

MICHAEL MEYER*, RÜDIGER SCHNURRE[†],
ROLF REISSBRODT[‡], WOLFRAM TROWITZSCH-KIENAST[†]

Computer-aided design of novel siderophores: Pyridinochelin

*Konrad-Zuse-Zentrum
[†] Technische Fachhochschule Berlin
[‡] Robert-Koch-Institut

Computer-aided design of novel siderophores: Pyridinochelin

Michael Meyer*, Rüdiger Schnurre†,
Rolf Reissbrodt‡, Wolfram Trowitzsch-Kienast†

Abstract

Pyridinochelin, a novel catecholate type siderophore, has been designed on the basis of the active analog enterobactin. Growth promotion tests indicate that this synthetic siderophore feeds various pathogenic bacteria effectively with iron even though it lacks one catecholate group compared to enterobactin. The superposition of the siderophore structures suggests that the structure of the skeleton connecting the catecholate groups might be an important factor for the iron transport.

Introduction

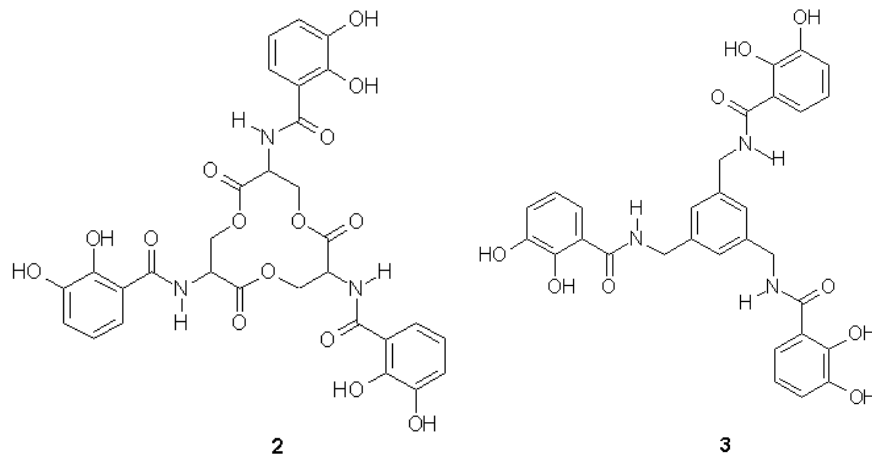
Bacteria produce siderophores, highly specific low molecular weight ligands, for iron supply.^{1a-f} We have designed novel ligands because it is a promising strategy to use conjugates of siderophores and antibiotics as a Trojan horse in order to increase the concentration of the latter only at the site of action.^{1f} Furthermore, siderophores can be used to promote the growths of bacteria to facilitate diagnostics. They can be used also in the medical treatment of patients suffering from an excess of iron ions and they are useful for the medication of viral infections. Cinatl et al. proved siderophores to interfere with the uridine uptake into the viral DNA.² Shanzer and others^{3a-e} demonstrated antibiotic activities of siderophores, especially for lipophilic complexing agents, against the *Plasmodium falciparum* causing malaria. Most applications require that a synthetic ligand has a specific structure to be able to form complexes with iron *and* to be recognized by the receptor.

We have designed and synthesized an effective novel iron transporter of the catecholate [2,3-dihydroxy benzoate(DHB)]-type termed pyridinochelin⁴ (Bis-2,3-dihydroxybenzoyl-2,6-dimethylamino-pyridin, **1**) using an active analog approach. The enterobactin receptor FepA seems to be a unique bacterial receptor of siderophores recognizing the DHB group in nature. The structure of FepA has been determined recently,⁵ but as the electron density in the putative enterobactin binding region was not well defined, structure based design techniques could not be used. Furthermore, these methods focus on the design of molecules which bind specifically to a protein with a high affinity,^{6a-e} but

*Konrad-Zuse-Zentrum für Informationstechnik Berlin, Takustr. 7, D-14195 Berlin

†Technische Fachhochschule Berlin, FB II, Luxemburger Str. 10, D-13353 Berlin

‡Robert-Koch-Institut, D-38855 Wernigerode



ligands derived this way might be poor iron transporters. Enterobactin (ent, **2**) provides an ideal basis for active analog modeling because the formation constant of $[\text{Fe}(\text{ent})]^{3-}$ is about 10^{52} while the affinity towards alkali and alkaline earth metals tends to be much lower. The structures of the $[\text{M}(\text{ent})]^{3-}$ anions do not have rotatable bonds eliminating the problem to find out the bioactive conformation from a set of potential conformations for active analog modeling.

2 is a natural catecholate-type siderophore with a trilactone ring anchoring skeleton consisting of three L-serine residues, which induce a Δ -configuration of the three bidentate catechol groups at the metal nucleus (right handed propeller). In addition to Fe^{III} , enterobactin forms isostructural complexes with Ga^{III} , Cr^{III} and V^{IV} .^{7a-e} These ions have been used often in theoretical and experimental studies as a model for iron, because the ionic radii are very similar to that of the ferric ion. The X-ray structure of the V^{IV} -enterobactin complexes^{7d,e} and the computed structures of Ga^{III} with **2** and MECAM, **3**,⁸ a synthetic siderophore with a non-chiral backbone, have been taken as active analogs.

Results and Discussion

Computations

The structures of the Ga^{III} complexes with the ligands **2** (Figure 1) and **3** have been optimized at HF/6-31G(d) level^{9a-c} with Gaussian94¹⁰ for the Δ -configuration. The V^{IV} ^{7d,e} and Ga^{III} complexes with **2** and **3** have been superimposed using a least squares fit of the metal atoms and all catechol oxygen atoms in order to generate a pseudo receptor with LUDI.^{11a-c} The pseudo receptor, a cavity which is complementary to the shape of the superimposed active siderophores described above, restricts the size of the ligands created by the program similar to the real binding pocket of the FepA receptor. Then interaction sites, donor or acceptor sites located at the corresponding atom positions within the cavity, are derived from the enterobactin metal complexes. In the next step pharmacophores assumed to be important for metal binding and transport were selected and placed inside the cavity. The selection of the phar-

macrophore was derived from previous iron uptake investigations with different ligands. These experiments have shown that the absolute configuration at the iron atom is essential for iron uptake. In contrast to the natural Δ -enterobactin, synthetical Λ -enantiomer enterobactin (left handed propeller) derived from D-serine does not support growth.^{1a} Consequently the first pharmacophore consists of the tris-catecholamide moiety adopting the Δ -configuration as in natural enterobactin complexes. We decided to leave the amide group at the catechol rings unchanged, because a previous study with derivatives of MECAM has shown that iron uptake is greatly reduced if the amide groups are alkylated or if the carbonyl and methylene functions are interchanged.^{1a} In the first case intramolecular hydrogen bonds between the amide hydrogen atom and the ortho-oxygen atom (Figure 1) were made impossible, the latter case might indicate that the carbonyl groups are involved in hydrogen bonding with FepA. Previous analogs of **2** retained three catecholate groups whereas we prove here that molecules with bis-catecholate moieties are efficient iron transporter in bacteria. This hypothesis has been derived from our previous conclusion that certain ligands are able to promote the growth of bacteria¹² even though they are only able to use two from three catecholate groups for the binding of a single metal ion.¹³ If less than 6 coordination groups are available, complexes with a ratio between the metal and the ligand different from 1:1 may be formed like in other siderophores^{1c} or water molecules may be added to form octahedral complexes.

In the final step a search for suitable fragments was performed in order to link the pharmacophore to a complete molecule with an anchoring skeleton. These fragments had to satisfy three conditions. They must be small enough to fit into the cavity, the atom types of the fragments should correspond to the interaction sites derived from the active molecules and third, the geometry of the fragments has to be suitable to link the pharmacophore without strain. The superposition of the most interesting fragment from the database, 2,4-dimethyl thiazole, with the skeleton shows that the nitrogen acceptor atom of this fragment corresponds to the serine side chain oxygen acceptor atom OG. The dimethyl thiazole geometry fits snugly into the skeleton geometry of $[M(\text{ent})]^{3-}$. The distance between both methyl group carbon atoms of 2,4-dimethyl thiazole is 4.932 Å at HF/6-31G(d) level, whereas the corresponding distance between the methylene carbon atoms is 4.649 ± 0.074 Å in the X-ray structure of $[V(\text{ent})]^{3-}$ and 4.712 Å in the calculated structure of $[Ga(\text{ent})]^{3-}$.

In contrast to the alkyl chains, aromatic ring linkers are more rigid and thus probably freeze the bioactive conformation. Therefore we computed the geometries of heteroaromatic linkers. The distances between both methyl group carbon atoms of 2,6-dimethyl pyridine is 4.825 Å and therefore this linker is able to fix the catecholamide groups of **1** in a conformation corresponding to the one of enterobactin metal complexes (Figure 2). Apart from the different number of catecholamide groups it is related to **3**⁸ having a mesitylene fragment as the skeleton. However **3** does not contain any acceptor atom in the skeleton, which probably leads to the less effective iron transport compared to enterobactin. In contrast, **1** has a single acceptor atom N, which can be superimposed on the serine side chain oxygen atom OG in the lactone ring (Figure 2). In the siderophore with a 2,4-dimethylthiazole linker even the carbonyl oxygen atom O is mimicked by the sulfur atom, but it is not known whether the carbonyl oxygen is involved in hydrogen bonds with FepA or not. We will report on the properties of other heterocyclic linkers in a further publication. In contrast to **1**

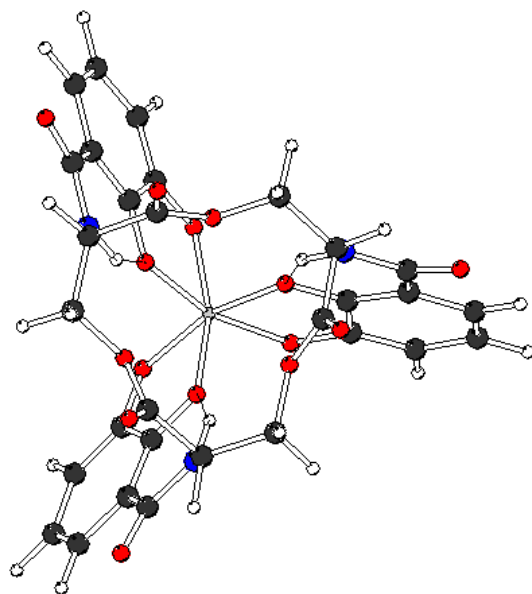


Figure 1: MOLSCRIPT¹⁹ representation of the calculated [Ga(ent)]³⁻ structure.

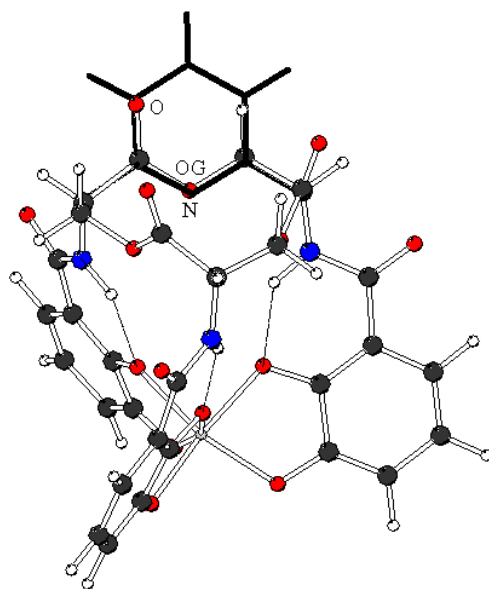
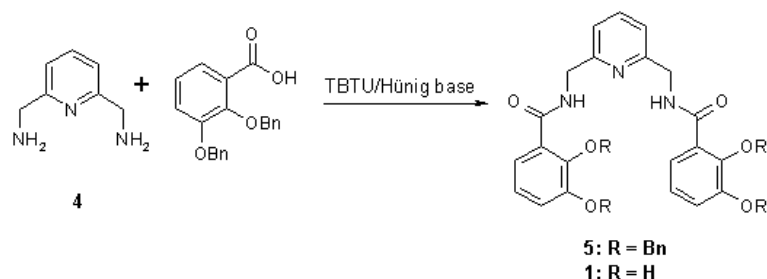
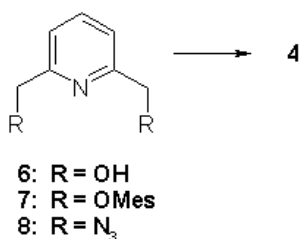


Figure 2: Superposition of the 2,6-dimethylpyridine linker (bold bonds) on the skeleton of the calculated [Ga(ent)]³⁻ structure.



Scheme 1. Synthesis of pyridinochelin **1**



Scheme 2. Alternative route to **4**

the phenyl rings of 3,5-bis(ortho-hydroxyphenyl)-1,2,4-triazole¹⁴ cannot adopt a conformation like the ones of $[\text{M}(\text{ent})]^{3-}$ and acceptor atoms corresponding to the carbonyl oxygen atoms are missing. Therefore the iron uptake through the FepA receptor with derivatives of this ligand might be less efficient, even though it is an excellent ligand for iron.

Synthesis and physico-chemical properties of pyridinochelin

To our surprise, screening the literature for our calculated disubstituted heterocyclic ligands did not result in any publication. Since the 2,6-bis-methylamino-pyridine **4** was a well known compound produced by the Gabriel synthesis from bis-2,6-bromomethyl-pyridin and phthalimide¹⁵, the synthesis of the pyridinochelin was straight forward. Providing **4** by the described way, we only had to combine the diamine **4** with the benzyl protected 2,3-dihydroxy benzoic acid¹² to get the protected di-amide **5**. For this purpose we used the TBTU/Hünig-base approach (Scheme 1). Deprotection to **1** was achieved smoothly by hydrogenation with the Pd/charcoal catalyst.

Because of the availability of the 2,6-bis-hydroxymethyl-pyridin **6**, we also prepared **4** on an alternative way by transforming **6** into the di-mesylate **7**, and that into the di-azide **8** (Scheme 2). We could isolate **4** in good yields by catalytic reduction on Pd/charcoal.

Physico-chemical data

Bis-2,3-dibenzoyloxybenzoyl-2,6-dimethylamino-pyridin **5**

¹H-NMR (*CDCl*₃, 400 MHz): δ (ppm) = 8.64 (t, J = 5.2 Hz, 2H, 2 x -CH₂-NH), 7.72 (m, 2H, DHB), 7.50 (t, J = 7.73, 1H, Pyridin-4-H), 7.42-7.31 (m, 11 H, arom).

H), 7.17-7.06 (m, 15 H, arom. H), 5.09 (s, 4H, 2 x -O-CH₂-), 4.99 (s, 4H, -O-CH₂-), 4.46 (d, J = 5.2 Hz, 4H, 2 x -CH₂-NH-).

¹³C-NMR (CDCl₃, 100.6 MHz): δ(ppm) = 165.28 (s, 2 x C=O), 156.69 (s, 2 x =C-O-CH₂-), 151.91 (s, 2 x =C-O-CH₂-), 146.90 [s, 2 x -C(=O)-C=], 137.26 (d, pyridin-C-4), 136.5 (s, 2 x benzyl-C-1), 136.2 (s, 2 x benzyl-C-1), 128.78, 128.67, 128.43, 128.38, 128.24, 127.71(all d, 28 x arom. C), 124.40 (d, 2 x arom. C-H), 123.3 (d, 2 x arom. C-H), 120.15 (d, 2 x arom. C-H), 117.07 (d, 2 x arom. C-H), 76.22 (t, 2 x -O-CH₂-phe), 71.34 (t, 2 x -O-CH₂-phe), 45.14 (t, 2 x -CH₂-NH-).

EI-MS : m/z (%) = 769 (39), [M]⁺, 678 (100) [M-benzyl].

for C₄₉H₄₃N₃O₆ calculated C 76.44, H 5.63, N 5.46, O 12.47, found C 76.52 H 5.66 N 5.31.

Bis-2,3-dihydroxybenzoyl-2,6-dimethylamino-pyridin 1

tlc (Kiesegel, Merck SiF₆₀, : 0.60 (DCM+10 % MeOH).

¹H-NMR (CD₃OD, 400 MHz): δ(ppm) = 7.76 (t, J= 8.6 Hz, partially exchanged, 2H, 2 x -NH-), 7.32, [m (broad), 5H, 3x pyridin-H, 2x DHB-6'-H), 6.99 (d, J= 8.3 Hz, 2H, 2 x DHB-4'-H), 6.70 (dd, J = 8.3 Hz, 2H, 2 x DHB-5'-H), 4.74 [s, (broad), 4H, 2 x -CH₂-NH-).

FAB-MS (negative mode):m/z (%) : 408.3 (100)[M-H]⁻, 272.4 (70)[M-H-DHB]⁻.

FAB-MS (positive mode):m/z (%) : 432.3 (100) [M+H+Na]⁺, 410.4 (85)[M+H]⁺, for C₂₁H₁₉N₃O₆ (409.40) calculated: C 61.61, H 4.68, N 10.26, O 23.45, found C 61.45, H 4.71, N 10.15.

Biological studies on pyridinochelin

Up to now it was an unwritten law, that an effective siderophore has to have a hexadentate ligand system. Consequently we gave the pyridinochelin only a little chance to be a good siderophore. But, in contrary, it turns out to be one of the best hitherto known synthetic siderophore. Table I outlines the extraordinary capacity of **1** as a siderophore. To test the siderophoric activity we used the protocol published by Reissbrodt et al.¹⁶ This test was performed as a growth promotion test, and strains of different bacteria were used, which lack their natural siderophores. The diameters of the growth zones measured in mm correlate with the siderophoric capacity showing its activity in the ng-range comparable with activities measured for enterobactin.

Some important conclusions can be drawn from the above results. Pyridinochelin cannot feed *E. coli* bacteria lacking the enterobactin *and* the tonB system. This proves **1** to use the tonB mediated enterobactin transport system. *Klebsiella pneumoniae* was said to possess also the enterobactin transport and utilization system, but **1** does not promote growth of these bacteria. Contrarily behaved **1** on *Mycobacterium smegmatis*. It very effectively promoted growth. However, enterobactin was unable to promote this strain,¹⁷ hence the effective uptake of **1** by *M. smegmatis* requires a different uptake system. The growth zones documented for the *Salmonella* species equal almost the zones generated by enterobactin. This is the first example for a synthetic siderophore with the same activity as for enterobactin. In addition, pyridinochelin also feeds mycobacteria quite effectively, one of the reasons for our studies. It should be noted, that **1** exhibits quite good activity against *Plasmodium falciparum*.¹⁸

Table I. The siderophoric capacity of Pyridinochelin 1 at diverse pathogenic bacteria with growth zones (GC)

Species	GC (mm)
<i>Pseudomonas aeruginosa</i> PAO 6609	16
<i>Yersinia enterocolitica</i> H 5030	24
<i>Aeromonas hydrophila</i> SB 22	lysis ^{a)} 14; 34
<i>Klebsiella pneumophila</i> KN 4401	0
<i>Salmonella typhimurium</i> TA 2700	44
<i>Salmonella typhimurium</i> WR 1223	45
<i>Salmonella typhimurium</i> enb-7	44
<i>Salmonella enteritidis</i> P 125109	40
<i>Escherichia coli</i> AB 2847	28
<i>Escherichia coli</i> (tonB)	0
<i>Mycobacterium smegmatis</i> 987	28

a) lysis means inhibition for higher concentrations of pyridinochelin, growth promotion with low concentrations only.

Conclusion

Pyridinochelin designed by active analog modeling acts as a surprisingly active synthetic siderophore for several pathogenic bacteria, even though it lacks one catecholamide group compared to enterobactin.

Acknowledgement

We thank Dr. H. J. Böhm and Dr. S. K. Buchanan for additional information about LUDI and the FepA receptor, respectively, and Dr. H.-D. Ambrosi, AnalytiCon AG Potsdam, for synthesis support.

References

- [1] (a) Raymond, K. N.; Müller, G., Matzanke, B. F. *Top. Curr. Chem.* 1984, 123, 49-102. (b) Raymond, K. N. *Pure & Appl. Chem.* 1994 66, 773-781. (c) J. B. Neilands, *J. Biol. Chem.* 1995, 270, 26723-26726. (d) Drechsel, H.; Jung, G. *J. Peptide Sci.* 1998, 4, 147-181. (e) Drechsel, H.; Winkelmann, G. in *Transition Metals in Microbial Metabolism* (Eds.: Winkelmann, G.; Carrano, C. J.), Harwood Academic Publishers, London, 1997; pp. 1-49. (f) Rosenberg, J. M.; Lin, Y.-M.; Lu, Y.; Miller, M. J. *Curr. Med. Chem.* 2000, 7, 159-197.

- [2] Cinatl Jr., J.; Hoffmann, F.; Cinatl, J.; Weber, B.; Scholz, M.; Rabenau, H.; Stieneker, F.; Kabickova, H.; Blasko, M.; Doerr, H.W. *Antiviral Research* 1995, 31, 23.
- [3] (a) Hider, R. C.; Liu, Z. *J. Pharm. Pharmacol.* 1997, 59-64. (b) Shanzer, A.; Libman, J.; Lytton, S. D.; Glickstein, H.; Cabantchik, Z. I. *Proc. Natl. Acad. Sci. USA*, 1991, 88, 6585-6589. (c) Raventos-Suarez, C.; Pollack, S.; Nagel R. L. *Am. J. Trop. Med. Hyg.* 1982, 31, 919-922. (d) Golenser, J.; Tsafack, A.; Amichai, Y.; Libman, J.; Shanzer, A.; Cabantchik, Z. I. *Antimicrob. Agents Chemother.* 1995, 61-65; (e) Jenett-Siems, K.; Kraft, C.; Koehler, I.; Siems, K.; Jas, G.; Ambrosi, H.; Trowitzsch-Kienast, W.; Mockenhaupt, F. P.; Bienzle, U.; Eich, E. Myxochelins, iron chelators with antimalarial properties, Symposium on Drugs against parasitic Diseases, Montpellier, France, 24.-16.5.1999, Abstract Book p. 95.
- [4] patent pending.
- [5] Buchanan, S. K.; Smith, B. S.; Venkatramani, L.; Xia, D.; Esser, L.; Palnitkar, M.; Chakraborty, R.; van der Helm, D.; Deisenhofer, J. *Nature Struct. Biol.* 1999, 6, 56-63.
- [6] (a) Böhm, H. J. *Prog. Biophys. molec. Biol.* 1996, 66, 197-210. (b) Böhm, H. J.; Klebe, G. *Angew. Chem.* 1996, 108, 2750-2778. (c) Marrone, T. J.; Briggs, J. M.; McCammon, J. A. *Annu. Rev. Pharmacol. Toxicol.* 1997, 37, 71-90. (d) Amzel, L. M. *Curr. Opin. Biotech.* 1998, 9, 366- 369; (e) Meyer, M.; Schomburg D. *Protein Interactions in Biotechnology*, Vol. 5a (Eds.: Rehm, H. J.; Reed G.), Wiley-VCH, Weinheim, 1999; pp. 88-108.
- [7] (a) Ised, S. S.; Kuo, G.; Raymond, K. N. *J. Am. Chem. Soc.* 1976; 98, 1763-1767. (b) Raymond, K. N.; Ised, S. S.; Brown, L. D.; Fronczek, F. R.; Nibert, J. H. *J. Am. Chem. Soc.* 1976, 98, 1767-1774. (c) Stack, T. D. P.; Karpishin, T. B.; Raymond, K. N. *J. Am. Chem. Soc.* 1992, 114, 1512-1514. (d) Karpishin, T. B.; Raymond, K. N. *Angew. Chem. Int. Ed. Engl.* 1992 31, 466. (e) Karpishin, T. B.; Dewey, T. M.; Raymond, K. N. *J. Am. Chem. Soc.* 1993,115, 1842- 1851.
- [8] Harris, W. R.; Raymond, K. N. *J. Am. Chem. Soc.* 1979, 101, 6534-6541.
- [9] (a) Hehre, W. J.; Ditchfield, R.; Pople, J. A. *J. Chem. Phys.* 1972, 56, 2257-2261. (b) Frand, M. M.; Pietro, W. J.; Hehre, W. J.; Binkley, J. S.; Gordon, M. S.; DeFrees, D. J.; Pople, J. A. *J. Chem. Phys.* 1982,77, 3654-3665. (c) Hariharan, P. C.; Pople, J. A. *Theoret. Chim. Acta* 1973, 28, 213-222.
- [10] Frisch, M. J.; Trucks, G. W.; Schlegel, H. B.; Gill, P. M. W.; Johnson, B. G.; Robb, M. A.; Cheeseman, J. R.; Keith, T. A.; Petersson, G. A.; Montgomery, J. A.; Raghavachari, K.; Al-Laham, M. A.; Zakrzewski, V. G.; Ortiz, J. V.; Foresman, J. B.; Cioslowski, J.; Stefanov, B. B.; Nanayakkara, A.; Challacombe, M.; Peng, C. Y.; Ayala, P. Y.; Chen, W.; Wong, M. W.; Andres, J. L.; Replogle, E. S.; Gomperts, R.; Martin, R. L.; Fox, D. J.; Binkley, J. S.; Defrees, D. J.; Baker, J.; Stewart, J. P.; Head-Gordon, M.; Gonzalez, C.; Pople J. A. *GAUSSIAN 94*, Revision B.3, Gaussian, Inc., Pittsburgh PA (1995).
- [11] (a) Böhm, H.-J., *J. Comput-Aided Mol. Design* 1992, 6, 61-78. (b) Böhm, H.-J. *J. Comput- Aided Mol. Design* 1992, 6, 593-606. (c) Böhm, H.-J. *J. Comput-Aided Mol. Design* 1996, 10, 265-272.
- [12] Ambrosi, H. D.; Hartmann, V.; Pistorius, D.; Reissbrodt, R.; Trowitzsch-Kienast, W. *Eur. J. Org. Chem.* 1998, 541-551.
- [13] Meyer, M.; Trowitzsch-Kienast, W. *J. Mol. Struct. (Theochem)* 1997, 418, 93-98.

- [14] Heinz, U.; Hergetschweiler, K.; Acklin, P.; Faller, B.; Lattmann, R.; Schnebli, H. *P. Angew. Chem. Int. Ed.* 1999, 38,2568-2570.
- [15] Buhleier, E; Wehner, W.; Vögtle, F. *Liebigs Ann. Chem.* 1978, 537-644.
- [16] Reissbrodt, R.; Heinisch, L.; Möllmann, U.; Rabsch, W.; Ulbricht, H. *BioMetals* 1993, 6, 155-162.
- [17] Matzanke, B.F.; Böhnke, R.; Möllmann, U.; Reissbrodt, R.; Schünemann, V.; Trautwein, A.X. *BioMetals* 1997, 10, 193-203.
- [18] K. Jenett-Siems, FU Berlin, personal communication.
- [19] Kraulis, P. J. *Appl. Crystallogr.* 1991, 24, 946-950.