Design, synthesis and biological evaluation of cyclic peptidotriazoles derived from BPC194 as novel agents for plant protection

Imma Güell,[1] Sílvia Vilà,[1] Esther Badosa,[2] Emilio Montesinos,[2]

Lidia Feliu,[1] Marta Planas[1]

[1]LIPPSO, Department of Chemistry, University of Girona, Campus Montilivi, 17003 Girona, Spain

[2]Laboratory of Plant Pathology, Institute of Food and Agricultural Technology-CIDSAV-XaRTA, University of Girona, Campus Montilivi, 17003 Girona, Spain

Correspondence to:

Lidia Feliu, LIPPSO, Department of Chemistry, University of Girona, Campus Montilivi, 17003 Girona, Spain; e-mail: lidia.feliu@udg.edu

Marta Planas: LIPPSO, Department of Chemistry, University of Girona, Campus Montilivi, 17003 Girona, Spain; e-mail: marta.planas@udg.edu

ABSTRACT

The search for novel antimicrobial agents to be used for plant protection has prompted us to design analogues incorporating non-natural amino acids. Herein, we designed and synthesized cyclic peptidotriazoles derived from the lead antimicrobial cyclic peptide c(Lys-Lys-Leu^3-Lys-Lys^5-Phe-Lys-Lys-Leu-Gln) (**BPC194**). In particular, Leu^3 and Lys^5 were replaced by a triazolyl alanine, a triazolyl norleucine or a triazolyl lysine. These peptides were screened for their antibacterial activity against *Xanthomonas axonopodis* pv. *vesicatoria*, *Erwinia amylovora* and *Pseudomonas syringae* pv. *syringae*, for their hemolysis and for their phytotoxicity. Results showed that the type of triazolyl amino acid and the substituent present at the triazole influenced the antibacterial and hemolytic activities. Moreover, the position of this residue was also crucial for the hemolysis. The lead compounds **BPC548** and **BPC550** exhibited high antibacterial activity (MIC of 3.1 to 25 µM), low hemolysis (19 and 26% at 375 µM, respectively) and low phytotoxicity. Therefore, these analogues could be used as new leads for the development of effective agents to control pathogenic bacteria responsible for plant diseases of economic importance.

Keywords:

Antimicrobial peptides; click chemistry; eukaryotic cytotoxicity; peptidomimetics; phytopathogenic bacteria

INTRODUCTION

Phytopathogenic bacteria cause major diseases in plants that have a great economic impact on agricultural communities worldwide.[1,2] These bacterial infections are mainly treated with copper compounds and antibiotics. Although the latter are highly effective, their use has been seriously hampered by the emergence of drug-resistant bacteria.[3,4] Therefore, there is an urgent need for new agents active against these pathogens and that are able to manage resistance problems. In this field, antimicrobial peptides are considered an excellent alternative to conventional antibiotics.[5-12] On the one hand, they show a wide spectrum of activity. On the other hand, their unique mechanism of action, which primarily involves the interaction with the bacterial membrane, makes it unlikely that bacteria build up resistance to this type of antibiotics.

In the light of the continuing need for new effective compounds, synthetic analogues maintaining the crucial features of native antimicrobial peptides have been designed which display remarkable activity.[6,7,13-19] For instance, the incorporation of unnatural amino acids, such as D-, biaryl or triazolyl residues,[17] has been reported as an efficient strategy to obtain synthetic antimicrobial peptides with comparable or improved biological activity against plant pathogens in respect to the native peptides.

Regarding triazolyl amino acids, these residues have been broadly used in drug discovery due to the structural properties and the straightforward synthesis of the 1,2,3-triazole ring.[20-29] This nitrogen heterocycle has been described to be resistant to metabolic degradation and it is considered as a powerful pharmacophore.[30,31] In fact, this ring is found in a wide variety of compounds with interesting biological activities, such as antimicrobial, antitumor, and antivirus agents. Moreover, the formation of this heterocycle can be easily achieved through an alkyne-azide cycloaddition under mild conditions that can be applied to derivatize peptide sequences. Various 1,2,3-triazole-containing peptides have been reported with a range of biological properties,[28] including HIV-1 cell entry inhibition,[32] protease inhibition,[20] or antimicrobial activity.[25,26,29]

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Despite the interesting biological properties displayed by peptidotriazoles, the use of these compounds to treat plant diseases caused by phytopathogenic bacteria has only been addressed in two reports by our group. First, we designed and synthesized linear undecapeptides derived from the antimicrobial peptide **BP100** bearing a triazolyl amino acid.[33] These linear peptidotriazoles were active against the bacteria *Xanthomonas axonopodis* pv. *vesicatoria*, *Erwinia amylovora*, and *Pseudomonas syringae* pv. *syringae* (MIC of 1.6-12.5 μM), low hemolytic (0-23% at 50 μM), and no phytotoxic. Recently, we described the synthesis of cyclic peptidotriazoles derived from the antimicrobial cyclic peptide c(Lys-Lys-Leu[3]-Lys-Lys[5]-Phe-Lys-Lys-Leu-Gln) (**BPC194**).[34,35] These sequences were designed by incorporating a triazolyl amino acid at the 3-position. In particular, Leu[3] was replaced by a triazolyl alanine, a triazolyl lysine, a triazolyl norleucine or a triazolyl glutamic acid. Cyclic peptidotriazoles with high antibacterial activity against the above phytopathogens (3.1-25 μM) and low hemolysis (0-30% at 375 μM) were identified, constituting good candidates for the design of new antimicrobial agents.

On the basis of these promising results, we considered it important to prepare novel cyclic peptidotriazoles. On the one hand, in the current report, we describe new analogues incorporating a triazolyl amino acid at position 3. On the other hand, a series of derivatives were designed and synthesized by replacing Lys[5] with a triazolyl alanine, a triazolyl norleucine or a triazolyl lysine. All compounds were evaluated against the above plant pathogenic bacteria, against erythrocytes to determine their hemolytic properties and also in tobacco leaves to assay the phytotoxicity.

MATERIALS AND METHODS

Chemicals and Instruments

Commercially available reagents were used throughout without purification. Solvents were purified and dried by passing them through an activated alumina purification system (MBraun SPS-800) or by conventional distillation techniques.

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All compounds were analyzed under standard analytical high performance liquid chromatography (HPLC) conditions with a Dionex liquid chromatography instrument. Detection was performed at 220 nm. Analysis was carried out using the Dionex instrument with a Kromasil 100 C_{18} (40 mm×4.6 mm, 3.5 μm) column with a 2-100% B linear gradient over 7 min at a flow rate of 1 mL/min. Solvent A was 0.1% aqueous trifluoroacetic acid (TFA), and solvent B was 0.1% TFA in CH_3CN.

Electrospray ionization (ESI) mass spectrometry (MS) analyses were performed with an Esquire 6000 ESI ion Trap LC/MS (Bruker Daltonics) instrument equipped with an electrospray ion source. The instrument was operated in the positive ESI(+) ion mode. Samples (5 μL) were introduced into the mass spectrometer ion source directly through an HPLC autosampler. The mobile phase (80:20 CH_3CN/H_2O at a flow rate of 100 μL/min) was delivered by a 1100 Series HPLC pump (Agilent). Nitrogen was employed as both the drying and nebulizing gas.

High resolution mass spectrometry (HRMS) spectra were recorded under conditions of ESI with a Bruker MicroTof-Q instrument (University of Zaragoza) or with a Bruker MicrOTOF-Q IITM instrument (University of Girona) using a hybrid quadrupole time-of-flight mass spectrometer. Samples were introduced into the mass spectrometer ion source directly through a 1100 Series Agilent HPLC autosampler (University of Zaragoza) or by direct infusion through a syringe pump (University of Girona) and were externally calibrated using sodium formate. The instruments were operated in the positive ESI(+) ion mode.

General Procedure for the Synthesis of Linear Peptidyl Resins 6-10

These peptidyl resins were synthesized manually by the solid-phase method using standard 9-fluorenylmethoxycarbonyl (Fmoc) chemistry. Fmoc-Rink-MBHA resin (0.3 mmol/g) (MBHA, 4-methylbenzhydrylamine) was used as solid support. Fmoc-Leu-OH, Fmoc-Lys(Boc)-OH, Fmoc-Lys(Mtt)-OH, Fmoc-Nle(ε-N₃)-OH,[35,36] Fmoc-Pra-OH, Fmoc-Phe-OH or Fmoc-Glu-OAll were

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used as amino acid derivatives (All, allyl; Boc, *tert*-butyloxycarbonyl; Mtt, 4-methyltrityl). Peptide elongation was performed by repeated cycles of Fmoc group removal, coupling and washings. Fmoc group removal was achieved with piperidine/*N,N*-dimethylformamide (DMF) (3:7, 2+10 min). Couplings of the Fmoc-amino acids (4 equiv) were mediated by 2-cyano-2-(hydroxyimino)acetate (Oxyma) (4 equiv) and *N,N'*-diisopropylcarbodiimide (DIPCDI) (4 equiv) in DMF at room temperature for 1 h under stirring. The completion of the reactions was checked by the Kaiser test.[37] After each coupling and deprotection step, the resin was washed with DMF (6×1 min) and CH_2Cl_2 (6×1 min), and air dried. After the fifth coupling, *N*-methylpyrrolidinone (NMP) was used instead of DMF. An aliquot of each resulting peptidyl resin was treated with TFA/H_2O/triisopropylsilane (TIS) (95:2.5:2.5) for 2 h at room temperature. Following TFA evaporation and diethyl ether extraction, the crude peptide was dissolved in H_2O, lyophilized, analyzed by HPLC, and characterized by mass spectrometry. The physical data can be found in the Supporting Information.

General Procedure for the Synthesis of the Alkynyl and Azido Cyclic Peptidyl Resins 1, 2, 4 and 5

The C-terminal allyl ester of the corresponding linear peptidyl resin was cleaved by treatment with Pd(PPh$_3$)$_4$ (3 equiv) in CHCl$_3$/AcOH/*N*-methylmorpholine (NMM) (3:2:1) under nitrogen and stirring for 3 h at room temperature. After this time, the resin was washed with tetrahydrofuran (THF) (3×2 min), NMP (3×2 min), *N,N'*-diisopropylethylamine (DIPEA)/CH_2Cl_2 (1:19, 3×2 min), sodium *N,N*-diethyldithiocarbamate (0.03 M in NMP, 3×15 min), NMP (10×1 min) and CH_2Cl_2 (3×2 min). Fmoc was removed with piperidine/DMF (3:7, 2+10 min) followed by washes with DMF (6×1 min) and CH_2Cl_2 (3×1 min). Cyclization was carried out by treating the resulting resin with [ethyl cyano(hydroxyimino)acetato-O^2]tri-1-pyrrolidinylphosphonium hexafluorophosphate (PyOxim) (5 equiv), Oxyma (5 equiv), and DIPEA (10 equiv) in NMP under stirring for 24 h.

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Following washes with NMP (6×1 min) and CH$_2$Cl$_2$ (6×1 min), an aliquot of the cyclic peptidyl resin was cleaved by treatment with TFA/H$_2$O/TIS (95:2.5:2.5) for 2 h. After TFA evaporation and diethyl ether extraction, the crude peptide was dissolved in H$_2$O, lyophilized, analyzed by HPLC, and characterized by mass spectrometry. The physical data can be found in the Supporting Information.

Synthesis of the Alkynyl Cyclic Peptidyl Resin 3

This alkynyl cyclic peptidyl resin was synthesized from the cyclic peptidyl resin c[Lys(Boc)-Lys(Boc)-Leu-Lys(Boc)-Lys(Mtt)-Phe-Lys(Boc)-Lys(Boc)-Leu-Glu(Rink-MBHA)]   which   was prepared from the linear peptidyl resin **10** following the general procedure described above for cyclic peptidyl resins **1**, **2**, **4** and **5**. The above cyclic peptidyl resin was treated with 1% TFA in CH$_2$Cl$_2$ upon which the solution became yellow. The mixture was stirred for 5 min at room temperature and the resin was then washed with CH$_2$Cl$_2$ (2×1 min), MeOH (2×1 min) and CH$_2$Cl$_2$ (2×1 min). The TFA treatment was repeated until the solution remained colorless. Then, the N^{ε}-amino group of the lysine residue at position 5 was acylated with propiolic acid using the conditions described in the general procedure for the coupling of Fmoc-protected amino acids. Following washes with NMP (6×1 min) and CH$_2$Cl$_2$ (6×1 min), an aliquot of the cyclic peptidyl resin was cleaved by treatment with TFA/H$_2$O/TIS (95:2.5:2.5) for 2 h. After TFA evaporation and diethyl ether extraction, the crude peptide was dissolved in H$_2$O, lyophilized, analyzed by HPLC, and characterized by mass spectrometry. The physical data can be found in the Supporting Information.

Synthesis of the Cyclic Peptidotriazoles

The corresponding alkynyl or azido cyclic peptidyl resin was swollen with CH$_2$Cl$_2$ (1×20 min) and DMF (1×20 min). Then, it was treated with an azide (5 equiv) or with an alkyne (5 equiv), respectively, in presence of ascorbic acid (5 equiv) and CuI (5 equiv) in piperidine/DMF (2:8). The

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reaction mixture was stirred for 5 h at room temperature. The resin was subsequently washed with sodium *N,N*-diethyldithiocarbamate (0.03 M in NMP, 3×3 min), DMF (6×1 min) and CH_2Cl_2 (1×20 min). The resulting cyclic peptidotriazole was cleaved from the resin with TFA/H_2O/TIS (95:2.5:2.5) for 2 h, and analyzed by HPLC and mass spectrometry. The physical data can be found in the Supporting Information.

Bacterial Strains and Growth Conditions

The following plant pathogenic bacterial strains were used: *Erwinia amylovora* PMV6076 (Institut National de la Recherche Agronomique, Angers, France), *Pseudomonas syringae* pv. *syringae* EPS94 (Institut de Tecnologia Agroalimentària, Universitat de Girona, Spain) and *Xanthomonas axonopodis* pv. *vesicatoria* 2133-2 (Instituto Valenciano de Investigaciones Agrarias, Valencia, Spain). All bacteria were stored in Luria Bertani (LB) broth supplemented with glycerol (20%) and maintained at 80 ºC. *E. amylovora* and *P. syringae* pv. *syringae* were scrapped from LB agar after growing for 24 h and *X. axonopodis* pv. *vesicatoria* after growing for 48 h at 25 ºC. The cell material was suspended in sterile water to obtain a suspension of 10^8 CFU ml^{-1}.

Antibacterial Activity

The minimum inhibitory concentration (MIC) of 15 compounds was evaluated at 1.6, 3.1, 6.2, 12.5, 25 and 50 μM as previously described.[35]

Hemolytic Activity

The hemolytic activity of 15 compounds was evaluated at 150, 250 and 375 μM by determining hemoglobin release from erythrocyte suspensions of horse blood (5% vol/vol) (Oxoid) using absorbance at 540 nm as previously described.[35] Three replicates for each peptide concentration were used.

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Phytotoxicity

BPC194 and the cyclic peptidotriazoles were evaluated for their phytotoxicity. Peptide solutions of 50, 100, and 200 μM were infiltrated (100 μL) into the mesophylls of fully expanded tobacco leaves. Six independent inoculations were carried out in a single leaf, and at least three independent inoculations were performed per peptide and concentration randomly distributed in different leaves and plants. Control infiltrations with water (negative control) or mellitin (positive control) at the same molar concentration were performed. The appearance of symptoms on the leaves was followed for 48 h after infiltration and measured as a lesion diameter.

RESULTS

Design of the Cyclic Peptidotriazoles

In a preliminar study we designed cyclic peptidotriazoles derived from c(Lys-Lys-Leu^3-Lys-Lys^5-Phe-Lys-Lys-Leu-Gln) (**BPC194**) by replacing the Leu at position 3 for amino acids incorporating a triazole ring at the side-chain.[35] In particular, Leu^3 was replaced by an alanine, a lysine or a norleucine residue incorporating an unsubstituted or substituted triazole ring at the side-chain. The evaluation of the antibacterial and hemolytic activities of these cyclic peptidotriazoles allowed the identification of sequences with a promising biological activity profile (MIC of 3.1 to 25 μM; hemolysis between 0 to 30% at 375 μM).

These results prompted us to broaden this study by preparing 22 new analogues with the general structure **I-IV** (Figure 1). These new cyclic peptidotriazoles were designed by incorporating at position 3 a triazolyl alanine (**I**) or a triazolyl norleucine (**II**), in which the triazole moiety was substituted with an aryl group having various electronic demands. Moreover, we decided to include in this study analogues obtained by replacing Lys^5 in **BPC194** with a triazolyl alanine (**III**, X = -CH$_2$-), a triazolyl lysine (**III**, X = -(CH$_2$)$_4$-NH-CO-) or a triazolyl norleucine (**IV**). Peptidotriazoles

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III and **IV** incorporated the triazole ring unsubstituted or substituted with an alkyl or an aryl group. Lys[5] was chosen because it was observed that this residue is involved in the interaction of **BPC194** with negatively charged model membranes.[38] Moreover, we have recently shown that the acylation of Lys[5] renders cyclic lipopeptides with significant antimicrobial activity against phytopathogenic bacteria and fungi.[39,40] Based on these findings and considering that the triazole ring is regarded as a powerful pharmacophore, we envisioned that the replacement of Lys[5] with a triazolyl amino acid could provide cyclic peptidotriazoles with remarkable antimicrobial activity.

Synthesis of Cyclic Peptidotriazoles

The synthesis of the cyclic peptidotriazoles of general structure **I-IV** followed the methodology previously described.[35] This procedure relied on the preparation of an azido or alkynyl cyclic peptidyl resin and its subsequent cycloaddition with the corresponding alkyne or azide, respectively (Scheme 1 and Table 1). Accordingly, to obtain compounds **I** and **III**, the alkynylpeptidyl resins **1-3** were required, whereas for the synthesis of derivatives **II** and **IV** the azido cyclic peptidyl resins **4** and **5** were prepared.

The synthesis of the cyclic peptidyl resins **1**, **2**, **4** and **5** was carried out using a standard three dimensional orthogonal Fmoc/*tert*-butyl (*t*Bu)/allyl (All) strategy. The protocol involved the preparation of the corresponding linear peptidyl resin **6-9** followed by on-resin head-to-tail cyclization (Scheme 1). Fmoc-Rink-MBHA was used as solid support and, after Fmoc removal with piperidine/DMF (3:7), Fmoc-Glu-OAll was coupled using DIPCDI and Oxyma. Sequential deprotection and coupling steps were carried out. Side-chain protection for Lys residues was as *tert*-butyl carbamate (Boc), Fmoc-Pra-OH was used to obtain peptidyl resins **6** and **7**, and Fmoc-Nle(ε-N$_3$)-OH[35,36] was employed for the synthesis of resins **8** and **9**. After completion of the linear sequences **6-9**, the C-terminal allyl ester was cleaved with Pd(PPh$_3$)$_4$ in CHCl$_3$/AcOH/NMM. Next, the Fmoc group was removed and cyclization was performed using PyOxim, Oxyma and DIPEA,

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leading to peptidyl resins **1**, **2**, **4** and **5**. The cyclic alkynyl peptidyl resin **3** was prepared following the same protocol (Scheme 1). However, in this case, to achieve the selective derivatization of Lys[5] with a propioloyl group, this residue was incorporated as Fmoc-Lys(Mtt)-OH. The 4-methyltrityl (Mtt) group can be removed with 1% TFA/CH_2Cl_2, conditions that do not compromise either the resin anchorage or the other side-chain protecting groups. Thus, synthesis of the linear sequence **10** and cyclization was followed by Mtt group removal and acylation with propiolic acid, rendering the alkynyl cyclic peptidyl resin **3**. Afterwards, an aliquot of resins **1-5** was subjected to acidolytic cleavage with TFA/H_2O/TIS (95:2.5:2.5) affording the expected cyclic peptides in HPLC purities >92%. Their identity was confirmed by ESI-MS.

With the cyclic peptidyl resins **1-5** in hand, we set out to examine the synthesis of the corresponding cyclic peptidotriazoles (Table 1). The triazole ring was formed through a cycloaddition reaction under the conditions previously described that involved the treatment of the alkynyl or azido cyclic peptidyl resin with an azide or an alkyne, respectively, in presence of ascorbic acid and CuI in piperidine/DMF for 5 h at room temperature.[35] After acidolytic cleavage, the crude reaction mixtures were analyzed by HPLC and ESI-MS. Results showed that 15 cycloadditions proceeded smoothly and the expected cyclic peptidotriazoles were obtained in HPLC purities ranging from 90 to 99%. Their identity was further confirmed by HRMS.

Antimicrobial Activity

Cyclic peptidotriazoles obtained in good purities were tested for in vitro growth inhibition of the plant pathogenic bacteria *X. axonopodis* pv. *vesicatoria*, *E. amylovora* and *P. syringae* pv. *syringae* (Table 2). For comparison purposes, the parent peptide **BPC194** and the previously reported cyclic peptidotriazoles **BPC458**, **BPC460**, **BPC516**, **BPC548**, **BPC692**, **BPC696** and **BPC700**[35] were also included in this study. Results showed that the 15 new derivatives were considerably active, displaying MIC < 50 μM against at least two pathogens. In agreement with previous reports,[35,41] *E.*

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amylovora was the least sensitive bacteria to these compounds, however five sequences exhibited MIC < 25 μM. Against *P. syringae* pv. *syringae,* six derivatives displayed MIC < 6.2 μM being more active than the parent peptide **BPC194**. Interestingly, two cyclic peptidotriazoles showed higher activity against *X. axonopodis* pv. *vesicatoria* (MIC of 1.6 to 3.1 μM) than **BPC194** and the previously reported sequences **BPC516** and **BPC548**, and nine peptidotriazoles were as active as **BPC194** (MIC of 3.1 to 6.2 μM) against this pathogen.

A different pattern of antibacterial activity was observed for the three sets of cyclic peptidotriazoles resulting from the replacement of Leu^3 with a triazolyl amino acid. The derivatives incorporating a triazolyl alanine synthesized in this study were more active than the previously reported **BPC458** and **BPC460** (MIC of 3.1 to 50 μM vs. 6.2 to >50 μM). The triazolyl norleucine peptidotriazoles displayed a similar activity, being **BPC548** (Nle^3(Tr-Ph-Me)) and **BPC550** (Nle^3(Tr-Ph-OMe)) the most active with MIC values against *P. syringae* pv. *syringae* lower than those of the parent peptide **BPC194** (MIC of 3.1 to 6.2 μM vs. 6.2 to 12.5 μM). No differences were observed among the derivatives incorporating a triazolyl lysine, which were slightly less active than **BPC194** with MIC values ranging from 6.2 to 25 μM.

The replacement of Lys^5 with a triazolyl amino acid rendered cyclic peptidotriazoles as active as those resulting from the substitution of Leu^3. Compared to **BPC194**, the analogues modified at position 5 with a triazolyl alanine **BPC512** (Ala^5(Tr-Nle)) and **BPC514** (Ala^5(Tr-Ph-NH$_2$)) were more active (MIC of 1.6 to 3.1 μM) against *X. axonopodis* pv. *vesicatoria*. The peptidotriazoles containing a triazolyl norleucine at position 5 **BPC562** (Nle^5(Tr-Ph)) and **BPC558** (Nle^5(Tr-Ph-OMe)) exhibited high activity against the three bacteria tested. In particular, **BPC562** (Nle^5(Tr-Ph)) was more active than **BPC194** against *P. syringae* pv. *syringae* (MIC of 3.1 to 6.2 μM). Concerning the peptides incorporating a triazolyl lysine at position 5, **BPC698** (Lys^5(CO-Tr-Nle)) displayed a remarkable activity against *X. axonopodis* pv. *vesicatoria,* being as active as **BPC194** (MIC of 3.1 to 6.2 μM).

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From all these antibacterial activity results highlighted **BPC548** (Nle^3(Tr-Ph-Me)), **BPC550** (Nle^3(Tr-Ph-OMe)) and **BPC562** (Nle^5(Tr-Ph)).

Hemolytic Activity

The hemolytic activity of the above cyclic peptidotriazoles was also tested. The percent hemolysis at 150, 250 and 375 µM is shown in Table 2. The parent peptide **BPC194** and the seven cyclic peptidotriazoles previously described, **BPC458**, **BPC460**, **BPC516**, **BPC548**, **BPC692**, **BPC696** and **BPC700**,[35] were also included in the assays. The analysis of the results showed that seven out of the 15 new compounds were very low hemolytic (≤ 6% at 375 µM) and three sequences displayed a hemolysis between 19 and 25% at this concentration.

Cyclic peptidotriazoles incorporating a triazolyl amino acid at position 3 were low hemolytic, all peptides exhibited a hemolysis ≤ 30% at 375 µM. Derivatives modified at position 5 were more hemolytic. However, four peptidotriazoles showed a hemolytic activity < 30% at 375 µM.

Phytotoxicity

The phytotoxicity of the cyclic peptidotriazoles was assayed by infiltrating 100 µL of a 50, 100, and 200 µM solution of each compound into the mesophyls of the leaves (Figure 2). **BPC194** was also included in these assays for comparison purposes and melittin was used as reference control. After 48 h, the latter led to a necrotic area of around 1.7 cm diameter at 200 µM. Interestingly, only **BPC562**, **BPC694** and **BPC696** caused a necrosis between 0.70-0.86 cm at this concentration, whereas **BPC194** and the rest of peptides were less phytotoxic leading to a size of the lesion between 0-0.57 cm.

DISCUSSION

Nowadays the search for new compounds to control plant diseases is crucial to overcome the problems associated with the traditional pesticides. Due to their biological activity and unique mode of action, synthetic antimicrobial peptides are considered a convenient alternative.[5-12] Along these lines, in this work we designed and synthesized cyclic decapeptides incorporating a triazolyl amino acid at position 3 or 5 of the lead peptide c(Lys-Lys-Leu³-Lys-Lys⁵-Phe-Lys-Lys-Leu-Gln) (**BPC194**), which displays high activity against the phytopathogenic bacteria *P. syringae* pv. *syringae, X. axonopodis* pv. *vesicatoria* and *E. amylovora*, and is low hemolytic.[34]

All the 15 cyclic peptidotriazoles of this study showed potent activity against at least two of the above bacteria tested. MIC values below 6.2 μM were obtained for 11 and six sequences against *X. axonopodis* pv. *vesicatoria* and *P. syringae* pv. *syringae*, respectively, and five peptides showed MIC values below 25 μM against *E. amylovora*. This differential susceptibility of bacteria to a given peptide has been previously observed for other families of linear and cyclic antimicrobial peptides[14,41-43] and of linear peptidotriazoles.[33] This result was ascribed to the different charge and lipid composition of the target microorganism membrane that would lead to different binding rates of peptides.

The position of the triazolyl amino acid did not influence the antibacterial activity. Accordingly, cyclic peptidotriazoles with a triazolyl amino acid at position 3 were similarly active than those bearing this residue at position 5. Regarding the type of triazolyl amino acid, in general, the derivatives containing a triazolyl alanine or a triazolyl norleucine were more active than those incorporating a triazolyl lysine.

The substituent at the triazole ring also influenced the antibacterial activity. The triazolyl alanine-containing peptides bearing an aromatic group at the triazole were active against the three pathogens. In contrast, those incorporating a 2-aminohexanoic group (**BPC460** and **BPC512**) were poorly active. The analogues containing a triazolyl norleucine displayed significant antibacterial

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activity. For the triazolyl lysine derivatives, the substituent on the triazole ring had a different effect on the antibacterial activity depending on whether this lysine residue was at position 3 or at position 5. When this residue was at position 3, the substituent did not exert any influence on the activity. In the case of the sequences modified at position 5, the presence of a 2-aminohexanoic acid (**BPC698**) increased the activity against *X. axonopodis* pv. *vesicatoria*, but decreased significantly the activity against *E. amylovora*. Taken all together, these results pointed out that the presence of a benzene ring favored the antibacterial activity. Moreover, there was no correlation between the activity and the electronic demand of this aromatic group. Accordingly, the peptidotriazoles with the best antibacterial activity incorporated a phenyl triazolyl moiety (**BPC548**, **BPC550**, and **BPC562**).

The position of the triazolyl amino acid as well as the substituent did influence the hemolytic activity. In particular, cyclic peptidotriazoles incorporating a triazolyl amino acid at position 3 were, in general, less hemolytic than those bearing this residue at position 5. Whereas for the former group all peptides exhibited a hemolysis $\leq 30\%$ at 375 μM, the hemolytic activity of the derivatives modified at position 5 ranged from 3-93% at this concentration. Among this last set, peptides **BPC512** and **BPC698** bearing a hydrophilic 2-aminohexanoic group at the triazole ring were the least hemolytic (3 and 5% at 375 μM, respectively). The analogues incorporating an aniline (**BPC514** and **BPC572**) also displayed low hemolysis (23 and 25% at 375 μM, respectively). In contrast, peptides that contain a more hydrophobic substituent at the triazole ring were significantly hemolytic (40-93% at 375 μM).

The above hemolysis data revealed that, as expected, there is a good correlation between hydrophilicity and low hemolysis.[15,33,40,42] Thus, the replacement of Leu[3] by a triazolyl amino acid led to low hemolytic cyclic peptidotriazoles due to the hydrophilic character of the triazole. In contrast, when the triazolyl amino acid was incorporated at position 5 the resulting compounds were, in general, highly hemolytic pointing out that the triazole is more hydrophobic than the free ε-amino group of Lys[5]. Accordingly, the cyclic peptidotriazoles bearing a triazolyl norleucine were

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more hemolytic than those containing a triazolyl alanine, which could be attributed to the longer aliphatic chain of the former. Moreover, the presence of polar groups at the triazole ring, such as a 2-aminohexanoic acid or an aniline moiety, rendered sequences displaying a low hemolysis.

Cyclic peptidotriazoles were considerably less phytotoxic than melittin in tobacco leaves at concentrations up to 20 to 60 fold higher than the MIC. No correlation can be excerpted between the structure of these peptides and their phytotoxicity. Moreover, in agreement with previous studies,[40] a high hemolysis cannot be related to a high phytotoxicity. Thus, we identified high hemolytic peptides causing low necrosis (i.e. **BPC558** and **BPC690**).

These biological activity results reinforce our previous observations on how subtle changes influence the antibacterial and hemolytic activity of this type of antimicrobial peptides.[34,40] Notably, the cyclic peptidotriazoles **BPC548** (Nle[3]-(Tr-Ph-Me)) and **BPC550** (Nle[3]-(Tr-Ph-OMe)), that showed high antibacterial activity against the three pathogens, were also low hemolytic and low phytotoxic.

CONCLUSION

This study provides important hints for the design and synthesis of biologically active cyclic peptidotriazoles and it demonstrates that the incorporation of a triazolyl amino acid into a cyclic antimicrobial peptide constitutes a good strategy to develop compounds active against plant pathogenic bacteria. In particular, the introduction of a triazolyl alanine, a triazolyl norleucine or a triazolyl lysine at position 3 or 5 of **BPC194** led to the identification of lead cyclic peptidotriazoles active against *X. axonopodis* pv. *vesicatoria*, *E. amylovora* and *P. syringae* pv. *syringae*, with low hemolysis and low phytotoxicity. The best derivatives could be used as new leads for further chemical modifications to discover new antimicrobial agents to be used in plant protection. Although the biological profile of **BPC194** was not improved, the identification of these lead

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peptidotriazoles could be useful for further optimization and development in order to ensure the success of future screening assays.

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SUPPORTING INFORMATION

Synthetic procedures and analytical data of linear peptides, of alkynyl and azido cyclic peptides, and of cyclic peptidotriazoles.

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FIGURE AND SCHEME LEGENDS

FIGURE 1 General structure of cyclic peptidotriazoles **I-IV**

FIGURE 2 Phytotoxicity of **BPC194** and the cyclic peptidotriazoles. Phytotoxicity was determined at 50, 100 and 200 μM, as the size of the lesions in infiltrated tobacco leaves. Phytotoxicity was compared to melittin (MEL). Vertical bars within each column indicate confidence interval at the mean.

SCHEME 1 Synthesis of cyclic peptidyl resins **1-5**

FIGURE 1

FIGURE 2

SCHEME 1

1, 4, 6, 8   (Aa)$_n$ = Lys(Boc)-Lys(Boc)
(Aa)$_m$ = Lys(Boc)-Lys(Boc)-Phe-Lys(Boc)-Lys(Boc)-Leu

2, 3, 5, 7, 9, 10   (Aa)$_n$ = Lys(Boc)-Lys(Boc)-Leu-Lys(Boc)
(Aa)$_m$ = Phe-Lys(Boc)-Lys(Boc)-Leu
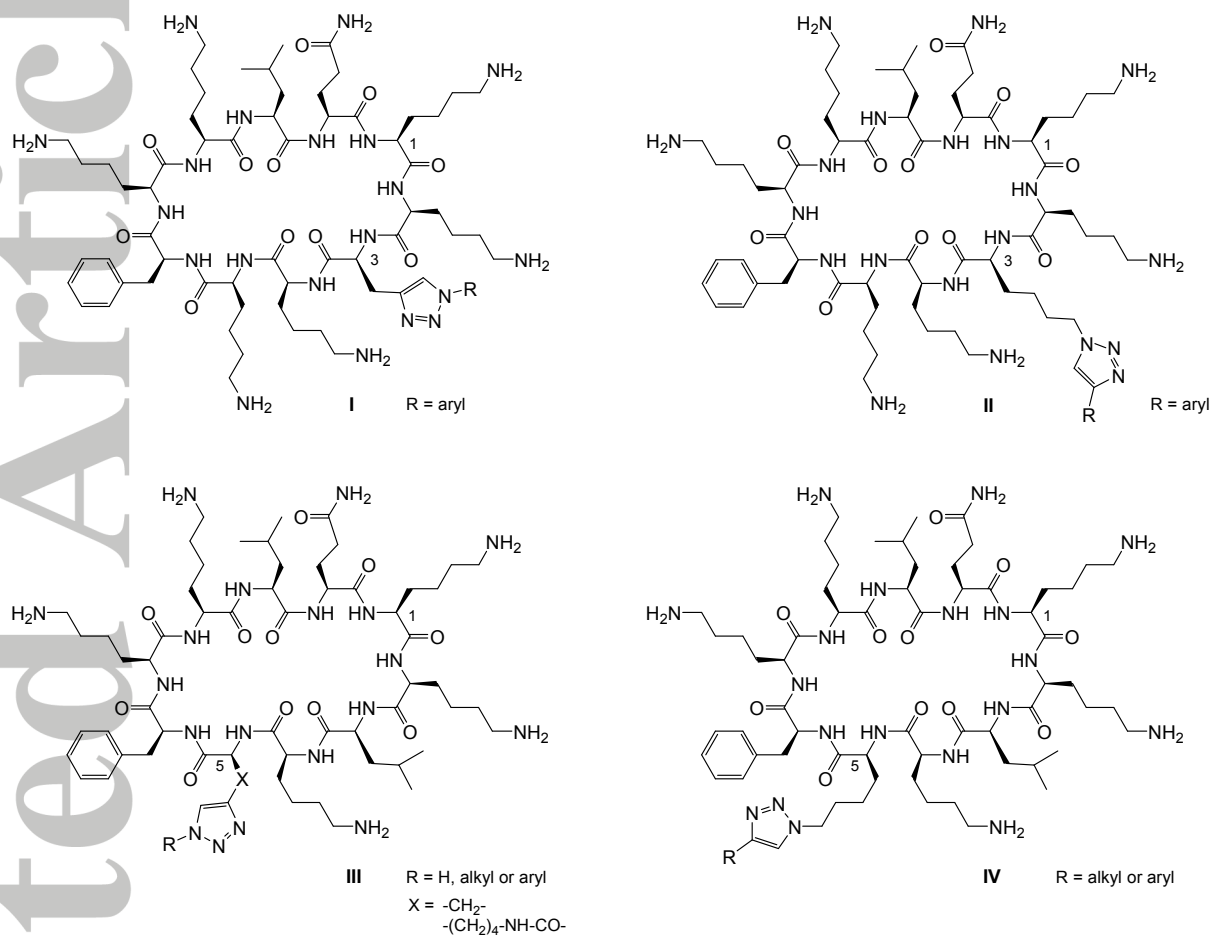
i) SPPS: a) Fmoc removal: piperidine/DMF (3:7), b) Amino acid coupling: Fmoc-amino acid, DIPCDI, Oxyma; ii) Pd(PPh$_3$)$_4$, CHCl$_3$/AcOH/NMM;
iii) Piperidine/DMF; iv) PyOxim, Oxyma, DIPEA; v) 1% TFA/CH$_2$Cl$_2$; vi) Propiolic acid, DIPCDI, Oxyma

TABLE 1 Synthesis of cyclic peptidotriazoles. Retention times and purities on HPLC, and HRMS data
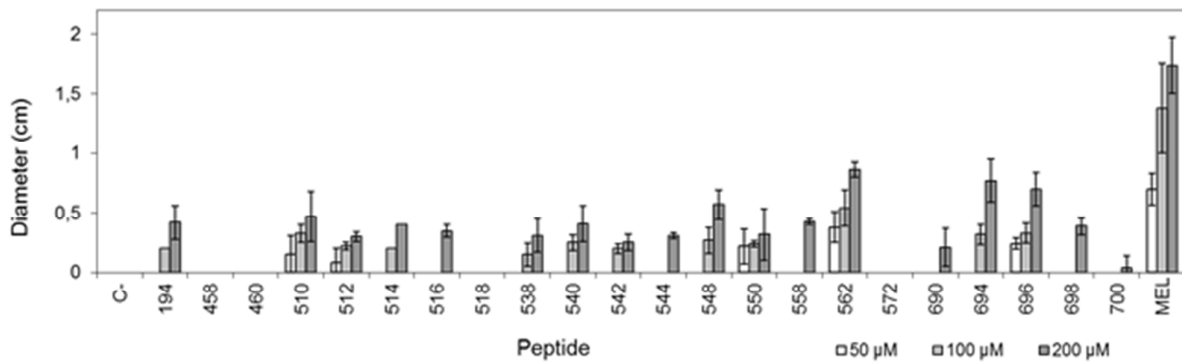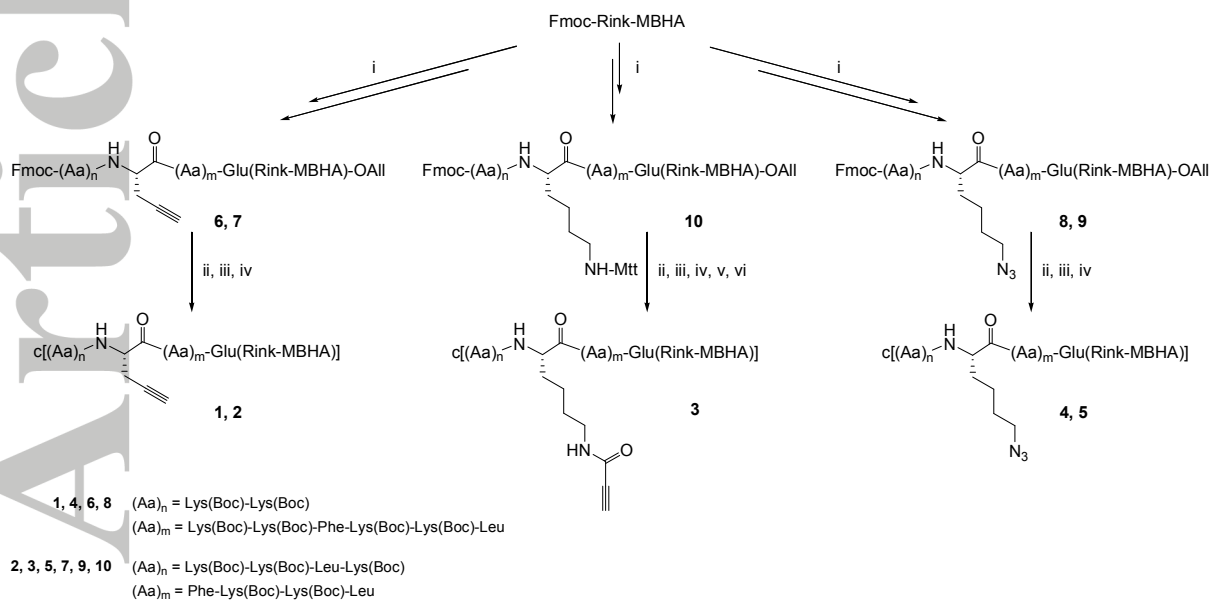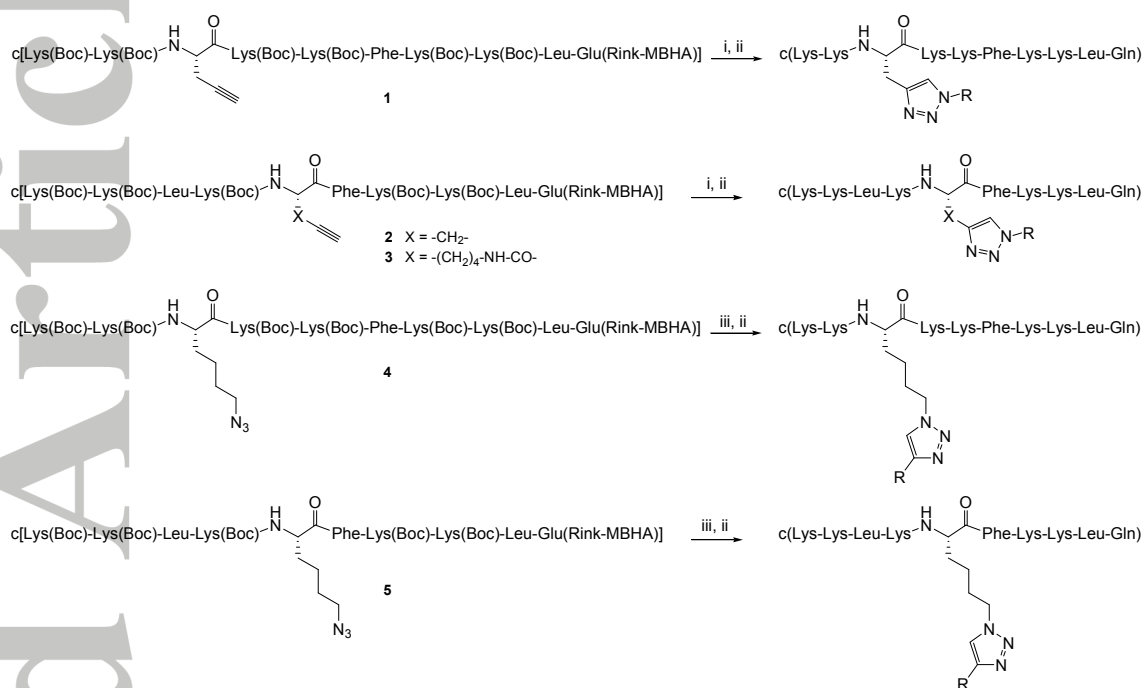

i) Azide, CuI, ascorbic acid, piperidine/DMF, 5 h, r.t.; ii) TFA/H$_2$O/TIS, 2 h; iii) alkyne, CuI, ascorbic acid, piperidine/DMF, 5 h, r.t.

Resin	Azide or alkyne	Compound	X	R	t_R (min)[a]	Purity (%)[b]	HRMS
Position 3							
1	p-NH$_2$-C$_6$H$_4$-N$_3$	**BPC518**	-	p-NH$_2$-C$_6$H$_4$-	5.90	98	693.9462 [M+2H]$^{2+}$
1	p-Me-C$_6$H$_4$-N$_3$	**BPC540**	-	p-Me-C$_6$H$_4$-	6.20	99	693.4490 [M+2H]$^{2+}$
1	p-MeO-C$_6$H$_4$-N$_3$	**BPC542**	-	p-MeO-C$_6$H$_4$-	6.09	96	701.4479 [M+2H]$^{2+}$
1	C$_6$H$_5$-N$_3$	**BPC544**	-	C$_6$H$_5$-	6.07	92	686.4421 [M+2H]$^{2+}$
4	p-NH$_2$-C$_6$H$_4$-C≡CH	**BPC538**	-	p-NH$_2$-C$_6$H$_4$-	5.83	95	714.9706 [M+2H]$^{2+}$
4	p-C$_5$H$_{11}$-C$_6$H$_4$-C≡CH	**BPC546**	-	p-C$_5$H$_{11}$-C$_6$H$_4$-	6.61	61	743.0065 [M+2H]$^{2+}$
4	p-MeO-C$_6$H$_4$-C≡CH	**BPC550**	-	p-MeO-C$_6$H$_4$-	6.23	99	722.4717 [M+2H]$^{2+}$
Position 5							
2	Bn-N$_3$	**BPC510**	-CH$_2$-	Bn-	6.53	98	685.9444 [M+2H]$^{2+}$
2	Boc-Nle(ε-N$_3$)-OH	**BPC512**	-CH$_2$-	Nle-	6.14	95	705.4623 [M+2H]$^{2+}$
2	p-NH$_2$-C$_6$H$_4$-N$_3$	**BPC514**	-CH$_2$-	p-NH$_2$-C$_6$H$_4$-	6.29	90	686.4411 [M+2H]$^{2+}$
2	p-Me-C$_6$H$_4$-N$_3$	**BPC564**	-CH$_2$-	p-Me-C$_6$H$_4$-	6.59	48	685.9422 [M+2H]$^{2+}$
2	p-MeO-C$_6$H$_4$-N$_3$	**BPC566**	-CH$_2$-	p-MeO-C$_6$H$_4$-	6.49	50	693.9410 [M+2H]$^{2+}$
2	C$_6$H$_5$-N$_3$	**BPC568**	-CH$_2$-	C$_6$H$_5$-	6.48	58	678.9377 [M+2H]$^{2+}$
5	p-C$_5$H$_{11}$-C$_6$H$_4$-C≡CH	**BPC554**	-	p-C$_5$H$_{11}$-C$_6$H$_4$-	7.46	54	734.9988 [M+2H]$^{2+}$
5	p-Me-C$_6$H$_4$-C≡CH	**BPC556**	-	p-Me-C$_6$H$_4$-	6.71	65	706.9708 [M+2H]$^{2+}$
5	p-MeO-C$_6$H$_4$-C≡CH	**BPC558**	-	p-MeO-C$_6$H$_4$-	6.72	93	714.9675 [M+2H]$^{2+}$
5	THPO-CH$_2$-C≡CH	**BPC560**	-	HO-CH$_2$-	6.68	52	676.9513 [M+2H]$^{2+}$
5	C$_6$H$_5$-C≡CH	**BPC562**	-	C$_6$H$_5$-	6.65	90	699.9620 [M+2H]$^{2+}$
5	p-NH$_2$-C$_6$H$_4$-C≡CH	**BPC572**	-	p-NH$_2$-C$_6$H$_4$-	6.34	99	707.4663 [M+2H]$^{2+}$
3	Na-N$_3$	**BPC690**	-(CH$_2$)$_4$-NH-CO-	H-	6.66	99	683.4390 [M+2H]$^{2+}$
3	Bn-N$_3$	**BPC694**	-(CH$_2$)$_4$-NH-CO-	Bn-	6.94	99	728.4670 [M+2H]$^{2+}$
3	Boc-Nle(ε-N$_3$)-OH	**BPC698**	-(CH$_2$)$_4$-NH-CO-	Nle-	6.20	99	498.9933 [M+3H]$^{3+}$

[a]HPLC retention time. [b]Percentage determined by HPLC at 220 nm from the crude reaction mixture.

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TABLE 2 Antimicrobial activity (MIC) against plant pathogenic bacteria and cytotoxicity

Peptide	Notation[a]	MIC (µM)			Hemolysis[c] (%)		
		Xav[b]	*Ea*[b]	*Pss*[b]	150 µM	250 µM	375 µM
BPC194		3.1-6.2	3.1-6.2	6.2-12.5	3 ± 0.6	3 ± 0.6	4 ± 0.7
BPC458	Ala³(Tr-Bn)	6.2-12.5	>50	6.2-12.5	2 ± 0.3	3 ± 0.5	5 ± 0.2
BPC460	Ala³(Tr-Nle)	25-50	>50	>50	0 ± 0.3	0 ± 0.3	0 ± 0.1
BPC518	Ala³(Tr-Ph-NH₂)	3.1-6.2	25-50	12.5-25	1 ± 0.1	1 ± 0.1	1 ± 0.1
BPC540	Ala³(Tr-Ph-Me)	6.2-12.5	25-50	3.1-6.2	2 ± 0.5	4 ± 0.4	6 ± 1
BPC542	Ala³(Tr-Ph-OMe)	3.1-6.2	25-50	3.1-6.2	1 ± 0.6	2 ± 0.2	4 ± 0.4
BPC544	Ala³(Tr-Ph)	3.1-6.2	25-50	3.1-6.2	1 ± 0.4	1 ± 0.3	2 ± 0.4
BPC516	Nle³(Tr-Ph)	3.1-6.2	25-50	6.2-12.5	5 ± 0.2	6 ± 0.4	8 ± 0.1
BPC538	Nle³(Tr-Ph-NH₂)	6.2-12.5	25-50	3.1-6.2	1 ± 0.8	1 ± 0.4	2 ± 0.4
BPC548	Nle³(Tr-Ph-Me)	3.1-6.2	12.5-25	3.1-6.2	12 ± 0.3	24 ± 1	26 ± 4
BPC550	Nle³(Tr-Ph-OMe)	3.1-6.2	12.5-25	3.1-6.2	7 ± 0.8	10 ± 0.8	19 ± 5
BPC692	Lys³(CO-Tr)	6.2-12.5	12.5-25	12.5-25	5 ± 0.7	5 ± 0.8	7 ± 0.2
BPC696	Lys³(CO-Tr-Bn)	6.2-12.5	12.5-25	12.5-25	19 ± 4	20 ± 5	30 ± 4
BPC700	Lys³(CO-Tr-Nle)	6.2-12.5	12.5-25	12.5-25	0 ± 2	0 ± 0.4	0 ± 0.1
BPC510	Ala⁵(Tr-Bn)	3.1-6.2	12.5-25	12.5-25	32 ± 2	35 ± 3	40 ± 4
BPC512	Ala⁵(Tr-Nle)	1.6-3.1	>50	25-50	2 ± 0.1	2 ± 0.2	3 ± 0.4
BPC514	Ala⁵(Tr-Ph-NH₂)	1.6-3.1	25-50	12.5-25	16 ± 0.9	19 ± 0.8	23 ± 2
BPC562	Nle⁵(Tr-Ph)	3.1-6.2	12.5-25	3.1-6.2	42 ± 5	51 ± 3	58 ± 1
BPC572	Nle⁵(Tr-Ph-NH₂)	3.1-6.2	25-50	6.2-12.5	8 ± 0.2	13 ± 0.2	25 ± 5
BPC558	Nle⁵(Tr-Ph-OMe)	3.1-6.2	12.5-25	6.2-12.5	54 ± 4	76 ± 8	90 ± 8
BPC690	Lys⁵(CO-Tr)	6.2-12.5	25-50	12.5-25	47 ± 7	67 ± 8	75 ± 7
BPC694	Lys⁵(CO-Tr-Bn)	6.2-12.5	12.5-25	12.5-25	57 ± 5	74 ± 15	93 ± 15
BPC698	Lys⁵(CO-Tr-Nle)	3.1-6.2	>50	25-50	3 ± 0.5	4 ± 1	5 ± 0.7

[a]The notation defines the triazolyl amino acid at position 3 or 5. The side chain substituent of the Ala, the Nle or the Lys residue is in parenthesis being: Tr, unsubsituted triazole; Tr-Bn, triazole bearing a benzyl group; Tr-Nle, triazole bearing a 2-aminohexanoic acid; Tr-Ph-NH₂, triazole bearing an aniline moiety; Tr-Ph-Me, triazole bearing a tolyl group; Tr-Ph-OMe, triazole bearing an anisole moiety; Tr-Ph, triazole bearing a phenyl group. [b]*Xav*, *Xanthomonas axonopodis* pv. *vesicatoria*; *Pss*, *Pseudomonas syringae* pv. *syringae*; *Ea*, *Erwinia amylovora*. [c]Percent hemolysis at 150, 250 and 375 µM plus confidence interval ($\alpha = 0.05$).

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