

Chelating ability of proctolin tetrazole analogue*

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The aim of the investigation was to establish the chelating ability of a new proctolin analogue of the sequence Arg-Tyr-LeuΨ[CN₄]Ala-Thr towards copper(II) ions. The insertion of the tetrazole moiety into the peptide sequence has considerably changed the coordination ability of the ligand. Potentiometric and spectroscopic (UV-Vis, CD, EPR) results indicate that the incorporation of 1,5-disubstituted tetrazole ring favours the formation of a stable complex form of CuH₂L. This 4N coordination type complex is the dominant species in the physiological pH range. The tetrazole moiety provides one of these nitrogens. The data indicate that Cu(II) ions are strongly trapped inside the peptide backbone. These findings suggest that Cu(II) can hold peptide chains in a bent conformation. This bent conformation may be essential for bioactivity of the tetrazole peptides.

Keywords: metal complexes, metalloptides, proctolin, tetrazole peptide analogue

Proctolin (Arg-Tyr-Leu-Pro-Thr) was the first neuropeptide isolated from insects (Brown & Starratt, 1975). This neuropeptide has been detected in various neuronal cell types of insects (Bishop *et al.*, 1981; O'Shea & Adams, 1986; Orchard *et al.*, 1989) and identified in neurones with a widespread distribution within the central nervous system of arthropods (Eckert *et al.*, 1981). In order to determine structure-activity relationships for the myotropic activity of proctolin in insects, many modifications of the peptide have been made (Konopińska *et al.*, 1988a; 1988b; Kuczer *et al.*, 1996; Konopińska & Rosiński, 1999; Woźnica *et al.*, 2004). We have previously described analogues of insect kinins, another class of insect neuropeptides, that retain very significant diuretic activity, modified with a 1,5-disubstituted tetrazole ring (Ψ[CN₄]) (Fig. 1), which preferentially forms the type VI β-turn (Zabrocki *et al.*, 1988; Nachman *et al.*, 2002; 2004). In this study, we have

prepared a tetrazole analogue of proctolin. Proctolin was modified at position 3–4 by replacing the Leu-Pro dipeptide unit with the tetrazole dipeptide LeuΨ[CN₄]Ala. The interaction of Cu(II) with proctolin may have physiological relevance because copper content is especially high in synaptosomal fluids which are rich in neuropeptides (Linder & Goode, 1991). Studies on the Cu(II)-exorphin systems have shown that these exogenous opiate-like peptides are efficient chelating agents (Chruscinska *et al.*, 1997; 1998; Łodyga-Chruscinska *et al.*, 1998; 1999). Moreover, insertion of the tetrazole ring can effectively stabilize the metallopeptide structure (Łodyga-Chruscinska *et al.*, 1999; 2000; 2004; Chruscinska *et al.*, 2001). It has been found that tetrazole ring can be directly involved in the Cu(II) binding and then very stable complex species with 3N or 4N co-ordination mode are formed at physiological pH. The complex formation is the result of simultaneous formation of

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Abbreviations: CD, circular dichroism; Dab, α,γ-diaminobutyric acid; EPR, electron paramagnetic resonance; HPLC, high performance liquid chromatography; UV-Vis, electron absorption spectroscopy.

five-membered chelate rings by consecutive nitrogen donors. The unusual feature found for the Cu(II)-tetrazole enkephalin analogue system has been the cooperative transition from a 2N to a 4N complex (Łodyga-Chruścińska *et al.*, 1999). The position of the tetrazole ring system in the peptide backbone plays a critical role in the metalloprotein molecule stabilization. The insertion of the tetrazole between amide groups leads to enhanced complex stability and results in a very effective peptide chelating agent. These previous studies led us to investigate copper(II) complexes of proctolin (Scheme 1a) and its tetrazole analogue (Scheme 1b) in order to evaluate the factors governing their chelating ability. The copper(II) coordination to several proctolin analogues modified in the fifth position of the peptide chain have been studied (Kowalik-Jankowska *et al.*, 2005). The presence of a proline residue in the fourth position of the proctolin analogues (RYLPP, RYLPI) led to the existence of the CuL and CuH₂L complexes with 2N {NH₂, N⁻, CO} and 3N {NH₂, 2N⁻, CO} coordination modes, respectively, over a wide pH range. The amine group of the Dab residue of the RYLP-Dab proctolin analogue was coordinated to copper(II) ions, and a 3N {NH₂, N⁻, CO, NH₂Dab} complex was found. In this paper, we report the results of combined potentiometric and spectroscopic (UV-Vis, CD and EPR) studies on the copper(II) complexes of proctolin and its tetrazole

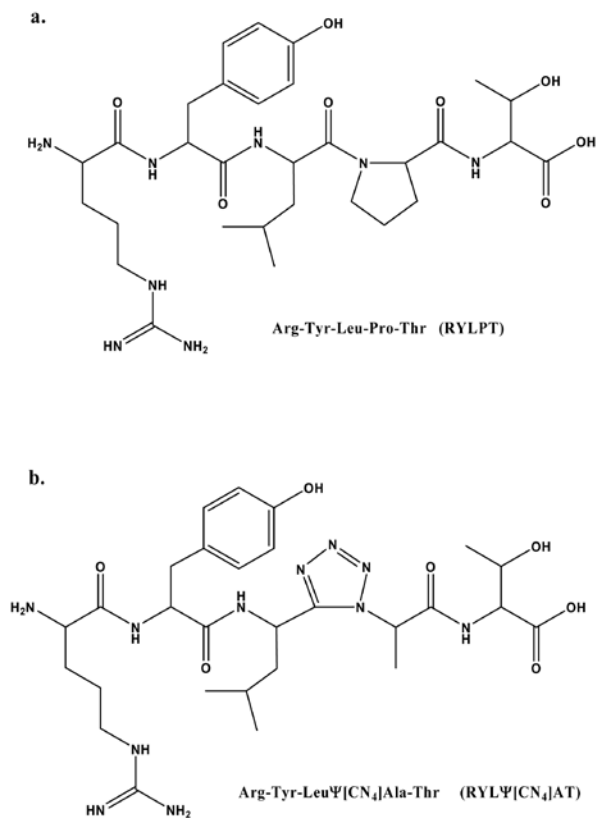
analogue. The results suggest that the presence of the tetrazole ring enhances the metal binding ability of the peptide.

MATERIALS AND METHODS

Reagents. The tetrazole proctolin analogue (RYLΨ[CN₄]AT) was synthesized according to a previously reported procedure (Zabrocki *et al.*, 1988). Their purity was verified by HPLC, mass spectrometry and potentiometry to be >99%. Proctolin (RYLPT) was purchased from Bachem and used without purification, Cu(NO₃)₂, KNO₃, HNO₃ and NaOH were Merck products and were used without further purification.

Potentiometric studies. Protonation and coordination equilibria were investigated by potentiometric titration in aqueous solution, over the pH 3–11 range, at a constant ionic strength using 0.1 M KNO₃ and at constant temperature (298 K) under argon atmosphere with a total volume of 1.5–2 cm³ of the sample. A 0.05 M solution of Cu(NO₃)₂ was used as the stock for the Cu(II) ion. An automatic titration set including autoburette meter (Molspin Ltd., Newcastle-upon-Tyne, UK), a semi-microcombined electrode (Russell CMAWL/S7) and an IBM-compatible PC were used to collect data. Alkali, about 0.1 M NaOH, free of CO₂ was added with a 0.250 cm³ micrometer syringe, which was calibrated by both weight titration and titration of standard materials. The electrode was calibrated for hydrogen ion activity. The relationship between activity and concentration was calculated daily by titration with HNO₃ (Irving *et al.*, 1967). Calculations were made with the aid of the SUPERQUAD computer program (Gans *et al.*, 1985). This allows the refinement of total ligand concentrations. Therefore we were able to confirm the purity of the peptide, in particular the absence of acetate, a frequent impurity in peptide samples or of other coordinating ions. In all cases triplicate titrations (about 500 experimental points in one set of measurements) were carried out at the Cu/L ratio of 1:1. The ligand concentration was 1 × 10⁻³ M. As usual, the stabilities of the metal complexes are reported as logarithms of the overall formation constants β_{pqr} = [M_pH_qL_r]/[M]^p[H]^q[L]^r, where M stands for the metal ion, H is proton and L the deprotonated form of the ligand (Table 1). The standard deviations quoted were computed by SUPERQUAD and refer to random errors only. They are, however, a good indication of the importance of a particular species in the equilibrium.

Spectroscopic studies. UV-Vis spectra were recorded with a Perkin-Elmer Lambda 11 spectrophotometer. Circular dichroism spectra were obtained with a Jobin-Yvon CD-6 dichrograph over the



Scheme 1. Proctolin (a) and its tetrazole analogue (b).

range 200–750 nm, using 1 and 0.05 cm cuvettes. The spectra are expressed as $\Delta\varepsilon = \varepsilon_l - \varepsilon_r$, where ε_l and ε_r are the molar absorption coefficients for left and right circularly polarized light, respectively. Electron paramagnetic resonance measurements were carried out with a Varian E-9 instrument at the X-band frequency (9.1 GHz) at 120 K; about 10% of ethanediol was added to the samples in order to obtain good glasses. Measurements were performed at the maximum concentration of each species found in titrations. The EPR parameters were read from the spectra (estimated uncertainties for A and g values are $1 \times 10^{-4} \text{ cm}^{-1}$ and 0.002, respectively, in the spectra of a single species).

RESULTS AND DISCUSSION

Protonation equilibria of free peptides

The protonation constants of the ligands are included in Table 1 together with some literature data for comparison. Two peptides studied (Scheme 1) contain an arginine at the first position of the peptide backbone. The arginine residue contains a very basic terminal nitrogen atom as a result of resonance stabilization of the protonated guanidine group ($\log K \approx 12$) (Clarke & Martell, 1970). Under the experimental conditions used, the pH is ≈ 11 and, at this value, the degree of deprotonation of the guanidine group is insignificant. Therefore the proton was considered as unionisable under

the conditions used and its protonation constant ignored. Both pentapeptides investigated contain three functional groups: the L-tyrosine phenolic, the N-terminal L-arginine α -ammonium and the C-terminal threonine carboxyl groups, which deprotonate over the pH range 3–11. The first protonation constant, $\log \beta_{\text{HL}} = 9.76$ or 9.82 for RYLPT and RYL Ψ [CN₄]AT, respectively, refers to the protonation of the tyrosine OH group. This value is comparable with those of proctolin analogues (Kowalik-Jankowska *et al.*, 2005) and other literature values (Kozłowski *et al.*, 1989; Lodyga-Chruscinska *et al.*, 1999; 2000). The protonation constant of N-terminal L-arginine α -ammonium group is similar to that found in RYLPP and RYLPI (Kowalik-Jankowska *et al.*, 2005). For the C-terminal carboxyl group, the acidity decreases in the tetrazole derivative compared to the parent peptide and its analogues. This may be related to the specific conformational changes induced by the tetrazole ring, which makes the deprotonation of C-terminal threonine carboxyl more favorable. The same tendency was also observed in the [Leu⁵]enkephalin tetrazole analogue (see Table 1).

Complex formation

The peptide Arg-Tyr-Leu-Pro-Thr

The stability constants and spectroscopic parameters of the copper(II) complexes of RYLPT and RYL Ψ [CN₄]AT ligands are collected in Tables 2 and 3.

The best fit for the data obtained from the experimental titration curves for the Cu²⁺-RYLPT

Table 1. Stability constants ($\log \beta$) and calculated protonation constants for RYLPT and RYL Ψ [CN₄]AT and comparable peptides at 298 K and I = 0.10 M (KNO₃)

Peptide $\log \beta$	HL	H ₂ L	H ₃ L
RYLPT	9.76 ± 0.01	16.71 ± 0.01	20.51 ± 0.01
RYL Ψ [CN ₄]AT	9.82 ± 0.01	16.82 ± 0.01	19.88 ± 0.01
RYLPP ^a	9.66	16.77	20.61
RYLPI ^a	9.54	16.73	20.69
YGG Ψ [CN ₄]FL ^b	9.86	17.08	20.60
AAAAA-NH ₂ ^c	8.04		
Stepwise protonation constants ($\log K$ values)	O ⁻ -Tyr	NH ₂	COO ⁻
RYLPT	9.76	6.95	3.80
RYL Ψ [CN ₄]AT	9.82	7.00	3.06
RYLPP ^a	9.66	7.11	3.84
RYLPI ^a	9.54	7.19	3.96
YGG Ψ [CN ₄]FL ^b	9.86	7.22	3.52
AAAAA-NH ₂ ^c		8.04	

^aKowalik-Jankowska *et al.* (2005); ^bLodyga-Chruscinska *et al.* (1999); ^cBal *et al.* (1993).

Table 2. Stability constants ($\log \beta$) of copper(II) complexes with RYLPT and RYL Ψ [CN₄]AT and comparable peptides at 298 K and I = 0.10 M (KNO₃)

Peptide $\log \beta$	CuHL	CuL	CuH ₁ L	CuH ₂ L	CuH ₃ L
RYLPT	13.70 ± 0.03	8.99 ± 0.01	1.89 ± 0.01	-7.81 ± 0.01	-18.90 ± 0.01
RYLΨ[CN₄]AT	13.60 ± 0.02	9.35 ± 0.02	2.66 ± 0.02	-6.65 ± 0.02	-17.03 ± 0.01
RYLPP ^a	13.75	8.67	1.54	-7.92	-18.49
RYLPI ^a	13.94	8.69	1.31	-8.36	
YGG Ψ [CN ₄]FL ^b	13.99	9.19	3.56	-5.90	-16.36
AAAAA-NH ₂ ^c		4.93	-0.66	-8.40	-16.37
$\log K^*$ ^d values and deprotonation constants for amide protons (pK) in Cu ²⁺ complexes	{NH ₂ , CO}	{NH ₂ , N ⁻ , CO}	{NH ₂ , 2N ⁻ , CO/N _{tetr} }	pK ₁	pK ₂
RYLPT	-3.01	-7.72	-14.82	4.71	7.10
RYLΨ[CN₄]AT	-3.22	-7.47	-14.16	4.25	6.69
RYLPP ^a	-3.02	-8.10	-15.23	5.08	7.13
RYLPI ^a	-2.78	-8.05	-15.40	5.27	7.35
YGG Ψ [CN ₄]FL ^b	-3.09	-7.89	-13.52	4.80	5.63
AAAAA-NH ₂ ^c	-3.11	-8.70	-16.44	5.59	7.74

^aKowalik-Jankowska *et al.* (2005); ^bLodyga-Chruscinska *et al.* (1999); ^cBal *et al.* (1993); ^d $\log K^* = \log \beta(\text{CuH}_j\text{L}) - \log \beta(\text{H}_n\text{L})$ (where the index *j* corresponds to the number of the protons in the coordinated ligand to metal ion and *n* corresponds to the number of protons coordinated to ligand).

system revealed the presence of five metal complex species: CuHL, CuL, CuH₁L, CuH₂L and CuH₃L (charges omitted for simplicity, Table 2, Fig. 2).

From the species distribution diagram one can see that the first species is formed above pH 5 (Fig. 2). It is a minor species with metal coordination to the N-terminal amino and nearby carbonyl groups {NH₂, CO}. This species usually shows up in the earlier steps of Cu(II) complexation by peptides (Sovago, 1990). The CuHL complex cannot be supported by UV-Vis or CD spectroscopy data due to its very low concentration but it was distinguished by a set of EPR parameters: $A_{||} = 166 \times 10^{-4} \text{cm}^{-1}$, $g_{||} = 2.307$ suggesting the {NH₂, CO} coordination mode (Table 3) (Lodyga-Chruscinska *et al.*, 1999; Pettit *et al.*, 1990). The CuHL species of the peptide with the tyrosyl oxygen protonated corresponds to the CuL of AAAAA-NH₂. The $\log K^*$ values for a 1N {NH₂, CO} complex of proctolin ligand and the complexes

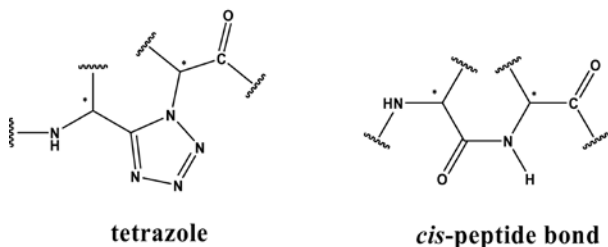


Figure 1. Structure of the tetrazole moiety (left), a mimic of a cis-peptide bond (right). Asterisks mark chiral centers.

of RYLPP, RYLPI and AAAAA-NH₂ are comparable to one another supporting the same set of donor atoms involved in copper(II) binding (Table 2). The next complex species with the stoichiometry CuL is dominant about pH 6. Its formation is a result of the CuHL deprotonation according to the equilibrium reaction: $\text{CuHL} \rightleftharpoons \text{CuL} + \text{H}^+$. The deprotonation constant $\text{p}K = 4.71$ (Table 2) indicates the deprotonation and coordination of the first amide nitrogen atom to Cu(II) ion (Lodyga-Chruscinska *et al.*, 1998; Kozłowski *et al.*, 1999).

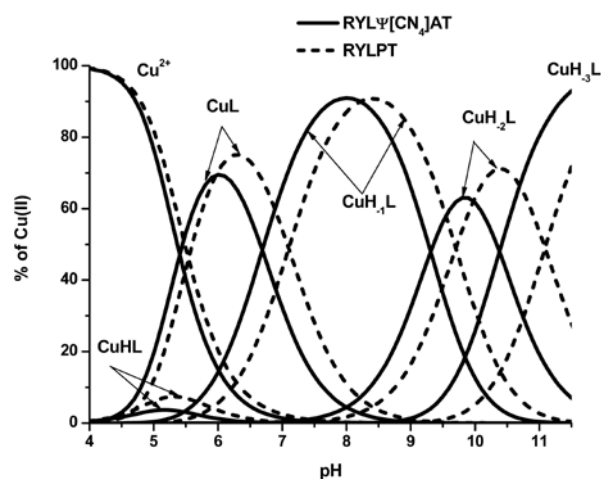


Figure 2. Species distribution of complexes formed in copper(II)-RYLPT (dashed lines) and RYL Ψ [CN₄]AT (solid lines) systems.

Cu(II) to peptide molar ratio 1:1, metal concentration 1 mM.

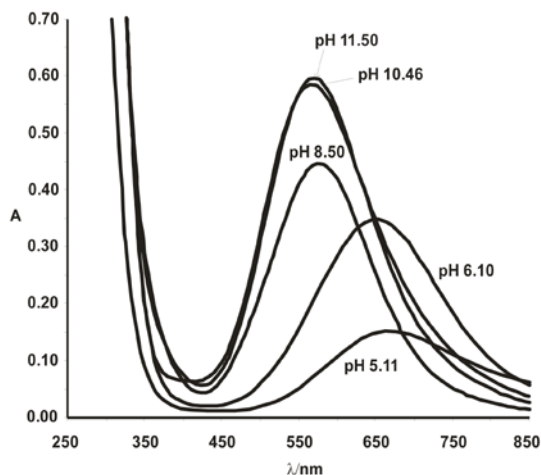


Figure 3. Electronic absorption spectra of Cu(II)-RYLPT system as a function of pH at 1 mM Cu(II) concentration and metal to ligand ratio 1:1.

The d-d transition energy at 649 nm measured from the electronic absorption spectra (Table 3, Fig. 3), the presence in CD spectra of the $N_{am}^- \rightarrow Cu^{2+}$ charge transfer transition at 338 nm (Table 3, Fig. 4) and the EPR parameters: $A_{||} = 175 \times 10^{-4} \text{ cm}^{-1}$, $g_{||} = 2.245$ support the $\{NH_2, N^-, CO\}$ coordination mode.

The CuL complex loses the next amide proton with increasing pH. The result of this process is the formation of $CuH_{-1}L$ as a major complex species at pH about 8 (Fig. 2). It has three nitrogen donor atom centers arranged in the equatorial plane of the tetragonal geometry around Cu(II) ion (Fig. 5a).

The hypsochromic shift of the d-d absorption maximum to 571 nm in UV-Vis and 580 nm in CD spectra and the EPR parameters: $A_{||} = 178 \times 10^{-4} \text{ cm}^{-1}$, $g_{||} = 2.209$ clearly indicate additional amide nitrogen atom binding and the $\{NH_2, N^-, N^-, CO\}$ coordination (Table 3) (Kozłowski & Micera, 1995). An increase in the pH above 9 results in the formation of a $CuH_{-2}L$ species (Fig. 2). This complex has a similar binding mode to that of $CuH_{-1}L$ as the CD, absorption and EPR parameters indicate (Table 3, Fig. 4). The protonation constant value of the $CuH_{-2}L$ complex ($\log \beta(CuH_{-1}L) - \log \beta(CuH_{-2}L) = 9.70$, see Table 2) is in good agreement with that corresponding to the Tyr side chain in the free ligand ($pK=9.76$). This indicates that in $CuH_{-2}L$ the tyrosine OH group is deprotonated. The phenolic O^- of the Tyr residue does not coordinate to the metal ion as was the case in C-terminally modified proctolin analogues (Kowalik-Jankowska *et al.*, 2005). This is supported by the absence of the characteristic charge transfer band at 400 nm in the UV-Vis or CD spectra (Figs. 3 and 4) (Livera *et al.*, 1988). The next species $CuH_{-3}L$ was detected by potentiometry above pH 11. The spectral parameters estimated at this pH range indicate that $CuH_{-3}L$ still has the 3N coordination with an additional depro-

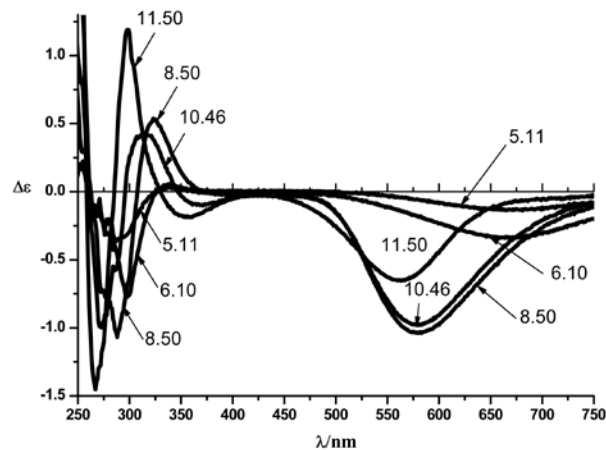


Figure 4. Circular dichroism spectra of Cu(II)-RYLPT system as a function of pH at 1 mM Cu(II) concentration and metal to ligand ratio 1:1.

tonated water molecule as OH^- in the fourth coordination position of the copper(II) ion (Table 3). The protonation constant of this species is 11.09 and corresponds to the deprotonation of the coordinated water molecule (Onindo *et al.*, 1995). The slight splitting observed in the CD spectra (Fig. 4) may reflect some constraints within the peptide backbone due to intraligand interactions following the deprotonation of the water molecule.

The peptide Arg-Tyr-Leu Ψ [CN₄]Ala-Thr

The stability constants and the species distribution diagram obtained for the tetrazole proctolin analogue is reported in Table 2 and Fig. 2. In the system involving Arg-Tyr-Leu Ψ [CN₄]Ala-Thr, the fit of the titration curves indicates the same species as were observed for the proctolin: $CuHL$, CuL , $CuH_{-1}L$, $CuH_{-2}L$ and $CuH_{-3}L$ (Fig. 2). The complex $CuHL$, with the usual 1N coordination mode, exists at a pH of about 5. It appears only as a minor species and therefore it has been not detected by UV-Vis, CD or EPR spectroscopy. However, with increasing pH, it evolves into the CuL complex which is the predominant one at a pH of about 6. The absorption band of the d-d transition at 685 nm and the CD parameters (Fig. 6, Table 3) clearly indicate the involvement of 2N coordination with the $\{NH_2, N^-, CO\}$ bonding mode (Kozłowski & Micera, 1995). The EPR parameters show an increase of the hyperfine splitting constant ($A_{||} = 184 \times 10^{-4} \text{ cm}^{-1}$) in comparison to those of RYLPT, RYLPP or RYLPI that may suggest a more stable tetragonal geometry around the copper(II) ion. Some stabilizing effect is also seen in the increase of $\log K^*$ value in comparison to that of proctolin and its analogues (Table 2). At pH 8 the $CuH_{-1}L$ complex was detected. It reveals a species with completely different coordination mode of copper(II) ion than that occurring in the parent peptide or its analogues (Fig. 5b).

Table 3. Spectroscopic parameters of proctolin and its tetrazole analogue

Peptide	Species Coordination mode	UV-Vis		CD		EPR	
		$\lambda(\text{nm})$	$\varepsilon(\text{M}^{-1}\text{cm}^{-1})$	$\lambda(\text{nm})$	$\Delta\varepsilon(\text{M}^{-1}\text{cm}^{-1})$	$A_{\parallel}(10^{-4}\text{cm}^{-1})$	g_{\parallel}
RYLPT	pH 5.11 CuHL {NH ₂ , CO}	-	-	668 ^a	-0.133	167	2.307
				229 ^c	+4.398		
				211 ^c	-1.610		
	pH 6.10 CuL {NH ₂ , N ⁻ , CO}	649	87	668 ^a	-0.327	175	2.245
				338 ^b	+0.071		
				291 ^d	-0.585		
				229 ^c	+6.897		
				205 ^c	+10.808		
	pH 8.50 CuH ₋₁ L {NH ₂ , N ⁻ , N ⁻ , CO}	571	149	580 ^a	-1.011	178	2.209
				324 ^b	+0.555		
				291 ^d	-1.072		
	pH 10.46 CuH ₋₂ L {NH ₂ , N ⁻ , N ⁻ , CO} Tyr-O ⁻ deprotonated	571	149	579 ^a	-0.957	178	2.209
				368 ^f	-0.072		
				315 ^b	+0.458		
				272 ^d	-1.020		
				208 ^c	+25.654		
	pH 11.50 CuLH ₋₃ {NH ₂ , N ⁻ , N ⁻ , OH ⁻ }	577	102	562 ^a	-0.628	200	2.199
				355 ^f	-0.161		
				302 ^b	+0.995		
				267 ^c	-1.453		
241 ^d				+7.692			
210 ^c				+27.766			
RYLΨ[CN ₄]AT	pH 6.19 CuL NH ₂ , N ⁻ , CO	685	71	725 ^a	-0.239	184	2.225
				573sh. ^a	-0.153		
				330 ^b	+0.154		
				293 ^d	-0.885		
				227 ^c	+9.296		
				205 ^c	+34.520		
	pH 8.39 CuH ₋₁ L NH ₂ , N ⁻ , N ⁻ , N _{tetr}	547	129	560 ^a	-0.605	201	2.204
				332 ^b	+0.281		
				291 ^d	-1.894		
				227sh. ^c	+18.894		
				207 ^c	+27.891		
	pH 10.10 CuH ₋₂ L NH ₂ , N ⁻ , N ⁻ , N _{tetr} Tyr-O ⁻ deprotonated	569	117	605 ^a	-0.430	201	2.204
				560sh. ^a	-0.350		
				323 ^b	+0.486		
				279 ^d	-1.449		
				227sh. ^c	+10.534		
	208 ^c	+31.125					
	pH 11.22 CuLH ₋₃ NH ₂ , N ⁻ , N ⁻ , OH	585	110	612 ^a	-0.320	194	2.196
				510 ^a	+0.088		
				361 ^f	-0.109		
304 ^b				+1.046			
273 ^d				-2.008			
238 ^c				+10.580			
210 ^c				+34.302			

^a d-d transition; ^b N⁻_{amide} → Cu²⁺ charge transfer transition; ^c intraligand transition; ^d NH₂ → Cu²⁺ charge transfer transition; ^e overlapping NH₂ → Cu²⁺ charge transfer transition and intraligand transition.

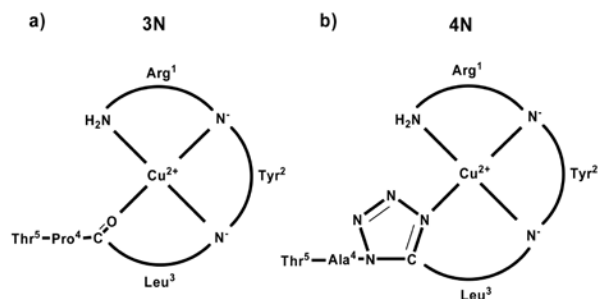


Figure 5. Arrangement of donor atoms identified on the basis of spectroscopic data in the CuH_{-1}L complex of RYLPT (a) and RYL Ψ [CN $_4$]AT (b).

The deprotonation constant of the CuL complex (pK value for $\text{CuL} \rightleftharpoons \text{CuH}_{-1}\text{L} + \text{H}^+$) equals 6.69 and corresponds very well to the deprotonation and coordination of the third amide nitrogen to copper(II) ion (Kozłowski *et al.*, 1999). The ligand with the phenolate group protonated coordinates the metal *via* an equatorial donor atom set of the $\{\text{NH}_2, \text{N}^-, \text{N}^-, \text{N}_{\text{tetrazole}}\}$ type. The coordination mode is supported by spectroscopic parameters (Table 3) (Lodyga-Chruscinska *et al.*, 1999). The value of $A_{11} = 200 \times 10^{-4} \text{ cm}^{-1}$ is comparable to that characteristic for the 4N species formed in the other oligopeptide–Cu(II) ion systems (Kozłowski & Micera, 1995). The band of the d-d transition energy seen in UV-Vis or CD spectra of the CuH_{-1}L complex is slightly above that typical of the 4N complexes of simple peptides (about 530 nm) but this is due to the different nature of the fourth coordinated nitrogen atom, since it is expected that different parameters should be obtained for different nitrogen donor atoms. The $\log K^*$ value for the CuH_{-1}L complex is 0.76, 1.07 and 1.24 orders of magnitude higher in comparison to those of RYLPT, RYLPP and RYLPI, respectively. Some gain in stability can arise from the replacement of the oxygen atom in the copper(II) coordination sphere by the nitrogen atom of tetrazole moiety (see Fig. 5) which exhibits a higher bonding

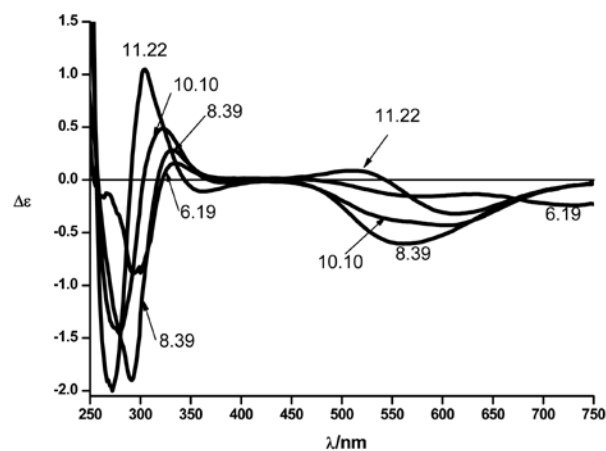


Figure 6. Circular dichroism spectra of $\text{Cu(II)-RYL}\Psi$ [CN $_4$]AT system as a function of pH at 1 mM Cu(II) concentration and metal to ligand ratio 1:1.

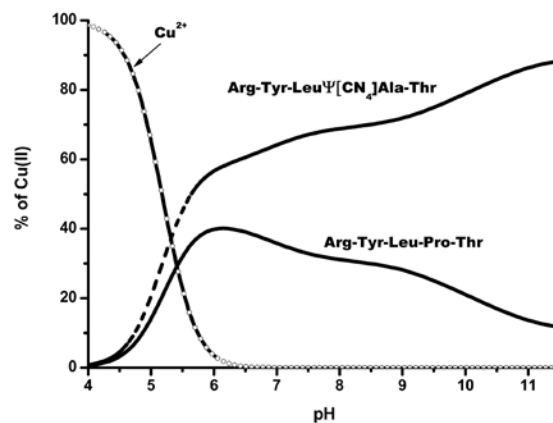


Figure 7. Competition diagram for proctolin (solid line) and its tetrazole analogue (dashed line) in binding of Cu(II) ions as a function of pH for 1:1:1 (ligand/metal/ligand) molar ratio and metal concentration 1 mM.

affinity for Cu(II) ion than that of oxygen. This finding could be also attributed to the conformation of the peptide tetrazole analogue that is well suited for metal ion coordination by deprotonated amide nitrogens and the tetrazole nitrogen. The formation of the next complex CuH_{-2}L at a pH of about 10 is a result of the Tyr side chain deprotonation. The spectroscopic parameters are almost the same as for the CuH_{-1}L species supporting the $\{\text{NH}_2, \text{N}^-, \text{N}^-, \text{N}_{\text{tetrazole}}\}$ coordination mode (Table 3). The last CuH_{-3}L complex species detected by potentiometric titrations is dominant above pH 11. The presence of the $\text{N}^- \rightarrow \text{Cu(II)}$ charge transfer band at 304 nm in the CD spectrum (Fig. 6, Table 3) supports an involvement of the amide nitrogens in the metal coordination. However, the red shift of the d-d transition band and the decrease in the EPR parameters indicate the presence of $\{\text{NH}_2, \text{N}^-, \text{N}^-, \text{OH}^-\}$ coordination (Table 3). In this complex the weakly basic nitrogen donor atom of the tetrazole moiety is substituted by the OH^- group of a deprotonated water molecule ($\text{pK} = 10.57$) (Lodyga-Chruscinska *et al.*, 1999). This coordination mode may lead to a distinct decrease in the geometric symmetry around the metal ion, which results in the splitting of the d-d band observed in the CD spectrum (Fig. 6).

CONCLUSIONS

The insertion of a tetrazole ring into the peptide sequence changes the coordination towards Cu(II) ion. Its insertion after the third amino-acid residue leads to the involvement of the tetrazole nitrogen in the metal binding as the fourth donor atom and a gain in the stabilization of the 4N complex species. The $\{\text{NH}_2, \text{N}^-, \text{N}^-, \text{N}_{\text{tetrazole}}\}$ coordination mode makes the tetrazole derivative a more effective chelating ligand than proctolin and its C-terminally modified analogues. The enhancement of the bind-

ing ability is clearly seen in the plot demonstrating the competition between RYLΨ[CN₄]AT and RYLPT ligands towards Cu(II) ions.

The results indicate that Cu(II) could hold the peptide chains in a biologically active bent conformation. The extra stability may be attributed to a metal ion-induced conformational organization of the peptide molecule, involving a β-turn, depending on the nature of the amino-acid residues. This bent conformation achieved may be essential for binding of the tetrazole peptides at the receptor site.

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