BioMetals **17:** 53–64, 2004.

Catecholates and mixed catecholate hydroxamates as artificial siderophores for mycobacteria

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Received 5 March 2003; accepted 19 March 2003; Published online: June 2003

Key words: catecholates, Gram-negative bacteria, hydroxamates, mycobacteria, siderophore analogue, synthesis

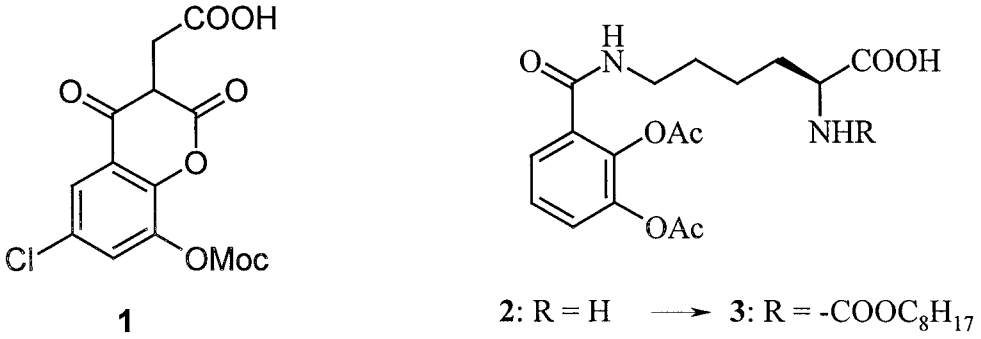
Abstract

Different mono-, bis- or triscatecholates and mixed mono- or biscatecholate hydroxamates were synthesized as potential siderophores for mycobacteria. Siderophore activity was tested by growth promotion assays using wild type strains and iron transport mutants of mycobacteria as well as Gram-negative bacteria. Some triscatecholates and biscatecholate hydroxamates were active in mutants of *Mycobacterium smegmatis* deficient in mycobactin and exochelin biosynthesis or exochelin permease, respectively, indicating an uptake route independent of the exochelin/mycobactin pathway. Structure activity relationships were studied. Ampicillin conjugates of some of these compounds were inactive against mycobacteria but active against Gram-negative bacteria.

Introduction

The iron supply by siderophores in mycobacteria follows a mechanism different to that in Gram-negative bacteria. Siderophores of Gram-negatives are excreted by bacterial cells to capture ferric ions and to transport these ions into the cell (Drechsel & Jung 1998). Mycobacteria demonstrate a biphasic mechanism for iron transport. They produce extracellular siderophores, the exochelins, to sequester the ferric ions and transfer them to the cell wall associated mycobactins which deliver the iron into the cell (Ratledge 1999). Therefore the conception of siderophore usage as shuttle vectors for antibiotics to overcome the bacterial membrane barrier as known for Gram-negative bacteria (Muñoz-Bellido *et al.* 1996) can not function in mycobacteria by the exochelin/mycobactin route. Hence siderophores are required, which transport iron directly into the bacterial cell. Mutants of *M. smegmatis* deficient in the exochelin/mycobactin iron transport system (Schumann *et al.* 1998; Schumann & Möllmann 2001) can be used to identify this type of independently acting siderophore compounds. In growth promotion assays with these mutants some active siderophore compounds were found, e.g., natural siderophores like aerobactin and other hydroxamates (Schumann & Möllmann 2001) and synthetic siderophores like biscatecholates based on diamino acids (Schnabelrauch *et al.* 2000), triscatecholates based on triamines (Heinisch *et al.* 2002a) and tripeptides of N^5-hydroxy-N^5-acetyl-L-ornithine, especially their iron complexes (Lin *et al.* 2001).

In this paper we report on the synthesis of mono-, bis- or triscatecholates and mixed mono- and biscatecholate hydroxamates and their siderophore activities in mycobacteria and Gram-negative bacteria. All compounds contain carboxylic substituents to construct antibiotic conjugates of active siderophores with ampicillin and other β-lactams. We synthesized compounds with acylated catecholate groups (both acetylated derivatives and 2,4-dioxo-1,3-benzoxazines as masked catecholates) and with free or acylated hydroxamate groups, respectively. The acylated derivatives were



Scheme 1. Chemical structures and synthesis of monocatecholates, Ac = $COCH_3$, Moc = $COOCH_3$.

synthesized with the aim to reduce pharmacological side effects. In Gram-negative bacteria acylated catecholates can act as siderophores like free catecholates obviously after cleavage by bacterial enzymes to the free catecholates (Heinisch *et al.* 2002a). Some of these tested compounds were used to synthesize antibiotic conjugates against Gram-negative bacteria (Heinisch *et al.* 2002b, c; Wittmann *et al.* 2002). The siderophore activity of the new analogues was studied by growth promotion assays with wild type strains and iron transport mutants as well as by the chromazurol S (CAS) assay (Schwyn & Neilands 1987).

Materials and methods

Structures and synthesis of the siderophore analogues

^{1}H-NMR spectra were recorded on a Bruker Advance DRX 300 MHz spectrometer. Chemical shifts δ are given in ppm. Mass spectra were recorded on a Finnigan MAT 95 XL sector field mass spectrometer equipped with fast-atom bombardment (FAB) and electrospray ionization (ESI), respectively. Column chromatography was accomplished using silicagel (Merck 60, 0.040–0.063 mm). Purification of the compounds by preparative HPLC was performed on an Abimed Gilson instrument equipped with an 115 UV detector (254 nm) and a Knauer Vertex reversed phase column (250 × 32 mm or 50 × 20 mm) packed with Eurospher 100-C18 (7 μm). The compound was eluated by a gradient of acetonitrile and water, beginning with ratio 30:70 (v/v) and achieving 80:20 (v/v) after a period of 20 min (flux rate 20 ml min or 10 ml min). Thin layer chromatography was carried out on silica gel plates (Merck 60 F254) applying UV detection. Solvents and reagents used were dried and purified by standard methods.

The following compounds were synthesized according to published procedures: 2,3-diacetoxybenzoyl chloride (Bergeron *et al.* 1980), 3,4-diacetoxybenzoyl chloride and 2,3-di(methoxycarbonyloxy)benzoyl chloride (Heinisch *et al.* 1992), 5-bromo-3,4-diacetoxybenzoyl chloride (Bird *et al.* 1992), N^2,N^6-bis-(2,3-dihydroxybenzoyl)-L-lysine **2** (Schnabelrauch *et al.* 2000), 1-benzyl N-[N^2,N^5-bis(2,3-diacetoxy-benzoyl)-*D*-ornithyl]-glutamate **15** (Wittmann *et al.* 2002), O-benzyl-N-methyl-hydroxylamine hydrochloride **16b**, O-benzyloxy-N-n-decyl-hydroxylamine hydrochloride **16c**, O-benzyl-N-(ethoxycarbonyl-propyl)-hydroxylamine **16d** (Sulsky *et al.* 1989) and O-benzoyl-N-methyl-hydroxylamine hydrochloride **16e** (Geffken 1986). O-Benzyl-hydroxylamine hydrochloride **16a** was purchased as a commercial product (Aldrich).

N^2-n-Octyloxycarbonyl-N^6-(2,3-diacetoxybenzoyl)-L-*lysine,* **3** $C_{26}H_{38}N_2O_9$
(522.60)

To a solution of N^6-(2,3-diacetoxybenzoyl)-L-lysine **2** (366 mg, 1 mmol) (prepared from N^2-Z-L-lysine and 2,3-diacetoxybenzoyl chloride and following catalytic hydrogenolysis) and triethylamine (282 μl, 2 mmol) in tetrahydrofuran (4 ml) and water (1 ml) at −5 °C a solution of n-octyl chloroformate (196 μl, 1 mmol) in tetrahydrofuran (3 ml) was added drop-wise with stirring. The mixture was stirred for 1 h at 0 °C and for 1 h at ambient temperature and then concentrated in vacuo. The residue was acidified with 1 M HCl to pH 3 and then extracted with ethyl acetate. The extract was washed with brine, dried over Na_2SO_4 and evaporated. To the residue petroleum ether was added to give **3** as a colourless solid, yield 10%. ^{1}H NMR (DMSO-d6) δ: 0.85 (t, 3H, CH_3); 1.20–1.75 (m, 18H, CH_2); 2.20 (s, 3H, $COCH_3$); 2.27 (s, 3H, $COCH_3$); 3.14 (m, 2H,

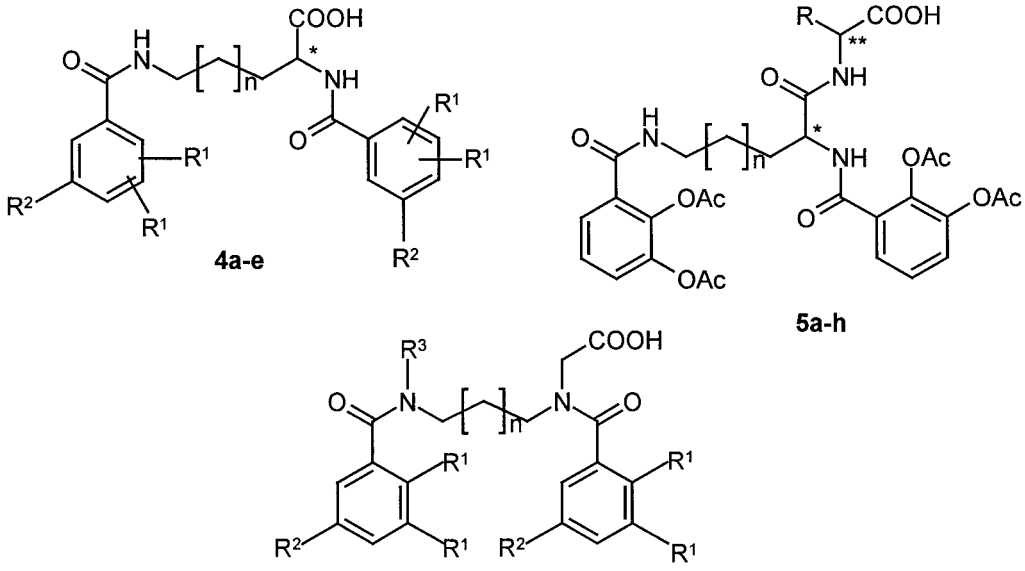


Figure 1. Chemical structures of biscatecholates, Ac = $COCH_3$, Moc = $COOCH_3$, Bn = benzyl, **4a**: R^1 = 3,4-OMoc, R^2 = H, n = 2, * = L, **4b**: R^1 = 3,4-OAc, R^2 = Br, n = 1, * = L, **4c**: R^1 = 2,3-OAc, R^2 = H, n = 1, * = L, **4d**: R^1 = 2,3-OAc, R^2 = H, n = 1, * = D, **4e** 95471: R^1 = 2,3-OAc, R^2 = H, n = 2, * = L, **5a**: n = 1,R = C_6H_5, * ,** = L, **5b**: n =1, R = CH_2OBn, * = L, ** = D, **5c**: n =1, R = CH_2OBn, *,** = L, **5d**: n = 1,R = tryptophanyl, *,** = L, **5e**: n = 1, R = tryptophanyl, * = L,** = D, **5f**: n = 1,R = $CH_2CH(CH_3)$, *,** = L, **5g**: n = 1, R = $(CH_2)_2$-COOBn, * = D,** = L, **5h**: n = 2, R = $(CH_2)_2$-COOBn, *,** = L, **6a**: R1 = OMoc, R^2 = 5-Cl, R^3 = CH_3, n = 1, **6b**: R^1 = OMoc, R^2 = 5-Br, R^3 = CH_3, n = 1, **6c**: R^1 = OAc, R^2, R^3 = H, n = 2.

CH_2); 3.91 (m, 3H, CH and CH_2); 7.25–7.45 (m, 6H, aromatic H); 8.30 (d, 1H, CONH). MS (FAB) 523.4 $[M+H]^+$.

*N^2,N^6-Bis-[3,4-di(methoxycarbonyloxy)-benzoyl]-*L*-lysine* **4a** $C_{28}H_{30}N_2O_{16}$ *(650.56)*

To a solution of L-lysine hydrochloride (0.91 g, 5 mmol) in 0.5 M aqueous sodium hydrogencarbonate (40 ml, 20 mmol) 3,4-di(methoxycarbonyloxy)-benzoyl chloride (3.36 g, 10 mmol) in tetrahydrofuran (10 ml) was added drop-wise in an ultrasonic bath at 0–5 °C. The mixture was stirred for 1 h at this temperature and then the tetrahydrofuran was evaporated. The aqueous solution was acidified to pH 2 with 2 M HCl at 0 °C and extracted with ethyl acetate. The organic layer was washed with brine, dried over Na_2SO_4, evaporated and dried in vacuo to give **4a** (50%) as a colourless solid. ^{1}H NMR (DMSO-d6) δ: 1.50–1.90 (m, 6H, 3× CH_2); 2.35 (s, 6H, $COCH_3$); 2.30 (s, 6H, $COCH_3$); 3.20 (m, 2H, CH_2); 4.40 (m, 1H, CH); 7.20–7.90 (m, 6H, aromatic H); 8.61 (t, 1H, CONH); 8.80 (d, 1H, CONH). MS (ESI-NI) 729.3 $[M-H]^-$.

*Bis-(5-bromo-3,4-diacetoxybenzoyl)-*L*-ornithine* **4b**, *$C_{27}H_{26}Br_2N_2O_{12}$ (730.32)*

Preparation according to **4a** from L-ornithine hydrochloride and 5-brom-3,4-diacetoxybenzoyl chloride, yield 50%, ^{1}H NMR (DMSO-d6) δ: 1.50–1.90 (m, 4H, 2 × CH_2); 2.31 (s, 6H, $COCH_3$); 2.37 (s, 6H, $COCH_3$); 3.20 (m, 2H, CH_2); 4.40 (m, 1H, CH); 7.78 (d, 2H, aromatic H); 8.12 (d, 2H, aromatic H); 8.63 (t, 1H, CONH); 8.82 (d, 1H, CONH). MS (ESI-NI) 729.3 $[M-H]^-$.

2L-N-(2,3-diacetoxybenzoyl)-glutamic acid 5-(N-n-decyl-N-hydroxy-amide) **12b**, *$C_{26}H_{38}N_2O_9$ (522.60)*

To a solution of 1-benzyl-N-(2,3-diacetoxybenzoyl)-L-glutamate (458 mg, 1 mmol) **11** (prepared from 1-benzyl L-glutamate and 2,3-diacetoxybenzoyl chloride) and N-methylmorpholine (112 μl) in tetrahydrofuran (10 ml) isobutyl chloroformate (130 μl, 1 mmol) was added at −20 °C with stirring. The mixture was stirred for 1 h at −10 °C and then a suspension of O-benzyl-N-n-decyl-hydroxylamine hydrochloride (263 mg, 1 mmol) **16c** and triethylamine (153 μl,

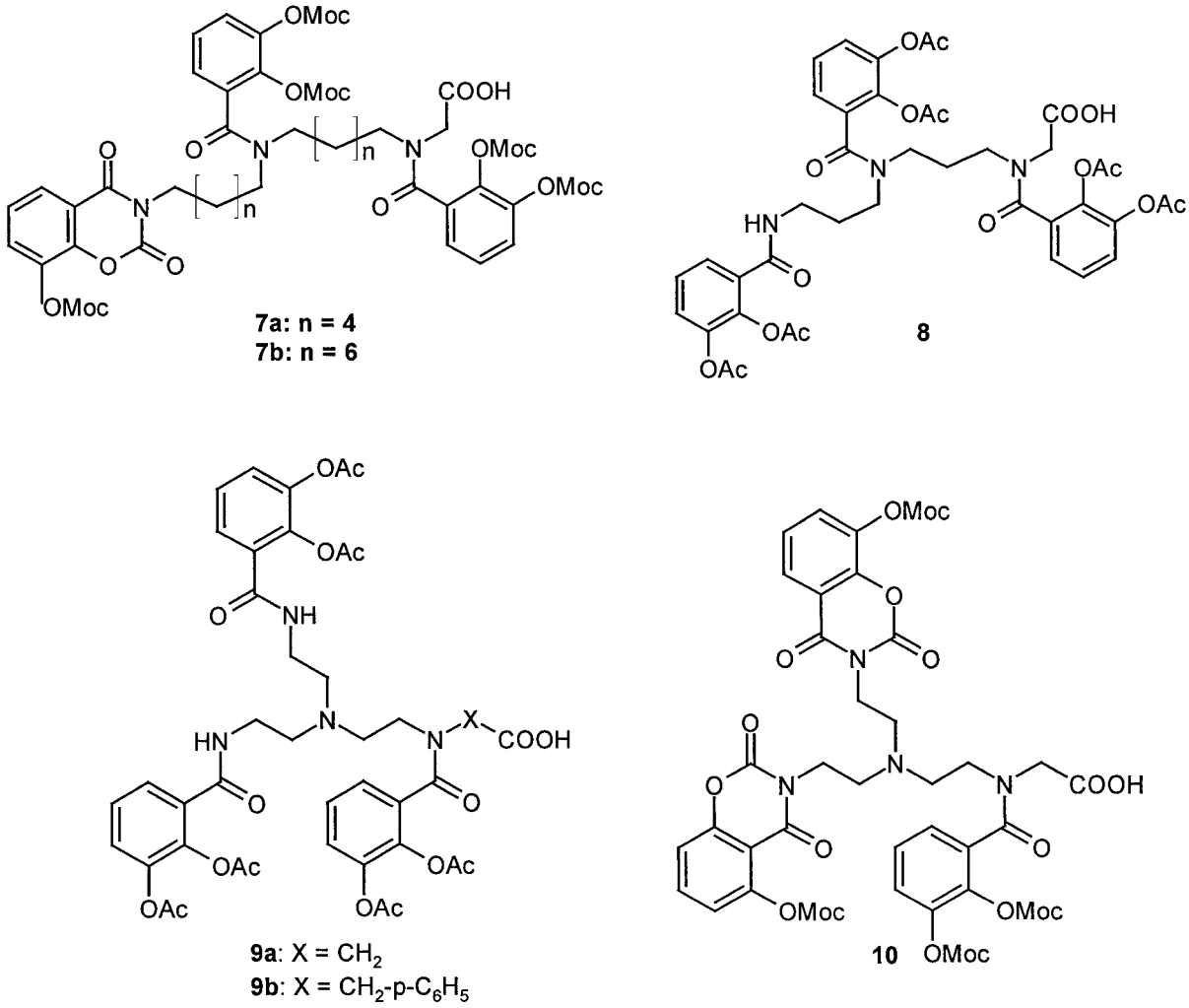
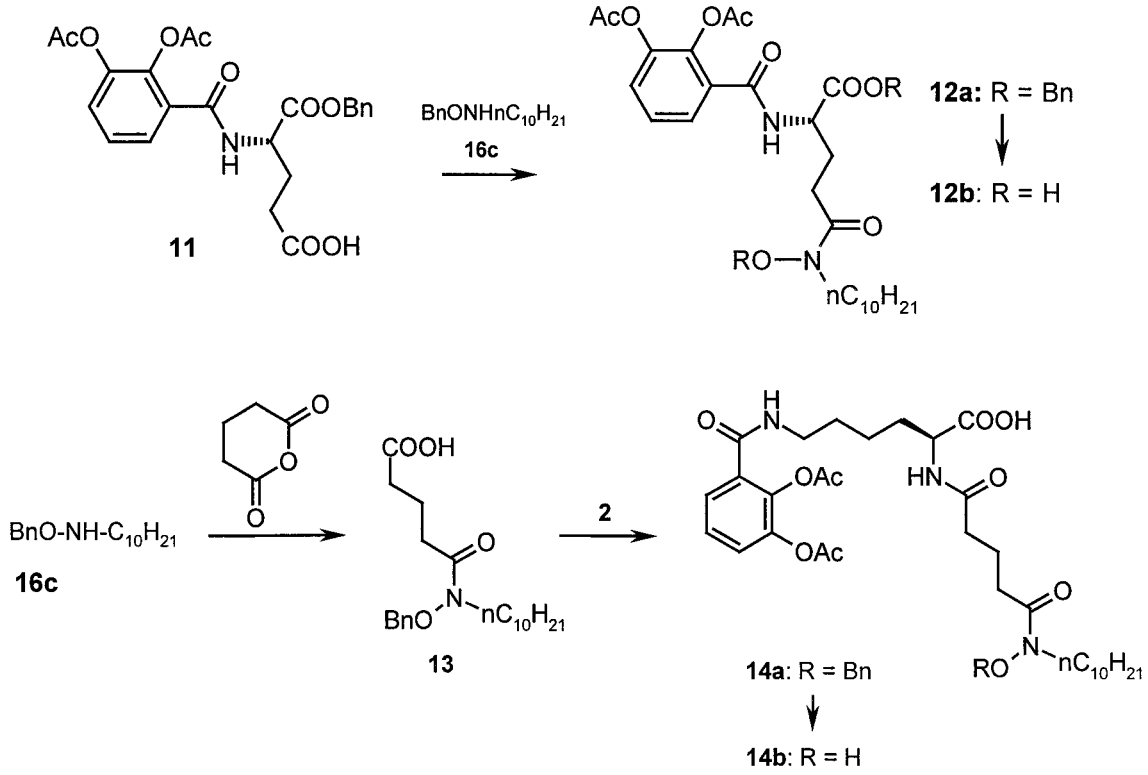


Figure 2. Chemical structures of triscatecholates **7–10**, Ac = $COCH_3$, Moc = $COOCH_3$.

1.1 mmol) in tetrahydrofuran (4 ml) and water (1 ml) was added. The mixture was stirred for 1 h at −10 ∘C and for 1 h at ambient temperature and then evaporated. To the residue ethyl acetate and water were added. The mixture was acidified carefully with 1 M HCl with shaking. The organic layer was separated, washed with brine, dried over Na_2SO_4 and evacuated to give the benzylester **12a** as a colourless solid, yield 50%, MS (FAB) 703.5 $[M+Na]^+$. The benzylester **12a** was hydrogenolysed in ethanol over Pd/C (10%) for 2 h at ambient temperature and atmospheric hydrogen pressure to give **12b** as colourless solid (234 mg, 90%). ^{1}H NMR (DMSO-d6) δ: 0.83 (t, 3H, CH_3); 1.22 (m, 14H, CH_2); 1.48 (m, 2H, CH_2); 1.89–2.00 (m, 2H, CH_2); 2.22 (s, 3H, $COCH_3$); 2.27 (s, 3H, $COCH_3$); 2.48 (m, 2H, CH_2); 3.45 (m, 2H, CH_2); 4.32 (m, 1H, CH); 7.37 (m, 2H, aromatic H); 7.47 (dd, 1H, aromatic H); 8.53 (d, 1H, NHCO). MS (FAB) 545.4 $[M+Na]^+$.

*N^2-[4-(N-Decyl-N-hydroxy-carbamoyl)-n-butanoyl]-N^6-(2,3-diacetoxybenzoyl)-*L*-lysine* **14b**
$C_{32}H_{49}N_3O_{10}$ (635.76)

Reacting glutaric O-benzyl-N-n-decyl-N-hydroxylamide **13** (378 mg, 1 mmol) (prepared from glutaric anhydride and O-benzyl-N-n-decyl-hydroxylamine hydrochloride **16c**) with N^6-(2,3-diacetoxybenzoyl)-L-lysine **2** (366 mg, 1 mmol) using the mixed anhydride method as described for compound **12b** gave the N-benzyloxy derivative **14a**, yield 30%, MS (ESI-NI) 724.6 $[M-H]^-$. Compound **14a** was hydrogenolysed in ethanol over Pd/C (10%) for 2 h at ambient temper-



Scheme 2. Synthesis of catecholate hydroxamate **12b** and **14b**, Ac = $COCH_3$ Bn = benzyl.

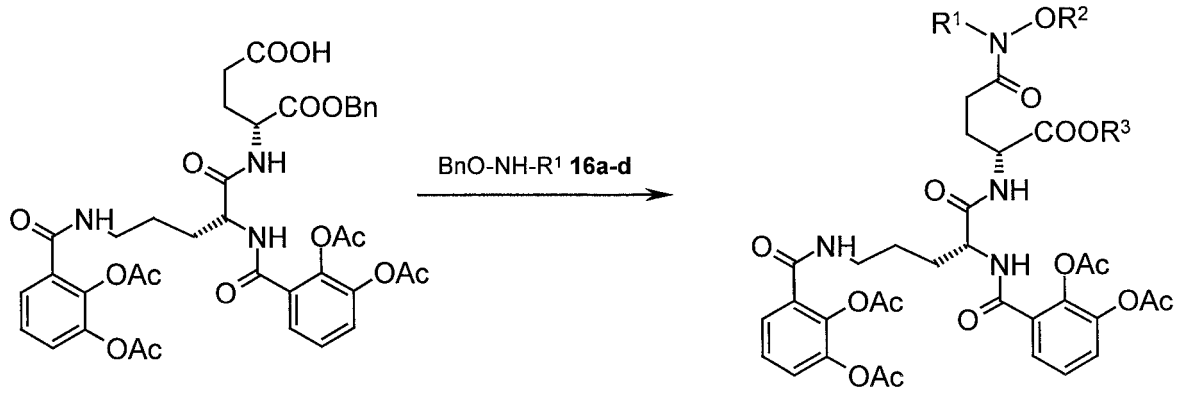
ature and atmospheric hydrogen pressure to give **14b** as colourless solid (180 mg, 80%). ^{1}H NMR (DMSO-d6) δ: 0.84 (t, 3H, CH_3); 1.20–1.70 (m, 24H, CH_2); 2.10 (m, 2H, CH_2); 2.21 (s, 6H, $COCH_3$); 3.34 (m, 2H, CH_2); 3.14 (m, 2H, CH_2); 3.44 (m, 2H, CH_2); 4.07 (m, 1H, CH); 7.35 (m, 2H, aromatic H); 7.41 (dd, 1H, aromatic H); 7.81 (d, 1H, NHCO); 8.31 (d, 1H, NHCO). MS (ESI-NI) 634.5 $[M-H]^-$.

N-[N^2,N5-Bis-(2,3-diacetoxybenzoyl)-D-*ornithyl]*-L-*glutamoyl 5-N-hydroxyamide* **18a** $C_{32}H_{36}N_4O_{15}$, *(716.66)*

Reacting 1-benzyl-N-[N^2,N^5-bis(2,3-diacetoxy-benzoyl)-*D*-ornithyl]-L-glutamate **15** (792 mg, 1 mmol) with O-benzyl-hydroxylamine hydrochloride **16a** (263 mg, 1 mmol) using the mixed anhydride method as described for compound **12b** gave the benzyl ester **17a** as light yellow solid, yield 40%. Compound **17a** was debenzylized by catalytic hydrogenolysis at ambient temperature and atmospheric pressure over 10% Pd/C to give **18a** as a colourless solid, yield 30%. ^{1}H NMR (DMSO-d6) δ: 1.45–2.16 (m, 8H, CH_2); 2.19 (s, 3H, $COCH_3$); 2.22 (s, 3H, $COCH_3$); 2.27 (s, 6H, $COCH_3$); 3.18 (m, 2H, CH_2); 4.15 (m, 1H, CH); 4.50 (m, 1H, CH); 7.30–7.50 (m, 6H, aromat.); 7.55 (dd, 1H, aromat.); 8.21 (d, 1H, NH); 8.26 (d, H, NHCO); 8.35 (d, 1H, NHCO). MS (ESI-NI) 715.1 $[M-H]^-$.

N-[N^2,N^5-Bis-(2,3-diacetoxybenzoyl)-D-*ornithyl]*-L-*glutamoyl-5-N-hydroxy-N-methyl-amide* **18b** $C_{33}H_{38}N_4O_{15}$ *(730.69)*

Reacting compound **15** and O-benzyl-N-methyl-hydroxylamine hydrochloride **16b** using mixed anhydride method as described for compound **12b** gave benzyl ester **17b** and following compound **18b** after hydrogenolysis as a colourless solid, yield 40%. ^{1}H NMR (DMSO-d6) δ: 1.45–2.06 (m, 6H, CH_2); 2.22 (s, 3H, $COCH_3$); 2.24 (s, 3H, $COCH_3$); 2.27 (s, 6H, $COCH_3$); 2.40 (m, 2H, CH_2); 3.06 (s, 3H, NCH_3); 3.18 (m, 2H, CH_2); 4.23 (m, 1H, CH); 4.52 (m, 1H, CH); 7.25–7.45 (m, 5H, aromat.); 7.56 (dd, 1H, aromat.); 8.24 (d + t, 2H, 2xNHCO); 8.33 (t, 1H, NHCO).



Scheme 3. Synthesis of biscatecholate hydroxamates **18a-d**, Ac = $COCH_3$. **16a, 17a, 18a**: R^1 = H, **16b, 17b, 18b**: R^1 = CH_3, **16c, 17c, 18c**: R^1 = n-$C_{10}H_{21}$, **16d, 17d, 18d**: R_1 = $(CH_2)_3COOC_2H_5$.

*N-[N^2, N^5-Bis-(2,3-diacetoxybenzoyl)-*D*-ornithyl]-*L*-glutamoyl 5-N-hydroxy-N-n-decyl-amide* **18***c* *$C_{42}H_{56}N_4O_{15}$ (856.93)*

Preparation according to **18a** from compound **15** and O-benzyl-N-n-decyl-hydroxylamine hydrochloride **16b** and hydrogenolysis of the obtained benzyl ester **17c** gave **18c** as a colourless solid, yield 40%. ^{1}H NMR (DMSO-d6) δ: 0,84 (t, 3H, CH_3); 1.15–2.06 (m, 20H, CH_2); 2.17 (d, 3H, $COCH_3$); 2.21 (d, 3H, $COCH_3$); 2.22 (s, 6H, $COCH_3$); 2.40 (m, 2H, CH_2); 3.19 (m, 2H, CH_2); 3.44 (m, 2H, CH_2); 4.22 (m, 1H, CH); 4.51 (m, 1H, CH); 7.25–7.45 (m, 5H, aromat.); 7.56 (dd, 1H, aromat.); 8.24 (d + t, 2H, 2xNHCO); 8.32 (d, 1H, NHCO). MS (FAB) 857.8 $[M+H]^+$.

*N-[N^2,N^5-Bis-(2,3-diacetoxybenzoyl)-*D*-ornithyl]-*L*-glutamoyl-5-N-hydroxy-N-ethoxy-carbonylpropyl-amide* **18d** *$C_{38}H_{46}N_4O_{17}$ (830.81)*

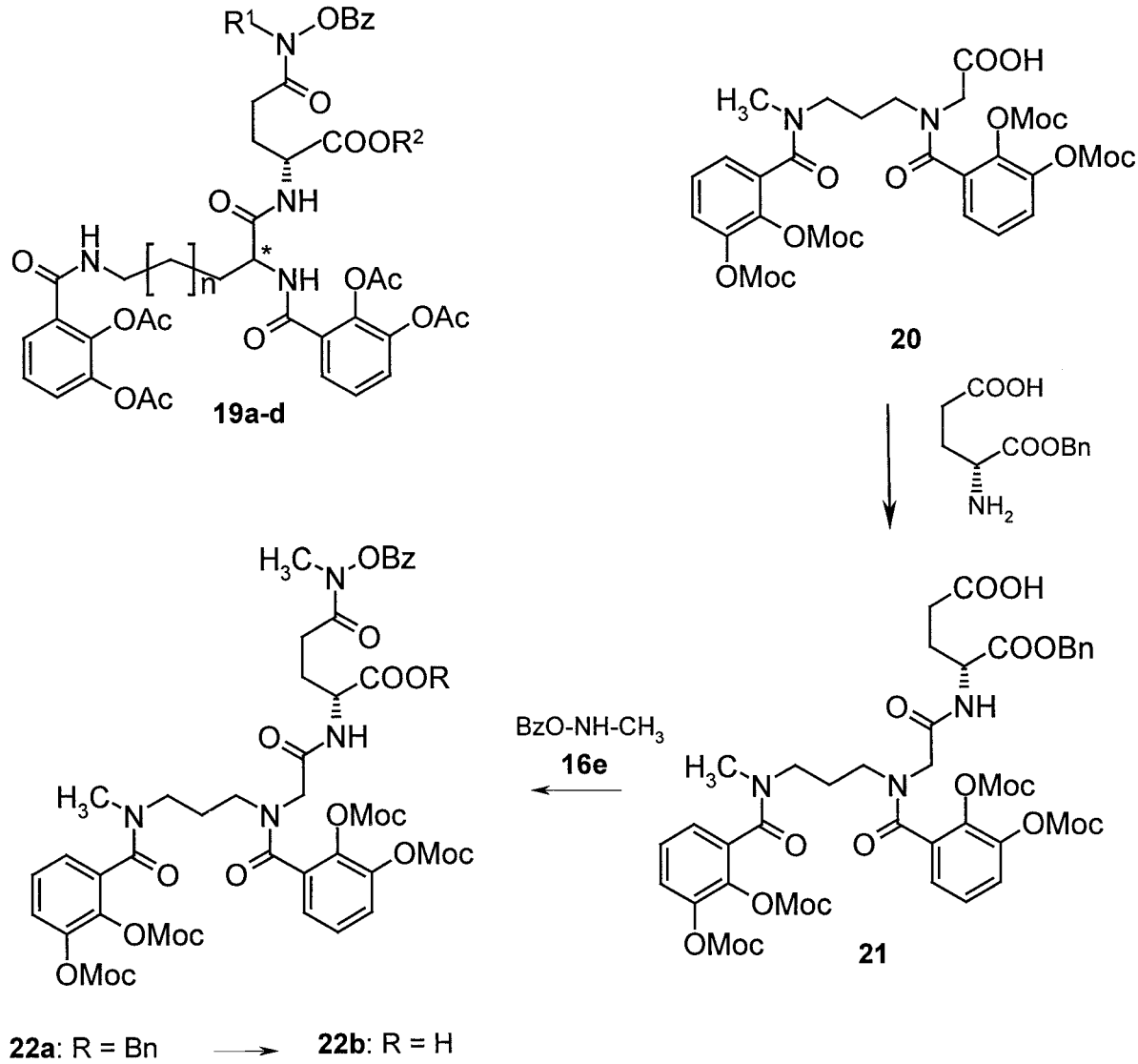
Preparation according to **18a** from compound **15** and O-benzyl-N-(ethoxycarbonyl-n-propyl)-hydroxylamine **16d** and hydrogenolysis of the obtained benzyl ester **17c** gave **18c** as a colourless solid, yield 30%. ^{1}H NMR (DMSO-d6) δ: 1.15 (t, 3H, CH_3); 1.45–2.06 (m, 10H, CH_2); 2.19 (s, 3H, $COCH_3$); 2.21 (s, 3H, $COCH_3$); 2.26 (s, 6H, $COCH_3$); 2.42 (m, 2H, CH_2); 3.18 (m, 2H, CH_2); 3.48 (m, 2H, CH_2); 4.03 (q, 2H, CH_2); 4.17 (m, 1H, CH); 4.50 (m, 1H, CH); 7.32–7.45 (m, 6H, aromat.); 7.53 (dd, 1H, aromat. H); 8.18 (d, 1H, NH); 8.25 (d, 1H, NHCO); 8.33 (t, 1H, NHCO). MS (ESI-NI) 828.9 $[M-H]^-$.

*N-[N^2, N^5-3,7-Bis-(2,3-dimethoxycarbonyloxybenzoyl)-3,7-diaza-octanoyl]-*L*-glutamic acid 5-(N-benzoyloxy-N-methyl)-amide* **22b**, *$C_{41}H_{44}N_4O_{20}$ (912.82)*

Reacting 1-benzyl N-[3,7-bis-(2,3-dimethoxycarbonyloxybenzoyl)-3,7-diaza-octanoyl]-L-glutamate **21** (870 mg, 1 mmol) (prepared analogously to compound **15** from 3,7-bis-[2,3-di(methoxycarbonyloxy)benzoyl]-3,7-di-aza octanoic acid **20** (Heinisch *et al.* 2002b)) and O-benzoyl-N-methyl-hydroxylamine hydrochloride (188 mg, 1 mmol) **16e** using the mixed anhydride method as described for compound **12b** gave the benzylester **22a** (456 mg, 50%). MS (ESI-NI) 1001.6 $[M-H]^-$. Compound **22a** was hydrogenolysed in ethanol over Pd/C (10%) for 2 h at ambient temperature and atmospheric hydrogen pressure to give **22b** as colourless solid (319 mg, 70%). ^{1}H NMR (DMSO-d6) δ: 1.75–3.40 (m, 10H, CH_2); 2.56 (s, 3H, NCH_3); 2.76 (s, 3H, NCH_3); 3.78 (m, 2H, CH_2COOH), 3.80 (m, 12H, OCH_3); 4.03 (m, 1H, CH); 7.24–8.00 (m, 11H, aromatic H); 8.31 (m, 1H, NHCO). MS (ESI-NI): 910.9 $[M-H]^-$.

Examination of siderophore activity

The compounds were tested for growth promotion activity in the wild type strain *Mycobacterium smegmatis* SG 987 and its mutant M10 (exochelin-), the wild type strain *M. smegmatis* mc^2155, its mutants B1 (exochelin-) and M24 (mycobactin-), and the M24 mutants B3 (mycobactin- and exochelin-) and U3 (mycobactin- and exochelin uptake-) (Schumann *et al.* 1998) by an agar diffusion assay under iron limited conditions (Schumann & Möllmann



Scheme 4. Chemical structures and synthesis of biscatecholate hydroxamates **19a-d, 20, 21** and **22a,b**, Ac = $COCH_3$, Moc = $COOCH_3$, Bz = benzoyl. **19a**: $R^1 = CH_3$, $R^2 = CH_2C_6H_5$, n = 1, **19b**: $R^1 = CH_3$, R^2 = H, n = 1, **19c**: R1 = CH_3, R^2 = cyclohexyl, n = 1, **19d**: $R^1 = CH_3$, R2 = cyclohexyl, n = 2.

2001). Siderophore activity in Gram-negative bacteria was tested by the same method using the wild type strains *Pseudomonas aeruginosa* ATCC 27853, SG 137, NTCC 10662 and ATCC 9027, *Escherichia coli* ATCC 25922 and the enterobactin-deficient mutant *Salmonella typhimurium* enb7 (JB Neilands, University of California, Berkeley, CA, USA). Iron complexing capacity was checked by the chromazurol-S (CAS) assay in an agar diffusion test according to Schwyn and Neilands (1987).

Results and discussion

Continuing our program in search of artificial siderophores for mycobacteria we synthesized a series of new catecholates and hydroxamates. We tested their activity in growth promotion assays including mutants deficient in mycobactin and exochelin biosynthesis and in exochelin permease. In this paper we report only on compounds which are active in the *M. smegmatis* mutants B1 (exochelin-), B3 (mycobactin- and exochelin-) and U3 (mycobactin- and exochelin permease-).

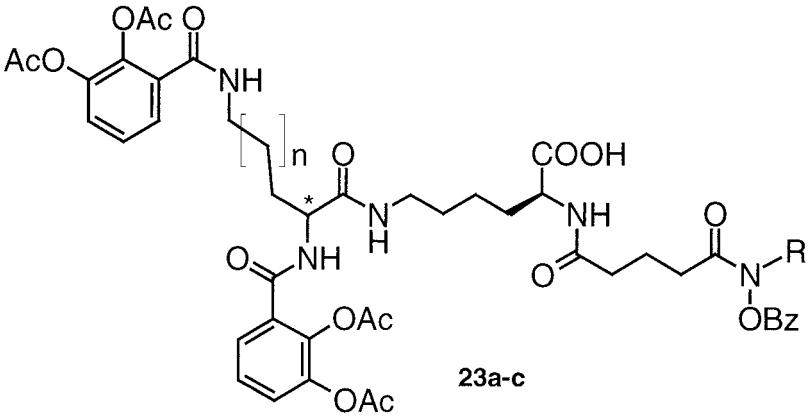


Figure 3. Chemical structures of biscatecholate hydroxamates **23a-c**, Ac = OCH_3, Bz = benzoyl, **23a**: R = CH_3, n = 2, * = L, **23b**: R = cyclohexyl, n = 2, * = L, **23c**: R = cyclohexyl, n = 1, * = D.

We studied monocatecholates based on amino acids, biscatecholates based on diamino acids, dipeptides and on (aminoalkyl)-amino acids, triscatecholates based on tri-aza alkanoic acids, monocatecholatehydroxamates and biscatecholate hydroxamates based on diamino acids and dipeptides. In these compounds the catecholate groups and in part also the hydroxamate groups are acylated (acetylated derivatives or 2,4-dioxo-1,3-benzoxazine derivatives as masked catecholates). Such compounds were active in Gram-negative bacteria. As published prior (Heinisch *et al.* 2002a) the acylated groups obviously must be changed by bacterial enzymes to free catecholates or hydroxamates thus allowing iron chelation. Catecholates and hydroxamates represent important iron chelating groups in bacterial siderophores. These groups use different siderophore receptors. Therefore mixed catecholate hydroxamates can use different uptake routes which improves the penetration into the cell.

Monocatecholates

We used two compounds, the 2,4-dioxo-1,3-benzoxazine derivative of glycine **1** (prepared according to Wittmann *et al.* 2000) and the N^2-n-octyloxycarbonyl-N^6-(2,3-diacetoxybenzoyl)-L-lysine **3**, prepared from N^2-Z-L-lysine and 2,3-diacetoxybenzoyl chloride by subsequent hydrogenolysis to N^6-(2,3-diacetoxybenzoyl)-L-lysine **2** and followed by reaction with n-octyl chloroformate (Scheme 1).

Biscatecholates

We synthesized two compounds with two catecholate moieties, the compounds with 3,4-acetoxybenzoyl groups **4a** and **b**. The biscatecholates 4c-e have been published (Wittmann *et al.* 2002). Additionally we prepared dipeptide derivatives with acylated biscatecholate moieties by the mixed anhydride method, starting from bis-2,3-diacetoxybenzoyl-L-ornithine **4c** by reaction with L-phenylalanine resulting in compound **5a**, with O-benzyl-D-serine resulting in compound **5b**, with O-benzyl-L-serine in **5c**, with L-tryptophan in **5d**, with D-tryptophan in **5e** and with L-leucine to **5f**. Furthermore we synthesized compound **5g** by reaction of bis-(2,3-diacetoxybenzoyl)-D-ornithine **4c** with 5-benzyl L-glutamate and compound **5h** from bis-2,3-diacetoxybenzoyl-L-lysine **4e** and 5-benzyl L-glutamate (Wittmann *et al.* 2001, 2002). Moreover we used three acylated biscatecholates based on di-aza alkanoic acids **6a-c** prepared according to a published procedure (Heinisch *et al.* 2002b) (Figure 1).

Triscatecholates

We used four types of acetylated triscatecholates based on linear tri-aza alkanoic acids (compound **8**), on tripodal tetra-aza alkanoic acid and on tetra-aza alkylbenzoic acid with 2,3-diacetoxy benzoyl substituents (compounds **9a,b**) as well as compounds **7a,b** and **10** with 2,3-dimethoxycarbonyloxybenzoyl and/or 8-methoxycarbonyloxy-2,4-dioxo-1,3-benzoxazine groups as masked catecholates (Heinisch *et al.* 2002c) (Figure 2).

Mixed catecholate hydroxamates

We synthesized two monocatecholate hydroxamates (Scheme 2), at first the N-2,3-diacetoxybenzoyl-glutamic acid 5- (N-n-decyl-N-hydroxyamide) **12b** prepared from 1-benzyl N-2,3-diacetoxybenzoyl-L-glutamate **11** by reaction with O-benzyl-N-n-decyl-hydroxylamine **16c** to the benzylester **12a** followed by catalytic hydrogenolysis to compound **12b**. Secondly, compound **14b** based on L-lysine was prepared from glutaroyl (N-n-decyl-N-hydroxy)-amide **13** and N^6-(2,3-diacetoxybenzoyl)-L-lysine **2**. Furthermore we synthesized two types of biscatecholate hydroxamates, at first the bis-(2,3-diacetoxybenzoyl)-D-ornithyl-L-glutamoyl N-hydroxymonoamides **18a-d**, prepared from bis-(2,3-diacetoxybenzoyl)-D-ornithine **4d** and 1-benzyl L-glutamate resulting derivative **15**, followed by reaction with O-benzylhydroxylamines **16a-d** resulting in the benzylesters **17a-d** and catalytic hydrogenolysis to compounds **18a-d**. Moreover, we used the biscatecholatebenzoylhydroxamates **19a-d**, prepared according to a published procedure (Wittmann *et al.* 2002) (Scheme 4). Compound **22b** was analogously prepared from biscatecholate **20** based on 3,7-di-aza octanoic acid (Heinisch *et al.* 2002b) by reaction with 1-benzyl L-glutamate to compound **21** followed by reaction with the O-benzoyl-N-methyl-hydroxylamine **16e** to the benzylester **22a** and by hydrogenolysis to compound **22b**. Finally we used three biscatecholatehydroxamates **23a-c** based on glutaric acid and prepared according to a published procedure (Wittmann *et al.* 2002) (Figure 3).

Investigation of Siderophore activity

To study the siderophore activity of the new compounds on mycobacteria we tested the growth promotion of different strains of *M. smegmatis*. As mentioned above the natural mycobacterial iron supply is characterized by ligand exchange from exochelin to mycobactin. This mechanism is not availably for the transport of antibiotics into the bacterial cell via siderophore conjugates. It needs an alternative route for direct transport of siderophores into the cell without ligand exchange. This mechanisms can be proofed using the wildtype strains SG 987 and mc^2155 in combination with the mutants M10 (exochelin-), M24 (mycobactin-), B1 (exochelin-), B3 (mycobactin- and exochelin-) and U3 (mycobactin- and exochelin uptake-) (Schumann *et al.* 1998; Schumann & Möllmann 2001) (Table 1).

Compounds **1, 3, 4a,c,d,e, 5b,d,h, 6a–c, 7a,b, 12b, 14b, 18a–d, 19a,c, 20, 23a**, which promote growth of the wild type strains and of the separate exochelin or mycobactin mutants but not of the double mutants B3 and U3 are active only by ligand exchange with exochelin or mycobactin and are no candidates for conjugation with antibiotics to transport the drugs directly into the cell. Compounds **4b, 5a, 5c, 5e, 5f** (biscatecholates), **8, 9a, 10** (triscatecholates) and **19b, 19d, 22b** and **23b** (biscatecholate hydroxamates) which promote additionally growth of mutant B3 but not U3 transport iron directly without ligand exchange with exochelin or mycobactin. But activity depends on the exochelin uptake permease. Compounds **8, 9a** and **5c, 5d** which promote the growth of both mutants B3 and U3 must be able to use a completely different uptake route and transport iron into mycobacteria independent of exochelin and mycobactin. Both of the latter types of siderophore compounds are suitable as shuttle vectors for antibiotic conjugates.

Additionally we tested the siderophore activity in Gram-negative bacteria by use of the wild type strains *P. aeruginosa* ATCC 27853, SG 137, NTCC 10662, ATCC 9027, *E. coli* ATCC 25922 and of the mutant *S. typhimurium* enb 7 (Table 2).

The results of the following compounds have been published prior and were demonstrated here again for comparison to the activity in mycobacteria: **6a–c** (Heinisch *et al.* 2002b), **7a,b, 8, 9a,b, 10** (Heinisch *et al.* 2002c), **19a–d, 20a–d 24a,b** (Wittmann *et al.* 2002). The monocatecholates **1** and **3** and the monocatecholate hydroxamates **12b** and **14b** exhibited only low activity. The biscatecholates **4b** and **5a–h** based on amino acids were active moderately. The triscatecholates **7a** and **b** with longer C-chains and benzoxazindione groups were strongly decreased in activity. The other triscatecholates were moderately (**8**) or highly (**9a,b, 10**) active siderophores. The biscatecholate hydroxamates **18a–d, 19a–d, 22b, 23a–c** were generally very efficient growth promotors.

In parallel to the growth promotion assays the relative iron complexing capacity of the siderophore derivatives was checked by the CAS assay according to Schwyn & Neilands (1987) where a positive reaction is associated with iron chelation (Table 2). This is one of the conditions for siderophore activity. There is evidence for a correlation of iron complexing capacity and siderophore activity of the compounds. Except for compounds **7a** and **b** all tested compounds were active in this assay.

Table 1. Growth promotion of *M. smegmatis* strains by the catecholate derivatives. Diameter of growth zones in mm, substance application 5 μg on paper discs of 6 mm in diameter.

Compound	SG 987 wild type	M10 exochelin$^-$	mc^{2}155 wild type	M24 mycobactin$^-$	B1 exochelin$^-$	B3 exochelin$^-$, mycobactin$^-$	U3 mycobactin$^-$, exochelin uptake$^-$
Monocatecholates							
1	22	30	30	0	40	0	0
3	25	24	30	n.d.	26	0	0
Biscatecholates							
4a	0	17	0	n.d.	25	0	0
4b	23	26	20	n.d.	25	20	0
5a	20	30	0	n.d.	30	20	0
5b	24	22	30	0	25	0	0
5c	21	25	25	n.d.	25	20	16
5d	24	25	22	n.d.	10	10	20
5e	22	25	0	0	21	19	0
5f	22	27	20	n.d.	23	18	0
5g	20	24	0	0	25	0	0
5h	22	18	25	20	27	0	0
6a	23	25	24	19	25	0	0
6b	23	23	25	18	25	0	0
6c	19	22	20	0	20	0	0
Triscatecholates, linear							
7a	25	17	28	0	28	0	0
7b	25	25	34	0	32	0	0
8	17	20	23	17	20	15	15
9a	19	28	19	23	26	28	25
Triscatecholates, tripodal							
9b	21	22	25	20	25	0	0
10	20	23	20	17	23	25	0
Catecholate hydroxamates							
12b	25	25	30	0	30	0	0
14b	25	25	0	0	30	0	0
Biscatecholate hydroxamates							
18a	20	19	0	0	22	0	0
18b	22	22	23	20	20	20	0
18c	32	32	35	17	35	0	0
18d	25	22	0	0	22	0	0
19a	20	20	25	0	27	0	0
19b	24	20	25	19	25	22	0
19c	27	24	25	0	28	0	0
19d	30	24	24	22	30	20	0
20	12	12	22	0	20	0	0
22b	16	18	19	18	22	16	0
23a	12	0	20	15	19	0	0
23b	30	28	34	28	30	20	0
23c	27	20	30	14	25	16	0
Mycobactin (2 mg)	15	15	14	16	15	15	16

n.d.= not determined.

Table 2. Growth promotion of wild type strains of Gram-negative bacteria and an *S. typhimurium* enterobactin (ent) mutant by the catecholate derivatives. Diameter of growth zones in mm, substance application 5 μg on paper discs of 6 mm in diameter, and results of the CAS assay.

	Pseudomonas aeruginosa				*S. typhimurium*	*E. coli*	
Compound	ATCC 27853	SG 137	NTCC 10662	9027	enb 7 ent$^-$	ATCC 25922	CAS[c] assay
Monocatedcholates							
1	0	12	0	15	0	0	+
3	0	15	11	0	0	15	+
Biscatedcholates							
4a	0	0	0	0	0	0	+
4b	20	18	18	20	0	20	+
5a	20	20	28	22	32	30	+
5b	18	25	15	25	30	30	+
5c	18	25	24	23	28	24	+
5d	20	25	30	19	32	30	+
5e	20	21	18	24	28	20	+
5f	22	30	25	25	33	25	+
5g	15	20	22	20	30	25	+
5h	23	25	30	20	26	30	+
6a	25	26	25	24	0	30	+
6b	22	25	27	20	0	32	+
6c	20	26	25	20	10	28	++
Triscatecholates, linear							
7a	0	18	18	0	0	0	n.d.
7b	0	12	0	20	18	20	−
8	18	18	20	17	0	0	+
Triscatecholates, tripodal							
9a	26	27	27	27	36	37	+
9b	30	31	28	25	36	29	+++
10	20	15	25	27	10	0	+
Catecholate hydroxamates							
12b	0	12	0	0	0	0	+
14b	14	16	15	14	0	20	+
Biscatecholate hydroxamates							
18a	22	27	22	25	32	25	+
18b	24	27	25	22	30	29	+
18c	20	20	20	22	20	29	+
18d	19	25	24	22	34	20	+
19a	16	20	22	20	27	19	+
19b	23	30	30	20	27	25	+
19c	19	23	20	20	27	29	++
19d	25	27	25	24	34	33	++
20	15	0	18	13	0	20	+
22b	15	19	20	15	0	24	+
23a	20	22	21	17	32	30	+
23b	22	24	25	27	25	34	+++
23c	16	24	20	20	20	28	++
Control	42[a]	30[a]	40[a]	32[a]	34[b]	33[b]	

[a]desferal, [b]ferricrocin [c] − no CAS reaction, + weak CAS reaction, ++ strong CAS reaction, +++ very strong CAS reaction.

We synthesized first conjugates with ampicillin as antibiotic moiety from compounds **5a, 5d, 5e, 8, 9a** and **b, 10** (Heinisch *et al.* 2002b, c; Wittmann *et al.* 2002). These type of conjugates did not show increased activity against the wild type strain of *M. smegmatis* SG 987 but against Gram-negative bacteria in an agar diffusion assay (data not shown).

Acknowledgements

The financial support of Bundesministerium für Bildung, Wissenschaft, Forschung und Technologie (Berlin, Germany, FZ 0311232) is gratefully acknowledged. We thank Antje Fritzsche, Christina Täumer and Silke Leonhardt for technical assistance with the synthesis, Andrea Perner and Renate Koch for mass spectra, Heike Heinecke for NMR spectra as well as Irmgard Heinemann and Monika Golembiewski for biological testing.

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