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Transition metal complexes of a cyclic pseudo hexapeptide: synthesis, complex formation and catalytic activities‡§

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Abstract: To contribute to a better understanding of metalloenzymes, we studied ion selectivity, complex formation tendencies and catalytic activities of linear and cyclic pseudopeptides. In this contribution, a linear and cyclic pseudo hexapeptide is described. The complex with transition metal ions and the sequence were designed using the programme COSMOS. Different routes of solid-phase synthesis were performed and compared using anchoring by *C*-terminus or a His side chain, using preformed pseudodipeptide building units or formation of *N*-functionalized peptide bond during stepwise assembly. The different strategies were compared regarding cyclization tendency, yield and purity. Side-chain anchoring to solid support favours the cyclization but leads to the formation of difficult to separate dioxopiperazine. Both routes require preformed building units. Complex-formation tendencies and selectivity for certain bivalent transition metal ions were experimentally estimated and compared to ones predicted theoretically. CD measurements indicate conformational changes by complex formation with different metal ions. Catalytic activities on oxidation of catechol and hydrolysis of bis-phosphate esters by some metal complexes of linear and cyclic peptide show only low catalytic activities compared to other model peptides and related metalloenzymes. The preference of the cyclic peptide for complexation of Ni^{2+} corresponds well to the predictions of COSMOS-NMR force field calculations. Copyright © 2008 European Peptide Society and John Wiley & Sons, Ltd.

Supporting information may be found in the online version of this article.

Keywords: solid-phase synthesis of cyclic pseudo hexapeptide; complexation of transition metal ions; catalytic activities

INTRODUCTION

Metalloenzymes are a widely spread and important group of enzymes, belonging to all major classes. They are reviewed in monographs [4] and many recent articles in various journals [5–7]. A large number of attempts have been undertaken to study the specific complex-formation tendencies with different metal ions and their catalytic mechanisms. Besides improved understanding of the role of metal ions in the enzymatic catalysis, a deeper and wider knowledge about the basic mechanisms is required for designing active enzyme mimetics. These synthetic catalysts could be designed, e.g. either for specific superoxid dismutase [8], nuclease and peptidase activity [9,10] or for such highly specific reactions such as asymmetric organic syntheses [11,12]. They could also be adapted to non-aqueous solvents with different polarities.

In addition to being involved in physiological processes, transition metal ions also play an important role in such pathophysiological processes as oligomerization of amyloid peptides [13] and perturbation of zinc-finger binding [14]. Their complexes can play a role as potential drugs [15]. Thus, complexes with peptides and pseudopeptides can act as dismutase [8], such as a metalloprotease [15]. Coupled to hormones as chelators for radioactive ions, they can be used for cancer treatment [16].

To estimate complex-formation tendencies and catalytic activities, we used small peptides as model compounds for our studies because natural metalloenzymes are proteins, in some cases very large proteins, and therefore more difficult to investigate. To predict the complex formation, tendency for a definite peptide with distinct metal ions continues to remain problematic. The transition metal ions differ only little in charge, radius or preferred coordination geometry. Proteins and especially peptides are flexible molecules existing in various conformational states. This flexibility seems to be a prerequisite for the very high catalytic activity

Abbreviations: BTSA, Bis-trimethyl silyl acetamide; CHES buffer, 2-(*N*-cyclohexylamino)ethanesulfonic acid; eq., equivalents; EtOH, ethanol; MeCN, acetonitrile; MeOH, methanol; MRE, mean residue ellipticity; iPrOH, isopropanol; rt., room temperature; tetracyclen, 1,4,7,10-tetraaza cyclododecane; TFFH, tetramethylfluoroformamidinium hexafluorophosphate; TIPS, Triisopropylsilane; TNBS, 2,4,6-trinitrobenzenesulfonic acid.

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of the proteoenzymes and allows furthermore complex formation with different metal ions [17], their so-called promiscuity [18].

Two different strategies are applicable to estimate the complex-formation tendency with a certain metal ion: (i) the complexes can be designed by related modelling programmes or (ii) some series of peptides can be synthesized and tested experimentally for their complex-formation tendencies. We used both theoretically and the experimentally means in this investigation.

For modelling of complexes, we used the programme COSMOS [19]. Besides such functional groups as imidazolyl, sulfhydryl, and amino groups, primarily amide bonds are involved in complex formation. To reduce the flexibility and therefore to enhance the efficiency of the modelling, we used a cyclic peptide. Derived from the active centre of metalloenzymes, we created a pseudopeptide with *N*-functionalized peptide bonds which should be able to contribute to the complex formation with their *N*-alkyl amino groups. To validate the modelled compounds, we estimated complexation tendency and catalytic activities for both cyclic and linear peptides. The results can be used to improve modelling of peptide chelates in both, complex-formation tendency and catalytic activity.

Synthesis of the designed cyclic pseudo hexapeptide is complex because of the cyclization step additionally to the formation of *N*-functionalized peptide bonds. To develop a favourable strategy, we investigated different synthetic approaches and compared them by yield and purity. The synthetic routes differ on the one hand in the coupling to the resin, either by *C*-terminal carboxyl group or by side chain of His and on the other hand by the preformation of the required pseudodipeptide units in solution or by their formation during the stepwise assembly on the resin.

MATERIALS AND METHODS

Materials

All chemicals, unless otherwise stated, were purchased from Fluka (Sigma-Aldrich Chemie, Seelze, Germany). Amino acid derivatives, coupling reagents (including TFFH) and 2-chlorotrityl chloride resin were purchased from Advanced ChemTec Europe (Bamberg, Germany) and Novabiochem (Bad Soden, Germany). TFFH was obtained from PE Biosystems (Weiterstadt, Germany). Fmoc-His(Bom)-OH, Fmoc-His(Trt)-OH were purchased from BACHEM. All materials and solvents were of reagent grade and were used without further purification with the following exceptions: DMF was first dried over molecular sieves and distilled from phthalic anhydride; DCM was stored over molecular sieves.

General

TLC was performed on precoated glass plates (silica gel 60 F_{254}, Merck, Darmstadt, Germany) with *n*-butanol/acetic acid/water (4/1/1) as the mobile phase. TLC detection was accomplished with UV-light, ninhydrine reagent and Cl_2/tolidine solution. The crude peptides were purified by semi-preparative reversed-phase HPLC on a Shimadzu LC-8A equipped with a KNAUER RP18 C5 column (32 × 250 mm) using isocratic condition (0.1% TFA in H_2O) for 20 min and subsequently a gradient from 0 to 45% solvent B (90% MeCN in H_2O and 0.1% TFA) over 120 min. Detection was accomplished at 220 nm.

Analytical HPLC was performed on a Shimadzu LC-10AT chromatograph with a Vydac 218TP C5 column (4.6 × 250 mm). Building units and intermediate peptides were eluted with the gradient 1 : 20–80% B in 60 min at a flow rate of 1.0 ml/min, A was 0.1% TFA in water and B 0.1% TFA in MeCN, free hexapeptides with gradient 2: 0–30% MeCN in 30 min, detection at 220 nm.

Molecular weight measurements were performed using MALDI-TOF MS. 4-Hydroxy-α-cyano-cinnamic acid 8 mg/1 ml 70% MeCN/water, containing 0.3% TFA was used as a matrix. The mixture of 1 µl prepared matrix and 1 µl peptide (in the range of 0.2 mM) was deposited on a test plate. The measurement was carried out on a Voyager DE-RP mass spectrometer (PerSeptive Biosystems, Framingham, MA, USA) equipped with a N_2 laser ($\lambda = 337$ nm). The peptides were also analysed by ESI-MS on a triple quadrupole instrument Quattro (Fisons Biotech VG, Altrincham, England).

Amino acid analysis was performed in an amino acid analysator 'Eppendorf-Biotronik LC 3000' after peptide hydrolysis with 6 N HCl at 110 °C for 24 h.

Synthesis of Building Units

(CH$_2$-CH$_2$-NH-Boc)Gly-OBzl 1. Method A: $BrCH_2COOBzl$ (14.2 g, 9.9 ml, 62 mmol) in 50 ml THF (dried with Na) was dropped slowly and under cooling into a solution of *N*-Boc ethylene diamine (10 g, 62 mmol) and TEA (17.3 ml, 124 mmol) in 100 ml THF. The reaction mixture was stirred overnight at rt. After cooling, the precipitated triethyl ammonium bromide was removed by filtration. The solvent was removed *in vacuo* and the remaining oil was dissolved in ethylacetate and washed with aqueous solutions of 0.1 M $KHSO_4$ and sat. $NaHCO_3$. After drying over $MgSO_4$, the solvent was removed and the obtained crude product purified by flash chromatography on silica gel 60 with $CHCl_3/CH_3OH$ 7 : 3 as eluent.

Method B: One eq. *N*-Boc ethylene diamine (10 g, 62 mmol), 1.1 eq. $BrCH_2COOBzl$ (15.6 g, 10.5 ml, 68 mmol), and 3 eq. TEA (18.8 g, 26 ml, 186 mmol) were dissolved in 20 ml toluene and cooled to −30 °C. Ag_2O (17.35 g) was added in many small portions with vigorous stirring. The reaction mixture was allowed to reach rt and was stirred overnight. After removing the precipitate by centrifugation, the solvent was removed; the obtained oil was dissolved in ethylacetate. The solution was washed (0.1 M $KHSO_4$, sat. $NaHCO_3$ and water), dried and evaporated. After washing with ether, the product was obtained as solid. Yield: 9 g (47%), homogeneous by HPLC.

HPLC gradient 1: $t_R = 14.8$ min; TLC: R_f(S1) = 0.83.

(CH$_2$-CH$_2$—NH-Boc)Gly-OH 2. (CH_2-CH_2–NH-Boc)Gly was obtained after hydrogenation of **1** in methanol with $Pd/BaSO_4$ twice for 4 h. Completeness of hydrogenation was followed by TLC.

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TLC: R_f(S1) = 0.65.

Mol wt calculated: 218.3 and found: 219, $[M + H]^+$; 241, $[M + Na]^+$.

Fmoc-(CH_2-CH_2—NH-Boc)Gly-OH 3. (CH_2-CH_2–NH-Boc)Gly (4 g, 2.3 mmol) and BTSA (17.9 ml, 9.2 mmol) were dissolved in 250 ml dry DCM and stirred overnight. After addition of DIPEA (3.8 ml, 2.86 mmol),

Fmoc-Cl (4.74 g, 2.29 mmol) was added in five portions with stirring at 0 °C. The reaction mixture was stirred additionally 2 h at rt. The solvent was removed *in vacuo*, the residue dissolved in ethylacetate and washed with 0.1 M $KHSO_4$ and water. After removing the solvent, the product was treated with hexane.

The obtained crude product (5.9 g, oil) was again purified by flash chromatography on silica gel 60 with $CHCl_3$/CH_3OH 7 : 3 as eluent, in portions of upto 1 g each.

Yield: 4.6 g (45%).

HPLC gradient 1: t_R = 33 min; TLC: R_f(S1) = 0.9.

Mol wt calculated: 440.5 and found: 463.1, $[M + Na]^+$ (in MALDI and ESI-MS).

Fmoc-Lys(Boc)-(CH_2-CH_2—NH-Boc)Gly-OBzl 4. Fmoc-Lys(Boc)-OH (1.13 g, 2.02 mmol), (CH_2-CH_2–NH-Boc)Gly-OBzl **1** (0.6 g, 1.62 mmol), TFFH (0.64 g, 2.02 mmol) and symmetrical collidin (0.65 ml, 4.05 mmol) were dissolved in dry DCM. The reaction mixture was refluxed for 2 h under an argon atmosphere. After cooling the mixture to rt, the solvent was removed *in vacuo*. The residue was dissolved in ethylacetate and washed (0.1 M $KHSO_4$,sat. $NaHCO_3$, and water). After drying over $MgSO_4$ the solvent was removed and the obtained crude product purified by flash chromatography on silica gel 60 with $CHCl_3$/CH_3OH 7 : 3 as eluent. After evaporation of the solvent, the residue was dissolved in tert. butanol. Lyophylization of the combined fractions gave a white solid. Yield: 1 g (81%).

HPLC gradient 1: t_R = 52.3 min.

Mol wt calculated: 759.9 and found: 781.1, $[M + Na]^+$.

The crude product (8 g) was purified by flash chromatography on silica gel 60 with ethylacetate/hexane 1 : 1 as eluent to give 4.6 g (46%).

Fmoc-Lys(Boc)-(CH_2-CH_2—NH-Boc)Gly-OH 5. The dipeptide building unit was obtained after hydrogenation of compound **4** (2 g, 2.63 mmol) with Pd/$BaSO_4$ (250 mg) in methanol twice for 12 h. Completeness of hydrogenation is estimated by HPLC. After removing the solvent the product was washed with hexane and ether, dissolved in water, centrifuged and lyophylized. Yield: 1.6 g (90%).

HPLC gradient 1: t_{Rr} = 40.8.

Mol wt calculated: 669.8 and found: 670, $[M + H]^+$; 691.0, $[M + Na]^+$.

Assembly of Linear Hexapeptide

NH_2-His-Lys-(CH_2-CH_2-NH_2)Gly-βAla-(CH_2-CH_2-NH_2)Gly-Gly-OH 6. The linear hexapeptide was synthesized by the solid-phase method on 2-chlorotrityl resin attached to the resin at the *C*-terminal Gly or side chain of His. In addition to these two anchoring sites, two other approaches were also performed to optimize the synthesis of the linear precursor.

In the approach A, the *N*-functionalized glycine derivative was formed using *Zuckemann's method* [20] to form pseudopeptides. The couplings of Fmoc-βAla and Fmoc-Lys(Boc)-OH to the secondary amines were performed using TFFH. In this case, the negative chloranil test was used to confirm the completeness of coupling. In the second approach B, the preformed building blocks Fmoc-(CH_2-CH_2-NH-Boc)Gly-OH **3** and Fmoc-Lys(Boc)-[(CH_2-CH_2-NH-Boc)]Gly-OH **6** were used. Each coupling was checked by TNBS and chloranil tests for primary and secondary amines, respectively and evaluated by the UV-spectrophotometric determination of Fmoc–piperidine adduct.

The unprotected linear peptide was obtained after removal from the resin, treatment with HCl in acetic acid, hydrogenation with Pd as described above and purification by semi-preparative HPLC. The linear peptide eluted at 15 min in a broad peak.

HPLC gradient 2: t_R = 4.4 min.

Mol wt calculated: 611.7 and found: 612.6, $[M + H]^+$; 634.6, $[M + Na]^+$; 650.6, $[M + K]^+$.

Amino acid analysis: Gly 1.1 (1.0), Lys 1.0 (1.0); (CH_2-CH_2-NH_2)Gly-OH eluted together with His, βAla with the same retention time as Phe.

CYCLIC HEXAPEPTIDE

Cyclo(His-Lys- (CH_2-CH_2-NH_2)Gly-βAla-(CH_2-CH_2-NH_2)Gly-Gly) 7

Cyclization in solution. After removal of the Fmoc group, the remaining 2.14 g chlorotrityl resin with *C*-terminal attached protected hexapeptide (0.2 mmol) were swollen in DCM and treated with 10 ml HFIP in DCM (20%) for 5 and 20 min. After thoroughly washing the resin with DCM, the combined solvents were evaporated. The residue (220 mg) was dissolved in 2 ml ethanol and precipitated with 200 ml ether (115 mg, 55%). The product is homogenous by HPLC (gradient 1, t_R = 23.6). This side-chain protected linear hexapeptide (112 mg, 0.11 mmol) was dissolved in 150 ml DMF, combined with PyBOP (170 mg, 0.33 mmol) in 50 ml DMF. Under vigorous stirring, 114 µl DIPEA (0.66 mmol) in 50 ml DMF was added dropwise in 1 h. The reaction mixture was allowed to stand overnight at rt. After evaporation of the solvent, the crude product was washed with water and treated for 2 h with a mixture of 1 ml of 6 N HCl and glacial acetic acid (1 : 1). The solvent was removed by lyophilization. To eliminate the BOM group, this product was hydrogenated for 4 h in 20% acetic acid with Pd/$BaSO_4$ (100 mg). After separation of the catalyst by centrifugation, addition of water and following lyophilization the product was purified by semi-preparative HPLC as described below under purification section. Yield: 71 mg (25%).

Cyclization on solid support. After removing of the *N*-terminal Fmoc, the *C*-terminal allyl ester was cleaved using the conventional method with $Pd^0(PPh_3)_4$ in DMF/THF/HCl 0.5 N/morpholine (2 : 2 : 1 : 0.9) under

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argon overnight [21]. The cyclization was performed twice using 5 eq. PyBOP/HOBt as coupling reagent and 10 eq. DIEA as a base; 3 h for each. The cyclization process was monitored by analytical HPLC.

The free cyclic peptide was obtained by the cleavage of the imidazole ring from the trityl group of the resin and simultaneously removing the Boc groups with TFA, 2.5% TIPS, and 2.5% H_2O for 2 h. Yield calculated from resin loading with linear precursor: 20%.

Purification and characterization of both cyclization products. The crude cyclic peptides from both strategies were purified by a semi-preparative RP-HPLC. The cyclic peptide eluted under the described conditions at 20 min.

Both cyclic compounds are homogeneous by HPLC and provide well developed spectra for $[M + H]^+$, $[M + Na]^+$ and $[M + K]^+$. Mol wt calculated: 593.7 and found: 594.7, $[M + H]^+$; 616.7, $[M + Na]^+$; 632.7, $[M + K]^+$.

HPLC gradient 2: $t_R = 4.9$ min.

Formation of Metal Complexes from Linear and Cyclic Hexapeptides

Peptide salt (5 mg) was dissolved in 50 ml water and given through 1 ml of the ion exchange resin Amberlite IRA 410 in the OH-form. After washing the resin with water, the combined solutions were lyophilized. Solutions of free peptide and metal ion were combined in the calculated ratios and incubated for 20 min before use.

Estimation of Relative Complex Stabilities by MALDI-TOF Mass Spectrometry

Experiments were carried out using 1 mM solutions of the ligands, $Cu(ClO_4)_2$, $Ni(ClO_4)_2$, $Zn(ClO_4)_2$, $Co(ClO_4)_2$ and $Mn(ClO_4)_2$ in deionized water. Equal amounts of a ligand solution and two metal salt solutions (typically 5 µl) were combined and shaken. One microlitre of the mixture was transferred to a MALDI plate using 4-nitroaniline as the matrix. For each ligand, all possible combinations of two metal salts were used, resulting in ten measurements per ligand, which adds some redundancy and control. Positive ion MALDI-MS was performed in reflectron mode with delayed extraction on a Voyager-DERP instrument (PerSeptive Biosystems, Framingham, MA, USA) equipped with a nitrogen laser ($\lambda = 337$ nm).

Estimation of Relative Complex Stabilities by ESI Mass Spectrometry

To confirm the independence of the MALDI results from the ionization method, peptides were additionally analysed by ESI-MS as described in previous publications [22,23]. Sample preparation was the same as above, 15 µl of each sample were diluted with 1 ml of methanol and injected into a Finnigan MAT 95 XL mass spectrometer using nitrogen as sheath gas.

Determination of Conditional Dissociation Constants of Copper(II)-Complexes

This method is based on the copper(II) catalysed oxidation of ascorbic acid, which can be followed spectrophotometrically at 265 nm. Since the ligand removes free copper(II) ions by complexation, it can be regarded as an inhibitor and the different oxidation rates in the presence of various concentrations of ligand and ascorbic acid can be used to determine a conditional dissociation constant. This method was first used to determine micro amounts of complexing reagents by Mottola *et al.* [24] and has been adapted by us with slight modification [22].

Estimation of Complex Formation Tendency by Circular Dichroism

CD spectra were recorded using a Jasco model 720 spectropolarimeter (Umstadt, Germany). The optical rotation was calibrated using D-camphorsulphonic acid at 290.5 nm. Unless otherwise stated, measurements of three scans were recorded using rectangular quartz cells of 0.1 cm path length, 2 nm bandwidth, 20 nm/min scan speed, 8 s time constant, and at 22 °C. The background spectra containing buffer and metal ion were recorded in the same solvent without peptide and subtracted subsequently. The spectra reported here were obtained as the average of three experiments. CD spectra were obtained with samples prepared from stock solutions of 1 mM peptide. Both hexapeptides **6** and **7** and were recorded in 25 mM borate buffer with a pH of 8.0 and in TFE. Complexes were only formed in buffer. The molar ellipticity is expressed in $mM^{-1} \times cm^{-1}$. Measurements of three scans were recorded using rectangular quartz cells of 0.1 cm path length, 2 nm bandwidth, 20 nm/min scan speed, 8 s time constant, and at 22 °C.

Catalytic Activities

Hydrolysis of Bis (4-nitrophenyl) phosphate. The hydrolysis of Bis (4-nitrophenyl) phosphate was measured according to Vance and Czarnik [25] in 70 mM CHES buffer ($I = 0.12$) at pH 9.2 and 50 °C. The release of 4-nitrophenolate was followed by increase of absorbance at 405 nm in buffered solution. Because of the enhanced temperature and a time of 72 h we carried out the incubation separately and used the spectrophotometer Ultospec 2000 (Pharmacia-Bio Tech.). The measurements were carried out at a substrate concentration of 10 mM. Both the Zn^{2+} complexes and $Zn(ClO_4)_2$ for blank reading were used in a concentration of 0.5 mM.

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Catechol oxidase activity. To form Cu^{2+} complexes were formed according to Rzepecki and Waite [26]. Equal volumes of 1 mM solutions of $Cu(ClO_4)_2$ and ligands were mixed and allowed to stand 20 min. at rt. The measurement requires two separate substrate solutions: Substrate A: 0.5 M Pro in 0.1 M phosphate buffer (pH 7.5). Substrate B: 0.01 M 4-methyl catechol in 0.02 M acetic acid, prepared from a frozen stored (−80 °C) stock aliquot by dilution 1 : 10. The concentration of substrate B in the cuvet was enhanced in certain steps from 0.2 to 1 mM, followed by measuring the enhancement of absorption at 258 nm ($\varepsilon = 4230\ M^{-1}\ cm^{-1}$), 35 °C over a period of 30 min. Because of the high rate of auto oxidation, all estimations were performed in triplicate parallel measurements, including blank (complex replaced by buffer). Concentrations in the cuvet are the following: 10 μM complex, 0.02 M Pro, 0.2 to 1 mM 4-methylcatechol, 0.3 mM oxygen in 0.1 mM phosphate buffer.

RESULTS AND DISCUSSION

Design, Synthetic Strategies and Chemical Characterization

Using the programme COSMOS [19] peptide structures were designed and their conformations were investigated using COSMOS-NMR force field. The final result was the sequence of the cyclic pseudo hexapeptide with the ability of complex formation. The search for fitting structures was carried out by performing MD simulations of pseudopeptides and optimizing the structures of a large number of MD snapshots. In these calculations, double-charged metal ions were allowed to move freely within the structure to find preferred binding sites and complex structures. For this aim, traditional force fields with fixed atomic charges cannot be used. Therefore, the electrostatic interactions within the COSMOS-NMR force field are calculated using atomic charges that are no longer fixed parameters but change with the positions of all atoms. Using the semi-empirical bond-polarization method (BPT) it was possible to include into the electrostatic part of the energy all mutual polarizations [27]. This feature is essential for complex compounds since their stability in many cases can only be understood by taking into account the polarization of the ligands by the charged metal ions [28,29]. Figure 1 shows the energetically lowest optimized structure of the pseudo hexapeptide complex. This cyclic peptide is predicted to form complexes with transition metal ions, preferably with Ni^{2+}. As seen in this figure, both *N*-alkyl amino groups are involved in complex formation.

Assembly of the pseudopeptides requires formation of branched dipeptide units with *N*-functionalized amide bonds. On the basis of our preceding synthetic studies at the backbone cyclized hormones bradykinin and somatostatin [30–33], we initiated two approaches to form these building units. Thus, we also assembled the hexapeptide with preformed dipeptide building units and tried to form these units on the resin. The routes to both preformed dipeptide building units **3** and **5** are shown in Scheme 1. The advantage of the solution synthesis of preformed units consists in the possibility to purify these units by precipitation or by flash chromatography. By assemblage of these units on the resin, the formation of the *N*-alkylated peptide bond

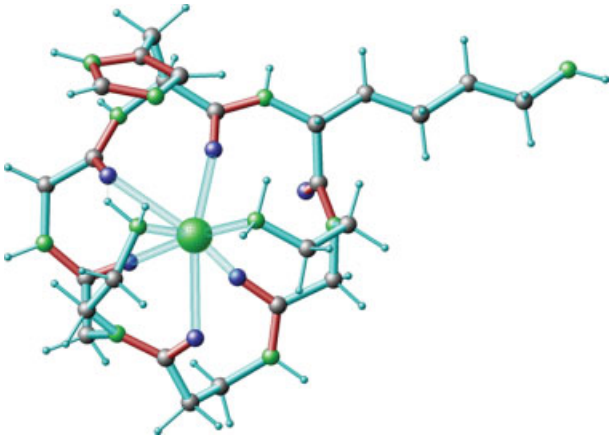
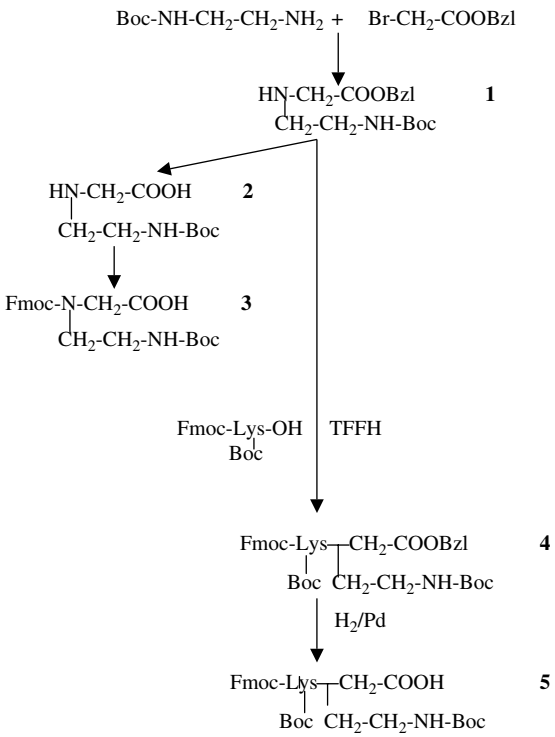


Figure 1 Optimized structure of the pseudo hexapeptide complex designed using programme COSMOS, taking into account the polarization of the ligand by the charged metal ions.



Scheme 1 Synthesis of preformed pseudo peptide building units in solution.

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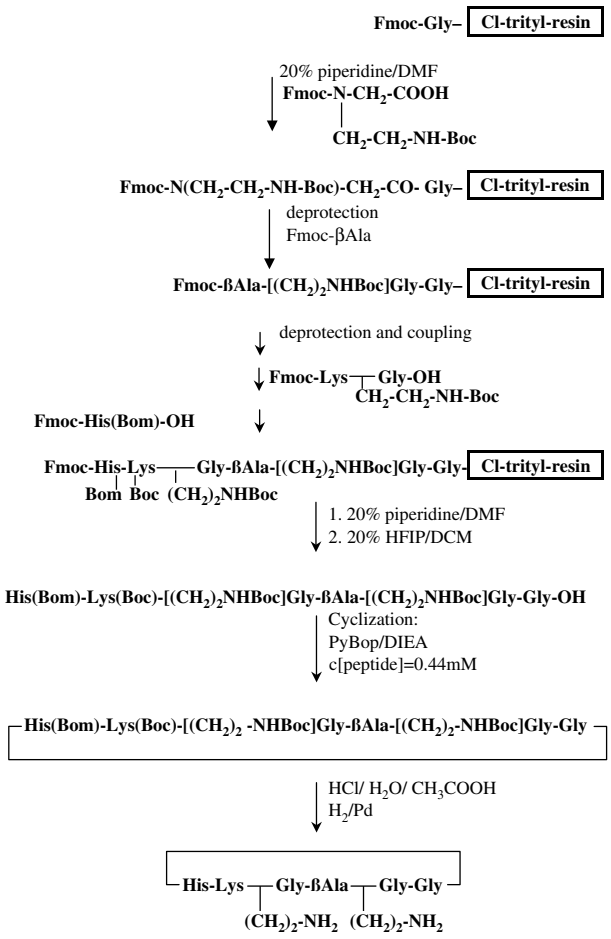
remains in many cases incomplete, but the coupling could be repeated several times to enhance the yield. In the special case of forming *N*-alkylated glycine via bromo acetic acid, the peptide is removed to some degree from the trityl or chlorotrityl resin by the acidity of the halogenated acetic acid.

The synthesis of unit **1** was performed in two different ways. The use of AgBr-formation as the driving force for *N*-alkylation in route 2 gives a higher yield, but requires a complete separation of colloidal AgBr, which is time-consuming. A third route to obtain compound **3** was described by Deuholm *et al.* [34].

Thus, the application of preformed building unit **3** gives better results with respect to yield and purity. Using unit **3,** the coupling of Lys(Boc)-OH to *N*-functionalized Gly residue gives nearly the same results as the coupling of dipeptide unit **6**.

The assembly of both linear and the cyclic hexapeptides was performed in two different ways. Both syntheses used 2-chlorotrityl resin. By the first route (Scheme 2), the *C*-terminal glycine was coupled to the resin and the peptide was assembled by the formation of the first *N*-alkylated peptide bond (between βAla and Gly) at the resin, followed by consecutive coupling of a preformed dipeptide building unit **5**. For side-chain protection of His, we tested in our approaches different groups (Boc, Trt, Dnp) and finally we preferred the Bom group because of its chemical stability and described low racemization rate. We checked this rate with Marfey's reagent and reduced the content of racemized product below 5% by changing the coupling conditions.



Scheme 2 Assembly of linear and cyclic pseudo hexapeptide on 2-chlorotrityl resin with coupling of preformed peptide (**3**) and dipeptide (**5**) building units.

The side chains remained protected by removal from the resin with hexafluoro isopropanol allowing cyclization between *N*- and *C*-terminus. The protected linear and the cyclic hexapeptides were purified by thoroughly washing with water. Cyclization was performed with good yield in highly diluted concentration in DMF (0.44 mM) with PyBOP. Hydrogenolytic removal of Bom group requires an active catalyst, otherwise degradation occurs. The elution profiles of semi-preparative HPLC for crude, linear and cyclic hexapeptides are very similar, showing as mean peak the desired peptide and a few hydrophobic fragments. No oligomerization during cyclization reaction could be detected (MS).

To cyclize the peptide on resin, we coupled a His derivative with the side chain to 2-Cl-Trt-resin [35] (Scheme 3). Both pseudo dipeptide units were also formed on the resin and preformed in solution. The preformed units **3** and **5** and the protected βAla and Lys derivative, respectively, were coupled by TFFH. After selective removal of *N*- and *C*-terminal protection, the side-chain protected peptide was cyclized on the resin. The peptide was obtained in good and nearly the same yield and purity, comparable to cyclization in solution.

Using bromo acetic acid, the first building unit HN(CH_2CH_2-NH-Boc)Gly was formed on the resin according to Zuckermann [20]. But the acidity of the halogenated acetic acid leads to increased cleavage from the resin. In contrast to the use of preformed building units, the coupling rates dropped only from the first amino acid to the last Fmoc-Lys(Boc)-OH to about 80%. Application of dipeptide building unit **5** enhances the yield of linear hexapeptide only slightly, compared to coupling of Fmoc-Lys(Boc)-OH to polymer bound HN(CH_2-CH_2-NH-Boc)Gly-peptide.

Formation of dioxo piparazine (DKP). We found two disadvantages in the strategy using a side-chain anchoring to the resin. The main disadvantage is the high tendency of dioxo piperazine formation by the reaction of the His-allyl ester with the free amino group of the second amino acid. Furthermore, accordingly to the literature the protection of His side chain by a trityl moiety can also lead to racemization [36,37].

The DKP formation at the dipeptide unit level remains a general problem for the head-to-tail cyclization with Fmoc/tBu/allyl strategy. To suppress DKP formation during the Fmoc removal from Fmoc-Gly-His(Trt-resin)-OAl, we reduced the deprotection time to 6 min and subsequently used a quick neutralization with

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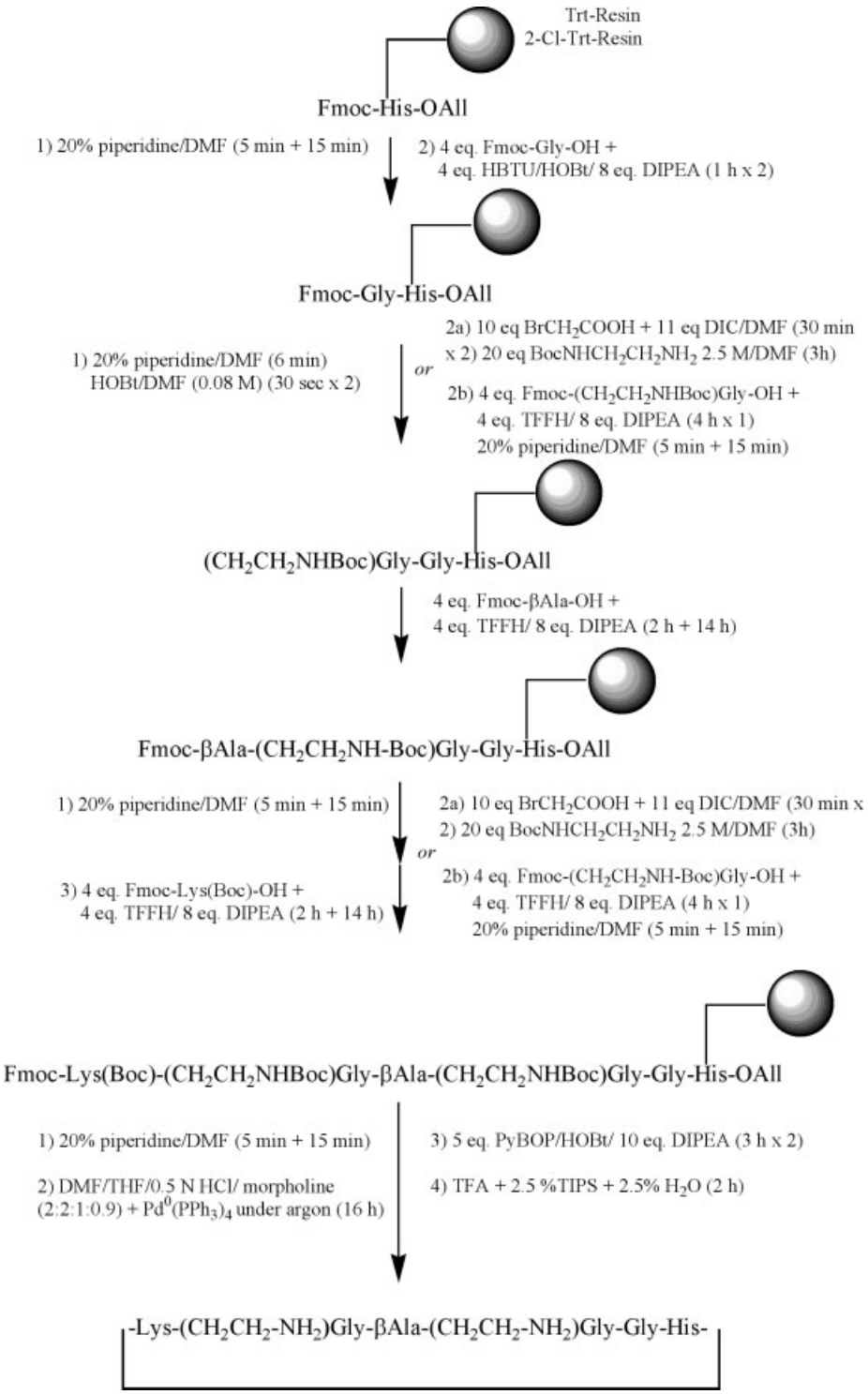

Scheme 3 Assembly of cyclic pseudo hexapeptide on trityl and 2-chlorotrityl resin, coupled through the His side chain.

HOBt/DMF and washing followed by an immediate next coupling step. As a result, 93% Fmoc group was removed and the Fmoc level of the next coupling was 80% compared to the loading of His(Trt-resin)-OAl. By this procedure, the DKP-level did not exceed 20%. We found that the semi-preparative HPLC could not separate the DKP from the peptide. Thus, the MALDI-TOF MS shows a peak of m/z 195 Da proving the existence of DKP in the purified fractions and the amino acid analysis shows enhanced values for Gly and His.

Cyclization. Cyclization in solution according to Scheme 2 was performed with PyBop in a dilution of 0.44 mM. Ring closure at the solid phase has been shown to have advantages over that performed in

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the solution. By our syntheses, the yield of the end product obtained with Scheme 3 is higher than that obtained by Scheme 2. With the loading of 0.4 mmol/g of the linear precursor, the ring closure occurred without dimerization or cyclodimerization. After semi-preparative HPLC purification, the cyclic peptide was obtained in a yield of 23%, calculated from the loading of the linear precursor.

The semi-preparative HPLC of linear and cyclic hexapeptides obtained from both strategies yields pure products, except for the incomplete separation of DKP from cyclic hexapeptide synthesized by Scheme 3. Thus the MS-spectra show, for both linear peptides and the cyclic peptide from Scheme 2, very well developed spectra with only three peaks: M, $[M + Na]^+$ and $[M + K]^+$ (Supplementary Information).

Conformational Investigations on Ligands and Complexes

CD indicates the shape of a peptide and allows studies about conformational changes. Because the formation of complexes with metal ions leads to more stabilized conformations, the CD curves should be altered. We performed such CD measurements to evaluate the shape of linear and cyclic hexapeptides and mainly to investigate the influence of complex formation with bivalent metal ions. CD curves of metal complexes represent an equilibrium of different states, e.g. different geometric forms and also oligomers.

The obtained CD curves show distinct differences between uncomplexed linear and cyclic peptides with respect to wavelength and sign of the peaks. Because the hexapeptides are unable to form classical α- and β-structures or a random coil, the obtained CD curves show only more or less ordered conformations. To exclude the influence of the pH value, the measurements were carried out in 25 mM borate buffer of pH 8.0. The solvent TFE favours the formation of H-bridges and of other non-covalent interactions leading to more ordered conformations. The CD curves of both free peptides show that effect (Figs. 2 and 3). TFE enhances the peak intensities irrespective of their sign. Complex formation with bivalent transition metal ions, except for Cu with the cyclic peptide, modifies only the basic spectra of linear and cyclic peptides without conversion into the other type. Thus, all complexes with the linear peptide have a minimum at about 220–230 nm and a maximum at about 190–205 nm, whereas complexes with the cyclic hexapeptide have their minima between 195 and 205 nm.

Copper^{2+} ions evoke at the cyclic peptide the most pronounced effect enhancing the intensity of both optima and shifting the negative peak from about 200 to 215 nm. The conformational influences of the other bivalent ions of Zn, Co, Ni on both the peptides are all in the same range. Only the transition metal Mn^{2+} shows, agreeing well with the MS the weakest influence on CD curves of linear and cyclic peptides. But, we have to keep in mind that the different coordination geometries (planar, pyramidal or tetrahedral), resulting from different metal ions, strongly contribute to the shape of the CD curve and to the molar ellipticity. Thus, the CD curves give no direct information about complex-formation tendencies. Copper ions seem to form different conformations and states of the complex with the cyclic hexapeptide compared to the other ions. For a better understanding of the unusual curve of the Cu complex, conformational studies with other methods are required, e.g. NMR and X-ray.

Complex-Formation Tendencies

Complex-formation tendencies of linear and cyclic pseudo hexapeptide with the bivalent ions Zn^{2+}, Ni^{2+}, and Cu^{2+} were estimated by a special MS method (comparison in pairs) [22] and for Cu^{2+} via the apparent dissociation constant [22,24].

MALDI- and ESI-MS spectra indicate a high tendency of the cyclic hexapeptide to form complexes with Ni^{2+} (Supplementary Information) and Cu^{2+}. Rank orders of complex-formation tendencies were estimated with the method of comparison in pairs estimating spectra from aqueous equimolar mixtures of two different metal ions with one ligand. Linear and cyclic peptides differ in their rank orders: linear peptide, Cu > Zn ~ Co ~ Ni; cyclic peptide, Ni > Co > Cu > Zn. Table 1 shows complex-formation tendencies qualitatively estimated by ESI-MS [22]. Also, this method indicates for the cyclic pseudohexapeptide the highest complex-formation tendency with Ni.

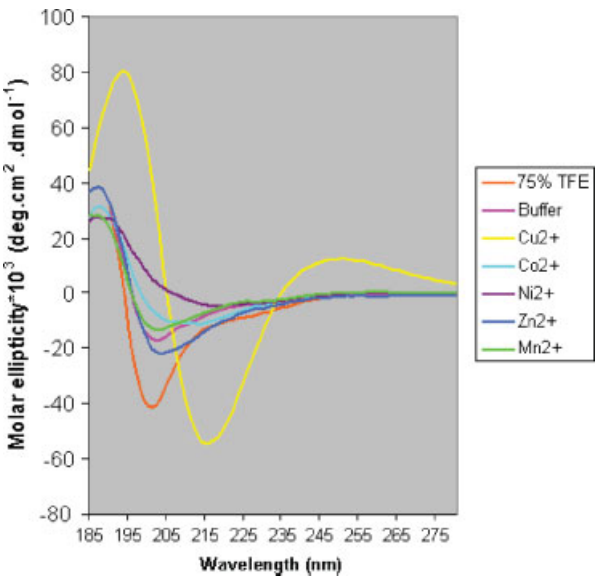

Figure 2 Circular dichroism spectra of linear pseudo hexapeptide in trifluoro ethanol (75%), in borate buffer (25 mM, pH = 8.0) and in complex with transition metal ions.

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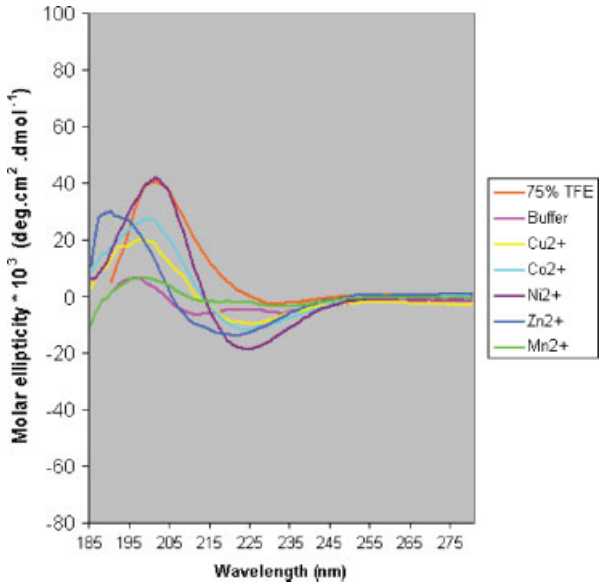

Figure 3 Circular dichroism spectra of cyclic pseudo hexapeptide in TFE (75%) and in borate buffer (25 mM, pH = 8.0) with certain transition metal ions.

Table 1 Metal complexes formed with linear and cyclic pseudo hexapeptide, estimated by ESI-MS

	ESI-MS		Complex-formation tendency
	Mol wt calcd	Mol wt found	
Cyclic hexapeptide	593.7	594.3	
Zn		656.3, 658.3	++
Ni		650.2, 652.3	++++
Co		651.3	++
Cu		656.3, 657.3	++
Linear hexapeptide	611.7	612.7	
Zn		674.4, 676.4	+++
Ni		668.4, 670.4	+++
Co		669.4	+++
Cu		673.4, 675.4	++++

The intensities of found peaks or pattern of complexes are indicated between + (low) and ++++ (very high)

To quantitatively evaluate the dissociation constants, we estimated the concentration of free Cu by an oxidation assay with ascorbic acid [24]. Because only free copper(II) catalyses oxidation of ascorbic acid, which can be followed spectrophotometrically, peptide ligands can be regarded as inhibitors and from various concentrations of ligands and ascorbic acid the conditional dissociation constants can be determined (Dixon plot). According to the rank order the linear hexapeptide has a slightly lower dissociation constant (3.1 μM) than the cyclic one (3.7 μM).

Catalytic Activities

Since it is difficult to predict or to design catalytic activities, we tested complexes of both the linear and the cyclic peptides for their ability to hydrolyse a phosphonic acid ester and to oxidize a catechol derivative. The catalytic activities were estimated under the same conditions as for phosphodiesterase [25] and catechol oxidase (tyrosinase assay) [26]. Hydrolytic activity was estimated from a Zn-complex, the oxidative activity from a complex with Cu^{2+}. Both catalytic

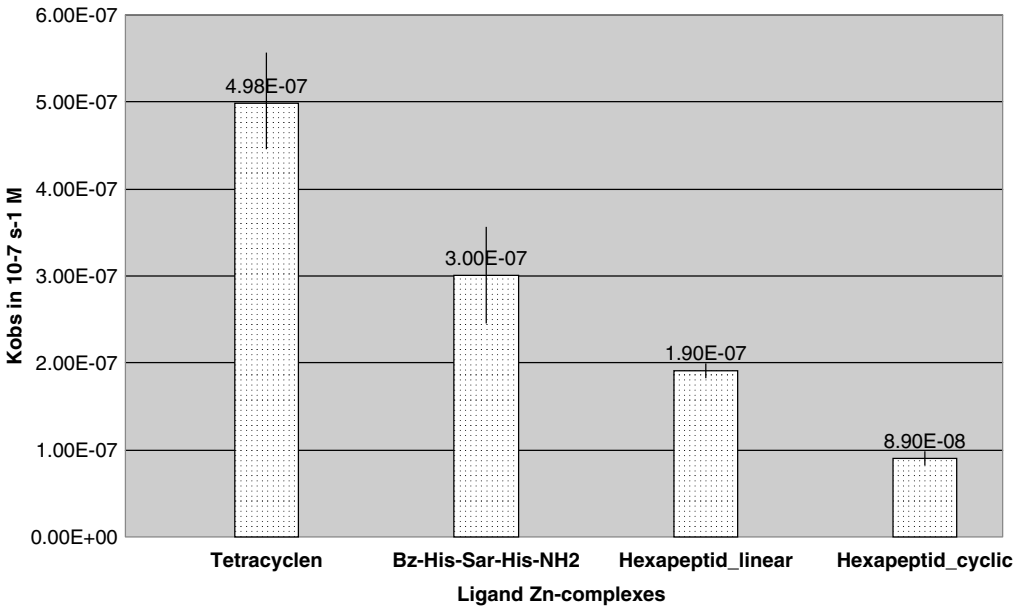

Figure 4 Phosphodiesterase-like hydrolytic activities of Zn-complexes of linear and cyclic pseudo hexapeptides compared to tetracyclen and a tripeptide from another series.

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Table 2 Catechol oxidase like activities of Cu^{2+} – complexes of linear and cyclic pseudo hexapeptide compared to monomeric and dimeric tripeptide from other series

	Sequence	K_M [M] 10^{-4}	k_{kat} [s^{-1}] 10^{-4}	k_{kat}/K_M [M^{-1} s^{-1}]
1 [41]	**Tyrosinase**	**2.5**	**$1 \cdot 10^7$**	
2	Pseudo hexapeptide linear	1.65 ± 0.6	0.66 ± 0.3	0.4 ± 0.2
3	Pseudo hexapeptide cyclic	11.7 ± 0.7	8.63 ± 0.6	0.74 ± 0.3
4 [38]	Bz-His-Sar-His-NH_2	2.42 ± 0.4	7.3 ± 0.5	3 ± 1
5[a]	H_2NCO-His ← Sar ← His ← L2 → His → Sar → His-$CONH_2$	1.4 ± 0.2	18.1 ± 0.1	12.8 ± 1

L2, Biphenylether dicarbonic acid.
[a] Manuscript submitted to JPS.

activities were compared to those of related enzymes, standard chelating compounds and to our most active peptides from other series [38]. Table 2 and Figure 4 clearly indicate that the catalytic (hydrolytic or oxidative) activities are low compared to the native enzymes, but also to 1,4,7,10-tetraaza cyclododecane [39] and linear monomeric [38] or dimeric tripeptides (publication submitted to this journal). Native enzymes belonging to the families of Zn-hydrolases or copper catechol oxidases have many times higher catalytic activities as the most synthetic peptides, including our peptides, independently from nearly the same affinity (K_M-value) to the substrate. Thus, the enzyme *E. coli* alkaline phophatase has a K_M from 21 mM and a k_{cat} from 80.5 s^{-1} [40], the enzyme tyrosinase a K_M-value from 25 mM and a k_{cat} from 10^3 s^{-1} [41]. Differences between linear and cyclic pseudo hexapeptide are only marginal. The cyclic hexapeptide is more active in the oxidative assay and the linear more active in the hydrolysis.

CONCLUSIONS

Both applied synthetic strategies allow assembly of the cyclic pseudo hexapeptide, each with intrinsic side reactions. Cyclization of linear precursors requires fully protected side chains. However, it remains a challenge to fully protect stabile and easily removable trifunctional moieties such as Lys, His and *N*-functionalized amino acids in combination with the linkage to the resin, and selective liberation of functional groups for cyclization. Because of the more convenient cyclization on the resin, this synthetic route was studied in more detail. Coupling of the His side chain to the resin by a trityl group fulfils the requirement for cyclization but has two disadvantages. Since the expected racemization of His is nearly undetectable, the formation of dioxo piperazine (DKP) must be taken into account. To avoid the formation of this side product, which seems difficult to be separated by the RP-HPLC method, a backbone linker attached onto an NH-group of Gly may be a better alternative for the Fmoc/tBu/allyl strategy of solid-phase cyclization. At the same time, this might be a good way to overcome the acid lability of Trt-resin which hampers the use of Zuckermann's method for stepwise assembly of *N*-functionalized derivatives.

Despite the different conformational shapes of linear and cyclic hexapeptide, as estimated by CD measurements, the complexes of transition metal ions show only marginal differences, except for Cu^{2+} and Mn^{2+}. The complexation with Cu leads, in the case of cyclic hexapeptide, to a more pronounced conformational change in the direction of an ordered structure than by the linear one. Mn^{2+} influences the shape of the CD curves only marginally, which agrees well with MS measurements for a very low complex-formation tendency. Despite the marginal differences found in the CD spectra for Zn, Co and Ni, their rank orders for complex-formation tendencies differ to some degree from those which were estimated with MS by comparison in pairs. They show the cyclic peptide as having a preference for Ni^{2+}, surprisingly similar to the computer design.

The catalytic activities, both hydrolase and oxidase, are rarely low compared to the standard compound tetracyclen or to active ones from our own pseudo peptide series. Also, the influence of cyclization on the catalytic activities seems to be marginal. From these results, we might draw the conclusion that as of now, experimental screening of peptides is more successful with regard to selective complexation than modelling. To design or predict catalytic activities remains unattainable. From our experiments, we learned that cyclization can in fact improve the ion selectivity but does not enhance the catalytic activities.

Supporting Information

Supporting information may be found in the online version of this article.

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