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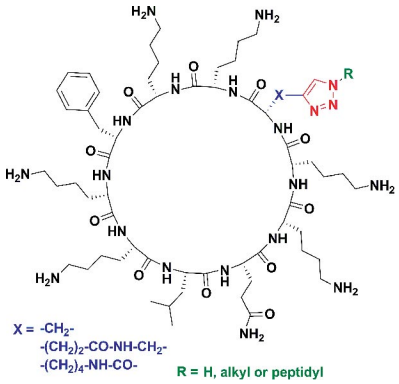
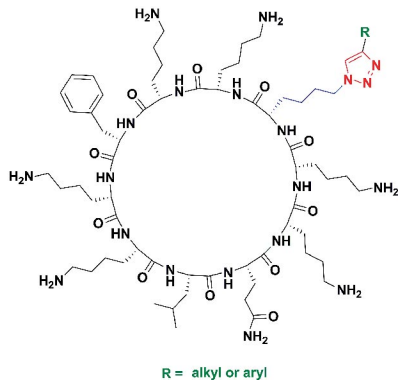
Antibiotics





Cyclic peptidotriazoles were synthesized on solid-phase from an alkyl or an azido peptidyl resin. Sequences active against phytopathogenic bacteria and with low hemolysis were identified.

I. Güell, S. Vilà, L. Micaló, E. Badosa, E. Montesinos, M. Planas,* L. Feliu* 1–12

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Synthesis of Cyclic Peptidotriazoles with Activity Against Phytopathogenic Bacteria

Imma Güell,[a] Sílvia Vilà,[a] Lluís Micaló,[a] Esther Badosa,[b] Emilio Montesinos,[b] Marta Planas,*[a] and Lidia Feliu*[a]

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Cyclic peptidotriazoles derived from the antimicrobial cyclic peptide c(Lys-Lys-Leu-Lys-Lys-Phe-Lys-Lys-Leu-Gln) (BPC194) were prepared by incorporating a triazolyl amino acid at the 3-position. The synthesis was accomplished on solid-phase and involved as the key step a copper-catalyzed cycloaddition reaction between a resin-bound alkyne or a resin-bound azide and a range of azides or alkynes in solution, respectively. This methodology was also applied to the synthesis of a conjugated peptide containing a cyclic and a linear peptidyl sequence linked through a triazolyl ring. Cyclic peptidotriazoles were obtained in excellent purities starting either from an alkynyl or an azido peptidyl resin. These compounds were screened in vitro for their growth inhibition of bacterial phytopathogens and for their cytotoxic effects on eukaryotic cells. Peptide sequences with high antibacterial activity and low hemolysis were identified, constituting good candidates for the design of new antimicrobial agents.

Introduction

The vulnerability of modern agriculture to diseases and pests has resulted in an intense research effort to develop new antimicrobial agents that are safe for the host organism and the environment, and that are unlikely to cause the emergence of resistant strains. Due to their broad spectrum of activity, low intrinsic cytotoxicity, and unique mechanism of action, antimicrobial peptides are considered to be suitable candidates for use in plant protection.[1]

Many synthetic antimicrobial peptides incorporating unnatural amino acids and maintaining the structural features of the natural sequences have been designed and have been reported to exhibit comparable or improved biological profiles.[2] In particular, our research group has reported linear undecapeptides incorporating a triazolyl amino acid derived from the antimicrobial peptide BP100. These peptidotriazoles displayed potent activity against plant pathogenic bacteria and fungi, low hemolysis, and high stability towards protease degradation.[3]

The 1,2,3-triazole ring has been increasingly used in drug discovery because of its favorable physicochemical properties and because of the general synthetic method that is now available.[4] In fact, there are a large number of compounds that contain a 1,2,3-triazole ring with interesting biological activities, such as anticancer, antimicrobial and antivirus agents. Concerning the formation of this ring, copper-catalyzed azide alkyne cycloaddition (CuAAC), first reported by Meldal and co-workers, regioselectively provides 1,4-disubstituted 1,2,3-triazoles.[4a,4c,4d,5] This reaction has been used for the macrocyclization or derivatization of peptide sequences, and as a method for linking peptide fragments, allowing the synthesis of biologically active peptidotriazoles.[4a,4b,5e]

The CuAAC has been successfully applied to the preparation of cyclic peptidotriazoles bearing the triazole ring in the peptide backbone.[6] Although very promising, modification of cyclic peptides by incorporating a triazole onto the side-chain of a selected residue has rarely been reported and has mainly been applied to obtain peptide conjugates.[7] Moreover, despite the potential interest of this type of cyclic peptidotriazoles, to the best of our knowledge their evaluation as antimicrobial agents has not been described.

During our current research into the development of new antimicrobial agents, we have identified cyclic decapeptides with high activity against the gram-negative plant pathogenic bacteria *Xanthomonas axonopodis* pv. *vesicatoria*, *Erwinia amylovora*, and *Pseudomonas syringae* pv. *syringae*.[8] The most active peptide, c(Lys-Lys-Leu-Lys-Lys-Phe-Lys-Lys-Leu-Gln) (BPC194), also showed minimal cytotoxicity and high stability to protease degradation. In this work, we decided to study the solid-phase synthesis of BPC194 analogues incorporating a 1,2,3-triazole ring at the side-chain of a selected residue and evaluate its influence on the

[a] Laboratori d'Innovació en Processos i Productes de Síntesi Orgànica (LIPPSO), Departament de Química, Universitat de Girona,
Campus Montilivi, 17071 Girona, Spain
Fax: +34-972-418150
E-mail: marta.planas@udg.edu, lidia.feliu@udg.edu
Homepage: http://www.lippso.com
[b] Laboratory of Plant Pathology, Institute of Food and Agricultural Technology-CIDSAV-Cer.t.A, Universitat de Girona,
Campus Montilivi, 17071 Girona, Spain

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biological activity. For this purpose, cyclic peptidotriazoles **I** and **II**, bearing a triazolyl amino acid at the 3-position, were designed (Figure 1). Their synthesis was envisaged from a resin-bound alkyne or a resin-bound azide, respectively. Subsequently, we studied the antibacterial activity of the synthesized cyclic peptidotriazoles against phytopathogenic bacteria and also their hemolytic activity.

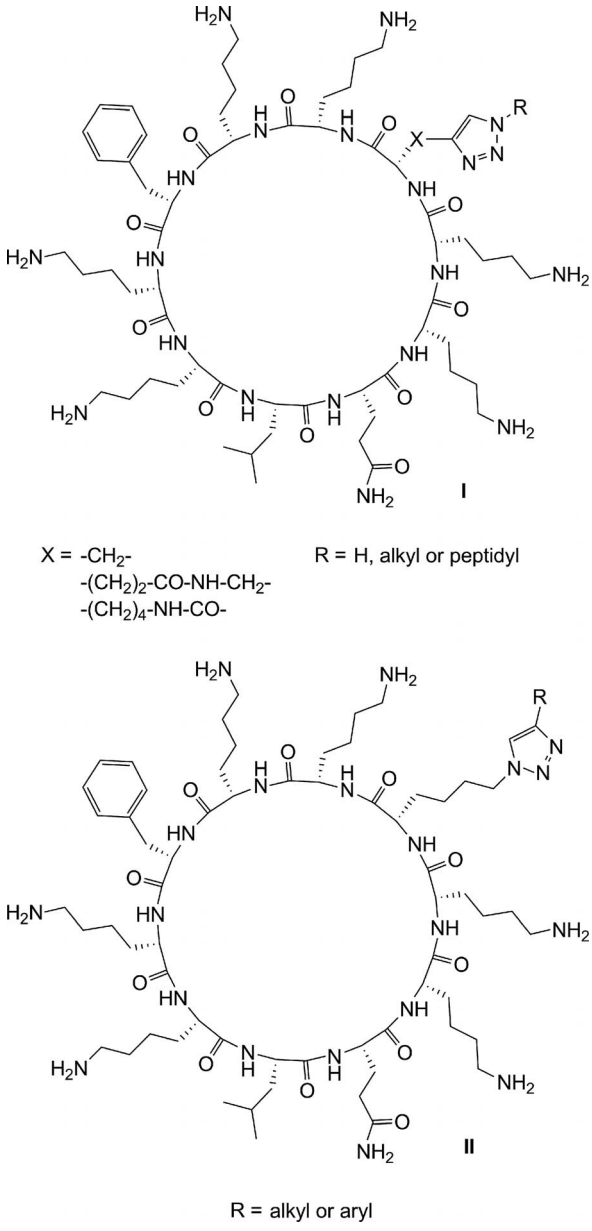


Figure 1. General structure of cyclic peptidotriazoles **I** and **II**.

Results and Discussion

Design of the Cyclic Peptidotriazoles

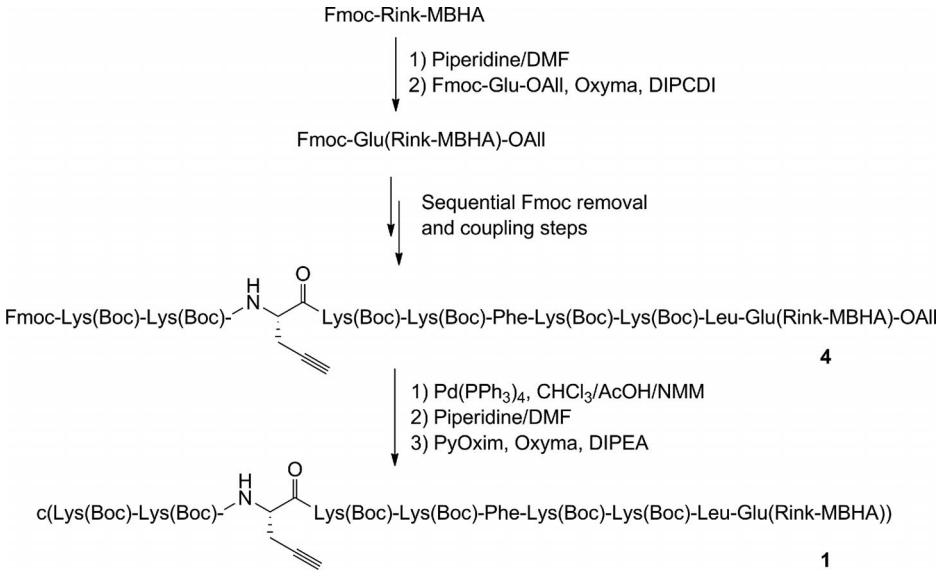
Cyclic peptidotriazoles with general structure **I** and **II** were designed based on the sequence of BPC194 c(Lys[1]-Lys-Leu[3]-Lys-Lys-Phe-Lys-Lys-Leu-Gln[10]) by replacing Leu[3] for residues incorporating a triazole at the side-chain (Figure 1). A Leu residue was selected because the cationic Lys residues are essential for the activity of these peptides. Moreover, among the two Leu residues present in the sequence, Leu[3] was chosen to be replaced because the fragment Lys[5]-Phe-Lys-Lys-Leu-Gln[10] was proven to be a structural requirement for high antibacterial activity.[8] In particular, for cyclic peptidotriazoles **I**, Leu[3] was substituted by an alanine, a glutamic acid or a lysine residue incorporating a 1,2,3-triazol-4-yl substituent at the side-chain. For cyclic peptidotriazoles **II**, Leu[3] was replaced with a norleucine residue bearing a 1,2,3-triazol-1-yl substituent at the side-chain. The triazole moiety was either unsubstituted or substituted with an alkyl, an aryl, or a peptidyl group.

Synthesis of Cyclic Peptidotriazoles I

The synthesis of cyclic peptidotriazoles **I** was based on a cycloaddition reaction between a resin-bound alkyne and an azide in solution. According to this strategy, the alkynyl cyclic peptidyl resins **1**–**3** were prepared (Scheme 1, Scheme 2, and Scheme 3). Resin **1** incorporated a propargylglycine at the 3-position whereas resin **2** contained a glutamic acid substituted with a propargylamine, and resin **3** incorporated a lysine bearing a propioloyl group.

The general strategy for the solid-phase synthesis of the alkynyl cyclic peptidyl resins **1**–**3** involved the preparation of a linear peptidyl sequence followed by head-to-tail cyclization. A three dimensional orthogonal 9-fluorenylmethoxycarbonyl (Fmoc)/*tert*-butyl (*t*Bu)/allyl (All) strategy was used.[9] Thus, resin **1** was prepared starting from Fmoc-Rink-MBHA (0.3 mmol/g) (Scheme 1). After Fmoc removal by treatment with piperidine/*N*,*N*-dimethylformamide (DMF) (3:7), Fmoc-Glu-OAll was coupled to the support by using ethyl 2-cyano-2-(hydroxyimino)acetate (Oxyma) (4 equiv.) and *N*,*N*-diisopropylcarbodiimide (DIPCDI) (4 equiv.) in DMF for 1 h. The linear sequence was then elongated by sequential Fmoc removal and coupling steps. Fmoc-Prg-OH was incorporated at the 3-position. Once the protected linear sequence **4** was completed, the allyl group was removed by treatment with [Pd(PPh_3)$_4$] (3 equiv.) in $CHCl_3$/AcOH/*N*-methylmorpholine (NMM) (3:2:1) for 3 h. Following Fmoc removal, cyclization was performed by using [ethyl cyano(hydroxyimino)-acetato-*O*[2]]tri-1-pyrrolidinylphosphonium hexafluorophosphate (PyOxim) (5 equiv.), Oxyma (5 equiv.), and diisopropylethylamine (DIPEA) (10 equiv.) in *N*-methyl-2-pyrrolidinone (NMP) for 24 h. An aliquot of resin **1** was subjected to acidolytic cleavage with trifluoroacetic acid (TFA)/H_2O/triisopropylsilane (TIS) (95:2.5:2.5) for 2 h, affording peptide c(Lys-Lys-Prg-Lys-Lys-Phe-Lys-Lys-Leu-Gln) (BPC466), which was analyzed by HPLC (96% purity) and characterized by mass spectrometry.

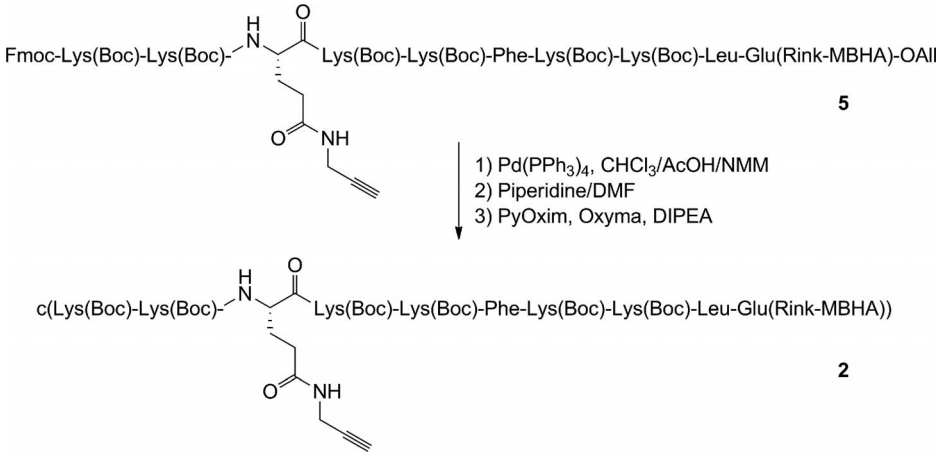
The synthesis of the alkynyl cyclic peptidyl resin **2** (Scheme 2) followed the same strategy described above for resin **1**. In this case, the preparation of the linear peptidyl



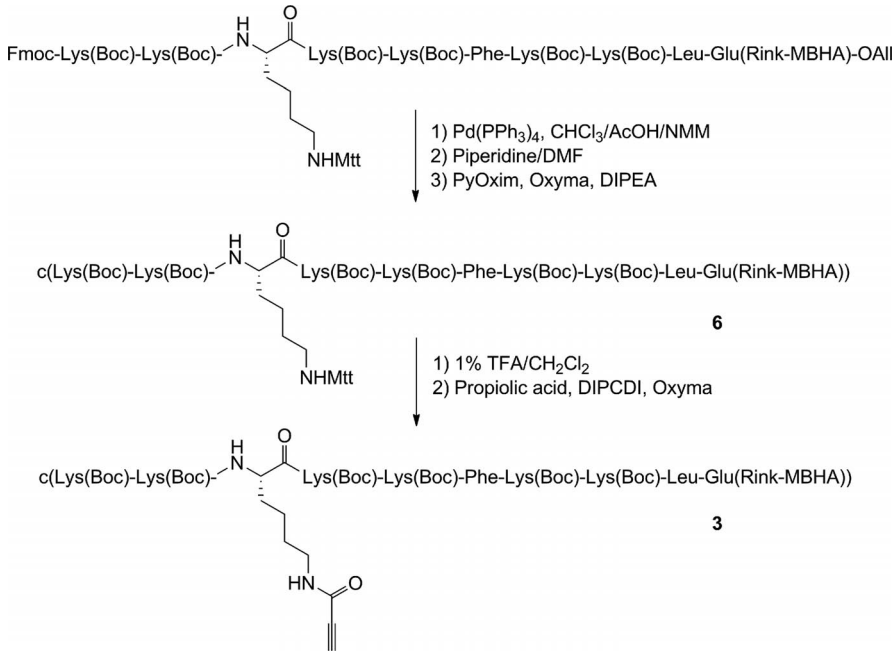
Scheme 1. Synthesis of the alkynyl cyclic peptidyl resin **1**.

resin **5** required the use of Fmoc-Glu(NH-CH_2-C≡CH)-OH as alkynyl-functionalized residue. This amino acid is not commercially available but was easily prepared from Fmoc-Glu-O*t*Bu by amidation of the side-chain carboxylic acid group with propargylamine followed by hydrolysis of the *t*Bu ester. The amidation was achieved by treatment of Fmoc-Glu-O*t*Bu with propargylamine, *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC), Oxyma, and DIPEA in anhydrous tetrahydrofuran (THF). The *t*Bu group was removed with TFA/CH_2Cl_2 (1:1). Fmoc-Glu(NH-CH_2-C≡CH)-OH was obtained as a white powder in 57% overall yield and was characterized by NMR spectroscopy and mass spectrometry. After assembly of the linear sequence **5**, allyl and Fmoc group removal followed by cyclization afforded the alkynyl cyclic peptidyl resin **2**. Acidolytic cleavage of an aliquot of this resin yielded c(Lys-Lys-Glu(NH-CH_2-C≡CH)-Lys-Lys-Phe-Lys-Lys-Leu-Gln) (BPC580) in 99% HPLC purity and was characterized by mass spectrometry.

Next, the alkynyl cyclic peptidyl resin **3**, with Lys^3 bearing a propioloyl group at the side-chain, was prepared by following the above procedure (Scheme 3). To achieve the selective acylation of Lys^3, this residue was incorporated with the N^{ε}-amino group protected with a 4-methyltrityl (Mtt) group. This group is highly acid labile and can be selectively removed without compromising the resin anchorage and the other side-chain protecting groups. Thus, we prepared the cyclic peptidyl resin **6**, which was then treated with 1% TFA in CH_2Cl_2 while stirring for 5 min at room temperature. This treatment was repeated until the yellow color produced by release of the Mtt cation disappeared. Following Mtt removal, the N^{ε}-amino group was acylated with propiolic acid under standard coupling conditions. An aliquot of the resulting resin was cleaved and the crude mixture was analyzed by HPLC and characterized by ESI-MS. Thus, c[Lys-Lys-Lys(CO-C≡CH)-Lys-Lys-Phe-Lys-Lys-Leu-Gln] (BPC468) was obtained in 99% purity.



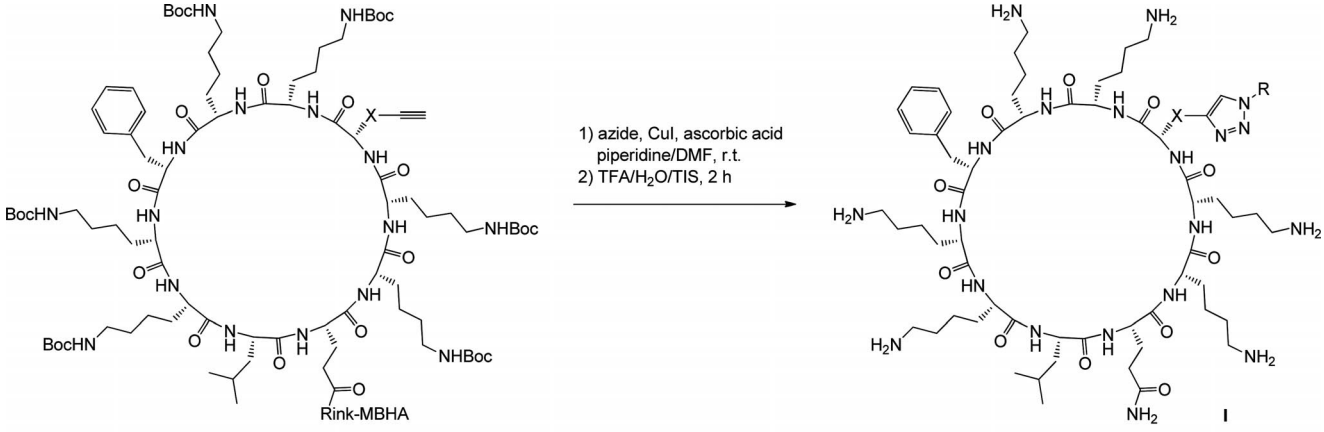
Scheme 2. Synthesis of the alkynyl cyclic peptidyl resin **2**.

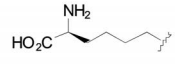
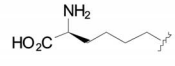
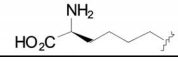


Scheme 3. Synthesis of the alkynyl cyclic peptidyl resin **3**.

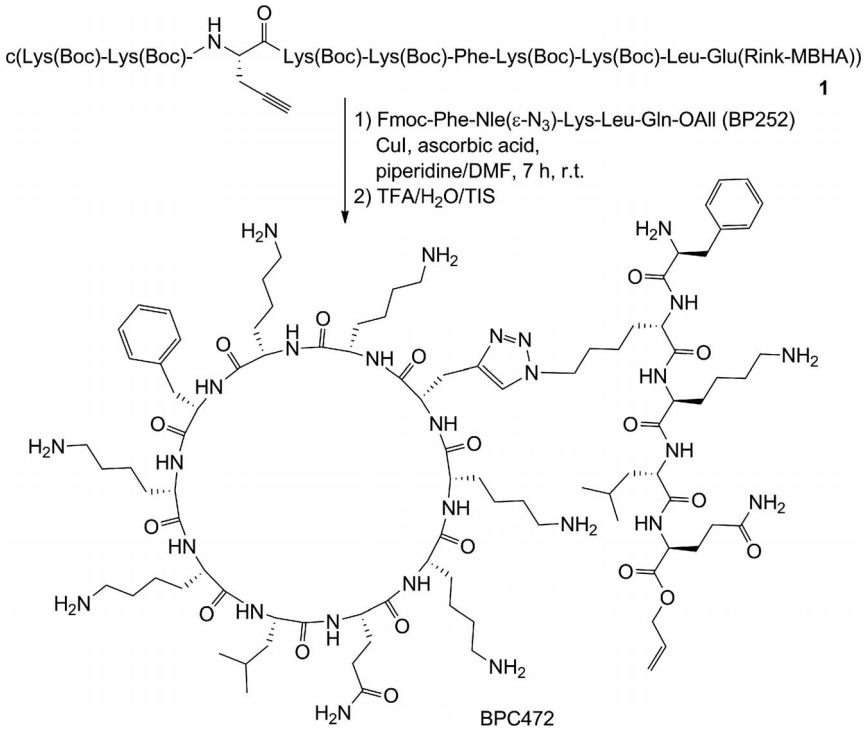
With the alkynyl cyclic peptidyl resins **1**–**3** in hand, we proceeded to subject them to a cycloaddition reaction with an azide to obtain the corresponding cyclic peptidotriazoles **I** (Table 1). Completion of the reactions was evaluated by ESI/MS analysis of the crude reaction mixtures after acidolytic cleavage of an aliquot of the resin. Resins **1**–**3** were

Table 1. Synthesis of cyclic peptidotriazoles **I**.



Resin	Azide[a]	Compound	X	R	Purity (%)[b]
1	NaN_3	BPC456	$-CH_2-$	H	97
1	BnN_3	BPC458	$-CH_2-$	Bn	94
1	Boc-Nle(ε-N_3)-OH	BPC460	$-CH_2-$		90
2	NaN_3	BPC532	$-(CH_2)_2-CO-NH-CH_2-$	H	98
2	BnN_3	BPC520	$-(CH_2)_2-CO-NH-CH_2-$	Bn	98
2	Boc-Nle(ε-N_3)-OH	BPC522	$-(CH_2)_2-CO-NH-CH_2-$		96
3	NaN_3	BPC692	$-(CH_2)_4-NH-CO-$	H	92
3	BnN_3	BPC696	$-(CH_2)_4-NH-CO-$	Bn	88
3	Boc-Nle(ε-N_3)-OH	BPC700	$-(CH_2)_4-NH-CO-$		-[c]

[a] Reaction time: For NaN_3: 2× 10 h; for BnN_3 and Boc-Nle(ε-N_3)-OH: 1× 5 h. [b] Percentage determined by HPLC analysis with detection at 220 nm of the crude reaction mixture. [c] The crude reaction contained a mixture of BPC700 and BPC468.



Scheme 4. Synthesis of the conjugated peptide BPC472.

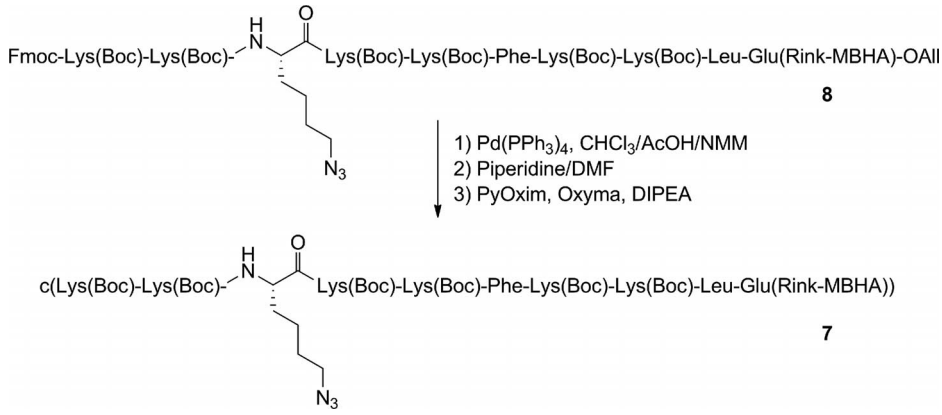
treated with NaN_3, BnN_3, and Boc-Nleu(ε-N_3)-OH (5 equiv.) in the presence of ascorbic acid (5 equiv.) and CuI (5 equiv.) in piperidine/DMF (2:8) while stirring at room temperature. Formation of the triazole moiety using BnN_3 was accomplished in 5 h, whereas cycloadditions with NaN_3 required two treatments of 10 h each to enable the reaction to reach completion. When using Boc-Nleu(ε-N_3)-OH, the expected cyclic peptidotriazoles were obtained from resins **1** and **2** after a 5 h treatment. In contrast, when resin **3** was subjected to these conditions, the reaction did not reach completion. ESI-MS analysis of the crude reaction mixture showed the expected cyclic peptidotriazole BPC700 together with the alkynyl cyclic decapeptide BPC468. Overnight treatment did not improve the results. Next, cyclic peptidotriazoles were individually cleaved from the resin with TFA/H_2O/TIS (95:2.5:2.5) and analyzed by HPLC and ESI-MS. Except for BPC700, cyclic peptidotriazoles were obtained in excellent purities (88–98%).

The above methodology was extended to the synthesis of a conjugated peptide containing a cyclic and a linear peptidyl sequence linked through a triazole ring. We chose as the model system the reaction between the alkynyl cyclic peptidyl resin **1** and the azido pentapeptide Fmoc-Phe-Nle(ε-N_3)-Lys-Leu-Gln-OAll (BP252) (Scheme 4). The pentapeptide BP252 was prepared by following the standard protocol previously described by incorporating Fmoc-Nle(ε-N_3)-OH at the 2-position. This amino acid is not commercially available and was synthesized from Fmoc-Lys(Boc)-OH.[10] After Boc removal with TFA/CH_2Cl_2 (1:1), azidation of Fmoc-Lys-OH was performed by treatment with TfN_3 in the presence of $NaHCO_3$ and $CuSO_4 \cdot 5H_2O$. The mixture was stirred overnight under pressure at room temperature. Fmoc-Nle(ε-N_3)-OH was obtained in 89% overall yield, which was characterized by NMR and mass spectrometry. This residue was then used for the synthesis of BP252 (91% purity). The click reaction between resin **1** and the azidopentapeptide BP252 (5 equiv.) was carried out as described previously in the presence of ascorbic acid (5 equiv.) and CuI (5 equiv.) in piperidine/DMF (2:8), while stirring at room temperature for 7 h. The resulting conjugated peptide BPC472 was cleaved from the resin with TFA/H_2O/TIS (95:2.5:2.5) and analyzed by HPLC and mass spectrometry; the peptide was obtained in 98% purity.

Synthesis of Cyclic Peptidotriazoles II

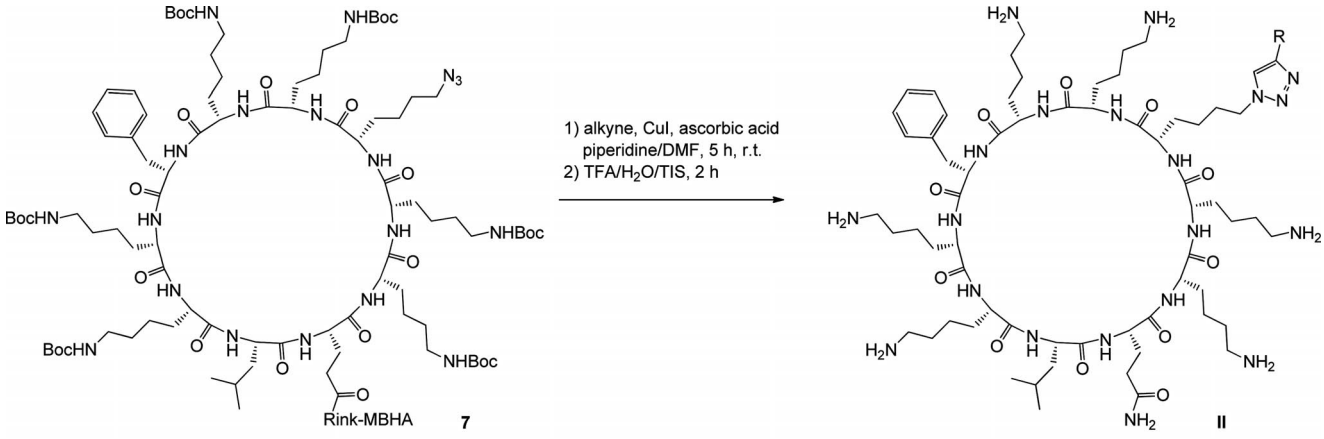
The synthesis of cyclic peptidotriazoles **II** (Figure 1) involved the cycloaddition reaction of resin-bound azide **7** and an alkyne in solution. The azido cyclic peptidyl resin **7** was prepared by following the Fmoc/*t*Bu/All strategy previously described (Scheme 5). First, the linear peptidyl resin **8** was prepared by incorporating Fmoc-Nle(ε-N_3)-OH at the 3-position followed by allyl and Fmoc group removal. Subsequent cyclization under standard conditions afforded the cyclic peptidyl resin **7**. Acidolytic cleavage of an aliquot of **7** yielded BPC464 in 94% HPLC purity, which was characterized by mass spectrometry.

Resin **7** was then subjected to a cycloaddition reaction with the alkynes phenylacetylene, *p*-ethynyltoluene and tetrahydro-2-(prop-2-ynyloxy)-2*H*-pyran (5 equiv.) in the presence of ascorbic acid (5 equiv.) and CuI (5 equiv.) in piperidine/DMF (2:8), while stirring for 5 h at room tem-



Scheme 5. Synthesis of the azido cyclic peptidyl resin **7**.

Table 2. Synthesis of cyclic peptidotriazoles **II**.



Alkyne	Compound	R	Purity (%)[a]
	BPC516		98
	BPC548		90
THPO	BPC552	HO	90

[a] Percentage determined by HPLC analysis with detection at 220 nm of the crude reaction mixture.

perature (Table 2). After acidolytic cleavage, the corresponding cyclic peptidotriazoles were obtained in excellent purities (90–98%) and were characterized by mass spectrometry.

Biological Activity

Cyclic peptidotriazoles were tested for in vitro growth inhibition of the plant pathogenic bacteria *E. amylovora*, *P. syringae* pv. *syringae* and *X. axonopodis* pv. *vesicatoria* at 3.1, 6.2, 12.5, 25 and 50 μM (Table 3). The results were compared to those obtained for BPC194. Most peptidotriazoles were considerably active against *X. axonopodis* pv. *vesicatoria* and *P. syringae* pv. *syringae*. Five sequences displayed the same MIC values as BPC194 (MIC of 3.1 to 6.2 μM) against *X. axonopodis* pv. *vesicatoria*. Against *P. syringae* pv. *syringae*, six sequences showed MIC values of less than 12.5 μM, with BPC548 being as active as BPC194 (MIC of 3.1 to 6.2 μM). *E. amylovora* was the least sensitive pathogen to these cyclic peptidotriazoles, with three sequences exhibiting MIC values of less than 25 μM. Interestingly, the conjugated peptide BPC472 was as active as BPC194 against *X. axonopodis* pv. *vesicatoria* (MIC of 3.1 to 6.2 μM).

In general, cyclic peptidotriazoles incorporating a triazolyl lysine (BPC692, BPC696) or a triazolyl norleucine (BPC516, BPC548, BPC552) were more active than those bearing a triazolyl alanine (BPC456, BPC458, BPC460, BPC472) or a triazolyl glutamine (BPC520, BPC522, BPC532). Regarding the substituent on the triazole ring, the introduction of a benzyl group did not significantly alter the antibacterial activity. In contrast, the incorporation of a 2-aminohexanoic group or a peptidyl chain resulted in

Table 3. Antibacterial activity (MIC) against three plant pathogenic bacteria and cytotoxicity of cyclic peptidotriazoles.

Peptide	MIC [μM]			Hemolysis [%][b]
	Xav[a]	*Ea*[a]	*Pss*[a]	(375 μM)
BPC194	3.1–6.2	6.2–12.5	3.1–6.2	17 ± 1.7
BPC456	6.2–12.5	>50	6.2–12.5	6 ± 0.5
BPC458	6.2–12.5	>50	6.2–12.5	5 ± 0.2
BPC460	25–50	>50	>50	0 ± 0.1
BPC472	3.1–6.2	>50	>50	6 ± 0.3
BPC516	3.1–6.2	25–50	6.2–12.5	8 ± 0.1
BPC520	3.1–6.2	>50	12.5–25	2 ± 0.2
BPC522	25–50	>50	25–50	3 ± 0.3
BPC532	12.5–25	>50	6.2–12.5	4 ± 0.4
BPC548	3.1–6.2	12.5–25	3.1–6.2	26 ± 4
BPC552	3.1–6.2	25–50	6.2–12.5	1 ± 1
BPC692	6.2–12.5	12.5–25	12.5–25	7 ± 0.2
BPC696	6.2–12.5	12.5–25	12.5–25	30 ± 4

[a] *Xav*, *Xanthomonas axonopodis* pv. *vesicatoria*; *Ea*, *Erwinia amylovora*; *Pss*, *Pseudomonas syringae* pv. *syringae*. [b] Percent hemolysis plus confidence interval (α = 0.05).

poorly active sequences (BPC460, BPC522, and BPC472). In terms of activity against the three bacteria tested, the best peptides were BPC516, BPC548, BPC552, BPC692, and BPC696.

We also evaluated the toxicity to eukaryotic cells of the cyclic peptidotriazoles; this was defined as the ability to lyse erythrocytes in comparison to melittin. Percent hemolysis at 375 μM is shown in Table 3. All compounds displayed low hemolytic activity. Ten out of 12 sequences displayed less than 10% hemolysis at 375 μM, being less hemolytic than the parent peptide BPC194. The cyclic peptidotriazole with an optimal balance between antibacterial and hemolytic activities was BPC548.

Conclusions

Analogues of the antimicrobial cyclic decapeptide BPC194, bearing a 1,2,3-triazole ring at the side-chain of the residue at the 3-position, were synthesized on solid-phase. Formation of the triazole moiety was performed by a copper-catalyzed cycloaddition reaction. The corresponding akynyl or azido peptidyl resins were prepared and treated with a range of azides or alkynes, respectively. Cyclic peptidotriazoles were obtained in excellent purities, and sequences with high antibacterial and low hemolysis were identified. The best cyclic peptidotriazole, BPC548, displayed a biological activity profile similar to that of the parent peptide. These results offer a good perspective for the further development of new antimicrobial agents.

Experimental Section

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. Flash chromatography purifications were performed on silica gel 60 (230–400 mesh, Merck).

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 with a Dionex instrument with a Kromasil 100 C_{18} (40 mm×4.6 mm, 3.5 μm) column and a 2–100% B linear gradient over 7 min at a flow rate of 1 mL/min (Solvent A: 0.1% aq. TFA; Solvent B: 0.1% TFA in CH_3CN).

ESI-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 (CH_3CN/H_2O, 80:20; flow rate: 100 μL/min) was delivered by a 1100 Series HPLC pump (Agilent). Nitrogen was employed as both the drying and nebulizing gas.

HRMS 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.

^1H and ^{13}C NMR spectra were measured with a Bruker 400 MHz NMR spectrometer. Chemical shifts are reported as δ values (ppm) directly referenced to the solvent signal.

Fmoc-Glu(NH-CH_2-C≡CH)-O*t*Bu: Propargylamine (110 μL, 1.56 mmol), Oxyma (404 mg, 2.84 mmol), DIPEA (720 μL, 4.22 mmol), and EDC (320 mg, 3.12 mmol) were sequentially added to a solution of Fmoc-Glu-O*t*Bu (600 mg, 1.42 mmol) in anhydrous THF (70 mL) under N_2. The reaction mixture was stirred at room temperature under N_2 and monitored by HPLC. After 24 h, further EDC (160 mg, 1.56 mmol), Oxyma (101 mg, 0.71 mmol), and DIPEA (125 μL, 0.73 mmol) were added and the mixture was stirred for an additional 24 h. The reaction was stopped by adding EtOH (2 mL). Removal of the solvents under vacuum gave a residue that was dissolved in EtOAc (50 mL), extracted with H_2SO_4 (0.5 M; 4× 50 mL), washed with distilled H_2O (2× 50 mL), dried with anhydrous $MgSO_4$, and concentrated. The crude product was purified by column chromatography (hexane/EtOAc, 2:1) to give Fmoc-Glu(NH-CH_2-C≡CH)-O*t*Bu as a a yellow oil (639 mg, 98% yield). R_f = 0.81 (hexane/EtOAc, 1:5); t_R = 8.94 min. IR (neat): $\tilde{\nu}$ = 3305.99 (≡CH, st), 2980.46 (C≡C, st), 1725.79, 1689.73 (C=O, st), 1637.08 (δ NH_2), 1083.99 (C–O, st), 738.99 (δ NH, opp), 646.23 (δ≡CH) cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$): δ = 1.51 [s, 9 H, $C(CH_3)_3$], 1.90–1.97 [m, 2 H, $CH_2(\beta)$, $CH_2(\gamma)$], 2.23–2.30 [m, 3 H, $CH_2(\beta)$, $CH_2(\gamma)$, C≡CH], 4.07–4.09 (m, 2 H, NCH_2), 4.24–4.27 [m, 2 H, CH_{Fmoc}, CH(α)], 4.42–4.51 (m, 2 H, OCH_2), 5.59 (d, J = 7.6 Hz, 1 H, NHCOO), 6.32 (br., 1 H, CONH), 7.36 (t, J = 7.6 Hz, 2 H, 2 CH_{Ar}), 7.45 (t, J = 7.6 Hz, 2 H, 2 CH_{Ar}), 7.63 (d, J = 7.6 Hz, 2 H, 2 CH_{Ar}), 7.81 (d, J = 7.6 Hz, 2 H, 2 CH_{Ar}) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 27.92 (CH_3), 29.06, 29.19, 32.23 [$CH_2(\beta)$, $CH_2(\gamma)$, CH_2N], 47.15 (CH_{Fmoc}), 53.80 [CH(α)], 66.93 (OCH_2), 71.52 (≡CH), 79.41 (C≡), 82.63 [$C(CH_3)_3$], 119.94 (CH_{Ar}), 119.97 (CH_{Ar}), 125.01 (CH_{Ar}), 125.06 (CH_{Ar}), 127.04 (2 CH_{Ar}), 127.70 (2 CH_{Ar}), 141.25 (C_{Ar}), 141.29 (C_{Ar}), 143.57 (C_{Ar}), 143.81 (C_{Ar}), 156.42 (NHCOO), 170.96 (COO*t*Bu), 171.71 (CONH) ppm. MS (ESI): m/z = 463.1 [M + H]$^+$, 485.1 [M + Na]$^+$.

Fmoc-Glu(NH-CH_2-C≡CH)-OH: Fmoc-Glu(NH-CH_2-C≡CH)-O*t*Bu (400 mg, 0.71 mmol) was dissolved in a solution of TFA/

CH_2Cl_2 (1:1, 10 mL) and stirred for 2 h at room temperature. Following TFA evaporation and diethyl ether extraction, Fmoc-Glu(NH-CH_2-C≡CH)-OH was obtained as a white powder (81 mg, 58% yield). R_f = 0.32 (EtOAc/NH_3/MeOH, 5:1:1); t_R = 7.70 min. IR (neat): $\tilde{\nu}$ = 3293.84 (≡CH, st), 2923.38 (C≡C, st), 1687.41 (C=O, st), 1640.36 (δ NH_2), 1536.22, 1448.67 (δ CH_2), 1085.73 (C–O, st), 738.22 (δ NH, opp), 620.39 (δ ≡CH) cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$): δ = 1.91–1.98 [m, 1 H, CH_2(β)], 2.12–2.24 [m, 1 H, CH_2(β)], 2.28–2.33 [m, 2 H, CH_2(γ)], 2.56 (t, J = 3.6 Hz, 1 H, ≡CH), 3.93 (d, J = 3.6 Hz, 2 H, NCH_2), 4.15–4.22 [m, 2 H, CH_{Fmoc}, CH(α)], 4.31 (dd, J = 9.4, 13.8 Hz, 1 H, OCH_2), 4.37 (dd, J = 9.4, 13.8 Hz, 1 H, OCH_2), 7.30 (td, J = 1.6, 9.8 Hz, 2 H, 2 CH_{Ar}), 7.38 (t, J = 9.8 Hz, 2 H, 2 CH_{Ar}), 7.64–7.68 (m, 2 H, 2 CH_{Ar}), 7.77 (d, J = 9.8 Hz, 2 H, 2 CH_{Ar}) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 28.54 (CH_2-β), 29.45 (NCH_2), 33.05 (CH_2-γ), 46.99 (CH_{Fmoc}), 54.91 (CH-α), 67.96 (OCH_2), 72.18 (≡CH), 80.51 (≡C), 120.86 (2 CH_{Ar}), 126.23 (2 CH_{Ar}), 128.13 (2 CH_{Ar}), 128.74 (2 CH_{Ar}), 142.51 (2 C_{Ar}), 145.10 (C_{Ar}), 145.27 (C_{Ar}), 158.62 (NHCOO), 174.46 (CONH), 175.35 (COOH) ppm. MS (ESI): m/z = 407.0 $[M + H]^+$. HRMS (ESI): calcd. for $C_{23}H_{23}N_2O_5$ 407.1601; found 407.1591 and calcd. for $C_{23}H_{22}N_2NaO_5$ 429.1421; found 429.1406.

Fmoc-Lys-OH: Fmoc-Lys(Boc)-OH (1.25 g, 3.40 mmol) was dissolved in a solution of TFA/CH_2Cl_2 (1:1, 75 mL) and stirred for 2 h at room temperature. Following TFA evaporation and diethyl ether extraction, Fmoc-Lys-OH was obtained as a white powder (1.22 g, 95% yield). R_f = 0.5 ($CHCl_3$/MeOH/AcOH, 5:3:1); t_R = 6.99 min. IR (neat): $\tilde{\nu}$ = 3066.47 (≡CH, st), 1670.64 (C=O, st), 1519.64 (C=C), 1449.63 (δ CH_2), 1181.57 (δ C–H, ip), 789.19 (γ CH_2), 738.60 (δ NH, opp) cm^{-1}. ^1H NMR (400 MHz, [D_6]DMSO): δ = 1.344–1.430 [m, 2 H, 2 CH_2(γ)], 1.48–1.71 [m, 4 H, 2 CH_2(β), 2 CH_2(δ)], 2.73–2.80 [m, 2 H, 2 CH_2(ε)], 3.90–3.96 [m, 1 H, CH(α)], 4.21–4.26 [m, 1 H, $CH_{(Fmoc)}$], 4.28–4.34 [m, 2 H, $CH_{2(Fmoc)}$], 7.33 (td, J = 0.7 7.2 Hz, 2 H, 2 CH_{Ar}), 7.42 (t, J = 7.2 Hz, 2 H, 2 CH_{Ar}), 7.63 (d, J = 7.2 Hz, 1 H, CH_{Ar}), 7.71–7.74 (m, 1 H, CH_{Ar}), 7.79 (br., 1 H, CONH), 7.89 (d, J = 7.2 Hz, 2 H, 2 CH_{Ar}) ppm. ^{13}C NMR (100 MHz, [D_6]DMSO): δ = 22.57 [CH_2(γ)], 26.51 [CH_2(β)], 30.17 [CH_2(δ)], 38.59 [CH_2(ε)], 46.66 [$CH_{(Fmoc)}$], 53.61 [CH(α)], 65.58 [$CH_{2(Fmoc)}$], 120.13 (CH_{Ar}), 120.14 (CH_{Ar}), 125.26 (2 CH_{Ar}), 127.07 (CH_{Ar}), 127.08 (CH_{Ar}), 127.65 (2 CH_{Ar}), 140.73 (C_{Ar}), 140.76 (C_{Ar}), 143.79 (C_{Ar}), 143.81 (C_{Ar}), 156.20 (CONH), 173.85 (COOH) ppm. MS (ESI): m/z = 369.1 $[M + H]^+$.

Fmoc-Nle(ε-N_3)-OH:[10] NaN_3 (883 mg, 13.58 mmol) was dissolved in a mixture of distilled H_2O (2 mL) and CH_2Cl_2 (3.5 mL). Triflic anhydride (Tf_2O) (460 μL, 2.72 mmol) was added slowly, and the resulting mixture was stirred for 2 h. The organic phase was removed and the aqueous phase was extracted with CH_2Cl_2 (2× 3 mL). The organic fractions containing TfN_3 were combined, washed with a saturated aqueous solution of Na_2CO_3 (6.5 mL), and used without further purification.

Fmoc-Lys-OH (500 mg, 1.36 mmol) was dissolved in distilled H_2O (4.5 mL) and MeOH (9 mL). Thereafter, $NaHCO_3$ (1.14 g, 13.58 mmol) and $CuSO_4$·$5H_2O$ (34 mg, 13 mmol) were added. TfN_3 in CH_2Cl_2 (9.5 mL) was then added and the mixture was stirred under pressure at room temperature. The reaction was monitored by HPLC. After 12 h, the organic solvents were removed under vacuum, and the remaining solution was diluted with distilled H_2O (36 mL) and acidified to pH 2 by the addition of aq. HCl. After extraction with EtOAc (4× 20 mL), the organic fractions were combined, washed with brine (20 mL), dried with anhydrous $MgSO_4$, and concentrated. The crude product was digested with pentane to give Fmoc-Nle(ε-N_3)-OH as a white powder (385 mg, 94% yield). R_f = 0.64 (CH_2Cl_2/MeOH, 7:1); t_R = 8.60 min. IR (neat): $\tilde{\nu}$ = 3381.58 (≡CH, st), 2095.48 (N≡N, st), 1701.11 (C=O, st), 1521.57 (C=C, st), 1450.02 (δ CH_2), 1190.06 (δ CH, ip), 739.37 (δ NH, opp) cm^{-1}. ^1H NMR (400 MHz, [D_6]-DMSO): δ = 1.34–1.43 [m, 2 H, 2 CH_2(γ)], 1.45–1.57 [m, 2 H, CH_2(δ)], 1.58–1.66 [m, 1 H, CH_2(β)], 1.68–1.77 [m, 1 H, CH_2(β)], 3.32 [t, J = 6.8 Hz, 2 H, CH_2(ε)], 3.91–3.97 [m, 1 H, CH(α)], 4.21–4.25 [m, 1 H, $CH_{(Fmoc)}$], 4.28–4.30 [m, 2 H, $CH_{2(Fmoc)}$], 7.42 (td, J = 0.9, 7.4 Hz, 2 H, 2 CH_{Ar}), 7.42 (t, J = 7.4 Hz, 2 H, 2 CH_{Ar}), 7.65 (d, J = 7.4 Hz, 1 H, CH_{Ar}), 7.73 (d, J = 7.4 Hz, 1 H, CH_{Ar}), 7.89 (d, J = 7.4 Hz, 2 H, 2 CH_{Ar}), 8.95 (s, 1 H, CONH) ppm. ^{13}C NMR (100 MHz, [D_6]DMSO): δ = 23.37 [CH_2(γ)], 28.29 [CH_2(δ)], 30.74 [CH_2(β)], 47.13 [$CH_{(Fmoc)}$], 50.98 [CH_2(ε)], 54.12 [CH(α)], 66.05 [$CH_{2(Fmoc)}$], 120.56 (CH_{Ar}), 120.58 (CH_{Ar}), 125.73 (CH_{Ar}), 125.76 (CH_{Ar}), 127.52 (2 CH_{Ar}), 128.10 (2 CH_{Ar}), 141.20 (C_{Ar}), 141.22 (C_{Ar}), 144.27 (C_{Ar}), 144.32 (C_{Ar}), 156.63 (CONH), 174.32 (COOH) ppm. MS (ESI): m/z = 395.1 $[M + H]^+$. HRMS (ESI): calcd. for $C_{21}H_{23}N_4O_4$ 395.1714; found 395.1727 and calcd. for $C_{21}H_{22}N_4NaO_4$ 417.1533; found 417.1544.

General Method for the Synthesis of Alkynyl and Azido Linear Peptidyl Resins: These peptidyl resins were synthesized manually by the solid-phase method using standard Fmoc chemistry. Fmoc-Rink-MBHA resin (0.3 mmol/g) was used as solid support. Fmoc-Leu-OH, Fmoc-Lys(Boc)-OH, Fmoc-Lys(Mtt)-OH, Fmoc-Nle(ε-N_3)-OH, Fmoc-Prg-OH, Fmoc-Glu(NH-CH_2-C≡CH)-OH, Fmoc-Phe-OH or Fmoc-Glu-OAll were used as amino acid derivatives. Peptide elongation was performed by repeated cycles of Fmoc group removal, coupling, and washings. Fmoc group removal was achieved with piperidine/DMF (3:7, 2 + 10 min). Couplings of the Fmoc-amino acids (4 equiv.) were mediated by Oxyma (4 equiv.) and DIPCDI (4 equiv.) in DMF at room temperature for 1 h while stirring. The completion of the reactions was checked by the Kaiser test.[11] 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, 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.

Fmoc-Lys(Boc)-Lys(Boc)-Prg-Lys(Boc)-Lys(Boc)-Phe-Lys(Boc)-Lys(Boc)-Leu-Glu(Rink-MBHA)-OAll (4): Synthesized following the procedure described above incorporating Fmoc-Prg-OH at the 3-position. After acidolytic cleavage of an aliquot of this resin, Fmoc-Lys-Lys-Prg-Lys-Lys-Phe-Lys-Lys-Leu-Gln-OAll was obtained in 85% purity. t_R = 6.24 min. MS (ESI): m/z = 767.5 $[M + 2H]^{2+}$, 1534.1 $[M + H]^+$.

Fmoc-Lys(Boc)-Lys(Boc)-Glu(NH-CH_2-C≡CH)-Lys(Boc)-Lys(Boc)-Phe-Lys(Boc)-Lys(Boc)-Leu-Glu(Rink-MBHA)-OAll (5): Synthesized following the procedure described above incorporating Fmoc-Glu(NH-CH_2-C≡CH)-OH at the 3-position. After acidolytic cleavage of an aliquot of this resin, Fmoc-Lys-Lys-Glu(NH-CH_2-C≡CH)-Lys-Lys-Phe-Lys-Lys-Leu-Gln-OAll was obtained in 91% purity. t_R = 6.41 min. MS (ESI): m/z = 802.5 $[M + 2H]^{2+}$, 1605.1 $[M + H]^+$.

Fmoc-Lys(Boc)-Lys(Boc)-Lys(Mtt)-Lys(Boc)-Lys(Boc)-Phe-Lys(Boc)-Lys(Boc)-Leu-Glu(Rink-MBHA)-OAll: Synthesized following the procedure described above incorporating Fmoc-Lys(Mtt)-OH at the 3-position. After acidolytic cleavage of an aliquot of this resin, Fmoc-Lys-Lys-Lys-Lys-Lys-Phe-Lys-Lys-Leu-Gln-OAll was obtained in 94% purity. t_R = 6.01 min. MS (ESI): m/z = 784.0 $[M + 2H]^{2+}$, 1566.0 $[M + H]^+$, 1588.0 $[M + Na]^+$.

Fmoc-Lys(Boc)-Lys(Boc)-Nle(ε-N_3)-Lys(Boc)-Lys-(Boc)-Phe-Lys(Boc)-Lys(Boc)-Leu-Glu(Rink-MBHA)-OAll (8): Synthesized following the procedure described above incorporating Fmoc-Nle(ε-N_3)-OH at the 3-position. After acidolytic cleavage of an aliquot of this resin, Fmoc-Lys-Lys-Nle(ε-N_3)-Lys-Lys-Phe-Lys-Lys-Leu-Gln-OAll was obtained in 77% purity. t_R = 6.61 min. MS (ESI): *m*/*z* = 1592.2 $[M + H]^+$.

Synthesis of Fmoc-Phe-Nle(ε-N_3)-Lys-Leu-Gln-OAll (BP252): The peptidyl resin Fmoc-Phe-Nle(ε-N_3)-Lys(Boc)-Leu-Glu(Rink-MBHA)-OAll was prepared following the general procedure described above for the synthesis of linear azido peptidyl resins. Acidolytic cleavage afforded Fmoc-Phe-Nle(ε-N_3)-Lys-Leu-Gln-OAll (BP252) in 91% purity. t_R = 8.29 min. MS (ESI): *m*/*z* = 951.4 $[M + H]^+$.

General Method for the Synthesis of Alkynyl and Azido Cyclic Peptidyl Resins 1–3 and 7: 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/NMM (3:2:1) under nitrogen and stirring for 3 h at room temperature. After this time, the resin was washed with THF (3× 2 min), NMP (3× 2 min), 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, while stirring for 24 h. Following washes with NMP (6× 1 min) and CH_2Cl_2 (6× 1 min), an aliquot of the cyclic peptidyl resin was cleaved by treatment with TFA/H_2O/TIS (95:2.5:2.5) for 2 h. Following TFA evaporation and diethyl ether extraction, the crude peptide was dissolved in H_2O, lyophilized, analyzed by HPLC, and characterized by mass spectrometry.

c[Lys(Boc)-Lys(Boc)-Prg-Lys(Boc)-Lys(Boc)-Phe-Lys(Boc)-Lys-(Boc)-Leu-Glu(Rink-MBHA)] (1): Synthesized from resin **4** following the procedure described above. After acidolytic cleavage of an aliquot of resin **1**, c(Lys-Lys-Prg-Lys-Lys-Phe-Lys-Lys-Leu-Gln) (BPC466) was obtained in 96% purity. t_R = 5.81 min. MS (ESI): *m*/*z* = 1253.0 $[M + H]^+$, 1274.9 $[M + Na]^+$. HRMS (ESI): calcd. for $C_{61}H_{108}N_{17}O_{11}$ 418.2799; found 418.2794 and calcd. for $C_{61}H_{107}N_{17}O_{11}$ 626.9163; found 626.9150.

c[Lys(Boc)-Lys(Boc)-Glu(NH-CH_2-C≡CH)-Lys(Boc)-Lys(Boc)-Phe-Lys(Boc)-Lys(Boc)-Leu-Glu(Rink-MBHA)] (2): Synthesized from resin **5** following the procedure described above. After acidolytic cleavage of an aliquot of resin **2**, c[Lys-Lys-Glu(NH-CH_2-C≡CH)-Lys-Lys-Phe-Lys-Lys-Leu-Gln] (BPC580) was obtained in 99% purity. t_R = 6.08 min. MS (ESI): *m*/*z* = 1324.0 $[M + H]^+$, 1346.0 $[M + Na]^+$.

c(Lys(Boc)-Lys(Boc)-Lys(CO-C≡CH)-Lys(Boc)-Lys(Boc)-Phe-Lys-(Boc)-Lys(Boc)-Leu-Glu(Rink-MBHA)) (3): Peptidyl resin c[Lys-(Boc)-Lys(Boc)-Lys(Mtt)-Lys(Boc)-Lys(Boc)-Phe-Lys(Boc)-Lys-(Boc)-Leu-Glu(Rink-MBHA)] (**6**) was prepared following the general procedure described above from Fmoc-Lys(Boc)-Lys(Boc)-Lys(Mtt)-Lys(Boc)-Lys(Boc)-Phe-Lys(Boc)-Lys(Boc)-Leu-Glu-(Rink-MBHA)-OAll. Peptidyl resin **6** was treated with 1% TFA in CH_2Cl_2 (the solution became yellow). The mixture was stirred for 5 min at room temperature and the resin was then washed with CH_2Cl_2 (2× 1 min), MeOH (2× 1 min) and CH_2Cl_2 (2× 1 min). TFA treatment was repeated until the solution remained colorless. The N^{ε}-amino group of the lysine residue at the 3-position was then acylated with propiolic acid using the conditions described above for the coupling of Fmoc-protected amino acids. After acidolytic cleavage of an aliquot of the resulting resin **3**, c[Lys-Lys-Lys-(CO-C≡CH)-Lys-Lys-Phe-Lys-Lys-Leu-Gln] (BPC468) was obtained in 99% purity. t_R = 5.98 min. MS (ESI): *m*/*z* = 1337.7 $[M + H]^+$, 1359.7 $[M + Na]^+$.

c[Lys(Boc)-Lys(Boc)-Nle(ε-N_3)-Lys(Boc)-Lys(Boc)-Phe-Lys(Boc)-Lys(Boc)-Leu-Glu(Rink-MBHA)] (7): Synthesized from resin **8** following the procedure described above. After acidolytic cleavage of an aliquot of resin **7**, c[Lys-Lys-Nle(ε-N_3)-Lys-Lys-Phe-Lys-Lys-Leu-Gln] (BPC464) was obtained in 94% purity. t_R = 6.00 min. MS (ESI): *m*/*z* = 1312.0 $[M + H]^+$, 1334.0 $[M + Na]^+$.

Synthesis of the Cyclic Peptidotriazoles: The corresponding alkynyl or azido cyclic peptidyl resin was swollen with CH_2Cl_2 (1× 20 min) and DMF (1× 20 min) and then treated with an azide (5 equiv.) or with an alkyne (5 equiv.), respectively, in the presence of ascorbic acid (5 equiv.) and CuI (5 equiv.) in piperidine/DMF (2:8). The 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) and analyzed by HPLC and mass spectrometry.

Cyclic Peptidotriazole BPC456: The alkynyl resin c[Lys(Boc)-Lys-(Boc)-Prg-Lys(Boc)-Lys(Boc)-Phe-Lys(Boc)-Lys(Boc)-Leu-Glu-(Rink-MBHA)] (**1**) was treated with NaN_3 following the general procedure described above. In this case, two treatments of 10 h were required. BPC456 was obtained in 97% purity. t_R = 5.87 min. MS (ESI): *m*/*z* = 1295.7 $[M + H]^+$. HRMS (ESI): calcd. for $C_{61}H_{109}N_{20}O_{11}$ 432.6189; found 432.6211 and calcd. for $C_{61}H_{108}N_{20}O_{11}$ 648.4248; found 648.4263.

Cyclic Peptidotriazole BPC458: The alkynyl resin c[Lys(Boc)-Lys-(Boc)-Prg-Lys(Boc)-Lys(Boc)-Phe-Lys(Boc)-Lys(Boc)-Leu-Glu-(Rink-MBHA)] (**1**) was treated with BnN_3 for 5 h following the general procedure described above. BPC458 was obtained in 94% purity. t_R = 5.98 min. MS (ESI): *m*/*z* = 1385.7 $[M + H]^+$, 1426.7 $[M + K]^+$. HRMS (ESI): calcd. for $C_{68}H_{115}N_{20}O_{11}$ 462.6346; found 462.6365 and calcd. for $C_{68}H_{114}N_{20}O_{11}$ 693.4482; found 693.4478.

Cyclic Peptidotriazole BPC460: The alkynyl resin c[Lys(Boc)-Lys-(Boc)-Prg-Lys(Boc)-Lys(Boc)-Phe-Lys(Boc)-Lys(Boc)-Leu-Glu-(Rink-MBHA)] (**1**) was treated with Boc-Nle(ε-N_3)-OH for 5 h following the general procedure described above. BPC460 was obtained in 90% purity. t_R = 5.85 min. MS (ESI): *m*/*z* = 1424.7 $[M + H]^+$. HRMS (ESI): calcd. for $C_{67}H_{120}N_{21}O_{13}$ 475.6453; found 475.6464 and calcd. for $C_{67}H_{119}N_{21}O_{13}$ 712.9643; found 712.9649.

Cyclic Peptidotriazole BPC472: The alkynyl resin c[Lys(Boc)-Lys-(Boc)-Prg-Lys(Boc)-Lys(Boc)-Phe-Lys(Boc)-Lys(Boc)-Leu-Glu-(Rink-MBHA)] (**1**) was treated with Fmoc-Phe-Nle(ε-N_3)-Lys-Leu-Gln-OH (BP252) for 7 h following the general procedure described above. BPC472 was obtained in 98% purity. t_R = 6.15 min. MS (ESI): *m*/*z* = 1981.6 $[M + H]^+$. HRMS (ESI): calcd. for $C_{96}H_{165}N_{27}O_{18}$ 496.0701; found 496.3234; calcd. for $C_{96}H_{164}N_{27}O_{18}$ 661.0910; found 661.4269 and calcd. for $C_{96}H_{163}N_{27}O_{18}$ 991.1329; found 991.6358.

Cyclic Peptidotriazole BPC516: The azido peptidyl resin c[Lys-(Boc)-Lys(Boc)-Nle(ε-N_3)-Lys(Boc)-Lys(Boc)-Phe-Lys(Boc)-Lys-(Boc)-Leu-Glu(Rink-MBHA)] (**7**) was treated with phenylacetylene for 5 h following the general procedure described above. BPC516 was obtained in 98% purity. t_R = 6.09 min. MS (ESI): *m*/*z* = 1414.0 $[M + H]^+$, 1436.0 $[M + Na]^+$. HRMS (ESI): calcd. for $C_{70}H_{119}N_{20}O_{11}$ 471.9784; found 471.9808 and calcd. for $C_{70}H_{118}N_{20}O_{11}$ 707.4639; found 707.4654.

Cyclic Peptidotriazole BPC520: The alkynyl resin c[Lys(Boc)-Lys(Boc)-Glu(NH-CH_2-C≡CH)-Lys(Boc)-Lys(Boc)-Phe-Lys(Boc)-Lys(Boc)-Leu-Glu(Rink-MBHA)] (**2**) was treated with BnN_3 for 5 h following the general procedure described above. BPC520 was obtained in 98% purity. t_R = 6.04 min. MS (ESI): *m*/*z* = 1457.0 [M + H]$^+$, 1479.0 [M + Na]$^+$. HRMS (ESI): calcd. for $C_{71}H_{120}N_{21}O_{12}$ 486.3136; found 486.3157 and calcd. for $C_{71}H_{119}N_{21}O_{12}$ 728.9668; found 728.9677.

Cyclic Peptidotriazole BPC522: The alkynyl resin c[Lys(Boc)-Lys(Boc)-Glu(NH-CH_2-C≡CH)-Lys(Boc)-Lys(Boc)-Phe-Lys(Boc)-Lys(Boc)-Leu-Glu(Rink-MBHA)] (**2**) was treated with Boc-Nle(ε-N_3)-OH for 5 h following the general procedure described above. BPC522 was obtained in 96% purity. t_R = 5.66 min. MS (ESI): *m*/*z* = 1496.1 [M + H]$^+$, 1518.1 [M + Na]$^+$. HRMS (ESI): calcd. for $C_{70}H_{125}N_{22}O_{14}$ 499.3243; found 499.3276 and calcd. for $C_{70}H_{124}N_{22}O_{14}$ 748.4828; found 748.4837.

Cyclic Peptidotriazole BPC532: The alkynyl resin c[Lys(Boc)-Lys(Boc)-Glu(NH-CH_2-C≡CH)-Lys(Boc)-Lys(Boc)-Phe-Lys(Boc)-Lys(Boc)-Leu-Glu(Rink-MBHA)] (**2**) was treated with NaN_3 following the general procedure described above. In this case, two treatments of 10 h were required. BPC532 was obtained in 98% purity. t_R = 5.79 min. MS (ESI): *m*/*z* = 1367.0 [M + H]$^+$, 1388.9 [M + Na]$^+$. HRMS (ESI): calcd. for $C_{64}H_{114}N_{21}O_{12}$ 456.3047; found 456.3005 and calcd. for $C_{64}H_{113}N_{21}O_{12}$ 683.9433; found 683.9449.

Cyclic Peptidotriazole BPC548: The azido peptidyl resin c[Lys(Boc)-Lys(Boc)-Nle(ε-N_3)-Lys(Boc)-Lys(Boc)-Phe-Lys(Boc)-Lys(Boc)-Leu-Glu(Rink-MBHA)] (**7**) was treated with *p*-ethynyltoluene for 5 h following the general procedure described above. BPC548 was obtained in 90% purity. t_R = 6.35 min. MS (ESI): *m*/*z* = 1428.2 [M + H]$^+$, 1450.1 [M + Na]$^+$. HRMS (ESI): calcd. for $C_{71}H_{121}N_{20}O_{11}$ 476.6502; found 476.6542 and calcd. for $C_{71}H_{120}N_{20}O_{11}$ 714.4717; found 714.4729.

Cyclic Peptidotriazole BPC552: The azido peptidyl resin c[Lys(Boc)-Lys(Boc)-Nle(ε-N_3)-Lys(Boc)-Lys(Boc)-Phe-Lys(Boc)-Lys(Boc)-Leu-Glu(Rink-MBHA)] (**7**) was treated with tetrahydro-2-(prop-2-ynyloxy)-2*H*-pyran for 5 h following the general procedure described above. BPC552 was obtained in 90% purity. t_R = 5.77 min. MS (ESI): *m*/*z* = 1368.1 [M + H]$^+$, 1386.1 [M + NH_4]$^+$, 1408.1 [M + K]$^+$. HRMS (ESI): calcd. for $C_{65}H_{117}N_{20}O_{12}$ 456.6381; found 456.6401 and calcd. for $C_{65}H_{116}N_{20}O_{12}$ 684.4535; found 684.4579.

Cyclic Peptidotriazole BPC692: The alkynyl resin c[Lys(Boc)-Lys(Boc)-Lys(CO-C≡CH)-Lys(Boc)-Lys(Boc)-Phe-Lys(Boc)-Lys(Boc)-Leu-Glu(Rink-MBHA)] (**3**) was treated with NaN_3 following the general procedure described above. In this case, two treatments of 10 h were required. BPC692 was obtained in 92% purity. t_R = 6.10 min. MS (ESI): *m*/*z* = 1380.8 [M + H]$^+$, 1402.8 [M + Na]$^+$. HRMS (ESI): calcd. for $C_{65}H_{117}N_{21}O_{12}$ 345.9792; found 345.9805, calcd. for $C_{65}H_{116}N_{21}O_{12}$ 460.9699; found 460.9706; and calcd. for $C_{65}H_{115}N_{21}O_{13}$ 690.9512; found 690.9513.

Cyclic Peptidotriazole BPC696: The alkynyl resin c[Lys(Boc)-Lys(Boc)-Lys(CO-C≡CH)-Lys(Boc)-Lys(Boc)-Phe-Lys(Boc)-Lys(Boc)-Leu-Glu(Rink-MBHA)] (**3**) was treated with BnN_3 for 5 h following the general procedure described above. BPC696 was obtained in 88% purity. t_R = 6.47 min. MS (ESI): *m*/*z* = 1470.9 [M + H]$^+$, 1492.9 [M + Na]$^+$. HRMS (ESI): calcd. for $C_{72}H_{123}N_{21}O_{13}$ 368.4910; found 368.4919, calcd. for $C_{72}H_{122}N_{21}O_{13}$ 490.9855; found 490.9861, and calcd. for $C_{72}H_{121}N_{21}O_{13}$ 736.4762; found 736.4739.

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 °C. *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 °C. The cell material was suspended in sterile water to obtain a suspension of 10^8 CFU mL^{-1}.

Antibacterial Activity: Lyophilized compounds were solubilized in sterile Milli-Q water to a final concentration of 1000 μM and filter sterilized through a 0.22 μm pore filter. For minimum inhibitory concentration (MIC) assessment, dilutions of the compounds were made to obtain a stock concentration of 500, 250, 125, 62.5 and 31.125 μM. Aliquots (20 μL) of each dilution were mixed in a microtiter plate well with the corresponding suspension of the bacterial indicator (20 μL), Trypticase Soy Broth (TSB) (BioMèrieux, France) (160 μL) to a total volume of 200 μL. Three replicates for each strain, compound, and concentration were used. Positive controls contained water instead of compound and negative controls contained compounds without bacterial suspension. Microbial growth was automatically determined by optical density measurement at 600 nm (Bioscreen C, Labsystem, Helsinki, Finland). Microplates were incubated at 25 °C with 20 sec shaking before hourly absorbance measurement for 48 h. The experiment was repeated twice. The MIC was taken as the lowest compound concentration with no growth at the end of the experiment.

Hemolytic Activity: The hemolytic activity of the compounds was evaluated by determining hemoglobin release from erythrocyte suspensions of fresh human blood (5% vol/vol). Blood was aseptically collected using a BD vacutainer K2E System with EDTA (Belliver Industrial State, Plymouth, U. K.) and stored for less than 2 h at 4 °C. Blood was centrifuged at 6000 g for 5 min, washed three times with TRIS buffer (10 mM TRIS, 150 mM NaCl, pH 7.2) and diluted. Compounds were solubilized in TRIS buffer to a stock concentration of 750, 500, and 300 μM (final concentrations tested were 375, 250 and 150 μM). Human red blood cells (65 μL) were mixed with the compound solution (65 μL) and incubated under continuous shaking for 1 h at 37 °C. The tubes were then centrifuged at 3500 g for 10 min. Aliquots (80 μL) of the supernatant were transferred to 100-well microplates (Bioscreen) and diluted with Milli-Q water (80 μL). Hemolysis was measured as the absorbance at 540 nm with a Bioscreen plate reader. Complete hemolysis was determined in TRIS buffer plus melittin at 100 μM final concentration (Sigma–Aldrich Corporation, Madrid, Spain) as a positive control. The percentage of hemolysis (*H*) was calculated by using the equation: $H = 100\times[(Op–Ob)/(Om–Ob)]$, where *Op* is the density for a given compound concentration, *Ob* for the buffer, and *Om* for the melittin positive control.

Supporting Information (see footnote on the first page of this article): Copies of HPLC, ESI-MS, IR, ^1H and ^{13}C NMR spectra of amino acid derivatives. Copies of HPLC and ESI-MS of alkynyl and azido linear and cyclic peptides. Copies of HPLC, ESI-MS and HRMS (ESI) of cyclic peptidotriazoles.

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