Coordination Chemistry Reviews 255 (2011) 1824–1833

Contents lists available at ScienceDirect

Coordination Chemistry Reviews

journal homepage: www.elsevier.com/locate/ccr

Review

Tetrazole peptides as copper(II) ion chelators

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Contents

1. Introduction 1824
2. Formation of metallopeptides. Peptides as chelating agents for metal ions 1825
3. Tetrazole peptide chelators 1825
   3.1. Copper(II) complexes with the tetrazole analogue of tetraalanine 1825
   3.2. Influence of the 1,5-disubstituted tetrazole ring on the chelating ability of enkephalins 1827
      3.2.1. Tyr-Gly$\Psi(CN_4)$Gly-Phe-Leu 1827
      3.2.2. Tyr-Gly(D-Ala)-Gly$\Psi(CN_4)$Phe-Leu 1829
      3.2.3. Tyr-D-Ala-Gly-Phe-Leu$\Psi(CN_4)CH_3$ 1830
   3.3. Influence of the 1,5-disubstituted tetrazole ring on the chelating ability of β-casomorphin-7 1830
      3.3.1. Biological studies *in vitro* 1832
4. Concluding remarks 1832
   Acknowledgements 1833
   References 1833

ARTICLE INFO

Article history:
Received 25 November 2010
Accepted 27 February 2011
Available online 26 March 2011

Keywords:
Tetrazole peptides
Enkephalins
Casomorphins
Copper complexes

ABSTRACT

The 1,5-disubstituted tetrazole ring a mimetic of the *cis*-amide bond is an unique element modifying the ability of peptides to chelate copper(II) ions. The position of the tetrazole ring system in the peptide backbone plays a critical role in the stabilization of the metallopeptide molecule. The insertion of a tetrazole between amide groups leads to enhancing the stability of the complex and to obtaining a very effective peptide chelating agent. These findings can provide important information for modeling biologically relevant peptide–metal binding sites. Some aspects of biological activity of tetrazole modified exogenous opioid peptides in the presence of copper(II) ions are also presented in this review.

1. Introduction

Research into complex systems containing metallopeptides is particularly useful for the rational design of coiled-coil protein structures [1–6]. Metallopeptide systems are unique and can effect the biological activity of peptides. Studies on the influence of peptide sequences and peptide bond modification on the structure and thermodynamic stability of metallopeptides are very important to understanding both the biological role of peptides and the influence of metal ions on protein conformation. Moreover, data obtained on the thermodynamic stability of metallopeptides can lead to the recognition of some peptides as ligands competitive to other ligands present in human body fluids.

This review centers on endogenous and exogenous opioid peptides. These peptides play a significant role in the regulation of physiological processes not only in the brain, but also in almost all tissues. They can act as hormones, neuromodulators and neurotransmitters [7–12]. Their proper functioning depends on many factors, and in particular on the presence of copper ions [13]. Copper is a trace element in the human body but plays a fundamental role in the biochemical processes occurring in the nervous system [14]. Therefore, the interactions between copper(II) ions and opioid peptides or their analogues were the focus of the reported work. The aim of reviewed studies was to investigate the coordinating effects of a new class of peptide chelators, tetrazole opioid peptide analogues [15–22], on copper(II) ions. The 1,5-disubstituted tetrazole ring is a *cis* amide bond surrogate [23–26]. Enkephalins and β-casomorphin-7 were subjected to tetrazole modification. These peptides exhibit the same receptor activity as morphine, and hence they could be useful as potential natural analgesics [27,28]. The tetrazole ring affects both the conformation of the pep-

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doi:10.1016/j.ccr.2011.02.023

tides and their interactions with opiate receptors [29]. Therefore, the influence of the *cis* -amide bond surrogate in metallopeptides was investigated from the chemical and biological points of view. The studied compounds were tetraalanine **(1)**, [Leu^5]enkephalin ([Leu^5]EK) (**2–5**), and β-casomorphin-7-amide (**6–8)** analogues:

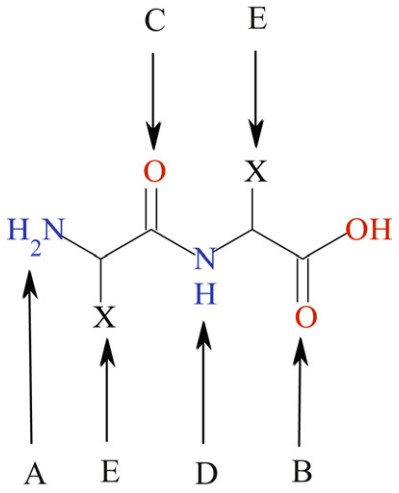
1. Ala-AlaΨ(CN_4)Ala-Ala
2. Tyr-GlyΨ(CN_4)Gly-Phe-Leu
3. Tyr-Gly-GlyΨ(CN_4)Phe-Leu
4. Tyr-D-Ala-GlyΨ(CN_4)Phe-Leu
5. Tyr-D-Ala-Gly-Phe-LeuΨ(CN_4)CH_3
6. Tyr-ProΨ(CN_4)Phe-Pro-Gly-Pro-Ile-NH_2
7. Tyr-Pro-PheΨ(CN_4)Ala-Gly-Pro-Ile-NH_2
8. Tyr-Pro-Phe-ProΨ(CN_4) Gly-Pro-Ile-NH_2

The examination of coordination equilibria in aqueous solution containing copper ions and peptide ligands was performed by using potentiometric measurements combined with spectroscopic techniques: electronic absorption (UV–vis), circular dichroism (CD) and electron paramagnetic resonance (EPR). Potentiometry enables the detection of different metallopeptide species formed in the solution as well as the determination of their thermodynamic stability and species distribution relative to solution pH. Spectroscopic data can be used to identify the type and number of peptide donor atoms involved in metal ion coordination. Thanks to the above methods, it has been possible to define the metal binding ability of tetrazole analogues of peptides. The most interesting problems to solve were: (i) how does the tetrazole ring, as a *cis*-amide bond surrogate, affect the formation and thermodynamic stability of complex species at different pH ranges? and (ii) what is the competitive copper binding efficiency of tetrazole peptides as compared to other opioid oligopeptides? In order to obtain the most accurate pattern of tetrazole functioning in metallopeptides, the authors investigated the chelating abilities of systems containing endogenous enkephalin Tyr-Gly-Gly-Phe-Leu [19], exogenous α-caseins Arg-Tyr-Leu-Gly-Tyr-Leu (casein 90–95) [15] and Arg-Tyr-Leu-Gly-Tyr-Leu-Glu (casein 90–96) [16], and β-casomorphins: bovine Tyr-Pro-Phe-Pro-Gly-Pro-Ile and human Tyr-Pro-Phe-Val-Glu-Pro-Ile [17,18].

2. Formation of metallopeptides. Peptides as chelating agents for metal ions

Peptides are effective and specific ligands for metal ions [30–33]. In the peptide molecule there are several potential coordinating sites (Scheme 1).

The most important donor center is the N-terminal amine nitrogen atom (A). The amine group is usually primary, although it can be secondary, as in the case of proline. The oxygen donor atoms occur in all peptides, the C-terminal carboxylate group (B) being the most effective. Carbonyl oxygen donors of the peptide bond (C) can also participate in metal ion coordination. Copper(II) and platinum metals promote the hydrogen ionization of the peptide bond (D) and form very stable N^-–metal bonding. Amino acid side chains can also contain different donor centers (E). The most important ones include histidine imidazole [34–40], cysteine sulfhydryl, aspartic carboxylate [41] and tyrosine phenolate [42]. The hydroxylate groups of serine or threonine, the amide carbonyl group of asparagine or the lysine amine side chain are seldom involved in metal coordination. The stability of the metallopeptide molecule depends not only on the interaction of the donor atoms directly involved in metal binding but also on the hydrophobic action of non-coordinating amino acid side chains [33]. Aliphatic or aromatic chains form a spatial shield preventing the hydrolysis of the N^-–metal bond and increasing the structural strength of the complex molecule [43]. Peptide conformation has a substantial impact on coordinating equilibria in the metal–peptide system both in thermodynamic and structural terms. Oligoglycine and oligoalanine are examples of simple peptides without side chains. In these systems, Cu(II) coordination starts from the N-terminal nitrogen atom, which is regarded as the "anchoring" site for the metal [30–33,44]. The adjacent carbonyl oxygen is the second donor atom to close the chelating ring. The deprotonation of consecutive amide nitrogen atoms in the presence of metal ions in a medium with increasing pH leads to the formation of successive N^-–Cu(II) bonds and eventually to the production of the $CuH_{-3}L$ complex. In this metallopeptide species four nitrogen atoms are bonded to the Cu(II) ion in three five-membered chelating rings (Fig. 1).



Scheme 1. Potential donor atoms in peptide molecule.

The involvement of amide nitrogen atoms in copper(II) coordination was also confirmed in the solid phase of complexes the di-, tri-, tetra- and pentaglycine [45–49]. The formation of the chelating ring by consecutive nitrogen donor atoms is a driving force for the coordination process leading to a decrease in the p*K* value of the first amide group by up to 10 log units as compared with the free peptide ligand [30,31,44]. In the case of Cu(II) peptide complexes, the deprotonation of the adjacent amide groups are usually well separated from each other. This indicates that the binding process occurs in a non-cooperative manner.

3. Tetrazole peptide chelators

3.1. Copper(II) complexes with the tetrazole analogue of tetraalanine

The tetrazole ring incorporated into the peptide backbone chain mimics the *cis*-amide bond conformation, i.e. the α-carbons of the amino acid residues connected by the tetrazole are placed at the same side of the C–N bond (Scheme 2).

Such a modification dramatically changes the ability of tetraalanine to coordinate to Cu(II) ions [22]. The incorporation of the 1,5-disubstituted tetrazole ring results in the absence of the consecutive amide nitrogen in copper bonding, just as in proline-containing peptides. In Pro peptides, the second proline nitrogen atom in the peptide backbone is unable to undergo ionization and participate in the formation of the $N^- \rightarrow$ Cu(II) bond. Therefore, it acts as a "break-point" in metal coordination [50–53]. The characteristic feature of the tetrazole moiety distinguishing it from the Pro residue is the presence of N(4) nitrogen atom as a potential donor center for a metal ion (Scheme 2). Potentiometric and spectroscopic results [22] revealed that tetrazole incorporation into the Ala^2-Ala^3

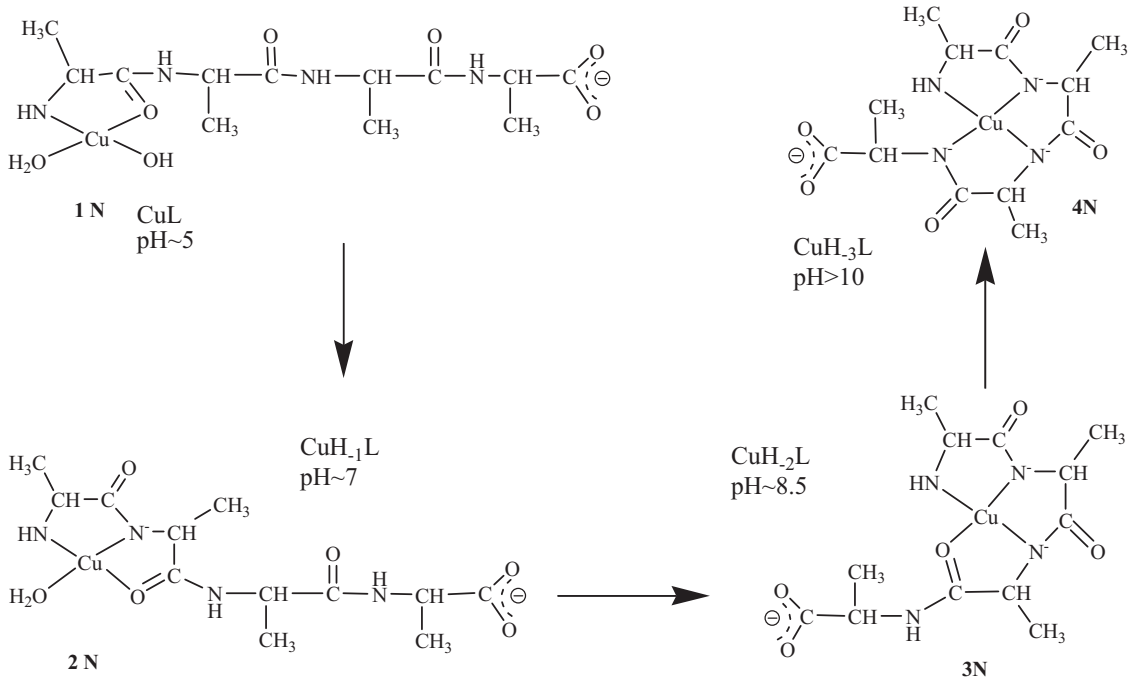
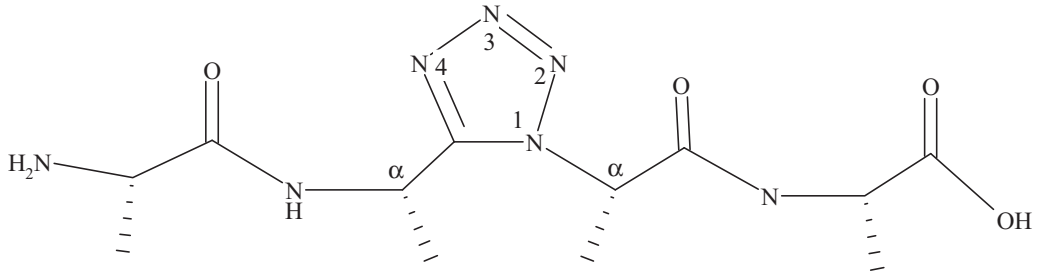


Fig. 1. Metallopeptide species formation in Cu(II)–tetraalanine system at different pH.



Scheme 2. Ala-AlaΨ(CN_4)Ala-Ala.

position of tetraalanine significantly raises the binding efficiency of Cu(II) ions at physiological pH region. This is reflected in increased complex stability constants as compared to other peptide ligands (Table 1).

The pH distribution curves of metallopeptides indicate that the complexing process starts at pH 4, which is one unit lower than in the Cu(II)–tetraalanine system. The first identified CuL complex exists in a higher excess than that observed for the Ala-Ala-Ala-Ala system [22]. This means that the tetrazole peptide analogue binds copper(II) ions in the low pH range more efficiently than the parent peptide (Fig. 2).

Spectroscopic data made it possible to determine the ligand donor atoms coordinating to metal ion [22]. The d–d electron transition bands observed in UV–vis and CD spectra are typical of the involvement of one nitrogen donor (1N) and three nitrogen donors (3N) in copper binding (Fig. 3). The EPR parameters confirm these coordination modes as well [22].

Table 1
Stability constants (as log β) of Cu(II) complexes in the tetrazole analogue of tetraalanine and several oligopeptide systems (T = 298 K, I = 0.1 mol dm^3 (KNO_3)).

Peptide	log β					Refs
	CuHL	CuL	$CuH_{-1}L$	$CuH_{-2}L$	$CuH_{-3}L$	
Ala-AlaΨ(CN_4)Ala-Ala	–	5.85	0.41	−7.98	–	[22]
Ala-Ala-Ala-Ala	–	4.77	−0.45	−8.09	−17.33	[54]
Gly-Gly-Gly-Gly	–	5.08	−0.42	−7.31	−16.60	[52]
Gly-Gly-Pro-Gly	–	4.95	−0.06	−9.46	−19.47	[52]

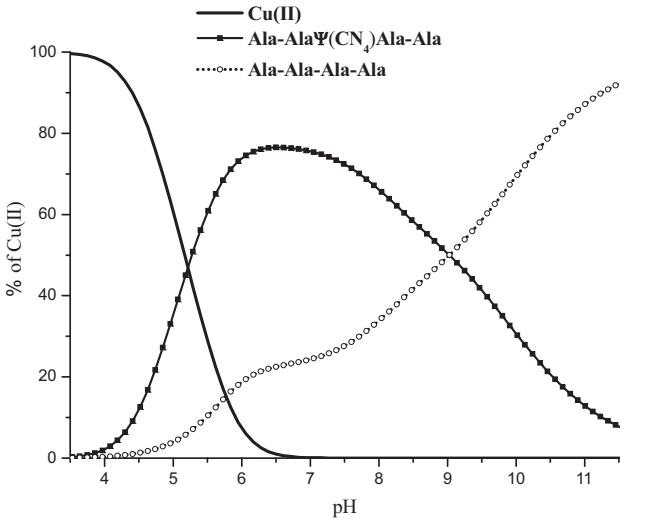


Fig. 2. Comparison of Cu(II) binding abilities of tetraalanine and its tetrazole analogue. Metal-to-ligands molar ratio 1:1:1; $c_{Cu(II)} = 10^{-3}$ M.

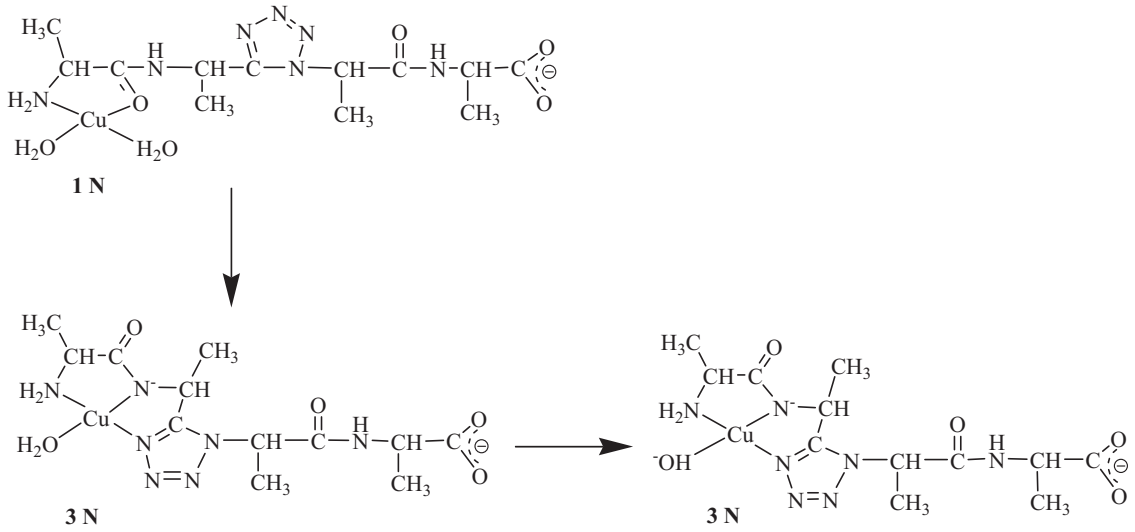


Fig. 3. The proposed scheme of consecutive metallopeptide species formation in Cu(II)–tetrazole tetraalanine system.

The presence of the tetrazole ring imposes a bent peptide structure (Fig. 4), which favors simultaneous coordination of two nitrogen atoms N^- and N_{tetr} in the $CuH_{-1}L$ species.

The CD spectrum in the visible range assigned to the complex splits into three bands, while only one band is observed for the 3N Cu(II)–AAAA complex (Fig. 5b).

The d–d splitting transition band indicates a distortion of the square-planar complex geometry. Such an effect was also observed in other oligopeptide Cu(II) complexes [55,56]. $CuH_{-2}L$ is formed in the alkaline pH range. In this complex the metal ion is still bounded to three nitrogen donors $\{NH_2, N^-, N_{tetr}\}$. Its formation probably results from deprotonation of the water molecule, which is suggested by the fact that the reaction $CuH_{-1}L \leftrightarrows CuH_{-2}L + H^+$ has a pK value of 8.28. The spectral parameters still indicate the 3N coordination mode although the rigidity of the metallopeptide molecule may be higher than that in $CuH_{-1}L$, as suggested by $\Delta\varepsilon$ values (Fig. 5a). Such structure rigidity may arise from intramolecule ligand interactions between the N- and C-terminal groups [22,55,56]. To summarize, one can conclude that the insertion of the 1,5-disubstituted tetrazole ring into the tetraalanine sequence leads to very efficient peptide chelator of copper(II) ions (Fig. 2). The distinguishing factor of the complexes is the formation of stable five-membered chelating rings with the three nitrogen atoms coordinated around Cu(II). The tetrazole ring imposes on the peptide molecule a specific bent conformation. This leads to: (i) cooperative deprotonation of the amide nitrogen and a simultaneous coordination of the tetrazole ring nitrogen and (ii) the distortion of the tetragonal geometry around the metal ion.

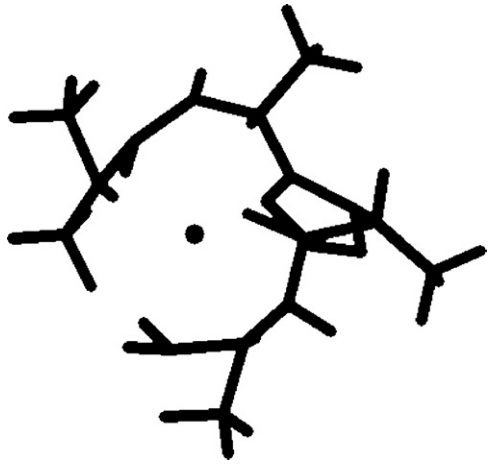


Fig. 4. The proposed spatial model of metallopeptide Cu(II)-Ala-Ala$\Psi(CN_4)$Ala-Ala obtained by minimization energy method.
Adapted from [22].

3.2. Influence of the 1,5-disubstituted tetrazole ring on the chelating ability of enkephalins

3.2.1. Tyr-Gly$\Psi(CN_4)$Gly-Phe-Leu

Enkephalins are a group of neuropeptides known as opioid peptides or endogenous opioids. Enkephalins are synthesized in neurons and stored in synapses inside the nerve terminals. The mechanism of their action can be affected by metal ion interactions. The enkephalin concentration in blood serum is related to copper(II) levels in the food intake. Decreased supply of Cu(II) ions leads to lower enkephalin levels in the blood serum is, whereas increased Cu(II) supply cancels the above enkephalin effect [57]. These results suggest that both the synthesis and the release of enkephalins depend on copper [58]. Opioid peptides can coordinate copper ions [59–61]. Cu(II) may hold the peptide chains in a biologically active bent conformation (β-turn) and influence the specificity and affinity of peptides to opiate binding sites [59]. Studies of several biologically active enkephalin analogues aimed to show how structural modification could affect the ability of the peptides to coordinate Cu(II) ions. Stable complexes are formed with three or four nitrogen atoms coordinating in the pH range of 7–9. Significant stabilization was found for the 3N species in [Leu^5]EK derivatives [61]. This enhanced stability was attributed to the metal ion-induced conformational organization of the peptide molecule, involving a β-turn, depending on the nature of the fifth amino acid residue.

The replacement of the –CONH group by the 1,5-disubstituted tetrazole ring in the enkephalin backbone leads to a significant change in the coordinating properties of thus modified peptides. The amide bond is of great importance in metallopeptides especially give that Cu(II) ions are capable of deprotonating the nitrogen atom and coordinating to it. The insertion of the tetrazole ring in [Leu^5]EK between Gly^2 and Gly^3 significantly modifies the chelating ability of the enkephalin [19]. The coordination mode of copper(II) ion changes with pH, the species forming at low pH is CuHL (Fig. 6).

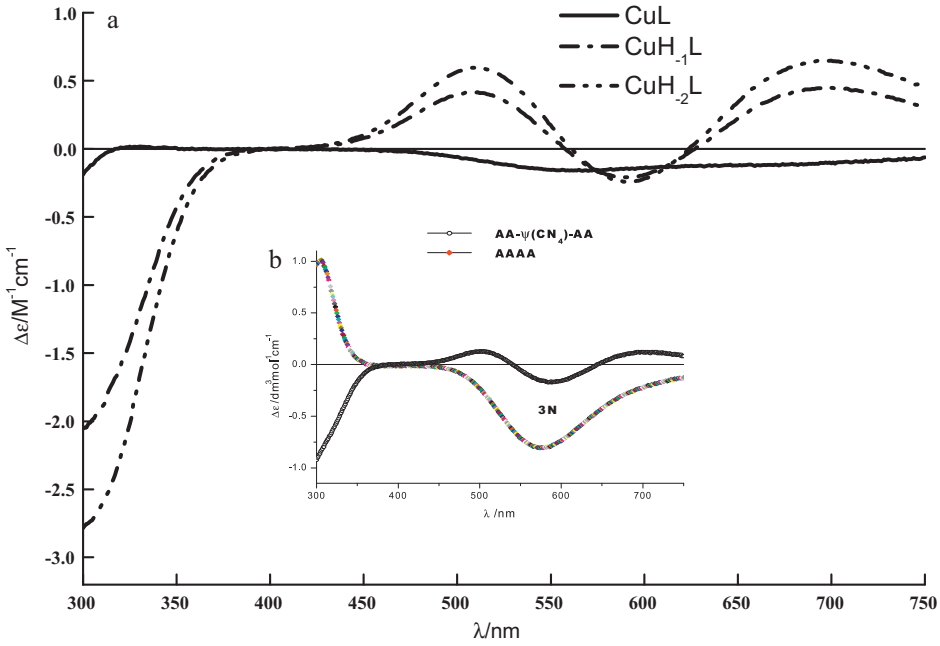


Fig. 5. CD spectra in visible region for: (a) for Cu(II)–AAΨ(CN$_4$)AA and (b) for 3N coordination mode in the parent and tetrazole analogue peptides. Adapted from [22].

It is a minor species with the metal coordinated to the N-terminal amino group and the nearby carbonyl groups and is usually observed in the early steps of Cu(II) complexation by peptides [32,62]. One can see from species distribution (Fig. 6) that the major form of the metallopeptide at pH 6–7 is the CuL complex. The structure of the species is totally different from that found in the other studied enkephalins [60,61]. Comparative analysis of spectral data reveals the involvement of three nitrogen atoms in copper binding [19]. The energy of d–d transitions observed both in absorption electronic and visible ranges of CD spectra at λ_{max} = 558 nm as well as the presence of c.t. transitions NH_2–Cu(II) and N^-_{amid}–Cu(II) at λ_{max} = 301 nm and 311 nm, respectively in the CD spectrum unequivocally indicate the 3N {NH_2, N^-, N_{tetr}} coordination mode (Fig. 7).

Corrected stability constant values (log K) for the selected 3N complexes (see Table 2) show a significant thermodynamic stability of the CuL form of the Tyr-GlyΨ(CN$_4$)Gly-Phe-Leu peptide.

The gain in CuL stabilization results from a higher affinity of the ligand to Cu(II) ions (as compared to unmodified enkephalins) due to the tetrazole ring insertion. Its presence in the peptide backbone favors deprotonation of adjacent amides in a cooperative manner and transformation of the 1N coordination mode into the 3N mode with a simultaneous formation of two very stable five-membered

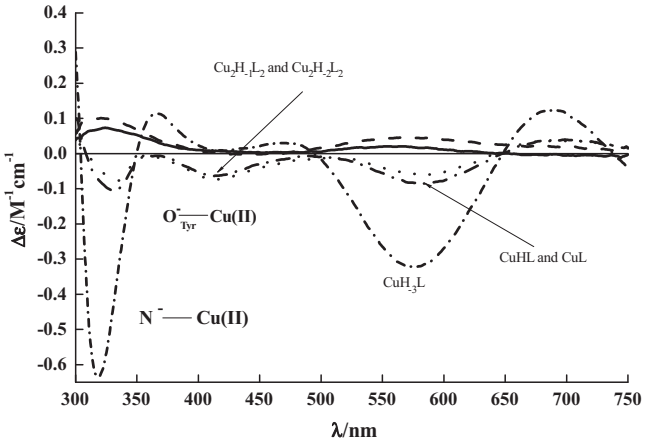


Fig. 7. CD spectra for Cu(II)-Tyr-GlyΨ(CN$_4$)Gly-Phe-Leu system. Metal-to-ligand molar ratio: 1/1; $c_{Cu(II)} = 1 \times 10^{-3}$ M. Adapted from [19].

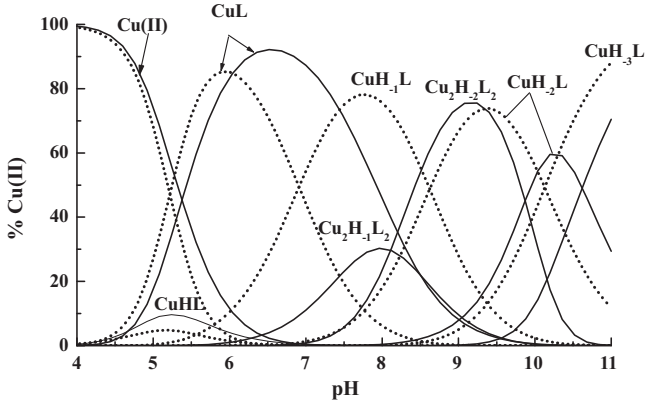


Fig. 6. Species distribution diagrams for (- - - - -) Tyr-Gly-Gly-Phe-Leu and (—) Tyr-GlyΨ(CN$_4$)Gly-Phe-Leu. Metal-to-ligand molar ratio: 1/1; $c_{Cu(II)} = 10^{-3}$ M. Adapted from [19].

Table 2
Values of corrected stability formation constants (log K) for the selected 3N and 4N complexes (log β from [19]).

Peptide	log K	
	3N	4N
Tyr-Gly-Gly-Phe-Leu	−7.55	−16.21
Tyr-D-Ala-Gly-Phe-Leu	−7.21	−15.43
Tyr-Gly-Ψ(CN$_4$)-Gly-Phe-Leu	−0.25	–
Tyr-Gly-Gly-Ψ(CN$_4$)-Phe-Leu	–	−6.30
Tyr-D-Ala-Gly-Ψ(CN$_4$)-Phe-Leu	–	−6.25
Tyr-D-Ala-Gly-Phe-LeuΨ(CN$_4$)CH$_3$	−7.28	−15.46

log K = log β_{CuH_jL} − log β_{HL} (K-corrected stability formation constant; log β_{HL}-protonation constant of Tyr phenolate group).

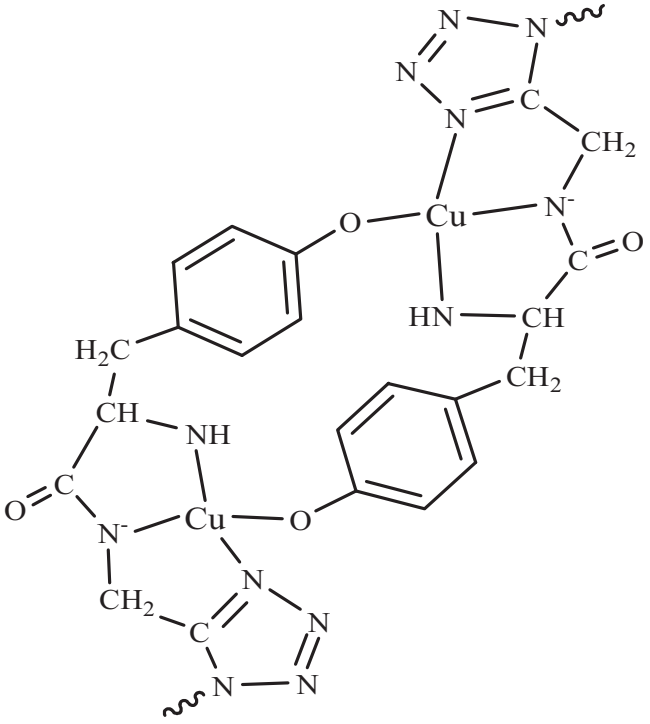


Fig. 8. The proposed structure of $Cu_2H_{-2}L_2$ species, L = Tyr-GlyΨ(CN_4)Gly-Phe-Leu.

chelating rings (Fig. 3). At alkaline pH the copper binding mode changes dramatically and strongly depends on the metal to ligand ratio (M/L). A ligand excess leads to the formation of CuL_2, $CuH_{-1}L_2$ and $CuH_{-2}L_2$ species, where the peptide molecule binds Cu(II) ions through nitrogen donor atoms of the $-NH_2$ amine group and the adjacent amide $-N^-$ groups [19]. $CuH_{-1}L_2$ and $CuH_{-2}L_2$ complexes can originate from CuL_2 as a result of the dissociation of one or two phenolate groups. It is clearly seen in the deprotonation constant values of pK = 9.74 and 10.60 during the formation of the complexes $CuL_2 \leftrightarrows CuH_{-1}L_2 + H^+$ and $CuH_{-1}L_2 \leftrightarrows CuH_{-2}L_2 + H^+$, respectively, as these values are close to that of the free ligand (pK_{Tyr} = 9.98). In the case of $CuH_{-2}L_2$, deprotonation of the water molecule cannot be excluded.

On the other hand, in an equimolar system (M/L = 1) at alkaline pH a new charge transfer band appears at λ_{max} = 400 nm in electronic absorption and CD spectra (Fig. 7). This band is characteristic of O^-_{Tyr}–Cu(II) charge-transfer transition and indicates the involvement of the Tyr phenolate group in metal coordination [39,51]. A species distribution diagram obtained from potentiometric titration shows the predominance of the metallopeptide dimeric form in the pH range of 8–9 (Fig. 6). In the dinuclear species the most likely donor sets are {(NH_2, N^-, N_{tetr})}, {(NH_2, N^-, N_{tetr}, O^-_{Tyr})}$_2$ for $Cu_2H_{-1}L_2$ and {(NH_2, N^-, N_{tetr}, O^-_{Tyr})}$_2$ for $Cu_2H_{-2}L_2$ (Fig. 8).

At above pH 9 two monomeric species are formed: $CuH_{-2}L$ and $CuH_{-3}L$. The significant increase in the intensity of the $N^- \rightarrow$ Cu(II) charge transfer band (~320 nm) seen in the CD spectrum (Fig. 7) suggests that in both monomeric complexes additional amide nitrogen atoms are involved in metal coordination. The same binding pattern was observed for proline containing peptides, in which the Pro residue in the peptide sequence acts as a break-point [63–65]. The N_{tetr} donor is weakly basic and thus sensitive to hydrolysis at high pH. Therefore, it is likely that at above pH 9 the Cu(II)–N_{tetr} bond is replaced by the C-terminal donor set {N^-, COO^-} of Leu residue resulting in the {NH_2, N^-, N^-, COO^-} coordination mode, as observed in the proline containing peptides [64,65]. This unusual coordination mode may lead to a distinct decrease in the geometric symmetry around the metal ion, which

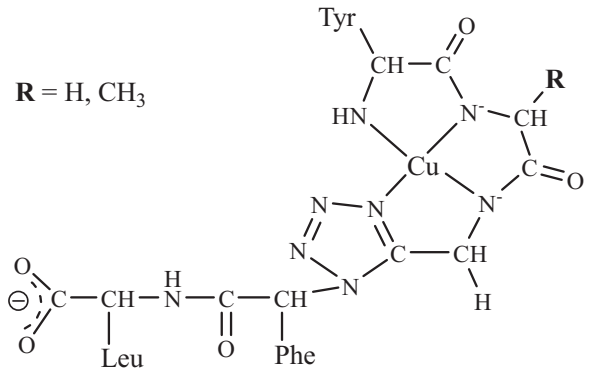


Fig. 9. Coordination mode in the 4N complex of Tyr-Gly(D-Ala)-GlyΨ(CN_4)Phe-Leu.

results in the splitting of the d–d band observed in the CD spectra (Fig. 7).

3.2.2. *Tyr-Gly(D-Ala)-GlyΨ(CN_4)Phe-Leu*

The position of the tetrazole moiety characterized by the $Gly^3\Psi(CN_4)Phe^4$ sequence makes the enkephalin analogue exceptionally efficient in coordinating Cu(II) ions [19]. Copper binding at pH 6 proceeds in a cooperative manner with the involvement of two amide nitrogen atoms. The position of the tetrazole enables to the simultaneous formation of three five-membered chelating rings (Fig. 9).

The fitting of the titration curves indicates that the dominant form is the $CuH_{-1}L$ complex, while CuL is present to a very small extent and the CuHL is below detection limit (Fig. 10).

Tetrazole ligands coordinate copper ions *via* an equatorial four nitrogen donor atom set {NH_2, N^-, N^-, N_{tetr}}. Spectral parameters support this type of coordination mode [19]. The specific arrangement of 4N around the Cu(II) ion favors very high stability of this $CuH_{-1}L$ metallopeptide form at pH ranging from 5 to 10. At above pH 10 the hydrolysis of the N_{tetr}–Cu(II) bond occurs and the $CuH_{-3}L$ is formed with the 3N coordination mode. In the latter complex, the weakly basic tetrazole nitrogen donor is substituted by the OH^- group. The thermodynamic stability of the $CuH_{-1}L$ species is very high as compared to the 4N species of the parent peptide (see pK Table 2). This means that modified enkephalins with the 1,5-disubstituted tetrazole ring inserted between the Gly^3 and Phe^4 sequence are efficient peptide chelators for copper(II) ions. The comparison of Cu(II) sequestration by [Leu]enkephalin and its

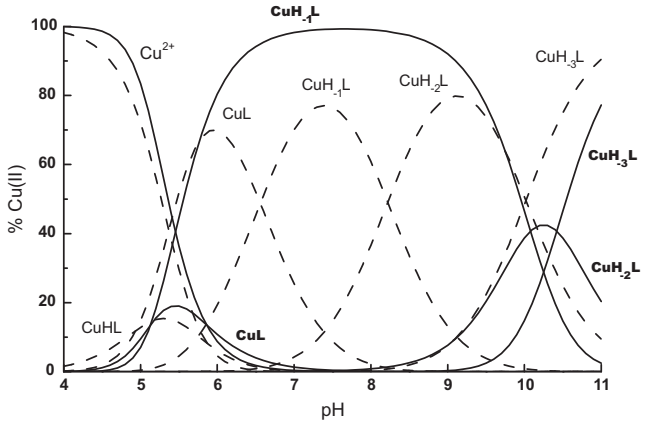


Fig. 10. Species distribution diagrams for (- - - - -)Tyr-D-Ala-Gly-Phe-Leu and (—) Tyr-D-Ala-GlyΨ(CN_4)Phe-Leu systems. Metal concentration $c_{Cu(II)} = 1 \times 10^{-3}$ M; metal to ligand ratio 1:1.
Adapted from [19].

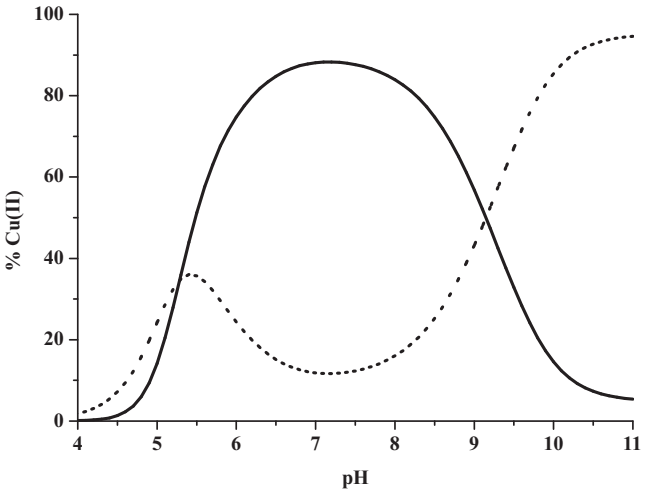


Fig. 11. Comparison of binding ability of ((- - - - -)Tyr-D-Ala-Gly-Phe-Leu) and (—) its tetrazole analog. Metal-to-ligands molar ratio 1:1.
Adapted from [19].

tetrazole analogue is the best illustration of the strong chelating ability of tetrazole peptides (Fig. 11).

One can argue that under physiological pH conditions tetrazole modified enkephalin could be a very competitive ligand for copper binding in biological fluids containing other bioligands.

3.2.3. Tyr-D-Ala-Gly-Phe-LeuΨ(CN$_4$)CH$_3$

The tetrazole ring incorporated at the C-terminal of the Leu residue in Tyr-D-Ala-Gly-Phe-LeuΨ(CN$_4$)CH$_3$ is not involved in copper(II) binding and actually has no effect on the thermodynamic stability or the type of complexes as compared to the parent peptide (Table 2). Such behavior of the tetrazole moiety could be explained by steric hindrance.

3.3. Influence of the 1,5-disubstituted tetrazole ring on the chelating ability of β-casomorphin-7

Peptides with opioid activity are found in partial enzymatic digests of proteins derived from foodstuffs [8]. β-Casomorphins (also called β-casein exorphins) are a family of exogenous opioid peptides originally isolated from an enzymatic digest of bovine β-casein. They differ from endogenous opioids in the Pro-Phe sequence following the Tyr N-terminus. β-Casomorphins display activity for specific receptor binding sites. In particular, C-terminal amidation of β-casomorphins enhances both their analgesic potency and receptor affinity [28]. Studies were performed on several β-casomorphin-7-amide analogues (Scheme 3) in order to find a correlation between their biological activity and complexing properties towards Cu(II) ions [21]. Generally, they revealed diversified biological activity [66].

β-Casomorphin-7 is a heptapeptide with a very special sequence which influences not only its biological activity but also its chelating effect on copper ions [17,18]. The normal mode of coordination achieved in regular opiate-like peptides [15,16] is broken because Pro residues do not possess an ionizable peptide proton [67]. However, the presence of Pro residues promotes very stable dimeric species involving the Tyr side-chain phenolic donor. The incorporated tetrazole ring, as a mimetic of the *cis* peptide bond leads to a local restriction of molecular flexibility and favors the β-turn conformation of the peptide [26]. Modification of β-casomorphin-7-amide was accomplished by inserting the tetrazole ring at a different site in the peptide backbone. Hence, investigation of the properties of tetrazole β-casomorphin-7-amide analogues

Table 3
Stability constants ($\log K_{CuHL}$) of Cu(II) complexes with: (**a**)YPFPGPI-NH$_2$, (**b**) YPΨ(CN$_4$)FPGPI-NH$_2$, (**c**) YPFΨ(CN$_4$)AGPI-NH$_2$ and (**d**) YPFPΨ(CN$_4$)GPI-NH$_2$ (at T = 298 K and I = 0.10 mol dm^{-3} (KNO$_3$)) ($\log \beta$ from [21]).

Peptide	$\log K_{CuHL}$ [a]
Tyr-Pro-Phe-Pro-Gly-Pro-Ile-NH$_2$ – **a**	5.11
Tyr-ProΨ(CN$_4$)Phe-Pro-Gly-Pro-Ile-NH$_2$ – **b**	4.40
Tyr-Pro-PheΨ(CN$_4$)Ala-Gly-Pro-Ile-NH$_2$ – **c**	5.60
Tyr-Pro-Phe-ProΨ(CN$_4$)Gly-Pro-Ile-NH$_2$ – **d**	5.04

[a] $\log K_{CuHL} = \log \beta_{CuHL} - \log \beta_{HL}$.

were primarily expected to explain how the position of the tetrazole ring can influence the type and stability of the complex species formed in the studied systems. The results of potentiometric and spectroscopic (UV–vis, CD and EPR) measurements have shown that in all peptide systems only monomeric species are formed in the pH range of 4–6 [21].

Spectroscopic parameters suggested in these species a coordination mode with the participation of the N-terminal amine and the adjacent carbonyl groups {NH$_2$, CO}. The comparison of $\log K$ values for the equilibrium Cu(II) + HL ⇌ CuHL of the tetrazole analogues and the parent peptide systems indicates that the lowest $\log K_{CuHL}$ is in the Tyr-ProΨ(CN$_4$)Phe-Pro-Gly-Pro-Ile-NH$_2$ system (Table 3).

This most probably results from the location of –Ψ(CN$_4$)– between Pro^2 and Phe^3, which significantly disfavors the coordination of the {NH$_2$, CO} donor set because of the steric hindrance and/or hydrophobic effects and leads to the destabilization of the complex. On the other hand, a tetrazole ring located in the central part of the ligand molecule (between Phe^3 and Ala^4 or Pro^4 and Gly^5) increases CuHL stability. This is particularly well seen in the case of analogue **c** (see Table 3).

At above pH 6 the peptides exhibit a different trend in forming dimeric species. Analogue **d** binds copper ions most effectively at neutral pH, as the Cu$_2$L$_2$ complex. The mass fraction of the dimeric forms is much lower in subsequent analogues [21]. Spectroscopic parameters indicate the 2× {NH$_2$, CO, O$^-$} donor set in this type of complexes, where each copper(II) ion is bound to the (NH$_2$, CO) end of one ligand and to the phenolate group of the other ligand. The Cu$_2$H$_{-2}$L$_2$ dimer formed in the Cu(II)-Tyr-ProΨ(CN$_4$)Phe-Pro-Gly-Pro-Ile-NH$_2$ system can possess another donor atom set [21]. In this case, the oligonuclear nature of the species is substantiated by a significant loss in the intensity of EPR signals consistent with a strong magnetic interaction between the metal ions. However phenolate coordination is not indicated by any charge transfer transition in the absorption spectrum. Most likely, the oligonuclear arrangement results from hydroxo bridging, *e.g.* units with {NH$_2$, CO} coordination are bridged by couples of OH$^-$ ions (Fig. 12).

Similar μ-hydroxo species are formed by peptides containing Pro at the second position [20]. Indeed, the Pro residue acts as a break-point in the sequence of coordination because the non-ionizable amide group is not a metal binding site. Therefore, unless stable macrochelate complexes are formed, hydrolytic species are favored [20].

Analogue **c** appeared to be the most efficient Cu(II) chelator at neutral pH. Potentiometric and spectroscopic data revealed the

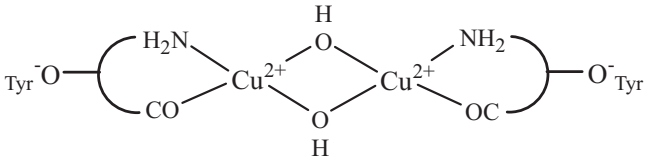
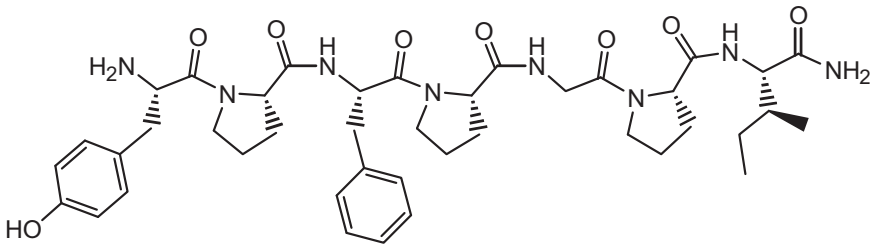
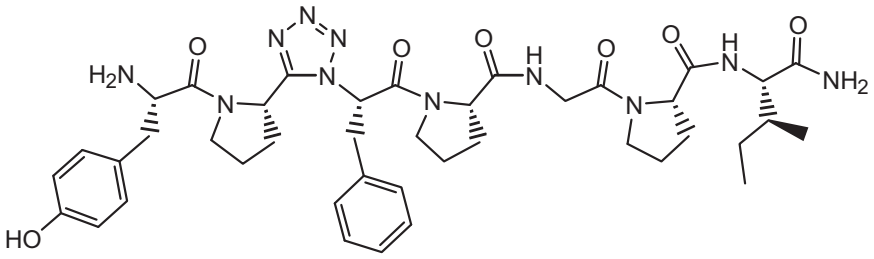


Fig. 12. The proposed donor set in the Cu$_2$H$_{-2}$L$_2$ of Tyr-ProΨ(CN$_4$)Phe-Pro-Gly-Pro-Ile-NH$_2$.

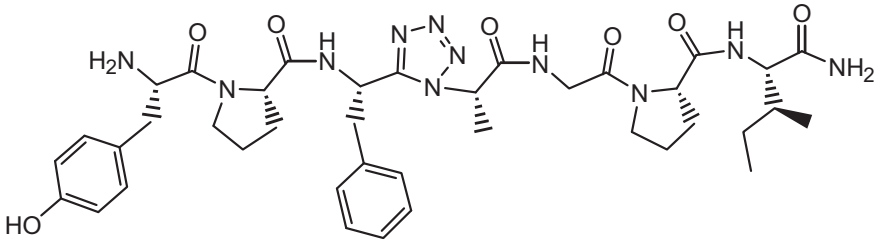
YPFPGPI-NH_2 -a



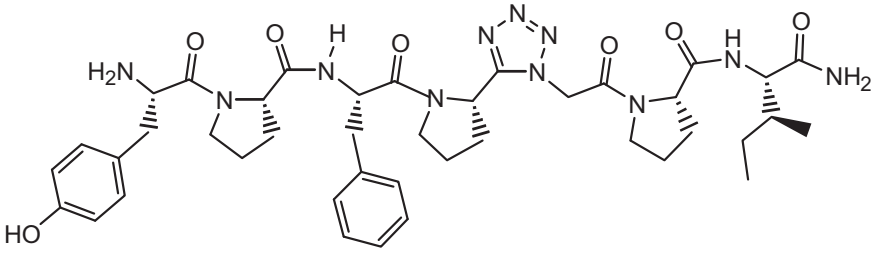
YP-Ψ(CN_4)-FPGPI-NH_2 -b



YPF-Ψ(CN_4)-AGPI-NH_2 -c



YPFP-Ψ(CN_4)-GPI-NH_2 -d



Scheme 3. Scheme of β-casomorphin-7-amide tetrazole analogues.

formation of $CuH_{-1}L$ and $CuH_{-2}L$ monomeric forms at this pH. Copper(II) is coordinated through the amino N of the N-terminus and the peptide N of the Phe residue. The coordination of the amide bond is clearly seen in the CD spectra, where the N^-–Cu(II) charge transfer (c.t.) band at 325 nm is observed [21]. The predominance of the $CuH_{-1}L$ monomeric species is substantiated by EPR spectra. However, the magnetic parameters are significantly different from those of the five-membered {NH_2, N^-} Cu(II) complexes of simple oligoglycines [68], and the higher $g_{\|}$ and lower $A_{\|}$ values indicate that the larger ring results in a considerable distortion. A simulation of secondary structures obtained with HyperChem reveals that the [–Ψ(CN_4)–] ring can lock the peptide backbone in a folded conformation that can bring close together the terminal amine and Phe amide nitrogen atoms and thus enable effective coordination to copper(II) [21]. This suggests that the Ψ(CN_4) moiety following the third amino acid is very favorable to a large (eight-membered) chelate ring spanning Tyr to Phe. The simulation also shows that the carbonyl oxygen adjacent to the amino group is in a position suitable for a long contact to the copper ion. Therefore, the site could impose distorted coordination to copper(II), resulting in unusual EPR parameters.

The results obtained for the complexes of this peptide series enable the estimation of the significant influence of the position of the tetrazole moiety in the peptide backbone on the complex stability of β-casomorphin analogues. The insertion of the tetrazole unit into position 3–4 (analogue **c**) has the biggest effect on the stability of the 2N complex, which is the dominating species at neutral pH. The tetrazole ring in position 2–3 (analogue **b**) or 4–5 (analogue **d**) at the β-casomorphin-NH_2 chain has a smaller impact on the stability of the complexes, although peptide **d** shows a tendency

Table 4
Histamine release from rat mast cells induced by tetrazole casomorphin analogues and/or Cu^{2+} ions.

Agent(s)	Concentration (M)	Histamine release (%) ± SEM
(**b**) Tyr-ProΨ(CN_4)Phe-Pro-Gly-Pro-Ile-NH_2	10^{-4}	0
	10^{-5}	0
	10^{-6}	0
(**b**) Tyr-ProΨ(CN_4)Phe-Pro-Gly-Pro-Ile-NH_2 z Cu(II)	10^{-4}:10^{-4}	23.3 ± 3.3
	10^{-5}:10^{-5}	19.0 ± 4.8
(**d**) Tyr-Pro-Phe-ProΨ(CN_4)Gly-Pro-Ile-NH_2	10^{-4}	12.7 ± 3.4
	10^{-5}	7.9 ± 1.9
	10^{-6}	5.0 ± 1.1
(**d**) Tyr-Pro-Phe-ProΨ(CN_4)Gly-Pro-Ile-NH_2 z Cu(II)	10^{-4}:10^{-4}	18.0 ± 3.9
	10^{-5}:10^{-5}	2.3 ± 0.7

Adapted from [21].

to coordinate metal ions in the dimeric species Cu_2L_2 at physiological pH values. The binding ability diagram reveals that the most effective Cu(II) binding tetrazole ligand is analogue **c** (Fig. 13).

It seems that the effectiveness of this ligand derives from its particular conformation, which is suitable for copper(II) 2N coordination at neutral pH. However, this conformational effect does not prevent the complexes from a hydrolytic attack by the bulk solution. The differences in the secondary structure of the studied peptides are also reflected in their biological activity.

3.3.1. *Biological studies in vitro*

Neuroendocrine peptide hormones regulate a number of important immune functions. These peptides influence the biology of different cells which are involved in of immunological reaction mechanisms [69–71]. Mast cells play a very important role in many immune processes [72–74]. These cells are distributed throughout connective tissues [72,73]. Taking into account these data, the action of selected β-casomorphin-7-amide analogues and their complexes on mast cells has been evaluated [21]. Experiments were done on rat peritoneal mast cells, which are commonly used in immunopharmacological studies [75]. Tetrazole derivative **b** does not activate rat mast cells to release histamine while derivative **d** evokes marked histamine release up to 12.7 ± 3.4%. Uncoordinated Cu(II) ions also activate histamine release but only in the presence of Ca^{2+} ions. Data collected in Table 4 show that the coordination of Cu(II) ions to the studied peptides might change histamine release by mast cells quite considerably.

In view of all the above results, one can conclude that the bent conformation resulting from Cu(II) coordination via macrochelate ring formation may be essential for the ability of tetrazole casomorphins to bind to opiate receptors.

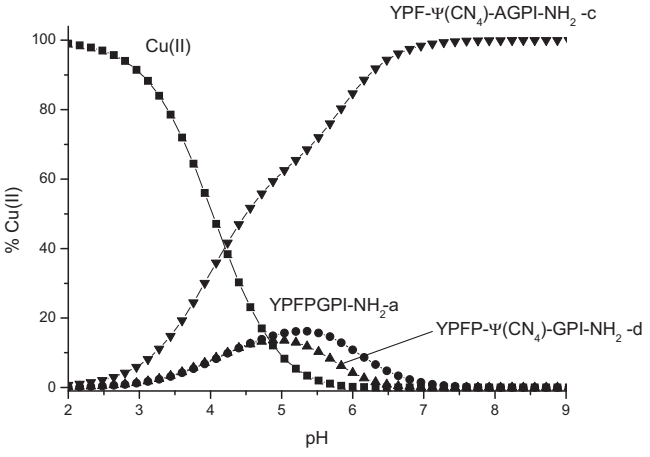


Fig. 13. Comparison of Cu(II) binding abilities of β-casomorphin tetrazole analogues.

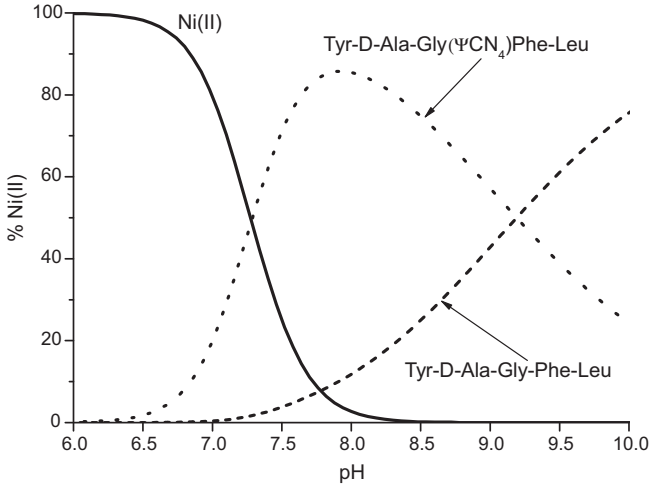


Fig. 14. Comparison of Ni(II) binding abilities of Tyr-D-Ala-Gly-Phe-Leu and its tetrazole analog. Metal-to-ligands molar ratio 1:1:1; $c_{Ni(II)} = 1 \times 10^{-3}$ M.
Adapted from [76].

4. Concluding remarks

The results indicate that the 1,5-disubstituted tetrazole ring has a crucial influence on the chelating ability of peptide ligands. Its incorporation into the peptide backbone chain significantly increases the metal binding efficiency of the ligand. Substantial Cu(II) sequestration by tetrazole peptide analogues at physiological pH, is clearly evident in the system of Tyr-Gly(D-Ala)-GlyΨ(CN_4)Phe-Leu. In the case of other metal ions, *e.g.* nickel or palladium, ligating sites should be generally the same as for copper(II) ion and characterized by a slower formation kinetics. A study undertaken on Ni(II)-Tyr-D-Ala-GlyΨ(CN_4)Phe-Leu system revealed that the interaction of nickel(II) with this peptide results in the formation of yellow colored (maximum transition band at λ_{max} = 401 nm), square-planar, diamagnetic species, [$NiH_{-1/-2}L$] [76]. The comparison of Ni(II) sequestration by [Leu]enkephalin and its tetrazole analogue illustrates stronger chelating ability of tetrazole peptide (Fig. 14). The coordination similarity one can expect also for palladium(II) ions as they reveal the same tendency to bind to the nitrogen donor atoms as Cu(II) and Ni(II) ions at neutral and alkaline pH [77].

The studies can contribute to the design of new complexing agents with high metal ion selectivity under biological conditions. The tetrazole ring imposes a bent conformation on the peptide molecules, making metallopeptide systems structurally very spe-

cific. This may result in metal-assisted modulation of biological activity, *e.g.* when neuropeptides interact with their receptors.

Acknowledgements

The author would like to express her gratitude to Prof. Dr Janusz Zabrocki and his research team for the synthesis of tetrazole peptides.

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