**Antimicrobial properties of derivatives of the cationic tryptophan-rich
hexapeptide PAF26**

Running title: Antimicrobial properties of derivatives of PAF26

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Abstract

Short antimicrobial peptides represent an alternative to fight pathogen infections. PAF26 is a hexapeptide identified previously by a combinatorial approach against the fungus *Penicillium digitatum* and shows antimicrobial properties towards certain phytopathogenic fungi. In this work, PAF26 was used as lead compound and its properties were compared with two series of derivatives, obtained by either systematic alanine substitution or N-terminal amino acid addition. The alanine scan approach underlined the optimized sequence of PAF26 in terms of potency and permeation capability, and also the higher contribution of the cationic residues to these properties. The N-terminal addition of amino acids resulted in new heptapeptides with variations in their antimicrobial characteristics, and very low cytotoxicity to human red blood cells. Positive (Arg or Lys) and aromatic (Phe or Trp) residue addition increased broad spectrum activity of PAF26. Noteworthy, addition of selected residues had specific effects on the properties of derivatives of PAF26.

Keywords

Tryptophan-rich cationic antimicrobial peptide; AMP; antifungal peptide; phytopathogenic fungi; *P. digitatum*; *B. cinerea*; *M. grisea*; *F. oxysporum*

Introduction

Antimicrobial peptides (AMP) are important components of an evolutionarily ancient mechanism of immunity, found in a wide range of organisms [1]. AMP differ in length, sequence and structure, but generally are amphipathic and a great number have positive charge and are referred as cationic antimicrobial peptides (CAMP). In many examples, these peptides are effective against microorganisms resistant to antibiotics or fungicides. In addition, AMP are unlikely to cause rapid emergence of resistance [2]. These facts and their short length, fast and efficient action against microbes and low toxicity to mammal cells have made them potential candidates as peptide drugs.

Rational design of AMP is an attractive approach to the improvement of antimicrobial properties. Agriculture could also greatly benefit from this emerging research area, with the identification, design and selection of peptides targeted to specific plant protection problems [3-6]. Soluble combinatorial libraries (SCL) represent an extensive source of molecular diversity for the *de novo* identification of lead AMP with new properties [7]. SCL have been used to identify novel peptides towards phytopathogenic fungi such as 66-10 hexapeptide (Ac-frlkfh-NH₂) [8] and its derivative heptapeptide 77-3 (Ac-frlkfhf-NH₂), which has activity against fungal strains of *Fusarium sambucinum* that are resistant to the fungicide thiabendazole (TBZ) [5]. In a previous work, we have used a synthetic D-hexapeptide library in a positional scanning format to identify AMP against selected phytopathogenic fungi that cause postharvest decay in fruits, such as *Penicillium digitatum* [6]. One of these peptides is PAF26 (Table 1), which showed strong activity against certain filamentous fungi and lower toxicity to *Escherichia coli* and *Saccharomyces cerevisiae* [6].

PAF26 is a tryptophan-rich CAMP with sequence similarities to other AMP [9-13]. It shares some properties with similar peptides, as absence of hemolytic activity [12,14]. PAF26 is active against strains resistant to fungicides and performed better than TBZ in experimental fruit decay tests [15]. Additionally, we have also demonstrated that PAF26 belongs to the class of AMP endowed with cell-penetrating properties [16,17], being capable to specifically interact with and locate inside target fungal cells [14]. PAF26 and similar peptides synthesized with either D- or L-enantiomers do not differ substantially in antimicrobial potency [9,11,14], which makes biotechnological production feasible.

In this work, we have used PAF26 as a lead in an optimization strategy to design two sets of peptides with single residue variations. The purpose was to analyze the effect of such variations in the antimicrobial properties of the resulting peptides. First, alanine substitution analogues addressed the influence of each residue on PAF26 antimicrobial properties. Secondly, we designed and compared novel heptapeptides obtained by addition of different N-terminal residues to PAF26 in terms of (i) spectrum of activity, (ii) specificity, (iii) microbicidal properties, and (iv) cytolysis of human red blood cells.

Materials and methods

Microorganisms. We used microorganisms that included fungal isolates of agricultural relevance (three distinct species of *Penicillium*, and *Alternaria* sp., *F. oxysporum*, *B. cinerea* and *M. grisea*) as well as fungal (*A. nidulans*), yeast (*S. cerevisiae*) and bacterial (*E. coli* and *B. subtilis*) model strains (see Supplemental Table 4). Fungi were cultured on potato dextrose agar (PDA) (Difco-BD Diagnostics, Sparks, MD) plates at 24 °C with the exception of *M. grisea*, which was maintained on rice flour medium. Conidia were collected and adjusted to the appropriate concentration. *S. cerevisiae* was grown in YPD (1% yeast extract, 1.5% peptone, 2% dextrose) at 30 °C and bacteria were grown in Luria-Bertani (LB) medium at 37 °C.

Peptides. Peptides used in this work (Table 1 and Supplemental Table 3) were purchased at >90% purity (GenScript Corporation, Piscataway, NJ). Peptides were acetylated at the N-terminus (Ac) and amidated at the C-terminus (NH₂). Stocks were prepared at 1 mM in 5 mM 3-(N-morpholino)-propanesulfonic acid pH 7 buffer and stored at -20 °C. Peptide concentrations were determined by absorbance at 280 nm.

Growth inhibition assays. The antimicrobial activities of the peptides were determined using a microtiter plate assay [6,18]. Growth was quantified as optical density (OD) at 492 nm. Potato dextrose broth (PDB) (Difco-BD Diagnostics) diluted one twentieth (5% PDB) was used as growth medium for fungi, and YPD diluted one tenth (10% YPD) for yeast, in both cases containing 0.003% (w/v) chloramphenicol. In

antibacterial assays, the medium was LB diluted one tenth (10% LB). Three replicates were prepared for each treatment.

The minimum inhibitory concentration (MIC) of a peptide for a given microorganism was the lowest peptide concentration that showed no growth at the end of the experiment. The IC_{50} of a peptide was the concentration required to obtain 50% inhibition of growth, and the value in each experiment was estimated by adjustment of the experimental data (SigmaPlot v 8.02, SPSS Inc., Chicago, IL). Statistical analyses were carried out with the software package StatGraphics Plus 4.0 (Manugistics Inc., Rockville, MD).

Membrane permeation assays. Membrane permeation was determined with the probe Sytox Green (SG) (Molecular Probes-Invitrogen Corp., Carlsbad, CA) and fluorometric measurement with a microplate reader (Fluoroskan Ascent FL, Labsystems, Finland) at an excitation of 485 nm and emission of 538 nm wavelengths [14]. Three replicates were prepared for each treatment. The FC_{50} of a peptide was defined as the concentration inducing 50% of the maximum fluorescence emission, and the values were calculated by adjustment of the experimental data as above.

Fungicidal and bactericidal activity assays. Assessment of peptide microbicidal activity was conducted as follows. In the case of *P. digitatum*, 2.5×10^4 conidia/ml were incubated with peptides in 5% PDB at 24 °C. After 1 day of incubation, 50 µl samples were spread onto peptide-free PDA plates to monitor colony forming units. *S. cerevisiae* and *E. coli* (5.0×10^5 CFU/ml) were incubated for 1 day with peptides in either 10% YPD at 30 °C or 10% LB at 37 °C, respectively, and 2.5 µl drops of samples were placed onto YPD or LB peptide-free plates. The lethal concentration (LC) of a peptide was defined as the lowest peptide concentration at which no growth or <1% of CFU was recovered after peptide treatment.

Hemolytic activity assay. The cytolytic activity of the peptides on human red blood cells was determined as release of hemoglobin monitored by absorbance at 415 nm [14]. Peptides were used at final concentrations of 1, 10 or 100 µM. Zero percent hemolysis and 100% hemolysis controls were determined in PBS and 0.1% Triton X-100, respectively.

Results and discussion

Antimicrobial properties of a series of alanine substitution analogues of PAF26.

We designed a set of six Ala substitution analogues of the cationic tryptophan-rich hexapeptide PAF26 (PAF26.r1a to PAF26.w6a, Table 1 and Supplemental Table 3). Distinct antimicrobial properties were determined and the results are summarized as IC_{50} , MIC and LC towards *P. digitatum* (Table 2). We observed lower activity for all the analogues, although the decrease was higher in the peptides with substitution of the positively charged residues (PAF26.r1a, .k2a and .k3a), which approximately four-fold higher MIC and LC, and higher significant differences in the IC_{50} .

We also used an assay based on the uptake of SG to quantify the permeation of *P. digitatum* mycelium promoted by the analogues. SG assays have been used to establish a link between antimicrobial activity of AMP and cell permeation [5,19]. Previously, we demonstrated that incubation of fungal hyphae with PAF26 resulted in uptake and increase in the fluorescence of SG [14].

We have quantified and compared the permeation capability of the Ala analogues with PAF26, by determining permeation dose-response curves in conjunction with inhibition curves (Fig. 1). Data allowed the calculation of FC_{50} as an estimate of the permeation capability of peptides (Table 2). In the case of PAF26 and the PAF26.f5a analog, the permeation curve paralleled that of growth inhibition (Fig. 1A and C), and both peptides had IC_{50} and FC_{50} values not significantly different (Table 2). Regarding the other five analogs, a noticeable result was the slight but consistently reproduced higher peptide concentrations needed to achieve 50% permeation (FC_{50}) than 50% inhibition (IC_{50}). This distinct effect could be visualized by plotting the relative activities for the six analogs as compared to PAF26 (Fig. 2). All the analogues except PAF26.f5a had losses of permeation capability (white bars) higher than losses of inhibition activity (black bars) (Fig. 2). Such differences among peptides are exemplified in a representative experiment (Fig. 1). PAF26.f5a initiates permeation and reaches maximum at concentrations similar to PAF26, in a curve that mirrors the inhibition response, while PAF26.r1a has a permeation curve shifted to higher peptide concentrations as compared to growth inhibition.

1 This differential effect of each Ala substitution in either the antimicrobial or the
2 permeation properties dissociate to some extent growth inhibition from permeation
3 among the different peptides, thus suggesting that PAF26 antimicrobial action is not
4 solely based on its ability to permeate target cells. This has been previously proposed
5 for other AMP [1,2,9,16,17], and also explored in the case of PAF26, for which it was
6 shown microscopically that produces growth alterations of mycelium in areas that are
7 not permeabilized [14]. Moreover, PAF26 is internalized by *P. digitatum* hyphae at very
8 low sub-inhibitory concentrations (i.e., 300 nM) that have no detectable effect on
9 growth, morphology or permeation [14].

10 Therefore, our Ala scanning approach showed that all the amino acid residues in
11 PAF26 contribute to some extent to its antifungal or permeation activities and that none
12 of them is dispensable for its properties towards *P. digitatum*. However, differences
13 among the analogs were observed. PAF26 has an amphipathic arrangement with three
14 N-terminal cationic residues followed by three aromatic and hydrophobic residues at the
15 C-terminus. Distinct independent parameters (Table 2 and Fig. 2) indicated that the
16 antimicrobial potency and permeation properties were more affected by substitutions of
17 the cationic rather than of the aromatic amino acids. The activity of CAMP, including
18 those rich in Trp as PAF26, is dependent on the ionic environment [10,13,20],
19 indicating that the initial interaction with microbes is electrostatic. In fact, confocal
20 microscope observations have shown that indolicidin and PAF26 primarily interact with
21 surfaces of hyphae [13,14]. The results of the Ala scan approach confirm the importance
22 of such electrostatic interaction for antimicrobial activity and permeation.

23 Our data indicate that the two Trp residues follow in relevance to the cationic
24 ones (Table 2). It has been reported that PAF26 interacts *in vitro* with membrane
25 mimetics and that substitution of Trp-4 for Pro decreases this interaction and
26 concomitantly also biological activity [21]. Finally, Phe-5 was found to be the least
27 significant residue since its replacement produced a peptide with modest differences as
28 compared to PAF26 and in fact its dose response curves were quite similar to that of
29 PAF26 (Fig. 1).

30 *Distinct activity profiles of heptapeptides derived from PAF26 by amino acid addition.*

31 Improvement in PAF26 antimicrobial potency and/or specificity could be
32 achieved by means of replacement for, or addition of, specific amino acids. We have
33 explored such scenario by addition of selected N-terminal residues and screening of the
34

1 resulting set of heptapeptides. Similar approaches have been used to improve other lead
2 AMP [5,9]. Amino acids highly represented in AMP databases [22] were chosen: the
3 positively charged residues Arg, Lys and His, the aromatic residues Trp, Phe and Tyr,
4 the aliphatic residues Leu and Ala, and hydrophilic residues Thr and Gln (Table 1). The
5 ten different resulting heptapeptides had 3-4 positive net charges at neutral pH and
6 distinct hydropathic indexes that indicate a hydrophilic character (Supplemental
7 Table 3).

8 An evaluation of antimicrobial potency and specificity was carried out against a
9 panel of selected microorganisms that include fungi of agronomic relevance as well as
10 the model filamentous fungus *A. nidulans*, the yeast *S. cerevisiae*, the Gram negative
11 bacteria *E. coli* and the Gram positive *B. subtilis*, in order to assay overall activity,
12 specificity, and bioactivity against reluctant fungi. The parameters IC₅₀ and MIC were
13 calculated (Supplemental Table 4). The previously described hexapeptide PAF34,
14 which differ from PAF26 in two amino acid residues (Table 1), was introduced as a
15 control of antimicrobial peptide with lower specificity than PAF26 [6].

16 Distinct inhibitory profiles of the heptapeptides were found (Supplemental
17 Table 4). Considering the microorganisms, *P. digitatum* was the most sensitive to AMP
18 based on the PAF26 lead, as expected given that this peptide was found in a
19 combinatorial screen against this fungus. On the other hand, the least sensitive
20 microorganisms were the phytopathogenic fungus *M. grisea*, the yeast *S. cerevisiae* and
21 the bacteria *E. coli*. The non-filamentous microorganism that has the susceptibility
22 pattern more similar to the filamentous fungi was the Gram positive bacterium
23 *B. subtilis*.

24 Taking into account the different peptides, the generally most active ones were
25 PAF38 (Arg) and PAF39 (Lys), a result in agreement with the above demonstrated
26 importance of cationic N-terminal residues in the activity of PAF26. However, our
27 study found no clear correlation between the antimicrobial potency/specificity and the
28 molecular weight, net charge, or hydrophilicity character of the peptides (Supplemental
29 Table 4), suggesting that more complex interactions between the amino acid residues
30 determine the antimicrobial properties. In other AMP, it has been shown that
31 appropriate position/clustering of residues are determinant for activity and selectivity
32 [12].

33 It was remarkable the higher activity against the economically important
34 *M. grisea* of PAF41 (Phe) and PAF42 (Trp), but not of PAF38 and PAF39. In the case

of *B. cinerea* and *F. oxysporum* a modest two-fold increase in the MIC was found in PAF40 (His) and PAF38, respectively, as compared to PAF26.

In most of the peptides the increase in antifungal activity correlates with an even higher increase in activity against the bacteria and yeast (Supplemental Table 4). In fact, the addition of cationic (PAF38, PAF39) or aromatic hydrophobic (PAF41, PAF42) residues showed a higher increase in antibacterial than in antifungal properties.

There were peptides that showed an improvement in the IC₅₀ but a deleterious effect on the MIC, for instance in the case of PAF45 (Thr), PAF46 (Gln) or PAF47 (Ala) against *P. digitatum*, emphasizing the existence of differently shaped dose-response curves and the need for considering different parameters when characterizing antimicrobials [23].

Microbicidal assays of heptapeptides derived from PAF26.

The killing capacity of peptides to conidia of *P. digitatum*, or cells of *E. coli* and *S. cerevisiae* was evaluated (Fig. 3). It has been previously shown that inhibitory and fungicidal properties against *P. digitatum* are not linked in selected AMP, as lactoferricin-derived peptides and melittin [14,18]. This experiment confirmed the reverse microbicidal properties of PAF26 and PAF34 (Fig. 3A). These two peptides have distinct activity profiles: PAF26 is more microbicidal than PAF34 to *P. digitatum*, while less to *S. cerevisiae* and *E. coli*.

Microbicidal data confirmed and extended the growth inhibition results described above. The cationic derivatives PAF38 and PAF39 showed a two-fold improvement of fungicidal activity against *P. digitatum* but also killed *S. cerevisiae* and *E. coli* more efficiently (Fig. 3B).

PAF40 has activity against *P. digitatum* and *S. cerevisiae* similar to the sequence-related PAF38, PAF39, PAF41 and PAF42, but a four-fold reduction in killing capacity to *E. coli* (Supplemental Table 4 and Fig. 3B). On the contrary, some of the peptides as PAF43 (Tyr) showed an increase in activity against bacteria while had activity against several fungi similar to PAF26 (Supplemental Table 4 and Fig. 3B). In fact, PAF40 and PAF43 showed reversal microbicidal properties towards *S. cerevisiae* and *E. coli* (Fig. 3B).

Also noticeable was the loss of fungicidal activity against conidia of *P. digitatum* of some peptides as PAF46 (Fig. 3B). We have reported that peptides derived from the antimicrobial motif of bovine Lactoferricin [10], which shown sequence

similarities with PAF26 and also contain a Gln residue, have IC₅₀ values very similar to PAF26 but a much lower fungicidal activity to *P. digitatum* [18]. This finding will guide future experiments to test whether Gln residues negatively impact fungicidal properties of CAMP while maintaining growth inhibition, and thus whether determinants of CAMP fungistatic and fungicidal properties differ.

Overall, our results indicate that some of the modifications resulted in broader antimicrobial activity, and therefore would be undesirable whenever filamentous fungi are the specific target of the antimicrobial approach. Also, data provide information to the development of novel peptides with broader activities. The detrimental loss of antifungal properties of some heptapeptides also demonstrates that the presence of the PAF26 sequence by itself does not guarantee high inhibitory activity, confirming that a specific arrangement of a peptide sequence and interactions between residues are also important for the properties of AMP [12,24].

Evaluation of hemolytic activity of heptapeptides derived from PAF26.

An evaluation of toxicity was performed by conducting hemolytic assays on human red blood cells at different peptide concentrations (1, 10 and 100 µM). All except one of the PAF26-derived peptides described in this work exhibited no hemolysis even at 100 µM (i.e., below the detection limit, which in our assay is 0.3% of the hemolysis of the control). The only peptide showing marginal toxicity was PAF42 (Trp) that produced 0.6% hemolysis at 100 µM. The well known toxic peptide Melittin (used as control) was 100% hemolytic at 100 µM and 38% at 10 µM. Data indicate that PAF heptapeptides are at least 1000 times less cytolytic to red blood cells than Melittin. It has been reported that PAF26 and Melittin are similarly active against *P. digitatum*, being Melittin more toxic to bacteria [6].

Conclusion

We have generated a series of peptides derived from PAF26, with different profiles of antimicrobial properties and negligible hemolysis. They are of interest in the development of novel AMP, adding to the catalog of compounds with potential application in agriculture and biomedicine. Selection of the most suitable peptide would depend on the importance for each particular use of potency and/or specificity.

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5

Figure legends**Fig. 1**

Dose-response curves of inhibition (black circles) and permeation (white triangles) activity of peptides PAF26 (A), PAF26.r1a (B) and PAF26.f5a (C) on *P. digitatum*. Data shown are the mean values $\pm$ SD of either OD (492 nm) or (FI) (538 nm).

Fig. 2

Relative antimicrobial and permeation activity of the Ala substitution analogs as compared to the parental peptide PAF26. Bars represent the relative IC₅₀ as measure of growth inhibition (black bars), FC₅₀ as measure of permeation (white bars), and LC as measure of fungicidal activity to conidia (stripped bars). Relative values were calculated taking the corresponding parameter of PAF26 as reference.

Fig. 3

Assessment of microbicidal activity of peptides against *P. digitatum* (left), *S. cerevisiae* (middle) and *E. coli* (right). Microorganism samples, either conidia (*P. digitatum*) or CFU (*S. cerevisiae* and *E. coli*), were treated with selected peptide concentrations (top) for 1 day, and spread (for the fungus) or applied as droplets (for the yeast and bacteria) onto peptide-free plates. Representative photographs are shown for (A) the previously described PAF26 and PAF34, and (B) for the novel heptapeptides described here (PAF38 to PAF47).

Table 1
Amino acid sequences of peptides.

Peptide	Sequence ^a
PAF26	Ac-rkkwfw-NH ₂
PAF34	Ac-rk w lfw-NH ₂
PAF26.r1a	Ac- a kkwfw-NH ₂
PAF26.k2a	Ac-r a kwfw-NH ₂
PAF26.k3a	Ac-rk a wfw-NH ₂
PAF26.w4a	Ac-rkk a fw-NH ₂
PAF26.f5a	Ac-rkkw a w-NH ₂
PAF26.w6a	Ac-rkkwf a -NH ₂
PAF38	Ac- r rkkwfw-NH ₂
PAF39	Ac- k rkkwfw-NH ₂
PAF40	Ac- h rkkwfw-NH ₂
PAF41	Ac- f rkkwfw-NH ₂
PAF42	Ac- w rkkwfw-NH ₂
PAF43	Ac- y rkkwfw-NH ₂
PAF44	Ac- l rkkwfw-NH ₂
PAF45	Ac- t rkkwfw-NH ₂
PAF46	Ac- q rkkwfw-NH ₂
PAF47	Ac- a rkkwfw-NH ₂

^a The D-amino acids are shown in lower case.
Residues distinct from PAF26 are in bold.

Table 2

Antimicrobial properties of PAF26 analogues towards *P. digitatum*.

Peptide	IC ₅₀ (μM) ^{a,b}	MIC (μM)	LC (μM)	FC ₅₀ (μM) ^{a,b}
PAF26	2.2 ± 0.3 (a)	4	16	1.7 ± 0.2 (a)
PAF26.r1a	6.3 ± 1.1 (c)	16	>64	11.5 ± 1.1 (c)
PAF26.k2a	5.6 ± 1.5 (c)	16	64	7.7 ± 2.4 (b)
PAF26.k3a	6.6 ± 2.1 (c)	16	>64	7.9 ± 3.0 (bc)
PAF26.w4a	3.2 ± 0.7 (ab)	8	32	6.3 ± 1.4 (b)
PAF26.f5a	2.8 ± 0.6 (ab)	8	32	2.0 ± 0.4 (a)
PAF26.w6a	3.7 ± 0.5 (b)	16	32	5.7 ± 0.6 (b)

^a Mean value ± standard deviation, calculated from independent experiments.^b Values with the same letter do not differ at 95% confidence level (Fisher's LSD procedure).

Figure 1 (Muñoz et al., 2007)

Figure 2 (Muñoz et al., 2007)

Figure 3 (Muñoz et al., 2007)

Supplemental Table 3

Amino acid sequences and properties of peptides used in this study.

Peptide	Sequence ^a	MW	Net charge ^b	GRAVY ^c	Source
PAF26	Ac-rkkwfw-NH ₂	991.2	3+	-1.883	[1]
PAF34	Ac-rk w lfw-NH ₂	976.2	2+	-0.600	[1]
PAF26.r1a	Ac- a kkwfw-NH ₂	906.1	2+	-0.833	This study
PAF26.k2a	Ac-r a kwfw-NH ₂	934.1	2+	-0.933	This study
PAF26.k3a	Ac-rk a wfw-NH ₂	934.1	2+	-0.933	This study
PAF26.w4a	Ac-rkk a fw-NH ₂	876.1	3+	-1.433	This study
PAF26.f5a	Ac-rkkw a w-NH ₂	915.1	3+	-2.050	This study
PAF26.w6a	Ac-rkkwf a -NH ₂	876.1	3+	-1.433	This study
PAF38	Ac- r rkkwfw-NH ₂	1147.4	4+	-2.257	This study
PAF39	Ac- k rkkwfw-NH ₂	1119.4	4+	-2.171	This study
PAF40	Ac- h rkkwfw-NH ₂	1128.3	3.1+	-2.071	This study
PAF41	Ac- f rkkwfw-NH ₂	1138.4	3+	-1.214	This study
PAF42	Ac- w rkkwfw-NH ₂	1177.4	3+	-1.743	This study
PAF43	Ac- y rkkwfw-NH ₂	1154.4	3+	-1.800	This study
PAF44	Ac- l rkkwfw-NH ₂	1104.4	3+	-1.071	This study
PAF45	Ac- t rkkwfw-NH ₂	1092.3	3+	-1.714	This study
PAF46	Ac- q rkkwfw-NH ₂	1119.3	3+	-2.114	This study
PAF47	Ac- a rkkwfw-NH ₂	1062.3	3+	-1.357	This study

^a The D-amino acids are shown in lower case. Residues distinct from PAF26 are in bold.^b Estimated at pH = 7.^c Peptide grand average of hydropathicity (GRAVY) index [2] was calculated using a web-based tool (<http://www.expasy.ch/>).

Reference List

- [1] B.López-García, E.Pérez-Payá, J.F.Marcos, Identification of novel hexapeptides bioactive against phytopathogenic fungi through screening of a synthetic peptide combinatorial library, *Appl. Environ. Microbiol.* 68 (2002) 2453-2460.
- [2] J.Kyte, R.F.Doolittle, A simple method for displaying the hydropathic character of a protein, *J. Molec. Biol.* 157 (1982) 105-132.

Supplemental Table 4
Growth inhibitory activity of synthetic peptides against different microorganism.

Microorganism ^a	Peptide ^b											
	PAF26	PAF34	PAF38	PAF39	PAF40	PAF41	PAF42	PAF43	PAF44	PAF45	PAF46	PAF47
<i>P. digitatum</i> PHI-26	2.2 (4)	6.9 (8)	1.4 (4)	2.3 (4)	1.7 (4)	1.7 (4)	1.6 (4)	2.8 (8)	1.5 (4)	1.4 (8)	1.6 (8)	1.9 (8)
<i>P. italicum</i> PHI-1	2.3 (4)	6.0 (8)	1.9 (4)	3.4 (8)	2.1 (4)	2.4 (4)	3.4 (4)	2.1 (4)	2.2 (4)	2.2 (4)	3.2 (8)	3.8 (8)
<i>P. expansum</i> PHI-65	3.2 (8)	6.9 (16)	2.7 (8)	3.5 (8)	4.2 (8)	4.2 (8)	4.4 (8)	4.4 (8)	2.3 (8)	4.5 (8)	4.1 (8)	4.7 (8)
<i>Alternaria</i> sp. PHI-44	4.6 (8)	8.1 (16)	2.4 (8)	4.5 (8)	7.0 (8)	5.1 (8)	5.1 (8)	6.8 (16)	6.9 (8)	5.5 (8)	7.0 (16)	5.7 (16)
<i>A. nidulans</i> biA1	3.6 (8)	7.2 (8)	2.2 (4)	2.1 (4)	2.3 (4)	2.4 (8)	2.6 (8)	3.8 (8)	2.3 (4)	3.7 (8)	2.4 (4)	2.2 (4)
<i>B. cinerea</i> #8	2.9 (8)	5.4 (16)	3.1 (8)	2.7 (8)	2.4 (4)	4.3 (8)	3.1 (8)	4.1 (8)	3.5 (8)	3.2 (8)	4.0 (8)	4.4 (8)
<i>F. oxysporum</i> CECT2866	4.5 (8)	7.5 (16)	2.3 (4)	2.3 (8)	2.5 (8)	2.7 (8)	2.4 (8)	4.1 (8)	3.7 (8)	4.1 (8)	3.4 (8)	4.1 (8)
<i>M. grisea</i> PR-9	(>32) ^c	9.4 (32)	(>32) ^c	(>32) ^c	(>32) ^c	9.0 (16)	6.7 (16)	(>32) ^c	(>32) ^c	(>32) ^c	(>32) ^c	(>32) ^c
<i>E. coli</i> DH5α	20.4 (64)	15.5 (32)	4.5 (8)	6.7 (8)	12.7 (32)	4.5 (8)	5.1 (8)	8.7 (16)	14.4 (16)	17.1 (32)	22.2 (64)	17.3 (32)
<i>B. subtilis</i> CECT498	4.0 (16)	5.6 (16)	2.0 (8)	2.6 (8)	4.1 (8)	2.1 (8)	1.1 (4)	2.5 (8)	2.5 (8)	4.3 (16)	4.8 (16)	5.4 (16)
<i>S. cerevisiae</i> FY1679	15.9 (32)	13.4 (32)	9.0 (16)	8.1 (16)	9.2 (16)	10.5 (16)	16.6 (32)	17.6 (32)	15.5 (32)	13.0 (32)	12.9 (32)	20.1 (32)

a For fungi, the assay mixture contained 2.5×10^4 conidia/ml and different peptide concentrations up to 32 μ M (0.25, 0.5, 1, 2, 4, 8, 16 and 32 μ M). For yeast and bacteria the assays contained 5.0×10^5 CFU/ml and different peptide concentrations up to 64 μ M (2, 4, 8, 12, 16, 24, 32, 48 and 64 μ M).

b Data are shown as IC₅₀ value, followed by MIC value in parenthesis, in μ M units. IC₅₀ is the mean value obtained from independent experiments. MIC is the minimum concentration that was completely inhibitory in all the experiments.

c The symbol ">" indicates some, but not complete, inhibition of growth at the highest concentration used. In these cases, the IC₅₀ was not calculated.