Natural Silicon Complexes

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The *E. coli* Siderophores Enterobactin and Salmochelin Form Six-Coordinate Silicon Complexes at Physiological pH**

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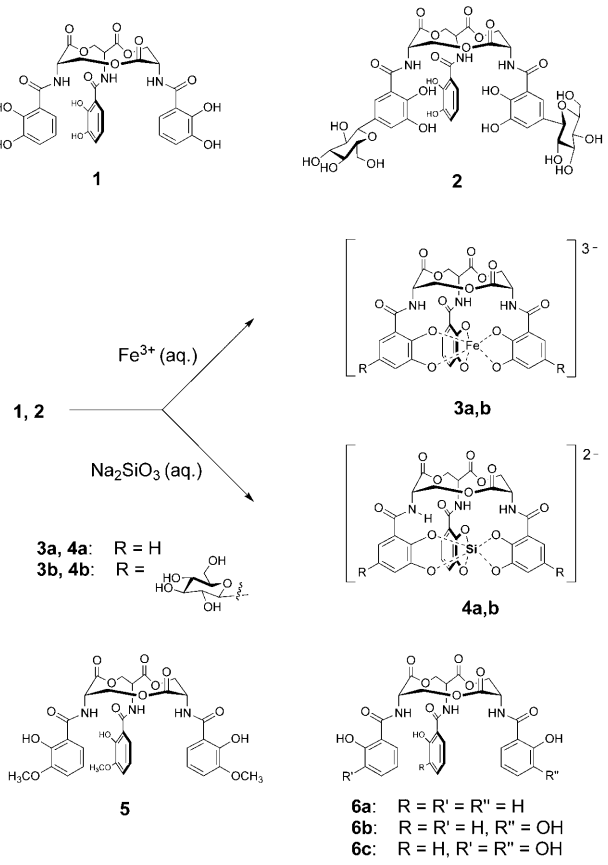
Dedicated to Professor Siegfried Blechert on the occasion of his 65th birthday

Iron is essential for nearly all organisms, because it is a key component of many metalloenzymes that catalyze redox reactions of critical importance for cellular growth. Typically, Fe^{3+} concentrations of 10^{-6}–10^{-5} M are required for growth of most bacterial species, but under aerobic conditions Fe^{3+} is not readily bioavailable because of the formation of poorly water-soluble polymeric iron aquo-hydroxo complexes. As a result, the concentration of soluble iron is as low as 10^{-10} M at pH 7.4.[1a] To extract iron from the environment, bacteria and fungi produce low-molecular-weight chelators, termed siderophores, which possess high Fe^{3+} affinity. The chelating moieties are typically catechol, hydroxamate, and carboxylate groups. Among them, enterobactin (Ent; **1**), produced by *E. coli* and *Salmonella*,[2] exhibits the highest binding constant observed thus far. In humans, iron is found in iron-binding proteins, such as transferrin and ferritin, and is a central constituent of myoglobin, hemoglobin, and P450-type monooxygenases. In all cases the iron complex formation constants are orders of magnitude lower than what is observed in bacterial siderophores. Consequently, protein-bound iron in humans can be extracted by siderophores which are therefore considered bacterial virulence factors.[3]

The twofold C-glycosylated enterobactin salmochelin (**2**, Scheme 1), isolated from uropathogenic *E. coli* and *Salmonella enterica*, was recently characterized.[4,5] Surprisingly, we have now found that enterobactin and salmochelin bind Si^{IV} with high affinity to afford the first examples of silicon complexes of natural products that are stable under physiological conditions. Moreover, our study suggests that Si^{IV} forms a six-coordinate complex with octahedral geometry.



Scheme 1. Structures of catecholate-type siderophores. Models of iron (**3**) and silicon complexes (**4**) of enterobactin (Ent, **1**) and salmochelin (Sal, **2**) synthesized and sequestered under low-iron conditions from *E. coli* and *Salmonella* strains.[2,5] Structures of synthetic model siderophores **5** and **6a–c**.

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Hypervalent silicon complexes, especially the hexacoordinate complexes, have been widely studied by inorganic chemists.[6,7] The syntheses of cationic,[8] anionic,[7] and also neutral[9,10] hexacoordinated silicon complexes have been reported. However, these compounds have not been thoroughly studied in a biological context. The report of a transient hexavalent silicon complex in the diatom *Navicula pelliculosa*[11] is an exception.

Silicon complexes of Ent (**1**) and Sal (**2**) were identified by close inspection of HPLC-ESI-MS chromatograms of filtrates from *E. coli* cultures grown in glass flasks; we observed a signal for $[M-H]^-$ (692 Da) with a mass difference of $\Delta m =$ 24 amu compared to enterobactin and a signal for $[M-H]^-$ (1016 Da) with a mass difference of $\Delta m = 24$ amu compared to salmochelin. Based on these mass spectrometric signatures, we assumed a close structural correlation of these compounds. The exact molecular masses of enterobactin, salmochelin, and their Si complexes, Si-Ent (**4a**) and Si-Sal (**4b**), were determined by high-resolution ESI orbitrap MS as $[M-H]^-$: 692.0809 (**4a**; $C_{30}H_{22}N_3O_{15}Si$) and $[M-H]^-$: 1016.1865 (**4b**; $C_{42}H_{42}N_3O_{25}Si$) (see the Supporting Information). Hence, compared to enterobactin and salmochelin, the Si-Ent and Si-Sal forms are accompanied by the formal loss of four protons upon Si binding. Incubating enterobactin (**1**) and salmochelin (**2**) with sodium silicate (Na_2SiO_3) under physiological conditions (pH 7.6) in a buffer solution reproducibly yielded Si-Ent and Si-Sal as monomeric complexes. Larger quantities of Si-Ent and Si-Sal were generated by this procedure and purified by solid-phase extraction (SPE) and preparative HPLC (see the Supporting Information).

One-dimensional (1D) ^{29}Si NMR spectra of Si-Sal (Figure 1) and Si-Ent (see the Supporting Information) each exhibit a single peak at -140 ppm which, in the majority of studied cases, is characteristic of hexaoxo silicon complexes,[12–14] ($\delta(^{29}Si) = -135$ to -145 ppm), whereas chemical shifts of $\delta = -71$ to -110 ppm are typical for tetraoxo silicon complexes and values of $\delta = -98$ to -110 ppm are common for pentaoxo silicon complexes. Accordingly, the NMR data suggest hexaoxo-coordinated silicon in the siderophore complexes. NMR spectra do not indicate the direct involvement of carbohydrate residues in Si complexation. Two distinctively different binding motifs, denoted as catecholate and salicylate binding, have been proposed for Fe^{3+}-enterobactin complexes,[15] with the degree of protonation determining the binding mode adopted. Comparison of the IR and NMR spectra (see the Supporting Information) of enterobactin and salmochelin with their silicon complexes argue for the involvement of all three catecholate residues in silicon binding. IR spectra show no shift in the carbonyl bands of the benzoic acid residues, rebutting their direct participation in complex formation.

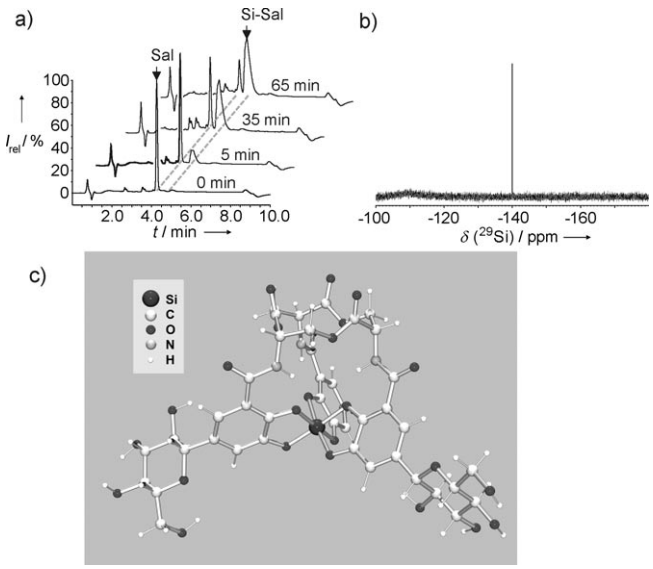

Figure 1. Investigation of the salmochelin-Si complex. a) In vitro transformation of salmochelin (**2**) into the Si-Sal (**4b**) monitored by HPLC-ESI-MS. The sequence of chromatograms shows the decrease of salmochelin and an increase of Si-Sal over time (100 mM Tris buffer, 10 mM Si^{IV} (Na_2SiO_3), pH 7.6). b) 1D ^{29}Si NMR spectrum of Si-Sal ([D_6]DMSO; standard: TMS) with a signal at $\delta(^{29}Si)$ -140 ppm. c) Calculated structure of the Si-Sal complex.

To further assess the possibility of a salicylate-binding mode, 3-methoxyenterobactin **5** (SER(3M)SAM) was synthesized following published procedures.[15] In contrast to enterobactin, **5** contains three blocked 3-hydroxy groups and it should coordinate Fe^{III} by formation of the corresponding salicylate-type complexes. In our studies the binding properties of both systems to Fe^{3+} and silicon were tested under the same conditions with aqueous solutions of $FeCl_3$ and Na_2SiO_3, respectively. In accordance with the literature, the binding of Fe^{3+} by **5** was observed with ESI mass spectrometry, whereas the Si complex formed only with enterobactin. In addition, time-dependent NMR experiments were performed by addition of $Si(OMe)_4$ to solutions of **1** and **5** in [D_6]DMSO. Formation of the Si-Ent complex (**4a**) was completed after 24 h, whereas the ^{1}H NMR spectra of **5** remained unchanged, which speaks against salicylate-type interactions.

To address the steric constraints imposed by the methoxy groups of **5**, we prepared a mixture of compounds **6a–c** and enterobactin (**1**; see the Supporting Information) and assayed for Si^{IV} binding. Direct ESI-MS measurements did not show signals indicative of silicon complexes of SERSAM (**6a**) or SERCAM(SAM)$_2$ (**6b**), which mainly rely on salicylate-type binding (see the Supporting Information). As an interpretation of these results we suggest the formation of anionic octahedral Si complexes of enterobactin and salmochelin as shown in Scheme 1. Final conclusions regarding the coordination geometry may result only from X-ray structure data obtained from the silicon complexes and more detailed studies with enterobactin and salmochelin derivatives.

To gain further insight into the different binding modes and their energetics, we carried out quantum chemical simulations combined with classical molecular-mechanics-based molecular dynamics simulations. We estimate the solution-phase free energy of binding to be 25 kcal mol^{-1} more negative for salmochelin than for enterobactin. Whereas the magnitude of this energy difference is likely exaggerated and unrealistic, this result suggests a fundamental trend that is interesting. The local structures for Si binding in enterobactin and salmochelin are identical in their Si–O bond lengths and coordination geometry (see the Supporting

Information), indicating that the glycosylation does not lead to an intrinsically stronger Si–O bond. The calculated structure of the Si-Sal complex is shown in Figure 1. Figure 2 presents our conceptual proposal of binding affinities under physiological conditions explaining the surprising result.

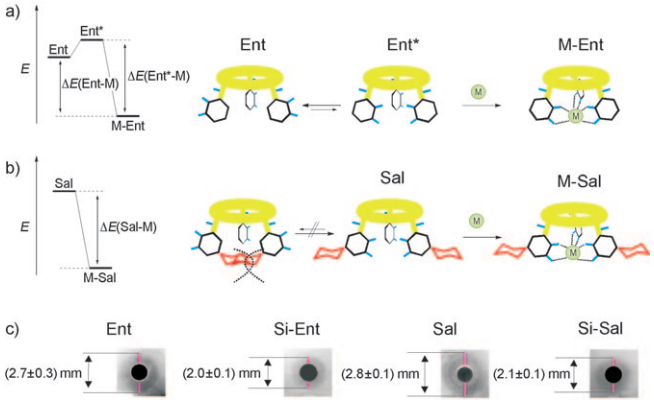

Figure 2. Conceptual model of silicon binding by a) enterobactin and b) salmochelin. c) Growth response of an *E. coli* strain in iron-deficient medium to enterobactin (Ent), salmochelin (Sal), Si-enterobactin (Si-Ent), and Si-salmochelin (Si-Sal).

In free enterobactin (**1**), the hydrophilic hydroxy groups point to the water-exposed exterior and thus a hydrophobic center forms in the molecule, comparable to the situation in the hydrophobic collapse of proteins. The glycosylation of salmochelin (**2**) at the C5 position of the phenyl moiety forces the hydroxy groups to point to this center, generating less solvated hydroxy moieties. Consequently, the driving force to expel a proton and bind the highly charged cation is increased in salmochelin (**2**) compared to enterobactin (**1**). To bind silicon, enterobactin has to first rotate the oxygen-carrying side of the catecholate moiety inwards, to afford the conceptual conformer Ent* (Figure 2). In salmochelin, the catecholate groups are already correctly orientated to bind silicon (Figure 2). Our calculations show that the difference in the gas-phase enthalpies of binding is 10 kcal mol^{-1} in preference for salmochelin, which we attribute to this rotational motion required for binding. A second energetic contribution comes from loss of solvation energy, which is expected to be more significant for enterobactin, as the exposure of the hydroxy groups to polar solvents on the reactant side should give much higher solvation energy for the reactant compared to the product, in which these hydrophilic groups are used to bind silicon. In the case of salmochelin this solvation penalty of silicon binding is expected to be much smaller because the hydroxy groups are already pointed inwards. Our calculations quantify this effect to be 16 kcal mol^{-1}.

Although these energies must be evaluated with some reservation, the underlying conceptual difference in binding is plausible and supports the conclusion that glycosylation of enterobactin should, in general, lead to a more effective binding of ions in polar, preferably aqueous solvents. Our proposed concept of binding highlights a simple and effective strategy for controlling the affinity of siderophores towards cations which may be amenable for further technical exploitation. Since the intrinsic binding energy between a cation and the contact ligand atom is fixed, the cation binding constant can be controlled by varying the binding pocket. However, at physiological conditions the improved solubility and therefore better availability of the glycosylated form of enterobactin may play a more dominant role in its binding abilities.

To further characterize the binding behavior of both enterobactin and salmochelin to silicon and iron, three experiments were performed: First, in a competition experiment, enterobactin and salmochelin were incubated separately in aqueous solutions containing iron and silicon in stoichometric concentrations. Since iron binding is considerably faster than silicon binding, this experiment showed predominantly iron binding with only traces of Si-Ent and Si-Sal. In the second experiment displacement of Si from already-formed silicon complexes was investigated. Si-Ent and Si-Sal were incubated separately with stoichometric amounts of Fe^{3+} solutions. No significant iron binding by displacement of Si^{IV} ions was detected. Finally, we wanted to obtain an estimate on the exchange rate of bound Si between bound and nonbound siderophore receptors. Therefore, Si-Ent was incubated with Sal, and Si-Sal was incubated with Ent. Results indicate very slow exchange rates for silicon between Si-Ent and Sal, and between Si-Sal and Ent. The results of these competition experiments are shown in the Supporting Information.

To better understand the scope of Si complexation by bacterial siderophores, aerobactin, vibriobactin, yersiniabactin, and other siderophores were tested for their ability to bind silicon (see the Supporting Information). In contrast to enterobactin and salmochelin, which both showed complete conversion to the silicon complexes, vibriobactin, desferricoprogen, and desferrioxamine formed only traces of silicon complexes. Aerobactin and yersiniabactin did not show any conversion with silicon, supporting the strong preference for catecholate groups in silicon binding.

Finally, the effect of Ent, Sal, Si-Ent, and Si-Sal on the growth of *E. coli* was tested in an EDDHA/4,4′-bipyridyl assay (see the Supporting Information). Enterobactin and salmochelin cause significant bacterial growth relative to their silicon complexes (Figure 2). Hence both Si-salmochelin and Si-enterobactin complexes could not be used for iron complexation by *Escherichia coli*, which is in accordance with the binding characteristics determined in vitro. Thus, we suggest that the inability of uptake is representative also for other *E. coli* or *Salmonella* strains.

In the last years inorganic chemists have made considerable progress in the synthesis of hypervalent silicon complexes,[14,16] and the formation and structure of these complexes is an area of continuing interest.[7] Inorganic chemists have reported on silicon complexation with catechols,[17] organosilanes,[16] thiocyanates,[18] and a large number of other ligands.[6,7] The finding of silicon complexes of the bacterial siderophores enterobactin and salmochelin in vivo is the first observation of the formation of a hypervalent silicon complex by natural products under physiological

conditions. These findings open up interesting aspects of silicon chemistry in view of biochemical considerations and pose new questions on the nature and function of siderophores, as well as the role of Si coordination chemistry and silicon in a biological context.

Silicon is not known to form complexes with secondary metabolites; this is in contrast to the metalloid boron, which forms the antibiotic boromycin (known since 1967)[19,20] and has been found more recently to form complexes with the siderophores vibrioferrin and petrobactin.[21] Our observation suggests that the binding of silicon by the siderophores enterobactin and salmochelin is not only possible under physiological conditions but moreover that the silicon complexes Si-Ent and Si-Sal may be formed in significant amounts in silicon-rich environments such as soil,[22] urine,[23] and seawater (Si^{IV} 2.9 ppm/Fe^{3+} 0.0034 ppm).[24] Recently, certain bacteria were observed to etch iron-silica minerals like hornblende (48 % SiO_2, 11 % Fe_2O_3),[22] and siderophores of the catecholate-type are plausible candidates for liberating Fe^{3+} ions from surrounding silicate in minerals. As enterobactin and salmochelin are mainly produced by pathogenic bacteria (*Salmonella*, uropathogenic *E. coli* strains) in animals and humans the biological function of silicon binding by enterobactin and salmochelin is unclear. Silica is actively excreted through the kidney's glomeruli into the urine, and silica content is 20–100 times higher in urine than in blood. The reported silicon concentrations in healthy individuals are in the range of 280 μM[23] and clearly exceed the concentration of iron, which is assumed to be in the range of 1 to 2 μM.[25] The high silicon concentration in urine may become relevant in urinary tract infections since this favors formation of Si-Ent and Si-Sal which may lower the formation of the respective iron complexes and thus the pathogenicity of the infection. This effect may not be very strong since iron binding was shown to be faster than silicon binding. Preliminary data, however, show that silica has no inhibitory effect on the growth of *E. coli* K-12 (which is able to form only enterobactin) under low-iron conditions. In addition, in uropathogenic *E. coli* strains growth may result from other siderophores for example, aerobactin or yersiniabactin, which do not form stable silicon complexes. Hence, by producing a set of structurally different siderophores, the organism may secure an iron supply in favor of other competing ions. The fundamental significance of silicon in biology is to confer mechanical stability in the form of silicates accumulated in plants, some sponges, and marine organisms such as diatoms and radiolaria.[20,21] Currently, "quorum-sensing" functions of Si siderophore complexes cannot be ruled out, as previously suggested for boron siderophores.[21] However, the finding of key enzymes related to enterobactin biosynthesis in the diatom *Thalassiosira pseudonana* and the recent finding of shared pathways in Si and Fe metabolism[26] indicate that a contribution of catecholates or similar structures to silicon metabolism may be assumed.[27] It is conceivable that silica uptake may occur following mechanisms similar to that of iron uptake.

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