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Solid-Phase Synthesis, Characterization, and Antibacterial Activities of Metallocene–Peptide Bioconjugates

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This work shows how the introduction of an organometallic group enhances and modifies the specificity of biologically active peptides. Ferrocene was chosen as an organometallic group because it has been shown to alter the pharmacodynamic profile of bioactive compounds. A comparison with the isosteric cobaltocenium group allows one to explore the influence of charge and redox potential on the biological activity of the conjugates. Arginine and tryptophan containing peptides H-WRWRWR-NH_2 and H-RWRWRW-NH_2 and the metallocene peptide bioconjugates [M]-C(O)-RWRWR-NH_2 and [M]-C(O)-WRWRW-NH_2, where [M] = $[Co(Cp)(C_5H_4)]^+$, $[Fe(Cp)(C_5H_4)]$ were prepared by solid-phase peptide synthesis (SPPS). They were purified by HPLC, characterized by ESIMS and NMR spectroscopy, and tested for antibacterial properties against Escherichia coli, Pseudomonas aeruginosa, *and* Staphylococcus aureus *using the minimum inhibitory concentration (MIC) test. In most cases, no metal-specific activity could be observed. However, the conjugate $[Fe(Cp)(C_5H_4)$-C(O)-WRWRW-$NH_2]$* **6** *was found to be particularly effective against the Gram-positive* S. aureus*. The activity of this metallocene–pentapeptide conjugate (7.1 μM) was even better than the 20 amino acid naturally occurring pilosulin 2, which was used as a positive control. Unlike all other compounds tested, which were most active against the Gram-negative* E. coli *strain, the ferrocene conjugate* **6** *was the only compound in this series that was most active against Gram-positive bacteria. Given the health concerns resulting from multidrug resistant* S. aureus *strains, the incorporation of metallocenes may provide a novel line of attack.*

Introduction

A wide variety of plants, insects, and animals possess antibacterial peptides as part of their innate immune system and host defense mechanisms. Interest in antimicrobial peptides (AMPs) has grown considerably in recent years and over 800 AMPs, both ribosomally and nonribosomally synthesized have been isolated.[1] The majority of AMPs are cationic. These peptides display an astonishing range of shapes and sizes; linear peptides with α-helical structures (for example, insect cecropins, frog magainins), β sheets with disulfide bridges (for example, α-, β-, and θ-defensins), and peptides with loop structures (for example, cow bactenecin).[2] The range and speed of activity make antibacterial peptides a potential new class of antibiotic drugs. The low incidence of bacterial resistance points towards novel mechanisms of action.[3] Furthermore, the acquisition of bacterial resistance to conventional antibiotics increases the urgency for developing effective substitutes.

Phosphatidylglycerol and phosphatidylethanolamine are major components of bacterial membranes and as the former has an overall negative charge, bacterial membranes typically carry a net negative charge. Incidentally, certain types of cancer cells have a significant proportion of the negatively charged phosphatidylserine on their surface membrane. A cationic AMP such as lactoferricin B has been shown to bind to the net negatively charged membrane of cancer cells.[4] This compound also exhibits antitumor properties. The antibacterial activity of an AMP is initiated when the cationic AMPs accumulate at the surface of the cell membrane, causing a modification of the biophysical properties of the membrane. The membrane integrity is compromised by disruption to the membrane potential, which leads to increased permeability, leakage of metabolites, and ultimately cell death. While the cationicity

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of an AMP can be attributed to the presence of Lys and Arg side chains, the guanidinium side chain of Arg seems to confer better antibacterial activity.[5] Amino acids with hydrophobic, aromatic side chains such as Trp, Tyr, and Phe also have an important role. The larger Trp, in particular, is known to have a preference for membrane interfaces.[6]

The susceptibility of AMPs to proteolysis and the high production cost are some of the limitations to their commercial use as antibiotics. Therefore, shorter, modified peptides with similar or enhanced activities would be desirable.[7] Recently, organometallic derivatives of biomolecules have attracted much attention.[8–10] The metal could confer an increased stability to the conjugate in biological media and, in certain cases, metal-specific modes of action were suggested, for example, by the lipophilicity[11] or redox properties of the metal complex.[12,13] For example, replacement of a phenyl ring in the well-known anticancer drug, tamoxifen, by the ferrocenyl group[9,14,15] results in a spectacular change of the pharmacodynamic profile which can be traced to the redox activity of the organometallic group.[10,12]

In early work, the antibacterial activities of the β-lactam antibiotics, penicillin and cephalosporin, were claimed to be enhanced by replacing aromatic groups with a ferrocenyl group; although no comprehensive data was provided.[16,17] In a recent paper, we identified metallocene-modified tri- to pentapeptides with antibacterial activities.[18] The most active compound was the ferrocenoyl peptide FcC(O)-WRRF-NH_2 with a minimum inhibitory concentration (MIC) of about 50 μM. Although it was encouraging to see that an organometallic derivative showed the best activity of all peptides tested, its activity is still at least one order of magnitude smaller than MIC values found for the most active naturally occurring AMPs. By adding metallocenes to more active peptide sequences per se, we hope to arrive at small, readily available artificial AMPs with an activity comparable to the best naturally occurring AMPs.

In this work, we have selected two Arg- and Trp-containing hexapeptide sequences which were shown to have good antibacterial properties[19] and modified them by replacing the N-terminal amino acid with a ferrocenyl and a cobaltocenium group. The lipophilic nature of the ferrocenyl moiety can be thought of as being a mimic for the bulky Trp residue, whereas the cobaltocenium group has the advantage of carrying a positive charge, and thus acts as a bulky Arg-mimetic. The positively charged cobaltocenium moiety is isostructural to the neutral ferrocene thus allowing assessment of an additional positive charge on the antibacterial activity.

Results

The metallocene peptide conjugates were prepared by solid-phase peptide synthesis (SPPS). The ferrocenyl and cobaltocenium groups can be conveniently attached to the solid support with a peptide bond by reacting the appropriate metallocene carboxylic acid, for example, ferrocene carboxylic acid, [Fe(Cp)(C_5H_4-COOH)], and cobaltocenium carboxylic acid hexafluorophosphate, [Co(Cp)(C_5H_4-COOH)]PF_6, with the free N-terminal amino group of the selected peptide. Furthermore, instead of isolating the free carboxylate peptide, the C-terminal was amidated (Rink amide resin) as this increases the overall positive charge on the peptide bioconjugate by eliminating a negative charge. Capping of the N-terminal results in a net loss of one unit of positive charge in the case of the ferrocenoyl bioconjugates, but the cobaltocenium analogues retain the overall charge of the peptide. The metallocene moieties are stable towards Fmoc and amino acid side-chain deprotection reagents, and to resin cleavage. It should be noted, however, that the ferrocenoyl peptides are stable only when phenol rather than water is used in the cleavage mixture. The structures of all compounds that were prepared are shown (**1**–**6**).

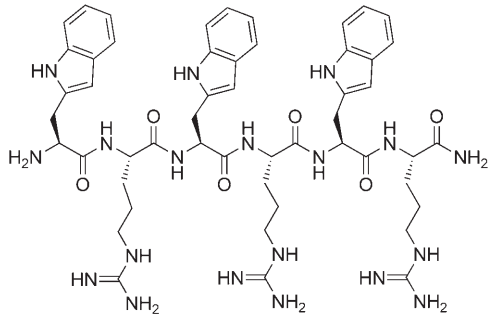
The compounds were purified by RP-HPLC and the retention times are listed in Table 1. These values reflect the polarity of the compounds, and correlate with the overall composition of charged and bulky substituents on the peptide. The identities of the compounds were verified by ESIMS. The $[M+H]^+$ peaks corresponded to the calculated values, but were detected in very low intensities. However, their identities could be confirmed by examining the 2^+ and 3^+ molecular ion peaks. The dications were the base peaks for the ferrocene derivatives **3** and **6**. For the cobaltocenium derivatives, no ions containing the TFA counter ion were detected in the chosen positive ion detection mode. The ESIMS (positive ion detection mode) spectra of the ferrocenoyl peptides (**3** and **6**) typically show a peak at *m/z* of 213. This can be attributed to the Fe(Cp)(C_5H_4-CO) fragment, which results from the decomposition of the molecular ion, and is quite commonly observed with ferrocenoyl peptides,[18,20] notwithstanding the fact that electron-spray ionization is usually considered a "soft" technique. This fragment is analogous to [Fe(Cp)(C_5H_4-CH_2)] which is frequently observed in the EI mass spectra of ferrocenyl amino acid derivatives of the type [Fe(Cp){C_5H_4-CH_2-N(Bzl)(Leu-H)}].[21]

Two-dimensional H,H-COSY and H,C-HETCOR NMR experiments were conducted to aid the assignment of amide protons (H^N, δ 7.6–9.0 ppm), alpha protons (H^α, δ 3.7–4.9 ppm), and alpha carbons (C^α, δ 51–54 ppm) of the peptide backbone, and side-chain resonances. The pres-

Table 1. Summary of HPLC retention times (t_R) and ESIMS data for **1**–**6**.

Compound	t_R [min][a]	Exact $Mass_{calcd}$	$[M+H]^+$	$[M+2H]^{2+}$	$[M+3H]^{3+}$
1	14.092	1043.57	1044.8	523.1	349.1[b]
2	12.950	1185.46[d]	1186.3	1072.6[c]	
3	15.562	1069.48	1070.7	536.0[b]	357.7
4	14.674	1043.57	1044.9	523.1	349.1[b]
5	15.597	1215.44[d]	Not observed	[c]	
6	18.875	1099.46	1100.7	551.0[b]	

[a] linear solvent gradient from 5% MeCN/95% H_2O/0.1% TFA to 95% MeCN/5% H_2O/0.1% TFA over 30 min, C18 column (60 Å/8 μm, 250×4.60 mm), flow rate 1 mL min^{-1}. [b] 100% intensity. [c] $[M-CF_3COO + nH]^{(n+1)+}$ peaks were detected where $n=0$, 1, 2, 3. [d] Calculated masses for compounds **2** and **5** are for TFA salts.



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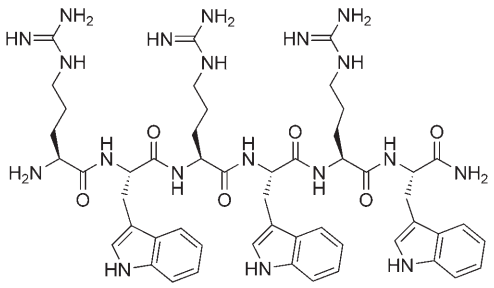

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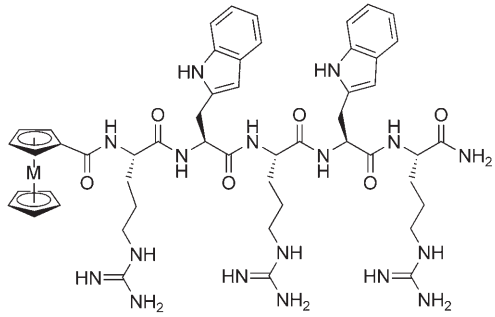
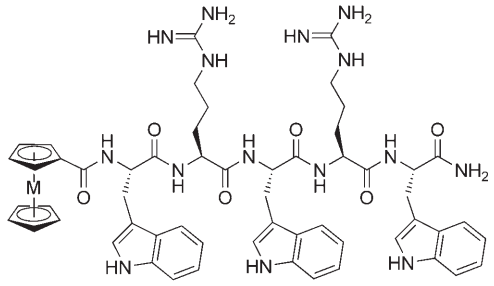


M = Co^+ **2**, Fe **3**



M = Co^+ **5**, Fe **6**

ence of the metallocene groups is immediately apparent from the ^{1}H NMR spectra of the bioconjugates, which show a characteristic intensity pattern of 1:1:2:5 for the Cp protons (Figure 1).[22,23] The coupling constants for the protons on the substituted Cp rings were not resolved and thus, the signals appeared as broad singlets. The Cp resonances of the cobaltocenium complexes are shifted downfield significantly (δ 5.2–6.4 ppm) compared with the spectra of the ferrocenoyl compounds (δ 4.2–4.8 ppm). The ferrocenoyl peptides decompose on standing for prolonged periods in dimethyl sulfoxide.

The compounds were prepared for bacterial testing by being weighed and dissolved in water to give approximately 1.2 mM solutions. As lyophilized peptides may contain anything from 10% to 50% water and salts by weight, the concentrations of these solutions were determined by spectrophotometric measurement at 280 nm.[24–27] In the case of the metallocene–peptides, the ferrocene and cobaltocenium moieties also absorb at 280 nm and separate experiments were carried out to determine the molar extinction coefficients of each metallocene chromophore (see Experimental Section).

The antibacterial properties of peptides **1**–**6** were tested against the Gram-negative *Escherichia coli* (ATCC 25922) and *Pseudomonas aeruginosa* (ATCC 27853) and the Gram-positive *Staphylococcus aureus* (ATCC 25923) by MIC testing. The MIC is defined as the maximum dilution, in other words, the lowest concentration, of the antibacterial compound that will still inhibit the visible growth of a test microorganism. Serial 1:1 dilutions of the peptides were made in bacterial growth media. The test bacteria were then added to the dilutions, incubated, and scored for growth or inhibition of growth. All the compounds were tested in parallel and in duplicate. The results are

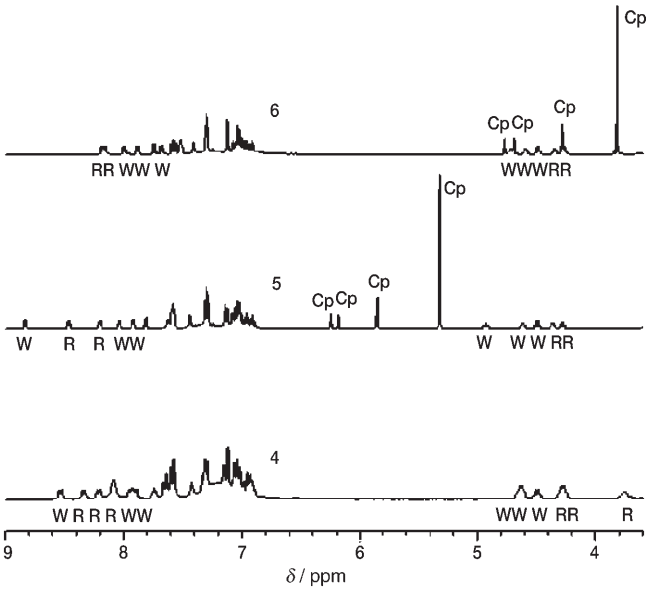


Figure 1. Comparison of ^{1}H NMR spectra (3.5 to 9.0 ppm) for **4**–**6**.

summarized in Table 2. Pilosulin 2, a 20 amino acid peptide, H-GLLSKFGRLARKLARVIPKV-NH_2, derived from the ant venom toxin pilosulin and known to be highly active, was used as the positive control.[28]

Care must be taken when comparing MIC results from different sources, because of the different strains of bacteria, growth media, and salt concentrations that can be used in the tests. It has been reported,[2] however, that the MIC values of the more effective peptides are in the range of 0.1–10 μg mL^{-1}.

Table 2. MIC results for the metallocene–peptide bioconjugates.[a]

Compound[b,c]	Units of Charge	Units of Bulk	Molar Mass	MIC [μM]		
				E. coli	*S. aureus*	*P. aeruginosa*
1, H-WRWRWR-NH_2	4	3	1044.22	3.9	16	16
				3.9	16	16
2, Cc^+CO-RWRWR-NH_2	4	3	1186.14	15	121	>121
				15	121	>121
3, FcCO-RWRWR-NH_2	3	3	1070.03	16	33/16	16
				16	33/16	16
4, H-RWRWRW-NH_2	4	3	1044.22	4.0	16	7.9
				2.0	7.9	7.9
5, Cc^+CO-WRWRW-NH_2	3	4	1216.16	6.8	27	27
				6.8	27	27
6, FcCO-WRWRW-NH_2	2	4	1100.06	57	7.1	28
				28	7.1	28
pilosulin 2	*7*	*1*	2221.8	2.0	16	4.0
				2.0	8.0	4.0

[a] Individual results from the duplicate experiments are given (lines 1 and 2 for each conjugate); see Experimental Section for details. [b] Fc = [Fe(Cp)(C_5H_4-)]. [c] Cc^+ = [Co(Cp)(C_5H_4-)]$^+$, counter ion is $CF_3CO_2^-$ for **2** and **5**.

Discussion

Herein, we describe the synthesis of novel peptide metallocene bioconjugates by SPPS. Very little work has been done previously on the solid-phase synthesis of organometallic bioconjugates. In the 1980s some ferrocenyl peptides and half-sandwich peptide complexes were isolated in low yields because of problems with decomposition.[29–36] More recently, labeling of DNA with ferrocene has been reported.[37–41] The solid-phase synthesis of Mo carbonyl complexes have also been reported; without biomolecules[42–44] and with [Leu^5]-enkephalin.[45] We have previously prepared metallocene–peptide conjugates with tri- to pentapeptides for antibacterial testing.[18] The optimized solid-phase synthetic methodology allowed us to prepare all conjugates reported herein in high yield and purity.

In general, there is currently keen interest in biologically active organometallic compounds. Ferrocenyl derivatives have recently become significant in drug design, as the replacement of an aromatic group by ferrocene has been shown to improve the activities of existing drugs. This is best exemplified by the antimalarial compound ferroquine[13,46–49] and the antitumor agent ferrocifen.[9,14,15] In both compounds the introduction of the organometallic ferrocenyl group enhances the activity of the compound and also changes the pharmacodynamic profile. Although much work has been devoted to ferrocene bioconjugates,[8,50] the closely related cobaltocenium group has, surprisingly, received considerably less attention. Its unique electrochemical properties have, however, been exploited in enzyme biosensors and DNA detection,[51,52] and in a recent study on the cellular uptake and directed nuclear delivery of a cobaltocenium-NLS peptide bioconjugate.[11]

Several models have been proposed to explain the integration of AMPs into the cell membrane, which appears to be responsible for their activity:[53] the barrel-stave mechanism,[54] the aggregate channel model,[55] and the carpet model.[56,57] Furthermore, AMPs may also enter the cell and inhibit cellular processes, possibly by binding to DNA or RNA. Strøm and coworkers have described the antibacterial activities of AMPs in terms of units of charge and units of bulk (the unit of bulk being at least the size of a phenyl group). According to their work, the minimum structural requirement for antibacterial activity was the presence of two cationic charges and two units of bulk for *S. aureus,* while *E. coli* requires an extra unit of bulk.[58] We have incorporated this motif in metallocene–peptide conjugates but were not able to simply correlate the antibacterial activity to charge and bulk in the same way.[18] Furthermore, a peptide such as Ac-RRWWFR-NH_2 (three units of charge and three units of bulk) was found to be weakly active against *E. coli* (MIC > 100 μM).[59] A combination of different factors such as peptide helicity, hydrophobicity, hydrophobic moment, peptide charge, and the size of the hydrophobic/hydrophilic domain influences the antibacterial activity and makes it difficult to predict.[60] To shed more light on their mechanism of action, the structures of naturally occurring peptides with antibacterial activity were actively investigated by several groups recently. NMR studies were carried out in micelles or lipid bilayers, so as to mimic the bacterial cell membrane.[61–63] By using TOCSY-trim experiments, Wang and coworkers identified a minimal motif from the human host defense peptide LL-37 which is still 13 amino acids long.[61] The structure of this minimal motif was solved by NMR methods and its activity against *E. coli* was found to be 40 μM.

In this work, antibacterial testing was performed against Gram-negative *E. coli* and *P. aeruginosa* and against Gram-positive *S. aureus* strains. We used a 20 amino acid sequence of the ant venom toxin pilosulin 2 as a positive control. In our experiments, pilosulin 2 was shown to be extremely active against *E. coli* (2 μM), the activity decreasing twofold for *P. aeruginosa* and at least twofold again for *S. aureus*. This is consistent with the biological properties reported previously, albeit under somewhat different conditions.[28] However, it has generally been observed that the minimum concentration for an AMP to exert antibacterial action falls in the micromolar range and that this is a result of the AMP's mode of action on the cellular membrane.[3,4] While MIC results for compounds **1** and **4** were previously reported as being 5 μM against *E. coli* and *S. aureus*, our results show that both **1** and **4** are more active against *E. coli*. Peptide **1** shows a fourfold decrease in activity for *S. aureus* and *P. aeruginosa*. Overall, peptide **4** is more active than **1**, although these two peptides each contain three Arg and three Trp residues. Substitution of the N-terminal Trp in **1**

with Cc^+ in **2** decreases the activity across all three strains, but especially so for *S. aureus* and *P. aeruginosa*. Specificity, however, against *E. coli* is retained. On the other hand, substitution of the N-terminal Trp by Fc in **3** does not change the activity against *S. aureus* and *P. aeruginosa* but does decrease the activity fourfold with respect to *E. coli*. So although **3** has one unit of charge less than **1** and **2**, this is apparently not detrimental to antibacterial activity. Substitution of the N-terminal Arg in **4** by Cc^+ also leads to a decrease in antibacterial activity for **5**, as was found for **2**. However, this decrease is less drastic and it is still very active against *E. coli* (6.8 μM or 8.3 μg mL^{-1}). All these MIC results are considerably better than the values reported for the optimized human LL-37 core peptide/minimal fragment mentioned above, which is still more than twice as long.[61] Furthermore, the metal-containing peptides are in most cases comparable in activity to the metal-free derivatives. Radical generation has been proposed as a mechanism of cytotoxicity of simple ferrocenes.[64,65] Such a mechanism, which should result in an enhancement of the activity of all ferrocene derivatives, does not seem to be in place for the ferrocene–peptide conjugates described herein.

The Gram-negative *E. coli* is most susceptible to the AMPs tested herein. As an exception, the ferrocene-containing bioconjugate **6** is the only one in the series with the highest activity against the Gram-positive *S. aureus*. This is of significance because of dangers posed by multiresistant strains of *S. aureus*. In addition, the MIC is in a very favorable range (7.1 μM or 7.7 μg mL^{-1}) and even more effective than the pilosulin 2 control. This is a most interesting case where ferrocene substitution not only enhances the activity of a purely organic compound, but has a different target specificity. Further antimicrobial testing is currently in progress.

Experimental Section

Peptide synthesis and purification. Solid-phase peptide syntheses were performed manually at room temperature using an Fmoc-protection strategy on a Rink Amide resin. The resin (200 mg, 0.13 mmol) was left to swell in CH_2Cl_2 for 1 h prior to use. N-terminal Fmoc deprotection was achieved using 20% *v/v* piperidine in DMF over 10 min. A mixture of Fmoc-aa-OH (4 equiv)/TBTU (4 equiv)/DIPEA (9 equiv) in DMF was used for each coupling step, whereas the metallocenes were attached using [Co(Cp)(C_5H_4-COOH)]PF_6 (4 equiv) or [Fe(Cp)(C_5H_4-COOH)] (4 equiv)/TBTU (4 equiv)/HOBt·H_2O (4 equiv)/DIPEA (9 equiv). Arg and Trp side chains were protected by Pbf and Boc, respectively. After the last deprotection step (**1**, **4**) or otherwise the last coupling step, the resin was washed with CH_2Cl_2 and dried under vacuum. Peptide (**1**, **4**) and peptide-cobaltocenium amides (**2**, **5**) were cleaved from the resin and deprotected using a mixture of TFA, H_2O, and TIS (95:2.5:2.5) for 3 h at room temperature. Peptide-ferrocenoyl amides (**3**, **6**) were cleaved and deprotected using a mixture of TFA, phenol, and TIS (85:10:5). The cleavage solutions were filtered and the peptides were precipitated by the addition of cold Et_2O. After centrifugation, the peptide precipitates were washed with cold Et_2O and dried in air. The peptides were then dissolved in H_2O/MeCN, filtered, and lyophilized. The peptides were purified using reverse-phase HPLC on a Varian C18 column (60 Å/8 μm, 250×10.0 mm) using a mixture of H_2O and MeCN, both containing 0.1% TFA, as eluent. The peptides were analyzed by RP-HPLC at 25 °C using an analytical Varian C18 column (60 Å/8 μm, 250× 4.60 mm) at 220 nm and 254 nm (Varian ProStar PDA detector). A flow rate of 1 mL min^{-1} starting at 5% MeCN/95% H_2O was used. The solvent gradient was increased linearly to 95% MeCN at 30 min and decreased to 5% MeCN at 35 min. The purities of the HPLC traces were greater than 97%. Pilosulin 2 was prepared as previously published.[28]

Peptide characterization. Mass spectra were measured by positive ion electrospray mass spectrometry on a Finnigan TSQ 700 spectrometer. NMR spectra were recorded on a Bruker DRX 300 spectrometer. The ^{1}H and ^{13}C chemical shifts were referenced to residual solvent protons. Two-dimensional H,H-COSY and H,C-HETCOR NMR experiments were conducted to aid assignment of signals. UV/Vis measurements were obtained using a Varian CARY 100 spectrophotometer.

H-WRWRWR-NH$_2$, ***1***. White solid. ^{1}H NMR (300 MHz), [D_6]DMSO, 25 °C: δ = 10.93 (s, 1H; $H^{\varepsilon1}{}_{Trp}$), 10.77 (s, 1H; $H^{\varepsilon1}{}_{Trp}$), 10.74 (s, 1H; $H^{\varepsilon1}{}_{Trp}$), 8.79 (d, ^3J = 8.0 Hz, 1H; $H^{N}{}_{Arg}$), 8.21 (d, ^3J = 7.4 Hz, 1H; $H^{N}{}_{Arg}$), 8.10 (d, ^3J = 7.6 Hz, 1H; $H^{N}{}_{Trp}$), 7.96–8.04 (m, 5H; $H^{N}{}_{Arg}$, 2×$H^{N}{}_{Trp}$, 2× $H^{\varepsilon}{}_{Arg}$/CO′NH_2), 7.54–7.69 and 6.84–7.35 (m; $H^{\delta1}{}_{Trp}$, $H^{\varepsilon3}{}_{Trp}$, $H^{\zeta2,3}{}_{Trp}$, $H^{\eta2}{}_{Trp}$, $H^{\eta}{}_{Arg}$, $H^{\varepsilon}{}_{Arg}$/CO′NH_2), 4.61 (m, 2H; 2×$H^{\alpha}{}_{Trp}$), 4.38 (m, 1H; $H^{\alpha}{}_{Arg}$), 4.29 (m, 1H; $H^{\alpha}{}_{Arg}$), 4.19 (m, 1H; $H^{\alpha}{}_{Arg}$), 4.04 (m, 1H; $H^{\alpha}{}_{Trp}$), 2.91–3.17 (m, 12H; $H^{\beta}{}_{Trp}$, $H^{\delta}{}_{Arg}$), 1.35–1.75 ppm (m, 12H; $H^{\beta}{}_{Arg}$, $H^{\gamma}{}_{Arg}$); ^{13}C NMR (75 MHz), [D_6]DMSO, 25 °C: δ = 173.2 (C=O), 171.4 (C=O), 170.8 (C=O), 170.6 (C=O), 170.1 (C=O), 168.4 (C=O), 158.8 ($C^{\zeta}{}_{Arg}$), 158.3 ($C^{\zeta}{}_{Arg}$), 156.8 ($C^{\zeta}{}_{Arg}$), 136.3 ($C^{\varepsilon2}{}_{Trp}$), 136.0 ($C^{\varepsilon2}{}_{Trp}$), 127.3 ($C^{\delta2}{}_{Trp}$), 125.0, 123.6, 120.8, 118.4, 118.2, and 111.2 ($C^{\delta1}{}_{Trp}$, $C^{\varepsilon3}{}_{Trp}$, $C^{\zeta2}{}_{Trp}$, $C^{\zeta3}{}_{Trp}$, $C^{\eta2}{}_{Trp}$), 109.8 and 106.7 ($C^{\gamma}{}_{Trp}$), 52.4, 52.3, and 52.2 (C^{α}), 29.6, 29.2, and 27.9 ($C^{\beta}{}_{Trp}$, $C^{\beta}{}_{Arg}$), 25.0 and 24.9 ppm ($C^{\gamma}{}_{Arg}$), $C^{\delta}{}_{Arg}$ masked by the solvent peak and not all C^{α} were resolved. ESI (pos.) for $C_{51}H_{69}N_{19}O_6$ (1043.6) *m/z*: 1044.6 [*M* + H]$^+$, 523.1 [*M* + 2H]$^{2+}$, 349.1 [*M* + 3H]$^{3+}$.

[Co(Cp)(η-C_5H_4)-C(O)-RWRWR-NH$_2$](CF$_3$CO$_2$), ***2***. Yellow solid. ^{1}H NMR (300 MHz), [D_6]DMSO, 25 °C: δ = 10.80 (s, 2H; 2×$H^{\varepsilon1}{}_{Trp}$), 8.76 (d, ^3J = 7.7 Hz, 1H; $H^{N}{}_{Arg}$), 8.23 (d, ^3J = 7.7 Hz, 1H; $H^{N}{}_{Arg}$), 8.12 (d, ^3J = 7.7 Hz, 1H; $H^{N}{}_{Trp}$), 8.04 (d, ^3J = 7.8 Hz, 1H; $H^{N}{}_{Arg}$), 7.99 (d, ^3J = 7.4 Hz, 1H; $H^{N}{}_{Trp}$), 7.76 (m, 1H; $H^{\varepsilon}{}_{Arg}$), 7.64 (m, 2H; CO′NH_2/$H^{\varepsilon}{}_{Arg}$), 7.56 (m, 2H; $H^{\delta1}{}_{Trp}$), 6.85–7.32 (m, 11H; $H^{\varepsilon3}{}_{Trp}$, $H^{\zeta2,3}{}_{Trp}$, $H^{\eta2}{}_{Trp}$, $H^{\eta}{}_{Arg}$, CO′NH_2/$H^{\varepsilon}{}_{Arg}$), 6.36 (pseudo s, 1H; H_{Cp}), 6.25 (pseudo s, 1H; H_{Cp}), 5.92 (pseudo s, 2H; H_{Cp}), 5.70 (s, 5H; H_{Cp}), 4.61 (m, 2H; 2×$H^{\alpha}{}_{Trp}$), 4.40 (m, 1H; $H^{\alpha}{}_{Arg}$), 4.28 (m, 1H; $H^{\alpha}{}_{Arg}$), 4.20 (m, 1H; $H^{\alpha}{}_{Arg}$), 2.95–3.15 (m, 10H; $H^{\beta}{}_{Trp}$, $H^{\delta}{}_{Arg}$), 1.35–1.85 ppm (m, 12H; $H^{\beta}{}_{Arg}$, $H^{\gamma}{}_{Arg}$); ^{13}C NMR (75 MHz), [D_6]DMSO, 25 °C: δ = 173.2 (C=O), 171.4 (C=O), 171.2 (C=O), 171.1 (C=O), 161.7 (C=O), 158.7 ($C^{\zeta}{}_{Arg}$), 158.4 ($C^{\zeta}{}_{Arg}$), 156.8 ($C^{\zeta}{}_{Arg}$), 136.0 ($C^{\varepsilon2}{}_{Trp}$), 127.4 ($C^{\delta2}{}_{Trp}$), 123.7, 123.6, 120.9, 118.4, 118.3, 118.2, 111.3, and 111.2 ($C^{\delta1}{}_{Trp}$, $C^{\varepsilon3}{}_{Trp}$, $C^{\zeta2}{}_{Trp}$, $C^{\zeta3}{}_{Trp}$, $C^{\eta2}{}_{Trp}$), 109.8 and 109.6 ($C^{\gamma}{}_{Trp}$), 92.7 ($C_{Cp\ ipso}$), 86.0 (C_{Cp}), 85.8 ($C_{Cp\ unsubstituted}$), 84.4 (C_{Cp}), 83.7 (C_{Cp}), 53.5, 53.3, and 53.2 ($C^{\alpha}{}_{Trp}$, $C^{\alpha}{}_{Trp}$, $C^{\alpha}{}_{Arg}$), 52.3 ($C^{\alpha}{}_{Arg}$), 52.0 ($C^{\alpha}{}_{Arg}$), 40.4, ($C^{\delta}{}_{Arg}$), 29.3, 28.3, and 27.8 ($C^{\beta}{}_{Trp}$, $C^{\beta}{}_{Arg}$), 25.4, 25.0, and 24.9 ppm ($C^{\gamma}{}_{Arg}$), 1×C=O_{amide} unresolved. ESI (pos.) for $C_{53}H_{67}CoF_3N_{17}O_8$ (1185.5) *m/z*: 1186.3 [*M* + H]$^+$, 1072.6 [*M*−CF_3CO_2]$^+$, 537.0 [*M* - CF_3CO_2 + H]$^{2+}$, 358.5 [*M*−CF_3CO_2 + 2H]$^{3+}$, 269.1 [*M*−CF_3CO_2 + 3H]$^{4+}$.

Fe(Cp)(η-C_5H_4)-C(O)-RWRWR-NH$_2$, ***3***. Yellow solid. ^{1}H NMR (300 MHz), [D_6]DMSO, 25 °C: δ = 10.81 (s, 1H; $H^{\varepsilon1}{}_{Trp}$), 10.78 (s, 1H; $H^{\varepsilon1}{}_{Trp}$), 8.18 (d, ^3J = 7.5 Hz, 1H; H^{N}), 8.03 (d, ^3J = 7.8 Hz, 1H; H^{N}), 7.97 (d, ^3J = 7.2 Hz, 1H; H^{N}), 7.88 (d, ^3J = 7.6 Hz, 1H; H^{N}), 7.80 (d, ^3J = 7.8 Hz, 1H; H^{N}), 7.67 (m, 2H; CO′NH_2/$H^{\varepsilon}{}_{Arg}$), 7.52–7.59 and 6.83–7.31 (m; $H^{\delta1}{}_{Trp}$, $H^{\varepsilon3}{}_{Trp}$, $H^{\zeta2,3}{}_{Trp}$, $H^{\eta2}{}_{Trp}$,$H^{\varepsilon3}{}_{Trp,}$ $H^{\eta}{}_{Trp}$, $H^{\varepsilon}{}_{Arg}$/CO′NH_2), 4.86 (pseudo s, 1H; H_{Cp}), 4.84 (pseudo s, 1H; H_{Cp}), 4.58 (m, 2H; 2×$H^{\alpha}{}_{Trp}$), 4.37 (pseudo

s, 2H; H_{Cp}), 4.20 (m, 3H; 3×H^{α}_{Arg}), 4.15 (s, 5H; H_{Cp}), 2.98–3.18 (m, 10H; H^{β}_{Trp}, H^{δ}_{Arg}), 1.37–1.72 ppm (m, 12H; H^{β}_{Arg}, H^{γ}_{Arg}); ^{13}C NMR (75 MHz), [D$_6$]DMSO, 25 °C: δ = 173.1 (C=O), 171.7 (C=O), 171.2 (C=O), 171.0 (C=O), 170.9 (C=O), 169.6 (C=O), 156.6 (C^{ζ}_{Arg}), 135.9 ($C^{\varepsilon 2}_{Trp}$), 127.2 ($C^{\delta 2}_{Trp}$), 123.4 ($C^{\delta 1}_{Trp}$), 121.9 ($C^{\eta 2}_{Trp}$), 118.2 ($C^{\zeta 3}_{Trp}$), 118.1 ($C^{\varepsilon 3}_{Trp}$), 111.1 ($C^{\zeta 2}_{Trp}$), 109.6 (C^{γ}_{Trp}), 75.6 ($C_{Cp\ ipso}$), 70.0 (C_{Cp}), 69.2 ($C_{Cp\ unsubstituted}$), 68.3 (C_{Cp}), 68.1 (C_{Cp}), 53.6 (C^{α}), 53.4 (C^{α}), 52.6 (C^{α}), 52.1 (C^{α}), 51.9 (C^{α}), 29.4, 28.3, and 27.5 (C^{β}_{Trp}, C^{β}_{Arg}), 25.2, 24.8, and 24.7 ppm (C^{γ}_{Arg}), C^{δ}_{Arg} obscured by solvent peak; ESI (pos.) for $C_{51}H_{67}FeN_{17}O_6$ (1069.5) *m/z*: 1070.7 [*M* + H]$^{+}$, 536.0 [*M* + 2H]$^{2+}$, 357.7 [*M* + 3H]$^{3+}$.

H-RWRWRW-NH$_2$, ***4***. White solid. ^{1}H NMR (300 MHz), [D$_6$]DMSO, 25 °C: δ = 10.79 (s, 1H; $H^{\varepsilon 1}_{Trp}$), 10.77 (s, 1H; $H^{\varepsilon 1}_{Trp}$), 10.73 (s, 1H; $H^{\varepsilon 1}_{Trp}$), 8.53 (d, ^3J = 7.2 Hz, 1H; H^{N}_{Trp}), 8.33 (d, ^3J = 7.7 Hz, 1H; H^{N}_{Arg}), 8.20 (d, ^3J = 7.4 Hz, 1H; H^{N}_{Arg}), 8.08 (m, 3H; H^{N}_{Arg}, CO′NH_2/H^{ε}_{Arg}), 7.94 (d, ^3J = 7.5, 1H; H^{N}_{Trp}), 7.90 (d, ^3J = 7.7, 1H; H^{N}_{Trp}), 7.73 (m, 1H; H^{ε}_{Arg}), 7.57–7.66 (m, 6H; $H^{\delta 1}_{Trp}$, $H^{\varepsilon 3}_{Trp}$), 7.41 (m, 1H; H^{ε}_{Arg}), 6.88–7.33 (m, $H^{\zeta 2,3}_{Trp}$, $H^{\eta 2}_{Trp}$, H^{η}_{Arg}, CO′NH_2/H^{ε}_{Arg}), 4.62 (m, 2H; 2×H^{α}_{Trp}), 4.49 (m, 1H; H^{α}_{Trp}), 4.27 (m, 2H; 2×H^{α}_{Arg}), 3.74 (m, 1H; H^{α}_{Arg}), 2.87–3.13 (m, 12H; H^{β}_{Trp}, H^{δ}_{Arg}), 1.35–1.75 ppm (m, 12H; H^{β}_{Arg}, H^{γ}_{Arg}); ^{13}C NMR (75 MHz), [D$_6$]DMSO, 25 °C: δ = 173.1 (C=O), 171.4 (C=O), 171.2 (C=O), 171.0 (C=O), 168.5 (C=O), 158.8, 158.4 and 156.8 (C^{ζ}_{Arg}), 136.1 and 136.0 ($C^{\varepsilon 2}_{Trp}$), 127.4, 127.3, and 127.2 ($C^{\delta 2}_{Trp}$), 123.8, 123.5, 120.9, 120.8, 118.5, 118.2, 115.2, and 111.2 ($C^{\delta 1}_{Trp}$, $C^{\varepsilon 3}_{Trp}$, $C^{\zeta 2}_{Trp}$, $C^{\zeta 3}_{Trp}$, $C^{\eta 2}_{Trp}$), 109.8 and 109.6 (C^{γ}_{Trp}), 53.4 (C^{α}_{Trp}), 53.3 (C^{α}_{Trp}), 53.2 (C^{α}_{Trp}), 52.5 (C^{α}_{Arg}), 52.3 (C^{α}_{Arg}), 29.2, 27.8, 27.7, and 27.5 (C^{β}_{Arg}, C^{β}_{Trp}), 24.9, 23.9, and 23.8 ppm (C^{γ}_{Arg}), C^{δ}_{Arg} obscured by solvent peak, 1×$C{=}O_{amide}$ and 1×C^{α} were not resolved. ESI (pos.) for $C_{51}H_{69}N_{19}O_6$ (1043.6) *m/z*: 1044.9 [*M* + H]$^{+}$, 523.1 [*M* + 2H]$^{2+}$, 349.1 [*M* + 3H]$^{3+}$.

[Co(Cp)(η-C$_5$H$_4$)-C(O)-WRWRW-NH$_2$](CF$_3$CO$_2$), ***5***. Yellow solid. ^{1}H NMR (300 MHz), [D$_6$]DMSO, 25 °C: δ = 10.90 (s, 1H; $H^{\varepsilon 1}_{Trp}$), 10.78 (s, 1H; $H^{\varepsilon 1}_{Trp}$), 10.74 (s, 1H; $H^{\varepsilon 1}_{Trp}$), 8.82 (d, ^3J = 8.4 Hz, 1H; H^{N}_{Trp}), 8.46 (d, ^3J = 7.8 Hz, 1H; H^{N}_{Arg}), 8.20 (d, ^3J = 7.5 Hz, 1H; H^{N}_{Arg}), 8.04 (d, ^3J = 7.4 Hz, 1H; H^{N}_{Trp}), 7.92 (d, ^3J = 7.6 Hz, 1H; H^{N}_{Trp}), 7.82 (m, 1H; $H^{\delta 1}_{Trp}$), 7.60 (m, 5H; $H^{\delta 1}_{Trp}$, H^{ε}_{Arg}, CO′NH_2), 7.44 (m, 1H; H^{ε}_{Arg}), 6.95–7.32 (m; $H^{\varepsilon 3}_{Trp}$, $H^{\zeta 2,3}_{Trp}$, $H^{\eta 2}_{Trp}$, H^{η}_{Arg}), 6.24 (pseudo s, 1H; H_{Cp}), 6.18 (pseudo s, 1H; H_{Cp}), 5.84 (pseudo s, 2H; H_{Cp}), 5.31 (s, 5H; H_{Cp}), 4.93 (m, 1H; H^{α}_{Trp}), 4.62 (m, 1H; H^{α}_{Trp}), 4.49 (m, 1H; H^{α}_{Trp}), 4.36 (m, 1H; H^{α}_{Arg}), 4.28 (m, 1H; H^{α}_{Arg}), 2.95–3.24 (m, 10H; H^{β}_{Trp}, H^{δ}_{Arg}), 1.38–1.75 ppm (m, 8H; H^{β}_{Arg}, H^{γ}_{Arg}); ^{13}C NMR (75 MHz), [D$_6$]DMSO, 25 °C: δ = 173.2 (C=O), 171.4 (C=O), 171.3 (C=O), 171.2 (C=O), 171.0 (C=O), 161.2 (C=O), 158.5 (C^{ζ}_{Arg}), 156.7 (C^{ζ}_{Arg}), 136.2 ($C^{\varepsilon 2}_{Trp}$), 136.0 ($C^{\varepsilon 2}_{Trp}$), 127.4 ($C^{\delta 2}_{Trp}$), 127.1 ($C^{\delta 2}_{Trp}$), 124.0, 123.5, 121.1, 120.9, 118.9, 118.5, 118.4, 118.3, 111.5, and 111.3 ($C^{\delta 1}_{Trp}$, $C^{\varepsilon 3}_{Trp}$, $C^{\zeta 2}_{Trp}$, $C^{\zeta 3}_{Trp}$, $C^{\eta 2}_{Trp}$), 110.4 and 109.8 (C^{γ}_{Trp}), 92.5 ($C_{Cp\ ipso}$), 86.0 (C_{Cp}), 85.5 ($C_{Cp\ unsubstituted}$), 84.3 (C_{Cp}), 83.5 (C_{Cp}), 53.7 (C^{α}_{Trp}), 53.3 (C^{α}_{Trp}), 53.2 (C^{α}_{Trp}), 52.4 (C^{α}_{Arg}), 52.3 (C^{α}_{Arg}), 40.4 (C^{δ}_{Arg}), 29.3 and 29.2 (C^{β}_{Arg}), 27.8 and 27.7 (C^{β}_{Trp}), 25.1 and 24.8 ppm (C^{γ}_{Arg}). ESI (pos.) for $C_{58}H_{65}CoF_3N_{15}O_8$ (1215.4) *m/z*: 1102.9 [*M*−CF_3CO_2]$^{+}$, 551.9 [*M*−CF_3CO_2 + H]$^{2+}$, 368.4 [*M*−CF_3CO_2 + 2H]$^{3+}$.

Fe(Cp)(η-C$_5$H$_4$)-C(O)-WRWRW-NH$_2$, ***6***. Yellow solid. ^{1}H NMR (300 MHz), [D$_6$]DMSO, 25 °C: δ = 10.83 (s, 1H; $H^{\varepsilon 1}_{Trp}$), 10.77 (s, 1H; $H^{\varepsilon 1}_{Trp}$), 10.71 (s, 1H; $H^{\varepsilon 1}_{Trp}$), 8.18 (d, ^3J = 8.0 Hz, 1H; H^{N}_{Arg}), 8.15 (d, ^3J = 7.6 Hz, 1H; H^{N}_{Arg}), 7.99 (d, ^3J = 7.4 Hz, 1H; H^{N}_{Trp}), 7.88 (d, ^3J = 7.6 Hz, 1H; H^{N}_{Trp}), 7.73 (d, ^3J = 7.7 Hz, 1H; $H^{\delta 1}_{Trp}$), 7.68 (d, 7.7 Hz, 1H; H^{N}_{Trp}), 7.57 (m, 2H; $H^{\delta 1}_{Trp}$), 7.52 (m, 2H; CO′NH_2/H^{ε}_{Arg}), 7.41 (s, 1H; H^{ε}_{Arg}), 7.27–7.32 and 6.90–7.20 (m, 20H; $H^{\delta 1}_{Trp}$, $H^{\varepsilon 3}_{Trp}$, $H^{\zeta 2,3}_{Trp}$, $H^{\eta 2}_{Trp}$, H^{η}_{Arg}), 4.76 (pseudo s, 1H; H_{Cp}), 4.73 (m, 1H; H^{α}_{Trp}), 4.68 (pseudo s, 1H; H_{Cp}), 4.59 (m, 1H; H^{α}_{Trp}), 4.48 (m, 1H; H^{α}_{Trp}), 4.34 (m, 1H; H^{α}_{Arg}), 4.28 and 4.26 (pseudo s overlapping with m, 3H; 2×H_{Cp}, H^{α}_{Arg}), 3.81 (s, 5H; H_{Cp}), 2.90–3.20 (m, 10H; H^{β}_{Trp}, H^{δ}_{Arg}), 1.35–1.71 ppm (m, 8H; H^{β}_{Arg}, H^{γ}_{Arg}); ^{13}C NMR (75 MHz), [D$_6$]DMSO, 25 °C: δ = 173.2 (C=O), 172.4 (C=O), 171.4 (C=O), 171.3 (C=O), 171.0 (C=O), 169.5 (C=O), 156.7 (C^{ζ}_{Arg}), 136.2 ($C^{\varepsilon 2}_{Trp}$), 136.0 ($C^{\varepsilon 2}_{Trp}$), 127.4 ($C^{\delta 2}_{Trp}$), 127.3 ($C^{\delta 2}_{Trp}$), 127.2 ($C^{\delta 2}_{Trp}$), 123.9, 123.5, 120.9, 118.6, 118.5, 118.3, 111.4, and 111.2 ($C^{\delta 1}_{Trp}$, $C^{\varepsilon 3}_{Trp}$, $C^{\zeta 2}_{Trp}$, $C^{\zeta 3}_{Trp}$, $C^{\eta 2}_{Trp}$), 110.5 and 109.8 (C^{γ}_{Trp}), 75.6 ($C_{Cp\ ipso}$), 70.0 (C_{Cp}), 69.2 ($C_{Cp\ unsubstituted}$), 68.5 (C_{Cp}), 67.9 (C_{Cp}), 53.7 (C^{α}_{Trp}), 53.3 (C^{α}_{Trp}), 53.3 (C^{α}_{Trp}), 52.4 (C^{α}_{Arg}), 52.2 (C^{α}_{Arg}), 40.5 (C^{δ}_{Arg}), 29.2 (C^{β}_{Arg}), 27.8 (C^{β}_{Trp}), 25.0 and 24.8 ppm (C^{γ}_{Arg}). ESI (pos.) for $C_{56}H_{65}FeN_{15}O_6$ (1099.5) *m/z*: 1100.7 [*M* + H]$^{+}$, 551.0 [*M* + 2H]$^{2+}$.

Antibacterial activity. The antimicrobial activity of the peptides were determined against *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), and *Staphylococcus aureus* (ATCC 25923) using the minimum inhibitory concentration (MIC) test. Serial 1:1 dilutions of each peptide were made in 96-well microtiter plates, to a final volume of 100 μL of Tryptic soy broth (15% *v/v* in 10 mM sodium phosphate buffer, pH 7.4) with the bacteria in the exponential phase; for *E. coli* 4×10^5 colony forming units (CFU) mL^{-1}, for *S. aureus* 2×10^5 CFU mL^{-1} and for *P. aeruginosa* 1×10^6 CFU mL^{-1}. Plates were incubated at 37 °C overnight (16 h) and the MIC was considered as the first well without visible growth. Each peptide was tested in duplicate. Pilosulin 2 (H-GLLSKFGRLARKLARVIPKV-NH$_2$) was used as the positive control.

The concentrations of the peptide solutions were verified by spectroscopic measurement at 280 nm, after determining the molar extinction coefficient of each metallocene chromophore. As dictated by the availability and solubility of related metallocene compounds, ε_{280nm} values of 10820 and 1840 cm^{-1} M^{-1} were obtained for cobaltocenium ([Co(Cp)(C$_5$H$_4$-COOH)]PF$_6$ in water) and for ferrocene (Fe(Cp)(C$_5$H$_4$-COOH)] in methanol), respectively. We estimate an error of not more than 10%. For Trp, an ε_{280nm} value of 5500 cm^{-1} M^{-1} was used.[24,26,27]

Reagents and materials. Ferrocene carboxylic acid was purchased from Fluka whereas [Co(Cp)(C$_5$H$_4$-COOH)]PF$_6$ was synthesized following a method reported in the literature.[66] The amino acids were obtained from NovaBiochem and only pure L-amino acids were used throughout; DIPEA, TBTU, and HOBt·H$_2$O from Iris Biotech; TFA and TIS from Acros, Tryptic soy broth from Acumedia.

Abbreviations

Peptides are consistently written from the N to C terminus in standard peptide nomenclature using one-letter codes for individual amino acids. aa = amino acid, ATCC = American Type Culture Collection, Boc = *tert*-butoxycarbonyl, DIPEA = *N*,*N*-diisopropylethylamine, Fmoc = 9-fluorenylmethoxycarbonyl, HOBt = *N*-hydroxybenzotriazole, Pbf = 2,2,4,6,7-pentamethyldihydrobenzofurane-5-sulfonyl, TBTU = 2-(1H-benzotriazol-1-yl)1,1,3,3-tetramethyl uronium, TFA = trifluoroacetic acid, TIS = triisopropylsilane.

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Keywords: antibacterial agents · ferrocene · medicinal organometallic chemistry · metallocenes · peptides · solid-phase peptide synthesis

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