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Highly Active Antibacterial Ferrocenoylated or Ruthenocenoylated Arg-Trp Peptides can be Discovered by an L-to-D Substitution Scan

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The rapid increase in resistance against common antibiotics calls for the development of novel antibiotics, particularly against multi-resistant bacteria such as the methicillin-resistant *Staphylococcus aureus* (MRSA). In this work, the two group 8 metallocenoyl derivatives ferrocenoyl (FcC(O)-) or ruthenocenoyl (RcC(O)-) were attached to the N-terminus of two libraries of short antimicrobial peptides (AMPs), resulting in organometallic-AMP derivatives with yet unparalleled antibacterial activities. In addition, these organometallic AMPs only cause limited lysis of human red blood cells (hRBCs). Our structure-activity relationship (SAR) study on these metallocenoylated peptides showed that specific combinations of L- and D-amino acid residues results in peptides with significantly improved antibacterial activity. Whereas the all-L FcC(O)-containing lead peptide had a MIC of 12 μM against MRSA, several peptides were found with MIC-values as low as 1.5–3 μM, a 4–8-fold increase in activity. For the RcC(O)-derivatized peptides a similar result was obtained: against MRSA an MIC of 5.8 μM for the all-L peptide could be lowered to 0.7 μM, an 8-fold improvement. In addition, exposure of human red blood cells with 112 μM of the most active peptides led to a maximum hemolysis of 6%, indicating prominent selectivity that can be used to realize antibiotics based on organometallic-AMPs. We have hereby performed a systematic and highly successful SAR optimization against the two crucial parameters, i.e. antibacterial activity and hemolysis. Importantly, some of the RcC(O)-derivatized peptides presented here are among the most active antibacterial peptides; per amino acid, they approach or even exceed the activity of vancomycin.

Introduction

Antimicrobial peptides (AMPs) are a prominent class of biologically active peptides that can have interesting and useful pharmacological properties.[1] They tend to have micromolar activity, can be selective for certain types of membranes, and have been found to be active against bacteria, fungi, tumors and viruses.[2] One major advantage of membrane-targeting AMPs over conventional single-target antibiotics is the usually much more difficult development of resistance; an active compound cannot simply be deactivated by a mutation in its biological target.[3] Therefore, these peptides hold great promise when it comes to fulfilling the urgent need of new antibiotics.[1] However, many membrane-targeting AMPs will also interact with mammalian membranes, causing strong hemolysis, which immediately prohibits effective derivatization towards clinical development.

Peptides with a high activity against bacteria - but no other pathogens like fungi or protozoae - are grouped in a sub-class called 'antibacterial' peptides. These peptides are typically composed of 15-50 amino acid residues and share a common distribution of functionalities resulting in an amphipathic molecule. This amphipathic nature has been shown to be important for their interaction with membranes.[4] They can kill bacteria by forming pores in the bacterial membrane, causing leakage of cellular components and killing of the bacteria. Much shorter AMPs composed of as little as five amino acid residues are not able to span the entire membrane, but have been shown to impact the membrane and thereby disturbing membrane functionality, leading to cell death even at low concentrations (μM). We recently reported on the mode of action (MoA) of one of these short AMPs, the RcC(O)-labeled peptide RcC(O)-WRWRW-NH_2, and showed its integration into the bacterial membrane, causing delocalization of essential membrane-associated proteins that are crucially involved in cell wall biosynthesis, respiration, and cell division.[5] Using the unique properties of ruthenium, we were able to apply atomic absorption spectroscopy to trace the localization of the ruthenium atom, and to confirm its abundant presence in the membrane of the bacterium, i.e. 89% of the peptide localized there.

An additional advantage of relatively small peptide-based antibiotics is the convenience to perform a comprehensive structure-activity relationship (SAR) study. Thereby it is subsequently possible to identify promising compounds, establish their specific activity against bacteria, compare their activity to the toxicity against erythrocytes and mammalian cell lines, validate their MoA, and finally optimize their properties. Whereas an MoA-elucidation can be a time-consuming ordeal, the chemical modification of a lead sequence is nowadays relatively convenient. A large number of compounds can be prepared and tested for their activity at the same time. Methods for tuning of the activity of AMPs involve a multivalent presentation of AMPs[6] and conjugation of AMPs to lipids[7,8] or other moieties targeting bacteria[9], which are now well established. A more recent addition to the class of performance-enhancing moieties is the covalent attachment of organometallic moieties.[10] Initial studies were directed at the conjugation of

cobaltocinium (Cc^+) and ferrocene (Fc) derivatives,[11] but we recently uncovered that the attachment of a ruthenocenoyl (RcC(O)-) moiety can produce very active antibacterial peptides with low activity against erythrocytes or human cancer cells.[12] Further, an elegant method potentially discovering peptides with an improved specificity is the inversion of the chiral centers within a peptide. In fact, a detailed SAR study on peptides can already be achieved by performing a simple systematic L-to-D substitution scan on all positions and correlating it to various activity parameters. The substitution of an L-amino acid residue for its D-enantiomer changes the orientation by which the functional groups 'branch of' from the peptide's backbone and has fundamental consequences for the activity, as was shown by Shai and coworkers for relatively long α-helical antibacterial peptides.[13] Additionally, there is a large conceptual space for structural optimization due to bacterial membranes markedly differing from mammalian ones.

In the present work, we systematically performed an L-to-D substitution scan on all positions of the McC(O)-WRWRW-NH_2 sequence. We were particularly interested in FcC(O)- or RcC(O)-peptides with enhanced antibacterial activity. For the SAR study, we prepared two libraries of McC(O)-derivatized AMPs in which each of the amino acid residues was either L or D; one library of 32 L-to-D scanned peptides was derivatized with the FcC(O)-group and the other with the RcC(O)-group. The crucial hemolytic activity against human red blood cells (hRBCs) was directly monitored to ensure possible clinical testing of AMPs as potential drug candidates. The fact that hemolytic activity of the first RcC(O)-peptide was already quite low, *i.e.* 60–70% hemolysis when human red blood cells (hRBCs) are treated with 193 µM of peptide (33 times higher than the MIC-value),[10] encouraged us to proceed with a full SAR study.

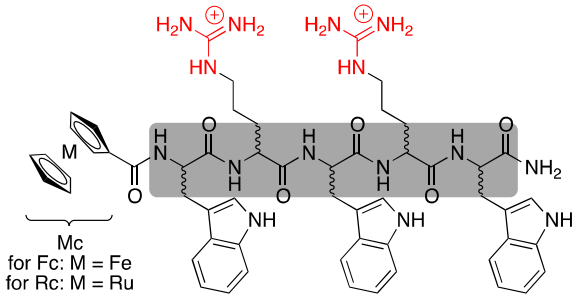

Figure 1. Structure of the metallocenoyl-derivatized AMPs for which a systematic L-to-D exchange scan of the five amino acid residues was performed (highlighted with the gray box).

Experimental Section

Synthesis of the L-to-D scanned peptides

All peptides were prepared manually by means of standard Fmoc-based solid phase peptide synthesis protocols in a split-and-split strategy. For this, two batches of ChemMatrix-Rink resin (loading: 0.6 mmol/g) were used, 0.6 g each. To one batch was coupled Fmoc-^{L}Trp(Boc)-OH and to the other Fmoc-^{D}Trp(Boc)-OH using TBTU, HOBt, and D*i*PEA in DMF (5 mL for 3 hrs). After removal of the Fmoc-group – using 20% piperidine in DMF (2 times 10 mL, 10 min), followed by washing with DMF (5 times 10 mL, 2 min) – each batch of resin was split in half. Using the above-mentioned coupling reagents alternative coupling cycles were used to couple either the L- or D-enantiomer of Fmoc-Arg(Pbf)-OH, or the L- or D-enantiomer of Fmoc-Trp(Boc)-OH. This process was repeated until the last tryptophan residue was attached and 32 batches containing all combinations of L- and D-amino acid residues were obtained. After splitting each batch in half and coupling of either FcC(O)OH or RcC(O)OH to the terminal amino group of the tryptophan residues, each of the 64 batches of resin was washed with DMF and DCM. Finally, the 64 peptides were cleaved from the resin using TFA/TIS/phenol – 92.5/5/2.5 (%, v/v/m) for the FcC(O)-peptides[14] and TFA/TIS/water – 92.5/5/2.5 (%, v/v/v) for the RcC(O)-peptides. Precipitation of the cleaved peptides in cold (-20 °C) Et_2O/*n*-hexane – 1/1 (%, v/v) and purification by semi-preparative HPLC on a C_{18}-column afforded 64 peptides in high (>99%) purity (see ESI). As buffers we used for A: water/MeCN/TFA – 95/5/0.1 (%, v/v/v), and for B: MeCN/water/TFA – 95/5/0.1 (%, v/v/v). MALDI-TOF MS analysis of all Fc- and Rc-derivatized diastereomeric peptides provided *m*/*z*-values that were all comparable to previously published values. MALDI mass spectra were obtained on a Bruker Ultraflex III MALDI-TOF instrument.

Antibacterial Activity

Antibacterial activity was tested against three Gram-negative bacterial strains (*Escherichia coli*, type DSM 30083; *Pseudomonas aeruginosa*, type DSM 50071; and *Acinetobacter baumannii*, type DSM 30007) and three Gram-positive strains (*Staphylococcus aureus*, type DSM 20231; Methicillin resistant *S. aureus* (MRSA), type ATCC 43300; and *Bacillus subtilis* 168, type DSM 402). This was done as described in detail in ref 8 and 12.[‡] The concentration of the peptides was calculated from the accurately measured volume to dissolve a sample and the amount of peptide that was used, taking in consideration the presence of one TFA-counterion (FW = 114.02) for each positive charge, i.e. two TFA-counterions for each peptide in this study. The FW for each peptide was taken to be 1327.44 for the FcC(O)-, and 1373.41 for the RcC(O)- peptides.

Hemolytic Activity

This assay was performed according to our previously described procedure[7] using 20 µL of each 1 mg/mL peptide stock solution in DMSO and 100 µL 5% hRBC suspension in PBS (pH 7.4). Final concentration of the peptide in each well was 121 µM, as a blank we used 20% DMSO and as positive control 2% triton X-100 in 10 µL DMSO.

Results

Synthesis and Stability

In a straightforward fashion and using well-established synthetic protocols 64 (2×2^5) metallocenoyl functionalized diastereomeric peptides were obtained. Even though the crude peptides already had a very high purity (>90%), each peptide was further purified by preparative HPLC in order to receive highly pure peptides (see ESI). Several of the HPLC-samples were analysed after they were stored on the bench for two weeks, and no disintegration of any of the peptides was observed by HPLC. Loss of a CpRu or CpFe fragment would leave a Cp-modified peptide that elutes earlier

than the parent peptide. This has previously been observed for FcC(O)-peptides that were cleaved in the presence of moist, which results in the more labile ferrociniumoyl-derivatized peptide. In our present case, no such degradation of the organometallic-peptide conjugate was ever observed, indicating high stability of the FcC(O)- and RcC(O)-WRWRW-NH_2 peptides.

Antibacterial Activity

Considering the observation that none of the synthesized peptides were significantly active against Gram-negative bacteria (table 1), the discussion below will focus only on the Gram-positive bacteria.

In general, this study firmly establishes that RcC(O)-derivatized peptides are more active than FcC(O)-conjugated peptides. In fact, most of the RcC(O)-peptides are between 2–4 times more active against *S. aureus* than their corresponding FcC(O)-derivatives. Hence, what was previously observed for one single peptide[11] has now been proven to be true for a whole family of peptides. Interestingly, the activities of the two sets of peptides follow each other quite well with only a few notable exceptions (chart 1, table 1). To be more specific, a large difference is seen between the RcC(O)- and FcC(O)-peptides of the DLLDL-isomer (entry 13, table 1), where the former is ~8 times more active than the latter. For the DDLLD peptide (entry 22, table 1), the FcC(O)-peptide seems to be as active as the RcC(O)-peptide.

Ferrocenoylated Peptides, FcC(O)-WRWRW-NH_2

The MIC-values of the ferrocenoyl-derivatized peptides were between 3–24 μM, demonstrating that these peptides are already very active against Gram-positive bacteria. Curiously, almost all diastereomeric peptides were as active or even more active than the parent all-L peptide. None of the diastereomeric FcC(O)-peptides shows a notable selective activity for one of the two *S. aureus* strains, and MRSA growth is efficiently inhibited by all peptides. Also, activity against *B. subtilis* is generally in the same range as the activity against *S. aureus*. The high similarity in activity is congruent with the phospholipid-based membrane bilayer being the primary target structure. Astoundingly, the most active peptides share a C-terminal ^{L}Arg-^{D}Trp-NH_2 dipeptide unit and have MIC values ranging from 3–6 μM. Assuming that the FcC(O)-LDLLD peptide, which was lost during purification, has similar activities (an assumption that is supported by the high activity of the corresponding RcC(O)- analogue (entry 9, table 1)), eight highly active peptides are identified here. This allows us to assess the effect of this C-terminal ^{L}Arg-^{D}Trp-dipeptide unit in greater detail.

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Table 1. Antibacterial activity (as MIC-values, in µM) of the RcC(O)- (*left* block) and FcC(O)- (*right* block) L-to-D substitution scanned WRWRW-pentapeptides.[a] For the RcC(O)-peptides, activities against Gram-negative and -positive bacteria are reported, as well as the corresponding retention times (min) on a C_{18}-column.[b] The FcC(O)-peptides were only active against Gram-positive bacteria.

		RcC(O)-WRWRW-NH$_2$								**FcC(O)-WRWRW-NH$_2$**		
		Gram-negative			Gram-positive					Gram-positive		
Entry	Chirality of the amino acid residues in McC(O)-WRWRW-NH$_2$	*E. coli*	*A. baumannii*	*P. aeruginosa*	*S. aureus*	*S. aureus* (MRSA)	*B. subtilis*	retention time (min)		*S. aureus*	*S. aureus* (MRSA)	*B. subtilis*
		MIC	MIC	MIC	MIC	MIC	MIC			MIC	MIC	MIC
1	**LLLLL**	47	**12–23**	93	5.8	5.8	2.9	20.1		12	12	6
2	**LLLLD**	93	93	n.a.	n.d.	n.d.	n.d.	19.8		6	3–6	3–6
3	**LLLDL**	47	47	n.a.	5.8	n.d.	2.9	19.9		6–12	12	3–6
4	**LLDLL**	47	93	>93	2.9	1.5–2.9	1.5	19.8		12	6–12	3
5	**LDLLL**	47	93	>93	n.d.	n.d.	n.d.	19.8		12	12	3–6
6	**DLLLL**	47	47	n.a.	n.d.	n.d.	n.d.	19.9		6–12	6	3–6
7	**LLLDD**	47	93	n.a.	2.9	2.9–5.8	1.5	19.7		12	12	3–6
8	**LLDLD**	93	n.a.	n.a.	2.9	2.9	1.5	19.4		3	3	3
9	**LDLLD**	93	47	n.a.	2.9	1.5	1.5	19.9		n.d.	n.d.	n.d.
10	**DLLLD**	93	93	n.a.	1.5-2.9	1.5	1.5	19.8		3	3–6	3
11	**LLDDL**	47	47	n.a.	2.9	1.5–2.9	1.5	19.8		6–12	6	3–6
12	**LDLDL**	93	93	n.a.	5.8	2.9–5.8	1.5	19.6		12–24	12–24	6
13	**DLLDL**	93	**23–47**	n.a.	**1.5**	**0.7–1.5**	1.5	19.7		12	12	6
14	**LDDLL**	47	93	n.a.	2.9	2.9	1.5	19.7		6	6	3
15	**DLDLL**	47	47–93	n.a.	1.5–2.9	1.5	1.5	19.7		6	6–12	3–6
16	**DDLLL**	93	47	n.a.	2.9	1.5	2.9	19.7		6–12	6–12	3
17	**LLDDD**	93	>93	n.a.	2.9	2.9	1.5	19.8		6–12	6	3
18	**LDLDD**	93	93	n.a.	2.9–5.8	2.9	1.5	19.8		12	12	3–6
19	**DLLDD**	93	93	n.a.	**1.5**	**0.7–1.5**	1.5	19.6		6	6	3
20	**LDDLD**	93	93	n.a.	2.9–5.8	2.9	**0.7–1.5**	19.5		3	3	**1.5–3**
21	**DLDLD**	>93	93	n.a.	**1.5**	**0.7–1.5**	1.5	19.5		1.5	3	3
22	**DDLLD**	93	93	n.a.	2.9–5.8	2.9	**0.7–1.5**	19.8		**1.5–3**	**1.5–3**	3
23	**LDDDL**	>93	93	n.a.	2.9–5.8	2.9	2.9	19.6		6–12	6	3
24	**DLDDL**	47–93	47–93	n.a.	**1.5**	**0.7–1.5**	1.5–2.9	19.9		6	6	3–6
25	**DDLDL**	93	>93	n.a.	5.8	2.9–5.8	1.5–2.9	19.4		6–12	6	**1.5–3**
26	**DDDLL**	47	>93	n.a.	2.9	2.9–5.8	**0.7–1.5**	19.6		12	12	3–6
27	**LDDDD**	47–93	47	n.a.	2.9	2.9–5.8	1.5	19.9		6	6–12	3–6
28	**DLDDD**	47	47	n.a.	**1.5**	**0.7–1.5**	**0.7–1.5**	19.8		n.d.	n.d.	n.d.
29	**DDLDD**	47–93	93	n.a.	**1.5**	**1.5**	**0.7–1.5**	19.8		6	6–12	3
30	**DDDLD**	47–93	47	>93	2.9	1.5	0.7	19.8		3	3–6	3
31	**DDDDL**	93	47–93	n.a.	2.9	1.5	1.5	19.9		6	6–12	3
32	**DDDDD**	**23–47**	93	>93	5.8	2.9	1.5	20.1		6	6–12	3

Notes: [a] MIC = Minimal Inhibitory Concentration, i.e. the lowest concentration at which bacterial growth is inhibited; n.a. means 'not active', referring to activity above 186 µM; n.d. means 'not determined' due to insufficient amounts; >93 means a MIC of 93–186 µM. [b] Analytical HPLC was performed on an automated HPLC system using a C_{18}-AQ RP column (250 × 4.6 mm) at a flow-rate of 1 mL/min. A linear gradient of 5% buffer B per min was started at 5 min of buffer A (A: H_2O/MeCN/TFA, 95:5:0.1, v/v/v; B: MeCN/H_2O/TFA, 95:5:0.1, v/v/v).

Ruthenocenoylated Peptides, RcC(O)-WRWRW-NH$_2$

As mentioned, the RcC(O)-derivatized peptides are up to 2–4 times more active against *S. aureus* than their corresponding FcC(O) analogues (for example entry 13, table 1). They also have the tendency to be slightly more active against MRSA than against the *S. aureus* wild-type strain. Interestingly, a common feature present in all but one of the most active RcC(O)-peptides is the N-terminal ^{D}Trp-^{L}Arg pattern; the one active peptide that is an exception has a ^{D}Trp-^{D}Arg-^{L}Trp motive (entry 29, table 1).

The difference in activity between the diastereomeric peptides is a factor of 4-8 against *S. aureus* wild-type strain. There is almost no difference in activity against *B. subtilis*: All diastereomeric peptides are very active with MIC-values at or below 2.9 µM. Again, almost all diastereomeric compounds are more active than the all-L peptide.

Comparison of FcC(O)- with RcC(O)-WRWRW-NH_2

This SAR study on metallocenoylated diastereomeric Arg-Trp peptides shows that, indeed, RcC(O)-functionalized peptides are more active than their FcC(O)-derivatized counterparts: None of the FcC(O)-peptides is significantly more active than the RcC(O)-containing analogue. Considering membrane interaction as the most important contributor to the antibacterial activity, we expect that the activity of other membrane targeting peptides may also be enhanced by attaching RcC(O)OH rather than the commercially available FcC(O)OH.

Within this specific set of diastereomeric peptides, patterns in the MIC values for each peptide can be conveniently identified using a radar-plot. For example, plotting of the MIC values for each diastereomeric peptide against the two *S. aureus* strains highlights the previously mentioned pattern that the most active FcC(O)-peptides share a C-terminal ^{L}Arg-^{D}Trp unit. In fact, their activity approaches the RcC(O)-derivatized analogue's levels (chart 1, green traces). It also becomes clear that the RcC(O)-peptides with a ^{D}Trp-^{L}Arg unit on their N-terminus are the most active peptides (chart 1, blue traces).

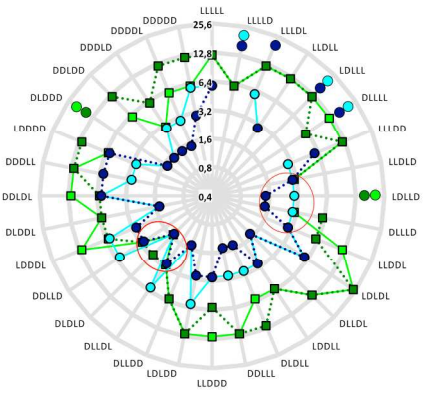


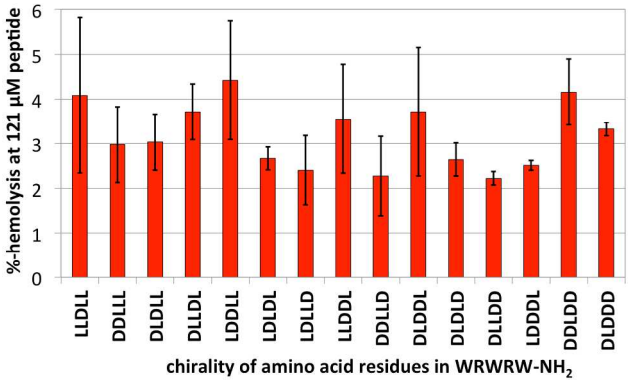
Chart 1. Radar plot of the antibacterial activity of the diastereomeric FcC(O)- (green) or RcC(O)- (blue) WRWRW-NH_2 peptides against *S. aureus* (filled) and MRSA (line). Not determined values are given as 'zero' and are highlighted by the colored circles; red circles highlight the most generally active peptides.

In the two cases where both patterns are combined in one peptide, *i.e.* in DLDLD and in DLLLD (entries 10 and 23 in table 1, respectively), the activities of both the FcC(O)- and the RcC(O)-derivatives are indeed very similar and amongst the highest found (chart 1, red circles). Studying the interaction of these peptides with model membrane systems could provide valuable information for a further optimization of the activity.[15] Also, comparing the MoA of the FcC(O)- and RcC(O)-derivatives that have the DDLLD and LLDDL configurations could provide clues as to why the DDLLD peptides are very similar in activity and the LLDDL peptides are so different.

Hemolytic Activity

Hemolysis was studied using a high concentration of a representative set of the most active peptides; since the hemolysis of the lead-sequence was already very low, we did not expect to see significantly higher levels of hemolysis. In fact, based on our recent finding that diastereomeric short AMPs can have significantly lower hemolytic potential than the all-L lead sequence[16], we were expecting to see only low levels of hemolysis. Thus, 121 µM of 15 RcC(O)-derivatized diastereomeric peptides were applied to freshly isolated hRBCs. This concentration is >20 times higher than the highest MIC-value against the Gram-positive bacteria, *i.e.* 5.8 µM. With only 1–6% hemolysis present, none of these peptides are very hemolytic (chart 2). The combination of high antibacterial potency with low hemolytic activity found in our new metallocene-AMPs opens a significant therapeutic window.

Chart 2. Bar-graph of the percentage hemolysis caused by 121 µM of RcC(O)-WRWRW-NH_2 peptides.



Apparent Lipophilicity: Retention Times

Lastly, different retention times were noticed for peptides with different combinations of L- and D-amino acid residues. Concerning the RcC(O)-derivatized peptides, a difference of about 0.7 min was observed. Using a radar-plot of the retention time against the chirality of the amino acids in the WRWRW-NH_2 sequence, it immediately becomes clear that all diastereomeric peptides are less lipophilic than the all-L or all-D peptides (chart 3). As could be expected, the pattern of the radar-plot has a plane of symmetry running inbetween LLLLL and DDDDD at the top, as well as DDLLL and LLDDD at the bottom. There is no obvious correlation between antibacterial activity and retention time, as was observed for lipidated versions of similar peptides.[7] This rules out higher lipophilicity of the Rc-derivatives as a potential explanation for their improved activity. Of the two peptides that were classified as most active, *i.e.* DLLLD and DLDLD, one is slightly less lipophilic than the other with a retention time of 19.8 min *vs* 19.5 min, respectively. A correlation between hemolysis and retention time is also not apparent. Importantly, there is no difference in lipophilicity of the two different metals in the peptide families: on a C_{18}-reversed phase column RcC(O)-derivatized peptides have the exact same retention time as FcC(O)-containing counterparts under identical conditions (see ESI).

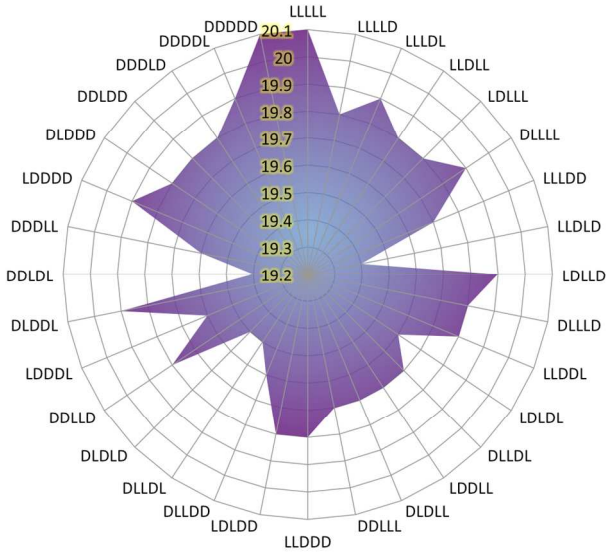


Chart 3. Filled-radar plot of the retention times (min) of the 32 diastereomeric RcC(O)-WRWRW-NH_2 peptides.

Discussion

Whereas the antibacterial activity of the all-L FcC(O)-WRWRW-NH_2 lead sequence could be increased 2–4 fold using an L-to-D substitution scan, substitution of an FcC(O)-moiety for an RcC(O) counterpart produced an 4–8 fold increase in activity. Thus, the effect of the metal on the metallocenoyl-group is on average twice as significant as the chirality of the amino acid residue in the WRWRW-NH_2 pentapeptide sequence. It is therefore valuable to discuss the role of the organometallic fragment in more detail.

Contribution of the Metallocenoyl Moiety

For this, the following properties of the organometallic fragment have to be considered:

- The redox potential of Fc (+450 mV, versus SCE)[17] undergoes a significant shift of about 210 mV towards more positive values in ferrocenoyl-derivatives, i.e. the metal becomes harder to oxidize.[18] Nevertheless, electron-donation to biological entities is still within the range of a few redox-enzymes, e.g. various cytochromes and ferredoxins.[19] This is not the case for RcC(O), because ruthenocene itself has a redox-potential of about ~700 mV. In principle, this could interact with rusticyanin, a redox-active enzyme that is found in the periplasmic space of the Gram-negative *Thiobacillus ferrooxidans* and has a redox-potential of +680 mV (versus Fe^{2+}/Fe^{3+}).[20] However, by analogy to ferrocene, the presence of an acyl group on ruthenocene probably pushes this potential outside the range covered by bacterial redox enzymes. In addition, the hydrophobic surrounding of the membrane probably pushes the redox-potential even further towards positive values, as it is the case in hydrophobic organic solvents.[21]
- Ferrocene is notably smaller than ruthenocene: the former has C-M bond-lengths of 204 pm *vs* 221 pm for the latter, a difference of 17 pm.[22] Using calculations, the difference in the height of the metallocene-derived π-systems of the sandwich complex FcC(O)NHMe *vs* RcC(O)NHMe is estimated to be as large as 0.5 Å.[23] This difference in size can be relevant in the biological world, because numerous interactions are tuned with sub-Ångstrom precision and could be the underlying cause for the differences in activity of the RcC(O)- *vs* FcC(O)-derivatized peptides. Nevertheless, the precise relevance of this difference in size within the context of a membrane targeting molecule remains to be established.
- The ruthenium ion in ruthenocene has been shown to act as H-bond acceptor in metal-hydrogen bonds.[24] The extended nature of the filled d-orbitals on the metal-center,[25] especially the d-orbitals that are not involved in Cp-binding and stretch out from between the two Cp rings (z^2, x^2-y^2, and xy), can bind to polarized hydrogen atoms. For the iron(II) ion in ferrocene the contracted nature of its d-orbitals does not allow such an interaction.[26] In α-ruthenocenylcarbinols the intramolecular Ru···HO bond energy is about 4.1 kcal/mol; this value will be different in our case, because an electron withdrawing C(O)NH-moiety is attached to one of the Cp rings of Rc. Even more, the precise chemical nature of the membrane-water interface will have implications for the intermolecular Ru···HO bond.[§] For comparison, H-bond energies are usually in the range of 3–7 kcal/mol and amide NH···O bonds are typically around 5 kcal/mol.[27]

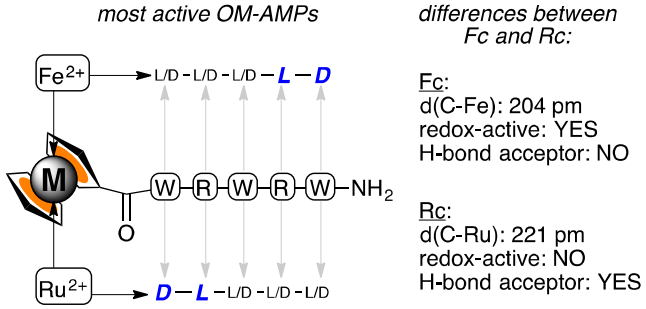


Figure 2. Schematic depiction of the antibacterial activity of the most active diastereomeric OM-AMPs (left) and the fundamental differences in the properties of the attached organometallic fragment (right).

Stability of the Metallocenoyl-Peptide Conjugates

Based on this knowledge of these two representatives of group 8 metallocene moieties and derivatives, we are now in a position to design experiments that help us gain a better understanding of the underlying fundamentals that determine the differences in activity. In those future experiments, particular attention should be directed at the study of the stability of the organometallic-bioconjugates. In case of these group 8 metallocenoyl peptides, degradation of the purified peptides was never observed, even not after storage of the HPLC sample (in water/MeCN – 1:1, v/v) for several weeks on the bench, or of the biological samples (concentrated solutions in DMSO) for several months. In combination with our previous observation that the all-L RcC(O)-WRWRW-NH_2 peptide accumulates in the membrane of the bacteria, we are convinced that under the conditions of the biological experiment, the peptide remains intact. Slow degradation of these peptide cannot be excluded at this point, but since the killing kinetics of the bactericidal all-L peptide is very fast, as is seen by an instant drop in the number of colony forming units (CFUs) of 2–3 log units, the observed activity of

the peptides is primarily related to the intact peptide, and not caused by a secondary effect caused by a disintegrated organometallic fragment.

Conclusions

The comprehensive two-parameter SAR study described in this work shows how the high antibacterial activity of group 8 metallocenoyl-derivatized AMPs can be enhanced simultaneously to successfully controlling their hemolytic activity. By combining certain L- and D-amino acid residues using an L-to-D substitution scan on all positions, diastereomeric peptides are identified that are 8 times more active than the lead sequence, having low micro molar activity. In fact, considering the all-L FcC(O)-peptide as the lead sequence, an 8-fold improvement is obtained in this optimization study. Comparing our most active AMPs with the activity of prominent antibacterial peptides like gramicidin S (2.8 µM, 1200 mu, 10 amino acids) and vancomycin (0.6 µM, 1447 mu, 7 amino acids and 2 sugar moieties), our peptides are among the most active antibacterial peptides (0.7 µM, 1145 mu, 5 amino acids) known to date. Importantly, none of the representative 15 diastereomeric peptides that were tested for hemolysis was significantly active when hRBCs were exposed to 121 µM of peptide, which is about 100 times higher than the lowest MIC-value obtained. This places these peptides among the most active against bacteria, but non-toxic towards human kind, short AMPs known.

Looking ahead, the role of the organometallic fragment is particularly interesting. Not only does the replacement of FcC(O) with RcC(O) result in a significant increase of antibacterial activity, we also observed that the most active FcC(O)-derivatized peptides share a C-terminal ^{L}Arg-^{D}Trp-NH_2 motive, whereas the most active RcC(O)-peptides share an N-terminal ^{L}Trp-^{D}Arg pattern. This shift in preference of a certain metallocene for a specific combination of L- and D-residues is likely to originate from the properties of the metal-ion in the sandwich complex. Future studies are directed at a precise determination of the role of these two organometallic moieties when attached to peptides, especially in a biological context leading up to clinical applications.

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† Electronic Supplementary Information (ESI) available: HPLC-traces of all Rc- and 10 Fc-derivatized peptides. See DOI: 10.1039/b000000x/

‡ The FcC(O)-derivatized peptides were also tested against *Candida albicans*. However, only low activity was determined for three peptides – *i.e.* 96 µM for FcC(O)-DLLDD, FcC(O)-LDDLD, and FcC(O)-LDLDD – whereby the RcC(O)-derivatives were not tested against the pathogenic fungus.

§ For comparison, the α-osmocenylcarbinol has an intramolecular $(Cp)_2Os\cdots HO$ bond with an energy of 5.0 kcal/mol but an intramolecular $(Cp)_2Os\cdots HOH$ bond with an energy of 11.7 kcal/mol.[18]

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