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Biological evaluation of Tyr6 and Ser7 modified drosocin analogues

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This paper is dedicated to the memory of our colleague Jacques van Boom, who died on July 31, 2004, at the age of 67

Abstract—An array of analogues of the cationic antimicrobial peptide drosocin was synthesized containing substitutions of Tyr6 and Ser7 in order to increase the proteolytic stability. Stabilizing the N-terminus with unnatural amino acids increased the serum stability of analogues by almost a factor 30 over an 8 h period.
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1. Introduction

Cationic antimicrobial peptides (CAPs) have found wide attraction as lead structures in combating bacterial infections,[1] and considerable progress in elucidating the mode of action of a variety of CAPs has been made in recent years.[2,3] It has been widely accepted that most CAPs, (e.g., polymyxin B)[4] exert their activity through adoption of a defined secondary structure upon contact with Gram-negative bacterial cell membranes. As a result, either bacterial cell lysis or disturbance of membrane transport events occurs, with bacterial cell death as a result. The fact that most 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 oligopeptide containing three PRP repeats and an *O*-glycosylation site at Thr11 (Table 1).[5]

The antibacterial activity of drosocin, as is the case with other members of the proline-rich CAPs (Table 1), appears not to be based on cell lysis. Rather, it is likely that drosocin activity toward bacterial strains is based on recognition of a specific intracellular target.[6] This observation is underscored by the finding that enantiomeric drosocin, composed of D-amino acids,[7] does not possess any antibacterial activity, whereas most enantiomeric CAPs do.[8] Drosocin, and related proline-rich CAPs, are further distinguished from other CAPs by the lack of toxicity toward human erythrocytes, and their apparent bias in activity toward Gram-negative bacteria.[5] These properties combined make drosocin a very attractive lead structure in the search for new and effective antibacterial agents.

A major hurdle in the development of drosocin-based antibacterial agents is the inherent instability of drosocin toward 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 large doses of the peptide.[9] Any strategy that leads to drosocin analogues with enhanced proteolytic stability without impairing antibacterial activity should therefore be an important step forward in the development of new antibacterial agents. With this observation in mind, we embarked on a program aimed at the development and evaluation of chemically modified drosocin analogues, the initial results of which are presented here.

2. Results and discussion

Our design of drosocin analogues is based on the reported finding that the first step in the proteolytic

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

	Sequence																				
Drosocin	G	—	K	P	R	P	Y	S	P	R	P	T[a]	S	H	P	R	P	I	R	V	
Pyrrhocoricin	V	D	K	G	S	—	Y	L	P	R	P	T[a]	—	P	P	R	P	I	Y	N	R	N
Formaecin I	G	—	R	P	N	P	V	N	N	K	P	T[a]	P	H	P	R	—	L				

[a] Glycosylation site.

Table 2. Synthetic peptides used in this study

#	Sequence[a]	HRMS
1	GKPRP YSPRP TSHPR PIRV	2197.220
2	GKPRP Y^{Me}SPRP TSHPR PIRV	2211.237
3	GKPRP YLPRP TSHPR PIRV	2223.280
4	GKPRP YTPRP TSHPR PIRV	2211.241
5	GKPRP Y^{D}SPRP TSHPR PIRV	2197.201
6	GKPRP Y$^{\beta}$SPRP TSHPR PIRV	2211.241
7	GKPRP FSPRP TSHPR PIRV	2195.236
8	GKPRP ^{D}YSPRP TSHPR PIRV	2197.201
9	GKPRP $^{\beta}$YSPRP TSHPR PIRV	2211.241
10	GKPRP FTPRP TSHPR PIRV	2195.236
11	GKPRP ^{D}Y^{D}SPRP TSHPR PIRV	2197.218
12	GKPRP $^{\beta}$Y$^{\beta}$SPRP TSHPR PIRV	2225.197
13	**Sar**-KPRP YTPRP TSHPR PIRV	2225.263
14	**βAla**-KPRP YTPRP TSHPR PIRV	2225.260
15	**Abu**-KPRP YTPRP TSHPR PIRV	2239.282
16	**Ava**-KPRP YTPRP TSHPR PIRV	2253.308
17	NTDGS TDYGI LQINS R	N/d[b]

[a] Modifications are highlighted in bold face. $^{\beta}$X = β^3-HX Sar = sarcosine, β*Ala* = β-alanine, Abu = γ-aminobutyric acid, Ava = δ-aminovaleric acid.
[b] Not determined.

Table 3. MIC values and serum stability data of drosocin analogues

#	MIC (μM)[a]	Intact (%)[b]
1	6.3	3
2	25	11
3	12.5	N/d[c]
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
13	6.3	77
14	3.1	87
15	6.3	76
16	6.3	84
17	>100	N/d

[a] Minimal inhibitory concentration against *E. coli* ATCC 11775.
[b] Percentage of intact peptide after 8 h of digestion in 25% human serum as determined by MALDI peak height of the corresponding peptide.
[c] Not determined.

degradation of drosocin in human serum comprises cleavage of the peptide bond at Tyr6-Ser7.[9] In order to simplify the synthetic procedure, we decided to omit the *O*-glycosyl modification at Thr11. Non-glycosylated drosocin **1** (Table 2) is about seven times less active than native drosocin,[5] but the expected gain in stability should largely compensate for this loss of activity. Based on these considerations, we prepared a series of drosocin analogues **2**–**16** (Table 2). The design of compounds **2**–**12** is based on modification of the labile Tyr6-Ser7 peptide linkage. This includes replacement of Ser7 with Thr (**4**, **10**), Leu (**3**) D-Ser (**5**, **11**), *N*-Me-Ser (**2**), or β^3-HSer (**6**, **12**) and replacement of Tyr6 with Phe (**7**,**10**), D-Tyr (**7**, **11**), or β^3-HTyr (**9**, **12**). Compounds **13**–**16** contain an extra modification at the N-terminus (vide infra). As a negative control, oligopeptide sequence **17** was included in the synthetic scheme.[10]

The preparation of the non-glycosylated linear peptides **1**–**17** was readily accomplished using standard Fmoc-based solid-phase peptide synthesis 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**, which was introduced manually. In this particular case, the N^{α}-methyl amino acid was introduced on the growing peptide chain using PyBOP and successive acylation with tyrosine was achieved using PyBroP.[11] All peptides were purified to homogeneity by reverse phase HPLC prior to biological assessment, and were obtained in reasonable to good overall yields (typical yields were in the range of 10–40%).

The MIC values of **1**–**12** against *E. coli* ATCC 11775[4b] are summarized in Table 3. Interestingly, none of the prepared peptides possess any haemolytic activity at concentrations up to 100 μM (data not shown). *N*-Methylation of the endopeptidase-sensitive peptide bond (peptide **2**) results in a considerably lower antibacterial activity as compared to the native form (**1**). In contrast, the drosocin analogues containing a single amino acid substitution of Tyr6 or Ser7 (that is, compounds **4**–**9**) display equal or better antibacterial activity. Analogue **3** however, which contains a Ser7 → Leu replacement to resemble the stable YLPRP pentamer present in the related CAP pyrrhocoricin[10] (Table 1), appeared to be completely inactive. The double mutant drosocin analogues **11** and **12** proved to have antimicrobial activity four times less than reference compound **1** (MIC 6.3 μM). The positive exception to this trend is represented by peptide **10** with a 3.1 μM minimal inhibitory concentration.

From these results we concluded that Ser7 → Thr substitution does not appear to have large consequences for antibacterial activity. On the premise that N-terminal capping of natural peptides often results in enhanced stability toward aminopeptidase activities, we prepared

a select panel of analogues **13**–**16**, equipped with a non-natural amino acid as the N-terminal residue (Table 2). Determination of the MIC value of these revealed no loss of activity upon replacement of the N-terminal glycine residue as compared to unglycosylated drosocin **1**. However, the positive effect of the Ser7 → Thr substitution (**4**, MIC 1.6 μM) seems to be (partly) compromised by subsequent Gly1 substitution (**13**–**16**, MIC values 3.1–6.3 μM).

As the next research objective, we set out to the evaluation of the proteolytic stability of non-glycosylated drosocin (**1**) and analogues **2** and **4**–**16** (Table 3). The degradation curves of **1** as well as selected examples are presented in Figure 1. The experiments were performed in 25% pooled human serum to increase peptide recovery and to allow monitoring of the degradation over an 8 h period. The relative abundance of the intact peptide was qualitatively determined by comparison of the MALDI mass spectral peak heights with those at $t = 0$.

Based on the results, non-glycosylated drosocin **1** and analogues **2** and **4**–**16** can be roughly divided into two categories. The first is represented by the peptides that are almost completely degraded after a digestion period of 8 h (that is, less than 5% intact peptide remains). In this group, analogues **5**, **9**, and **12** accompany reference compound **1.** The other peptides form the second category and display improved serum stability. Interestingly, all analogues comprising single natural 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 8 h of digestion in pooled 25% human serum, compared to only 3% for **1**. The improved serum stabilities and antimicrobial activities found for these peptides **4**, **7**, and **10** illustrate that substitution of one or two amino acids by structurally 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 demonstrated a proteolytic stability similar to **1**. Remarkably, completely 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 8 h 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. However, *N*-methylation of the N-terminal glycine, yielding a sarcosine residue (**13**), decreased susceptibility toward aminopeptidase activity in the assay. Moreover, a very significant increase in proteolytic stability is acquired by substitution of the Gly1 residue in peptides based on analogue **4**. Replacement of this glycine residue with βAla (**14**), Abu (**15**) or Ava (**16**) results in almost 30-fold increase in stability over the 8 h period, leaving an impressive average of ~80% of the peptide intact.

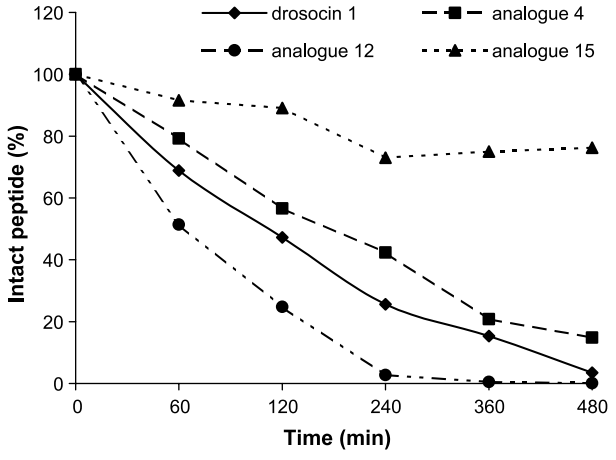

Figure 1. Selected degradation curves (average of three experiments).

In addition to the percentage intact peptide after 8 h 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 2 h of digestion were identified (Table 4), together with their respective intensities.[12] The most abundant early degradation product of non-glycosylated drosocin (**1**) is lacking six N-terminal residues (i.e., cleavage between Tyr6 and Ser7), which is fully consistent with the literature data.[9] The corresponding cleavage product from drosocin analogues **2**–**12** is considerably less abundant, or even fully absent, after the same time span. As mentioned previously, the proteolytic stabilities of peptides **5**, **9**, and **12** are similar to **1**. The low serum stability of these three analogues is reflected by the noticeable high abundances of proteolytic fragments –N1, –N3 and –N5, rather than fragment –N6.[13] N-terminal stability is obviously increased within N-terminally modified analogues of peptide **4**, **13**–**16**, as

Table 4. Abundances of major degradation products

#	Degradation products[a]				
	–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
13	0	0	0	**1**	1
14	0	0	0	**2**	0
15	0	0	0	**2**	1
16	0	0	0	**1**	1

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

no –N1, –N3 or –N5 fragments were detected. This correlates with their increased serum stability with respect to their precursor peptide **4**.

3. Conclusions

In conclusion, we have demonstrated that simple substitution of selected amino acid residues at sites that are susceptible to proteolytic cleavage, results in drosocin analogues with remarkably enhanced stability, while leaving the desired antibacterial properties intact. N-terminal modifications on the selected analogue **4** led to the identification of drosocin analogues **13**–**16** with even further improved activity/stability ratios. The in-depth study of the drosocin analogues presented here with respect to different bacterial strains and to their propensity to clear infections from relevant animal models is currently under investigation.

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12. Only fragments with molecular weight of 800 or more were taken into consideration. The relative abundance of these fragments was calculated under the assumption that the detector sensitivity was uniform for all fragments.
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**.