New cationic amphiphilic compounds as potential antibacterial agents

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1.1 | Introduction

Cationic antimicrobial peptides (CAPs) have found attraction as lead structures in combating bacterial infections,[1] and much research effort has been directed in recent years towards the elucidation of their mode of action.[2,3] From the amassed data it becomes apparent that a majority of CAPs exert their activity through adoption of a defined secondary structure upon contact with anionic bacterial cell membrane components. As a result, either bacterial cell lysis or disturbance of membrane transport events occurs, with bacterial cell death as a result. The fact that a large number of CAPs appear to be indiscriminate to cell type and are often equally effective in killing mammalian cells normally limits their use to topical applications.

Drosocin, a CAP isolated from *Drosophila melanogaster*, is a 19-mer peptide containing three Pro-Arg-Pro repeats and an *O*-glycosylation site at Thr11 (Table 1).[4] The antibacterial activity of drosocin, like other members of the proline-rich CAPs (Table 1), appears not to be based on cell lysis. Rather, drosocin's activity towards bacteria is based on inhibition of a specific intracellular heat-shock protein named DnaK resulting in cell death.[5] This finding explains the observation that *all-D* drosocin, composed of D-amino acids,[6] does not possess any antibacterial activity, in contrast to most *all-D* CAPs.[7] Drosocin, and related proline-rich CAPs, are further distinguished from other CAPs by the lack of toxicity towards human erythrocytes and their apparent bias in activity towards Gram-negative bacteria.[4] These properties combined make drosocin a very attractive lead structure in the search for new and effective antibacterial agents.

TABLE 1 | Sequences of drosocin and selected related Pro-rich CAP family members.

CAP	Sequence
Drosocin	G - K P R P Y S P R P T* S H P R P I R V
Pyrrhocoricin	V D K G S - Y L P R P T* - P P R P I Y N R N
Formaecin I	G - R P N P V N N K P T* P H P R - L

* Glycosylation site

A major hurdle in the development of drosocin-based antibacterial agents is the inherent instability of drosocin towards proteolytic activities present in mammalian sera. In fact, drosocin and its congeners are degraded in sera at such a rate that effective treatment of bacterial infections would require relatively large doses of the peptide.[8] Any strategy that leads to drosocin

analogues with enhanced proteolytic stability without impairing antibacterial activity should therefore be an important step forwards in the development of new antibacterial agents.

1.2 | First Generation Drosocin Analogues

1.2.1 | Design & Synthesis

The reported finding that the first step in the proteolytic degradation of drosocin in human serum comprises cleavage of the peptide bond[8] between Tyr6 and Ser7 was an incentive to design a series of drosocin (DRC) analogues (**2-12**, Table 2) in which either of the two or both positions at the cleavage site were substituted. This included replacement of Ser7 with Leu (**3**), Thr (**4**, **10**), D-Ser (**5**, **11**), *N*-Me-Ser (**2**) or β^3-HSer (**6**, **12**; see Figure 1) and replacement of Tyr6 with Phe (**8, 10**), D-Tyr (**7**, **11**) or β^3-HTyr (**9**, **12**). As a negative control, unrelated peptide **17** was included in the array.[9]

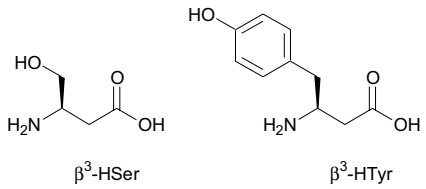

FIGURE 1 | Structures of β^3-HSer and β^3-HTyr used in this study; in this nomenclature, H indicates a homoamino acid with the amine function at the ß-carbon and the sidechain connected to the 3rd backbone carbon atom. Fmoc derivatives of both amino acids are commercially available.

Although non-glycosylated DRC (nG-DRC **1**, Table 2) is about 7 times less active than native drosocin,[4] it was decided to omit the *O*-glycosyl modification at Thr11 to simplify the synthetic procedure. The preparation of the non-glycosylated linear peptides in Table 2 was accomplished using standard Fmoc-based SPPS techniques using the appropriate Fmoc-amino acid building blocks and PyBOP/DiPEA as the activator system. Automated peptide synthesis was employed in all instances except for the Tyr6-*N*-Me-Ser7 stretch in **2** that was introduced manually. In this particular case, the N^{α}-methyl amino acid was introduced on the growing peptide chain using PyBOP and successive elongation with tyrosine was achieved using the stronger activator

PyBroP.[10] All peptides were purified to homogeneity by reverse phase HPLC prior to biological assessment.

TABLE 2 | Synthetic peptides used in this study.

#	Sequence[A]	HRMS [M+H]$^+$	
		Calculated	Found
1	GKPRP **Y** SPRP TSHPR PIRV	2197.234	2197.220
2	GKPRP **$Y^{Me}S$**PRP TSHPR PIRV	2211.250	2211.237
3	GKPRP Y **L**PRP TSHPR PIRV	2223.294	2223.280
4	GKPRP Y **T**PRP TSHPR PIRV	2211.250	2211.241
5	GKPRP Y **^{D}S**PRP TSHPR PIRV	2195.255	2195.236
6	GKPRP Y **^{ß}S**PRP TSHPR PIRV	2211.250	2211.241
7	GKPRP **F** SPRP TSHPR PIRV	2181.240	2181.251
8	GKPRP **^{D}Y** SPRP TSHPR PIRV	2197.234	2197.201
9	GKPRP **^{ß}Y** SPRP TSHPR PIRV	2211.250	2211.241
10	GKPRP **F T**PRP TSHPR PIRV	2195.255	2195.236
11	GKPRP **^{D}Y ^{D}S**PRP TSHPR PIRV	2197.234	2197.218
12	GKPRP **^{ß}Y ^{ß}S**PRP TSHPR PIRV	2225.266	2225.197
17	NTDGS T DYGI LQINS R	1753.8[B]	1753.7[B]

[A] Modifications are highlighted in bold face, [Me] = *N*-methylated amino acid [D] = D-amino acid, [ß] = ß^3-homo-amino acid; [B] not HRMS.

1.2.2 | Antibacterial Activity

The results of the analysis of the antibacterial activity of DRC analogues **1-12** against *E. coli* ATCC 11775 are summarized in Table 3.[11] None of the prepared peptides possess any hemolytic activity at concentrations up to 300µM. *N*-Methylation of the endopeptidase-sensitive peptide bond (peptide **2**) results in a considerably lower antibacterial activity as compared to the unglycosylated native form (**1**).

In contrast, the DRC analogues containing a single amino acid substitution of Tyr6 or Ser7 (compounds **4-9**) display equal or better antibacterial activity. Analogue **3** however, which contains a Ser7→Leu replacement to resemble the stable Tyr-Leu-Pro-Arg-Pro (YLPRP) pentamer present in the related CAP pyrrhocoricin[9] (Table 1), displayed a loss in activity. The double mutant DRC analogues **11** and **12** were four times less active than reference compound **1**. The positive exception is represented by peptide **10** with a 3.1µM minimal inhibitory concentration.

TABLE 3 | MIC (minimal inhibitory concentration) values of drosocin analogues and serum stability assay.

Compound	MIC (μM)[A]	Intact (%)[B]
1 (nG-DRC)	6.3	3
2	25	11
3	12.5	n/d
4	1.6	15
5	3.1	1
6	3.1	31
7	3.1	19
8	6.3	16
9	3.1	2
10	3.1	26
11	25	17
12	12.5	<1
17	>100	n/d

[A] Against *E. coli* ATCC 11775; [B] Percentage of intact peptide after 8h of digestion in 25% human serum as determined by MALDI-MS peak height of the corresponding peptide; n/d - not determined.

1.2.3 | Serum Stability

The next research objective was to evaluate the proteolytic stability of non-glycosylated drosocin (**1**) and analogues **2** and **4-12**. The experiments were performed in 25% pooled human serum to increase peptide recovery and to allow monitoring of the degradation over an 8h period. The relative abundance of the intact peptide was determined by comparison of the MALDI mass spectral peak heights with those at t=0.[8,12]

Based on the results, non-glycosylated drosocin and analogues **1**, **2** and **4-12** can be roughly divided into two categories. The first is represented by the peptides that are almost completely degraded after a digestion period of eight hours (that is, less than 5% intact peptide remains). In this group, analogues **5**, **9**, and **12** accompany reference compound **1.** Peptides **2**, **4**, **6-8, 10** and **11** form the second category and display improved serum stability. Interestingly, all analogues comprising single proteogenic amino acid replacements (**4**, **7**, and **10**) appear to be more stable than parent peptide **1**. On average, 20% of intact peptide was detected after eight hours of digestion in serum, compared to only 3% for **1**. The improved serum stability and antimicrobial activity found for these peptides (**4**, **7** and **10**) illustrate that substitution of one or two amino acids by closely related ones can already be beneficial.

The analogues having substitutions with β^3- or D-amino acids feature distinct differences in serum stability. For instance, the serum stability was improved ten times by the introduction of β^3-serine moiety at position 7 (**6**), while the other two β^3-amino acid containing analogues (**9**, **12**)

demonstrated a proteolytic stability similar to **1**. The opposite result was observed for the D-amino acid containing analogues **5**, **8** and **11**. Here, 16 and 17% of the peptide remained intact after 8h of proteolytic digestion in the case of analogues **8** and **11**, respectively, whereas a single L- to D-serine replacement in **5** did not improve the serum stability at all.

In addition to the percentage of intact peptide after 8h of proteolytic digestion, early degradation products provide useful information about the serum stability of the peptides tested. For this purpose, the degradation fragments formed after two hours of digestion were identified (Table 4), together with their respective intensities.[12]

The most abundant degradation product of non-glycosylated DRC (**1**) after 2h is lacking 6 *N*-terminal residues (*i.e.* cleavage between Tyr6 and Ser7), which is consistent with literature data.[8] This particular cleavage product is considerably less abundant from drosocin analogues **2**-**12** or even fully absent.[13] *N*-methylation (in **2**) yields the lowest increase in stability of the susceptible amide bond. As mentioned previously, the proteolytic stabilities of peptides **5**, **9** and **12** are similar to **1**, but the low serum stability of these analogues is reflected by the high abundances of proteolytic fragments –N1, –N3 and –N5, rather than fragment –N6 which is the most abundant cleavage product in **1**.

TABLE 4 | Abundances of major degradation products.

	Degradation products[A]				
Compound	–N1	–N3	–N5	–**N6**	–N8
1	2	1	0	**5**	3
2	1	1	1	**3**	0
4	1	1	1	**1**	1
5	5	3	2	**1**	0
6	1	1	1	**1**	1
7	2	1	1	**2**	2
8	1	1	1	**0**	0
9	3	2	3	**0**	0
10	1	1	1	**2**	2
11	2	1	1	**1**	0
12	4	3	5	**0**	1

[A] Major degradation products are displayed as missing N-terminal residues after 2h digestion in 25% human serum, *e.g.* –N3 indicates loss of the first three N-terminal residues. The relative MALDI-MS spectral peak heights were measured and divided into 6 classes (not detected (0), 1-25mm (1), 26-50mm (2), 51-75mm (3), 76-100mm (4) and >100mm (5)). Numbers are averages of three experiments.

1.3 | Second Generation Drosocin Analogues

Based on the results of the 1st generation DRC analogues, attention was focussed on further stabilization of DRC analogues against proteolytic inactivation. Fragments missing some of the N-terminal residues were encountered in the 1st generation analogues (see Table 4) as a result of aminopeptidase activities. Due to its favorable outcome in the stability/antimicrobial tests and its completely proteogenic composition, compound **4** (Ser7→Thr) was selected as lead structure for further elaboration. On the premise that N-terminal capping of natural peptides may result in enhanced stability towards aminopeptidase activities, the N-terminal Gly residue in **4** was replaced subsequently by sarcosine (Sar, *N*-methylglycine) and the lengthened Gly derivatives β-alanine (βAla), γ-aminobutyric acid (Abu) and δ-aminovaleric acid (Ava).

TABLE 5 | 2nd Generation DRC analogues containing N-terminal modifications.

Compound	Sequence[A]	HRMS Calculated	HRMS Found
13	**Sar** KPRP Y**T**PRP TSHPR PIRV	2225.266	2225.263
14	**ßAla**KPRP Y**T**PRP TSHPR PIRV	2225.266	2225.260
15	**Abu** KPRP Y**T**PRP TSHPR PIRV	2239.281	2239.282
16	**Ava** KPRP Y**T**PRP TSHPR PIRV	2253.671	2253.308

[A] Modifications are highlighted in bold face.

The resulting set of doubly modified DRC analogues **13-16** (Table 5) was subjected to identical stability and antimicrobial assays as the 1st generation analogues. Obtained MIC values are summarized in Table 6. Compared to parent peptide **4** (MIC 1.6µM), its derivatives **13-16** have on average slightly reduced MIC values, although comparison with DRC (**1**, MIC 6.3µM) shows that these 2nd generation analogues are as potent as the natural non-glycosylated CAP. None of the 2nd generation DRC analogues displayed hemolytic activity up to concentrations of 300µM. N-terminal stability is increased within these N-terminally modified analogues as no –N1, –N3 or –N5 fragments were detected (Table 6). The overall stability is increased dramatically as ~80% of these peptides remains intact after 8h incubation in serum – for analogue **14** an increase of ~5.5-fold compared with peptide **4** and ~30-fold regarding peptide **1** (see also Figure 2 for selected degradation curves).

TABLE 6 | MIC values of 2nd generation DRC analogues and serum stability assay. For comparison, data of lead structure **4** is added.

			Degradation Products after 2h				
Compound	**MIC (μM)[A]**	**Intact (%)[B]**	-N1	-N3	-N5	**-N6**	-N8
4	1.6	15	1	1	1	**1**	1
13	6.3	77	0	0	0	**1**	1
14	3.1	87	0	0	0	**2**	0
15	6.3	76	0	0	0	**2**	1
16	6.3	84	0	0	0	**1**	1

[A] Minimal inhibitory concentration against *E. coli* ATCC 11775; [B] Percentage of intact peptide after 8h of digestion in 25% human serum as determined by MALDI-MS peak height of the corresponding peptide.

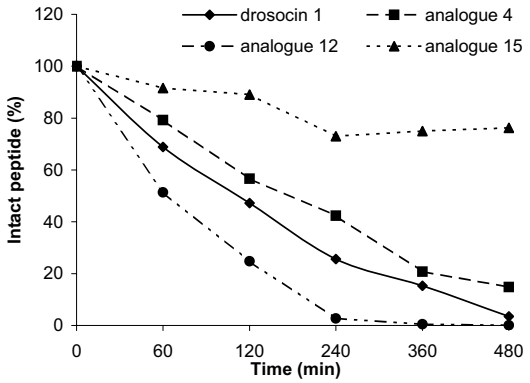

FIGURE 2 | Selected degradation curves (averages of three experiments) of nG-DRC **1** and analogues **4**,**12** and **15**.

1.4 | Conclusion

Substitution of selected amino acid residues at sites that are susceptible to proteolytic cleavage results in DRC analogues with enhanced stability, while leaving the antibacterial properties intact. In the 1st generation DRC analogues, both antimicrobial activity and stability were increased with respect to native nG-DRC **1**. Analogue **4** was selected as lead for the design of a 2nd generation of analogues containing a modified N-terminus to overcome unwanted aminopeptidase inactivation. N-terminal modifications on **4** led to the synthesis of a 2nd generation of DRC analogues (**13**-**16**) with almost 30-fold increase in stability compared to nG-

DRC **1**, while being equally potent. For future studies towards the design of DRC-based drugs, analogues **13-16** can serve as a new lead structures.

1.5 | Experimental Section

1.5.1 | General

LCMS analyses were performed using a Jasco 900 LC system with detection at 214 and 254nm connected to a Perkin Elmer Q-TOF monoquad mass spectrometer in positive ion mode. Crude peptides were purified on a ÄKTA Explorer HPLC system. In all cases, Alltech Alltima C_{18} analytical (150x4.6mm or 250x4.6mm) and semi-preparative (250x10.0mm) were used. Applied buffer systems – Jasco: A. H_2O; B. MeCN; C. 0.1% aq. TFA (always 10% C present in the gradients); ÄKTA: A. 0.1% TFA in 5% MeCN/H_2O; B. 0.1% TFA in 80% MeCN/H_2O. Eluents were degassed before use and kept under Ar flow. MALDI-MS analyses were performed on a Bruker Biflex III mass spectrometer. Electrospray tandem mass analyses were performed on a Q-TOF mass spectrometer (Micromass) at a cone voltage of 20V.

1.5.2 | SPPS

Peptides were synthesized using either an ABI-433A (50µmol scale, Applied Biosystems) synthesizer or Syro 2000 (10µmol scale, MultiSyntech) using customized Fmoc-based protocols. Amino acid residues were used in 5-fold excess, using PyBOP (5eq.)/DiPEA (10eq., ABI-433A) or BOP (5eq.)/DiPEA (10eq., Syro 2000) as activating systems and 20% piperidine/NMP as Fmoc-deprotecting mixture. The first amino acid, Fmoc-Val-OH (5eq.) was manually coupled to TentaGel PHB resin (0.24mmol/g) with DIC (5eq.) and DMAP (0.1eq.) in DMF for 2h. Loading was established by UV determination of the amount of Fmoc-chromophore released after treatment of a sample with 20% piperidine/DMF for 10min. Loading was 0.20mmol/g (85%). *N*-Methyl amino acids (4eq.) were coupled manually with BOP (4eq.)/HOBt (4eq.)/DiPEA(8eq.) in DMF. Amino acids to be reacted with *N*-methyl amino acids were also coupled manually, using the amino acid (5eq.) and preactivation with PyBroP (5eq.) and DiPEA (10eq.) in DMF. The potent activating reagent HATU worked equally efficient in acylations of *N*-methyl amino acids. In case the chloranil test was positive, coupling was repeated. After removal of the N-terminal Fmoc group, crude peptides were treated with TFA/TIS/H_2O (95/2.5/2.5, v/v/v) for cleavage from the resin and removal of protecting groups, and subsequently precipitated in Et_2O. Centrifugation and decantation of solvents yielded the crude products. After HPLC purification, the target peptides were obtained: **1** (nG-DRC), yield 42%, purity 96%, HPLC Rt 9.34min; **2** (S7^{Me}S), yield 5%, purity 95%, HPLC Rt 9.29min; **3** (S7L), yield 65%, purity 99%, HPLC Rt 9.36min; **4** (S7T), yield 11%, purity 98%, HPLC Rt 9.20min; **5** (S7$_D$S), yield 11%, purity 99%, HPLC Rt 9.20min; **6** (S7βS), yield 5%, purity >99%, HPLC Rt 9.18min; **7** (Y6F), yield 12%, purity 99%, HPLC Rt 9.49min; **8** (Y6$_D$Y), yield 7%, purity 90%, HPLC Rt 9.32min; **9** (Y6βY), yield 12%, purity 99%, HPLC Rt 9.14min; **10** (Y6F/S7T), yield 11%, purity 99%, HPLC 9.50min; **11** (Y6$_D$Y/S7$_D$S), yield 13%, purity 99%, HPLC Rt 9.24min; **12** (Y6βY/S7βS), yield 6%, purity 91%, HPLC Rt 9.18min; **13** (G1Sar/S7T), yield 3.1mg (1,41µmol, 14%), purity >99%, HPLC Rt 9.59min; **14** (G1βAla/S7T), yield 2.5mg (1.41µmol, 11%), purity 98%, HPLC Rt 9.56min; **15** (G1Abu/S7T), yield 2.3mg (1.02µmol, 10%), purity 97%, HPLC Rt 9.55min; **16** (G1Ava/S7T), yield 1.6mg (0.72µmol, 7%), purity 98%, HPLC Rt 9.56min.

1.5.3 | Antibacterial Assay

Antibacterial assays were performed in sterilized round-bottom 96-well plates with a final volume of 110µL as follows. The bacteria (*E. coli* ATCC 11775) were grown in nutrient broth E (NBE; beef extract 1g/L, yeast extract 2g/L, peptone 5g/L and NaCl 5g/L) and kept at 4^0C. Lyophilized peptides were dissolved in NBE to give a concentration of 400µM and filtered using 0.22µm filter discs. A 16h culture in NBE was adjusted to

5×10^6 CFU/mL and transferred to the micro titer plate wells (10µL) containing each 100µL of a serial 2-fold dilution (200-0.4µM) of the tested peptide in NBE. Plates were incubated while gently shaking at 37^0C for 24h. Next, 80µL suspension of each well was transported into a flat-bottom 96-well plate. The absorbance was measured at 600nm using a µQuant micro plate spectrophotometer (Bio-Tek Instruments). All peptides were measured in quadruplo. The peptide with sequence NTDGSTDYGILQINSR was used as negative control.

1.5.4 | Hemolysis Assay

The hemolytic activity of the peptides was determined in quadruplo. Human blood was collected in EDTA-tubes and centrifuged to remove the buffy coat. The residual erythrocytes were washed three times in 0.85% saline. Serial dilutions of the peptides in saline were prepared in sterilized round-bottom 96-well plates using 100µL volumes (100-0.2µM). Red blood cells were diluted with saline to 1/25 packed volume of cells and 50µL of the resulting cell suspension was added to each well. Plates were incubated while gently shaking at 37^0C for 4h. Next, the micro titer plate was quickly centrifuged and 50µL supernatant of each well was transported into a flat-bottom 96-well plate. The absorbance was measured at 405nm using a µQuant micro plate spectrophotometer (Bio-Tek Instruments). The A_{blank} was measured in the absence of additives and 100% hemolysis (A_{tot}) in the presence of 1% Triton X-100 in saline. The percentage of hemolysis is determined as $(A_{pep}-A_{blank})/(A_{tot}-A_{blank})\times100$.

1.5.5 | Serum Stability Assay

The serum stability studies were carried out in triplicate. Briefly, 10µL of an aqueous peptide solution (0.8mg/mL) was added to 1mL freshly pooled 25% human serum in PBS. The mixtures were thermostated at 37^0C under gentle stirring. At t=0, 1, 2, 4, 6 and 8h, 100µL of each mixture was taken out and precipitated in 230µL 15% aq. TCA. Samples were stored at 0^0C for 20min and centrifuged for 5min at 0^0C. The supernatants (250µL of each) were immediately stored at -80ºC until analyzed by MALDI mass spectrometry. The spectra were recorded in the linear mode, using α-cyanohydroxycinnamic acid in 50% MeCN in 0.1% aqueous TFA. Serum control samples consisted of 100µL of pooled 25% human serum solution in PBS precipitated in 230µL 15% aq. TCA. Peptide reference samples consisted of 10µL of the peptide stock solution diluted in 1mL H_2O, of which 100µL was added to 230µL 15% aq. TCA. MALDI peak heights were measured from prints that were generated using standard procedures.[12]

1.6 | Notes & References

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12. The relative abundance of these fragments was calculated under the assumption that both the detector sensitivity and MALDI susceptibility was uniform for all fragments. In Table 4, only fragments with molecular weight of 800 or more were taken into consideration
13. The following degradation products were also detected: –C1,N1 for analogue **7** (abundance 1), –C2,N5 for analogues **2**, **6** and **9** (abundance 3, 1 and 1 respectively) and –N10 (abundance 2) for **16**.

CHAPTER 2 | Safety-Catch Synthesis & Biological Evaluation of Polymyxin B1 and Analogues

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