Short thesis for the degree of Doctor of Philosophy (PhD)

Coordination chemistry behaviour of various tau-transition metal complexes

by Bettina Diána Balogh

Supervisor: Prof. Dr. Katalin Várnagy

UNIVERSITY OF DEBRECEN

Doctoral School of Chemistry

Debrecen, 2023.

I. INTRODUCTION AND THE AIM OF THE WORK

Improving the quality of life of patients suffering from neurodegenerative disorders has become a major challenge for health and social care systems. Although, the molecular background of these diseases has yet to be elucidated, the abnormal changes and subsequent aggregation of certain proteins presumably play a crucial role in neuronal death. Amyloid β and tau proteins are associated with the development of Alzheimer's disease. These proteins contain several potential metal binding sites, such as the terminal amino group, histidyl and cysteinyl side chains. Great number of publications imply that the coordination of metal ions might contribute to the misfolding of proteins, hence they might play an important role in the development of neurodegeneration. These interactions can be modelled by such systems that contain a transition metal ion as central ion and oligopeptides as ligands. One of the research fields of the *Bioinorganic Research Group* at the *University of Debrecen* aims to gain a deeper understanding of the interactions between biogenic metal ions and proteins involved in neurodegenerative disorders. During the years of PhD, I was involved in the systematic studies focusing on the tau protein.

The aim of my work was to study the complex formation processes of various tau fragments containing histidyl and cysteinyl side chains, primarily in the presence of copper(II) and zinc(II) ions. As the characterization of nickel(II)-peptide complexes can contribute to understand the more complex coordination chemistry behaviour of copper(II)-peptide systems, the nickel(II) complexes of the ligands were also investigated.

*My aim was to study the complex formation processes of the **tau(326-333)** fragment and its mutants in order to gain information about the metal binding affinity of the neighbouring histidines derived from the R3 domain of the protein. I also planned to investigate the solution equilibrium properties of the **tau(320-333)** derivatives containing histidyl and cysteinyl side chains in order to provide a more realistic picture of the interaction between tau and zinc(II) ions. The comparison of the metal binding ability of anchoring groups derived from different chemical environments was also a goal of my research.*

In order to study the potential selectivity of different metal binding sites I intended to investigate the complex formation processes of the ***tau(30-34)(327-332)*** *chimeric peptide containing adjacent histidyl residues and the –TMH– sequence that proved to be a very efficient copper(II) binding moiety. Comparing the metal binding affinity of tau fragments with that of the* ***HAVAHHH-NH$_2$*** *peptide was also an aim of my work. Besides the three neighbouring imidazole rings, this ligand contains a terminal amino group and an N-terminal histidyl residue; and with the involvement of these donor groups stable "histamine-like" coordinated complexes can be formed.*
Furthermore, the study of the effect of the ***tau(326-333) KL*** *derivative on the aggregation of the Aβ(1-42) was also my objective.*

II. EXPERIMENTAL METHODS

Some of the studied ligands (Ac-GNIHHKAG-NH_2, Ac-GNGHHKPG-NH_2, Ac-GNGHAKPG-NH_2, Ac-GNGAHKPG-NH_2 and Ac-TMHQDNIHHKP-NH_2) were purchased from *SynPeptide Co. Ltd.* (China). Tau derivatives containing both histidyl and cysteinyl side chains (Ac-SKCGSLGNIHHKPG-NH_2 and Ac-SKCGSLGNIHHHKPG-NH_2) as well as the HAVAHHH-NH_2 ligand were synthetised at the *University of Debrecen*, while the native *tau(326-333)* fragment (Ac-GNIHHKPG-NH_2) and its derivative containing the –KLVFF– motif were prepared at CNR-IBB (Catania, Sicily). The ligands were synthetised by **solid phase peptide synthesis** using a microwave-assisted Liberty 1™ peptide synthesizer (*CEM*, Matthews, NC). Rink Amide AM resin was used as solid phase in order to obtain C-amidated peptides. During the synthesis of the ligands the Fmoc/*t*Bu method was used and the activation of the carboxyl group of the amino acids was carried out according to the TBTU/HOBt/DIPEA strategy. In order to obtain N-terminally protected peptides the free amino terminus was treated with acetic anhydride. The schematic structures of the investigated ligands are shown in *Figure 1*. The purity of the peptides was checked by high-performance liquid chromatography; the correct sequence was confirmed by ESI- or MALDI-MS experiments.

pH-potentiometric measurements were used to check the purity of the studied peptides and determine the protonation constants of the ligands, as well as the stability constants of the copper(II), nickel(II), zinc(II) and mixed metal complexes. The experiments were carried out in aqueous samples or 70% (V/V) DMSO-water mixture at 298 K and at constant ionic strength of 0.20 M KCl. The metal to ligand ratio varied between 1:3 and 3:1. Protonation constants of the peptides and stability constants of the metal complexes were calculated by means of general computational programs (PSEQUAD and SUPERQUAD). Based on the calculated values the species distribution diagrams of the corresponding systems were plotted using the MEDUSA program.

Various spectroscopic techniques were used in order to investigate the coordination environment around the metal ion.

UV-visible spectrophotometric studies were carried out to gain information about the quantity and quality of the donor groups coordinated to the metal ion. The measurements were carried out at

room temperature in wavelength ranges 200-900 and 200-400 nm. Absorption spectra were recorded at different pH values using a Perkin Elmer Lambda 25 double beam and a VWR UV-1600PC single beam scanning spectrophotometer and quartz cuvettes with 1.000 cm path length.

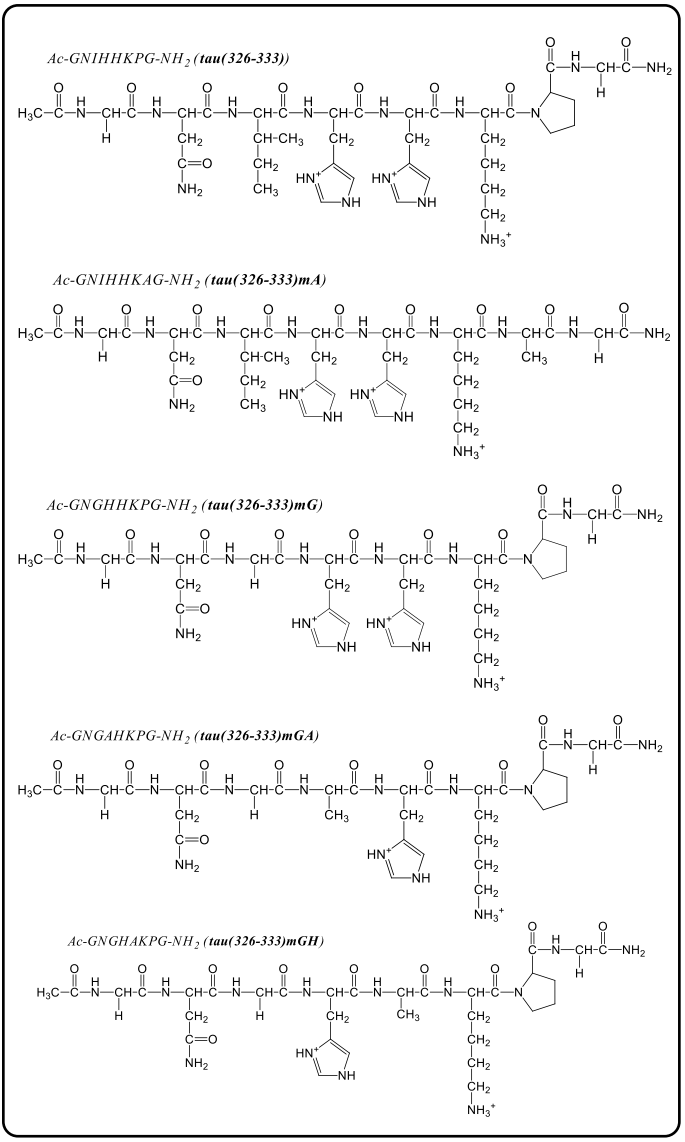
Circular dichroism spectroscopy is a widely used technique to study the structure of optically active copper(II)- and nickel(II)-peptide complexes. CD spectra usually give us more information than we can obtain from the UV-Vis absorption spectra as the line broadening of the electronic transitions are rather strait. CD spectroscopy is a useful experimental method to determine the primarily metal binding site and estimate the ratio of the coordination isomers. The CD spectra were recorded on a JASCO-810 spectropolarimeter in the wavelength range 220-800 nm using quartz cuvettes with 0.100 and 1.000 cm cell length. Experimental conditions (metal to ligand ratio, concentration, pH range, ionic strength) were similar to those applied for pH-potentiometric measurements.

Electron spin resonance spectroscopy was used to investigate the structure of paramagnetic copper(II) complexes as the hyperfine structure of the ESR spectra ($A_{\parallel}$ and $g_{\parallel}$) is sensitive to even slight structural changes of the complexes. The ESR studies were carried out at the University of Catania (*Università di Catania*) on a Bruker Elexsys E500 CW-ESR spectrometer at 150 K and different pH values. A small amount of methanol (up to 10%) was added to the samples in order to increase spectral resolution.

Mass spectrometry studies (ESI-MS and MALDI-MS) were used to identify the synthesized ligands and some of their metal complexes. Some of the mass spectra were recorded by *Dr. Gizella Csire* and *Dr. Lajos Nagy* on a Bruker MicroTOF-Q spectrometer (*Bruker Daltonik*, Bremen, Germany). The spectra of the copper(II) and zinc(II) complexes of the Ac-TMHQDNIHHKP-NH_2 ligand were registered by *Dr. Giuseppe di Natale* on a Q Exactive (Orbitrap) MS instrument (*ThermoFisher Scientific*) at CNR-IBB.

The kinetics of amyloid formation was followed by **Thioflavin T assay**. Experiments were carried out in Corning 96-well nonbinding surface plates. Time traces were recorded for 24 hours using a Varioskan plate reader (*ThermoFisher*, Waltham, MA) with λ_{ecc} = 440 nm and λ_{em} = 485 nm at 37 °C. All ThT curves represent the average

of three independent experiments. The experimental data were evaluated by *Michele F.M. Sciacca* and his co-workers.



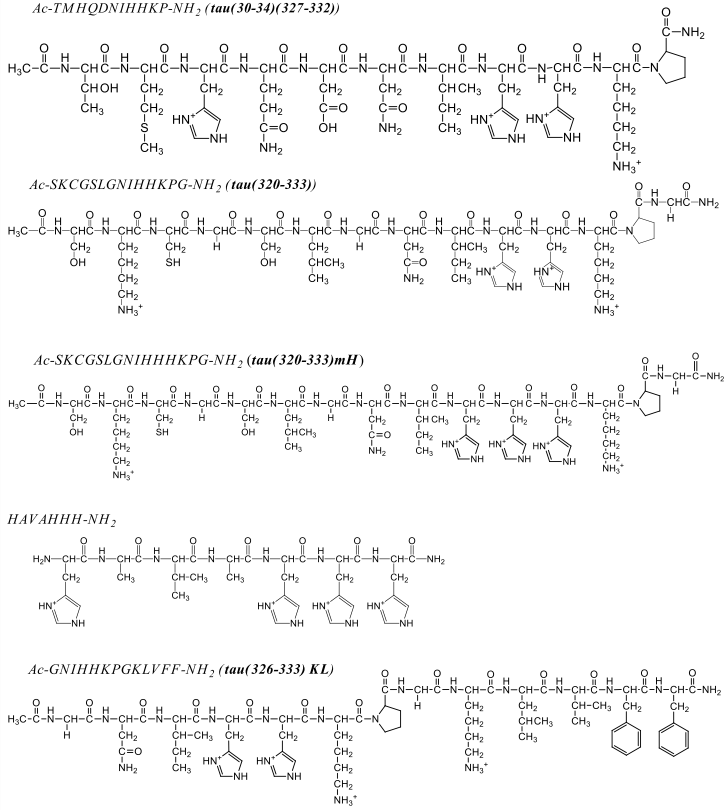


Figure 1. The structural formulae of the investigated ligands

III. NEW SCIENTIFIC ACHIEVEMENTS

3.1. The complex formation processes of the *tau(326-333)* ligand and its various mutants were investigated in the presence of copper(II), nickel(II) and zinc(II) ions. Copper(II) and nickel(II) ions are able to promote the deprotonation of peptide nitrogen atoms and coordination isomers are formed in alkaline samples. The presence of the prolyl residue does not prevent the amide coordination toward the C-termini, resulting in the formation of dinuclear complexes.

- In acidic samples various mononuclear complexes are formed and the three transition metal ions are coordinated by the histidyl side chain(s). In the presence of excess of ligand a bis(ligand) copper(II) species is formed in this pH range.
- The increase of pH results in the metal ion induced deprotonation and coordination of the amide functions, resulting in the formation of coordination isomers. Our studies revealed that either histidyl residue can be anchoring site for copper(II) binding and the two isomers are present in an almost equal ratio, while nickel(II) is predominantly bound by the His330 imidazole nitrogen at high pH.
- The zinc(II) complexes of *tau(326-333)* derivatives containing two histidyl residues do not undergo hydrolytic processes. ESI-MS studies suggest the formation of amide coordinated, mononuclear complexes in alkaline samples.
- Although the *tau(326-333)* derivatives are able to bind only one nickel(II) ion and only mononuclear zinc(II) complexes are formed, the studied ligands are able to coordinate two copper(II) ions. The proline in the peptide chain does not prevent the amide coordination toward the C-termini, however, it affects the stoichiometry of the complexes. The presence of the prolyl residue results in the deprotonation of only one amide group toward the C-termini as a maximum, while the absence of proline enables the coordination of three peptide nitrogen atoms to both copper(II) ions.

3.2. The solution equilibrium studies of the *tau(320-333)* derivatives were carried out in the presence of nickel(II) and zinc(II) ions. In the mononuclear complexes both metal ions are

predominantly bound by the thiolate moiety, while in the mixed metal complexes the nickel(II) ion is coordinated by the thiolate group and the zinc(II) ion is bound by one of the histidyl residues.

- Nickel(II) is coordinated by the side chain donor groups of the *tau(320-333)* derivatives in acidic and neutral samples. Above pH 7 amide coordinated species are formed in which the metal ion is predominantly bound by the thiolate moiety and the preceding deprotonated amide nitrogens (~ 70%). It suggests that the cysteinyl residue is the preferred binding site for nickel(II) even in the presence of two or three neighbouring histidines. At twofold metal ion excess one nickel(II) is bound by the –SKCG– part of the peptide and the other metal ion is coordinated by imidazole nitrogen(s) of the –HH(H)– moiety.
- In the presence of zinc(II) ions highly stable complexes are formed in which the metal ion is coordinated by S and N donor atoms. Amide nitrogens presumably cannot displace the thiolate function and with increasing pH hydrolytic processes take place instead of the deprotonation of peptide NH groups.
- Mixed nickel(II)/zinc(II) species are formed only in slightly alkaline solution. Below pH 8 mononuclear zinc(II) complexes are the dominant species which is another proof of their outstanding stability. In the mixed metal complexes the nickel(II) ion is bound by the –SKCG– sequence and the zinc(II) ion is coordinated by one of the histidyl residues and the neighbouring amide functions, even though in the mononuclear species the thiolate sulphur atom is the main anchoring site for zinc(II) coordination.

3.3. The complex formation processes of the *tau(30-34)(327-332)* chimeric peptide containing the His32 and adjacent His329-His330 residues were investigated in order to study the potential selectivity of the different metal binding sites. His32 proved to be the primary anchoring site for copper(II) even in mixed copper(II)/zinc(II) species, while the neighbouring imidazolyl residues serve as zinc(II) binding sites.

- In acidic samples of the equimolar copper(II)–***tau(30-34)(327-332)*** system the His330 histidine seems to have the highest affinity for copper(II) binding. The involvement of the peptide NH functions in the coordination of the metal ion probably changes the metal ion affinity of the binding sites around the histidyl moieties.

Thus, the His32 imidazole nitrogen becomes the main (~ 80%), albeit not exclusive, anchoring group for copper(II) coordination at physiological pH as well as in alkaline solution.

- In the presence of metal ion excess all imidazolyl groups become independent metal binding sites, resulting in the formation of di- and even trinuclear species, confirming the outstanding copper(II) binding ability of the studied ligand.
- In the physiological pH range presumably a tridentate imidazole-N coordinated zinc(II) complex is formed. The involvement of the carboxylate group of the aspartic acid side chain in the coordination of the metal ion might also contribute to the exceptional stability of this species. In alkaline solution the formation of amide coordinated zinc(II) complexes is assumed with one of the adjacent histidyl residues being the main anchoring site.
- The results obtained for the mixed copper(II)/zinc(II) complexes support that the N-terminal imidazole nitrogen atom is the main anchoring site for copper(II) coordination at physiological pH while the zinc(II) ions are accumulated at one of the adjacent histidyl residues if both metal ions are present in the solution (*Figure 2*).

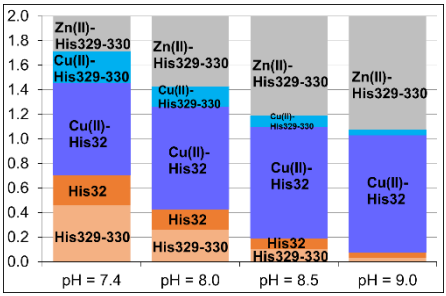


Figure 2. Distribution of Cu(II) and Zn(II) ions between His32 and His329-His330

3.4. The HAVAHHH-NH_2 peptide enabled us to investigate the effect of the "histamine-like" coordination on the complex formation processes. The N-terminal part of the ligand proved to be the primary binding site for all three studied transition metal ions in slightly acidic samples but the increase of pH resulted in the formation of coordination isomers of copper(II) and nickel(II) species.

- The "histamine-like" binding mode of the copper(II), nickel(II) and zinc(II) complexes formed in acidic media is presumably supported by the coordination of the C-terminal histidyl residues.
- Not even the combined binding of four or five donor groups can prevent the hydrolysis of zinc(II) ions in alkaline solution.
- In the presence of copper(II) and nickel(II) ions the increase of pH is accompanied by the metal ion induced deprotonation and coordination of amide functions.
- In the case of copper(II) the [NH_2, N^-, N^-, N^-] and [N^-, N^-, N^-, N(Im)] coordinated species are formed in an equal ratio, while the amino nitrogen is the preferred binding site for nickel(II).
- In the presence of metal ion excess dinuclear copper(II) and nickel(II) complexes are formed, however the pH range of their formation differs. In the copper(II)–nickel(II)–HAVAHHH-NH_2 ternary system not only mixed metal species but also numerous coordination isomers coexist.

3.5. The studied ligands enabled us to compare the metal binding ability of anchoring groups derived from different chemical environments. The "histamine-like" coordination of the metal ion proved to be the most preferred binding mode for copper(II) and nickel(II) in the physiological pH range, while in the case of zinc(II) the S,N donor atoms provided the most favourable coordination environment.

- Although thermodynamically stable nickel(II)- and zinc(II)-peptide complexes are formed, the copper(II) species are undoubtedly the most stable ones.
- The "histamine-like" coordination of the copper(II) and nickel(II) ions is presumably supported by the coordination of the neighbouring histidyl side chains.

- The –TMH– sequence provides a favourable coordination environment for copper(II) even in neutral samples (*Figure 3*). It might be due to the contribution of adjacent threonyl and/or methionyl residue(s). The sequence also proved to be the most efficient copper(II) binding motif in alkaline samples.

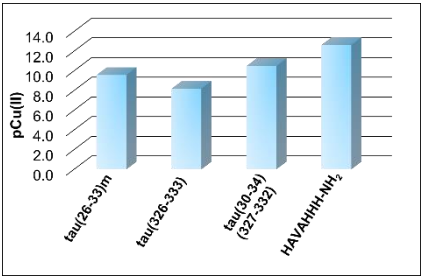


Figure 3. The pCu(II) values of the investigated ligands at pH = 7.4

- The outstanding copper(II) binding affinity of the –TMH– motif is further confirmed by the fact that the Ac-ATMHQD-NH_2 hexapeptide is able to displace the *tau(326-333)mA* from its metal complexes to form stable copper(II) species.
- The thiolate function of the cysteine provides the most favourable coordination environment for the amide coordinated nickel(II) complexes (*Figure 4/a*).
- Both the "histamine-like" binding mode and the coordination via cysteine side chain are preferred for zinc(II) at physiological pH, especially if it is supported by the coordination of other donor groups (e.g., histidine imidazole nitrogen atoms). In alkaline samples the [$(N^-)_x$, N(Im)] coordinated zinc(II) complexes are the dominant species (*Figure 4/b*).

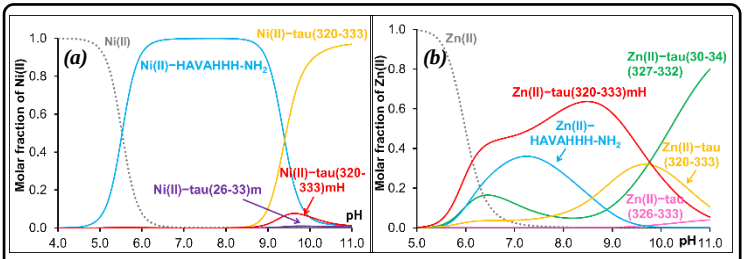


Figure 4. Distribution of Ni(II) *(a)* and Zn(II) ions *(b)* between the studied ligands

3.6. The effect of the *tau(326-333) KL* derivative on the aggregation of the *Aβ(1-42)* was investigated. We were also interested in the influence of the –KLVFF– sequence on the complex formation processes of the *tau(326-333)* peptide in the presence of copper(II) ions. We concluded that neither the presence of the pentapeptide motif, nor the different solvent had a significant effect on the structure of the complexes.

- Due to the presence of the hydrophobic –KLVFF– residue the solution equilibrium studies of the *tau(326-333) KL* ligand were carried out in 70% (V/V) DMSO-water mixture. Our results suggest that the same donor groups (histidine imidazole and deprotonated peptide nitrogen atoms) are involved in the complex formation regardless of the solvent and the presence of the additional pentapeptide motif.
- **The effect of the *tau(326-333) KL* derivative on the aggregate-on of the *Aβ(1-42)* was also investigated.** Even though the *tau(326-333) KL* ligand does not form amyloid aggregates in the explored 24 hours' time range, it also shows only limited anti-aggregation properties.

IV. POSSIBLE APPLICATION OF THE RESULTS

There are numerous unanswered questions related to the development of neurodegenerative disorders, including Alzheimer's disease. We do not understand completely the role of metal ions in the onset of AD and the main binding site of the transition metal cations of the tau has yet to be clarified. The results described in *Chapter 3* contribute to the area of basic research in the field of (bio)coordination chemistry. Understanding the interactions between the tau protein and copper(II) as well as zinc(II) ions might give us a deeper insight into the molecular background of the neurodegenerative disorders and provide sound scientific basis for future *in vivo* studies.

The high copper(II) binding affinity of the –TMH– sequence might be our most interesting finding from a practical point of view as the protein deposits of AD's brain are known to contain metal ions. Small molecules containing this motif might be sufficient copper(II) chelators. It is interesting to note that derivatives containing the aforementioned motif form significantly less stable zinc(II) complexes, indicating that small molecules containing the –TMH– sequence could meet the selectivity requirements of the chelators.

UNIVERSITY of DEBRECEN

UNIVERSITY AND NATIONAL LIBRARY
UNIVERSITY OF DEBRECEN
H-4002 Egyetem tér 1, Debrecen
Phone: +3652/410-443, email: publikaciok@lib.unideb.hu

Registry number: DEENK/443/2022.PL
Subject: PhD Publication List

Candidate: Bettina Diána Balogh
Doctoral School: Doctoral School of Chemistry
MTMT ID: 10069142

List of publications related to the dissertation

Foreign language scientific articles in international journals (3)

1. **Balogh, B. D.**, Szakács, B., Di Natale, G., Tabbi, G., Pappalardo, G., Sóvágó, I., Várnagy, K.: Copper (II) binding properties of an octapeptide fragment from the R3 region of tau protein: A combined potentiometric, spectroscopic and mass spectrometric study.
J. Inorg. Biochem. 217, 1-13, 2021. ISSN: 0162-0134.
DOI: http://dx.doi.org/10.1016/j.jinorgbio.2021.111358
IF: 4.336

2. **Balogh, B. D.**, Szunyog, G., Lukács, M., Szakács, B., Sóvágó, I., Várnagy, K.: Thermodynamics and structural characterization of the nickel(II) and zinc(II) complexes of various peptide fragments of tau protein.
Dalton Trans. 50 (40), 14411-14420, 2021. ISSN: 1477-9226.
DOI: http://dx.doi.org/10.1039/D1DT02324A
IF: 4.569

3. **Balogh, B. D.**, Bihari, Z., Buglyó, P., Csire, G., Kerekes, Z., Lukács, M., Sóvágó, I., Várnagy, K.: Metal binding selectivity of an N-terminally free multihistidine peptide HAVAHHH-NH.
New J. Chem. 43 (2), 907-916, 2019. ISSN: 1144-0546.
DOI: http://dx.doi.org/10.1039/C8NJ04538K
IF: 3.288

DEBRECENI EGYETEM * Egyetemi és Nemzeti Könyvtár *

UNIVERSITY of DEBRECEN

UNIVERSITY AND NATIONAL LIBRARY
UNIVERSITY OF DEBRECEN
H-4002 Egyetem tér 1, Debrecen
Phone: +3652/410-443, email: publikaciok@lib.unideb.hu

List of other publications

Foreign language scientific articles in international journals (1)

4. Székely, E., Csire, G., **Balogh, B. D.**, Erdei, J. Z., Király, J. M., Kocsi, J., Pinkóczy, J., Várnagy, K.: The Role of Side Chains in the Fine-Tuning of the Metal-Binding Ability of Multihistidine Peptides.
Molecules. 27, 1-29, 2022. ISSN: 1420-3049.
DOI: http://dx.doi.org/10.3390/molecules27113435
IF: 4.927 (2021)

Total IF of journals (all publications): 17,12
Total IF of journals (publications related to the dissertation): 12,193

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.

13 October, 2022