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Selective growth promotion and growth inhibition of Gram-negative and Gram-positive bacteria by synthetic siderophore-β-lactam conjugates

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Conjugates of a carbacephalosporin with hydroxamate, spermexatol, N^{α},N^{ϵ}-bis(2,3-dihydroxybenzoyl)-L-lysine, mixed catecholate/hydroxamate and cyanuric acid-based siderophores were investigated for their potential to promote growth of siderophore indicator strains of Gram-negative and Gram-positive bacteria under iron depleted conditions, for their antibacterial activity and for their ability to use iron transport pathways to penetrate the Gram-negative bacterial outer membrane. The selective growth promotion of enterobacterial and pseudomonas strains by hydroxamate, spermexatol and mixed catecholate–hydroxamate siderophore-based conjugates bearing a L- or D-amino acid spacer was correlated with TonB dependent uptake routes. The preferred outer membrane siderophore receptor used in *Escherichia coli* was found to be Fiu, followed by Cir. Antagonistic effects of siderophores administered with the conjugates to determine antibacterial activity confirmed the active transport of conjugates via siderophore receptors. All of the conjugates were still able to diffuse through the porin proteins OmpC and OmpF. Nevertheless, strong inhibition of *E. coli* and *Pseudomones aeruginosa* outer membrane mutants DC2 and K799/61 compared to the parent strains indicated inefficient penetrability of all types of conjugates tested. *Mycobacterium smegmatis* SG 987 was able to use all of the siderophore-cephalosporin conjugates as growth promotors. Consequently there was no growth inhibition of this strain.

Keywords: antibacterial activity, antibiotics, conjugates, growth promotion, outer membrane penetration, siderophores

Introduction

One of the strategies employed by bacteria to assimilate physiologically essential iron is the expression of siderophores and of the complementary uptake and transport systems. Siderophores chelate ferric iron. The Fe^{3+}-siderophore complex is taken up by a cascade of iron-regulated outer membrane proteins (IROMPs), the TonB protein, periplasmatic proteins and cytoplasmatic proteins (Winkelmann 1991, Wooldridge and Williams 1993). Recognition and transport of ferric-siderophores are more or less specific and effective for bacteria. Evidence suggests that the metal center is the key to microbial recognition, and receptor and transport proteins often do not require the full siderophore structure. The existence of albomycin and ferrimycin (Winkelmann *et al.* 1987) which incorporate both a siderophore component and a toxic agent, prompted attempts to

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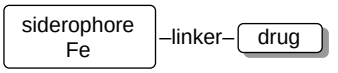
prepare mimics by conjugation of siderophores or siderophore components directly to antibacterial drugs. The aim of synthesizing siderophore–antibiotic conjugates is to circumvent the permeation barrier of the bacterial cell envelope by utilization of the ferric-siderophore transport pathways and to smuggle drugs into the cell by assistance of the siderophore–moiety. The general structure of these conjugates is as follows:



Although conceptually a variety of drugs can be incorporated into the conjugates, studies described here focus on β-lactam antibiotics (carbacephems indicated as R and R′ in the following structures) a relatively recent class of potent β-lactam antibiotics. The effect of siderophore-β-lactam conjugates depends on i) the presence of ferric-siderophore mediated transport routes into the bacterial cell, ii) binding of the β-lactam moiety to the penicillin binding proteins, iii) resistance against and circumventing the action of β-lactamases (which is supported by facilitated transport), iv) iron chelating capability of the siderophore moiety, its recognition by outer membrane receptors and transport by the iron-regulated membrane proteins. The last point might have a favorable effect on growth promotion or might additionally inhibit growth of the bacteria by complexation and withholding of iron from the cell. Depending on the balance of the influencing effects, siderophore-β-lactam conjugates could exert growth promoting as well as growth inhibiting activity.

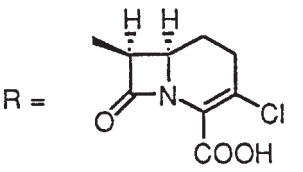
A number of siderophore–carbacephalosporin conjugates were synthesized and described earlier (Dolence *et al.* 1991a,b; McKee *et al.* 1991; Ghosh & Miller 1993; Miller & Malouin 1993; Ghosh & Miller 1994). Various types of these synthetic siderophores linked to carbacephalosporin directly or by phenylglycyl-type spacers (as known for Lorabid®) were selected and tested in this study for growth promotion in cross-feeding tests and for antibacterial activity by agar diffusion tests against a number of Gram-negative and Gram-positive bacteria.

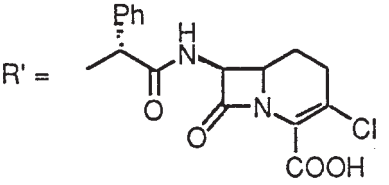
Materials and methods

Siderophore–carbacephalosporin conjugates

Five different groups of siderophore–carbacephalosporin conjugates are shown below.

β-lactams used:

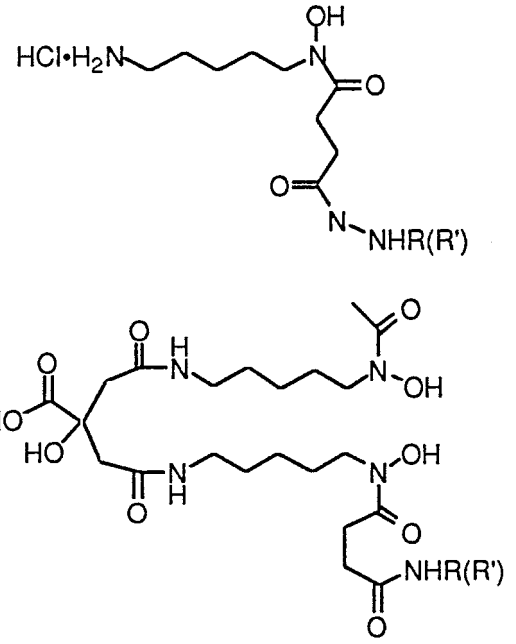




R = Carbacephalosporin

R′ = Carbacephalosporin with (**a**) D- or L-phenylglycine spacer, Ph = phenyl (D-shown) or (**b**) D- or L-*p*-hydroxyphenylglycine spacer, Ph = *p*-hydroxyphenyl

I. Hydroxamate-based siderophore–carbacephalosporin conjugates

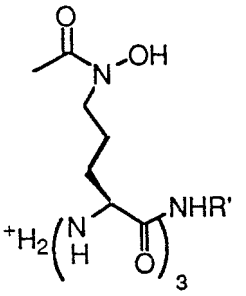


1 (R), identical with compound **44**
2 (R′ = **a**, D-isomer), identical with compound **45**

described by Ghosh & Miller 1993

3 (R), identical with compound **49**
4 (R′ = **a**, D-isomer), identical with compound **51**

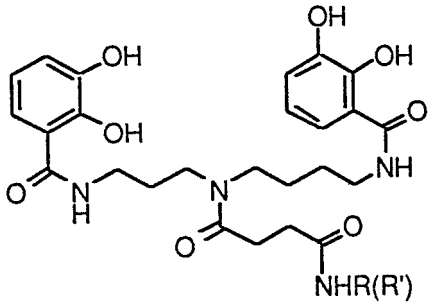
described by Ghosh & Miller 1993



5 (R′ = **a**, D-isomer), identical with compound **1**

described by Dolence *et al.* 1991a

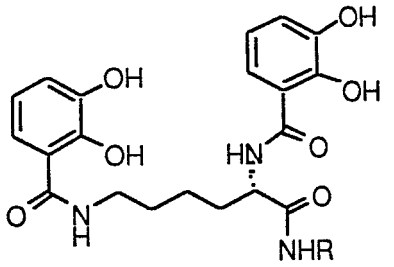
II. Spermexatol-based siderophore–carbacephalosporin conjugates



6 (R), identical with compound **37**
7 (R′ = **b**, L-isomer) identical with compound **37d**
8 (R′ = **b**, D-isomer) identical with compound **37c**
9 (R′ = **a**, L-isomer) identical with compound **37b**
10 (R′ = **a**, D-isomer) identical with compound **37a**

described by Miller & Malouin 1994

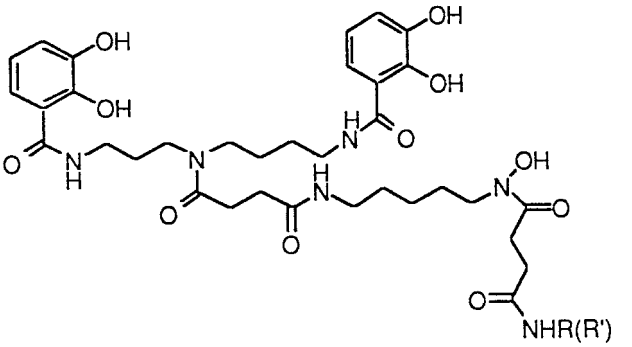
III. N^{α}, N^{ϵ}-bis(2,3-dihydroxybenzoyl)-L-lysine based carbacephalosporin conjugate



11 (R) identical with compound **7**

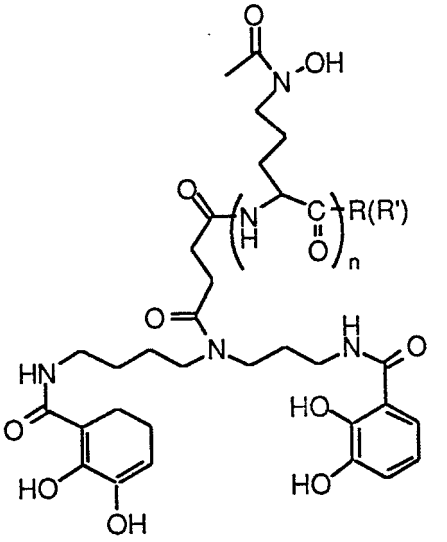
described by McKee *et al.* 1991

IV. Mixed hydroxamate-catecholate based siderophore–carbacephalosporin conjugates



12 (R) identical with compound **3a**
13 (R′ = **a**, D-isomer) identical with compound **3b**

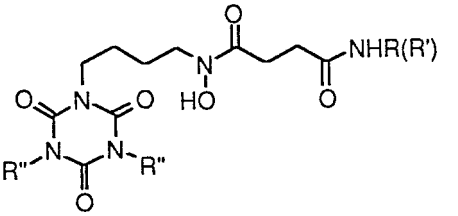
described by Ghosh *et al.* 1996



14 (R′ = **a**, D-isomer, $n = 1$) identical with compound **4a**
15 (R′ = **a**, D-isomer, $n = 3$) identical with compound **4b**

described by Ghosh *et al.* 1996

V. Cyanuric acid based siderophore–carbacephalosporin conjugates



R" = $-(CH_2)_4N(OH)COCH_3$

16 (R) identical with compound **20**
17 (R′ = **a**, D-isomer) identical with compound **21**

described by Ghosh & Miller 1994

Siderophore cross-feeding tests

Siderophore cross-feeding tests with Gram-negative bacteria and *Aureobacterium flavescens* JG-9 were performed as described by Reissbrodt *et al.* (1993). Siderophore cross-feeding tests with *Mycobacterium smegmatis* SG 987 were performed according to Reissbrodt *et al.* (1997). *Listeria monocytogenes* EGD was tested by use of a modified minimal medium of Welshimer 1963 containing (g l^{-1}) KH_2PO_4 (3.28); $Na_2HPO_4 \cdot 12H_2O$ (20.68); $MgSO_4$ (0.2) Glucose (10); Casamino Acids (Difco) (20.0); Agar (Oxoid) (10.0); Riboflavine (1 mg); Biotin (0.1 mg); Thiamine · HCl (1 mg). EDDHA 50 μM was added to obtain iron-deprived conditions. Filter paper discs were loaded with 5 μg each of the siderophore-β-lactam conjugates.

The evaluation of tests is indicated as: 0 = no growth; (+) = faint growth; + = growth zone < 15 mm; ++ = 15–20 mm; +++ = 21–29 mm; ++++ = ⩾ 30 mm; (lysis): inhibition zone, subsequent growth zone.

Antibacterial activity

Antibacterial activity of the compounds was determined by an agar diffusion test. The Gram-positive and Gram-negative test organisms used were from culture collections (*Bacillus subtilis* ATCC 6633, *Salmonella gallinarum* ATCC 9184) or from the stock of the institute (*M. smegmatis* SG 987 =HKI 0056; *Escherichia coli* SG 458). Penetration mutants and parent strains of *E. coli* DC2 and DC0 were described by Richmond *et al.* (1976), *Pseudomonas aeruginosa* K799/WT and K799/61 were from Zimmermann (1979). The siderophore indicator strains used are listed in Table 1.

Assay plates were prepared by suspending 10^6 CFU ml^{-1} of the test organisms in melted and tempered Mueller-Hinton Medium Difco). After solidification of the agar medium in petri dishes, wells of 9 mm in diameter were cut out and each was filled with 50 μl of the test sample at a concentration of 100 μg ml^{-1}. Inhibition zones were read after incubation at 37 °C for 18 h.

Influence of siderophores on antibacterial activity of siderophore–carbacephalosporin conjugates

P. aeruginosa K799/61 was suspended in Mueller-Hinton Agar as described above. According to the methods of Zähner (1960), dried filter paper strips loaded with 100 μg of 2,3- or 3,4-dihydroxybenzoic acid or 10 μg of ferricrocin as siderophores and 10 μg of the siderophore–carbacephalosporin conjugates **5.** and **13.**, respectively were placed cross-wise onto the solidified agar surface. After incubation overnight at 37 °C inhibition zones of growth were documented.

Results

The following types of siderophore–carbacephalosporins (see Materials and methods) were tested in cross-feeding tests against Gram-negative and Gram-positive siderophore-indicator strains:

Group	Type of siderophore–carbacephalosporins	Tested compounds
I.	Hydroxamate-based siderophore–carbacephalosporin conjugates	**1–5**
II.	Spermexatol-based siderophore–carbacephalosporin conjugates	**6–10**
III.	N^{α},N^{ϵ}-bis(2,3-dihydroxybenzoyl)-L-lysine carbacephalosporin conjugate	**11**
IV.	Mixed catecholate–hydroxamate based carbacephalosporin conjugates	**12–15**
V.	Cyanuric acid based siderophore–carbacephalosporin conjugates	**16–17**

Selective and unspecific growth promotion could be detected (Tables 2–5). The hydroxamate-type siderophore–carbacephalosporin conjugates (group I) strongly promoted growth of *K. pneumoniae* KN 4401, to a weaker extent *S. typhimurium* enb-7 and either did not or weakly promoted growth of the other Gram-negative bacteria tested. The group of spermexatol-based siderophore carbacephalosporins did not stimulate growth of *K. pneumoniae* KN 4401, *S. typhimurium* enb-7, the *E. coli* siderophore indicator strains, but very strongly promoted growth of *P. mirabilis* 12, *P. vulgaris* 718 and *M. morganii* SBK 3 (Tables 2, 4). Out of the compounds of group I, the tripeptide of N^5-acetyl-N^5-hydroxy-L-orthinine-containing hydroxamate-based siderophore–carbacephalosporin conjugate (**5**) strongly promoted growth of all of the Gram-negative bacteria (except *P. mirabilis* 12 and *M. morganii* SBK 3) and Gram-positive bacteria tested.

N^{α}-N^{ϵ}-bis (2,3-dihydroxybenzoyl)-L-lysine carbacephalosporin (**11**) strongly stimulated growth of all of the tested Gram-negative and Gram-positive bacteria except the *E. coli* strains. Growth of *Y. enterocolitica* H 5030 was promoted only weakly by all of the conjugates. Strong stimulation was demonstrated by compound **5** only. Among the group of mixed catecholate–hydroxamate-type siderophore–carbacephalosporin conjugates, compounds **12–14** did not promote growth of *S. typhimurium* enb-7 nor the *E. coli* strains, but strongly stimulated growth of *K. pneumoniae* KN 4401, *P. mirabilis* 12, *P. vulgaris* 718 and *M. morganii* SBK 3. Compound **15** of group IV strongly promoted growth of all the Gram-negative siderophore indicator strains except the TonB mutant *E. coli* IR112. Thus, growth promotion

Table 1. List of siderophore indicator strains, porin and receptor mutants

Indicator strain	Iron related marker	Detection of	Origin
E. coli H1443	aroB	enterobactin, (DHBS)[2,3,] DHBA, ferrichrome, coprogen, none of the ferrioxamines	K. Hantke (University of Tübingen, Germany)
E. coli IR112	aroB tonB	DHBA	V. Braun (University of Tübingen, Germany)
E. coli BR 158	aroB, tonB	DHBA	V. Braun (University of Tübingen, Germany)
E. coli H1876	aroB cir, fiu, fepA	ferrichrome, coprogen, not enterobactin, amonabactin	K. Hantke (University of Tübingen, Germany)
E. coli H1728	cir, fiu		K. Hantke (University of Tübingen, Germany)
E. coli H1877	fepA, fiu		
E. coli H1875	fepA, cir		
E. coli 41/2	fepA, cir, fhuA		
E. coli MS 172	fhuE		
E. coli HK 9/7	fhuA, fhuE		
E. coli KB4	ompF		K. Hantke (University of Tübingen, Germany)
E. coli KB5	ompC		
E. coli PLB3268	ompF overexpressed		
Salmonella typhimurium enb-7	ent (class II)	enterobactin, (DHBS)[2,3,] DHBA, ferrichrome, ferrioxamine and other hydroxamate-type siderophores not alcaligin, aerobactin	J. B. Neilands (University of California, Berkeley, CA, USA)
Klebsiella pneumoniae KN4401	ent, iuc	most of the phenolate-type and hydroxamate-type siderophores except amonabactins	P. Williams (University of Nottingham, UK)
Yersinia enterocolitica H5030	yb	yersiniabactin, ferrichrome, ferrioxamines, enterobactin	K. Hantke (University of Tübingen, Germany)
Proteus mirabilis 12	wild-type	α-keto acids; α-hydroxy acids, enterobactin, amonabactin, not ferrichrome or ferrioxamine	Robert Koch-Institute, Wernigerode
P. vulgaris 718	wild-type	similar to *P. mirabilis* 12	Robert Koch-Institute, Wernigerode
Morganella morganii SBK 3	wild-type	rhizoferrin, enterobactin, amonabactin, aerobactin, not ferrichrome or ferrioxamine	Robert Koch-Institute, Wernigerode

Table 1. Continued

Indicator strain	Iron related marker	Detection of	Origin
Pseudomonas aeruginosa PAO 6609	pvd	enterobactin, amonabactin, ferrichrome, ferrioxamine E, coprogen, pyoverdines	J.-M Meyer *et al.* (University of Strasbourg, France)
P. aeruginosa K437	pvd, pyo 90 kDa OMP (FpvA)	as the parent PAO 6609, uptake of pyoverdine diminished	K. Poole *et al.* (University of Kingston, Canada)
P. aeruginosa K407	pvd, 80 kDa OMP (PefA)	as the parent PAO 6609, uptake of enterobactin diminished	K. Poole *et al.* (University of Kingston, Canada)
P. aeruginosa 690	pvd, FpvA	as the parent PAO 6609, not pyoverdine	K. Poole *et al.* (University of Kingston, Canada)
Listeria monocytogenes EKD	wild-type	ferrioxamines ferrichrome	A. Bubert (Theodor Boveri-Institute of Biosciences, Würzburg, Germany)
Aureobacterium flavescens JG-9	hydroxamate siderophore auxotroph	hydroxamate type siderophores including alcaligin except aerobactin, nannochelin	P. J. Szaniszlo (University of Texas, Austin, TX, USA)

activity obviously requires the presence of active iron transport mechanisms.

The cyanuric acid-based siderophore–carbacephalosporin conjugates (group V) exhibited good growth promotion of *K. pneumoniae* KN 4401, *E. coli* H 1443 and *E. coli* H 1876 but not *E. coli* IR 112 and not or weakly for the other enterobacteriaceae and pseudomonads tested. Growth of *S. typhimurium* enb-7 was strongly promoted by cyanurate conjugate **16** but not by related conjugate **17**. Most of the siderophore–carbacephalosporins somewhat promoted growth of the *P. aeruginosa* siderophore-indicator strains. Compounds **5**, **11**, **13**, and **15** were superior to the other compounds. Growth of *M. smegmatis* SG 987 was strongly promoted by the siderophore–carbacephalosporins **5** to **17**, and, to a weaker extent, by compounds **1** to **4**. The siderophore conjugates only weakly stimulated growth of *L. monocytogenes* EGD (except for compound **5**). Growth of *A. flavescens* JG-9 was not stimulated by any of the siderophore–carbacephalosporins tested.

Table 2. Growth promotion of *S. typhimurium* and *E. coli* siderophore-indicator strains by siderophore–carbacepholosporin conjugates

Group	sidero-phore	*S. typhimurium* enb-7	*E. coli* H1443	*E. coli* IR112	*E. coli* H1876
I	**1**	++	+	0	+
	2	++	+	0	+
	3	++	+	0	+
	4	(lysis) ++	0	0	0
	5	++++	++++	0	++++
II	**6**	0	(+)	0	0
	7	(lysis) ++	0	0	0
	8	0	0	0	0
	9	0	0	0	0
	10	0	0	0	0
III	**11**	(lysis) ++++	(++)	0	0
IV	**12**	0	0	0	0
	13	0	0	0	0
	14	0	0	0	0
	15	(lysis) ++++	(lysis) ++++	0	(lysis) ++++
V	**16**	(lysis) ++++	(lysis) +++	0	(lysis) ++++
	17	0	(lysis) +++	0	(lysis) +++

The antibacterial activity of the siderophore–carbacephalosporin conjugates was checked with the compounds alone and in combination with sulbactam to demonstrate the influence of β-lactamases on activity. Wild-type strains of Gram-positive and Gram-negative bacteria, porin mutants of *E. coli* and penetration mutants of *E. coli* and *P. aeruginosa* were used (Tables 6 and 7). Most of the siderophore–carbacephalosporin conjugates exhibited significantly lower activity than Azlocillin, a ureido penicillin without an attached siderophore.

Table 3. Growth promotion of Gram-negative siderophore-indicator strains by siderophore–carbacephalosporin conjugates

Group	Siderophore	*K. pneumoniae* KN4401	*Y. enterocolitica* WA H 5030	P. mirabilis 12	*P. vulgaris* 718	*M. morganii* SBK 3
I	**1**	(++)	+	0	0	0
	2	+++	+	+	++	+
	3	+++	+	+	+	+
	4	+++	+	0	0	0
	5	+++	+++	0	++	0
II	**6**	0	+	+++	++++	++++
	7	0	+	++++	++++	++++
	8	0	+	(lysis) ++++	++++	++++
	9	0	+	(lysis) ++++	++++	++++
	10	0	+	(lysis) +++	++++	++++
III	**11**	++++	+	++++	++++	++++
IV	**12**	(lysis) ++++	+	(lysis) ++++	(lysis) ++++	++++
	13	(lysis) ++	+	(lysis) ++++	(lysis) ++++	++++
	14	(lysis) +++	+	++++	++++	+++
	15	++++	+	++++	++++	++++
V	**16**	(lysis ++++	0	+	+	+
	17	(lysis) ++++	+	0	0	0

Table 4 Growth promotion of *P. aeruginosa* siderophore-indicator strains by siderophore–carbacephalosporin conjugates

		P. aeruginosa			
Group	Siderophore	PAO 6609	K407	K437	690
I	**1**	+	(+)	+	(+)
	2	+	(+)	+	(+)
	3	+	(+)	+	(+)
	4	+	0	+	++
	5	+++	(++)	++	++
II	**6**	++	(+)	++	(+)
	7	++	(+)	++	++
	8	+	(++)	++	++
	9	++	(+)	++	+++
	10	++	(+)	++	+++
III	**11**	+++	(++)	+++	+++
IV	**12**	++	(+)	++	(++)
	13	+++	(++)	+++	(++)
	14	++	++	+++	++
	15	+++	+++	+++	++
V	**16**	++	+	+	+
	17	+	(+)	+	+

Table 5. Growth promotion of Gram-positive siderophore-indicator strains by siderophore–carbacephalosporin conjugates

Group	Siderophore	*M. smegmatis* 987	*L. monocytogenes* EGD
I	**1**	+	++
	2	+	+
	3	++	+
	4	++	0
	5	+++	+++
II	**6**	+++	0
	7	+++	++
	8	+++	++
	9	+++	+
	10	+++	0
III	**11**	++++	0
IV	**12**	+++	0
	13	++++	0
	14	+++	0
	15	+++	0
V	**16**	+++	++
	17	+++	+

Only compounds **8**, **10**, and **13** were comparable to azlocillin in susceptibility to *S. gallinarum* ATCC 9184. Addition of sulbactam slightly enhanced their activity. Of the Gram-positive bacteria tested, *M. smegmatis* SG 987 was not inhibited by any of the compounds including azlocillin (data not shown). *B. subtilis* ATCC 6633 was slightly inhibited by the compounds of group I (except compound **3**) and of group V. Addition of sulbactam slightly enhanced activity here too (Tables 6 and 7).

Compound **13**, the 7β-[[[[[N^5-[[N^1,N^8-bis[2,3-bis (hydroxy)benzoyl]spermidine-N^4yl]succinyl]-N^1-(hydroxy)-1,5-diaminopentyl]-N^1-yl]succinyl]-D-

Table 6. Antibacterial activity of the siderophore–carbacephalosporin conjugates against wild-type test strains and penetration mutants (inhibition zones in mm)

Group	Compounds	*B. subtilis* ATCC 6633	*S. gallinarum* ATCC 9184	*E. coli* SG 458	*E. coli* DC 0	*E. coli* DC 2	*P. aeruginosa* KW 799/WT	*P. aeruginosa* KW 799/61
I	**1**	10.5	0	0	0	0	0	A
	2	10.5	0	0	0	0	0	0
	3	0	0	0	0	0	0	0
	4	10.5	0	0	0	0	A24	28
	5	A	0	26.5P	0	30	0	24
II	**6**	0	A12	12P	0	0	0	12
	7	0	0	0	0	0	0	11.5
	8	0	21.5	16/21.5p	15.5P	24	13P	29
	9	0	0	11.5P	0	13.5P	0	18
	10	A11.5	22.5	17.5/22.5p	18	26	13P	30
III	**11**	0	0	0	0	0	0	0
IV	**12**	0	0	12A	0	0	A	13
	13	10	23.5	18.5	13	17	13P	22.5
	14	0	0	14P	0	0	15P	22
	15	0	0	0	0	0	0	21
V	**16**	10.5	0	0	0	0	0	0
	17	10.5	0	0	0	0	0	12A
control	Azlocillin	29	22.5	32	17.5	31	26	38

p: colonies within the inhibition zone
P: many colonies within the inhibition zone
A: slight indication of inhibition

Table 7. Antibacterial activity of the siderophore–carbacephalosporin conjugates in combination with 0.5 mM sulbactam (S) against wild-type test strains, penetrations mutants and porin mutants (inhibition zones in mm, abbreviations see Table 6)

Group	Compounds	*B. subtilis* ATCC 6633	*S. gallinarum* ATCC 9184	*E coli* DC 0	*E coli* DC 2	*P. aeruginosa* KW 799/WT	*P. aeruginosa* KW 799/61
I	**1** + S	12.5P	0	0	0	0	17p
	2 + S	15p	0	0	0	0	16p
	3 + S	0	0	0	0	0	0
	4 + S	13P	0	11.5P	0	A25	28
	5 + S	14p	13.5P	13.5P	33	0	26
II	**6** + S	0	15/17P	13P	17	0	18p
	8 + S	14.5	23/25P	16.5	24.5	23P	32
IV	**12** + S	12.5P	17.5	13p	16	A18	21
	13 + S	16	21.5	16.5	22.5	11/20P	29
	14 + S	0	12.5	12p	13.5	17P	24.5
	15 + S	0	0	0	0	0	20.5
V	**16** + S	12P	0	0	0	0	0
	17 + S	13P	12.5	0	0	0	16p
control	Azlocillin + S	32	25	25.5	32	39	26

phenylglycyl]amino]-1-carba-3-chloro-3-cephem-4-carboxylic acid, exhibited the broadest activity against both Gram-positive and Gram-negative bacteria tested. Compounds **1** to **3** of group I were devoid of antibacterial activity against Gram-negative bacteria. Only the porin mutant *E. coli* PLB 3268 and the *P. aeruginosa* penetration mutant KW 799/61 were inhibited moderately when combined with sulbactam. Generally the penetration mutants were considerably more sensitive than the complementary wild-type strains. The penetration mutant of *P. aeruginosa* K799/61 and the *E. coli* K-12 mutant PLB 3268 which over produces the OmpF porin protein were inhibited by nearly all of the

compounds tested (Table 7). Obviously, considering the activity against the penetration mutants *E. coli* DC2 and *P aeruginosa* KW799/61 in comparison with the wild-type strains DC0 and KW799/WT as well as with the *E. coli* porin protein mutants, the low activities of the conjugates, especially against *P. aeruginosa*, are due to inefficient penetration through the cell membrane.

As anticipated, based on the superb antibiotic activity of Lorabid®, which contains a D-phenylglycyl side chain, the same D-amino acid spacer in compounds **8**, **10** and **13** enhanced the antibacterial activity over compounds **7** and **9** containing the corresponding L-amino acid spacer.

Activity against the *E. coli* K-12 mutant H1443 with normal expression of outer membrane siderophore receptors (Table 8) is comparable to the activity against *E. coli* DC0 (Tables 6 and 7) and is decreased for some of the receptor mutants and the TonB mutant (Tables 2 and 8).

Testing the influence of siderophores on the antibacterial activity of siderophore–carbacephalosporin conjugates against *P. aeruginosa* K799/61, 3,4-dihydroxy benzoic acid induced a 'butterfly' formed inhibition of the antibacterial activity of compound **13**, but had no influence on the activity of compound **5**. However, the antibacterial activity of compound **5** was reduced by ferricrocin in an analogous study. Addition of 2,3-dihydroxybenzoic acid did not influence the antibacterial activity of either compound (Figure 1(a) and (b)).

Discussion

Most of the siderophore–carbacephalosporin conjugates tested in this study function as growth factors providing iron to bacterial cells under iron limited conditions. This is contradictory to the expected antibacterial effectiveness. All of the compounds promoted growth of *M. smegmatis* SG 987. The catecholate-type and cyanuric acid-type siderophore conjugates were especially effective growth stimulants for this organism. However, none of the conjugates inhibited growth of this strain. This could be due to β-lactamase activity together with slow penetration through the extraordinarily thick and hydrophobic cell envelope. Probably the compounds did not enter the mycobacterial cell. Iron might have been transferred into the cell by ligand exchange with exochelin and/or mycobactin (Ratledge & Marshall 1972) resulting in growth promoting effect.

The tripeptide N^5-acetyl-N^5-hydroxy-L-ornithine-D-phenylglycyl-carbacephalosporin (**5**) was the most powerful growth promoting agent for the Gram-negative and Gram-positive bacteria tested under iron restricted conditions. This compound was taken up via tonB exhibiting an active transport. As shown with *E. coli* H 1876 (Table 2), the siderophore effectiveness of compound **5**, as well as of the other hydroxamate-based siderophore–carbacephalosporins, was independent of the IROMPs Cir, Fiu and FepA, outer membrane receptors for catecholate-type siderophores. As indicated by

Table 8. Antibacterial activity of the siderophore–carbacephalosporin conjugates in combination with 0.5 mM sulbactam (S) against *E. coli* K-12 iron transport mutants (inhibition zone in mm, abbreviations see Table 6)

Group	Compounds	H 1443	H 1728	H 1875	H 1876	H 1877	BR 158	41/2	MS 172	HK 9/7
I	**1** + S	0	0	0	0	0	0	0	0	0
	2 + S	0	0	0	0	0	0	0	0	0
	3 + S	0	0	0	0	0	0	0	0	0
	4 + S	0	0	0	0	0	0	0	0	0
	5 + S	13	12.5p	13p	12.5p	12p	12.5P	12p	13p	12p
II	**6** + S	12	12P	12p	12P	12P	10/13P	11	10	12.5
	7 + S	–	–	–	–	–	–	12p	0	12.5
	8 + S	19	12P	18	12P	14	9.5	16	19	19.5
	9 + S	–	–	–	–	–	–	12p	13	14.5
	10 + S	–	–	–	–	–	–	17	20	20.5
III	**11** + S	–	–	–	–	–	–	0	0	12p
IV	**12** + S	14	0	14.5	0	11	0	13	14	14.5
	13 + S	18	11.5	17	12P	13.5	11.5	15.5	16	17.5
	14 + S	13.5	0	14	0	0	0	12p	11.5	12
	15 + S	0	0	0	0	0	0	0	0	0
V	**16** + S	0	0	0	0	0	9.5	0	0	0
	17 + S	0	0	0	0	0	10	0	0	0
control	Azlocillin + S	25	23	25	24.5	24	23.5	20	19.5	20.5

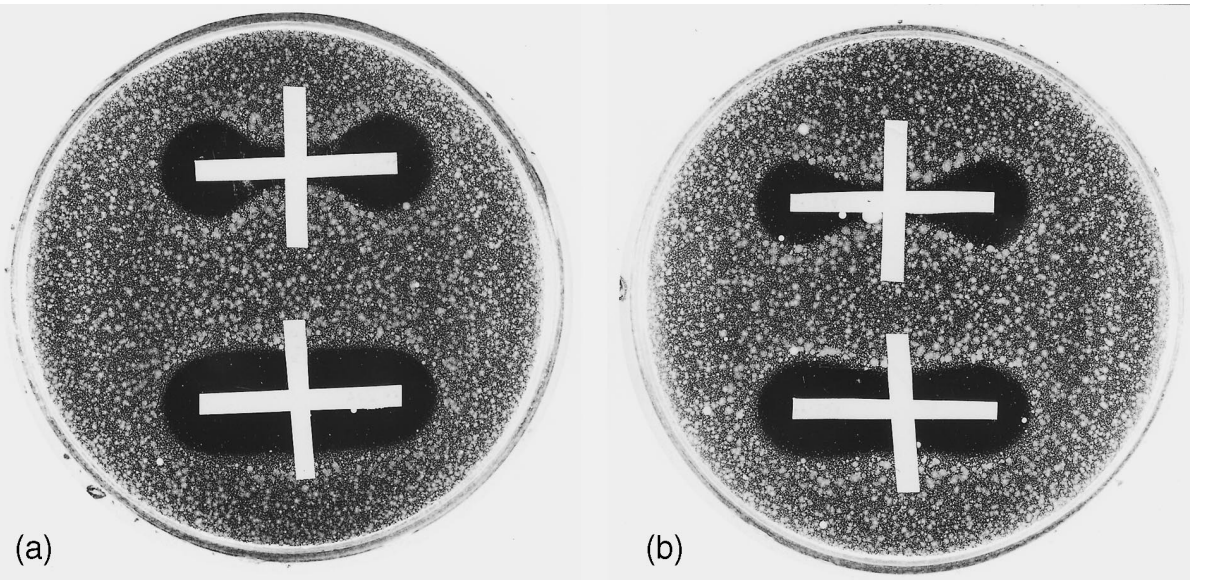


Figure 1. Agar diffusion test of the influence of siderophores on the antibacterial activity of compounds (a) **13** and (**b**) **5**. Horizontal filter paper strips contain the siderophore–carbacephalosporin conjugates, vertical filter paper strips were loaded with 3,4-dihydroxybenzoic acid (top) (**a**) or with ferricrocin (**b**) and 2,3-dihydroxybenzoic acid (bottom).

P. aeruginosa K437 and 690 (Table 4) uptake of compound **5** also seems to be independent of the pyoverdin receptor FpvA. The siderophore moiety of compound **5**, the tripeptide hydroxamate, as well as its derivatives, where D-phenylglycyl and *p*-hydroxyphenylglycyl amino acid residues were incorporated, strongly promoted growth of *S. flexneri* SA240 (SA iucD:Tn5) (Dolence *et al.* 1991a). Compound **5** was anticipated to be transported by the ferrichrome hydroxamate iron transport system (FhuA functioning as receptor). It showed a significant delay in onset of growth of the β-lactam hypersensitive strain *E. coli* X580 (Dolence *et al.* 1991b). Interestingly, the tripeptide hydroxamate moiety with the incorporated D-phenylglycyl spacer linked to oxamajin exhibited growth promoting activity to *S. flexneri* SA100 (Dolence *et al.* 1991b).

The use of *E. coli* mutants, missing receptors for hydroxamate-type siderophores, to detect uptake routes for the hydroxamate-based siderophore–carbacepholosporin conjugates was not successful. This might have been due to the specificity in recognition of hydroxamates and to the lower target activity of the conjugates in *E. coli* compared to *P. aeruginosa* as demonstrated by the inhibition of the penetration mutant DC2 and K799/61, respectively.

The hydroxamate-based siderophore–carbacephalosporin conjugates **1**–**4** exhibited only weak growth promoting activity, except of *K. pneumoniae* KN 4401, and very poor growth inhibitory activity overall. They weakly inhibited the penetration mutants of *E. coli* and *P. aeruginosa* only. Compounds **3** and **4**, when incubated with *E. coli* X580 in Luria broth, caused delayed growth, even though this media is iron sufficient. Compound **4** appeared to be a more effective growth inhibitor, perhaps because it contains the phenylglycyl-spacer of Lorabid® (Ghosh & Miller 1993).

The N^{α},N^{ϵ}-*bis*(2,3-dihydroxybenzoyl)-L-lysine carbacephalosporine (**11**) was also found to be a powerful siderophore for most of the bacteria tested except for *L. monocytogenes*. The lysine-based siderophore moiety was isolated from *Azotobacter vinelandii* and its siderophore activity is known (Corbin & Bulen 1969). Nevertheless, due to its very poor inhibitory effect (Table 6, 8) this compound's access to the target PBPs seemed to be hindered.

Among the enterobacteriaceae tested, the spermexatol-based siderophore–carbacephalosporin conjugates (group II) functioned as selective growth factors of the *Proteus-Morganella* group. The conjugates of group II did not promote growth of *E. coli* and *S. typhimurium* under iron limited conditions but were antibacterially active. The compounds with the D-configuration of the phenylglycine spacer (**8**) or a D-4-hydroxyphenylglycyl-spacer (**10**) were considerably more active than the compounds with the L-configuration. The antibacterial effectivity of those conjugates on *E. coli* X580 also had been shown by Miller & Malouin (1994).

The mixed catecholate–hydroxamate-based carbacephalosporin conjugates **12** and **13**, containing the 2,3-bis-hydroxybenzoyl spermidine residue, functioned as poor growth factors for *E. coli* and *S. typhimurium* but were more effective for *K. pneumoniae*, and *Proteus-Morganella* bacteria and the pseudomonads. N^4-Substituted spermidines with 2,3-dihydroxybenzoyl groups also were found to be weak siderophores for these bacteria (Reissbrodt *et al*. 1997). With the exception of compound **13**, which also contains a D-phenylglycyl spacer, the inhibitory activity of these conjugates was found to be low. Enhancement of the antibiotic effectiveness of compound **13** by combination with sulbactam suggested that it is a well transported conjugate with good access to the target PBPs (Tables 6, 7, 8).

Neutralization of antibacterial activity of conjugate **13** by 3,4-dihydroxybenzoic acid (Figure 1a) and of conjugate **5** by ferricrocin (Figure 1b) suggested utilization of an iron uptake mechanism facilitated by the siderophore moiety of the compounds. 3,4-Dihydroxybenzoic acid is known to supply *P. aeruginosa* with iron in contrast to 2,3-dihydroxybenzoic acid which did not stimulate growth (Reissbrodt *et al*. 1993). The ability of 3,4-dihydroxybenzoic acid and ferricrocin to facilitate iron acquisition by *P. aeruginosa* is competitive with iron supplementation by the siderophore–carbacephalosporin conjugates. This could be, at least in part, a competition at the cell surface receptor level, as conjugate **5** and other conjugates with hydroxamate-based siderophore moieties were not antagonized in their antibacterial activity by the catecholate type siderophore 3,4-dihydroxybenzoic acid or by enterobactin (data not shown) but by the hydroxamate-type siderophore ferricrocin. This is in agreement with the conclusion of Dolence *et al*. (1991b) that compound **5** is taken up by the ferricrocin receptor fhuA. On the other hand, conjugates with catecholate or mixed-type siderophores were strongly antagonized by 3,4-dihydroxybenzoic acid.

Cyanuric acid-based siderophore conjugates were broadly acting growth factors that were especially effective for *S. typhimurium*, *E. coli*, *K. pneumoniae* and *M. smegmatis*. No significant antibacterial activity was seen for compounds of group V by the agar diffusion method. These compounds have been shown to cause delayed growth of approximately 5 h (**16**) and 10 h (**17**) of the hypersensitive *E. coli* X580 in Luria broth compared to the control. No growth was seen of *E. coli* X850 with compounds **16** and **17** in EDDHA (100 μg ml^{-1}) supplemented iron deficient culture medium over 30 h (Ghosh & Miller 1994).

The IROMPs Cir and Fiu as well as TonB are involved in uptake and with it in activity of the spermexatol-based conjugates and the mixed catecholate-hydroxamate-based conjugates as exhibited by the decreased activity against the cir, fiu double mutants H1728 and H1876, the TonB mutant BR 158, the cir mutants H1875 and 41/2 and the fiu mutant H1877. The difference in sensitivity of H1877 and H1875 indicated a preferred recognition and uptake of the conjugates by Fiu.

Colonies grown in inhibition zones (Tables 6, 7, 8) suggested the formation of spontaneous mutants. This property was previously described for siderophore-antibiotic conjugates (e.g. Watanabe *et al* 1987). Most often, these mutants were recognized as TonB mutants or mutants of other iron related markers. They were characterized by a loss of virulence and by decreased viability.

This study gives evidence for active transport of some of the siderophore–carbacephalosporin conjugates via TonB dependent uptake routes. This is a principle strategy to overcome bacterial membrane-mediated resistance mechanisms. Further investigations will be necessary to optimize structure-activity relationships.

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