

## Siderophore activity of chemically synthesized dihydroxybenzoyl derivatives of spermidines and cystamide

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**Chemically synthesized dihydroxybenzoyl derivatives of spermidine and cystamide containing two-, three- and four-bidentates with the hydroxyl groups in 2,3 or 3,4 position were examined in cross-feeding tests using Gram-negative siderophore indicator strains carrying different iron-related markers, and two *Mycobacterium* spp. The catecholates were unable to feed tonB mutants of *E. coli* and *S. typhimurium* as well as the fepA, fiu, cir mutant of *E. coli*, pointing to a tonB- and fepA, cir, fiu-dependent transport. Bis(2,3-dihydroxybenzoyl)derivatives promoted *Salmonella* spp, *E. coli*, *K. pneumoniae* and *P. aeruginosa* strains significantly better than did 3,4-dihydroxybenzoyl derivatives. *N*<sup>4</sup>-substituted spermidines acted more effectively than non-substituted derivatives. Bis(2,3-dihydroxybenzoyl) cystamide was superior to the other catecholates tested in growth promotion of Gram-negative bacteria. The two four-bidentates and the tri-bidentate reacted to *K. pneumoniae* in an inhibitory mode. The position of the hydroxyl groups did not significantly influence the growth promotion of *M. smegmatis* and *M. fortuitum* in the cases of substituted spermidines and of cystamides.**

**Keywords:** dihydroxybenzoylcystamides, dihydroxybenzoylspermidines, Gram-negative bacteria, *Mycobacterium* spp, siderophore activity

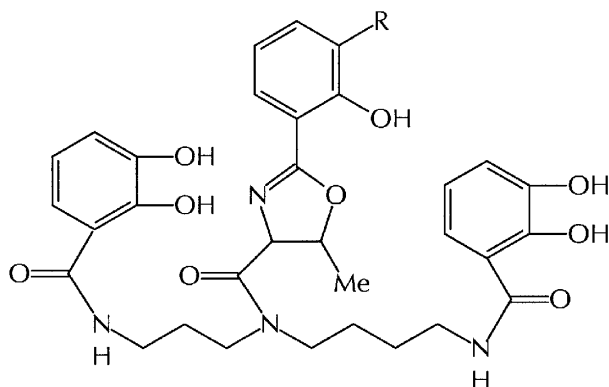
### Introduction

Iron is essential in cellular redox chemistry, and therefore for the survival of microorganisms. Consequently, the extreme insolubility ( $10^{-38}$  M) of ferric ions existing under oxidizing conditions at physiological pH has forced bacteria to evolve elaborate systems for acquiring extracellular iron. To overcome the extreme insolubility of iron, many microorganisms synthesize and excrete powerful ferric ion-selective agents called siderophores. These sequestering agents facilitate active transport of chelated iron into cells. The transport system consists of receptor proteins (so called iron regulated outer membrane proteins, IROMPs), the tonB

protein (which plays a crucial role in energy-coupled transport processes) as well as the ExbB, C proteins, and special cytoplasmic proteins. Most microorganisms utilize hydroxamate-type, catecholate-type and polycarboxylate-type siderophores (Matzanke 1991, Wooldridge & Williams 1993). The most effective siderophores contain three bidentate ligands for complete octahedral coordination of the ferric ion. Recognition of siderophores depends on different parts and structural features of the siderophore molecule: (i) the molecule as an entity; (ii) the geometry and stability of the backbone; (iii) the chirality or geometry at the dentate centre; and (iv) peripheral groups.

Naturally occurring and synthetic ferric ion chelators are currently of great interest because of their role in microorganisms and plants, their potential applications in the treatment of iron overload diseases (Hider *et al.* 1994, Pippard 1994), and their role as part of siderophore antibiotics (Miller &

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**Figure 1.** Structures of parabactin (R = H) and agrobactin (R = OH).

Malouin 1993), as biomimetic iron carriers for further use in malaria therapy (Shanzer & Libman 1991) and, more recently, as selective growth factors of nutrient media (Reissbrodt *et al.* 1996). Polyamines serve as the backbone for a number of natural catechol-containing siderophores. Parabactin (Figure 1), isolated from *Paracoccus denitrificans*, and agrobactin (Figure 1), from the plant pathogen *Agrobacterium tumefaciens*, contain a spermidine base substituted with dihydroxybenzoyl groups at the primary nitrogens and threonine-derived oxazoline residues at the central secondary nitrogen (Peterson *et al.* 1980, Figure 1). *Vibriobactin* (Griffith *et al.* 1984), from *Vibrio cholerae*, utilizes the same dihydroxybenzoyl and oxazoline residues for iron chelation, but incorporates them into the symmetrical norspermidine. Early indications that these siderophores had a potential for the treatment of iron overload promoted the synthesis of a number of catechol analogues. Spermaxin and spermaxinol were synthesized and examined (Sharma *et al.* 1989, McKee *et al.* 1991) microbiologically as growth promoters, and also as being parts of siderophore-antibiotic conjugates of carba-cephalosporin and oxamazin.

In this study we report on new spermidine derivatives, their synthesis and effects on Gram-negative bacteria, and on two *Mycobacterium* spp. We also discuss the synthesis and growth promoting effects of dihydroxybenzoyl cystamides on these bacteria.

## Materials and methods

### Bacterial strains

The Gram-negative siderophore indicator strains and the two mycobacterial strains used in the growth promo-

tion experiments are characterized and listed in Tables 1 and 2.

### Chemically synthesized siderophores

The catecholates were prepared as shown in the following paragraphs; they are partially described by Bricard (1993). The following methods were used for analysis.

Proton NMR spectra were obtained in  $\text{CDCl}_3$ ,  $\text{CD}_3\text{OD}$  or  $\text{DMSO}-d_6$  solutions with their signals as internal standards – 7.24, 4.78 (HDO) and 2.49 ppm – using a Bruker AM 200 or AM 250 spectrometer. Carbon-13 NMR spectra were recorded on the same equipment using the centre line of  $\text{CDCl}_3$  as an internal standard (77.0 ppm). Elemental analyses were performed by the CNRS Microanalysis Service at Gif-sur-Yvette, France. Infrared spectra were recorded on a Perkin-Elmer 883. Routine mass spectra were recorded in the electron impact (EI) or chemical ionization (CI) mode on a Ribermag R-10-10 at the University of Paris-Sud Facility (Orsay, France). High resolution mass spectra were taken on a Kratos MS-80 at ICSN, CNRS, Gif-sur-Yvette, France. TLC was conducted using Merck Silica Gel 60  $\text{F}_{254}$  precoated plastic sheets. Column chromatography was performed using Merck Silica Gel (70–230 mesh). All new compounds gave satisfactory microanalysis and/or high resolution data of the molecular parent ion peak.

### Bis(3,4- and 2,3-dihydroxybenzoyl) cystamides 2 and 3.

These were prepared from 3,4- or 2,3-dimethoxybenzoylchloride by reaction with cystamide hydrochloride yielding 54% (3,4-derivative) or 53% (2,3-derivative) of pure diamides. These compounds were demethylated by use of boron tribromide with a yield of 96% (2,3-derivative) and 98% (3,4-derivative); Figure 2.  $\text{C}_{18}\text{H}_{20}\text{O}_6\text{N}_2\text{S}_2$ , MW = 424..

### Bis(3,4-dihydroxybenzoyl) cystamide 2

NMR ( $\text{CD}_3\text{OD}$ ):  $^1\text{H}$ : -1.8 (t, 4H) :  $2 \times -\text{CH}_2-\text{S}$ ; -2.5 (t, 4H):  $2 \times -\text{CH}_2-\text{N}$ ; -5.65 (d, 2H),  $J = 10 \text{ Hz}$  :  $\text{CH}-5$ ; -6.0 (d, 2H),  $J = 2 \text{ Hz}$  :  $\text{CH}-2$ ; -6.1 (dd, 2H),  $J = 10 \text{ Hz}$  and  $J = 2 \text{ Hz}$  :  $\text{CH}-6$ .

$^{13}\text{C}$ : 37.9 ( $2 \times -\text{CH}_2-\text{S}$ ), 40.9 ( $2 \times -\text{CH}_2-\text{N}$ ), 115.80 and 116.10 ( $\text{CH}-2$  and  $\text{CH}-5$ ), 121.40 and 124.20 ( $-\text{CH}-6$  and  $-\text{C}-1$ ), 146.40 and 151.20 ( $\text{C}-3$  and  $\text{C}-4$ ), 171.60 ( $\text{CO}$ ).

Mass spectrometry: EI:  $m/z = 213 (\text{M}/2 + \text{H})^+$ , 137 ( $\text{CO}-\text{C}_6\text{H}_3(\text{OH})_2)^+$ . CI/ $\text{NH}_3$ :  $m/z = 425 (\text{M}-\text{H})^+$ . IR ( $\text{CHCl}_3$ ):  $\nu (\text{cm}^{-1})$ : 3400 (OH bound amide), 3378 (NH bound amide), 1658 ( $-\text{CO}$  amide).

### Bis(2,3-dihydroxybenzoyl) cystamide 3.

NMR ( $\text{CD}_3\text{OD}$ ):  $^1\text{H}$ : -2.8 (t, 4H) :  $2 \times -\text{CH}_2-\text{S}$ ; -3.5 (t, 4H):  $2 \times -\text{CH}_2-\text{N}$ ; -6.55 (d, 2H),  $J_{\text{ortho}} = 8 \text{ Hz}$  :  $\text{CH}-5$ ; -6.8 (dd, 2H),  $J_{\text{ortho}} = 8 \text{ Hz}$ ,  $J_{\text{meta}} = 1.4 \text{ Hz}$  :  $\text{CH}-6$ ; -7.1 (dd, 2H),  $J_{\text{ortho}} = 8 \text{ Hz}$ ,  $J_{\text{meta}} = 1.4 \text{ Hz}$  :  $\text{CH}-4$ .

**Table 1.** Cross-feeding tests of the cystamide and spermidine derivatives on Gram-negative bacteria

Indicator strain	Iron related marker	Detection of	2	3	4	5	6	7	8	9	10	11
<i>S. typhimurium</i> enb-7	ent (class II)	Enterobactin, (DHBS) <sub>2,3</sub> , DHBA, ferrichrome, ferrioxamines and other hydroxamate-type siderophores, not alcaligin	+	+++	-	-	+	+	+	+	+	++
<i>S. typhimurium</i> TA 2700	ent (class I)	Enterobactin, (DHBS) <sub>2,3</sub> , myxochelins A, B, C, not siderophores of hydroxamate type	+	+++	-	-	-	-	+	-	-	-
<i>S. typhimurium</i> SR 1001	enb-7, but tonB	DHBA	-	-	-	-	-	-	-	-	-	-
<i>S. stanleyville</i> 207/81	wild strain ent, iuc	Common indicator like enb-7, not aerobactin	-	+++	-	-	-	+	-	++	++	++
<i>E. coli</i> AB 2847	aro B	Enterobactin, (DHBS) <sub>2,3</sub> , DHBA, ferrichrome, coprogen	+	+++	-	-	-	++	-	+	++	-
<i>E. coli</i> H 1876	fiu, cir, fepA	Ferriochrome, coprogen, not enterobactin, (DHBS) <sub>2,3</sub> , monocatecholates	-	-	-	-	-	-	-	-	-	-
<i>E. coli</i> LG 1522	ent, iuc, fepA	Aerobactin	-	-	-	-	-	-	-	-	-	-
<i>E. coli</i> IR 122	aroB, tonB	DHBA	-	-	-	-	-	-	-	-	-	-
<i>K. pneumoniae</i> KN 4401	ent, iuc	Most of the phenolate-type and hydroxamate-type siderophores except amonabactin	lysis	+++	-	-	-	+	lysis	lysis	+	+++
<i>Y. enterocolitica</i> H 5030	yb	Yersiniabactin	n.d.	n.d.	++	++	-	-	n.d.	n.d.	-	+
<i>P. aeruginosa</i> PAO 6609	pvd	Pyoverdines, enterobactin, ferrioxamines, ferrichrome, DHBA	-	+++	-	-	(+)	+	+	++	++	-

- no growth zone; + growth zone to 15 mm; ++ growth zone to 15–25 mm; +++ growth zone to > 25 mm; (+) faint growth; lysis means inhibition zone around the filter paper disc within the weak growth of the indicator strain; n.d. not determined.

**Table 2.** Cross-feeding tests of the cystamide and spermidine derivatives involving two *Mycobacterium* spp

Indicator strain	Detection of	2	3	4	5	6	7	8	9	10	11
<i>M. smegmatis</i> 987	mycobactins, mycoexochelin	++	+	-	-	++	++	+	+	-	+
<i>M. fortuitum</i> 10605	mycobactins, mycoexochelin	+++	+++	-	-	+++	++	++	++	lysis	-

- no growth zone; + growth zone to 15 mm; ++ growth zone to 15–25 mm; +++ growth zone to > 25 mm; lysis means inhibition zone around the filter paper disc within the weak growth of the indicator strain.

<sup>13</sup>C: 38.37 (2 × -CH<sub>2</sub>-S), 39.84 (2 × -CH<sub>2</sub>-N), 111.63 (C-1), 118.77 (CH-6), 119.61 and 119.66 (CH-4 and CH-5), 147.24 (CH-3), 150.17 (CH-2), 171.53 (CO).

Mass spectrometry: EI: *m/z* = 213 (M/2 + H)<sup>+</sup>. CI/NH<sub>3</sub>: *m/z* = 425 (M + H)<sup>+</sup>. IR (CHCl<sub>3</sub>): *n* (cm<sup>-1</sup>): 3400 (bound OH), 3373 (bound amide), 1657 (CO amide).

#### N<sup>1</sup>,N<sup>8</sup>-bis(3,4- and 2,3-dihydroxybenzoyl) spermidines **4** and **5**.

N<sup>1</sup>,N<sup>8</sup>-bis(3,4- or 2,3-dimethoxybenzoyl) spermidines were synthesized following the procedure described by Sharma *et al.* (1989) and demethylated with boron tribromide. The yield of **4** was quantitative (97%). The same procedure was successfully applied to the 2,3-isomer **5** (98% yield); see Figure 3. C<sub>21</sub>H<sub>27</sub>O<sub>6</sub>N<sub>3</sub>, MW = 417.

#### N<sup>1</sup>,N<sup>8</sup>-bis(3,4-dihydroxybenzoyl) spermidine **4**.

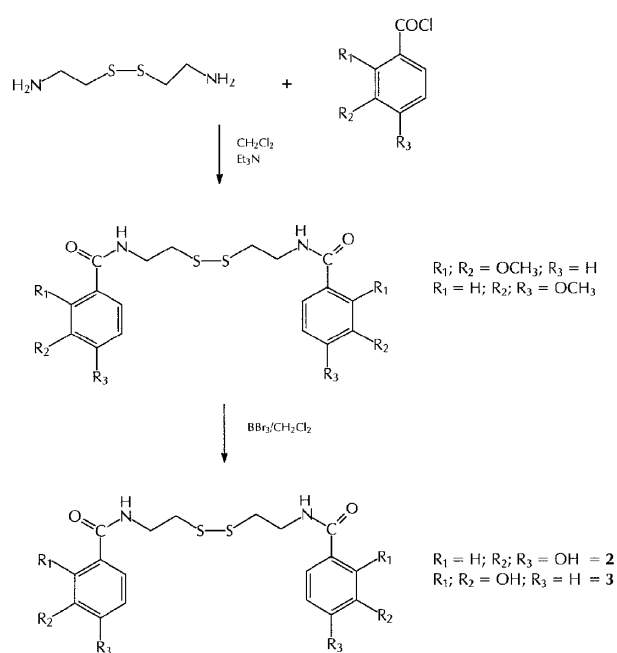
NMR (CDCl<sub>3</sub>): <sup>1</sup>H: -1.2 (m, 4H) : -CH<sub>2</sub>-CH<sub>2</sub>-; -1.45 (q, 2H): CH<sub>2</sub>-CH<sub>2</sub>N; -2.5 (m, 4H) : 2 × CH<sub>2</sub>N secondary amide; -2.9 (m, 4H) : 2 × CH<sub>2</sub>NH amide; -6.25 (dd, 2H) : 2 × CH-6; -6.7 (m, 4H) : (2 × 6H-2) and (2 × CH-5).

<sup>13</sup>C: 24.63, 27.06 and 27.46 (CH<sub>2</sub>), 37.83 and 41.01 (CH<sub>2</sub>-N), 46.46 (CH<sub>2</sub>-NH amide), 115.98 (CH-2), 116.13 (CH-5), 121.49 (CH-6), 125.00 (C-1), 146.54 (C-3), 150.81 (C-4), 171.42 and 171.70 (C=O amide).

Mass spectrometry: CI/NH<sub>3</sub>: *m/z* = 499, 497 (M+HBr)<sup>+</sup>, 418 (M + H)<sup>+</sup>, 417 M<sup>+</sup>.

#### N<sup>1</sup>,N<sup>8</sup>-bis(2,3-dihydroxybenzoyl) spermidine **5**.

NMR (CD<sub>3</sub>OD): <sup>1</sup>H: -0.9 (m, 4H) : -CH<sub>2</sub>-CH<sub>2</sub>-; -1.2 (m, 2H)



**Figure 2.** Synthesis of the dihydroxybenzoyl cystamides **2** and **3**.

:  $-\text{CH}_2-$ ; -2.25 (m, 4H) :  $2 \times -\text{CH}_2\text{N}$ ; -2.6 (m, 4H) :  $2 \times -\text{CH}_2\text{NH}$  amide; -5.9 (t, 2H) :  $2 \times \text{CH}$ -5; -6.11 (d, 2H) :  $2 \times \text{CH}$ -6; -6.4 (dd, 2H) :  $2 \times \text{CH}$ -4.

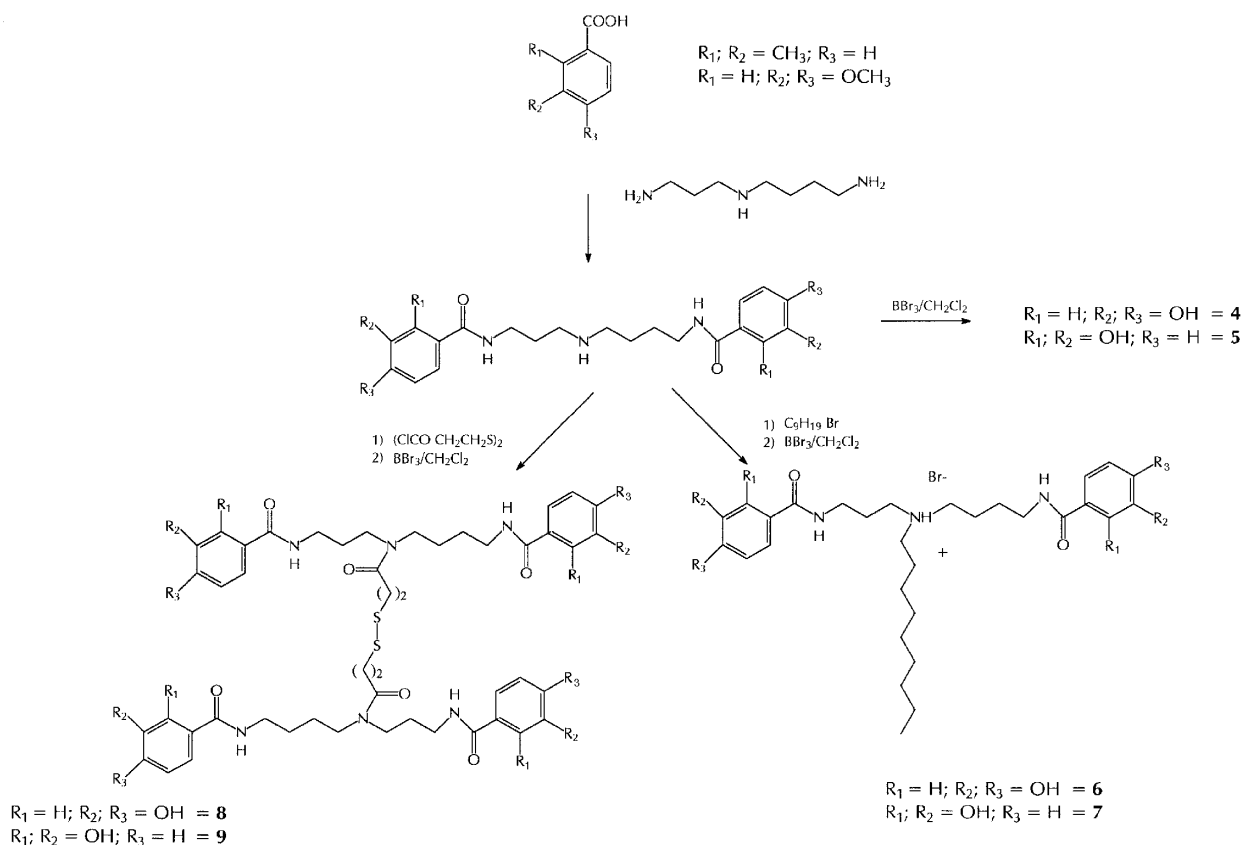
$^{13}\text{C}$ : 116.25 ( $\text{C}$ -1), 118.89 ( $\text{CH}$ -6), 119.72 ( $\text{CH}$ -4 and  $\text{CH}$ -5), 147.14 ( $\text{C}$ -3), 150.05 ( $\text{C}$ -2), 172.11 ( $2 \times \text{C}=\text{O}$  amide).

Mass spectrometry:  $\text{Cl}/\text{NH}_3$  :  $m/z$  = 499, 497 ( $\text{M} + \text{HBr}$ )<sup>+</sup>, 418 ( $\text{M} + \text{H}$ )<sup>+</sup>.

*N*<sup>4</sup>-*n*-nonyl-*N*<sup>1</sup>,*N*<sup>8</sup>-bis(3,4- and 2,3-dihydroxybenzoyl) spermidines **6** and **7**.

*N*<sup>1</sup>,*N*<sup>8</sup>-bis(3,4- and 2,3-dimethoxybenzoyl) spermidines were *N*<sup>4</sup>-alkylated by use of *n*-bromononane; both derivatives were subsequently demethylated with boron tribromide (Figure 3). Purification was by chromatography over silica-gel ( $\text{CH}_2\text{Cl}_2/\text{MeOH}$  5 to 7.5%).  $\text{C}_{34}\text{H}_{53}\text{O}_6\text{N}_3$ ,  $\mu\text{W}$  = 599.

*N*<sup>4</sup>-*n*-nonyl-*N*<sup>1</sup>,*N*<sup>8</sup>-bis(3,4-dimethoxybenzoyl) spermidine **6**. NMR ( $\text{CDCl}_3$ ):  $^1\text{H}$ : -0.8 (t, 3H) :  $-\text{CH}_3$ ; -1.2 (s, 12H) :  $-\text{CH}_2 \times 6$ ; -1.5–1.8 (m, 6H) :  $-\text{CH}_2-\text{CH}_2$ ;  $-(\text{C}_4) - \text{CH}_2-$  ( $\text{C}_3$ ); -2.0 (m, 2H) :  $-\text{CH}_2-\text{CH}_2-\text{C}_7\text{H}_{15}$ ; -2.9 (m, 6H) :  $3 \times -\text{CH}_2\text{N}$  amine; -3.3–3.55 (m, 4H) :  $2 \times -\text{CH}_2-\text{NHCO}$ ; -3.8 (2s, 12H):  $4 \times -\text{OCH}_3$ ; -6.75 (2d, 2H) :  $2 \times \text{CH}$ -6; -7.45 (m, 4H) :  $2 \times \text{CH}$ -5 and  $2 \times \text{CH}$ -2; -8.2 (t, 2H) :  $\text{NH}$  amine.



**Figure 3.** Synthesis of the *N*<sup>1</sup>,*N*<sup>8</sup>-bis(dihydroxybenzoyl) spermidines (**4,5**), of the *N*<sup>4</sup>-*n*-nonyl-*N*<sup>1</sup>,*N*<sup>8</sup>-bis(dihydroxybenzoyl) spermidines (**6,7**) and of the *N*<sup>4</sup>*N*<sup>4</sup>-dithiopropionyl-bis(*N*<sup>1</sup>,*N*<sup>8</sup>-dihydroxybenzoyl) spermidines (**8,9**).

$^{13}\text{C}$ : 13.76 ( $-\text{CH}_3$ ), 21.11, 22.26, 23.55, 24.01, 26.30, 26.53, 28.79, 29.04 and 31.40 ( $10 \times -\text{CH}_2-$ ), 37.07 and 38.32 ( $-\text{CH}_2\text{N}$  amide), 50.81, 52.17 and 52.58 ( $3 \times -\text{CH}_2\text{N}$ , tertiary amine), 55.57 and 55.79 ( $4 \times -\text{OCH}_3$ ), 110.23 ( $\text{CH}-5$ ), 110.96 ( $\text{CH}-2$ ), 120.06 and 120.23 ( $\text{CH}-6$ ), 126.26 ( $\text{C}-1$ ), 148.30 ( $\text{C}-3$ ), 151.24 and 151.43 ( $\text{C}-4$ ), 167.01, 167.38 ( $2 \times \text{C}=\text{O}$  amides).

Mass spectrometry: CI:  $m/z$  = 487 ( $\text{M}-\text{C}_8\text{H}_{16}$ ).

$N^4$ -*n*-nonyl- $N^1,N^8$ -bis(2,3-dimethoxybenzoyl) spermidine **7**.

NMR ( $\text{CDCl}_3$ ):  $^1\text{H}$ : -0.75 (t, 3H) :  $-\text{CH}_3$ ; -1.15 (s, 12H) :  $6 \times -\text{CH}_2$ ; -1.3–1.6 (m, 6H) :  $3 \times -\text{CH}_2$ ; -1.8 (q, 2H) :  $\text{CH}_2$ ; -2.45–2.6 (m, 6H) :  $3 \times -\text{CH}_2\text{N}$ , tertiary amine; -3.3–3.45 (m, 4H) :  $2 \times -\text{CH}_2\text{NH}$  amide; -3.75 (s, 12H) :  $3 \times -\text{OCH}_3$ ; -6.9–7.0 (m, 4H) :  $2 \times \text{H}-5$  and  $2 \times \text{H}-6$ ; -7.5 (m, 2H) :  $2 \times \text{H}-4$ ; -8.0 (2t, 2H) :  $\text{NH}$  amide.

$^{13}\text{C}$ : 13.90 ( $\text{CH}_3$ ), 22.43, 22.87, 25.21, 25.83, 27.06, 27.27, 29.00, 29.11, 29.28 and 31.59 ( $10 \times \text{CH}_2$ ), 37.60 and 38.85 ( $\text{CH}_2\text{-NH}$  amide), 51.31, 52.96, 53.25 ( $3 \times \text{CH}_2\text{N}$ , tertiary amine), 55.83 ( $\text{OCH}_3$ ), 115.03 ( $\text{CH}-4$ ), 122.33 ( $\text{CH}-6$ ), 124.10 ( $\text{CH}-5$ ), 126.58 ( $\text{C}-1$ ), 147.20 ( $\text{C}-3$ ), 152.35 ( $\text{C}-2$ ), 165.09, 165.35 ( $2 \times \text{C}=\text{O}$  amide).

Mass spectrometry: CI/ $\text{NH}_3$  :  $m/z$  = 601 ( $\text{M} + 2\text{H}$ )<sup>+</sup>, 600 ( $\text{M} + \text{H}$ )<sup>+</sup>.

$N^4,N^4$ -dithiodipropionyl-bis[ $N^1,N^8$ -bis(3,4-dihydroxybenzoyl) spermidine] **8** and the 2,3-dihydroxybenzoyl derivative **9**.

These were prepared from  $N^1,N^8$ -bis(3,4- or 2,3-dimethoxybenzoyl) spermidine by reaction with dithiodipropionyl chloride and demethylation by boron tribromide (Figure 3).  $\text{C}_{30}\text{H}_{45}\text{O}_6\text{N}_3$ , MW = 543.

$N^4,N^4$ -dithiodipropionyl-bis[ $N^1,N^8$ -bis(3,4-dihydroxybenzoyl) spermidine] **8**.

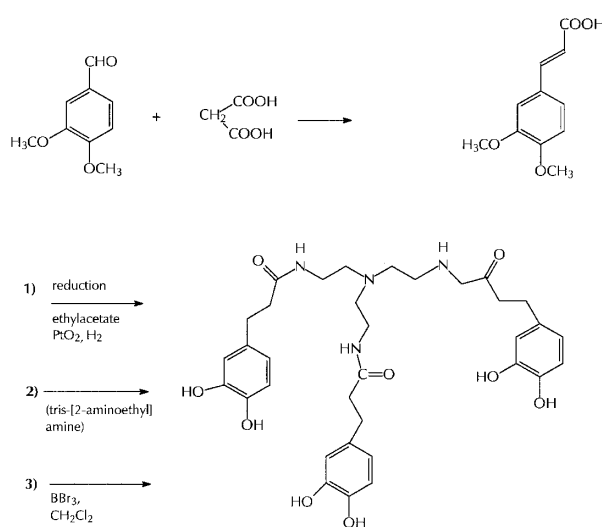
NMR ( $\text{CD}_3\text{OD}$ ):  $^1\text{H}$ : -1.4–1.8 (m, 12H) :  $6 \times \text{CH}_2$ ; -2.5 (m, 4H) :  $-\text{CH}_2\text{-CO-N}$  tertiary amide; -2.7 (t, 3H) :  $\text{CH}_2\text{-S}$ ; -3.1 (m, 16H) :  $4 \times \text{CH}_2\text{-NH}$  secondary amide and  $4 \times \text{CH}_2\text{-N}$  tertiary amide; -6.6 (dd, 4H) :  $4 \times \text{CH}-5$ ; -7.1 (m, 4H) :  $4 \times \text{CH}-6$ ; -7.15 (d, 4H) :  $4 \times \text{CH}-2$ .

$^{13}\text{C}$ : 25.72 ( $\text{CH}_2$ ), 28.05 ( $\text{CH}_2$ ), 33.17 ( $\text{CH}_2\text{-C}=\text{O}$ ), 34.45 ( $\text{CH}_2\text{-S}$ ), 38.71 and 38.95 ( $2 \times \text{CH}_2\text{-NCO}$  tertiary amide), 41.11 ( $\text{CH}_2\text{-NHCO}$ ), 47.72 ( $\text{CH}_2\text{-NHCO}$ ), 115.75 and 116.04 ( $4 \times \text{CH}-5$  and  $4 \times \text{CH}-2$ ), 123.97 and 124.44 ( $2 \times 2 \times \text{CH}-6$ ), 124.44 and 125.02 ( $4 \times \text{C}-1$ ), 146.12 ( $4 \times \text{C}-3$ ), 150.67 ( $4 \times \text{C}-4$ ), 170.81 and 171.04 ( $-\text{CON}$  tertiary amide), 173.20 and 173.48 ( $-\text{CONH}$  secondary amide).

Mass spectrometry: CI/ $\text{NH}_3$  :  $m/z$  = 425 ( $\text{MH}^+$ ).

$N^4,N^4$ -dithiodipropionyl-bis[ $N^1,N^8$ -bis(2,3-dihydroxybenzoyl) spermidine] **9**.

NMR ( $\text{CD}_3\text{OD}$ ):  $^1\text{H}$ : -1.1–1.5 (m, 12H) :  $6 \times \text{CH}_2$ ; -2.3–2.45 (m, 4H) :  $-\text{CH}_2\text{-CO-N}$  tertiary amide and  $\text{CH}_2\text{-S}$ ; -2.95 (m, 16H) :  $4 \times \text{CH}_2\text{-NH}$  secondary amide and  $4 \times \text{CH}_2\text{-N}$  tertiary amide; -6.25 (t,  $J$  = 8 Hz, 4H) :  $4 \times \text{CH}-5$ ; -6.5 (dd, 4H),  $J$  = 8 Hz and  $J$  = 1.4 Hz :  $4 \times \text{CH}-6$ ; -6.8 (dd, 4H),  $J$  = 8 Hz and  $J$  = 1.4 Hz :  $4 \times \text{CH}-2$ .



**Figure 4.** Synthesis of tris-*N*-(2-aminoethyl)-[3-(3,4-dihydroxyphenyl)propionamido] amine (**10**).

Mass spectrometry: CI/ $\text{NH}_3$  :  $m/z$  = 506 ( $\text{M}/2 + \text{H}$ )<sup>+</sup>.

Tris-*N*-(2-aminoethyl)-[3-(3,4-dihydroxyphenyl)propionamido] amine **10**.

This was prepared from 3,4-dimethoxycinnamic acid by reduction to 3-(3,4-dimethoxyphenyl) propionic acid, followed by condensation with tris(2-aminoethyl)amine and demethylation with boron tribromide (Figure 4).  $\text{C}_{33}\text{H}_{42}\text{O}_9\text{N}_4$ , MW = 638.

NMR ( $\text{CD}_3\text{OD}$ ):  $^1\text{H}$ : -2.1–2.3 (t, 6H) :  $3 \times \text{CH}_2\text{CO}$ ; -2.4–2.6 (m, 6H) :  $3 \times \text{CH}_2$  benzylic; -2.9–3.3 (m, 12H) :  $3 \times \text{CH}_2\text{NH}$  and  $3 \times \text{CH}_2\text{N}$ ; -6.2 (m, 3H) :  $3 \times \text{CH}-6$ ; -6.4–6.5 (m, 6H) :  $3 \times \text{CH}-2$  and  $3 \times \text{CH}-5$ .

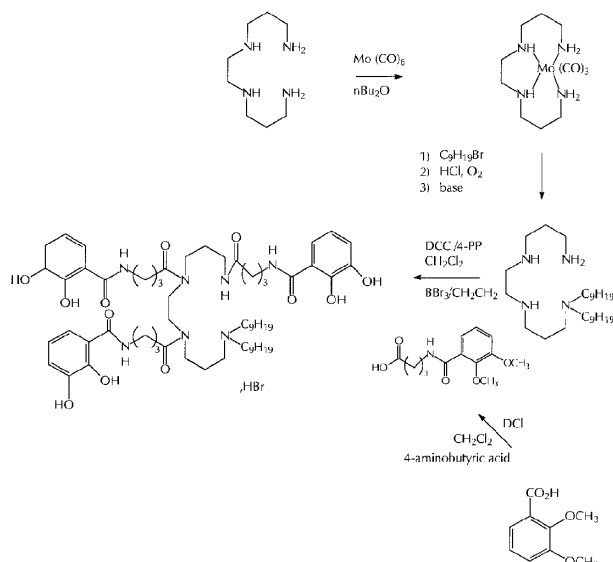
$^{13}\text{C}$ : 31.6 ( $3 \times \text{CH}_2\text{CO}$ ), 35.7 ( $3 \times \text{CH}_2\text{N}$ ), 38.6 ( $3 \times \text{CH}_2$  benzylic), 55.3 ( $3 \times \text{CH}_2\text{NH}$ ), 116.5 ( $3 \times \text{CH}-2$  and  $3 \times \text{CH}-5$ ), 120.7 ( $3 \times \text{CH}-6$ ), 133.5 ( $3 \times \text{C}-1$ ), 144.3 ( $3 \times \text{C}-3$ ), 145.9 ( $3 \times \text{C}-4$ ), 177.2 ( $3 \times \text{CO}$ ).

Mass spectrometry: CI/ $\text{NH}_3$  :  $m/z$  = 638.

1-Di-*n*-nonyl-5,8,12-tris[4-(2,3-dihydroxybenzoyl)butyryl]-1,5,8,12-tetraazadodecane hydrobromide **11**.

This was prepared by a complexation procedure of 1,5,8,12-tetraazadodecane with  $\text{Mo}(\text{CO})_6$ , selective *N*-di-alkylation with 1-bromononane, tri-acylation with 4-pyrrolidinopyridine, and demethylation by boron tribromide (Figure 5).  $\text{C}_{59}\text{H}_{91}\text{O}_{12}\text{N}_7$ , MW = 1090.

NMR ( $\text{CD}_3\text{OD}$ ):  $^1\text{H}$ : -0.3 (t, 6H),  $J$  = 9 Hz :  $2 \times \text{CH}_3$ ; -0.8 (m, 30H) :  $2 \times (\text{CH}_2)_7$  and  $\text{CH}_2$ ; -1.2 (m, 4H) :  $2 \times \text{CH}_2$ ; -1.4 (m, 10H) :  $2 \times \text{N-CH}_2\text{-CH}_2\text{-CH}_2\text{N}$  and  $3 \times (\text{C}=\text{O})\text{-CH}_2\text{-CH}_2$ ; -1.9 (m, 6H) :  $3 \times -\text{CH}_2\text{-C}=\text{O}$ ; -2.6 (m, 6H) :  $3 \times -\text{CH}_2\text{-NH-CO}$  (aromatic); -2.95 (m, 16H) :  $8 \times -\text{CH}_2\text{NHCO}$  (aliphatic); -6.2 (t, 3H),  $J_{\text{ortho}}$  = 6 Hz :  $3 \times \text{CH}-5$ ; -6.4 (d, 3H),  $J_{\text{ortho}}$  = 6 Hz :  $3 \times \text{CH}-6$ ; -6.8 (m, 3H) :  $3 \times \text{CH}-4$ .



**Figure 5.** Synthesis of 1-di-n-nonyl-5,8,12-tris[4-(2,3-dihydroxybenzoyl)butyryl]-1,5,8,12-tetraazadodecane hydrobromide **11**.

$^{13}\text{C}$ : 14.4 ( $-\text{CH}_3$ ), 23.5, 24.8, 25.5, 25.9, 26.1, 26.6, 27.5, 30.1, 30.3, 32.8 and 32.1 ( $-\text{CH}_2$ ), 39.9 ( $\text{CH}_2\text{N}$ ), 54.0 ( $\text{CH}_2\text{NHCO}$ ), 54.3 ( $\text{CH}_2\text{-NH-CO}$ ), 116.4 ( $-\text{C}-1$ ), 119.0 ( $\text{CH}-5$ ), 119.7 ( $\text{CH}-4$  and  $\text{CH}-6$ ), 147.1 ( $\text{C}-2$ ), 150.1 ( $\text{C}-3$ ), 171.6 ( $\text{CONH}$  aliphatic), 175.3 and 176.8 ( $\text{CONH}$  aromatic).

Mass spectrometry:  $\text{Cl}/\text{NH}_3$  :  $m/z$  = 1090 ( $\text{M}^+$ ), 1091 ( $\text{MH}^+$ ).

### Cross-feeding tests

Cross-feeding tests with Gram-negative bacteria were performed according to Reissbrodt *et al.* (1993). Filter paper discs were impregnated with 5  $\mu\text{g}$  of the catechol compounds and placed onto the surface of the bioassay plates. The same procedure was used in the case of cross-feeding tests involving both the mycobacterial strains. Here, the bioassay medium was prepared according to Hall & Ratledge (1982) by precipitation of a glycerol-asparagine medium with 2% aluminium oxide, supplemented again with trace elements ( $\text{Zn}^{2+}$ ,  $\text{Mn}^{2+}$ ,  $\text{Mo}^{6+}$ ,  $\text{Cu}^{2+}$ ,  $\text{Co}^{2+}$ ,  $\text{Mn}^{2+}$ ) and also with  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ . This medium was used with 10  $\mu\text{M}$  (final concentration) EDDHA [ethylenediamine-di-(*o*-phenylacetic acid)]. The cross-feeding plates were checked with natural siderophores, a positive one and a negative one each for control. All trials were performed in triplicate. The cross-feeding plates of Gram-negative bacteria were incubated for 24 h at 37°C and read. For the *Mycobacterium* spp, the plates were incubated at 37°C for 24 h and observed for up to six days during storage at room temperature. Mycobactin J (purchased from Rhône Mérieux, Lyon, France; 2  $\mu\text{g}$  per disc) was used for positive control. It

exhibited a growth zone of 20–25 mm in diameter around the filter paper disc.

## Results

The production of the dihydroxybenzoyl cystamides **2** and **3**, and of the dihydroxybenzoyl spermidines **4**, **5**, **6**, **7**, **8**, **9**, as well as **10** and **11**, is described in Materials and methods and shown in Figures 2 to 5.

A number of Gram-negative bacteria, being well defined in their ability to transport and utilize natural siderophores (so called siderophore indicator strains) showed different reactions in cross-feeding tests involving these compounds. None of the chemically synthesized catecholates could be utilized by the tonB mutants *S. typhimurium* SR 1001 and *E. coli* IR 112. Furthermore, *E. coli* LG 1522, the aerobactin indicator strain, could not be stimulated (Table 1). This strain served as a control. *E. coli* H 1876, lacking the IROMPs *fiu*, *cir* and *fepA*, was likewise not stimulated by any of the compounds tested in this study. The *Salmonella*, *E. coli* and *P. aeruginosa* siderophore indicator strains were not stimulated either by **4** or **5**, the dihydroxybenzoyl spermidine compounds without any further substituent at the central  $N^4$ -atom. However, the growth of *Y. enterocolitica* H 5030 was promoted without any differences by both these compounds. When comparing the three spermidine derivatives possessing an n-nonylchain (**6**, **7**) or an ethylamine-3,4-dihydroxyphenylpropionic acid group (**10**) as substituents at the central- $N^4$  (Figures 3 and 4), it was found that **10** promoted *Salmonella stanleyville* 207/81 and *P. aeruginosa* PAO 6609 better than did the other two compounds. Compounds **7** and **10** behaved similarly in promoting *E. coli* AB 2847, *K. pneumoniae* KN 4401 and *S. typhimurium* enb-7. No growth promotion was detected for *S. typhimurium* TA 2700, the enterobactin indicator strain, or for *Y. enterocolitica* H 5030. The  $N^4$ -n-nonyl- $N^1$ , $N^8$ -bis(3,4-dihydroxybenzoyl) spermidine **6** stimulated only *S. typhimurium* enb-7 and (weakly) *P. aeruginosa* PAO 6609.

The  $N^4$ , $N^4$ -dithiodipropionyl-bis( $N^1$ , $N^8$ -2,3-dihydroxybenzoyl) spermidine **9** (Figure 3) stimulated in particular *S. stanleyville* 207/81 and *P. aeruginosa* PAO 6609, and, to a lesser extent, *S. typhimurium* enb-7 and *E. coli* AB 2847. The 3,4-dihydroxybenzoyl derivative **8** showed weaker to absent growth promotion of these indicator strains. This compound weakly promoted *S. typhimurium* TA 2700. Both these compounds caused a lysis zone on *K. pneu-*



*moniae* KN 4401, which means an inhibition of this strain. Compound **11** effectively promoted *S. typhimurium* enb-7, *S. stanleyville* 207/81 and, in particular, *K. pneumoniae* KN 4401, but was unable to feed either *E. coli* AB 2847 or *P. aeruginosa* PAO 6609 and exhibited only a weak promotion of the growth of *Y. enterocolitica* H 5030.

When comparing the growth promoting effects of the dihydroxybenzoyl cystamides, the 2,3-dihydroxybenzoyl compound **3** was found to be superior to the 3,4-dihydroxybenzoyl compound **2** (Figure 2, Table 1). 2,3-Dihydroxybenzoyl cystamide **3** appeared to be the most powerful of the chemically synthesized catecholates tested in this study. This compound was taken up by the common *Salmonella* siderophore indicators, and by *E. coli* AB 2847, *K. pneumoniae* KN 4401, *P. aeruginosa* PAO 6609 and *S. typhimurium* TA 2700. The 3,4-dihydroxybenzoyl cystamide **2** inhibited *K. pneumoniae* KN 4401, as demonstrated by a lysis zone around the filter paper disc.

In contrast to the cross-feeding tests on Gram-negative bacteria, the 3,4-dihydroxybenzoyl compounds **2**, **6** and **8** reacted equally or better in growth promotion of both the mycobacterial strains tested (Table 2) as compared with their corresponding 2,3-dihydroxybenzoyl derivatives. However, the spermidine catecholates without a substituent at the central- $N^4$  did not promote growth of the mycobacterial strains, while compound **11** was weakly or not at all stimulatory; **10** reacted in an inhibitory mode only like an antibiotic.

## Discussion

Naturally or chemically synthesized siderophores in the form of the siderophore moiety of antibiotic conjugates act by smuggling antibiotics into the cell via siderophore transport systems. For their application, beside other parameters, detailed information about structure–function relationships is needed. Growth promotion of Gram-negative as well as of Gram-positive bacteria by such compounds, which has been confirmed in cross-feeding tests, will yield precious information about those qualities (Reissbrodt & Rabsch 1994).

All the chemically synthesized catecholates (Figures 2 to 5) tested in this study and utilized by any of the siderophore indicator strains passed through the outer membrane of *E. coli* and *S. typhimurium* by means of an energy-coupled, tonB-dependent active transport process as seen in the non-stimulated tonB mutants, *S. typhimurium* SR

1001 and *E. coli* IR 112 (Table 1). These tonB mutants can grow with 2,3-dihydroxybenzoic acid or its *o*-acetyl derivative (Reissbrodt *et al.* 1993), but not with any of the well known natural siderophores of the hydroxamate or catecholate type. Lack of growth of any of the compounds on *E. coli* H 1876 (fepA, cir, fiu) pointed to a transport route which could be utilized, for example, by monocatecholates such as 2,3-dihydroxybenzoylserine (Hantke 1990) or 2,3-dihydroxybenzhydrazone (Reissbrodt *et al.* 1993). The IROMPs Cir and Fiu are also involved in the transport of catechol substituted cephalosporins (Curtis *et al.* 1988, Nikaido & Rosenberg 1990) and of a 1,5-dihydroxy-4-pyridone substituted cephalosporin (Tatsumi *et al.* 1995). The dihydroxybenzoyl spermidines **4** and **5**, lacking a substituent at the central- $N^4$  atom, fed only the *Y. enterocolitica* H 5030-siderophore indicator strain. However, a spermidine compound similar to **5** (without a substituent at  $N^4$ ) was isolated from *Vibrio vulnificus* (Okujo *et al.* 1994) and behaved positively in cross-feeding tests. This compound contained an additional oxazoline group at position  $N^1$ , linked to a simple phenol group. This result pointed again to the significance of oxazoline groups occurring in natural polyamine-based siderophores, in iron transport of microorganisms. Obviously, *Yersinia enterocolitica* H 5030 possesses an uptake and utilization system for these compounds. Both compounds function independently of the position of their hydroxyl groups. Further experiments should be undertaken to determine the selectivity of these compounds as possible growth factors in nutrient media of pathogenic *Y. enterocolitica* strains.

The following effects on Gram-negative siderophore indicator strains were seen in the series of the  $N^4$ -substituted bis(dihydroxybenzoyl) spermidines **6**, **7** and **10**. The compound with tri-bidentate oxygen-containing ligands (**10**) seems to be more effective than molecules with two bis-bidentate ligands (**6**, **7**). The effectiveness of the last two compounds as compared with that of **4** and **5** could be explained by the introduction of the lipophilic structures based on the nonyl chain at  $N^4$ . Compound **7** seems to be comparable in its microbiological activity to spermaxin and spermaxatol. This has also been examined in cross-feeding tests on *V. cholerae* and *E. coli* mutants (Sharma *et al.* 1989). The 2,3-dihydroxybenzoyl derivative **7** was more effective than the 3,4-dihydroxybenzoyl derivative. Monocatecholates of dihydroxybenzoic acid, and of glyoxylic acid benzhydrazones, behaved similarly regarding the position of the two hydroxyl groups (Reissbrodt *et al.* 1993). However, 3,4-dihydroxybenzoic acid

and its derivatives preferred to promote growth of *P. aeruginosa* under iron-depleted conditions. In this study, a similar effect of *P. aeruginosa* PAO 6609 using the bis-dihydroxybenzoyl bidentates to show preference for the 3,4-dihydroxybenzoyl derivatives was not detected. Probably, the conformation of bis-bidentates containing 2,3-dihydroxybenzoyl groups fits better than that of bis-bidentates with 3,4-dihydroxybenzoyl groups. This is also indicated by the fact that  $^{55}\text{Fe(III)}$ -2,3-dihydroxybenzoic acid and  $^{55}\text{Fe(III)}$ -*N*-2,3-dihydroxybenzoyl-L-serine were taken up by *P. aeruginosa* (Screen *et al.* 1995).

The  $N^4$ -n-nonyl-substituted lipophilic bis(dihydroxybenzoyl) spermidine derivatives have shown their potential as antimalarial drugs (Ramiandrasoa *et al.* 1994). Compound **6** showed a 60-fold higher *in vitro* activity than Desferal<sup>®</sup>. Compound **6** was more active against *Plasmodium falciparum* than was the 2,3-dihydroxybenzoyl derivative **7**. Probably the utilization of siderophores by bacteria and their effectiveness in controlling other organisms are associated with different targets and metabolic pathways.

The  $N^4N^4$ -dithiopropionyl-bis( $N^1,N^8$ -dihydroxybenzoyl) spermidines **8** and **9** containing four bidentate groups and a disulfide bridge (Figure 3) behaved similarly to **6** and **7** in respect of the position of the hydroxyl groups (2,3 position > 3,4 position) in growth promotion (Table 1). Since the dithiopropionyl bridge is quite hydrophilic, iron uptake mechanisms other than those thought to be involved in the case of  $N^4N^4$ -nonyl-bis(dihydroxybenzoyl) spermidines should be considered. Additionally, *K. pneumoniae* KN 4401 was lysed by these compounds, perhaps by an intracellular influence on iron metabolism. By contrast to this, compound **11**, a tri-bidentate polyamine catecholate linked with ethylenediamine strongly promoted this strain. Probably, the lipophilic bis-nonylamide facilitated the transport of this compound, which was also well used by *S. typhimurium* enb-7, *S. stanleyville* 207/81 and *K. pneumoniae* KN 4401.

Hitherto, no naturally occurring siderophore was detected that contained a cystamide bridge between two dihydroxycatecholates. Thiazole as a sulfur-containing part of, for example, pyochelin has been described (Matzanke 1991). On the other hand, the complex binding constant of 3,4-dihydroxybenzoyl cystamide (**2**, Figure 2) was shown to be  $10^{43.4}$ , i.e. within a range known for enterobactin. The 2,3-dihydroxybenzoyl cystamide (**3**) functions as a very effective siderophore in non-selective promotion of the common salmonella strains, and of *E. coli*, *K. pneu-*

*moniae* and *P. aeruginosa* (Table 1). Its utilization by *S. typhimurium* TA 2700 indicates a transport route similar to those of enterobactin, the linear dimer and trimer of 2,3-dihydroxybenzoyl-L-serine, and of myxochelins. 3,4-Dihydroxybenzoyl cystamide reacted to a significantly lesser degree and inhibited *K. pneumoniae* KN 4401, perhaps by influencing the intracellular iron metabolism based on the high complex binding constant and resulting absence of utilization.

Iron uptake by mycobacteria is a more complex process than hitherto believed and a number of separate routes may co-exist, as they do in Gram-negative bacteria such as *E. coli* and *S. typhimurium*. Ratledge & Marshall (1972) proposed a mechanism for the uptake of iron by *M. smegmatis*, involving the mediation of mycobactin, a lipid soluble iron-binding agent, in shuttling iron across the boundary levels of the cell. Mycoexochelin occurs extracellularly to act as a scavenger. Thus, mycobactin is probably not directly involved in the transport of iron from ferri-mycoexochelin at physiological concentrations (Stephenson & Ratledge 1979). Specific iron-regulated envelope proteins are also produced by several mycobacteria as potential receptors in iron uptake (Hall *et al.* 1987). As yet, none of the well known natural siderophores could be assigned to any of those proteins.

The detected positive cross-feeding tests presently inform only about a growth promotion of the two mycobacterial strains studied. The spermidine derivatives **6** and **7**, as well as the cystamide derivatives **2** and **3**, were the most powerful compounds tested. They produced growth zones in the same range as seen for mycobactin. Inhibition of *M. fortuitum* by tris-(3,4-dihydroxyphenyl) spermidine **10** may be based on an influence on the intracellular iron metabolism as a possible new strategy of antimycobacterial drugs. Further experiments are necessary to confirm this hypothesis.

The structure-function relationships shown in this study may contribute to the synthesis of new siderophores designed as more or less selective promoters or inhibitors of bacterial growth, thus opening new strategies in diagnosis and therapy.

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