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Potential scorpionate antibiotics: Targeted hydrolysis of lipid II containing model membranes by vancomycin–TACzyme conjugates and modulation of their antibacterial activity by Zn-ions

H. Bauke Albada [a], Christopher J. Arnusch [a,b], Hilbert M. Branderhorst [a], Anne-Marie Verel [c], Wouter T. M. Janssen [c], Eefjan Breukink [b], Ben de Kruijff [b], Roland J. Pieters [a], Rob M. J. Liskamp [a,*]

[a] Department of Medicinal Chemistry and Chemical Biology, Utrecht Institute for Pharmaceutical Sciences, Utrecht University, PO Box 80082, 3508 TB Utrecht, The Netherlands
[b] Department of Chemical Biology and Organic Chemistry, Institute of Biomembranes, Bijvoet Center for Biomolecular Research, Science Faculty, Utrecht University, Padualaan 8, 3584 CH Utrecht, The Netherlands
[c] Department of Medical Microbiology, University Medical Center Utrecht, Heidelberglaan 100, 3584 CX Utrecht, The Netherlands

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ABSTRACT

The antibiotic vancomycin—that binds lipid II in the bacterial cell membrane—was conjugated to a mono- and tetravalent mimic of the tris-histidine catalytic triad of metalloenzymes. Targeted hydrolysis by the conjugate was observed using model membranes containing lipid II, and in vitro MIC-values of the targeted mimic constructs could be modulated by Zn-ions.

The increasing number of reports concerning multi-drug resistant pathogens found in hospitals urges the scientific community to explore novel approaches to fight pathogens.[1] An exciting approach is the conjugation of an existing drug to a molecule with a different activity, ideally leading to more powerful drugs.[2] Noteworthy examples include the targeting of tumors,[2a,3] targeted hydrolysis of DNA and proteins,[4] and enzymatic degradation of bacterial adhesins.[5] We consider the last two examples as the first step toward the development of hydrolytic antibiotics. Based on our interest in both the synthesis of novel antibiotics[6] and in the synthesis of artificial enzyme mimics,[7] we chose to conjugate the antibiotic vancomycin[8] as a targeting moiety to a close structural mimic of a tris-histidine catalytic triad often found in the active site of metallohydrolases.[9]

Vancomycin is a glycopeptide that binds strongly to the tripeptide part (Lys-D-Ala-D-Ala) of lipid II, which is present in both the mature and immature cell wall of bacteria.[8] This binding of vancomycin to the tripeptide sequence prevents the peptidoglycan part of the cell wall from maturing, leading to lysis of the cell and bacterial cell death. Previously, a hybrid approach using fragments of nisin and vancomycin resulted in restoring the activity toward vancomycin resistant *Enterococci*.[6b]

However, an alternative mode of action found in nature for defense against pathogens is exemplified in lysozyme, a powerful example of an antibacterial hydrolytic enzyme. Hydrolytic enzymes are crucial for life since they catalyze the hydrolysis of several bonds in bio-molecules, thereby facilitating the re-use of building blocks like amino acids and nucleotides.[10] Bonds that can be hydrolyzed by these enzymes include those found in peptides, esters and phosphate esters. Therefore, hydrolytic enzymes have served as a tremendous source of inspiration in the construction of small hydrolysis catalysts.[11] Especially mimicry of metallohydrolases has resulted in active hydrolysis catalysts,[12] some of which even showed hydrolysis of peptide-bonds.[13] Related to this, the application of synthetic enzyme mimics in hydrolytic antibiotics has several advantages over the use of enzymes. Mimics are smaller, thereby reducing immunogenic reactions and allowing a better bio-distribution, they can be modified to cleave other substrates and to fit pharmacokinetic demands, and they usually have a broader substrate scope, which allows hydrolysis of more than one bio-molecular entity. We recently reported a close structural mimic of the tris-histidine triad, which we now name TACzyme,[9] a motif commonly found in metalloenzyme active sites.[14] Many

* Corresponding author. Tel.: +31 30 2537396; fax: +31 30 2536655.
E-mail address: r.m.j.liskamp@uu.nl (R.M.J. Liskamp).

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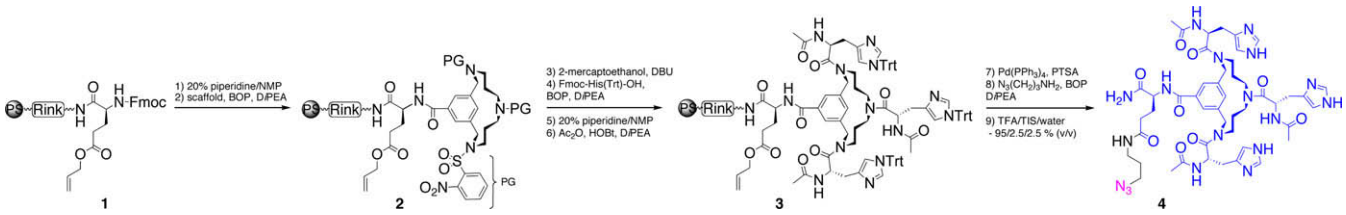
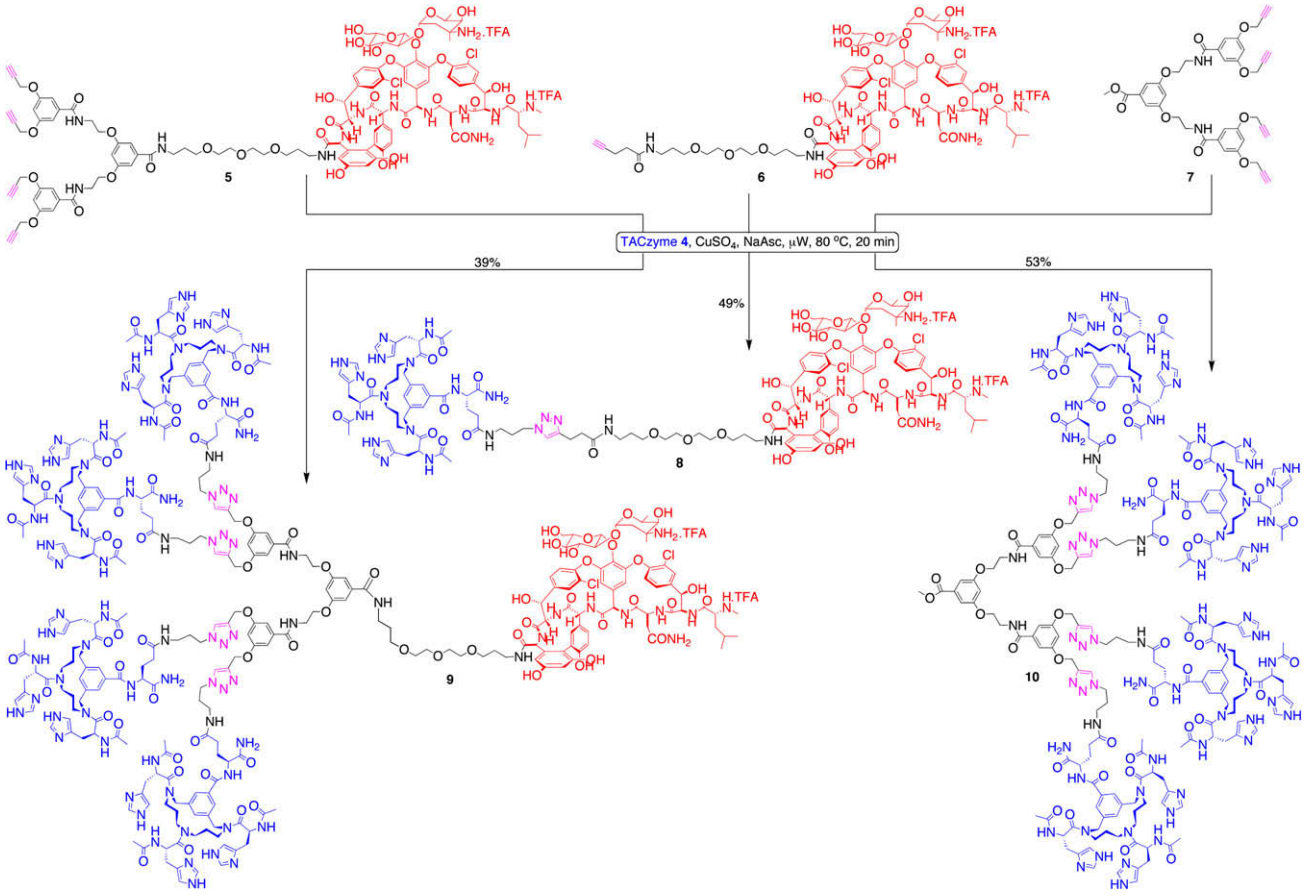


Scheme 1. Solid-phase synthesis of TACzyme **4**. See Supplementary data for details.



Scheme 2. Synthesis of TACzyme–vancomycin conjugates **8** and **9** and TACzyme dendrimer **10**. See Supplementary data for procedures.

of the metalloenzymes hydrolyze peptide-bonds, esters and phosphate-esters.[15] We wanted to target this system as an initial attempt toward the design of a hydrolytic antibiotic mimic. Therefore, we conjugated the TACzyme to vancomycin using click chemistry.[16]

The synthesis consisted of two parts: preparation of an azide functionalized TACzyme (**4**, Scheme 1) and alkyne-functionalized vancomycin derivatives (**5** and **6**, see Scheme 2). The azide functionalized TACzyme **4** was prepared by solid-phase synthesis similar to previously described procedures.[9] In this modified approach, Fmoc-Glu(OAll)-Rink linker containing resin **1** was loaded with a protected TAC-scaffold to give resin-bound scaffold **2**. The amines of the scaffold could be functionalized toward the construction of the tris-histidine triad. After this, the allyl-protected carboxylic acid of resin-bound triad **3** was functionalized with an azide-containing amine in order to allow further conjugation to alkyne containing vancomycin derivatives. Introduction of the azide was performed after synthesis of the triad, preventing thiolate-mediated reduction of the azide during *o*-NBS removal.[17] Acidic cleavage of the TACzyme from the resin afforded azide functionalized TACzyme **4**.

Vancomycin derivatives **5** and **6** were prepared from unprotected vancomycin and a mono- or tetra-alkyne functionalized ethyleneglycol-spacer, respectively,[18] using EDCI, HOAt and D*i*PEA. A tetravalent construct was prepared since we anticipated a positive multivalency effect with respect to the hydrolytic activity of this construct.[19] Also, many metallohydrolases and their mimics affect cleavage of the substrate by the cooperative action of more than one metal-centre.[14b,15] The target constructs were prepared by conjugation of the TACzyme to the vancomycin derivatives using copper-catalyzed click-chemistry (Scheme 2).[16] These new constructs, of which the potential mode of action resembles the attacking strategy of a scorpion, were called 'scorpionate antibiotics' (**8** and **9**). One part targets specifically lipid II, and the other part 'stings' the target by its hydrolytic activity. We also prepared multivalent TACzyme **10** without the vancomycin targeting part.

Non-conjugated TACzyme compounds **4** and **10** as well as vancomycin conjugated constructs **8** and **9** were tested in a model-sys-

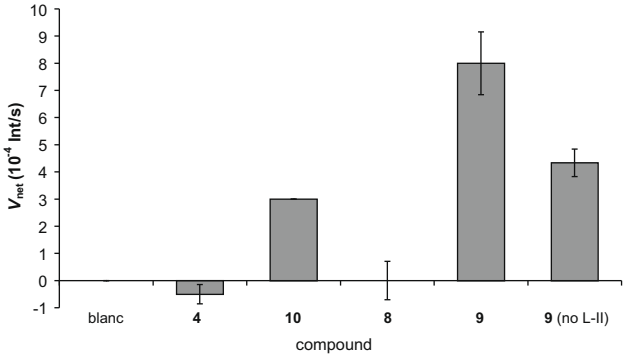


Chart 1. Hydrolytic activity for TACzyme conjugates in targeted hydrolysis using a di-acetyl carboxyfluorescein containing model system (see graphical abstract for schematic representation of this system).

tem to determine the efficacy of targeted hydrolysis. We measured the rate of hydrolysis of the acetyl-groups of di-acetylated carboxyfluorescein (DACF) covalently bound to DOPE[20] (DACF–DOPE). This modified DOPE was mixed with DOPC[20] resulting in model membranes composed of a 50:50 mixture of DOPC:DACF–DOPE. These vesicles also contained 0.1% lipid II, the target of vancomycin.[8,21] An increase in fluorescence as result of hydrolysis was measured upon addition of 20 μM TACzyme equivalents corresponding to 20 μM of **4** and **8**, and to 5 μM of 9 and 10 (Chart 1). We observed that the rate of hydrolysis by tetra-TACzyme-vancomycin **9** using vesicles containing no lipid II, denoted by '**9** (no L-II)' in Chart 1, was only marginally higher than the rate of tetravalent-TACzyme **10** using vesicles containing lipid II. In case of targeting to lipid II, the multivalency effect was especially clear considering that mono-TACzyme–vancomycin **8** showed hydrolytic activity similar to that of TACzyme **4** and the blanc, whereas the activity of tetra-TACzyme–vancomycin **9** was significantly higher. Even though there was only 0.1% of lipid II present in the membranes, the enhanced hydrolytic activity of tetra-TACzyme–vancomycin **9** conclusively showed the concept of targeted hydrolysis.

The antibiotic activity of the molecules was assessed by determination of the MIC values towards one vancomycin sensitive enterococcus (VSE) and one vancomycin resistant enterococcus (VRE) bacterial strain (Table 1). Compounds **4** and **10**, which lacked vancomycin, did not show any activity toward both VRE and VSE (MIC>256 mg/L), indicating that targeting is crucial for activity. In contrast, vancomycin conjugated constructs **8** and **9** showed interesting differences in activity toward VSE: monovalent TACzyme construct **8** was almost seven times more active than tetravalent TACzyme containing construct **9**. Thus, even though the hydrolytic activity of **9** is higher (vide supra) than that of **8**, the added steric bulk may have a negative effect on the antibacterial activity. However, the presence of $ZnSO_4$ in the medium had a beneficial effect on the antibacterial activity of the constructs: the activity of monovalent TACzyme **8** doubled and tetravalent TACzyme **9** became

Table 1
Minimum inhibitory concentration (MIC) values (in μM) of vancomycin (**V**) and conjugates **5**, **6**, **8** and **9**[a] and of TACzyme derivatives **4** and **10**

	Without $ZnSO_4$					With $ZnSO_4$[b]			
	V	**5**	**6**	**8**	**9**[c]	**4**	**10**	**8**	**9**[c]
VSE	0.3	1.6	1.0	5.8	40	na	na	2.9	5
VRE	86	3.2	na	na	na	na	na	92.8	na

[a] MIC values (μM) toward VSE (15A797) and VRE (15A799). Methods and strains are given in the Supplementary data (na = not active).
[b] 100 μM $ZnSO_4$ present in the medium.
[c] Wells containing **9** were turbid.

even eight times more active. Whether this is due to increased hydrolytic activity as result of added $ZnSO_4$ remains unclear at this point.[22] Importantly, it was found that $ZnSO_4$ itself did not act as an inhibitor of bacterial growth when non-conjugated **4** and **10** were evaluated in the presence of $ZnSO_4$. Although the coupling of TACzyme **4** to vancomycin derivatives **5** and **6**, leading to **8** and **9**, had a negative effect on the antibacterial activity of the latter, the presence of $ZnSO_4$ in the medium partly restored the antibacterial activity. With respect to the effect of hydrophobic extensions on vancomycin it is interesting to note that vancomycin-tetra-alkyne **5** showed higher activity toward VRE than vancomycin. The hydrophobic dendrimer increased the activity from 86 to 3.2 μM, whereas it even decreased activity toward VSE (Table 1). This is another example that addition of hydrophobic extensions on vancomycin, in our case on the C-terminus, can increase its activity.[23]

In summary, the versatile conjugation of a TACzyme mimic of a hydrolytic enzyme to vancomycin for the construction of potential novel antibiotics is described. The synthesis of the constructs was straightforward and very convenient, resulting in potential scorpionate antibiotics of high purity. Targeting of hydrolysis was demonstrated using a model system and a clear multivalency effect was observed. Although multivalent construct **9** was more active in the model membrane system than monovalent construct **8**, the added steric bulk may have led to reduction of the antibacterial activity of the entire construct in bacterial inhibition assays. In line with this assumption, we anticipate that the attachment of more powerful hydrolysis catalysts to an antibiotic will ultimately lead to very active hydrolytic antibiotics. Nevertheless, the type of positively charged constructs described here might be useful in other antimicrobial applications, for example the neutralization of the negative charge of lipopolysaccharide or lipoteichoic acid.[24] We also found that alkyne-functionalized vancomycin construct **5** had increased activity toward VRE as compared to vancomycin. Thus, hydrophobic extensions of the C-terminus may be beneficial and lead to vancomycin derivatives with increased activity.[23]

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Supplementary data

Supplementary data (experimental procedures, methods and analyses of the constructs) associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2009.05.064.

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