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ARTICLE

Evaluation of the antibacterial and antibiofilm activity of novel CRAMP-vancomycin conjugates with diverse linkers†

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We report the design, synthesis and antibacterial activity analysis of conjugates of vancomycin and cathelicidin-related antimicrobial peptides (CRAMP). Vancomycin inhibits the nascent peptidoglycan synthesis and is highly active against Gram-positive bacteria, whereas Gram-negative bacteria are generally insensitive due to a protective outer membrane. CRAMP is known to translocate across the Gram-negative outer membrane by a self-promoted uptake mechanism. Vancomycin-CRAMP conjugates were synthesized using click chemistry with diverse hydrophilic and hydrophobic linkers, with CRAMP functioning as a carrier peptide for the transfer of vancomycin through the outer membrane. Small hydrophobic linkers with an aromatic group result in the most active conjugates against planktonic Gram-negative bacteria, while maintaining the high activity of vancomycin against Gram-positive bacteria. These conjugates thus show a broad-spectrum activity, which is absent in CRAMP or vancomycin alone, and which is strongly improved compared to an equimolar mixture of CRAMP and vancomycin. In addition, these conjugates also show a strong inhibitory activity against *Salmonella* Typhimurium biofilm formation.

Introduction

The increase and spread of antibiotic resistance is a major challenge for the healthcare at a global scale. The massive use of antibiotics, often in an inadequate and inappropriate way, has led to an increasing incidence of multidrug-resistant bacteria both in hospitals and the community. Simultaneously, the discovery of new classes of antibiotics drastically diminished after the so-called golden age of antibiotic discovery during the 1960s. Since 1987, no novel classes of antibiotics have been reported.[1] To meet the societal need for new antibiotics, there is a global call to policymakers to install a new regulatory framework with incentives to reinvigorate antibiotic development in an economically viable way.[2]
Natural products have been a pivotal source for many successful antibiotics and are currently revisited in antibiotic drug discovery. However, the diversity of natural products that is nowadays explored is much larger than ever. Antimicrobial peptides (AMPs) represent such a new source of potent, broad-spectrum antibacterials. They are isolated from throughout the animal and plant kingdom. Animals and plants protect themselves against pathogenic microorganisms such as bacteria, fungi, viruses and protozoa to which they are continuously exposed. They are generally protected from infections by these microbes by a variety of responses produced by their innate immune system, which includes the production and release of AMPs.[3] During the past decades, a large number of naturally occurring AMPs (also called host defense peptides) have been isolated. Their abundance, tissue distribution and *in vitro* activity confirm an important role in innate immunity and host defense.[4-6] The peptides do not only possess the ability to directly kill invaders, but also stimulate effector molecules of the host immune system.[7]
Cathelicidins are an important class of AMPs. They have been identified in several species ranging from fish, amphibians, reptiles to mammals.[7-9] Members of the cathelicidin family are distinguished by a highly conserved *N*-terminal cathelicidin domain and a more variable C-terminal cathelicidin antimicrobial peptide domain.[7,8,10,11] In humans and mice only one cathelicidin is expressed, hCAP18/LL-37 and cathelicidin-related antimicrobial peptide (CRAMP), respectively. They act rapidly on Gram-positive and Gram-negative bacteria and thus contribute to the protection against a wide variety of pathogens. In Gram-negative bacteria, the positively charged cathelicidins interact with the lipopolysaccharide (LPS) cation

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binding sites with high affinity, leading to the competitive displacement of the stabilizing divalent cations. The outer membrane is disrupted and cathelicidins can pass the outer membrane in a so-called self-promoted uptake process. Interaction with the negatively charged cytoplasmic membrane leads to a transition of the cathelicidins from an unstructured to a structured form. They aggregate in clusters that perforate the cytoplasmic membrane. The membrane integrity is disrupted, resulting in a rapid cell death.[12] Generally, AMPs that target the cytoplasmic membrane are less prone to resistance development given the relatively immutable nature of the membrane.[13]

In this study, we covalently link mouse CRAMP to vancomycin. Vancomycin is a glycopeptide and it is used for the treatment of Gram-positive bacterial infections, including methicillin-resistant *Staphylococcus aureus* (MRSA), multiple resistant enterococci and *Clostridium difficile* infections.[14] Vancomycin binds to the C-terminal D-Ala-D-Ala of the pentapeptide of lipid II and the nascent peptidoglycan, inhibiting both transpeptidation and transglycosylation during the peptidoglycan synthesis. Vancomycin is only active against Gram-positive bacteria as it cannot penetrate the Gram-negative outer membrane given its large size.[15] We reasoned that a direct coupling of CRAMP and vancomycin may improve antibacterial activity against Gram-negative species if the CRAMP moiety could act as a carrier molecule for the transport of vancomycin through the outer membrane. After passage across the outer membrane, both the vancomycin and CRAMP moiety may exert their specific antibacterial action. AMPs often have only a moderate affinity for the target membrane. Since vancomycin has a high affinity for lipid II, which is embedded in the cytoplasmic membrane, vancomycin is hypothesized to act as a high-affinity targeting molecule for CRAMP towards the cytoplasmic membrane, again resulting in an increased antibacterial effect against Gram-negative bacteria. Similarly, the targeting effect of vancomycin could also contribute to an improved activity against Gram-positive species. The targeting principle has been shown for conjugates of Magainin-2 and its truncated version with vancomycin. The conjugates showed enhanced membrane permeabilizing activity against large unilamellar vesicles with embedded lipid II molecules. Also the antibacterial activity of conjugates against Gram-positive vancomycin-resistant enterococci was improved.[16] In another report, the antimicrobial peptides anoplin and temporin L were conjugated via click chemistry to vancomycin. The vancomycin targeting function led to an enhanced membrane disruption of large unilamellar vesicles (LUVs) for anoplin but not for temporin L.[13] An additional argument for our approach is that the combination of two active molecules in a single molecule is always preferred over mixtures, both from the clinical and regulatory perspective.

Specifically, CRAMP (H-KIGEKLKKIGQKIKNFFQKLVPQPEQ-NH_2) was conjugated with vancomycin using various aliphatic and aromatic linkers of different length with minimal steric bulk of the linker group, and without affecting the binding affinity of CRAMP and vancomycin (Figure 1 and Table 2). In total, 23 CRAMP-vancomycin conjugates were synthesized for analysis of antibacterial and antibiofilm activity. We demonstrate that specific CRAMP-vancomycin conjugates using short hydrophobic linkers with an aromatic group have an enhanced antibacterial activity against Gram-negative bacteria compared to CRAMP or vancomycin alone, or a 1:1 mixture of them. In addition, they show good antibacterial activity against Gram-positive species and prevent *S.* Typhimurium biofilm formation.

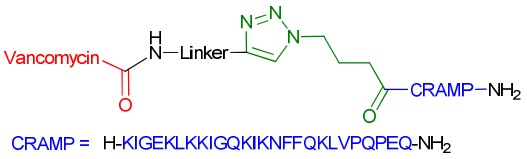

Figure 1: CRAMP vancomycin conjugate covalently linked via various linkers

Results and discussion

A mixture of CRAMP and vancomycin does not inhibit bacterial growth better than its separate compounds. A set of four Gram-positive (*Bacillus cereus*, *Bacillus subtilis*, *Micrococcus luteus*, *Staphylococcus aureus*) and four Gram-negative bacterial species (*Escherichia coli*, *Pseudomonas putida*, *Salmonella* Typhimurium, *Yersinia enterocolitica*) were selected to screen for inhibition of bacterial growth. We initially analyzed the minimum inhibitory concentration (MIC) for CRAMP, vancomycin and an equimolar (1:1) mixture of CRAMP and vancomycin (Table 3). Generally, we observed that the MIC of the mixture was equal to the MIC of vancomycin for Gram-positive species and equal to the MIC of CRAMP for Gram-negative species. This was expected given the ineffectiveness of vancomycin against Gram-negative bacteria and the higher sensitivity of Gram-positive bacteria for vancomycin than for CRAMP. Only for *S.* Typhimurium and *Y. enterocolitica*, a four-fold lower MIC was observed for the mixture in comparison to CRAMP alone, which might indicate a potential synergy when both molecules are added as a mixture.

A short hydrophobic linker to conjugate vancomycin and CRAMP shows most promise. In our approach, CRAMP was initially linked to vancomycin using a diamino 4,7,10-Trioxa-1,13-tridecanediamine derivative based linker **10a** (Table 2). This linker was selected to enhance the solubility of the final compound. Synthesis of CRAMP was carried out by applying the strategy of solid phase peptide synthesis (SPPS) using rink amide resin. Subsequently, the conjugate was synthesized using the highly efficient and widely used CuAAC click chemistry, which is a copper-mediated coupling between an azide and an alkyne.[17, 18] We firstly coupled 4-azidobutanoic acid **3** on the N-terminus of CRAMP which was on the solid support (Scheme 1). Simultaneously, the C-terminally modified vancomycin-linker-alkyne derivative **11a** (Scheme 1, Table 2) was prepared by standard solution-phase synthesis using EDC/HOAt as coupling reagents and linker **10a** and was purified using preparative HPLC. To couple this vancomycin-

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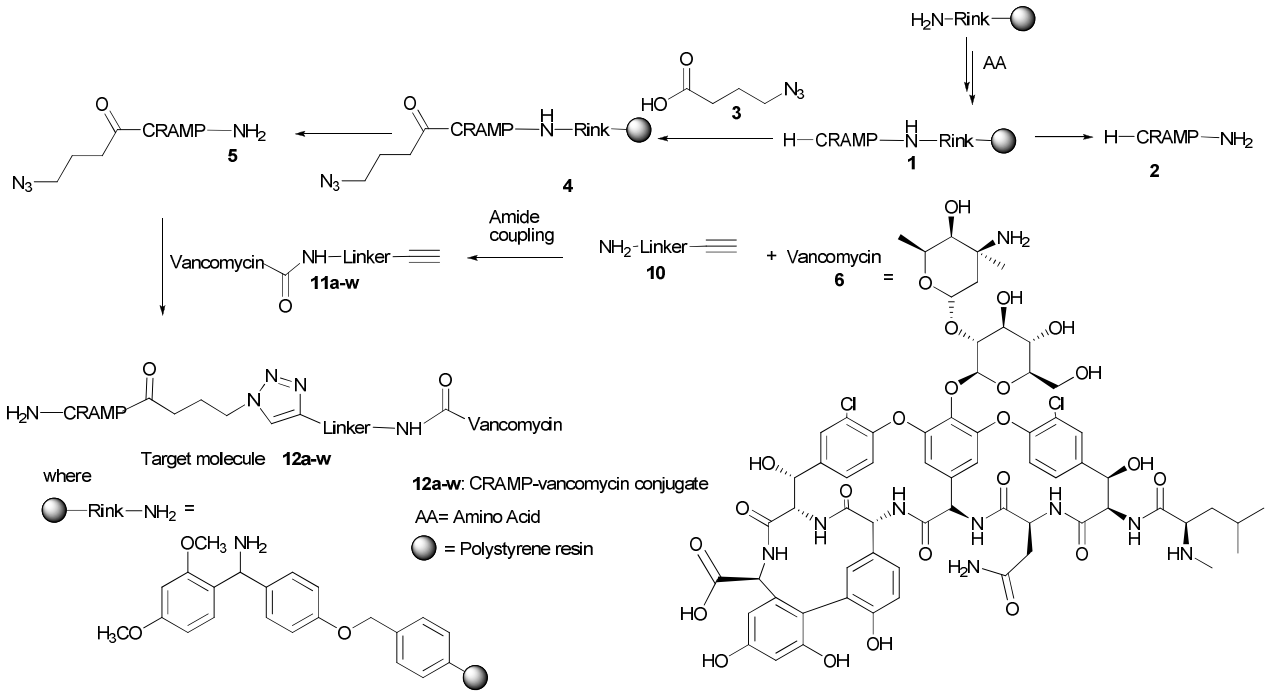
TEG linker **11a** with CRAMP-butyl azide derivative **5** via click reaction, different conditions were tested (Table 1). Firstly, conjugation was evaluated by dissolving the functionalized precursors in an aqueous solution (DMF/H_2O; 1:9) with addition of sodium ascorbate and $CuSO_4{\cdot}5H_2O$ and under microwave irradiation. This approach turned out to be rather sluggish and isolation of the product was not possible due to very small amount of product formation (entry 1, Table 1). In addition, it was noted that the use of tris [(1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl]amine (TBTA) ligand along with $CuSO_4{\cdot}5H_2O$/sodium ascorbate did not significantly improve the outcome of the reaction (entry 2, Table 1). Several conditions were evaluated unsuccessfully, employing various copper sources like $Cu(OAc)_2$ (entry 3, Table 1), CuI (entry 4, Table 1) and $Cu(ACN)_4PF_6$ (entry 5, Table 1). $Cu(OAc)_2$ and $Cu(ACN)_4PF_6$ made the reaction mixture sluggish with unreacted starting materials and a very small amount of desired product, along with other unidentified compounds. Ruthenium catalyzed click condition was also tested but resulted in the decomposition of starting materials (Entry 7, Table 1). Using CuI, iodination of vancomycin linker was observed, giving rise to the formation of iodinated CRAMP-vancomycin conjugate along with desired product **12a** in a very small amount contaminated with impurities although exact position of iodination could not be defined. Finally, optimal conditions were obtained with $CuSO_4{\cdot}5H_2O$ and sodium ascorbate at 40 ^{o}C in DMF/H_2O (1:9) for 20h (entry 6, Table 1). However, LC-MS and HPLC analysis surprisingly showed two major peaks of the same mass, which could not be separated.

The obtained CRAMP-vancomycin conjugate **12a** was tested against the Gram-positive and Gram negative bacterial species (Table 3). It was found that **12a** showed inhibitory activity against all species tested, however, **12a** was comparatively less active than vancomycin in case of Gram-positive species and less or equally active than CRAMP in case of Gram-negative bacteria (Table 3). Therefore it was hypothesized that the length and/or the nature of the TEG linker was not optimal for proper interaction of vancomycin and CRAMP to their respective targets. Therefore, shorter linkers (**10b** and **10c**, entry 2 & 3; Table 2) and a longer linker **10d** (Table 2, entry 4; for synthesis refer to the SI, Scheme 3) and the corresponding CRAMP-linker-vancomycin conjugates **12b-d** were synthesized following the same strategy as for CRAMP-vancomycin conjugate **12a**. However, this resulted again in two major peaks with similar mass in the LC-MS spectrum. We could isolate these two major peaks (annotated as **12b-d** and **12b'-d'**, respectively) in all three cases by preparative HPLC. These isolated products **12b-d** and **12b'-d'** were tested against the same bacterial species. Compound **12b'** showed the highest inhibitory activity, which was improved compared to **12a**, specifically for *B. subtilis* (four-fold), *M. luteus* (eight-fold), *E. coli* (four-fold) and *Y. enterocolitica* (four-fold). For Gram-positive bacteria, **12b'** showed comparable activity as vancomycin (except for *B. cereus* with a four-fold higher MIC for **12b'**); for Gram-negative bacteria, **12b'** performs equally or better (*Y. enterocolitica*) than CRAMP alone. Compounds with linker **10c** and especially **10d** showed less inhibitory activity compared to the compound with linker **10a** (Table 3). From these results, it was concluded that short and hydrophobic linkers such as **10b** may have the highest potential.



Scheme 1. General scheme for the synthesis of the target molecule (the carboxyl group attached to vancomycin is part of vancomycin itself i.e. the C-terminus of its heptapeptide backbone)

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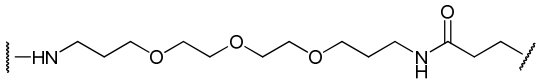
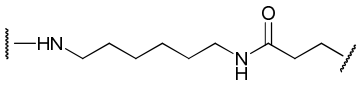
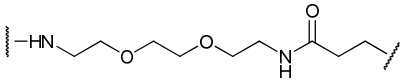
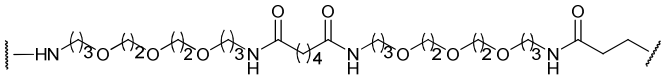
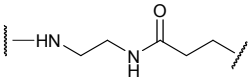
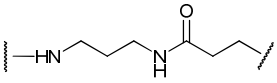
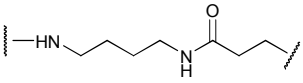
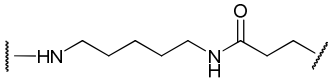
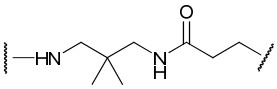
Table 1. Optimization of CuAAC reaction

Entry	Catalyst (ligand)	Base	Solvent	Temp	Time	LC-MS Conversion **12a** (%)
1	$CuSO_4 \cdot 5H_2O$	Sodium ascorbate	DMF/H_2O (1:9)	70°C (MW)	0.5h	5
2	$CuSO_4 \cdot 5H_2O$, (TBTA)	Sodium ascorbate	DMF/H_2O (1:9)	70°C (MW)	0.5h	5
3	$Cu(OAc)_2$	Sodium ascorbate	H_2O/*t*-BuOH (1:1)	40°C	30h	10
4	CuI	DIPEA	DMF	40°C	24h	10
5	$Cu(ACN)_4PF_6$	--	MeOH	rt	20h	40
6	$CuSO_4 \cdot 5H_2O$	Sodium ascorbate	DMF/H_2O (1:9)	40°C	20h	**63**
7	$CpRu(PPh_3)_2Cl_2$	--	DMF	40°C	24h	0

Table 2. Structure of linkers used for the synthesis of CRAMP-vancomycin conjugates

Entry	Product	—NH—Linker—	Vancomycin linker	Final product
1	**10a**		**11a**	**12a**
2	**10b**		**11b**	**12b** & **12b'**
3	**10c**		**11c**	**12c** & **12c'**
4	**10d**		**11d**	**12d** & **12d'**
5	**10e**		**11e**	**12e** & **12e'**
6	**10f**		**11f**	**12f** & **12f'**
7	**10g**		**11g**	**12g** & **12g'**
8	**10h**		**11h**	**12h** & **12h'**
9	**10i**		**11i**	**12i** & **12i'**

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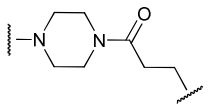
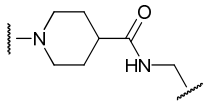
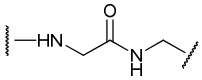
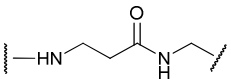
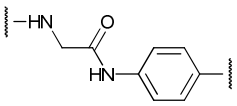
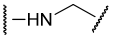
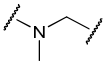
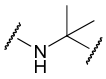
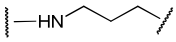
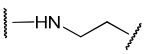
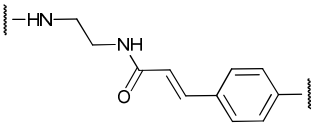
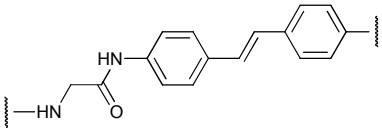
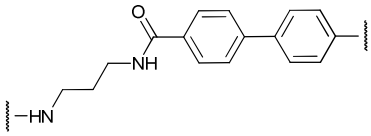
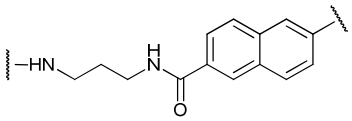
10	**10j**		**11j**	**12j** & **12j'**
11	**10k**		**11k**	**12k** & **12k'**
12	**10l**		**11l**	**12l**& **12l'**
13	**10m**		**11m**	**12m** & **12m'**
14	**10n**		**11n**	**12n** & **12n'**
15	**10o**		**11o**	**12o** & **12o'**
16	**10p**		**11p**	**12p** & **12p'**
17	**10q**		**11q**	**12q** & **12q'**
18	**10r**		**11r**	**12r** & **12r'**
19	**10s**		**11s**	**12s** & **12s'**
20	**10t**		**11t**	**12t** & **12t'**
21	**10u**		**11u**	**12u** & **12u'**
22	**10v**		**11v**	**12v** & **12v'**
23	**10w**		**11w**	**12w & 12w'**

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Table 3. Antibacterial activity of CRAMP-vancomycin conjugates

		MIC (μM)							
		Gram-positive species				Gram-negative species			
Entry	Product	*B. cereus* LMG 9610	*B. subtilis* PSB3	*M. luteus* ATCC4689	*S. aureus* ATCC6538	*E coli* XL-1 Blue MRF'	*P. putida* G1	*S.* Typhimurium LT2	*Y. enterocolitica* ATCC9610
1	**CRAMP**	25	25	50	> 100	3	12.5	100	50
2	**V**	1.5	1.5	1.5	≤ 0.75	100	100	100	100
3	**C+V**	1.5	1.5	1.5	≤ 0.75	3	12.5	25	12.5
4	**12a**	6	6	12.5	nd	12.5	12.5	>100	50
5	**12b**	3	6	1.5	nd	6	25	>100	25
6	**12b'**	6	1.5	1.5	nd	3	12.5	>100	12.5
7	**12c**	3	25	25	nd	12.5	25	>100	25
8	**12c'**	3	12.5	12.5	nd	6	25	>100	12.5
9	**12d**	6	50	50	nd	25	100	>100	100
10	**12d'**	25	25	50	nd	25	50	>100	50
11	**12e**	6	6	1.5	nd	nd	>100	>100	>100
12	**12e'**	1.5	12.5	3	nd	6	25	>100	12.5
13	**12f**	6	6	1.5	nd	nd	50	>100	100
14	**12f'**	1.5	12.5	3	nd	6	12.5	100	6
15	**12g**	6	6	1.5	nd	nd	100	>100	100
16	**12g'**	3	6	3	nd	6	12.5	100	6
17	**12h**	12.5	12.5	3	nd	nd	>100	>100	>100
18	**12h'**	1.5	6	3	nd	6	12.5	>100	6
19	**12i**	6	6	1.5	nd	nd	>100	>100	100
20	**12i'**	1.5	6	3	nd	3	12.5	>100	6
21	**12j**	6	3	1.5	nd	nd	100	>100	100
22	**12j'**	1.5	6	3	nd	6	25	>100	6
23	**12k**	6	3	1.5	nd	nd	>100	>100	>100
24	**12k'**	1.5	3	3	nd	3	12.5	50	6
25	**12l**	6	3	3	nd	nd	>100	100	>100
26	**12l'**	3	12.5	3	nd	6	12.5	100	12.5
27	**12m**	6	3	3	nd	nd	>100	>100	>100
28	**12m'**	3	12.5	6	nd	6	12.5	100	12.5
29	**12n**	6	3	3	nd	nd	>100	>100	100
30	**12n'**	1.5	6	3	nd	3	6	100	3
31	**12o**	3	3	1.5	nd	nd	100	>100	50
32	**12o'**	3	6	3	nd	6	12.5	100	6
33	**12p**	6	3	3	nd	nd	>100	>100	>100
34	**12p'**	1.5	12.5	3	nd	6	>100	>100	6
35	**12q**	6	3	3	nd	nd	100	>100	100
36	**12q'**	6	12.5	3	nd	6	>100	>100	12.5
37	**12r**	6	6	1.5	nd	nd	>100	>100	100
38	**12r'**	3	12.5	3	nd	6	25	>100	12.5
39	**12s**	6	3	3	nd	nd	>100	>100	>100
40	**12s'**	6	25	6	nd	12.5	>100	>100	12.5
41	**12t**	1.5	≤ 0.75	nd	6	3	3	**1.5**	1.5
42	**12t'**	3	1.5	nd	12.5	6	6	6	6
43	**12u**	6	1.5	nd	25	6	6	3	6
44	**12u'**	3	≤ 0.75	nd	3	6	12.5	6	6
45	**12v**	3	≤ 0.75	nd	6	3	6	3	3
46	**12v'**	3	1.5	nd	6	6	6	**1.5**	6
47	**12w**	1.5	≤ 0.75	nd	3	1.5	6	**≤ 0.75**	3
48	**12w'**	1.5	≤ 0.75	nd	6	3	3	**1.5**	3

MIC (μM): lowest concentration of an antibacterial agent that completely inhibits growth of the microorganism as detected by the unaided eye; (nd): bioactivity not determined; V: vancomycin; C+V: mixture of CRAMP and vancomycin in a 1:1 ratio.; in case of the equimolar mixture (C+V), the concentration represents the concentration of the individual compounds

Conjugates with short linkers comprising an aromatic group show broad inhibitory activity against Gram-negative and Gram-positive species. Based on these findings, we synthesized nine shorter hydrophobic linkers **10e-m**, and five

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aromatic linkers; **10n** and **10t-w** (Table 2), according to the reported procedures[18-20] using shorter commercially available building blocks (refer to the SI: Table 2 & 3; Scheme 2, 5 & 6) and utilized five commercially available shorter linkers **10o-s** (Table 4, refer to the SI). Subsequently, these linkers were coupled to vancomycin *via* its C-terminus to obtain vancomycin-linker conjugates **11e-w** (Table 2), resulting in CRAMP-vancomycin conjugates **12e-w** and **12e'-w'** *via* CuAAC. This new series of CRAMP-vancomycin conjugates was analyzed for inhibitory activity on the panel of species. From the entire series conjugates with aromatic linkers (**12t-w**/**12t'-w'** except **12n**/**12n'**) showed the strongest inhibitory activity against Gram-negative strains, which was stronger than the mixture of individual components. This improvement was most prominent for *S.* Typhimurium (up to 32-fold for **12w** in comparison to the mixture and 128-fold compared to vancomycin or CRAMP alone), followed by *Y. enterocolitica* (up to 8-fold for **12t** in comparison to the mixture, and 16-32-fold compared to CRAMP and vancomycin alone, respectively). In case of Gram-positive species, the aromatic conjugates outperformed vancomycin against *B. subtilis*, but there was no conjugate with improved activity against all Gram-positive species tested compared to vancomycin. Nevertheless, the aromatic conjugates represent a new class of antibacterial compounds that have broad activity against Gram-negative and Gram-positive species (MIC < 10 μM), which is neither the case for CRAMP or vancomycin alone nor for the equimolar mixture of both individual molecules.

CRAMP-vancomycin conjugation effectively prevents *S.* Typhimurium biofilm formation. A large majority of bacterial infections is biofilm-related.[21] Therefore we evaluated the prevention of *S.* Typhimurium biofilm formation for the complete set of CRAMP-vancomycin conjugates. Inhibition is expressed as IC_{50}, which corresponds to the concentration of the compound that is needed to inhibit biofilm formation by 50%. In addition, a 95% confidence interval is calculated (Table 4). CRAMP or vancomycin alone have high IC_{50} values, however a mixture of CRAMP and vancomycin appears to be even more efficient (IC_{50} = 0.36 μM) in biofilm prevention. This result is consistent with lower synergy at the 1:1 ratio of CRAMP and vancomycin, since the Interaction Index I < 1 (I = $IC_{50}C+V/IC_{50}CRAMP + IC_{50}C+V /IC_{50}V = 0.57$).[22] Although most conjugates of CRAMP and vancomycin have slightly higher IC_{50} values than the mixture, the results indicate that they still show potent biofilm inhibitory activity.[23]

Table 4. Influence of CRAMP-vancomycin conjugates **12a-w** and **12a'-w'** on the prevention of biofilm formation of *Salmonella* Typhimurium (expressed as IC_{50} in μM)

		Biofilm inhibition (IC_{50} in μM)	
Entry	Product	IC_{50}	95% confidence interval
1	**CRAMP**	0.88	0.75 to 1.06
2	**V**	2.29	1.87 to 2.80
3	**C+V**	0.36	0.32 to 0.40
4	**12a**	1.41	1.06 to 1.87
5	**12b**	1.86	1.23 to 2.78
6	**12b'**	0.64	0.25 to 1.65
7	**12c**	1.86	1.52 to 2.25
8	**12c'**	1.97	1.33 to 2.93
9	**12d**	1.32	0.95 to 1.83
10	**12d'**	3.38	2.44 to 4.68
11	**12e**	0.33	0.19 to 0.59
12	**12e'**	3.46	2.14 to 5.59
13	**12f**	1.29	0.87 to 1.90
14	**12f'**	1.38	1.07 to 1.78
15	**12g**	0.76	0.28 to 2.10
16	**12g'**	1.79	1.24 to 2.59
17	**12h**	2.18	0.79 to 6.03
18	**12h'**	1.10	0.62 to 1.96
19	**12i**	1.01	0.69 to 1.48
20	**12i'**	1.13	0.77 to 1.66
21	**12j**	0.62	0.45 to 0.86
22	**12j'**	2.00	1.57 to 2.46
23	**12k**	2.34	1.55 to 3.53
24	**12k'**	1.43	1.16 to 1.76
25	**12l**	0.71	0.43 to 1.20
26	**12l'**	0.99	0.57 to 1.71
27	**12m**	~1.56	nd
28	**12m'**	2.18	0.76 to 6.24
29	**12n**	1.27	0.83 to 1.96
30	**12n'**	1.42	1.02 to 1.97
31	**12o**	0.35	0.24 to 0.53
32	**12o'**	0.88	0.56 to 1.40
33	**12p**	1.71	0.72 to 4.05
34	**12p'**	2.53	1.33 to 4.81
35	**12q**	0.41	0.30 to 0.56
36	**12q'**	1.57	0.90 to 2.70
37	**12r**	1.10	0.58 to 2.07
38	**12r'**	1.41	0.40 to 5.00
39	**12s**	0.38	0.18 to 0.79
40	**12s'**	~6.7	nd
41	**12t**	1.56	1.30 to 1.85
42	**12t'**	5.00	3.43 to 7.28
43	**12u**	8.64	5.97 to 12.50
44	**12u'**	10.77	5.97 to 12.50
45	**12v**	1.88	1.25 to 2.84
46	**12v'**	7.27	4.47 to 11.84
47	**12w**	0.91	0.58 to 1.45
48	**12w'**	1.60	1.34 to 1.92

IC50 in (μM): concentration of inhibitor needed to inhibit biofilm formation by 50%; (nd): not determined; V: vancomycin; C+V: mixture of CRAMP and vancomycin in 1:1 ratio; ~: approximately.

Structural differences between 12 and 12' compounds related to isomerization of CRAMP affect inhibitory activity against Gram-negative species. When comparing the inhibitory activity of the two compounds **12** and **12'** against planktonic bacteria, a high variability among the species tested is observed. For Gram-negative species, **12'** is often more active (up to 8-16-fold) than **12**, whereas for Gram-positive species **12'** is mostly slightly less active against *B. subtilis* and *M. luteus*. We tried to identify the structural differences between the two compounds **12** and **12'** obtained after CuAAC reaction. As this reaction was performed using Cu, which is known to give rise to the 1,4 triazole product selectively, there was a negligible chance for the formation of the 1,5-cycloadduct. It was indeed confirmed by ^{1}H NMR that the 1,4-triazole was formed in both cases. Therefore it was considered that some isomerisation took place under CuAAC reaction conditions. To verify if isomerisation is occurring in the peptide or in vancomycin part, they were treated separately with $CuSO_4{\cdot}5H_2O$ (3.0 equiv) and sodium ascorbate (6.0 equiv) in H_2O/DMF (4.5:0.5) upon stirring for 20h at 40°C. The reactions

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were monitored by mass and LC-MS. The mass spectrum did not show any changes, while in the LC-MS chromatogram of the reaction with CRAMP-butyl azide **5** (Figure 2 and 3) a new peak was observed with a retention time of 28.18 min with similar intensity. The reaction with the vancomycin-linker **11** did not show any change in the LC-MS chromatogram. Hence, it was clear that some changes were occurring in CRAMP-butyl azide **5**. The same observation was made when only CRAMP **2** was treated. Apparently only CRAMP was interacting with $CuSO_4{\cdot}5H_2O$ and/or sodium ascorbate, leading to the formation of isomerised compound with the same mass.

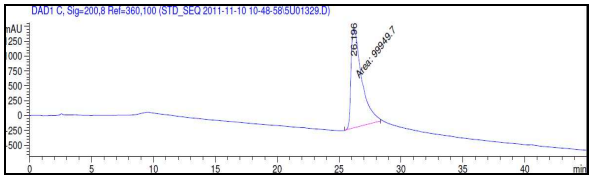

Figure 2. LC-MS chromatogram of CRAMP-butyl azide **5** before reaction with $CuSO_4{\cdot}5H_2O$ and sodium ascorbate (retention time 26.19 min, method 0-60% ACN/H_2O in 40 min)

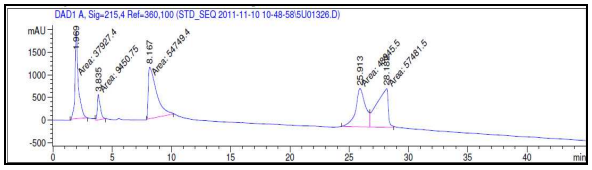

Figure 3. LC-MS chromatogram of CRAMP-butyl azide **5** after reaction with $CuSO_4{\cdot}5H_2O$ and sodium ascorbate (a new peak was observed at a retention time of 28.18 min, method 0-60% ACN/H_2O in 40 min)

Isomerisation is not due to helicity distortion or racemization. To our knowledge, this is the first report of the generation of two isomers of a peptide under CuAAC reaction conditions. One obvious explanation could be distortion of the helicity of the peptide due to the interaction with $CuSO_4{\cdot}5H_2O$. To confirm this, both compounds corresponding with the two peaks in the LC-MS chromatogram of CRAMP were isolated by preparative HPLC, and a circular dichroism (CD) spectrum was recorded along with this of pure CRAMP (Figure 3, SI). According to the CD spectrum, both compounds were found to be α-helical in TFE (trifluoroethanol) and random coiled in pure water hence excluding the helicity distortion hypothesis (Figure 3 & 4, SI). An alternative explanation could be attributed to the racemization of the amino acids of the peptide to some extent, resulting in the formation of diastereoisomers. To confirm this, the enantiomeric purity of the each amino acid in both the isolated peptides (corresponding with the two peaks in the LC-MS chromatogram of CRAMP upon treatment with $CuSO_4{\cdot}5H_2O$/sodium ascorbate) was analyzed by determination of the optical purity of each amino acid of the sequence by hydrolyzing in a 6N HCl solution. This hydrolysis caused the suitable derivatisation of the free amino acids whereby racemisation is accomplished by deuterium exchange in the α-C position followed by gas chromatographic separation of the enantiomers of the peptide via GC-MS (Table 7, refer to the SI). No enantiomeric formation was observed in amino acids, indicating that the observation of the two peaks could not be explained in this way.

Isomerization of CRAMP is caused by exposure to sulphate anions. Trying to avoid the problem, the amount of copper sulphate was reduced. This led to incomplete conversion. To determine the amount of copper sulphate needed for the generation of the side compound, a set of reactions was carried out starting with 0.1 to 2.0 equiv of $CuSO_4{\cdot}5H_2O$. It was observed that even 0.1 equiv of $CuSO_4{\cdot}5H_2O$ was sufficient for the generation of the second peak in the chromatogram although to a lesser extent. To investigate the role of the oxidation state of copper, CRAMP was treated with $CuSO_4{\cdot}5H_2O$ in the absence of sodium ascorbate. However, in this case the side compound was also generated, indicating that the oxidation state of the copper ion does not play any role in this transformation of the peptide. To further confirm this, CuI was used as a copper source instead of $CuSO_4{\cdot}5H_2O$. Surprisingly, in this case there was no generation of second peak-compound in the LC-MS chromatogram of CRAMP. Also, increasing the amount of CuI from 2.0 to 4.0 equiv did not cause a second peak. Similar results were obtained with CuCl and $CuCl_2$. Hence, it was proven that the oxidation state of copper does not play any role in the generation of the second peak-compound. It was also confirmed that not all copper sources lead to the generation of the double peak compound as chloride and iodide salts did not cause any change. Apparently, only $CuSO_4{\cdot}5H_2O$ was causing this problem. According to the literature, anions (especially divalent anions) might interact with peptides.[24] They could bind to the positively charged residue of the peptide and cause either aggregation or nanostructure formation.[24] The mass of SO_4^{2-} was observed by LC-MS in one of the isolated peaks of CRAMP after reaction with $CuSO_4{\cdot}5H_2O$. X-ray fluorescence (XRF) analysis suggested that SO_4^{2-} is trapped in the isolated compounds of CRAMP (probably at the positively charged lysine residues) after treatment with $CuSO_4{\cdot}5H_2O$ (Table 5). However, the exact position of the binding site could not be defined. CRAMP was also treated with another sulphate source, i.e. Na_2SO_4, and as expected the two peaks appeared again in the LC-MS chromatogram. Notably, when these two compounds were isolated by preparative HPLC and treated again with $CuSO_4{\cdot}5H_2O$ to analyze if further conversions into two peaks occur, this was indeed observed, although, the rate of conversion was now rather slow. To investigate the possibility of nanofibre formation or aggregation of CRAMP in the presence of sulphate anions, atomic force microscopy (AFM) measurements were performed (Figure 4). Although there were some differences in both images, nanofibre formation or aggregation of the peptide could not be confirmed unambiguously. Therefore, at this stage, we have come to the conclusion that the sulphate anion binds to the positively charged amino acid residue(s), changing the conformation of the peptide.

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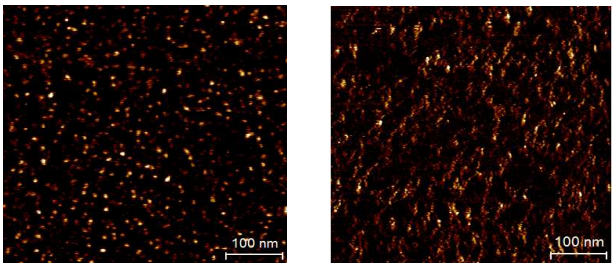

Figure 4a **Figure 4b**

Figure 4. AFM-image of CRAMP in the absence of $CuSO_4 \cdot 5H_2O$ (Figure 4a) and in the presence of $CuSO_4 \cdot 5H_2O$ (Figure 4b)

Table 5: Percentage of Cu and S present in CRAMP samples

Samples	Amount of Cu	Amount of S
CRAMP pure (before treatment with $CuSO_4 \cdot 5H_2O$)	0.006%	0.110%
CRAMP peak-1 compound	0.010%	0.250%
CRAMP peak-2 compound	0.009%	0.267%

Experimental

All the experimental procedures and schemes are given in the supporting information**.**

Conclusions

We aimed to synthesize a compound with broad-spectrum antibacterial activity by conjugating an antibacterial peptide with self-promoted uptake properties (CRAMP) and an antibiotic that targets the cell wall synthesis (vancomycin). We envisioned that CRAMP could function as a carrier molecule for the transport of vancomycin through the outer membrane of Gram-negative bacteria, while vancomycin could exert a targeting function for CRAMP to enhance the affinity of CRAMP for the cytoplasmic membrane. Short and hydrophobic linkers bearing an aromatic group resulted in conjugates with the best inhibitory properties. Whereas the effect was most prominent for Gram-negatives, the conjugates with these linkers also maintained their activity against some of the tested Gram-positive bacteria comparable to vancomycin alone. Biofilm formation was also strongly prevented with these conjugates. Molecular mechanistic studies will be needed in the future to elucidate if the carrier function, the targeting function or both simultaneously explain the improved antibacterial properties of the best conjugates.

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Notes and references

Electronic Supplementary Information (ESI) is available, including detailed experimental procedure and characterization of compounds etc.

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