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Syntheses and Iron Binding Affinities of the *Bacillus anthracis* Siderophore Petrobactin and Sidechain-Modified Analogues

Nikolas Bugdahn[a] and Markus Oberthür*[a,b]

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An improved synthesis of petrobactin and analogues has been developed that provides reliable access to siderophores in sufficient quantities for biological studies. The determination of the iron affinity of different petrobactin derivatives showed that functionalization at the central amino group of the spermidine sidechain has only a minor influence on the iron binding properties of the siderophore. Accordingly, such derivatives are promising starting points for the development of biological probes to study bacterial siderophore transport, a possible new target for antibiotics.

Introduction

The acquisition of the essential element iron is a difficult task for all organisms. Despite its widespread abundance in the earth's upper crust, the environmental concentration of Fe^{III} ions is rather low because iron is sequestered in the form of insoluble oxides and hydroxides.[1] Consequently, organisms have developed a number of acquisition and storage strategies to enable iron homeostasis. Whereas humans simply rely on their diet to supply sufficient quantities of iron for all essential cellular functions, smaller organisms, such as bacteria[2,3] and fungi,[4,5] were forced to come up with more ingenious solutions to this problem. The most abundant strategy features the secretion of siderophores, which are polar, low molecular weight molecules with exceptionally high binding affinities for Fe^{III}.[6,7] Upon iron binding, for example by removal from iron storage proteins within a host, the siderophore-iron complex is internalized by dedicated import systems,[8,9] and the iron is extracted from the complex by either reductive or hydrolytic processes for further use.

Inhibition of the siderophore cycle offers a potentially new target for antibacterial and antifungal agents, because siderophore-mediated iron uptake is essential for both the survival and the virulence of many pathogens.[10] In addition to blocking their biosynthesis,[11] interfering with siderophore uptake would provide an additional means by which to deplete the cytoplasmic iron concentration. So far, however, only a limited number import systems have been characterized biochemically and structurally.[8,9,12–14]

We have recently developed an identification strategy for siderophore-binding proteins that is based on the actual siderophore-binding event during affinity chromatography rather than on sequence homologies.[15,16] For our initial studies, we selected petrobactin (**1**, Figure 1), a siderophore that is produced by different bacteria, most importantly by the two pathogens *Bacillus cereus*[17] and *Bacillus anthracis*.[18,19] As a proof of concept, we prepared a biotinylated petrobactin derivative **2** (Figure 1) in which the biotin

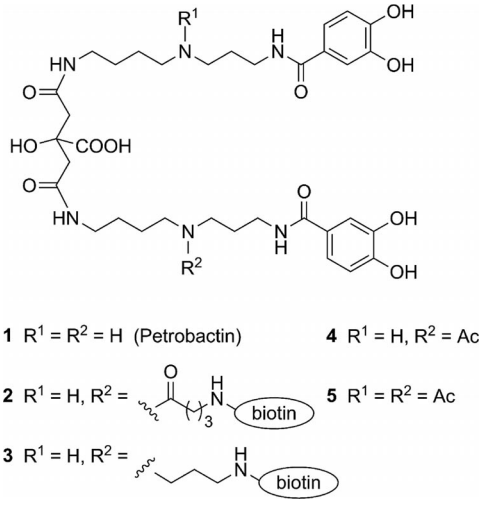

Figure 1. Structure of petrobactins used in this study to determine the influence of sidechain substitution on iron binding. The functionalized petrobactins **2** and **3** contain a biotin group connected to a short linker that is attached to the central amino group of the spermidine sidechain through amidation or alkylation, respectively. Mono- and di-$N^{4'}$-acetyl petrobactin **4** and **5**, respectively, were prepared as model compounds to assess the general influence of N-acylation as well as steric effects.

[a] Fachbereich Chemie, Philipps-Universität Marburg, Hans-Meerwein-Straße, 35032 Marburg, Germany
E-mail: oberthuer@chemie.uni-marburg.de
www.uni-marburg.de/fb15
[b] Current address: Deutsches Textilforschungszentrum Nord-West, Universität Duisburg-Essen, NETZ/DTNW gGmbH, Carl-Benz-Str. 199, 47057 Duisburg, Germany
E-mail: oberthuer@dtnw.de
www.dtnw.de

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group is attached to the central side chain amine through a γ-butyric acid linker.

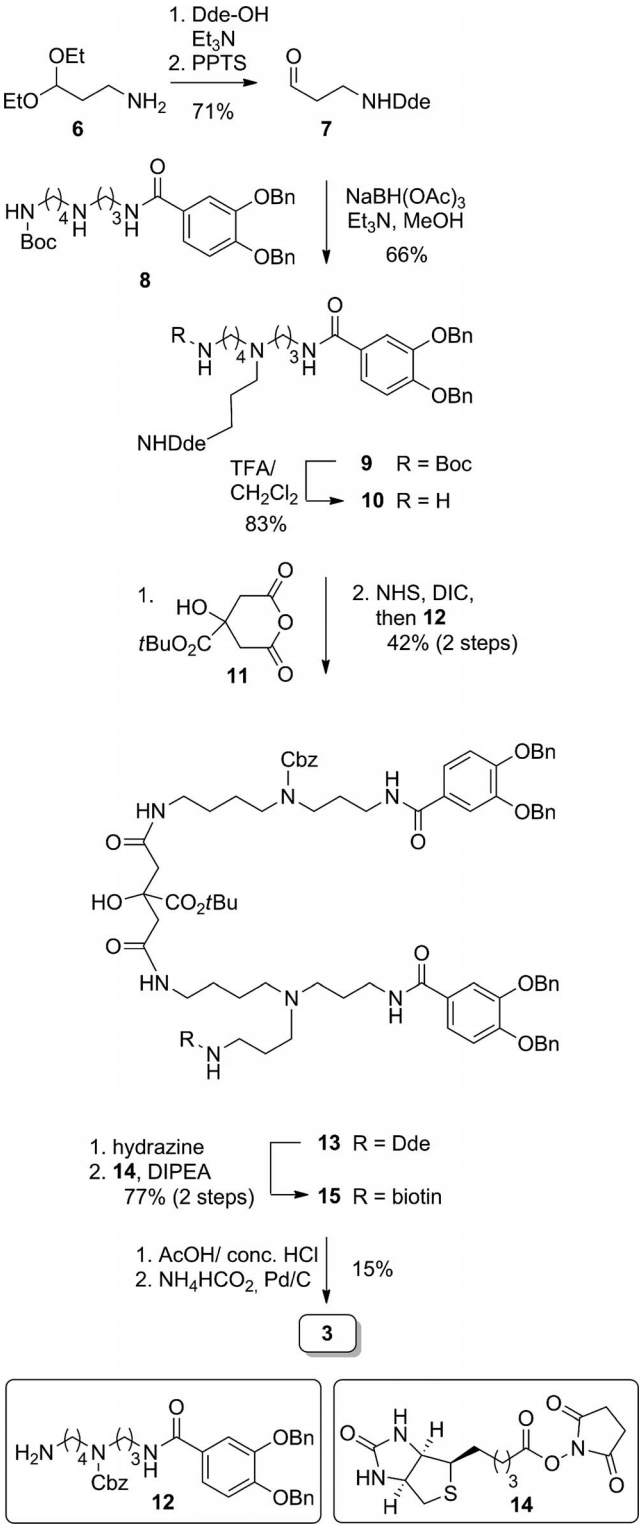
Immobilization of this derivative on streptavidin-modified agarose and subsequent affinity chromatography of crude cell extracts then led to the identification of the petrobactin-binding protein FpiA and the corresponding petrobactin uptake system *fpiABCD* in the model organism *Bacillus subtilis*.[15,20] Notwithstanding the success of our protein fishing experiments, information regarding the iron-binding abilities of such modified petrobactins was still lacking. Accordingly, we prepared a set of additional derivatives carrying substituents at this position. We then determined their iron-binding affinity, which for the first time allowed quantitative assessment of the influence that a sidechain modification exerts on iron binding. This information will help the design of functionalized petrobactins for other possible applications, for example pathogen capture[21,22] and siderophore-mediated cargo delivery.[23–26]

Results and Discussion

Petrobactin (**1**) is a citric acid derived siderophore that carries two spermidine sidechains with 3,4-catecholic end groups. For our binding protein capture experiments, we modified the central amino group of one of the sidechains with a biotin group through a linker (see derivative **2**, Figure 1). The spermidine chains are presumably not directly involved in the iron complexation process, but provide a suitable orientation of the primary ligands, i.e., the two catechols and the hydroxyl and carbocylic acid groups of the citric acid moiety. Nevertheless, it was not clear at the beginning of our studies in which way a modification at this position might influence the stability of the Fe^{III} complex either sterically or electronically. This information is, of course, vital for the design of future petrobactin probes because iron binding is required for the biological function. To study possible effects in more detail, we prepared biotinylated petrobactin **3**, in which the linker is of similar size, but attached to the spermidine sidechain through alkylation instead of amidation of N-4′. As a consequence, derivative **3** is a closer analogue of petrobactin (**1**) than amide **2** because the sidechain amine retains a similar geometry (tetrahedral) and basicity to that in **1**. To study possible steric effects of amides attached to the spermidine sidechains, we also prepared two $N^{4'}$-acetyl analogues **4** and **5**, which carry acyl groups of minimal size, by an improved synthetic route.

Synthesis of Petrobactins

N-Alkyl-linked biotinyl petrobactin **3** was prepared by analogy to the procedure already developed for **2** (Scheme 1),[15] which features the opening of a cyclic citric acid anhydride with the linker containing side chain amine, followed by attachment of the regular spermidine side chain. It should be noted that the addition of two different sidechains to the citric acid core leads to diastereomeric mixtures when additional stereogenic centers are present within at least one of the side chains (e.g., biotin derivatives **2** and **3**, Figure 1), or to racemic mixtures when two different achiral sidechains are used, for example the mono-Ac derivative **4** (Figure 1). For our exploratory experiments regarding iron binding and the isolation of binding proteins



Scheme 1. Synthesis of biotinyl petrobactin **3**. Dde-OH = acetyldimedone, PPTS = pyridinium *para*-toluenesulfonate, NHS = *N*-hydroxysuccinimide, DIC = diisopropylcarbodiimide.

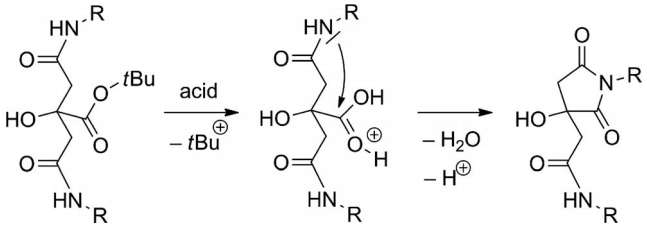
from crude cell extracts, however, this limitation was deemed acceptable because it simplified the synthesis dramatically.

The synthesis of **3** started with monoalkylation of the known spermidine side chain **8**[15] by reductive amination with 1-(4,4-dimethyl-2,6-dioxocyclohexylidene)ethyl (Dde) protected β-amino aldehyde **7**, which, in turn, was obtained from commercially available acetal **6** (71%, two steps). The Dde group[27] is stable under a variety of reaction conditions and can be removed selectively by using hydrazine. Optimal yields for the reductive amination (66%) were obtained when $NaBH(OAc)_3$ (1.8 equiv.) in anhydrous MeOH was used as the reducing agent and molecular sieves (3 Å) and Et_3N (→ pH 8) were added to the reaction mixture. The use of $NaBH_3CN$ (2 equiv.), on the other hand, gave significantly lower yields. Following removal of the Boc group [trifluoroacetic acid (TFA)/CH_2Cl_2], primary amine **10** was treated with cyclic anhydride **11**, which afforded the corresponding crude monoacid. Activation of the carboxylic acid as the *N*-hydroxysuccinimide (NHS) ester and coupling with the regular side chain amine **12**[15] then gave the fully assembled petrobactin derivative **13** carrying the Dde-protected linker (42%, two steps).

Removal of the Dde group with hydrazine and subsequent reaction with biotin NHS ester **14** then led to the protected biotinyl petrobactin **15** in good yields (77%, two steps). Finally, global deprotection was achieved by using conditions established previously in a two-step procedure, which afforded pure biotinyl petrobactin **3** following purification by reverse-phase (RP) HPLC.

Although this final sequence easily afforded enough material for our studies, the quite low yields (15%) of the deprotection steps left room for further improvement. In addition to some losses during the RP HPLC purification process due to the highly polar nature of the product, the removal of the *tert*-butyl ester was judged to be the most problematic step. So far, our synthetic efforts relied on citric acid building blocks in which the central carboxylic ester was blocked with a *tert*-butyl group, similar to the two total syntheses that had been published previously.[28–30] The advantages of the central *tert*-butyl ester in citric acid systems are twofold: its ease of preparation[31] and its stability against various nucleophiles as a result of steric hindrance. A major drawback, however, becomes apparent when the protecting group has to be removed. Under the acidic conditions required for deprotection of the *tert*-butyl ester, intramolecular attack of one of the amide nitrogen atoms on the central carboxy group is a common side reaction (Scheme 2).[28,32,33]

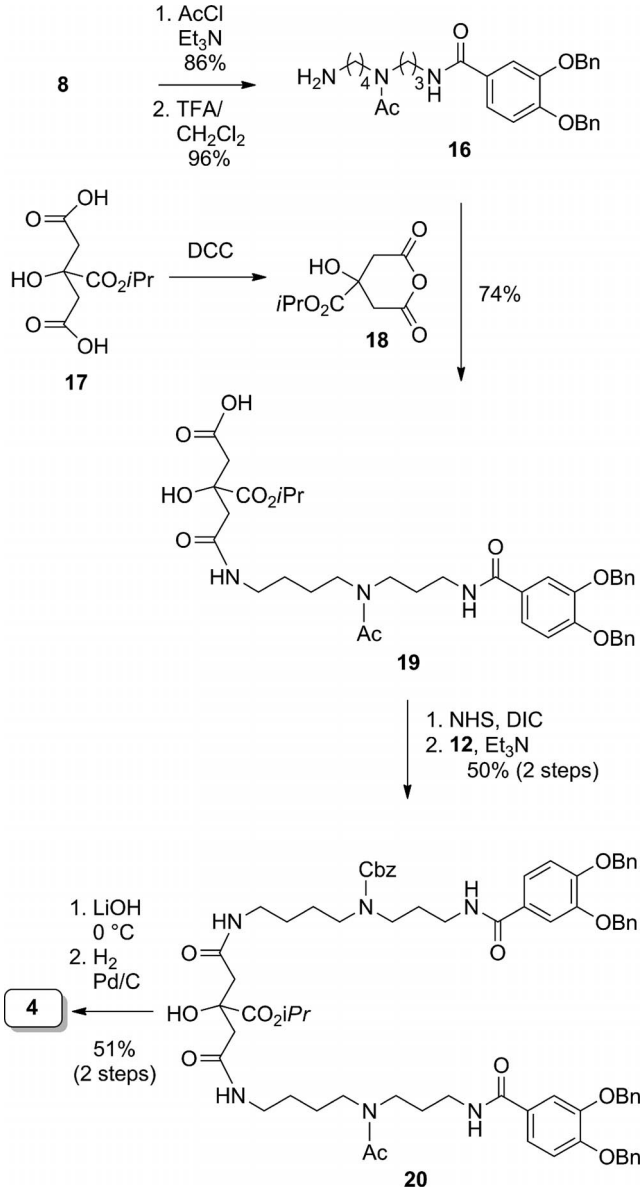
This imide formation was observed for different siderophores even under neutral conditions,[34] but is greatly facilitated by protonation of the carbonyl group. Accordingly, the removal of the *tert*-butyl ester requires careful optimization of reaction and work-up conditions. In addition, in our hands, previously successful deprotection protocols for a specific system were found to be difficult to reproduce from time to time. Therefore, we wanted to develop a different protecting group strategy for the synthesis of additional petrobactin derivatives that might also lead to a further improved route to petrobactin (**1**) itself. After a careful survey of the available literature, we decided to explore the use of corresponding isopropyl derivatives, which are also easily accessible and were reported to be stable in the presence of primary amines. The removal of such citric acid isopropyl esters by treatment with aqueous hydroxide solution was successfully achieved by the Miller group without imide formation in the case of the siderophore schizokinen,[32] but was never tested in the petrobactin system.



Scheme 2. Problematic imide formation by intramolecular attack of one of the amide nitrogen atoms during acidic removal of the *tert*-butyl ester.

The practicality of the isopropyl group was first established in the synthesis of mono-$N^{4'}$-acetyl petrobactin derivative **4** (Scheme 3) in a sequence similar to that used for the preparation of **2**. For the generation of an appropriate N^4-acetylated spermidine, petrobactin side chain **8** was first acetylated (AcCl), followed by removal of the Boc group with TFA to afford amine **16** in good overall yields (82%, two steps). Reaction with the isopropyl-protected cyclic anhydride **18**, obtained from 2-isopropyl citrate **17**[32,35] (Scheme 4) by activation with *N*,*N*-dicyclohexylcarbodiimide (DCC),[36] afforded the corresponding monoacid **19** in good yields (74%). This acid was then coupled with spermidine **12** as described for the preparation of **2** to afford protected $N^{4'}$-acetyl petrobactin **20**. Gratifyingly, the isopropyl ester proved to be easily removable by saponification of **20** with aqueous lithium hydroxide without any formation of the imide side product. Removal of the Cbz and Bn groups by hydrogenolysis (45 bar, Pd/C) then afforded mono-$N^{4'}$-Ac petrobactin **4** in high purity after RP HPLC purification (51%, two steps).

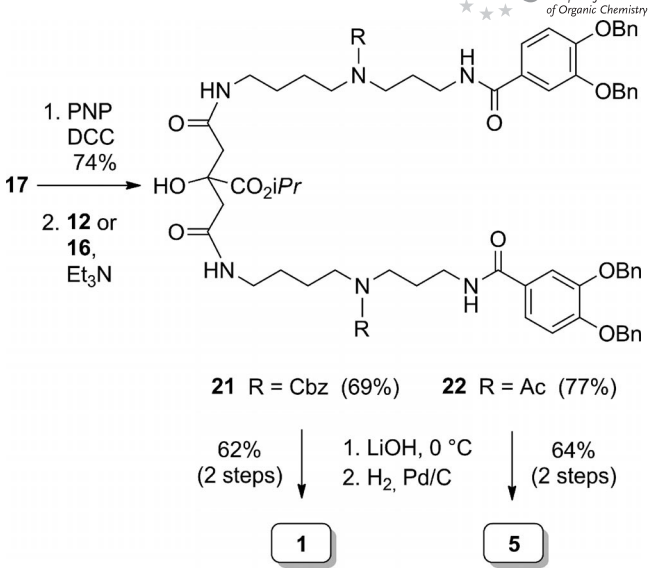
Based on these encouraging results, we also modified our established route to symmetric petrobactins, including **1**, by using isopropyl-protected citric acid building block **17**. This led to an improved total synthesis of petrobactin (**1**) itself, as well as other symmetric petrobactins such as di-$N^{4'}$-Ac derivative **5** (Scheme 4). After some exploratory studies, we found that optimal yields for amide coupling reactions were obtained when **17** was activated by treatment with *para*-nitrophenol and DCC.[32] The active ester was easily isolated by acidic and base extractions and filtration through a short pad of silica gel. Reaction of this diester with 2.4 equiv. of sidechain amine **12** then delivered the fully protected petrobactin **21** in a good overall yield (69%). Again, the isopropyl ester was saponified cleanly by treatment with aqueous lithium hydroxide without imide formation. Removal of the Cbz and Bn groups by hydrogenolysis



Scheme 3. Synthesis of mono-$N^{4'}$-Ac petrobactin **4**.

(55 bar, Pd/C) then gave petrobactin (**1**) in high purity after RP HPLC purification (62%). Our optimized synthesis is a further improvement of our initial approach using the *tert*-butyl derivative and afforded petrobactin (**1**) in ten steps and 20.2% overall yield starting from 3,4-dihydroxybenzoic acid. Because only five chromatographic separations are required, the process is amenable to scale-up and offers easy access to gram quantities of the siderophore. This is especially noteworthy because the isolation of petrobactin (**1**) in quantities of more than 100 mg by fermentation is not practical for many laboratories because of safety issues and the low production levels within the producing organisms.[37]

Finally, the synthesis of the diacetyl derivative di-$N^{4'}$-Ac petrobactin **5** proved that this reaction sequence also proceeds in reliably good yields with other spermidine sidechains. Thus, reaction of the bis-*para*-nitrophenyl ester of



Scheme 4. Synthesis of petrobactin (**1**) and di-$N^{4'}$-Ac petrobactin **5**. PNP = *para*-nitrophenol.

17 with the $N^{4'}$-acetylated sidechain amine **16** afforded protected petrobactin **22** even in slightly better yields (77%). Saponification and hydrogenolysis then led to the deprotected product **5** in 62% yield after purification by RP HPLC.

Determination of the Iron Binding Affinities of Modified Petrobactins

Previous to our studies, the iron binding affinity of petrobactin (**1**) was determined by using two different approaches,[38,39] however, no information was available that showed to what extent structural modifications of the petrobactin skeleton altered the stability of the iron complex. The precise determination of the stability constant for the formation of a siderophore-iron complex is difficult for various reasons. Direct measurement of the formation constant is usually not possible because no significant amount of unbound iron is present at equilibrium. Therefore, the iron affinity of the siderophore is often determined by using a competition assay against ethylenediaminetetraacetic acid (EDTA) or another ligand for which the formation constant of the iron complex is known.[40] In addition, the determination of "standard" pH independent binding constants that are usually reported requires the knowledge of all acidity constants of dissociable protons within the siderophore, because the overall formation constant $\beta_{Fe-siderophore}$ is expressed in terms of a fully deprotonated ligand. For some siderophores, these acidity constants are known, but often only estimated values are used, because factors such as complex aggregation or precipitation and degradation of the siderophore makes their experimental determination difficult. In the case of petrobactin (**1**), for example, oxidation of the 3,4-dihydroxybenzamide residue at elevated pH prevents an accurate determination of the pK_a values of the catecholic hydroxyl groups.[41] Accordingly, the group of Butler has shown that only three of the

eight dissociation constants of petrobactin can be accurately determined by potentiometric measurements, which, in turn, made a precise calculation of a pH independent binding constant using estimated values difficult.[39]

To overcome these obstacles, the "pM" scale has been introduced and is being increasingly used to compare the binding affinities of a variety of structurally unrelated siderophores for iron (M = Fe^{III}) or other metals.[42] The pFe^{III} value describes the concentration of unbound iron $-\log[Fe^{III}]$ in equilibrium with the metal-ligand complex(es) at fixed concentrations of ligand and metal ([siderophore] = 10 μM, $[Fe^{III}]$ = 1 μM) and at a physiological pH of 7.4. By definition, the pFe^{III} value is independent of the nature and protonation state of the metal-ligand complexes formed under these conditions and is proportional to the free energy released by metal ligand binding. For the determination of the pFe^{III} values for different petrobactin derivatives, we used a spectrophotometric EDTA competition assay by following a protocol developed by Abergel et al.[38] By using this method, we determined a pFe^{III} value for petrobactin (**1**) of 23.4 ± 0.16, which is close to the value obtained by the Raymond group (23.0)[38] under similar conditions. The pFe^{III} values for various sidechain modified analogues that were obtained by this method are shown in Table 1. All acylated analogues (**2**, **4**, and **5**) proved to have a slightly higher binding affinity than petrobactin (**1**). In addition, there was no dramatic change even when both sidechain amines were acylated, as demonstrated by the very similar value obtained for diacetate **5** and monoacetate **4**. Finally, derivative **3**, in which the biotin residue was attached through an alkyl linkage, has a pFe^{III} value close to that of petrobactin (**1**). Despite these small differences, all pFe^{III} values are within a close range (23.42–24.60), i.e., one can conclude that a structural modification of petrobactin at the secondary amine of the spermidine sidechain has no significant influence on iron binding.

Table 1. pFe^{III} values determined for petrobactin (**1**) and analogues **2**–**5**.

Entry	Siderophore	pFe^{III}
1	**1**	23.42 ± 0.16
2	**2**	24.60 ± 0.08
3	**3**	23.55 ± 0.09
4	**4**	23.90 ± 0.14
5	**5**	23.84 ± 0.09

Accordingly, functionalized derivatives of this type are valid probes for biological experiments. We could already show that biotinyl derivative **2** can be successfully used as an immobilized ligand for the isolation of siderophore-binding proteins through affinity chromatography, which led to the identification of the siderophore-binding protein FpiA in *B. subtilis*. Interestingly, protein retention was achieved despite the fact that the binding affinity of FpiA for the modified ligand **2** dropped quite substantially by three orders of magnitude compared with petrobactin (**1**) itself [18 μM (**2**) vs. 51 nM (**1**)].[15] With alkylated petrobactin **3** in hand, we were now able to show that, similar to iron complexation, a change of the linker type (acyl vs. alkyl) has only a minor influence on the binding of the modified siderophore by purified FpiA [2.4 μM (**3**) vs. 18 μM (**2**)]. Despite these similarities, the solubility of the various iron complexes at physiological pH can differ quite substantially. Compound **3**, with the amine linker, was better soluble in pH 7.4 buffer than derivatives that contain an amide linker.[43] Therefore, possible probes for biological experiments have to be chosen carefully to obtain reliable data.

Conclusions

The exchange of the previously used citric acid 2-*tert*-butyl ester for the corresponding isopropyl derivative **17** has vastly improved the overall yield and efficiency of our synthetic route to petrobactin (**1**), a siderophore that is produced by different bacteria, most notably by two pathogens *B. cereus* and *B. anthracis*. Because the deprotection of the isopropyl ester is much less prone to imide formation, our synthesis can reliably provide quantities of **1** that are difficult to obtain by fermentation. In addition, this flexible route makes functionalized petrobactin derivatives for chemical and biological studies easily available. Accordingly, further analogues, for example, fluorescent derivatives or photoaffinity probes, should be accessible in a straightforward way. Finally, we were able to determine the previously unknown influence of structural modifications to petrobactin on the iron binding affinity of the siderophore. Our data shows that both alkylation and acylation of the spermidine sidechain has only a minor effect on iron binding, which makes this type of petrobactin analogues promising tools for the study of iron homeostasis in pathogenic bacteria.

Experimental Section

General Remarks: All reactions were performed under an argon atmosphere at ambient pressure unless indicated otherwise. All reactions leading to unprotected petrobactin and petrobactin analogues were performed under the exclusion of light. Spermidine sidechain **12** and biotinylated petrobactin **2** were prepared as described.[15] Commercial reagents were used as received. All extracts were dried with $MgSO_4$ and evaporated below 40 °C under reduced pressure. Thin-layer chromatography (TLC) was performed on aluminum plates coated with silica gel 60 F_{254}. Detection was carried out by fluorescence quenching under UV light (λ = 254 nm) or by staining with 20% H_2SO_4 or ninhydrin solution followed by heating to ca. 300 °C. Flash chromatography was performed on silica gel 60 (0.040–0.063 mm). For chromatography solvents that contained NH_4OH, a solution of 25wt.-% (NH_4OH/H_2O) was used. Preparative high-performance liquid chromatography was performed by using a Nucleodur 100–5 C18 ec column (25 mm × 250 mm) or a Luna 5 μm C18 column (4.60 × 250 mm). Chemical shifts (δ) are given in ppm referring to the solvent residual signal. In many cases, *cis*/*trans* rotational isomers were observed even at elevated temperatures. In the ^1H NMR spectroscopic data, peaks for the same H-atoms corresponding to such rotamers are combined and listed with a subscript (e.g., $2\times s_{cis/trans}$). The integral given corresponds to that of the combined peaks. The signals were assigned with the aid of COSY, HMBC and HMQC spectra. All coupling patterns were characterized according to their actual appearance (phenome-

nological data) and not according to the multiplicity that is theoretically expected. All coupling constants are $^3J_{H,H}$ couplings unless indicated otherwise and are the actual values observed for each signal and not averaged values. Mass spectra were recorded with a LTQ FT spectrometer; the resolution was set to 100.000.

***N*-{[1-(4,4-Dimethyl-2,6-dioxocyclohexylidene)ethyl]-3-oxopropyl}-amine (7):** To a solution of acetyldimedone (Dde-OH, 0.99 g, 5.5 mmol)[44] and Et_3N (0.48 mL, 3.5 mmol) in EtOH (30 mL) was added freshly activated molecular sieves (3 Å) and the mixture was stirred at room temperature for 10 min. 3,3-Diethoxy-1-propylamine (**6**, 1.4 mL, 8.8 mmol) was added in one portion. The mixture was stirred at 25 °C for 16 h, filtered through a pad of Celite, and concentrated under reduced pressure. The resulting residue was dissolved in EtOAc, extracted with aq. citric acid (20%), aq. NaOH (1 M), and brine. The combined organic phases were dried, filtered, and concentrated to dryness to afford Dde-protected acetal (1.66 g, 97%) as a yellow oil, which was used directly in the next reaction step.

To a solution of this crude acetal (1.65 g, 5.30 mmol) in acetone/H_2O (4:1, 60 mL), a catalytic amount of pyridinium toluene-4-sulfonate was added and the solution was stirred at 50 °C for 36 h. After the solvents were removed in vacuo, the colorless residue was dissolved in EtOAc and extracted with brine, aq. NaOH (1 M), and water. The solution was dried ($MgSO_4$), filtered, and the solvents were evaporated to dryness. The oily residue was purified by flash chromatography (CH_2Cl_2/MeOH, 20:1→10:1) to give **7** (0.89 g, 71%) as a clear oil. R_f = 0.40 (CH_2Cl_2/MeOH, 10:1). ^{1}H NMR (300 MHz, [D$_6$]DMSO): δ = 1.01 (s, 6 H, 2× CH_3^{Dde}), 2.35 (s, 4 H, 2× CH_2^{Dde}), 2.58 (s, 3 H, CH_3^{Dde}), 2.90 (t, $J_{1,2}$ = 6.6 Hz, 2 H, 2-H$_2$), 3.71 (q, J = 6.3 Hz, 2 H, 1-H$_2$), 9.83 (s, 1 H, 3-H), 13.55 (br. s, 1 H, NH^{Dde}) ppm. ^{13}C NMR (75 MHz, [D$_6$]DMSO): δ = 17.7, 28.2, 30.1, 36.3, 43.0, 52.8, 108.1, 173.7, 198.1, 198.2 ppm. HRMS (ESI): calcd. for $C_{13}H_{19}N_1O_3Na$ [M + Na]$^+$ 260.1257; found 260.1260.

***N*^{1}-[3′,4′-Bis(benzyloxy)benzoyl]-*N*^{8}-*tert*-butyloxycarbonyl-*N*^{4}-(*N*-{1-[(4,4-dimethyl-2,6-dioxocyclohexylidene)ethyl]}aminopropyl)-spermidine (9):** To a solution of aldehyde **7** (270 mg, 1.14 mmol) and amine **8**[15] (0.49 g, 0.88 mmol) in anhydrous MeOH (20 mL) was added freshly activated molecular sieves (3 Å) and Et_3N until pH 8 was reached. The resulting mixture was stirred for 1 h at room temperature before $NaBH(OAc)_3$ (335 mg, 1.58 mmol) was added in one portion. The mixture was stirred for 48 h, then the resulting slurry was filtered through a pad of Celite and H_2O (5 mL) was added to quench the reaction. The solvents were removed under reduced pressure and the resulting oil was dissolved in EtOAc and extracted with satd. aq. $NaHCO_3$ and brine. The organic layer was dried and filtered, the solvent was removed in vacuo, and the oily residue was purified by flash chromatography (SiO_2; CH_2Cl_2/MeOH/NH_4OH, 10:1:0→10:1:0.1) to afford spermidine derivative **9** (452 mg, 66%) as an amorphous solid. R_f = 0.35 (CH_2Cl_2/MeOH, 10:1). ^{1}H NMR (600 MHz, [D$_6$]DMSO): δ = 0.92 (s, 6 H, 2× CH_3^{Dde}), 1.33–1.39 (m, 13 H, 6-H$_2$, 7-H$_2$, CH_3^{Boc}), 1.60–1.66 (m, 2 H, 2-H$_2$), 1.66–1.73 (m, 2 H, 10-H$_2$), 2.24 (s, 4 H, 2× CH_2^{Dde}), 2.30–2.38 (m, 2 H, 5-H$_2$), 2.38–2.46 (m, 4 H, 3-H$_2$, 9-H$_2$), 2.47 (s, 3 H, CH_3^{Dde}), 2.86–2.91 (m, 2 H, 8-H$_2$), 3.24 (q, $J_{1,2}$ = 6.5 Hz, 2 H, 1-H$_2$), 3.43 (q, J = 6.4 Hz, 2 H, 11-H$_2$), 5.16, 5.19 (2× s, 4 H, 2× CH_2^{Bn}), 6.76 (t, $J_{NH,8}$ = 5.3 Hz, 1 H, 8-NH), 7.11 (d, $J_{5,6}$ = 8.5 Hz, 1 H, 5-H^{Ar}), 7.30–7.47 (m, 11 H, 2× Ph, 6-H^{Ar}), 7.55 (d, $^4J_{2,6}$ = 1.98 Hz, 1 H, 2-H^{Ar}), 8.24–8.27 (m, 1 H, 1-NH), 13.25 (t, J = 5.2 Hz, 11-NH) ppm. ^{13}C NMR (150 MHz, [D$_6$]-DMSO): δ = 17.2, 23.7, 26.3, 26.6, 27.4, 27.8, 28.2, 29.6, 37.6, 39.3, 40.7, 50.3, 51.0, 52.4, 52.9, 69.9, 70.2, 77.3, 106.9, 113.2, 113.3, 120.6, 127.4, 127.5, 127.8, 128.3, 128.4, 136.9, 137.1, 147.6, 150.5, 155.5, 165.4, 172.6, 196.3 ppm. HRMS (ESI): calcd. for $C_{46}H_{63}N_4O_7$ [M + H]$^+$ 783.4691; found 783.4690.

***N*^{1}-[3′,4′-Bis(benzyloxy)benzoyl]-*N*^{4}-(*N*-{1-[(4,4-dimethyl-2,6-dioxocyclo-hexylidene)ethyl]}aminopropyl)spermidine·2TFA (10):** A solution of amine **9** (0.46 g, 0.58 mmol) in CH_2Cl_2 (12 mL) was stirred at 0 °C for 30 min. A solution of TFA (1.4 mL) in CH_2Cl_2 (12 mL) was added dropwise over 1 h and the mixture was stirred for an additional 30 min at 0 °C before it was allowed to reach room temperature. The solution was stirred for 12 h at room temperature, then coevaporated with toluene (4×) and $CHCl_3$ (2×), to afford amine **10** (di-TFA salt, 438 mg, 83%) as an amorphous solid. R_f = 0.10 (CH_2Cl_2/MeOH, 10:1). ^{1}H NMR (300 MHz, [D$_6$]DMSO): δ = 0.93 (s, 6 H, 2× CH_3^{Dde}), 1.49–1.72 (m, 4 H, 6-H$_2$, 7-H$_2$), 1.84–2.02 (m, 4 H, 2-H$_2$, 10-H$_2$), 2.26 (s, 4 H, 2× CH_2^{Dde}), 2.47 (s, 3 H, CH_3^{Dde}), 2.76–2.88 (m, 2 H, 8-H$_2$), 3.06–3.18 (m, 6 H, 3-H$_2$, 5-H$_2$, 9-H$_2$), 3.31 (q, $J_{1,2}$ = 5.9 Hz, 2 H, 1-H$_2$), 3.48 (q, J = 6.5 Hz, 2 H, 11-H$_2$), 5.17, 5.20 (2× s, 4 H, 2× CH_2^{Bn}), 7.13 (d, $J_{5,6}$ = 8.5 Hz, 1 H, 5-H^{Ar}), 7.29–7.47 (m, 11 H, 2× Ph, 6-H^{Ar}), 7.57 (d, $^4J_{2,6}$ = 1.92 Hz, 1 H, 2-H^{Ar}), 7.83 (br. s, 3 H, 8-NH_3^+), 8.53 (t, $J_{NH,2}$ = 5.4 Hz, 1 H, 1-NH), 9.70 (br. s, 1 H, 4-NH^+), 13.26 (t, J = 5.3 Hz, 11-NH) ppm. ^{13}C NMR (75 MHz, [D$_6$]DMSO): δ = 17.3, 20.1, 26.3, 26.6, 27.4, 27.8, 29.6, 36.4, 38.2, 40.7, 48.9, 49.0, 51.3, 52.4, 69.9, 70.2, 107.1, 113.2, 113.3, 120.8, 127.4, 127.5, 127.8, 128.3, 128.4, 136.9, 137.1, 147.8, 150.5, 165.9, 173.0, 196.6 ppm. HRMS (ESI): calcd. for $C_{41}H_{55}N_4O_5$ [M + H]$^+$ 683.4167; found 683.4165.

1-(*N*$^{1′}$-[3′′′,4′′′-Bis(benzyloxy)benzoyl]-*N*$^{4′}$-{1-[(4,4-dimethyl-2,6-dioxocyclohexylidene)ethyl]aminopropyl}spermidinyl)-3-{*N*$^{1′′}$-[3′′′,4′′′-bis(benzyloxy)benzoyl]-*N*$^{4′′}$-benzyloxycarbonyl-spermidinyl}-2-*tert*-butyl Citrate (13): To a solution of citric acid anhydride **11**[36] (0.20 g, 0.86 mmol) and freshly activated molecular sieves (3 Å) in DMF (2 mL), was added amine **10** (di-TFA salt, 0.36 g, 0.40 mmol) and Et_3N (0.46 mL, 3.4 mmol). The mixture was stirred at room temperature for 36 h, filtered through a pad of Celite, and the solvents were evaporated to dryness. The oily residue was dissolved in EtOAc, extracted with aq. HCl (1 M, 2×) and brine, dried, and filtered. The organic layer was concentrated in vacuo and purified by flash chromatography (SiO_2; CH_2Cl_2/MeOH/AcOH, 10:1:0.1) to give the monoacid (234 mg) as a clear oil; R_f = 0.05 (CH_2Cl_2/MeOH, 10:1).

To a solution of this acid (0.23 g, 0.26 mmol) in anhydrous THF (5 mL) was added *N*-hydroxysuccinimide (45 mg, 0.39 mmol) and diisopropylcarbodiimide (73 μL, 0.47 mmol). The mixture was stirred at room temperature for 18 h, then the solvents were evaporated to dryness to afford the crude *N*-hydroxysuccinimide ester, which was used for the next step without further purification.

To a mixture of amine **12** (TFA salt, 0.30 g, 0.42 mmol) and Et_3N (0.65 mL, 4.7 mmol) in anhydrous CH_2Cl_2 (5 mL) was added the crude *N*-hydroxysuccinimide ester dissolved in anhydrous dioxane (3 mL). The mixture was stirred at room temperature for 72 h, then the solvents were evaporated to dryness. The residue was dissolved in EtOAc and extracted with aq. HCl (1 M), aq. NaOH (1 M), and brine. The organic phase was dried, filtered, and concentrated to dryness. The obtained residue was purified by flash chromatography (SiO_2; CH_2Cl_2/MeOH, 10:1) to give petrobactin derivative **19** (248 mg, 42%, 2 steps) as an amorphous solid. R_f = 0.30 (CH_2Cl_2/MeOH, 10:1). ^{1}H NMR (400 MHz, [D$_6$]DMSO): δ = 0.91 (s, 6 H, 2× CH_3^{Dde}), 1.30–1.42 (m, 13 H, *t*Bu, 7′-H$_2$, 7′′-H$_2$), 1.43–1.59 (m, 4 H, 6′-H$_2$, 6′′-H$_2$), 1.60–1.80 (m, 6 H, 2′-H$_2$, 2′′-H$_2$, 10′-H$_2$), 2.23 (s, 4 H, 2× CH_2^{Dde}), 2.35–2.48 (m, 2 H, CH_aH^{citr}), 2.46 (s, 3 H, CH_3^{Dde}), 2.55 (d, ^2J = 14.7 Hz, 2 H, CHH_b^{citr}), 2.95–3.05 (m, 4

H, 8′-H_2, 8″-H_2), 3.15–3.35 (m, 12 H, 1′-H_2, 1″-H_2, 3′-H_2, 3″-H_2, 5′-H_2, 5″-H_2), 3.35–3.50 (m, 4 H, 9′-H_2, 11′-H_2), 5.02–5.08 (br. s, 2 H, CH_2^{Cbz}), 5.15, 5.19 (2× s, 8 H, 4× CH_2^{Bn}), 5.65 (s, 1 H, OH^{citr}), 7.11 (d, $J_{5,6}$ = 8.5 Hz, 2 H, 2× 5-H^{Ar}), 7.30–7.48 (m, 27 H, 2× 6-H^{Ar}, 5× Ph), 7.55 (m, 2 H, 2× 2-H^{Ar}), 7.90 (m, 2 H, 8′-NH, 8″-NH), 8.29 (br. s, 2 H,1′-NH, 1″-NH), 13.24 (t, $J_{NH,11}$ = 5.1 Hz, 1 H, 11′-NH) ppm. ^{13}C NMR (100 MHz, [D$_6$]DMSO): δ = 17.2, 23.8, 24.2, 26.3, 26.8, 27.4, 27.8, 29.6, 36.9, 38.1, 40.6, 43.2, 44.8, 46.5, 49.3, 50.2, 51.9, 52.3, 69.6, 69.9, 70.2, 73.9, 80.1, 106.9, 113.2, 113.3, 120.6, 127.3, 127.4, 127.7, 127.8, 128.1, 128.3, 128.4, 128.5, 136.9, 137.0, 147.7, 150.6, 155.2, 165.5, 169.3, 172.2, 172.7, 196.3 ppm. HRMS (ESI): calcd. for $C_{87}H_{108}N_7O_{15}$ $[M + H]^+$ 1490.7898; found 1490.7905.

1-{$N^{1'}$-[3‴,4‴-Bis(benzyloxy)benzoyl]-$N^{4'}$-[*N*-(biotinyl)aminopropyl]spermidinyl}-3-{$N^{1''}$-[3‴,4‴-bis(benzyloxy)benzoyl]-$N^{4''}$-benzyloxycarbonyl-spermidinyl}-2-*tert*-butyl Citrate (15): To a solution of Dde-protected petrobactin **13** (0.24 g, 0.16 mmol) in EtOH (5 mL), was added hydrazine (0.11 mL, 3.4 mmol) at 0 °C. The mixture was stirred at room temperature for 16 h, then toluene (10 mL) was added and the mixture was evaporated to dryness to give the crude amine as a clear oil.

The amine was dissolved in DMF (5 mL), biotin *N*-hydroxysuccinimide ester (**14**, 80 mg, 0.23 mmol) and *i*Pr_2EtN (80 μL, 0.47 mmol) were added, and the mixture was stirred for 48 h. After evaporation to dryness, purification of the residue by flash chromatography (SiO_2; CH_2Cl_2/MeOH/NH_4OH, 10:1:0.1) gave biotinylated petrobactin **15** (202 mg, 77%) as an amorphous solid. R_f = 0.60 (CH_2Cl_2/MeOH/NH_4OH, 10:1:0.1). HPLC: t_R = 29.91 min (C18, 4.6 mm × 150 mm, CH_3CN/H_2O/0.1% TFA, flow rate: 0.6 mL min^{-1}, gradient: 10–90% over 45 min). ^{1}H NMR (600 MHz, [D$_6$]DMSO): δ = 1.26–1.38 (m, 17 H, *t*Bu, 6′-H_2, 6″-H_2, 7′-H_2, 15′-H_2), 1.41–1.54 (m, 7 H, 7″-H_2, 10′-H_2, 14′-H_2, 16′-H_a), 1.56–1.66 (m, 3 H, 2′-H_2, 16′-H_b), 1.68–1.78 (m, 2 H, 2″-H_2), 2.02–2.08 (m, 2 H, 13′-H_2), 2.30–2.40 (m, 6 H, 3′-H_2, 5′-H_2, 9′-H_2), 2.45 (d, ^2J = 14.6 Hz, 2 H, CH_aH^{citr}), 2.52–2.59 (m, 3 H, CHH_b^{citr}, 18′-H_a), 2.79 (dd, ^2J = 12.5, $J_{18,19}$ = 5.2 Hz, 1 H, 18′-H_b), 2.96–3.08 (m, 7 H, 8′-H_2, 8″-H_2, 11′-H_2, 17′-H), 3.18–3.27 (m, 8 H, 1′-H_2, 1″-H_2, 3″-H_2, 5″-H_2), 4.08–4-11 (m, 1 H, 21′-H), 4.26–4.28 (m, 1 H, 19′-H), 5.02–5.07 (m, 2 H, CH_2^{Cbz}), 5.15, 5.18, 5.19 (s, 8 H, 4× CH_2^{Bn}), 5.65 (s, 1 H, OH^{citr}), 6.34 (s, 1 H, 19′-NH), 6.39 (s, 1 H, 21′-NH), 7.11 (d, $J_{5,6}$ = 8.6 Hz, 2 H, 2× 5-H^{Ar}), 7.28–7.47 (m, 27 H, 2× 6-H^{Ar}, 5× Ph), 7.56 (d, $^2J_{2,6}$ = 2.0 Hz, 2 H, 2× 2-H^{Ar}), 7.74 (br. s, 1 H, 11′-NH), 7.90 (t, $J_{8,NH}$ = 5.2 Hz, 2 H, 8′-NH, 8″-NH), 8.30 (br. s, 2 H, 1′-NH, 1″-NH) ppm. ^{13}C NMR (100 MHz, [D$_6$]DMSO): δ = 24.0, 25.1, 25.3, 26.3, 26.9, 27.4, 28.0, 28.2, 28.6, 34.9, 35.2, 36.8, 37.7, 38.1, 38.4, 39.5, 43.2, 44.7, 46.4, 51.0, 51.1, 53.4, 55.4, 59.1, 60.9, 65.9, 69.8, 70.2, 73.9, 80.1, 113.2, 113.3, 120.6, 127.3, 127.4, 127.5, 127.6, 127.8, 128.3, 128.4, 136.9, 137.0, 147.6, 150.5, 155.5, 162.6, 165.4, 169.3, 172.2, 174.1 ppm. HRMS (ESI): calcd. for $C_{87}H_{110}N_9O_{15}S_1$ $[M + H]^+$ 1552.7837; found 1552.7807.

1-{$N^{1'}$-[3‴,4‴-Bis(hydroxy)benzoyl]-$N^{4'}$-[*N*-(biotinyl)aminopropyl]spermidinyl}-3-{$N^{1''}$-[3‴,4‴-bis(hydroxy)benzoyl]spermidinyl} Citrate (3): To a solution of protected biotinylated petrobactin **15** (81 mg, 52 μmol) in AcOH (3 mL) was added a mixture of conc. HCl (0.5 mL) and AcOH (3 mL) at 15 °C with vigorous stirring. The mixture was stirred at room temperature for 3 h, then the solvents were removed by coevaporation with toluene (3×) and $CHCl_3$ (3×), and the obtained crude monoacid was used for the next step without further purification. HPLC showed complete removal of the *tert*-butyl ester [t_R = 28.25 min (C18, 4.6 mm × 150 mm, CH_3CN/H_2O/0.1% TFA, flow rate: 0.6 mL min^{-1}, gradient: 10–90% over 45 min)].

The residue was dissolved in methanol (10 mL) and ammonium formate (82 mg, 1.3 mmol) and Pd/C (30%, 25 mg) were added, and the resulting mixture was heated to reflux for 4 h. The mixture was filtered through a pad of Celite and the solvents were evaporated to dryness to give an amorphous solid. The residue was purified by preparative HPLC (column: C18, 4.60 mm × 250 mm solvent: CH_3CN/H_2O/0.1% TFA, flow rate: 1.1 mL min^{-1}, gradient: 5–35% over 35 min) and the appropriate fractions were lyophilized to give biotinylated petrobactin **3** (7.6 mg, 15%) as an amorphous solid. HPLC: t_R = 11.39 min. ^{1}H NMR (600 MHz, [D$_6$]DMSO): δ = 1.23–1.35 (m, 2 H, 15′-H_2), 1.38–1.45 (m, 6 H, 7′-H_2, 7″-H_2, 16′-H_2), 1.45–1.63 (m, 6 H, 6′-H_2, 6″-H_2, 14′-H_2), 1.71–1.77 (m, 2 H, 2″-H_2), 1.78–1.87 (m, 4 H, 2′-H_2, 10′-H_2), 2.05–2.10 (m, 2 H, 13′-H_2), 2.50–2.53 (m, 2 H, CH_aH^{citr}, 18′-H_a), 2.56–2.61 (m, 3 H, CHH_b^{citr}), 2.81 (dd, ^2J = 12.4, $J_{18,19}$ = 5.1 Hz, 1 H, 18′-H_b), 2.87–2.95 (m, 4 H, 3″-H_2, 5″-H_2), 3.03–3.15 (m, 13 H, 3′-H_2, 5′-H_2, 8′-H_2, 8″-H_2, 9′-H_2, 11′-H_2, 17′-H), 3.25–3.30 (m, 4 H, 1′-H_2, 1″-H_2), 4.10–4.15 (m, 1 H, 21′-H), 4.28–4.35 (m, 1 H, 19′-H), 5.55 (br. s, 1 H, OH^{citr}), 6.38, 6.40 (2× s, 2 H, 19′-NH, 21′-NH), 6.76 (d, $J_{5,6}$ = 7.8 Hz, 2 H, 2× 5-H^{Ar}), 7.18 (dd, $J_{5,6}$ = 8.3, $^4J_{2,6}$ = 1.5 Hz, 2 H, 2× 6-H^{Ar}), 7.27 (br. s, 2 H, 2× 2-H^{Ar}), 7.93 (t, $J_{13,NH}$ = 5.5 Hz, 1 H, 12′-NH), 7.99 (t, $J_{8,NH}$ = 5.4 Hz, 2 H, 8′-NH, 8″-NH), 8.28–8.32 (m, 3 H, 1′-NH, 1″-NH, 4″-NH), 9.10 (br. s, 2 H, 2× 3-OH^{Ar}), 9.19 (br. s, 1 H, 4′-NH^+), 9.53 (br. s, 2 H, 2× 4-OH^{Ar}), 12.63 (br. s, 1 H, CO_2H^{citr}) ppm. ^{13}C NMR (125 MHz, [D$_6$]-DMSO): δ = 20.3, 22.9, 23.8, 25.2, 26.0, 26.1, 26.2, 28.0, 28.2, 35.1, 35.6, 36.1, 36.3, 37.7, 39.5, 43.3, 44.8, 46.5, 49.9, 50.2, 51.6, 55.4, 59.2, 61.1, 73.5, 114.8, 115.1, 118.9, 125.3, 125.4, 144.8, 148.5, 162.7, 166.6, 166.7, 169.5, 169.6, 172.6, 175.0 ppm. HRMS (MALDI): calcd. for $C_{47}H_{72}N_9O_{13}S_1$ $[M + H]^+$ 1002.797; found 1002.793.

N^1-[3′,4′-Bis(benzyloxy)benzoyl]-N^4-acetyl Spermidine·TFA (16): To a solution of amine **8** (700 mg, 1.25 mmol) and Et_3N (0.28 mL, 1.5 mmol) in CH_2Cl_2 (20 mL) was added AcCl (0.11 mL, 1.5 mmol) at 0 °C and the resulting mixture was stirred at room temperature for 18 h. The reaction was quenched by the addition of H_2O (50 mL), and the solution was extracted with CH_2Cl_2 (3×). The organic layer was dried and evaporated, and the residue was purified by flash chromatography (SiO_2; CH_2Cl_2/MeOH, 20:1) to give the N^4-acetyl derivative (647 mg, 86%) as an amorphous solid.

To a solution of acetyl derivative (647 mg, 1.07 mmol) in CH_2Cl_2 (20 mL) was added a solution of TFA (3 mL) in CH_2Cl_2 (30 mL) dropwise over 1 h at 0 °C. After stirring for 30 min at 0 °C, the mixture was stirred for 4 h at room temperature. Coevaporation with toluene (4×) and $CHCl_3$ (2×) then afforded amine **16** (TFA salt, 633 mg, 96%) as an amorphous solid. R_f = 0.05 (CH_2Cl_2/MeOH, 10:1). ^{1}H NMR (300 MHz, [D$_6$]DMSO): δ (*cis*/*trans* rotamers were observed) = 1.42–1.60 (m, 4 H, 6-H_2, 7-H_2), 1.65–1.82 ($m_{cis/trans}$, 2 H, 2-H_2), 1.95, 2.00 (2× s, 3 H, CH_3^{Ac}), 2.72–2.88 (m, 2 H, 8-H_2), 3.16–3.34 (m, 6 H, 1-H_2, 3-H_2, 5-H_2), 5.16, 5.20 (2× s, each 2 H, CH_2^{Bn}), 7.11–7.14 ($m_{cis/trans}$, 1 H, 5-H^{Ar}), 7.32–7.48 (m, 11 H, 2× Ph, 6-H^{Ar}), 7.56 (br. s, 1 H, 2-H^{Ar}), 7.66–7.80 (br. s, 3 H, 8-NH_3^+), 8.35, 8.42 (2× $t_{cis/trans}$, $J_{NH,1}$ = 5.4 Hz, 1 H, 1-NH) ppm. ^{13}C NMR (75 MHz, [D$_6$]DMSO): δ = 21.1, 24.1, 26.6, 28.2, 36.0, 38.4, 42.1, 43.8, 45.8, 47.1, 69.5, 69.8, 112.9, 113.0, 120.2, 127.1, 127.4, 127.5, 127.8, 128.1, 128.9, 136.9, 137.0 ppm, no C=O signals observed (broad). HRMS (ESI): calcd. for $C_{30}H_{38}N_3O_4$ $[M + H]^+$ 504.2857; found 504.2851.

1-{$N^{1'}$-[3″,4″-Bis(benzyloxy)benzoyl]-$N^{4'}$-acetyl-spermidinyl}-2-isopropyl Citrate (19): Preparation of cyclic anhydride **18**:[32,35,36] To a solution of 2-isopropyl citrate (**17**, 586 mg, 2.50 mmol) in THF (15 mL) was added freshly activated molecular sieves (3 Å) and

DCC (516 mg, 2.50 mmol), and the resulting suspension was stirred for 1 h at room temperature. The solution was filtered through a pad of Celite and the solvent was removed under reduced pressure to afford the cyclic anhydride **18** (460 mg, 82%), which was used directly in the next reaction step.

To a solution of the anhydride **18** (460 mg, 2.00 mmol) in DMF (2 mL) was added molecular sieves (3 Å) and a solution of amine **16** (TFA salt, 882 mg, 1.43 mmol) and Et_3N (0.79 mL, 5.72 mmol) in DMF (5 mL). The resulting mixture was stirred for 36 h at room temperature, filtered, and the solvents evaporated. The residue was dissolved in EtOAc and extracted with aq. HCl (1 M). The organic layer was dried, filtered, and evaporated, and the residue was purified by flash chromatography (SiO_2; CH_2Cl_2/MeOH/HOAc, 20:1:0.1→10:1:0.1) to afford monoacid **19** (755 mg, 74%) as a clear oil. R_f = 0.05 (CH_2Cl_2/MeOH/HOAc, 20:1:0.1). ^{1}H NMR (300 MHz, [D$_6$]DMSO): δ (*cis*/*trans* rotamers were observed) = 1.15 (d, J = 6.2 Hz, 6 H, 2× CH_3^{iPr}), 1.28–1.54 (m, 4 H, 6′-H_2, 7′-H_2), 1.62–1.82 (m$_{cis/trans}$, 2 H, 2′-H_2), 1.93, 1.99 (2× s$_{cis/trans}$, 3 H, CH_3^{Ac}), 2.42–2.53, 2.56–2.68, 2.72–2.82 (3× m, 4 H, 2× CH_2^{citr}), 2.94–3.12 (m, 2 H, 8′-H_2), 3.14–3.33 (m, 6 H, 1′-H_2, 3′-H_2, 5′-H_2), 4.87 (sept, J = 6.7 Hz, 1 H, CH^{iPr}), 5.16, 5.19 (2× s, 2 H, CH_2^{Bn}), 5.95 (br. s, 1 H, OH^{citr}), 7.12 (d, $J_{5,6}$ = 8.5 Hz, 1 H, 5-H^{Ar}), 7.30–7.48 (m, 11 H, 2× Ph, 6-H^{Ar}), 7.57 (br. s, 1 H, 2-H^{Ar}), 7.98 (q, $J_{NH,8}$ = 5.4 Hz, 1 H, 8′-NH), 8.30, 8.36 (2× t$_{cis/trans}$, $J_{NH,1}$ = 5.5 Hz, 1 H, 1′-NH), 12.10 (br. s, 1 H, CO_2H^{citr}) ppm. ^{13}C NMR (75 MHz, [D$_6$]DMSO): δ = 21.0, 21.3, 26.3, 27.6, 28.9, 36.7, 38.2, 42.8, 43.0, 46.1, 48.0, 67.8, 69.9, 70.2, 73.3, 113.3, 120.5, 127.3, 127.5, 127.7, 127.8, 128.4, 136.9, 137.0, 147.6, 150.6, 165.3, 165.6, 169.0, 169.4, 171.1, 172.4 ppm. HRMS (ESI): calcd. for $C_{39}H_{48}N_3O_{10}$ [M – H]$^-$ 718.3340; found 718.3344.

1-{*N*$^{1′}$-[3′′′,4′′′-Bis(benzyloxy)benzoyl]-*N*$^{4′}$-acetyl-spermidinyl}-3-{*N*$^{1′′}$-[3′′′,4′′′-bis(benzyloxy)benzoyl]-*N*$^{4′′}$-benzyloxycarbonyl-spermidinyl}-2-isopropyl Citrate (20): To a solution of acid **19** (0.70 g, 0.97 mmol) in anhydrous THF (5 mL) was added *N*-hydroxysuccinimide (167 mg, 1.46 mmol) and DIC (275 μL, 1.75 mmol). The mixture was stirred at room temperature for 16 h, then the solvents were evaporated to dryness to afford the crude *N*-hydroxysuccinimide ester, which was used for the next step without further purification.

To a mixture of amine **12** (TFA salt, 1.24 g, 1.75 mmol) and Et_3N (2.43 mL, 17.5 mmol) in anhydrous CH_2Cl_2 (10 mL) was added a solution of the crude NHS ester in anhydrous dioxane (7 mL). The mixture was stirred at room temperature for 48 h, then evaporated to dryness. The residue was dissolved in EtOAc and extracted with aq. HCl (1 M), aq. NaOH (1 M), and brine. The organic phase was dried, filtered, and concentrated. The residue was purified by flash chromatography (SiO_2; CH_2Cl_2/MeOH, 20:1→10:1) to give petrobactin derivative **20** (628 g, 50%, 2 steps) as an amorphous solid. R_f = 0.35 (CH_2Cl_2/MeOH, 10:1). ^{1}H NMR (500 MHz, [D$_6$]DMSO): δ (*cis*/*trans* rotamers were observed) = 1.13 (d, J = 6.3 Hz, 6 H, 2× CH_3^{iPr}), 1.27–1.53 (m, 8 H, 6′-H_2, 6′′-H_2, 7′-H_2, 7′′-H_2), 1.64–1.80 (m, 4 H, 2′-H_2, 2′′-H_2), 1.93, 1.99 (2× s$_{cis/trans}$, 3 H, CH_3^{Ac}), 2.44–2.50, 2.55–2.62 (2× m, each 2 H, 2× CH_2^{citr}), 2.95–3.06 (m, 4 H, 8′-H_2, 8′′-H_2), 3.16–3.29 (m, 12 H, 1′-H_2, 1′′-H_2, 3′-H_2, 3′′-H_2, 5′-H_2, 5′′-H_2), 4.84 (sept, J = 6.2 Hz, 1 H, CH^{iPr}), 5.02–5.08 (m, 2 H, CH_2^{Cbz}), 5.16, 5.19 (2× br. s, 8 H, 4× CH_2^{Bn}), 5.73 (s, 1 H, OH^{citr}), 7.09–7.14 (m, 2 H, 2× 5-H^{Ar}), 7.29–7.48 (m, 27 H, 5× Ph, 2× 6-H^{Ar}), 7.54–7.57 (m, 2 H, 2× 2-H^{Ar}), 7.90–8.04 (2× m$_{cis/trans}$, 2 H, 8′-NH, 8′′-NH), 8.25–8.36 (2× m$_{cis/trans}$, 2 H, 1′-NH, 1′′-NH) ppm. ^{13}C NMR (125 MHz, [D$_6$]DMSO): δ = 21.2, 21.3, 25.7, 26.2, 26.4, 27.6, 28.6, 36.9, 37.9, 38.1, 42.4, 43.2, 44.2, 44.7, 45.6, 47.6, 66.0, 67.6, 69.8, 70.2, 73.8, 113.2, 113.3, 120.5, 120.6, 127.2, 127.3, 127.4, 127.5, 127.8, 128.3, 128.4, 136.9, 137.0, 147.7, 150.6, 155.6, 165.3, 165.4, 169.0, 169.2, 169.3, 172.6 ppm. HRMS (ESI): calcd. for $C_{75}H_{88}N_6O_{14}Na$ [M + Na]$^+$ 1319.6251; found 1319.6233.

1-{*N*$^{1′}$-[3′′′,4′′′-Bis(hydroxy)benzoyl]-*N*$^{4′}$-acetyl-spermidinyl}-3-{*N*$^{1′′}$-[3′′′,4′′′-bis(hydroxy)benzoyl]spermidinyl} Citrate (4): Protected petrobactin **20** (0.27 g, 0.21 mmol) was dissolved in a mixture of THF/H_2O (15 mL, 2:1) and cooled to 0 °C. After the addition of LiOH (13 mg, 0.25 mmol), the mixture was stirred for 60 min at this temperature. Strongly acidic ion exchange resin (Dowex 50WX8-100, 50–100 mesh) was added and the mixture was stirred for 5 min, filtered, and the solvents evaporated to dryness. The residue was used for the next step without further purification.

The crude acid was dissolved in EtOH/H_2O (20 mL, 10:1) and Pd/C (30%, 80 mg) was added. The mixture was stirred vigorously at room temperature for 72 h under a H_2 atmosphere (35 bar), then the mixture was filtered through a pad of Celite, concentrated, and the residue was purified by preparative HPLC (column: C18, 4.60 mm × 250 mm, solvent: CH_3CN/H_2O/0.1% TFA, flow rate: 1.10 mL min^{-1}, gradient: 5→35% CH_3CN over 35 min). Appropriate fractions (t_R = 8.0–9.1 min) were lyophilized to give monoacylated petrobactin **4** (81 mg, 51%, two steps) as an amorphous solid. ^{1}H NMR (500 MHz, [D$_6$]DMSO): δ (*cis*/*trans* rotamers were observed) = 1.25–1.59 (m, 8 H, 6′-H_2, 6′′-H_2, 7′-H_2, 7′′-H_2), 1.65, 1.75 (2× quint.$_{cis/trans}$, J = 7.2 Hz, 1 H, 2′-H_2), 1.80 (quint., J = 7.1 Hz, 2′′-H_2), 1.95, 1.99 (2× s$_{cis/trans}$, 3 H, CH_3^{Ac}), 2.48–2.52, 2.53–2.65 (2× m, each 2 H, 2× CH_2^{citr}), 2.85–2.95 (m, 4 H, 3′-H_2, 5′-H_2), 3.00–3.10 (m, 4 H, 8′-H_2, 8′′-H_2), 3.14–3.29 (m, 8 H, 1′-H_2, 1′′-H_2, 3′′-H_2, 5′′-H_2), 6.73–6.77 (m, 2 H, 2× 5-H^{Ar}), 7.15–7.19 (m, 2 H, 2× 6-H^{Ar}), 7.25–7.28 (m, 2 H, 2× 2-H^{Ar}), 7.92–7.99 (m, 2 H, 8′-NH, 8′′-NH), 8.09, 8.15 (2× t$_{cis/trans}$, $J_{NH,1}$ = 5.6 Hz, 1 H, 1′-NH), 8.26–8.32 (m, 3 H, 1′′-NH, 4′′-NH_2^+), 9.10 (br. s, 2 H, 2× 3-OH^{Ar}), 9.50 (br. s, 2 H, 2× 4-OH^{Ar}) ppm. ^{13}C NMR (125 MHz, [D$_6$]DMSO): δ = 21.7, 23.4, 25.2, 26.1, 26.5, 28.1, 36.6, 37.1, 38.1, 38.6, 42.9, 43.7, 44.7, 45.2, 46.9, 73.9, 115.3, 115.4, 115.5, 119.2, 119.4, 125.9, 126.3, 145.3, 148.7, 148.9, 167.1, 169.6, 169.9, 170.0, 175.4 ppm. HRMS (ESI): calcd. for $C_{36}H_{53}N_6O_{12}$ [M + H]$^+$ 761.3716; found 761.3712.

1,3-Bis-{*N*$^{1′}$-[3′′,4′′-bis(benzyloxy)benzoyl]-*N*$^{4′}$-benzyloxycarbonyl-spermidinyl}-2-isopropyl Citrate (21): Synthesis of bis(*p*NP) ester: To a solution of 2-isopropyl citrate (**17**, 800 mg, 3.42 mmol)[32,35] in anhydrous CH_3CN (34 mL) were added *p*-nitrophenol (1.23 g, 8.80 mmol) and DCC (1.83 g, 8.80 mmol) at 0 °C, and the mixture was then stirred at room temperature for 20 h. After complete formation of the active ester was observed by TLC (R_f = 0.40; CH_2Cl_2/MeOH, 10:1), the solvent was removed under reduced pressure and the yellow residue was purified by flash chromatography (SiO_2; CH_2Cl_2/MeOH, 10:1) to give the bis(*p*NP) ester (1.22 g, 74%).

To a solution of amine **12** (TFA salt, 0.43 g, 0.60 mmol) in anhydrous DMF (2 mL) was added Et_3N (0.14 mL, 1.0 mmol) at 0 °C and the mixture was stirred for 15 min. This solution was added dropwise to a solution of the bis(*p*NP) ester (122 mg, 0.250 mmol) in anhydrous DMF (2 mL) in the presence of molecular sieves (3 Å), and the mixture was stirred at room temperature for 18 h. The mixture was filtered, evaporated to dryness, and the residue was dissolved in EtOAc, extracted with satd. aq. Na_2CO_3 (2×), aq. HCl (1 M), and brine. The organic layer was dried, and the residue obtained after evaporation was purified by flash chromatography (SiO_2; CH_2Cl_2/MeOH, 20:1) to give protected petrobactin **21** (240 mg, 69%) as an amorphous solid. R_f = 0.30 (CH_2Cl_2/MeOH, 10:1). ^{1}H NMR (400 MHz, [D$_6$]DMSO): δ = 1.12 (d, J = 6.3 Hz, 6 H, CH_3^{iPr}), 1.28–1.36 (m, 4 H, 2× 7′-H_2), 1.42–1.51 (m, 4 H, 2× 6′-H_2), 1.69–1.77 (m, 4 H, 2× 2′-H_2), 2.40 (d, ^2J = 14.6 Hz, 2

H, CH_aH^{citr}), 2.58 (d, ^2J = 14.6 Hz, 2 H, CHH_b^{citr}), 2.95–3.06 (m, 4 H, 2× 8′-H_2), 3.17–3.28 (m, 12 H, 2× 1′-H_2, 2× 3′-H_2, 2× 5′-H_2), 4.83 (quint, J = 6.3 Hz, 1 H, CH^{iPr}), 5.01–5.08 (m, 2 H, CH_2^{Cbz}), 5.15 (s, 4 H, 2× CH_2^{Bn}), 5.19 (s, 4 H, 2× CH_2^{Bn}), 5.73 (s, 1 H, OH^{citr}), 7.11 (d, $J_{5,6}$ = 8.5 Hz, 2 H, 2× 5-H^{Ar}), 7.25–7.47 (m, 32 H, 6× Ph, 2× 6-H^{Ar}), 7.56 (br. s, 2 H, 2× 2-H^{Ar}), 7.90–7.95 (br. s, 2 H, 2× 8′-NH), 8.26–8.32 (br. s, 2 H, 2× 1′-NH) ppm. ^{13}C NMR (100 MHz, [D$_6$]DMSO): δ = 21.3, 25.0, 25.6, 26.3, 27.9, 28.6, 36.9, 38.1, 43.2, 44.3, 44.7, 46.0, 46.4, 66.0, 67.6, 69.8, 70.2, 73.8, 113.2, 113.3, 120.6, 127.3, 127.4, 127.5, 127.6, 127.8, 128.3, 128.4, 136.9, 137.0, 147.7, 150.6, 155.3, 165.5, 169.3, 172.6 ppm. HRMS (ESI): calcd. for $C_{81}H_{92}N_6O_{15}$NNa [M + Na]$^+$ 1411.6513; found 1411.6526.

1,3-Bis-{$N^{1'}$-[3′′,4′′-bis(hydroxy)benzoyl]spermidinyl} Citrate (Petrobactin, 1): Protected petrobactin **21** (124 mg, 89.7 μmol) was dissolved in a mixture of THF/H_2O (10 mL, 2:1) and cooled to 0 °C. After LiOH (6.0 mg, 0.13 mmol) was added, the mixture was stirred for 40 min, then strongly acidic ion exchange resin (Dowex 50WX8-100, 50–100 mesh) was added, and the mixture was stirred for 10 min, filtered, and the solvents evaporated to dryness to give the crude monoacid (90 mg).

The monoacid was dissolved in EtOH/H_2O (10 mL, 10:1) and Pd/C (10%, 10 mg) was added. The mixture was stirred vigorously at room temperature for 36 h under a positive H_2 pressure (55 bar). After filtration through a pad of Celite, the mixture was concentrated under reduced pressure and the residue was purified by preparative HPLC (column: C18, 25 mm × 250 mm; solvent: CH_3CN/H_2O/0.1% TFA, flow rate: 16 mL min^{-1}, gradient: 5–10% CH_3CN over 10 min, 10–50% CH_3CN over 30 min). The appropriate fractions (t_R = 14.4 min) were lyophilized to give petrobactin (**1**; 40 mg, 62%, two steps) as an amorphous solid. The NMR and HRMS data were identical to those obtained previously.[15]

1,3-Bis-{$N^{1'}$-[3′′,4′′-bis(benzyloxy)benzoyl]-$N^{4'}$-acetyl-spermidinyl}-2-isopropyl Citrate (22): To an ice-cooled solution of **16** (TFA salt, 0.32 g, 0.52 mmol) in anhydrous DMF (4 mL) was added Et_3N (74 μL, 1.0 mmol), and the mixture was stirred for 15 min. This solution was added dropwise to a solution of the bis(*p*NP) ester of **17** (0.12 g, 0.25 mmol, prepared as described for the preparation of **21**) in anhydrous DMF (2 mL) that contained molecular sieves (3 Å). After stirring at room temperature for 18 h, the mixture was filtered and the solvents evaporated. The residue was dissolved in EtOAc, extracted with satd. aq. Na_2CO_3 (2×), aq. HCl (1 M), and brine. The organic layer was dried, filtered, and evaporated, and the residue was purified by flash chromatography (SiO_2; CH_2Cl_2/MeOH, 10:1) to give the protected di-$N^{4'}$-acetyl petrobactin **22** (231 mg, 77%) as an amorphous solid. R_f = 0.30 (CH_2Cl_2/MeOH, 10:1). ^{1}H NMR (600 MHz, [D$_6$]DMSO): δ (*cis*/*trans* rotamers were observed) = 1.14 (d, J = 6.2 Hz, 6 H, CH_3^{iPr}), 1.27–1.52 (m, 8 H, 2× 6′-H_2, 2× 7′-H_2), 1.67, 1.76 (2× quint$_{cis/trans}$, J = 7.2 Hz, 4 H, 2× 2′-H_2), 1.94, 1.99 (2× s$_{cis/trans}$, 6 H, 2× CH_3^{Ac}), 2.45–2.49, 2.55–2.61 (2× m$_{cis/trans}$, 4 H, 2× CH_2^{citr}), 2.96–3.08 (m, 4 H, 2× 8′-H_2), 3.17–3.29 (m, 12 H, 2× 1′-H_2, 2× 3′-H_2, 3′′-H_2, 2× 5′-H_2, 5′′-H_2), 4.84 (sept, 1 H, J = 6.2 Hz, CH^{iPr}), 5.16, 5.19 (2× s, each 4 H, 4× CH_2^{Bn}), 5.74 (br. s, 1 H, OH^{citr}), 7.11, 7.12 (2× d, $J_{5,6}$ = 8.6 Hz, 2 H, 2× 5-H$^{Ar}{}_{cis/trans}$), 7.30–7.47 (m, 22 H, 4× Ph, 2× 6-H^{Ar}), 7.55, 7.56 (2× d$_{cis/trans}$, $^2J_{2,6}$ = 2.0 Hz, 2× 2-H^{Ar}), 7.91–7.98 (m, 2 H, 2× 8′-NH_c), 8.28, 8.33 (2× t$_{cis/trans}$, $J_{NH,1}$ = 5.6 Hz, 2 H, 2× 1′-NH) ppm. ^{13}C NMR (125 MHz, [D$_6$]DMSO): δ = 21.2, 21.3, 21.4, 24.8, 25.7, 26.2, 26.4, 27.6, 28.6, 36.7, 36.8, 37.9, 38.1, 42.4, 43.2, 44.2, 45.6, 47.6, 67.7, 68.8, 70.2, 73.8, 113.2, 113.3, 120.5, 120.6, 127.2, 127.3, 127.5, 127.6, 127.8, 128.2, 128.3, 128.4, 136.9, 137.1, 147.6, 150.6, 165.3, 165.7, 169.0, 169.2, 169.3, 169.4, 172.6 ppm. HRMS (ESI): calcd. for $C_{69}H_{84}N_6O_{13}$Na [M + Na]$^+$ 1227.5989; found 1227.6001.

1,3-Bis-{$N^{1'}$-[3′′,4′′-bis(hydroxy)benzoyl]-$N^{4'}$-acetyl-spermidinyl} Citrate (5): Protected di-$N^{4'}$-acetyl petrobactin **22** (200 mg, 166 μmol) was dissolved in a mixture of THF/H_2O (15 mL, 2:1) and cooled to 0 °C. LiOH (11 mg, 0.25 mmol) was added and the mixture was stirred for 40 min. Strongly acidic ion exchange resin (Dowex 50WX8-100, 50–100 mesh) was added and the mixture was stirred for 10 min, filtered, and the solvents evaporated. The crude monoacid (200 mg) was used for the next step without further purification.

The monoacid was dissolved in EtOH/H_2O (20 mL, 10:1), Pd/C (10%, 120 mg) was added, and the mixture was stirred vigorously at room temperature for 36 h under a H_2 atmosphere (45 bar). After filtration through a pad of Celite, the mixture was concentrated and the residue was purified by preparative HPLC (column: C18, 4.60 mm × 250 mm; solvent: CH_3CN/H_2O/0.1% TFA, flow rate: 1.10 mL min^{-1}, gradient: 5→35% CH_3CN over 35 min). Appropriate fractions (t_R = 8.0–9.1 min) were lyophilized to give di-$N^{4'}$-acetyl petrobactin **5** (85 mg, 64%) as an amorphous solid. ^{1}H NMR (600 MHz, [D$_6$]DMSO): δ (*cis*/*trans* rotamers were observed) = 1.28–1.45 (m, 6 H, 2× 7′-H_2, 2× 6′-H_a), 1.52–1.59 (m, 2 H, 2× 6′-H_b), 1.60–1.68, 1.72–1.80 (2× m$_{cis/trans}$, each 2 H, 2′-H_2), 1.94, 1.98 (2× s$_{cis/trans}$, 6 H, 2× CH_3^{Ac}), 2.45–2.52, 2.54–2.66 (2× m$_{cis/trans}$, each 2 H, 2× CH_2^{citr}), 2.98–3.08 (m, 4 H, 2× 8′-H_2), 3.14–3.28 (m, 12 H, 2× 1′-H_2, 2× 3′-H_2, 2× 5′-H_2), 5.32 (br. s, 1 H, OH^{citr}), 6.74 (d, $J_{5,6}$ = 8.3 Hz, 2 H, 2× 5-H^{Ar}), 7.16, 7.17 (2× dd$_{cis/trans}$, $J_{5,6}$ = 8.3, $^4J_{2,6}$ = 2.1 Hz, 2 H, 2× 6-H^{Ar}), 7.25, 7.26 (2× d$_{cis/trans}$, $^4J_{2,6}$ = 2.3 Hz, 2 H, 2× 2-H^{Ar}), 7.92–7.96 (m, 2 H, 2× 8′-NH), 8.08, 8.14 (2× t$_{cis/trans}$, $J_{NH,1}$ = 5.6 Hz, 2 H, 2× 1′-NH), 9.06 (br. s, 2 H, 2× 3-OH^{Ar}), 9.40 (br. s, 2 H, 2× 4-OH^{Ar}), 12.52 (br. s, 1 H, CO_2H^{citr}) ppm. ^{13}C NMR (125 MHz, [D$_6$]DMSO): δ = 21.2, 24.7, 25.6, 26.3, 26.4, 27.6, 28.6, 36.6, 37.9, 38.2, 42.4, 43.1, 44.2, 45.7, 47.6, 73.5, 114.8, 114.9, 115.0, 118.7, 118.8, 125.8, 125.9, 144.8, 148.2, 165.9, 166.2,169.1, 169.4, 169.5, 174.9 ppm. HRMS (ESI): calcd. for $C_{38}H_{55}N_6O_{13}$ [M + H]$^+$ 803.3822; found 803.3804.

Determination of Iron Binding Affinities; General Remarks: An Ultrospec 3100 pro spectrophotometer was used for the EDTA competition experiments. A UV/Vis range from 200 to 700 nm with a data pitch of 1.0 nm and a scan speed of 1800 nm/min was used. The samples were mixed and equilibrated for at least 16 h prior to the competition experiment. All operations prior to the measurement were performed under strict safe-light conditions.

Determination of the pFe^{III} Values: For petrobactin derivatives **1**–**5**, a spectrophotometric EDTA competition assay was performed by following the general protocol developed by Abergel et al.[38] The petrobactin derivatives were dissolved in HEPES buffer and an equimolar Fe^{III} chloride solution was added, giving a final concentration of 0.2 mM. The UV/Vis measurements were performed as a batch assay by adding different EDTA concentrations (EDTA amounts of 1:10 to 25:1) of the same volume and equilibration for at least 16 h. The composition of each batch was determined by measuring a complete UV/Vis spectrum (buffer/EDTA as background) and deconvolution by using the UV/Vis spectra of the pure iron complexes of each derivative. A plot of log[EDTA]/[sid] (sid = petrobactin derivative) against log[Fe-EDTA]/[Fe-sid] directly provided the difference of pFe^{III} values of both ligands, ΔpFe^{III} = pFe^{III} (sid) – pFe^{III} (EDTA), when the data is fitted to Equation (1) derived by Abergel et al.[38] and the known pFe^{III} value of EDTA (23.42) is used.

$$\log[\text{Fe-EDTA}]/[\text{Fe-sid}] = \log[\text{EDTA}]/[\text{sid}] - \Delta\text{pFe}^{III} \quad (1)$$

Plots of log[EDTA]/[sid] against log[Fe-EDTA]/[Fe-sid] that were used for the determination of pFe^{III} of the different ligands are included in the Supporting Information (Figure S1).

Fluorescence Spectroscopy: General experimental details and the calculation of the protein-binding constants were performed as described previously.[15] A plot of the fluorescence intensity of FpiA against the concentration of ligand **3** is included in the Supporting Information (Figure S2).

Supporting Information (see footnote on the first page of this article): ^{1}H and ^{13}C NMR spectra of all new compounds. For compound **16**, a $^{1}H/^{13}C$-HSQC spectrum is shown because of the peak broadening observed in the ^{13}C spectrum. Plots of log[EDTA]/[sid] against log[Fe-EDTA]/[Fe-sid] for ligands **1**–**5**. Fluorescence intensity plot of FpiA against ligand **3**.

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