Introduction

Bacteria produce siderophores, highly specific low molecular weight ligands, for iron supply (Raymond et al., 1984; Raymond, 1994; Neilands, 1995; Drechsel and Jung, 1998; Drechsel and Winkelmann, 1997; Rosenberg et al., 2000). 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 (Rosenberg et al., 2000). Furthermore, siderophores can be used to promote the growth 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. Doerr et al. (1995) proved siderophores to interfere with the uridine uptake into the viral DNA. Shanzer and others demonstrated antibiotic activities of siderophores, especially for lipophilic complexing agents, against the Plasmodium falciparum causing malaria (Hider and Liu, 1997; Shanzer et al., 1991; Raventos-Suarez et al., 1982; Golensker et al., 1995; Jenett-Siems et al., 1999). 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 Pyridinochelin (bis-2,3-dihydroxybenzoyl-2,6-dimethyl-amino-pyridine (I)) using an active analog approach. Enterobactin (ent,2) provides an ideal basis for active analog modeling because the formation constant of [Fe(ent)]³⁺ is about 10⁴² while the affinity towards alkali and alkaline earth metals tends to be much lower. The structures of the [M(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. The enterobactin receptor FepA seems to be an unique bacterial receptor of siderophores recognizing the DHB group in nature. The structure of FepA has been determined recently (Buchanan et al., 1999), but as the electron density in the putative binding region of 2 (Scheme 1) 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 (Bohm, 1996; Bohm and Klebe, 1996; Marrone et al., 1997; Amzel, 1998; Meyer and Schomburg, 1999), but ligands derived this way might be poor iron transporters. Another complication is the high degree of protein flexibility involved in iron transport, because the FepA is believed to act like an air lock with two hatches in iron transport through the pore.
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), 2 forms isostructural complexes with Ga(III), Cu(III) and V(IV) (Ized et al., 1976; Raymond et al., 1976; Stack et al., 1992; Karpishin et al., 1993). 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.

Results and Discussion

Computations

The structures of the Ga(III) complexes with the ligands 2 (Fig. 1) and 3 have been optimized at HF/6-31G(d) level (Hehre et al., 1972; Francel et al., 1982) with Gaussian94 (Frisch et al., 1995) for the Δ-configuration. In order to generate a pseudoreceptor restricting the size of putative ligands, the V(IV) (Karpishin et al., 1993) and Ga(III) complexes with 2 and MECAM 3 (Harris and Raymond, 1979), a synthetical siderophore with a non-chiral backbone, have been superimposed using a least squares fit of the metal atoms and all catechol oxygen atoms. Default parameters have been used for both programs, except for a maximal RMS of 0.04 nm in the active analog mode of LUDI (Böhm, 1992; Böhm, 1992a; Böhm, 1996a).

Interaction sites, donor or acceptor sites located at the corresponding atom positions within the receptor, are derived from the 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 pharmacophore was derived from previous iron uptake investigations with different ligands. These experiments show that the absolute configuration at the iron atom is essential for iron uptake. In contrast to the natural Δ-enterobactin, synthetical Δ-enantio enterobactin (left handed propeller) derived from D-serine does not support growth (Raymond et al., 1984). Consequently the first pharmacophore consists of the tris-catecholamide moiety adopting the Δ-configuration as in natural 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 (Raymond et al., 1984). In the first case intra molecular hydrogen bonds between the amide hydrogen atom and the ortho-oxygen atom (Fig. 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 transporters in bacteria. This hypothesis has been derived from our previous conclusion that certain ligands are able to promote the growth of bacteria (Ambrosi et al., 1998) even though they are only able to use two from three catecholate groups for the binding of a single metal ion (Meyer and Trowitzsch-Kienast, 1997). 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 (Nellands, 1995) 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 proposed by LUDI, 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(ent)]3. The distance between both methyl group carbon atoms of 2,4-dimethyl thiazole is 0.4932 nm at HF/6-31G(d) level, whereas the corresponding distance between the methylene carbon atoms is 0.4649 ± 0.0074 nm in the X-ray structure of [V(ent)]3 and 0.4712 nm in the calculated structure of [Ga(ent)]3.

In contrast to the alkyl chain linkers, aromatic rings are more rigid and thus probably freeze the bioactive conformation. Therefore we computed the geometries of further potential heteroaromatic linkers. The distances between both methyl group carbon atoms of 2,6-dimethyl pyridine is 0.4825 nm and therefore this linker is able to fix the catecholamide groups of 1 in a conformation corresponding to the one of complexes with 2. Apart from the different number of catecholamide groups it is related to 3 (Harris and Raymond, 1979) having a mesitylene fragment in the skeleton. However 3 does not contain any acceptor atom in the skeleton, which probably leads to the less effective iron transport compared to 2. 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 (Fig. 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. As 1 shows probably multiple binding sites and ligand orientations at the experimental structure of FepA (Buchanan et al., 1999) and some relevant coordinates are missing in the protein structure, we could not compare the pseudoreceptor with the biopolymer structure. In contrast to 1 selected as a first test of our hypothesis, the phenyl rings of 3,5-bis(ortho-hydroxyphenyl)-1,2,4-triazole (Heinz et al., 1999) cannot adopt a conformation like the ones of [M(ent)]3 and acceptor atoms corresponding to the carbonyl oxygen atoms are missing.

Synthesis and physicochemical properties
Since the 2,6-bis-methylamino-pyridine (4) was a well known compound produced by the Gabriel synthesis from bis-2,6-bromomethyl-pyridine and phthalimide (Buhleier et al., 1978), the synthesis of 1 was straight forward (Scheme 2). Providing 4 by the described way, we only had to combine the
diamine-4 with the benzyl protected 2,3-dihydroxy benzoic acid (Ambrosi et al., 1998) to get the protected di-amide 5. For this purpose we used the TBTU/Hünig-base approach [TBTU = 0-(benzo-triazol-1-yl)-N,N,N',N'-tetramethyluronium tetra-fluoroborate]. Deprotection to 1 was achieved smoothly by hydrogenation with the Pd charcoal catalyst. Because of the availability of the 2,6-bis-hydroxymethyl-pyridine (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 3). We could isolate 4 in good yields by catalytic reduction on Pd charcoal.

Scheme 3. Alternative route from 6 to 4 with the intermediates di-mesylate 7 and di-azide 8. 4 is used in the synthesis pathway to 1.

Bis-2,3-dihydroxybenzoyl-2,6-dimethylanilino-pyridine (1): mp 210 °C; tlc (Kieselgel, Merck SiF₆₀, 0.60 (DCM+10% MeOH); ¹H-NMR (CD₂OD, 600 MHz) δ (ppm) = 7.79 (t, J = 7.75 Hz, 1H, pyridine 4-H), 7.33 (d,J = 7.76 Hz, 2H, pyridine 3-H and 5-H), 7.31 (dd, J₁ = 1.36 Hz, J₂ = 7.85 Hz, 2H, 2 × DHB 4-H or 6-H), 6.99 (dd, J₁ = 1.35 Hz, J₂ = 7.85 Hz, 2H, 2 × DHB 6-H or 4-H), 6.76 (t, J = 7.98 Hz, 2H, 2 × DHB-H-5), 4.74 (s, 4H, 2 × -CH₂-NH-); ¹³C-NMR (CD₂OD, 300.1 MHz): δ (ppm) = 171.5 (2 × amide-CO), 158.7 (pyridine C-2 and C-6), 150.2 and 147.3 (2 × DHB C-2 and C-3), 1329.2 (pyridine C-4), 121.1, 119.8 and 118.9 (2 × DHB C-4, C-5, C-6 and pyridine C-3 and C-5), 116.7 (2 × DHB C-1), 45.4 (2 × -CH₂-NH); UV λ_max (ε) = 315 nm (5.400); 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]⁺; Anal. (C₂₁H₁₆N₃O₆) C calcd., 61.61; found 61.45; H: calcd., 4.68; found, 4.71; N: calcd., 10.26; found, 10.15.

Bis-2,3-dibenzoylbenzoyl-2,6-dimethylanilino-pyridine (5): mp 116 °C; ¹H-NMR (CDCl₃, 400 MHz) δ 8.64 (t, J = 5.2 Hz, 2H, 2 × -CH₂-NH), 7.72 (m, 2H, DHB), 7.50 (t, J = 7.73, 1H, Pyridine-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 × -O-CH₂-), 4.99 (s, 4H, -O-CH₂-), 4.46 (d, J = 5.2 Hz, 4H, 2 × -CH₂-NH-); ¹³C-NMR (CDCl₃, 100.6 MHz) δ (ppm) = 165.28 (s, 2 × C=O), 156.69 (s, 2 × =C-0-CH₂-), 151.91 (s, 2 × =C-0-CH₂-), 146.90 (s, 2 × =C-0-CH₂-), 137.26 (d, pyridine-C-4), 136.5 (s, 2 × benzyl-C-1), 136.2 (s, 2 × benzyl-C-1), 128.78, 128.67, 128.48, 128.38, 128.24, 127.71 (all d, 28 × arom. C), 124.40 (d, 2 × arom. C-H), 123.3 (d, 2 × arom. C-H), 120.15 (d, 2 × arom. C-H), 117.07 (d, 2 × arom. C-H), 76.22 (t, 2 × -O-CH₂-phe), 71.34 (t, 2 × -O-CH₂-phe), 45.14 (t, 2 × -CH₂-NH-); EI-MS m/z (%) 769 (39), [M]⁺, 678 (100)[M-benzyl]; Anal. (C₄₉H₄₅N₉O₆) C calcd., 76.44; found., 76.52; H: calcd., 5.63; found, 5.66; N: calcd., 5.46; found, 5.51.

Biological studies

To determine the siderophoric activity we carried out growth promotion tests by seeding the iron-starved siderophore-indicator strains listed in Table 1 into iron-depleted nutrient agar media (Reissbrodt et al., 1993). Each batch of these plates was controlled with natural siderophores. Filter paper discs were loaded with 1 µg of 1 and 2, as estimated by UV-spectroscopy. Cross-feeding
plates with these dried discs on the surface were incubated at least 20 h at of 37 °C (S. typhimurium, Klebsiella pneumonia, E. coli, M. smegmatis) and 30 °C (P. aeruginosa, Y. enterocolitica), respectively. All tests were performed at least twice. Cross-feeding tests yield the growth promotion of 1 relative to 2.

Tetradentate siderophores like azotochelin and aminobactins have been described previously (Duhme et al., 1998; Telford et al., 1998). But up to now it was an unwritten law that an effective siderophore for iron transport has to have a hexadentate ligand system. Consequently we gave 1 only a little chance to be a good siderophore. But, in contrast, it turns out to be one of the best hitherto known synthetic siderophores, even better than 2. Table I outlines the extraordinary capacity of 1 as a siderophore determined with growth promotion tests (Reissbrodt et al., 1993). The diameters of the growth zones measured in mm correlate with the siderophoric capacity showing its activity in the nanogram-range comparable with activities measured for 2. In contrast, Bis 2,3-dihydroxybenzoyl-2-methylamino pyridine lacking one catecholamide group relative to 1 is completely inactive (data not shown).

Some important conclusions can be drawn from the above results. 1 cannot feed E. coli bacteria lacking the enterobactin and the tonB system. This proves 1 to use besides the FepA receptor the tonB mediated enterobactin transport system. Klebsiella pneumonia was said to possess also the enterobactin transport and utilization system but 1 does not promote the growth of these bacteria. In contrast, 1 very effectively promoted growth of Mycobacterium smegmatis. However, 2 is unable to promote this strain (Matzanke et al., 1997), hence the effective uptake of 1 by M. smegmatis requires a different uptake system.

Amonobactins, tetradentate ligands, cannot singly satisfy the octahedral coordination sphere of iron. It is the M_{2}L_{3} complex which fully satisfies the coordination geometry of the ferric ion. The behaviour of the 1:1 ferric aminobactin complexes should be comparable to that observed for the dihydroxybenzoylserine linear dimer derived from 1 (Telford et al., 1998). 1 and aminobactin promoted a number of Gram-negative bacteria as S. typhimurium, E. coli, E. agglomerans, Y. enterocolitica, and also to M. smegmatis. Checking the growth promotion of 1 in a cross-feeding test in comparison with freshly isolated 2 produced by the FepA mutant E. coli AN 311 (Langman et al., 1972), S. typhimurium enb-7 exhibited 1 to be superior since the growth zone was about 50% larger.

**Conclusion**

We have described a rational strategy for the design of novel siderophores. The derived model suggests that acceptor atoms at the skeleton might be important for efficient iron transport, whereas only two catecholamide groups are required. 1 designed this way acts as a surprisingly active synthetic siderophore for several pathogenic bacteria. The growth zones documented for the Salmonella species equal at least the zones generated by 2. This is the first example for a synthetic tetradentate siderophore with the same activity as 2. In addition, 1 also feeds mycobacteria quite effectively, one of the objectives for our studies. It should be noted, that 1 exhibits quite good activity against a multi-resistant strain of Plasmodium falciparum (Jenett-Siems, 2000).
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