. Chemical structure figures, bond length and bond angle values were determined by the Mercury 3.0 program.

Redox properties of the ligands and complexes were investigated with Metrohm 746 VA Trace Analyzer and BASi Epsilon EClipse type **cyclic voltammeter**s (CV). Glassy carbon (CHI104) working electrode, Pt wire auxiliary electrode and Ag/AgCl reference electrode were used. Concentration of the samples was 1 mM and water or water : methanol = 1 : 1 mixture were used as solvent. As an inert electrolyte KNO_3 was used in aqueous solutions and $[NBu_4][BF_4]$ was used in methanolic solutions and the concentration of the salt was 0.2 M in all cases. The system was calibrated with the aqueous solution of $K_3[Fe(CN)_6]$ and all samples were degassed for five minutes with argon gas. The pH was adjusted with 0.2 M HCl and 0.2 M KOH solutions for pH-dependence studies.

Collaboration with various domestic and international research groups provided us the opportunity for **biological screening** of the compounds.

Lipofilicity measurements were carried out to model how the compounds can get through the cell membrane. Mixture of *n*-octanol and buffer (PBS, pH = 7.4) was used for the measurements. Studies were performed by Dr. Orsolya Dömötör at the Department of Inorganic and Analytical Chemistry, University of Szeged for quinizarin compounds. Concentration of the samples was in the range of 20-100 μM, and the studied wavelength region was 200-500 nm for both quinizarin, and flavonol compounds.

Study of the compounds *binding to human serum albumin (HSA)* was performed to obtain information about transport possibilities of the compounds in the blood-stream. The binding process was monitored using *spectrophotometric* and

spectrofluorimetric measurements. Measurements were performed with the help of Dr. Orsolya Dömötör at the Department of Inorganic and Analytical Chemistry, University of Szeged using an Agilent Cary 8454 type spectrofotometer and Hitachi F-4500 type spectrofluorimeter. Warfarin (W) and danzil-glycine (DG) were used as marker molecules. Excitation wavelength were 295 nm for quenching experiments, 310 nm for W-displacement measurements, and 380 nm for DG-displacement 380 nm. Each sample contained 1 μM HSA, 1 μM marker molecule and 0-15 μM studied compound. Samples were dissolved in PBS buffer (pH = 7.4) thermostated at 37 ^{o}C for 30 min before the measurement. After the absorption correction of the spectra, the binding constants were determined by the PSEQUAD program.

Antibacterial activity of the compounds was tested against various bacteria at the University of Dublin and at the University of Brno. The measurements were performed by Dr. Deirdre Fitzgerald-Hughes and the research group of Prof. Jana Kasparkova using 18 h or 24 h incubation time, respectively.

DNA-binding properties of the compounds were examined by *spectrophotometric*, *ethidium bromide displacement*, *viscosimetric* and *DNA-melting point*, measurements. *DNA-cleaving* and *enzyme-inhibitory* properties of the compounds were also tested. Studies were performed by the research group of Prof. Jana Kasparkova.

SRB assay was used to study the *anticancer activity* of the flavonol compounds under both hypoxic and normoxic conditions. Assays were performed at the University of Leiden. MTT assays of quinolone and quinizarine compounds were carried out by by the research group of Prof. Jana Kasparkova.

III. STUDIED LIGANDS

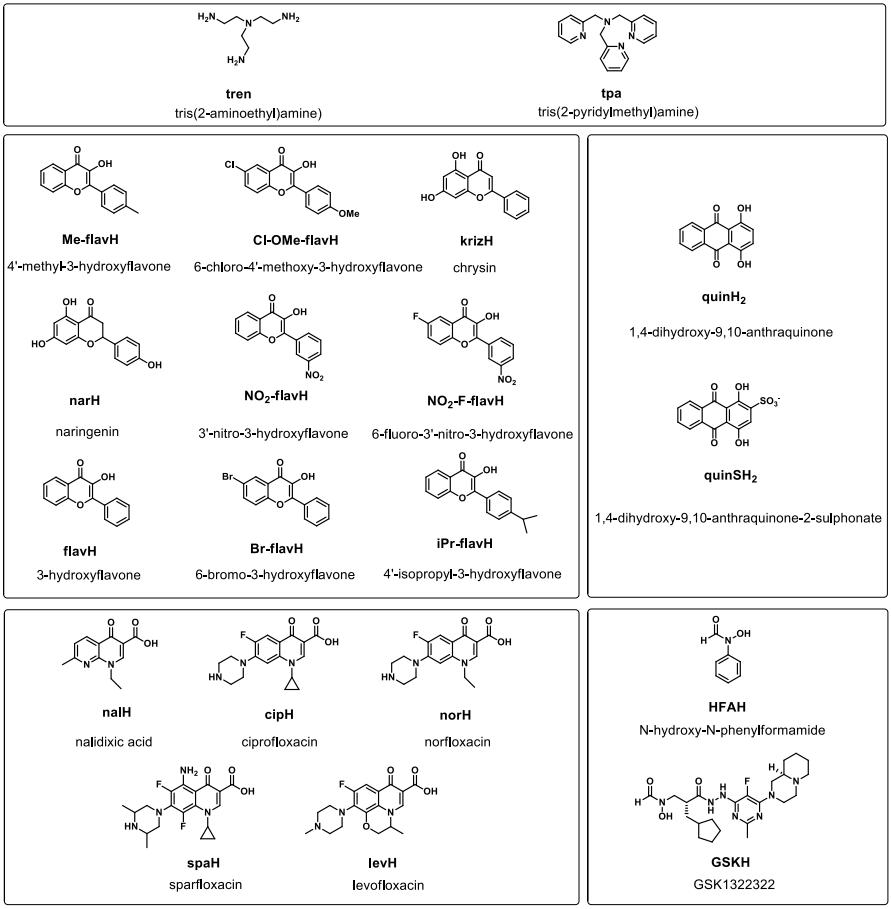

Figure 1. Structures and abbreviations of the studied ligands.

IV. NEW SCIENTIFIC RESULTS

4.1 New chalcone and flavonol type ligands containing electron withdrawing substituents were synthesized and characterized.

4.1.1 The list of the studied flavonol ligands was extended by the synthesis of compounds having electron withdrawing substituents to study the effect of the various types of functional groups on the redox properties of the complexes. Reaction of properly substituted hydroxyacetophenones and 3-nitrobenzaldehydes afforded the desired hydroxychalcone derivates. 3'-nitro-3-hydroxyflavone type compounds were synthesized by Algar-Flynn-Oyamada reaction of the hydroxychalcone derivates.

4.1.2 The products were characterized by using NMR, IR and elemental analysis techniques. Chemical formula and high purity of the compounds were confirmed by all the analytical results.

4.1.3 Single crystals of the synthesized NO_2-F-flavH, GSKH and Me-flavH ligands were grown. Molecular structures of the compounds were confirmed by X-ray diffraction studies.

4.2 New $[Co(4N)(O,O)]^{n+}$ type Co(III) complexes were synthesized and characterized.

4.2.1 Differently substituted flavonols, qunizarines, quinolones and retrohydroxamic acid based molecules were selected as (O,O) type ligands and tren and tpa tripodal amines were used as 4N donor ligands for the synthesis of the Co(III) complexes. $[Co(4N)(Cl)_2]^+$ type precursors were synthesized before the synthesis of the complexes. General scheme of the synthesis is illustrated in ***Figure 2*** by the example of $[Co(tren)Cl_2]^+$, $[Co(tren)(flav)]^{2+}$, $[(Co(tren))_2(quin)]^{4+}$.

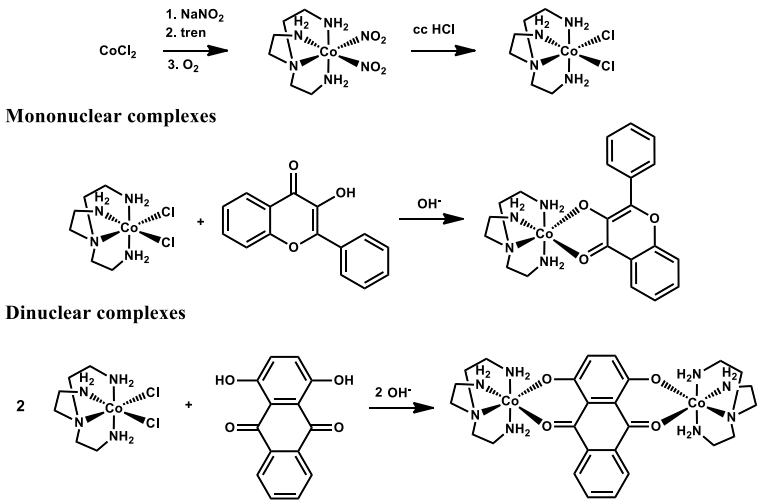

Figure 2. Scheme of the synthesis of the complexes.

4.2.2 The obtained complexes were characterized by NMR, IR, ESI-MS and elemental anlysis techniques. As an illustration ^{1}H NMR spectrum of $[Co(tren)(nor)](PF_6)_2$ is shown in ***Figure 3***. Formation of dinuclear complexes for quinizarin compounds and mononuclear complexes for retrohydroxamic acid based, quinolone and flavonol type bioligands was determined.

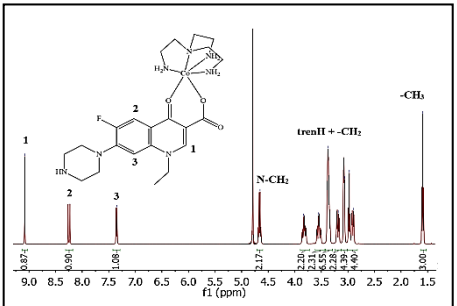

Figure 3. ^{1}H NMR spectrum of $[Co(tren)(nor)](PF_6)_2$ in D_2O.

4.2.3 In many cases the formation of isomers was observed based on the NMR results. For mononuclear complexes the formation of two different geometric isomers was noticed depending on whether the tertiary N atom of the 4N donor ligand is *cis* or *trans* to the aromatic substituent of the (O,O) ligand. Similar isomers can be formed for the dinuclear complexes too, but because of the two binding sites of the bioligand two symmetric and one asymmetric isomers can be differentiated.

4.2.4 Due to the presence of an $–NH_2$ group in chelating position in sparfloxacin for this ligand a (N,O) chelating mode was also possible instead of the expected (O,O) coordination. The coordination mode was confirmed by ^{19}F NMR studies that showed sparfloxacin to coordinate to Co(III) *via* its O donor atoms too, like all the other bioligands.

4.2.5 Presence of mixed counter ions and solvent molecules in the complexes was determined based on elemental analysis data. The involvement of the carbonyl group was confirmed using IR by comparing the peaks related to υ(C=O) vibration in the spectra of the complexes with that of the free ligands. Identity of the complexes was further prooved by ESI-MS studies. All the spectra displayed the correct isotopic pattern confirming the identity of the particles.

4.2.6 Molecular structures of eleven complexes were assessed by single crystal analysis. Coordination of the ligands to the central metal ion *via* the O donor atoms of the carboxyl group and the deprotonated hydroxyl group were found in all cases resulting in complexes with octahedral geometry. As an illustration molecular structure of $[Co(tren)(NO_2\text{-F-flav})]^{2+}$ determined by X-ray diffraction is shown in ***Figure 4.***

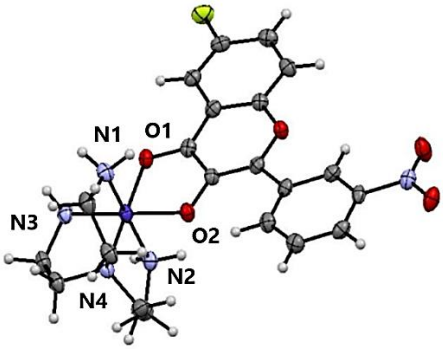

Figure 4. Molecular structure of $[Co(tren)(NO_2\text{-F-flav})]^{2+}$. Counter ions and solvent molecules are omitted for clarity.

4.3 Redox properties of the complexes were explored by cyclic voltammetric measurements.

4.3.1 Major differences were found between the redox behaviour of the tren and tpa analogues. Reduction potential values of the tren containing complexes were observed to be significantly more negative than those of the tpa containing ones, making the tren complexes harder to be reduced. Furthermore, voltammogramms of tpa complexes showed reversible reduction in contrast to the tren analogues (***Figure 5***). Both of the above differences between the complexes of the two 4N donor ligands can be explained with the π-back bonding character of tpa because of its aromatic N donor atoms resulting in higher stabilization of the lower oxidation state Co(II) form.

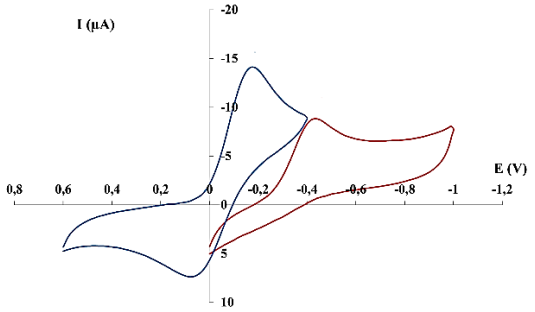

Figure 5. Registered cyclic voltammograms of [Co(tpa)(lev)]$(ClO_4)_2$ (**blue**) and [Co(tren)(lev)]$(PF_6)_2$ (**red**). Solvent: water:MeOH = 1:1, reference electrode: Ag/AgCl, scan rate: 200 mV/s.

4.3.2 Since the E_{pc} values of the corresponding flavonol and quinolone containing complexes fell into similar potential range the different coordination modes involving six- and five-membered chelate rings were found to have no significant influence on the redox properties of the complexes. On the contrary, complexes of narH and chrysH found to be less reducible than the other flavonol containing complexes. This can be interpreted by the different position of the coordinating –OH group in their structure.

4.3.3 Different substituents of the complexed flavonols and quinolones found to have much less effect on the redox properties of the Co(III) complexes than the type of the 4N ligand. E_{pc} values of the complexes were shifted to the more negative potential range by functional goups with electron withdrawing character mainly in case of flavonolato complexes.

4.3.4 Since E_{pc} values of tren analogues fell close to the potential range of cellular reductants these type of complexes can be suitable for biological approaches. Irreversible reduction processes of flavonol

and quinolone containing complexes make these compounds remarkable candidates for selective release of the bioligand in tumor tissues.

4.4 Some biological properties and antibacterial activity of several $[Co(4N)(O,O)]^{n+}$ type complexes containing quinolone and retrohydroxamic based ligands were explored.

4.4.1 Significant antibacterial activity of $[Co(tren)(cip)](ClO_4)_2$, $[Co(tpa)(cip)](ClO_4)_2$ and $[Co(tpa)(GSK)](PF_6)_2$ complexes was revealed on all the tested cell lines. The GSK complex showed higher activity than the free ligand against antibiotic-sensitive *S. aureus* bacteria and found to be more effective on resistant bacteria than the well known antibiotic ciprofloxacin.

4.4.2 In the case of ciprofloxacin containing complexes higher activity against the tested *E. coli* bacteria was determined for the tpa analogue over the tren complex. This can be connected to the higher DNA binding and topoisomerase II inhibiting ability of the tpa complex over the tren analogue.

4.5 Anticancer activity and some biological property of quinizarin and flavonol complexes were explored.

4.5.1 HSA-bindig capabilty of quinizarin compounds was revealed by spectrophotometric and spectrofluorimetric studies. Both $quinSH_2$ and $quinH_2$ ligands were found to bind to HSA, however, no interaction was detected between the protein and the studied complexes.

4.5.2 Anticancer activity of quinizarin containing complexes was found to be higher on all the tested cell lines than the used antitumor agent, carboplatin. Complexes remained active on cisplatin-resistant cell line too, in contrast with carboplatin. The tpa analogue showed higher efficacy over the tren analogue similarly as for the

ciprofloxacin complexes. This difference might be related to the more lipophilic character and DNA cleaving capability of the tpa complex being enhanced in the presence of reducing agents.

4.5.3 Anticancer activity of flavonol containing complexes were explored under both normoxic and hypoxic conditions. In good agreement with the previously mentioned results of the other type of complexes tpa complexes displayed higher activity than the tren analogues and found to be more effective than the corresponding free ligand. However, hypoxia-selectivity was observed in the case of two tren complexes. These results might be explained by the higher cell uptake values of the tpa complexes in connection to their more lipophilic character. However, the reduction potentials of the tpa complexes are probably too positive for hypoxia-selectivity.

V. POSSIBLE APPLICATIONS OF THE RESULTS

Thirty two new $[Co(4N)(O,O)]^{n+}$ type Co(III) complexes were synthesized during this research. Flavonols, quinizarines, quinolones and retrohydroxamic acid based molecules were selected as (O,O) type ligands and tren and tpa tripodal amines as 4N donor ligands. The products were characterized by NMR, IR, MS and elemental analysis techniques. Molecular structures of 3 ligands and 11 complexes were confirmed by single crystal analysis. Redox properties of the complexes were studied by cyclic voltammetric measurements and their biological properties and activity were also tested in the framework of domestic and international collaborations.

During my research several Co(III) complexes of bioactive drugs were synthesized and found to be as active as the free ligand or had even enhanced activity on the tested cell lines. Based on these results the use of the Co(III) complexes can be an option to overcome the resistance problem and to enhance the efficiency of drug molecules for both antitumor and antibacterial agents. The results indicate that both selectivity and high efficency together might be achieved by the use of proper ligands. The results of this study might provide support to the development of novel complexes for medical applications.

PUBLICATIONS

Articles related to the dissertations (4)

1. *Máté Kozsup*, Etelka Farkas, Attila Bényei, Jana Kasparkova, Hana Crlikova, Viktor Brabec, Péter Buglyó
   Synthesis, characterization and biological evaluation of Co(III) complexes with quinolone drugs
   J. Inorg. Biochem **193** (2019) 94-105
   IF: 3.212 (2019)

2. *Máté Kozsup*, Orsolya Dömötör, Sándor Nagy, Etelka Farkas, Éva A. Enyedy, Péter Buglyó
   Synthesis, characterization and albumin binding capabilities of quinizarin containing ternary cobalt(III) complexes
   J. Inorg. Biochem **204** (2020) 110963
   IF: 3.212 (2019)

3. Hana Crlikova, Hana Kostrhunova, Jitka Pracharova, *Máté Kozsup*, Sándor Nagy, Péter Buglyó, Viktor Brabec, Jana Kasparkova
   Antiproliferative, DNA binding and cleavage properties of dinuclear Co(III) complexes containing the bioactive quinizarin ligand
   J. Biol. Inorg. Chem. **25** (2020) 339-350
   IF: 3.246 (2019)

4. *Máté Kozsup*, Donal M. Keogan, Deidre Fitzgerald-Hughes, Éva A. Enyedy, Branden Twamley, Péter Buglyó, Darren Griffith
   Synthesis and characterization of Co(III) complexes of N-formylhydroxylamines and antibacterial activity of a Co(III) peptide deformmylase inhibitor complex
   Dalton Trans. **49** (2020) 6980 – 6988
   IF: 4.174 (2019)

Articles not related to the dissertations (2)

1. Péter Buglyó, Krisztina Lénárt, *Máté Kozsup,* Attila Bényei, Éva Kováts, Imre Sóvágó, Etelka Farkas
   $[Pd(en)(H_2O)_2]^{2+}$ and $[Pd(pic)(H_2O)_2]^{2+}$ complexation by monohydroxamic acids: A solution equilibrium and solid state approach
   Polyhedron, **100** (2015) 392-399
   IF: 1.926
2. Péter Buglyó, István Kacsir, *Máté Kozsup*, Imre Nagy, Sándor Nagy, Attila Bényei, Éva Kováts, Etelka Farkas
   Tuning the redox potentials of ternary Co(III) complexes containing various hydroxamates
   Inorg. Chim. Acta, **472** (2018) 234-242
   IF: 2.304

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Registry number: DEENK/367/2020.PL
Subject: PhD Publication List

Candidate: Máté Kozsup
Doctoral School: Doctoral School of Chemistry
MTMT ID: 10072622

List of publications related to the dissertation

Foreign language scientific articles in international journals (4)

1. Crlikova, H., Kostrhunova, H., Pracharova, J., **Kozsup, M.**, Nagy, S., Buglyó, P., Brabec, V., Kasparkova, J.: Antiproliferative, DNA binding, and cleavage properties of dinuclear Co(III) complexes containing the bioactive quinizarin ligand.
J. Biol. Inorg. Chem. 25 (2), 339-350, 2020. ISSN: 0949-8257.
DOI: http://dx.doi.org/10.1007/s00775-020-01765-4
IF: 3.246 (2019)

2. **Kozsup, M.**, Dömötör, O., Nagy, S., Farkas, E., Enyedy, É. A., Buglyó, P.: Synthesis, characterization and albumin binding capabilities of quinizarin containing ternary cobalt(III) complexes.
J. Inorg. Biochem. 204, 1-9, 2020. ISSN: 0162-0134.
DOI: http://dx.doi.org/10.1016/j.jinorgbio.2019.110963
IF: 3.212 (2019)

3. **Kozsup, M.**, Keogan, D. M., Fitzgerald-Hughes, D., Enyedy, É. A., Twamley, B., Buglyó, P., Griffith, D. M.: Synthesis and characterisation of Co(III) complexes of N-formyl hydroxylamines and antibacterial activity of a Co(III) peptide deformylase inhibitor complex.
Dalton Trans. 49 (21), 6980-6988, 2020. ISSN: 1477-9226.
DOI: http://dx.doi.org/10.1039/D0DT01123A
IF: 4.174 (2019)

4. **Kozsup, M.**, Farkas, E., Bényei, A., Kasparkova, J., Crlikova, H., Brabec, V., Buglyó, P.: Synthesis, characterization and biological evaluation of Co(III) complexes with quinolone drugs.
J. Inorg. Biochem. 193, 94-105, 2019. ISSN: 0162-0134.
DOI: http://dx.doi.org/10.1016/j.jinorgbio.2019.01.005
IF: 3.212

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List of other publications

Foreign language scientific articles in international journals (2)

5. Buglyó, P., Kacsir, I., **Kozsup, M.**, Nagy, I., Nagy, S., Bényei, A., Kováts, É., Farkas, E.: Tuning the redox potentials of ternary cobalt(III) complexes containing various hydroxamates. *Inorg. Chim. Acta. 472*, 234-242, 2018. ISSN: 0020-1693.
DOI: http://dx.doi.org/10.1016/j.ica.2017.07.026
IF: 2.433

6. Buglyó, P., Lénárt, K., **Kozsup, M.**, Bényei, A., Kováts, É., Sóvágó, I., Farkas, E.: [Pd(en)(H2O)2]2+ and [Pd(pic)(H2O)2]2+ complexation by monohydroxamic acids: a solution equilibrium and solid state approach.
Polyhedron. 100, 392-399, 2015. ISSN: 0277-5387.
DOI: http://dx.doi.org/10.1016/j.poly.2015.08.036
IF: 2.108

Total IF of journals (all publications): 18,385
Total IF of journals (publications related to the dissertation): 13,844

The Candidate's publication data submitted to the iDEa Tudóstér have been validated by DEENK on the basis of the Journal Citation Report (Impact Factor) database.

01 December, 2020

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