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Mapping of the Modular Closthioamide Architecture Reveals Crucial Motifs of Polythioamide Antibiotics

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Abstract: Closthioamide, the first known secondary metabolite from an anaerobic microorganism (*Clostridium cellulolyticum*), represents a highly potent antibiotic that is active against methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Enterococcus faecalis* (VRE) at nanomolar concentrations. To unveil structure–activity relationships of the unusual polythioamide natural product we have designed a synthetic grid to access analogues with altered terminal aromatic moieties, diverse *p*-phenyl substituents, different types and sizes of aliphatic spacers, varying numbers of thioamide residues, and diverse sizes and symmetries of the poly-β-thioalanyl backbone. A library of 28 closthioamide analogues was tested against a panel of human pathogenic bacteria. We found that aromatic terminal groups, the defined length of the spacer groups, the presence of all six thioamide residues and the modular arrangement of the β-thioalanyl units play essential roles for the antibiotic activity of closthioamide, yet there is a degree of freedom in the symmetry of the molecule. This study yields the first insights into pivotal structural motifs and the structural space of this new family of antibiotics, a prerequisite for the development of these promising antibiotics.

Introduction

The dramatic emergence and dissemination of resistant bacterial pathogens during the past decades is one of the most challenging problems in current healthcare.[1] Indeed, alarming numbers of nosocomial and community-acquired infections caused by multidrug-resistant bacteria and the lack of novel antimicrobial agents in the drug discovery pipelines could indicate a beginning second pre-antibiotic era.[2] Despite the urgent need for new antibiotics, only a small number of antibacterials has been approved as therapeutics, and practically all of them share their scaffolds with established pharmaceuticals.[3] This is particularly worrying as in the battle against the rapid resistance development, new antibiotics with novel architectures and different modes of action are urgently needed. Whereas natural products are still the major source of inspiration,[3] screening for new leads is hampered by frustratingly high rediscovery rates. A promising approach to bypass this problem is the genomics-guided search for natural products in yet unexplored organisms.[4] With the advent of full genome sequencing the overlooked biosynthetic potential of strictly anaerobic bacteria, for example, *Clostridium* species, has become evident.[4c] However, all biosynthesis genes investigated to date were silent under laboratory conditions and apparently need to be triggered by specific signals.[5] Addition of an aqueous soil extract to *C. cellulolyticum* provides the cues for pathway activation and led to the discovery of the first antibiotic, closthioamide (**1 a**, CTA, see Figure 1) from an anaerobe.

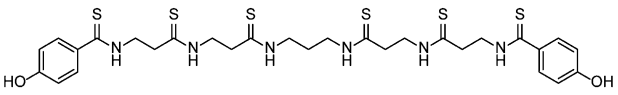

Figure 1. Structure of closthioamide (CTA, **1 a**).

Closthioamide features a symmetric linear backbone linked by six thioamide moieties, an entity that is, although well established in diverse fields of medicinal chemistry,[6] extremely scarce among natural products.[7] Moreover, CTA shows potent antimicrobial activity against infamous pathogens such as methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Enterococcus faecalis* (VRE) at nanomolar concentrations.[7,8] Thus, the chemical structure of CTA is not only fully unprecedented, but also represents a novel, yet unexplored class of antibiotics.

Using convergent peptide coupling and polythionation techniques we designed a highly efficient synthetic route towards CTA, which confirmed the potent antibiotic activity.[8] Furthermore, genetic engineering of the producer strain allowed for the isolation of seven congeners of CTA that enabled first insights into the importance of the number of thioamide moiet-

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ies and the overall size of the molecule for the antibacterial activity.[9] We also found that CTA specifically chelates Cu^{I} ions in a compact and symmetric manner involving all six thioamide moieties in the metal complex.[10] Yet, insights into the structural requirements for antibacterial activity have remained elusive. Here we report the first systematic study to unveil structural features of polythioamides that are crucial for antibacterial activity.

Results and Discussion

With the modular structure and the convergent synthetic approach at hand, we mapped the structure of CTA to reveal residues that could have an impact on antibacterial activity. Specifically, we aimed to elucidate the role of: 1) the terminal aromatic substituents, 2) the *p*-phenyl substituents, 3) the type and size of aliphatic spacers, 4) the number of thioamide residues, and 5) the size and symmetry of the poly-β-thioalanyl backbone (Figure 2). To tackle this ambitious goal we synthesized 28 closthioamide derivatives, analogues and homologues using a modular approach (Scheme 1, Table 1).

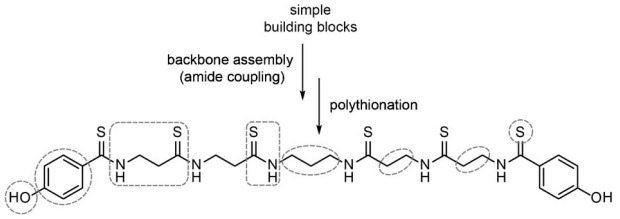


Figure 2. General synthetic approach to access CTA derivatives and sites of structural diversification (boxed areas).

Taking advantage of the established polythionation approach, we designed a synthetic grid that allows the flexible design of polythioamides. In brief, using peptide synthesis techniques, diamines **11a**, **b** were coupled with *N*-Boc protected amino acids (**10a**, **b**) to afford dicarbamates **9a**–**c**. Terminal groups were introduced as carboxylic acids **4a**–**d** (Scheme 1, right) or as 3-amidopropanoic/4-amidobutanoic acids (**5a**–**f**, Scheme 1, middle), obtained from coupling of β-alanine benzylester or benzyl γ-aminobutyrate with carboxylic acids and subsequent hydrogenolysis (see Supporting Information). After deprotection, coupling of diamides **9a**′–**c**′ with **5a**–**f** or of the homologue tetraamide **8**′ with **4a**–**d** or **5f** afforded closamide derivatives **3b**–**h** and **3n**–**t** (for selected precursor pairs see Table 1). Thionation with Lawesson's reagent[11] in pyridine gave the corresponding thioamides (**2b**–**h**, **n**–**t**). Deprotection of the *O*-cyclohexylated products (**2n**, **o**, **p**, **s**) with trifluoromethanesulfonic acid (TFMSA) in trifluoroacetic acid (TFA)[8] and of the *O*-*tert*-butylated derivatives (**2f**, **r**, **t**) with cerium(III) chloride and sodium iodide[12] yielded phenolic closthioamide derivatives (**1n**–**t**) and the aliphatic alcohol **1f**. The benzylamine derivative **1g** was synthesized through deprotection of the corresponding *tert*-butyl carbamate **2g** with hydrogen chloride in dry dioxane. All derivatives were tested in vitro for antibacterial activities and antiproliferative and cytotoxic effects according to standardized procedures.

Table 1. Library of synthesized symmetrically substituted closthioamide derivatives, analogues and precursors (see Scheme 1).

Series		Precursor pair		A	R[a]	*m*	*n*	*r*	*s*
2,3	**b**	**9a**	**5a**	-$(CH_2)_3$-	phenyl	2	2	1	1
2,3	**c**	**9a**	**5b**	-$(CH_2)_3$-	3-pyridyl	2	2	1	1
2,3	**d**	**9a**	**5c**	-$(CH_2)_3$-	4-pyridyl	2	2	1	1
2,3	**e**	**8**	**4a**	-$(CH_2)_3$-	4-oxazolyl	2	2	1	1
1–3	**f**	**8**	**4d**	-$(CH_2)_3$-	-$(CH_2)_2$-OH	2	2	1	1
1–3	**g**	**8**	**4c**	-$(CH_2)_3$-	4-(NH_2-CH_2)-ph·HCl	2	2	1	1
2,3	**h**	**8**	**4b**	-$(CH_2)_3$-	4-MeO-ph	2	2	1	1
1–3	**n**	**9b**	**5d**	-$(CH_2)_2$-	4-OH-ph	2	2	1	1
1–3	**o**	**9c**	**5d**	-$(CH_2)_3$-	4-OH-ph	3	2	1	1
1–3	**p**	**9a**	**5e**	-$(CH_2)_3$-	4-OH-ph	2	3	1	1
1–3	**r**	**11a**	**7a**	-$(CH_2)_3$-	4-OH-ph	–	–	0	0
1–3	**s**	**11a**	**5d**	-$(CH_2)_3$-	4-OH-ph	2	–	0	1
1–3	**t**	**8**	**5f**	-$(CH_2)_3$-	4-OH-ph	2	2	2	1

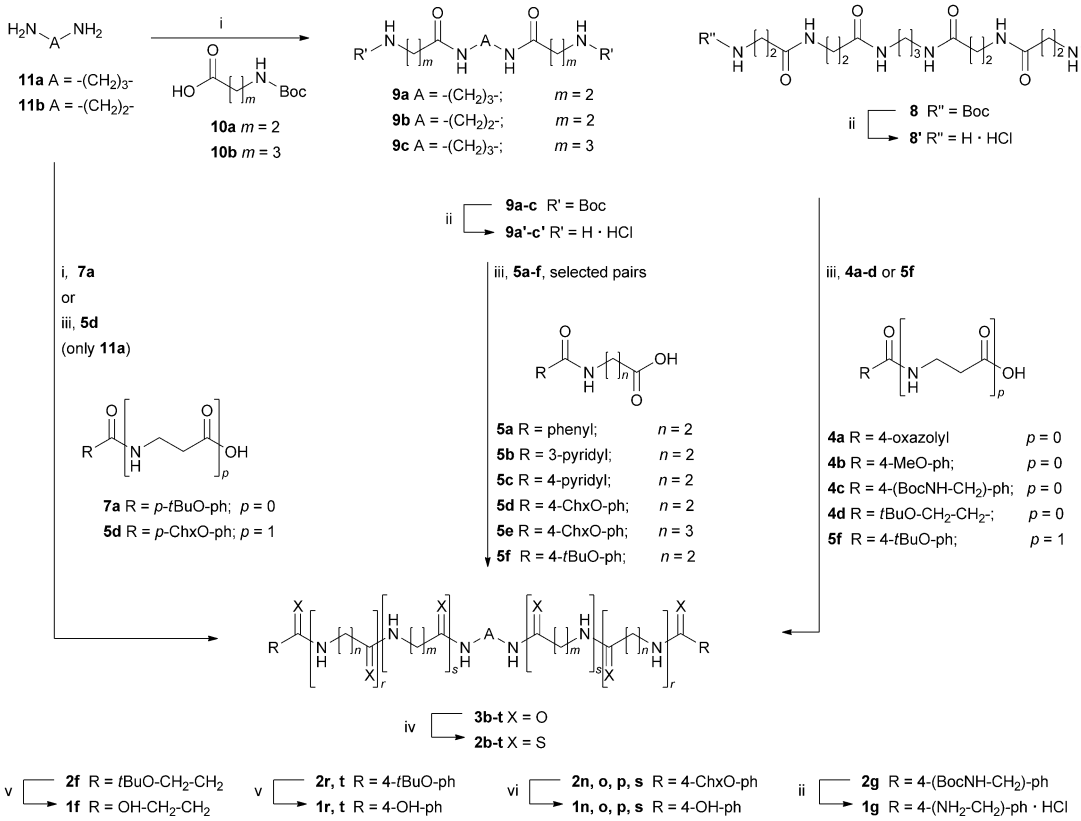
[a] Target residue; ph = phenylene; for experimental details see the Supporting Information.

Importance of the terminal aromatic substituents

To evaluate the impact of the terminal aromatic substituents, a focused library of derivatives bearing phenyl, heterocyclic and aliphatic moieties was synthesized (Table 2, entries 2–7). All derivatives were less potent than CTA. However, the least affected candidate was the phenyl derivative (**2b**) with submicromolar activities against the Gram-positive test strains. The pyridyl substituted derivatives **2c** and **2d**, showed reduced activities, and the 4-oxazolyl (**2e**) and aliphatic analogues (**2f**, **1f**) were nearly inactive in the assays. These findings indicate that the terminal six-membered aromatic rings are crucial. In particular a phenolic group confers stronger antibacterial activities than heterocycles or aliphatics. However, we found that the *p*-hydroxyl substituent in CTA is not totally essential for antibacterial activity.

The impact of the *p*-phenyl substituent

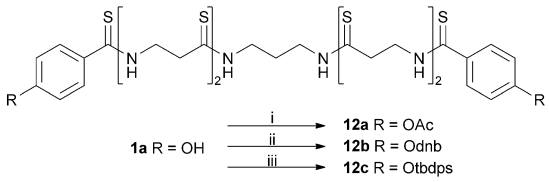
Since the *p*-hydroxyl moiety of CTA strongly contributes to the antimicrobial activity, we reasoned that either steric, polar or electronic effects are responsible for the increased activity. In order to pinpoint the type of effects, we introduced a series of substituents including ethers and esters into the CTA architecture (Table 2, entries 8–18). We either incorporated the sub-



Scheme 1. Modular approach for the synthesis of symmetric closthioamide derivatives and analogues. Reagents: i) EDC, HOBt, DMF; ii) dioxane/HCl; iii) EDC, HOBt, Hünig's base, DMF; iv) Lawesson's reagent, pyridine; v) $CeCl_3$, NaI, acetonitrile; vi) TFMSA, TFA; ph = phenylene; see Table 1 (for more details see Supporting Information).

stituents before thionation or employed formal semisynthetic strategies starting from CTA (**1a**), taking advantage of the moderate reactivity of both phenolic groups. Derivatives **12a–d** were readily available using modified standard procedures for the derivatization of phenols to afford esters **12a**, **b** or silyl ether **12d** (Scheme 2).

When sterically demanding substituents (*tert*-butyl, cyclohexyl, tbdms, tbdps) were installed, we observed a complete loss of activity (Table 2, compounds **2g**, **k**, **m**, **12c**, **d**). A plausible explanation would be reduced membrane permeability. Monodeprotection of the *tert*-butyl analogue **2k** afforded asymmetric mono-*tert*-butyl derivative **1k**, which showed good activity against *S. aureus* and *E. faecium* similar to the phenyl derivative **2b**. To further evaluate the effect of polarity and steric demand, the methylether (**2h**), fluoro-analogue (**2i**) and acetate (**12a**) were synthesized and tested. Even in the sub-micromolar range, all three derivatives were active against the Gram-positive test strains. The methyl ether (**2h**) showed very good activity against *S. aureus* and *E. faecium* and even better activity against *E. coli* than CTA. Furthermore, the methyl ether showed drastically increased antiproliferative activity. In contrast, the more polar benzylamine derivative **1g** was only moderately active. We therefore concluded that small, electron-donating *p*-substituents promote the antimicrobial activity, and that a hydroxyl group is not strictly required.

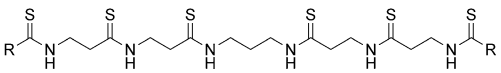


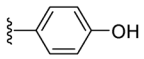
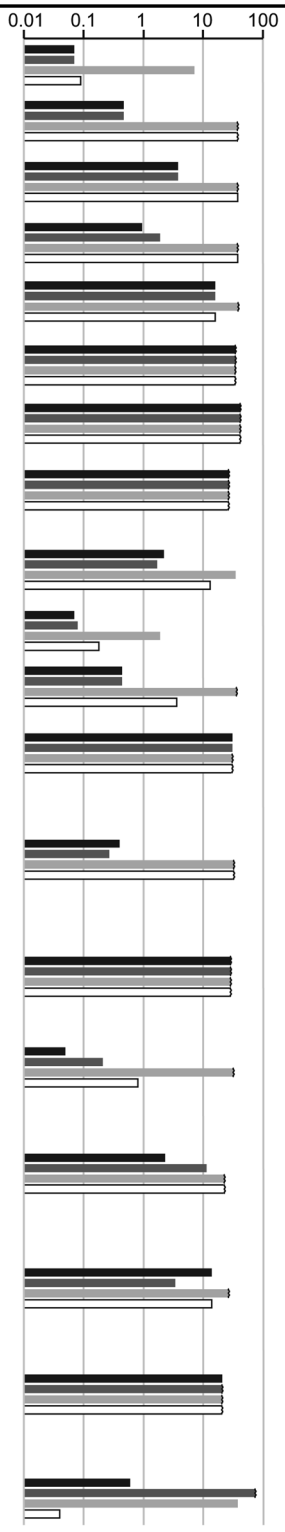
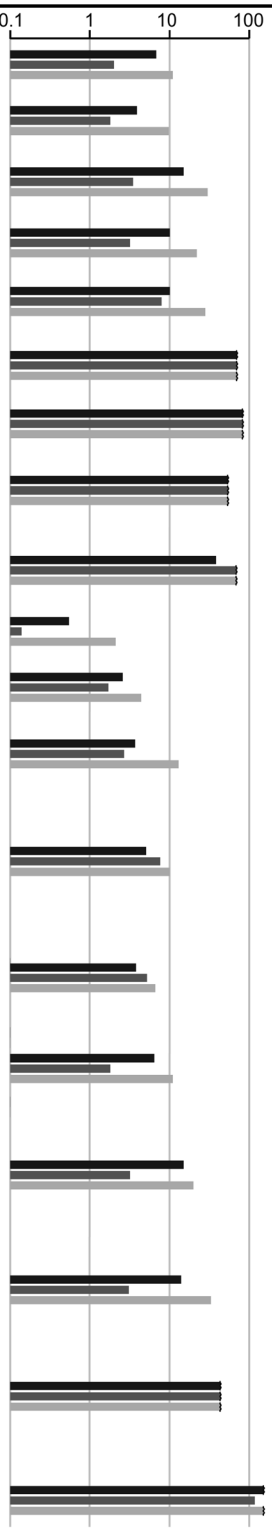
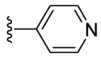
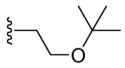
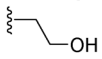
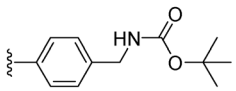
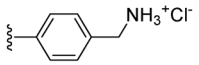
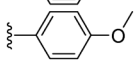
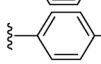
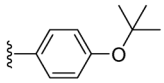
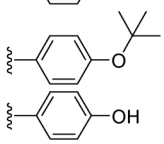
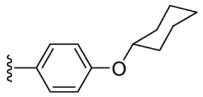
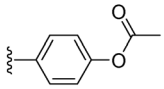
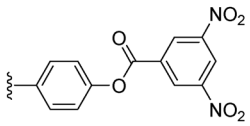
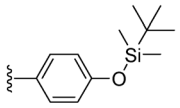
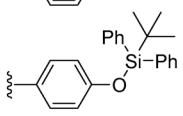
Scheme 2. Semisynthetic approaches for phenolic CTA derivatives. Reagents: i) Ac_2O, DMAP, pyridine; ii) 3,5-dinitrobenzoyl chloride, Hünig's base, acetonitrile; iii) tbdpsCl, imidazole, DMF; dnb = 3,5-dinitrobenzoyl (for more details see the Supporting Information).

Importance of the aliphatic spacers

Derivatives with a modified aliphatic backbone were synthesized using the corresponding diamides **9b**, **c** and carboxylic precursors **5d**, **e** as described above. β-Thioalanyl moieties were replaced for γ-thioaminobutyryl moieties, and the diaminopropane bridge was substituted by ethylenediamine. These modifications (see derivatives **1n–p**) resulted in a dramatic decrease or even complete loss of antibacterial activity. Thus, we concluded that the length of each aliphatic spacer group plays a pivotal role for antibiotic activity (Table 3).

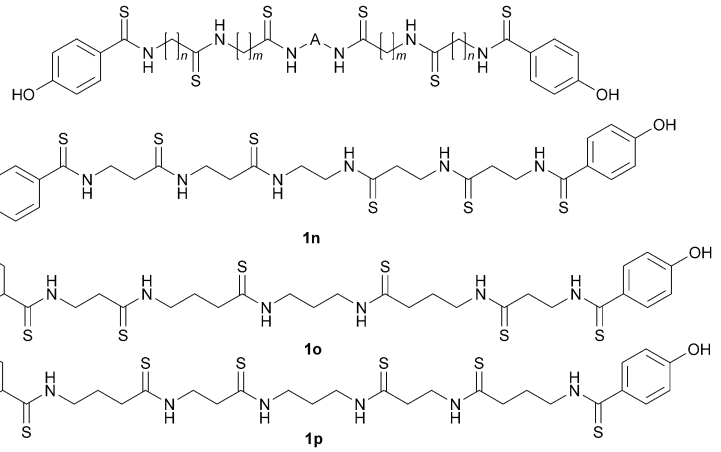
Table 2. Influence of the terminal moieties on the corresponding bioactivity values.



Entry	Compound	R	Antimicrobial activity (MIC [μM]) ■ *S. aureus* ■ *E. faecium* ■ *E. coli* □ *E. coli*[a]	Antiproliferative/cytotoxic activity (GI_{50}/CC_{50} [μM]) ■ HUVEC ■ K562 ■ HeLa
1	**1a** (CTA)			
2	**2b**			
3	**2c**			
4	**2d**			
5	**2e**			
6	**2f**			
7	**1f**			
8	**2g**			
9	**1g**			
10	**2h**			
11	**2i**[9]			
12	**2k**[10]			
13	**1k**[b]			
14	**2m**[8]			
15	**12a**			
16	**12b**			
17	**12c**[10]			
18	**12d**			
19	Cip			

[a] Drugs efflux system deficient strain; [b] mono-deprotected (for experimental details see the Supporting Information); Cip = ciprofloxacin.

Table 3. Influence of the aliphatic spacer moieties on the corresponding bioactivity values.

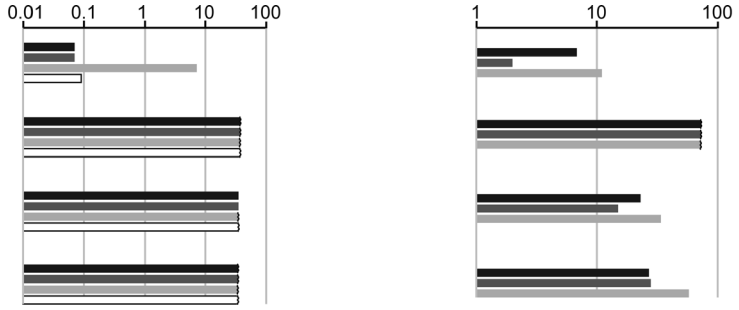


1n

1o

1p

Compound	A	*m*	*n*	Antimicrobial activity (MIC [μM]) ■ *S. aureus* ■ *E. faecium* ■ *E. coli* □ *E. coli*[a]	Antiproliferative/cytotoxic activity (GI_{50}/CC_{50} [μM]) ■ HUVEC ■ K562 ■ HeLa
1a	-$(CH_2)_3$-	2	2		
1n	-$(CH_2)_2$-	2	2		
1o	-$(CH_2)_3$-	3	2		
1p	-$(CH_2)_3$-	2	3		



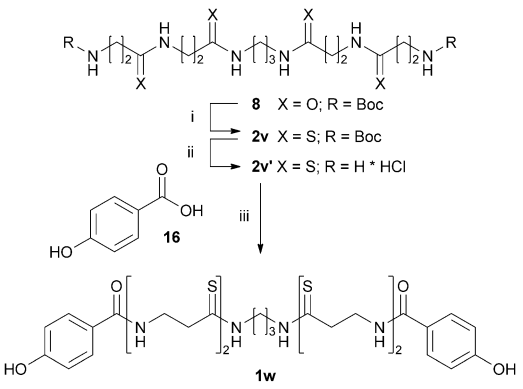
[a] Drugs efflux system deficient strain.

Influence of the number of thioamide residues

Partial hydrolysis of CTA in *C. cellulolyticum* cultures provided two derivatives (closthioamide B and C) each with one internal β-thioalanyl sulfur replaced by oxygen.[9] These analogues showed drastically reduced antibacterial activity compared to CTA (i.e., one order of magnitude per thioamide moiety).[9] To investigate whether the terminal (aromatic) thioamide functionalities equally contribute to the antibacterial bioactivity, we synthesized the symmetric partially thionated closthioamide analogue **1w** (Scheme 3).

Tetraamide **8** was efficiently transformed into the polythioamide **2v** using our established general thionation protocol.

As for derivative **2g**, careful handling of the acid-sensitive thioamides was crucial for the selective removal of Boc residues. After successful deprotection in dry dioxane/hydrogen chloride, **2v′** was coupled with *p*-hydroxybenzoic acid (**16**) to afford closthioamide analogue **1w**. Like closthioamides B and C, analogue **1w** showed only weak antibiotic activity. Taken together, these data suggest a cooperative effect of all six thioamide functionalities of CTA (Table 4).

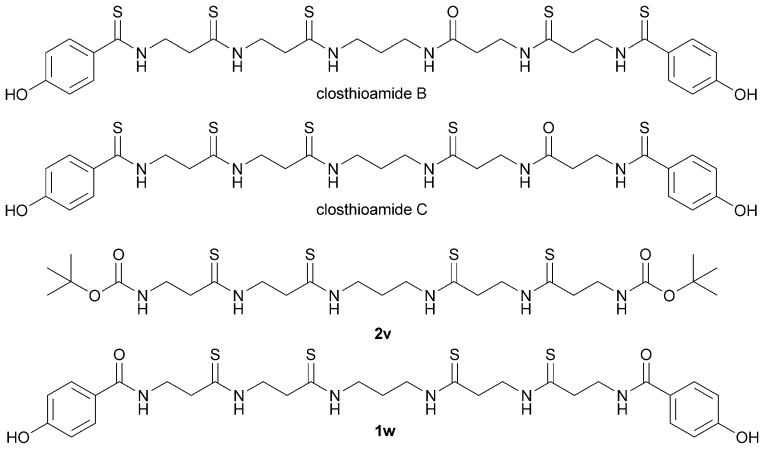


Scheme 3. Synthesis of partially thionated closthioamide **1w**. Reagents: i) Lawesson's reagent, pyridine; ii) dioxane/HCl; iii) EDC, HOBt, Hünig's base, DMF (for more details see Supporting Information).

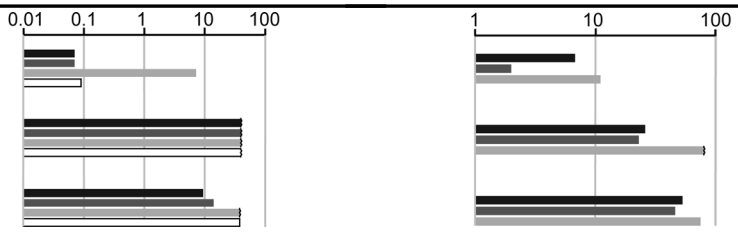
Impact of the size of the poly-β-thioalanyl backbone

The presence of β-thioalanyl moieties and six intact thioamides proved to be a prerequisite for submicromolar antimicrobial activities of CTA. However, it remained to be clarified whether or not the size and symmetry of the poly-β-thioalanyl back-

Table 4. Influence of the position of thioamide functionalities on the corresponding bioactivity values.



Compound	ΣC=S	ΣC=O	Antimicrobial activity (MIC [μM])	Antiproliferative/cytotoxic activity (GI_{50}/CC_{50} [μM])
			■ *S. aureus* ■ *E. faecium* ■ *E. coli* □ *E. coli*[a]	■ HUVEC ■ K562 ■ HeLa
1a	6	0		
2v	4	2		
1w	4	2		

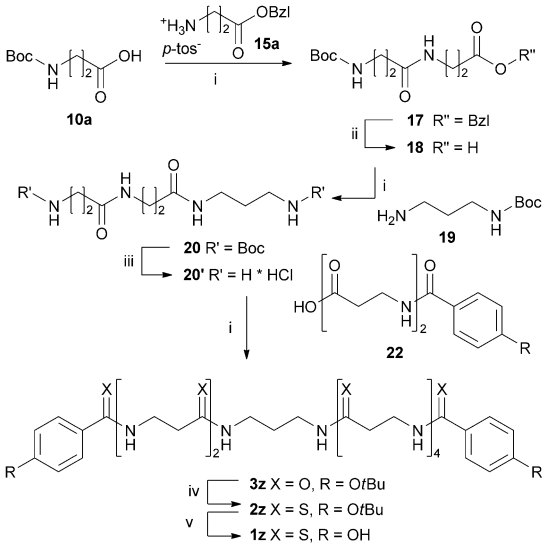


[a] Drugs efflux system deficient strain.

bone have impact on the antibiotic potency. We first synthesized symmetric homologues **1r**–**t** (with 0, 2 or 6 β-thioalanyl residues) employing the modular fusion approach (Scheme 2 and Table 1). Compounds **1r** and **1s** were prepared from diamine **11a** and carboxylic acids **7a** or **5d** (Scheme 2, left), whereas octathioamide **1t** was obtained by fusing tetraamide **8** and acid **5f** (Scheme 2, right).

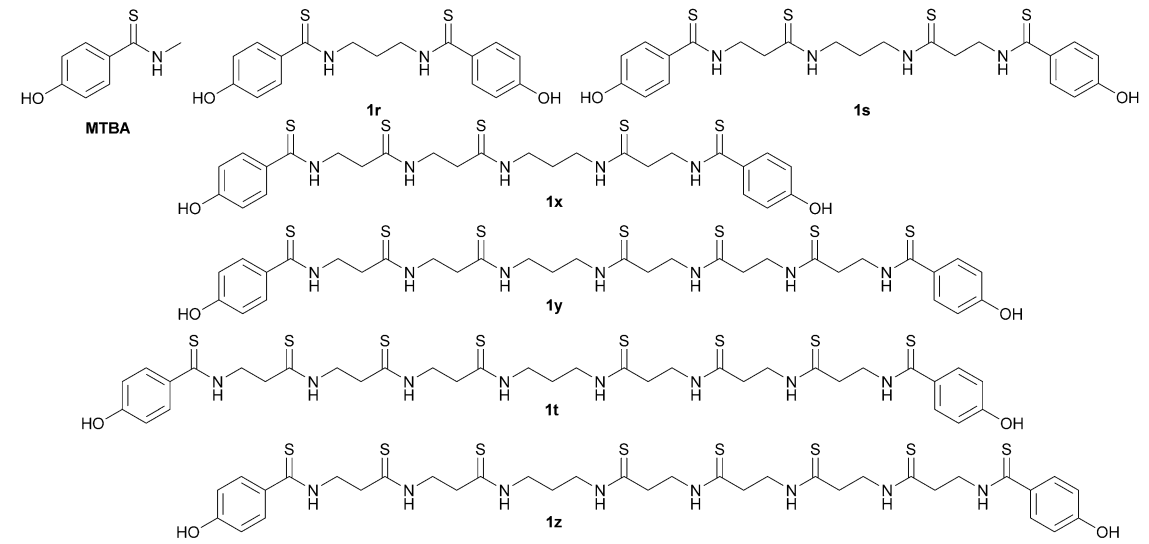
The biological assays revealed that antimicrobial activities of these analogues increase with the number of β-thioalanyl units (Table 5). Interestingly, despite its smaller overall size the tetrathioamide **1s** has an even higher activity than the partially thionated derivative **1w**. Among these derivatives octathioamide **1t** is the most potent compound, but has slightly lower activity against *S. aureus* and substantially reduced activity against *E. coli* compared to CTA. These results indicate that four β-thioalanyl residues (=CTA, **1a**) confer maximal activity to the molecule. Next, we investigated asymmetric analogues with an odd number (three and five) of β-thioalanyl units. These derivatives (**1x**, **y**) were isolated in trace amounts as by-products in the total synthesis of CTA when using crude peptide precursors including incorrect peptide coupling products (that is, traces of unprotected β-alanine or incomplete coupling; see the Supporting Information).[8] Notably, both compounds proved to be highly active against all Gram-positive bacteria tested. The heptathioamide **1y** showed similar potency against *E. coli* as CTA. This finding was quite unexpected, as the octathioamide **1t** is nearly inactive against *E. coli* (Table 5).

We therefore concluded that a trithioamide residue (two β-thioalanyl moieties and an aromatic thioamide) installed at the diaminopropane bridge could be the key to highly potent antimicrobial polythioamides.

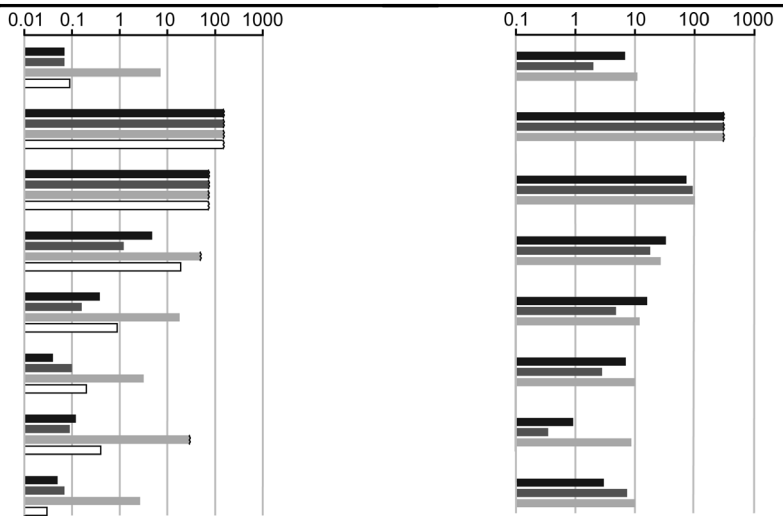


Scheme 4. Synthesis of asymmetrically substituted closthioamide **1z**. Reagents: i) EDC, HOBt, Hünig's base, DMF; ii) H_2, $Pd(OH)_2$ (C), MeOH; iii) dioxane/HCl; iv) Lawesson's reagent, pyridine; v) $CeCl_3$, NaI, acetonitrile; *p*-tos$^-$=*p*-toluenesulfonate (for more details see Supporting Information).

Table 5. Influence of the size and symmetry of the poly-β-thioalanyl backbone on bioactivity.



Compound	Sulfur atoms	Antimicrobial activity (MIC [μM])				Antiproliferative/cytotoxic activity (GI_{50}/CC_{50} [μM])		
		S. aureus	*E. faecium*	*E. coli*	*E. coli*[a]	HUVEC	K562	HeLa
1a	6							
MTBA[b]	1							
1r	2							
1s	4							
1x	5							
1y	7							
1t	8							
1z	8							



[a] Drugs efflux system deficient strain; [b] MTBA = 4-hydroxy-*N*-methylbenzothioamide (synthesized).[13]

To test this hypothesis, we developed a synthetic route for the asymmetric octathioamide isomer **1z** (Scheme 4). Starting with *N*-Boc-protected β-alanine we prepared dipeptide **17**. After deprotection, coupling to mono-*N*-Boc-protected diaminopropane (**19**) afforded the asymmetric dicarbamate **20**. Boc cleavage and coupling with diamide precursor **22** (see the Supporting Information) yielded the asymmetric closamide homologue **3z**. Thionation and *tert*-butyl cleavage eventually gave the asymmetric closthioamide homologue **1z**. This compound proved to be particularly active against the Gram-positive test strains (Table 5).

These results illustrate that the number of β-thioalanyl units in the polythioamide chain can be modified to some degree without impairing the antibiotic potency. This is remarkable as already the removal of one methylene group in the chain (e.g., Table 3, **1n**) results in a complete loss of bioactivity. We therefore conclude that β-thioalanyl moieties are the major feature of this kind of polythioamide antibiotics, yet a symmetric architecture is not a prerequisite for antibacterial action.

Conclusion

The synthesis and biological evaluation of 28 closthioamide derivatives, analogues and homologues of the highly potent *Clostridium*-derived antibiotic closthioamide was reported in this study. Our results provide the first insight into structure–activity relationships of this novel type of antibiotic. Aromatic terminal groups equipped with small *p*-substituents and the length of each spacer group in the polythioamide chain play pivotal roles for antibiotic activity of closthioamide. Further-

more, we have shown that all thioamide sulfurs of CTA contribute to the antimicrobial activity. Finally, we have unequivocally shown the importance of the modular arrangement of β-thioalanyl units and that the symmetric structure is not a strict requirement in this class of antibiotics (Figure 3).

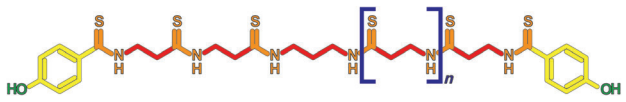


Figure 3. Heat map for structural freedom of antimicrobial polythioamides; red = no freedom, intermediate colors = limited freedom, blue = variable.

Taken together, our findings are an important addition to the yet small body of knowledge on this unusual new class of antibiotics. Beyond granting better understanding of the design rules for closthioamide, our findings pave the way to the development of polythioamides as potential therapeutics to treat bacterial infections.

Experimental Section

For experimental details see the Supporting Information.

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