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# The configuration of the chiral carbon atoms in staphyloferrin A and analysis of the transport properties in *Staphylococcus aureus*

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**Abstract**

Staphyloferrin A, the iron-transporting siderophore of *Staphylococci,* contains two citric acid residues linked to a D-ornithine backbone, having thus three chiral centers. While the chirality of the backbone can be determined after hydrolysis, the chirality of the two citryl residues can only be determined from the intact staphyloferrin A molecule by circular dichroism spectra. The chirality of the quarternary carbon atoms of citryl residues in fungal rhizoferrin and bacterial *enantio*-rhizoferrin have been determined previously to be R,R and S,S respectively. The present investigation shows that of the three chiral centers in staphyloferrin A, the citryl residues can be assigned an S,S-configuration by comparison with synthetic analogs, confirming a common chirality among the bacterial *enantio*-rhizoferrin and staphyloferrin A. This suggests that the bacterial carboxylates originate from a common biosynthetic pathway leading to an S,S-configuration, while the fungal rhizoferrin possessing an R,R-configuration must have a different biosynthetic origin. Growth promotion tests with staphylococci revealed that the S,S-configuration of staphyloferrin A and *enantio*-rhizoferrin enabled iron uptake, while the fungal rhizoferrin with R,R-configuration was not utilized.

## Introduction

The siderophore staphyloferrin A isolated from staphylococci is composed of two molecules of citric acid bound amidically to the amino groups of ornithine. The $C_\alpha$-atom of ornithine was determined to have R-configuration by chiral amino acid analysis (Konetschny-Rapp *et al.* 1990). The chirality of the citryl residues has not yet been reported. In an effort to determine the chirality at these centers we report here a comparison of the circular dichroism spectra of staphyloferrin A with that of rhizoferrin (Drechsel *et al.* 1992), *enantio*-rhizoferrin (Münzinger *et al.* 1999) and some synthetic analogues. The CD effects of the rhizoferrins are simplified as they contain putrescine instead of D-ornithine and thus lack the chirality in the diamino bridge. The CD effects from the quaternary carbons and from the $C_\alpha$-atom in staphyloferrin should be largely independent of each other, since they are separated by at least 3 atoms and are not part of a common chromophore. Therefore, the CD spectra of staphyloferrin A can be assumed to be a combination of almost separate effects originating from the D-ornithine in the bridge and from the two quaternary carbon atoms of the citric acid residues. Several analogues of known chirality and representing the various structural features of stapyloferrin A with increasing fidelity were prepared and examined by pH-dependent CD spectroscopy (Fig. 1) to determine the effects of the neighbouring groups on the CD spectra.

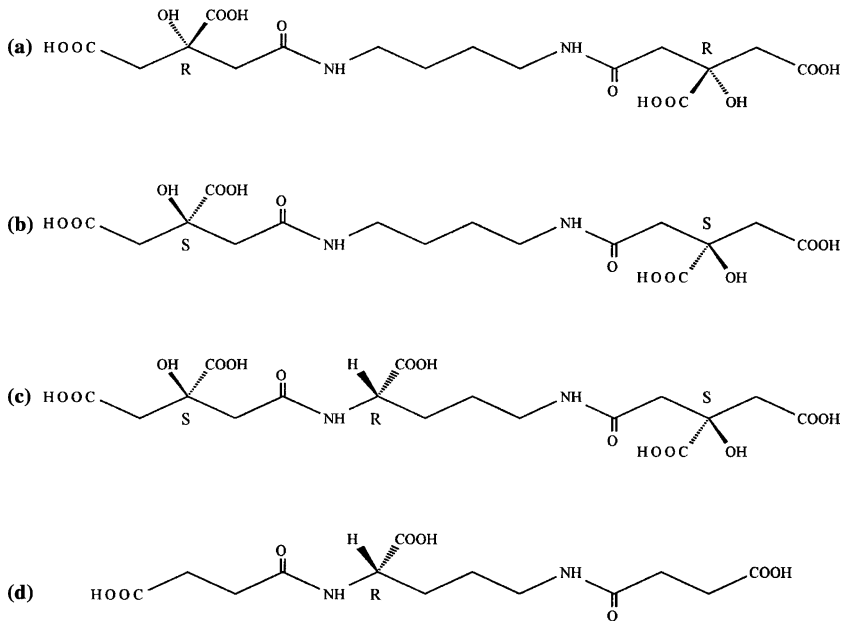


*Figure 1.* Structures of staphyloferrin A and of reference compounds: A = R,R-rhizoferrin, B = S,S-rhizoferrin, C = staphyloferrin A, D = N,N disuccinyl-D-ornithine.

## Materials and methods

### *Chemicals and instruments*

D-ornithine hydrochloride, D-leucine and N-acetyl-D-leucine were purchased from Nova Biochem, succinic anhydride was purchased from Aldrich. Staphyloferrin A was isolated form *S. hyicus* DSM 20459 (Konetschny-Rapp *et al.* 1990); rhizoferrin was isolated from *Cunnighamella elegans* (Drechsel *et al.* 1992) and *enantio*-rhizoferrin was isolated from *Ralstonia pickettii*, DSM 6297 as described earlier (Münzinger *et al.* 1999). The purity of the compounds was checked by HPLC (Shimadzu, LC-10) equipped with gradient controller and automatic sampler, using a $C_{18}$ reversed-phase column (Nucleosil, 5 $\mu$m, 250 × 4.6 mm) and a gradient of acetonitrile in water (3–8%) plus 0.1% trifluoroacetic acid. CD spectra were recorded on a Jasco 720 CD-spectrometer (Japan Spectroscopic Company Ltd., Tokyo, Japan) equipped with a personal computer for data acquisition, storage and processing.

### *Strains and growth conditions*

The Staphylococcus strains, *S. aureus* RN4220, *S. carnosus* TM300 and *S. xylosus* C2a were kindly provided by Fritz Götz, Institut für Mikrobiologie, Mikrobielle Genetik, Tübingen, Germany. The strains were grown on TY medium, containing per litre, 8 g tryptone, 5 g yeast extract, and 5 g NaCl.

### *Synthesis of chiral analogs of staphyloferrin A*

Succinyl-D-leucine: 20 mg of D-leucine and 100 mg of succinic anhydride (6.5 eq.) were dissolved in 4 ml dioxane / water (1:1). The pH was adjusted to 8 by the addition of aqueous sodium hydroxide. The reaction mixture was stirred at room temperature overnight, the pH adjusted to 7 and the neutral solution lyophilized. The crude material was purified by gel filtration on Biogel P2 (Biorad, München), and examined by ESI-MS. Fractions containing the target mass were checked for purity by HPLC. Fractions containing pure product were used for CD-spectroscopy.

Disuccinyl-D-ornithine: 20 mg D-ornithine hydrochloride and 48 mg of succinic anhydride (4 eq.) were dissolved in 2 ml of dioxane / water (1:1) and isolated and purified as described for succinyl-D-leucine.

### *CD-spectroscopy*

Succinyl-D-leucine was dissolved in bidistilled water to a starting concentration of 5.34 mM. The

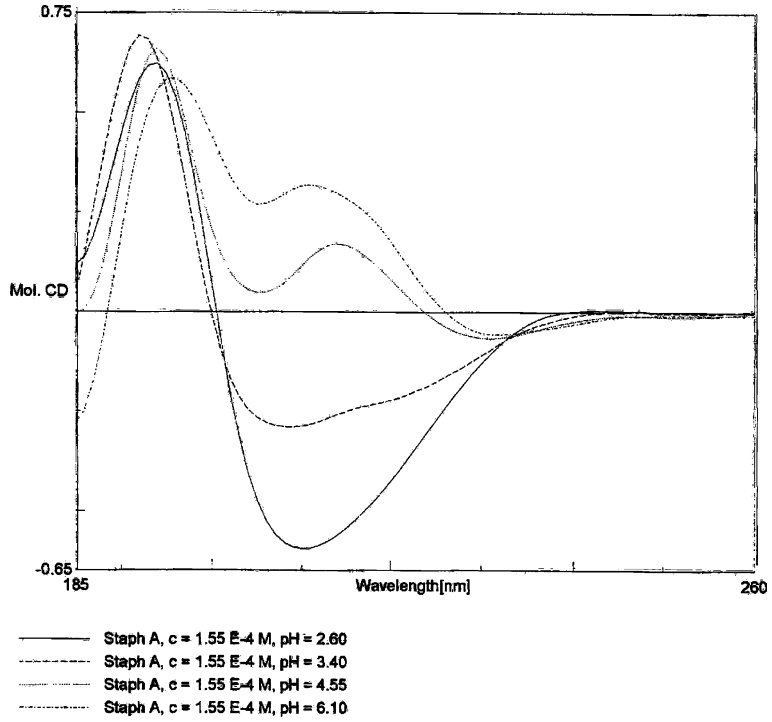


*Figure 2.* CD spectra of staphyloferrin A at different pH.

resulting pH was measured and the CD spectrum of the initial sample was recorded in a quartz-cuvette with a pathlength of 10 mm. The solution was retrieved, its pH adjusted by stepwise addition of aqueous hydrochloric acid and a CD spectrum at each pH value recorded. The reported ellipticities were corrected for volume changes during the titration. Disuccinyl-D-ornithine was dissolved in bidistilled water to a starting concentration of 17.7 mM. The resulting pH was measured and the CD spectrum of the initial sample was recorded in a quartz-cuvette with a pathlength of 5 mm and treated as above.

*Growth promotion tests*

Growth promotion tests were performed according to Deiss *et al.* (1998), using bipyridyl and EDDHA (150 $\mu$M) as iron binding agents and 10 $\mu$l/plate of an overnight culture of the Staphylococcus test strain. Sterile aqueous solutions (100 $\mu$M) of staphyloferrin A, rhizoferrin and *enantio*-rhizoferrin were prepared and 10 $\mu$l each were added to sterile filter disks and placed on seeded soft agar (0.6%) medium. Growth promotion was recorded after incubation at 37 °C overnight. For comparison hydroxamate siderophores (ferrioxamine B, ferrichrysin and coprogen, 100 $\mu$M) were also tested.

## Results

The CD spectrum of staphyloferrin A contains a narrow positive CD-effect at 190–200 nm, which is virtually independent of pH, and a pH sensitive, broad band at 200–230 nm, which is negative at acidic pH and changes into a positive CD effect at weakly acidic to neutral pH (Fig. 2). The structural basis of these two CD effects is, first, the chirality at the quaternary carbon of the citryl residues and, secondly that at ornithine-$C_\alpha$. These two contributing factors can be separated with the help of suitably chosen analogs. The chirality of two citryl residues on an achiral backbone can be measured with the compounds rhizoferrin (Fig. 3) and *enantio*-rhizoferrin (Fig. 4). The chiral citric acid residues of rhizoferrin, which have R,R-configuration, produce a sharp negative CD effect at. 200–210 nm, scarcely dependent on pH with

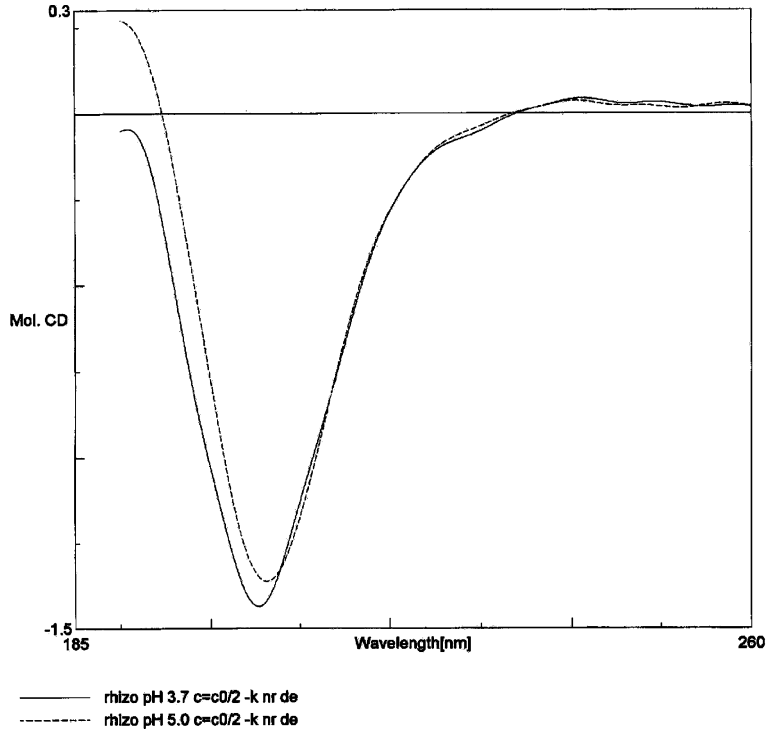


*Figure 3*. CD spectra of rhizoferrin at different pH.

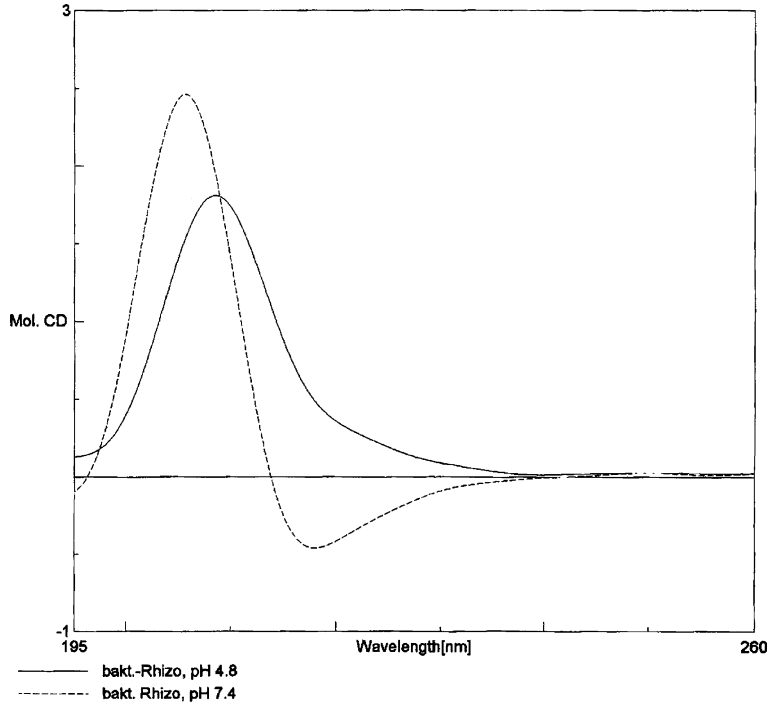


*Figure 4*. CD spectrum of *enantio*-rhizoferrin at different pH.

*enantio*-rhizoferrin showing the expected mirror image.

The first and second reference compounds, N-acetyl-D-leucine (Fig. 5a) and succinyl-D-leucine (Fig. 5b) have a negative CD effect at 205–230 nm, most prominent at pH 2, decreasing with increasing pH. At neutral and basic pH, the CD effect produces an S-shaped curve with a positive amplitude at 205–210 nm and a smaller negative effect at 220 nm. The third reference compound, disuccinyl-D-ornithine (Fig. 5c), shows its negative maximum at 215–230 nm, most prominent at pH 2 and decreasing with increasing pH. In addition to this slight bathochromic shift (compared to N-acetyl-D-leucine and succinyl-D-leucine), there is also a new positive CD effect at 235–245 nm at pH above 6.

The CD effect of staphyloferrin A in the short wavelength region, around 200 nm, resembles closely the CD effect of rhizoferrin, with a slight hypsochromic shift of about 10 nm. For both siderophores, this band is rather narrow and – virtually – independent of pH. Since the sign is positive, we conclude that the citryl residues in staphyloferrin A like in *enantio*-rhizoferrin have opposite configuration as in rhizoferrin, therefore having S-configuration. The CD effects of staphyloferrin A at longer-wavelengths resemble, with respect to broadness and pH-dependency, the CD effects of reference compounds containing amides of D-leucine and D-ornithine as expected given that staphyloferrin A contains D-ornithine.

The CD spectrum of staphyloferrin A can therefore be explained as the combination of the CD effects of two citryl residues with S-configuration, with opposite sign as in the CD spectrum of rhizoferrin, and the CD effect originating from D-ornithine in the backbone of the molecule.

As is evident from the present investigation, carboxylate siderophores of bacterial origin, i.e. staphyloferrin A and *enantio*-rhizoferrin, have a common stereochemical criterion: the chirality of the citryl residues is S, S. In contrast to this, the fungal carboxylate siderophore rhizoferrin, along with analogs produced by directed fermentation (Drechsel *et al.* 1995), always have R,R-configuration at the citryl residues. This points to a common biosynthetic pathway in fungal siderophore producers, which is different from the biosynthetic pathway in bacteria.

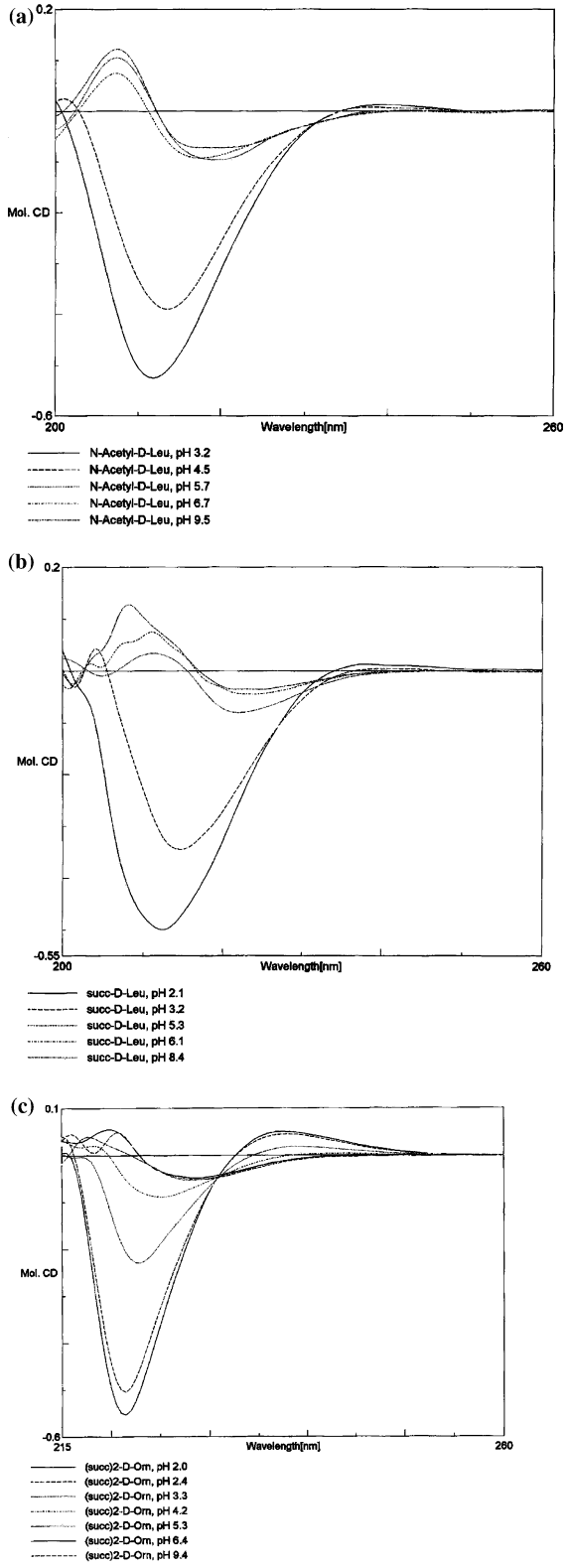


*Figure 5.* CD spectra of N-acetyl-D-leucine (a), succinyl-D-leucine (b) and disuccinyl-D-ornithine (c).

*Biological studies with carboxylate siderophores*

Although it has been shown earlier that staphyloferrin A functions as a siderophore in Staphylococci, other carboxylate siderophores, like rhizoferrin and *enantio*-rhizoferrin, have not been tested so far. As shown in Figure 6, growth promotion tests with Staphylococci (*S. aureus, S. carnosus, S. xylosus*) revealed that *enantio*-S,S-rhizoferrin functioned as a good siderophore, similar to staphyloferrin A, while R,R-rhizoferrrin was ineffective, suggesting specific structural requirements of the ferric rhizoferrin complexes during recognition by the putative Fe-carboxylate transport system(s) or the subsequent siderophore utilization enzymes in Staphylococci.

## Discussion

For verifying the contribution of diacylated D-ornithine, a set of several analogs was examined by pH-dependent CD-spectroscopy. The first approximation was done with the commercially available N-acetyl- D-leucine, which resembles the substructure in staphyloferrin A with an amidated, therefore neutral, carboxy group and an aliphatic side chain. The amidation is essential, since a change of charge along the pH series of measurements will usually have a profound effect on the neighbouring chiral center. The second and third analogues were synthesized according to the structural requirements. Succinyl-D-leucine represents an analogous substructure of the citryl-ornithine residue present in staphyloferrin A, and gave a net negative charge to the molecule. Di-succinyl-D-ornithine approximated the target molecule even closer, using the same bridge component as is present in staphyloferrin A and acylating the diamino part on both sides with net negative, acidic residues. The structural approximation is depicted in Figure 1.

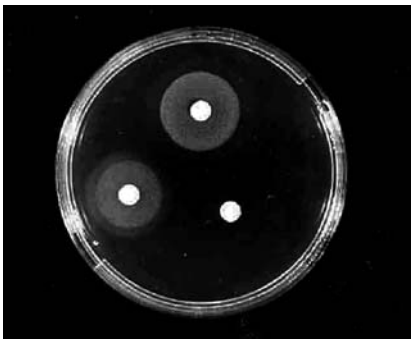

*Figure 6*. Growth promotion tests with *enantio*-S,S-rhizoferrin (left) and R,R-rhizoferrin (right) as well as ferrioxamine B (upper) as a control.

The expression of ferric hydroxamate transport systems in Staphylococci has recently been demonstrated (Sebulsky *et al*. 2000) showing that a variety of hydroxamate type siderophores including ferrichrome, aerobactin and ferrioxamine are taken up by a binding-protein dependent ferric hydroxamate transport system in *S. aureus*. Thus hydroxamate siderophores are taken up, although staphylococci have not been shown to biosynthesize or excrete any of these hydroxamates. The actual siderophores of Staphylococci have been shown earlier to be staphyloferrin A and B, both belonging to the carboxylate type (Drechsel *et al*. 1991, 1993). Besides staphyloferrin A and B, other carboxylate type siderophores have been isolated, such as rhizoferrin from zygomycetous fungi like Rhizopus (Drechsel et al 1991; Thieken & Winkelmann 1992; Winkelmann 1992) and *enantio*-rhizoferrin from *Ralstonia pickettii* (Münzinger *et al*. 1999). Although the fungal and bacterial rhizoferrins have the same constitution, the chirality of the two citryl residues is different, i.e. R,R for the fungal rhizoferrins and S,S for the bacterial rhizoferrin (Münzinger *et al*. 1999)

Due to the fact that staphyloferrin A contains three chiral centers, an unequivocal assignment of the citryl chiralities by direct CD-measurements was not possible. The present circular dichroism approach of analyzing the chiral contribution by comparison to N(alpha)acyl-ornithine analogs clearly showed that the two citryl residues have S,S configuration. This is in agreement with the S,S-configuration of *enantio*-rhizoferrin isolated from the bacterium *Ralstonia pickettii* (formerly *Pseudomonas pickettii*), suggesting a common biosynthetic pathway of carboxylate siderophores in gram-positive and gram-negative bacteria. The observed identical stereochemistry of the two citryl residues in staphyloferrin A and *enantio*-rhizoferrin correlates well with the observed iron transport and utilization properties, assuming recognition of analogous ferric complexes during uptake by the putative carboxylate transport system(s) in Staphylococci.

Based on the opposite stereochemistry of fungal ferric rhizoferrin and the bacterial ferric *enantio*-rhizoferrin as determined in previous publications (Drechsel *et al*. 1992, Münzinger *et al*. 1999) we conclude that a specific configuration of the iron carboxylate complexes is required for uptake or subsequent processing in Staphylococcus and that an analogous complex configuration must be assumed for uptake of ferric staphyloferrin A and ferric *enantio*-rhizoferrin. Although a *lambda* configuration was suggested previously for the ferric staphyloferrin A complex by direct CD measurements (Konetschny-Rapp *et al*. 1990), our results of the corresponding S,S-configuration of the citryl residues in staphyloferrin A as well as in *enantio*-rhizoferrin (*delta* ferric complex) would suggest a common configuration (''delta'') with the ferric staphyloferrin A complex. This suggestion is supported by a biological growth promotion test which shows uptake of ferric staphyloferrin A and *enantio*-rhizoferrin in staphylococci but not of ferric rhizoferrin. It should be noted that the assignment of *lambda*- or *delta*-configuration is based on a formal $D_3$ symmetry about the metal, as found in tris-bidentate complexes such as hydroxamates. These assignments are not strictly applicable for bis-tridentate complexes, as are rhizoferrins (Carrano *et al*. 1996) and staphyloferrin A, the configurations of which have to be assigned by the *syn*- / *fac*-nomenclature or, more generally, by IUPAC conventions clockwise or counter-clockwise (Leigh 1990; Drechsel & Winkelmann 1997). Beyond that, the involvement of the $C_\alpha$-carboxyl group in coordination to the metal center cannot be excluded and should have a profound influence on the CD-spectrum of ferric staphyloferrin A.

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