Journal of Peptide Science
J. Peptide Sci. **4**: 147–181 (1998)

REVIEW ARTICLE

Peptide Siderophores

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Received 17 May 1997
Accepted 20 June 1997

Abstract: Siderophores are low molecular weight iron chelators, produced by virtually all bacteria, fungi and some plants. They serve to deliver the essential element iron, barely soluble under aerobic conditions, into microbial cells. Siderophores are therefore important secondary metabolites which are very often based on amino acids and their derivatives. Biosynthesis, transport, regulation and chemical synthesis of natural siderophores and their analogues is of considerable interest for the protein and peptide chemist. This review gives an overview of the structural classes of peptidic siderophores, along with data on their biosynthesis. On a number of representative examples, strategies and schemes of their chemical synthesis are described.

Keywords: peptide; siderophore; microbial metabolites; iron; complex

Research on peptides and proteins is the centrepiece of biosciences, spanning a large range from immunology to enzymology. The functions of peptides lie primarily in the field of messengers, being hormones, neurotransmitters, regulators of genetic expression, antigens and mediators of immunologic reactions, among others. Peptidic building blocks are, however, also found in siderophores, low molecular weight iron chelators, produced by microbes and some plants. They function extracellularly, complexing and transporting inorganic ferric iron. The study of siderophores combines almost all fields of peptide and protein biochemistry: research on the genetics and enzymology of regulation, biosynthesis and receptor-mediated uptake as well as on the structural and physicochemical characterization of the low molecular weight iron complexes.

While organic biomolecules can often be synthesized from a variety of different precursors, the metal supply is in any case essential for growth and proliferation of cells. This is valid for all kinds of trace elements, but has been especially well investigated in the case of iron. Higher organisms acquire iron through feeding on lower organisms that have already incorporated iron. For prime producers, such as bacteria, fungi and plants, iron bioavailability is limited by the inherently low solubility of

Abbreviations: Aloc, allyloxycarbonyl-; AMP, adenosine monophosphate; Asp(OH), hydroxyaspartic acid; CTHPMD, 4-carboxy-3,4,5,6-tetrahydropyrimidine; DCC, dicyclohexylcarbodiimide; DEAD, diethylazodicarboxylate; DIC, diisopropylcarbodiimide; DHP, dihydropyran; EEDQ, *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline; HSAB, hard and soft acids and bases; Hse, homoserine; NADPH, nicotinamide adenine dinucleotide; PCC, pyridinium chlorochromate; THP, tetrahydropyranyl-.

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CCC 1075-2617/98/030147-35

ferric ions. Under aerobic conditions, iron is precipitated in the form of $Fe(OH)_3$ with a solubility product of 10^{-39}, which limits the concentration of ferric ions at pH 7 to about 10^{-18} M. This is far below the level of demand for the iron supply of living cells. Therefore, microorganisms had to evolve methods of solubilization and uptake of mineral iron. This is accomplished by biosynthesis and excretion of low molecular weight iron chelators, named siderophores.

Siderophore production is known in bacteria, fungi and plants. Siderophore production and usage is especially well investigated in Gram-negative enterobacteria, where the transport across the outer membrane and the energy coupling is known on the molecular and genetic level. In *Escherichia coli*, seven iron transport systems have been described [1]. Bacterial siderophores are structurally more diverse than fungal siderophores. Catecholate siderophores, containing 2,3-dihydroxybenzoic acid, are exclusively found in bacteria. Bacteria also produce the other structural classes of siderophores, hydroxamates and carboxylates. Fungi predominantly synthesize hydroxamates, and the recently discovered carboxylate rhizoferrin in the zygomycetous class. Fungal siderophores never contain 2,3-dihydroxybenzoic acid. Although the biosynthesis of a number of fungal siderophores is well understood, the identification of specific receptors has not yet been accomplished. The siderophores produced by plants are named phytosiderophores. They have been isolated from gramineous plants and contain neither hydroxamate nor catecholate groups. One exception to the ubiquitous importance of iron should not be omitted: *Lactobacillus plantarum* does not require iron [2]: the organism obviously manages to activate all its enzymatic functions with metals other than iron.

After complexing iron, the ferric siderophores are taken up into the cell. Highly specific receptors in the outer membrane of bacteria recognize the specific siderophore as well as its chirality. They transport the ferric complexes into the periplasm in an active and energy-dependent way. The ferric ions once collected here are then handed over to the intracellular transport and storage components and finally incorporated into proteins to fulfil their enzymatic functions.

The release of iron from the ferric siderophore complexes deserves special attention because of the high equilibrium constants of complex formation. Typical formation constants are 10^{23} to 10^{25} for carboxylates [3], 10^{29} to 10^{32} for trihydroxamates [4] and range up to 10^{52} for enterobactin and alterobactin [5]. Therefore, internal storage compounds cannot directly compete with most siderophores. On the contrary, siderophores of pathogenic bacteria are able to scavenge iron from the host's storage compounds, as has been demonstrated for pyoverdin [6] and amonabactin [7]. Release of iron from ferric siderophores in the cell interior often proceeds through degradation of the ligand or reduction of ferric to ferrous ion with much less affinity to the siderophore ligand. Also, acidification in certain cell compartments can shift the balance between competing donor groups, depending on their pK_a.

This review will focus on the siderophores, the extracellular iron scavengers. Their building blocks are often peptidic in the sense that they contain amino acids, often derivatized in a special way. Their biosynthesis is usually not ribosomal, but carried out by specific enzymes. For a secondary metabolite to be classified as siderophore, three conditions must be met: (1) iron-regulated biosynthesis; (2) ferric ion chelating capability; and (3) active transport through the cell membrane.

Iron-regulated biosynthesis means that the siderophores are only produced under conditions of iron starvation. Addition of soluble iron will switch off the enzymes involved in synthesis (and transport) of siderophores. Producer strains that secrete, for example, 2,3-dihydroxybenzoic acid under any growth conditions, cannot be regarded as true siderophore producers. This must not be confused with optimized fermentation procedures, in which often constitutive mutants are selected for easier control of feeding parameters and enhanced yield.

Iron chelating capability can be proven by physicochemical means. When transition metals form complexes, charge transfer bands usually appear which give the complexes visible colour. In case of ferric complexes, charge transfer bands are between 350 and 650 nm, depending on the class of ligands. Therefore, ferric siderophores can be visually detected as yellow-brown to red-brown compounds. UV-vis spectroscopy and circular dichroism allows these effects to be quantified and complex stability to be monitored under different pH and metal/ligand ratios.

Active transport is verified by the uptake of radiolabelled iron (^{55}Fe assay). Cells are incubated for increasing time intervals with ferric radiolabelled siderophores, washed, and the internalized radioisotopes are measured in a scintillation counter. This will give a kinetic uptake curve, from which rate

of uptake and saturation can be read. The uptake can be stopped by addition of azide or cyanide, proving the energy dependence of the transport process.

The nomenclature of siderophores and their ferric complexes is unfortunately largely unsystematic. In the beginning of siderophore research, the scientists were delighted by the colourful compounds, and colour actually played a role in facilitating detection and workup of these new substances. The names were chosen accordingly; for instance, ferrichrome, ferrirubin, which only denotes a reddish-coloured, iron-containing substance. Since this nomenclature includes the iron, the metal-free ligand has to be designated as desferri-siderophore, e.g. desferriferrichrome. This principle is also applied to iron-containing, siderophore-related antibiotics, such as the ferrimycins. Another principle, still useful with new substances today, designates the siderophores with the producer. This was done first with enterobactin, isolated from *Enterobacteriaceae*. In this case, however, alternative names persist: enterobactin and enterochelin denote the same substance (the metal-free ligand), isolated by two competing research groups [8, 9]. This also shows a second aspect: the names of siderophores are usually composed of two parts. The first relates to the producer, and the second could in principle be used for describing the chemical class of the siderophore. However, this opportunity of systematization has unfortunately not been exploited. In enterobactin/enterochelin, the interchangeability of the suffix can be seen. One can find more examples when viewing names within chemically related subclasses of siderophores: aerobactin, nannochelin, schizokinen are all citrate-based dihydroxamates. The reason why names cannot easily be changed is that the substances are already named at the stage of isolation, purification and the optimization of the fermentation procedure, when the chemical structure is still unknown or not yet fully resolved. An especially unfortunate example of unsystematic nomenclature is the similarity in the names of rhizobactin DM4, a carboxylate siderophore, and rhizobactin 1021, a citrate/dihydroxamate mixed type siderophore of the aerobactin-family (see below). Another detail of siderophore nomenclature relates to the precise spelling of the compounds' names. Structures that have been discovered and originally described in France, usually carry an 'e' at the end, for example 'pyoverdine'. This is, however, being abandoned in international literature, thus the preferred name today is 'pyoverdin'.

Coordination chemistry of siderophore

Iron in its trivalent state has d^5 electronic configuration. Therefore, its complexes are preferentially hexacoordinate, quasi octahedral. The donor atoms in ferric siderophore complexes are primarily oxygen and, rarely, heterocyclic nitrogen. This is due to ferric ion being a 'hard' ion in terms of the HSAB principle, and preferentially seeks similar coordination partners [10, 11] The ligand field is weak, and the ferric siderophore complexes are high spin complexes, with a ^6S ground state. This implies that there are no spin-allowed d–d transitions, and the colour of the complexes is solely due to metal-to-ligand charge transfer. From the wavelengths of the absorption maxima, conclusions can be drawn on the types of complex. The charge transfer bands are at 340 nm for carboxylates, around 425 nm for tris-hydroxamates, 470 nm for bis-hydroxamates, 515 nm for monohydroxamates, and around 480 nm for tris-catecholates, 560 nm for bis-catecholates and 680 nm for mono-catecholates.

Sixfold coordination is, in the field of siderophores, usually achieved by trisbidentate ligands. We first assume, as the simplest example, coordination by three molecules of catechol around a ferric ion. On an octahedral arrangement, the complex can have two different configurations, a left-handed propeller, termed Λ-configuration, and a right-handed propeller, the Δ-configuration (Figure 1). With monomeric catechol ligands, the two complexes are exact mirror images of each other and form a racemic mixture. When the catechol moieties are bound to a chiral backbone, the Λ- and Δ-configurations around the metal centre are diastereomers, and a preference for one of the configurations occurs. This is found in enterobactin, where L-serine residues form the anchoring skeleton of catecholate donor functions and introduce a pre-

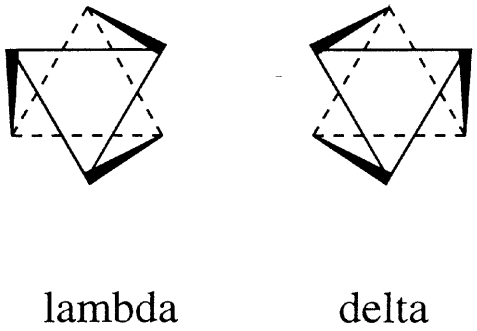


Figure 1 Λ-configuration, left, and Δ-configuration, right, of a tris-bidentate complex.

dominant Δ-configuration on the ferric complex. The Δ-complex is stereospecifically recognized by the enterobactin receptor, as has been demonstrated by comparison with the synthetically obtained Λ-*enantio*-enterobactin [12].

When the complex is formed with hydroxamate functions, the donor groups are asymmetric, and *cis*- and *trans*-complexes are possible in addition to the Λ- and Δ-configurations. In ferrioxamines, where there are three hydroxamates in a (mostly) linear, asymmetric backbone of diamines and dicarboxylic acids, the hydroxamate groups are distinguishable by their sequence position. Therefore, *cis*/*cis*, *cis*/*trans*, *trans*/*cis* and *trans*/*trans* isomers, relative to the N-terminal hydroxamate, occur in both the Λ- and the Δ-configurations. Since the N-terminal hydroxamate itself can also be oriented in two different directions, altogether 16 isomers arise (Figure 2). Since the backbone does not contain any chiral centre, the whole ligand is achiral and Λ- and Δ-configurations are equally represented in a racemic mixture. The different *cis*- and *trans*-complexes can, however, be separated, as has been done with kinetically inert chromium complexes of coprogen, where geometrical as well as optical isomers have been resolved [13]. Spectroscopically, the Λ- and Δ-configurations can be distinguished by circular dichroism. The main feature of the CD spectra, the Cotton effect at the wavelength of the iron-to-ligand charge transfer band, is exactly reversed between Λ- and Δ-complexes. The *cis*- and *trans*-isomers of complexes differ slightly in the shape and exact wavelength of their UV–vis spectra.

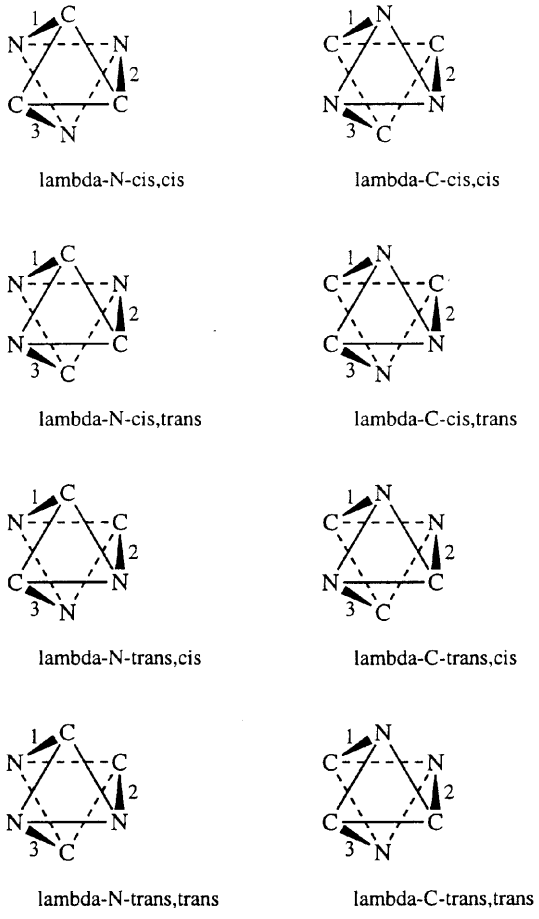


Figure 2 Stereiosomers of ferrioxamines.

The stability of ferric siderophore complexes is crucial to their role of iron transport agents. In the natural environment, competition between a considerable number of different organisms will favour those microbes that are most effective in organizing their supply of nutrients. Therefore, siderophores need to be very strong chelators that can scavenge iron in the diluted medium outside the cell, in competition with other molecules. Polycarboxylic acids, amino acids, α-keto- and α-hydroxyacids are present in the medium as nutrients or catabolic end products. These are indeed capable of complexing ferric ion, as has been shown in the *Proteus* group of bacteria, where α-keto- and α-hydroxyacids function as iron transport agents [14]. Therefore, the electrostatic attraction between trivalent iron and deprotonated hydroxy groups, which is represented in the enthalpic contribution to the free energy of the complexation reaction,

$$Fe^{3+} + L^{3-} \rightleftarrows FeL$$

$$K = \frac{[FeL]}{[Fe^{3+}][L^{3-}]}$$

$$K = e^{-\Delta G/RT}$$

$$\Delta G = \Delta H - T\Delta S$$

is not the outstanding feature of siderophores. What makes siderophores peculiarly well-suited ligands is the availability of – usually – six coordination sites in one single molecule. This is expressed in the entropic part of the free energy of complex formation, and is called the chelate effect.

The complex formation constants of siderophores range up to $K_f = 10^{52}$, as in the case of enterobactin, an 'ideal' tricatecholate siderophore. Trihydroxamates such as the ferrichromes have formation constants around 10^{30}, dihydroxamate/carboxy-

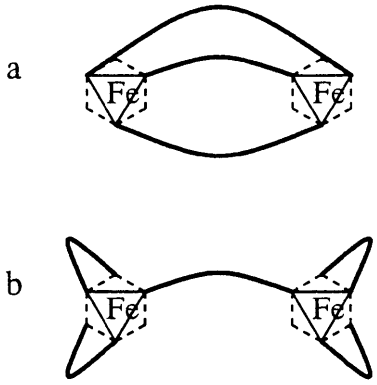


Figure 3 Bridged dinuclear iron complexes: (a) rhodotorulic acid; (b) dimerum acid.

lates such as aerobactin, 10^{23}, and pure carboxylates, e.g. rhizoferrin, 10^{21}.

When a siderophore has less than six coordination groups available, the requirements of ferric ions are fulfilled by forming other than 1:1 complexes. The dihydroxamates rhodotorulic acid and dimerum acid form Fe_2L_3 complexes, with bridging ligands between the iron centres (Figure 3). The stability of these complexes [15] is comparable to (synthetic) dihydroxamates and a little less than the carboxylate/dihydroxamate mixed type siderophore aerobactin.

In the above equations, we assumed the ligand as fully deprotonated. The protonation of the ligand will, however, be a competitive reaction to metal complexation. Therefore, the basicity of the ligand has to be taken into account [4] and the constant of complex formation, K, has to be adjusted to K_{eff}:

$$\log K_{\text{eff}} = \log K + n \log \alpha_{\text{L}}$$

$$Fe^{3+} + L^{n-} \rightleftarrows FeL^{(3-n)+}$$

$$K = \frac{[FeL]^{(3-n)+}}{[Fe^{3+}][L^{n-}]}$$

by a potential series on the protonation steps of the ligand:

$$\alpha_{\text{L}}{}^{-1} = [1 + [H^+]\beta_1^{\text{H}} + [H^+]^2\beta_2^{\text{H}} + \ldots + [H^+]^n\beta_n^{\text{H}}]$$

$$\beta_n^{\text{H}} = \frac{[H_nL]}{[H^+]^n[L]}$$

From this it becomes clear that the effectiveness of different siderophores depends on the pk_a of their donor groups. Catecholates dissociate at pH 9–10, hydroxamates at approx. 8–9, and carboxylates have pK_a values of 3.5–5. Noteworthy is the group of carboxylate siderophores, which, although the poorest ligands with respect to their constant of complex formation, become especially competitive at mildly acidic pH, which is typically the growth medium of producers of carboxylate siderophores. Carboxylates have been shown to form stable ferric complexes down to a pH of approximately 4 [16, 17].

Structures of siderophores

Peptide siderophores will be defined in the broadest possible sense as siderophores that contain amino acids and – in most cases – peptide (amide) bonds. This is a novel viewpoint in the field of siderophores, which have traditionally been classified by their producers [18], or by the chemical nature of their donating groups [19]. The restriction to amino acids means that some groups of siderophores will be omitted. These usually contain diamines instead of amino acids, which can be N-hydroxylated to form hydroxamates (e.g. the ferrioxamines, schizokinen), or simply serve as carriers for other chelating substructures (e.g. the rhizoferrins, staphyloferrin B). The use of diamines as building blocks for siderophores is especially widespread in fungi. In this review, we will start with siderophore structures most closely related to peptides, and continue into structures with less similarity. On several examples of siderophores, we will give details on what is known on the biosynthesis of siderophores.

Siderophores Containing Oligopeptides

Siderophores most closely related to peptides contain amino acids in a sequence of α-amino acids only linked with – underivatized – peptide bonds between carboxy groups and α-amino groups. The side chains are available for complexation to ferric ions.

Members of the ferrichrome family are cyclohexapeptides (two exceptions, see below) of three side-chain derivatized L-ornithines and three other amino acids, either glycine or L-serine or L-alanine. The third amino acid of this latter amino acid triplet is always glycine. Ferrichrome [20] is cyclo-triglycine-tri-N^5-hydroxy-N^5-acetyl-L-orithine (Figure 4). Ferricrocin [21] carries a serine in the second position of the amino acid triplet. Ferrichrysin [22] has Ser-Ser-Gly in its cyclic backbone. The acylation of the ornithine side chains may also vary. Ferrichrome A is acylated with *trans*-2-methyl-propene-dicar-

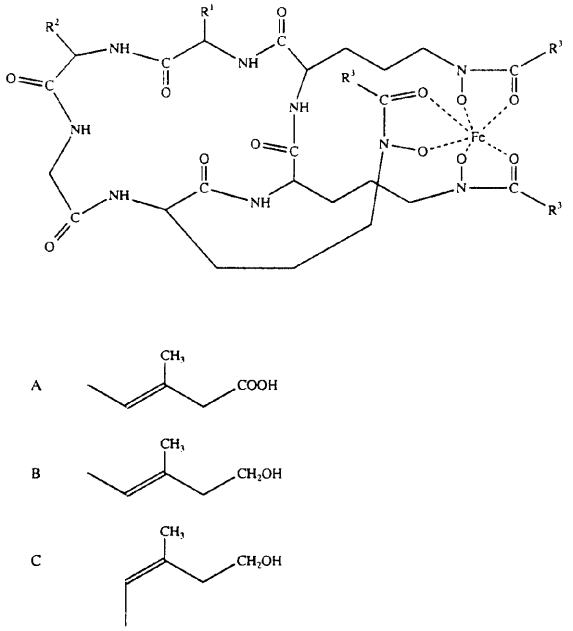


Figure 4 The peptide hydroxamate siderophore family of the ferrichromes: Ferrichrome, $R^1 = R^2 = H$, $R^3 = CH_3$; Ferrichrome A, $R^1 = R^2 = H$, $R^3 = A$; Ferricrocin, $R^1 = H$, $R^2 = CH_2OH$, $R^3 = CH_3$; Ferrichrysin, $R^1 = R^2 = CH_2OH$, $R^3 = CH_3$; Ferrirubin, $R^1 = R^2 = CH_2OH$, $R^3 = B$; Ferrirhodin, $R^1 = R^2 = CH_2OH$, $R^3 = C$; Asperchromes, $R^1 = R^2 = CH_2OH$, $R^3 = B$ and CH_3 (mixture).

boxylic acid [23]. On the sequence of ferrichrysin, ferrirubin is acylated with *trans*-anhydromevalonic acid, ferrirhodin is acylated with *cis*-anhydromevalonic acid [24]. In asperchromes, the homogeneity of acylations is disrupted, (in most cases: *trans*-) anhydromevalonic acid and acetyl residues are mixed [25]. In asperchrome C, three *trans*-anhydromevaloyl residues occur, one of which is acetylated at the hydroxy group [26]. Tetraglycylferrichrome, as a singular exception, is a cycloheptapeptide from three N^5-hydroxy-N^5-acetyl-L-ornithines and four glycines [27]. Des(diserylglycyl)ferrirhodin is the acyclic analogue of ferrirhodin [28].

The ferrichrome family of siderophores is produced by fungi [18]. Its biosynthesis starts with hydroxylation of ornithine to N^5-hydroxy-L-ornithine. This monooxygenase is a flavin containing NADPH : oxygen oxidoreductase, specific on L-ornithine [29]. N^5-hydroxy-L-ornithine is acylated to the different monohydroxamic acids, N^5-acyl-N^5-hydroxy-L-ornithine. The acyl residues are introduced from acyl-CoA. Several acetyl-transferases have been isolated, e.g. from *Ustilago sphaerogena* [30], *Aspergillus quadricinctus* [31] and *Rhodotorula glutinis* [32]. With respect to the N-hydroxy component, the acetyl transferases from *Aspergillus* and *Rhodotorula* have low substrate specificity. Anhydromevalonic acid is produced by stereospecifically *cis*- or *trans*-eliminating dehydratases. In *Penicillium chrysogenum*, the substrate for *cis*-anhydromevalonic acid is D-mevalonic acid [33]. In the synthesis of ferrichrome, the acetylhydroxamic acids and the tripeptide are assembled to the complete siderophore by ferrichrome synthetase, a large multienzyme complex of 10^6 Da [34, 35].

Pyoverdins and pseudobactins are a class of fluorescent peptide siderophores that are the main, but not the only, siderophores of the *Pseudomonas fluorescens* group of the Pseudomonadaceae [36]. The conspicuous fluorescence of these compounds arises from a chromophore that is derived from 2,3-diamino-6,7-dihydroxyquinoline (Figure 5(a)). The chromophore is variably acylated with succinic acid (amide), malic acid (amide), 2-oxo-glutaric acid or glutamic acid (Table 1) and connected to a peptide chain of 6–12 amino acids, roughly half of which have D-configuration. The acylations on the chromophore have been studied with respect to culture time, pH and iron content. Succinic acid has been found to be an artefact, resulting from hydrolysis of its amide. 2-Oxo-glutaric acid and glutamic acid are in an enzymatically controlled equilibrium [37]. The occurrence of D-amino acids in the pyoverdin family makes the peptidic residues resistant to degradation by proteases, as has been found for pseudobactins [38]. In the oligopeptide there is at least one N^5-acyl-N^5-hydroxy-ornithine which serves as the second bidentate metal coordinating group beside the catecholate substructure in the chromophore. The third bidentate ligand is most often provided by *threo*-β-hydroxy aspartic acid, in a few cases by a second N^5-acyl-N^5-hydroxy-ornithine. Beyond these requirements of metal coordinating side chains, the peptide sequence is very variable, often containing unusual amino acids such as *allo*-threonine and diaminobutyric acid (Table 1). In a number of cases, the C-terminus is cyclized onto basic or hydroxy-amino acids, for instance in pyoverdin Py Pf 12 (Figure 5(b)). When N^5-acyl-N^5-hydroxy-ornithine is the C-terminal amino acid, it is often, but not always, acylated by its own carboxy group, forming *cyclo*-N^5-hydroxy-ornithine. Azoverdins, isolated from *Azomonas macrocytogenes*, are very similar to pyoverdins. They share the chromophore of the pyoverdins, derived from 2,3-diamino-6,7-dihydroxy-quinoline. The peptide sequence is (L)-Hse-(D)-AcOHOrn-(D)-Ser-(L)-AcOHOrn-(D)-Hse-(L)-CTHPMD.

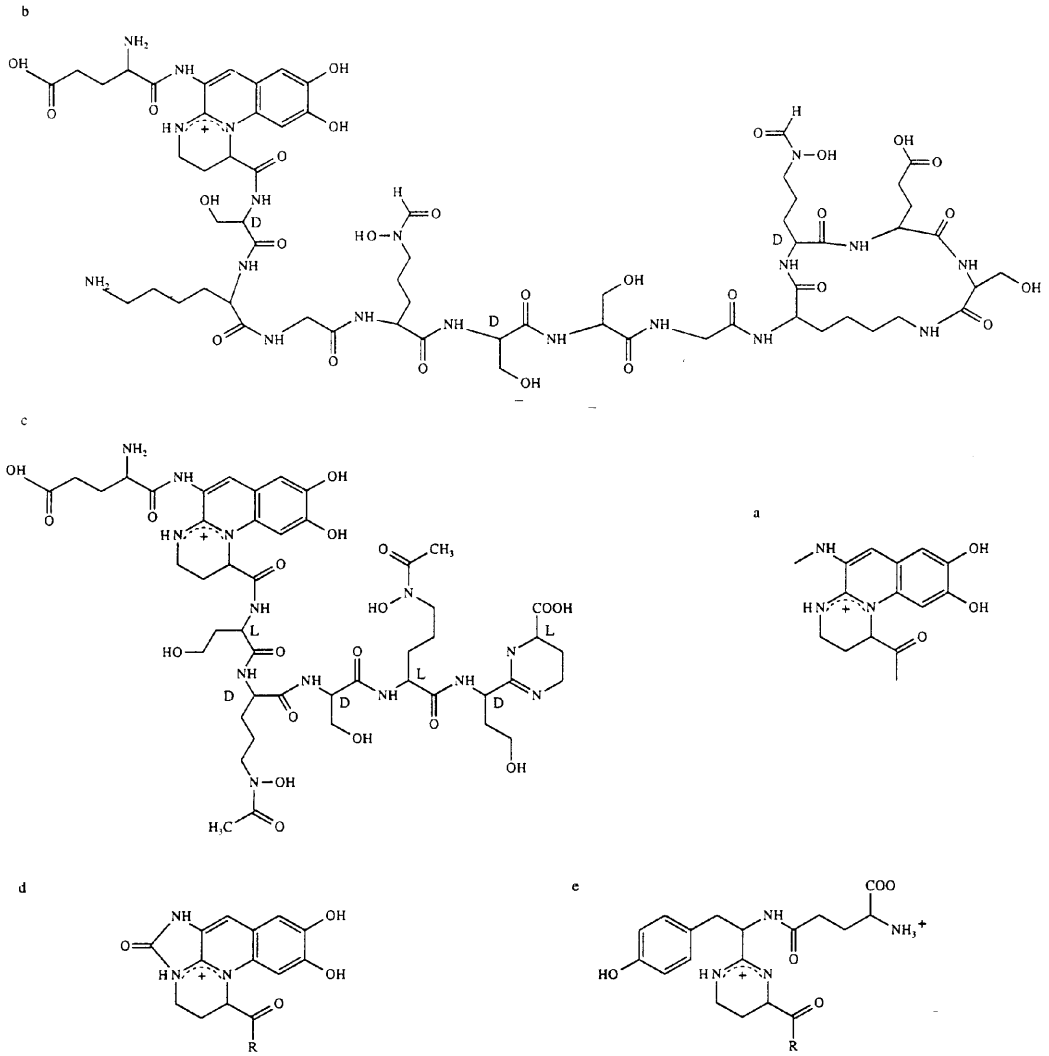


Figure 5 (a) Chromophore of the pyoverdins. (b) Pyoverdin Pf12. (c) Azoverdins. (d) Chromophore of the azotobactins. (e) Chromophore of the ferribactins.

The C-terminus is a cyclization of homoserine and 2,4-diaminobutyric acid. The cyclic amidine belongs to the tetrahydropyrimidine family: 2-homoseryl-4-carboxy-3,4,5,6-tetrahydropyrimidine (Figure 5(c)) [39].

From Azotobacteriaceae several related siderophores have been isolated [40–43] that show a high similarity to pyoverdins. Azotobactins share the peptidic component with the pyoverdins, but differ in the type of chromophore attached on the N-terminus. The carboxylic acid of the pyoverdin chromophore is missing; instead there is an extra imidazolone ring (Figure 5(d)).

Ferribactins [44] are non-fluorescent siderophores from pseudomonads, which are presumably precursors of the pyoverdins. Ferribactins and pyoverdins isolated from the same producer strain are identical in their peptide sequence [36]. Ferribactin from *Pseudomonas aptata*, for example, lacks the chromophore of pyoverdin Py Pap, but contains a cyclization product of Tyr/Dab instead (Figure 5(e)), which is linked through Glu to the peptide chain.The γ-carboxy group of Glu is amidically linked to the cyclized Tyr/Dab, the α-carboxy group is bound to the N-terminus of the oligopeptide [46].

The biosynthetic steps of pyoverdin production are being intensively studied. As can be expected from the occurrence of D-amino acids in the peptide sequence of siderophores from the pyoverdin family, the synthesis of the oligopeptides does not proceed

Table 1 Structures of Pyoverdins and Pseudobactins

	R = *N*-acyl residue of chromophore	Peptide sequence	Amino acids forming cyclic substructures
Ps	Succinic acid (amide), 2-oxoglutaric aid	Lys-Asp(OH)-Ala-aThr-Ala-cOrn(OH)	cOrn(OH)
Ps 7SR1	Malic acid (amide)	Ser-Gly-Ala-Orn(AcOH)-Ser-Thr-Asp(OH)-Ser	Ser-Ser
Ps A214	Succinic acid (amide)	Ser-Ala-Gly-Ser-Ala-Asp(OH)-aThr-Orn(AcOH)	–
Py I-III	2-Oxo-glutaric acid, succinic acid (amide)	Asn-Orn(FoOH)-Lys-Thr-Ala-Ala-Orn(FoOH)-Lys	Lys-Thr
Py C-E (Pa)	2-Oxo-glutaric acid, succinic acid (amide)	Ser-Arg-Ser-Orn(FoOH)-Lys-Orn(FoOH)-Thr-Thr	Thr-Lys
Py Gm	2-Oxo-glutaric acid, succinic acid (amide)	Ala-Lys-Gly-Gly-Asp(OH)-Gin-Ser-Ala-Ala-Ala-Ala-cOrn(OH)	–
Py Pf 12	2-Oxo-glutaric acid, glutamic acid, succinic acid (amide)	Ser-Lys-Gly-Orn(FoOH)-Ser-Ser-Gly-Lys-Orn(FoOH)-Glu-Ser	Ser-Lys
Py R	Succinic acid (amide)	Ser-Dab-Orn(FoOH)-Gln-Gln-Orn(FoOH)-Gly	Ser/Dab
Ps 589a	Malic acid (amide)	Asp-Lys-Asp(OH)-Ser-Thr-Ala-Glu-Ser-cOrn(OH)	–
Py Pt 2192	2-Oxo-glutaric acid, succinic acid (amide)	Ser-Lys-Ser-Ser-Thr-Ser-Orn(AcOH)-Thr-Ser-cOrn(OH)	–
Py Pf	Malic acid (amide), succinic acid (amide)	Ser-Dab-Gly-Ser-Asp(OH)-Ala-Gly-Ala-Gly-cOrn(OH)	Ser/Dab
Py Pap3	Succinic acid (amide)	Ala-Lys-Thr-Ser-Orn(AcOH)-cOrn(OH)	–
Py Pp1,2	Succinic acid (amide)	Ser-Thr-Ser-Orn-Asp(OH)-Gln-Dab-Ser-aThr-cOrn(OH)	Gln/Dab
Py PaT II	Succinic acid (amide)	Ser-Orn(FoOH)-Orn-Gly-aThr-Ser-cOrn(OH)	–
Py Pp2,3	Malic acid (amide), succinic acid (amide)	Asp-Orn(BuOH)-Dab-Thr-Gly-Ser-Ser-Asp(OH)-Thr	–
Py Pf_{13525}	2-Oxo-glutaric acid, glutamic acid, succinic acid (amide)	Ser-Lys-Gly-Orn(FoOH)-Lys-Or(FoOH)$_n$-Ser	Ser-Lys
Py Pf_{17400}	Succinic acid (amide)	Ala-Lys-Gly-Gly-Asp(OH)-Gln-Dab-Ser-Ala-cOrn(OH)	Gln/Dab

Fo = formyl, Ac = acetyl, cOrn(OH) = *cyclo*-N^5-hydroxyornithine, Asp(OH) = β-hydroxyaspartic acid, aThr = *allo*-threonine.

on the ribosomes. The genes involved in synthesis and uptake of pyoverdins are clustered in the *pvd* region of the genome. They are regulated by *pvdS*, the promotor region that matches the consensus binding site of *E. coli Fur* protein. Involvement of *Fur* proteins has been proven in *Pseudomonas putida* [47]. The transcription of the *pvdS* gene is therefore iron-repressible [48]. The translation product of *pvdD*, a gene involved in the biosynthesis of pyoverdins, is a protein of 2448 residues and a molecular mass of approximately 270 kDa, which has high similarity with peptide synthetases from a wide range of bacterial and fungal species [49]. The amino acid components of the peptide chain are activated as their adenylates [50]. Intrinsic limitations of the synthetase complex or deficiency on certain amino acids might be the reason for the heterogeneity of pyoverdin siderophores isolated from each single producer strain [51]. Another gene from the *pvd* gene cluster, *pvdA*, encodes for a peptide with 426 residues and a molecular mass of 47.7 kDa. It functions as an L-ornithine-N^5-oxidase [52] and thus provides part of the side-chain hydroxamates of the pyoverdin-type siderophores. Recently, a forth gene, *pvdE*, essential for pyoverdin synthesis in *Pseudomonas aeruginosa*, has been identified. Its deduced amino acid sequence is similar to membrane transporter proteins of the ATP-binding cassette [53]. For the biosynthesis of the chromophore of the pyoverdins, a hypothesis has been put forward [54]. In this proposal, tyrosine is hydroxylated to 3,4-dihydroxyphenylalanine and 2,4,5-trihydroxyphenylalanine. The latter is condensed with L-2,4-diaminobutyric acid and cyclized to 5-amino-2,3-dihydro-8,9-dihydroxy-1H-pyrimido-[1,2a] quinoline-1-carboxylic acid, the chromophore of the pyoverdins. This pathway has gained credibility by the identification of a side-product,

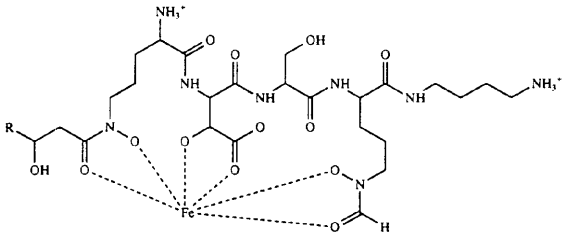


Figure 6 The hydroxamate siderophores ornibactins are acylated linear tetrapeptide amides: Ornibactin C4, R = CH_3–; Ornibactin C6, R = CH_3–$(CH_2)_2$–; Ornibactin C8, R = CH_3–$(CH_2)_4$–.

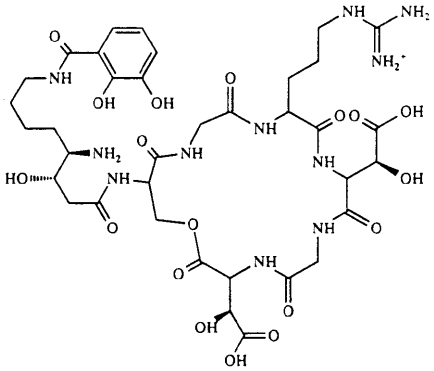


Figure 7 Alterobactin A has a 19-membered depsipeptide structure.

pseudoverdine. It is a coumarine derivative and arises from cyclization of 2,4,5-trihydroxyphenylalanine in the absence of L-2,4-diaminobutyric acid [54]. It lacks the peptide part of the pyoverdins, consisting of a chromophore only. It does complex iron, but does not restore growth to *pvd* mutants or mediate iron uptake [55]. The chromophore of the pseudobactins starts from tyrosine, as has been proven by isotope labelling. 3,4-Dihydroxyphenylalanine was not incorporated showing that hydroxylation occurs only after incorporation of tyrosine [56].

Ornibactins are a group of homologous siderophores resembling the pyoverdin-type siderophores in their oligopeptidic component [57]. They lack a chromophore, and instead carry different β-hydroxy fatty acids on their N-hydroxylated side chain of the N-terminal ornithine [58]. The C-terminus is amidated by putrescine (Figure 6). Iron binding occurs through two acylated N^5-hydroxy-L-ornithines and D-*threo*-β-hydroxyaspartic acid.

Alterobactins are siderophores from marine bacteria (*Alteromonas luteoviolacea*) similar to pseudomonads. Alterobactin A [5] is a hexapeptide lactone with the sequence Ser-Gly-Arg-β-HOAsp-Gly-β-HOAsp (Figure 7). The N-terminal serine is acylated with 3-hydroxy-4,8-diamino-octanoic acid, which in turn forms a terminal amide with 2,3-dihydroxybenzoic acid. Iron-binding groups are the catecholate-moiety and both β-HOAsp, thus forming trisbidentate complexes with ferric ions. The formation constant of the ferric complexes is reported to be in the range of 10^{49} to 10^{53}, exceptionally high and comparable to enterobactin, the strongest known iron chelator. Alterobactin B is the open chain analogue with a hydrolysed lactone bond.

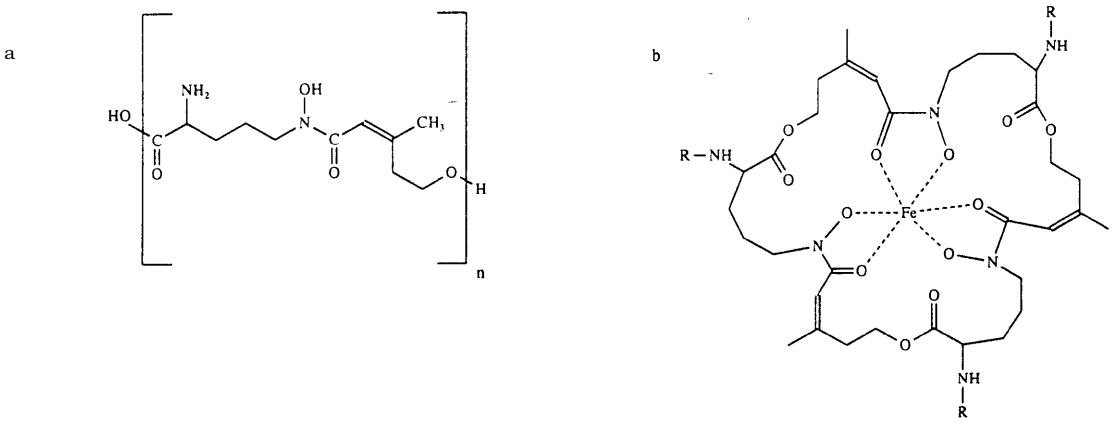


Figure 8 Fusarinines are cyclic siderophores containing N^5-hydroxyornithine, N^5-acylated with *cis*-anhydromevalonic acid: Fusarinine A, $n=2$, Fusarinine B, $n=3$; (b) Fusarinine C, R=H; Triacetylfusarinine C, R=$COCH_3$.

Siderophores with Side-Chain-Connected Amino Acids Along Their Backbones

Fusarinines are a class of hydroxamate siderophores composed of N^5-acyl-N^5-hydroxy-L-ornithine and anhydromevalonic acid. They have been isolated from fungi of the genera *Fusarium*, *Giberella*, *Aspergillus*, *Penicillium* and many others [59–62]. The monomeric unit, obtained as a degradation product of fusarinines [63], is *cis*-fusarinine, in which N^5-hydroxy-L-ornithine is N^5-acylated by *cis*-5-hydroxy-3-methyl-2-pentenoic acid. *cis*-Fusarinine is therefore a monohydroxamate. For the formation of hexadentate complexes, siderophores should ideally contain three of these building blocks. Fusarinine A is the linear dimer and fusarinine B the linear trimer of *cis*-fusarinine (Figure 8(a)) [64].

N^{α}-diacetylfusarine A and N^{α}-triacetylfusarine B are acetylated on the α-amino groups. The cyclic triester of *cis*-fusarinine is fusigen (fusarinine C). The free α-amino group of the ornithine residues in fusigen can be acetylated: *N*, *N′*, *N″*-triacetylfusigen or *N*, *N′*, *N″*-triacetylfusarinine C (Figure 8(b)) [65].

Fusigen synthetase is an enzyme of 600–800 kDa and a pH optimum of 7.0 [66]. Its substrate is activated N^5-*cis*-anhydromevalonyl-N^5-hydroxy-L-ornithine [67]. Since no dimers and trimers are found, the cyclic triester is presumably synthesized from monomers bound to the enzyme as adenylates or thioesters. The biosynthesis of the earlier precursors, *cis*-anhydromevalonic acid and N^5-hydroxy-L-ornithine, has been discussed in the context of the ferrichrome-type siderophores (above).

Coprogens are a group of fungal trihydroxamate siderophores containing diketopiperazine rings. Two molecules of N^5-acyl-N^5-hydroxy-ornithine are joined together head to head, forming the six-membered diketopiperazine ring. One of the acyl residues is *trans*-anhydromevalonic acid, which in turn is esterified with N^5-acyl-N^5-hydroxy-yornithine (Figure 9). Therefore, two terminal acylations and one or two acetylations or two methylations on the α-amino group of the ester-bound N^5-acyl-N^5-hydroxy-ornithine allow for diversity in the coprogen family (Figure 9). Desferricoprogen B (desferri-desacetylcoprogen) is twice terminally acylated with *trans*-anhydromevalonic acid [68]. Desferricoprogen is twice terminally acylated with *trans*-anhydromevalonic acid and α-acetylated [69]. Desferrineocoprogens differ from coprogen in being once or twice terminally acetylated instead of carrying *trans*-anhydromevalonic acid [70]. Desferridimethylcopro-

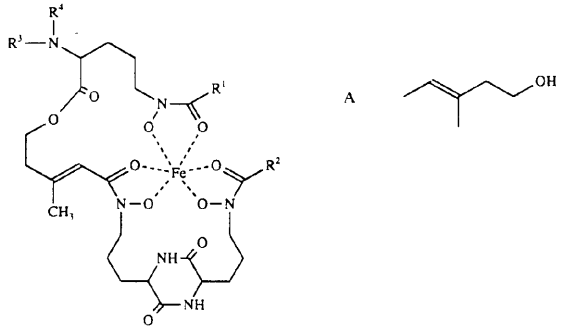


Figure 9 Coprogens have a diketopiperazine ring of two N^5-acylated-N^5-hydroxylated ornithines as a basic structure: coprogen, $R^1=H$, $R^2=COCH_3$, $R^3=R^4=A$; coprogen B, $R^1=R^2=H$, $R^3=R^4=A$; neocoprogen I, $R^1=H$, $R^2=COCH_3$, $R^3=CH_3$, $R^4=A$; neocoprogen II, $R^1=H$, $R^2=COCH_3$, $R^3=R^4=CH_3$; N^{α}-dimethylcoprogen, $R^1=R^2=CH_3$, $R^3=R^4=A$.

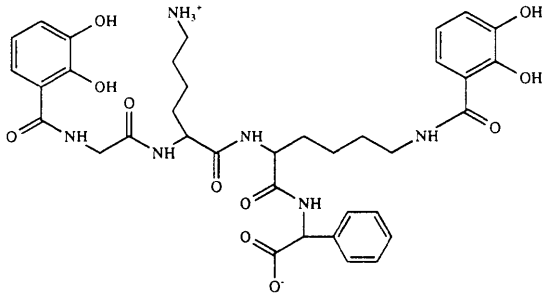


Figure 10 Amonobactins are tri- and tetrapeptide siderophores containing 2,3-dihydroxybenzoic acid. Phenylalanine can be substituted by tryptophane, glycine can be omitted.

gens are twice α-methylated [71]. Desferrihydroxycoprogens have 4-hydroxy-*trans*-anhydromevalonic acid as one of the terminal acylations [72].

Amonabactins are a group of bacterial catecholate siderophores in which two molecules of lysine are joined head to head, without forming a diketopiperazine ring. Their amino groups are acylated with 2,3-dihydroxybenzoic acid, with or without the insertion of a glycine residue (Figure 10). The open carboxy group of one of the lysines can be amidated with phenylalanine or tryptophan. Studies of amonabactin negative mutants suggest that biosynthetic genes are located in two distinct groups, one synthesizing 2,3-dihydroxybenzoic acid, the other assembling the latter with the amino acids into the complete siderophore. Tryptophane- and phenylalanine-containing variants may be synthesized by the same novel enzyme that inserts either Phe or Trp into amonabactin [73]. The biosynthesis of 2,3-dihydroxybenzoic acid is different from, but functionally related to, the 2,3-dihydroxybenzoic acid operon of *E. coli* [74]. The biosynthetic gene *amoA*, cloned from *Aeromonas hydrophila* [75], corresponds to isochorismate synthetase in *E. coli*.

Exochelins are the recently discovered exo-siderophores of the mycobacteria. Exochelins MS [76] and MN [77] contain side-chain connected N^5-hydroxyornithines in their backbone. In exochelin MS, the N-terminal lysine is formylated, the second and third hydroxamate are formed by N^5-acyl-N^5-hydroxyornithine in the backbone (Figure 11(a)). Exochelin MN has a cyclo-N-hydroxyornithine at the C-terminus and another hydroxamate group on N^5-hydroxy-N^2-methylornithine in the backbone (Figure 11(b)). Exochelins are structurally similar to mycobactins, lipophilic membrane-bound iron shuttle compounds of mycobacteria, described below.

The biosynthesis of these exochelins has been shown to be different from the biosynthetic pathway of mycobactins, since they are no precursors to mycobactins. The formylation of exochelin MS is presumably effected by an enzyme with some homology to phosphoribosylglycine amide formyltransferases (GAR enzymes) and methionyl-tRNA formyltransferase [78].

Siderophores with Amino Acids Acting as Functional Carriers for Chelating Groups

Enterobactin (also named enterochelin) is one of the best-studied siderophores because it has the highest

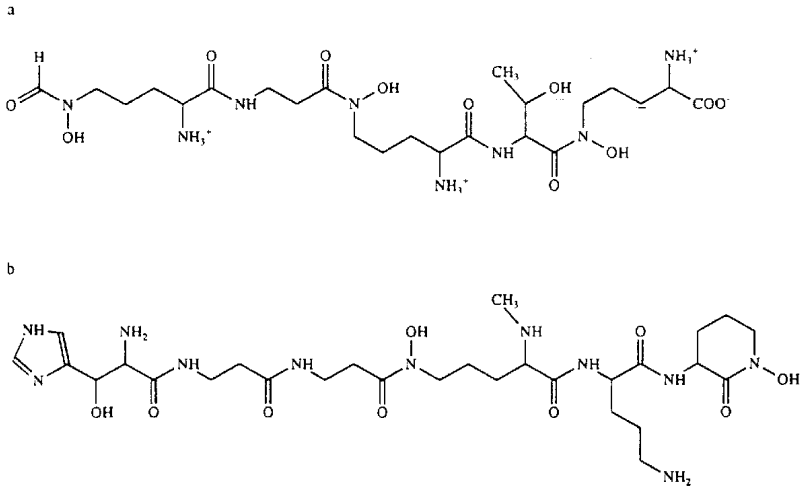


Figure 11 Exochelins MS and MN are linear penta- and hexapeptide hydroxamate siderophores: (a) exochelin MS; (b) exochelin MN.

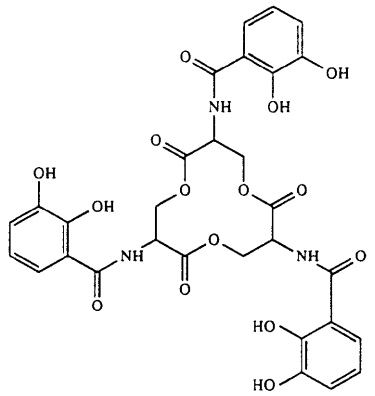


Figure 12 Enterobactin is a cyclic triester of serine, acylated with 2,3-dihydroxybenzoic acid.

reliably reported iron-binding capacity of all siderophores. It is a tricatecholate siderophore with a formation constant of its ferric complex of 10^{52}. Originally isolated from *E. coli* [8, 9], enterobactin has been found in many other enterobacteriaceae. The chelating groups are provided by three residues of 2,3-dihydroxybenzoic acid anchored on a skeleton of the cyclotriester of L-serine (Figure 12). By three internal H-bonds of the amide protons to the lactone oxygen of the same serine, a cavity for complexing ferric ions is preformed in the free ligand. When complexing ferric ions, the H-bonds are shifted to H-bonds between the amide protons and the catecholate oxygens (*ortho* in the aromatic ring) [79]. It is therefore the prototype of hexadentate siderophores and has been the target of an extensive study by synthetic analogues [79].

The biosynthesis of enterobactin is well investigated. 2,3-Dihydroxybenzoic acid is formed from chorismate by the gene products of *entABC*. EntC transforms chorismate into isochorismate [80], EntB cleaves off pyruvate, yielding 2,3-dihydro-2,3-dihydroxy-benzoic acid. EntA is 2,3-dihydro-2,3-dihydroxy-benzoate dehydrogenase [81]. The gene products of *entDEF* first link 2,3-dihydroxybenzoic acid to L-serine, then assemble the monomers to the cyclic trimer. EntE activates 2,3-dihydroxybenzoate as adenylate. EntF likewise produces the adenylate of L-serine, then cleaves off AMP and binds L-serine as thioester [1]. Both activated precursors are then processed by EntD [82], yielding enterobactin.

Protochelin, azotochelin and aminochelin are closely related catecholate siderophores, in which 2,3-dihydroxybenzoate is amidically bound to a framework of lysine and/or putrescine. Protochelin [83] is a tricatecholate, containing L-lysine, amidically linked to putrescine. Three molecules of 2,3-dihydroxybenzoate are bound to the terminal amino groups of lysine and putrescine and to the α-amino group of lysine (Figure 13(a)). Azotochelin [84], formally a fragment of protochelin, contains only L-lysine, both amino functions carrying 2,3-dihydroxybenzoate residues (Figure 13(b)). Myxochelin A is the lysinol analogue of azotochelin [85]. Aminochelin [86] is 2,3-dihydroxybenzoyl-putrescine, therefore the complementary fragment of protochelin (Figure 13(c)).

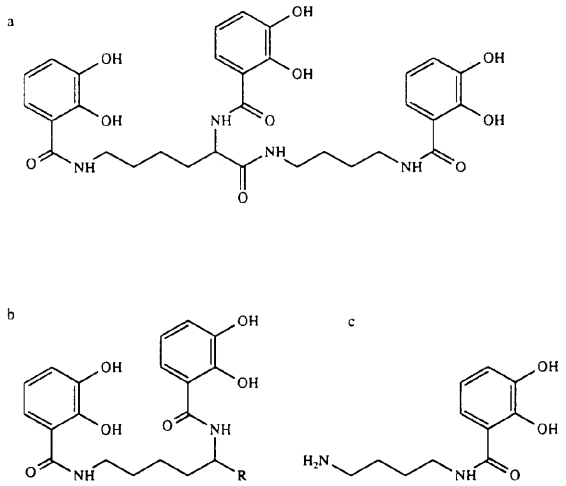


Figure 13 (a) Protochelin, (b) azotochelin, (c) aminochelin.

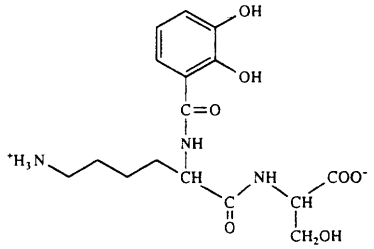


Figure 14 Chrysobactin.

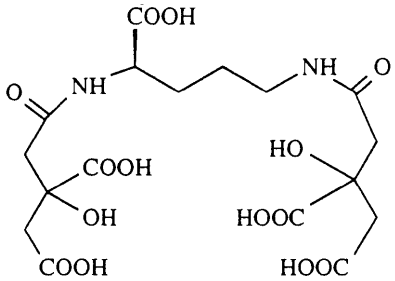


Figure 15 Staphyloferrin A, a citrate-based carboxylate siderophore.

Chrysobactin [14] is not related to the protochelin group, since it contains D-lysine. It is N^2-(2,3-dihydroxybenzoyl)-D-lysyl-L-serine, a functionalized

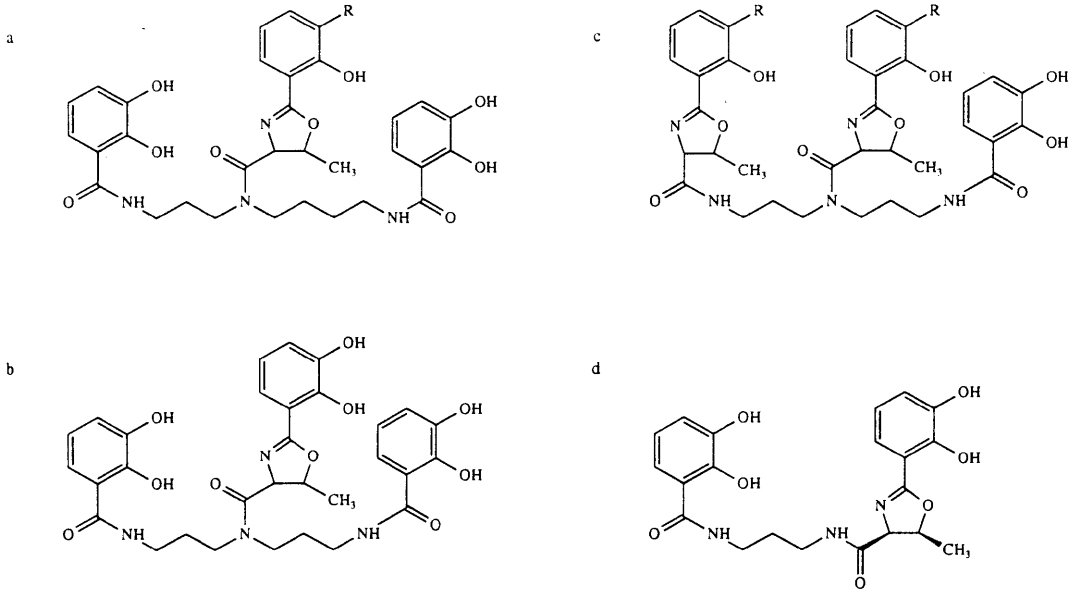


Figure 16 Siderophores containing oxazoline rings: (a) agrobactin, R = OH, parabactin: R = H; (b) Fluvabactin; (c) vibriobactin, R = OH, vulnibactin, R = H; (d) Serratiochelin.

dipeptide (Figure 14). Staphyloferrin A is a member of the carboxylate class of siderophores. It is N^2-N^5-dicitryl-D-ornithine [88], thus the basic amino acid is a difunctional carrier for the iron-chelating citric acid (Figure 15).

Siderophores Containing Cyclized Amino Acids

Agrobactin and parabactin (Figure 16(a)) are similar to the already-mentioned siderophores of the protochelin group. In agrobactin [89, 90], three molecules of 2,3-dihydroxybenzoic acid are located on a backbone of spermidine, the middle benzoyl moieties linked through a 5-methyloxazoline ring. Parabactin [91] is not a tricatecholate, since the middle aromatic residue is 2-hydroxybenzoic acid (salicylic acid). Since iron complexation requires six coordination groups, participation of the oxazoline nitrogen has been assumed [92, 93]. Fluvibactin [94] is the analogue of agrobactin, but based on norspermidine (Figure 16(b)). Vibriobactin [95] and vulnibactin [96] are similarly based on a backbone of norspermidine, but carry two 5-methyloxazoline moieties, one terminally and one in the middle of the molecule (Figure 16(c)). Vibriobactin resembles agrobactin in being a tricatecholate, whereas vulnibactin is a dicatecholate/salicylate.

Serratiochelin [97] can be regarded as an incomplete relative of agrobactin or vibriobactin, since it is based on 1,3-diaminopropane. One molecule of 2,3-dihydroxybenzoic acid is directly bound to the diamine, the other is linked through 5-methyloxazoline (Figure 16(d)). The configurations of the 5-methyloxazoline rings in agrobactin, parabactin, serratiochelin and vibriobactin are 4S, 5*R*, therefore these siderophores all contain L-threonine.

The biosynthesis of parabactin has been shown to involve dibenzoylation of spermidine on its terminal amine groups. This product then reacts with L-threonine and salicylic acid. Finally, cyclodehydration yields the siderophore [98]. In the case of agrobactin, the X-ray structure showed the relative configuration of the protons in the oxazoline ring to be *trans* [99]. If L-threonine is assumed to be incorporated in the biosynthesis of agrobactin (as is the case in parabactin), the methyl-oxazoline ring contains L-(*R*)-*allo*-threonine. If L-(*S*)-threonine is assumed to be the biologically active precursor [100], cyclization involves inversion at the β-carbon, presumably through imine formation on the benzoylamide of threonine.

Acinetobactin [101] is a monocatecolate/monohydroxamate siderophore. It contains 2,3-dihydroxybenzoic acid and 5-methyloxazoline-4-carboxylic acid, amidated with N^1-hydroxyhistamine (Figure 17(a)). Anguibactin [102, 103] is the thiazoline analogue of acinetobactin, the heterocycle derived from cysteine instead of threonine (Figure 17(b)).

A histidine decarboxylase has been identified as a necessary enzyme for the biosynthesis of anguibactin and histamin has been demonstrated to be a precursor for anguibactin [104]. The genes for the

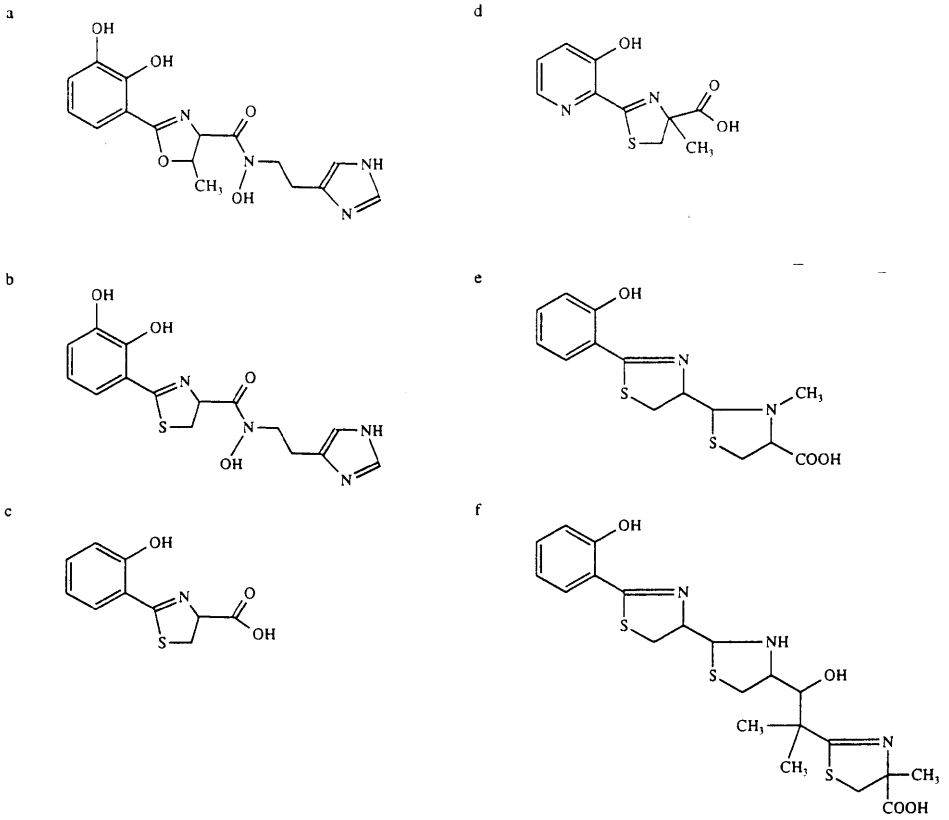


Figure 17 Siderophores containing oxazoline, thiazoline, thiazolidine and imidazole rings: (a) acinetobactin; (b) anguibactin; (c) aeruginic acid, (d) desferriferrithiocin; (e) pyochelin; (f) yersiniabactin.

precursor 2,3-dihydroxybenzoic acid are located on the chromosome of *Vibrio anguillarum*, whereas the expression of anguibactin is plasmid-mediated [105]. The protein AngR, positively regulating anguibactin biosynthesis, has an EntE-like enzymatic function [106].

Aeruginic acid [107] lacks the histamine residue of anguibactin and contains salicylic acid instead of 2,3-dihydroxybenzoic acid (Figure 17(c)). In desferriferrithiocin [108], salicylic acid is substituted for 3-hydroxy-pyridine-2-carboxylic acid. The five-membered heterocycle is 4-methylthiazoline, originating from L-α-methylcysteine (Figure 17(d)).

More than one cyclized amino acid is found in pyochelin and yersiniabactin. Pyochelin [109] is composed of salicylic acid, a thiazoline ring (from L-cysteine [110]) and *N*-methylthiazolidine-4-carboxylic acid (from L-α-methylcysteine [110]; Figure 17(e). Yersiniabactin [111, 112] contains a thiazoline ring, an unmethylated thiazolidine ring and terminates with 4-methyl-thiazoline-4-carboxylic acid (Figure 17(f)).

Salicylic acid has been demonstrated to be a precursor in the biosynthesis of pyochelin [113]. Four genes for the biosynthesis of salicylic acid, *pchA* to *pchD*, have been identified recently in *Pseudomonas aeruginosa*, with *pchDCBA* forming a single transcriptional unit [114]. PchA is a 56 kDa protein with similarity to isochorismate synthase, while PchB transformed an *entB* defective mutant of *E. coli* into a salicylate producer [115]. PchC is a 28 kDa thioesterase, while PchD is a 60 kDa protein similar to EntE of *E. coli*, a 2,3-dihydroxybenzoate-AMP ligase. The pathway to salicylic acid can therefore be assumed to lead from chorismate to isochorismate and then to salicylate plus pyruvate, catalysed consecutively by the iron-repressible PchA and PchB proteins. Salicylate is then activated as adenylate [114], coupled to cysteine, and the latter is cyclized to a thiazoline ring. The enzymes involved in the incorporation of salicylic acid into the siderophore have limited specificity, since a number of analogues could be incorporated as well [116]. The incorporation of 3-hydroxypicolinic acid is note-

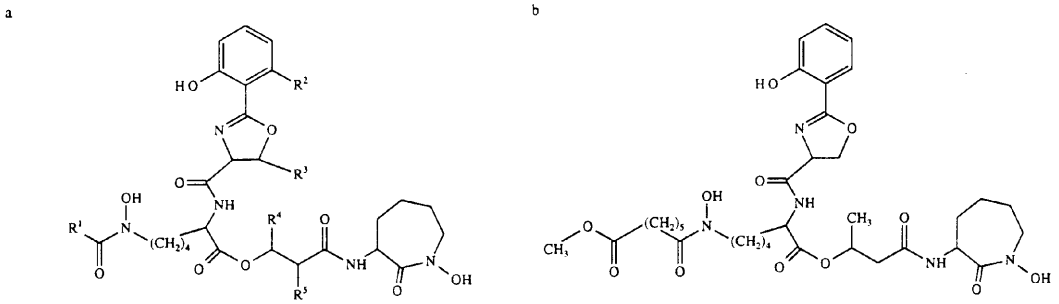


Figure 18 (a) Mycobactins: $R^1 = CH_3(CH_2)_n$, $R^2 = H/CH_3$, $R^3 = H/CH_3$, $R^4 = H/CH_3/C_2H_5$, $R^5 = H/CH_3$. (b) Exochelins and carboxymycobactins.

worthy, which is also found in the siderophore desferriferrithiocin (see above and Figure 17(d)).

Mycobactins are a group of closely related siderophores that were isolated from mycobacteria [117–119] much earlier than the exochelins, described above. Since they are acylated with long chain fatty acids (C_{12}–C_{18}), they are insoluble in aqueous media and not excreted as exosiderophores. They probably serve as intermediate carriers, firmly located close to the cytoplasmic membrane [120]. Mycobactins (Figure 18(a)) are easily cleaved at an ester bond into two fragments, mycobactic acid and cobactin. Mycobactic acid is composed of salicylic acid (or 5-methylsalicylic acid), cyclized L-serine (or cyclized L-threonine), N^6-hydroxy-L-lysine and a mixture of fatty acids, often α-β-unsaturated. Cobactin contains a C_4- or C_5-hydroxyacid and cyclo-N^6-hydroxy-L-lysine. Through the two molecules of acylated N^6-hydroxy-L-lysine, the mycobactins are dihydroxamates. Nevertheless, their affinity for ferric ions is very high: $pK_F = 36$ [119], comparable to trihydroxamates.

A group of exochelins with structures analogous to the mycobactins has recently been described. Instead of fatty acids, they contain shorter saturated or longer monounsaturated or saturated [121] dicarboxylic acids (C3–C9), terminating in methyl esters [122] or, in the case of carboxymycobactins, open carboxy groups [123] (Figure 18(b)). As well as the exochelins MS and MN (see above), these are exosiderophores, exereted into the culture media.

A siderophore with a very unusual cyclization is maduraferrin [124]. It is composed of salicylic acid, β-alanine, glycine, L-serine, N^5-hydroxy-N^5-methyl-L-ornithine and L-hexahydropyridazine-3-carboxylic acid. The pyridazine ring system originates from two molecules of ornithine, forming a diketopiperazine ring. One ornithine is cyclized N^5 to N^2. The other ornithine is N^5-hydroxylated and acylated by the C-terminus of the tripeptide β-Ala-Gly-Ser, the N-terminus of which is acylated with salicylic acid (Figure 19).

Siderophores Containing Amino Acids of Unusual Structures

Rhizobactin DM4 [125] is the first representative of the carboxylate class of siderophores, to which, among the peptidic siderophores, also belongs staphyloferrin A (see above). It is composed of alanine, its amino group ethylene-bridged to the amino group of lysine, the side chain of which is acylated with L-malic acid (Figure 20).

The schizokinen/acrobactin family of siderophores is characterized by citric acid with amino acids or diamines amidated on both terminal carboxy groups. Schizokinen, arthrobactin, rhizo-

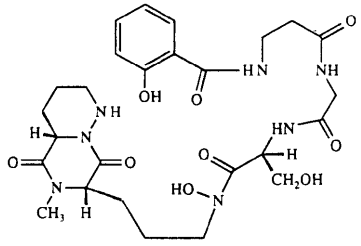
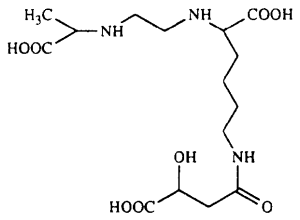


Figure 19 Maduraferrin.



Rhizobactin DM4.

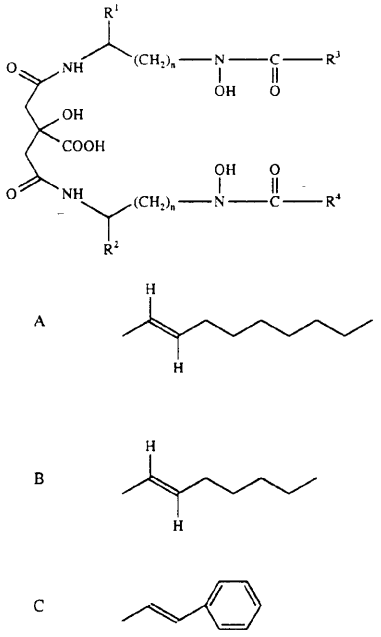


Figure 21 Aerobactin and nannochelins are citrate-containing dihydroxamate siderophores. (a) Aerobactin: $R^1 = R^2 = COOH$, $R^3 = R^4 = CH_3$, $n = 4$. (b) Nannochelin A: $R^1 = R^2 = COOCH_3$, $R^3 = R^4 = C$, $n = 4$. Nannochelin B: $R^1 = COOCH_3$, $R^2 = COOH$, $R^3 = R^4 = C$, $n = 4$. Nannochelin A: $R^1 = R^2 = COOH$, $R^3 = R^4 = C$, $n = 4$.

bactin 1021 and acinetoferrin contain diamines and therefore are not considered in this review. Acrobactin contains citric acid, terminally amidated with two molecules of ornithine, the side-chain amino groups of which are N-hydroxylated and acetylated (Figure 21(a)). It is therefore a symmetric dihydroxamate, the fifth and sixth coordination groups for ferric ions being provided by the carboxy and the hydroxy group on the quaternary carbon of citric acid. The nannochelins carry cinnamoyl residues instead of acetylations. In nannochelin C, both carboxy groups of lysine are free, nannochelin B is a monomethylester and nannochelin A is a dimethylester (Figure 21(b)).

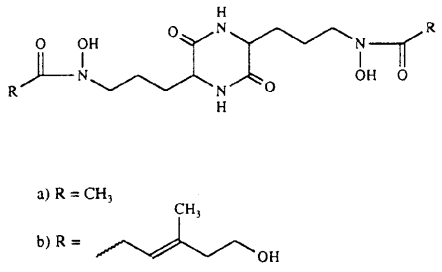


Figure 22 Siderophores containing diketopiperazine rings from N^5-acylated-N^5-hydroxylated ornithine: (a) rhodotorulic acid; $R = CH_3$; (b) dimerum acid, R = A.

The biosynthesis of aerobactin has been well studied. The biosynthetic genes are located on the ColV-plasmid [126, 127], a recognized virulence factor of pathogenic *E. coli* strains [128]. Four genes and their corresponding polypeptide products have been identified [129]. AerA, a 50 kDa protein, is a lysine-N^6-oxidase [130]. AerB, 35 kDa, is N^6-hydroxylysine : acetylCoA N^6-acetyltransferase [131]. The first molecule of N^6-hydroxylysine is condensed with citric acid by AerD, 60 kDa, the second amidation is performed by AerC, 62 kDa.

Rhodotorulic acid [132] consists of two molecules of *N*-acetyl-*N*-hydroxy-L-ornithine joined 'head to head', i.e. condensation of their α-amino and α-carboxy groups yields a diketopiperazine ring (Figure 22(a)), which is also present in the structurally related coprogens (see above). Dimerum acid [68] differs only in the type of acylations of the side chains. Instead of acetic acid it contains *trans*-anhydromevalonic acid (Figure 22(b)). Both siderophores are dihydroxamates. Therefore, their complexes with ferric ions are dimeric, Fe_2L_3, with bridging ligands.

The biosynthesis of N^5-acetyl-N^5-hydroxy-L-ornithine has been described in the context of ferrichromes (see above). In *Rhodotorula pilimanae*, N-hydroxylation of L-ornithine occurs exclusively

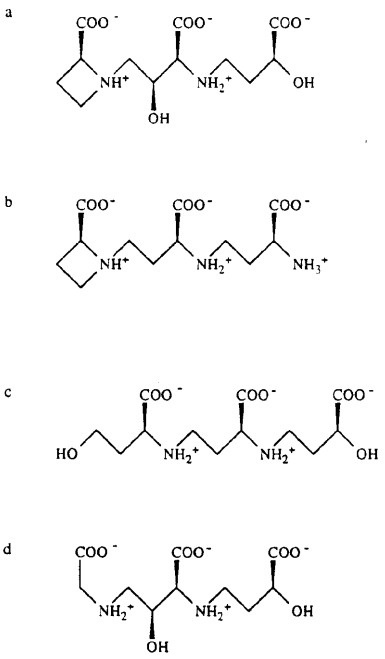


Figure 23 Phytosiderophores: (a) mugineic acid; (b) nicotianamine; (c) avenic acid; (d) distichonic acid.

from molecular oxygen, as has been demonstrated by isotope labelling experiments [133]. The *N*-acetyltransferase of *Rhodotorula pilimanae* is less specific, accepting also N^6-hydroxylysine, N-methyl-hydroxylamine and even hydroxylamine [134]. Two molecules of N^5-acetyl-N^5-hydroxy-L-ornithine are then condensed by rhodotorulic acid synthetase in the presence of ATP, with a pH optimum of 8.5 [135].

The phytosiderophores around mugineic acid contain α-amino acid substructures, but no amide bonds. Instead, their amino acid components are linked through *N*-alkylations. The nitrogen atoms are not hydroxylated, ferric ions are complexed by carboxy and hydroxy groups. Mugineic acid (Figure 23(a)) [136], 2′-deoxymugineic acid [137], 3-hydroxymugineic acid [138] and isomugineic acid [139], isolated from barley, wheat and rye, as well as nicotianamine (Figure 23(b)) [140] from tobacco, contain an azetidine ring. Avenic acid (Figure 23(c)) [141] from oat and distichonic acid (Figure 23(d)) [138] from beer barley possess open-chain structures.

Chemical Synthesis

There are various reasons for the chemical synthesis of siderophores. Preparing the structures identical to the native compounds is primarily a proof of the reported structure. Synthesizing those siderophores that have medical or industrial applications has to compete with optimized fermentation procedures. Even for comparatively simple structures, enantiomeric purity can be difficult to achieve in chemical synthesis, so that microbiological production is more economic. Chemical synthesis is, however, unrivalled when structural analogues must be prepared that are unknown in natural systems.

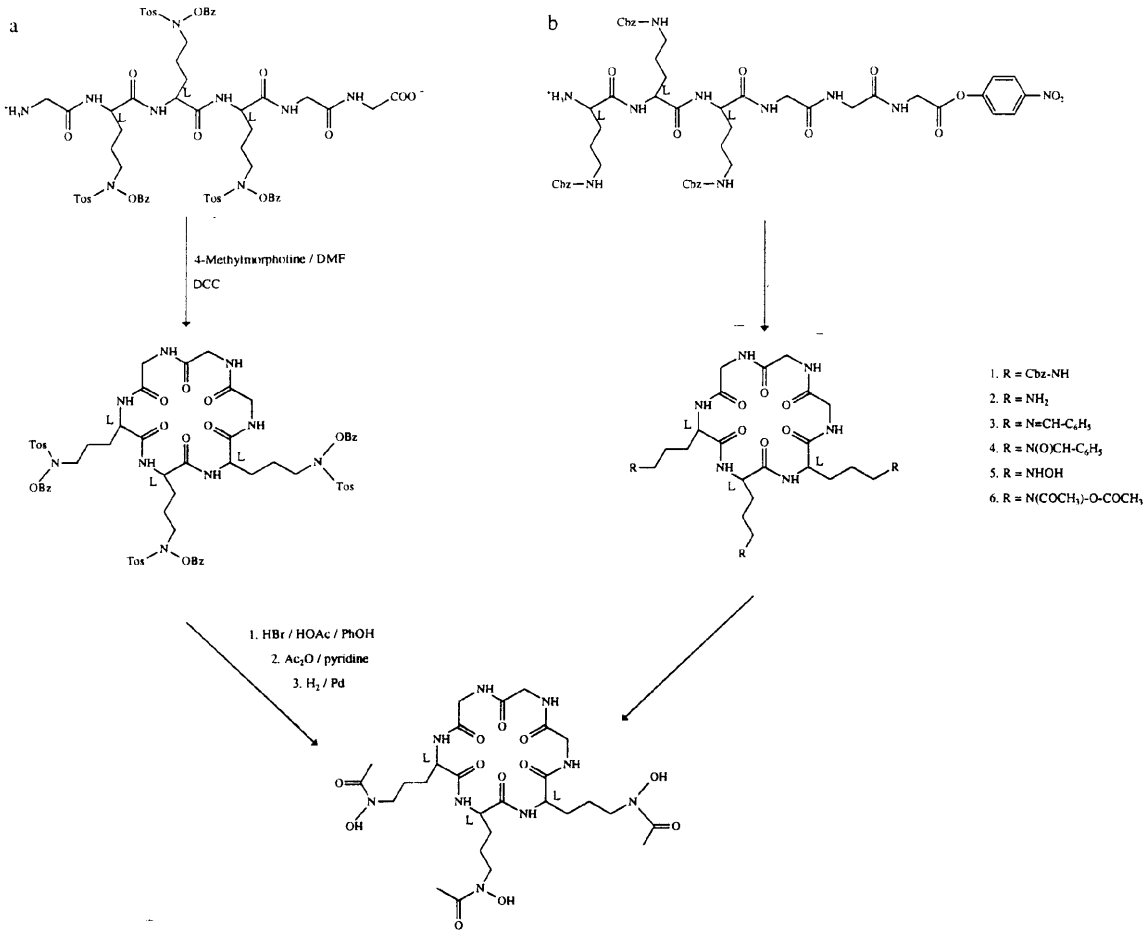


Figure 24 (a) Synthesis of ferrichrome, using protected N^5-hydroxyornithine. (b) Synthesis of ferrichrome, through oxaziridines.

Chiral siderophores can be prepared that are the opposite enantiomer of the natural product. With *enantio*-ferrichrome [142] and *enantio*-enterobactin, the stereospecificity of receptor recognition has been studied. Both *enantio*-siderophores form ferric complexes with opposite chirality compared to the natural product, and both receptors do not transport the *enantio*-ferric complexes. Methylene homologues of natural siderophores and synthetic analogues have been prepared to study the equilibrium of differently bridged dimers [143]. The exceptional coordination properties of enterobactin have been explained with the help of synthetic analogues [144].

Using a selection of several representative and instructive examples we will give an overview of synthetic possibilities for different classes of compounds. We do not intend to mention or describe every synthesis that has been used to obtain native siderophores or their analogues.

Following the same sequence as previously concerning the structures and biosynthesis of siderophores, we start with the oligopeptidic siderophores. Desferriferrichrome is a cyclohexapeptide, three residues of which are based on the unusual amino acid N^5-hydroxy-L-ornithine. The procedures of stepwise coupling of activated amino acids onto a growing peptide chain can be applied when a suitably protected derivative of N^5-hydroxy-L-ornithine can be found. Since acetylation of ornithine interferes with the hydroxy group on N^5-hydroxy-L-ornithine, the side chain must be orthogonally protected. For this purpose, N^5-benzyloxy-N^5-tosyl-L-ornithine [145, 146] has been used. The linear hexapeptide Gly-Orn*-Orn*-Orn*-Gly-Gly is synthesized (Figure 24(a)), cyclized and the tosyl-group removed selectively with HBr/CH_3COOH. The amino functions are then acetylated and finally the *O*-benzylations are removed by hydrogenation. Sequence-modified analogues of ferrichromes have been synthesized using N^5-benzyloxy-N^5-butyloxycarbonyl-L-ornithine [147]. Another synthetic scheme is the formation of the N^5-hydroxy group on the side-chain amine of ornithine. In this approach (Figure 24(b)), the linear hexapeptide is built with side-chain protected ornithines and cyclized. After removing the protecting groups, benzaldimines are formed and oxidized to oxaziridines. Upon cleavage, the oxaziridine rings yield N^5-hydroxyornithines which are acetylated twice. Finally the acetyl ester is cleaved off, yielding N^5-hydroxy-N^5-acetyl-ornithine. Both syntheses were performed with L-ornithine as well as D-ornithine, the latter providing access to *enantio*-ferrichrome [148]. By substituting glycine for serine and by different acylations, other members of the ferrichrome family are accessible as well. What remains

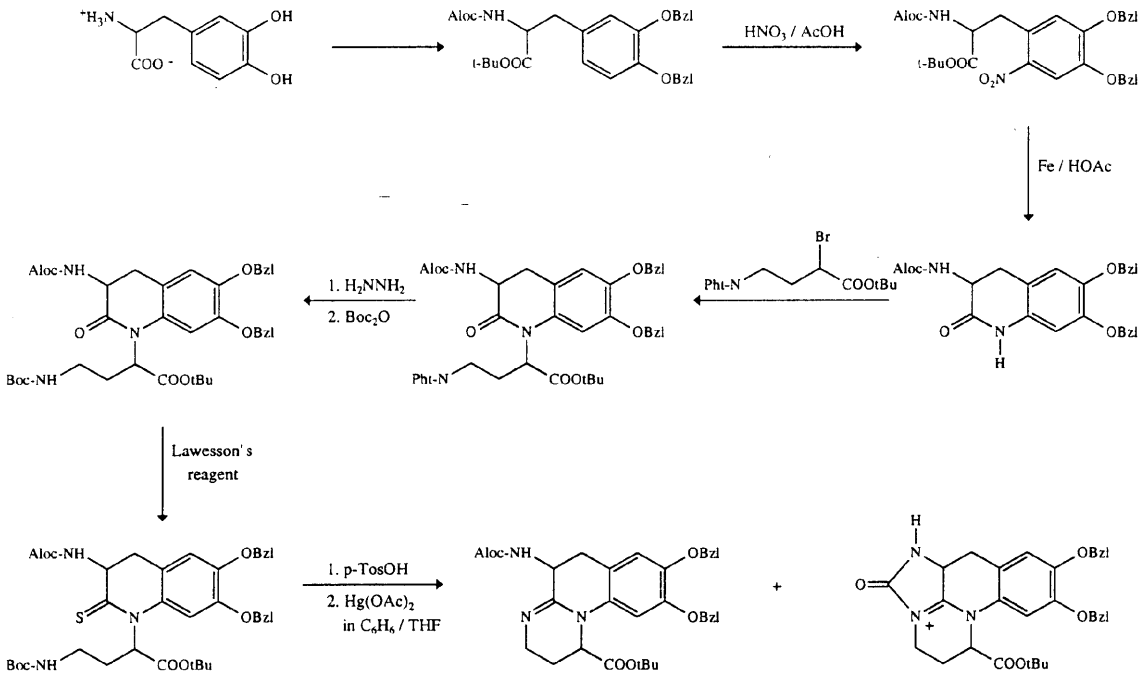


Figure 25 Synthesis of the chromophore of azotobactins.

complicated is the occurrence of mixed acylations, as in the asperchromes.

Pyoverdine-type siderophores, including pseudobactins, azoverdins, azotobactins and ferribactins, contain acetylated or formylated N^5-hydroxyornithine, D-amino acids and β-hydroxyaspartic acid. Their oligopeptidic parts can be imagined to be synthesized by similar procedures, and the different chromophores attached to it. The chromophores themselves can also be synthesized chemically, as is exemplified by the synthesis of the chromophores of pseudobactin and azotobactin, starting from dihydroxyphenylalanine (Figure 25). The choice of protection groups and observed side reactions are discussed in the original paper [149]. The dibenzylether of N^{α}-Aloc-protected DOPA-*t*-butylester is nitrated in the *ortho* position. Reduction of the nitro group, cleavage of the butyl ester and lactamization is carried out in one step with iron in acetic acid. The bicyclic intermediate is reacted with the butyl ester of N^{γ}-phthaloyl-protected γ-amino-α-bromobutyric acid. The phthaloyl protection of the product was exchanged against Boc-protection and the thioamide formed with Lawesson's reagent. The Boc-group was carefully removed with retaining the *t*-butyl ester. Cyclization with

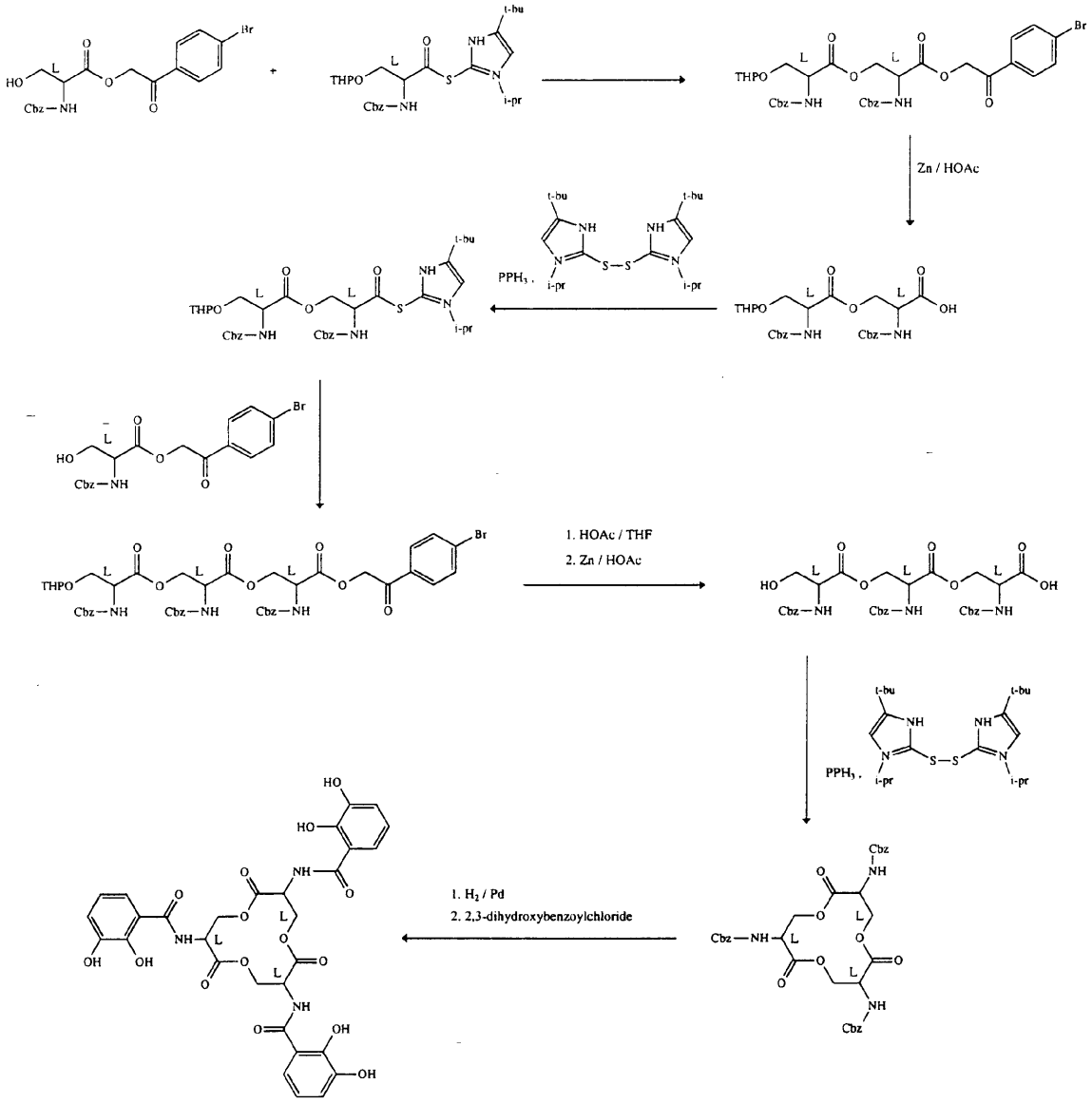


Figure 26 (a) Synthesis of enterobactin by the method of thioesters. (b) Synthesis of enterobactin on organotin template.

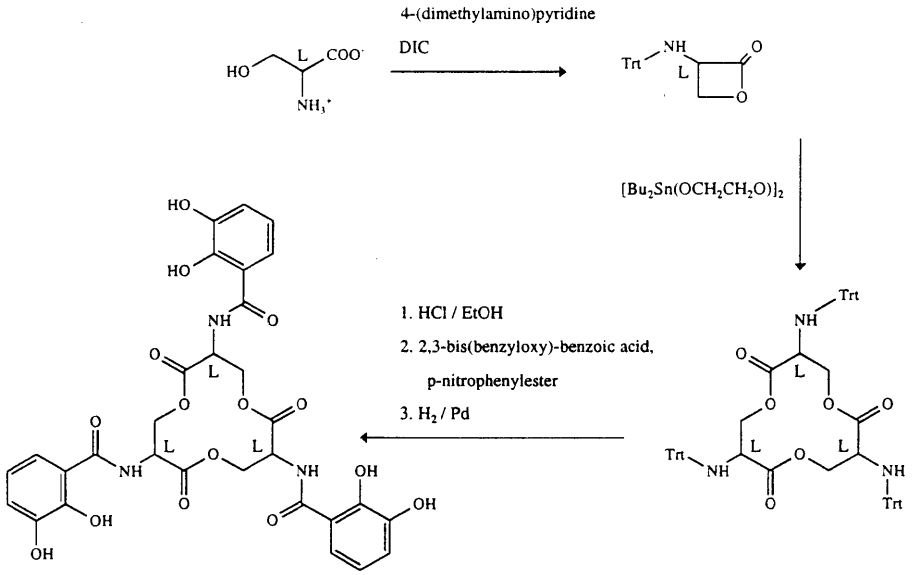


Figure 26(b)

mercuric acetate yielded a mixture of the chromophores of the azotobactins and of the pseudobactins. Exclusive formation of the chromophore of the pseudobactins can be effected by converting the Aloc protection of the α-amino group into Boc protection on the stage of the bicyclic intermediate. Final cyclization is carried out on the N^{7}-Troc-protected thioketone, obtained analogously with Lawesson's reagent [149]. The protected chromophores can be converted to the differently acetylated chromophore variants of the pyoverdines, pseudobactins and azotobactins and coupled to the respective peptide chains of these siderophores.

The group of siderophores in which amino acids serve as anchoring groups for chelating moieties mainly contain 2,3-dihydroxybenzoic acid.

Chrysobactin is a functionalized dipeptide and can be synthesized using the usual methods of peptide chemistry. N^{6}-*Z*-protected D-lysine is coupled with the *p*-nitrophenylester of 2,3-dibenzyloxybenzoic acid and then reacted with L-serine-benzylester by EEDQ. Finally, the benzyl groups are removed by hydrogenation [87].

Enterobactin, based on a cyclic triester of serine, differs considerably from the amide-forming methods of peptide chemistry. The historical synthesis by Corey and Bhattacharya (Figure 26(a)) uses activation of the carboxy group of serine as thioester. The first molecule of serine is side-chain protected by tetrahydropyranyl group, N-protected by benzyloxycarbonyl group and activated by the thioester with 4-*t*-butyl-l-isopropyl-2-mercapto-imidazol. The second serine is condensed onto the thioester as *Z*-Ser-*p*-bromophenacylester. The phenacyl group is then removed with zinc and acetic acid and the carboxy terminus again activated as thioester. After coupling with the third serine (as *Z*-Ser-*p*-bromophenacylester), the protecting groups on C-terminus and 'O-terminus' are removed and the linear triester is cyclized through activation as thioester. Finally, the *Z*-groups are removed from the cyclic triester by hydrogenation and the amino groups are amidated with 2,3-dihydroxybenzoylchloride. By using D-serine, *enantio*-enterobactin could be obtained as well [151, 152]

A shorter way to obtain enterobactin makes use of a template mechanism with tin (Figure 26(b)) [153]. Serine is *N*-tritylated and cyclized to its β-lactone with diisopropylcarbodiimide. The dimeric stannoxane $(Bu_2Sn(OCH_2CH_2O))_2$ is employed to cyclize three molecules of the serine-lactone into the triester. After removal of the trityl protecting groups the amino groups are acylated with 2,3-dihydroxybenzoic acid, protected as benzyloxyethers and activated as *p*-nitrophenylester. Hydrogenolysis of the benzyl protecting groups yields enterobactin.

Agrobactin, parabactin and vibriobactin are examples of siderophores with cyclized amino acids. Their backbone structures are the triamines spermidine and norspermidine, which have to be acylated differently on the two primary and on the secondary amine. Bergeron and McManis [152] have described an elegant method for providing orthogonally diprotected triamines (Figure 27), which can

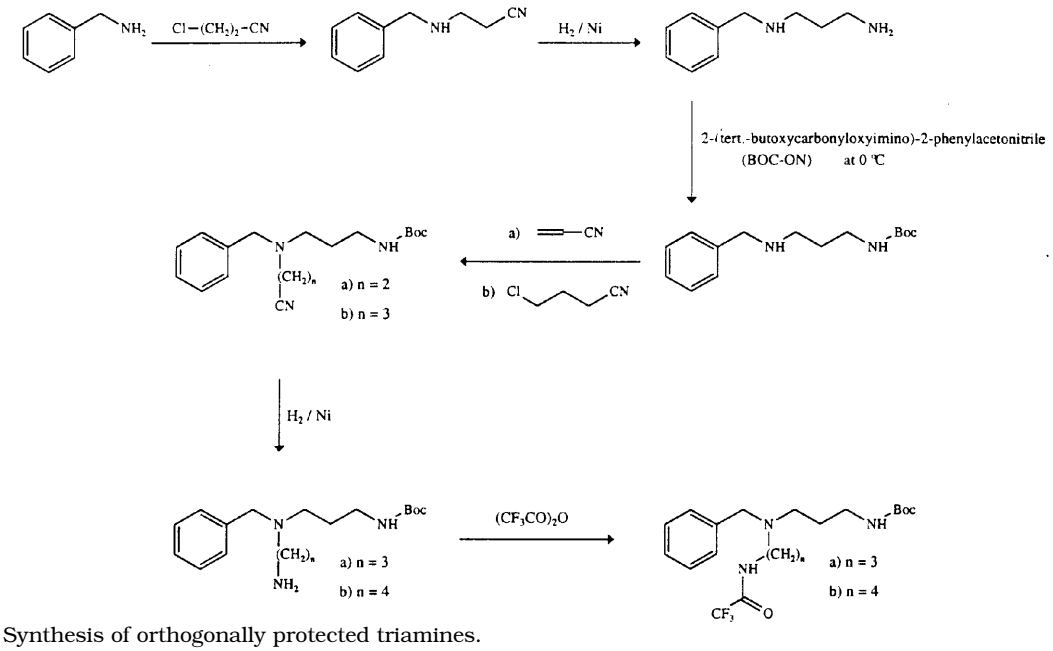


Figure 27 Synthesis of orthogonally protected triamines.

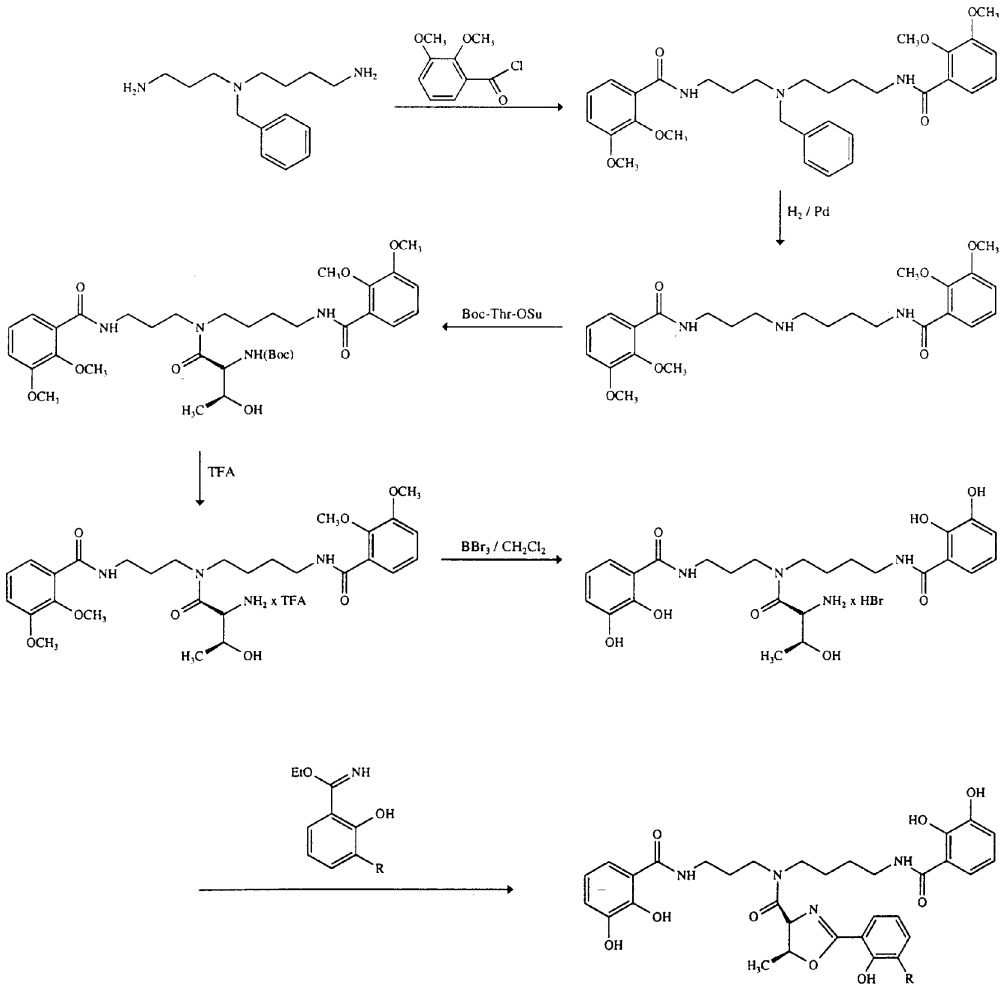


Figure 28 Synthesis of agrobactin (R = OH) and parabactin (R = H).

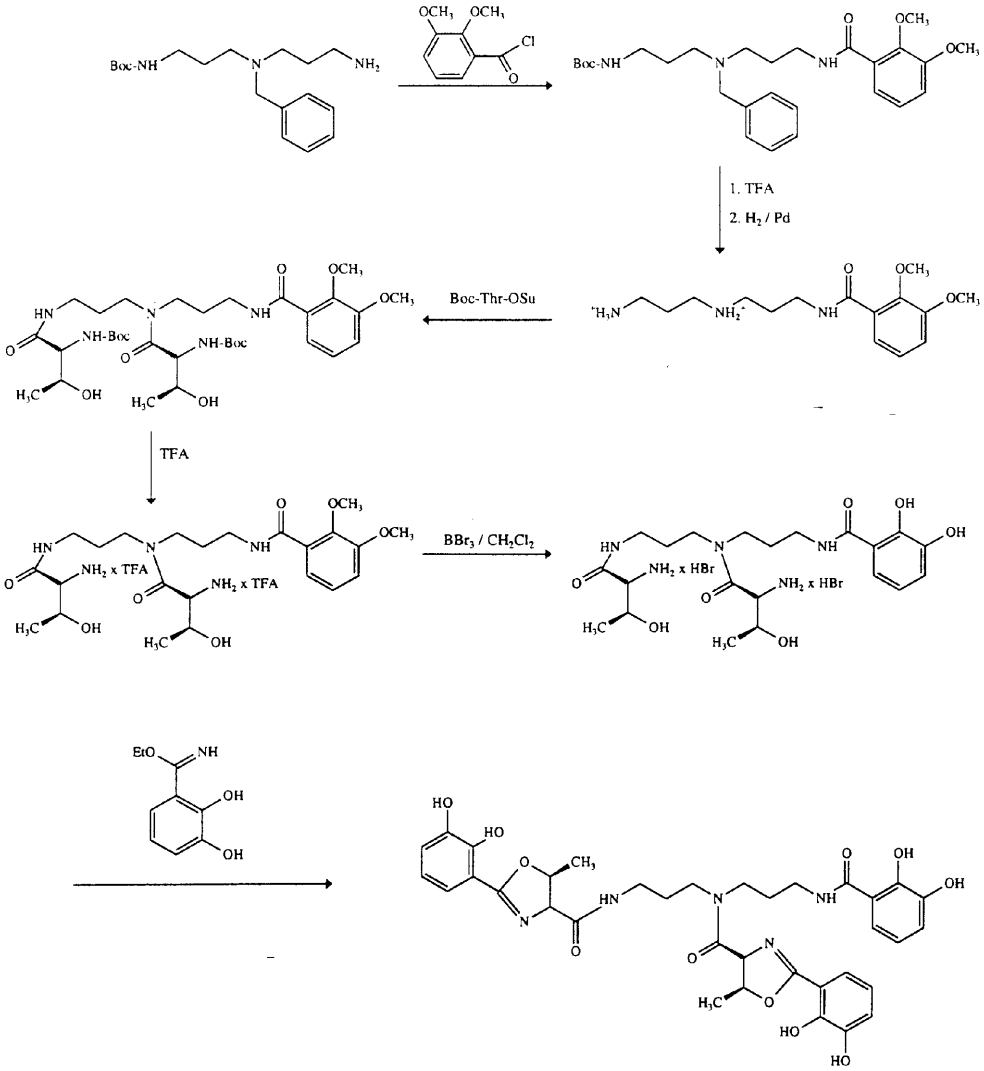


Figure 29 Synthesis of vibriobactin.

serve as building blocks for the whole class of siderophores with triamine backbones.

Monobenzyldiamines are synthesized from the corresponding diamine (in excess) and benzaldehyde in a reductive amination. The product is mono-*t*-butoxycarboxylated with one equivalent of 2-(*t*-butoxycarbonyloxyimino)-2-phenylacetonitrile (Boc-ON) at 0°C. These orthogonally diprotected diamines are converted to the desired triamines by addition on the double bond of acrylonitrile or nucleophilic substitution on 4-chlorobutyronitrile, the nitriles being finally reduced with Raney nickel.

For the synthesis of agrobactin [154] and parabactin [155] (Figure 28), the diprotected triamine spermidine (from the synthetic scheme shown in Figure 27) is treated briefly with trifluoroacetic acid to yield spermidine, benzylated at the secondary amine function. This is reacted with 2,3-dimethoxybenzoylchloride to the diamide. The benzyl protection group is removed by hydrogenolysis and the secondary amine acylated with *N*-*t*-butyloxycarbonyl-L-threonine. The *t*-butyloxycarbonyl group is removed with trifluoroacetic acid and the methylethers of the catecholates cleaved with borontribromide in dichloromethane. Condensation with ethyl-2-hydroxybenzimidate or ethyl-2,3-dihydroxybenzimidate and simultaneous cyclization of the threonine yields parabactin or agrobactin, respectively. Ethyl-2-hydroxy-benzimidate can be made from 2-cyanophenol in ethanolic HCl. 2,3-Dihydroxyethylimidate is obtained through *O*-alkylation of 2,3-dibenzoylated benzoylamide with triethyloxonium hexafluorophosphate in dichloromethane [152].

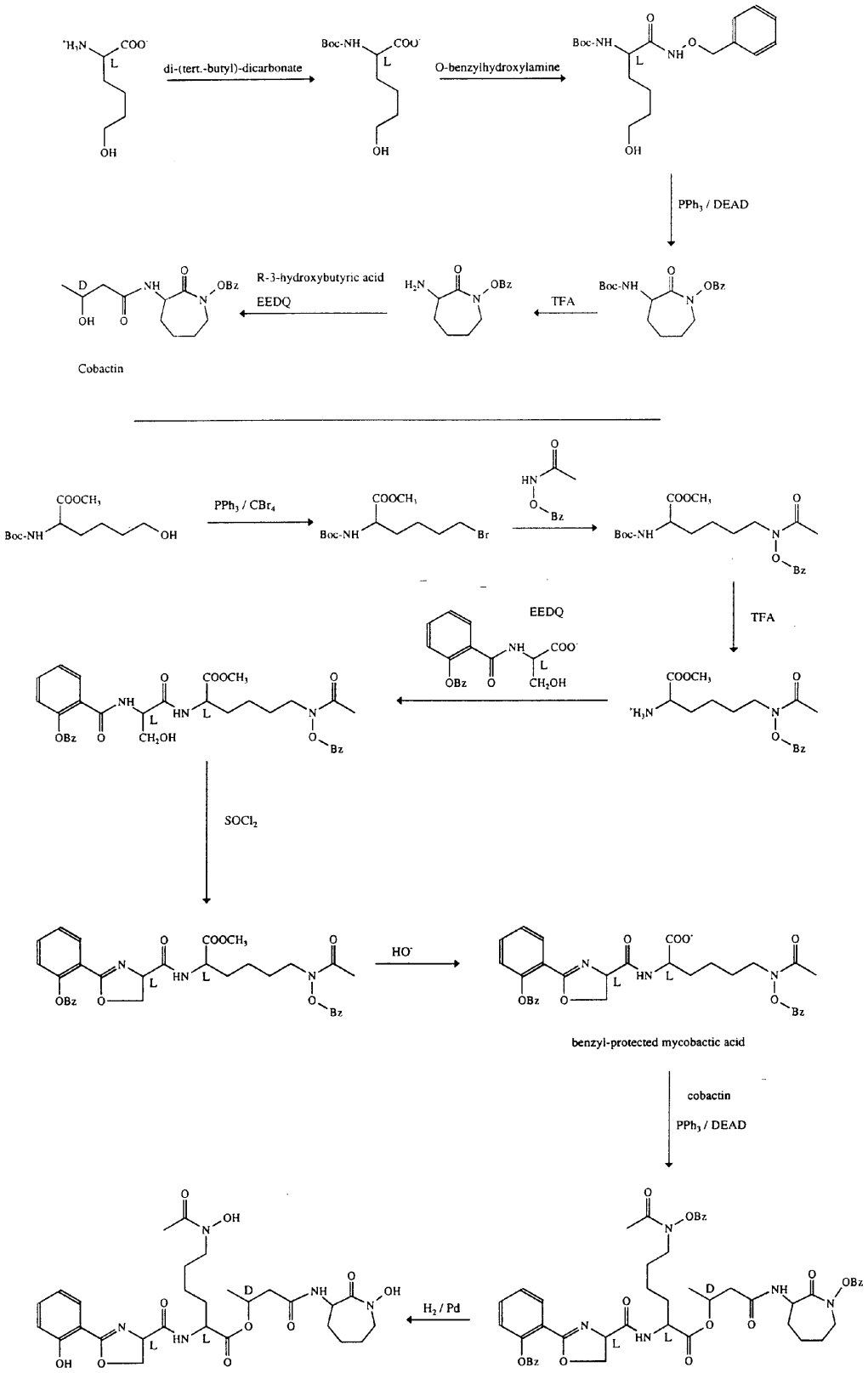


Figure 30 Synthesis of mycobactin S2.

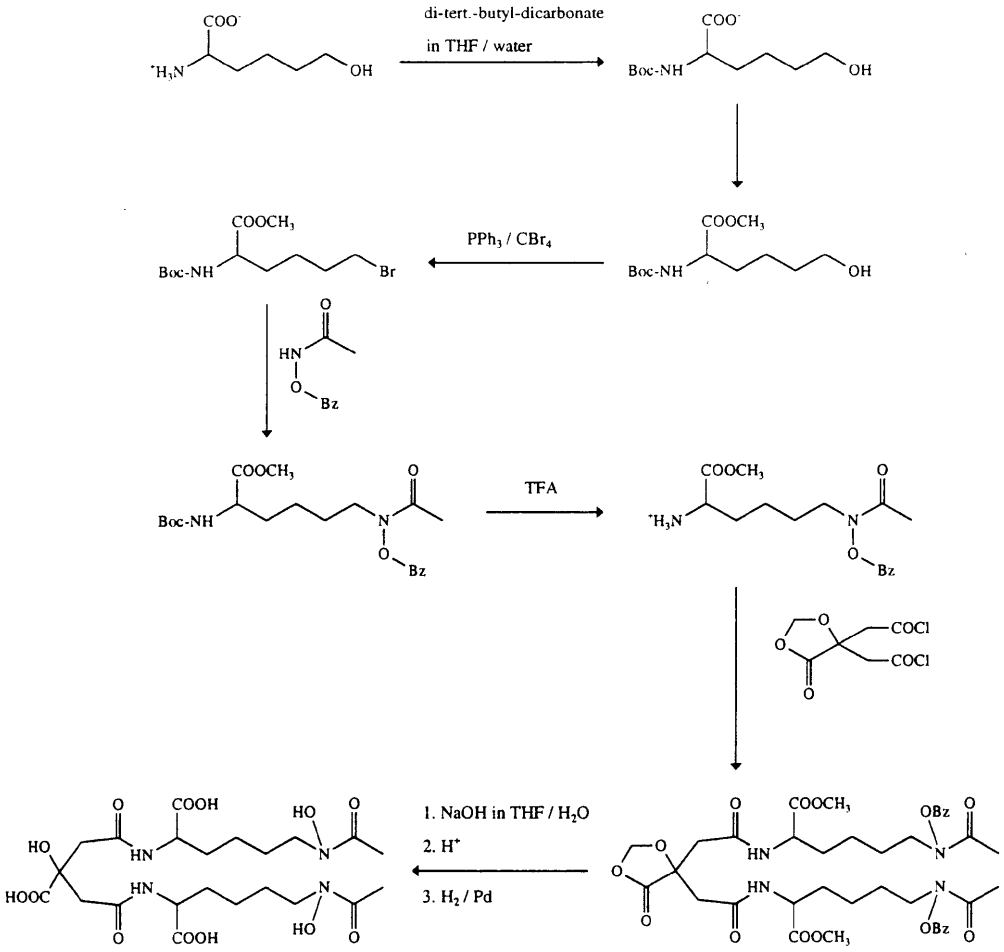


Figure 31 Synthesis of aerobactin.

For the synthesis of vibriobactin [156] (Figure 29), the Boc-/benzyl-protected norspermidine is first terminally amidated with 2,3-dimethoxybenzoyl chloride. After deprotection with trifluoroacetic acid and H_2/Pd, N-Boc-L-serine is coupled to the free amine groups. As in the synthesis of agro-/parabactin, the Boc-protection on threonine and the methylethers on the catechol are removed and 2,3-dihydroxyethylimidate condensed onto the threonines, which cyclize in the same step. On using 2-hydroxyethylimidate, one would be able to obtain vulnibactin as well.

Since mycobactins are mixed siderophores, containing both 2-hydroxybenzoylamide oxazoline and hydroxamate functions, their synthesis employs principles from both classes of compounds. Mycobactin S2 will serve as a convenient example [157], since it does not contain fatty acid acylations. The complete siderophore is constructed from the two main fragments, mycobactic acid and cobactin, already mentioned in the structural part of this review. Cobactin contains cyclo-*N*-hydroxy-lysine, and the synthetic principle can also be employed for the synthesis of cyclo-*N*-hydroxy-ornithine, found in numerous siderophores of the pyoverdin group (see above). *N*-Boc-protected ε-hydroxy-L-norleucine is condensed with *O*-benzyl-hydroxylamine and cyclized with DEAD and triphenylphosphine (Figure 30). After removal of the *t*-butyloxycarbonyl-protecting group, the benzyl-protected *N*-hydroxylactame is amidated with D-3-hydroxybutyric acid. To obtain mycobactic acid, *O*-benzyl-protected salicylic acid is activated as *p*-nitrophenylester and coupled to L-serine. The synthesis is continued to the dipeptide by coupling the methyl ester of N^6-acetyl-N^6-benzyloxy-L-lysine to the carboxy group of serine with the help of EEDQ. The cyclization of serine to the oxazoline-derivative is achieved with thionylchloride. After saponification of the methyl ester, the benzyl-protected mycobactic acid is coupled to the

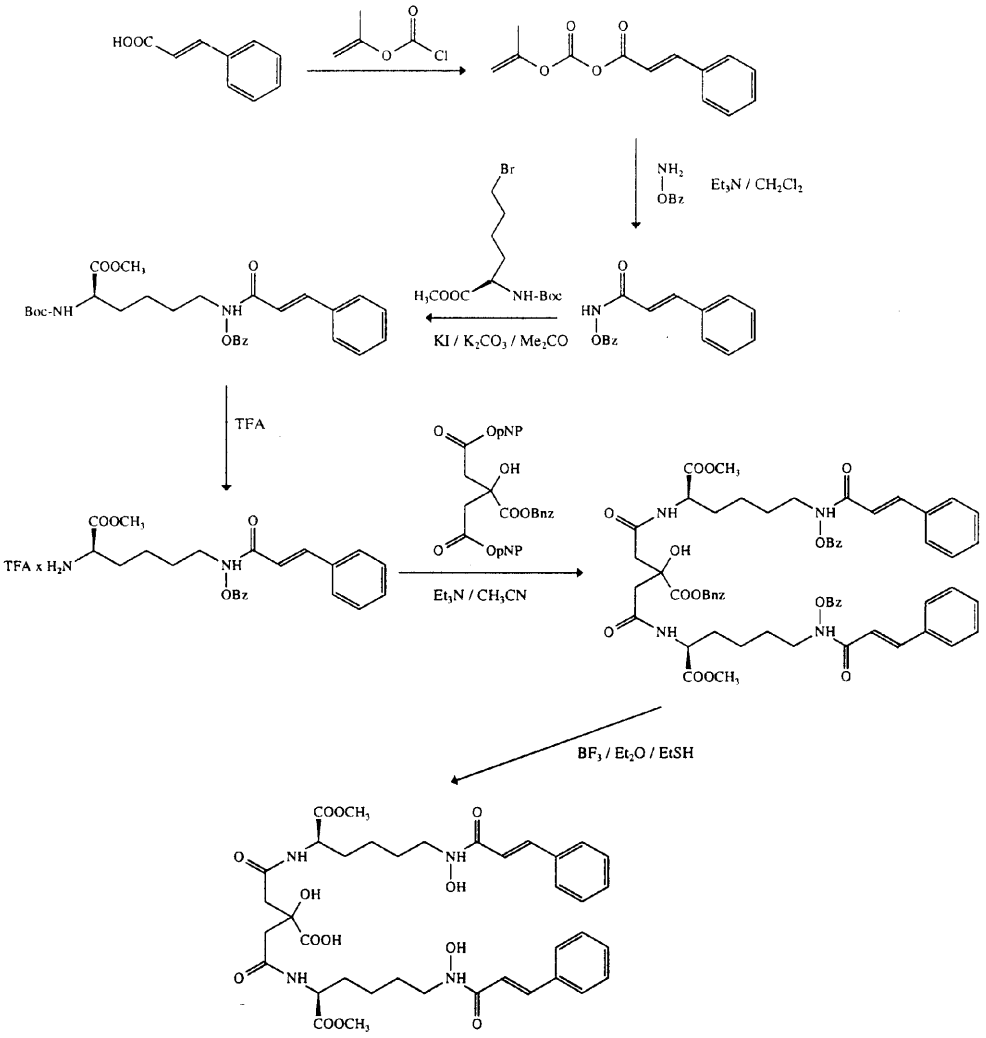


Figure 32 Synthesis of nannochelin A.

benzyl-protected cobactin with DEAD/triphenylphosphine. Hydrogenation yields mycobactin S2.

The aerobactin family of siderophores contains citric acid, symmetrically bonded to lysines, which are side-chain hydroxylated and acylated. The synthesis of aerobactin [158] (Figure 31) starts from ε-hydroxy-L-norleucine, which is N^{α}-Boc-protected and converted to its methyl ester. The hydroxy group is exchanged to bromine with tetrabromomethane and triphenylphosphine. The bromide is reacted with *N*-acetyl-*O*-benzyl-hydroxylamine in a nucleophilic substitution. After removal of the Boc protection group, the resulting N^{6}-benzyloxy-N^{6}-acetyl-L-lysine-methylester is reacted with anhydromethylenecitryldichloride, yielding fully protected aerobactin. The methylene anhydride of the citryl moiety and the methyl esters on the lysine carboxy groups are removed by treatment with sodium hydroxide in water/THF. Finally, the benzyl groups are removed by hydrogenation, affording free aerobactin.

The synthesis of nannochelin A [159] (Figure 32) starts with the coupling of *O*-benzylhydroxylamine to cinnamic acid, activated as mixed anhydride with isopropenyl chloroformate. The *N*-cinnamoyl-*O*-benzylhydroxylamine is reacted with *N*-Boc protected L-ε-bromonorleucine [158] and the amine protection removed with TFA. Two molecules of the aminoester are coupled to terminally bis-*p*-nitrophenyl-activated citric acid, the middle carboxy group of which is benzyl-protected. Removal of the benzyl protecting groups is carried out with borontrifluoride etherate.

Diketopiperazine formation being an undesired side-reaction of peptide synthesis, the syntheses of rhodotorulic acid and dimerum acid, which contain diketopiperazine rings, are especially noteworthy. In

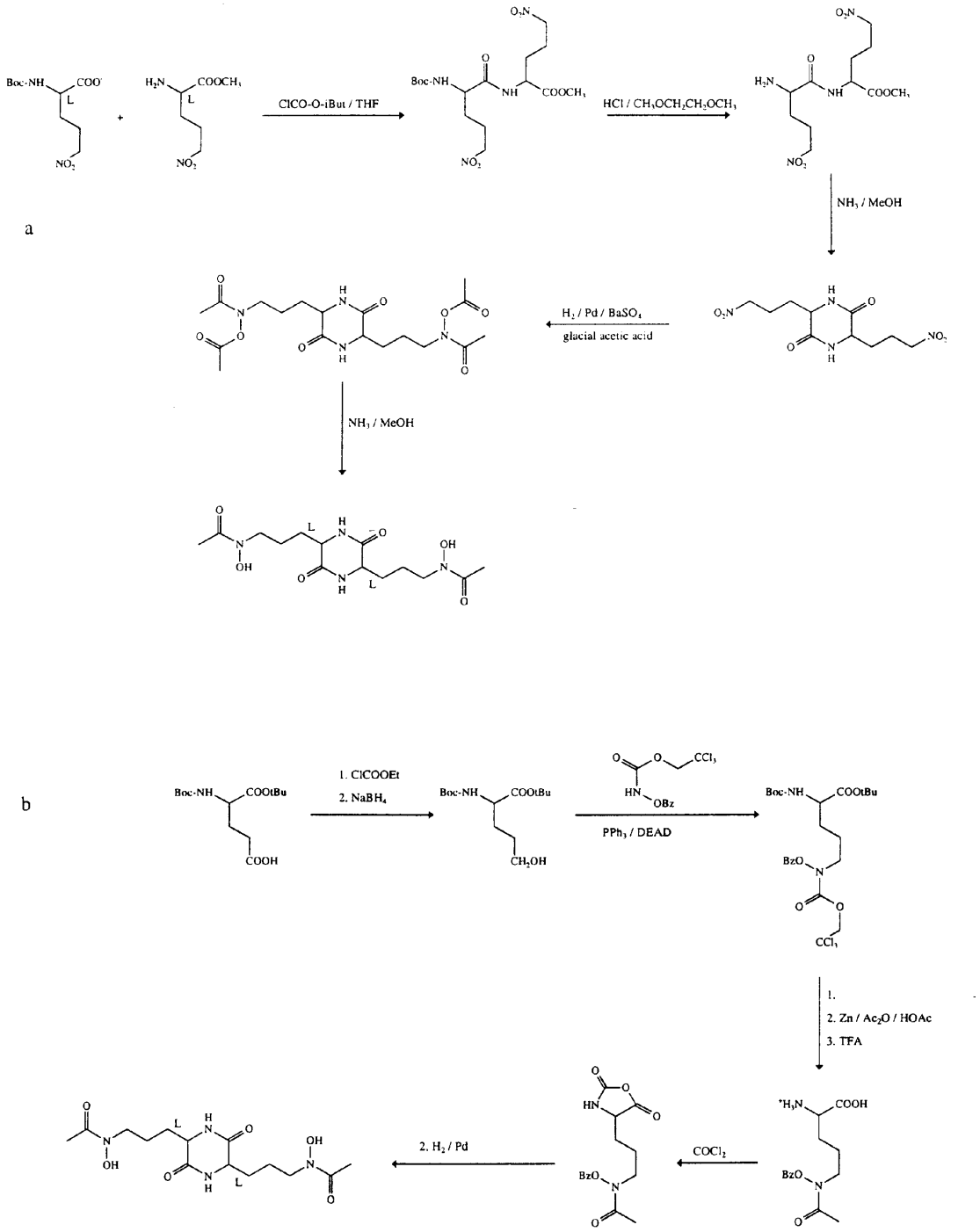


Figure 33 (a) Synthesis of rhodotorulic acid from 5-nitronorvalin. (b) Synthesis of rhodotorulic acid through protected N^5-hydroxyornithine.

the older synthetic approaches to these siderophores (Figure 33(a)) the diketopiperazine ring was constructed first, by cyclization of the dipeptide of 5-nitronorvalin methylester (synthesis described in [160]) with ammonia/methanol. The amino groups were then converted to hydroxylamines by reduction on $Pd/BaSO_4$ in glacial acetic acid. The dihydroxylamine can be acetylated to rhodotorulic acid or acylated with (*E*)-*O*-acetyl-Δ^2-anhydromevalonic acid to yield dimerum acid [161].

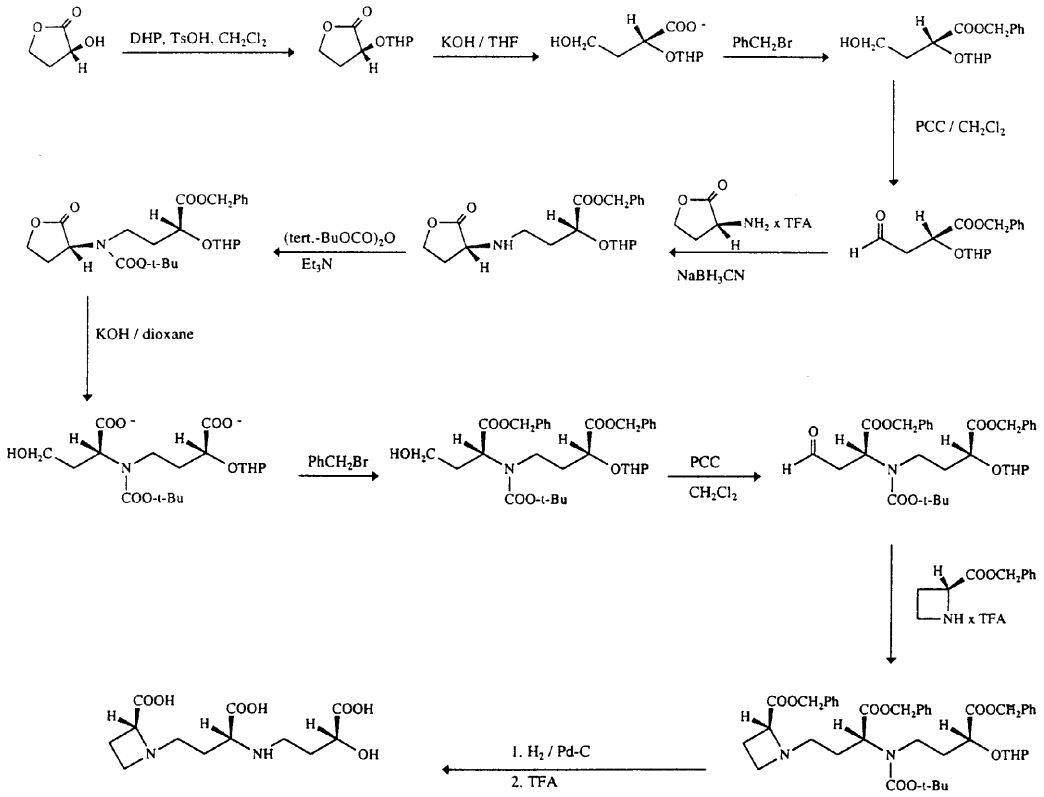


Figure 34 Synthesis of 2′-deoxymugineic acid.

Another route to diketopiperazine siderophores [162] makes the diketopiperazine ring in the last step (Figure 33(b)). *N*-Boc-L-glutamic acid α-*t*-butyl ester is reduced to the corresponding alcohol with sodium borohydride. The alcohol is reacted with *N*-((2,2,2-trichloroethoxy)carbonyl)-*O*-benzylhydroxylamine with the help of DEAD triphenyl-phosphine, resulting in fully protected N^5-hydroxy-L-ornithine. After removal of the 2,2,2-trichloroethoxycarbonyl-protecting group, the *O*-benzylamine is acetylated. The *N*-Boc group and the *t*-butyl ester are removed with trifluoroacetic acid and the amino acid dimerized to the diketopiperazine ring with Leuchs anhydride. Hydrogenolysis finally yields rhodotorulic acid.

The phytosiderophores differ from the other siderophores in that they contain *N*-alkylated amino acids, lacking amide bonds and esters. Therefore, the amino acid constituents cannot be joined by the usual coupling procedures of peptide chemistry. Instead, *N*-alkylation is effected by reductive amination, as in the synthesis of 2′-deoxymugineic acid (Figure 34) [163]. The lactone of L-malic acid is protected on its α-hydroxy function by the tetrahydropyranyl group. Then the lactone is opened and the carboxy group benzylated. The γ-hydroxy group is oxidized to the aldehyde with pyridinium chlorochromate and coupled to the amino group of L-homoserine lactone through reductive animation with cyanoborohydride. The secondary amine of this intermediate is Boc-protected and the synthetic steps of the first sequence repeated. The lactone is opened, the carboxy groups are benzylated and the γ-hydroxy group oxidized to the aldehyde with pyridinium chlorochromate. Reductive amination with cyanoborohydride couples the aldehyde to the amino group of benzyl-protected L-azetidine carboxylic acid. Hydrogenation removes the benzyl groups, and protolysis removes the Boc-protection and yields 2′-deoxymugineic acid.

Conclusions and Perspectives

The biosynthesis and transport of siderophores provide access to the element iron, the importance of which for microbes is comparable to the availability of oxygen for higher organisms. In some cases, the availability of the receptor alone, without the corresponding synthesis of a siderophore, allows the use of 'exogenous' siderophores for iron supply. These exogenous siderophores can be excreted by

different, competing microorganisms, thus establishing an opportunistic relationship between the two microbial strains. The phenomenon of 'cross-feeding' on siderophores can have more profound effects in the case of medical use of siderophores. Desferri-ferrioxamin B, a siderophore composed of succinic acid and *N*-hydroxylated diamines and therefore not discussed in the field of peptide siderophores, is used as 'Desferal'™ for the treatment of pathological iron overload. The high concentration of this siderophore in the patients' blood has occasionally led to severe, rapidly lethal mucormycosis [164, 165]. The reason is the ability of aspergillus, rhizopus and mucor strains of fungi to 'crossfeed' on ferrioxamine B [166, 167], originally a siderophore of streptomycetous bacteria.

Since DesferaI is not orally applicable and has severe side effects and limitations, research is directed at finding other clinically applicable compounds comparable to or even better than Desferal™. The research on clinically useful, especially orally applicable, chelators extends into the field of synthetic compounds [168, 169], for which siderophores and their complexation mechanisms can serve as lead structures for drug development.

The synthetic methods of peptide chemistry not only serve to verify structural results, but also provide access to a large number of analogues [147, 170–172] and stereochemical counterparts [148, 173]. With these analogues, it is possible to study the complexation kinetics and equilibria, the structure of complexes, the structural prerequisites for metal complexation and the extent of receptor specificity. *Enantio*-compounds and isotopically or fluorescent-labelled siderophores are valuable tools for exploring the stereospecificity of receptor recognition [142, 174, 175] or to isolate receptor proteins.

The importance of the iron uptake for microbes has prompted researchers to investigate the possibilities to fight medically relevant microrganisms at this crucial point. The receptor-mediated uptake of iron can be imagined to be blocked, for example by delivering kinetically and redox inert metal complexes or by synthetic ligand analogues that remain bound to the receptor. The discovery of natural conjugates between siderophores and antibiotics, e.g. the ferrimycins [176], the albomycins [177], the danomycins [178] and the salmycins [179], has led to synthetic analogues. Desferri-ferrioxamin B has been coupled to penicillins [180], cephalosporins [181], sulfonamides [182], and other antibiotics [183]. Most promising results have been obtained by antibiotics that are already effective at the periplasm of Gram-negative bacteria, since receptor-mediated iron transport is well characterized only across the outer membrane. Recently, the chemical functionalization of antibiotics with incomplete siderophores, simply by attaching catecholate or hydroxamate ligand functionalities, has been explored [184, 185].

Siderophores are yet another class of natural compounds in which amino acids are frequently encountered. The analytical as well as the synthetic techniques of protein and peptide chemistry combine well with the methods of microbiology, genetics and analytical organic chemistry to solve the remaining questions and to open up new fields of interdisciplinary cooperation.

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