DOI: 10.1002/cbic.200500220

Preparation of Cyclo-Phen-Type Ligands: Chelators of Metal Ions as Potential Therapeutic Agents in the Treatment of Neurodegenerative Diseases

Christophe Boldron,[a] Ingrid Van der Auwera,[b] Céline Deraeve,[a] Heinz Gornitzka,[c] Stefaan Wera,[b] Marguerite Pitié,*[a] Fred Van Leuven,[d] and Bernard Meunier*[a]

Many recent studies have shown the major role played by metal ions (copper, zinc, iron, …) in the modification of the folding and/or the aggregation of proteins that leads to serious pathologies. Several neurodegenerative diseases (Alzheimer's disease, spongiform encephalopathies, Parkinson's disease, Huntington's disease, …) involve similar disastrous interactions between metal ions and proteins.[1]

In the case of Alzheimer's disease, the pathology is associated with the aggregation of β-amyloid peptides (Aβ) in the brain, which leads to the formation of amyloid plaques. The accumulation of redox-active metal ions in these amyloid plaques is probably responsible for the oxidative stress which induces neuronal lesions in the brain that result in irreversible loss of intellectual faculties.[2]

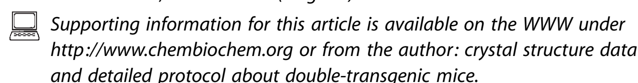
The use of a metal ligand like Clioquinol led to improvements in patients suffering from Alzheimer's disease and indicated that therapeutic approaches are possible with metal ion chelators in neurodegenerative diseases.[3] Among Cu/Zn chelators capable of solubilizing Aβ from post mortem brain tissue, interesting results were obtained with bathophenanthroline and bathocuproine, two ligands based on the 1,10-phenanthroline structure.[4] However, due to the presence of sulfate residues, these chelators are too hydrophilic to cross the blood brain barrier; consequently their use in vivo cannot be envisaged.

[a] *Dr. C. Boldron, C. Deraeve, Dr. M. Pitié, Dr. B. Meunier*
Laboratoire de Chimie de Coordination du CNRS
205 route de Narbonne, 31077 Toulouse Cedex 4 (France)
Fax: (+33) 561-55-30-03
E-mail: pitie@lcc-toulouse.fr
bmeunier@lcc-toulouse.fr

[b] *Dr. I. Van der Auwera, Dr. S. Wera*
NV reMYND,
Minderbroederstraat 12, 3000 Leuven (Belgium)

[c] *Dr. H. Gornitzka*
Laboratoire d'Hétérochimie Fondamentale et Appliquée (UMR 5069)
Université Paul Sabatier,
118 route de Narbonne, 31062 Toulouse Cedex 04 (France)

[d] *Dr. F. Van Leuven*
LEGT-EGG, K.U. Leuven
Herestraat 49, 3000 Leuven (Belgium)



Supporting information for this article is available on the WWW under http://www.chembiochem.org or from the author: crystal structure data and detailed protocol about double-transgenic mice.

© 2005 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim

Chelators must indeed have particular properties in order to be used as potential drugs in the treatment of neurodegenerative diseases. They must have a low molecular weight and be poorly or not charged in order to be able to cross the blood brain barrier. They must also have a structure that can be altered to adjust the chelation selectivity in order to target certain metal ions (a strong nonspecific chelation would result in a general depletion of metal ions, including those of metalloenzymes, which are essential for the organism).

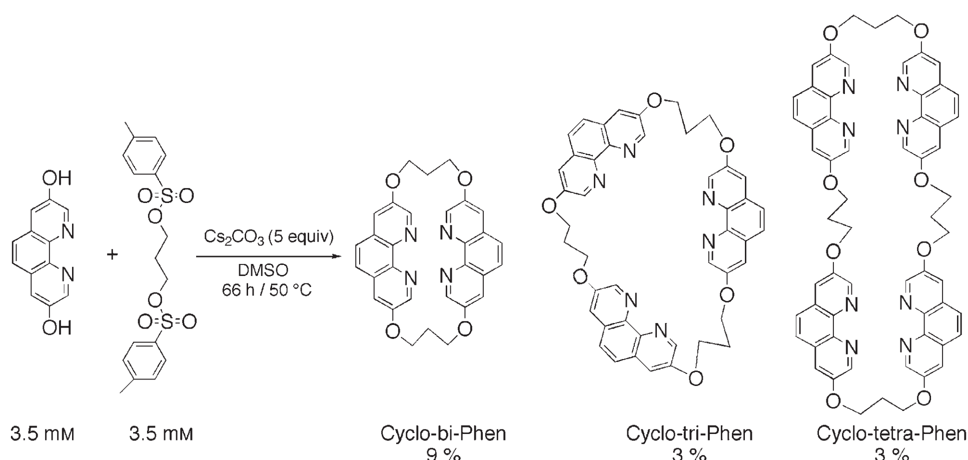
Once the chelator is in the brain, the molecule must be able to complex the metal ions present in excess in the pathogen proteins so as to allow their dissolution and their elimination. From here two evolutions can be proposed: i) if the metal–chelator complex is hydrophobic enough, it can be exported to the blood system, leading to its ultimate excretion;[3] or ii) it has also been proposed that the complex will contribute to the redistribution within the brain of metal ions sequestered in the plaques in order to restore their normal homeostasis and thus to stop the amyloid cascade.[5] Whatever the explanation of the role of chelator in brain, in both cases, the presence of an exogenous chelator will be beneficial and contribute to limiting the evolution of the disease.

Our recent work on Clip-Phen-type ligands and nucleic acid cleavage has demonstrated the benefit of complexing copper with two connected phenanthroline ligands.[6] These ligands can be structurally modulated, and Clip-Phen ligands with two Phen entities linked at their C3 carbon by a three-methylene bridge were particularly active. Interestingly, their activity was not altered by the presence of a large excess of physiological ions like Mg^{II} as was that of ligands that selectively chelate some metals (copper ions in our previous experiments). We therefore decided to prepare new cyclic uncharged ligands called "Cyclo-Phen", small and sufficiently hydrophobic to be able to cross the barriers: first the intestinal barrier, so that these molecules will be orally active, and then the blood brain barrier to finally coordinate the metal ions (copper in preference) that are present in excess in the pathogen amyloid aggregates.

We chose to connect two phenanthroline entities at the C3 and C8 positions with an alkoxy linker including three methylenes, in agreement with the structure of the most efficient linkage in the Clip-Phen series (on C3 of Phen). The second bridge is identical and is linked on the C8 carbon of Phen in order to obtain a compound with a C2 symmetry.

The Cyclo-bi-Phen macrocycle was prepared by using a bistosylate linker. The catalytic metathesis approach for macrocyclization cannot be used due to the structure of the desired linker, and different attempts at other classical synthesis strategies failed in our hands:[7] i) the reaction between 3,8-dihalogenoPhen and propanediolate through a synthetic strategy analogous to the preparation of Clip-Phen, although it is a classical method of preparing cyclic derivatives of 2,6-pyridine[8] (due to the specific problems associated with the position of the substituents on the Phen that induce poor chemical reactivity and reduction ability under the experimental conditions used);[6,9] ii) the fixation of one or two linker(s) on a first Phen derivative and, then, the reaction with a second Phen residue; iii) the use of a template effect by complexation of halogeno- (or hydroxy-)Phen precursor to different metals before the cyclization reaction in order to form $(\text{ligand})_2$–metal entities favoring Cyclo-bi-Phen synthesis.

Cyclo-bi-Phen was successfully prepared by treatment of 1,10-phenanthroline-3,8-dihydroxylate with propane-1,3-diol-di-*para*-tosylate at high dilution (3.5 mM) in order to favor the synthesis of Cyclo-bi-Phen and to decrease polymerization reactions (Scheme 1).[9,10] The replacement of propane-1,3-diol-di-*para*-tosylate by 1,3-dibromopropane or of Cs_2CO_3 by NaH, a change of solvent, or the use of higher concentrations decreased the yield. The structure of Cyclo-bi-Phen was confirmed by X-ray analysis of single crystals (Figure 1). Two other cyclic ligands, Cyclo-tri-Phen and Cyclo-tetra-Phen with, respectively, 3 and 4 phenanthroline residues, were also isolated as minor products. Both trimer and tetramer were also characterized by X-ray analysis.



Scheme 1. Synthesis of "Cyclo-Phen".

Three different chelating agents were then tested in double-transgenic mice mimicking Alzheimer's disease: Cyclo-bi-Phen, 3-propyl-Clip-Phen[6,9] (its analogue in the Clip-Phen series), and Clioquinol, chosen because it has shown activity in decreasing amyloid deposits in vivo (Figure 2).

Preliminary toxicity studies in wild-type male FVB/NCrl mice were performed. The drugs, initially dissolved in DMSO in the presence of 2.6 equivalents of HCl and then diluted in water, were tested at 10 $mg\,kg^{-1}$ by intraperitoneal injection (i.p.) on three consecutive days. At day 4, all mice survived and they

 © 2005 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim

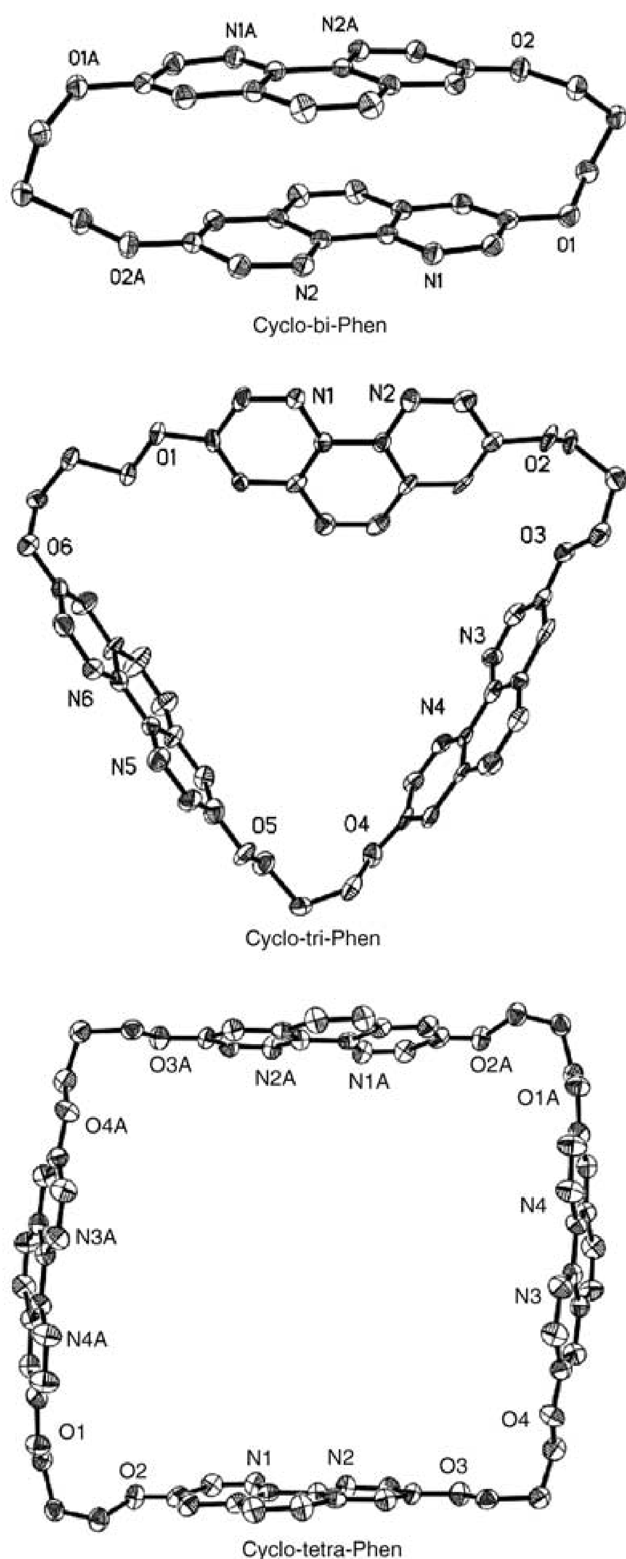


Figure 1. Molecular structures of Cyclo-bi-Phen (CCDC 268177), Cyclo-tri-Phen (CCDC 268178), and Cyclo-tetra-Phen (CCDC 268179).

were sacrificed and analyzed: no gross anatomical problems were observed in the stomach, spleen, kidneys, liver, heart, lungs, or peritoneum. Further tests included five wild-type mice treated on days 1 and 3 with 50 mg kg^{-1} per day by i.p. injection that showed 100% survival after 30 days. Therefore the compounds appeared nontoxic under these conditions at a concentration of 10 mg kg^{-1}.

Subsequently, double-transgenic APP[V717I]×PS1[A246E] female mice were treated with the drugs. This well-established double-transgenic mouse model was chosen since it shows virtually all aspects of the amyloid pathology of Alzheimer's disease: accumulation of Aβ peptides in the brain and cerebrospinal fluid, memory deficit, and amyloid plaque formation.[11] Moreover, diverse experimental therapies, ranging from vaccination to β-sheet breakers, have been shown to affect relevant parameters in this mouse model.[12]

The molecules, dissolved in DMSO/groundnut oil (1:4), were administered by i.p. injection (5 mg kg^{-1} for both Phen derivatives or 10 mg kg^{-1} for Clioquinol) three times per week over a nine-week period to six-month-old double-transgenic mice. Ten double-transgenic animals were treated with each Phen derivative, whereas nine transgenic mice received vehicle and nine transgenic mice received Clioquinol. During the nine-week period, two animals were lost in each Phen-treatment group and three in the Clioquinol group, as opposed to none in the vehicle control group; this indicates some toxicity associated with longer-term metal-chelating therapy. Mean body weight increased by about 2 g in all groups, possibly due to the use of oil as vehicle.

After nine weeks of treatment, the animals were sacrificed, amyloid peptides levels were assayed in one brain hemisphere, and amyloid plaque load was determined in the other hemisphere. None of the treatments appeared to affect the levels of soluble Aβ40 or Aβ42. Remarkably, treatment with 3-propyl-Clip-Phen increased the brain levels of insoluble Aβ40 and Aβ42 about twofold (from 54.7 ± 8.8 ng g^{-1} to 112.6 ± 13.1 ng g^{-1}, $p = 0.005$ for Aβ40 and from 265 ± 43 ng g^{-1} to 624 ± 91 ng g^{-1}, $p = 0.007$ for Aβ42), whereas neither Clioquinol nor Cyclo-bi-Phen affected these parameters in a statistically significant way. A similar picture emerged from the immunohistochemical staining of amyloid plaques. Here all treatments remained without significant effect, except 3-propyl-Clip-Phen, which increased total plaque load from 8.8 ± 1.6% to 15.5 ± 2.3% ($p = 0.03$). Thus, the previously observed ability of Clioquinol to reduce the load of insoluble Aβ and to increase the quantity of soluble β-amyloid peptide was not observed here.[3] These differences were probably due to the differences between experimental conditions (mono- versus double-transgenic mice, i.p versus oral drug administration, variations in dosing protocols, etc.).

Brain sections were subsequently analyzed by staining with Thioflavin-S, a dye with an affinity for β-sheet structures that specifically stains dense (so-called senile) amyloid plaques, as opposed to the immunological staining used above, which does not discriminate between senile and diffuse plaques.[13] Surprisingly, both Clioquinol and Cyclo-bi-Phen specifically reduced Thioflavin-S-positive plaque load by about 30–40% (Figure 2), although this is statistically insignificant, probably due to the small number of mice included in this study (from control levels of 1.0 ± 0.2% to 0.7 ± 0.2%; $p = 0.26$ for Clioquinol and to 0.6 ± 0.2%; $p = 0.1$ for Cyclo-bi-Phen). 3-Propyl-Clip-

 © 2005 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim

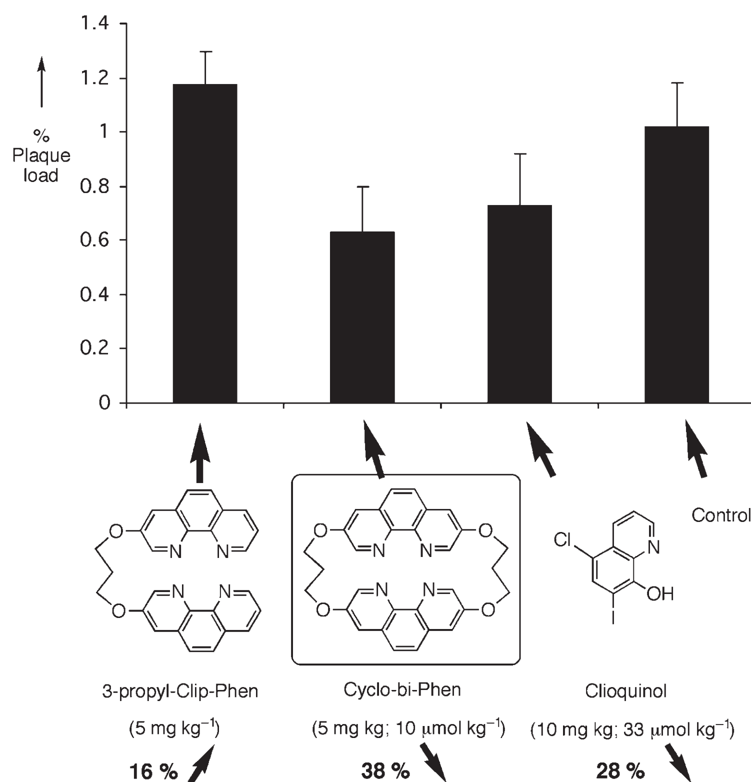


Figure 2. Quantitative analysis by histology of Thioflavin-S staining of the amyloid load in the subiculum of double-transgenic APP[V717I]×PS1[A246E] mice treated with 3-propyl-Clip-Phen, Cyclo-bi-Phen, or Clioquinol by intraperitoneal injection three times per week over nine-week periods.

Phen showed a tendency to increase Thioflavin-S-positive plaque load to $1.2\pm0.3\,\%$ ($p=0.5$).

Hence chemically related metal chelators appear to affect plaque load in opposite directions. Moreover, taking into consideration the difference in molecular weight of the two chelators that both decrease plaque load (504 for Cyclo-bi-Phen and 305 for Clioquinol), these results were obtained with $9.9\,\mu mol\,kg^{-1}$ of Cyclo-bi-Phen and $32.8\,\mu mol\,kg^{-1}$ of Clioquinol. We therefore conclude that Cyclo-bi-Phen exerts its effect at a threefold lower level of the drug.

The data obtained on the reduction of Thioflavin-S-stained amyloid deposits are of particular interest since Thioflavin-stained plaques might be selectively neurotoxic[14] through the induction of oxidative stress, a deleterious phenomenon, possibly related to the complexation of redox active metals by Aβ-peptides.[15]

Selective chelation of Cu^{II}, Zn^{II}, and, to a lesser extent, Fe^{III} by Aβ-peptides has been related to an increase in amyloid deposition. Our strategy being aimed at the removal of these metal ions, we estimated the stability constants of Cyclo-bi-Phen (L) for Cu^{II} and Zn^{II} spectrophotometrically through competition experiments with different ligands with known binding constants for copper and zinc.[16] Similar values to those obtained for Clioquinol were observed (Table 1) and were in accordance with our attempts to prepare a ligand that had a mildly "complexing ability", capable of removing metals from Aβ and thereby decreasing the formation of amyloid plaques. After treatment with one equivalent of $CuCl_2$ or of $ZnCl_2$, complex formation was confirmed by electrospray mass spectrometry, and $[M-Cl]^+$ complexes were observed ($m/z=602$ and 603 for $LCuCl_2$ and $LZnCl_2$, respectively). The partition of Cyclo-bi-Phen between octanol and a physiological aqueous phase was also measured; this resulted in an observed $\log D_{7.4}$ value of 2.7, which is in accordance with our attempts to prepare a ligand exhibiting a significant hydrophobicity.

In conclusion, the reduction of the plaque load observed with Cyclo-bi-Phen indicates that the Cyclo-Phen derivatives can be considered as drug candidates in the treatment of neurodegenerative diseases in which an over-loading of metal ions in the brain has been evoked as being one of the main factors in the pathologies related to metal-related misfolding of proteins.

Table 1. Comparison of some physicochemical properties of Cyclo-bi-Phen and Clioquinol. $D_{7.4}$ is the distribution coefficient of the ligands between octan-1-ol and Tris·HCl buffer (20 mM pH 7.4, 150 mM NaCl). $K_{Cu^{II}}$ and $K_{Zn^{II}}$ are the stability constants of the ligands.

	M_W	$\log D_{7.4}$	$\log K_{Cu^{II}}$	$\log K_{Zn^{II}}$
Cyclo-bi-Phen	504	2.7	10 ± 1	6 ± 1
Clioquinol	305	3.8	8.9 [a]	7 [a]

[a] From ref. [3].

Experimental Section

Synthesis of Cyclo-Phen: Caesium carbonate (2.22 g, 6.83 mmol) was added to 3,8-dihydroxy-1,10-phenanthroline hydrobromide[9] (0.40 g, 1.37 mmol) dissolved in anhydrous DMSO (310 mL) under nitrogen. Then a solution of 1,3-propanediol di-*para*-tosylate (0.53 g, 1.37 mmol) in anhydrous DMSO (80 mL) was added over 1 h, before the mixture was heated for 48 h at 50 °C under nitrogen and with vigorous stirring. The volume was reduced to 100 mL, then aqueous ammonia (40 mL, pH 10) was added, and cyclized products were extracted with CH_2Cl_2 (2×40 mL). The organic phase was washed with aqueous ammonia (pH 10), evaporated, and dried under vacuum. Chromatography on silica gel (eluent 1% triethylamine (TEA) in $CHCl_3$) afforded Cyclo-bi-Phen (31 mg, 0.06 mmol, 9%) as a white powder. A mixture of Cyclo-tri-Phen and Cyclo-tetra-Phen was then eluted from the column with $CHCl_3/CH_3OH$/TEA (94:5:1). After evaporation of the solvent, the two products were dissolved in $CHCl_3/CH_3OH$ (9:3), and Cyclo-tetra-Phen was precipitated by the addition of CH_3OH (6 volumes). The supernatant was evaporated, and a flash chromatography on silica gel (eluent 1% TEA in $CHCl_3$) afforded Cyclo-tri-Phen (14 mg, 0.013 mmol, 3%) as a white powder. Pure Cyclo-tetra-Phen was obtained from crystallization in $CHCl_3/CH_3OH$ (3:1) as white crystals (10 mg, 0.01 mmol, 3%). For each ligand, white crystals suitable for X-ray analysis were obtained from recrystallization in $CHCl_3/CH_3OH$. Cyclo-bi-Phen: ^1H NMR (250 MHz in $CDCl_3/CD_3OD$ 3:1): $\delta=2.12$ (m, 4H), 4.15 (m, 4H), 4.35 (m, 4H), 6.98

 © 2005 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim

(d, ^{4}J(H,H) = 3 Hz, 4 H), 7.19 (s, 4 H), 8.21 (d, ^{4}J (H,H) = 3 Hz, 4 H); elemental analysis calcd (%) for $C_{30}H_{24}N_4O_4\cdot 0.6\,H_2O$: C 69.92, H 4.93, N 10.87; found C 70.01, H 4.94, N 10.53; MS (ES > 0): 505 $[M+H]^+$. Cyclo-tri-Phen: ^{1}H NMR (250 MHz in $CDCl_3/CD_3OD$ 3:1): $\delta = 2.21$ (quint, ^{3}J(H,H) = 5 Hz, 6 H), 4.20 (t, ^{3}J(H,H) = 5 Hz, 12 H), 7.26 (d, ^{4}J-(H,H) = 3 Hz, 6 H), 7.36 (s, 6 H), 8.50 (d, ^{4}J(H,H) = 3 Hz, 6 H); elemental analysis calcd (%) for $C_{45}H_{36}N_6O_6\cdot CHCl_3$: C 63.05, H 4.23, N 9.59; found C 62.61, H 4.57, N 9.01; MS (ES > 0): 757 $[M+H]^+$. Cyclo-tetra-Phen: ^{1}H NMR (250 MHz in $CDCl_3/CD_3OD$ 3:1) : $\delta = 2.31$ (m, 8 H), 4.20 (m, 16 H), 7.37 (d, ^{4}J(H,H) = 3 Hz, 8 H), 7.49 (s, 8 H), 8.54 (d, ^{4}J(H,H) = 3 Hz, 8 H); elemental analysis calcd (%) for $C_{60}H_{48}N_8O_8\cdot 2\,CHCl_3$: C 59.68, H 4.04, N 8.98; found C 59.78, H 3.62, N 8.56; MS (ES > 0): 1009 $[M+H]^+$.

Animal studies were approved by the Ethical Commission on Animal Testing (K.U. Leuvan, Belgium) under Project number P04026.

Acknowledgements

PALUMED S. A. and the CNRS are gratefully acknowledged for their financial support. We thank Dr. L. Azema for his technical help.

Keywords: amyloid · chelates · drug design · neurodegenerative diseases · phenanthroline

[1] a) P. M. Doraiswamy, A. E. Finefrock, *Lancet Neurol.* **2004**, *3*, 431 – 434; b) K. J. Barnham, C. L. Masters, A. I. Bush, *Nat. Rev. Drug Discovery* **2004**, *3*, 205 – 214.
[2] a) M. P. Mattson, *Nature* **2004**, *430*, 631 – 639; b) M. Citron, *Nat. Rev. Neurosci.* **2004**, *5*, 677 – 685.
[3] R. A. Cherny, C. S. Atwood, M. E. Xilinas, D. N. Gray, W. D. Jones, C. A. McLean, K. J. Barnham, I. Volitakis, F. W. Fraser, Y.-S. Kim, X. Huang, L. E. Goldstein, R. D. Moir, J. T. Lim, K. Beyreuther, H. Zheng, R. E. Tanzi, C. L. Masters, A. I. Bush, *Neuron* **2001**, *30*, 665 – 676.
[4] a) R. A. Cherny, K. J. Barnham, T. Lynch, I. Volitakis, Q.-X. Li, C. A. McLean, G. Multhaup, K. Beyreuther, R. E. Tanzi, C. L. Masters, A. I. Bush, *J. Struct. Biol.* **2000**, *130*, 209 – 216; b) R. A. Cherny, J. T. Legg, C. A. McLean, D. P. Fairlie, X. Huang, C. S. Atwood, K. Beyreuther, R. E. Tanzi, C. L. Masters, A. I. Bush, *J. Biol. Chem.* **1999**, *274*, 23 223 – 23 228.
[5] T. A. Bayer, S. Schäfer, A. Simons, A. Kemmling, T. Kamer, R. Tepest, A. Eckert, K. Schüssel, O. Eikenberg, C. Sturchler-Pierrat, D. Abramowski, M. Staufenbiel, G. Multhaup, *Proc. Natl. Acad. Sci. USA* **2003**, *100*, 14 187 – 14 192.
[6] M. Pitié, C. Boldron, H. Gornitzka, C. Hemmert, B. Donnadieu, B. Meunier, *Eur. J. Inorg. Chem.* **2003**, *8*, 644 – 652.
[7] a) C. Kaes, A. Katz, M. W. Hosseini, *Chem. Rev.* **2000**, *100*, 3553 – 3590; b) H. An, J. S. Bradshaw, R. M. Izatt, *Chem. Rev.* **1992**, *92*, 543 – 572; c) G. R. Newkome, J. D. Sauer, J. M. Roper, D. C. Hager, *Chem. Rev.* **1977**, *77*, 513 – 597.
[8] G. R. Newkome, H. C. R. Taylor, F. R. Fronczek, V. K. Gupta, *J. Org. Chem.* **1986**, *51*, 970 – 974.
[9] C. Boldron, M. Pitié, B. Meunier, *Synlett* **2001**, *10*, 1629 – 1631.
[10] E. Weber, H.-P. Josel, H. Puff, S. Franken, *J. Org. Chem.* **1985**, *50*, 3125 – 3132.
[11] a) I. Dewachter, J. van Dorpe, L. Smeijers, M. Gilis, C. Kuipéri, I. Laenen, N. Caluwaerts, D. Moechars, F. Checler, H. Vanderstichele, F. Van Leuven, *J. Neurosci.* **2000**, *20*, 6452 – 6458; b) I. Dewachter, J. van Dorpe, K. Spittaels, I. Tesseur, C. Van Den Haute, D. Moechars, F. Van Leuven, *Exp. Gerontol.* **2000**, *35*, 831 – 841.
[12] a) B. Permanne, C. Adessi, G. P. Saborio, S. Fraga, M.-J. Frossard, J. van Dorpe, I. Dewachter, W. A. Banks, F. van Leuven, C. Soto, *FASEB J.* **2002**, *16*, 860 – 862; b) R. Etcheberrigaray, M. Tan, I. Dewachter, C. Kuipéri, I. Van der Auwera, S. Wera, L. Qiao, B. Bank, T. J. Nelon, A. P. Kozikowski, F. van Leuven, D. L. Alkon, *Proc. Natl. Acad. Sci. USA* **2004**, *101*, 11141 – 11146; c) D. Frenkel, I. Dewatcher, F. van Leuven, B. Solomon, *Vaccine* **2003**, *21*, 1060 – 1065; d) R. Zurbriggen, *J. Mol. Neurosci.*, in press.
[13] K. R. Bales, T. Verina, R. C. Dodel, Y. Du, L. Altstiel, M. Bender, P. Hyslop, E. M. Johnstone, S. P. Little, D. J. Cummins, P. Piccardo, B. Ghetti, S. M. Paul, *Nat. Genet.* **1997**, *17*, 263 – 264.
[14] B. Urbanc, L. Cruz, R. Le, J. Sanders, K. Hsiao Ashe, K. Duff, H. E. Stanley, M. C. Irizarry, B. T. Hyman *Proc. Natl. Acad. Sci. USA* **2002**, *99*, 13 990 – 13 995.
[15] a) M. E. McLellan, S. T. Kajdasz, B. T. Hyman, B. J. Bacskai, *J. Neurosci.* **2003**, *23*, 2212 – 2217; b) Y. Matsuoka, M. Picciano, J. La Francois, K. Duff, *Neuroscience* **2001**, *104*, 609 – 613.
[16] The method for determining the stability constants will be reported elsewhere.

Received: May 26, 2005
Published online on October 6, 2005

 © 2005 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim