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New synthetic catecholate-type siderophores with triamine backbone

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Abstract

New analogues of triscatecholate siderophores based on linear or tripodal triamines with or without spacer groups or lipophilic and hydrophilic substituents were synthesized. The catecholate moieties were prepared in OH-forms, as acetylated compounds or masked as 8-methoxycarbonyloxy-2,4-dioxo-1,3-benzoxazine derivatives. Some of the new compounds were active as siderophores tested by growth promotion assays using various Gram-negative bacteria and mycobacteria under iron limitation and by CAS-assay. Structure-activity-correlations have been studied.

Introduction

Triscatecholates represent an important group of bacterial siderophores (Drechsel & Jung 1998). One of them is enterobactin with a cyclic trilactone backbone. It is produced by enteric bacteria and is one of the most powerful iron chelators. Other triscatecholate siderophores contain a linear polyamine backbone based on spermidine (1,5,10-triaza-n-decane) or norspermidine (1,5,9-triaza-n-nonane). Examples are agrobactin produced by *Agrobacterium tumefaciens* (Ong *et al*. 1979), fluvabactin (Yamamoto *et al*. 1993) from *Vibrio fluvialis*, Vibriobactin (Griffith *et al*. 1984) from Vibrio cholerae and Vulnibactin (Okujo *et al*. 1994) from *Vibrio vulnificus*. Some synthetic analogues of these siderophores based on spermidine or norspermidine were published (Bergeron *et al*. 1981; Weitl *et al*. 1979; Buckley *et al*. 1994; Miyasaka *et al*. 1987). Similar compounds with lipophilic alkyl groups were synthesized for potential use in radiopharmaceutical metal complexes (Weitl & Raymond 1981). Other analogues as myxochelin derivatives C, D, E and F with a tripodal polyamine structure (Ambrosi *et al*. 1998) and a triscatecholate of tris-(aminomethyl)-ethane (Cheraiti *et al*. 1999) are powerful siderophores. Analogues based on tris-(aminoethyl)-amine were synthesized to simulate the optimal octahedral structure of enterobactin iron complexes forming a propeller like configuration (Shanzer *et al.* 1996; Tor *et al*. 1992). Siderophore analogues are tools in the study of structure specifity of siderophore receptors. These studies are a basis for the design of siderophore components as shuttle vectors for antibiotics to overcome penetration mediated resistance (Arisawa *et al.* 1991). Another potential application for siderophore analogues is in the therapy of malaria (Pradines *et al*. 1996) or as antiinflammatory agent. In this paper we report on the syntheses and siderophore activities of novel triscatecholate siderophore analogues with triamine scaffolds (bis-(aminoalkyl)-amines or tris-(aminoethyl)-amine). We studied the influence of different alkyl chains of the triamine backbone and of different spacer groups on the geometric structure allowing an optimal iron complex formation. We synthesized compounds with free and acylated catechol groups (acetylated derivatives and 2,4-dioxo-1,3-benzoxazines as masked catechols) and with lipophilic or hydrophilic substituents. The

Dedicated to Professor Udo Gräfe on the occasion of his 60th birthday.

siderophore activity of the new siderophore analogues was studied by the chromazurol-S (CAS) assay and by growth promotion assays with various Gram-negative bacteria and mycobacteria.

Materials and methods

Synthesis of the siderophore analogues

^{1}H-NMR spectra were recorded on a Bruker Advance DRX 300 MHz spectrometer. The chemical shifts δ ar given in ppm. The coupling constants J are reported in Hz. High resolution mass spectra were obtained by a Finnigan MAT 95 XV high resolution mass spectrometer with fast-atom bombardment (FAB) and electrospray ionization (ESI), respectively. Column chromatography was accomplished using silicagel (Merck 60, 0.040–0.063 mm). Purifications of the compounds by preparative HPLC were performed on an Abimed Gilson instruments 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 column 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). The purity of the new compounds were checked by thin layer chromatography carried out with precoated silica plates (Merck 60 F254) applying UV detection. Solvents and reagents used were dried and purified by standard methods.

The following compounds have been synthesized according to published procedures: 2,3-diacetoxybenzoyl chloride **2** (Bergeron *et al.* 1980), 2,3-di(methoxycarbonyloxy)benzoyl chloride **3** and (8-methoxycarbonyloxy-3,4-dihydro-2,4-dioxo-1,3-benzoxazin-3-yl)-acetic acid **10a** (Wittmann *et al.* 2000).

1,5,9-Tris-(2,3-diacetoxybenzoyl)-1,5,9-triaza-n-nonane ***4a,*** $C_{39}H_{41}N_3O_{15}$ (791.8)

To a solution of 262 mg (2 mmol) bis-(3-aminopropyl)-amine and 0,84 ml (6 mmol) triethylamine in 20 ml dichlormethane at 0 °C was added dropwise with stirring 1.54 g (6 mmol) 2,3-diacetoxybenzoyl chloride **2** in 10 ml dichlormethane. The mixture was stirred for 30 min at this temperature and then the solvent was evaporated. The residue was dissolved in ethyl acetate, the solution was washed with 1 M HCl and then with brine, dried over Na_2SO_4, filtered and evaporated affording a colourless foam of **4a** (1.0 g, 63%). According to the ^{1}H NMR spectra the substance **4a** consists of a mixture of rotamers which will be identically at 360 K. ^{1}H-NMR (DMSO-d6): 1,66–1,75 (m, 4H, CCH_2); 2.19–2.27 (5× s, 18H, $COCH_3$), 3.11–3.25 (m, 8H, NCH_2), 7.22–7.36 (m, 9H, aromatic CH), 8.23, 8.27 (2× t, at 360 K s, 2H, NHCO). MS (FAB): 792.1 $[M+H]^+$, MS (ESI): 814.0 $[M+Na]^+$.

1,8,15-Tris-(2,3-diacetoxybenzoyl)-1,8,15-triaza-n-pentadecane ***4b,*** $C_{45}H_{53}N_3O_{15}$ *(875.9)*

The compound was prepared analogously to **4a** from bis-(6-aminohexyl)-amine and **2**, yield 66%. ^{1}H-NMR (DMSO-d6): 1.14–1.49 (m, 16H, CCH_2) 2.19, 2.20, 2.21, 2.25, 2.27, (5× s, 18H, $COCH_3$), 3.07, 3.09, 3.16 (2× dd, 1× dt, at 360 K *s*, 8H, NCH_2), 7.19–7.42 (m, 9H, aromatic CH), 8.23, 8.30 (2H, 2× t, at 360 K s, NHCO). MS (FAB): 876.4 $[M+H]^+$, MS (ESI): 898.1 $[M+Na]^+$.

1,10,19-Tris-(2,3-diacetoxybenzoyl)-1,10,19-triaza-n-nonadecane ***4c,*** $C_{49}H_{61}N_3O_{15}$ *(932.9)*

The compound was prepared analogously to **4a** from bis-(8-aminooctyl)-amine and **2**, yield 60%, MS (FAB): 932.6 $[M+H]^+$, MS (ESI): 954.1 $[M+Na]^+$.

N,N-Bis-[3-(8-Methoxycarbonyloxy-2,4-dioxo-1,3-benzoxazin-3-yl)-propyl]-N-2,3-di(methoxy-carbonyloxy)-benzamide ***5a****,* $C_{37}H_{33}N_3O_{19}$ *(823.7)*

To a solution of 262 mg (2 mmol) bis-(3-aminopropyl)-amine **1c** and 0.84 ml (6 mmol) triethylamine in 10 ml dichlormethane at 0 °C was dropped with stirring a solution of 1.73 g (6 mmol) 2,3-di(methoxycarbonyloxy)benzoyl chloride **3** in 10 ml dichlormethane. The solvent was evaporated, the residue dissolved in ethyl acetate and washed with 1 M HCl and brine. The organic phase was dried with Na_2SO_4, filtered, evaporated and the residue dried *in vacuo*. The colourless foam was stirred over night with 0.7 ml triethylamine in 8 ml acetonitrile. Then the solution was evaporated and the residue dissolved in ethyl acetate. The solution was washed with 1 M HCl and water, dried and evaporated. The residue was purified by column chromatography affording a colourless foam of **5a** (360 mg, 22%). ^{1}H-NMR (DMSO-d6): 1.91–1.98 (m, 4H, CCH_2), 3.13–3.49 (m, 4H, NCH_2), 3.66–3.91 (m, 16H, $OCOOCH_3$, $CONCH_2$), 7.15–7.87 (m, 9H, aromatic H). MS (FAB): 824.1 $[M+H]^+$.

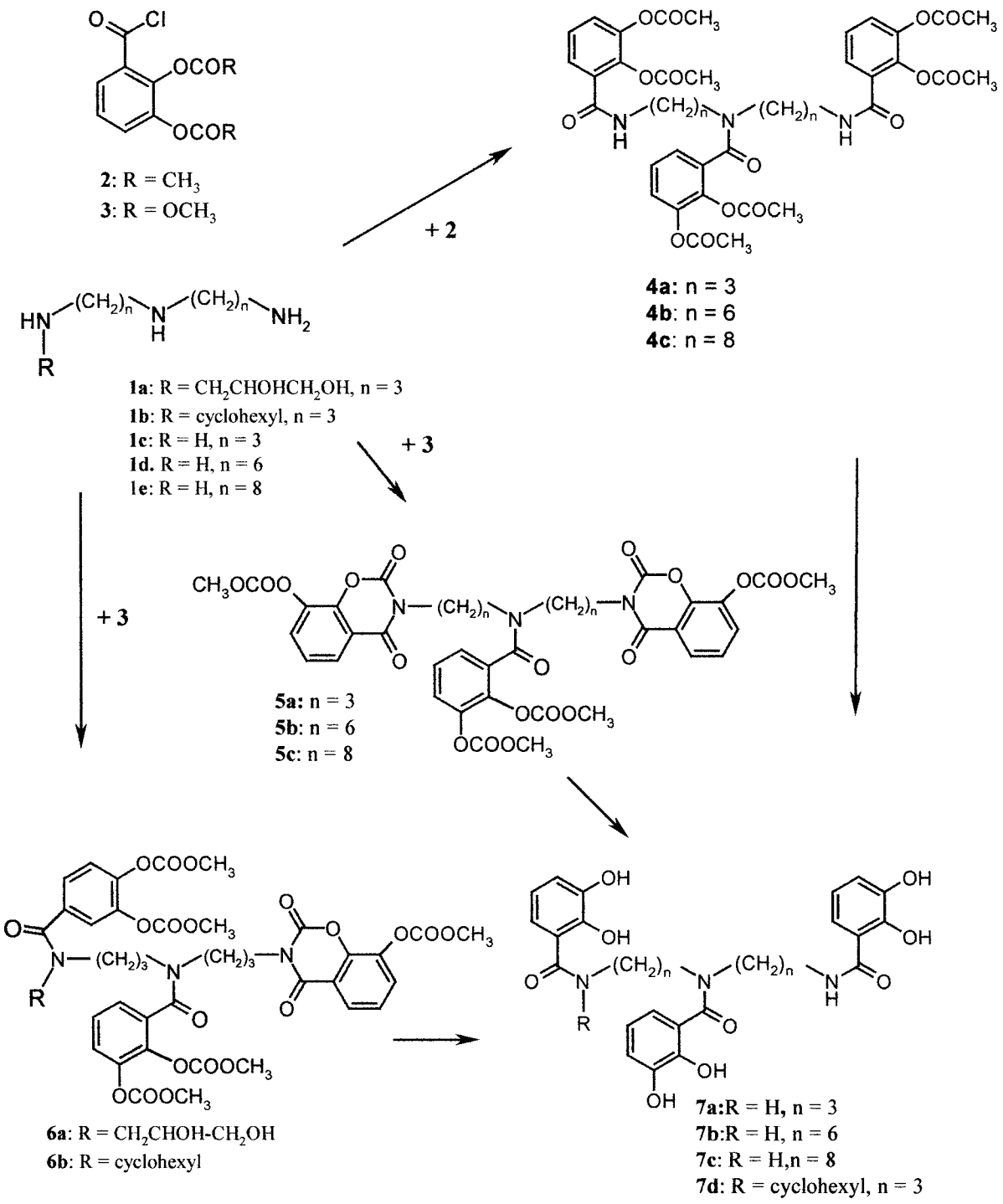

Fig. 1. Synthesis of catechol derivatives of bis-(aminoalkyl)-amines.

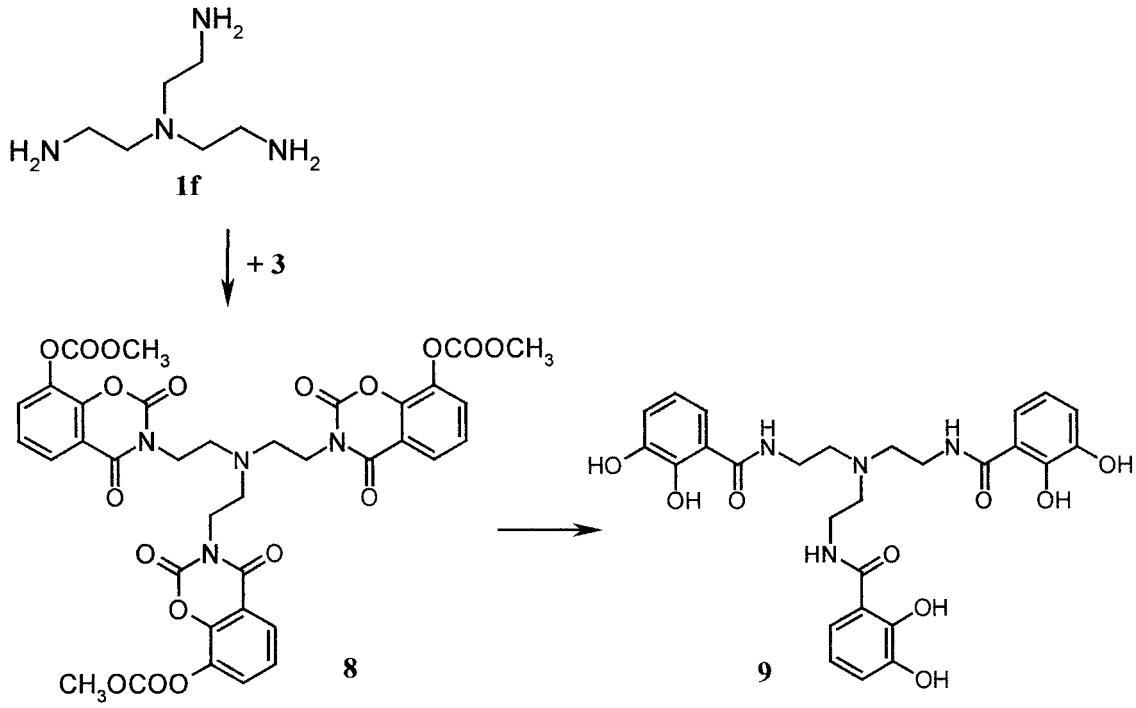

Fig. 2. Synthesis of catechol derivatives of tris-(aminoethyl)-amine.

N,N-Bis-[6-(8-Methoxycarbonyloxy-2,4-dioxobenzoxazin-3-yl)-n-hexyl]-2,3-di(methoxy-carbonyloxy)benzamide ***5b****,* $C_{43}H_{45}N_3O_{19}$ *(907.9)*

The compound was prepared analogously to **5a** from bis-(6-amino-n-hexyl)-amine **1d** and **2**. The colourless foam was purified by preparative HPLC affording **5b** as colourless solid foam (25%). ^{1}H-NMR (DMSO-d6): 1.09–1.51 (m, 16H, CCH_2), 3.73–3.98 (m, 20H, NCH_2 and $COOCH_3$), 7.40–7.47 (m, 5H, aromatic CH), 7.77–7.80 (dd, 2H, aromatic CH), 7.86–7.89 (m, 2H, aromatic CH) . MS (ESI): 908.1 $[M+H]^+$, 931.0 $[M+Na]^+$.

N,N-Bis[8-(8-Methoxycarbonyloxy-2,4-dioxo-1,3-benzoxazin-3-yl]-n-octyl-2,3-di-(methoxy-carbonyloxy)-benzamide ***5c****,* $C_{47}H_{53}N_3O_{19}$ *(964.0)*

The compound was prepared analogously to **5a** from bis-(8-amino-n-octyl)-amine **1e** and **3**. The colourless foam was purified by prearative HPLC, yield 30%. ^{1}H-NMR: (DMSO-d6): 1.04–3.52 (m, 32H, 16× CH_2), 3.79, 3.81, 3.90 (3 × s, 12H, $OCOOCH_3$), 6.95–7.47 (m, 7H, aromatic CH), 7.78 (d, J = 8, 1H, aromatic CH), 7.88 (d, J = 8, 1H, aromatic CH), MS (FAB): 964.3 ($[M+H]^+$.

D,L-10,11-*Dihydroxy-4,8-bis-(di-methoxy-carbonyloxy-benzoyl)-1-(8-methoxycarbonyloxy-2,4-dioxo-1,3-benzoxazin-3-yl)-4,8-diaza-n-undecane* ***6a****,* $C_{41}H_{43}N_3O_{22}$ *(929.8)*

a: (D,L-11,12-Dihydroxy-1,5,9-triaza-n-dodecane **1a,** $C_{12}H_{27}N_3$, (213.4): A solution of 5 g (55,5 mmol) D,L-glyceraldehyde in 25 ml methanol was dropped at 0 °C to a stirred solution of 8.01 g (61 mmol) bis-(3-aminopropyl)-amine in 50 ml methanol. The solution warmed up to ambient temperature. Under nitrogen 250 mg of palladium on activated charcoal was added and afterwards hydrogen at 1 atm. The mixture was shaken over night, then filtered over celite and washed with methanol. Evaporation in vacuo afforded 12.9 g of a crudy oil. The product was purified by preparation of the tris-Z-derivative, separation of impurities by preparative HPLC and following hydrogenolysis over 10% Pd/C at ambient temperature affording pure **1a** (0.66 g, 6%). **6a**: A solution of 0.366 g (1.27 mmol) of **3** in 3 ml of dry tetrahydrofurane was added to a stirred mixture of 0.261 g (1.27 mmol) of **1a** and 5 ml saturated aqueous sodium hydrogencarbonate solution. After 15 min the mixture was warmed to ambient temperature and stirred for 30 min. The solvent was evaporated, the residue acidified with diluted

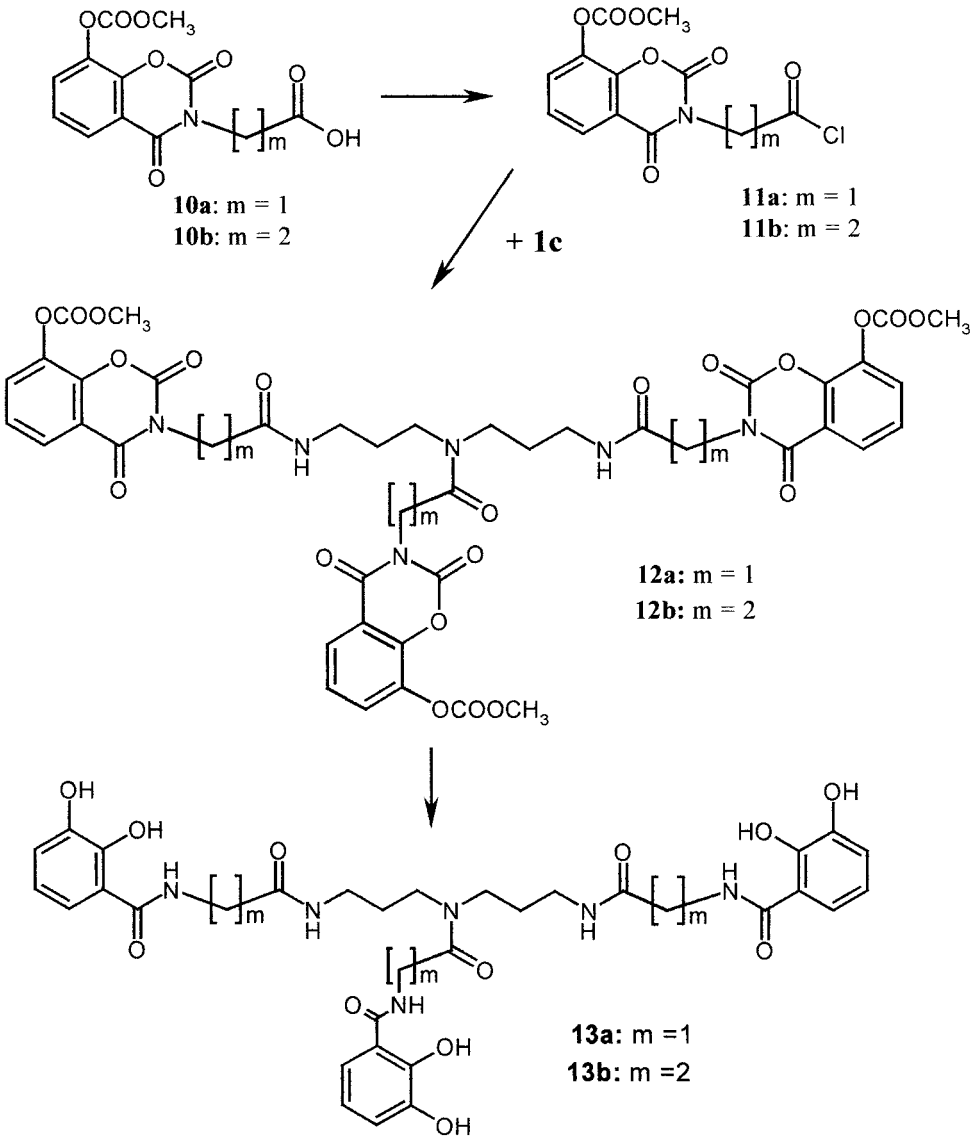

Fig. 3. Preparation of catechol derivatives of bis-(3-aminopropyl)-amine.

HCl and extracted with 4 × 40 ml ethyl acetate. The residue was neutralized with 2 × 15 ml brine, dried over Na_2SO_4 and evaporated. The residue was purified by preparative HPLC to yield **6a** as a colourless foam (0.206 g, 17%). ^{1}H-NMR: (DMSO-d6): 1.70–1.97 (m, 4H, CH_2), 3.06–3.99 (m, 28H, CH_3O and CH_2N), 7.14–7.97 (m, 9H, aromatic CH), MS (ESI): 930.4 ($[M+Na]^+$.

1-Cyclohexyl-1,5-bis-(di-methoxycarbonyloxy-benzoyl)-8-(8-methoxycarbonyloxy-2,4-dioxo-1,3-benzoxazin-3-yl)-1,5-diaza-n-octane ***6b,*** *$C_{44}H_{47}N_3O_{20}$ (937.9)*

a: 1-Cyclohexyl-1,5,9-triaza-n-nonane ***1b***, $C_{12}H_{27}N_3$ (213,4). A solution of 1.96 g (20 mmol) cyclohexanone in 20 ml methanol was dropped at 0 °C to a stirred solution of 2.62 g (20 mmol) bis-(3-aminopropyl)-amine in 20 ml methanol. The solution was warmed up to ambient temperature and stirred for 1 h. To the mixture 300 mg Pd/C (10%) was added under nitrogen and afterwards hydrogen under 70 atm.

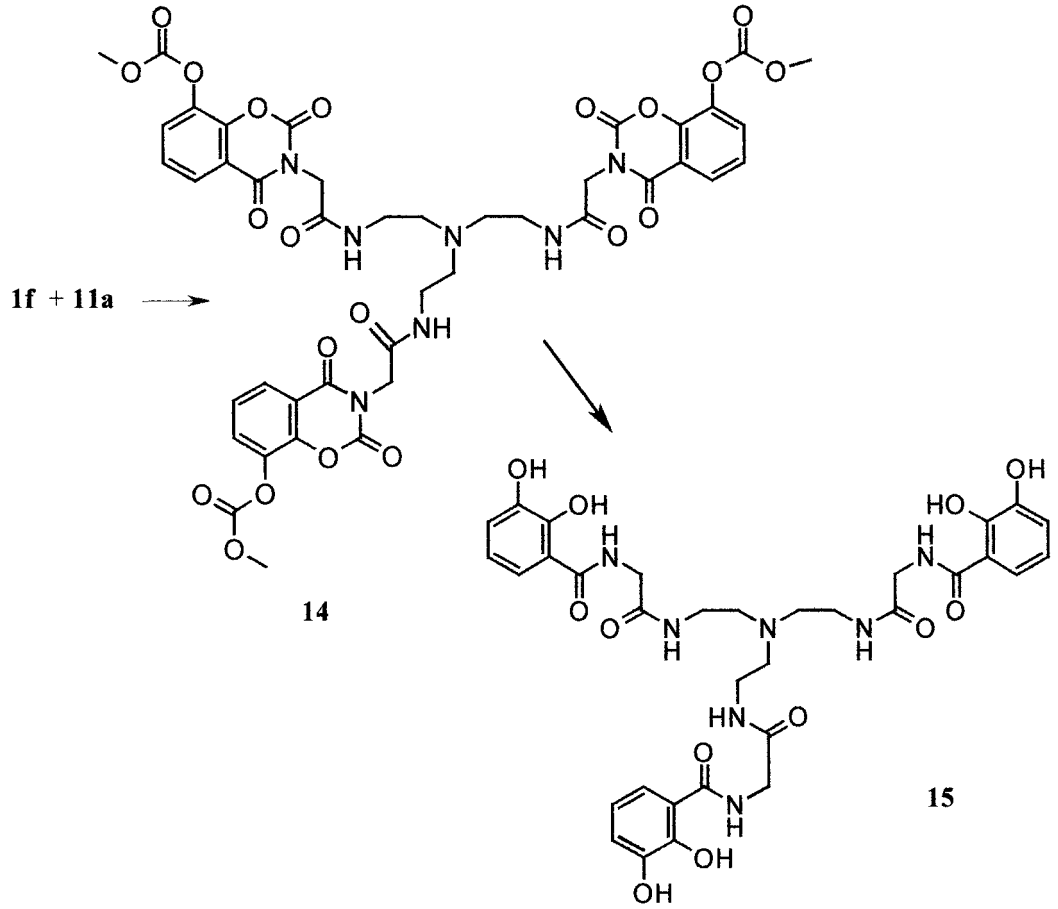

Fig. 4. Synthesis of catechol derivatives of tris-(2-aminoethyl)-amine.

The mixture was shaken for 4.5 h, filtered over celite and washed with methanol. Evaporation in vacuo afforded 3.8 g of a crude oil, which was purified via its Z-derivative according to **6a** to given pure **1b** (1.4 g, 33%). b. **6b**: The compound was prepared analogously to **6a** from **1b** and **3**, yield 38%. ^{1}H-NMR: (DMSO-d6): 0.90–2.07 (m, 14H, CH_2), 3.07–3.49 (m, 9H, CHN, CH_2N), 3.71–3.91 (m, 15H, $COOCH_3$), 7.12–7.89 (m, 9H, aromatic CH), MS (ESI): 938.5 ($[M+H]^+$; 960.6 ($[M+Na]^+$.

1,5,9-Tris-(2,3-dihydroxy-benzoyl)-1,5,9-triaza-n-nonane ***7a****, $C_{27}H_{29}N_3O_9$ (539.6)*

To 7 ml 2M NaOH under nitrogen 200 mg (0.25 mmol) **4a** was added. The suspension was stirred at room temperature for 1 h and then carefully neutralised with 2M HCl. The solid was washed with water affording **7a** as colourless solid (85 mg, 63%). ^{1}H-NMR (DMSO-d6): 1.74–1.82 (m, 4H, CCH_2), 2.93–3.48 (m, 8H, NCH_2), 6.48–7.23 (m, 9H, aromatic CH), 8.57; 8.62 (d, 2H, NHCO); 8.75 (s, 1H, OH), 9.06 (s, 2H, OH), 9.47 (s, 1H, OH); 12.69; 12.75 (s, 2H, OH). MS (FAB): 540.3 $[M+H]^+$.

1,8,15-Tris-(2,3-dihydroxybenzoyl)-1,8,15-triaza-n-pentadecane ***7b,*** *$C_{33}H_{41}N_3O_9$ (623.7)*

The compound was prepared analogously to **7a** from **4b**, yield 80%. ^{1}H-NMR (DMSO-d6): 0.78–1.47 (m, 16H, CCH_2), 3.0–3.2 (m, 8H, NCH_2), 6.47 (dd, J_1 = 8, J_2 = 1.5 1H, aromatic CH), 6.59–6.68 (m, 3H, aromatic CH), 6.75 (dd, J_1 = 7, J_2 = 1, 5, 1H, aromatic CH), 6.89 (dd, J_1 = 8, J_2 = 0, 3, 2H, aromatic CH), 7.25 (2H, d, J = 8, aromatic CH), 8.70, 9.30, 9.05, 12.83 (s, 8H, OH, NHCO), MS (FAB): 624.2 $([M+H]^+$.

1,10,19-Tris-(2,3-dihydroxy-benzoyl)-1,10,19-triaza-n-nonadecane ***7c,*** *$C_{37}H_{49}N_3O_9$ (679.8)*

The compound was prepared analogously to **7a** from **4c**, yield 71%. ^{1}H-NMR (DMSO-d6): 1.03–1.49 (m, 24H, CCH_2), 3.05–3.30 (m, 8H, NCH_2), 6.73–7.44 (m, 9H, aromatic CH), 8.51, 8.71, 9.05, 9.44, 12.92 (s, 8H, OH, NHCO). MS (FAB): 680.3 $[M+H]^+$.

1-Cyclohexyl-1,5,9-tris-(2,3-dihydroxy-benzoyl)-1,5,9-triaza-n-nonane ***7d,*** *$C_{33}H_{39}N_3O_9$ (621.69)*

The compound was prepared analogously to **7a.** from **6b**, yield 63%. ^{1}H-NMR (DMSO-d6): 0.89 (m, 14H, CH_2), 3.31 (m, 9H, NCH, NCH_2), 6.35–7.26 (m, 9H, aromatic CH), 8.46; 8.76; 9.05, 9.44, 9.46, 12.67, 12.78 (s, 7H, OH, NHCO), MS (ESI): 622.7 $[M+H]^+$, 644.7 $[M+Na]^+$.

N,N′,N″-Tris-[2-(8-Methoxycarbonyloxy-2,4-dioxobenzoxazin-3-yl)-ethyl]-amine **8,** *$C_{36}H_{30}N_4O_{18}$ (806.7)*

To a solution of 146 mg (1 mmol) tris-(2-aminoethyl)-amine and 0.42 ml (3 mmol) triethylamine in 12 ml tetrahydrofurane at −20 °C 864 mg (3 mmol) 2,3-di(methoxycarbonyloxy)benzoyl chloride **3** in 10 ml tetrahydrofurane was added dropwise with stirring. The mixture was stirred 1 h at 0 °C, 1 h at room temperature and then filtered. The solution was evaporated. To the residue ethyl acetate and water was added. The mixture was acidified with 1 M HCl, shaken and the organic phase washed with water, dried and evaporated affording a yellow foam of **8** (322 mg, 40%). ^{1}H-NMR (DMSO-d6): 2.67–2.77 (m, 6H, NCH_2), 3.79–4.04 (m, 15H, $CONCH_2$ and $COOCH_3$), 7.38–7.92 (m, 9H, aromatic CH). MS (ESI): 807.1 $[M+H]^+$, 804.1 $[M-2H]^-$.

N,N′,N″-[Tris-(2,3-dihydroxy-benzoyl)-2-aminoethyl]-amine ***9,*** *$C_{27}H_{30}N_4O_9$ (554.6)*

322 mg (0.4 mmol) **8** was added to 16 ml 0.5 M NaOH under N_2. The suspension was stirred under N_2 at room temperature for 1 h and then neutralized with 2 M HCl. The solid was purified by preparative HPLC affording a colourless solid of **9** (22 mg, 10%). ^{1}H-NMR (DMSO-d6): 3.30–3.87 (m, 12H, NCH_2), 6.60 (t, J = 7,50, 1H, aromatic H), 6.95 (d, J = 7.40, 1H, aromatic H), 7.29 (d, J = 7.40, 1H, aromatic H), 9.03; 9.21, 12.19 (s, 9H, OH, NHCO). MS (ESI): 555.2 $[M+H]^+$, 553.3 $[M-H]^+$.

3-(8-Methoxycarbonyloxy-2,4-dioxo-1,3-benzoxazin-3-yl)-propionic acid ***10b,*** *$C_{13}H_{11}NO_8$ (309.2)*

The compound was prepared analogously to 8-methoxycarbonyloxy-2,4-dioxo-1,3-benzoxazine-3-yl)-acetic acid **10a** (Wittmann *et al.* 2000) from *β*-alanine and **3** in aqueous $NaHCO_3$-solution and recrystallized from ethyl acetate affording colourless crystals (55%, mp 140–144 °C). ^{1}H-NMR (DMSO-d6): 2.58 (t, J = 4,5, 2H, CH_2), 3.90 (s, 3H, $OCOOCH_3$), 4.05 (t, J = 8.0, 2H, NCH_2), 7.46 (t, J = 8,0, 1H, aromatic CH), 7.80 (dd, J_1 = 8,0, J_2 = 1,3, 1H, aromatic CH), 7.82 (dd, J_1 = 8.0, J_2 = 1,3, 1H, aromatic CH), 12.39 (1H, s, COOH), MS (ESI): 332.0 $[M+Na]^+$, 308.0 $[M-H]^-$.

(8-Methoxycarbonyloxy-2,4-dioxo-1,3-benzoxazin-3-yl)-acetyl-chlorid ***11a*** *$C_{12}H_8ClNO_7$ (309.2)*

The mixture of 1.62 g (5.52 mmol) (8-methoxycarbonyloxy-2,4-dioxo-1,3-benzoxazin-3-yl)-acetic acid (***10a***), 1.52 g PCl_5 and 14 ml tetrachlormethane was boiled 30 min until HCl development was finished. The solution was filtered and evaporated. The residue was recrystallized from tetrachlormethane affording yellow crystals of **11a** 1.27 g, 73%, mp 99–101 °C.

(8-Methoxycarbonyloxy-2,4-dioxo-1,3-benzoxazin-3-yl)-propionyl-chloride **11b** *$C_{13}H_{10}ClNO_7$ (327.7)*

The compound was prepared analogously to **11a** affording as a yellow oil, yield 92%. MS (FAB): 326.9. $[M]^+$.

1,5,9-Tris-[(8-Methoxycarbonyloxy-2,4-dioxo-1,3-benzoxazin-3-yl)-acetyl]-1, 5,9-triaza-n-nonane ***12a,*** *$C_{42}H_{38}N_6O_{21}$ (962.8)*

To a solution of 131 mg (1 mmol) bis-(3 aminopropyl)-amine **1c** and 0.42 ml (3 mmol) triethylamine in 10 ml dichlormethane was added dropwise at −20 °C 939 mg (3 mmol) **11a** in 10 ml dichlormethane. The mixture was stirred for 1 h at 0 °C and 1 h at ambient temperature. Then the solvent was evaporated and to the residue ethyl acetate/water was given. The mixture was acidified with 1 M HCl and shaken. The organic phase was washed with NaCl-water, dried over Na_2SO_4 and evaporated affording colourless foam of **12a** (129 mg, 20%). ^{1}H-NMR (DMSO-d6): 1.59–1.75 (m, 4H, CCH_2) 3.02–3.27 (m, 8H, NCH_2), 3.81–3.84 (m, 9H, $COOCH_3$), 4.41 (s, 2H, NCH_2), 4.45 (s, 2H,

NCH_2), 4.72 (s, 2H, NCH_2), 7.43–7.89 (m, 9H, aromatic CH), 8,15, 8,32 (2× t, 1H, NHCO), MS (ESI): 985.4 $[M+Na]^+$, 961.2 $[M-H]^-$.

1,5,9-Tris-[(8-Methoxycarbonyloxy-2,4-dioxo-1,3-benzoxazine-3-yl)-propionyl]-1,5,9-triaza-n-nonane ***12b*** *$C_{45}H_{44}N_6O_{21}$ (1004.9)*

The compound was prepared analogously to **12a** as a colourless foam, yield 16%. ^{1}H-NMR (DMSO-d6): 1.50–1.65 (m, 4H, CCH_{2a}) 2.39–2.48 (m, 4H, $COCH_2$), 2.64 (t, 2H, $COCH_2$), 2.99 (t, 4H, NCH_2), 3.19 (t, 4H, NCH_2), 3.79–3.82 (m, 9H, $COOCH_3$), 4.01–4.05 (m, 6H, NCH_2), 7.39–7.47 (m, 3H, aromatic CH), 7.74–7.90 (m, 6H, aromatic CH), 7.94, 7.98 (t, 2H, NHCO), MS (ESI): 1027.5 $[M+Na]^+$.

1,5,9-[Tris-(2,3-dihydroxy-benzoyl) glycyl]-1,5,9-triaza-n-nonane ***13a****, $C_{33}H_{38}N_6O_{12}$ (710.7)*

To 104 mg (0.11 mmol) **12a** suspended in 3 ml water 3 ml 2 M NaOH under nitrogen was added and the mixture was stirred for 1 h. After acidification with 2 M HCl the formed precipitate was washed with water affording **13a** as a colourless solid (25 mg, 33%). ^{1}H-NMR (DMSO-d6): 1.61, 1.74 (2× t, 4H, CCH_2), 3.06–3.16 (m, 8H, NCH_2), 3.86, 3.88 (dd, 4H, NCH_2CO), 4.13 (d, 2H, NCH_2CO), 6.68 (dd, $J_1 = 7.7$; $J_2 = 7.6$; 3H, aromatic CH), 6.90 (d, J = 7.5, 3H, aromatic CH), 7.30 (d, J = 7.6, 3H, aromatic CH), 7.98; 8.09 (2× t, 2H, NHCO), 9.01 (m, 3H, NHCO), 9.23 (s, 3H, OH), 12.29 (s, 3H, OH). MS (ESI): 733.2 $[M+Na]^+$, 709.2 $[M-H]^-$.

1,5,9-[Tris-(2,3-dihydroxy-benzoyl)propionyl]-1,5,9-triaza-n-nonane ***13b,*** *$C_{36}H_{44}N_6O_{12}$ (752.8)*

The compound was prepared analogously to **13a** as a colourless solid (21%). ^{1}H NMR (DMSO-d6): 1.60–1.71 (m, 4H, CCH_2) 2.38 (t, 2H, CH_2CON), 2.55 (m, 4H, CH_2CONH), 3.03–3.45 (m, 10H, NCH_2), 6.64 (m, 3H, aromatic CH), 6.88 (d, 3H, aromatic CH), 7.23 (d, 3H, aromatic CH), 7.86; 7.93 (2× t, 2H, NHCO), 8.75 (m, 3H, NHCO), 9.14 (s, 3H, OH), 10.43 (s, 3H, OH). MS (ESI-PI): 775.5 $[M+Na]^+$, (ESI-NI): 751.7 $[M-H]^+$.

N,N,N-Tris-[2-(8-Methoxycarbonyloxy-2,4-dioxo-1,3-benzoxazin-3-yl)-acetaminoethyl]-amine ***14,*** *$C_{42}H_{39}N_7O_{21}$ (977.8)*

To a solution of 146 mg (1 mmol) tris-(2-aminoethyl)-amine and 0.42 ml (3 mmol) triethylamine in 5 ml tetrahydrofurane was added dropwise at −20 °C the solution of 939 mg (3 mmol) **11a** in 12 ml tetrahydrofurane. The mixture was stirred for 1 h at 0 °C and 1 h at ambient temperature. The precipitate was filtered and the solution was evaporated. To the residue ethyl acetate and water was added, acidified with 1 M HCl and shaken. The organic phase was washed with brine, dried and concentrated. By addition of ligroine a colourless precipitate of **14** (155 mg, 16%). was afforded. ^{1}H-NMR (DMSO-d6): 3.20–3.50 (m, 12H, NCH_2), 3.80–3.90 (m, 15H, $COOCH_3$ $NHCH_2CO$), 7.40–8.10 (m, 12H, aromatic CH, NHCO). MS (ESI): 978.4 $[M+H]^+$, 977.3 $[M-H]^-$.

N,N,N-[Tris-(2,3-dihydroxy-benzoyl)-glycyl]-(2-aminoethyl)-amine ***15,*** *$C_{33}H_{39}N_7O_{12}$ (725.7)*

To 200 mg (0.20 mmol) **14** suspended in 3 ml water 2.3 ml 2 M NaOH was added under N_2 and the mixture was stirred for 1 h at room temperature. After acidification with 2 M HCl the formed precipitate was washed with water affording **15** (37 mg, 25%). ^{1}H-NMR (DMSO-d6): 2.50–3.50 (m, 12H, NCH_2), 3.80–3.95 (m, 6H, $COCH_2N$), 6.35–7.40 (m, 9H, aromatic CH), 7.90–8.20 (m, 3H, NHCO), 9.01–9.53 (m, 6H, 3-OH, NHCO), 12.10–12.20 (m, 3H, 2-OH). MS (ESI): 726.2 $[M+H]^+$, 724.2 $[M-H]^-$.

Examination of the siderophore activity

The synthesized compounds were tested for their siderophore activities by growth promotion tests (Reissbrodt *et al.* 1993) using various wild type bacteria and mutants that are well defined in their ability to transport and utilize natural siderophores (siderophore indicator strains). The following indicator strains were used: *Pseudomonas aeruginosa* PAO 6609 (pyoverdin-), *Escherichia coli* AB 2847 (aroB-), Klebsiella KN4401, *Salmonella typhimurium* enb7 (enterobactin-), *S. typhimurium* TA 2700 (enterobactin-'fhuC')(Rabsch 1998), *Yersinia enterocolytica* H 5030 and *Morganella morganii* SBK3 (wild type).

Synthetic siderophore mimetics have to compete *in vivo* with the endogenous bacterial siderophores for the rare iron and for the siderophore receptors to

Table 1. Growth promotion of the catechol derivatives on Gram-negative indicator strains. Diameter of growth zone (mm), substance application 5 μg.

Compound	*P. aerug.* PAO 6609	*E. coli* AB 2847	*Klebsiella* KN 4401	*S. typhimurium* enb 7	*S. typhimurium* TA 2706	*Y. enteroc.* H 5030	*M. morganii* SBK 3
4a	25	0	0	0	0	0	29
4b	22	16	0	7	0	16	34
4c	0	10	n.t.	0	n.t.	8	n.t.
5a	20	24	0	0	0	0	28
5b	0	8	0	12	0	8	20
5c	0	0	0	10	0	8	0
6a	0	0	0	12	0	0	28
6b	0	0	0	15	0	0	0
7a	25	0	30	16	0	12	34
7b	20	8	0	10	0	20	32
7c	6	0	20	6	0	0	8
7d	0	0	0	0	0	0	7
8	22	18	0	0	0	0	30
9	35	14	0	0	0	26	16
12a	26	0	0	22	24	0	25
12b	17	0	0	0	0	10	32
13a	32	10	0	24	32	10	26
13b	32	20	27	24	0	20	32
14	20	8	0	12	0	0	32
15	30	12	0	10	0	0	48
control	30[b]	28[a]	26[b]	28[b]	30[c]	18[a]	22[d]

[a] ferrichrome, [b] ferrioxamine B, [c] enterobactin, [d] 2,3-dihydroxybenzylidene-1,3,5-trimethyl-aniline (Reissbrodt et al., 1993).

be transported into the cells. To be efficient there is a prerequisite for a siderophore structure to function as antibiotic shuttle vector. To proof it, we used in a second series of experiments the following Gram-negative wild type strains grown under iron limitation: *P. aeruginosa* ATCC 27853, SG 137, NTCC 10662, ATCC 9027, K799/WT and *E. coli* ATCC 25922.

Additionally we proofed the new compounds for growth promotion of mycobacteria. In contrast to Gram-negative bacteria and to other Gram-positive bacteria siderophore iron uptake in mycobacteria includes ligand exchange between the extracellular siderophore exochelin and the membran associated siderophore mycobactin. For the use of siderophores as antibiotic shuttle vector only a direct transport into the bacterial cell without ligand exchange is acceptable. To identify, whether the synthetic siderophore analogues supply iron to mycobacteria by ligand exchange with exochelin or mycobactin, via the exochelin permease or by an independent route we used the following set of strains: the wild types *Mycobacterium smegmatis* SG 987 and mc^2 155, the mutants *M. smegmatis* M10 (exochelin-), M24 (mycobactin-) and 3 mutants of *M. smegmatis* mc^2 155 generated by gene replacement: B1 (blocked in exochelin biosynthesis), B3 (blocked in mycobactin and exochelin biosynthesis) and U3 (blocked in mycobactin biosynthesis and exochelin uptake) (Schumann *et al*. 1998; Schumann & Möllmann 2001).

Results and discussion

Synthesis of catechol derivatives from bis-(aminoalkyl)-amines

We synthesized triscatecholates from three linear triamines carrying alkyl chains of different length, bis-(3-aminopropyl)-amine (1,5,9-triazanonane), bis-(6-aminohexyl)-amine (1,8,15-triazapentadecane) and bis-(8-aminooctyl)-amine (1,10,19-triazanonadecane). Reaction with 2,3-diacetoxybenzoyl chloride **2** afforded the acetylated triscatecholates **4a–c** and reaction with 2,3-di(methoxycarbonyloxy) benzoyl chloride **3** the 2,4-dioxo-1,3-benzoxazine derivatives **5a–c**,

Table 2. Growth promotion of the catechol derivatives on Gram-negative bacteria with iron limitation. Diameter of growth zone (mm), substance application 5 μg.

Compound	P. aerug. ATCC 27853	P. aerug. SG 137	P. aerug. NTCC 10662	P. aerug. ATCC 9027	P. aerug K799/WT	E. coli ATCC 25922	CAS assay[c]
4a	24	23	22	25	23	25	++
4b	20	25	25	15	n.t.	24	+
4c	0	0	0	0	n.t.	0	+
5a	19	15	19	16	n.t.	15	+
5b	0	0	0	0	n.t.	0	−
5c	0	0	0	0	n.t.	0	−
6a	22	25	27	11	21	28	++
6b	19	15	20	15	13	20	+
7a	23	34	25	25	n.t.	29	++
7b	17	20	17	15	n.t.	27	++
7c	13	19	0	0	n.t.	12	+
7d	25	15	27	22	22	26	++
8	18	15	19	16	19	24	+
9	27	29	30	30	30	32	+
12a	18	19	23	17	20	19	−
12b	17	19	21	15	20	18	−
13a	25	35	25	30	27	29	+
13b	26	33	25	28	25	31	+
14	17	12	19	22	24	27	+
15	24	25	28	30	27	34	++
control	42[a]	30[a]	40[a]	32[a]	35[a]	33[b]	

[a] desferal, [b] ferricrocin (2 μg).
[c] – no CAS reaction, + weak CAS reaction, ++ strong CAS reaction.

in which the catechol moiety is masked as a heterocyclic structure. As basic triamines with hydrophilic or lipophilic groups we prepared D,L-11,12-dihydroxy-1,5,9-triaza-n-dodecane **1a** and 9-cyclohexyl-1,5,9-triaza-n-nonane **1b** from bis-(3-aminopropyl)-amine and D,L-glyceraldehyde or cyclohexanone, respectively, by hydrogenation with Pd/C (10%). From **1a** or **1b** and **3** we prepared analogously to **5** the 2,4-dioxo-1,3-benzoxazine-3-yl derivatives **6a** and **b** to investigate the influence of hydrophilic or lipophilic substituents on siderophore activities. Compounds **4, 5** or **6** were deprotected to the free catecholates **7a–d** by reaction with sodium hydroxide under nitrogen. The syntheses are outlined in Figure 1.

Catecholderivates of tris-(aminoethyl)-amine

In the same manner we prepared the tris-(2,4-dioxo-1,3-benzoxazine-3-yl) derivative **8** from tris-(2-aminoethyl)-amine **1f** and **3.** This compound was afforded by reaction with sodium hydroxide under nitrogen to the free catecholate **9** (figure 2), which was synthesized already by an other method and published without biological data (Rodgers *et al.* 1987).

Catechol derivatives with spacer groups

To synthesize analogs with spacer groups we used the (2,4-dioxo-1,3-benzoxazine-3-yl)-alkanoic acids **10a** (Wittmann *et al.* 2000) and **b** derived from glycine or β-alanine and **3**. The corresponding chlorides **11a** and **b** react with bis-(3-aminopropyl)-amine to give the 2,4-dioxo-1,3-benzoxazine-3-yl-derivatives **12a** and **b**, which were transformed to the free catecholates **13a** and **b** by aqueous sodium hydroxide under nitrogen.

Analogously we synthesized the compound **14** from tris-(2-aminoethyl)-amine **1f** and **11a.** Reaction with sodium hydroxide under nitrogen afforded compound **15**, respectively. A homologue compound was published (Shanzer *et al.* 1996; Tor *et al.* 1992) as enterobactin analogue with the known propeller conformation like enterobactin realized by H-bonds between the side chains. The same conformation can be assumed for compound **15**.

Table 3. Growth promotion of the catechol derivatives on test strains of mycobacteria. Diameter of growth zone (mm), substance application 5 μg.

Compound	SG 987	M10	mc^2 155	M24	B1	B3	U3
4a	20	20	0	0	18	0	0
4b	28	25	30	0	28	12	18
4c	24	23	26	0	24	22	17
5a	0	0	15	0	0	0	0
5b	0	0	15	0	12	0	0
5c	0	0	11	0	11	0	0
6a	15	0	15	0	17	0	0
6b	19	0	32	0	27	0	0
7a	24	22	25	22	25	0	0
7b	22	21	31	15	32	17	0
7c	20	17	25	13	23	12	14
7d	19	0	30	0	15	0	0
8	13	13	15	9	0	0	0
9	16	11	0	0	18	0	0
12a	15	14	14	0	10	0	0
12b	11	0	0	0	13	0	0
13a	18	0	0	0	12	0	0
13b	11	0	0	0	21	21	0
14	13	13	15	9	14	0	0
15	15	14	0	0	16	0	0
mycobactin (2μg)	15	15	14	16	15	15	16

Siderophore activity

The results of the growth promotion assays on indicator strains are given in Table 1. Most of the compounds were active on *P. aeruginosa* PAO 6609 and *M. morganii* SBK3 and to a lower extend or not active on the other test strains. Among the catecholates based on linear triamines **4–7** the derivatives **4a**, **5a** and **7a** (derivatives of 1,5,9-triazanonane) gave the best results, The derivatives **4b**, **5b** and **7b** of 1,8,15-triazapentadecane were slightly less active. Compounds **4c**, **5c** and **7c** with the longest basic chain, the derivatives of 1,10,19-triaza-nonadecane exhibited very low or no activity. Low differences were found for activities between the free catecholates **7a, b** and the acylated or heterocyclic masked catecholates **4a, b** and **5a, b.** However **5a** promoted bacterial growth significantly stronger than **5b.**

The compounds with additional hydrophilic or lipophilic substituents **6a, 6b** and **7d** were low or not active except the activity of **6a** on the strain *M. morganii* SBK3.

The activities of the tripodal derivatives **8** and **9** are comparable with the activities of the linear compounds. The used spacer groups increased the siderophore activities, especially the compounds **13a** and **b** strongly promoted most of the siderophore indicator strains. The tripodal compound **15** showed the highest activity on *M. morganii* SBK3.

Results of the growth promotion tests with bacterial wild type strains under iron limitation are given in Table 2. The compounds with longer alkyl chains show lower siderophore activities, especially the acetyl derivative **4c** + **7c**. The benzoxazine-dione derivatives **5b** and **5c** were not active. Highly active were compounds **4a** and **7a** with propylene chains and especially the compounds with spacer groups **13a** and **b** and the derivatives of tris-(aminoethyl)-amine **9** and **15** carrying free catechol groups. The compounds with additional hydrophilic or lipophilic substituents **6a, 6b** and **7d** showed in this test more activity then the basic substances **4a** and**7a**.

The results of growth promotion test on mycobacteria are given in Table 3. Here we found higher siderophore activities of compounds with longer alkyl chains, having free **(7b, c)** or acetylated catechol groups (**4b, c**), but no activities of compounds with benzoxazine groups **5a–c**. The more lipophilic com-

pounds **4b, 4c**, the compounds with free catechol groups, **7b**, **7c** and the compound **13b** with spacer group were active on the mutant B3 (exocheline -, mycobactine -) and **4b, 4c, 7c** also on the mutant U3 (mycobactine -, exocheline permease-) indicating a Fe-siderophore uptake independent on ligand exchange with exochelin/myco bactin or on exochelin permease. In conclusion compounds with more hydrophilic structures (shorter alkylene group as on **4a**, **7a**, **8**, **9**) and compounds with spacer groups. **12–15** were more active as siderophores on Gram-negative strains then on mycobacteria. Otherwise compounds with more hydrophobic structures (longer alkylene group as on **4b**, **4c**, **7b**, **7c**) or compounds with cyclohexyl substituent (**6b**, **7d**) were more active on mycobacteria then on Gram-negative strains.

In parallel to the growth promotion assays the relative iron complexing capacity of the siderophore derivatives was checked by the chromazurol-S (CAS) assay according to Schwyn & Neilands (1987), where a positive reaction is associated with iron chelation (Table 2). Most of the compounds, including the acylated ones **4a–c**, but not **5b, 5c, 12a** and **12b,** showed positive results in this test.

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