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Synthesis and activity of *p*-azidobenzoyloxyferricrocin, a photoactivatable analog of ferrichrome

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Abstract

p-azidobenzoyloxy desferriferricrocin (AF) **2**, a photoactivatable analog of ferrichrome, was prepared by selective acylation of the serine group of ferricrocin **1** in two steps: transesterification of ferricrocin followed by demetallation. A model compound, (L) 2-benzyloxycarbonylamino-3-*p*-azidobenzoyloxy N-isopropyl propionamide **8**, was separately synthesized in order to set up optimal transesterification conditions to avoid α, β-elimination or epimerization of serine. Binding of iron-loaded AF (FeAF) to the FhuA outer membrane receptor protein of *Escherichia coli* AB2847 was demonstrated by inhibition of ferrichrome transport, interference with the infection by the bacteriophage ϕ80 and with killing of cells by albomycin and colicin M. FeAF transported iron only weakly which indicates that the photoaffinity moiety is incompatible with transport or intracellular iron release from the siderophore.

Abbreviations: AF – azidobenzoyldesferriferricrocin; FeAF – azidobenzoylferricrocin; Z – benzyloxycarbonyl; t-Bu – tertiobutyl; AzB – azidobenzoyl; SuA – succinic acid, iPrA – isopropylamine; DCC – dicyclohexylcarbodiimide; TFA – trifluoroacetic acid; AzBNOSu – *p*-azidobenzoic acid N-hydroxysuccinimide ester; OHOrn – N^{δ}-hydroxyornithine.

Introduction

Most nutrients used by gram-negative bacteria move across bacterial outer-membranes through non specific channels called porins (Nikaido & Vaara 1985). A similar mechanism of diffusion is thought to concern most antibiotics used against bacteria. However, some nutrients are too large to undergo efficient diffusion through the porin channels. These compounds, including iron-siderophore complexes, are taken up by specific receptors present in the outer membrane of the bacteria. At least seven proteins, whose production is regulated by the availability of ferric iron in the environment, have been identified in the outer membrane of *Escherichia coli*. Four of them, encoded by the *fepA*, *fecA*, *fhuA* and *fhuE* genes, have been identified as the receptors for ferric enterobactin, ferric citrate, ferrichrome and coprogen, respectively (Neilands, 1982; Braun & Hantke 1997; Braun *et al.* 1998).

A common method used to characterize protein receptors and ultimately to study the siderophore-receptor interactions during iron transport is photoaffinity labeling (Bailey *et al.* 1986). The photoaffinity labels are siderophore derivatives that retain binding affinity for the receptor and which possess a photoactivatable group capable of covalently binding the siderophore moiety to the receptor. The covalent binding of the siderophore to a receptor prevents transport *via* the concerned receptor. There are only three

examples of photoaffinity labeling studies on receptors involved in siderophore-mediated iron transport in microorganisms. In the first, *p*-azidobenzoyl coprogen B was used to block the siderophore-mediated iron-transport system of *Neurospora crassa* (Bailey *et al.* 1986). In the second, *p*-azidobenzoyl ferrioxamine B containing a radio-nuclide was used to identify the receptor protein FoxB, the receptor of ferrioxamine B in *Escherichia coli* (Nelson *et al.* 1992). In the third, a *p*-azidobenzoylated analog of pyoverdin PaA was used to characterize FpvA, the pyoverdin receptor of *Pseudomonas aeruginosa* (Ocaktan *et al.* 1996).

Ferrichrome is taken up by *E. coli* K-12 by a genetically well-defined transport system that consists of the FhuA protein located in the outer membrane, the FhuD binding protein in the periplasmic space between the outer membrane and the cytoplasmic membrane, the FhuB protein in the cytoplasmic membrane and the FhuC ATPase associated with the inner side of the cytoplasmic membrane (Braun *et al.* 1998). Attempts to identify ferrichrome binding sites of FhuA by introducing point mutations (Killmann & Braun, 1992), deletion of segments (Killmann *et al.* 1998) and insertion of foreign peptides (Carmel *et al.* 1990; Koebnik & Braun 1993) did not reveal single amino acid residues as binding sites. Rather it appeared that several amino acid side chains located in different parts of the protein contribute to ferrichrome binding. Photoaffinity labeling by a ferrichrome derivative offers an alternative approach for the identification of ferrichrome binding sites not only of FhuA but also of FhuD and presumably also of FhuB. The Fhu transport system tolerates structural alterations in ferrichrome such as those contained in ferricrocin in which one of the glycine residues of ferrichrome [cyclic hexapeptide consisting of $(\text{glycine})_3$-N^{δ}-hydroxy-N^{δ}-$\text{acetylornithine})_3$] is replaced by a serine residue. Coupling of a succinate residue to the hydroxyl group of the serine residue yielded the negatively charged ferricrocinyl succinate that transported iron with the same rate as ferricrocin and ferrichrome (Coulton *et al.* 1979). It was therefore likely that substitution of the serine hydroxyl group by a photoaffinity label would yield a transport-active ferricrocin derivative. Since FhuA (Braun *et al.* 1973; Boulanger *et al.* 1996; Locher & Rosenbusch 1997) and FhuD of *Escherichia coli* were purified, it was of interest to synthesize a photoaffinity labeled analog of ferrichrome. The *in vivo* labeled proteins could be cleaved with proteases and binding sites of the photoaffinity label identified on the resulting peptides.

Materials and methods

Melting points are uncorrected and determined on a Büchi SPM-20 instrument. 1 D and 2 D NMR spectra were recorded either on an AC-200 MHz or an ARX 500 MHz instrument (Bruker, Wissembourg, France) as described in Salah El Din *et al.* (1997). The electron impact mass spectra were recorded on a Finnigan MAT TSQ 700 Instrument (Fisons Instruments, Manchester, UK).

TLC was performed on silica gel 60 F254 plates (Merck, Darmstadt, Germany). Elemental analyses were performed by the Service de Microanalyse of the Institut Charles Sadron (CNRS) in Strasbourg.

4-azidobenzoyloxyferricrocin 2

To a solution of ferricrocin **1** (50 mg, 65 μmol) in absolute pyridine (1 mL) the N-hydroxysuccinimide ester of 4-azidobenzoic acid (2 equivalents) was added. The reaction mixture was stirred for 48 h at 60 °C, then dried under reduced pressure and the residue purified by chromatography on a silicagel column eluted first with methylene chloride/ethylacetate (9.5:0.5, v:v) to remove the excess of 4-azidobenzoic acid ester, then with methylene chloride/methanol 9:1 (v/v) to yield 4-azidobenzoyloxyferricrocin **2** (28 mg, 46%).

TLC : $R_f = 0.55$ (methylene chloride/methanol, 8.5 : 1.5, v/v).

4-azidobenzoyloxy desferriferricrocin 3

A solution of 8-hydroxyquinoline (50 mg in 2 mL of dichlormethane) was added to 25 mg of 4-azidobenzoyloxyferricrocin **2** dissolved in 5 mL of water. The mixture was stirred overnight and the aqueous layer was separated and washed four times with dichloromethane and finally concentrated under reduced pressure. After lyophilization a colourless powder was obtained.

TLC : $R_f = 0.44$ (methylene chloride/methanol, 8.5 : 1.5, v/v).

NMR (see Tables 1 and 2).

2-benzyloxycarbonylamino-3-t-butyloxy-1-N-isopropylpropionamide 6

N-hydroxysuccinimide ester **5** was prepared according to Anderson *et al.* (1964). Seventy mg (0.34 mmol) of dicyclohexyl-carbodiimide (Lancaster, Synthesis Ltd, Bischheim, Strasbourg, France) in 0.4 mL of

Table 1. Protons chemical shifts (ppm) of *p*-azidobenzoyl desferriferricrocin 3 in $(CD_3)_2SO$

Residue	NH	Hα	Hβ	Hγ	Hδ	Others
Gly1	5.51	3.40				
Ser	8.39	4.38	**4.55**			H3,5; 7.15 H2,6; 7.88 (azidobenzoyl)
Gly2	8.55	3.70				
OHOrn1	7.84	3.96	1.56	1.45	3.38	CH_3, 1.88 (N-acetyl)
OHOrn2	7.95	4.10	1.58	1.50	3.30–3.45	CH_3, 1.88 (N-acetyl)
OHOrn3	8.18	3.90	1.65	1.52	3.40	CH_3, 1.88 (N-acetyl)

Table 2. ^{13}C chemical shifts (ppm) of *p*-azidobenzoyl desferriferricrocin 3 in $(CD_3)_2SO$

Residue	CO	Cα	Cβ	Cγ	Cδ	Others
Gly1	168.50	42.47				
Ser	169.64	54.15	63.34			
Gly2	169.02	43.03				
OHOrn1	170.17	51.69	28.61	22.65	46.51	3 *C*H_3; 20.33
OHOrn2	170.30	52.65	28.71	22.78	46.71	3 CH_3*CO*,
OHOrn3	171.65	52.99	29.49	23.28	48.58	171.89
p-AzB		C1	C2 & C6	C3 & C5	C4	
	164.65	125.89	131.15 & 131.25	119.24 & 119.30	144.54	

dioxane were added to 100 mg (0.34 mmol) of Z-ser(Bu^t)OH **4** (Bachem, Biochimie SARL, Voisins-le-Bretonneux, France) and 39 mg (0.34 mmol) of N-hydroxysuccinimide (Sigma-Aldrich, Saint Quentin-Fallavier, France) in 1 mL dioxane cooled on ice. The reaction mixture was stored for 16 h in the refrigerator and the dicyclohexylurea formed was removed by filtration and washed with dioxane.

The filtrate containing almost pure N-hydroxysuccinimide ester **5** was concentrated under reduced pressure to yield a yellow oil (100 mg, 83%) which was dissolved in methylene chloride (1 mL) and treated with isopropylamine (40 μL, 0.68 mmol, Sigma-Aldrich) for 45 min at room temperature. The N-hydroxysuccinimide formed was filtered off and the filtrate purified by chromatography on a silicagel column (Geduran SI 60, Merck) with a mixture of methylene chloride/ethyl acetate 9:1 (v/v) to yield 2-benzyloxycarbonylamino-3-*t*-butyloxy-1-N-isopropylpropionamide **6** (96 mg, 85%).

TLC : $R_f = 0.45$ (methylene chloride/ethyl acetate 8.5 : 1.5 (v/v).

m.p = 116–117°.
NMR: see Tables 3 and 4.

EI − MS m/z 336 (4%)(M^+), 279 (12%), 250 (39%), 159 (20%), 91 (100%).

Calculated for			
$C_{18}H_{28}N_2O_4$	C% 64.34	H% 8.33	N% 8.33
Found	C% 64.28	H% 8.41	N% 8.33

2-benzyloxycarbonylamino-3-hydroxy-1-N-isopropylpropionamide 7

Seventy mg of compound **6** were dissolved in anhydrous trifluoroacetic acid (1 mL) and the solution kept at room temperature for 16 h. The solvent was removed by evaporation under reduced pressure, and the residue dissolved in methylene chloride and purified by chromatography on a silicagel column eluted

with a methylene chloride/methanol mixture 9:1 (v/v) to yield 52 mg of 2-benzyloxycarbonylamino-3-hydroxy-1-N-isopropylprop ionamide **7** (89%).

TLC : $R_f = 0.4$ (methylene chloride/methanol, 8 : 2, v/v).

m.p = 133–134°.
NMR: see Tables 3 and 4.

EI − MS m/z 280 (9%)(M^+), 250 (6%), 194 (7%), 177 (39%), 91 (100%).

Calculated for			
$C_{14}H_{20}N_2O_4$	C% 60.00	H% 7.14	N% 10.00
Found	C% 59.99	H% 7.05	N% 9.70

2-benzyloxycarbonylamino-3-(4′-azidobenzoyloxy)-1-N-isopropylpropionamide 8

To a solution of compound **7** (50 mg, 0.18 mmol) in absolute pyridine (1 mL) N-hydroxysuccinimide ester of 4-azidobenzoic acid (Galardy *et al.* 1974) (47 mg, 0.36 mmol) was added. The reaction mixture was stirred for 48 h at 60 °C, then dried under reduced pressure and the residue purified by chromatography on a silicagel column eluted first with methylene chloride/ethylacetate (9.5:0.5, v:v) to remove the excess of 4-azidobenzoic acid ester, and then with methylene chloride/methanol 9:1(v/v) to yield 2-benzyloxycarbonylamino-3-(4′-azidobenzoyloxy)-1-N-isopropylpropionamide **8** (50 mg, 66%, m.p = 160-162 °C).

TLC : $R_f = 0.52$ (methylene chloride/methanol, 8.5 : 1.5, v/v).

NMR: see Tables 3 & 4.

EI − MS m/z 425 (3%)(M^+), 399 (1%), 339 (12%), 177 (39%), 91 (100%).

Calculated for			
$C_{21}H_{23}N_5O_5$	C% 59.29	H% 5.41	N% 16.47
Found	C% 59.45	H% 5.52	N% 16.47

Photodecomposition of p-azidobenzoyloxyferricrocin

Photolysis was performed in 50 mM MOPS, pH 7.4 buffer at 23 °C with a bench lamp emitting at 254 nm (VL-C, 4W, Bioblock Scientific, Illkirch, France. The samples were kept in a quartz cuvette of 1 cm optical pathway (Hellma, France) and irradiated with the light source maintained at 12 cm. A UV spectrum of this solution was determined every 10 seconds.

Transport assays

Cells of *E. coli* AB2845 *aroB malT tsx thi* were grown overnight on tryptone-yeast extract (TY) agar plates, collected, washed and suspended in transport buffer which consisted of M9 salts, 0.4% glucose and 0.25 mM nitrilotriacetate to an optical density at 578 nm of 0.5, corresponding to approximately 5×10^8 cells per mL, as described previously (Killmann & Braun 1993). FeAF was prepared with a 2.5-fold molar excess of AF over Fe, and $^{55}Fe^{3+}$ AF and $^{55}Fe^{3+}$ ferrichrome were prepared with a five-fold surplus of the siderophores over $^{55}Fe^{3+}Cl_3$. Samples of 0.1 mL were withdrawn from the 1 mL culture, and mixed with 3 mL 0.1 M LiCl, filtered through cellulose nitrate filters (0.45 μm, Sartorius, Göttingen, Germany) and washed with 3 mL 0.1 M LiCl. The filters were dried at 60 °C for 15 min and then counted in a liquid scintillation counter. The concentrations of the compounds are given in the figure legends.

Covalent binding of FeAF to cells was determined by using a cell suspension in M9 transport medium spread in a thin layer that was exposed to UV irradiation at 254 nm for 2 min prior to the transport assays. Under these conditions cells retained 80% of their transport activities.

Interference of FeAF and ferrichrome with ligand binding to the FhuA protein

Cells (10^8) of *E. coli* AB2847 pregrown in TY medium were suspended in 0.1 mL 0.9% NaCl and incubated for 20 min in 3 ml TY soft agar that contained 20 μM ferrichrome or FeAF and then spread over 20 mL TY agar plates. Serial dilutions of the FhuA ligands (4 μL) were dropped on the agar plates and the final dilutions were scored which no longer inhibited growth of the cells.

Results

Synthesis and structural data

Synthesis of *p*-azidobenzoyloxydesferriferricrocin **3** was performed in two steps from ferricrocin after transesterification of ferricrocin **1** with the N-hydroxysuccinimide ester of 4-azidobenzoic acid, followed by demetallation of the iron complex with good yields (Figure 1).

Table 3. Protons chemical shifts (ppm) of compounds 4 to 8 in $CDCl_3$ (for the lettering see Figure 2)

Residue		4	5	6	7	8
Ser:	Ha	4.45	4.85	4.12	4.12	4.55
	Hb	**3.54, 3.87**	**3.65, 3.96**	**3.32, 3.77**	**3.36, 3.99**	**4.05, 4.55**
	Hd	5.60	5.69	5.70	5.85	5.75
Z:	Hf, s,2H,	5.12	5.13	5.11	5.12	5.08
	Ph, s,5H	7.35	7.35	7.34	7.35	7.31
R_1=	SuA Hi, s,4H		2.81			
R_1=	iprA Hk			6.36	6.37	6.31
	Hl, 1H			4.04	4.06	4.55
	Hm, 6H			1.12	1.11	1.09
R_2=	*t*-Bu Hh, s,9H	1.17	1.18	1.17		
R_2=	H n				3.13	
R_2=	*p*-AzB					7.93, 7.97
H2',d,2H						6.99, 7.03
H3',d,2H						

Table 4. ^{13}C chemical shifts (ppm) of compounds 4 to 8 in $CDCl_3$ (for the lettering see Figure 2)

Residue			4	5	6	7	8
Ser:	Ca		54.32	53.43	54.48	55.27	54.45
	Cb		61.7	61.66	61.96	**62.93**	**64.91**
	COc		174.91	168.58	169.27	170.01	165.6
Z:	COe		156.28	155.87	156.01	156.5	156.5
	Cf		67.26	67.45	67.05	67.39	67.36
	C1		136.18	136.06	136.30	136.06	136.07
	C2		128.56	128.6	128.59	128.65	128.61
	C3		128.17	128.11	128.16	128.39	128.36
	C4		128.23	128.3	128.24	128.13	128.11
R_1=	SuA:	Ci		25.62			
		COj		166.65			
R_1=	iPrA:	Cl			41.59	41.70	41.86
		Cm			22.72	22.57	22.57
R_2=	*t*-Bu:	Cg	74.13	74.10	74.05		
		Ch	27.27	27.2	27.46		
R_2=	*p*-AzB:	COo					167.49
		C1′					125.87
		C2′					131.57
		C3′					118.95
		C4′					145.31

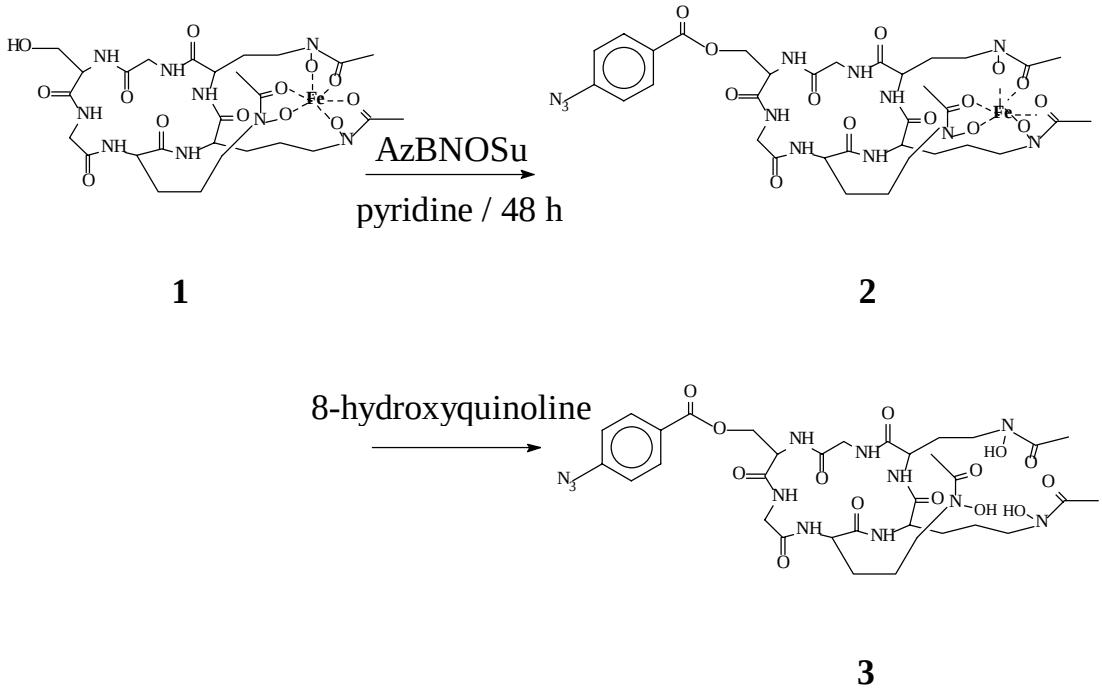
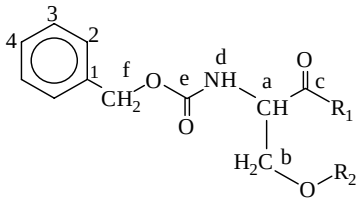


Figure 1. Scheme of the synthesis of *p*-azidobenzoyldesferriferricrocin.

The transesterification reaction was first performed on the readily synthetically available (see below) 2-benzyloxycarbonyl amino-3-hydroxy-N-isopropyl propionamide **7**, in order to establish reaction conditions which avoid secondary reactions such as α, β-elimination or epimerization of serine (Schluter *et al.* 1974) and yielded (66%) 2-benzyloxycarbonyl amino-3-(4′-azidobenzoyloxy)-N-isopropyl propionamide **8** (Figure 2).

The ferricrocin model **7** which had only one hydroxyl functional group capable of undergoing the transesterification reaction, was easily prepared starting from the commercially available Z-Ser(*t*-Bu)-OH in a 3-step sequence (Figure 2). The carboxylic group of the serine derivative **4** was first transformed into the corresponding N-hydroxysuccinamide ester **5**, using N-hydroxysuccinamide and dicyclohexylcarbodiimide (DCC) in dioxane (Anderson *et al.* 1964). Treatment of **5** with isopropylamine in methylene chloride afforded compound **6** in 85% overall yield, which in the presence of anhydrous trifluoroacetic acid gave 2-benzyloxycarbonylamino-3-hydroxy-N-isopropylpropionamide **7** (89%).

The structures of compounds **4** to **8** were determined by ^{1}H and ^{13}C NMR spectrum and by mass



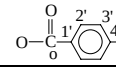
4	R_1 =	OH	R_2 =	$C(CH_3)_3$
5	R_1 =		R_2 =	$C(CH_3)_3$
6	R_1 =	$NHCH(CH_3)_2$	R_2 =	$C(CH_3)_3$
7	R_1 =	$NHCH(CH_3)_2$	R_2 =	H
8	R_1 =	$NHCH(CH_3)_2$	R_2 =	

Figure 2. Structures of the various serine derivatives **4**–**8**.

spectroscopy. The chemical shifts are reported in Tables 3 and 4.

The photoactivatable analog, *p*-azidobenzoyloxy ferricrocin **2** was prepared in the same way as compound **8**, starting from ferricrocin **1** (yield 38% after purification). Desferriferricrocin was not used as a starting material in order to prevent

the secondary reactions on the hydroxamate groups occurring, and *p*-azidobenzoyloxydesferriferricrocin **3** was obtained as a colorless powder by treatment of *p*-azidobenzoyloxy ferricrocin **2** with 8-hydroxyquinoline (Keller-Schierlein & Déer 1963) with an overall yield of 85% (Figure 1). All the protons and carbon signals were assigned from two-dimensional NMR correlations (HOHAHA, ^{1}H-^{1}H, and ^{1}H-^{13}C correlations) (Tables 1 and 2).

The chemical shifts of the protons Hα and Hβ of serine in compounds **4** to **8** were affected by the change in the groups close to the CHα and CH$_2\beta$ of serine. In compounds **4**, **5** and **6**, the neighbourhood was strongly perturbed changing from acid to ester and amide whereas the CH$_2\beta$-group of serine remained bound to the *t*-butyl group by an ether bond: the chemical shifts of Hβ protons of serine were found to be shifted in the 0.1 to 0.3 ppm range. On the contrary, the differences in chemical shifts of the same protons Hβ for compounds **6**, **7** and **8** were much higher when the CH$_2$ group was substituted by an ether, an alcohol or an ester group. In this latter case, the chemical shifts were strongly affected for the proton Hα (0.4 ppm) and even more so for the Hβ protons (0.6 ppm). Conversely, in the ^{13}C spectra the chemical shift of carbon Cβ of serine underwent a 3 ppm shift.

In *p*-azidobenzoyloxy desferriferricrocin **3** the chemical shifts of Hβ protons of serine and the corresponding carbon atom had values very close to those of the model molecule **8** (4.55 ppm for the protons and 63.34 ppm for the carbon ^{13}C), confirming that the introduction of the *p*-azidobenzoyl group had occurred on the hydroxyl group of serine (Tables 1 and 2).

Photodecomposition of p-azidobenzoylferricrocin

The absorption spectrum of the ferric complex of *p*-azidobenzoyloxy ferricrocin **2** (Figure 3) showed two peaks in the visible to ultraviolet range: a broad peak at about 420 nm ($\epsilon_{420} = 1300$ M^{-1} cm^{-1} typical of ferric trihydroxamate siderophores (Coulton *et al.* 1979; Wong *et al.* 1983) and a sharp peak at 265 nm ($\epsilon_{265} = 35000$ M^{-1} cm^{-1}) typical of an aryl azido group.

The photodecomposition of **2** in 50 mM MOPS pH 7.4 buffer under irradiation at 254 nm was characterized by the decrease of the absorption band of the arylazido group at 265 nm. The half-life of the compound deduced from the course of this photodecomposition process was about 1 min.

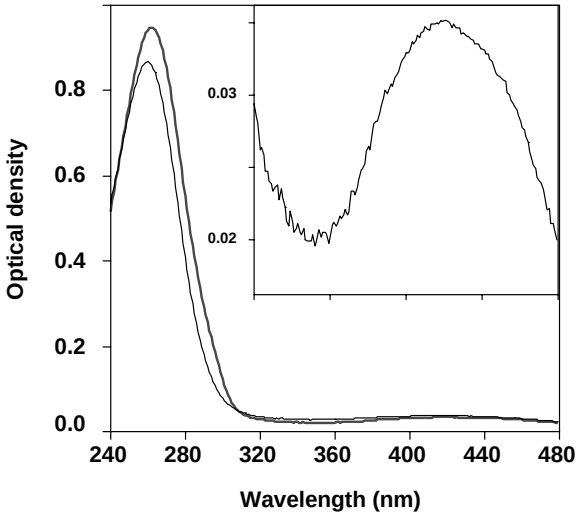


Figure 3. Photodecomposition at 254 nm of *p*-azidobenzoylferricrocin **2** in 50 mM MOPS, pH 7.4 buffer and at a distance of 12 cm from the UV-lamp. Before irradiation (thicker line) and after 20 seconds irradiation (thinner line). **Inset**: The magnified 320–480 nm region of the absorption spectrum of *p*-azidobenzoylferricrocin.

Functional studies

As a first attempt to examine the biological activity of *p*-azidobenzoyloxy ferricrocin (AF) inhibition of [^{55}Fe^{3+}]ferrichrome transport was determined. If iron-loaded AF (FeAF) binds to the same sites of the FhuABD proteins as ferrichrome it should interfere with ferrichrome-mediated iron transport. The experiments were performed in dim red light to avoid inactivation of AF. For the transport studies *E. coli* AB2847 was used which is mutated in the *aroB* gene and therefore does not synthesize its own siderophore enterobactin. Prior to the transport assay cells were grown in a rich medium with sufficient iron supply to measure transport with metabolically active cells. A 10-fold surplus of FeAF over ferrichrome reduced the iron transport rate by about 40% (Figure 4). Under the same conditions unlabeled ferrichrome entirely inhibited [^{55}Fe^{3+}]ferrichrome transport (Figure 4). Illumination of FeAF in the presence of cells for 2 min at 254 nm prior to the addition of [^{55}Fe^{3+}]ferrichrome reduced the [^{55}Fe^{3+}]ferrichrome transport rate by approximately 55% (Figure 5). This value does not contain the 20% reduction of the [^{55}Fe^{3+}]ferrichrome transport rate of illuminated cells without added FeAF.

Interference of AF with ferrichrome transport could occur on the FhuA, FhuD, and FhuB proteins and during cytoplasmic release of ^{55}Fe^{3+} from fer-

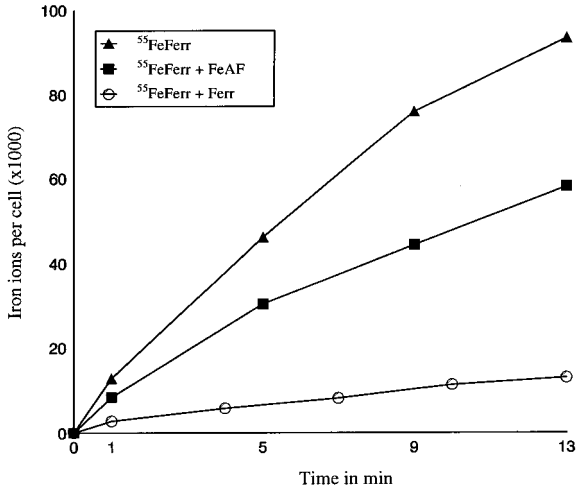


Figure 4. Inhibition of [$^{55}Fe^{3+}$]ferrichrome transport by unlabeled FeAF and unlabeled ferrichrome, respectively. Cells of *E. coli* AB2847 were incubated for 5 min with 10 μM FeAF (10 μM ferrichrome) and then with 1 μM [$^{55}Fe^{3+}$]ferrichrome. Samples were withdrawn after the times indicated, filtered, washed twice with 3 mL 0.1 M LiCl and the radioactivity on the filters counted in a liquid scintillation counter.

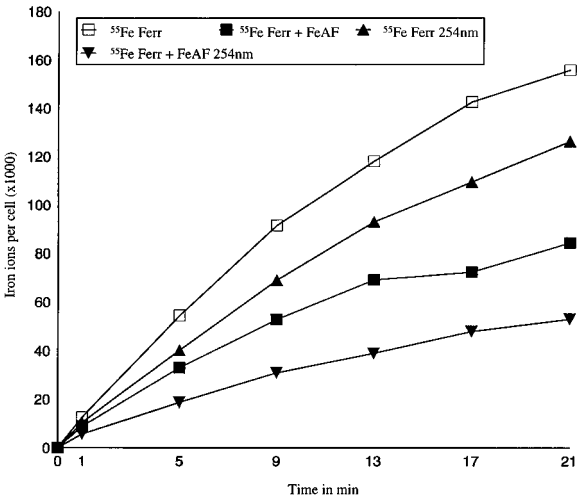


Figure 5. Inhibition of [$^{55}Fe^{3+}$]ferrichrome transport by illumination of *E. coli* AB2847 for 2 min at 254 nm in the presence of 10 μM FeAF after which transport was started by addition of 1 μM [$^{55}Fe^{3+}$] ferrichrome.

richrome. We therefore examined whether FeAF transports iron into cells of *E. coli* AB2847. Uptake of ^{55}FeAF was very slow (Figure 6). A 2 min illumination prior to the measurement of iron uptake did not affect the transport rate. A fivefold higher concentration of $^{55}Fe^{3+}$ (5 μm) increased iron uptake (in 21 min from 50 000 to 105 000 ions per cell) which was completely inhibited by 25 μm ferrichrome. The high

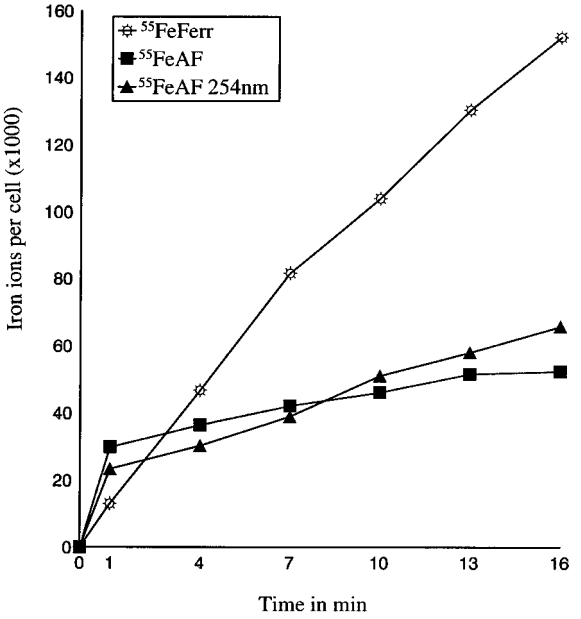


Figure 6. Comparison of the transport rates of $^{55}Fe^{3+}$AF and [$^{55}Fe^{3+}$] ferrichrome with and without illumination at 254 nm.

intial value of 50 000 counts was caused by unspecific binding of ^{55}FeAF since it was not reduced in the presence of ferrichrome and stayed constant during the experiment. These data show that FeAF transport use the same protein(s) as ferrichrome transport and that AF serves as a weak iron carrier in *E. coli* AB2847.

To see whether FeAF binds to FhuA competition experiments were performed with colicin M, the bacteriophage ϕ80 and with the antibiotic albomycin which all use FhuA as receptor to enter cells. FeAF, and ferrichrome as a control, were dissolved in nutrient soft agar at a final concentration of 20 μM. Cells (10^8) of *E. coli* AB2847 were added and after 20 min incubation 10-fold serial dilutions of the FhuA ligands were added. The final dilutions which resulted in a clear zone of growth inhibition were recorded. In the presence of FeAF sensitivity of cells to colicin M was reduced 10-fold, in the presence of ferrichrome 100-fold, to phage ϕ80 10-fold for both iron siderophores, and to albomycin 100 and 10-fold, respectively. Since FhuA is the only ferrichrome-specific transport constituent common to all these FhuA ligands these data demonstrate binding of FeAF to FhuA.

Discussion

Ferricrocin is structurally very closely related to ferrichrome and mediates with the same rate iron-uptake through the specific FhuABCD transport proteins of *Escherichia coli*. *p*-azidobenzoyloxydesferriferricrocin (AF) displays some inhibition of ferrichrome transport which suggests interference with ferrichrome binding to transport proteins. Binding of FeAF to the FhuA outer membrane receptor was demonstrated by inhibition of the entry of FhuA ligands which use only FhuA of the FhuABD transport proteins. Competition of FeAF with binding of the FhuA ligands also demonstrated the specificity of the binding. This does not exclude interference of FeAF with the interaction of ferrichrome with FhuD. Binding of ferrichrome to FhuD and induction of a conformational change has been shown (Köster & Braun 1990; Rohrbach *et al.* 1995). No data exist whether ferrichrome also binds to FhuB, and binding to the FhuC ATPase is not expected since ATPases of related ABC transporters can replace each other which excludes substrate specificity (Boos & Shuman 1998; Schneider & Hunke 1998). Ideally a photoaffinity label may be used to trace the path of ferrichrome from FhuA to FhuD to FhuB. The isolation of peptides of all three proteins with covalently bound FeAF would allow to determine the binding sites and to see whether they are of similar structure. These experiments require a higher specific activity of the synthesized compound than was available for the preliminary experiments of this study and FeAF should be radiolabeled in AF since the iron is lost during preparation of the peptides. However, it is likely that the active sites are composed of amino acid side chains that are distantly located in the polypeptides which would limit the information about the active sites of the proteins obtained by photoaffinity labeling.

Only a low iron transport rate by AF was observed. This could have several reasons. FeAF may only slowly be translocated across the outer membrane, and/or the periplasm and the cytoplasmic membrane, or iron is not released from AF in the cytoplasm. Inhibition of any of these steps would give a negative result in the transport assays.

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References

Anderson GW, Zimmerman JE, Callahan FM. 1964 The use of esters of N-hydroxysuccinimide in peptide synthesis. *J Am Chem Soc* **86**, 1839–1842.

Bailey CT, Kime-Hunt EM, Carrano CJ, Huschka HG, Winkelmann G. 1986 A photoaffinity label for the siderophore-mediated iron transport system in *Neurospora crassa*. *Biochim Biophys Acta* **883**, 299–305.

Boos W, Shuman H, 1998 Maltose/maltodextrin system of *Escherichia coli*: transport, metabolism, and regulation. *Microb Mol Biol Rev* **62**, 204–229.

Boulanger P, Le Maire M, Bonhivers M, Dubois S, Desmadril M, Letellier L. 1996 Purification and structural and functional characterization of FhuA, a transporter of the *Escherichia coli* outer membrane. *Biochemistry* **35**, 14216–14224.

Braun V, Hantke K. 1997 Receptor-mediated bacterial iron transport. In: Winkelmann G, Carrano CJ, eds. *Transition Metals in Microbial Metabolism.* Amsterdam, Harwood Academic Publishers, 81–116.

Braun V, Schaller K, Wolff H. 1973 A common receptor protein for phage T5 and colicin M in the outer membrane of *Escherichia coli*. *Biochem Biophys Acta* **323**, 87–97.

Braun V, Hantke K, Köster W. 1998 Bacterial iron transport: mechanisms, genetics and regulation. In: Sigel A, Sigel S, eds. *Metal Ions in Biological Systems. Iron Transport and Storage in Microorganisms, Plants and Animals*. New York, Marcel Dekker, 67–145.

Carmel G, Hellstern D, Henning D, Coulton JW. 1990 Insertion mutagenesis of the gene encoding the ferrichrome-iron receptor of *Escherichia coli*. *J Bacteriol* **172**, 1861–1869.

Coulton JW, Naegeli HU, Braun, V. 1979 Iron supply of *Escherichia coli* with polymer-bound ferricrocin. *Eur J Biochem* **99**, 39–47.

Galardy RE, Craig LC, Jamieson JD, Printz MP. 1974 Photoaffinity labeling of peptide hormone binding sites. *J Biol Chem* **249**, 3510–3518.

Keller-Schierlein W, Deer A. 1963 Zur Konstitution von Ferrichrysin und Ferricrocin. *Helv Chem Acta* **46**, 1907–1920.

Killmann H, Braun V. 1992 An aspartate deletion mutation defines a binding site of the multifunctional FhuA outer membrane receptor of *Escherichia coli*. *J Bacteriol* **174**, 3479–3486.

Killmann H, Braun V. 1993 Conversion of the FhuA transport protein into a diffusion channel through the outer membrane of *Escherichia coli*. *EMBO J* **12**, 3007–3016.

Killmann H, Herrmann C, Wolff H, Braun V. 1998 Identification of a new site for ferrichrome transport by comparison of the FhuA proteins of *Escherichia coli*, *Salmonella paratyphi B*, *Salmonella typhimurium*, and *Pantoea agglomerans*. *J Bacteriol* **180**, 3845–3852.

Koebnik R, Braun V. 1993 Insertion derivatives containing segments of up to 16 amino acids identify surface- and periplasm-exposed regions of the FhuA outer membrane receptor of *Escherichia coli*. *J Bacteriol* **175**, 828–839.

Köster W, Braun V. 1990 Iron(III) hydroxamate transport into *Escherichia coli*. Substrate binding to the periplasmic FhuD protein. *J Biol Chem* **265**, 21407–21410.

Locher KP, Rosenbusch JP. 1997. Oligomeric states and siderophore binding of the ligand-gated FhuA protein that forms channels across *Escherichia coli* outer membranes. *Eur J Biochem* **247**, 770–775.

Neilands JB. 1982. Microbial envelope proteins related to iron. *Annu Rev Microbiol* **36**, 285–309.

Nelson M, Carrano CJ, Szaniszlo PJ. 1992. Identification of the ferrioxamine B receptor FoxB in *Escherichia coli*. *BioMetals* **5**, 37–46.

Nikaido H, Vaara M. 1985 Molecular basis of bacterial outer-membrane permeability. *Microbiol Rev* **45**, 1–32.

Ocaktan A, Schalk I, Hennard C, Linget-Morice C, Kyslik P, Smith AW, Lambert PA, Abdallah MA. 1996 Specific photoaffinity labeling of a ferripyoverdin outer-membrane receptor of *Pseudomonas aeruginosa*. *FEBS Lett* **396**, 243–247.

Rohrbach MR, Braun V, Köster W. 1995 Ferrichrome transport *in Escherichia coli* K-12: altered substrate specificity of mutated periplasmic FhuD and interaction of FhuD with the integral membrane protein FhuB. *J Bacteriol* **177**, 7186–7193.

Salah El Din AM, Kyslik P, Stephan D, Abdallah MA. 1997 Bacterial siderophores: Structure elucidation and ^{1}H, ^{13}C, ^{15}N two-dimensional NMR assignments of pyoverdin G4R and related siderophores synthesized by a nitrogen-fixing strain of *Pseudomonas putida*. *Tetrahedron* **53**, 12539–12552.

Schluter DN, Mamantov G, Vercellotti J. 1974. Reactivity of N-acetyl-3-O-*p*-tolylsulfonyl-D,L-serine methyl ester: nucleophilic displacement by water at C-3 versus elimination. *Carbohydrate Res* **38**, 333–338.

Schneider E, Hunke S. 1998 ATP-binding-cassette (ABC) transport systems: functional and structural aspects of the ATP-hydrolyzing subunits/domains. *FEMS Microbiol Rev* **22**, 2–20.

Wong GB, Kapel MJ, Raymond KN, Matzanke B, Winkelmann G. 1983 Coordination chemistry of microbial iron-transport compounds: 24. Cheracterization of coprogen and ferricrocin, two ferric hydroxamate siderophores. *J Am Chem Soc* **105**, 810–815.