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# Specific interactions of bovine and human β-casomorphin-7 with Cu(II) ions

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**Abstract**

Complex formation between Cu(II) and human and bovine β-casomorphin heptapeptides, Tyr-Pro-Phe-Val-Glu-Pro-Ile and Tyr-Pro-Phe-Pro-Gly-Pro-Ile, respectively, was investigated by pH potentiometry and spectroscopic (CD, EPR and electronic absorption) techniques. The results showed the critical impact of Pro residues on the complex equilibria formed. The presence of the Pro residue at the second position leads to formation of very stable dimeric species in which two metal ions co-ordinate to N-terminal $\{NH_2,C=O\}$ binding sites of one peptide molecule and the deprotonated phenolic oxygen of the second ligand molecule. The presence of two additional hydrophobic residues on the C-terminal makes heptapeptide molecule much more effective ligand than its pentapeptide N-terminal fragment. 

## 1. Introduction

β-Casomorphins (also called β-casein exorphins) belong to the family of exogenous opioid peptides originally isolated from an enzymatic digests (peptone) of the bovine milk protein β-casein [1]. They all contain the common N-terminal amino acid sequence {Tyr-Pro-Phe-Pro} differing from that of other endogeneous opioid peptides {Tyr-Gly-Gly-Phe} and possess preferential μ-receptor agonist activity opposite to the Δ-receptor preference of enkephalins.

Apart from their possible role as neuro-transmitters and neuro-modulators, β-casomorphins may also influence neutral maturation, since pre-natal and post-natal administration of opiate receptor agonists can alter the development of various neutral pathways [2]. The β-casomorphins sequence seems to be of general occurrence in all β-caseins so far investigated, although the structure of human β-casein slightly differs from that of its bovine counterpart. The difference occurs within the β-casomorphin sequence responsible for opioid activity. Thus, although the N-terminal tripeptide structure essential for opioid activity is conserved, human β-caseins are less potent than bovine β-casomorphins in the GPI assay [3].

An earlier work on the opioid peptide β-casomorphin-5 isolated from casein and a number of its fragments containing Tyr and Pro residues showed a very specific co-ordination to Cu(II) ions [4]. Pro residues, which encourage the presence of bent structures (β-turns) within the peptide chain, contains a secondary nitrogen so that when incorporated within a peptide sequence, it does not possess an ionizable peptide proton and cannot form a Cu–N bond. It therefore induces a break in the normal mode of co-ordination achieved by regular peptides such as tetraalanine [5–9]. In the latter case co-ordination consists of the deprotonation and binding of the N-terminal amino group at low pH, followed by stepwise deprotonation and binding of successive peptide nitrogens as the pH is raised until square-planar geometry of 4N donors is produced with the tetrapeptide held in a tight, circular conformation. The presence of Pro residue in the chain leads to very specific modes of binding. Cu(II) ions start its co-ordination at N-terminal amino group and successive deprotonated amide nitrogen till it reaches Pro secondary nitrogen.

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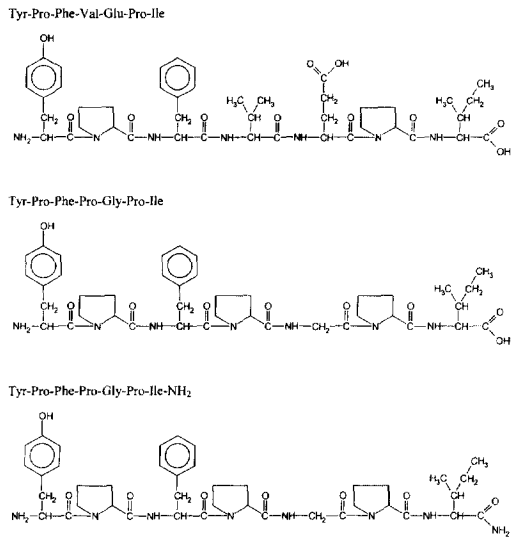


Scheme 1.

It was shown that the stabilization of the complex species co-ordinated through the amide nitrogens of residues being on the C-terminal side of the proline residue or through the phenolic oxygens of Tyr side chains results in either large chelate rings or dimeric arrangements [10]. In the core of β-casomorphin-5 [4] the presence of Pro residues leads to promotion of very stable dimeric species involving Tyr side-chain phenolic donor.

This study was undertaken in order to examine the Cu(II) co-ordination ability of the human β-casomorphin-7, Tyr-Pro-Phe-Val-Glu-Pro-Ile (YPFVEPI) and bovine casomorphin, Tyr-Pro-Phe-Pro-Gly-Pro-Ile (YPFPGPI) as well as its amide Tyr-Pro-Phe-Pro-Gly-Pro-Ile-$NH_2$ (Scheme 1).

A comparison with the similar pentapeptide system studied earlier is made to determine, how the length of the peptide chain influences the complex stability in the systems studied.

## 2. Experimental

The bovine and human heptapeptides were purchased from Bachem and used without further purification. L-Tyrosylglycine was a Sigma product.

### 2.1. Synthesis of β-casomorphin-7-amide

Synthesis β-casomorphin-7-amide was performed by the manual solid-phase technique using MBHA resin (0.56 mg/g). Nα-Boc (tert-butyloxycarbonyl) protection [11] and BOP (benzotriazol-1-yl-oxy-tris(dimethylamino)-phosphonium hexafluorophosphate) activation method [12] was used during synthesis. Benzyl protecting group was used for tyrosine phenol. The following steps were performed in each cycle: (i) addition of protected amino acid in DMF (dimethylformamide) (3 equiv.), (ii) addition of BOP and DIPEA (diisopropylethylamine) (3 equiv.) and mixing for 2–3 h, (iii) washing with DMF (3 × 2 min), (iv) washing with DCM (dichloromethane) (3 × 2 min), (v) monitoring of reaction completion with the ninhydryn test (for proline residues acylation couplings were repeated anyway), (vi) Boc deprotection with 50% (v/v) TFA (trifluoroacetic acid) in DCM (5, 25 min), (vii) washing with DCM (3 × 2 min), (viii) washing with DMF (3 × 2 min). After final deprotection the resin was washed with DCM (3 × 2 min), MeOH (3 × 2 min) and $Et_2O$ (3 × 2 min) and dried in desiccator overnight. Peptide cleavage from the resin was accomplished by HF treatment for 1 h at 0°C (10 $cm^3$ of HF plus 1 $cm^3$ of anisole per 1 g of resin). After evaporation of HF the resin was washed with $Et_2O$ and subsequently several times with 20% acetic acid. Crude peptide was then obtained in solid form by lyophilization (of acetic acid extract, Peptide was purified by reversed-phase HPLC on Vydac C-18 column) in 20–50% B linear gradient in 25 min (A – 0.05% TFA in water, B – 0.038% TFA in 90% $CH_3CN$). Homogeneity of purified β-casomorphin-7-amide was confirmed by analytical RP HPLC. Structure of obtained compound was confirmed by correct mass-peak in FAB-MS (MW-788.9 calculated for $C_{41}H_{56}N_8O_8$, found 789.8-MW+$H^+$).

## 3. Potentiometric studies

Stability constants for complexes of $H^+$ and Cu(II) were calculated from titration curves obtained using total volumes of about 1.5 $cm^3$. Alkali was added from a 0.25 $cm^3$ micrometer syringe which had been calibrated by both weight titration and the titration of standardized materials. Experimental details were: peptide concentration 0.002 mol $dm^{-3}$, copper(II) ($Cu(NO_3)_2$) concentration 0.001 mol $dm^{-3}$; ionic strength 0.1 ($KNO_3$); pH-metric titrations on a MOLSPIN pH-meter system using a micro-combined glass/calomel electrode (Russell, TR/CMAWL/TB) calibrated in concentration using $HNO_3$; temperature 25°C. Calculations were made with the aid of the SUPERQUAD computer program [13] which allows for the refinement of total ligand concentrations. Titration data confirmed the purity of the peptide, in particular, the absence of acetate.

## 4. Spectroscopic measurements

Anisotropic X-band EPR spectra (9.15 GHz) of frozen solutions were recorded at 120 K, using a Varian E-9 and Bruker ESP 300E spectrometers after addition of ethanediol to ensure good glass formation. Circular dichroism (CD) spectra were obtained with a Jobin–Yvon CD-6 spectropolarimeter. Concentrations used in the spectroscopic measurements were the same as those given for pH-metric titrations.

## 5. Results and discussion

Over the measurable pH range the human heptapeptide behaves as a $H_4L$ and bovine heptapeptides as $H_3L$ (acid form) and $H_2L$ (amide) acids, respectively (Table 1). The ionizable protons belong to the carboxylic ($pK = 3.56$ and 4.44 for Ile terminals and $pK = 4.68$ for Glu side-chain), amino ($pK = 7.34$ for human and 7.31 and 7.24 for bovine peptides) and phenolic ($pK = 9.73$ for human and 9.80, and 9.69 for bovine peptides) groups. The values are comparable to those measured previously on β-casomorphin-5 [4]. It is also worthy to note that the acidity of the phenolic proton is comparable to that measured for N-terminal tyrosine residues [5].

### 5.1. Cu(II)-human peptide complexes

The values calculated for the formation constants for Cu(II) complexes obtained for the best fit of the potentiometric data are collected in Table 1. The species distribution for the selected models are shown in Fig. 1. The chemical model obtained from the potentiometric titrations is strongly supported by the spectroscopic data (Table 1). The $CuLH_2$ and CuHL species involve N-terminal $\{NH_2, C{=}O\}$ peptide donors in metal ion binding and differ from each other by protonation of a side-chain of Glu residue. The $pK$ of $CuH_2L \rightarrow CuHL + H^+$ reaction of 4.97 is very close to the $pK$ value of $\gamma$-$COO^-$ (4.68). The d–d transition around 760 nm and a weak CD band at 270 nm ($NH_2 \Rightarrow Cu(II)$ charge transfer transition) support the co-ordination of one amino group nitrogen [5,6,10] and an adjacent carbonyl oxygen, which closes chelate ring. Proline residue acts as a break-point to $Cu^{2+}$ co-ordination when inserted into an oligopeptide chain, particularly in the second position [5–9], stabilizing the species with the $\{NH_2, CO\}$ binding mode. Also in β-casomorphin this arrangement makes the $\{NH_2, CO\}$ binding mode much more favored than that in e.g. Gly–Gly, and the formation of a bis-complex $CuH_2L_2$ in the presence of excess ligand is likely. The 1× and 2× $\{NH_2, CO\}$ donor sets involved in the CuHL and $CuH_2L_2$ complexes, respectively, result in EPR spectra which are in a fairly good agreement with those of the analogous species [5,6,10]

Above pH 6 in the absorption and CD spectra new charge transfer band is observed around 400–420 nm indicating the involvement of Tyr side chain phenolate in metal ion co-ordination [10]. The involvement of the phenolate leads to formation of the $Cu_2L_2$ dimeric species, which predominates in the pH region 7–9. The formation of dimeric complexes is characteristic for oligopeptides containing the N-terminal tyrosine residue. For example L-tyrosylglycine forms a dinuclear complex $Cu_2H_{-2}L_2$ where each metal ion is bound to the ($NH_2$, $N^-$,$COO^-$) donor set of one ligand molecule and to the phenolate group of the other peptide molecule [14]. This arrangement is likely to be favored by the stacking interactions between the aromatic rings [15].

The magnetic interactions between two copper(II) ions in the dimeric complex has been reported to be responsible for a decrease of the EPR signal intensity [16] and/or for the appearance of the half-field transition [5,10,17]. A distinct indication for the Cu(II)–Cu(II) interactions following the phenolate bridging was also obtained from the EPR spectra analysis of the L-tyrosylglycine-Cu(II) system. The weak EPR spectrum which is the result of the formation of $Cu_2H_{-2}L_2$ species, exhibits parallel hyperfine features typical of a dimeric entity involving two $I = \frac{3}{2}$ equivalent paramagnetic centers. In Cu(II) dimers, the hyperfine splitting is expected to consist of seven components with a coupling constant which is about one-half of that expected for a monomeric complex with the same co-ordination set. Actually, at least five lines are distinguished in the spectrum of $Cu_2H_{-2}L_2$ species of L-tyrosylglycine, with $A_{\|} = 67 \times 10^{-4} cm^{-1}$ (Table 1). Noticeably, the formation of $Cu_2L_2$ in the Cu(II)-β-casomorphin system yields EPR features analogous to those measured with Cu(II) L-tyrosylglycine (Table 1). Thus, the spectroscopic data support the dimer complex structure involving a couple of ligands, each of which bridges two copper ions by co-ordinating one metal ion via the $\{NH_2, CO\}$ donor set and the other metal ion via phenolate oxygen of Tyr side chain.

Above pH 8 the best fit of the potentiometric data suggests the formation of the monomeric species again. The d–d transition moves to higher energy reaching 540 nm at pH 9.2. This may suggest the involvement of the additional nitrogen atoms in metal ion co-ordination [6]. In the CD spectra one observes the diminishing of the phenolate charge transfer transition and appearance of the new charge transfer band around 320 nm corresponding to amide nitrogen to Cu(II) charge transfer transition [5–10]. The transition about 250–270 nm characteristic for N-terminal amine co-ordination is present till pH~10.5. Thus, according to the spectroscopic data the dimeric complex transforms into a monomeric species with the $\{NH_2, 2N^-\}$ co-ordination. The co-ordination of the two or three amide nitrogens in metal ion binding requires involvement of amide nitrogens inserted between two break-point Pro residues. According to potentiometric data above pH 8 there are two monomeric species, $CuH_{-1}L$ and $CuH_{-2}L$. The $pK$ for the deprotonation of the former complex of 9.88 suggests that proton leaves phenolate oxygen ($pK = 9.72$, Table 1). The $CuH_{-2}L$ stoichiometry obtained from the titration curves suggests the involvement of two amide nitrogens in the binding of Cu(II) ion.

The simultaneous binding of metal ion to N-terminal amino nitrogen and amide nitrogens of central part of the heptapeptide ligand leads to formation of macrocyclic chelate rings.

### 5.2. Cu(II)-bovine peptide complexes

The co-ordination equilibria in the Cu(II)-YPFPGPI solutions are distinctly different than those described above. The two complexes $CuH_2L$ (with protonated Ile terminal carboxyl and Tyr groups) and CuL (Tyr side chain protonated) are similar to those obtained for

Table 1
Stability constants (log $\beta$) and spectroscopic parameters

| Species | log $\beta$ | Visible | | EPR | | CD | | Donor set |
|---|---|---|---|---|---|---|---|---|
| | | $\lambda$ (nm) | $\varepsilon$ ($dm^3mol^{-1}cm^{-1}$) | $g_\parallel$ | $A_\parallel$ ($10^{-4}cm^{-1}$) | $\lambda$ | $\Delta\varepsilon$ | |
| YPFVEPI | | | | | | | | |
| $H_4L$ | 25.31(1) | | | | | | | |
| $H_3L$ | 21.75(1) | | | | | | | |
| $H_2L$ | 17.07(1) | | | | | | | |
| HL | 9.73(1) | | | | | | | |
| $CuH_2L$ | 19.61(3) | 760 | 23 | | | 698[a] | −0.140 | $\{NH_2, CO\}$ |
| | | | | | | 270[b] | +0.280 | |
| | | | | | | 257 | +0.466 | |
| CuHL | 14.68(2) | 715 | 41 | 2.329 | 161 | 698[a] | −0.353 | $\{NH_2, CO\}$ |
| | | | | | | 251[b] | +3.900 | |
| $CuH_2L_2$ | 28.70(2) | | | 2.278 | 162 | | | $2 \times \{NH_2, CO\}$ |
| $Cu_2L_2$ | 20.17(4) | 696 | 54 | 2.259 | 88 | 698[a] | +0.212 | $\{NH_2, CO, O^-_{Tyr}\}$ |
| | | | | | | 415[c] | −0.037 | |
| | | | | | | 252[b] | +2.178 | |
| $Cu_2H_{-1}L_2$ | 11.68(6) | | | | | | | $\{NH_2, CO, O_{Tyr}\}$ |
| | | | | | | | | $\{NH_2, CO, OH^-\}$ |
| YPFPGPI | | | | | | | | |
| $H_3L$ | 21.55(1) | | | | | | | |
| $H_2L$ | 17.11(1) | | | | | | | |
| HL | 9.80(1) | | | | | | | |
| $CuH_2L$ | 20.85(2) | | | | | | | |
| CuHL | 16.80(1) | 760 | 31 | | | 720[a] | −0.027 | $\{NH_2, CO\}$ |
| | | | | | | 274[b] | +0.101 | |
| $Cu_2L_2$ | 25.24(5) | | | | | | | |
| $Cu_2H_{-1}L_2$ | 19.52(4) | 700 | 63 | 2.308 | 133 | 713[a] | −0.101 | $\{NH_2, CO, O^-_{Tyr}\}$ |
| | | 394 | 224 | | | 274[b] | +0.248 | |
| | | | | | | 253 | +0.331 | |
| $Cu_2H_{-2}L_2$ | 11.13(4) | 700 | 79 | | | | | |
| | | 394 | 341 | | | | | |
| YPFPGPI-$NH_2$ | | | | | | | | |
| $H_2L$ | 16.93(1) | | | | | | | |
| HL | 9.69(1) | | | | | | | |
| CuHL | 14.80(1) | | | 2.306 | 123 | | | $\{NH_2, CO\}$ |
| $CuH_2L_2$ | 28.90(1) | 688 | 24 | | | | | $2 \times \{NH_2, CO\}$ |
| | | 395 | 130 | | | | | |
| $Cu_2L_2$ | 20.64(2) | 648 | 55 | | | 725[a] | −0.189 | $\{NH_2, CO, O^-_{Tyr}\}$ |
| | | | | | | 273[b] | +0.320 | |
| | | | | | | 251 | +0.491 | |
| $Cu_2H_{-1}L_2$ | 12.75(1) | 648 | 60 | 2.271 | 176 | 702[a] | −0.112 | |
| | | 407 | 175 | | | 421[c] | −0.071 | |
| | | | | | | 274[b] | +0.125 | |
| | | | | | | 250 | +0.289 | |
| $Cu_2H_{-2}L_2$ | 3.69(1) | 648 | 68 | | | | | |
| L-Tyr-Gly | | | | | | | | |
| CuL | | | | 2.244 | 184 | | | $\{NH_2, N^-, COO^-\}$ |
| $Cu_2H_{-2}L_2$ | | | | 2.234 | 67 | | | $\{NH_2, N^-, COO^-, O^-_{Tyr}\}$ |
| $CuH_{-2}L$ | | | | 2.243 | 151 | | | $\{NH_2, N^-, COO^-, OH^-\}$ |
| $CuH_{-3}L$ | | | | 2.217 | 203 | | | $\{NH_2, N^-, OH^-\}$ |

[a] d–d transition.
[b] $NH_2 \rightarrow Cu(II)$ CT transition.
[c] $O^-_{Tyr} \rightarrow Cu(II)$ CT transition.

human peptide. The formation of much more stable $Cu_2L_2$ dimeric species by acid form of bovine peptide, however, prevents in the latter case the formation of the $CuH_2L_2$ complex (Table 1, Fig. 1(a) and (b)). The dimer species formed by acid form of bovine peptide is around 5 orders of magnitude more stable than that of human peptide (Table 1). The amide analogue of bovine $\beta$-casomorphin-7 behaves below pH 7 very similarly to

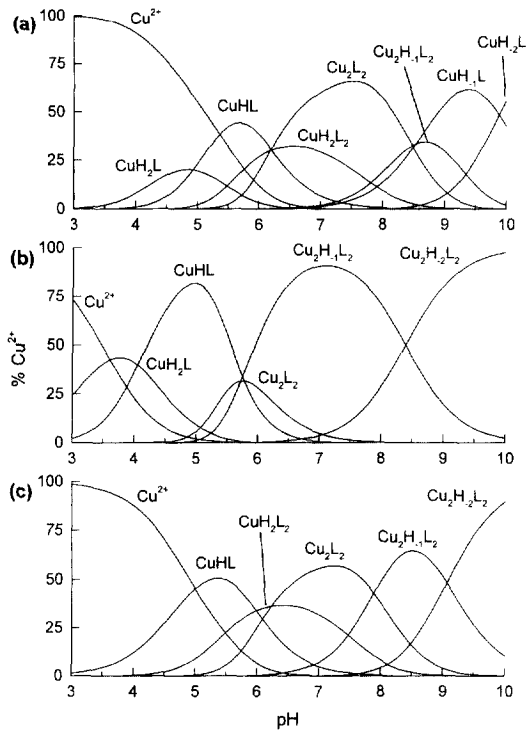


Fig. 1. Species distribution curves for (a) $Cu^{2+}$-YPFVEPI, (b) $Cu^{2+}$-YPFPGPI, (c) $Cu^{2+}$-YPFPGPI-$NH_2$ systems. The metal-to-ligand molar ratio is 1:2, metal concentration is $1 \times 10^{-3}$ mol $dm^{-3}$.

the human peptide (Table 1, Fig. 1(a) and (c)). This could suggest that three Pro residues in bovine peptide may induce peptide conformation promoting e.g. the direct interaction of metal ion with C-terminal Ile carboxylate. Above pH 9 both bovine peptide analogues are removed from the co-ordination sphere of metal ion by hydrolysis process, while human peptide is bound to Cu(II) ion even at pH 11 (vide supra). In bovine peptides the presence of three Pro residues within the peptide sequence prevent the involvement of the amide nitrogens in the metal ion co-ordination and in strongly basic solutions Cu(II) ions undergo complete hydrolysis to $[Cu(OH)_4]^{2-}$ species.

The set of Cu(II) complexes with bovine heptapeptide is similar to that obtained earlier for Cu(II)-$\beta$-casomorphin-5 (Tyr-Pro-Phe-Pro-Gly) [4]. The major differences, however occur in the complex stability constants. The heptapeptide is distinctly more effective ligand than the pentapeptide. For example the stability constant of $Cu_2L_2$ species of heptapeptide with log $\beta = 25.24$ is about 6 orders of magnitude higher than that of pentapeptide analogue (log $\beta = 19.02$, [4]) although the co-ordination modes in both species are the same. This much higher efficiency of longer peptide in metal ion binding may derive from its larger hydrophobicity [18].

## 6. Conclusions

$\beta$-casomorphins, both human and bovine are very specific ligands for Cu(II) ions. Although the N-terminal Tyr is a basic binding site for metal ions the Pro residues inserted into the peptide sequence have critical impact on the co-ordination equilibria at pH above 5. Human peptide having two Pro residues inserted into second and sixth positions is able to involve amide nitrogens of internal residues to co-ordinate to Cu(II) ion, while three Pro of bovine peptide at positions 2, 4 and 6 prevent such co-ordination. It is interesting to note that amide analogue of bovine peptide is unable to interact with Cu(II) ions via C-terminal amide nitrogen. The very interesting result obtained for bovine peptides. The elongation of the peptide chain from pentapeptide, $\beta$-casomorphin-5 to heptapetide, $\beta$-casomorphin-7, has very critical impact on complex stability constants. The longer peptide forms much more stable complexes suggesting that higher hydrophobicity and likely other conformation may increase effectiveness of the metal ion binding ability of the peptides.

## References

[1] E. Schlimme, H. Meisel, H. Frister, in: Milk Proteins, Steinkopff Verlag Darmsdadt, Springer, New York, 1989, pp. 143–149.
[2] G.E. Handelman, J. Physiol. Paris 80 (1985) 268.
[3] V. Brantl, Eur. J. Pharm. 106 (1985) 213.
[4] G. Formicka-Kozłowska, L.D. Pettit, I. Steel, B. Hartrodt, K. Neubert, P. Rekowski, G. Kupryszewski, J. Inorg. Biochem. 22 (1984) 155.
[5] H. Kozłowski, M. Bezer, L.D. Pettit, M. Bataille, B. Hecquet, J. Inorg. Biochem. 18 (1983) 231.
[6] L.D. Pettit, J.E. Gregor, H. Kozłowski, in: R.W. Hay, J.R. Dilworth, K.B. Nolan (Eds.), Perspectives in Bioinorganic Chemistry, JAIPress, London, 1991, pp. 1–41.
[7] L.D. Pettit, I. Steel, G. Formicka-Kozłowska, T. Tatarowski, M. Bataille, J. Chem. Soc., Dalton Trans. (1985) 535.
[8] M. Bezer, L.D. Pettit, I. Steel, M. Bataille, S. Djemil, H. Kozłowski, J. Inorg. Biochem. 20 (1984) 13.
[9] C. Livera, L.D. Pettit, M. Bataille, J. Krembel, W. Bal, H. Kozłowski, J. Chem. Soc., Dalton Trans. (1988) 1357.
[10] L.D. Pettit, I. Steel, T. Kowalik, H. Kozłowski, M. Bataille, J. Chem. Soc., Dalton Trans. (1985) 1201.
[11] H. Kappeler, R. Schwyzer, Helvetica Chim. Acta 44 (1961) 1136.
[12] J.A. Fehrentz, B. Castro, Synthesis (1983) 676.
[13] P. Gans, A. Sabatini, A. Vacca, J. Chem. Soc., Dalton Trans. (1985) 1195.
[14] R.J.W. Hefford, L.D. Pettit, J. Chem. Soc., Dalton Trans. (1981) 1331.
[15] O. Yamauchi, K. Tsujide, A. Odani, J. Am. Chem. Soc. 107 (1985) 659.
[16] B. Radomska, I. Sóvágó, T. Kiss, J. Chem. Soc., Dalton Trans. (1990) 289.
[17] T. Kiss, Z. Szücs, J. Chem. Soc., Dalton Trans. (1986) 2443.
[18] W. Bal, G.N. Chmurny, B.D. Hilton, P.J. Sadler, A. Tucker, J. Am. Chem. Soc. 118 (1996) 4727.